2015

Division of Intramural Research

ANNUAL REPORT

Eunice Kennedy Shriver National Institute of Child Health and Human Development

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** nominee*

Jerome F. Strauss, III, MD, PhD, *chair*
7/1/2009 – 6/30/2016
Obstetrics, Gynecology & Genetics

Dean, School of Medicine, Virginia Commonwealth University, Executive Vice President for Medical Affairs, VCU Health System, Richmond, VA

Rita J. Balice-Gordon, PhD
7/1/2013 – 6/30/2018
Neuroscience

Vice-President and Head, Integrative Neuroscience and Circuitry, Neuroscience Research Unit, Pfizer, Inc., Cambridge, MA

Jeanne Brooks-Gunn, PhD
7/1/2013 – 6/30/2018
Developmental Psychology & Behavioral Science

Virginia and Leonard Marx Professor of Child Development, Teachers College and College of Physicians and Surgeons, Columbia University, New York, NY

P. Michael Conn, PhD
7/1/15 – 6/30/20
Cell & Molecular Biology, Neuroscience, & Endocrinology

Senior Vice President for Research, Associate Provost, Professor of Internal Medicine and Cell Biology/Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX

Laurinda Jaffe, PhD
7/1/2011 – 6/30/16
Signal Transduction

Professor and Chair, Department of Cell Biology, University of Connecticut Health Center, Farmington, CT

Frances E. Jensen, MD *
7/1/2015 – 6/30/2020
Neuroscience

Professor and Chair, Neurology Department, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Antonios G. Mikos, PhD*
7/1/2013 – 6/30/2018
Bioengineering & Biophysics

Louis Calder Professor of Bioengineering, Chemical & Biomolecular Engineering Director, Center for Excellence in Tissue Engineering Director, J.W. Cox Laboratory for Biomedical Engineering, Rice University, Houston, TX

Tarun B. Patel, PhD
7/1/2010 – 6/30/2016
Cell & Molecular Biology, Biochemistry and Pharmacology

Provost, Vice President of Academic Affairs, Albany College of Pharmacy and Health Sciences, Albany, NY

Scott A. Rivkees, MD *
7/1/2015 – 6/30/2020
Pediatric Endocrinology

Professor, Nemours Eminent Scholar and Chair, Department of Pediatrics, University of Florida, Physician in Chief, Shands Hospital for Children, Gainesville, FL

Yoel Sadovsky, MD
7/1/2014 – 6/30/2019
Reproductive Biology, Obstetrics, & Gynecology

Director, Magee-Womens Research Institute, Elsie Hilliard Chair of Women's Health, Professor of Obstetrics, Gynecology and Reproductive Sciences and Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Magee-Womens Research Institute, Pittsburgh, PA

Lilianna "Lila" Solnica-Krezel, PhD
7/1/2012 – 6/30/2017
Developmental Biology

Professor and Head, Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO

Joan A. Steitz, PhD
7/1/2011 – 6/30/2016
Biochemistry & Molecular Biology

Sterling Professor of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Susan S. Taylor, PhD
7/1/2014 – 6/30/2019
Structural Biology & Molecular Biochemistry

Professor of Chemistry and Biochemistry, Professor of Pharmacology, University of California, San Diego, La Jolla, CA

Eric Vilain, MD, PhD*
7/1/2015 – 6/30/2020
Molecular & Human Genetics

Professor, Departments of Human Genetics, Pediatrics, & Urology, Chief, Division of Medical Genetics, Department of Pediatrics, University of California, Los Angeles, CA

Michelle A. Williams, DSc
7/1/2011 – 6/30/2016
Epidemiolology

Professor and Chair, Department of Epidemiology, Harvard School of Public Health, Boston, MA

Message *from the* Scientific Director

The 2015 annual report of the Division of Intramural Research (DIR) for the Eunice Kennedy Shriver National Institute of Child Health and Human Development is now accessible electronically, in two ways, on the web and on your cell phones or tablets, at: **http://annualreport.nichd.nih.gov**

You are invited to search the report site, whether to review our medical and scientific discoveries of the past year, to see what work a colleague may currently be engaged in, or to identify a laboratory where you may wish to collaborate or refer a student. For potential postdoctoral fellows, graduate students, and clinical fellows, the report is fully searchable. It offers you an introduction to a panoply of research endeavors in NICHD's DIR.

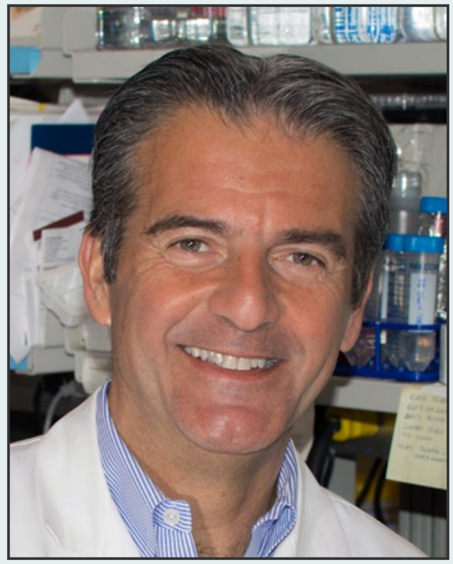
NICHD intramural investigators represent a broad array of basic, translational, and clinical researchers. Our work is reflected in a new mission statement:

> To plan and conduct the Institute's laboratory and clinical research programs to seek fundamental knowledge about the nature and behavior of living systems through basic, clinical, and population-based research and determine how to apply such knowledge to illuminate developmental origins of health and disease and help ensure that women and men have good reproductive health, that children are born healthy, and that people develop to live healthy and productive lives.

We use a range of model systems in the areas of developmental biology, molecular and cellular biology, neurosciences, structural biology, imaging, behavior, and biophysics. Investigators take advantage of our resources in a 19,000-tank zebrafish core facility, working with a number of other animal models, from fruit flies to rats and mice, and supported by a wide array of core services, such as imaging, proteomics and molecular genomics. Each investigator participates in at least one, and typically more, affinity groups. The affinity groups are intended to be team-based and future-oriented—to build on thematic interests while responding to rapidly shifting scientific priorities as new knowledge is uncovered. We are looking closely at how work at the bench may translate to the bedside, through our translational research affinity group.

In particular, I invite you to read through the selection of our Clinical Research Protocols listed in this flyer and to consider how we may collaborate, through the NIH U01 grant mechanism at the NIH Clinical Center. The support of this program can lead to our next new success in therapeutics, the next miracle drug, if we combine expertise, take advantage of our NIH infrastructure and our patient population, whether on rare disorders or the most persistent problems affecting human health. **http://clinicalcenter.nih.gov/translational-research-resources/U01/**

The DIR researchers whose names appear in this publication are dedicated mentors and accomplished investigators. Link to their reports on the web to learn about their work in 2015. I also invite you to reach out to me with your ideas and proposals for collaborative initiatives we may undertake together. At our core, the scientists of the DIR seek to uncover fundamental answers to our existence, the basic science that underpins life. We seek out the connections



Constantine Stratakis, MD, D(med)Sci, *Scientific Director*

Brenda R. Hanning, *Deputy Director, Liaison & Training*

Carol Carnahan, *Staff Assistant to the Scientific Director*

Renee Gethers, *Program Support Assistant*

Sara K. King, *Scientific Program Analyst – Board of Scientific Counselors* to human health and to disease, whether very rare disorders or perturbing and persistent health challenges, on behalf of the American public and the global community.

Sincerely yours,

Constantine A. Stratakis, MD, DMSci
NICHD, NIH

Goals and objectives

The intramural Office of Education was established in September 2004 to support the training needs of intramural scientists, fellows, and students at all levels. This is achieved through recruitment and development of academic support programs; support of accreditation; contributions to mentoring, evaluation, and career guidance; and creation of new training initiatives. Additional areas of involvement include career development programming, networking among fellows and alumni, grantsmanship, and the enhancement of fellows' competitiveness for awards, as well as support of new tenure-track investigators.

Notable accomplishments of the past year

In spring 2015, the Division of Intramural Research gave its eighth Mentor of the Year awards to investigator Karl Pfeifer and postdoctoral fellow Chad McCormick. FARE (Fellows Award for Research Excellence) 2016 awards went to a record 28 applicants. The NICHD Scholars Developing Talent program, established in 2011, added three new postbaccalaureate fellows, Nicholas Johnson (lab of Mary Lilly), Rim Mehari (lab of Jack Yanovski), and Miles Oliva (lab of Paul Love); Nicole Millan (lab of Erin Wolff) and PhD student Dezmond Taylor-Douglas (Yanovski lab) continue for a further year. Office of Education program activities included the annual grantsmanship workshop, career and public speaking workshops, among others. Our annual "Becoming an Effective Scientist" course, organized and taught by fellows for postbaccalaureate trainees, entered its tenth year. The eleventh annual meeting of fellows took place at the Smithsonian Museum of the American Indian and featured keynote speakers Graham Chedd of the Alan Alda Center for Communicating Science and Toby Freedman of Synapsis Search on careers in the life sciences. Our TmT competition was held in conjunction with NHGRI and NIDCR this year; it emphasizes the importance to scientists of communicating their research in an accessible way. Contestants participated in speaking workshops and were judged by internal and external panels of judges, leading to a first-place award for Kathryn Tabor from the lab of Harold Burgess. A video clip of this winning talk was presented to the NICHD Director's advisory council. Along with continuing scientific orientations for fellows, led by Yvette Pittman, the Individualized Development Plan was actively promoted for all fellows, using myIDP on the Science Careers web site. The Fellows Intramural Grant Supplement (FIGS) continues to recognize grant applicants and awardees with stipend increases; and the Fellows Recruitment Incentive Award, launched in 2013, promotes the recruitment of postdoctoral fellows from backgrounds under-represented in science. The *NICHD Connection,* a monthly newsletter run and written by fellows, will publish its 67th issue in December 2015; it continues to highlight programs and fellows' scientific successes.

Contact

For further information, contact **pittmanyv@mail.nih.gov** or **hanningb@mail.nih.gov**.

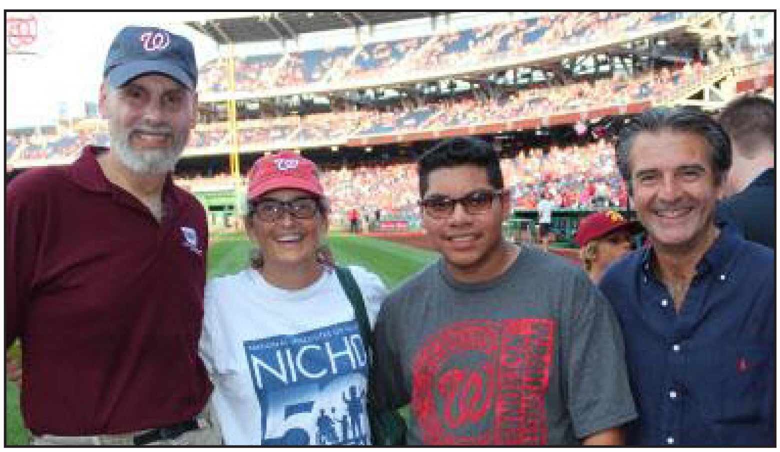
Brenda Hanning, *Director, Office of Education*

Yvette R. Pittman, PhD, *Associate Director*

Office of the Clinical Director, NICHD

The NICHD intramural clinical research program currently includes 98 protocols with five main areas of focus: (1) adult, pediatric, and reproductive endocrinology; (2) human genetics; (3) normal growth and development; (4) national/international public health; and (5) women's health. The protocols are conducted by 36 NICHD principal investigators and 90 NICHD associate investigators. The NICHD clinical protocol portfolio spans the spectrum from Natural History to therapeutic trials. Sixteen protocols involve an investigational drug or device. Five protocols support our teaching mission. Approximately half the protocols include pediatric patients.

The conduct of studies is guided by two entities administered by the Office of the Clinical Director: the NICHD Institutional Review Board (IRB) and the NICHD Data Safety Monitoring Committee (DSMC). The NICHD IRB is chaired by Gilman Grave, MD, and has 13 members and three alternates. The composition of the NICHD IRB is diverse, both in terms of medical and ethical expertise and affiliation. The IRB has specific expertise in reproductive endocrinology, gynecology, pediatrics, endocrinology, genetics, and the ethics of human subject research. NICHD's IRB is a resource for other institutes that have protocols involving children or women's health and supports the National Children's Study. The NICHD DSMC is chaired by Frank Pucino, PharmD, and has five other members. Both committees possess expertise in issues related to clinical trials, ethics, pediatrics, genetics, and reproductive medicine.



NICHD staff enjoy HHS night at National's ballgame.
Take me out to the ballgame!

HHS night at the National's ballpark with one of our NICHD patients and NICHD Director, Dr. Alan Guttmacher and NICHD Scientific Director, Dr. Constantine Stratakis.

CONTACT

For more information, email **fdporter@helix.nih.gov** or visit **http://science.nichd.nih.gov/confluence/display/ocd/Home**.

Forbes D. Porter, MD, PhD, *Clinical Director*
Meg Keil, PhD, *Associate Director, Nursing and Protocol Navigation*
Maryellen Rechen, BS, RN, *Special Assistant to the Clinical Director*
Donna Peterson, BS, RN, *Protocol and Institutional Review Board (IRB) Coordinator*
Simona Bianconi, MD, *Staff Clinician*
Jenny Blau, MD, *Staff Clinician*
Andrew Demidowich, MD, *Staff Clinician*
Karan Adams, CRNP, *Nurse Practitioner*
Sheila Brady, CRNP, *Nurse Practitioner*
Lee Ann Keener, RN, *Research Nurse*
Margarita Raygada, PhD, *Genetic Counselor*
Craig Abbott, PhD, *Statistician*
Denise Phillips, *Clinical Research Coordinator*
DuShon Hutchinson, *Patient Care Coordinator*
Fathy Majadly, BS, *Patient Specimen Coordinator*
Loc Trinh, *Research Chemical Engineer*

Clinical Trials *at* NICHD

Numerous clinical protocols are run by the NICHD, Division of Intramural Research (for a complete listing, please visit https://www.clinicaltrials.gov). The following is a list of investigators within the DIR who recruit patients, and their contact information. For detailed information on all related research projects, please check the individual investigator's listing in the report or the DIR website at http://dir.nichd.nih.gov.

PEDIATRIC ENDOCRINOLOGY AND OBESITY

» Studies on endocrine, genetic, and other pediatric disorders that are associated with the predisposition to develop obesity and diabetes. Patients may be referred to Dr. Jack Yanovski at **yanovskj@mail.nih.gov** or 301-496-4168.
» Investigations on endocrine complications faced by pediatric cancer survivors. Additional studies to improve clinical care for pediatric patients with many types of endocrine cancers including pheochromocytoma, Cushing disease, and thyroid cancer. Patients may be referred to Dr. Maya Lodish at **lodishma@mail.nih.gov**.
» Research on endocrine, genetic, and other pediatric disorders that are associated with the predisposition to endocrine and other tumors, abnormal development in fetal or later life and may affect the pituitary, the adrenal and other related organs. Patients may be referred to Dr. Constantine Stratakis at **stratakc@mail.nih.gov** or to Ms. Elena Belyavskaya at 301-496-0862.
» Evaluation of patients with endocrine disorders that are associated with excess androgen, including different forms of congenital adrenal hyperplasia. Patients may be referred to Dr. Deborah Merke at **dmerke@nih.gov** or Ms. Terri McHugh at 301-451-0399.
» Clinical and genetic studies of patients with disorders of puberty and reproduction, including early and late entry into puberty, and amenorrhea or infertility due to central hypogonadism, including isolated GnRH deficiency. Patients may be referred to Dr. Angela Delaney at **delaneya@mail.nih.gov**.

ADULT ENDOCRINOLOGY

» Patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma (PHEO) and paraganglioma (PGL). Patients may be referred to Dr. Karel Pacak at **karel@mail.nih.gov**.

REPRODUCTIVE ENDOCRINOLOGY AND WOMEN'S HEALTH

» Research on uncommon reproductive disorders, including developmental abnormalities of the female reproductive tract. Patients may be referred to Dr. Alan DeCherney at **decherna@mail.nih.gov**, or a clinical research nurse at 301-435-7926. (Please note that this protocol does not include disorders for which assisted reproduction is indicated or has been advised.)
» Research on reproductive disorders affecting the endometrium (such as recurrent implantation failure) using endometrial biopsy. Patients can contact Jackie Nottidge at **jacqueline.nottidge@nih.gov** or 301-435-7926.
» Research on reproductive function in sickle cell disease. Patients can contact Jackie Nottidge at **jacqueline.nottidge@nih.gov** or 301-435-7926.
» Research on ovarian stem cells and how they impact infertility. Patients can contact Jackie Nottidge at **jacqueline.nottidge@nih.gov** or 301-435-7926.
» Fertility preservation (oocyte freezing) for women scheduled to undergo gonadotoxic therapies such as chemotherapy and radiation. Patients can contact Jackie Nottidge at **jacqueline.nottidge@nih.gov** or 301-435-7926.
» Research on endometriosis and pain, HPV vaccination after stem cell transplant, and genital graft versus host disease. Patients may be referred to Dr. Pamela Stratton at **strattop@mail.nih.gov** or her research line at 301-496-1190.
» Investigation of the role of the neuropeptide kisspeptin on female reproductive physiology. Patients may be referred to Dr. Angela Delaney at **delaneya@mail.nih.gov**.

GENETICS

» Counseling and research on patients with suspected or diagnosed genetic disorders. Patients and their families receive comprehensive evaluations, counseling, and risk assessment. Patients may be referred to Dr. Margarita Raygada at **raygadam@mail.nih.gov** or call 301-451-8822.

- Studies on patients with genetic disorders related to altered cholesterol metabolism. This includes patients with Smith-Lemli-Opitz syndrome (SLOS) and Niemann-Pick Disease, type C (NPC). Patients may be referred to Dr. Forbes Porter at **fdporter@mail.nih.gov**, Ms. Lee Ann Keener (301-594-2005), or Ms. Nicole Farhat (301-594-1765).
- Studies on children with osteogenesis imperfecta, both dominant and recessive forms. Current protocols focus on natural history of secondary features of OI in pulmonary function, audiology, and neurology as well as on identification of causative genetic mutations. Patients may be referred to Dr. Joan Marini at **oidoc@helix.nih.gov**.
- Studies to identify novel genetic causes of idiopathic growth disorders using whole-exome sequencing. Subjects will include children and adults with a clearly recognizable phenotype that includes either short stature or tall stature and a pedigree that strongly suggests a monogenic inheritance. Patients may be referred to Dr. Jeffrey Baron at **baronj@cc1.nichd.nih.gov**.
- Studies on patients with genetic disorders related to altered copper transport. This includes patients with Menkes disease, MEDNIK syndrome, Huppke-Brendel syndrome, ATP7A-related distal motor neuropathy, and Wilson disease. Patients may be referred to Dr. Stephen Kaler at **kalers@mail.nih.gov** or Ms. Maryellen Rechen at **rechenma@mail.nih.gov**.
- Studies on patients with genetic disorders related to lysosomal storage. This includes patients with Alpha-mannosidosis and Mucopolysaccharidosis type 3B (Sanfillipo B). Patients may be referred to Dr. Stephen Kaler at **kalers@mail.nih.gov** or Ms. Kristen Stevens at **kristen.stevens@nih.gov**.

MEDICAL BIOPHYSICS AND NOVEL IMAGING TECHNIQUES

- Studies with healthy subjects to evaluate the age-dependent characteristics of cervical structure and collagen noninvasively by illumination of the cervix area using polarized light. The study is important to evaluate pregnant women who are at risk of early cervical dilation during pregnancy. Subjects may be referred to Dr. Amir Gandjbakhche at **amir@helix.nih.gov**.
- Research with normal volunteers to calibrate imaging modalities such as multispectral imaging, laser Doppler and thermal imaging, and algorithms to quantify skin chromophores such as blood oxygenation and blood volume. Currently, the imaging modality has been used in the clinic to study the therapeutic response of Kaposi's sarcoma patients. Subjects may be referred to Dr. Amir Gandjbakhche at **amir@helix.nih.gov**.

COGNITIVE AND SOCIAL-EMOTIONAL DEVELOPMENT

- Studies on the processes by which the risk for psychopathology is transmitted from clinically depressed mothers to their children, over time and across several domains of child development, and how this risk can be modified by various contextual factors. The depressed group comprises mothers with major depression, minor depression, and dysthymia at 5 months postpartum. Contact Dr. Marc Bornstein at **marc_h_bornstein@nih.gov**.

NICHD-NIDDK Inter-Institute Endocrine Training Program

The Inter-Institute Endocrinology Training Program (IETP) seeks to train internal medicine physicians to become first-rate endocrinologists dedicated to investigative careers. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Diabetes Branch, Metabolic Diseases Branch, and Clinical Endocrinology Branch), the National Institute of Child Health and Human Development (NICHD, Program in Developmental Endocrinology and Genetics, Program in Reproductive and Adult Endocrinology), and the National Institute of Dental and Craniofacial Research (NIDCR, Skeletal Clinical Studies Section) participate in the program, with faculty from all three institutes.

Clinical and research training

Clinical training occurs largely in the first year. At any one time, fellows are responsible for five to ten patients on the inpatient service of the NIH. Under the supervision of the endocrine faculty, the trainee has complete responsibility for all aspects of a patient's care. Fellows make daily rounds, discuss patients with the attending physicians, and participate in management decisions related to both patient care and clinical investigation. Although all patients are admitted under peer-reviewed research protocols, there are many other aspects of diagnosis and patient-care that fall entirely under the discretion of the endocrine fellows. During the second and third year, emphasis is placed on learning how to develop research questions, which enables fellows to investigate unusual disorders or particular scientific questions, and on maintaining clinical expertise. Fellows are also encouraged to participate in specific hypothesis-driven protocols.

The second and third year are spent primarily in laboratory or clinical research under the mentorship of a senior investigator in one of the several endocrinology branches of the NIH. During this research period, active clinical experience continues through bi-weekly continuity outpatient clinics (general endocrinology as well as diabetes clinics) and participation in clinical conferences. In addition, fellows on the endocrine service serve as consultants to the other services within the Clinical Center, where patients are not selected with regard to endocrine problems. Thus, fellows gain experience with several common problems of endocrine disease that may occur in any general medical ward. Clinical research activities include programs in all the areas of endocrine and metabolic diseases. Study design, outcome measures, statistical analysis, and ethical and regulatory issues are stressed.

The IETP provides a comprehensive training experience that involves not only the NIH clinical branches working in Endocrinology but also the Georgetown University Hospital and Washington Hospital Center, in the Washington area. The basic and clinical endocrine research facilities at the NIH are among the most extensive and highly regarded in the world. Thus, the fellowship is ideal for physicians who seek a broad education in both research and clinical endocrinology.

Monica C. Skarulis, MD, *Director, Inter-Institute Endocrine Training Program*
Michael T. Collins, MD, *Associate Director (NICHD), Inter-Institute Endocrine Training Program, NIDCR Senior Investigator, CSDB, NIDCR*
Jenny E. Blau, MD, *Assistant Director, Inter-Institute Endocrine Training Program, NICHD*
Lynnette Nieman, MD, *Senior Investigator, Director of Human Subjects Research*
Constantine Stratakis, MD, D(med) Sci, *Senior Investigator, Program on Developmental Endocrinology and Genetics, NICHD*
Karel Pacak, MD, PhD, DSc, *Senior Investigator, Program in Reproductive and Adult Endocrinology, NICHD*
Andrew Demidowich, MD, *Staff Clinician, NICHD*
Susmeeta Sharma, MD, *Senior Fellow*
Ricardo Correa, MD, *Clinical Fellow*
Smita Jha, MD, *Clinical Fellow*
Ijeoma Muo, MD, *Clinical Fellow*
Firas Riyazuddin, MD, *Clinical Fellow*
Fady Hannah-Shmouni, MD, *Clinical Fellow*
Sri Tella, MD, *Clinical Fellow*

PUBLICATIONS

1. Jha S, Wang Z, Laucis N, Bhattacharyya T. Trends in Media Reports: Oral bisphosphonate prescriptions and hip fractures 1996-2012: an ecological analysis. *J Bone Miner Res* 2015;30:2179–87.
2. Correa R, Salpea E, Stratakis C. Carney Complex: review literature update. *Eur J Endocrinol* 2015;M85–97.
3. Correa R, Zilbermint M, Berthon A, Espiard S, Batsis M, Papadakis GZ, Xekouki P, Lodish MB, Bertherat J, Faucz FR, Stratakis CA. ARMC5 gene shows extreme genetic variance in primary macronodular adrenocortical hyperplasia. *Eur J Endocrinol* 2015;173:435–440.
4. Correa R, Ortega C. Moving into the field of scientific publishing from the perspective of young doctors. *Archivos de Medicina* 2015;2.
5. Muo IM, Miller M, Goldberg AP. Considerations in favor of the use of fish oil for cardiovascular disease prevention and treatment in older adults. *J Aging: Res Clin Practice* 2014;3(4):191–195.

FACULTY

Kenneth Berman, MD, *Director, Endocrine Training Program, Washington Hospital Center, Washington, DC*
Rebecca Brown, MD, *Diabetes and Metabolic Disease Branch, NIDDK*
Alan H. DeCherney, MD, *Program in Reproductive and Adult Endocrinology, NICHD, Bethesda, MD*
Rachel Gafni, MD, *Craniofacial and Skeletal Diseases Branch, NIDCR, Bethesda, MD*
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James C. Reynolds, MD, *Nuclear Medicine Department, NIH Clinical Center, Bethesda, MD*
William F. Simonds, MD, *Metabolic Diseases Branch, NIDDK, Bethesda, MD*
Joseph Verbalis, MD, *Director, Endocrine Training Program, Georgetown University Medical Center, Washington, DC*
Lee S. Weinstein, MD, *Metabolic Diseases Branch, NIDDK, Bethesda, MD*

CONTACT

Adult Inter-Institute Endocrine Training Program (IETP)
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10 Center Drive, MSC 1613
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Phone: 301-496-1913
Fax: 301-480-4517

Program Coordinator: Sandra Wallace, **sandraw@mail.nih.gov**
Program Director: Dr. Monica Skarulis, **monicas@mail.nih.gov**

Online Application: **https://www.aamc.org/services/eras/**

Maternal-Fetal Medicine Fellowship

The goal of the Fellowship is to train individuals to provide specialized patient care in Maternal-Fetal Medicine as well as to prepare candidates for a career in academic medicine as physician scientists. It is a three-year training program. There is the possibility of completing a combined Maternal-Fetal Medicine and Human Genetics Fellowship, and candidates can opt to complete a PhD, which is based in the Department of Physiology at Wayne State University.

The clinical rotations (18 months) include: maternal-fetal medicine or high-risk obstetric service, obstetrical ultrasound, reproductive genetics, labor and delivery, anesthesia/intensive care unit, fetal echocardiography, and elective rotations. The program is housed at Hutzel Women's Hospital (with 5,000 deliveries per year), Detroit, and Faculty Members are Wayne State University appointees as well as Attendings of the Detroit Medical Center. The Perinatology Research Branch (PRB) is also housed at the Detroit Medical Center in Detroit, MI. The program is approved for seven positions, two of which are funded by the Perinatology Research Branch and the remainder by the Detroit Medical Center. The Fellowship emphasizes clinical, translational, and basic research (18 months are dedicated to research). Ideal candidates for our Program are well trained individuals from a university program wishing to pursue a career in academic medicine, who thrive in a rigorous and challenging environment and are goal-oriented and self-motivated.

The Fellowship emphasizes a multi-disciplinary approach to complications of pregnancy. There is a strong emphasis on the prenatal diagnosis of congenital anomalies with ultrasound, and our graduates are expected to be proficient in two-dimensional and three-dimensional ultrasound, fetal echocardiography, advanced imaging techniques such as Doppler, as well as ultrasound-guided invasive procedures such as amniocentesis. Opportunities for laboratory-based research and training are available in the fields of parturition, reproductive immunology, placental pathology and biology, biomarker discovery, and systems biology in reproduction, which are Units related to the Perinatology Research Branch. Alternative opportunities are available at the C.S. Mott Center of Wayne State University.

Primary areas of interest of the PRB are the mechanisms responsible for obstetrical disease, prediction and prevention of preterm birth, prenatal diagnosis of congenital anomalies, the role of infection and inflammation in perinatal disease, fetal growth and development, placental pathology, and the use of high-dimensional biology techniques to identify biomarkers for preterm labor, preterm PROM (premature rupture of membranes), preeclampsia, fetal death, and IUGR (intrauterine growth restriction).

Detailed information about the training program is available at the following website: **http://www.med.wayne.edu/prb**. The website contains information about each current fellow (as well as the faculty) and his/her publications and awards. The Director of the Fellowship is Dr. Lami Yeo, and the Associate Director for the PRB is Dr. Roberto Romero, Chief of the Perinatology Research Branch and Head of the Program in Perinatal Research and Obstetrics. The Program is sponsored by the Perinatology Research Branch, Detroit Medical Center, and Wayne State University. Fellows are employees of the Detroit Medical Center (DMC), and program oversight is with the Office of Graduate Medical Education of the DMC.

Medical or Pediatric Genetics Training at the Program in Developmental Endocrinology and Genetics

Clinical fellowship training in Medical or Pediatric Genetics is offered as a single program or in conjunction with training in Pediatric Endocrinology (and/or Internal Medicine and Endocrinology) at the Program in Developmental Endocrinology and Genetics (PDEGEN). Training in genetics is offered through PDEGEN's participation in the Medical Genetics fellowship programs of the National Institutes of Health (NIH) led by the National Human Genome Research Institute (NHGRI).

The PDEGEN also sponsors a combined medical genetics and pediatric endocrinology fellowship that leads to certification by both the American Board of Medical Genetics and the American Board of Pediatrics Sub-Board on Pediatric Endocrinology after 5–6 years of training (after approval by each Board).

Graduates of pediatrics or combined pediatrics/internal medicine residency programs, approved by the ACGME (Accreditation Council for Graduate Medical Education), in the United States who are either citizens or legal residents (green-card holders) of this country are eligible. We encourage applicants with previous PhD training or graduates of an MD/PhD program to apply for this unique fellowship, which aims to bridge two highly relevant sub-specialties of pediatric medicine: genetics and endocrinology.

This is an excellent opportunity for a physician-scientist in training who wishes to take advantage of the exciting opportunities offered by the NIH Clinical Center, the hundreds of state-of-the-art research laboratories at the NIH campus, and the commitment of NIH leadership to training initiatives on translational research.

The program is headed by:

Dr. Constantine A. Stratakis, MD, DMedSci
Scientific Director, NICHD, NIH, and
Director, Pediatric Endocrinology Training Program, NICHD, NIH
10 Center Drive, Building 10, Room 9D42, MSC 1830
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and

Dr. Forbes D. Porter, MD, PhD
Clinical Director, NICHD, and
Program Director, PDGEN, NICHD, NIH
Email: **fdporter@mail.nih.gov**

Pediatric Endocrinology Inter-Institute Training Program

The fellowship in Pediatric Endocrinology is a three-year, ACGME–accredited program. Applicants must have completed a residency in Pediatrics or Medicine/Pediatrics and be eligible to sit for the American Board of Pediatrics certification examination. Three fellows are accepted per year. The fellowship is based at the National Institutes of Health Clinical Center, which is one of the largest and most sophisticated research institutions in the United States. The program is conducted in partnership with Children's National Health System in Washington, DC. The fellowship is designed to provide clinical and research exposure that permits the development of academic Pediatric Endocrinologists with experience in both clinical and bench research.

Program structure

The Pediatric Endocrinology Fellowship at NIH consists of one year of clinical training, and two years of combined clinical and research training.

First year

A typical training schedule for first-year fellows includes six months at the NIH clinical research center, four months at Children's National Health Systems (CNHS), one month at The Johns Hopkins University Hospital, Baltimore, MD, and one month at Georgetown University Hospital, Washington, DC. Continuity clinics are held once a week and alternate between the NIH outpatient pediatric endocrine clinic and the Diabetes and general endocrine outpatient clinics at CNHS. In addition, multi-disciplinary clinics in long-term follow-up for childhood cancer survivors, bone health, polycystic ovarian syndrome, disorders of sexual development, obesity, thyroid nodules, and cancer are offered. The Clinical Center maintains clinical research protocols involving the treatment of adrenal and pituitary tumors, congenital adrenal hyperplasia, McCune-Albright syndrome, disorders of sexual development, obesity, Cushing's syndrome, and others.

Second and third years

During the second and third years, mandatory clinical responsibilities are limited to a half-day continuity clinic per week and inpatient pediatric endocrine consultation on an on-call basis for three months per year. Fellows learn how to develop a research protocol, conduct a study, evaluate the results, and create a presentation or manuscript suitable for publication. Fellows may choose to work in a laboratory setting, clinical setting, or both, and they perform state-of-the-art basic and clinical research closely supervised by internationally known mentors. During the first year, a research mentor is chosen and the fellow's progress is monitored by a Scholarship Oversight Committee. The overwhelming majority of our fellows go on to present their work at national and international meetings and chose academic careers following graduation.

Additional information

The following URL provides more detailed information about the program:
https://science.nichd.nih.gov/confluence/display/pe/Home

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Jeffrey Baron, MD, *Head, Section on Growth and Development, NICHD*
Rebecca Brown, MD, *Senior Clinical Fellow, Clinical Endocrinology Branch, NIDDK*
Angela Delaney, MD, *Assistant Clinical Investigator and Head, Unit on Genetics of Puberty and Reproduction, NICHD*
Rachel Gafni, MD, *Craniofacial and Skeletal Diseases Branch, NIDCR*
Margaret Keil, MS, CRNP, *Director, Pediatric Endocrine Clinical Services, NICHD*
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Youn Hee Jee, MD, *Senior Fellow, NICHD*
Fetima Worthington, *Program Coordinator, NICHD*

Application information

Applications are submitted through ERAS. The application must contain three letters of reference, medical school transcripts, USMLE scores, a personal statement, and a CV. The program participates in the NRMP match; pediatric endocrinology is now part of the fall subspecialty match. Applications must be submitted by August 31st, and interviews are conducted from September through November. Applicants must register with both NRMP and ERAS (**http://www.nrmp.org**, **https://www.aamc.org**).

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Reproductive Endocrinology and Infertility Training Program

The Intramural NICHD Reproductive Endocrinology and Infertility Training Program sponsors three-year clinical fellowships in Reproductive Endocrinology and Infertility, which are accredited by the American Board of Obstetrics and Gynecology. The objective of this graduate medical education program is to train clinicians to serve as researchers and future leaders in the field of reproductive endocrinology with a view toward advancing basic, translational, and clinical science in reproduction. The program was started in 1978 and has since trained over 65 physicians in reproductive endocrinology, many of whom have become leaders in the reproductive sciences. Upon completion of the rigorous scientific, clinical, and surgical curriculum, fellows may apply to the American Board of Obstetrics and Gynecology for certification in the subspecialty of Reproductive Endocrinology. The Fellowship in Reproductive Endocrinology and Infertility comprises faculty from four institutions: the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), the Uniformed Services University of the Health Sciences (USUHS), Walter Reed National Military Medical Center (WRNMMC), and the Shady Grove Fertility Center. The program accepts graduates of U.S. residencies in Obstetrics and Gynecology and has the mission to train reproductive endocrinology and infertility fellows who will serve as faculty in military, government, and academic institutions in order to establish and maintain high standards of training for students and residents in obstetrics and gynecology and to provide evidence-based, cutting-edge treatments to couples with infertility. Program graduates have become assistant, associate, and tenured professors and departmental chairs.

Fellows in the program rotate on clinical services of NICHD–supported intramural graduate medical programs in Medical and Pediatric Endocrinology as well as in Medical Genetics. The clinical training is robust. Thus, within the unique environment of the NIH, fellows participate in evaluation and management of rare and challenging endocrine conditions on the NIH Reproductive Endocrine Teaching Service. Clinical and surgical rotations take place at the Walter Reed National Medical Center and the Shady Grove Fertility Center. Research is strongly emphasized. Trainees in the program must complete a thesis project and may choose among any research laboratory in the Institute's intramural research program. To complete the research project, fellows are given 20 months of protected research time. The curriculum includes two university-based graduate courses: one in biostatistics and the other in reproduction. In the past year, faculty and fellows published 70 peer-reviewed articles. Over the past five years, graduates of the program published an average of five peer-reviewed manuscripts associated with the training program, and several trainees have received national recognition for excellence in research.

The three-year training program is structured to capitalize on the particular strengths and resources of each participating institution. Specifically, infertility services and operative care are provided by the busy clinical services at Walter Reed Bethesda Hospital, the NIH Clinical Center, and the Shady Grove Fertility Center. Experience in the Assisted Reproductive Technologies (ART) is provided by rotations in the newly renovated, state-of-the-art Walter Reed Bethesda ART facility and at Shady Grove Fertility Center. Fellows also obtain

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Nicole Banks, MD, *Clinical Fellow*
Matthew T. Connell, DO, *Clinical Fellow*
Katherine Green, MD, *Clinical Fellow*
Mae W. Healy, MD, *Clinical Fellow*
Terrence D. Lewis, MD, PhD, *Clinical Fellow*
Carter Monique Owen, MD, *Clinical Fellow*
George Patounakis, MD, PhD, *Clinical Fellow*
Torie Plowden, MD, MPH, *Clinical Fellow*
G. Donald Royster, IV, MD, *Clinical Fellow*

REI Fellows in-training

Left to right: G. Donald Royster, IV, Mae Wu Healy, Matthew Connell, George Patounakis, Katherine Green, Nicole Banks, Carter Monique Owen. *In Front:* Terrence Lewis. *Not shown:* Torie Plowden

medical endocrine, pediatric endocrine, and genetic clinical training through rotation on the active inpatient services at the NIH Clinical Center. The program staff and fellows see 1,500 patients in the NIH Clinical Center in addition to conducting 40 surgeries and 50 oocyte retrievals per year. Outstanding research training is available either though NIH intramural laboratories at the NICHD or at the Uniformed Services University of the Health Sciences. The program is intended to achieve synergistic interaction between the four sponsoring institutions and provide fellows with an experience and resources not available from a single institution.

Requirements for enrollment include graduation from a residency in Obstetrics and Gynecology in the United States that is accredited by the American Board of Obstetrics and Gynecology and an active medical license in the United States. Selection is competitive, and prospective candidates must register with the National Resident Matching Program (NRMP). Three positions are approved for a complement of nine fellow trainees. Trainees may meet criteria for the NIH Loan Repayment Program (LRP) for outstanding educational debt.

Didactic instruction

Structured training includes a series of introductory seminars geared to the first year fellows that takes place from July to September of the first year. These introductory seminars provide a historical perspective and basic understanding of the practice of Reproductive Endocrinology. There is a weekly NIH teaching rounds conference where challenging cases are reviewed and discussed with faculty and fellows. In addition, all faculty and fellows of all years are expected to attend the weekly Pre-operative and Fellows' conference. Fellows also attend weekly Branch research conferences sponsored by the Program in Reproductive and Adult Endocrinology (PRAE) at NICHD and present updates on thesis work at the weekly "Research in Progress Conference" at NIH. Core ACGME training objectives are covered in special NIH grand rounds and via courses at NIH or Walter Reed Bethesda. NIH Endocrine Grand Rounds provides additional training in medical, pediatric, and reproductive endocrine conditions. Regular attendance at a monthly journal club is expected. Finally, fellows regularly attend ART clinical meetings where management of patients pursing ART is discussed and outcomes are reviewed. Furthermore, fellows are encouraged to participate in didactic training offered at national meetings, such as the *American Society for Reproductive Medicine*, the *Society for Reproductive Investigation* and the *Society for the Study of Reproduction*. Moreover, fellows are encouraged to attend specialty meetings in their chosen interest areas, such as a Keystone meeting on hormone action.

PUBLICATIONS

1. Chaiworapongsa T, Romero R, Korzeniewski SJ, Chaemsaithong P, Hernandez-Andrade E, Segars JH, DeCherney A, McCoy MC, Kim C, Yeo L, Hassan S. Pravastatin to prevent recurrent fetal death in massive perivillous fibrin deposition of the placenta (MPFD). *J Matern Fetal Neonatal Med* 2015;28:1-8.
2. Connell MT, Szatkowski JM, Terry N, DeCherney AH, Propst AM, Hill MJ. Timing luteal support in assisted reproductive technology: a systematic review. *Fertil Steril* 2015;103(4):939-946.
3. Devine K, Mumford SL, Wu M, DeCherney AH, Hill MJ, Propst. Diminished ovarian reserve in the United States assisted reproductive technology population: diagnostic trends among181,536 cycles from the Society for Assisted Reproductive Technology Clinic Outcomes Reporting System. *Fertil Steril* 2015;104(3):612-619.
4. Hill MJ, Royster GD, Healy MW, Richter KS, Levy G, DeCherney AH, Levens ED, Suthar G, Widra E, Levy MJ. Are good patient and embryo characteristics protective against the negative effect of elevated progesterone level on the day of oocyte maturation? *Fertil Steril* 2015;103:1477-84.
5. Yu B, Russanova VR, Gravina S, Hartley S, Mullikin JC, Ignezweski A, Graham J, Segars JH, DeCherney AH, Howard BH. DNA methylome and transcriptome sequencing in human ovarian granulosa cells links age-related changes in gene expression to gene body methylation and 3'-end GC density. *Oncotarget* 2015;6:3627-3643.

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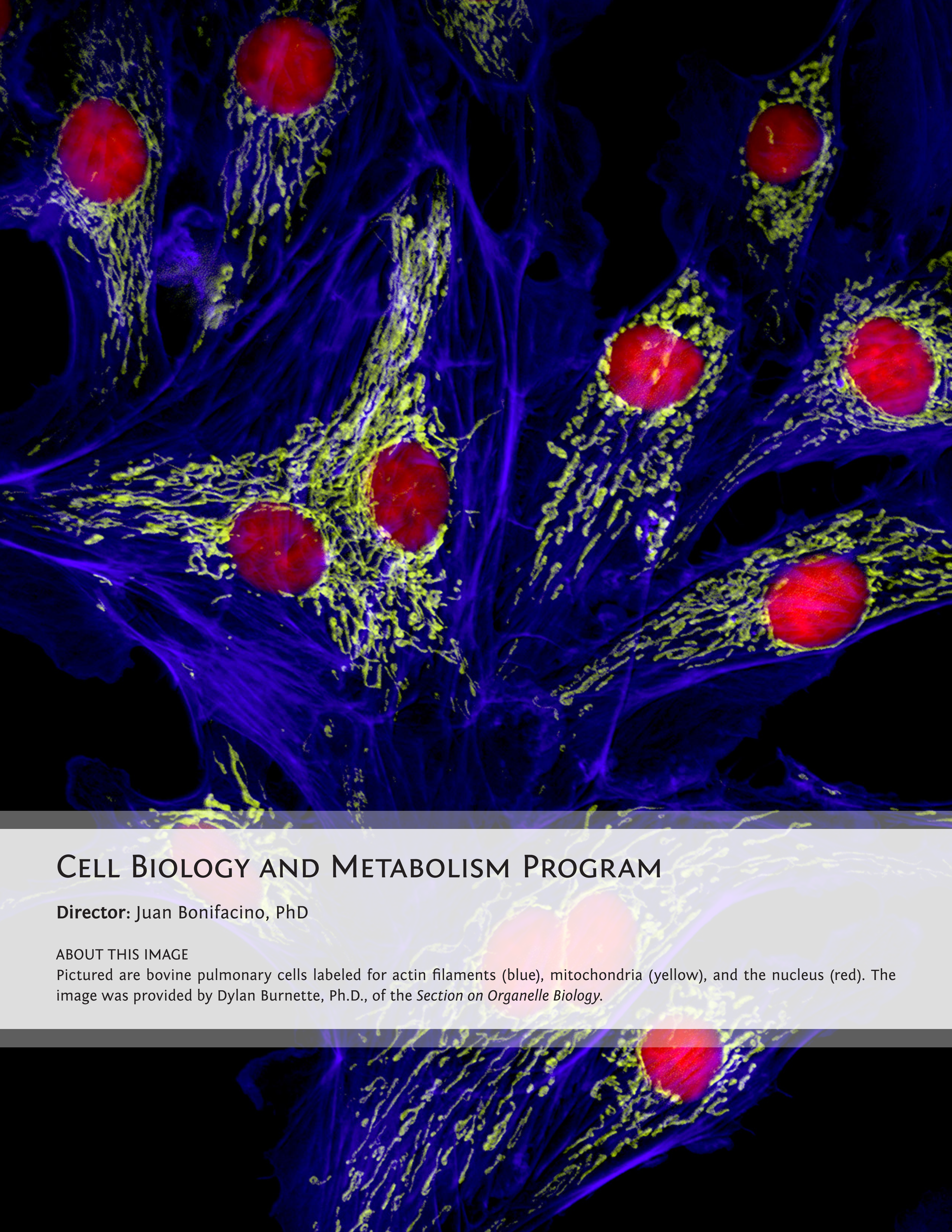

Cell Biology and Metabolism Program

Director: Juan Bonifacino, PhD

ABOUT THIS IMAGE

Pictured are bovine pulmonary cells labeled for actin filaments (blue), mitochondria (yellow), and the nucleus (red). The image was provided by Dylan Burnette, Ph.D., of the *Section on Organelle Biology*.

Cell Biology and Metabolism Program

The *Cell Biology and Metabolism Program (CBMP)* conducts research in various areas of molecular cell biology, including the mechanisms of intracellular protein traffic, organelle biogenesis, host-pathogen interactions, the biology of small non-coding RNAs and small proteins, the regulation of the cell cycle during oogenesis, and the structural and functional properties of integral membrane proteins. Knowledge gained from the study of basic cellular processes is applied to the elucidation of the causes of human diseases, including disorders of protein traffic as well as neurodegeneration and microbial pathogenesis.

The *Unit on Structural and Chemical Biology of Membrane Proteins*, headed by Anirban Banerjee, conducts atomic-level investigations into processes at the biological membrane. Broadly, the Unit is interested in both the mechanisms of action of several membrane proteins and the role of the surrounding lipid environment in modulating the proteins' structure and function. To approach these issues, members of the laboratory employ a combination of X-ray crystallography and biochemical and biophysical studies with peptide and protein chemistry. As part of a global understanding of these processes, the Unit is currently pursuing two questions: how small peptidic toxins, which are components of animal venoms, interact with K^+ channels; and the structural basis of the functions of mitochondrial inner-membrane transporters that are involved in iron homeostasis.

The *Section on Intracellular Protein Trafficking*, led by Juan Bonifacino, focuses on the molecular machinery involved in protein sorting to endosomes, lysosomes, lysosome-related organelles, and various domains of the plasma membrane (i.e., polarized sorting) and on the diseases that result from dysfunction of this machinery. Research over the past year elucidated the role of clathrin and the adaptor protein 1 (AP-1) complex in sorting of transmembrane receptors to the somato-dendritic domain of hippocampal neurons. In addition, the Section uncovered the structural mechanism by which the small GTPase Arf1 both recruits the AP-1 complex to membranes and activates it for recognition of sorting signals. Additional structural analyses revealed the mechanisms of signal recognition by the related AP-3 complex. In a further project, *in vitro* reconstitution analyses showed that transmembrane-domain interactions between the Vpu protein of HIV-1 and the host cell co-receptor CD4 promote CD4 polyubiquitination through a combination of increased ubiquitination and decreased deubiquitination.

The *Section on Gamete Development*, led by Mary Lilly, examines cell-cycle regulation during oogenesis. Over the past year, the Section explored how metabolism influences meiotic progression and gamete differentiation. From these studies, members of the Section determined that the multiprotein SEA/GATOR complex defines a newly discovered upstream module of TORC1 regulation that controls meiotic progression and oocyte growth. Specifically, they elucidated opposing functions for the SEA/GATOR–complex components Npr2/Npr3 and Mio/Seh1 in the regulation of TORC1 activity during *Drosophila* oogenesis. Furthermore, they characterized three additional components of the SEA/GATOR complex and determined how these proteins influence both TORC1 signaling and meiotic progression in the female germline.

The *Section on Organelle Biology*, headed by Jennifer Lippincott-Schwartz, continued to investigate cellular processes by using novel fluorescence imaging approaches combined with quantitative analysis and mathematical modeling. Among the areas of investigation were: (1) mitochondrial regulation of cell polarization; (2) cell-shape control by cellular adherence and contractile systems; (3) autophagy's role(s) in ensuring cell survival and longevity during starvation; (4) Rab10 and myosin-Va mediation of insulin-stimulated GLUT4 storage vesicle translocation in adipocytes; (5) primary cilia utilization of glycoprotein-dependent adhesion mechanisms to stabilize long-lasting cilia-cilia contacts; (6) endoplasmic reticulum (ER) stress–dependent clearance of misfolded GPI–anchored proteins from the ER; (7) computational modeling of cytokinetic abscission driven by ESCRT-III polymerization; (8) super-resolution analysis of HIV-1 formation and budding; (9) plasticity of the asialoglycoprotein receptor deciphered by ensemble FRET imaging and single-molecule–counting PALM imaging; (10) tool development for accelerating image reconstruction during Bayesian analysis of single-molecule fluorescence blinking/bleaching; and (11) spatial analysis of PALM datasets to analyze complex patterns of protein organization at the plasma membrane.

The *Unit on Microbial Pathogenesis*, headed by Matthias Machner, analyzes host-pathogen interactions during Legionnaires' disease, a potentially fatal pneumonia caused by the bacterium *Legionella pneumophila*. Of particular interest are bacterial proteins, so-called effectors, that are delivered into the host-cell cytosol, where they alter signaling processes in order to create conditions favorable for the survival and replication of the pathogen. The Unit continued its in-depth analysis of the bacterial infection cycle by identifying novel host targets for translocated effectors and determining their importance for *Legionella* virulence. By chemically modifying Rab proteins, small GTPases that function as key regulators of vesicle traffic in eukaryotic cells, major progress was made in the characterization of effectors that exploit intracellular cargo transport.

The *Section on Environmental Gene Regulation*, headed by Gisela Storz, studies small, regulatory RNAs in *Escherichia coli*. Many of these bacterial RNAs act analogously to eukaryotic miRNA and siRNAs to regulate mRNA stability and translation. In addition to identifying further small RNAs and characterizing their functions, the Section helped develop general tools for the study of the RNA regulators. The Section also pursues a project to identify and characterize another category of largely overlooked genes that encode small proteins of less than 50 amino acids. Systematic screens for growth conditions that lead to increased expression and for phenotypes associated with null mutations, combined with the identification of co-purifying proteins, are yielding insights into the physiological roles of these small proteins, such as the regulation of a major drug-efflux pump.

Structural and Chemical Biology of Membrane Proteins

Cell membranes lie at the heart of cellular compartmentalization. Integral membrane proteins, which are embedded in cell membranes, perform critically important functions, exemplified by the propagation of electrical signals along the cellular surface, exchange of material between two cellular compartments, and response of a cell to numerous signaling cues. We are interested in the structural basis of the functions of several integral membrane protein families. Our approach calls for a combination of X-ray crystallography with functional analyses and a range of biochemical and biophysical techniques. In addition to solving high-resolution structures and using them to guide functional experiments, we also carry out experiments to investigate the role of membrane lipids in modulating the structure and function of membrane proteins. The experiments will provide novel insights into the structure and function of the membrane proteins and thus lead to new discoveries in the cellular processes in which they participate. Malfunctioning of the processes causes a wide range of human disorders such as multiple sclerosis, ataxia, and various forms of neurodegenerative disease, to name but a few. Thus, in the process, we will also gain important insights into the biological underpinnings of these diseases.

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Eric Christenson, PhD, *Postdoctoral Fellow*
Pramod Kumar, PhD, *Postdoctoral Fellow*
Chul-jin Lee, PhD, *Postdoctoral Fellow*
Mitra Rana, PhD, *Postdoctoral Fellow*
Raffaello Verardi, PhD, *Postdoctoral Fellow*

Structural studies of toxin block of K^+ channels

Potassium-selective channels (K^+ channels) are a large, diverse group of integral membrane proteins, crucial for proper cellular functioning. Toxins from animal venoms are able to specifically inhibit ion conduction by specific K^+ channel subtypes by binding to different parts of the channel; the toxins have thus emerged as indispensable tools in neuroscience. However, there is a dearth of available co-crystal structures of K^+ channel–toxin complexes, and thus the structural bases for recognition of specific K^+ channels by specific toxins remain obscure. Currently, we are pursuing the structures of K^+ channels with toxins, specifically, dendrotoxin, a component of snake venom. Given that different parts of K^+ channels are targeted by different toxins, the structure of each distinct class of toxin–channel complexes will lead to insights into unique aspects of this very important class of ion channels and their role in cellular physiology.

Molecular mechanism of iron transport

We are focusing on mitochondrial inner membrane transporters that bring iron into mitochondria. Subsequently, the iron is used in the biosynthesis of heme, a central component of the heme in hemoglobin, myoglobin, and cytochromes, and in the biosynthesis of iron-sulfur clusters, important cofactors required for proteins involved in a wide range of cellular activities, namely, electron transport in respiratory chain complexes, regulatory sensing, photosynthesis, and DNA repair. We are currently using heterologous expression to obtain enough purified material for biochemical and biophysical characterization.

Structural and chemical biology approach to design novel antibiotics

Antibiotic-resistant pathogenic bacteria pose a major threat to our healthcare systems. In the face of this challenge, there is a pressing need to identify new targets for combating antibiotic-resistant bacteria and to identify and develop therapeutic leads that can result in clinically useful drugs. Clinically approved antibiotics that are currently in use mostly target bacterial cytosolic enzymes and the ribosome. Integral membrane proteins are, however, a largely uncharted territory for antibiotic development, owing to the difficulty in handling and purification, and importantly, to the lack of structural information. In collaboration with Clifton Barry's lab, we propose to combine fragment-based drug discovery (FBDD) with high-throughput screening (HTS), together with high-resolution structural analyses, to target integral membrane proteins involved in bacterial cell-envelope biosynthesis and to develop leads for novel antibacterial therapies. In the process, we hope to make fundamental discoveries regarding the mechanistic underpinnings of bacterial cell-envelope biosynthesis.

ADDITIONAL FUNDING

» NIH Director's Challenge Award

COLLABORATORS

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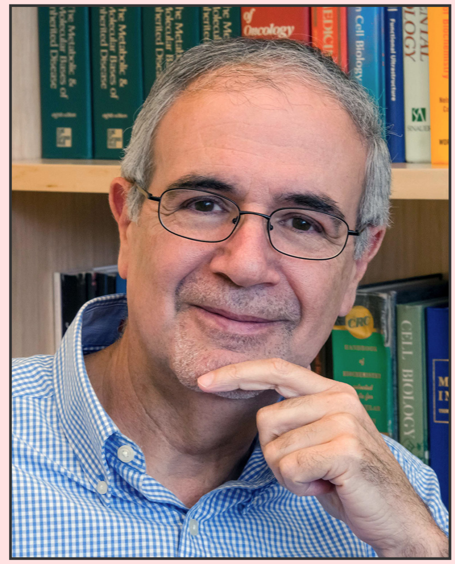
Protein Sorting in the Endosomal-Lysosomal System

We investigate the molecular mechanisms by which transmembrane proteins (referred to as cargo) are sorted to different compartments of the endomembrane system in eukaryotic cells. The system consists of an array of membrane-enclosed organelles including the endoplasmic reticulum (ER), the Golgi apparatus, the trans-Golgi network (TGN), endosomes, lysosomes, lysosome-related organelles (LROs) (e.g., melanosomes), and various domains of the plasma membrane in polarized cells (e.g., epithelial cells and neurons). Transport of cargo between these compartments is mediated by carrier vesicles or tubules that bud from a donor compartment, translocate through the cytoplasm, and eventually fuse with an acceptor compartment. Work in our laboratory focuses on the molecular machineries that mediate these processes, including (1) sorting signals and adaptor proteins that select cargo proteins for packaging into the transport carriers, (2) microtubule motors that drive movement of the transport carriers and other organelles through the cytoplasm, and (3) tethering factors that promote fusion of the transport carriers to acceptor compartments. We study the machineries in the context of different intracellular transport pathways, including endocytosis, recycling to the plasma membrane, retrograde transport from endosomes to the TGN, biogenesis of lysosomes and LROs, and polarized sorting in epithelial cells and neurons. We apply knowledge gained from our research to the elucidation of disease mechanisms, including congenital disorders of protein traffic such as the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS) and the neuro-cutaneous disorder MEDNIK syndrome. In addition, we study how the molecular mechanisms of protein transport are exploited by intracellular pathogens such as HIV-1.



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Loreto Cuitiño, PhD, *Visiting Fellow*

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Jing Pu, PhD, *Visiting Fellow*

Dylan Britt, BSc, *Postbaccalaureate Student*

Xiaolin Zhu, RN, *Technician*

An AP-1/clathrin pathway for the sorting of transmembrane receptors to the somatodendritic domain of hippocampal neurons

A major focus of our research is processes mediated by recognition of sorting signals in the cytosolic tails of transmembrane proteins by adaptor proteins that are components of protein coats (e.g., clathrin coats). Two types of sorting signal—tyrosine-based and dileucine-based—participate in various sorting events, including endocytosis, transport to lysosomes and melanosomes, and sorting to the basolateral surface of polarized epithelial cells. In previous work, we found that tyrosine-based signals bind to a conserved site on the mu1, mu2, and mu3 subunits of three hetero-tetrameric adaptor protein (AP) complexes, AP-1, AP-2, and AP-3, respectively. Dileucine-based signals, on the other hand, bind to a different site, a site that spans the surface of two subunits—in the case of the AP-1 complex the gamma-sigma1 subunits, of the AP-2 complex the alpha-sigma2 subunits, and of the AP-3 complex the delta-sigma3 subunits. In recent years, we extended our studies to the role of signal-adaptor interactions in the process of polarized sorting in neurons. Neurons are highly polarized cells with distinct somatodendritic and axonal domains. The plasma membrane of each of these domains possesses a distinct set of transmembrane proteins, including receptors, channels, transporters, and adhesion molecules. We found that several transmembrane proteins, including the transferrin receptor (TfR), the Coxsackie virus and adenovirus receptor (CAR), and the Nipah virus fusion glycoprotein (NiV-F), are sorted to the somatodendritic domain by interaction of tyrosine-based signals with the mu1A subunit of AP-1. More recently, we discovered that a different set of proteins, including the copper transporter ATP7B and the SNARE VAMP4, also undergo sorting to the somatodendritic domain, but in this case through recognition of dileucine-based signals by the gamma1-sigma1 subunits of AP-1. Together with previous work on epithelial cells, these findings establish the AP-1 complex as a global regulator of polarized sorting in different cell types. Defects in polarized sorting likely underlie the pathogenesis of several neuro-cutaneous disorders caused by mutation in sigma1 subunit isoforms, such as the MEDNIK syndrome (sigma1A), Fried/Pettigrew syndrome (sigma1B), and pustular psoriasis (sigma1C).

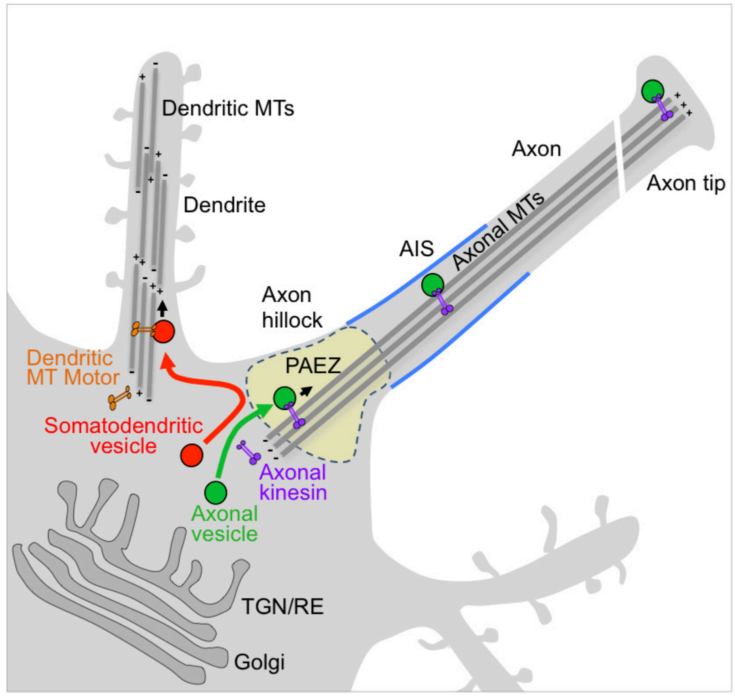

Figure 1. Sorting of somatodendritic and axonal vesicles at the pre-axonal exclusion zone (PAEZ)

Sorting of dendritic and axonal vesicles at the pre-axonal exclusion zone (PAEZ)

Polarized sorting of newly-synthesized proteins to the somatodendritic and axonal domains of neurons occurs by selective incorporation into distinct populations of vesicular transport carriers. An unresolved issue is how the vesicles themselves become sorted to their corresponding neuronal domains. Previous studies led to the conclusion that the axon initial segment (AIS) is an actin-based filter that selectively prevents passage of somatodendritic vesicles into the axon. We found, however, that most somatodendritic vesicles fail to enter the axon at a more proximal region in the axon hillock named the "pre-axonal exclusion zone" (PAEZ). Forced coupling of a somatodendritic cargo protein to an axonally directed kinesin is sufficient to drive transport of whole somatodendritic vesicles through the PAEZ toward the distal axon. Based on these findings, we proposed that polarized sorting of transport vesicles occurs at the PAEZ and depends on the ability of the vesicles to acquire an appropriately directed microtubule motor.

BORC: a novel multisubunit complex that regulates lysosome positioning and motility

In another line of research, we recently obtained unexpected insights into the mechanisms of lysosome positioning and motility. The research was an outgrowth of our previous work on the biogenesis of LROs such as melanosomes. Years ago, we discovered that mutations in AP-3 cause eye color defects in *Drosophila* and the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS) type 2 (HPS-2) in humans. Other types of HPS are caused by mutations in subunits of the hetero-octameric BLOC-1 and the heterodimeric BLOC-3 complexes. AP-3 mediates sorting of transmembrane proteins to melanosomes, but the functions of BLOC-1 and BLOC-3 are less well understood. In experiments aimed at identifying proteins that interact with BLOC-1, we made the surprising discovery of a related hetero-octameric complex named BORC (for BLOC-one-related complex). Biochemical analyses showed that BORC comprises three subunits that are shared with BLOC-1 (named BLOS1, BLOS2, and Snapin) and five unique subunits (named KXD1, MEF2B, Myrlysin, Lyspersin, and Diaskedin). Further studies revealed that BORC is associated with late endosomes and lysosomes, where it functions to recruit the small GTPase Arl8, which initiates a chain of interactions that drives kinesin-dependent movement of lysosomes toward the peripheral cytoplasm. Mutations in BORC cause collapse of the lysosomal population into the pericentrosomal area. In addition, BORC–mutant cells exhibit defective autophagic flux, probably a result of the inability of lysosomes to reach autophagosomes in the cell periphery. The cells also display reduced spreading and migration, likely caused by impaired lysosome-dependent delivery of adhesion and signaling molecules to the plasma membrane. Given the critical importance of cell adhesion and motility in tumor growth, invasion, and metastasis, the BORC pathway of lysosome dispersal could be an attractive target for pharmacologic inhibition in cancer therapeutics.

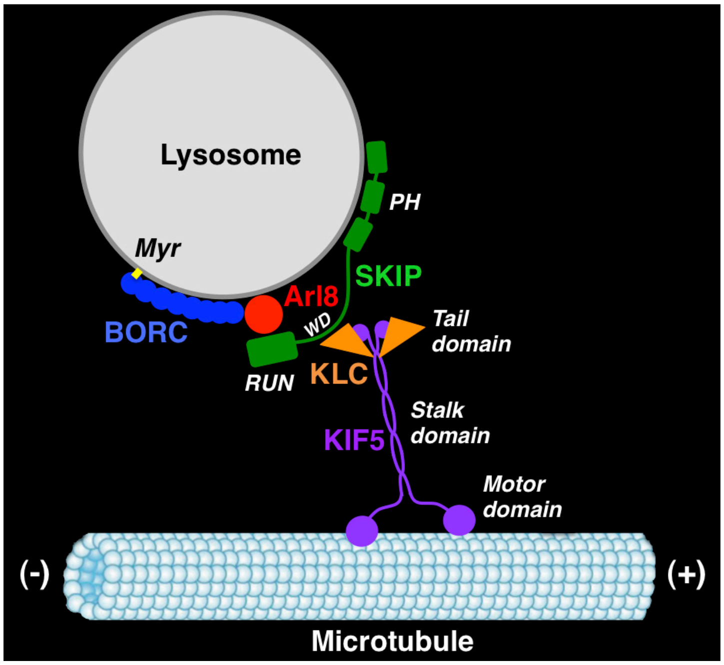

Figure 2. BORC regulates kinesin-dependent movement of lysosomes to the cell periphery.

GARP and EARP: multisubunit tethering complexes involved in endosomal retrieval pathways

Recycling of endocytic receptors to the cell surface involves passage through a series of membrane-bound compartments by mechanisms that are poorly understood. In particular, prior to our work, it was unknown whether endocytic recycling requires the function of multisubunit tethering complexes, as is the case for other intracellular trafficking pathways. In the course of studies on the Golgi-associated retrograde protein (GARP) complex, we discovered a related complex named endosome-associated recycling protein (EARP). The two complexes share the Ang2 (also known as Vps51), Vps52, and Vps53 subunits, but whereas GARP comprises a fourth subunit named Vps54, EARP contains a previously uncharacterized protein named Syndetin, a divergence that determines differential localization of GARP to the TGN and EARP to recycling endosomes. Importantly, we found that EARP is involved in recycling of internalized proteins to the plasma membrane. The findings should contribute to the understanding of the pathogenesis of progressive cerebello-cerebral atrophy type 2, a neurodegenerative disorder caused by mutations in Vps53, which in light of our results could result from impairment of both GARP–mediated retrograde transport to the TGN and EARP–mediated recycling to the plasma membrane.

Mechanisms of CD4 downregulation by HIV-1 Nef

In previous work, we found that the HIV-1 accessory protein Nef connects surface CD4 to both the endocytic and lysosomal targeting machineries, leading to efficient and sustained removal of CD4 from host cells early during infection. The role of Nef in CD4 internalization involves an interaction with the AP-2 clathrin adaptor. Subsequent to induction of CD4 internalization by an AP-2/clathrin pathway, Nef promotes delivery of internalized CD4 to the multivesicular body pathway (MVB) for eventual degradation in lysosomes. In collaboration with Luis daSilva, we recently found that this targeting depends on a direct interaction of Nef with Alix/AIP1, a protein associated with the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery, which assists with cargo recruitment and intraluminal vesicle formation in MVBs. We showed that Nef interacts with both the Bro1 and V domains of Alix. Depletion of Alix or overexpression of the Alix V domain impaired lysosomal degradation of CD4 induced by Nef. In contrast, V-domain overexpression did not prevent cell surface removal of CD4 by Nef or protein targeting to the canonical, ubiquitination-dependent MVB pathway. We also showed that the Nef-Alix interaction occurs in late endosomes that are enriched in internalized CD4. Together, the results indicated that Alix functions as an adaptor for the ESCRT–dependent, ubiquitin-independent targeting of CD4 to the MVB pathway induced by Nef.

ADDITIONAL FUNDING

» Intramural AIDS Targeted Antiviral Program (IATAP)

PUBLICATIONS

1. Mattera R, Farías GG, Mardones GA, Bonifacino JS. Co-assembly of viral envelope glycoproteins regulates their polarized sorting in neurons. *PLoS Pathogens* 2014; 10:e1004107.
2. Jain S, Farías GG, Bonifacino JS. Polarized sorting of the copper transporter ATP7B in neurons mediated by recognition of a dileucine signal by AP-1. *Mol Biol Cell* 2015; 26:218–228.

3. Pu J, Schindler C, Jia R, Jarnik M, Backlund P, Bonifacino JS. BORC, a multisubunit complex that regulates lysosome positioning. *Dev Cell* 2015; 33:176–188.
4. Schindler C, Chen Y, Pu J, Guo X, Bonifacino JS. EARP is a multisubunit tethering complex involved in endocytic recycling. *Nat Cell Biol* 2015; 17:639–650.
5. Farias GG, Guardia CM, Britt DJ, Guo X, Bonifacino JS. Sorting of dendritic and axonal vesicles at the pre-axonal exclusion zone. *Cell Rep* 2015; 13:1221–32.

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Cell Cycle Regulation in Oogenesis

The long-term goal of our laboratory is to understand how the cell-cycle events of meiosis are coordinated with the developmental events of gametogenesis. Chromosome mis-segregation during female meiosis is the leading cause of miscarriages and birth defects in humans. Recent evidence suggests that many meiotic errors occur downstream of defects in oocyte growth and/or the hormonal signaling pathways that drive differentiation of the oocyte. Thus, an understanding of how meiotic progression and gamete differentiation are coordinated during oogenesis is essential to studies in both reproductive biology and medicine. We use the genetically tractable model organism *Drosophila melanogaster* to examine how meiotic progression is instructed by the developmental and metabolic program of the egg.

In mammals, studies on the early stages of oogenesis face serious technical challenges in that entry into the meiotic cycle, meiotic recombination, and the initiation of the highly conserved prophase I arrest all occur during embryogenesis. By contrast, in *Drosophila* these critical events of early oogenesis all take place continuously within the adult female. Easy access to the early stages of oogenesis, coupled with available genetic and molecular genetic tools, makes *Drosophila* an excellent model for studies on meiotic progression and oocyte development.

To understand the regulatory inputs that control early meiotic progression, we are working to determine how the oocyte initiates and then maintains the meiotic cycle within the challenging environment of the ovarian cyst. Our studies focus on questions that are relevant to the development of all animal oocytes. What strategies does the oocyte use to protect itself against inappropriate DNA replication? How does the oocyte inhibit mitotic activity before meiotic maturation and the full growth and development of the egg? How does cell-cycle and metabolic status within the ovarian cyst influence the differentiation of the oocyte? To answer these questions, we have undertaken studies to determine the basic cell-cycle program of the developing ovarian cyst.

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The SEA/GATOR complex: integrating developmental and metabolic signals in oogenesis

We are interested in how metabolism influences oocyte growth, development, and quality. Target of Rapamycin Complex 1 (TORC1) is a primary regulator of cell growth and metabolism, responding to multiple signals including nutrient availability, energy status, and growth factors. In the past year, we defined the role of the GATOR complex, a highly conserved upstream regulator of TORC1, in the regulation of meiotic progression, genomic stability, endomembrane dynamics, and oocyte development using *Drosophila*. We demonstrated that the GATOR1 complex down-regulates TORC1 activity to protect oocytes during times of amino acid scarcity and plays an essential role in the regulation of meiotic entry, genomic stability, and oocyte growth. We also found that the GATOR2 components Mio, Seh1, and Wdr24 oppose the activity of the GATOR1 complex in the female germline to promote growth in later stages of oogenesis. We thus conclude that the tight regulation of TORC1 activity by the GATOR1 and GATOR2 complexes is critical to oocyte development, meiotic progression, and genomic stability. Our data strongly suggest that the important role of nutrient stress pathways in the regulation of gametogenesis has been conserved from single-cell to multicellular animals.

In addition to our studies of oogenesis, we undertook a comprehensive characterization of the GATOR2 complex. We defined tissue-specific functions for several GATOR2 complex components. Surprisingly, our data support a dual role for the GATOR2 complex in the regulation of cellular metabolism and endomembrane dynamics.

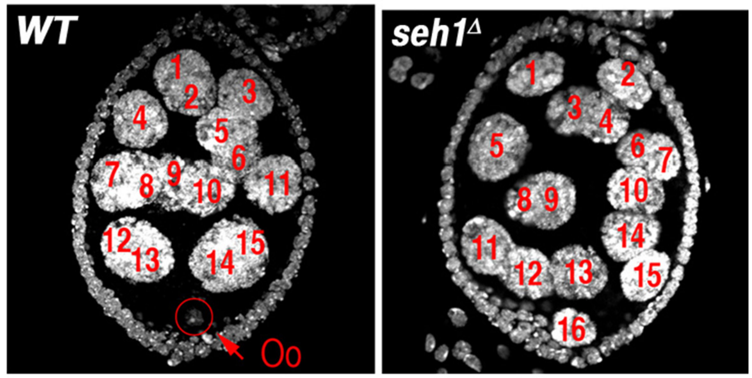

Figure 1. The GATOR1 component Seh1 is required for maintenance of the meiotic cycle.
Wild-type (WT) and *seh1*$^{\Delta}$ egg chambers stained with the DNA dye DAPI. The *seh1*$^{\Delta}$ egg chamber has 16 polyploid nurse cells but no oocyte (Oo).

The meiotic regulators Mio and Seh1 are components of the GATOR2 complex.

In earlier studies, we identified two genes, *missing oocyte* (*mio*) and *seh1*, that regulate meiotic progression and the maintenance of the oocyte fate. Both *mio* and *seh1* are highly conserved from yeast to humans. In *mio*- and *seh1*-null mutants, oocytes enter the meiotic cycle and progress to pachytene. The meiotic cycle, however, is not maintained. Ultimately, a large fraction of *mio* and *seh1* oocytes withdraw from meiosis, enter the endocycle, and become polyploid (Figure 1). Genetic and phenotypic analyses indicate that *mio* and *seh1* act early in oogenesis, before the formation of the synaptonemal complex (SC) and meiotic recombination. Using biochemical strategies, we determined that Mio and Seh1 are components of a large multiprotein complex called the Seh1–associated (SEA) complex in yeast and the GATOR complex in higher eukaryotes. Recently, the SEA/GATOR complex was shown to be an upstream regulator of TORC1 activity. We found that TORC1 kinase activity is dramatically reduced in the ovaries of *mio*- and *seh1*-null mutants. Reduced TORC1 activity correlates with the strong reduction in growth observed in *mio*$^{-}$ and *seh1*$^{-}$ mutant ovaries. In contrast, *mio* and *seh1* appear to have little effect on TORC1 activity and growth in most somatic tissues. Thus, there is a unique tissue-specific requirement for the TORC1 activators Mio and Seh1 in the female germline.

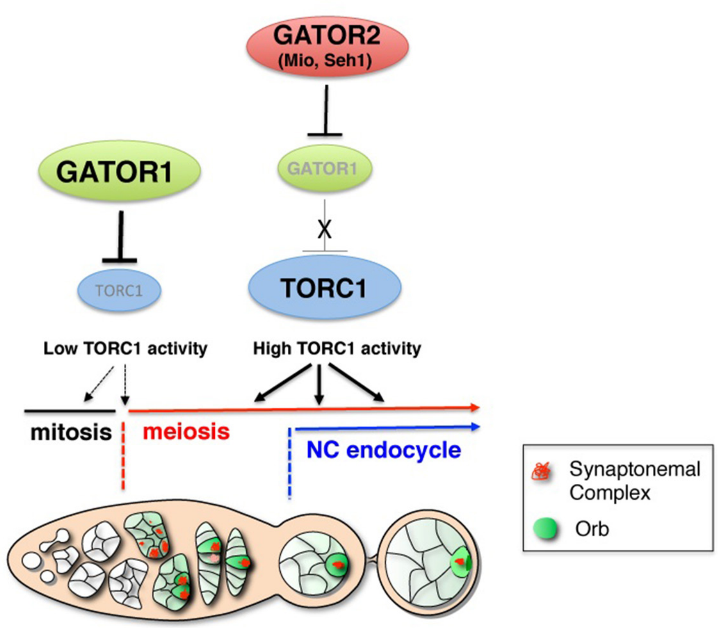

Figure 2. A model for the role of the GATOR1 and GATOR2 complexes during *Drosophila* oogenesis
The GATOR1 complex down-regulates TORC1 activity to promote the mitotic/meiotic transition after precisely four mitotic cyst divisions. Low TORC1 activity promotes the mitotic/meiotic transition. Whether the inhibition of TORC1 activity by the GATOR1 complex is required throughout the ovarian cyst divisions or precisely at the mitotic/meiotic juncture is currently undetermined and thus is presented as dashed arrows. After meiotic entry, the Mio and Seh1 components of the GATOR2 complex oppose the activity of GATOR1 complex, thus preventing the constitutive inhibition of TORC1 during later stages of oogenesis. High TORC1 activity is required to drive the nurse cell endoreplication cycle as well as for the maintenance of the meiotic cycle into later stages of oogenesis.

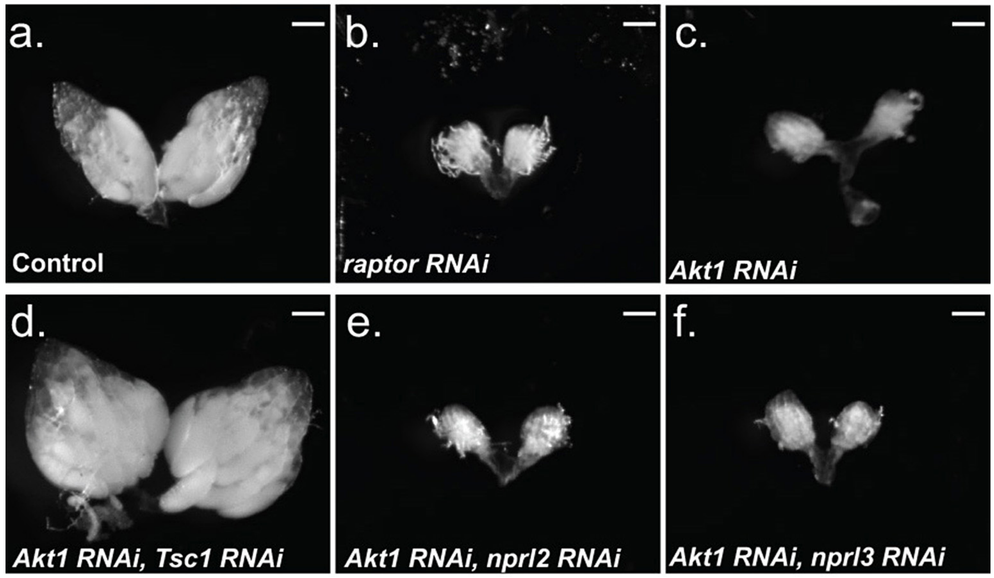

Figure 3. The TORC1 inhibitor Tsc1 is downstream of Akt1 in the female germline.
a. *nanos-GAL4; UAS-mCherry RNAi,* b. *nanos-GAL4; UAS-raptor RNAi,* c. *nanos-GAL4; UAS-Akt1 RNAi,* d. *nanos-GAL4; UAS-Akt1 RNAi/UAS-Tsc1 RNAi,* e. *nanos-GAL4; UAS-Akt1 RNAi/UAS-nprl2 RNAi*, and f. *nanos-GAL4; UAS-Akt1 RNAi/UAS-nprl3 RNAi* flies were cultured on standard fly media with wet yeast two days prior to dissection. The germline-specific driver *nanos-GAL4* was used to drive expression of the RNAi constructs. (a, d) 100% of the ovaries examined contained mature (stage 13–14) egg chambers. (b, c, e, f) 0% of the ovaries examined contained egg chambers beyond stage 5. (a–f) N>50 ovaries. Note that, while knocking down *Tsc1* in the female germline results in a block to oogenesis, the co-depletion of the TORC1 inhibitor Tsc1, the downstream target of Akt1, restores oogenesis. Thus, Tsc1 is epistatic to Akt1 in the female germline. Size bar 50 µm.

The GATOR2 component Wdr24 uses TORC1–dependent and –independent pathways to regulate cellular metabolism.

To better define the *in vivo* role of the GATOR complex, we used whole-animal studies to dissect the function of the GATOR2 component Wdr24. Surprisingly, we found that Wdr24 regulates both TORC1–dependent and –independent processes. First, Wdr24 is a critical effector of the GATOR2 complex; Wdr24 promotes TORC1 activity and cellular growth in all tissues, which is in stark contrast to the GATOR2 components Mio and Seh1, which primarily function to promote TORC1 activity in the female germline. Second, Wdr24 is required for the TORC1–independent regulation of lysosome function and dynamics, representing a surprising, and previously unrecognized, role for the GATOR2 complex. Consistent with a role in the regulation of lysosomes, we showed that Wdr24 localizes to the surface of lysosomes and autolysosomes. Our results represent the first *in vivo* examination of the requirement for Wdr24 in a metazoan and provide a framework for future studies on the dual role of the GATOR2 complex in the regulation of cellular metabolism and endomembrane dynamics.

A conserved nutrient stress pathway regulates meiotic entry during oogenesis.

Over the past year, we defined a conserved pathway that controls the transition from the mitotic cycle to the meiotic cycle (Figure 2). In single-cell eukaryotes, the pathways that monitor nutrient availability are central to initiating the meiotic program and gametogenesis. A master regulator of metabolism in eukaryotes, TORC1 integrates information from multiple upstream signaling pathways. In *Saccharomyces cerevisiae*, an essential step in the transition to the meiotic cycle is the down-regulation of TORC1 by the Iml1/GATOR1 complex in response to amino acid starvation. How metabolic inputs influence early meiotic progression and gametogenesis remains poorly understood in metazoans. We demonstrated that, as is observed in yeast, the Iml1/GATOR1 complex inhibits TORC1 activity to slow cellular metabolism and drive the mitotic/meiotic transition in developing ovarian cysts. In *iml1* germline depletions, ovarian cysts undergo an extra mitotic division prior to meiotic entry. The TORC1 inhibitor Rapamycin can suppress this extra mitotic division. Thus, high TORC1 activity delays the mitotic/meiotic transition. Conversely, mutations in *Tor*, which encodes the catalytic subunit of the TORC1 complex, result in premature meiotic entry. Thus, the levels of TORC1 activity control the timing of the mitotic/meiotic decision in a metazoan. Taken together, our data indicate that the role of the Iml1/GATOR1 complex in the regulation of meiotic entry has been conserved from single cell to multicellular animals. The data strongly suggest that catabolic metabolism, triggered by low TORC1 activity, is a conserved feature of early meiosis in many eukaryotes.

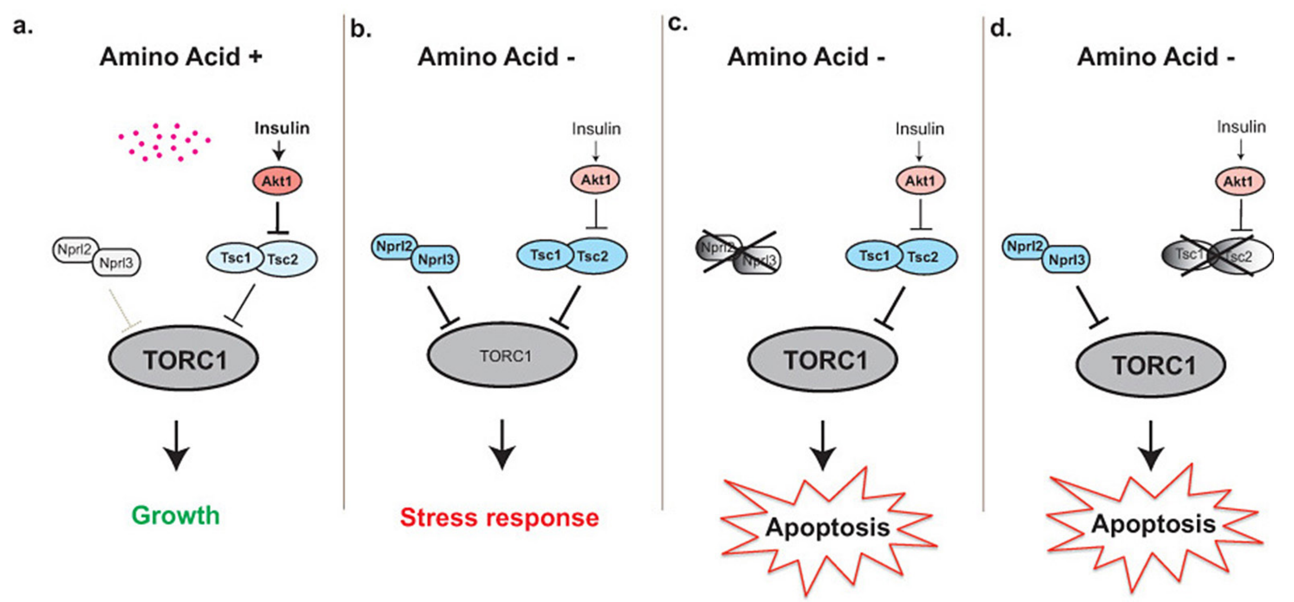

Figure 4. The TORC1 inhibitors Nprl2/3 and Tsc1/2 prevent apoptosis and protect young oocytes during amino acid starvation.

a. In the presence of amino acids, the TORC1 inhibitors Nprl2/3 are inactive while the insulin pathway inhibits the activity of Tsc1/2. Thus, TORC1 activity is high, driving anabolic metabolism and growth.
b. In the absence of amino acids, Nprl2/3 functions to inhibit TORC1 activity. Amino acid starvation also results in reduced insulin signaling, leading to activation of the Tsc1/2 complex owing to the reduced activity of its inhibitor Akt1. Together, Nprl2/3 and Tsc1/2 sufficiently inhibit TORC1 activity to induce an essential stress response.
c. In *nprl2* or *nprl3* knockdowns, cells fail to adequately down-regulate TORC1 activity in response to amino acid starvation, triggering apoptosis.
d. In *Tsc1* knockdowns, TORC1 activity also remains inappropriately high during amino acid starvation, triggering apoptosis.

The GATOR1 components Nprl2 and Nprl3 mediate an adaptive response to amino acid starvation in *Drosophila*.

In yeast, the Nitrogen permease regulators 2 and 3 (Npr2 and Npr3) are components of the Iml1/GATOR1 complex; they mediate an essential response to amino acid limitation upstream of TORC1. In mammals, the Npr2 ortholog, Nprl2, encodes a putative tumor suppressor gene, which inhibits cell growth and enhances sensitivity to numerous anticancer drugs including cisplatin. However, the precise role of Nprl2 and Nprl3 in the regulation of metabolism in metazoans remains poorly defined. We determined that the central importance of Nprl2 and Nprl3 in the response to amino acid starvation has been conserved from single celled to multicellular animals. We found that, in *Drosophila*, Nprl2 and Nprl3 physically interact and are targeted to lysosomes and autolysosomes, the sites of TORC1 activation. Using oogenesis as a model system, we found that Nprl2 and Nprl3 inhibit TORC1 signaling in response to amino acid starvation. Moreover, the inhibition TORC1 by Nprl2/3 is critical to the preservation of female fertility during times of protein scarcity. In young egg chambers, the failure to down-regulate TORC1 in response to amino acid limitation triggers apoptosis (Figure 3). Thus, our data suggest the presence of a metabolic checkpoint that initiates a cell death program in the oocyte when, during periods of amino acid and/or nutrient scarcity, TORC1 activity remains inappropriately high.

The TSC1/2 complex is a TORC1 inhibitor that is required to maintain baseline levels of TORC1 activity in most cell types. As observed with *nprl2* and *nprl3*, we found that depleting *Tsc1* in the female germline renders oogenesis acutely sensitive to amino acid stress. Yet the TSC1/2 complex is not activated by amino acid starvation. One possible model to explain these data is that, in the female germline, the TSC1/2 complex is required to maintain baseline levels of TORC1 activity while Nprl2/Nprl3 are required to specifically down-regulate TORC1 activity in response to amino acid scarcity. Therefore, both TORC1 inhibitor pathways are required to repress TORC1 activity in order to prevent oocytes from being shunted into the apoptotic pathway in response to amino acid scarcity (Figure 4). We predict that, in many tissues, the two independent TORC1–inhibitory pathways work in concert to fine-tune TORC1 activity in response to various developmental and environmental inputs. We also found that Tsc1 is a critical downstream effector of Akt1 in the female germline.

ADDITIONAL FUNDING

» NICHD Director's Challenge Award

PUBLICATIONS

1. Wei Y, Reveal B, Reich J, Laursen W, Senger S, Akar T, Iida-Jones I, Cai W, Jarnik M, Lilly MA. TORC1 regulators Iml1/GATOR1 and GATOR2 control meiotic entry and oocyte development in Drosophila. *Proc Natl Acad Sci USA* 2014; 111:E5670–E5677.
2. Wei Y, Lilly MA. The TORC1 inhibitors Nprl2 and Nprl3 mediate an adaptive response to amino-acid starvation in Drosophila. *Cell Death Differ* 2014; 21:1460–1468.
3. Kassis JA, Lilly MA. PRC2 goes solo in the Drosophila female germline. *Dev Cell* 2013; 26:329–330.

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Interplay between Membrane Organelles, Cytoskeleton, and Metabolism in Cell Organization and Function

We investigate the global principles underlying cell behavior at both small and large spatial scales. At the small scale, we employ the super-resolution imaging techniques of photoactivated localization microscopy (PALM), interferometric 3D PALM, single-particle tracking PALM, and pair-correlation PALM to map the spatial organization, stoichiometry, and dynamics of proteins associated with various membrane-bound compartments and with the cytoskeleton. We also employ fluorescence photobleaching, photoactivation, fluorescence correlation, and fluorescence energy transfer methods to measure protein-protein interactions, protein turnover rates, and protein association rates. Such approaches allow us to assay cellular functions, including receptor stoichiometry and protein clustering and diffusion behavior at the nanometric scale in living cells. At the large scale, we investigate how complex behaviors of cells arise, such as cell crawling, polarization, cytokinesis, and viral budding. We study these complex behaviors by quantitatively analyzing diverse intracellular processes, including membrane trafficking, autophagy, actin/microtubule dynamics, and organelle assembly/disassembly pathways, which undergo dramatic changes as cells alter their behavior and organization throughout life. To assist these efforts, we combine various fluorescence-based imaging approaches, including total internal reflection fluorescence (TIRF) microscopy imaging and spinning-disk and laser-scanning confocal microscopy, with FRAP (fluorescence recovery after photobleaching), FLIP (fluorescence loss in photobleaching), and photoactivation to obtain large image data sets. We process the data sets computationally to extract biochemical and biophysical parameters, which can be related to the results of conventional biochemical assays. We then use the results to generate mechanistic understanding and predictive models of the behavior of cells and subcellular structures (including endoplasmic reticulum, Golgi, cilia, endosomes, lysosomes, autophagosomes, and mitochondria) under healthy and pathological conditions.

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Carolyn Ott, PhD, *Postdoctoral Fellow*
Christopher Obara, PhD, *Postdoctoral Fellow*
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Prabuddha Sengupta, PhD, *Postdoctoral Fellow*
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Aubrey Weigel, PhD, *Postdoctoral Fellow*
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Prasanna Satpute, PhD, *Volunteer*
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Actomyosin organization and its diverse functions

Cytotoxic T lymphocytes (CTLs) kill target cells by secreting granules containing perforin and granzymes into the immunological synapse (the site of contact formed between the CTL and target cell). We used spinning disk confocal and lattice light sheet microscopy to obtain unprecedented spatial and temporal resolution of the actin cytoskeleton and its role in both facilitating and limiting CTL secretion. We saw dynamic lamellapodial projections and a rearward flow of actin in migrating CTLs as they engaged a target cell. The synapse then formed in two stages: concentration of T cell receptors (TCRs) in the plasma membrane (PM) through lateral translocation (1 minute), followed by vesicular delivery of intracellular TCRs as the centrosome reached the synapse (6 minutes). Prior to synapse formation, a continuous actin meshwork underlies the entire PM; however, local clearing occurred as both the centrosome and granules docked. After several vesicles fused, the actin meshwork reappeared and secretion ceased. Actin clearance and reappearance correlated with the loss and gain of PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) in the contact zone. We concluded that the CTL contact zone is like a radially symmetric leading edge, with the distal region of

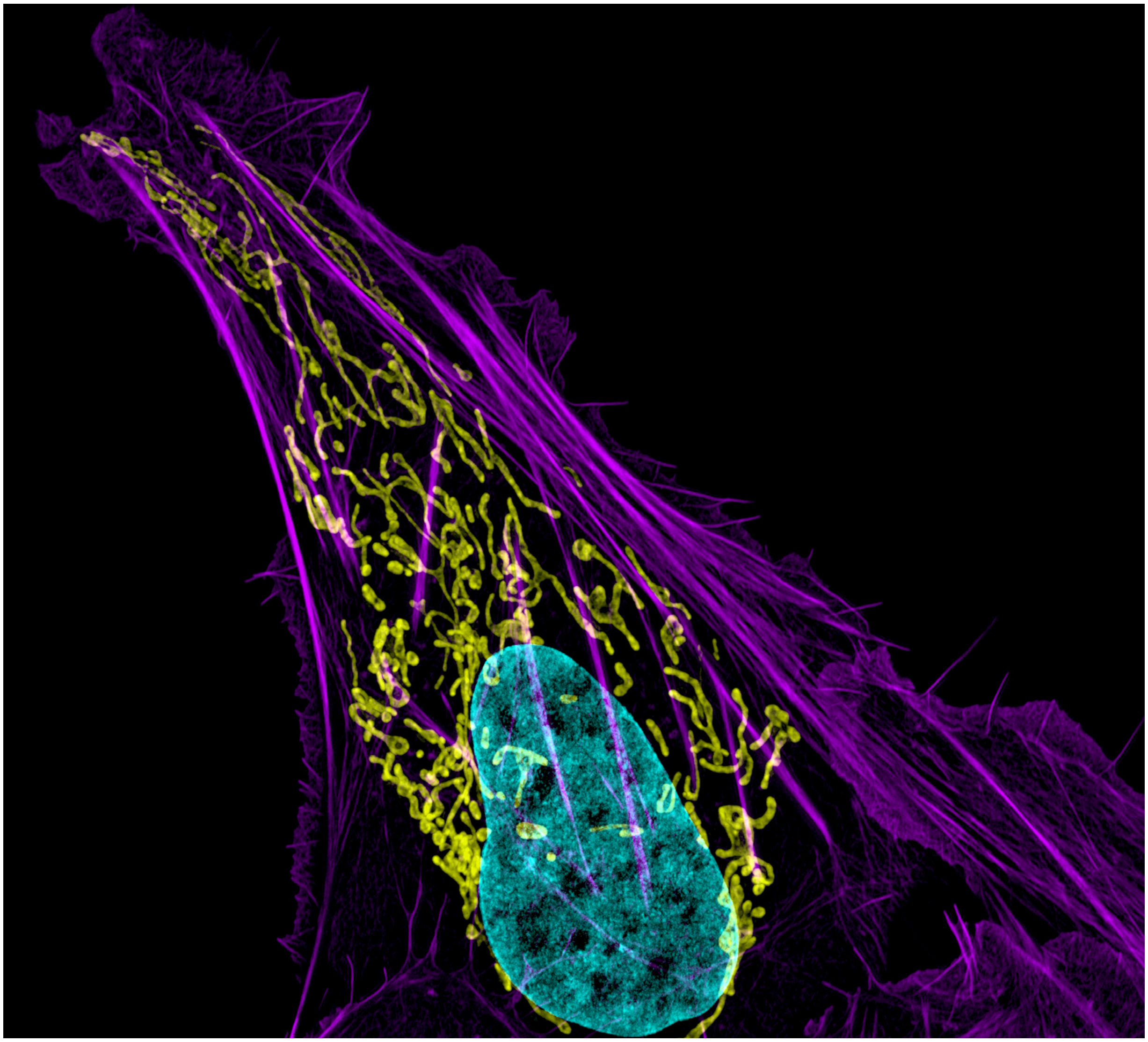

Figure 1. Mitochondrial and actin organization in a crawling cell
Structured illumination microscope (SIM) image of a U2OS (osteoblastoma) cell showing mitochondria stained with MITO-FRP (*pink*), actin stained with Alexa Fluor 488® phalloidin (*green*), and DNA stained with Hoechst 33342 (*blue*)

protrusive actin polymerization being analogous to the lamellipodium, and the more central region, enriched in integrins and myosin IIA, analogous to the lamellum. The spatial-temporal regulation of actin in the contact zone serves to coordinate TCR docking and the timing of granule secretion.

In a second project, we examined the role of the actin cytoskeleton in regulating overall motion within the cytoplasm. We reasoned that ensemble forces from actomyosin activity could have a large effect on global motion within the cytoplasm, making these forces a critical readout of the dynamic state of the cell. To measure these forces and test how they control the motion of cytoplasmic components, we collaborated with physicist David Weitz, who devised a new methodology called force-spectrum-microscopy (FSM) to quantify force fluctuations within the cytoplasm. The technique combines measurements of the random motion of probe particles with independent micro-mechanical measurements of the cytoplasm. Increased cytoplasmic force fluctuations substantially enhanced intracellular movement of small and large components, including organelles. Cytoplasmic force fluctuations varied between cell types and were three times larger in malignant cells than in normal cells. Separately, in close collaboration with Christoph Schmidt, we found that force generation by the actin cytoskeleton surrounding the basal body causes previously undocumented active primary cilia movements, which could be

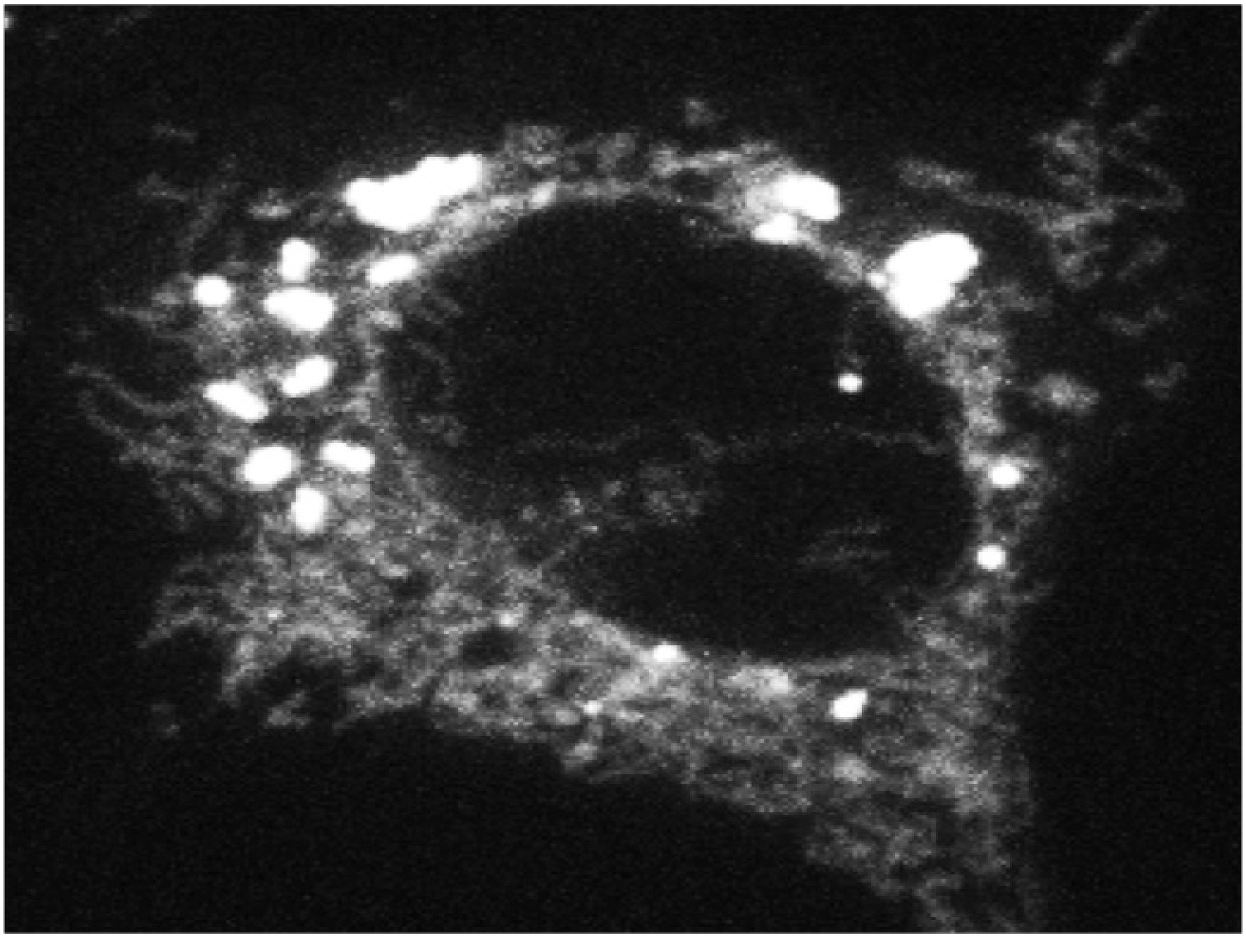

Figure 2. Fatty acid trafficking from lipid droplets to mitochondria

Confocal microscopy of a cell whose lipid droplets were labeled with a fluorescent fatty acid probe and then starved to visualize movement of the fatty acid from lipid droplets to mitochondria

important for tuning and calibrating ciliary sensory functions. The results of these studies reveal that actomyosin dynamics are a critical readout of proper cell health and have major effects on diverse cellular functions.

Mitochondrial dynamics

To study mammalian cell adaptation to nutrient starvation, we examined the interplay between mitochondrial fusion dynamics, autophagy, fatty acid (FA) trafficking, and lipid droplets (LDs). Given that cells appear to adapt to nutrient starvation by shifting their metabolism from reliance on glucose metabolism to utilization of mitochondrial FA oxidation, we developed an assay to investigate how FAs become mobilized and delivered to mitochondria. Using a pulse-chase labeling method to visualize movement of FAs in live cells, we demonstrated that starved cells primarily use LDs as a conduit to supply mitochondria with FAs for β-oxidation. This occurred through lipase-mediated FA mobilization from mitochondria-associated LDs, rather than autophagy (contrary to the pathway used by yeast cells). Autophagy contributed to the altered metabolic scheme by recovering lipids from degraded organelles, which could be used to refill LDs. Notably, mitochondrial tubulation was essential for distribution of FAs throughout the mitochondrial network. Defects in mitochondrial fusion led to massive alterations in cellular FA routing. Not only were non-metabolized FAs redirected to and stored in LDs, they were excessively expelled from cells. Given that FAs are toxic at high levels and serve as signaling molecules at low levels, the results suggest that defects in mitochondrial dynamics and FA trafficking pathways may underlie the pathologies of many metabolic diseases such as diabetes and obesity.

In a separate mitochondria-related project, we uncovered new machinery regulating mitochondrial fission. Seminal work from others showed that, before mitochondrial division by the dynamin-related protein Drp1, endoplasmic reticulum (ER) tubules encircle and constrict mitochondria. Constriction results from actin polymerization controlled by the ER–localized formin protein INF2. However, how ER tubules recognize mitochondria and facilitate fission is unclear. In investigating this question, we discovered a novel mitochondria-localized actin-nucleating protein, Spire1C, which interacts with INF2 on the ER. Cooperation between Spire1C and INF2 enhanced actin assembly selectively at ER/mitochondria intersections, facilitating mitochondrial constriction. We are proposing, therefore, that, during mitochondrial division, a Spire1C–INF2 interaction tethers the ER to mitochondria and mediates actin polymerization, resulting in mitochondrial constriction.

ADDITIONAL FUNDING

» Pharmacology Research Associate Training Program

PUBLICATIONS

1. Manor U, Bartholomew S, Golani G, Christenson E, Kozlov M, Higgs H, Spudich J, Lippincott-Schwartz J. A mitochondria-anchored isoform of the actin-nucleating spire protein regulates mitochondrial division. *eLife* 2015; 4:08828.
2. Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen BC, Gawden-Bone C, van Engelenburg S, Legant W, Gao L, Davidson MW, Betzig E, Lippincott-Schwartz J, Griffiths GM. Actin depletion initiates events leading to granule secretion at the immunological synapse. *Immunity* 2015; 42:864–876.
3. Rambold AS, Cohen S, Lippincott-Schwartz J. Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics. *Dev Cell* 2015; 32:678–692.
4. Battle C, Ott CM, Burnette DT, Lippincott-Schwartz J, Schmidt CF. Intracellular and extracellular forces drive primary cilia movement. *Proc Natl Acad Sci USA* 2015; 112:1410–1415.
5. Huang R, Xu Y, Wan W, Shou X, Qian J, You Z, Liu B, Chang C, Zhou T, Lippincott-Schwartz J, Liu W. Deacetylation of nuclear LC3 drives autophagy initiation under starvation. *Mol Cell* 2015; 57:456–466.

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Deciphering the Virulence Program of *Legionella pneumophila*

Our main research goal is to obtain mechanistic insight into the virulence strategies of microbial pathogens. As a model organism we use the bacterium *Legionella pneumophila*, the causative agent of a potentially fatal respiratory infection known as Legionnaires' disease. Contrary to what its name may imply, Legionnaires' disease occurs in individuals of all ages, including children who receive respiratory therapy, newborns who recently underwent surgery or under-water birth, and children who are immune-compromised. We are committed to the in-depth analysis of mechanisms that allow *L. pneumophila* to exploit the human host and cause disease. Insights gained from these studies will ultimately improve our ability to better diagnose, prevent, and fight Legionnaires' disease and related illnesses, thereby contributing to the success of NICHD's mission.

Upon inhalation of contaminated water droplets, *L. pneumophila* enters the lung and is phagocytosed (taken up) by alveolar macrophages, specialized immune cells. Instead of being degraded by these cells, the pathogen establishes a protective membrane compartment, the Legionella-containing vacuole (LCV). Within this intravacuolar niche, *L. pneumophila* can replicate to high numbers before it kills the host cell and infects neighboring cells.

Intracellular survival of *L. pneumophila* depends on the activity of more than 300 proteins, or effectors, that are injected into the host cell, where they create conditions favorable for infection. *L. pneumophila* mutants that are defective in effector protein delivery fail to escape endolysosomal degradation, underscoring the key role of microbial effectors for bacterial virulence. Our goal is to obtain a detailed mechanistic insight into the regulation and function of *L. pneumophila* effectors by investigating host-pathogen interactions at a molecular, cellular, and structural level. Deciphering the virulence program of this dangerous pathogen will set the stage for the development of novel therapeutics aimed at treating or preventing Legionnaires' disease and related illnesses.

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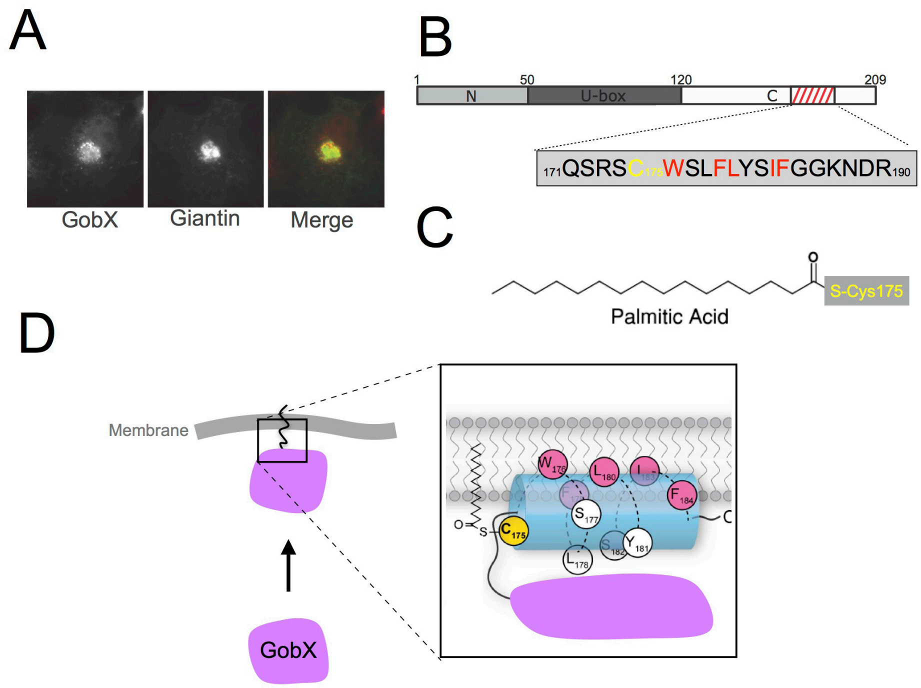
L. pneumophila exploits host-mediated S-palmitoylation for effector localization.

Although the existence within a camouflaged membrane-enclosed compartment provides several benefits to *L. pneumophila*, it also creates certain challenges. For example, how are microbial effector proteins, once they have been delivered into the host cytosol, directed towards the correct host target or organelle where they can then exhibit their function? Thus far, only few targeting mechanisms have been described, including the binding of effectors either to specific proteins or to phospholipids (References 1 and 2).

Our study of the effector protein GobX from *L. pneumophila* revealed yet another targeting strategy that exploits a form of lipidation called S-palmitoylation (Figure 1A) (Reference 3). S-palmitoylation is the covalent attachment of palmitic acid, a hydrophobic C-16 carbohydrate chain, to cysteine residues of proteins. The reaction is catalyzed by eukaryotic palmitoyl transferases (PATs), also known as DHHC proteins owing to a conserved catalytic aspartate-histidine-histidine-cysteine motif. More than

Figure 1. GobX exploits S-palmitoylation for Golgi localization.
A. Intracellular localization of GobX. Transiently transfected COS-1 cells producing GFP-GobX were labeled with antibody directed against the Golgi marker giantin. The merged image (*right*) shows GobX in green and giantin in red. B. Schematic illustration of the domain organization of GobX. Numbers indicate amino acid positions. The minimal Golgi-localizing fragment (residue 171–190) is indicated by the hatched box. The amino acid sequence of this region is shown in single letter code, with C175 highlighted in yellow and hydrophobic residues of the amphipathic helix in red. C. Chemical structure of palmitic acid covalently attached to C175. D. Model of membrane association and S-palmitoylation of GobX by PATs. Color coding as in (B).



500 mammalian proteins have been shown to be covalently modified with palmitic acid, making S-palmitoylation a post-translational modification of great importance. Despite the abundance of S-palmitoylated proteins in eukaryotic cells, the consensus sequence for this post-translational modification had remained unclear.

We discovered that the *L. pneumophila* GobX specifically localizes to the Golgi compartment of host cells in a process that requires host-mediated S-palmitoylation. A surprisingly short stretch of 20 amino acids (aa) within the C-terminal domain of GobX was sufficient for Golgi targeting (Figure 1B). Upon closer examination, we identified a cysteine residue at position 175 (C175) that was essential for Golgi localization. Using metabolic labeling and Click chemistry, we discovered that C175 was the target of host cell–mediated S-palmitoylation (Figure 1C). Substitution of C175 by either serine or alanine prevented S-palmitoylation and, thus, proper intracellular targeting of GobX. Notably, C175 was not the only residue within the 20 aa region of GobX that was essential for Golgi localization. Using site-directed mutagenesis, we identified several additional residues that were equally important for S-palmitoylation (highlighted red in Figure 1B). Interestingly, each of these important residues was hydrophobic in nature and positioned at one side of a predicted alpha helix in GobX, giving it an amphipathic character. We propose that the amphipathic helix can peripherally associate with Golgi membranes, thus bringing C175 into proximity with the DHHC active site of Golgi-resident PATs in order for S-palmitoylation to occur (Figure 1D).

Our in-depth analysis of GobX not only revealed a novel targeting mechanism for *L. pneumophila* effectors, but also provided the first evidence that the consensus motif for S-palmitoylation may be structure- rather than sequence-based (the amphipathic nature of the helix is more important than its exact sequence), explaining why no consensus sequence for S-palmitoylation had previously been identified in eukaryotes.

Host-pathogen interaction profiling using self-assembling human protein arrays

Despite many years of intense studies by several groups, *L. pneumophila* effectors of unknown function vastly outnumber those that have been well characterized. The identification of host targets has remained particularly challenging owing to the lack of

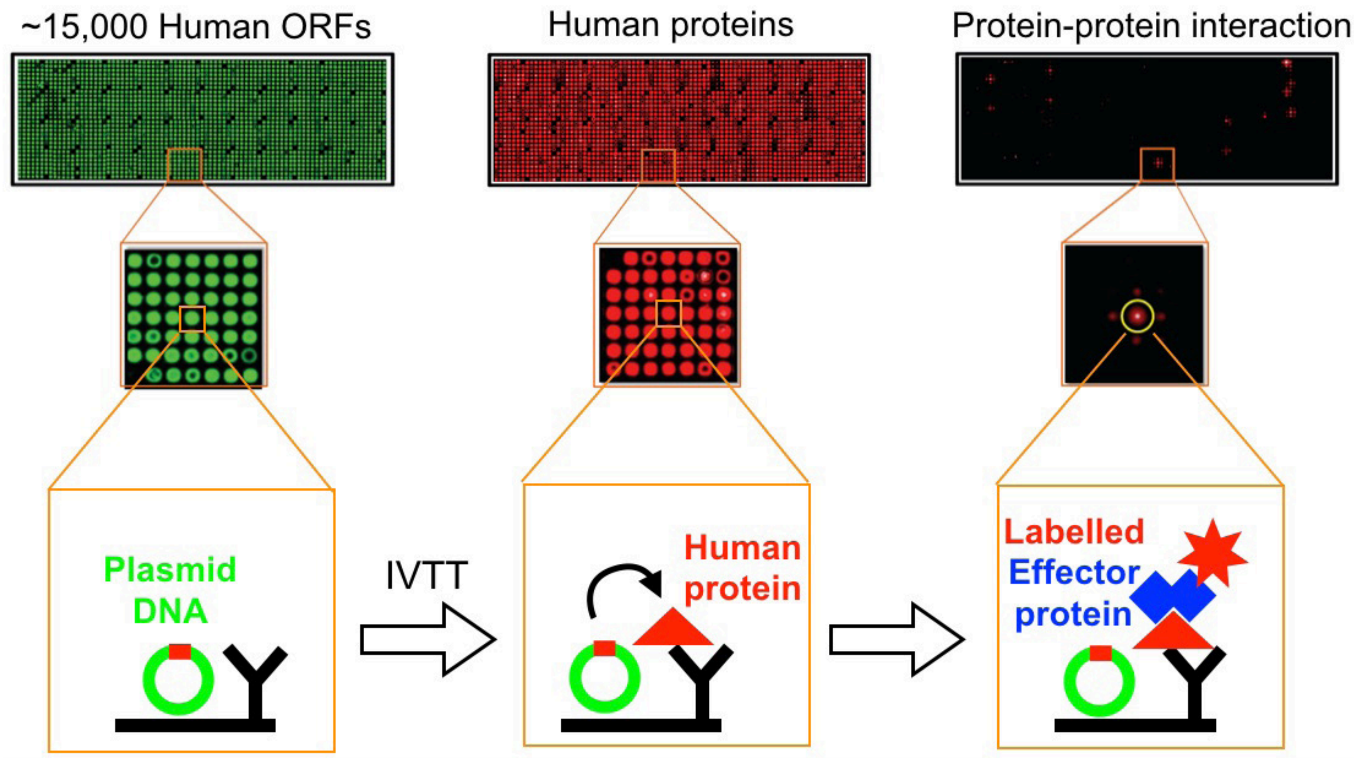


Figure 2. Flow scheme of NAPPA fabrication and protein interaction assay
NAPPA arrays are converted from DNA to protein through *in vitro* transcription/translation. Newly synthesized tagged human proteins are captured on the array by a tag-specific antibody, and binding of HaloTag-query protein to its target on NAPPA is detected using Alexa660-labeled Halo-ligand. Plasmid cDNA on the array is stained green, proteins red.

simple detection tools that avoid abundance biases while providing an open format for experimental modifications. Together with the group of Joshua LaBaer, we established an improved protein-protein interaction platform called Nucleic Acid-Programmable Protein Array (NAPPA) (Reference 4). For this array, thousands of genes encoding tagged human bait proteins are printed on an aminosilane-coated slide (Figure 2). At the time of assay, the proteins are freshly synthesized through *in vitro* transcription/translation (IVTT) and displayed *in situ* using co-spotted anti-tag antibodies.

We developed an improved NAPPA by introducing the HaloTag (Promega) at the C-terminus of the bacterial query protein. HaloTag is a modified haloalkane dehalogenase designed to covalently bind to synthetic Halo-ligands (haloalkanes). Once applied to NAPPA, binding of a HaloTag query protein to its interactor(s) can be specifically detected among thousands of proteins using an Alexa660-labeled Halo-ligand. In a proof-of-concept study, we probed the NAPPA with the *L. pneumophila* effectors SidM or LidA and identified most of the known targets but also potential novel interaction candidates, a subset of which we confirmed in independent *in vitro* pull-down and *in vivo* cell-based assays, thus providing further insight into how these effectors may discriminate between different host Rab GTPases.

In addition, by combining NAPPA with a chemical labeling technique called Click (Copper(I)-catalyzed azide-alkyne Huisgen cycloaddition) chemistry (in collaboration with Howard Hang), we generated a modified screening platform capable of identifying human targets of post-translational modifications such as AMPylation (adenylylation) (Reference 4). We identified the previously reported AMPylation targets of SidM, namely Rab1 and Rab35, and also discovered several novel AMPylation targets, whose contribution to a successful *L. pneumophila* infection has yet to be determined. Our improved NAPPA platform shows broad applicability and can be adapted for the high-throughput analysis of effectors from a variety of pathogens.

ADDITIONAL FUNDING

» NICHD Director's Challenge Innovation Award

PUBLICATIONS

1. Gaspar AH, Machner MP. VipD is a Rab5-activated phospholipase A1 that protects Legionella pneumophila from endosomal fusion. *Proc Natl Acad Sci USA* 2014; 111:4560–4565.
2. Lucas M, Gaspar AH, Pallara C, Rojas AL, Fernández-Recio J, Machner MP, Hierro A. Structural basis for the recruitment and activation of the Legionella phospholipase VipD by the host GTPase Rab5. *Proc Natl Acad Sci USA* 2014; 111:E3514–3523.
3. Lin YH, Doms AG, Cheng E, Kim B, Evans TR, Machner MP. Host cell-catalyzed S-palmitoylation mediates Golgi targeting of the Legionella ubiquitin ligase GobX. *J Biol Chem* 2015; 290:25766–25781.
4. Yu X, Decker KB, Baker K, Neunuebel MR, Graves M, Westcott N, Hang H, LaBaer J, Qiu J, Machner MP. Host-pathogen interaction profiling using self-assembling human protein arrays. *J Proteome Res* 2015; 14:1920–1936.

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Currently, we have two main interests: identification and characterization of small noncoding RNAs and identification and characterization of small proteins of less than 50 amino acids. Both small RNAs and small proteins have been overlooked because they are not detected in biochemical assays and the corresponding genes are poorly annotated and missed in genetic screens. However, mounting evidence suggests that both classes of small molecules play important regulatory roles.

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Identification and characterization of small regulatory RNAs

During the past 15 years, we have carried out several different systematic screens for small regulatory RNA genes in *Escherichia coli*. The screens have included computational searches for conservation of intergenic regions and direct detection after size selection or co-immunoprecipitation with the RNA–binding protein Hfq. We recently examined small RNA expression using deep sequencing to further extend our identification of small RNAs, particularly antisense RNAs (Reference 1).

A major focus of the group has been to elucidate the functions of the small RNAs that we and others have identified. Early on, we showed that the OxyS RNA, whose expression is induced in response to oxidative stress, acts to repress translation through limited base pairing with target mRNAs. We discovered that OxyS action is dependent on the Sm-like Hfq protein, which functions as a chaperone to facilitate OxyS RNA base pairing with its target mRNAs. Recently, we carried out an extensive mutational studies of Hfq (Reference 2). The analysis revealed that amino acids on three different RNA interaction surfaces—the proximal face, the distal face, and the rim of the ring-shaped hexamer—differentially impact Hfq association with small RNAs and their mRNA targets (Figure 1).

It is now clear that Hfq–binding small RNAs, which act through limited base pairing, are integral to many different stress responses in *E. coli*. For example, we showed that the Spot 42 RNA, whose levels are highest when glucose is present, plays a broad role in catabolite repression by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse non-preferred carbon sources. It was previously reported that the transcription factor Sigma(E) maintains membrane homeostasis in part by inducing synthesis of two small regulatory RNAs that down-regulate synthesis of abundant membrane porins. We discovered a third Sigma(E)-dependent small RNA, MicL, that is transcribed from a promoter located within the coding sequence of the *cutC* gene (Reference 3). MicL possesses features typical of Hfq–binding small RNAs but surprisingly targets only a single mRNA, which encodes the outer membrane lipoprotein Lpp, the most abundant protein of the cell. Interestingly, we found that the copper-sensitivity phenotype previously ascribed to inactivation of the *cutC* gene is actually derived from the loss of MicL and elevated Lpp levels. The observation raised the possibility that other phenotypes currently attributed to protein defects are the result of deficiencies in unappreciated regulatory RNAs and prompted us to ask questions about the evolution of base-pairing small RNAs

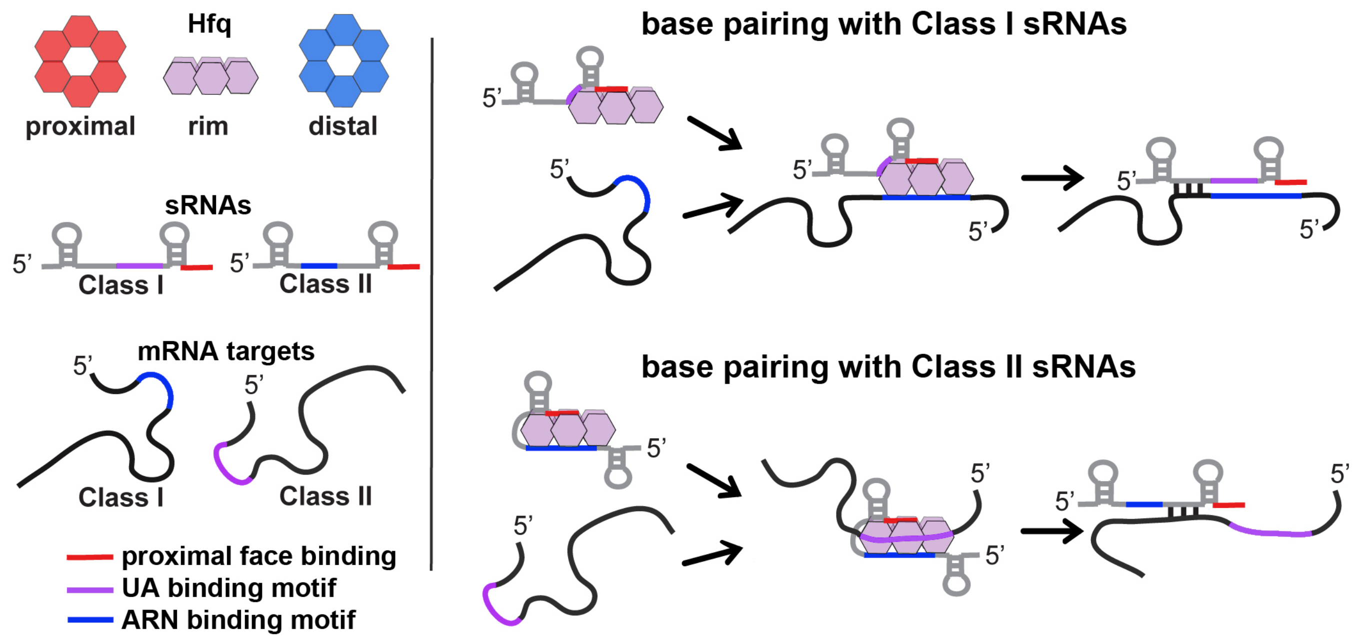

Figure 1. Model of RNA binding to Hfq

The cartoon model of the Hfq hexamer depicts the three RNA binding surfaces of Hfq: proximal face (*red*), rim (*purple*), and distal face (*blue*). For sRNAs and mRNAs, elements in red, purple and blue represent sequences that bind to the proximal face, the rim, and the distal face, respectively. The model depicts two alternative pathways for binding and regulation by sRNA:mRNA pairs. Class I sRNAs utilize a U–rich rho-independent terminator for binding to the proximal face and a UA binding motif for interaction with the rim. mRNA targets regulated by this class of sRNAs utilize ARN binding motifs for interacting with the distal face of Hfq. Class II sRNAs utilize the U–rich rho-independent terminator for binding to the proximal face of Hfq and an ARN binding motif in the 5′ end of the sRNA for binding to the distal face. mRNA targets regulated by this class of sRNAs contain UA–binding motifs that allow for binding to the rim of Hfq.

(Updegrove TB, Shabalina SA, Storz G, *FEMS Microbiol Rev* 2015;39:379).

In addition to small RNAs that act via limited base pairing, we have been interested in regulatory RNAs that act by other mechanisms. For example, early work showed that the 6S RNA binds to and modulates RNA polymerase by mimicking the structure of an open promoter. In a more recent study, we discovered that a broadly conserved RNA structure motif, the yybP-ykoY motif, found in the 5′ UTR of the *mntP* gene encoding a manganese exporter, directly binds to manganese, resulting in a conformation that liberates the ribosome-binding site (Reference 4). Remarkably, we were able to recapitulate the manganese-dependent activation of translation *in vitro*. We also found that the yybP-ykoY motif responds to manganese ions in *Bacillus subtilis*. The identification of the yybP-ykoY motif as a manganese ion sensor suggests that the genes that are preceded by this motif and that encode a diverse set of poorly characterized membrane proteins have roles in metal homeostasis.

Studies to further characterize other Hfq–binding RNAs, antisense RNAs, and small RNAs that act in ways other than base pairing are ongoing.

Identification and characterization of small proteins

In our genome-wide screens for small RNAs, we found that several short RNAs encode small proteins. The correct annotation of the smallest proteins is one of the greatest challenges of genome annotation, and perhaps more importantly, few annotated short ORFs have been confirmed to correspond to synthesized proteins. Although these proteins have largely been missed, the few small proteins that have been studied in detail in bacterial and mammalian cells have been shown to have important functions in signaling and in cellular defenses (Storz et al., *Annu Rev Biochem* 2014;83:18.1-18.25). We thus established a project to identify and characterize proteins of less than 50 amino acids.

We used sequence conservation and ribosome binding site models to predict genes encoding small proteins, defined as having 16–50 amino acids, in the intergenic regions of the model *E. coli* genome. We tested expression of these predicted as well as previously annotated small proteins by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and assaying for synthesis using immunoblot assays. The approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids are synthesized. We have now initiated complementary biochemical approaches to identify additional small proteins.

Remarkably, more than half the newly discovered proteins are predicted to be transmembrane proteins, an observation that prompted us to examine the localization, topology, and membrane insertion of the small proteins. Biochemical fractionation showed that, consistent with the predicted transmembrane helix, the small proteins are generally most abundant in the inner membrane fraction. We found examples of both N_{in}-C_{out} (N-terminal$_{in}$–C-terminal$_{out}$) and N_{out}-C_{in} orientations as well as dual topology in assays of topology-reporter fusions to representative small transmembrane proteins. In addition, fractionation analysis of small protein localization in strains depleted of SecE or YidC uncovered differential requirements for these membrane insertion pathways. Thus, despite their diminutive size, small proteins display considerable diversity in topology, biochemical features, and insertion pathways.

We now are employing many of the approaches the group has used to characterize the functions of small regulatory RNAs to elucidate the functions of the small proteins. Systematic assays for the accumulation of tagged versions of the proteins showed that many small proteins accumulate under specific growth conditions or after exposure to stress. We also generated and screened bar-coded null mutants and identified small proteins required for resistance to cell-envelope stress and acid shock. In addition, the attached sequential peptide affinity tag is being exploited to identify co-purifying complexes. The combination of these approaches is giving insights into when, where, and how the small proteins are acting.

For example, we found that synthesis of a 42-amino acid protein, now denoted MntS (formerly the small RNA gene *rybA*), is repressed by high levels of manganese through MntR. In recent studies, we showed that MntS helps manganese activate a variety of enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese and bacteriostasis under manganese-rich conditions (Reference 5). These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP.

We also discovered that the 49–amino acid inner membrane protein AcrZ (formerly named YbhT) associates with the AcrAB-TolC multidrug efflux pump, which confers resistance to a wide variety of antibiotics and other compounds. Co-purification of AcrZ with AcrB, in the absence of both AcrA and TolC, two-hybrid assays, and suppressor mutations indicate that the interaction occurs through the inner membrane protein AcrB. Mutants lacking AcrZ are sensitive to many of, but not all, the antibiotics transported by AcrAB-TolC. Such differential antibiotic sensitivity suggests that AcrZ may enhance the ability of the AcrAB-TolC pump to export certain classes of substrates. The work, together with our ongoing studies of other small proteins, suggests that many are acting as regulators of larger membrane protein complexes.

PUBLICATIONS

1. Thomason MK, Bischler T, Eisenbart SK, Förstner KU, Zhang A, Herbig A, Nieselt K, Sharma CM, Storz G. Global transcriptional start site mapping using dRNA-seq reveals novel antisense RNAs in Escherichia coli. *J Bacteriol* 2015; 197:18–28.
2. Schu DJ, Zhang A, Gottesman S, Storz G. Alternative Hfq-sRNA interaction modes dictate alternative mRNA recognition. *EMBO J* 2015; 34:2557–2573.
3. Guo MS, Updegrove TB, Gogol EB, Shabalina SA, Gross CA, Storz G. MicL, a new sE-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein. *Genes Dev* 2014; 28:1620–1634.
4. Dambach M, Sandoval M, Updegrove TB, Anantharaman V, Aravind L, Waters LS, Storz G. The ubiquitous yybP-ykoY riboswitch is a manganese-responsive regulatory element. *Mol Cell* 2015; 57:1099–1109.
5. Martin JE, Waters LS, Storz G, Imlay JA. The Escherichia coli small protein MntS and exporter MntP optimize the intracellular concentration of manganese. *PLoS Genet* 2015; 11:e1004977.

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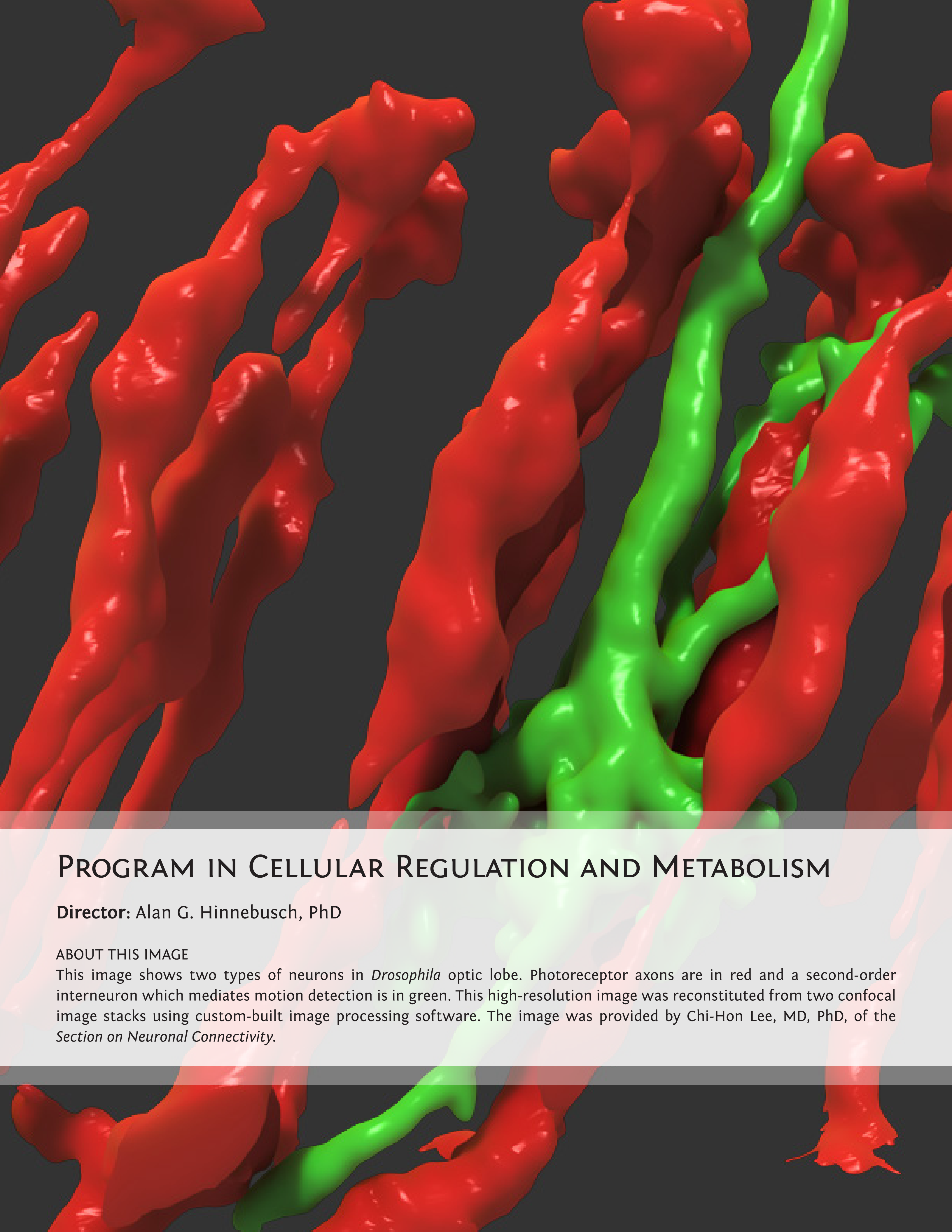


Program in Cellular Regulation and Metabolism

Director: Alan G. Hinnebusch, PhD

ABOUT THIS IMAGE

This image shows two types of neurons in *Drosophila* optic lobe. Photoreceptor axons are in red and a second-order interneuron which mediates motion detection is in green. This high-resolution image was reconstituted from two confocal image stacks using custom-built image processing software. The image was provided by Chi-Hon Lee, MD, PhD, of the *Section on Neuronal Connectivity*.

Program *in* Cellular Regulation and Metabolism

The seven investigators in this program, all belonging to the *Laboratory of Gene Regulation and Development (LGRD)*, share a common interest in using genetically tractable model organisms and a combination of genetics, biochemistry, cell biology, and structural biology to elucidate the molecular basis of processes fundamentally important in cell biology or animal development. Three groups employ yeast as a model system, with two studying molecular mechanisms of transcriptional and translational control of gene expression in budding yeast, and a third studying transposition of mobile elements in fission yeast. Two groups employ the fruit fly *Drosophila* to investigate development and function of neural circuits, including synaptic specificity in the visual system and assembly and growth of synapses at the neuromuscular junction. The anuran *Xenopus laevis* serves as a model system for the work of the remaining two groups, one of which focuses on spindle assembly and chromosome segregation and the roles of covalent protein modification by SUMO in regulating these events, and the other on transcriptional programming during the postembryonic formation of adult stem cells.

The *Section on Cell Cycle Regulation*, headed by Mary Dasso, studies cellular pathways that assure the integrity of genomic transmission during each cell division in higher eukaryotes, particularly mechanisms of mitotic chromosome segregation. The group showed that proteins previously described for their functions in interphase nuclear-cytoplasmic protein trafficking are critical for correct spindle assembly and mitotic progression and is studying the details of these functions, as well as mitotic roles of the SUMO protein modification pathway. The laboratory recently described the function of IRBIT, a novel inhibitor of ribonucleotide reductase (RNR). RNR supplies deoxynucleotide triphosphates for DNA replication, and its uncontrolled activity is associated with malignant transformation and tumor cell growth. Members of the Section showed that IRBIT is essential for proper cycle progression and genomic integrity, and they are currently exploring IRBIT's physiological activities and regulation.

The *Section on Protein Biosynthesis*, headed by Thomas Dever, is characterizing the structure and function of translation factors and stress-responsive eIF2α kinases that control cellular protein synthesis. Recently, the group showed that the baculovirus pseudokinase PK2 inhibits an insect eIF2a kinase through a proposed lobe-swapping mechanism. Members of the Section also identified an eIF2a–docking motif conserved among cellular and viral proteins that targets protein phosphatase PP1 to dephosphorylate eIF2a. They demonstrated that the hypusine-containing protein eIF5A promotes translation elongation by facilitating the reactivity of poor substrates like proline, and they characterized a human X-linked intellectual disability syndrome caused by a mutation in translation factor eIF2γ.

Members of the *Section on Nutrient Control of Gene Expression*, headed by Alan Hinnebusch, study fundamental mechanisms of transcriptional and translational control of gene expression in budding yeast. Recently, they demonstrated conformational rearrangements in the small (40S) ribosomal subunit that accompany transition from the scanning to start codon selection stages of translation initiation, implicated the 40S protein uS7 directly in this process, and showed that the DEAD–box helicase Ded1/Ddx3 selectively promotes initiation on mRNAs with structured 5′ untranslated regions. They established that Rli1/ABCE1 is required *in vivo* for ribosome recycling, the last step of protein synthesis, and blocks aberrant reinitiation by unrecycled ribosomes in 3′ untranslated regions of all mRNAs. They also demonstrated that the chromatin remodeling complex SWI/SNF, histone acetyltransferase complex SAGA, and Hsp70 co-chaperone Ydj1 functionally cooperate in evicting promoter nucleosomes genome-wide and selectively stimulating transcription of the most highly expressed genes in cells.

The *Section on Neuronal Connectivity*, headed by Chi-Hon Lee, investigates the assembly and function of chromatic circuits in *Drosophila*. The group combines imaging and genetic approaches to determine the mechanisms by which optic lobe neurons elaborate dendrites in stereotypic patterns to synapse with appropriate partners. Members of the Section recently identified several molecular cues that control different aspects of dendritic patterning, including dendritic receptive field sizes and planar projection directions. They demonstrated that dendritic patterning defects lead to the formation of erroneous synaptic connections. To study visual functions, they mapped the visual circuits involved in innate and learned color-driven behaviors and identified the neuro-pathways from photoreceptors to peripheral and to higher visual centers.

Henry Levin heads the *Section on Eukaryotic Transposable Elements*, which analyzes the integration of LTR retrotransposons, retroviruses, and DNA transposons into the chromosomes of host cells. Recently, the laboratory developed new methods of deep sequencing that yield ultra-dense profiles of integration. Dense maps of retrotransposon Tf1 integration in

Schizosaccharomyces pombe demonstrated that the promoters of stress-response genes are actively targeted. In other studies, dense maps of HIV-1 integration in cultured human cells reveal that cancer-related genes are among the most frequently disrupted. In a surprising turn, the laboratory found that HIV-1 integration is directed by the host factor LEDGF to highly spliced genes. The laboratory also adapted the Hermes transposable element from the housefly to generate maps of unprecedented density in *S. pombe*. The profiles of Hermes integration yielded comprehensive sets of essential genes and genes with roles in heterochromatin formation.

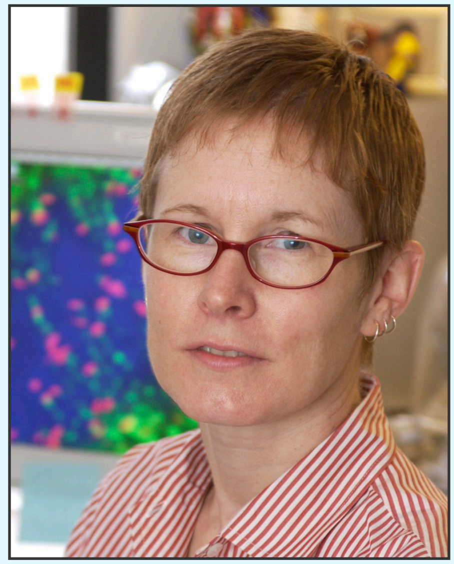
The *Unit on Cellular Communication*, headed by Mihaela Serpe, investigates molecular mechanisms that regulate synapse development. The group focuses on glutamatergic synapses and uses the *Drosophila* neuromuscular junction (NMJ) model system. Recently, the group identified Neto (Neuropillin and Tolloid-like) as an essential component of the NMJ ionotropic glutamate receptor (iGluR) complexes required for clustering of these receptors at synaptic sites. The laboratory established that Neto (1) engages in extracellular interactions that stabilize iGluRs at synaptic sites and trigger postsynaptic differentiation, (2) mediates intracellular interactions that anchor postsynaptic density components and sculpt iGluRs postsynaptic composition, and (3) modulates iGluRs' function, but not their assembly or surface delivery.

The *Section on Molecular Morphogenesis*, headed by Yun-Bo Shi, uses the *Xenopus* model to study the gene-regulatory mechanisms controlled by thyroid hormone receptor (TR) that establish the postembryonic developmental program in vertebrates. Using the TALEN-mediated gene knockdown approach, members of the Section revealed novel functions of TRα in development, i.e., regulating premetamorphic tadpole growth rate and controlling the timing of metamorphosis. They further demonstrated that the histone methyltransferase Dot1L is a direct TR target gene and is required for premetamorphic tadpole growth and survival but not embryogenesis. They also identified and characterized candidate thyroid hormone–regulated genes involved in adult intestinal stem cell development during metamorphosis.

Chromosome Segregation in Higher Eukaryotes

We are interested in mechanisms of chromosome segregation, defects in which lead to aneuploidy, i.e., an abnormal number of chromosomes. Several common birth defects, such as Down's syndrome, result from aneuploidy arising during meiotic cell divisions. Moreover, aneuploidy arising from mitotic divisions is a hallmark of many types of solid tumors. During interphase, chromosomes are enclosed within nuclei, and exchange of all molecules between this compartment and the rest of the cell occurs through nuclear pore complexes (NPCs). Surprisingly, NPC proteins and proteins involved in trafficking of molecules into and out of the nucleus have important roles in chromosome segregation; we are investigating these roles at a molecular level. Our studies concentrated on a GTPase called Ran and on a family of small ubiquitin-like modifiers (SUMOs), which are indispensable for mitotic chromosome segregation. We also recently reported that the IRBIT protein is an inhibitor of ribonucleotide reductase; IRBIT works through a novel mechanism and is vital for genomic integrity.

The ultimate goals of our studies are to understand how these pathways enable accurate chromosome segregation and to discover how they are coordinated with each other and with other aspects of cell physiology.



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Mitotic roles of nuclear pore complex proteins

NPCs consist of about thirty distinct proteins called nucleoporins. Kinetochores are proteinaceous structures that assemble at the centromere of each sister chromatid during mitosis and serve as sites of spindle microtubule attachment. The relationship between NPCs and mitotic kinetochores is surprisingly intimate but poorly understood. During interphase, several kinetochore proteins stably bind to NPCs (e.g., Mad1, Mad2, Mps1). During mitosis, metazoan NPCs disassemble, and at least a third of nucleoporins associate with kinetochores, including the RanBP2 complex and the Nup107-160 complex. We showed that the complexes play important roles in kinetochore function. Additional nucleoporins that do not associate with kinetochores have also been shown to have important mitotic roles, including Nup214, Nup98, and TPR.

Much of our current work concerns the RanBP2 complex. The mammalian RanBP2 complex consists of RanBP2 (a large nucleoporin that is also known as Nup358), SUMO-1–conjugated RanGAP1 (the activating protein for the Ran GTPase), and Ubc9 (the conjugating enzyme for the SUMO family of ubiquitin-like modifiers). The mammalian RanBP2 complex associates with kinetochores in a microtubule-dependent manner that also requires Crm1, a Ran-dependent nuclear export receptor. Disruption of RanBP2 association with kinetochores causes defective mitotic spindle assembly. Additional observations suggest an *in vivo* role of RanBP2 in interphase microtubule organization. We are also analyzing the association of RanBP2 and RanGAP1 in invertebrate species, particularly the fly *Drosophila melanogaster*. Flies mediate the association of RanBP2 and RanGAP1 through a biochemically distinct set of interactions that do not require SUMOylation or Ubc9. Elucidation of the mechanism of this association will not only allow us to test the importance of RanBP2 complex formation in a non-vertebrate

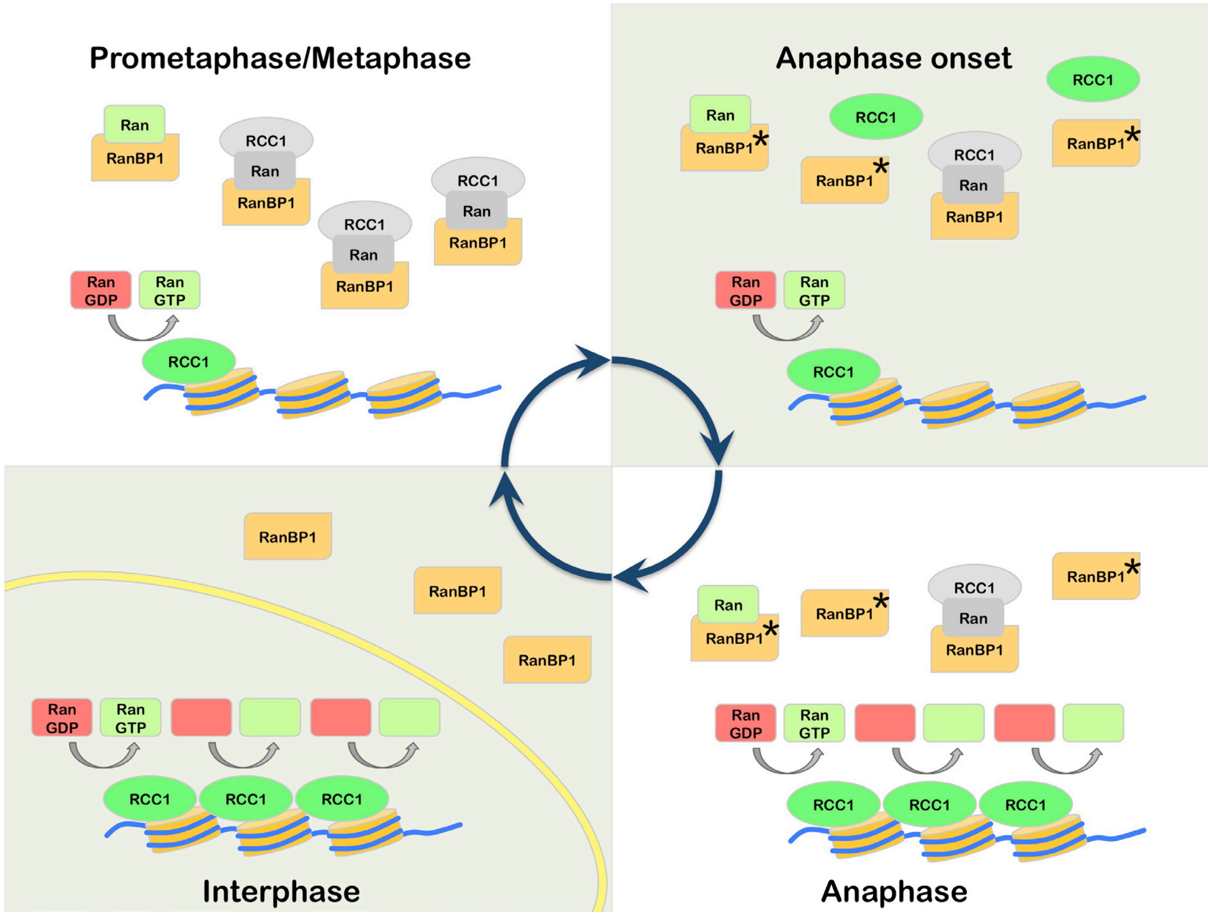

Figure 1. Model for mitotic regulation of RCC1 by RanBP1
Prometaphase: RCC1 is partitioned between an active, chromatin-bound pool (*green*) and an inactive pool (*grey*), associated with RRR complexes that also contain RanBP1 and nucleotide-free Ran (*grey*). Anaphase Onset: Phosphorylation of RanBP1 on Ser 60 (*asterisk*) releases RCC1 from the RRR complex. The free RCC1 is then recruited to chromatin. Anaphase: The high level of chromatin-bound RCC1 promotes enhanced levels of Ran-GDP (*red*) to Ran-GTP (*green*) exchange on chromatin. Interphase: Nuclear envelope (*yellow*) formation physically separates RCC1 from RanBP1, preventing RRR complex assembly and inhibition of RCC1.

system, but also provide a convenient alternative mechanism for the formation of this complex, to help us understand the complex's importance in vertebrate cells.

Mitotic regulation of the Ran GTPase

Ran is a Ras-family GTPase that plays critical roles in many cellular processes including nucleo-cytoplasmic transport, nuclear envelope assembly, and mitotic spindle assembly. Ran alternates between GDP– and GTP–bound forms. In interphase cells, GTP–bound Ran (Ran-GTP) is the major form in nucleus while GDP–bound Ran (Ran-GDP) is the predominant form in cytoplasm. The asymmetrical distribution of Ran-GTP and Ran-GDP drives cargo transport between the nucleus and cytoplasm through karyopherins, a family of nuclear transport carrier proteins that bind to Ran-GTP. In mitosis, after nuclear envelope breakdown, Ran-GTP is concentrated in the region close to mitotic chromatin, while Ran-GDP is the major form distal to chromatin. The Ran-GTP gradient guides mitotic spindle assembly by releasing spindle assembly factors (SAFs) from karyopherins based on local Ran-GTP concentrations. The conversion of Ran-GDP to Ran-GTP in cells is catalyzed by a Ran–specific guanine exchange factor (RanGEF), called RCC1 (Regulator of chromosome condensation 1) in vertebrates. The capacity of RCC1 to bind to chromatin establishes the asymmetrical distribution of Ran-GTP in interphase as well as the chromatin-centered Ran-GTP gradient in mitosis. Interestingly, RCC1's association with chromatin is not static during the cell cycle and is regulated in a particularly dramatic fashion during anaphase in vertebrate systems. The regulation has not been

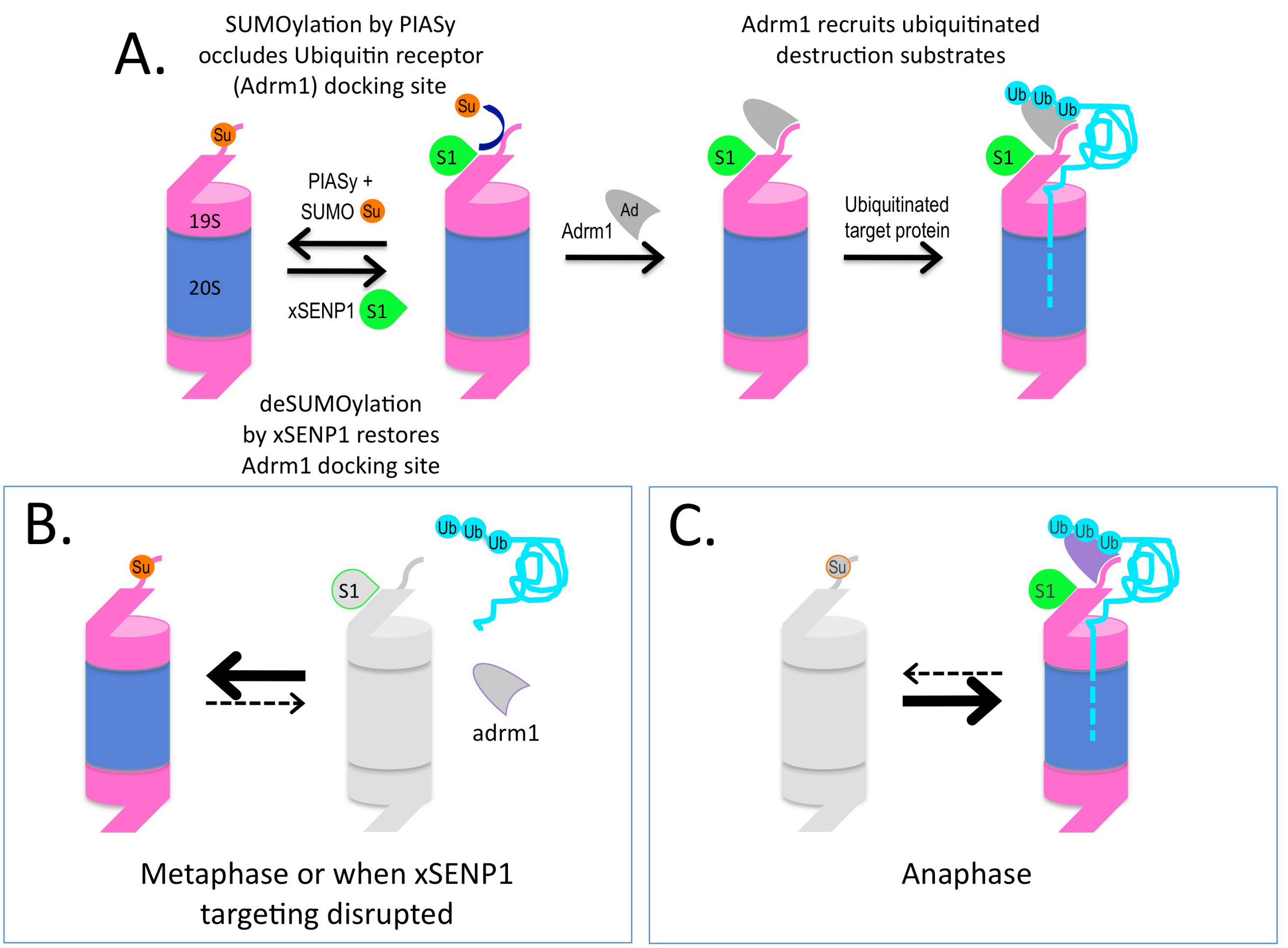

Figure 2. Working model for how SUMOylation regulates Adrm1 binding to the proteasome
A. Psmd1 becomes SUMO2/3 (Su)–conjugated by the SUMO ligase PIASy on Psmd1's C-terminus (shown as an extension from 19S-RP), causing occlusion of the Adrm1 (Ad)–binding site. The modification is antagonized by active xSENP1 (S1). Removal of the SUMO allows Adrm1 recruitment and the degradation of Adrm1–dependent ubiquitinated substrates (Ub). B. When xSENP1 targeting is disrupted, Adrm1 may be unable associate with the 19S-RP, depriving the proteasome of the ability to degrade a subset of targets. We are currently testing whether this mechanism controls proteasome activity during metaphase. C. We will also test whether changes upon anaphase onset could promote the degradation of important mitotic substrates through enhanced Psmd1 deSUMOylation.

correlated with post-translational modifications of RCC1, and the underlying molecular mechanism has not been reported.

RanBP1 is a highly conserved Ran-GTP–binding protein that acts as co-activator of RanGAP1 and can form a heterotrimeric complex with Ran and RCC1 *in vitro*. We found that RCC1 not associated with chromosomes during mitosis is sequestered and inhibited in RCC1/Ran/RanBP1 heterotrimeric complexes and that the sequestration is crucial for normal mitotic spindle assembly. In addition, RanBP1 complex formation competes with chromatin binding to regulate the distribution of RCC1 between the chromatin-associated and soluble fractions. Moreover, we identified a cell cycle–dependent phosphorylation on RanBP1 that modulates RCC1/Ran/RanBP1 heterotrimeric complex assembly and releases RCC1 to bind to chromatin; the phosphorylation is directly responsible for controlling RCC1 dynamics during anaphase. Together, our findings demonstrate novel roles of RanBP1 in spindle assembly and RCC1 regulation in mitosis (Reference 3) (Figure 1).

SUMO-family small ubiquitin-like modifiers in higher eukaryotes

SUMOs are ubiquitin-like proteins (Ubls) that become conjugated to substrates through a pathway that is biochemically

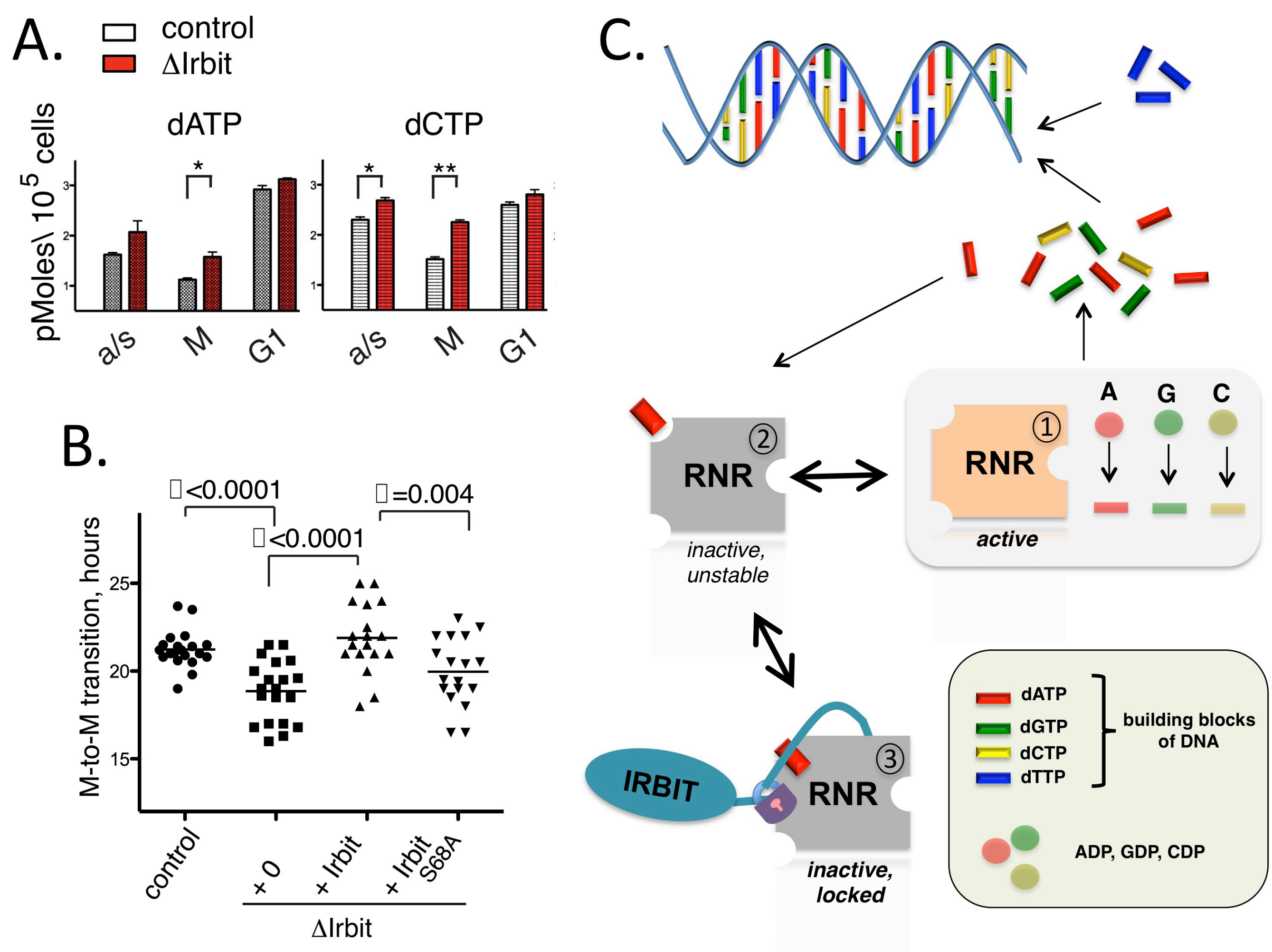

Figure 3. IRBIT, a novel regulator of ribonucleotide reductase in higher eukaryotes

A. dNTP levels in control and IRBIT–depleted HeLa cells in mitotic (M) or G1 phases; a/s – asynchronous population. B. Cell-cycle progression of HeLa cells with or without IRBIT. The Y-axis indicates timing between anaphase and prometaphase of the next cell cycle of an individual daughter cell (n=20). Values are presented as the mean ± SEM. Statistical differences were evaluated by Student's t test ($P < 0.01$). IRBIT-S68A is compromised in RNR binding because it cannot be phosphorylated within a critical regulatory motif. C. Schematic model for IRBIT inhibition of RNR. Top: RNR generates dNTPs, building blocks of DNA, from corresponding nucleotide precursors. Middle: dATP binds loosely to RNR and inactivates it. Bottom: IRBIT recognizes the RNR-dATP complex and stabilizes it by preventing dATP dissociation.

similar to ubiquitination. SUMOylation is involved in many cellular processes, including DNA metabolism, gene expression, and cell-cycle progression. Vertebrate cells express three major SUMO paralogs (SUMO-1–3): mature SUMO-2 and SUMO-3 are 95% identical to each other, while SUMO-1 is 45% identical to SUMO-2 or SUMO-3 (where they are functionally indistinguishable, we collectively call SUMO-2 and SUMO-3 SUMO-2/3). Like ubiquitin, SUMO-2/3 can be assembled into polymeric chains through the sequential conjugation of SUMOs to each other. Many SUMOylation substrates have been identified. SUMOylation promotes a variety of fates for individual targets, dependent upon the protein itself, the conjugated paralog, and whether the conjugated species contains a single SUMO or SUMO chains.

SUMOylation is dynamic owing to rapid turnover of conjugated species by SUMO proteases. Both post-translational processing of SUMO polypeptides and deSUMOylation are mediated by the same family of proteases that play a pivotal role in determining the spectrum of SUMOylated species. This group of proteases is called Ubl–specific proteases (Ulp) in yeast and Sentrin-specific proteases (SENP) in vertebrates. There are two yeast Ulps (Ulp1p and Ulp2p/Smt4p) and six mammalian

SENPs (SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7). SENP1, SENP2, SENP3, and SENP5 form a Ulp1p–related sub-family, while SENP6 and SENP7 are more closely related to Ulp2p. Yeast Ulps have important roles in mitotic progression and chromosome segregation. We defined the enzymatic specificity of the vertebrate SENP proteins and analyzed their key biological roles.

Ulp1p localizes to NPCs and is encoded by an essential gene; it is important for SUMO processing, nucleocytoplasmic trafficking, and late steps in the ribosome biogenesis pathway. Humans possess two NPC–associated SENPs: SENP1 and SENP2. While SENP2 is dispensable for cell division, mammalian SENP1 was recently shown to play an essential role in mitotic progression. Notably, we found that frogs possess a single NPC–bound SUMO protease, xSENP1, allowing us to examine the function of SUMO proteases at the NPC through analysis of a single protease (Reference 4). We determined the interaction partners of the enzyme throughout the cell cycle, using *Xenopus* egg extracts (XEEs). We found that xSENP1 associates strongly with Psmd1, the largest subunit of the proteasome 19S–regulatory particle (19S-RP). Proteasomes are complex ATP–dependent proteases that mediate the degradation of many cellular proteins typically targeted for destruction by ubiquitination. Ubiquitinated degradation substrates are fed into the proteasome's catalytic 20S core particle (20S-CP) through the 19S-RP. Psmd1 plays a key structural role in the 19S-RP and acts as a binding site for the recruitment of other proteasome subunits, including Adrm1. Adrm1 is one of two subunits that directly recruit ubiquitinated substrates to the proteasome. While many proteasomal subunits have been found in proteomic screens for SUMOylation substrates, no role of these modifications has been reported. We mapped SUMOylation sites within Psmd1 and found that modification of a critical lysine adjacent to the Adrm1–binding domain regulates Adrm1 association with Psmd1. Our findings suggest that Psmd1 SUMOylation controls proteasome composition and function, providing a new mechanism for the regulation of ubiquitin-mediated protein degradation through the SUMO pathway. We speculate that the mechanism may allow the regulated degradation of critical mitotic substrates (Figure 2) and are currently testing the model.

Yeast Ulp2p is nucleoplasmic and not essential for vegetative growth but important for chromosome segregation. Ulp2p acts particularly in disassembly of poly-SUMO chains. We demonstrated that human SENP6 is a vertebrate Ulp2p–related enzyme that similarly prefers substrates containing multiple SUMO-2/3 moieties. We analyzed the mitotic role of SENP6 and found it to be essential for accurate chromosome segregation. Mitotic defects observed in the absence of SENP6 reflected the loss of inner kinetochore proteins, including components of the CENP-H/I/K/M and CENP-O centromere protein complexes. The findings demonstrate a novel function of the SUMO pathway in inner kinetochore assembly, which finely balances the incorporation and degradation of components of the kinetochore's inner plate. We are currently analyzing, at a biochemical level, how SUMO controls the deposition of CENP-H/I/K/M.

IRBIT, a ribonucleotide reductase inhibitor with essential roles is genomic stability

Ribonucleotide reductase (RNR) provides deoxynucleotide triphosphates (dNTPs) for genomic and mitochondrial DNA replication and repair. Uncontrolled RNR activity has been associated with malignant transformation and tumor cell growth. RNR is subject to allosteric regulatory mechanisms in all eukaryotes, as well as to control by small protein inhibitors in budding and fission yeast. The key role of RNR in DNA synthesis has made it a target for both anticancer and antiviral therapy.

We discovered that the human IRBIT protein forms a dATP–dependent complex with RNR (Reference 5). The interaction appears to be closely conserved across a wide variety of metazoan species. dATP binds to the large subunit of RNR at two sites: dATP binding at the low-affinity activity site (A-site) inhibits RNR, while dATP binding to the specificity site (S-site) alters the enzyme's substrate preferences. We found that A-site occupancy by dATP is critical for IRBIT–RNR binding, suggesting that IRBIT recognizes inactive, dATP–bound RNR subunit R1 and stabilizes the enzyme in its inhibited state. Notably, formation of the IRBIT–RNR complex is highly dependent upon the phosphorylation status of IRBIT under physiological conditions.

HeLa cells showed an imbalanced pool of dNTP after IRBIT depletion (Figure 3A). We found that this effect was most pronounced during the mitotic phase of the cell cycle, but that dNTP pools were less sensitive during G_1 phase. Consistent with these findings, we observed that IRBIT bound to RNR more strongly during mitosis than during G_1 phase. Live imaging of tissue culture cells demonstrated that IRBIT depletion causes an acceleration of the cell cycle, as well as much greater variation in between individual cells in overall cell cycle duration (Figure 3B). As observed in previous reports testing the consequences of dNTP pool perturbation, IRBIT loss was associated with changes in DNA replication patterns and disruption of genomic stability.

Together, our findings demonstrate an IRBIT–dependent mechanism for RNR regulation in higher eukaryotes, which acts by enhancing allosteric RNR inhibition by dATP (Figure 3C). We are currently working to understand the physiological role of IRBIT in the context of development and disease mechanisms and to exploit this mechanism to target RNR in therapeutic contexts.

Phosphorylation of *Xenopus* p31^{comet} by IKK-beta potentiates mitotic checkpoint exit.

Given that it prevents chromosome mis-segregation and averts genomic instability, the spindle assembly checkpoint (SAC) is among the most important cellular surveillance pathways. To do this, the SAC inhibits premature separation of mitotic sister chromatids by monitoring interactions between kinetochores (KTs) and spindle microtubules (MTs). SAC components are essential in vertebrate cells because organisms that undergo open mitosis must re-establish KT-MT attachments after nuclear envelope breakdown (NEBD) in each cell division. During the process, unattached KTs transiently invoke the SAC during the interval between NEBD and MT attachment, so that subsequent SAC silencing plays a key role in determining the timing of anaphase onset. While SAC silencing is thus critical for mitotic progression in metazoan cells, it remains poorly understood. The p31^{comet} protein, found in higher eukaryotes, plays an important role in SAC silencing. We used *Xenopus* egg extracts (XEEs) to investigate the mitotic roles and regulation of p31^{comet}. Our observations suggest that endogenous p31^{comet} is important for anaphase timing in this system and is regulated through mitotic phosphorylation. While several well established mitotic kinases did not efficiently modify p31^{comet} *in vitro*, IKK-beta (Inhibitor of nuclear factor k-B kinase-beta) was an effective p31^{comet} kinase. Depletion or inhibition of IKK-beta delayed mitotic exit of XEEs, and a phosphomimetic p31^{comet} mutant showed increased activity in SAC silencing. Together, our experiments suggest that p31^{comet} contributes to the timing of anaphase onset in XEE through antagonism of the SAC and that IKK-beta modifies p31^{comet} to enhance its activity. Several previous reports established that IKK-beta plays a clear but under-appreciated role of within mitosis, a role that is distinct from its function in cellular stress and inflammatory signaling pathways. Our findings are among the first mechanistic insights into the nature of this role.

PUBLICATIONS

1. Mo M, Arnaoutov A, Dasso M. Phosphorylation of Xenopus p31(comet) potentiates mitotic checkpoint exit. *Cell Cycle* 2015; 14:3978–85.
2. Ryu H, Yoshida MM, Sridharan V, Kumagai A, Dunphy WG, Dasso M, Azuma Y. SUMOylation of the C-terminal domain of DNA topoisomerase IIa regulates the centromeric localization of Claspin. *Cell Cycle* 2015; 14:2777–2784.
3. Zhang MS, Arnaoutov A, Dasso M. RanBP1 governs spindle assembly by defining mitotic Ran-GTP production. *Dev Cell* 2014; 31:393–404.
4. Ryu H, Gygi SP, Azuma Y, Arnaoutov A, Dasso M. SUMOylation of Psmd1 controls Adrm1 interaction with the proteasome. *Cell Rep* 2014; 7:1842–1848.
5. Arnaoutov A, Dasso M. Enzyme regulation. IRBIT is a novel regulator of ribonucleotide reductase in higher eukaryotes. *Science* 2014; 345:1512–1515.

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Mechanism and Regulation of Eukaryotic Protein Synthesis

We study the mechanism and regulation of protein synthesis, focusing on GTPases and protein kinases that control this fundamental cellular process. We use molecular-genetic and biochemical studies to dissect the structure-function properties of the translation initiation factors eIF2, a GTPase that binds methionyl-tRNA to the ribosome, and eIF5B, a second GTPase that catalyzes ribosomal subunit joining in the final step of translation initiation. We also investigate stress-responsive protein kinases that phosphorylate eIF2alpha, viral regulators of these kinases, and how cellular phosphatases are targeted to dephosphorylate eIF2alpha. Our recent studies elucidated how an eIF2gamma mutation that is associated with intellectual disability impairs eIF2 function and revealed how eIF2 binds to methionyl-tRNA and the ribosome. Our studies also demonstrated that the hypusine-containing protein eIF5A promotes translation elongation by stimulating the peptidyl transferase activity of the ribosome and facilitating the reactivity of poor substrates such as proline.

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Molecular analysis of eIF2alpha phosphorylation, dephosphorylation, and viral regulation

The translation factor eIF2 is composed of three distinct subunits. Phosphorylation of the eIF2alpha subunit is a common mechanism for down-regulating protein synthesis under stress conditions. Four distinct kinases phosphorylate eIF2alpha on Ser51 under different cellular stress conditions. GCN2 responds to amino acid limitation, HRI to heme deprivation, PERK to ER stress, and PKR to viral infection. Consistent with their common activity to phosphorylate eIF2alpha on Ser51, the kinases show strong sequence similarity in their kinase domains. Phosphorylation of eIF2alpha converts eIF2 from a substrate to an inhibitor of its guanine-nucleotide exchange factor eIF2B. The inhibition of eIF2B impairs general translation, slowing the growth of yeast cells and, paradoxically, enhancing the translation of the GCN4 mRNA required for yeast cells to grow under amino-acid starvation conditions.

We previously used structural, molecular, and biochemical studies to define how the eIF2alpha kinases recognize their substrate. In collaboration with Frank Sicheri, we obtained the X-ray structure of eIF2alpha bound to the catalytic domain of PKR. Back-to-back dimerization enables each PKR protomer to engage a molecule of eIF2alpha in the crystal structure. Using site-directed mutagenesis studies, we demonstrated that a common mode of back-to-back dimerization is required for activation of PKR, GCN2, and PERK, and we proposed an ordered mechanism of PKR activation by which catalytic domain dimerization triggers autophosphorylation, which in turn is required for specific eIF2alpha substrate recognition. Our mutagenesis studies revealed that the position of the Ser51 residue in free eIF2alpha is restricted and that docking of eIF2alpha onto PKR helix alphaG disrupts a hydrophobic network and induces a conformational change that enables Ser51 to move by about 20 Å to engage the phospho-acceptor binding site of the kinase. We propose that the protected state of Ser51 in free eIF2alpha prevents promiscuous phosphorylation and attendant translational regulation by heterologous kinases, yet enables Ser51 phosphorylation upon binding of eIF2alpha to one of the canonical eIF2alpha kinase.

While the protein kinases GCN2, HRI, PKR, and PERK specifically phosphorylate eIF2alpha on Ser51 to regulate global and gene-specific mRNA translation, eIF2alpha is dephosphorylated by the broadly acting serine/threonine protein phosphatase 1 (PP1). In mammalian cells, the regulatory subunits GADD34 and CReP target PP1 to dephosphorylate eIF2alpha; however, as there are no homologs of these targeting subunits in yeast, it was unclear how GLC7, the functional homolog of PP1 in yeast, is recruited to dephosphorylate eIF2alpha. We recently showed that a novel N-terminal extension on yeast eIF2gamma binds to GLC7 and targets it to dephosphorylate eIF2alpha. Truncation or point mutations designed to eliminate the PP1–binding motif in eIF2gamma impaired eIF2alpha dephosphorylation both *in vivo* and *in vitro*. Moreover, replacement of the N-terminus of eIF2gamma with the GLC7–binding domain from GAC1 or fusion of heterologous dimerization domains to eIF2gamma and GLC7 maintained eIF2alpha phosphorylation at basal levels. Taken together, our results indicate that, in contrast to the paradigm of distinct PP1 targeting or regulatory subunits, the unique N-terminus of yeast eIF2gamma functions in *cis* to target GLC7 to dephosphorylate eIF2alpha (Reference 1).

Over the last year, we continued our studies on eIF2alpha dephosphorylation and reconstituted human GADD34 function in yeast cells. We mapped a novel eIF2α-binding motif to the C-terminus of GADD34 in a region distinct from where PP1 binds to GADD34. Point mutations altering the 19–residue eIF2α–binding motif impaired the ability of GADD34 to interact with eIF2α, promote eIF2α dephosphorylation, and suppress PKR toxicity in yeast. Interestingly, the eIF2α–docking motif is conserved among several viral orthologs of GADD34, and we showed that it is necessary for the proteins produced by African swine fever virus, Canarypox virus, and Herpes simplex virus to promote eIF2α dephosphorylation. Taken together, our data demonstrate that GADD34 and its viral orthologs direct specific dephosphorylation of eIF2α by interacting with both PP1 and eIF2α through independent binding motifs (Reference 2).

When expressed in yeast, human PKR phosphorylates the alpha subunit of eIF2 on Ser51, causing inhibition of protein synthesis and yeast cell growth. To subvert the anti-viral defense mediated by PKR, viruses produce inhibitors of the kinase. The poxviral protein E3L binds to double-stranded RNA and inhibits PKR by sequestering activators and forming heterodimers with the kinase. We previously showed that a Z-DNA–binding domain near the N-terminus of E3L, but not its Z-DNA–binding activity, is critical for E3L inhibition of PKR. We are currently characterizing mutations in PKR that confer resistance to E3L inhibition. Similarly, we previously discovered that the insect baculovirus PK2 protein is an eIF2alpha kinase inhibitor. PK2 structurally mimics the C-terminal lobe of a protein kinase domain. Using a genetic screen in yeast, and together with collaborators in Canada and Japan, we characterized mutations that enhance the ability of PK2 to inhibit eIF2 kinases. The mutations cluster to a surface of PK2 that, in bona fide protein kinases, forms the catalytic cleft through interactions with a kinase N-lobe. Yeast two-hybrid and protein-interaction assays revealed that PK2 associates with the N-lobe of PKR. Using yeast-based assays, we showed that PK2 was most effective in inhibiting an insect HRI–like kinase, and our collaborators showed that knockdown of the HRI–like kinase in insects rescued viral defects associated with loss of PK2. We propose an inhibitory mechanism whereby PK2 engages the N-lobe of an eIF2α kinase domain to create a nonfunctional pseudokinase domain complex, possibly through a lobe-swapping mechanism (Reference 3).

Analysis of an eIF2gamma mutation that links intellectual disability with impaired translation initiation

We have a long-standing interest in the structure-function properties of eIF2, and recently our studies have enabled us to provide insights into human disease. While protein synthesis is known to play a critical role in learning and memory in diverse model systems, human intellectual disability syndromes have not been directly associated with alterations in protein synthesis. Moreover, the consequences of partial loss of eIF2gamma function or eIF2 integrity are unknown in mammals, including humans. Our collaborators Lina Basel-Vanigaite and Guntram Borck identified a human X-chromosomal neurological disorder characterized by intellectual disability and microcephaly. Mapping studies identified the causative mutation as a single base change resulting in a missense mutation in eIF2gamma (encoded by *EIF2S3*). Biochemical studies of human cells overexpressing the eIF2gamma mutant and of yeast eIF2gamma with the analogous mutation revealed a defect in binding of the eIF2beta subunit to eIF2gamma. Consistent with this loss of eIF2 integrity, the mutation in yeast eIF2gamma impaired translation start codon selection and eIF2 function *in vivo* in a manner that was suppressed by overexpression of eIF2beta. The findings directly link intellectual disability with impaired translation initiation and provide a mechanistic basis for the human disease as a result of partial loss of eIF2 function (Borck et al., *Mol Cell* 2012;48:641-646). Over the past year, we have been characterizing new mutations in eIF2gamma that cause intellectual disability.

Molecular analysis of the hypusine-containing protein eIF5A

The translation factor eIF5A, the only protein containing the unusual amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl) lysine], was originally identified based on its ability to stimulate a model assay for first peptide–bond synthesis. However, the precise cellular role of eIF5A was unknown. Using molecular-genetic and biochemical studies, we previously showed that eIF5A promotes translation elongation and that this activity is dependent on the hypusine modification. Given that eIF5A is a structural homologue of the bacterial protein EF-P, we proposed that eIF5A/EF-P is a universally conserved translation elongation factor.

Recently, it was shown that EF-P promotes translation of polyproline sequences by bacterial ribosomes. Using *in vivo* reporter assays, we showed that eIF5A in yeast stimulates the synthesis of proteins containing runs of three or more consecutive proline residues. Consistently, the expression of native yeast proteins containing homopolyproline sequences was impaired in eIF5A mutant strains. To support the *in vivo* findings, we used reconstituted yeast *in vitro* translation assays to monitor the impact of eIF5A on protein synthesis. We found that the synthesis of polyproline peptides, but not of polyphenylalanine peptides, was critically dependent on addition of eIF5A. Toe-printing experiments revealed that addition of eIF5A relieved ribosomal stalling during translation of three consecutive proline residues *in vitro*. Consistent with the functions of eIF5A in promoting peptide bond synthesis, directed hydroxyl radical probing experiments localized eIF5A binding to near the E site of the ribosome, with the hypusine residue of eIF5A adjacent to the acceptor stem of the P-site tRNA. Thus, we propose that eIF5A, like its bacterial orthologue EF-P, stimulates the peptidyl-transferase activity of the ribosome and facilitates the reactivity of poor substrates such as proline (Reference 4).

Analysis of HAC1 mRNA translational control

In addition to studying translation factors and regulators, we also studied the impact of mRNA structure on translation. The HAC mRNA in yeast encodes a transcription factor that up-regulates genes that control protein homeostasis. Base-pairing interactions between sequences in the intron and the leader of the HAC1 mRNA represses Hac1 protein production under basal conditions. An unusual cytoplasmic splicing of the intron by the Ire1 kinase-endonuclease, activated under conditions of ER stress, relieves the inhibition and enables Hac1 synthesis. Using random and site-directed mutations, we showed that disruption of the base-pairing interactions derepresses translation of the unspliced HAC1 mRNA. With our collaborator Madhusudan Dey, we also showed that insertion of an in-frame AUG start codon upstream of the base-pairing interaction releases the translational block, demonstrating that an elongating ribosome can disrupt the interaction. Moreover, overexpression of the translation initiation factor eIF4A, a helicase, enhanced production of Hac1 from an mRNA containing an upstream AUG start codon at the beginning of the base-paired region. As the point mutations that enhanced Hac1 production resulted in an increased percentage of the HAC1 mRNA associating with polysomes, we conclude that the 5′ UTR–intron interaction represses translation initiation on the unspliced HAC1 mRNA (Reference 5).

PUBLICATIONS

1. Rojas M, Gingras AC, Dever TE. Protein phosphatase PP1/GLC7 interaction domain in yeast eIF2 bypasses targeting subunit requirement for eIF2 dephosphorylation. *Proc Natl Acad Sci USA* 2014; 111:E1344–1353.
2. Rojas M, Vasconcelos G, Dever TE. An eIF2alpha-binding motif in protein phosphatase 1 subunit GADD34 and its viral orthologs is required to promote dephosphorylation of eIF2alpha. *Proc Natl Acad Sci USA* 2015; 112:E3466–3475.
3. Li JJ, Cao C, Fixsen SM, Young JM, Ono C, Bando H, Elde NC, Katsuma S, Dever TE, Sicheri F. Baculovirus protein PK2 subverts eIF2alpha kinase function by mimicry of its kinase domain C-lobe. *Proc Natl Acad Sci USA* 2015; 112:E4364–E4373.
4. Gutierrez E, Shin BS, Woolstenhulme CJ, Kim JR, Saini P, Buskirk AR, Dever TE. eIF5A promotes translation of polyproline motifs. *Mol Cell* 2013; 51:35–45.
5. Sathe L, Bolinger C, Mannan MA, Dever TE, Dey M. Evidence that base-pairing interaction between intron and mRNA leader sequences inhibits initiation of HAC1 mRNA translation in yeast. *J Biol Chem* 2015; 290:21821–21832.

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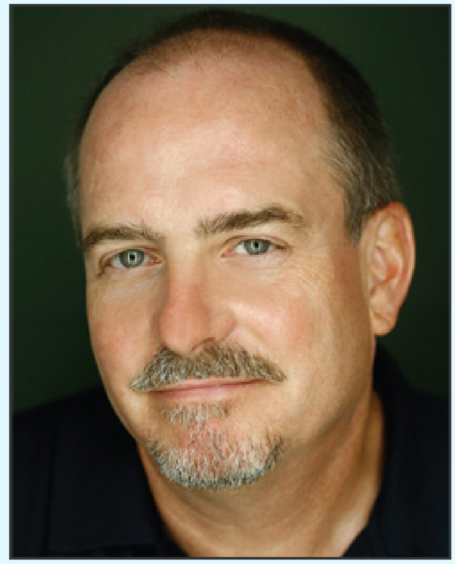
Transcriptional and Translational Regulatory Mechanisms in Nutrient Control of Gene Expression

We study molecular mechanisms of gene regulation at the translational and transcriptional levels, using the regulation of amino acid–biosynthetic genes in budding yeast as a model system. Transcription of these and many other genes is coordinately induced by the transcriptional activator Gcn4 in response to starvation of any amino acid. Expression of *GCN4* is coupled to amino acid levels by a conserved translational control mechanism involving upstream open reading frames (uORFs) in *GCN4* mRNA. Ribosomes translate the 5′-most uORF (uORF1) and, under non-starvation conditions, reinitiate translation at uORFs 2, 3, or 4 and then dissociate from the mRNA, keeping *GCN4* translation repressed. In starvation conditions, the reinitiating ribosomes bypass uORFs 2–4 and reinitiate at *GCN4* instead, owing to lowered availability of the ternary complex (TC)—comprised of initiation factor 2 (eIF2), GTP, and initiator Met-tRNAi—which binds to the small (40S) ribosomal subunit to assemble a 43S preinitiation complex (PIC). TC abundance is reduced in starved cells by phosphorylation of the alpha subunit of eIF2 (eIF2α) by Gcn2, a protein kinase conserved in all eukaryotes, converting eIF2 from substrate to inhibitor of its guanine nucleotide exchange factor (GEF) eIF2B. Hence, GCN4 translation is an *in vivo* indicator of impaired TC loading on 40S subunits. We previously exploited this fact to isolate mutations in subunits of eIF2B that constitutively derepress *GCN4* (Gcd^{-} phenotype) by lowering TC assembly in the absence of eIF2 phosphorylation. More recently, we used the Gcd^{-} selection to identify domains/residues in eIF1, eIF1A, and eIF3, and also residues of 18S rRNA located near the 'P' decoding site of the 40S subunit, that participate in rapid TC recruitment *in vivo*. In collaboration with Jon Lorsch's group, we demonstrated that segments/residues in eIF1, eIF1A, and 18S rRNA, which are implicated genetically in TC recruitment, also stimulate this reaction in a fully reconstituted *in vitro* system.

We also investigate the roles of various eIFs and the 40S subunit in scanning the mRNA 5′ untranslated region and in accurately identifying the AUG initiation codon. The studies exploit a genetic selection for mutations that elevate initiation at near-cognate UUG start codons (Sui^{-} phenotype) or suppress this aberrant initiation event (Ssu^{-} phenotype). In this way, we showed that eIF1A and the c-subunit of eIF3 make critical contributions to accurate AUG recognition, and localized these functions to the unstructured N- and C-tails of eIF1A and the N-terminal domain of eIF3c. Also in collaboration with Lorsch's group, we provided strong evidence that dissociation of eIF1 from the PIC is a critical step in AUG recognition, indicating that eIF1 serves as a 'gate-keeper' necessary to suppress initiation at non-AUG triplets. Biochemical analysis revealed that eIF1 stabilizes an 'open' conformation of the 40S subunit conducive to scanning and loading of TC in a metastable state[$P_{(OUT)}$], which enables inspection of successive mRNA triplets in the P site for complementarity with the anticodon of Met-tRNAi. However, eIF1 must dissociate from the PIC for AUG selection, as the absence of eIF1 favors the closed, scanning-arrested conformation of the 40S subunit with TC more tightly bound [$P_{(IN)}$ state]. We recently established that basic residues in eIF1's helix-1 and β-hairpin-1 mediate 40S binding and stabilize the open, $P_{(OUT)}$ complex to promote Met-tRNAi recruitment and accurate



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AUG selection *in vivo*. Previously, we showed that 10–amino acid repeats in the C-terminal tail (CTT) of eIF1A (dubbed "scanning enhancers" or SE elements) function together with eIF1 to stabilize the open, $P_{(OUT)}$ complex but must be antagonized by scanning-inhibitory (SI) elements in the N-terminal tail (NTT) and helical domain of eIF1A to achieve rearrangement to the closed, $P_{(IN)}$ complex and start codon recognition. In collaboration with Lorsch's group, we recently established that the eIF1A CTT moves towards the N-terminal domain (NTD) of eIF5 in response to AUG recognition, and that this movement—which depends on the eIF1A SE elements—is required for P_i release from eIF2. We proposed that accommodation of $tRNA_i$ in the $P_{(IN)}$ state triggers eIF1 displacement, which in turn triggers movement of the eIF1A-CTT and P_i release.

The eIF4F complex, which binds to the mRNA 5′ cap structure, is thought to resolve mRNA secondary structure to enhance recruitment of the 43S PIC and scanning of the mRNA leader. We recently dissected the NTD of eIF4G, the scaffolding subunit of eIF4F—harboring binding sites for cap-binding factor eIF4E, poly(A)-tail-binding factor PABP, and RNA helicase eIF4A—and identified conserved elements (RNA1, boxes 1–3) that functionally overlap to promote eIF4G interaction with mRNA and PABP, thereby activating mRNA for PIC attachment. We also identified a novel role for cofactor eIF4B in promoting eIF4AV/eIF4G association (eIF4F assembly) *in vivo* and, in collaboration with Lorsch's group, showed that binding of eIF4B near the mRNA entry channel of the 40S subunit is likely to be crucial for stimulating eIF4F function in PIC recruitment.

In the arena of transcriptional control, we previously defined multiple clusters of hydrophobic residues that constitute the transcriptional activation domain of Gcn4, identified co-activators required for gene activation by Gcn4 *in vivo*, and defined the molecular program for recruitment of nucleosome-modifying and -remodeling enzymes and adaptor proteins that remove repressive chromatin structure and recruit TATA–binding protein and RNA polymerase II (Pol II) to Gcn4 target promoters. We also demonstrated co-transcriptional recruitment of the histone acetyltransferase (HAT) complexes SAGA and NuA4 to transcribed coding sequences and showed that they cooperate to enhance transcription-associated histone eviction and Pol II elongation *in vivo*. We described a two-stage recruitment mechanism for NuA4, involving the serine-5–phosphorylated C-terminal domain (CTD) of Pol II and methylated histones, and more recently we extended this mechanism to include the histone deacetylase (HDA) complex Rpd3C(S). In addition, we showed that the HDAs Rpd3, Hos2, and Hda1 have overlapping functions in deacetylating coding-sequence nucleosomes and obtained evidence that histone acetylation is a key determinant of co-transcriptional nucleosome disassembly.

More recently, we elucidated the roles of the Pol II CTD kinases Kin28 and Bur1 and the C-terminal repeats (CTRs) of the Spt5 subunit of DSIF in recruitment of transcription elongation factor Paf1C. We showed that Ser5-CTD phosphorylation by Kin28 enhances recruitment of Bur1 near promoter regions where Bur1 contributes to Ser2–CTD phosphorylation. We then established that Kin28 enhances Paf1C recruitment by: (1) promoting Bur1 recruitment via the phosphorylated Pol II CTD, with attendant phosphorylation of Spt5 CTRs by Bur1; and (2) collaborating with Bur1 to generate Ser2–/Ser5–diphosphorylated Pol II CTD repeats. The resulting phosphorylated CTD and CTR repeats bind to distinct Paf1C subunits to enable efficient recruitment of the entire complex to elongating Pol II.

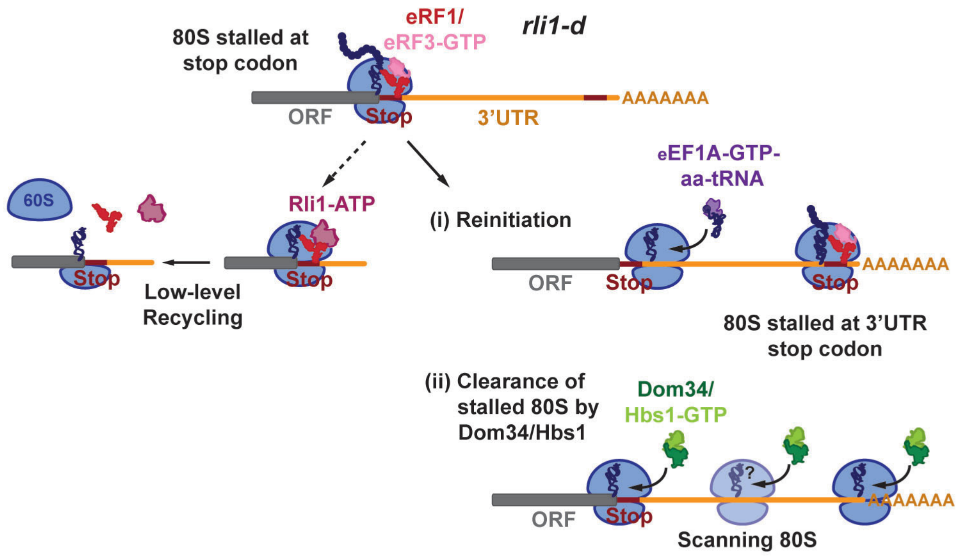
Biochemical evidence for conformational changes in the P site and mRNA entry channel evoked by AUG recognition in reconstituted PICs

It is thought that the scanning PIC assumes an open conformation and that AUG recognition evokes a closed state that arrests scanning with more stable Met-tRNAi binding [$P_{(IN)}$ state], with attendant displacement of the eIF1A-CTT from the P site and dissociation of eIF1 from the 40S subunit. We obtained physical evidence for these conformational rearrangements by comparing patterns of directed hydroxyl radical cleavage (DHRC) of rRNA by Fe(II)-BABE tethered to unique cysteine residues engineered in eIF1A, in PICs reconstituted with mRNA with AUG or near-cognate (AUC) start codons. Key rRNA residues in the P site displayed reduced cleavage in AUG versus AUC PICs, suggesting that accessibility of these rRNA residues is reduced by their increased interaction with Met-tRNAi in the $P_{(IN)}$ state. The cleavage data also indicate that AUG recognition evokes dissociation of eIF1 from its 40S binding site, a constricted conformation of the entry channel, and displacement of the eIF1A-CTT from near the P-site.

Structures of yeast preinitiation complexes reveal conformational changes from mRNA scanning to start-codon recognition.

In colloboration with Venki Ramakrishnan's lab and Jon Lorsch's group, we obtained cryo-EM reconstructions of yeast PICs that represent different stages of the initiation pathway, at 3.5–6.1 Å resolution. These include 40S-eIF1-eIF1A complexes and

Schematic model depicting the fate of post-termination ribosomal complexes (post-TCs) on depletion of Rli1/ABCE1 in an *rli1-d* degron mutant



Recognition of the stop codon of the main open-reading frame (ORF) at each gene by translation termination factors eRF1/eRF3-GTP (*top row*) is followed by release of the completed polypeptide and dissociation of eRF3-GDP (not depicted). Any residual Rli1 remaining in the *rli1-d* cells could bind to post-TCs (ternary complexes) and catalyze dissociation of the 60S subunit (*middle row, left*). However, many post-TCs are not recycled owing to Rli1 deficiency, migrate a short distance from the stop codon, reinitiate translation, and frequently terminate at a 3′ UTR stop codon to produce a 3′ UTR–encoded polypeptide (*middle row, right*). Such reinitiation events appear to be diminished by ribosome rescue factor Dom34, potentially because post-TC ribosomes are rescued at the main ORF stop codons or as they begin scanning. Any ribosomes that reach the 3′ UTR/poly(A) boundary by reinitiation or scanning are also rescued by Dom34.

partial yeast 48S PICs in open and closed conformations (py48S-open and py48-closed). The structures provide a wealth of new information about conformational changes occurring in the transition from scanning to AUG selection. Comparing the 40S-eIF1-eIF1A complex with the free 40S reveals rotation of the 40S head that might promote TC binding to form the 43S PIC. The py48S-open, formed using mRNA with AUC start codon, reveals an upward shift of the 40S head that widens the mRNA entry channel and opens its latch—consistent with our eIF1A/Fe(II)-BABE cleavage data—which should facilitate mRNA insertion into the binding cleft to form the scanning PIC. Moreover, the P site is widened and lacks tRNAi contacts with the 40S body present in canonical 80S-tRNAi complexes. By contrast, py48S-closed, formed with mRNA(AUG), reveals downward head movement that closes the latch, clamps the mRNA into the binding cleft, and fully encloses tRNAi in the P site. The eIF1A NTT assumes a structured conformation and interacts with the AUG:anticodon duplex, consistent with its role in stabilizing $P_{(IN)}$. eIF1 is repositioned on the 40S and deformed to prevent a clash with tRNAi, likely as a prelude to eIF1 dissociation from the 40S subunit. Both py48S-open and -closed complexes reveal eIF2beta and portions of the eIF3 complex. The eIF3 trimeric subcomplex eIF3b-CTD/eIF3i/eIF3g-NTD resides on the subunit-interface surface of the 40S and appears to lock mRNA into the 40S binding cleft. eIF2beta interacts with tRNAi and segments of eIF1 and eIF1A exclusively in py48S-open, which could stabilize binding of TC and eIF1 to the scanning PIC prior to AUG recognition. A portion of eIF2alpha domain-I projects into the entry channel and contacts the beta-hairpin of uS7/Rps5 and nucleotides just upstream of the AUG codon, including the key –3 nucleotide of the Kozak consensus sequence for efficient AUG selection, consistent with a proposed role for eIF2alpha in start codon recognition.

Exit-channel beta-hairpin of 40S ribosomal protein Rps5/u7 is a critical determinant of efficient and accurate translation initiation.

In the py48S complexes described above, the beta-hairpin of 40S protein Rps5/uS7 protrudes into the mRNA exit-channel, contacting the TC and mRNA context nucleotides; however, its importance in AUG selection was unknown. We identified substitutions in beta-strand 1 (E144R) and a nearby C-terminal residue (R225K) of yeast Rps5 that reduced bulk initiation, conferred leaky scanning of an upstream AUG, and lowered initiation fidelity by exacerbating the effect of poor context of the eIF1 start codon to thereby reduce eIF1 abundance. After reinstating WT eIF1 abundance with an extra copy of its gene (*SUI1*), both Rps5 substitutions were found to suppress UUG initiation, consistent with their discrimination against the poor-context SUI1 AUG codon. Consistent with this, *in vitro* analysis of mutant ribosomes showed that E144R greatly destabilized $P_{(IN)}$, increasing the dissociation rate (k_{off}) of TC from reconstituted 43S-mRNA PICs with AUG or UUG start codons. Other substitutions in beta-hairpin loop residues also elevated initiation fidelity, suppressing UUG initiation in cells expressing Sui^- variant eIF2beta-S264Y; one such mutant (R148E) destabilized P-in at UUG, but not AUG, start codons in PICs reconstituted with the eIF2beta-S264Y form of eIF2. Thus, the Rps5 beta-hairpin is crucial for efficient and accurate start codon recognition *in vivo*.

Genome-wide analysis of translational efficiency reveals distinct but overlapping functions of yeast DEAD-box RNA helicases Ded1 and eIF4A.

RNA helicases eIF4A and Ded1 are believed to resolve mRNA secondary structures that impede ribosome attachment to the mRNA or subsequent scanning to the start codon, but whether they perform unique or overlapping functions *in vivo* is poorly understood. We compared the effects of mutations in Ded1 or eIF4A on global translational efficiencies (TEs) in yeast by ribosome footprint profiling. Despite similar reductions in bulk translation, inactivation of a cold-sensitive Ded1 mutant substantially reduced the TEs of over 600 mRNAs, whereas inactivation of a temperature-sensitive eIF4A mutant yielded fewer than 40 similarly impaired mRNAs. Ded1–dependent mRNAs exhibit greater than average 5′ UTR length and propensity for secondary structure, implicating Ded1 in scanning though structured 5′ UTRs. Reporter assays confirmed that cap-distal stem-loop insertions raised dependence on Ded1 but not on eIF4A for efficient translation. Our findings suggest that Ded1 is critical to promote scanning through secondary structures in 5′ UTRs, while eIF4A promotes a step of initiation common to virtually all yeast mRNAs.

Rli1/ABCE1 recycles terminating ribosomes and controls reinitiation in 3′ UTRs *in vivo*.

Ribosome recycling occurs after release of completed polypeptides at stop codons, and involves dissociation of the 60S subunit from the 80S post-termination complex (post-TC). Rli1/ABCE1 (translation initiation factor/ATP-binding cassette E1) catalyzes this reaction *in vitro*, but it was unknown whether Rli1 is essential for recycling *in vivo*. Ribosome profiling of yeast cells depleted of Rli1 revealed accumulation of 80S ribosomes at the stop codons and adjoining 3′ UTRs of most genes, consistent with defective recycling that allows ribosomes to proceed into 3′ UTRs. The distribution of footprints indicates that 3′ UTR ribosomes are translating, with footprint peaks at 3′ UTR stop codons in all three frames, which are generally diminished by in-frame upstream stop codons and exhibit the atypical long fragment sizes characteristic of ribosomes at canonical stop codons. Moreover, histidine starvation evokes footprint peaks at 3′ UTR histidine codons upstream from prominent stop codon–stall sites in the same reading frame. Novel 3′ UTR translation products were detected by western analysis after inserting epitope tags immediately before stop codon-stall sites. The small sizes of the tagged 3′ UTR translation products, combined with their sequences determined by mass spectrometry, indicate that un-recycled post-TCs migrate a short distance from the stop codon and reinitiate translation without apparent codon preference. In a double mutant lacking also the 80S rescue factor Dom34, Rli1 evokes a dramatic increase in 3′ UTR ribosomes at densities comparable to the upstream coding sequences, indicating that Dom34 is responsible for rescuing unrecycled 80S ribosomes. Overexpressing Dom34 in wild-type eliminates the low-level 80S occupancy in 3′ UTRs and the expression of a tagged 3′ UTR reinitiation product, indicating that native Rli1 is insufficient to prevent all 3′ UTR translation by post-TCs in normal cells. Thus, Rli1 is required for ribosome recycling *in vivo* and acts with Dom34 to prevent aberrant reinitiation and translation of 3′ UTRs (see model in Figure).

Analysis of factors mediating nucleosome disassembly at Gcn4 target gene promoters *in vivo*

A key unsolved aspect of transcriptional activation by Gcn4 is how it mediates the eviction of nucleosomes that occlude promoter DNA sequences and block access by GTFs (general transcription factors) and Pol II. Indeed, the mechanism of this key step of gene activation and the impact of defective nucleosome eviction on transcription are not fully understood for any yeast genes. Previous studies implicated histone chaperones, chromatin remodelers, or histone acetyltransferases in the remodeling or eviction of nucleosomes from the promoters of certain yeast genes, but it was unclear whether these co-factors function broadly in nucleosome eviction, given that co-factor requirements at most yeast promoters are unknown. Eviction of promoter nucleosomes is considered to be rate-limiting for transcriptional activation, but the consequences of impaired nucleosome eviction on transcription have not been analyzed genome-wide. We addressed these questions by analyzing histone H3 eviction for the hundreds of genes in the Gcn4 transcriptome on induction of Gcn4 in a large panel of mutants lacking one or more co-factors implicated at particular yeast genes. By conventional chromatin immunoprecipitation analysis (ChIP) of four canonical Gcn4 target genes, *ARG1, HIS4, ARG4,* and *CPA2,* we excluded a requirement for several co-factors implicated previously at other genes (e.g., Asf1, Nap1, RSC) and implicated the remodeler SWI/SNF (Snf2), HAT Gcn5, and Hsp70 co-chaperone Ydj1 in nucleosome eviction at these Gcn4 target genes. Expanding our analysis genome-wide by H3 ChIP-Seq, we found that Snf2, Gcn5, and Ydj1 collaborate in evicting H3 from the −1 and +1 promoter nucleosomes and from the intervening nucleosome-depleted region (NDR) at a large fraction of the Gcn4 transcriptome. The three cofactors were found to function similarly at virtually all yeast promoters. Surprisingly, however, defective H3 eviction in co-factor mutants was coupled with reduced transcription (Pol II densities measured by Rpb3 ChIP-Seq) for a subset of genes only, which included the induced Gcn4 transcriptome and the most highly expressed subset of constitutively expressed yeast genes. In fact, the most weakly expressed genes displayed an increase in transcription relative to other genes in response to global attenuation of nucleosome eviction. Thus, we established that steady-state eviction of promoter nucleosomes is required for maximal transcription of highly expressed genes, and that Gcn5, Snf2, and Ydj1 function broadly in this step of gene activation, but discovered unexpectedly that some other aspect of transcriptional activation is more generally rate-limiting for transcription of most genes in amino acid–deprived yeast.

PUBLICATIONS

1. Zhang F, Saini AK, Shin BS, Nanda J, Hinnebusch AG. Conformational changes in the P site and mRNA entry channel evoked by AUG recognition in yeast translation preinitiation complexes. *Nucleic Acids Res* 2015; 43:2293–2312.
2. Sen ND, Zhou F, Ingolia NT, Hinnebusch AG. Genome-wide analysis of translational efficiency reveals distinct but overlapping functions of yeast DEAD-box RNA helicases Ded1 and eIF4A. *Genome Res* 2015; 25:1196–1220.
3. Young DJ, Guydosh NR, Zhang F, Hinnebusch AG, Green R. Rli1/ABCE1 recycles terminating ribosomes and controls reinitiation in 3'UTRs in vivo. *Cell* 2015; 162:872–884.
4. Llacer JL, Hussain T, Marler L, Aitken CE, Thakur A, Lorsch JR, Hinnebusch AG. Conformational differences between open and closed states of the eukaryotic translation initiation complex. *Mol Cell* 2015; 59:399–412.
5. Visweswaraiah J, Pittman Y, Dever TE, Hinnebusch AG. The ß-hairpin of 40S exit channel protein Rps5/uS7 promotes efficient and accurate translation initiation in vivo. *eLife* 2015; 4:e07939.

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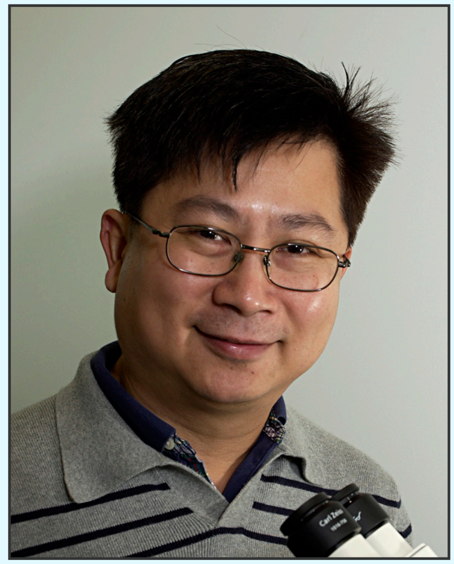
Assembly and Function of Chromatic Circuits in *Drosophila*

Using the *Drosophila* visual system as a model, we study how neurons form complex yet stereotyped synaptic connections during development and how the assembled neural circuits extract visual attributes, such as color and motion, to guide animal behaviors. To study visual circuit functions, we combine structural and functional approaches to map visual circuits. By targeted manipulation of neuronal activity, we identified specific neurons that are functionally required for color-driven behaviors. Using both light- and electron-microscopy (EM) studies, we mapped their synaptic circuits. For circuit development, we focus on the formation of synaptic connections between the chromatic photoreceptors and their synaptic partners in the medulla neuropil. We used high-resolution imaging techniques and genetic manipulations to delineate the molecular mechanisms that control dendritic patterning and synaptic specificity of the medulla neurons.



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Mapping color-vision circuits

Most visual animals utilize spectral information in two major ways: color vision, which differentiates spectral compositions independent of brightness, provides animals, from insects to primates, with great power for object recognition and memory registration and retrieval; innate spectral preference, on the other hand, is intensity-dependent and reflects individual species' specific ecological needs. Using a combination of genetic, histological, electrophysiological, and behavioral approaches, we study how visual circuits process chromatic information to guide behaviors in *Drosophila*. The receptor mechanism for color vision and spectral preference is well understood but not post-receptor processing of chromatic information. Our strategy is to: (1) identify key neuronal types and to map their synaptic connections; (2) examine the functional requirement of identified neurons for color vision and spectral preference behaviors; and (3) determine the neurotransmitter and receptor systems utilized in synaptic circuits.

Using molecular genetics and histology, we mapped the synaptic circuits of the chromatic photoreceptors R7 and R8 and their synaptic target neurons, Tm and Dm neurons, in the peripheral visual system. The medulla projection (Tm) neurons, analogous to vertebrate retinal ganglion cells, relay photoreceptor information to higher visual centers while the distal medulla (Dm) neurons connect photoreceptors to Tm neurons. Using a modified GRASP (GFP reconstitution across synaptic partners) method, we characterized the synaptic connections between photoreceptors and the Tm/Dm neurons. We found that the chromatic photoreceptors R7 (UV-sensing) and R8 (blue/green-sensing) provide inputs to a subset of first-order interneurons. The first-order interneurons Tm9, Tm20, and Tm5c receive direct synaptic inputs from R8s while Tm5a/b receive direct synaptic inputs from R7s. The Tm neurons relay spectral information from the medulla to the lobula, the deep visual processing center. In addition to the direct pathways from photoreceptors to Tm neurons, the amacrine neuron Dm8 receives inputs from multiple R7s and provides input for Tm5a/b/c.

To assign neurons to innate spectral preference, we systematically inactivated various first-order interneurons and examined the behavioral consequences.

We had previously found that the amacrine Dm8 neurons, which receive UV–sensing R7 photoreceptor inputs, are both required and sufficient for animals' innate spectral preference to UV light. We found that inactivating Tm5c, one of Dm8's synaptic targets, abolished UV preference, indicating that Tm5c is the key downstream targets for spectral preference. Using the single-cell transcript profiling technique, we found that both Dm8 and Tm5c express the vesicular glutamate transporter (VGlut), suggesting that they are glutamatergic. RNAi-knockdown of VGlut in Dm8 or Tm5c significantly reduced UV preference, suggesting the critical functions of the glutamatergic output of Dm8 and Tm5c. We further found that Tm5c expresses four kainite-type glutamate receptors and that RNAi–knockdown of these receptors, which prevents Tm5c from receiving Dm8 inputs, significantly reduced UV preference. Thus, the R7s→Dm8→Tm5c connections constitute a hard-wired glutamatergic circuit for detecting dim UV light.

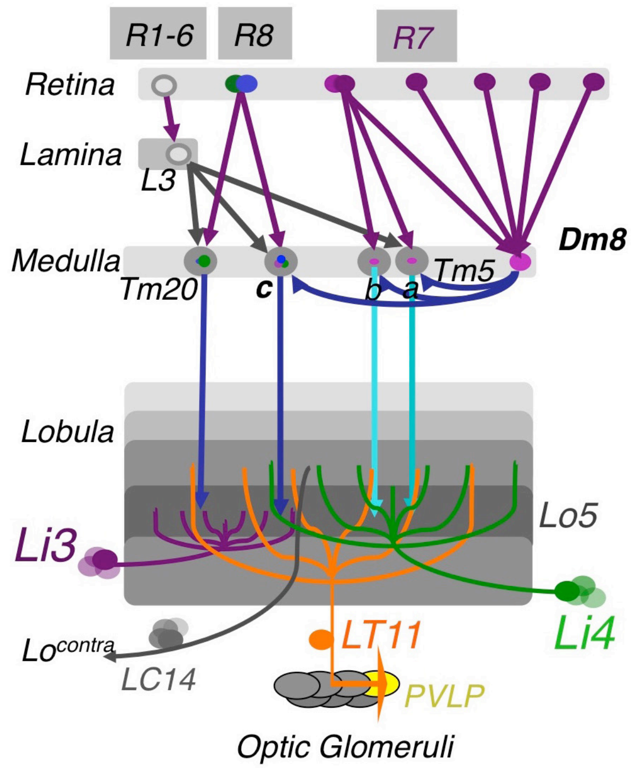

Figure 1. The chromatic circuit in *Drosophila*
Fly vision is mediated by three types of photoreceptors, R1–6, R7, and R8, each responding to a specific spectrum of light and connecting to different synaptic partners in the lamina and medulla neuropils. The chromatic photoreceptors R7 and R8 provide inputs for the amacrine neuron Dm8 and the transmedulla neurons Tm5a/b/c and Tm20. The transmedulla neurons transmit visual signals to four types of lobular neurons, LT11, LC14, Li3, and Li4, in the higher visual center.

To identify color-vision circuits, we developed a novel aversive operant conditioning assay for intensity-independent color discrimination. We conditioned single flies to discriminate between equiluminant blue or green stimuli. We found that wild-type flies can be trained to avoid either blue or green while mutants lacking functional R7 and R8 photoreceptors cannot, indicating that color entrainment requires the function of the narrow-spectrum photoreceptors R7 and/or R8. Inactivating all four types of first-order interneurons, Tm5a/b/c and Tm20, abolishes color learning. However, inactivating different subsets of these neurons is insufficient to block color learning, suggesting that true color vision is mediated by parallel pathways with redundant functions. The apparent redundancy in learned color discrimination sharply contrasts with innate spectral preference, which is dominated by a single pathway, R7→Dm8→Tm5c.

To determine how color information is processed in the higher visual center, we set out to identify the lobula neurons that receive direct synaptic inputs from the chromatic Tm neurons Tm5a/b/c and Tm20. We first collected and characterized available Gal4 lines for lobula expression. Second, we used our modified GRASP method to examine potential contacts between chromatic Tm neurons and the dendrites of candidate lobula neurons. We identified four types of lobula neuron that form synaptic contacts with chromatic Tm neurons: two novel lobula-intrinsic neurons, Li3 and Li4, and two lobula projection neurons, LT11 and LC14. Each LT11 elaborates a large dendritic tree to cover all the Lo4–6 lobula layers and projects an axon to optic glomeruli in the central brain. Each Li4 extends dendrites to cover about 60% of the Lo5 lobula layer. Using the single-cell GRASP method we had developed, we further characterized the synaptic connections at single-cell resolution and found that both Li4 and LT11 neurons receive inputs from all four chromatic Tm neurons. Our anatomical study suggests that the first relay in the lobula involves both spatial and chromatic integration.

Dendritic development of *Drosophila* optic lobe neurons

Assembling a functional neural circuit requires guiding axons and dendrites to appropriate regions to form synaptic connections during development. Neurodevelopmental disorders, such as Fragile X syndrome and Rett syndrome, have distinct defects in dendritic morphologies, including alteration of branching patterns, loss of branches, and changes in spine shapes and numbers. Many mental diseases, such as mental retardation, schizophrenia, and autism spectrum disorder, have hidden developmental origins, and their neurological and cognitive deficits, characteristic of neural miscommunication, are likely caused by dendritic defects. It is unclear however, how genetic defects cause dendritic patterning defects, resulting in erroneous connections and functional deficit.

We use the *Drosophila* visual neurons as a model to study dendrite development in the central nervous system. Like the vertebrate cortex and retina, the *Drosophila* optic lobe is organized in columns and layers, suggesting that fly visual neurons and vertebrate cortex neurons face similar challenges in routing their dendrites to specific layers and columns during development. In addition, the *Drosophila* visual system has several unique advantages: (1) the medulla neurons extend dendritic arbors in a lattice-like structure, facilitating morphometric analysis; (2) the synaptic partnership is known; (3) genetic tools for labeling specific classes of medulla neurons and determining their connectivity are available; (4) sensitive behavioral assays are available for quantifying functional deficits. To analyze the dendritic patterns of the medulla neurons, we developed several new techniques: a dual-imaging technique for high-resolution imaging; a registration technique to standardize and to compare dendritic patterns; and a modified GRASP method for visualizing bona fide synaptic connections at the light-microscopic level.

Using these techniques, we first analyzed the dendritic morphologies of four types of medulla neurons, Tm1, Tm2, Tm9 and Tm20. We identified four dendritic attributes: (1) over 80% of dendritic branches arise from one or two primary branching nodes in the medulla M2–3 layers; (2) dendrites project from the primary branching points in an either anterior or posterior direction (planar directions) in a type-specific fashion; (3) dendrites terminate in specific layers (layer-specific distribution) in a type-specific fashion; (4) the dendrites of four types of Tm neurons are largely confined to single medulla columns. By cluster analyses, using either PCA (principle component analysis) or information theory–based t-SNE (t-distributed stochastic neighboring embedding) algorithm, we found that layer-specific distribution of dendritic terminals and planar projection directions are the most important type-specific attributes and that they are sufficient to differentiate the Tm neurons. In sharp contrast, standard morphometric parameters, such as branch numbers and bifurcation topologies, are similar among these Tm neurons, and these parameters are incapable of

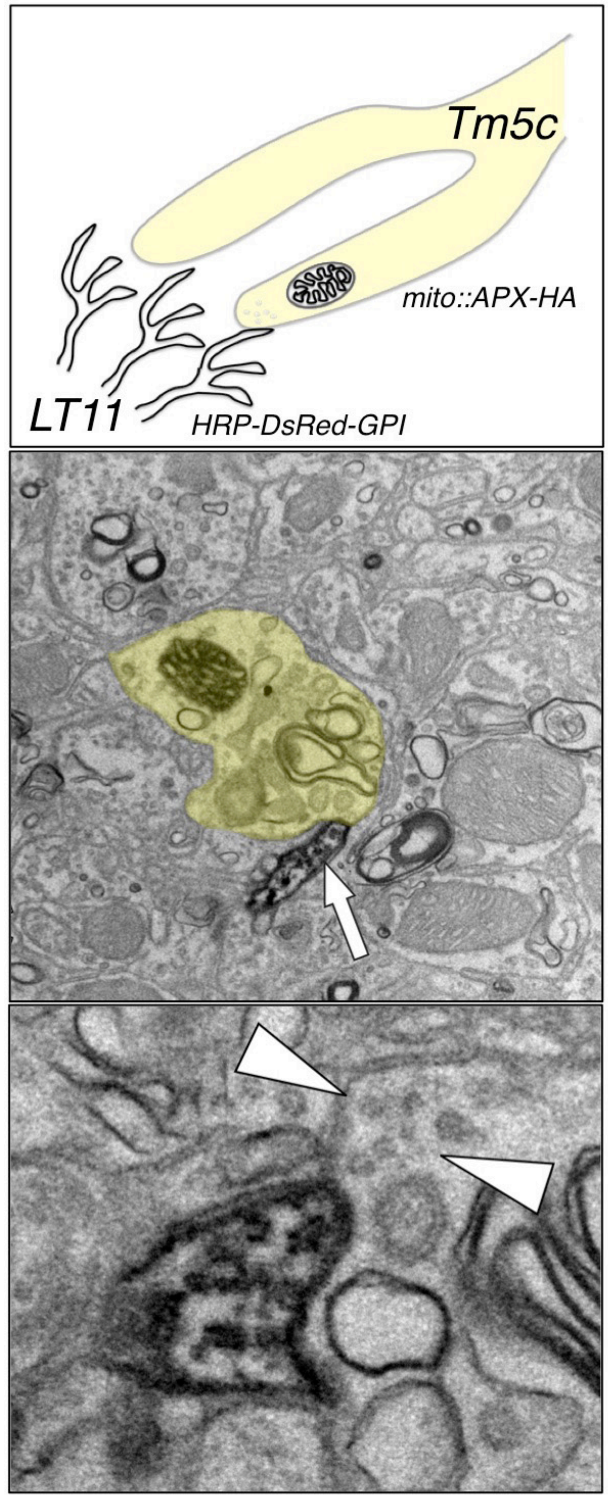

Figure 2. "Two-color" double-labeling EM
To map the synaptic circuits in complex neuropil, we developed a double-labeling method that allows the visualization of both pre- and post-synaptic partners at the ultrastructural level. We tag the presynaptic (Tm5c) mitochondria with an engineered ascorbate peroxidase and the dendritic membrane (LT11) with a membrane-tethered horseradish peroxidase. We are thus able to identify both presynaptic and postsynaptic partners in a single EM preparation.

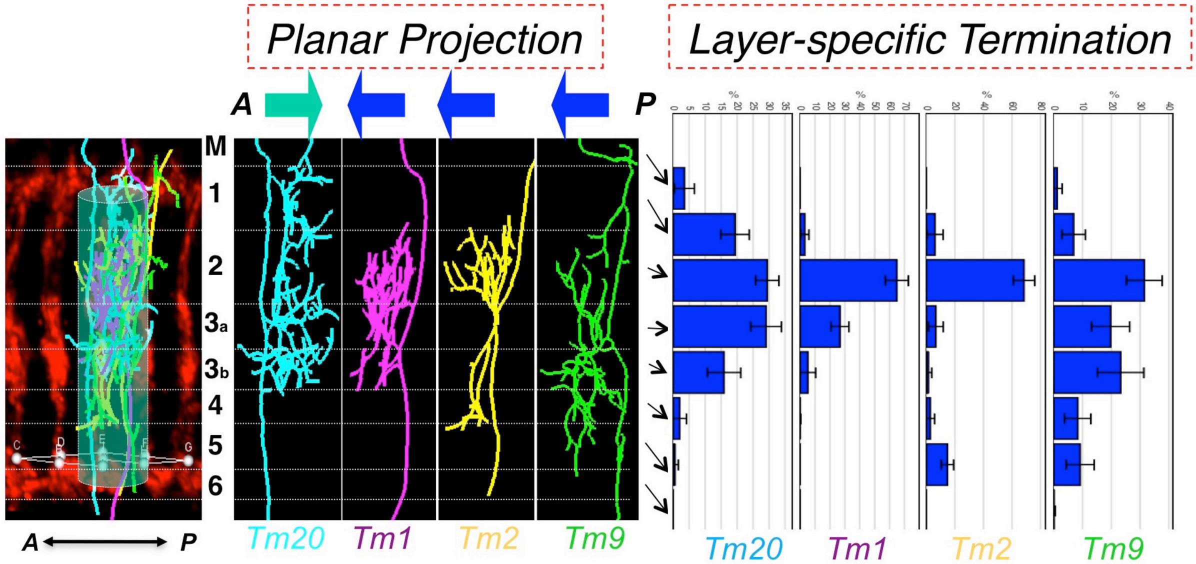

Figure 3. Type-specific dendritic attributes

By registering dendritic trees to a standard array structure, we identified two key dendritic attributes of medulla neurons: planar projections and layer-specific termination. Planar projections are the directions (anterior or posterior) of dendritic segments projecting from the axons to the medulla columns they innervate. Layer-specific termination refers to the locations of the dendritic terminals in specific medulla layers (M1–M6). The two attributes define medulla neuronal types.

categorizing Tm neurons' dendritic patterns. Furthermore, the other two attributes, layer-specific location of primary branching nodes and the dendritic field sizes, differentiate Tm1/2/9/20 from other Tm neurons and Dm8 neurons.

To determine the molecular mechanisms controlling dendritic patterning during development, we screened loss-of-function mutations for morphological defects in Tm20 and Dm8 dendrites. We identified mutations that specifically affect distinct aspects of dendritic patterning, including layer-specific location of primary branching nodes, planar projection directions, and dendritic field sizes. We focused on the components of the TGF-beta/Activin signaling pathway, which specifically affect the size of the dendritic fields of Tm20 and Dm8. Single-cell mosaic analyses revealed that mutant Tm20 lacking Activin signaling components, such as the receptor Baboon and the downstream transcription factor Smad2, elaborated an expanded dendritic tree spanning several medulla columns. Morphometric analyses based on a Kaplan-Meier non-parametric estimator further showed that *baboon* and *smad2* mutations significantly reduced dendritic termination frequency. Using a modified GRASP method, we found that the expanded dendritic tree of mutant Tm20 forms aberrant synaptic contacts with several neighboring R8 photoreceptors. In contrast, wild-type Tm20 forms synaptic connections with single R8 photoreceptors in its cognate column. Thus, the loss of Activin signaling in Tm20 neurons not only affects the size of their dendritic fields but also results in the formation of incorrect synaptic connections. Similarly, removing Baboon or Smad2 in Dm8 neurons resulted in expanded dendritic fields as compared with the wild-type. Conversely, overexpressing a dominant active form of Baboon in developing Dm8 resulted in reduced dendritic field sizes. Thus, Activin signaling negatively regulates the sizes of dendritic fields of Tm20 and Dm8.

To search for the source of TGF-beta ligands for Tm20 and Dm8, we carried out *in situ* hybridization and RT-PCR for all four potential ligands, Activin, Dawdle, Myoglianin, and Maverick, and found Activin expression in developing R7 and R8 photoreceptor neurons. RNAi–mediated knockdown of Activin in R7s and R8s caused abnormal expansion of dendritic fields of Dm8 and Tm20, respectively. The results indicate that photoreceptors R7 and R8 provide Activin specifically for their respective synaptic targets, Dm8 and Tm20. Interestingly, while the R7 and R8 growth cones were only a few micrometers apart, the Activin they provide is incapable of replacing that of the other, suggesting that Activin acts at a very short range. In summary, we found that photoreceptor-derived Activin controls the dendritic development of their respective synaptic target neurons, suggesting that anterograde Activin signaling coordinates afferent target development.

PUBLICATIONS

1. Ting C-Y, McQueen PG, Pandya N, Lin T-Y, Yang M, Reddy OV, O'Connor MB, McAuliffe M, Lee C-H. Photoreceptor-derived activin promotes dendritic termination and restricts the receptive fields of first-order interneurons in Drosophila. *Neuron* 2014; 81:830–846.
2. Karuppudurai T, Lin T-Y, Ting C-Y, Pursley R, Melnattur KV, Diao F, White BH, Macpherson LJ, Gallio M, Pohida T, Lee C-H. A hard-wired glutamatergic circuit pools and relays UV signals to mediate spectral preference in Drosophila. *Neuron* 2014 81:603–615.
3. Melnattur KV, Pursley R, Lin T-Y, Ting C-Y, Smith PD, Pohida T, Lee C-H. Multiple redundant medulla projection neurons mediate color vision in Drosophila. *J Neurogenet* 2014; 28:374–88.
4. Lin T-Y, Luo J, Shinomiya K, Ting C-Y, Lu Z, Meinertzhagen IA, Lee C-H. Mapping chromatic pathways in the Drosophila visual system. *J Comp Neurol* 2015; 524:213–27.
5. Macpherson LJ, Kearney PJ, Zaharieva EE, Lin T-Y, Turan Z, Lee C-H, Gallio M. Dynamic labelling of neural connections in multiple colours by trans-synaptic fluorescence complementation. *Nat Commun* 2015; 6:10024.

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Inherently mutagenic, the integration of retroviral and retrotransposon DNA is responsible for many pathologies, including malignancy. Given that some chromosomal regions are virtually gene free while others encode genes essential for cellular processes, the position of integration has great significance. Recent studies showed clearly that integration occurs into specific types of sequences and that the targeting patterns vary depending on the retrovirus or retrotransposon. Currently, there is great interest in such patterns, in part because understanding the mechanisms that position HIV-1 insertions may lead to new antiviral therapies. In addition, retrovirus-based vectors are now being used for gene therapy. Early gene therapy vectors had patterns of integration that activated oncogenes and caused leukemia in patients. Therefore, to gauge the risks associated with new gene therapy vectors, it is essential that we characterize in detail the positions of integration and understand the mechanisms that position such integration.

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Ultra-high throughput sequencing of transposon integration with serial number technology provides a saturated profile of target activity in *Schizosaccharomyces pombe*.

Tf1 integration is directed to the promoters of RNA pol II–transcribed genes and such integration can increase transcription. This raises important questions about the impact of integration, namely, what distinguishes the preferred promoters from those with less integration. Previously, we addressed these questions by isolating cells with integration of Tf1-*neo* and sequencing 73,000 integration sites using ligation-mediated PCR (Guo and Levin, *Genome Res* 2010;20:239-248). Over 90% of integration occurred within intergenic sequences that contained promoters, and most of the integration occurred in just 30% of promoters. This high level of variation was not the result of selection because the same pattern was obtained in diploid cells. Genes regulated by environmental stress are enriched in the preferred targets of integration. However, to understand factors that promote integration bias and to precisely define the integration pattern, we needed to overcome a serious limitation of the methods used to deep-sequence integration. High-throughput sequence runs of integration libraries produce hundreds of millions of sequence reads with large numbers of duplicates. The duplicate reads are typically the result of PCR amplification but could also arise from multiple integration events at the same chromosomal nucleotide. No method existed to distinguish between PCR bias and independent integration, so we and others published only the position of integration, not the number of independent events. The approach was reasonable until integration data sets became so large that rare sites of integration could no longer be distinguished from high frequency positions.

To overcome this limitation in sequencing, we developed a technology that measures the number of independent integration events that occur at single nucleotide positions. The technology, termed the serial number system, is based on randomizing eight base pairs in the tip of the Tf1 LTR. Each independent integration event is tagged with the "serial number" of the individual Tf1 element that was inserted. The serial numbers were introduced

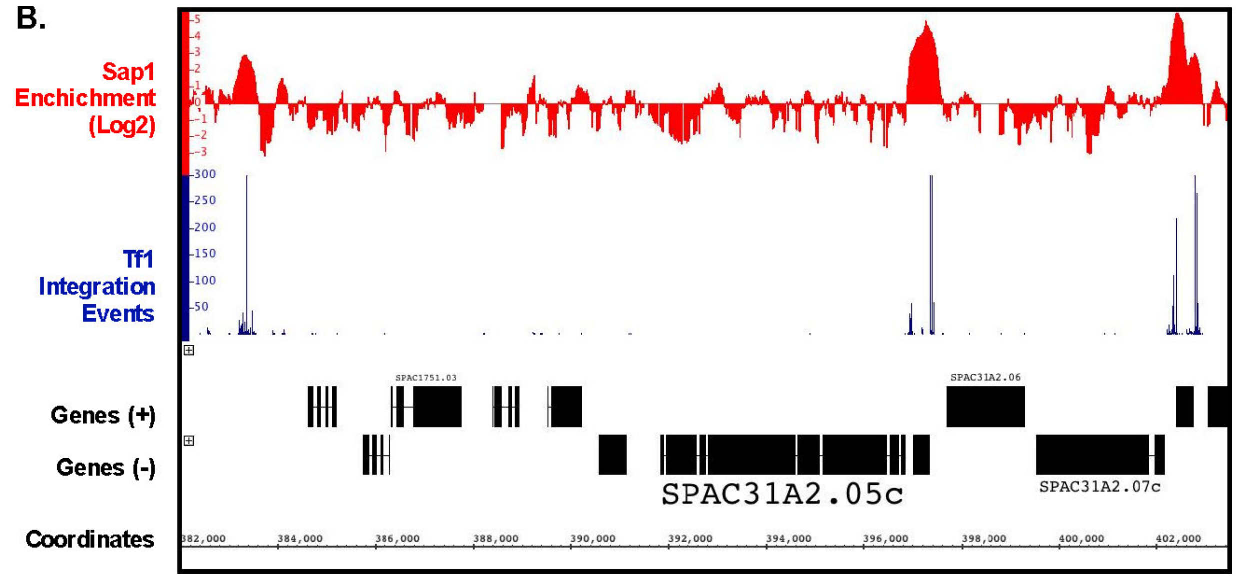

Figure 1. Serial Number integration data correlates with the position of Sap1 enrichment from ChIP-seq data.
A representative segment of chromosome 1 is shown.

in a library of 250,000 clones of the Tf1-*neo* expression plasmid. The eight base-pair serial number can record as many as 65,000 independent insertions at each nucleotide of the *S. pombe* genome. Our first application of the technique detected 1.0 million independent insertions in diploid cells distributed among 130,000 positions (Reference 1). The integration numbers at individual positions varied over two orders of magnitude. Linear regression of independent replicas showed that we obtained a highly reproducible measure of integration in the intergenic sequences. The serial number data show the wide range of integration that occurred at individual nucleotides. The data confirmed the strong preference for promoter sequences and the levels of bias that favored specific promoters, including stress-response promoters. The advantage of the serial number data is that the quantitative measures of individual sites allowed us to study what distinguishes sites with high numbers of integrations. One way to understand what features account for the high numbers of integration at specific chromosomal positions is to compare the nucleotide frequencies flanking positions with high numbers of integration to those with little integration. Logo analysis of the 150 positions with the highest numbers of integrations showed markedly higher nucleotide specificity with bit scores that were five times higher than the nucleotide composition flanking insertion sites with average numbers of integrations. This led us to ask how much influence insertion site sequence had on the genome-wide profile of integration. By ranking all integration events by the frequency of their repeated insertion, we found that sequence preference contributed to the efficiency of integration for 75% of the events. Importantly, we found that the 75% of the integration events occurred at just 33% of the total positions. Thus, the bulk of integration activity occurred at sites with a sequence signature. The sequence signature at high frequency insertion positions is just one determinant of the integration process. As described in the next section the quantitative integration data show that Sap1 is another key feature of strong integration sites.

Single nucleotide specific targeting of the Tf1 retrotransposon promoted by the DNA-binding protein Sap1 of *S. pombe*

While the serial number system identified specific sequences that contributed to integration efficiency, sequence did not account for the selection of promoters. We had tested the transcription factors known to activate stress-response promoters and found they do not contribute to the efficiency or position of Tf1 integration. However, a recent study of Switch activating protein 1 (Sap1), an essential DNA–binding protein in *S. pombe*, showed that Sap1 binds to genomic positions where Tf1 integration occurs. In order to determine whether Sap1 plays a role in Tf1 retrotransposition, we studied *S. pombe* with the temperature-sensitive mutant *sap1-1* (Reference 2). At permissive temperature Tf1, transposition is reduced ten-fold compared with wild-type *sap1+*, and the defect was not the result of decreases in levels of Tf1 proteins or cDNA. The data argue that Sap1 contributes to the integration of Tf1. A mutation that results in 10-fold less integration might be expected to cause off-

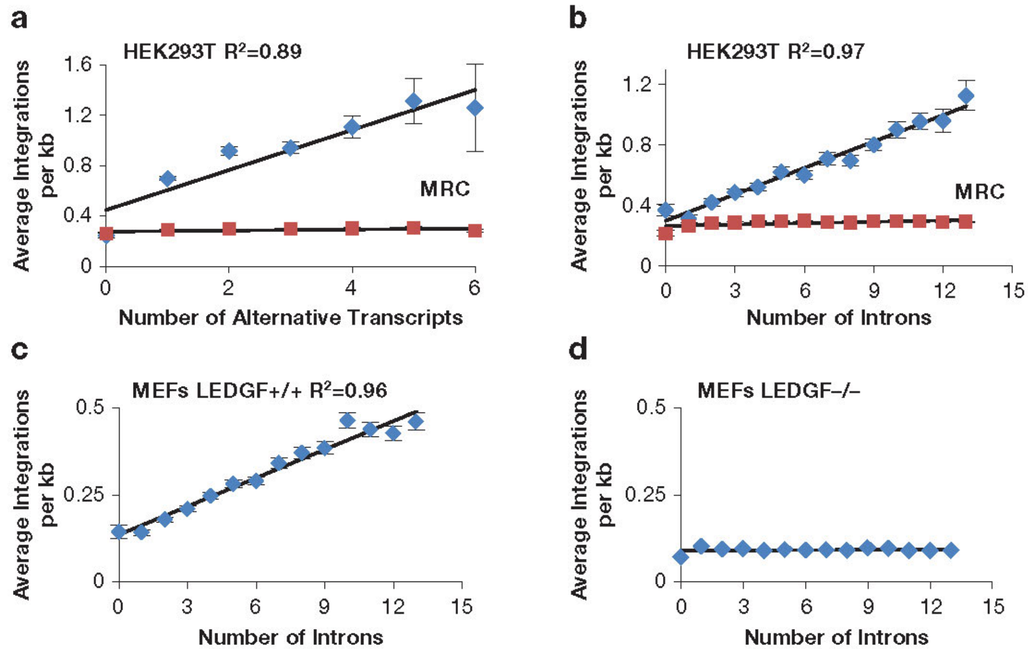

Figure 2. Integration density in transcription units correlates with amounts of splicing.
The numbers of HIV-1 integrations per kb in transcription units correlates with the amount of splicing (A and B). The preference for highly spliced transcription units depends on LEDGF (C and D).

target integration. However, serial number sequencing of integration in cells with the *sap1-1* mutation showed position changes in just 10% of the integration events.

In another approach to determine whether Sap1 contributes to integration, we compared the integration data from the serial number system with previously published maps of Sap1 binding created with ChIP-seq. Analysis of the ChIP-seq data showed that 6.85% of the *S. pombe* genome was bound by Sap1. Importantly, we found that 73.4% of Tf1 insertions occurred within these Sap1–bound sequences (Reference 2). An example of this close association can be seen in a segment of chromosome 1 (Figure 1). Another important observation is that a strong correlation was observed between levels of integration in intragenic sequences and the amount of Sap1 bound (R^2=0.98). If Sap1 were directly responsible for positioning Tf1 integration, we would expect integration to take place at specific nucleotide positions relative to the nucleotides bound by Sap1. Using the ChIP-Seq data, we were able to identify a Sap1–binding motif, which closely resembled previously published motifs. We used the FIMO program of the MEME Suite to perform genomic searches, which identified 5,013 locations that matched this motif. The alignment of all these motifs revealed that 82% of all integration events cluster within 1 kb of this motif. Importantly, 43% of all integrations occurred within 50 bp of the motif and they had two dominant positions: 9 bp upstream and 19 bp downstream of the motif. The clustering of inserts at the Sap1 motif would be expected to occur if Sap1 covers its binding site on the DNA and directs integration to either side of the protein. Thus far, we have been unable to detect a direct interaction between Sap1 and Tf1 integrase (IN) with pull-down assays. However, our two-hybrid assays detected a strong Sap1–IN interaction. The two-hybrid result together with the strong alignments of integration with Sap1 motif sequence and the reduction in integration in the *sap1-1* mutant argue that Sap1 plays an important role in Tf1 integration.

A Long Terminal Repeat retrotransposon of *Schizosaccharomyces japonicus* integrates upstream of RNA pol III transcribed gene.

Transposable elements (TEs) are common constituents of centromeres. However, it is not known what causes this relationship. *Schizosaccharomyces japonicus* contains 10 families of Long Terminal Repeat (LTR)-retrotransposons, elements that cluster in centromeres and telomeres. In the related yeast, *Schizosaccharomyces pombe*, the LTR-retrotransposons Tf1 and Tf2 are distributed in the promoter regions of RNA pol II–transcribed genes. Sequence analysis of TEs indicates that Tj1 of *S.*

japonicus is related to Tf1 and Tf2 and uses the same mechanism of self-primed reverse transcription. Thus, we wondered why these related retrotransposons localized in different regions of the genome.

To characterize the integration behavior of Tj1, we expressed it in *S. pombe* (Reference 3). We found Tj1 was active and capable of generating *de novo* integration in the chromosomes of *S. pombe*. The expression of Tj1 is similar to Type C retroviruses in that a stop codon at the end of Gag must be present for efficient integration. Seventeen inserts were sequenced, thirteen occurred within 12 bp upstream of tRNA genes and three occurred at other RNA pol III–transcribed genes. The link between Tj1 integration and RNA pol III transcription is reminiscent of Ty3, an LTR-retrotransposon of *Saccharomyces cerevisiae*, which interacts with TFIIIB and integrates upstream of tRNA genes. The integration of Tj1 upstream of tRNA genes and the centromeric clustering of tRNA genes in *S. japonicus* demonstrate that the clustering of this TE in centromere sequences is the result of a unique pattern of integration (Reference 3).

Retrotransposon Tf1 induces genetic adaptation to environmental stress.

Schizosaccharomyces pombe possesses a compact genome that tightly restricts retrotransposon expression under normal growth conditions. However, when retrotransposon Tf1 is expressed, it integrates into promoters of RNA Pol II–transcribed genes and, in many cases, this increases transcription of adjacent genes. The result, together with the Tf1 preference for stress-response promoters, led to the idea that Tf1 could be beneficial to its host by creating a pool of new alleles necessary for the host to survive changing environmental conditions. We tested the hypothesis by studying the Tf1 response to a stress such as exposure to cobalt and studying the fitness of cells with genomic insertions of Tf1 when exposed to cobalt.

Diverse cultures containing Tf1 integrated at 39,500 positions were grown competitively in cobalt. Cells with Tf1 at 141 positions greatly increased in proportion suggesting that the integrations improved growth in cobalt. Analysis of the positions and reconstruction of strains with single insertions indicate that Tf1 integration improved growth in cobalt by inducing key regulators of the TOR pathway. The results provide strong evidence that retrotransposons have the potential to promote evolution, and they identified mechanisms that mitigate the toxicity of cobalt.

Integration profiling: a whole-genome analysis of sequence function

The existing genome-wide methods for testing gene function consist largely of microarray hybridization and deep sequencing of RNA, techniques that infer function from patterns of gene expression. Despite the valuable information produced by these methods, they do not provide a direct demonstration of gene function. To address this need, we developed integration profiling, a simple method capable of directly probing the function of the single-copy sequences throughout the genome of a haploid eukaryote. With transposons that readily disrupt ORFs (open reading frames) and sequencing technology that can position over 250 million insertions per reaction, the analysis of a single culture can identify which sequences in a eukaryotic genome are functional. In previous work, we found that the ‘cut and paste’ DNA transposon Hermes from the housefly is highly active in *S. pombe*. The high rate of integration and the disruption of ORFs mean that Hermes is suitable for mutagenesis studies. With integration profiling, large populations of cells with transposon insertions are grown for many generations, depleting the culture of cells that have insertions in genes important for division. In one experiment, we passaged cells for 74 generations until 13.4% of the cells in the final culture contained an integrated copy of Hermes. We determined the positions of the insertions in the culture by ligation-mediated PCR followed by Illumina sequencing. We identified 360,000 unique insertion events that produced an average of one insertion for every 29 bp of the *S. pombe* genome (Reference 4). A survey of known essential genes revealed very few insertions per ORF, whereas neighboring nonessential gene ORFs had high numbers of insertions. Recently, a consortium systematically deleted the ORFs of *S. pombe* in heterozygous diploids and, after sporulation, designated which ORFs were essential (Kim et al., *Nat Biotechnol* 2010;28:617). Using these designations, we plotted the distribution of integration densities separately for the nonessential and essential ORFs. We also graphed the integration densities of a subclass of nonessential genes that, when deleted, resulted in small colonies. Clearly, the essential ORFs had significantly fewer insertions/kb than the nonessential ORFs, indicating that the integration profiles did indeed discriminate between essential and nonessential ORFs (Reference 4). Importantly, the nonessential ORFs required for full colony growth had intermediate densities of integration, indicating that intermediate levels of integration may be used to identify nonessential genes that nevertheless contribute to growth. The principal discrepancy between the designations made by the consortium and the Hermes integration is the group of 200 ORFs designated as nonessential, which exhibited very low levels of integration. Using PCR and DNA blotting, we found that the majority of these consortium designations were incorrect because the genes had not been successfully deleted. The results validate integration profiling as an accurate method for measuring gene function (Reference 4).

We extended the use of integration profiling to identify genes important for the formation of heterochromatin. Our initial strain contained a copy of *ura4* (gene encoding orotidine monophosphate decarboxylase) within the centromeric sequence. The heterochromatin present in the centromeric sequence silenced the expression of *ura4* and, as a result, allowed cells to grow in the presence of 5-fluorooritic acid (FOA). We then induced Hermes transposition and passaged cultures for many generations. Disruption of genes required for heterochromatin allowed *ura4* to be expressed and, as a result, inhibited growth in a medium containing FOA. To identify the positions that tolerated disruption, we sequenced the integration sites of cells in the final culture. Our data set of one million integration positions contained, on average, one insertion for every 8 bp of the genome. We found that approximately 200 genes contained significantly fewer insertions than the remainder of the genome. Importantly, this gene set contained the majority of genes previously shown to contribute to heterochromatin formation. To test directly their contribution to heterochromatin and to characterize their mode of action, we are now analyzing candidates identified by integration profiling that have not previously been studied.

LEDGF/p75 interacts with mRNA splicing factors and targets HIV-1 integration to highly spliced gene.

The promise of immunotherapy of cancer and treatment of other diseases with gene therapy relies on retroviral vectors to stably integrate the corrective/therapeutic sequences in the genomes of the patient's cells. First-generation gene therapy used vectors derived from gamma retroviruses that were successful in correcting X-linked severe combined immunodeficiency (SCID-X1). However, the integration pattern had a bias for promoter sequences that resulted in the activation of proto-oncogenes and progression to T-cell leukemia. Such adverse outcomes led to the use of lentivirus vectors for recent gene-therapy treatments. This switch to HIV-1–based vectors has occurred despite a fundamental lack of information about integration levels at specific genes, including proto-oncogenes. Structural and biochemical data show that HIV-1 IN interacts with the host factor LEDGF/p75 (a chomatin-binding protein and transcription coactivator), and the interaction favors integration in the actively transcribed portions of genes (transcription units). However, little is known about how LEDGF/p75 recognizes transcribed sequences and whether cancer genes are favored.

To measure integration levels in individual transcription units and to identify the determinants of integration-site selection, we generated a high-density map of the integration sites of a single-round HIV-1 vector in HEK293T cells (Reference 5). Improvements in sequencing methods allowed us to map 961,274 independent integration sites; most of the sites occurred in just 2,000 transcription units. Importantly, the 1,000 transcription units with the highest numbers of integration sites were highly enriched for cancer-associated genes, which raised concerns about the safety of using lentivirus vectors in gene therapy. Analysis of the integration site densities in transcription units (integration sites per kb) revealed a striking bias that favored transcription units that produced multiple spliced mRNAs and with transcription units that contain high numbers of introns (Figures 2A and 2B) (Reference 5). The correlations were independent of transcription levels, size of transcription units, and length of the introns. Analysis of previously published HIV-1 integration site data showed that integration density in transcription units in mouse embryonic fibroblasts also correlated strongly with intron number and that the correlation was absent from cells lacking LEDGF (Figures 2C and 2D). The data suggest that LEDGF/p75 not only tethers HIV-1 integrase to chromatin of active transcription units but also interacts with mRNA splicing factors. To test this, our collaborators used tandem MS to identify cellular proteins from nuclear extracts of HEK293T cells that interacted with GST-LEDGF/p75. The proteomic experiments found that LEDGF/p75 interacted with many components of the splicing machinery, including the small nuclear ribonucleic proteins (snRNP) SF3B1, SF3B2, and SF3B3 of U2 (a small nuclear RNA component of the spliceosome), U2–associated proteins PRPF8 and U2SURP, a factor of the U5 snRNP (SNRNP200), and many hnRNPs (heterologous ribonucleoproteins) that are associated with alternative splicing. The broad range of interactions with splicing factors suggested that LEDGF/p75 might contribute to splicing reactions. To test this, we performed RNAseq on HEK293T cells that were altered with TALEN endonucleases to truncate or delete the gene for LEDGF/p75, *PSIP1*. Analysis of the 11,000 transcription units that produced two or more spliced mRNA products showed that bi-allelic deletion of LEDGF/p75 significantly changed the ratio of spliced products of 4,305 transcription units (Reference 5). The results, together with our finding that integration in highly spliced transcription units was dependent on LEDGF, provide strong support for a model in which LEDGF/p75 interacts with splicing machinery and directs integration to highly spliced transcription units.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Chatterjee AG, Esnault C, Guo Y, Hung S, McQueen PG, Levin HL. Serial number tagging reveals a prominent sequence preference of retrotransposon integration. *Nucleic Acids Res* 2014; 42:8449–8460.
2. Hickey A, Esnault C, Majumdar A, Chatterjee A, Iben J, McQueen P, Yang A, Mizuguchi T, Grewal S, Levin HL. Single nucleotide specific targeting of the Tf1 retrotransposon promoted by the DNA-binding protein Sap1 of Schizosaccharomyces pombe. *Genetics* 2015; 201:905–24.
3. Guo Y, Singh P, Levin HL. A long terminal repeat retrotransposon of Schizosaccharomyces japonicus integrates upstream of RNA pol III transcribed genes. *Mob DNA* 2015; 6:19.
4. Guo Y, Park JM, Cui B, Humes E, Gangadharan S, Hung S, Fitzgerald PC, Hoe KL, Grewal SI, Craig NL, Levin HL. Integration profiling of gene function with dense maps of transposon integration. *Genetics* 2013; 195:599–609.
5. Singh P, Plumb M, Ferris A, Iben J, Wu X, Fadel H, Poeschla E, Hughes S, Kvaratskhelia M, Levin HL. LEDGF/p75 interacts with mRNA splicing factors and targets HIV-1 integration to highly spliced genes. *Genes Dev* 2015; 29:12.

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Mechanisms of Synapse Assembly, Maturation and Growth during Development

Synapse development is a highly orchestrated process coordinated by intercellular communication between the pre- and postsynaptic compartments and by neuronal activity itself. Our long-term goal is to elucidate the molecular mechanisms, particularly those involving cell-cell communication, that regulate formation of functional synapses during development and fine-tune them during plasticity and homeostasis. We focus on three key processes in synaptogenesis: (1) trafficking of components to the proper site, (2) organizing those components to build synaptic structures, and (3) maturation and homeostasis of the synapse to optimize its activity. We address the molecular mechanisms underlying these processes using a comprehensive set of approaches including genetics, biochemistry, molecular biology, super-resolution imaging, and electrophysiology recordings in live animals and reconstituted systems.

The *Drosophila* neuromuscular junction (NMJ) is a powerful genetic system in which to study mechanisms of synapse assembly and homeostasis. The fact that individual NMJs in flies can be reproducibly identified from animal to animal and are easily accessible for electrophysiological and optical analysis makes them uniquely suited for *in vivo* studies on synapse assembly, growth, and plasticity. Furthermore, the great richness of genetic manipulations that can be performed in *Drosophila* permits independent control of individual synaptic components in distinct cellular compartments. Moreover, the fly NMJ is a glutamatergic synapse similar in composition and physiology to mammalian central synapses. The *Drosophila* NMJ can thus be used to analyze and model defects in the structural and physiological plasticity of glutamatergic synapses, which are associated with a variety of human pathologies from learning, memory deficits, to autism. The similarity in architecture, function, and molecular machinery supports the notion that studying the assembly and development of fly glutamatergic synapses will shed light on their human counterparts.

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Drosophila Neto, an obligatory auxiliary subunit for NMJ glutamate receptors

Drosophila iGluRs are heterotetrameric complexes composed of three shared subunits, GluRIIC, GluRIID, and GluRIIE and either GluRIIA (type-A receptors) or GluRIIB (type-B). The shared subunits are essential for viability—without any of them the animals are completely paralyzed and die as late embryos. We recently discovered Neto (Neuropillin and Tolloid-like), a non-channel essential component of the NMJ glutamate receptor complexes required for clustering of these receptors at synaptic sites. Through live-imaging studies we showed that Neto clusters at nascent NMJs at the time when iGluRs begin to accumulate and cluster. Our genetics and biochemical analyses indicate that Neto forms a complex with the iGluRs on the muscle membrane and that the Neto/iGluRs complexes traffic together to synaptic sites, where they form stable aggregates. By controlling the clustering and trafficking of functional iGluR complexes, Neto directly controls synapse assembly, organization and maintenance of post-synaptic densities (PSDs), and NMJ functionality.

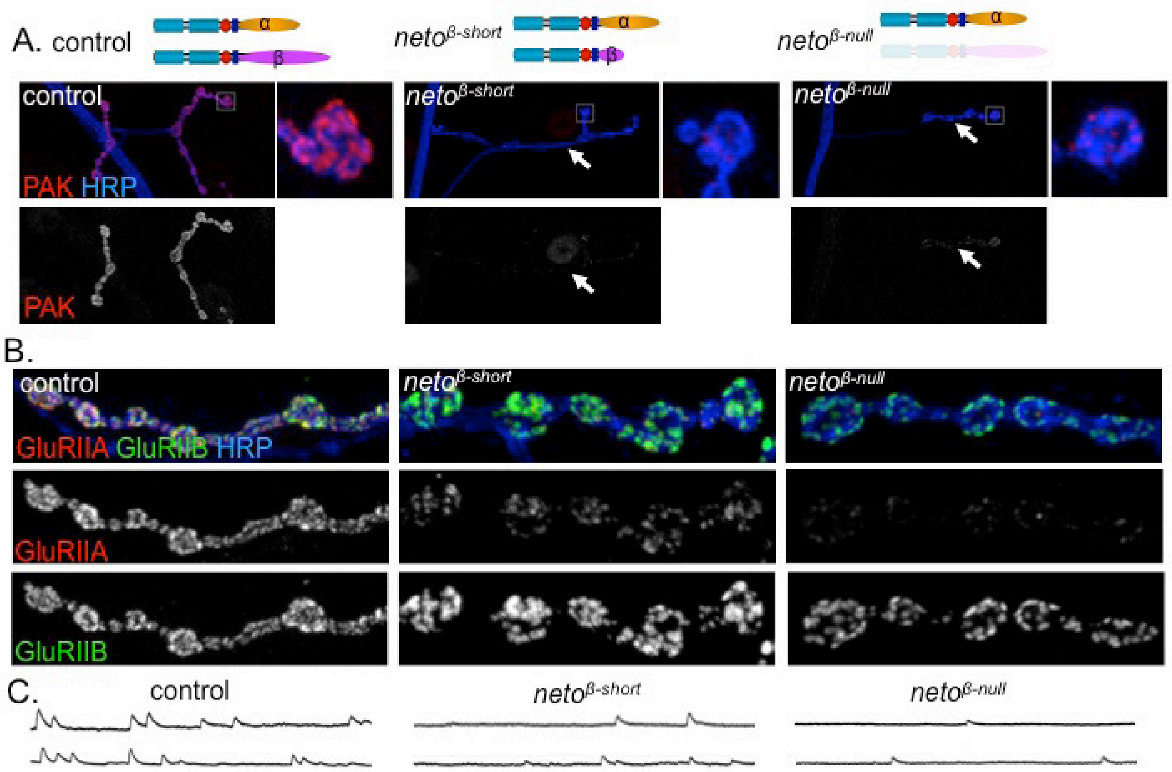

Figure 1. Neto-β–mediated intracellular interactions shape postsynaptic composition.

A. Confocal images of NMJs and bouton details in larvae of indicated genotypes labeled for P21-activated kinase (PAK) (*red*) and horse radish peroxidase (HRP) (*blue*). HRP marks neuronal membranes. In the absence of Neto-β or parts of its intracellular domain, PAK fails to be recruited at synaptic locations (*arrows*).

B. Confocal images of boutons in larvae of indicated genotypes labeled with HRP (*blue*) and for iGluR subunits GluRIIA (*red*) and GluRIIB (*green*). PAK is a postsynaptic component critical for the synaptic stabilization of a specific iGluR subtype, the type-A receptors. In the absence of PAK, the GluRIIA synaptic levels are drastically diminished, while the GluRIIB levels remain relatively constant.

C. Traces of spontaneous miniature potentials recorded from muscle 6 of third instar larvae indicate that neto-β mutants have reduced mini amplitudes, consistent with their reduced GluRIIA/GluRIIB synaptic ratio.

Neto belongs to a family of highly conserved proteins sharing an ancestral role in formation and modulation of glutamatergic synapses. Neto proteins are multidomain, transmembrane proteins with two extracellular CUB [for complement C1r/C1s, UEGF, BMP-1 (bone morphogenetic protein-1)] domains followed by an LDLa (low-density lipoprotein receptor domain class A) motif, a single transmembrane pass, and highly divergent intracellular parts. CUB domains are BMP–binding, protein-interaction domains that could promote aggregation via self-association and/or extracellular interactions. CUB–containing proteins have been implicated in the formation of multi-molecular complexes, including acetylcholine receptor aggregates in *C. elegans*.

Using Neto as our entry point, we set out to elucidate the molecular mechanisms underlying the synaptic recruitment of iGluRs and their incorporation in stable neural clusters. Given that Neto does not have any catalytic activities, we hypothesized that Neto controls synapse development by interacting with iGluRs and/or with other proteins critical for synapse development. During the last year, we focused on several key questions in iGluR cell biology and began dissecting the individual steps of iGluR assembly, surface delivery, trafficking and stabilization at synaptic locations, function, and postsynaptic composition.

Neto prodomain removal enables iGluR stabilization and postsynaptic differentiation.

Our analysis of the Neto prodomain revealed a requirement for Neto in the iGluR stabilization at synaptic sites and initiation of postsynaptic differentiation. In *Drosophila* and other species that utilize L-glutamate as a neurotransmitter at their NMJs, Neto activities are restricted by an inhibitory prodomain, which must be removed by Furin-mediated proteolysis. When the prodomain cleavage is blocked, Neto is properly targeted to the muscle membrane and engages the iGluR complexes *in vivo*, but fails to enable the incorporation of iGluRs in stable clusters at synaptic locations. The uncleavable-Neto/iGluR complexes have very weak and diffuse synaptic distribution but retain some function, that is, these NMJ have a relatively normal response to evoked potentials. However, the NMJs lack any distinguishable postsynaptic structures, as if their postsynaptic differentiation were never initiated. Thus, synapse activity alone does not trigger postsynaptic differentiation; instead, Neto-dependent iGluR clustering initiates an active process of recruitment of PSD components and assembly of postsynaptic structures. The recruitment of PSD components is likely attributable to Neto-mediated intracellular interactions (see below). Although the molecular nature of the iGluR clustering mechanism remains to be determined, our results make it clear that the process requires the removal of the Neto prodomain, presumably to unmask its protein-interaction domains.

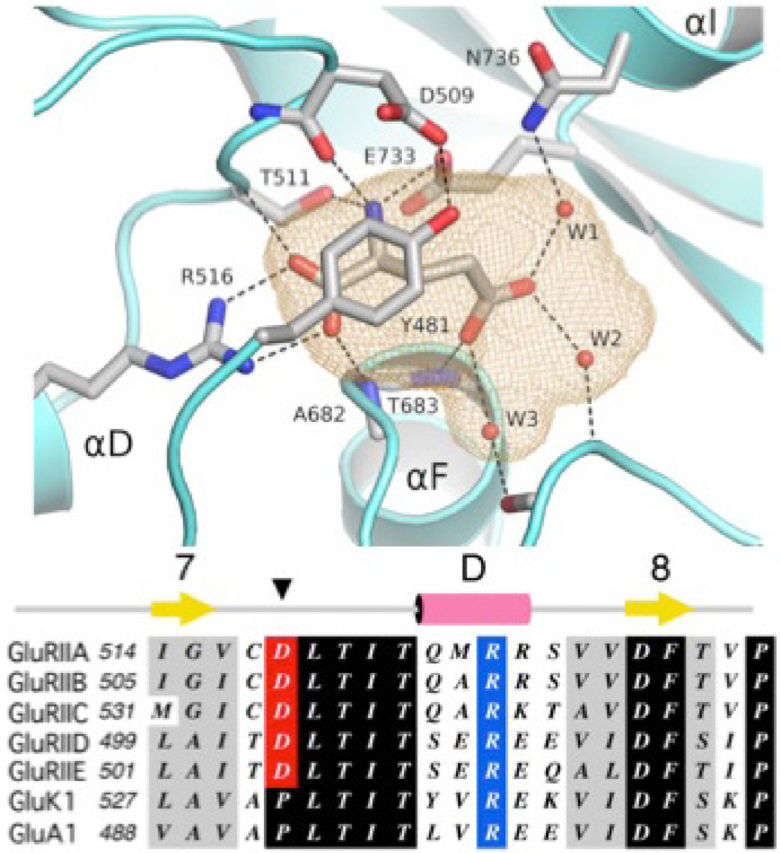

Figure 2. Structural basis for ligand selectivity of *Drosophila* iGluRs

Neto-β orchestrates postsynaptic development at the *Drosophila* NMJ.

Drosophila neto encodes two isoforms, Neto-α and Neto-β, with different cytoplasmic domains, generated by alternative splicing. The cytoplasmic domains are rich in putative phosphorylation motifs and docking sites and are highly divergent among Neto proteins, probably reflecting cell/tissue-specific roles. In flies, RT-PCR indicates that both Neto isoforms are expressed in larval carcasses. However, knockdown of Neto-β in the muscle altered the NMJ morphology, whereas knockdown of Neto-α had only a mild effect on the NMJ development.

To understand the role of Neto-β in synapse development, we generated *neto*-β isoform–specific mutants by imprecise excision of a transposable element and isolated a *neto*-β genetic null allele (*neto*-β^{null}) and a truncation of the cytoplasmic domain (*neto*-β^{short}). Both *neto*-β mutants have locomotor defects and reduced viability. During larval stages, Neto-β–defective NMJs have reduced mini frequency and amplitudes but normal evoked potentials as a result of a compensatory increase in presynaptic release. Our analysis of the *neto*-β mutants reveals that Neto is directly involved in the recruitment of postsynaptic components and organization of postsynaptic structures. The *neto*-β synapses have reduced iGluR synaptic clusters, in particular the type-A subtypes, and lack P21–activating kinase (PAK), an important PSD component (Figure 1). PAK has been previously implicated in the stabilization of type-A receptors at postsynaptic locations and in the recruitment of other postsynaptic components, such as Dlg. Indeed, *neto*-β mutants have defective postsynaptic structures, including small PSDs and diminished subsynaptic reticulum. Our developmental studies indicate that Neto-β controls PAK recruitment to synaptic sites. Neto-α can rescue the lethality of *neto*^{null} mutants, but fails to recruit PAK at synaptic locations, confirming the need for

Neto-β in this function. Given that PAK has been implicated in stabilization of selective iGluR subtypes, Neto-β emerges as a critical player required for synapse plasticity. Thus, Neto engages in intracellular interactions that regulates the iGluR subtype composition by preferentially recruiting and/or stabilizing selective receptor subtypes.

Neto is required for functional iGluRs in heterologous systems.

Given that the synaptic recruitment of iGluRs is also influenced by receptor activity, Neto may regulate iGluR clustering partly by modulating the receptor function. Studies on isolated receptors are necessary to distinguish between a role for Neto on iGluR distribution and/or receptor function. The reconstitution of functional iGluRs in heterologous systems has been a powerful tool for the analysis of receptor function in other species but has not been previously achieved for the *Drosophila* NMJ receptors. In collaboration with Mark Mayer, we recently reported the first functional reconstitution of *Drosophila* NMJ iGluRs in *Xenopus laevis* oocytes. Using this system, we found that Neto increases the glutamate-activated currents by several orders of magnitude, but has only a modest effect on the surface delivery of the iGluRs (up to four-fold increase). Genetic studies indicate that synaptic recruitment of iGluRs requires heterotetramers. We found that only iGluR heterotetramers are efficiently delivered at the cell surface, indicating that the assembly of heterotetramers is required for surface expression.

The Neto/iGluR complexes reconstituted in *Xenopus* oocytes recapitulate the properties of endogenous NMJ receptors: high permeability to Ca^{2+}, block by polyamines, low affinity for glutamate, and no response to AMPA, kainate, or NMDA. To understand the structural basis for this unique ligand-binding profile, we solved the structure of the GluRIIB ligand-binding domain complex with glutamate (Figure 2). X-ray diffraction data at a resolution of 2 Å revealed a classic back-to-back dimer assembly with glutamate bound in a large cavity, similar in size to that of AMPA and kainate receptors. However, several steric clashes owing to residues conserved in the *Drosophila* receptors prevent the binding of AMPA and kainate and thus explain the unique ligand-binding properties observed for these receptors.

Lack of synaptic iGluRs may reflect defects in receptor assembly, surface expression, synaptic trafficking and stabilization, and/or modulation of iGluR function. Our findings pave the way to understanding the changes in receptor function before interpreting the compound phenotypes observed *in vivo*.

PUBLICATIONS

1. Kim Y-J, Igiesuorobo O, Ramos CI, Bao H, Zhang B, Serpe M. Prodomain removal enables Neto to stabilize glutamate receptors at Drosophila neuromuscular junction. *PLoS Genet* 2015; 11:e1004988.
2. Ramos CI, Igiesuorobo O, Wang Q, Serpe M. Neto-mediated intracellular interactions shape postsynaptic composition at the Drosophila neuromuscular junction. *PLoS Genet* 2015; 11:e1005191.
3. Han TH, Poorva D, Mayer ML, Serpe M. Functional reconstitution of Drosophila NMJ glutamate receptors. *Proc Natl Acad Sci USA* 2015; 112:6182–6187.

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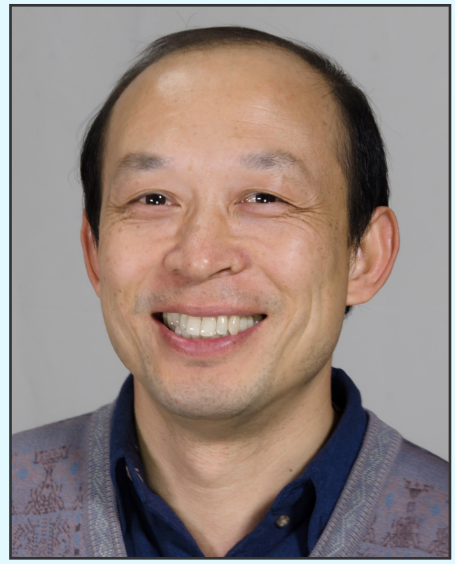
Thyroid Hormone Regulation of Vertebrate Postembryonic Development

This laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during postembryonic development. The main model is the metamorphosis of *Xenopus laevis* and *X. tropicalis*, two highly related species, that offer unique but complementary advantages. The control of their developmental process by TH offers a paradigm to study gene function in postembryonic organ development. During metamorphosis, diverse organs undergo vastly different changes. Some, like the tail, undergo complete resorption, while others, such as the limb, are developed *de novo*. The majorities of larval organs persist through metamorphosis but are dramatically remodeled to function in a frog. For example, tadpole intestine is a simple tubular structure consisting primarily of a single layer of larval epithelial cells. During metamorphosis, it is transformed into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles, a process that occurs through specific larval epithelial cell death and *de novo* development of the adult epithelial stem cells followed by their proliferation and differentiation. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both *in vivo*, by using genetic approaches or hormone treatment of whole animals, and *in vitro* in organ cultures offer an excellent opportunity to first study the developmental function of TH receptors (TRs) and the underlying mechanisms *in vivo* and second to identify and functionally characterize genes that are critical for organogenesis, in particular those responsible for the formation of adult organ-specific stem cells during postembryonic development in vertebrates. Our studies revealed likely conserved mechanisms in adult intestinal stem cell development in vertebrates, which prompted us to develop a mouse model to complement the amphibian system for gene knockout studies.



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Adapting TALEN and CRISPR technologies for studying GENE function during metamorphosis

In the past, gene knockout and knockdown in *Xenopus* tadpoles had been very difficult, if not impossible. We recently successfully adapted the TALEN (transcription activator-like effector nucleases, artificial restriction enzymes) and CRISPR (clustered regularly interspaced short palindromic repeats, a gene-editing tool) technologies to knockdown endogenous genes in *Xenopus* tadpoles by microinjecting TALEN and CRISPR RNAs into fertilized eggs (References 1 and 2). Importantly, we showed that we could achieve very high efficiencies of mutations in the target genes in the resulting premetamorphic tadpoles, making it possible to carry out functional studies directly on these tadpoles, the so called F0 genetic studies, which eliminates the need for F1 or F2 generation animals, thus allowing much faster determination of gene function. This has enabled us to investigate the roles of TRα and a histone methyltransferase, a likely TR coactivator as described below.

A novel function for TRα during development

We developed a TALEN that could mutate the *X. tropicalis* gene encoding TRα with over 90% efficiency by injecting TALEN mRNAs into fertilized eggs, making it possible to analyze the role of TRα in the resulting F0 animals (Reference 3). Consistent with our dual function model for TR, we observed that knocking down TRα accelerated animal development, with the knockdown animals reaching the onset of metamorphosis earlier (Figure 1). On the other hand, they were resistant to exogenous triiodothyronine (T3) treatment and had delayed natural metamorphosis. Thus, our studies directly demonstrated a critical role of endogenous TRα both in mediating the metamorphic effect of T3 during metamorphosis and in preventing precocious initiation of metamorphosis when T3 is absent. Surprisingly, we also found that TRα knockdown enhanced tadpole growth in premetamorphic tadpoles. This novel function of unliganded TRα (as little T3 is present during premetamorphosis) appears to be attributable to increased growth hormone gene expression. Further analyses suggest that the two functions of unliganded TRα during metamorphosis, i.e., regulating tadpole growth rate and the time to reach the onset of metamorphosis, are independent of each other. While this is the first direct evidence for a critical role of unliganded TR in vertebrate development, TRa knockout studies suggest that unliganded TRa is important for postembryonic regulation of heart rate and gene expression. Similarly, deleting the gene encoding the T3-inactivating enzyme type 3 deiodinase causes auditory defects, arguing for the importance of maintaining a very low level of T3, which would lead to more unliganded TR, for cochlear development. Thus, unliganded TR may also be important for postembryonic development in mammals.

Identification of the histone methyltransferase Dot1L as a direct target gene of TR and its essential role in premetamorphic tadpole growth

We earlier identified Dot1L, the only histone methyltransferase (HMT) capable of methylating histone H3K79 *in vitro*, as a direct target gene of T3. Interestingly, the level of H3 lysine79 (H3K79) methylation is strongly enhanced by T3 at TR target genes, suggesting that Dot1L is upregulated by TR and, in turn, functions as a TR coactivator. To investigate Dot1L function, we generated a Dot1L–specific TALEN that was extremely efficient in mutating Dot1L when expressed in *X. tropicalis* fertilized eggs, creating animals with almost no Dot1L and little H3K79 methylation (Reference 1). We observed that Dot1L knockdown had no apparent effect on embryogenesis, whereas it severely retarded tadpole growth and led to tadpole lethality before metamorphosis. The findings suggest that Dot1L and H3K79 methylation play an important role in tadpole growth and development prior to metamorphosis. Our analyses further revealed interesting similarities between *Xenopus* and mouse development and suggested the existence of two separate phases of vertebrate development with distinct requirements for epigenetic modifications.

Requirement of T3-induced Sonic hedgehog paracrine signaling for adult intestinal development

We have demonstrated that T3 activates the transcription of the Sonic hedgehog (*shh*) gene directly at the transcriptional level during intestinal metamorphosis and that exogenous Shh promotes cell proliferation in intestinal organ cultures. To determine the role of Shh during metamorphosis, we treated premetamorphic tadpoles with the Shh inhibitor cyclopamine and observed that it specifically inhibited the expression of the Shh response genes *snai2* and *twist1* (Reference 4). More importantly, cyclopamine reduced the proliferation of both developing adult stem cells in the epithelium and cells in the other intestinal tissues at the climax of metamorphosis, leading to delayed/incomplete remodeling of the intestine at the end of metamorphosis. We further revealed that both Snai2 and Twist1 were strongly upregulated specifically in the connective tissue during intestinal metamorphosis, suggesting that Shh signals the connective tissue to promote stem cell proliferation and the formation of the adult intestine. Consistently, we found that the Shh receptor Patched (Ptc)-1 and the signaling protein Smoothened (Smo), as well as the downstream transcription factors Gli1, Gli2, and Gli3, were all transiently up-regulated in the mesenchymal tissues, but not the epithelium, where Shh was induced by T3, during intestinal metamorphosis. We also showed, in intestinal organ cultures, that overexpression of Shh enhanced the expression of Ptc-1, Smo, and Glis even in the absence of T3, indicating that Shh regulates the components of its own pathway during intestinal remodeling. Thus, Shh signaling induced by T3 is required for adult intestinal stem-cell proliferation and functions via a paracrine mechanism.

Essential role of System L1 amino acid/thyroid hormone transporter in mouse development

The frog model offers a unique opportunity to identify and functionally characterize novel genes that are involved in the development of the adult intestine, particularly the adult stem cells. Interestingly, in all vertebrates, the formation/maturation of the adult intestine takes place around the time when plasma T3 levels are high. T3 or TR deficiency in mouse leads to abnormal intestinal morphology and a reduction in stem cell proliferation in the adult, and liganded TRa1 regulates stem cells during mouse intestinal maturation and homeostasis. Furthermore, we showed that many genes with peak levels of expression at the climax of metamorphosis are upregulated during mouse intestinal maturation. Our earlier studies suggest a conserved role of the histone 4 methyltransferase PRMT1 in stem cell development across vertebrates. Thus, we hypothesized that the formation of adult intestinal stem cells utilize conserved mechanisms, including regulation by T3 and the involvement of conserved T3 target genes during vertebrate development. To test our hypothesis, we initiated studies to take advantage of the ability to generate conditional knockout mice to investigate the developmental roles of the mouse homologs of the novel stem cell genes that we have discovered.

One of the genes, *Lat1*, that we previous discovered as a T3 response gene in the intestine during metamorphosis encodes the catalytic subunit (light chain) of the heterodimeric System L1 amino acid transporter. Interestingly, when LAT1 was co-expressed with the heavy chain (CD98hc) of the transporter, it mediated the intracellular uptake of T3 and, more importantly, enhanced transcriptional activation by TR in the presence of T3. Thus, LAT1 is activated by T3 and, in turn, may function to enhance the effect of T3 on adult intestinal development. We since showed that targeted disruption of the gene encoding CD98hc to specifically inactivate the heavy chain's ability to form a functional transporter with LAT1 led to mouse embryonic lethality, suggesting that LAT1 transporter activity is critical for organogenesis in mouse. In addition, we generated a mouse line with Floxed *Lat1* gene, which, when crossed with a mouse expressing the Cre recombinase, leads to the inactivation of *Lat1* gene. When it was crossed with mice expressing Cre driven by a global promoter, we observed that homozygous *Lat1* knockout animals were embryonic lethal (Reference 5), consistent with the findings on *Cd98hc* mutant animals. The findings suggest that LAT1 is critical for mouse organogenesis. Thus, it will be of interest to investigate whether LAT1 affects the development of the adult mouse intestinal stem cells by functioning as a T3 transporter.

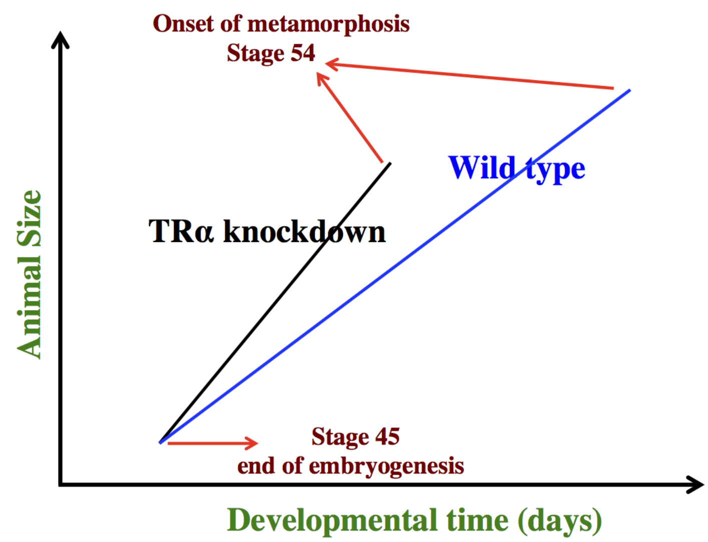

Figure 1. Schematics showing the dual effects of TRα knockdown on premetamorphic development in *Xenopus tropicalis*

TRα knockdown has little effect on embryogenesis, and the resulting tadpoles are normal by feeding stage (stage 45). Once feeding begins, the animals grow differently, with the knockdown ones growing faster, and thus larger when compared to wild-type siblings at the same age (in days) (compare the vertical axis values of the lines for the knockdown and wild-type animals at any given position along the horizontal axis between stages 45 and 54). The knockdown animals also have faster development, reaching developmentally more advanced stages than do wild-type siblings at the same age (in days). Thus, the knockdown animals reach stage 54, the onset of metamorphosis, at younger age (see the horizontal axis locations for the upper end of the lines). Interestingly, when the animals at stage 54 are compared, the wild-type ones are larger than the knockdown siblings, even though the latter grow faster. This is because the wild-type animals take longer to reach metamorphosis (stage 54). The extra growth time needed to reach stage 54 enables the wild-type ones to catch up and surpass the knockdown ones in size. The results indicate that the effects of TR on growth and development are not dependent on each other (as otherwise, the size of the animals at stage 54 would be identical between wild type and knockdown).

ADDITIONAL FUNDING

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PUBLICATIONS

1. Wen L, Fu L, Guo X, Chen Y, Shi Y-B. Histone methyltransferase Dot1L plays a role in postembryonic development in Xenopus tropicalis. *FASEB J* 2015 29:385–393.
2. Wang F, Shi Z, Cui Y, Guo X, Shi Y-B, Chen Y. Targeted gene disruption in Xenopus laevis using CRISPR/Cas9. *Cell Biosci* 2015 5:15.
3. Wen L, Shi Y-B. Unliganded thyroid hormone receptor a controls developmental timing in Xenopus tropicalis.

Endocrinology 2015 156:721–734.
4. Wen L, Hasebe T, Miller TC, Ishizuya-Oka A, Shi Y-B. A requirement for hedgehog signaling in thyroid hormone-induced postembryonic intestinal remodeling. *Cell Biosci* 2015 5:13.
5. Poncet N, Mitchell FE, Ibrahim AFM, McGuire VA, English G, Arthur SC, Shi Y-B, Taylor PM. The catalytic subunit of the System L1 amino acid transporter (Slc7a5) facilitates nutrient signaling in mouse skeletal muscle. *PLoS One* 2014 9:e89547.

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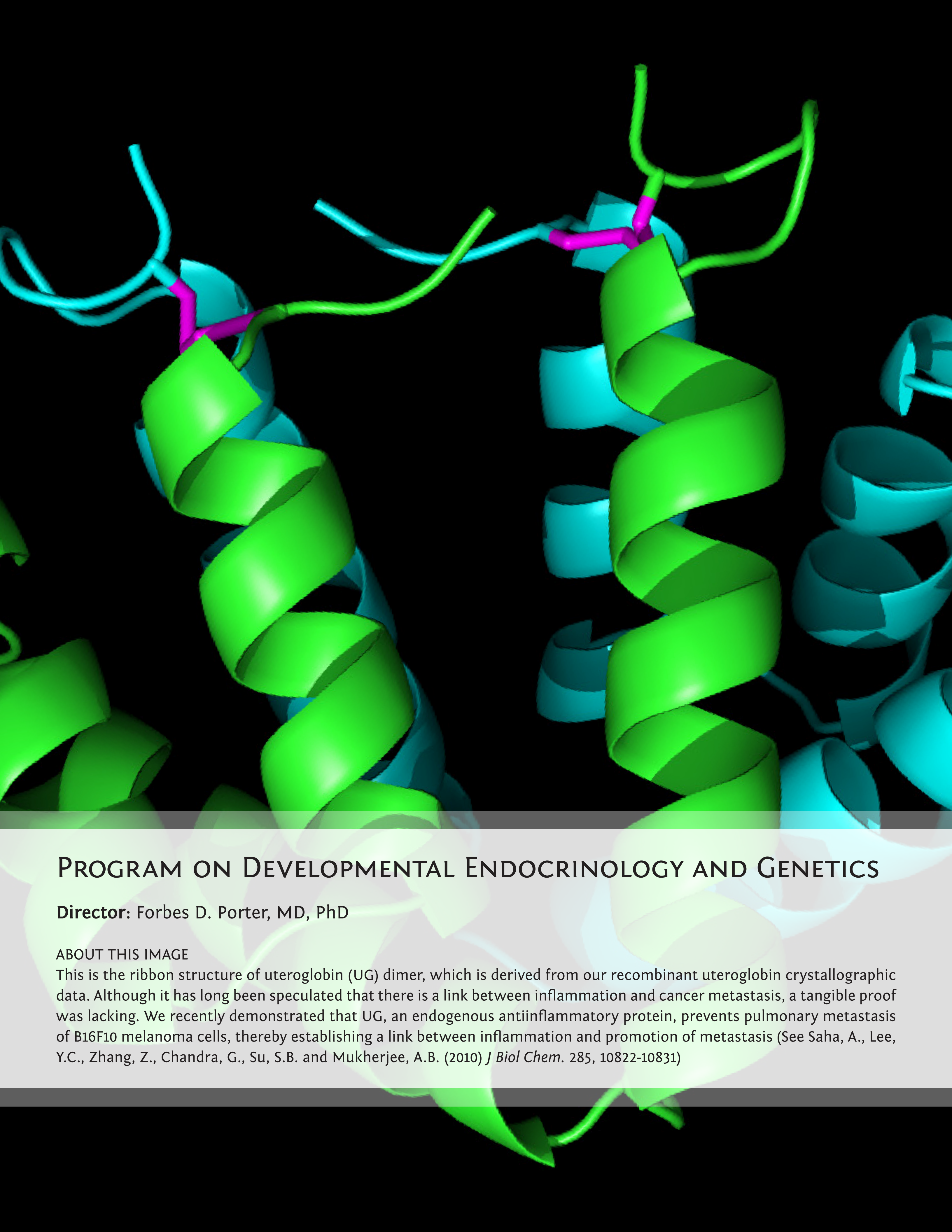

Program on Developmental Endocrinology and Genetics

Director: Forbes D. Porter, MD, PhD

ABOUT THIS IMAGE

This is the ribbon structure of uteroglobin (UG) dimer, which is derived from our recombinant uteroglobin crystallographic data. Although it has long been speculated that there is a link between inflammation and cancer metastasis, a tangible proof was lacking. We recently demonstrated that UG, an endogenous antiinflammatory protein, prevents pulmonary metastasis of B16F10 melanoma cells, thereby establishing a link between inflammation and promotion of metastasis (See Saha, A., Lee, Y.C., Zhang, Z., Chandra, G., Su, S.B. and Mukherjee, A.B. (2010) *J Biol Chem*. 285, 10822-10831)

Program *on* Developmental Endocrinology and Genetics

The primary mission of the *Program on Developmental Endocrinology and Genetics (PDEGEN)* is to carry out basic, translational, and clinical research focusing on endocrine and genetic disorders. Research conducted in the PDEGEN includes projects designed to improve our understand and treatment of childhood growth disorders, glycogen storage diseases, disorders of puberty and reproduction, lysosomal storage diseases, disorders of drug metabolism, inborn errors of cholesterol synthesis, endocrine tumors, and childhood obesity. A second goal of the PDEGEN is to provide training in these areas of research in order to help educate the next generation of scientists. The education is provided both to clinical science trainees participating in an accredited pediatric endocrinology fellowship training program and to basic science trainees in a variety of disciplines.

Jeffrey Baron's *Section on Growth and Development* investigates the cellular and molecular mechanisms governing childhood growth and development. The basic research performed in this Section focuses on the mechanisms that allow rapid cell proliferation and body growth in young children and that subsequently suppress proliferation, causing body growth to slow and eventually halt by adulthood. The group discovered that juvenile growth deceleration is attributable in part to a multi-organ genetic program, which involves the down-regulation of a large set of growth-promoting genes. Recent studies focused on the molecular mechanisms orchestrating this program. Elucidating such growth-limiting mechanisms not only provides insight into childhood growth disorders but also has broader medical applications because disruption of these regulatory systems contributes to oncogenesis; conversely, transient therapeutic suspension of these regulatory systems in adult cells might be used to achieve tissue regeneration. For the Section's clinical research, one major focus involves the use of high-throughput sequencing and other molecular-genetic approaches to study patients with growth disorders, including both growth failure and overgrowth.

Janice Chou's *Section on Cellular Differentiation* conducts research to delineate the pathophysiology of glycogen storage disease type Ia (GSD-Ia), deficient in G6Pase-alpha (or G6PC), GSD-Ib, deficient in the glucose-6-phosphate transporter (G6PT or SLC37A4), and glucose-6-phosphatase-beta (G6Pase-beta or G6PC3) deficiency, and to develop gene therapies for these disorders. Chou's group showed that neutrophils express the G6PT/G6Pase-beta complex and that inactivation of G6PT or G6Pase-beta leads to the enhanced neutrophil apoptosis that underlies the neutropenia in G6Pase-beta deficiency and in GSD-Ib. Chou's group further showed that G6Pase-beta is essential for energy homeostasis in neutrophils and macrophages. G6Pase-beta deficiency prevents recycling of glucose from the endoplasmic reticulum to the cytoplasm, leading to neutrophil/macrophage dysfunction. More recently, Chou's group showed that the mechanism of neutrophil dysfunction in GSD-Ib arises from activation of the HIF-1alpha/PPAR-gamma pathway. Chou's Section also developed mouse models of GSD-Ia, GSD-Ib, and G6Pase-beta deficiency. Using GSD-Ia mice, the Section developed an adeno-associated virus (AAV) vector–mediated gene transfer that corrects hepatic G6Pase-alpha deficiency and prevents chronic hepatocellular adenoma formation. The group further show that AAV–treated mice expressing 3% or more of normal hepatic G6Pase-alpha activity do not develop age-related obesity or insulin resistance. The study suggests that full restoration of normal G6Pase-alpha activity would not be required to confer significant therapeutic benefits in GSD-Ia by gene therapy. The AAV vector developed by Chou's group is the leading candidate in clinical trials for the treatment of human GSD-Ia.

Angela Delaney's *Unit on Genetics of Puberty and Reproduction* investigates the mechanisms responsible for pubertal onset in children. In collaboration with the Reproductive Endocrine Unit (REU) at the Massachusetts General Hospital, and under the mentorship of William Crowley, one of the world's leading experts on disorders of gonadotropin-releasing hormone (GnRH) secretion, Delaney is conducting translational research on the neuroendocrine and genetic control of GnRH secretion and its regulation of gonadotropin secretion and gonadal physiology. The collaboration aims to phenotypically and genetically characterize subjects with isolated hypogonadotropic hypogonadism (IHH) and other, more common disorders of puberty. The Unit is using insights gained from the investigation of this clinically and genetically heterogeneous group of patients to explore the biological pathways that may contribute to the reactivation of GnRH secretion at puberty. In collaboration with the REU, a variety of molecular techniques are used to further characterize the known genetic defects causing IHH, as well as to identify new genes responsible for the regulation of pubertal onset. Such approaches will help define the developmental physiology of pubertal development in order to gain a deeper understanding of human disorders of puberty and reproduction. Additional clinical studies are aimed at exploring neurocognitive and other outcomes of delayed exposure to sex hormones at or before the normal time of puberty.

Maria Dufau's *Section on Molecular Endocrinology* investigates the molecular basis of hormonal regulation of gonadal function, focusing on: (1) modes of transcriptional repression and derepression of receptors for human luteinizing hormone (LHR); (2) functions of novel short prolactin receptor (PRLR) inhibitory forms, identified by the Section, in physiological regulation and cancer and modalities of transcriptional regulation/expression of PRLR in breast cancer; (3) mechanisms involved in Leydig cell function and intracrine and paracrine androgen actions in the progression of spermatogenesis, and the regulation/functions of GRTH/DDX25, an androgen-regulated RNA helicase, essential for spermatogenesis and discovered by this group. The Section revealed the essential role of Positive Coactivator 4 (PC4), which associates with Sp1 at the LHR promoter, in the formation/assembly of the pre-initiation complex in LHR transcription. The Section found that histone 3 (H3) is recruited to the PC4 complex during the activation state of the receptor and that, in the complex, H3 is in an acetylated form. The sites of H3 acetylation and the impact of the PC4:acH3 complex on chromatin structure are under study. Other work demonstrated conformational determinants required for the inhibitory action of the PRLR short form (SF) on prolactin-induced signaling through the long form (LF). Studies revealing the essential role of the D1 domain of the PRLR on the SF configuration for its inhibitory action on LF–mediated function provide the basis for developing drugs of potential use in the treatment of advanced breast cancer. Also, the group provided direct evidence for local actions of prolactin independent of estradiol, with participation of the estrogen receptor in up-regulation of PRLR transcription/expression in breast cancer cells, which is of relevance to states that are refractory to therapy with aromatase inhibitors. Gonadotropin-regulated testicular helicase (GRTH/Ddx25), present in Leydig and meiotic/haploid germ cells, is a multifunctional protein that participates in nuclear transport of specific messages essential for the progress of spermatogenesis and protects Leydig cells from gonadotropin-mediated overstimulation of androgen. The group established the first connection between paracrine actions of androgen and two relevant germ cell genes essential for spermatogenesis: Germ Cell Nuclear Factor (GCNF) and *GRTH/Ddx25*. A transgenic animal model developed in the laboratory offers insights for the development of a male contraceptive based on the indirect blockade of the actions of androgens on GRTH expression in germ cells without affecting other aspects of androgen action.

Anil Mukherjee's *Section on Developmental Genetics* conducts both laboratory and clinical investigations into those hereditary neurodegenerative lysosomal storage disorders (LSDs) mostly affecting children. Current research in this Section focuses on the molecular mechanism(s) of pathogenesis of a group of neurodegenerative LSDs called neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease. Mutations in at least 13 different genes (called *CLNs*) cause various types of NCLs. At present, there is no effective treatment for any of the NCL types. The infantile NCL (or INCL) is an autosomal recessive LSD caused by mutations in the *CLN1* gene, which encodes the lysosomal depalmitoylating enzyme palmitoyl-protein thioesterase-1 (PPT1). PPT1 catalyzes the cleavage of thioester linkage in palmitoylated (S-acylated) proteins (constituent of ceroid), facilitating their degradation in lysosomes. Thus, PPT1 deficiency causes accumulation of ceroid in lysosomes, leading to INCL. Recently, Mukherjee and his colleagues concluded a bench-to-bedside clinical trial using a combination of cysteamine bitartrate and *N*-acetylcysteine. More recently, the group identified a thioesterase-mimetic small molecule, *N-tert*-butyl hydroxylamine, which arrests neuropathology and extends lifespan in a mouse model of INCL and is thus a potential drug for treating INCL. To conduct these studies, the Section uses gene knock-out and knock-in technologies as well as biochemical, molecular, and neurobiological techniques. Mechanism-based translational research is emphasized.

Ida Owens' *Section on Genetic Disorders of Drug Metabolism* studies the biology of the endoplasmic reticulum (ER)–based UDP-glucuronosyltransferase (UGT) isozymes. The Section's studies have shown that UGT isozymes convert lipophilic endogenous and exogenous substrates such as dietary aromatic-like therapeutics, environmental pro-carcinogens, and contaminants derived from pyrolysates to water-soluble excretable, non-toxic glucuronides; neurotoxic bilirubin is the most important endogenous substrate, followed by genotoxic catechol estrogens and elevated levels of dihydrotestosterone (DHT). Previously, the group discovered that each UGT isozyme examined requires protein kinase C–based phosphate signaling at an Src (a tyrosine kinase that is an inhibitor of apoptosis) active site that controls broad chemical detoxification of various chemical subtypes. Using Src-free cells, group discovered that the human prostate luminal-cell UGT-2B15, with IYG at amino-acid positions 98–100, is also constantly undergoing Src–based phosphorylation signaling to prevent ER–based apoptosis. By contrast, the prostate basal cell UGT-2B17, with the rare TYS at amino-acid positions 98–100, undergoes increased activity in Src–free cells. Exchange of IYG and TYS between the two isozymes at amino-acid positions 98–100 leads to the exchange of these activities. Hence, caspase-based loss of Src activity at position 98–100 leads to apoptosis for the resident luminal cells. Transfection studies indicate that the IYG Src site in UGT-2B15 elicits apoptosis more easily than the consensus IYA Src site. Human cancerous PC3 prostate cells transfected with UGT-2B15 were far less able to generate apoptotic activity than normal COS-1 cells transfected with UGT-2B15 or transfected LNCaP cells.

Forbes D. Porter's *Section on Molecular Dysmorphology* studies a group of human and mouse malformation syndromes attributable to inborn errors of cholesterol synthesis. The most common of these disorders is the Smith-Lemli-Opitz syndrome (SLOS). The Section studies both basic science and clinical aspects of SLOS, with the goal of developing and testing therapeutic interventions for SLOS. The Section also studies basic and clinical aspects of Niemann-Pick disease, type C (NPC). The group has maintained an ongoing Natural History trial for NPC and SLOS since 2006 and 1998 respectively. The NPC Natural History trial was designed to investigate biochemical markers and clinical aspects of NPC that could be used as outcome measures in a future clinical trial. In collaboration with the National Center for Translational Medicine, the Section is completing a Phase 1/2 trial of intrathecal 2-hydroxypropyl-β-cyclodextrin in NPC1 subjects and is now involved in a multicenter, multinational Phase 2b/3 trial to establish clinical efficacy in a controlled trial. In collaboration with extramural investigators, the Section was awarded a U01 grant to evaluate the safety and efficacy of histone deacetylase inhibitor therapy in NPC1 subjects.

Constantine Stratakis' *Section on Endocrinology and Genetics* investigates the genetic and molecular mechanisms leading to disorders affecting the pituitary gland and adrenal cortex, with emphasis on those that are developmental, hereditary, and associated with other conditions. The laboratory has made significant discoveries over the last two decades. The first studies led to the identification of the main regulator of the cAMP signaling pathway, regulatory subunit-type 1A (R1a) of protein kinase A (PKA, encoded by the *PRKAR1A* gene on chromosome 17q22-24), as responsible for primary pigmented nodular adrenocortical disease (PPNAD) and the Carney complex, a multiple endocrine neoplasia (MEN) whose main endocrine manifestation is PPNAD. Stratakis then described isolated micronodular adrenocortical disease (iMAD), a disorder likely to be inherited in an autosomal dominant manner and unrelated to the Carney complex or to other MENs. In 2006, a genome-wide association (GWA) study led to the identification of mutations in the phosphodiesterases (PDE) PDE11A, a dual specificity PDE, and PDE8B, a cAMP–specific PDE (encoded by the *PDE11A* and *PDE8B* genes, respectively) in iMAD. Stratakis also studied primary macronodular adrenocortical hyperplasia (PMAH) and, as part of this work, a new gene was identified (*ARMC5*), which, when mutated, causes more than a third of the known PMAH cases. Members of the laboratory are now characterizing mouse, fruit fly, and fish models of ARMC5. Recently, the laboratory identified genes encoding two other subunits of PKA as involved in endocrine tumors: *PRKACA* in various forms of bilateral adrenocortical hyperplasia and *PRKACB* in a form of Carney complex that is not associated with *PRKAR1A* mutations. Animal model studies are essential for the investigation and confirmation of each of the identified new genes in disease pathogenesis. Cells from tumors or other lesions from animals with R1a deficiency showed elevated beta-catenin expression and/or aberrant Wnt signaling and similarities to adult stem cells or cancer stem cells in other models of dysregulated Wnt signaling. The laboratory continues to investigate the pathways involved in early events in tumor formation in the pituitary gland and the adrenal cortex and/or the tissues affected by germline or somatic defects of the cAMP/PKA and related endocrine signaling defects, employing animal models and transcriptomic and systems biology analyses. The group continues to accrue patients under several clinical protocols, identify unique patients and families with rare phenotypes such as Carney Triad, and/or explore (mostly on a collaborative basis) various aspects of endocrine and related diseases. Paramount to these investigations is the availability of modern genetic tools such as copy number variation (CNV) analysis, comparative genomic hybridization (CGH), whole-exome sequencing (WES), and DNA sequencing (DSeq). As part of these clinical protocols, much clinical research is also being done, consisting mostly of observations of new associations, description of novel applications or modifications, and improvements in older diagnostic methods, tests, or imaging tools. The most recent discovery of the laboratory was the identification of the defect that explains the vast majority of cases of early pediatric overgrowth or gigantism: Stratakis and his group identified the gene *GPR101*, which encodes a G protein–coupled receptor, which was overexpressed in patients with elevated growth hormone (GH). Patients with GPR101 defects have a condition that Stratakis named X-LAG for X-linked acrogigantism caused by Xq26.3 genomic duplication and characterized by early-onset gigantism resulting from excess GPR101 function. Employing a variety of molecular techniques and animal models, ongoing work focuses on GPR101 ligands and molecular targets.

Jack Yanovski's *Section on Growth and Obesity* studies metabolic and behavioral factors involved in body weight regulation and body composition during childhood in an effort to develop etiology-specific prevention and treatment approaches for pediatric obesity. The Section has conducted multiple randomized clinical trials in adults and children involving pharmacotherapy and behavior as well as investigations into genetic causes of obesity. The Section's use of laboratory feeding paradigms has allowed it to conduct quantitative assessments of psychological constructs such "loss of control over eating" and "eating in the absence of hunger" in a reproducible fashion so that treatments directed at improving specific behaviors can be studied. The Section's recent work on the roles of appetite-regulating genes has focused on the melanocortin 3 receptor and brain-derived neurotrophic factor, elucidating the roles of these factors for human obesity by studying individuals with rare genetic abnormalities, such as the WAGR Syndrome, individuals with non-syndromic obesity, and murine models of gene dysfunction. Ongoing studies attempt to identify factors that predispose children to hyperphagic behaviors, including binge eating and related disorders, and to use this knowledge to develop rational, defect-specific prevention and treatment strategies for pediatric obesity.

In addition to research groups, PDEGEN also supports the Pediatric Endocrine fellowship program led by Constantine Stratakis and Maya Lodish. The fellowship in Pediatric Endocrinology is a three-year ACGME–accredited program providing comprehensive training in clinical patient management and guidance in the development of research skills. The NICHD program is based at one of the largest and most sophisticated research institutions in the United States, the NIH Clinical Center, which maintains clinical research protocols investigating the treatment of adrenal and pituitary tumors, congenital adrenal hyperplasia, precocious puberty, idiopathic juvenile osteoporosis, Cushing's syndrome, obesity, and others. Other institutions that participate in this training program include The Johns Hopkins University (JHU) Department of Pediatrics, Division of Pediatric Endocrinology; The Children's National Medical Center (CNMC), Division of Pediatric Endocrinology; and the co-sponsoring institution, Georgetown University (GU), Department of Pediatrics. The facilities make available to the fellows pediatric endocrine, diabetes, oncology, metabolic, bone disorders, and other pediatric subspecialty clinics and consult services, as well as general pediatric inpatient and intensive care units.

We investigate the cellular and molecular mechanisms governing childhood growth and development. We are especially interested in the mechanisms that allow rapid proliferation and hence rapid body growth in young mammals and subsequently suppress proliferation, thus setting a fundamental limit on the adult body size of the species. A goal of this work is to gain insight into the many human genetic disorders that cause childhood growth failure and overgrowth. Investigation of the identified growth-limiting mechanisms may lead to broader medical applications, because disruption of the mechanisms may contribute to oncogenesis, and conversely transient therapeutic suspension of growth-limiting mechanisms in adult cells might be used to achieve tissue regeneration.

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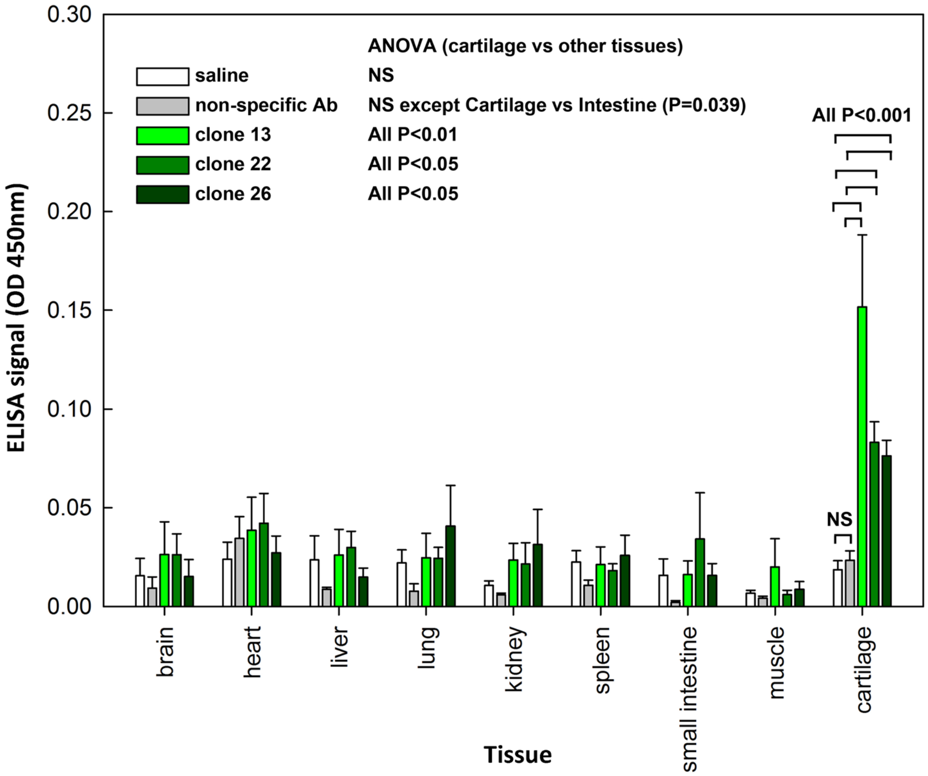
Mechanisms limiting childhood growth

Body growth is rapid in infancy but subsequently slows and eventually ceases. The decline in growth rate during juvenile life occurs across mammalian species, including humans. In each species, growth slows concordantly in major organs to maintain body proportions. The growth deceleration results primarily from a decline in cell proliferation. We previously showed that the decline in cell proliferation is attributable in part to a growth-limiting genetic program that includes the postnatal down-regulation of many growth-promoting genes in multiple organs. We recently sought to explore the role of microRNAs (miRNAs) in the regulation of this growth-limiting genetic program (Reference 1).

We first performed microarray analysis to identify changes in miRNA expression during early postnatal life. Because miRNAs negatively regulate gene expression, we focused on miRNAs that were up-regulated with age and thus might contribute to the previously observed down-regulation of multiple mRNAs with age. We paid particular attention to miRNAs that were commonly up-regulated with age in multiple organs and thus more likely to contribute to the regulation of a genetic program that occurs simultaneously in each of these organs. The expression microarray analysis revealed that all members of the miR-29 family, miR-29a, miR-29-b, and miR-29-c, were strongly up-regulated with age in multiple organs in mice, findings that we confirmed by real-time PCR. Bioinformatic analysis showed that target sequences of the miR-29 family of microRNAs were overrepresented among genes that are down-regulated with age in multiple organs. We next focused on three predicted miR-29 target genes (*Igf1*, *Imp1*, and *Mest*), which are all growth-promoting. Using a luciferase-based assay and site-directed mutagenesis of the seed region, we found evidence that all three genes are indeed regulated by miR-29.

Taken together, the findings suggest that up-regulation of miR-29 during juvenile life helps orchestrate a juvenile multi-organ genetic program that involves the down-regulation of many growth-promoting genes with age. Therefore, the evidence supports the hypothesis that up-regulation of the miR-29 family of microRNAs serves as a regulatory mechanisms that allows rapid proliferation and body growth in early life but then suppresses proliferation, causing the rate of body growth to slow and eventually approach zero in adulthood.



Homing of selected matrilin-3–binding antibody fragments to cartilage *in vivo*

Saline, non-specific antibody fragment, or purified antibody fragments 13, 22, and 26 were injected intravenously in 3-week-old mice. After 24 hours, various organs were collected and homogenized and tissue lysates used to coat plastic wells. The presence of antibody fragments was detected by ELISA with an anti-Fc antibody. All three antibody fragments were detected in cartilage, but not in non-cartilaginous organs. Saline and non-specific antibody served as negative controls. Data represent mean +/– SEM from five independent experiments (n=5).

Genes that control human linear growth

Children grow taller because their bones grow longer. Bone elongation occurs at the growth plate, a thin layer of cartilage found near the ends of juvenile bones. Consequently, many of the genes that control human height are expressed in and function in the growth plate. Based on this concept, we developed bioinformatic methods to identify genes that affect human height through local actions in the growth plate. The approach synthesizes data from our expression microarray studies of the growth plate, human disease databases, and a mouse knockout phenotype database. In collaboration with other research groups, we used the analytic approach to help identify genes that determine human height within loci detected by genome-wide association studies (Reference 2). In other collaborations, the method has been used to help identify human gene deletions that affect stature.

The work has important clinical implications because mutations in the genes that control growth lead to growth failure. Currently, for many children who experience linear growth failure, the etiology cannot be determined. Identifying new genes that control growth will likely allow us to determine the genetic cause of growth failure in many of these children.

Targeted treatment approaches for growth plate disorders

Mutations in hundreds of genes that are required for growth plate function give rise to disorders of skeletal growth, including the skeletal dysplasias, in which the bones are short and malformed, causing major disability. In addition to genetic disorders, acquired endocrine, nutritional, or inflammatory disorders can also impair bone growth at the growth plate, resulting in severe short stature.

Current treatment options for growth plate disorders are limited. Recombinant human growth hormone (GH) is used, but the efficacy is often suboptimal, particularly for children with skeletal dysplasias. Moreover, GH treatment carries the potential risks of increased intracranial pressure, slipped capital femoral epiphysis, insulin resistance, and promotion of malignancies. Because systemic administration of GH has limited efficacy and significant known and potential adverse effects, better treatments for growth plate disorders are needed.

Recent studies identified many paracrine factors that positively regulate growth plate chondrogenesis and therefore might be used therapeutically, including Indian hedgehog, bone-morphogenetic proteins, C-type natriuretic peptide, and WNT proteins. However, the development of these molecules into effective treatment has been hampered by their mechanism of action; such growth factors are produced locally and act locally in the growth plate, and thus do not lend themselves to systemic therapeutic approaches. We envisioned that these locally acting molecules could be targeted to the growth plate by linking them to cartilage-binding proteins, such as antibody fragments. When administered systemically, the hybrid molecules would be preferentially taken up by growth plate cartilage, and thus might greatly augment the therapeutic effect on the target organ while diminishing adverse effects owing to action on other tissues. Similarly, growth-promoting endocrine factors, such as GH and insulin-like growth factor-I, might be linked to cartilage-binding polypeptides and thereby targeted to the growth plate. Targeted endocrine therapy could potentially enhance the therapeutic effects on chondrogenesis and reduce effects on non-target tissues, thereby decreasing potential risks such as malignancy.

To develop cartilage-targeting therapy, we sought to identify polypeptides that home to cartilage tissue (Reference 3). In collaboration with scientists at the NCI, we employed a yeast display human antibody library and selected high-affinity binders to matrilin-3, an extracellular matrix protein expressed with high tissue specificity in cartilage. We identified antibody fragments that bind with high affinity to matrilin-3, as well as to cartilage tissue *in vitro*. *In vivo*, the antibody fragments homed specifically to cartilage tissue in mice (Figure).

Coupling the antibody fragments to endocrine and paracrine factors that stimulate chondrogenesis could be used to direct these potent molecules specifically to cartilage tissue and thus has the potential to open up new pharmacological approaches to treat childhood skeletal growth disorders.

Links between childhood growth and oncogenesis

When disrupted, many of the mechanisms that regulate mammalian body growth can contribute to the development of malignancies. We studied the role of two heparin-binding growth factors, pleiotrophin and midkine, in the regulation both of normal body growth and in the unregulated growth of malignancies. Recently, we measured midkine concentrations in fine-needle aspirate (FNA) samples from benign and malignant thyroid nodules to explore the possibility that midkine measurement might aid in the evaluation of thyroid nodules (Reference 4). Midkine was measured using a high-sensitivity sandwich ELISA and normalized to thyroglobulin concentration in the sample to adjust for tissue content in the aspirate. We found that, in FNA samples, the midkine/thyroglobulin ratio in papillary thyroid cancer was greater than in benign thyroid nodules, raising the possibility that the approach might provide adjunctive diagnostic or prognostic information to complement existing approaches.

ADDITIONAL FUNDING

» Thrasher Research Fund Early Career Award to Dr. Youn Hee Jee (2014, ongoing): “The Role of Heparin Binding Growth Factors in Fetal and Childhood Growth”
» Clinical Center Genomic Opportunity Program (2104, ongoing): “Genetic Causes of Childhood Growth Failure”
» Merck-Serono Grant for Growth Innovation to Julian Lui (2014, ongoing): “Cartilage-Targeted Therapeutics for Growth Disorders”
» Endocrine Scholars Award in Growth Hormone Research to Dr. Julian Lui (2015, ongoing): “Cartilage-Targeted IGF-I for Treatment of Growth Disorders

PUBLICATIONS

1. Kamran F, Andrade AC, Nella AA, Clokie SJ, Rezvani G, Nilsson O, Baron J, Lui JC. Evidence that upregulation of microRNA-29 contributes to postnatal body growth deceleration. *Mol Endocrinol* 2015; 29:921–932.
2. Wood AR, Esko T, Yang J, et al. (GIANT Consortium). Defining the role of common variation in the genomic and biological architecture of adult human height. *Nat Genet* 2014; 46:1173–1186.
3. Cheung CS, Zhu Z, Lui JC, Dimitrov D, Baron J. Human monoclonal antibody fragments targeting matrilin-3 in growth plate cartilage. *Pharm Res* 2015; 32:2439–2449.
4. Jee YH, Celi FS, Kebebew E, Sampson M, Sacks DB, Remaley AT, Baron J. Midkine concentrations in fine-needle aspiration of benign and malignant thyroid nodules. *Clin Endocrinol (Oxf)* 2015; 83:977–84.
5. Nilsson O, Weise M, Landman EB, Meyers JL, Barnes KM, Baron J. Evidence that estrogen hastens epiphyseal fusion

and cessation of longitudinal bone growth by irreversibly depleting the number of resting zone progenitor cells in female rabbits. *Endocrinology* 2014; 155:2892–2899.

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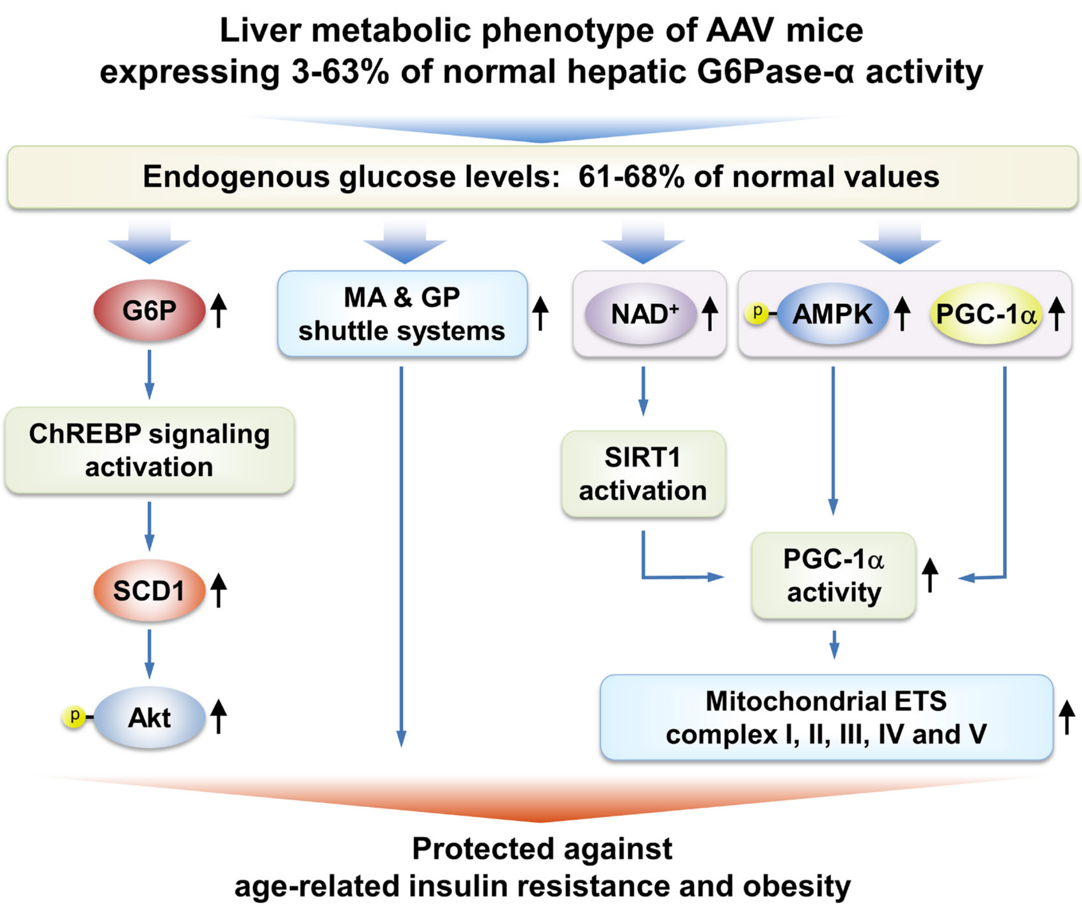
We conduct research to delineate the pathophysiology of glycogen storage disease type I (GSD-I) and glucose-6-phosphatase-beta (G6Pase-beta or G6PC3) deficiency, and to develop novel therapies for these disorders. There are two subtypes of GSD-I: GSD-Ia, deficient in G6Pase-alpha (or G6PC); and GSD-Ib, deficient in the glucose-6-phosphate (G6P) transporter (G6PT or SLC37A4). A third disease, G6Pase-beta deficiency, also known as severe congenital neutropenia syndrome type 4, is not a glycogen storage disease but biochemically a GSD-I–related syndrome (GSD-Irs). G6Pase-alpha and G6Pase-beta are endoplasmic reticulum (ER)–bound G6P hydrolases, with active sites lying inside the lumen, which depend upon G6PT to translocate G6P from the cytoplasm into the ER lumen. The G6PT/G6Pase-alpha complex maintains interprandial glucose homeostasis while the G6PT/G6Pase-beta complex maintains energy homeostasis and functionality of neutrophil and macrophages. GSD-Ia and GSD-Ib patients manifest the common metabolic phenotype of impaired glucose homeostasis, not shared by GSD-Irs. GSD-Ib and GSD-Irs patients manifest the common myeloid phenotype of neutropenia and myeloid dysfunction, not shared by GSD-Ia. Neutrophils express the G6PT/G6Pase-beta complex, and inactivation of G6PT or G6Pase-beta leads to the enhanced neutrophil apoptosis that underlies neutropenia in GSD-Ib and GSD-Irs. The G6PT/G6Pase-beta complex is also essential for energy homeostasis in neutrophils. A deficiency in either G6PT or G6Pase-beta prevents recycling of glucose from the ER to the cytoplasm, leading to neutrophil dysfunction. There is no cure for either GSD-Ia, GSD-Ib, or GSD-Irs. Animal models of the three disorders are available, which we are exploiting to both delineate the disease more precisely and develop new treatment approaches, including gene therapy.

Mice expressing reduced levels of hepatic G6Pase-α activity do not develop age-related insulin resistance or obesity.

We have shown that gene therapy mediated by rAAV8-G6PC, a recombinant adeno-associated virus pseudotype 2/8 (rAAV8) vector expressing human G6Pase-alpha directed by the human *G6PC* promoter/enhancer, normalizes blood glucose homeostasis in *G6pc* knockout ($G6pc^{-/-}$) mice for 70–90 weeks. The treated $G6pc^{-/-}$ mice, which express 3–63% of normal hepatic G6Pase-alpha activity (AAV mice), produce endogenous hepatic glucose at 61–68% levels of wild-type littermates, have a leaner phenotype, and exhibit fasting blood insulin levels more typical of young adult mice. We showed that, unlike wild-type mice, the lean AAV mice have elevated caloric intake but do not develop age-related obesity or insulin resistance. Pathway analysis showed that signaling by hepatic ChREBP, a glucose-activated transcription factor that improves glucose tolerance and insulin signaling, is activated in AAV mice. In addition, several longevity factors in the calorie restriction pathway, including the NADH shuttle systems, NAD^+ concentrations, and the AMPK/sirtuin1/PGC-1alpha pathway, are up-regulated in the livers of AAV mice. Our study suggests that full restoration of normal G6Pase-alpha activity by gene therapy will not be required to confer significant therapeutic benefits in GSD-Ia. The finding that a moderate reduction of hepatic G6Pase-alpha activity in mice may promote a leaner phenotype and prevent the development of age-related



Mechanisms that underlie protection of AAV mice against age-related obesity and insulin resistance
Several signaling pathways in the livers of AAV mice contribute to the beneficial metabolic phenotype. The ChREBP-SCD1-Akt signaling pathway, activated by elevated hepatic G6P levels, leads to improved insulin signaling and glucose tolerance. Increases in the expression of the malate-aspartate (MA) and glycerol-3-phosphate (GP) shuttle systems, which couple mitochondrial ATP synthesis to the regeneration of NAD^+, confer insulin sensitivity and resistance to age-related obesity. Activation of the AMPK/SIRT/PGC-1α pathway, which leads to increased expression of mitochondrial electron transport chain complexes and protects against age-related mitochondrial dysfunction, has three components: (1) increased expression of PGC-1α, a master regulator of mitochondrial biogenesis; (2) PGC-1α activation via deacetylation by SIRT1, which is activated by elevated cellular concentrations of NAD^+; and (3) PGC-1α activation via phosphorylation by p-AMPK.

decline in insulin sensitivity lends weight to the suggestion that G6Pase-alpha constitutes a potential pharmaceutical target for treating type 2 diabetes and provides insight into the safety margin in such activity-reducing therapies.

Molecular mechanisms of neutrophil dysfunction in GSD-Ib

The underlying cause of GSD-Ib neutropenia is an enhanced neutrophil apoptosis, but patients also manifest neutrophil dysfunction of unknown etiology. Previously, we show G6PT interacts with the enzyme G6Pase-beta to regulate the availability of G6P/glucose in neutrophils. A deficiency in G6Pase-β activity in neutrophils impairs both their energy homeostasis and functionality. We showed that G6PT–deficient neutrophils from human GSD-Ib patients are similarly impaired, an energy impairment that is characterized by reduced glucose uptake and diminished levels of intracellular G6P, lactate, ATP, and NADPH, while functional impairment is reflected in lowering of neutrophil respiratory burst, of chemotaxis, and of calcium mobilization. We also showed that the expression and membrane translocation of the NADPH oxidase subunit $p47^{phox}$ is downregulated in G6PT–deficient neutrophils, explaining why respiratory burst activity is impaired. We further showed that the HIF-1alpha/PPAR-gamma pathway, which directly impacts neutrophil respiratory burst, chemotaxis, and calcium mobilization, is activated in G6PT–deficient neutrophils. We also found that exposing human G6PT–deficient neutrophils to a PPAR-gamma antagonist improves their function. Taken together, our results demonstrate that the underlying cause of neutrophil dysfunction in GSD-Ib arises from impaired neutrophil energy homeostasis and activation of the HIF-1alpha/PPAR-gamma pathway. The insight into the etiology of neutrophil dysfunction in GSD-Ib should facilitate the development of novel therapies for this disorder.

Functional analysis of mutations in GSD-Irs

The enzyme G6Pase-beta is embedded in the ER membrane and catalyzes the hydrolysis of G6P to glucose and phosphate. To date, 33 separate G6Pase-beta mutations have been identified in GSD-Irs patients, but only the p.R253H and p.G260R missense mutations have been characterized functionally for pathogenicity. We functionally characterized 16 of the 19 known missense mutations, using a sensitive assay based on a recombinant adenoviral vector–mediated expression system to demonstrate pathogenicity. Twelve missense mutations completely abolish G6Pase-beta enzymatic activity while the p.M116V, p.T118R, p.S139I, and p.R189Q mutations retain 1.1%, 1.3%, 49%, and 45%, respectively, of wild-type G6Pase-beta activity. A database of residual enzymatic activity retained by the G6Pase-beta mutations will serve as a reference for evaluating genotype-phenotype relationships.

Long-term safety and efficacy of liver-directed gene therapy in murine GSD-Ia

GSD-Ia is characterized by impaired glucose homeostasis and the long-term complication of hepatocellular adenoma (HCA,) which may undergo malignant transformation to hepatocellular carcinoma (HCC). In a long-term study, we showed that 70–90 week-old rAAV-G6PC–treated *G6pc*$^{-/-}$ mice expressing 3% or greater of normal hepatic G6Pase-α activity maintain glucose homeostasis and show no evidence of HCA/HCC. Despite the encouraging results, to translate rAAV–mediated gene therapy in murine GSD-Ia to the clinic, the safety concerns associated with administering large doses of the rAAV vector must be addressed. We therefore generated rAAV-co-G6PC, a rAAV8 vector expressing a codon-optimized (co) human G6Pase-alpha (co-G6PC) directed by the human *G6PC* promoter/enhancer, and investigated the impact of codon optimization strategies on translation efficiency. In a 66–80-week study, we showed that the rAAV-co-G6PC vector is more efficacious than the rAAV-G6PC vector in directing hepatic G6Pase-alpha expression. As expected, the treated mice expressing 3% or more of normal hepatic G6Pase-α activity display normal metabolic profiles, maintain normoglycemia over a 24-hour fast, show no evidence of HCA/HCC, and are protected against age-related obesity and insulin resistance. Among the twenty-six 66–80-week-old rAAV8-G6PC- and rAAV8-co-G6PC–treated *G6pc*$^{-/-}$ mice, twelve expressed hepatic G6Pase-alpha activity 3% or more of normal hepatic G6Pase-α activity. One mouse expressing 0.9% of normal hepatic G6Pase-alpha activity developed HCA and two mice expressing 0.9% and 1.3% of normal hepatic G6Pase-alpha activity, respectively developed HCC, establishing the threshold of transgene expression required to avoid HCA/HCC. We further showed that ChREBP signaling, which improves glucose tolerance and insulin sensitivity, is activated in all rAAV–treated mice but that activation of ChREBP signaling is significantly attenuated in the HCC nodules.

Liver-directed gene therapy for murine GSD-Ib

We showed that systemic administration of rAAV-CBA-G6PT, a rAAV8 vector expressing human G6PT directed by the chicken b-actin (CBA) promoter/CMV enhancer, delivered the G6PT transgene to the liver and normalized metabolic abnormalities in murine GSD-Ib. However, the five transduced GSD-Ib mice that lived to age 52–70 weeks expressed less than 4% of wild-type hepatic G6PT activity and two mice developed HCA, with one undergoing malignant transformation. Studies have shown that the choice of transgene promoter not only impacts targeting efficiency and tissue-specific expression, but also the level of immune response or tolerance to the therapy. We therefore examined the safety and efficacy of rAAV8-G6PT, a rAAV8 vector expressing human G6PT directed by the tissue-specific human *G6PC* promoter/enhancer. Among the fifteen 60–78 week-old rAAV–treated *G6pt*$^{-/-}$ mice expressing 2–62% of wild-type hepatic G6PT activity, only one mouse expressing 6% of normal hepatic G6PT activity developed HCA. The rAAV–treated mice, including the HCA–bearing mouse, displayed normal hepatic fat storage, normal glucose tolerance profiles, and maintained normoglycemia over a 24-hour fast. The rAAV–treated mice also exhibit a leaner phenotype and are protected against age-related insulin resistance. We further showed that activation of hepatic ChREBP signaling, which improves glucose tolerance and insulin sensitivity, is one mechanism that protects the rAAV-GPE-G6PT–treated *G6pt*$^{-/-}$ mice against age-related obesity and insulin resistance.

ADDITIONAL FUNDING

» The Children's Fund for Glycogen Storage Disease Research, 2014

PUBLICATIONS

1. Jun HS, Weinstein DA, Lee YM, Mansfield BC, Chou JY. Molecular mechanisms of neutrophil dysfunction in glycogen storage disease type Ib. *Blood* 2014; 123:2843–2853.
2. Lin SR, Pan CJ, Mansfield BC, Chou JY. Functional analysis of mutations in a severe congenital neutropenia syndrome caused by glucose-6-phosphatase-ß deficiency. *Mol Genet Metab* 2015; 114:41–45.
3. Kim GY, Lee YM, Cho JH, Pa CJ, Jun HS, Springer DA, Mansfield BC, Chou JY. Mice expressing reduced levels of hepatic glucose-6-phosphatase-a activity do not develop age-related insulin resistance and obesity. *Hum Mol Genet* 2015; 24:5115–5125.
4. Chou JY, Jun HS, Mansfield BC. Type I glycogen storage diseases: disorders of the glucose-6-phosphatase/glucose-6-phosphate transporter complexes. *J Inherit Metab Dis* 2015; 38:511–519.
5. Lee YM, Kim GY, Pan CJ, Mansfield BC, Chou JY. Minimal hepatic glucose-6-phosphatase-a activity required to sustain survival and prevent hepatocellular adenoma formation in murine glycogen storage disease type Ia. *Mol Genet Metab Reposts* 2015; 3:28–32.

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We are interested in identifying the initiating factors for pubertal onset in children. Our long-term goal is to define the developmental physiology of pubertal development, in order to increase our understanding of human disorders of puberty and reproduction. In collaboration with the Reproductive Endocrine Unit (REU) at the Massachusetts General Hospital (MGH), we are conducting translational research on the neuroendocrine and genetic control of gonadotropin-releasing hormone (GnRH) secretion and its regulation of gonadotropin secretion and gonadal physiology. We use molecular, cellular, and biochemical techniques, as well as comprehensive clinical phenotyping of human subjects to identify and characterize biological pathways that may contribute to the reactivation of GnRH secretion at puberty and to explore diagnostic techniques and treatment of disorders of puberty and reproduction.

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The role of gonadotropin pulsations in the regulation of puberty and fertility

At one extreme of pubertal development, deficiency of GnRH results in a spectrum of rare clinical disorders of isolated GnRH deficiency (IGD), also known as idiopathic hypogonadotropic hypogonadism (IHH), which presents with delayed, incomplete, or absent sexual maturation. Defining the physiology of GnRH secretion is critical to understanding the clinical heterogeneity of IGD, particularly in light of emerging gene discoveries that aim to elucidate genotype-phenotype correlations. Non-reproductive phenotypic features have been identified in some individuals, including anosmia, auditory defects, and skeletal, neurological, and renal anomalies. The additional features may be the key to determining the developmental function of genes implicated in this spectrum of disorders.

Our clinical protocol (**http://gp.nichd.nih.gov**), which is a multicenter study in collaboration with the REU at MGH, identified a broad range of luteinizing hormone (LH) pulsatility patterns and other features, which are being investigated in the context of genetic variants, where identified, in order to deepen our understanding of the ontogeny of these disorders. Our phenotyping efforts established that uterine anomalies may represent a novel non-reproductive feature of IGD, which is now being investigated in our genetic study (see below) to determine whether there is a common molecular cause for these phenotypes. We have also initiated a pilot study to determine the prevalence of psychiatric disorders and symptoms of negative emotional states in our cohort, compared with healthy controls, in order to determine whether there are previously unidentified psychological features in need of further investigation.

As a result of the phenotyping efforts pioneered by our collaborators at MGH, several rare phenotypic categories have been described in men with IGD, including adult-onset IHH (Nachtigall et al., *N Engl J Med* 1997;336:410) and reversal of the disorder after a period of treatment (Raivio et al., *N Engl J Med* 2007;357:863). However, the frequency of these phenotypes in women, in whom the disorder is five times less common, is unknown. Phenotypic characterization of females is further complicated by the possibility that they may be experiencing functional hypothalamic amenorrhea (HA), a hormonally similar condition that occurs in association with risk factors such as nutritional deprivation, exercise, or significant stress. We are working to characterize these phenotypic sub-categories in our cohort of females, in combination with our genetic analysis below, in order to provide insight into the phenotypic spectrum of GnRH–deficient disorders in women.

Neurocognitive effects of sex hormone deficiency at or before puberty

There is little evidence for the neurocognitive effects of delayed puberty. We therefore performed neurocognitive testing and structural and functional MRI on subjects with IGD, comparing them with healthy controls matched for age, sex, and race. Accounting for gender, our preliminary findings suggested that, in both sexes, pubertal sex steroid deficiency contributes to persistent structural and functional brain differences as well as to neurocognitive deficits, primarily involving spatial ability and recognition memory, providing direct evidence in humans of the critical spatiotemporal role played by appropriately timed pubertal sex steroids during normal brain development. Final analysis of the preliminary cohort in this study is under way.

The molecular basis of inherited reproductive disorders

Human and animal models have identified several genes responsible for IGD, but more than half of patients with clinical evidence of the disorder do not have a detectable mutation. In addition, there is significant clinical heterogeneity among affected individuals, including members of the same family harboring the same mutations, which is often explained by oligo-digenic inheritance patterns. Whole-exome sequencing (WES) was performed in the Molecular Genomics Lab (NICHD) on 28 probands participating in our genetic research protocol (**http://ird.nichd.nih.gov**), including several extended families, to identify novel genes responsible for IGD. Data analysis is under way, and our findings are likely to yield important insights into additional pathways involved in the regulation of GnRH secretion. We also performed WES in several families with IGD and known uterine anomalies, based on our discovery that several patients have this phenotypic combination. Analysis of these data has the potential to identify a new non-reproductive feature of IGD, as well as a novel molecular pathway involved in the regulation of GnRH secretion and uterine development.

We have also been investigating genetic variants in 14 genes known to cause IGD in a subgroup of individuals with functional GnRH deficiency, resulting from HA, as described above. We aim to determine whether variants in the IGD genes are over-represented in individuals with HA, compared with the general population. If the data support our hypothesis, this would provide further evidence that heterozygous variants in these genes confer an increased susceptibility to developing HA in the setting of physiologic stressors, such as nutritional deficiency, extreme exercise, or psychological stress.

At the other extreme of pubertal development are patients with premature reactivation of hypothalamic GnRH secretion, resulting in idiopathic central precocious puberty (CPP). There is evidence that familial cases account for anywhere from 20–45% of CPP, with most studies describing autosomal dominant inheritance patterns. Far less is known about the molecular basis of CPP, and it was only recently that convincing evidence for a causative mutation was identified, using WES, in the *MKRN3* gene (Abreu AP et al., *N Engl J Med* 2013;368:2467). Candidate gene approaches have not been successful in identifying the molecular basis of this disorder, and an unbiased approach to gene discovery seems more likely to achieve the goal of identifying novel candidate genes responsible for premature GnRH secretion in CPP. We are now actively recruiting familial cases of idiopathic CPP to undergo WES analysis. We have established collaborations with investigators both locally and internationally to increase enrollment, and we anticipate performing WES analysis on this cohort in the coming year.

Examining the genetic characteristics of subjects with pubertal disorders will reveal insights into the mechanisms underlying the reawakening of the hypothalamic-pituitary-gonadal axis at puberty. This will provide opportunities for new diagnostic capabilities and therapeutic interventions for disorders of puberty and reproduction.

Blockade of kisspeptin signaling in women

The neuropeptide hormone kisspeptin potently stimulates secretion of GnRH. While single doses of kisspeptin stimulate the reproductive endocrine axis, animal models suggest that continuous administration of kisspeptin paradoxically suppresses the reproductive endocrine axis temporarily through desensitization of the kisspeptin receptor. By administering 24-hour infusions of kisspeptin to healthy women and to patients with reproductive disorders, we hope to learn more about the role of kisspeptin both in normal physiology and in pathological conditions, such as polycystic ovary syndrome (PCOS), a common condition characterized by ovulatory dysfunction and hyperandrogenism. Among other disturbances of hormonal regulation, patients with PCOS have high-amplitude, high-frequency LH pulses, which may contribute to the oligo-anovulation characteristic of this disorder.

In collaboration with Stephanie Seminara, and funded through an NIH Bedside-to-Bench Award, we are investigating healthy postmenopausal women to determine the safety of continuous kisspeptin administration in women and the proper dose and conditions required to achieve desensitization of the kisspeptin receptor. Once these conditions have been established, we plan to administer the peptide to women with PCOS to determine whether abnormal kisspeptin signaling is involved in these disturbed endocrine dynamics, as greater understanding of how kisspeptin modulates GnRH secretion in this condition could lead to novel therapeutic interventions for this patient population.

ADDITIONAL FUNDING

» NICHD DIR Molecular Genomics Lab Sequencing Award, 2014: Novel Gene Discovery in Inherited Reproductive Disorders (ongoing)
» NICHD DIR Molecular Genomics Lab Sequencing Award, 2015: Novel Gene Discovery in Inherited Reproductive Disorders (ongoing)

PUBLICATIONS

1. Delaney A, Padmanabhan V, Rezvani G, Chen W, Forcinito P, Cheung CS, Baron J, Lui JC. Evolutionary conservation and modulation of a juvenile growth-regulating genetic program. *J Mol Endocrinol* 2014 52(3):269–277.

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Receptors and Actions of Peptide Hormones and Regulatory Proteins in Endocrine Mechanisms

We investigate the molecular basis of peptide hormone control of gonadal function, with particular emphasis on the structure and regulation of the genes encoding the luteinizing hormone receptor (LHR) and prolactin (PRL) receptor (PRLR). We also investigate the regulatory mechanism(s) involved in the progress of spermatogenesis and the control of Leydig cell (LC) function. Our studies focus on the regulation of human *LHR* transcription (nuclear orphan receptors, epigenetics, DNA methylation, second messengers, repressors, corepressors, and coactivators), as well as on the multiple-promoter control of *hPRLR* gene transcription. We are elucidating the functions of two inhibitory short forms of prolactin receptors and their impact on the long form of the receptor as well as their relevance to physiological regulation and breast cancer. We also investigate novel gonadotropin-regulated genes relevant to the progression of testicular gametogenesis, LC function, and other endocrine processes. We focus on the function and regulation of the gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), an essential post-transcriptional regulator of spermatogenesis that was discovered, cloned, and characterized in our laboratory. The various functions of GRTH/DDX25 provide a fertile ground for the development of a male contraceptive.

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The luteinizing hormone receptor

The luteinizing hormone receptor (LHR) is expressed primarily in the gonads, where it mediates luteinizing hormone (LH) signals that regulate cyclic ovarian changes or testicular function. The LRH transcription gene is regulated by complex and diverse networks, in which coordination and interactions between regulatory effectors are essential for silencing/activation of *LHR* expression. The proximal Sp1 site of the promoter recruits histone (H) deacetylases and the Sin3A corepressor complex that contributes to the silencing of *LHR* transcription. Site-specific acetylation/methylation-induced phosphatase release serves as an on switch for Sp1 phosphorylation at Ser641, which causes p107 repressor release from Sp1, recruitment of Transcription Factor II B (TFIIB) and RNA polymerase II (Pol II), and transcriptional activation. Maximal derepression of the gene is dependent on DNA demethylation of the promoter, H3/acetylation, and HDAC/Sin3 A release. Positive Cofactor 4 (PC4) has an important role in the formation assembly of the preinitiation complex (PIC) in trichostatin A (TSA)–mediated *LHR* transcription (Kavarthapu et al., *Endocrinology* 2013;154:2200). It is recruited by Sp1 following TSA treatment and acts as a coactivator. However, PC4 does not participate in TSA release of phosphatases, Sp1 phosphorylation, or release of repressor or complexes. Although TFIIB recruitment is dependent on PC4, we ruled out TFIIB as its direct target and acetylation of PC4 in the activation process. However, we demonstrated TSA–induced acetylation of PC4–interacting proteins, identified as acetylated H3 by mass spectrometry, and PC4's presence in the complex in association with chromatin at the promoter was demonstrated by ChIP/reChIP. The role of these interactions on chromatin structure and their participation in the assembly of the PIC and transcriptional activation are under investigation. Immunoprecipitated flag-tagged PC4/H3 complexes in transfected MCF-7 cells analyzed by immunoblotting revealed H3-acetylated at K9, 14, 18, 23, 27 and 36 pull-

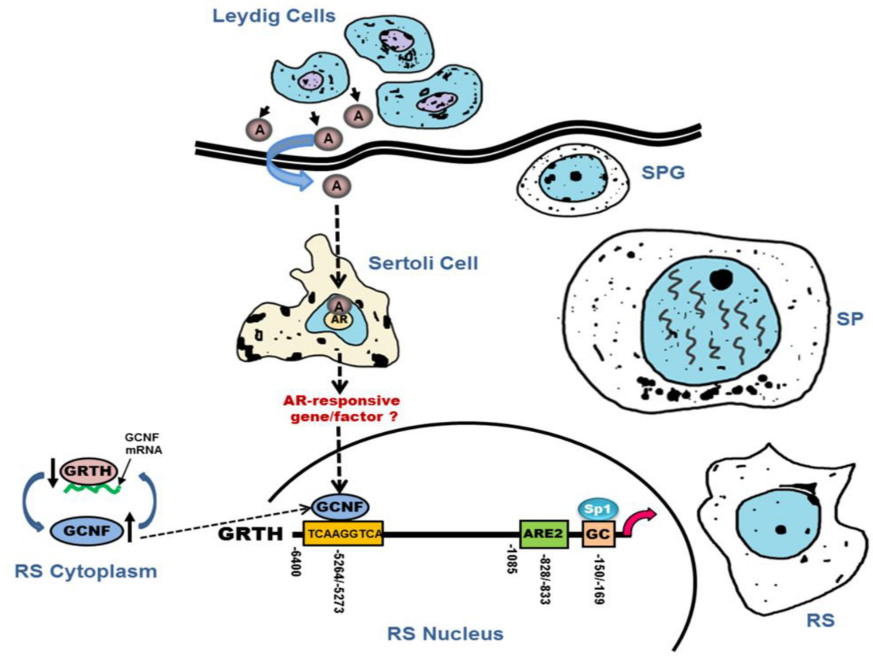

Figure 1. Germ Cell Nuclear Factor (GCNF) regulates transcription of gonadotropin-regulated testicular helicase (GRTH/DDX25) in testicular germ cells—the androgen connection. GCNF regulation of GRTH transcription/expression in testicular germ cells links androgen action and germ cell activation. Androgen (A) synthesized and released from Leydig cells (interstitial testis compartment) binds to androgen receptors (AR) in Sertoli cells (somatic cells located in tubule compartment; they provide structural support and nutrients to germ cells). A/AR complex enters into the nucleus of Sertoli cells, where it activates AR–responsive genes/factors (classical pathway). In addition, the non-classical pathway could mediate this GRTH activation by the transfactor GCNF. Both pathways could lead to activation of kinases and/or phosphatases or other modifiers for recruitment of coactivators or release of repressors of GCNF to induce transcription/expression of GRTH. Sertoli-mediated events/signals in response to androgen are in turn passed onto germinal cells [Round spermatids (RS)], where they activate GCNF, a germinal cell–specific transfactor, that binds to the upstream 5′ region of the *GRTH* gene and promotes its transcription/expression. Association of GRTH with GCNF mRNA at cytoplasmic sites and its inhibitory effect on GCNF message stability indicate that the helicase plays a role in the regulation of is own transcriptional regulator in germ cells (Reference 4). SPG, spermatogonia; SP, spermatocytes.

down by the Flag Ab. To elucidate the physiological impact of PC4 on Sp1–directed transcription in gonads, we are generating a PC4-floxed mice to be bred with transgenic mice expressing tissue-specific Cyp17 Cre (Cyp17 is a steroidogenic enzyme specifically expressed in Leydig cells and ovarian cells).

Gonadotropin-regulated testicular RNA helicase

Gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) is a testis-specific member of the DEAD-box family of RNA helicases present in LCs and meiotic germ cells. It is a multi-functional protein essential for the completion of spermatogenesis. Males lacking GRTH are sterile owing to the absence of sperm as a result of the failure of round spermatids to elongate. Besides its intrinsic RNA helicase activity, it is a shuttling protein that exports specific mRNAs from the nucleus to cytoplasmic sites. Our studies demonstrated the essential participation of GRTH–mediated export/transport of mRNAs in the structural integrity of the Chromatoid Body (storage/processing of mRNAs) and their transit/association to actively translating polyribosomes, where GRTH may regulate translational initiation of genes. We identified mRNAs that are associated with GRTH at polysomal sites of spermatocytes and round spermatids of the mouse testis. The reduction in mRNAs associated at these sites in studies comparing knockout (KO) with wild-type (WT), which is not detected at the total cellular level but evident in the cytoplasm with abolition of protein expression, reflects the importance of the transport function of GRTH to relevant sites and underscores its impact on protein synthesis. The multiple functions of GRTH to regulate post-transcriptional events, including processing, exporting, and storage of RNA, have been viewed as essential for controlling the availability of specific transcripts such as Tp1/2 (transition proteins 1/2) and Prm1/2 (protamines 1/2) for translation during the progression of spermatogenesis. These chromatin remodelers, which are essential for spermatid elongation and completion of spermatogenesis and whose RNAs associate with GRTH, failed to express in GRTH–null mice with impaired nuclear export of mRNA. Our recent studies provided insights into the association of TP2 expression via binding of its mRNA to GRTH protein. Two of the conserved RNA binding motifs of the DEAD-box family of RNA helicases Ia (PTRELA) and V (ARGID) are essential for GRTH binding to 3′ UTR of Tp2 mRNA. Nucleotide sequences within 1–47 and 78–127 downstream of the TGA stop codon of the Tp2 transcript are important for binding to GRTH (Reference 3). The study provides the basis for a deeper understanding of the contribution of GRTH to the regulation of the expression of genes as a component of mRNP particles.

GRTH is regulated by LH through androgen at the transcriptional level in LCs (direct) and germ cells (indirect), where GRTH's expression is both cell- and stage-specific. The helicase displays a novel negative autocrine control of androgen production in LCs by preventing overstimulation of the LH–induced androgen pathway through enhanced degradation of the StAR protein (Steroidogenic Acute Regulatory Protein) and, in this manner, controls the degree of cholesterol transport to the mitochondria and its availability for steroidogenesis. Our studies revealed the mechanism by which androgen (A)/androgen receptor (AR) regulates the expression of the *GRTH* gene in the LC. Through its activation of *GRTH* transcription, androgen/AR signaling in LCs participates in an autocrine regulatory mechanism with a major impact on LC steroidogenic function.

Our development of transgenic mice model carrying a *GRTH* 5′ flanking region–GFP reporter provided a unique *in vivo* system for differential elucidation of regulatory regions upstream in the *GRTH* gene that direct its expression (upstream) in germ cells (pachytene spermatocytes and round spermatids) and downstream in LCs and its regulation by A/AR (directly) in LC and indirectly in germ cells (Kavarthapu at al., *Endocrinology* 2013;154:2200). We identified a functional binding site for the germ cell–specific transcription factor Germ Cell Nuclear Factor (GCNF) in the *GRTH* 5′ UTR distal region (Reference 4). GCNF is a member of the orphan nuclear receptor superfamily that is expressed in nucleus of spermatocytes and spermatids of adult mice. By contrast, the proximal region of GRTH 5′ UTR directs basal GRTH expression and androgen-induced intracrine expression in LCs through a functional androgen-response element (ARE). In the transgenic animal model, the AR antagonist Flutamide blocked GRTH–GFP expression in LCs and germ cells (GC), demonstrating direct (intracrine regulation by androgen/AR in LCs) and indirect effects of androgen/AR in GC, which do not express AR, through paracrine regulation by androgen/AR in Sertoli cells (Reference 4). Upon treatment with Flutamide, GCNF protein expression was significantly reduced in GC, indicating the presence of a regulatory network for an androgen GCNF–upstream 5′ UTR sequence to regulate GRTH expression in GC. ChIP analysis further demonstrated the association of GCNF with the GRTH sequence spanning the GCNF–binding site; the interaction was abolished in round spermatids by antagonist treatment. Flutamide treatment of WT mice caused selective reduction of GCNF and GRTH in round spermatids. GCNF knock-down in seminiferous tubules (dark zone, round spermatid–rich) caused a reduction in GRTH expression. Exposure of tubules to Flutamide caused a reduction in GCNF and GRTH expression while androgen exposure induced a significant increase. Moreover, GRTH associates with GCNF mRNA. Its absence in GRTH–null mice and in seminiferous tubules with GRTH knock-down caused increase on GCNF expression and mRNA stability (12 h KO versus 5 h WT), indicative of negative autocrine post-transcriptional regulation of GCNF by GRTH (Figure 1). The studies clearly established that GCNF present in germ cells is regulated by androgen in round spermatids and that this transfactor in turn regulates GRTH at the transcriptional level. Such regulation, most likely from Sertoli cells, could result from classical or non-classical A/AR–mediated actions. These *in vivo* and *in vitro* models link androgen action to GC through GCNF, as a transcriptionally and post-transcriptionally regulated transfactor that controls transcription/expression of GRTH. The studies provided, for the first time, a connection between androgen action and two relevant germ cell genes, *GCNF* and *GRTH*, which are essential for the progress of spermatogenesis, and established their relationship (Figure 1). Studies on the molecular regulatory aspects of androgen on GCNF transcription/expression and function provide valuable links and clearly facilitate what could be a quite difficult search for identification of A/AR–mediated regulated gene product(s) in Sertoli cells affecting germinal cell function and spermatogenesis.

The prolactin receptor

The human prolactin receptor (PRLR) mediates the diverse cellular actions of prolactin (PRL). PRL plays a major role in the proliferation and differentiation of breast epithelium and is essential for stimulation and maintenance of lactation. It has also been implicated in the development of breast cancer, tumor growth, and chemo-resistance. *PRLR* expression is controlled at the transcriptional level by several promoters (one generic, [PIII], and five human-specific [hPN1–hPN5]), which were defined and characterized in our laboratory. Each promoter directs transcription/expression of a specific non-coding exon 1 (E1–3, hEN1–hEN5), a common non-coding exon 2, and coding exons (E3–E11). Transcription of *PRLR* in breast cancer cells is directed by the preferentially utilized PIII, which lacks an estrogen-response element. BRET (bioluminescence resonance energy transfer) studies revealed ERα constitutive homodimers. Complex formation of the ERα dimer (non-DNA bound) with the transcription factors Sp1 and C/EBPβ, bound to their cognate sites at the PIII promoter, is required for basal (constitutive

ERα homodimers) and E2–induced transcriptional activation/expression of the human *PRLR* gene (Reference 1).

In tumoral breast PRL causes cell proliferation by activating its cognate receptor. Exacerbation of PRL's actions in breast cancer resulting from increase receptor expression can explain resistance to estrogen inhibitors in breast cancer. Our studies in MCF7 and T47D cells reveal stimulation by PRL of *PRLR* transcription, mRNA, and protein in the absence of E2, which is abolished by mutation of a GAS site (Stat5 DNA-recognition motif), by Stat5 siRNA, or by an ER antagonist (ICI). This indicates the participation of the ERα in PRLR transcription via PRL/PRLR/Stat5. PRL/PRLR induces phosphorylation of ERα through the JAK2/PI3K/MAPK/ERK– and the HER2–activated pathways (Reference 1). Increased recruitment of phospho-ERα to Sp1 and C/EBPβ bound at promoter sites is essential for PRL–induced receptor transcription. Direct evidence for local actions of PRL independent of E2 is provided in the up-regulation of *PRLR* transcription/expression via the Stat5/ER activation loop with requisite participation of signaling mechanisms (Reference 2). These studies, which demonstrated a central role ERa in PRLR receptor up-regulation, are of relevance in states refractory to aromatase inhibitors, in which cancer progression can be fueled by endogenous PRL. Therapies that inhibit the function of PRL or PRLR, combined with inhibitors of various signaling pathways, could reverse resistance in breast cancer. Moreover, a combination therapy targeting ER and PRLR directly can offer an additional avenue to eliminate constitutive activation of ER and of PRLR by endogenous prolactin.

In current studies we are addressing the role of EGFR in the up-regulation of the PRLR, given that most breast cancers that become resistant to endocrine therapy have elevated expression/activation of EGFR and its family member ERBB2. Other studies demonstrated the essential role of the D1 domain of the PRLR short-form structure and its inhibitory action on PRL–induced long-form function. Changes in PRLR structure and dimerization affinity are triggered by single mutations in D1, providing avenues for breast cancer treatment (Reference 2). Collaborative studies demonstrated that indirect cross-talk of membrane initiates GnRH/GnRHR and E2/ERa signaling in immortalized GnRH neurons (GT1–7cells). Although there was no evidence of direct interaction between ERs and GnRH-R, GnRH agonist reduced ERa homodimerization, which led us to postulate that signaling events initiated by GnRH agonist influence ER dimerization and/or availability (Reference 5).

PUBLICATIONS

1. Kavarthapu R, Tsai Morris CH, Dufau ML. Prolactin induces up-regulation of its cognate receptor in breast cancer cells via transcriptional activation of its generic promoter by cross-talk between ERa and STAT5. *Oncotarget* 2014; 5:9079–9091.
2. Kang JH, Hassan SA, Zhao P, Tsai Morris CH, Dufau ML. Impact of subdomain D1 of the short form S1b of the human prolactin receptor on its inhibitory action on the function of the long form of the receptor induced by prolactin. *Biochim Biophys Acta* 2014; 1840:2272–2280.
3. Yang, R, Tsai-Morris CH, Kang JH, Dufau ML. Elucidation of RNA binding regions of gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) to transcripts of a chromatin remodeling protein essential for spermatogenesis. *Horm Mol Biol Clin Investig* 2015; 22:119–130.
4. Kavarthapu R, Dufau ML. Germ Cell Nuclear (GCNF/RTR) regulates transcription of gonadotropin-regulated testicular RNA helicase (GRTH/DDX25) in testicular germ cells—the androgen connection. *Mol Endocrinol* 2015; 29:1792–804.
5. Chason RJ, Kang J-H, Gerkowicz SA, Dufau ML, Catt KJ, Segars JH. GnRH agonist reduces estrogen receptor dimerization in GT1-7 cells: Evidence for cross-talk between membrane-initiated estrogen and GnRH signaling. *Mol Cell Endocrinol* 2015; 404:67–74.

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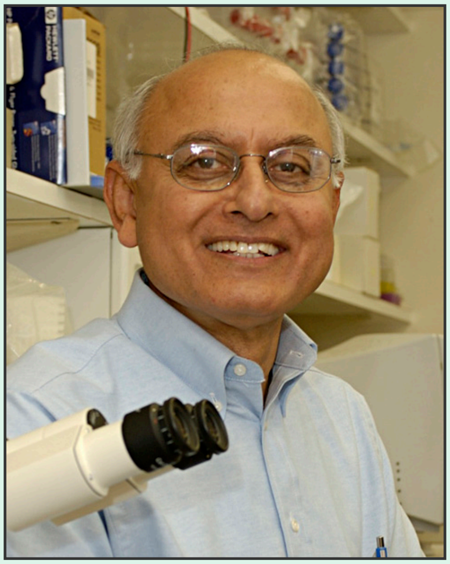
Childhood Neurodegenerative Lysosomal Storage Disorders

The Section on Developmental Genetics conducts both laboratory and clinical investigations into neurodegenerative lysosomal storage disorders (LSDs) primarily affecting children. Our current research focuses on understanding the molecular mechanism(s) of pathogenesis of a group of hereditary childhood neurodegenerative LSDs called neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease. Mutations in at least 13 different genes underlie various types of NCLs, and the list continues to grow. Currently, there is no effective treatment for any of the NCL types. The infantile NCL (or INCL) is an autosomal recessive disease caused by mutations in the *CLN1* gene, which encodes palmitoyl-protein thioesterase-1 (PPT1), a lysosomal depalmitoylating enzyme. Numerous proteins, especially in the brain, undergo palmitoylation, which is a post-translational modification of polypeptides in which a long-chain saturated fatty acid (predominantly palmitate) is attached to specific cysteine residues by thioester linkage. Palmitoylation (also called S-acylation) of proteins plays important roles in signal transduction pathways, and constitutive and controlled turnover of palmitate regulates membrane association and intracellular trafficking of signaling GTPases. Moreover, numerous receptors, transporters, and ion channels are S-acylated with diverse functional consequences, including protein-protein interactions, stability, and the regulation of assembly and trafficking. Palmitoylation is catalyzed by DHHC (Asp-His-His-Cys)-palmitoyl-acyltransferases (PATs), and the removal of palmitate (depalmitoylation) is catalyzed by palmitoyl-protein thioesterases (PPTs). Twenty three palmitoylacyl transferases are encoded in the mammalian genome; four thioesterases (depalmitoylating enzymes) have been characterized. While S-acylation regulates the function of many important proteins requiring membrane anchorage, the proteins must also be depalmitoylated for recycling or for degradation by lysosomal hydrolases. Therefore, dynamic palmitoylation (palmitoylation-depalmitoylation), essential for steady-state membrane localization and signaling by many proteins, requires the interplay of both the PATs and the PPTs. Several years ago, it was discovered that mutations in a lysosomal thioesterase palmitoyl-protein thioesterase-1 (PPT1) cause accumulation of palmitoylated proteins (constituents of ceroid) in the lysosomes, leading to the devastating neurodegenerative LSD INCL. However, the precise molecular mechanism of INCL pathogenesis remains largely unclear. Children afflicted with INCL are normal at birth, but exhibit signs of psychomotor retardation by 11 to 18 months of age. By two years of age, they are completely blind as a result of retinal degeneration and, by the age 4, manifest no brain activity and remain in a vegetative state for several more years before eventual death. These grim facts underscore the urgent need for the development of rational and effective therapeutic strategies not only for INCL but also for all NCLs. Thus, our goals are to first understand the molecular mechanism(s) of pathogenesis through innovative laboratory investigations and, second, apply the knowledge gained to develop novel therapeutic strategies for INCL and possibly for other types of Batten diseases.

In our laboratory studies on INCL, we use cultured cells from patients as well as from *Ppt1*–knockout ($Ppt1^{-/-}$) mice, which recapitulate virtually all clinical and pathological features of the disease. During the past several years, we discovered that PPT1 deficiency causes endoplasmic reticulum (ER) and



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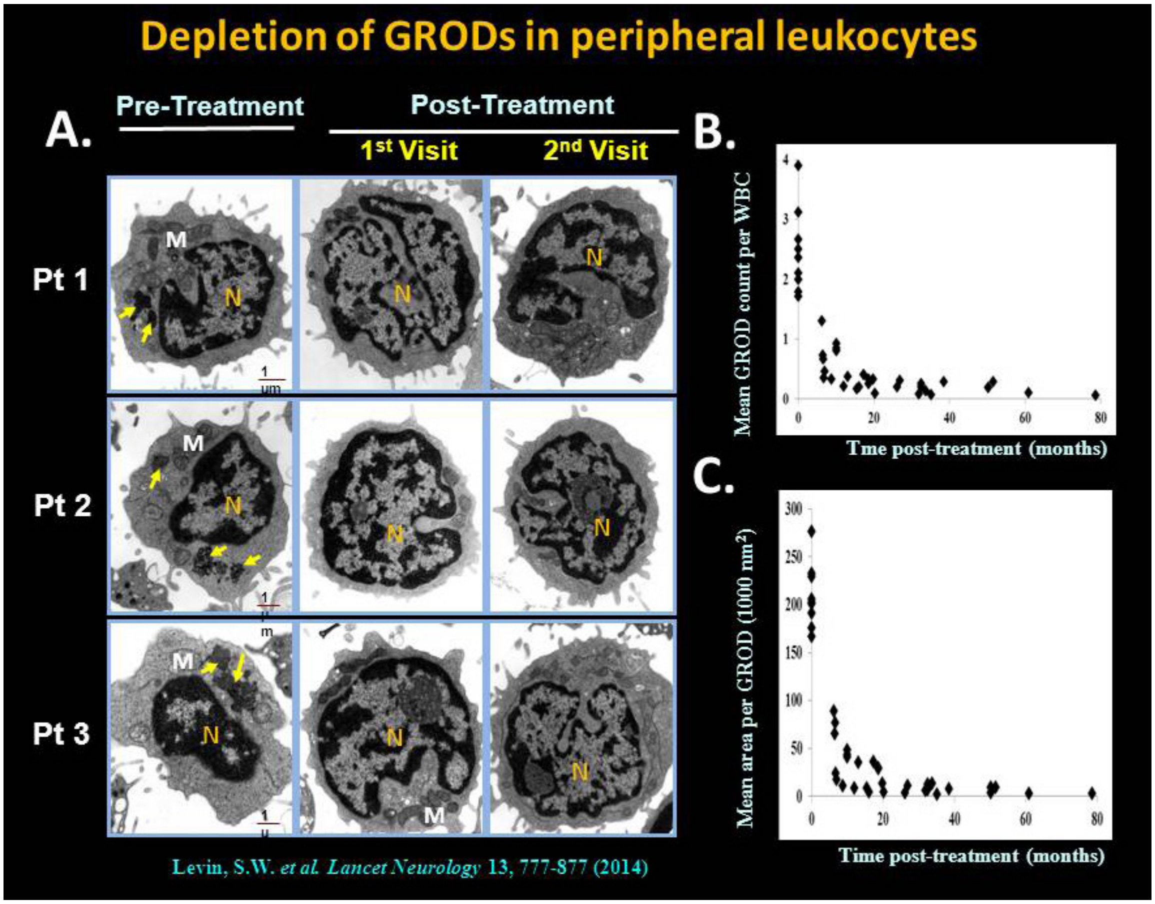

Depletion of lysosomal ceroid (GRODs) in INCL patients by a combination of cysteamine bitartrate and *N*-acetylcysteine

Oral cysteamine bitartrate and *N*-acetylcysteine combination mediates depletion of lysosomal ceroid (GRODs) from peripheral leukocytes of patients with INCL. Transmission electron-microscopic analyses of peripheral white blood cells from all patients were carried out at all visits, both pre-treatment and post-treatment. GRODs are irregularly shaped dark extranuclear structures that are distinct from mitochondria, secretory granules, and other membrane-enclosed cellular organelles. A. Representative micrographs from three patients are shown at three time points (pre-treatment and two post-treatment). Note that GRODs (*pale green arrows*) are readily detectable only in the pre-treatment image as characteristically dark, dense structures with irregular edges (clearly distinguishable from the nucleus [N] and mitochondria [M] at high magnification). B. Number of GRODs per cell and (C.) area of the GRODs for all nine patients together declined over time; the reduction began from the first visit after initiation of treatment. M=mitochondria. N=nucleus. GRODs=granular osmiophilic deposits. WBC=white blood cell.

oxidative stress, which at least in part contribute to neuropathology in INCL. Moreover, we delineated a mechanism by which PPT1 deficiency may disrupt the recycling of the synaptic vesicles (SVs), causing progressive loss of the SV pool size that is required for maintaining uninterrupted neurotransmission at nerve terminals. We also developed a noninvasive method, using MRI and MRS (magnetic resonance spectroscopy), to evaluate the progression of neurodegeneration in *Ppt1*$^{-/-}$ mice. The method permits repeated evaluations of potential therapeutic agents in treated animals. In addition, in collaboration with the NEI, we are conducting studies to determine whether electro-retinography can be used to assess the progressive retinal deterioration in *Ppt1*$^{-/-}$ as well as in *Ppt1*–knock-in (KI) mice, which carry the most common nonsense mutation found in the U.S. INCL patient population. We also discovered that the blood-brain barrier is disrupted in *Ppt1*$^{-/-}$ mice and that this pathology is ameliorated by treatment with resveratrol, which has anti-oxidant properties. More recently, we identified and characterized a non-toxic, thioesterase-mimetic, antioxidant small molecule, *N*-(*tert*-Butyl)hydroxylamine (NtBuHA), which mediates ceroid depletion, preserves motor function and modestly extends lifespan. Such compounds are potential therapeutic targets for INCL.

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From left to right: Maria B. Bagh, Ashleigh Bouchelion, Arnab Datta, Sondra W. Levin, Anil B. Mukherjee, Vinay Patil, and Zhongjian (Gary) Zhang.

Generation of a mouse model carrying the most common nonsense mutation in the *Cln1* gene

Nonsense mutations account for 5–70% of all genetic disorders. In the U.S., nonsense mutations in the *CLN1* gene (also known as the *PPT1* gene) underlie more than 40% of INCL cases. To evaluate nonsense suppressors *in vivo*, we sought to generate a reliable mouse model of INCL carrying the most common *Ppt1* nonsense mutation (c.451C→T) found in the U.S. patient population. We knocked-in (KI) the c.451C→T nonsense mutation in the *Ppt1* gene in C57 embryonic stem (ES) cells using a targeting vector in which LoxP flanked the Neo cassette; the cassette was removed from targeted-ES cells by electroporating *Cre*. Two independently targeted ES clones were injected into blastocysts to generate syngenic C57 KI mice, obviating the necessity for extensive back-crossing. Generation of *Ppt1*-KI mice was confirmed by DNA sequencing, which showed the presence of c.451C→T mutation in the *Ppt1* gene. The mice are viable and fertile, although they developed spasticity (a “clasping” phenotype) at a median age of six months. Autofluorescent storage materials accumulated throughout the brain regions and in visceral organs. Electron-microscopic analysis of the brain and the spleen showed granular osmiophilic deposits. Elevated neuronal apoptosis was particularly evident in cerebral cortex, and abnormal histopathological and electro-retinographic (ERG) analyses attested to striking retinal degeneration. Progressive deterioration of motor coordination and behavioral parameters continued until eventual death. Our findings show that *Ppt1*-KI mice reliably recapitulate the INCL phenotype, providing a platform for testing the efficacy of existing and novel nonsense-suppressors alone or in combination with other drugs *in vivo*.

Discovery of a pathogenic link between two of the most lethal NCLs

In multicellular organisms, the lysosome is the major degradative organelle responsible for disposing off the damaged macromolecules and organelles from the cell. It has been reported that impaired lysosomal degradative capability leads to pathogenesis of many neurodegenerative disorders including LSDs. As noted above, neurodegeneration is a devastating manifestation in the majority of the more than 50 LSDs. Moreover, lysosomal degradative capability has been reported to be impaired in several late-onset neurodegenerative diseases such as Alzheimer’s, Huntington’s, and Parkinson’s diseases. Cathepsin D (CD) is a major lysosomal aspartic protease in lysosomes. Lysosomal CD activity catalyzes degradation and clearance of exogenous as well as endogenous macromolecules and damaged organelles delivered to the lysosome. Intracellular accumulation of undegraded long-lived proteins and other macromolecules leads to the pathogenesis of many neurodegenerative disorders. Paradoxically, both CD overexpression and CD deficiency have been reported to underlie neurodegenerative diseases. However, despite intense studies, this paradox has remained unresolved until now.

NCLs (Batten disease) are the most common (1 in 12 500 births), autosomal recessive, neurodegenerative LSDs, mostly affecting children. Mutations in 13 different genes (called CLNs) underlie various types of NCLs. Among all the NCLs, the infantile NCL (INCL) and congenital NCL (CNCL) are the most devastating diseases. Although the inactivating mutations in the *CLN1* gene encoding palmitoyl-protein thioesterase-1 (PPT1) cause INCL, mutations in the *CLN10/Ctsd* gene encoding CD underlie CNCL. We sought to determine whether there is a pathogenic link between INCL and CNCL. The synthesis of CD occurs in the endoplasmic reticulum (ER) as a pre-propeptide with a molecular mass of about 50 kDa. The cleavage of the leader peptide in the ER generates the 48 kDa precursor of mature-CD (pro-CD). In the Golgi complex, attachment of mannose 6-phosphate to pro-CD facilitates the protein's binding to endosomal/lysosomal sorting receptors. The receptor-ligand complexes then exit the trans-Golgi network in clathrin-coated intermediates and fuse with the endosomal system. The low pH of the late endosomal lumen facilitates dissociation of the receptor-ligand complexes and allows the ligand (i.e., pro-CD) to be delivered to lysosome. The pro-CD then undergoes further proteolytic cleavage by cathepsin B (CB) and cathepsin L (CL), respectively, which generates the 31 and 14 kDa fragments, non-covalent dimerization of which constitutes the mature, catalytically active CD. We used *Cln1*$^{-/-}$/*Ppt1*$^{-/-}$ mice, which recapitulate virtually all clinical and pathological features of INCL, to test for a pathogenic link between INCL and CNCL. Our results show that despite Cln10/Ctsd overexpression, defective processing of pro-CD to mature-CD in lysosome leads to lysosomal CD deficiency causing neuropathology in INCL. Because CD deficiency underlies CNCL, we propose that CD deficiency in the lysosome is the link between INCL and CNCL. Furthermore, our results suggest that *N-tert*-(Butyl)hydroxylamine (NtBuHA) may have therapeutic potential for patients with INCL.

A combination of cysteamine bitartrate and *N*-acetylcysteine for INCL patients: a bench-to-bedside study

Previously, we discovered that both cysteamine- and *N*-acetylcysteine–mediated ceroid depletion appeared to counteract the pathological changes in cultured cells from INCL patients. On the basis of these results, we conducted a bench-to-bedside clinical trial to determine whether the combination of oral cysteamine bitartrate and *N*-acetylcysteine is beneficial for patients with NCL. Children between 6 months and 3 years of age suffering from INCL with any two of the seven most lethal *PPT1* mutations were eligible for inclusion in this pilot study. All patients were recruited from physician referrals. Patients received oral cysteamine bitartrate (60 mg/kg per day) and *N*-acetylcysteine (60 mg/kg per day) and were assessed every 6–12 months until they had an isoelectric electroencephalogram (EEG, attesting to a vegetative state) or were too ill to travel. Patients were also assessed by electro-retinography, brain MRI and MRS, and electron-microscopic analyses of leukocytes for granular osmiophilic deposits (GRODs). Children also underwent physical and neurodevelopmental assessments on the Denver scale. Outcomes were compared with the reported natural history of INCL and that of affected older siblings. This trial was registered with ClinicalTrials.gov, number NCT00028262.

Between March 14, 2001, and June 30, 2012, we recruited ten children with INCL; one child was lost to follow-up after the first visit and nine patients (five girls and four boys) were followed for 8 to 75 months. MRI showed abnormalities similar to those in previous reports; brain volume and *N*-acetyl aspartic acid (NAA) decreased steadily, but no published quantitative MRI or MRS studies were available for comparison. None of the children acquired new developmental skills, and their retinal function declined progressively. Average time to isoelectric EEG (52 months, SD 13) was longer than reported previously (36 months). At the first follow-up visit, peripheral leukocytes in all nine patients showed virtually complete depletion of GRODs. Parents and physicians reported less irritability, improved alertness, or both in seven patients. No treatment-related adverse events occurred apart from mild gastrointestinal discomfort in two patients, which disappeared when liquid cysteamine bitartrate was replaced with capsules. The study was completed and the results recently published. Our findings suggest that combination therapy with cysteamine bitartrate and *N*-acetylcysteine is associated with delay of isoelectric EEG, depletion of GRODs, and subjective benefits, as reported by parents and physicians. Our systematic and quantitative report of the natural history of patients with INCL provides a platform for evaluating experimental therapeutic strategies in the future.

MR spectroscopy to evaluate disease progression in INCL

As part of the pilot study to evaluate treatment benefits of cysteamine bitartrate and *N*-acetylcysteine, we quantitatively measured brain metabolite levels using magnetic resonance spectroscopy (MRS). A subset of two patients from a larger treatment and follow-up study underwent serial quantitative single-voxel MRS examinations of five anatomical sites. Three echo times were acquired in order to estimate metabolite T2 [quantification of the absolute concentration of metabolites using long-echo-time (TE) acquisition schemes]. Measured metabolite levels included a correction for partial volume of cerebrospinal fluid. Comparison of INCL patients was made to a reference group composed of asymptomatic and minimally symptomatic Niemann-Pick disease type C patients. In INCL patients, *N*-acetylaspartate (NAA) was abnormally low at all locations upon initial measurement, and further declined throughout the follow-up period. In the cerebrum (affected early in the disease course), choline and myo-inositol were initially elevated and fell during the follow-up period, whereas in the cerebellum and brainstem (affected later), choline and myo-inositol were initially normal and rose subsequently. Choline and myo-inositol levels in our patients are consistent with patterns of neuro-inflammation observed in two INCL mouse models. Low, persistently declining NAA was expected based on the progressive, irreversible nature of the disease. Progression of metabolite levels in INCL has not been previously quantified; therefore the results of this study serve as a reference for quantitative evaluation of future therapeutic interventions.

Suppression of agrin-22 production and synaptic dysfunction in Cln1$^{-/-}$ mice

Oxidative stress in the brain is highly prevalent in many neurodegenerative disorders including LSDs causing neurodegeneration. Despite intense studies, a precise mechanism linking oxidative stress to neuropathology in specific neurodegenerative diseases remains largely unclear. As mentioned in the introduction, INCL is a devastating neurodegenerative lysosomal storage disease caused by mutations in the *CLN1* gene encoding palmitoyl-protein thioesterase-1. Previously we reported that in the brain of *Cln1*$^{-/-}$ mice, which mimic INCL, and in postmortem brain tissues from INCL patients, elevated oxidative stress is readily detectable. We used molecular, biochemical, immunohistological, and electrophysiological analyses of brain tissues of *Cln1*$^{-/-}$ mice to study the role(s) of oxidative stress in mediating neuropathology. Our results show that, via upregulation of the transcription factor CCAAT/enhancer binding protein-δ, oxidative-stress in the brains of *Cln1*$^{-/-}$ mice stimulated expression of serpin a1, which is an inhibitor of a serine protease, neurotrypsin. Moreover, in these mice suppression of neurotrypsin activity by serpin a1 inhibited the cleavage of agrin (a large proteoglycan), which substantially reduced the production of agrin-22, which is essential for synaptic homeostasis. Direct whole-cell recordings at the nerve terminals of *Cln1*$^{-/-}$ mice showed inhibition of Ca^{2+} currents, attesting to synaptic dysfunction. Treatment of the mice with the thioesterase-mimetic small molecule NtBuHA elevated agrin-22 levels. Our findings provide insight into a novel pathway linking oxidative stress with synaptic pathology in *Cln1*$^{-/-}$ mice and suggest that NtBuHA, which raised agrin-22 levels, may ameliorate synaptic dysfunction in this devastating neurodegenerative disease.

PUBLICATIONS

1. Levin SW, Baker EH, Zein WM, Zhang Z, Quezado ZM, Miao N, Gropman A, Griffin KJ, Bianconi S, Chandra G, Khan OI, Caruso RC, Liu A, Mukherjee AB. Cysteamine bitartrate and N-acetylcysteine for patients with infantile neuronal ceroid lipofuscinosis: a pilot study. *Lancet Neurol* 2014 13:777–787.
2. Bouchelion A, Zhang Z, Li Y, Qian H, Mukherjee AB. Mice homozygous for c.451C > T mutation in Cln1 gene recapitulate INCL phenotype. *Ann Clin Transl Neurol* 2014 1:1006–1023.
3. Peng S, Xu J, Pelkey KA, Chandra G, Zhang Z, Bagh MB, Yuan X, Wu L-G, McBain CJ, Mukherjee AB. Suppression of agrin-22 production and synaptic dysfunction in Cln1$^{-/-}$ mice. *Ann Clin Transl Neurol* 2015; 2:1085–1104.
4. Chandra G, Bagh MB, Peng S, Saha A, Sarkar C, Moralle M, Zhang Z, Mukherjee AB. Cln1 gene disruption in mice reveals a common pathogenic link between two of the most lethal childhood neurodegenerative lysosomal storage disorders. *Hum Mol Genet* 2015 14:5416–5432.
5. Baker EH, Levin SW, Zhang Z, Mukherjee AB. Evaluation of disease progression in INCL by MR spectroscopy. *Ann Clin Transl Neuro* 2015 2:797–809.

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Molecular Biology, Regulation, and Biochemistry of UDP–Glucuronosyltransferase Isozymes

UDP–glucuronosyltransferase (UGT) isozymes–distributed primarily in liver, kidney, the gastrointestinal tract, and steroid-responsive tissues–are known to carry out the essential function of converting innumerable structurally diverse lipophilic endogenous substrates, such as neurotoxic bilirubin, catechol estrogens, and dihydrotestosterone, and dietary aromatic-like therapeutics to water-soluble excretable glucuronides. Most importantly, environmental pro-carcinogens and contaminants derived from pyrolysates are converted to avoid chemical toxicities. Our studies demonstrated that each UGT isozyme so far examined requires on-going regulated phosphate signaling, which enables an active site to convert an unspecified number of substrates. Recently, further studies showed that the human prostate luminal-cell UGT-2B15 and basal-cell UGT-2B17, which are 97% identical, have an additional Src or Src/PKCε–partnership phosphorylation site, respectively, at position 98–100. We found that the two isozymes exhibit opposite behavior when their Src sites are compromised: UGT-2B15 becomes polyubiquitinated, thus exhibiting a pro-apoptotic effect, while the activity of UGT-2B17 is elevated by 50%. Our studies will thus continue to detail and understand the specific reactions involved in human prostate–luminal cell apoptosis and de-ubiquitination. In collaboration with ongoing research within the NCI, NIDCR, and with researchers at the University of Maryland, we will also carry out basic studies to better understand prostate cancer development.

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Prostate-distributed mouse Ugt2b34 and Ugt2b36 control estrogenic metabolites.

To establish an *in vivo* mammary-gland model that prevents depurination by 4-OH-catecholestrogens associated with the initiation of carcinogenesis, we pursued studies to identify mouse homologs of the highly effective human UGT-2B7. Using sequence analysis, we found that mouse Ugt-2b34 and Ugt-2b36 homologs avidly metabolize the test agent 4-hydroxyestrone, with Ugt-2b35 expressing trivial activity. Unlike low K_m UGT-2B7 (14M), Ugt-2b34 and Ugt-2b36 metabolized 4-hydroxyestrone with 90M K_m and 430M K_m, respectively. Unexpectedly, the mouse isozymes are distributed primarily in male hormone–responsive tissues, whereas human UGT-2B7 is found primarily in female hormone–responsive tissues. Also, we found that Ugt-2b34 metabolizes the non-classical estrogenic DHT metabolite ADT-diol at a greater rate than DHT, which is not known to be estrogenic. Notably, UGT-2B7 does not metabolize xeno-estrogens; Ugt-2b34 and Ugt-2b36 did, however, metabolize bisphenol A (BPA) and diethylstilbestrol (DES) at superior rates. We also found, through real-time PCR–based analysis of estrogen receptor alpha (*Esr1*) gene knockout in mouse prostate, 50% and 63% lower Ugt2b34 mRNA and Ugt2b36 mRNA levels, respectively, than in controls. However, estrogen receptor beta (*Esr2*) knockout (KO) revealed a 2.7/3.3-fold increase in Ugt-2b34 mRNA and Ugt-2b36 mRNA, respectively, in prostate. *Esr1* KO completely suppressed Ugt-2b34 and Ugt-2b36 mammary-gland mRNA; *Esr2* KO caused a 12-fold increase in Ugt-2b34 mRNA without affecting Ugt-2b36 mRNA. Hence, according to tissue-distribution studies, it appears that male mice benefit from both Ugt isoforms, while females benefit from only one Ugt. Our findings for Ugt-2b34 and Ugt-2b36 suggest that the two mouse isozymes are intrinsically programmed to protect against a more complex environment than are human high-activity UGT-2B7 and low-activity UGT-2B4 isozymes (Raychoudhuri A et al., *Biosci Rep* 2015;in press).

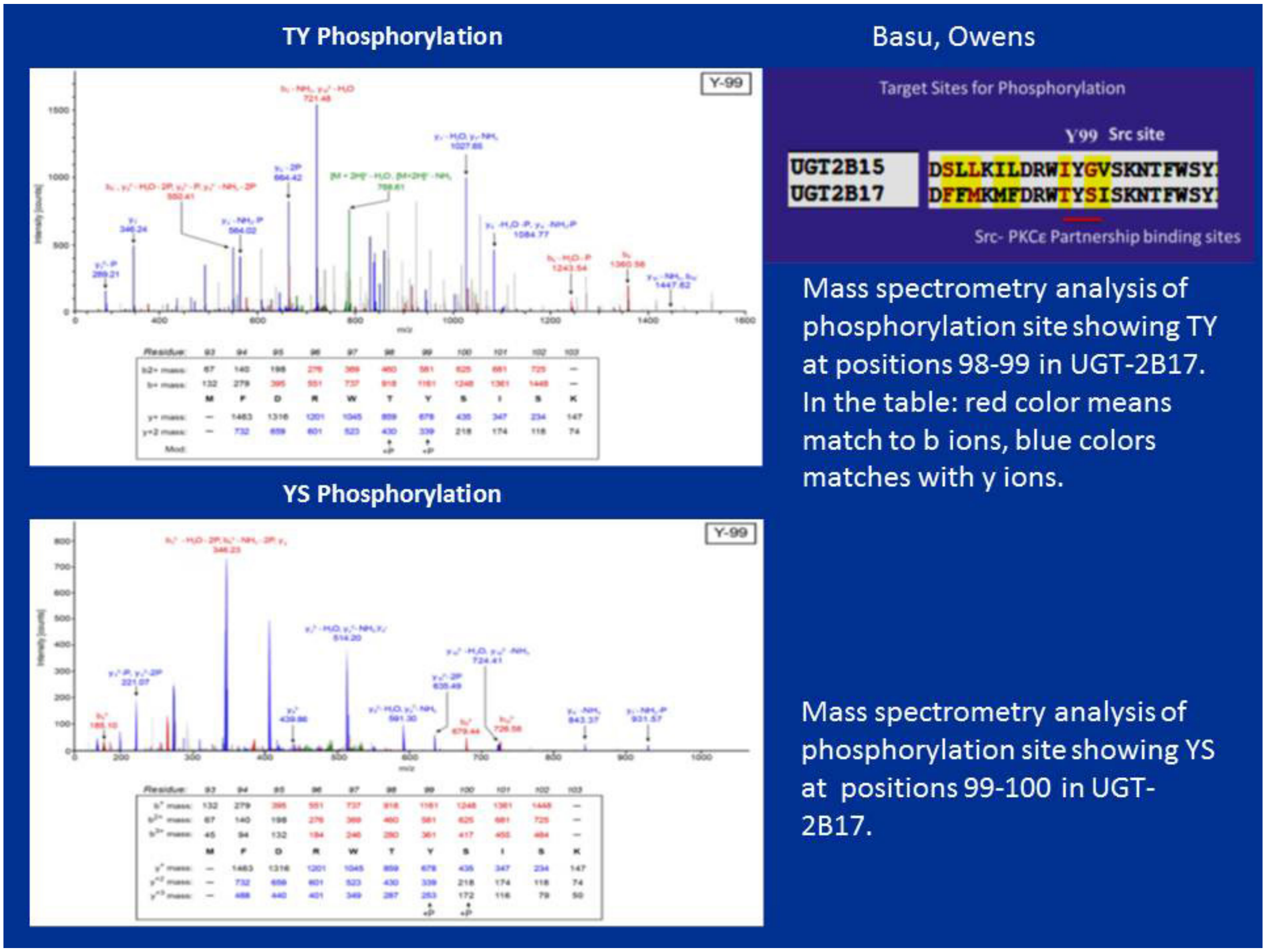
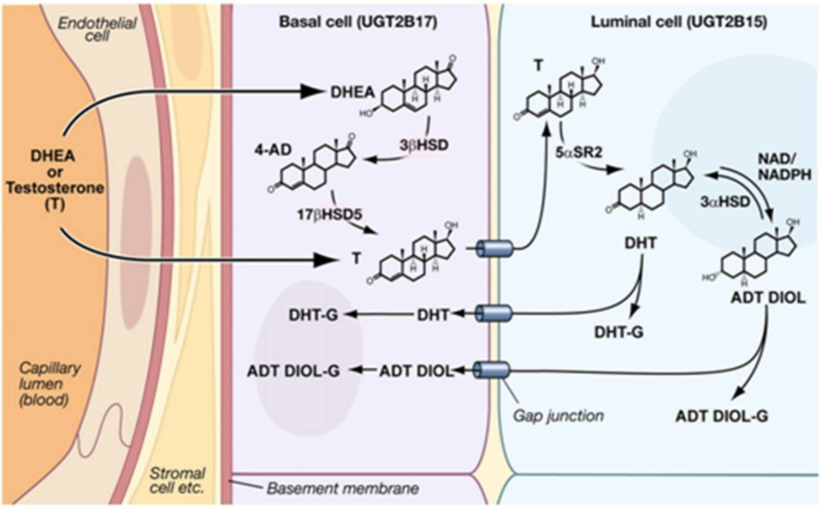


Figure 1. Mass-spectrometric analysis of TpYpSp phosphorylation of UGT-2B17
Phosphorylation of UGT-2B17 at 98–100 (TYS) residues

PUBLICATIONS

1. Basu NK, Kole L, Basu M, Chakraborty K, Mitra PS, Owens IS. The major chemical detoxifying system of UDP-glucuronosyltransferases requires regulated phosphorylation supported by protein kinase C. *J Biol Chem* 2008; 283:23048–23061.
2. Banerjee R, Pennington MW, Garza A, Owens IS. Mapping the UDP glucuronic acid binding site in UDP-glucuronosyltransferase-1A10 by homology-based modeling: confirmation with biochemical evidence. *Biochemistry* 2008; 47:7385–7392.
3. Mitra PS, Basu NK, Owens IS. Src supports UDP-glucuronosyltransferase-2B7 detoxification of catechol estrogens associated with breast cancer. *Biochem Biophys Res Commun* 2009; 382:651–656.
4. Mitra PS, Basu NK, Basu M, Chakraborty S, Saha T, Owens IS. Regulated phosphorylation of a major UDP-glucuronosyltransferase isozyme by tyrosine kinases dictates endogenous substrate selection for detoxification. *J Biol Chem* 2011; 286:1639–1648.
5. Chakraborty SK, Basu NK, Jana S, Basu M, Raychoudhuri A, Owens IS. Protein kinase Calpha and Src kinase support human prostate-distributed dihydrotestosterone-metabolizing UDP-glucuronosyltransferase 2B15 activity. *J Biol Chem* 2012; 287:24387–24396.



Distribution of normal prostate steroidogenic and UGT isozymes as presented in our publication , Chakraborty et. al, J. Biol. Chem, 287 : 24387, 2012. Based on studies cited, prostate DHT synthesis and its metabolism are summarized in the schematic. The EM shows 1:1 stratification of human prostate basal/luminal cells with intervening gap junctional structures that likely allow movement of small molecules between the two cells.

Figure 2. Predicted small-molecule movements and enzymatic reactions in prostate epithelial cells

Image taken from S. K. Chakraborty et al., *J Biol Chem* 2012;287:24387.

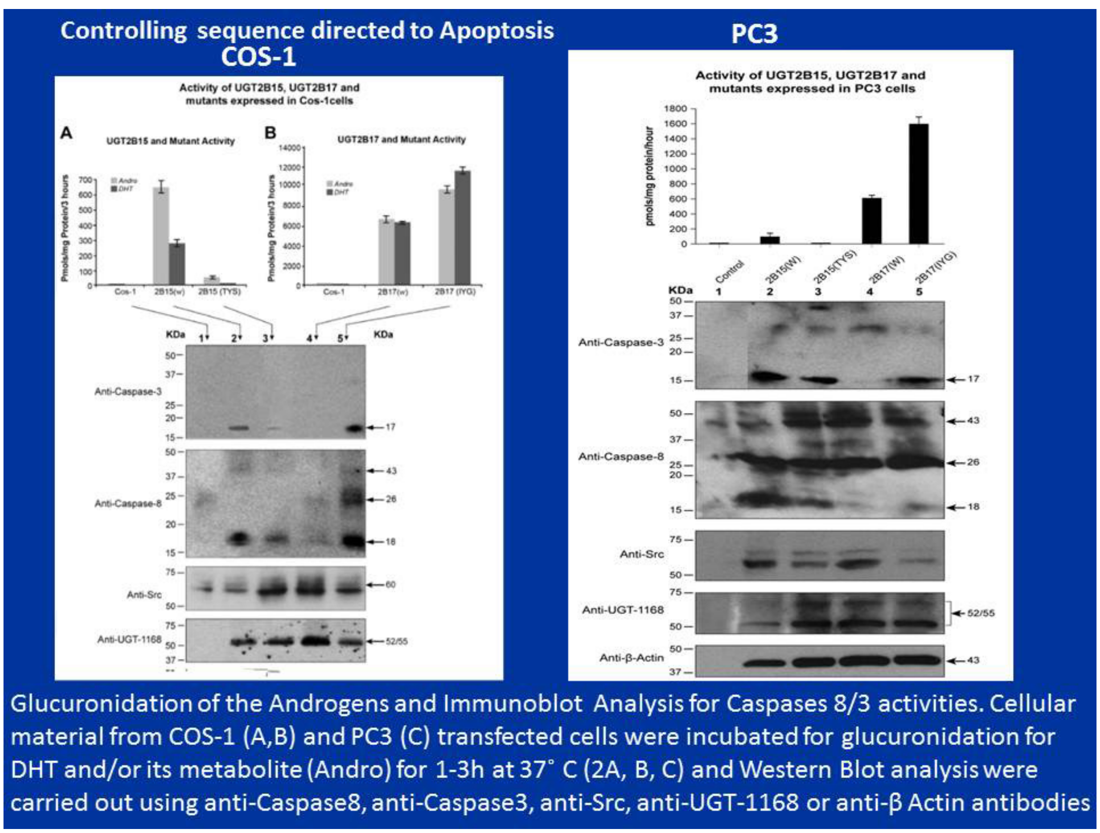
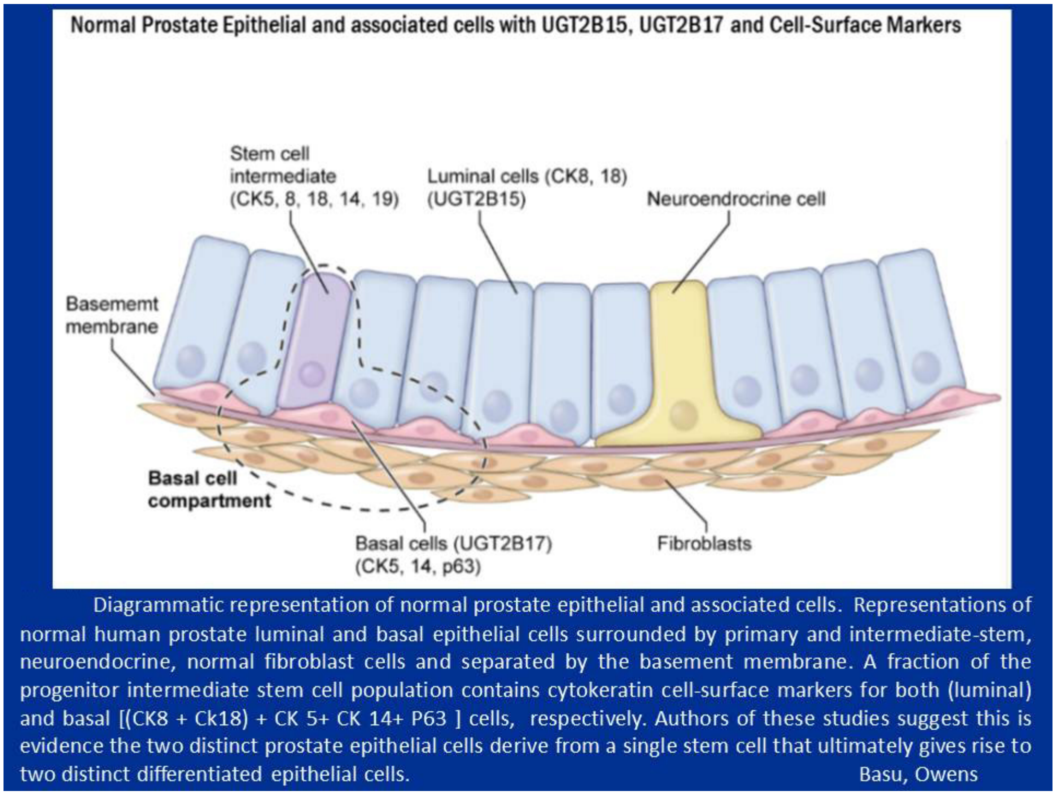


Figure 3. Controlling sequences for apoptosis

Controlling sequences for pro-apoptosis and anti-apoptosis in UGT-2B15 and UGT-2B17 expressed in COS-1 cells and the aggressive PC3 prostate cell line

Figure 4. Diagrammatic representation of normal prostate epithelial and associated cells

Normal prostate epithelial and associated cells with UGT-2B15, UGT-2B17, and cell surface markers.



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We study the molecular, biochemical, and cellular processes that underlie genetic disorders resulting from impaired cholesterol homeostasis. The disorders include malformation/cognitive impairment syndromes resulting from inborn errors of cholesterol synthesis, neurodegenerative disorders resulting from impaired intracellular cholesterol and lipid transport, and Autistic Spectrum disorder (ASD) associated with low cholesterol. Human malformation syndromes attributable to inborn errors of cholesterol synthesis include Smith-Lemli-Opitz syndrome (SLOS), lathosterolosis, desmosterolosis, X-linked dominant chondrodysplasia punctata type 2 (CDPX2), and the CHILD syndrome. Neimann-Pick disease type C (NPC) results from impaired intracellular transport of cholesterol and lipids, leading to neuronal loss. Our basic research uses mouse models of these genetic disorders to understand the biochemical, molecular, cellular, and developmental processes that underlie the birth defects and clinical problems encountered in affected patients. Our clinical research focuses on translating basic findings to the clinic. Natural history trials of both SLOS and NPC1 are ongoing. Our emphasis on both basic and clinical research allows us to integrate laboratory and clinical data in order to increase our understanding of the pathological mechanisms underlying both SLOS and NPC, with the goal of improving clinical care of these patients. Therapeutic trials have been conducted for both disorders, and we are conducting a Phase I therapeutic trial of 2-hydroxypropyl-B-cyclodextrin for NPC. A multicenter, multinational clinical efficacy trial of 2-hydroxypropyl-B-cyclodextrin was initiated in the fall of 2015.

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Inborn errors of cholesterol synthesis

SMITH-LEMLI-OPITZ SYNDROME (SLOS)

SLOS is an autosomal recessive, multiple-malformation syndrome characterized by dysmorphic facial features, cognitive impairment, hypotonia, poor growth, and variable structural anomalies of heart, lungs, brain, limbs, gastrointestinal tract, and genitalia. The SLOS phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die as result of multiple major malformations, while mild SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol.

Our laboratory initially cloned the human 3β-hydroxysterol Δ7-reductase gene (*DHCR7*) and demonstrated mutations of the gene in SLOS patients. Together with others, we have so far identified over 100 mutations of *DHCR7*. We also used gene targeting in murine embryonic stem cells to produce multiple SLOS mouse models, including a null deletion and a hypomorphic point mutation. Mouse pups homozygous for the null mutation (*Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$) exhibit variable craniofacial anomalies, are growth-retarded, feed poorly, appear weak, and die during the first day of life because they fail to feed. Thus, we were not able to use them to study postnatal brain development, myelination, or behavior or to test therapeutic interventions. For this reason, we developed a mis-sense allele (*Dhcr7*^{T93M}). The T93M mutation is the second most common mutation found in SLOS patients. *Dhcr7*$^{T93M/T93M}$ and *Dhcr7*$^{T93M/\Delta3\text{-}5}$ mice are viable and demonstrate SLOS with a gradient of biochemical severity (*Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$>*Dhcr7*$^{T93M/\Delta3\text{-}5}$>*Dhcr7*$^{T93M/T93M}$). We used *Dhcr7*$^{T93M/\Delta3\text{-}5}$ mice to test the efficacy of therapeutic interventions on tissue sterol profiles. As expected, dietary cholesterol therapy improved the sterol composition in peripheral tissues but not in the central nervous system. Treatment of mice with the statin simvastatin improved the biochemical defect in both peripheral and central nervous system tissue, suggesting that simvastatin therapy may be used to treat some of the behavioral and learning problems in children with SLOS. Most recently, we developed a zebrafish model for SLOS that will allow us to study the impact of aberrant cholesterol synthesis on behavior. Characterization of induced pluripotent stem cells from SLOS patients demonstrated a defect in neurogenesis. The defect in neurogenesis results from inhibition of Wnt signaling due to a toxic effect of 7-DHC

Figure 1.
Dr. Porter and one of our patients. Neurological exams in children frequently involve "playing" with the child.

As part of our clinical studies on SLOS, we identified a novel oxysterol, 27-hydroxy-7-dehydrocholesterol (27-7DHC), derived from 7-DHC in SLOS patients. We therefore investigated whether 27-7DHC contributes to the pathology of SLOS and found a strong negative correlation between plasma 27-7DHC and cholesterol levels in these patients. In addition, previous work showed that low cholesterol levels impair hedgehog signaling. Therefore, we hypothesized that increased 27-7DHC levels would have detrimental effects during development in response to suppression of cholesterol levels. To test our hypothesis, we produced SLOS mice (*Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$) expressing a *CYP27* (sterol 27-hydroxylase) transgene. *CYP27Tg* mice display increased *CYP27* expression and elevated 27-hydroxycholesterol levels but normal cholesterol levels. While *Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$ mice are growth-retarded, exhibit a low incidence of cleft palate (9%), and die during the first day of life, *Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$*:CYP27Tg* embryos are stillborn and have multiple malformations, including growth retardation, micrognathia, cleft palate (77%), lingual and dental hypoplasia, ankyloglossia, umbilical hernia, cardiac defects, cloacae, curled tails, and limb defects. We observed autopod defects (polydactyly, syndactyly, and oligodactyly) in 77% of the mice. Consistent with our hypothesis, sterol levels were halved in the liver and 20-fold lower in the brain tissue of *Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$*:CYP27Tg* than in *Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$ embryos. The fact that 27-7DHC plays a role in SLOS may explain some of the phenotypic variability and may lead to development of a therapeutic intervention. The project is a good example of the benefits of integrating clinical and basic science to both understand the pathology of SLOS and develop potential therapeutic interventions.

To gain insight into pathophysiological processes contributing to SLOS, we completed a series of proteomic experiments to identify proteins that are differentially expressed in the cortex of *Dhcr7*$^{\Delta3\text{-}5/\Delta3\text{-}5}$ embryos. Functional analysis demonstrated alterations in the Rho/Rac-LIMK-Cofilin pathway that result in altered dendrite and axon formation. The defect in neuronal development may contribute to the cognitive deficits found in SLOS.

We are conducting a longitudinal Natural History trial. Given that SLOS patients have a cholesterol deficiency, they may be treated with dietary cholesterol supplementation. To date, we have evaluated over 75 SLOS patients. As part of a Bench-to-Bedside proposal, we continue, in collaboration with Fran Platt's laboratory, to study impaired cholesterol and glycosphingolipid transport in SLOS and to investigate novel therapeutic interventions.

One reason for studying rare genetic disorders is to gain insight into more common disorders. Most patients with SLOS exhibit autistic characteristics. We are currently collaborating with other NIH and extramural groups to evaluate this further.

Lathosterolosis, desmosterolosis, and HEM dysplasia

Lathosterol 5-desaturase catalyzes the conversion of lathosterol to 7-dehydrocholesterol, representing the enzymatic step immediately preceding the defect in SLOS. Thus, to gain a deeper understanding of the roles of reduced cholesterol versus elevated 7-dehydrocholesterol in SLOS, we disrupted the mouse lathosterol 5-desaturase gene (*Sc5d*) by using targeted homologous recombination in embryonic stem cells. *Sc5d*$^{-/-}$ pups are stillborn, present with micrognathia and cleft palate, and exhibit limb-patterning defects. Many of the malformations in the mutant mice resemble malformations in SLOS and are consistent with impaired hedgehog signaling during development. Biochemically, the mice exhibit markedly elevated lathosterol levels and reduced cholesterol levels in serum and tissue.

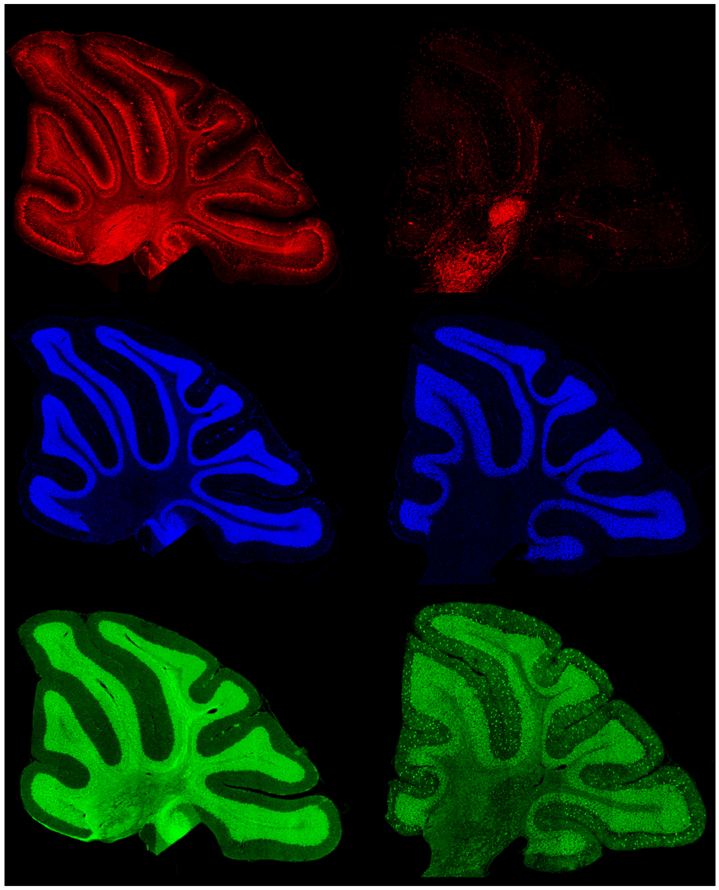

Figure 2. Comparison of control and late-stage NPC1 mouse cerebellum

Left: A sagittal cerebellar section from a control mouse is depicted, using fluorescent staining against a neuronal marker SMI32 (*red*), nuclear counterstain DAPI (*blue*), and lipid GM1 (*green*). Right: A comparable section from an *Npc1*–mutant mouse; note the extreme loss of Purkinje neurons (*red*), the perturbation of myelination (deep cerebellar tissue in *green*), and the green punctate accumulations of GM1 in the outermost cerebellar layer that are characteristic of the endo-lysosomal lipid storage found in this disorder.

A goal of producing a lathosterolosis mouse model was to gain phenotypic insight for the purpose of identifying a corresponding human malformation syndrome. We identified a human infant patient with lathosterolosis, a malformation syndrome not previously described in humans. Biochemically, fibroblasts from the patient show reduced cholesterol and elevated lathosterol levels. Mutation analysis showed that the patient is homozygous for a single A→C nucleotide change at position 137 in *SC5D*, resulting in a mutant enzyme in which the amino acid serine is substituted for tyrosine at position 46. Both parents are heterozygous for the mutation. The infant's phenotype resembled severe SLOS. Malformations found in both the human patient and the mouse model include growth failure, abnormal nasal structure, abnormal palate, micrognathia, and postaxial polydactyly. A unique feature of lathosterolosis is the clinical finding of mucolipidosis in the affected infant, which is not reported in SLOS and may help distinguish SLOS clinically from lathosterolosis. Lathosterolosis, which is a lysosomal storage disorder, may be replicated in embryonic fibroblasts from the *Sc5d*–mutant mouse model. To distinguish pathological changes attributable to reduced cholesterol in lathosterolosis from those that are a consequence of elevated 7-DHC in SLOS, we are comparing proteomic changes in the *Sc5d*–mutant mouse model with those in the SLOS mouse model. We recently developed induced pluripotent stem cells from lathosterolosis fibroblasts. Their characterization is in progress.

Desmosterolosis is another inborn error of cholesterol synthesis that resembles SLOS. It results from a mutation in the 3β-hydroxysterol Δ24-reductase gene (*DHCR24*). DHCR24 catalyzes the reduction of desmosterol to cholesterol. We disrupted the mouse *Dhcr24* gene by using targeted homologous recombination in embryonic stem cells. Surprisingly, although most *Dhcr24* mutant mice die at birth, the pups are phenotypically normal.

Others have shown that mutations of the lamin B receptor (LBR) cause HEM (hydrops, ectopic calcification, moth-eaten skeletal) dysplasia in humans and ichthyosis in mice. LBR has both lamin B–binding and sterol Δ14-reductase domains. Although only a minor sterol abnormality has been reported, it was proposed that LBR is the primary sterol Δ14-reductase and that impaired sterol Δ14-reduction underlies HEM dysplasia. However, *DHCR14* also encodes a sterol Δ14-reductase. To test the hypothesis that LBR and DHCR14 are redundant sterol Δ14-reductases, we obtained ichthyosis mice (*Lbr Sc5d*$^{-/-}$) and disrupted *Dhcr14*. *Dhcr14 Sc5d*$^{-/-}$ mice are phenotypically normal. We found no sterol abnormalities in either *Lbr Sc5d*$^{-/-}$ or *Dhcr14 Sc5d*$^{-/-}$ tissues at 1 or 21 days of age. We then bred the mice to obtain compound mutant mice. *Lbr Sc5d*$^{-/-}$*:Dhcr14 Sc5d*$^{-/-}$ and *Lbr Sc5d*$^{-/-}$*:Dhcr14*$^{+/-}$ died *in utero*. *Lbr*$^{+/-}$*:Dhcr14 Sc5d*$^{-/-}$ mice appeared normal at birth but, by 10 days of age, were growth-retarded and neurologically abnormal (with ataxia and tremors) and, consistent with a demyelinating process, evidenced vacuolation and swelling of myelin sheaths in the spinal cord upon pathology evaluation. We observed neither vacuolation nor swelling of myelin sheaths in either *Lbr Sc5d*$^{-/-}$ or *Dhcr14 Sc5d*$^{-/-}$ mice. In contrast to *Lbr Sc5d*$^{-/-}$ mice, *Lbr*$^{+/-}$*:Dhcr14 Sc5d*$^{-/-}$ mice had normal skin and did not display the Pelger-Huët anomaly. Peripheral tissue sterols were normal in all three mutant mice, although we found significantly elevated levels (50% of total sterols) of cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol in brain tissue from 10-day-old *Lbr*$^{+/-}$*:Dhcr14 Sc5d*$^{-/-}$ mice. In contrast, we observed relatively small transient elevations in Δ^{14}-sterol levels in *Lbr Sc5d*$^{-/-}$ and *Dhcr14*$^{\Delta 4\text{-}7/\Delta 4\text{-}7}$ brain tissue. Our data support the notion that HEM dysplasia and ichthyosis result from impaired lamin B receptor function rather than from impaired sterol Δ14-reduction. Impaired sterol Δ14-reduction gives rise to a novel murine phenotype for which a corresponding human disorder has yet to be identified.

Niemann-Pick disease, type C1

Niemann-Pick disease, type C1 (NPC1) is a neurodegenerative disorder that results in ataxia and dementia. In view of the dementia, it has been referred to as childhood Alzheimer's disease. The disorder is caused by a defect in intracellular lipid and cholesterol transport. Initially, as part of a Bench-to-Bedside award, we began a clinical protocol to identify and characterize biomarkers that could be used in a subsequent therapeutic trial. The project also received support from the Ara Parseghian Medical Research Foundation and Dana's Angels Research Trust. We have enrolled over 85 NPC1 patients into a longitudinal Natural History trial. The goals of the trial are to identify (1) a blood-based diagnostic/screening test, (2) biomarkers that can be used as tools to facilitate development and implementation of therapeutic trials, and (3) clinical symptoms/signs that may be used as efficacy outcome measures in a therapeutic trial.

Currently, the average time from first symptom to diagnosis, the 'diagnostic delay,' in our cohort of NPC patients is on the order of four to five years. In collaboration with Daniel Ory, we found elevated levels of non-enzymatically produced oxysterols in NPC1 patients. As well as a potential biomarker that may be used to follow therapeutic interventions, the oxysterols will likely form the basis of a blood-based diagnostic test that will significantly shorten the diagnostic delay encountered by NPC1 patients.

In addition to our Natural History study, we completed a randomized, placebo-controlled, cross-over trial to investigate the safety and efficacy of *N*-acetyl cysteine (NAC) in NPC1. The goal was to determine whether NAC treatment would reduce oxidative stress and subsequently decrease levels of the non-enzymatically produced oxysterols. In collaboration with the Therapeutics of Rare and Neglected Disease Program of NCATS, we are conducting a phase I therapeutic trial of lumbar intrathecal cyclodextrin therapy in NPC1. We are now transitioning to a multicenter, multinational phase 2b/3 trial.

In collaboration with Daniel Ory and Frederick Maxfield, our group was awarded an NIH U01 grant to test the safety and potential efficacy of an HDAC inhibitor, vorinostat, in adult NPC1 patients. The collaboration also includes scientists from Notre Dame and has been supported by the Ara Parseghian Medical Research Foundation.

To complement the clinical work, we have begun to apply molecular and proteomic approaches to both mouse and human biomaterials to identify biological pathways disrupted in NPC1. We identified several blood and cerebral spinal fluid proteins and are in the process of validating the biomarkers as potential outcome measures to be used as tools in the development of therapeutic interventions.

ADDITIONAL FUNDING

- U01HD079065 A Phase 1 Dose Escalation Study of Vorinostat in Niemann-Pick C1 Disease
- NICHD Directors Award: Phenotypic, electrophysiological investigation and treatment of hippocampal pathology in a mouse model of Niemann-Pick disease, type C1
- NICHD Directors Award: Identification and electrophysiological characterization of iPS derived neurons from Smith–

Lemli–Opitz syndrome patients
- » Ara Parseghian Medical Research Foundation
- » Dana's Angels Research Trust

PUBLICATIONS

1. Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, Olzeski D, Sidhu R, Dietzen DJ, Fu R, Wassif CA, Yanjanin NM, Marso SP, House J, Vite C, Schaffer JE, Ory DS. Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease. *Sci Transl Med* 2010; 2:56ra81.
2. Cluzeau CVM, Watkins-Chow DE, Fu R, Borate B, Yanjanin NM, Dail MK, Davidson CD, Walkley SU, Ory DS, Wassif CA, Pavan WJ, Porter D. Microarray expression analysis and identification of serum biomarkers for Niemann-Pick Disease, type C1. *Hum Mol Genet* 2012; 21:3632-3646.
3. Cologna SM, Jiang X-S, Backlund PS, Cluzeau CVM, Dail MK, Yanjanin NM, Siebel S, Toth CL, Jun H, Wassif CA, Yergey AL, Porter FD. Quantitative proteomic analysis of Niemann-Pick Disease, type C1 cerebellum identifies protein biomarkers and provides pathological insight. *PloS One* 2012; 7(10):e478.
4. Sparks SE, Wassif CA, Goodwin H, Conley SK, Lanham DC, Kratz LE, Hyland K, Gropman A, Tierney E, Porter FD. Decreased cerebral spinal fluid neurotransmitter levels in Smith-Lemli-Opitz syndrome. *J Inherit Metab Dis* 2014; 37:415-420.
5. Wassif CA, Cross JL, Iben J, Sanchez-Pulido L, Cougnoux A, Platt FM, Ory DS, Ponting CP, Bailey-Wilson JE, Biesecker LG, Porter FD. High incidence of unrecognized visceral/neurological late-onset Niemann-Pick disease, type C1 predicted by analysis of massively parallel sequencing data sets. *Genet Med* 2015; 18:41-8.

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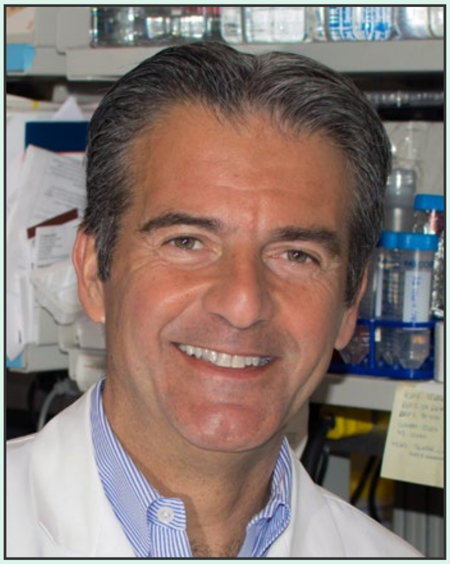
Molecular Genetics of Endocrine Tumors and Related Disorders

The project "Molecular Genetics of Endocrine Tumors and Related Disorders" was started in the late 1990s. The goal has always been to identify molecular pathways involved in the first steps of tumor formation. Our approach was to study patients with rare endocrine conditions, mostly inherited, identify the causative genes, and then study the involved signaling pathways in the hope of translating the derived knowledge into new therapies for such patients. The derived knowledge could also be generalized to conditions that were not necessarily inherited, e.g., to more common tumors and diseases caused by defects in the same molecular pathways. The approach has indeed led to fruitful research over the last two decades.

Our first studies led to the identification of the main regulator of the cAMP signaling pathway, the regulatory subunit-type 1A (R1a) of protein kinase A (PKA, encoded by the *PRKAR1A* gene on chromosome 17q22-24), as responsible for primary pigmented nodular adrenocortical disease (PPNAD) and the Carney complex, a multiple endocrine neoplasia (MEN) whose main endocrine manifestation is PPNAD. We then focused on clinically delineating the various types of primary bilateral adrenal hyperplasias (BAH). We described isolated micronodular adrenocortical disease (iMAD), a disorder likely to be inherited in an autosomal dominant manner and unrelated to the Carney complex or to other MENs. The identification of *PRKAR1A* mutations in PPNAD led to the recognition that non-pigmented forms of BAHs existed, and a new nomenclature was proposed, which we first suggested in 2008 and has since become used worldwide.

In 2006, a genome-wide association (GWA) study led to the identification of mutations in the phosphodiesterases (PDE) PDE11A, a dual specificity PDE, and PDE8B, a cAMP–specific PDE (encoded by the *PDE11A* and *PDE8B* genes, respectively) in iMAD. Following the establishment of cAMP/PKA involvement in PPNAD and iMAD, we and others found that elevated cAMP levels and/or PKA activity and abnormal PDE activity may be found in most benign adrenal tumors (ADTs), including the common adrenocortical adenoma (ADA). We then found *PDE11A* and *PDE8B* mutations or functional variants in adrenocortical cancer (ACA) and in other forms of adrenal hyperplasia such as massive macronodular adrenocortical disease (MMAD), also known as ACTH–independent adrenocortical hyperplasia (MMAD/AIMAH). Germline *PDE11A* sequence variants may also predispose to testicular cancer (testicular germ cell tumors or TGCTs) and prostate cancer, indicating a wider role of this pathway in tumor formation on cAMP–responsive, steroidogenic, or related tissues. Ongoing work with collaborating NCI laboratories aims to clarify the role of PDE in predisposition to these tumors. It is clear from these data, however, that there is significant pleiotropy of *PDE11A* and *PDE8B* defects. The histo-morphological studies that we performed on human adrenocortical tissues from patients with these mutations showed that iMAD is highly heterogeneous and thus likely to be caused by defects in various genes of the cAMP/PKA signaling pathway or its regulators and/or downstream effectors.

Similarly, the G protein–coupled receptor (GPCR)–linked MMAD/AIMAH disease includes a range of adrenal phenotypes from very similar



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(continued)

to iMAD to the *GNAS*–caused primary bimorphic adrenocortical disease (PBAD) and McCune-Albright syndrome, caused by somatic mutations of the *GNAS* gene (encoding the G protein–stimulatory subunit alpha or Gsa). Although a few of the patients with MMAD/AIMAH have germline *PDE11A*, *PDE8B*, or somatic *GNAS* mutations, others have germline fumarate hydratase (*FH*), menin (*MEN1*), and adenomatous polyposis coli (*APC*) mutations, pointing to the range of possibilities and the pathways that may be involved. Particularly interesting among these are *FH* mutations associated with mitochondrial oxidation defects that have been linked to adrenomedullary tumors. This led us to investigate a disorder known as the Carney Triad, the only known disease that has among its clinical manifestations both adrenocortical (ADA, MMAD/AIMAH) and medullary tumors (pheochromocytomas [PHEOs] and paragangliomas [PGLs]), in addition to hamartomatous lesions in various organs (pulmonary chondromas and pigmented and other skin lesions) and a predisposition to gastrointestinal stromal tumors or sarcomas (GISTs). A subgroup of patients with PHEOs, PGLs, and GISTs were identified as harboring mutations in succinate dehydrogenase (SDH) subunits B, C, and D (encoded by the *SDHB*, *SDHC*, and *SDHD* genes, respectively); the patients also rarely have adrenocortical lesions, ADAs, and/or hyperplasia, and their disease is known as the dyad or syndrome of PGLs and GISTs or, as named by a group of pathologists and now in wide use, the Carney-Stratakis syndrome (CSS).

This year, MMAD/AIMAH was renamed primary macronodular adrenocortical hyperplasia (PMAH) after it was discovered that it depends on adrenoglandular ACTH production, at least occasionally. As part of this work, a new gene was identified (*ARMC5*) that, when mutated, causes more than a third of the known PMAH cases (Reference 1). The function of the gene is unknown, and we have embarked on a project to characterize it further, including studying mouse, fruit fly, and fish models. The *ARMC5* gene has a beta-catenin–like motif.

PPNAD appears to be less heterogeneous and is mostly caused by *PRKAR1A* mutations, but up to one third of patients with the classic features of PPNAD do not have *PRKAR1A* mutations, deletions, or 17q22-24 copy-number variant (CNV) abnormalities. A subset of these patients may have defects in other molecules of the PKA holoenzyme, and their study is important for understanding how PKA works and the tissue specificity of each defect. For patients with disorders that are yet to be elucidated on a molecular level, we continue to delineate the phenotypes and identify the responsible genetic defects through a combination of genomic and transcriptomic analyses.

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Recently, we identified genes encoding two other subunits of PKA as involved in endocrine tumors: *PRKACA* in BAH and *PRKACB* in a form of Carney complex that is not associated with *PRKAR1A* mutations. Our laboratory is now investigating these two genes.

Animal model studies are essential for the investigation and confirmation of each of the identified new genes in disease pathogenesis. Furthermore, such studies provide insight into function that can be tested quickly in human samples for confirmation of its relevance to human disease. One excellent example of such a bench-to-bedside (and back) process in the last review cycle was the identification, from a variety of animal experiments, of Wingless/int (Wnt) signaling as one of the downstream effectors of tumor formation in the context of increased cAMP/PKA activity. Both our laboratory and our collaborators found somatic beta-catenin (*CTNNB1*) mutations in large ADAs that formed in the background of PPNAD

Members of the Section in Endocrinology and Genetics in September 2015 during one of their lab meetings

caused by germline *PRKAR1A* mutations. Our transcriptomic studies had previously identified the WNT1–inducible signaling pathway protein 2 (WISP2) as the main molecule overexpressed in food-dependent Cushing's syndrome caused by MMAD/AIMAH, and our recent micro-RNA studies showed that genes that regulate WNT signaling were major targets of micro-RNAs, which were found dysregulated in both PPNAD and MMAD/AIMAH. Cells from tumors or other lesions from animals with R1a deficiency showed elevated beta-catenin expression and/or aberrant WNT signaling and similarities to adult stem cells or cancer stem cells in other models of dysregulated WNT signaling. However, it appears that beta-catenin activation in R1a–deficient cells is preceded by yet unknown molecular abnormalities that take place within the still benign and R1a–haploinsufficient tissues in the early stages of tumor formation.

We continue to investigate the pathways involved in early events in tumor formation in the adrenal cortex and/or the tissues affected by germline or somatic defects of the cAMP/PKA and related endocrine signaling defects, employing animal models and transcriptomic and systems biology analyses. Understanding the role of the other PKA subunits in this process is essential. An example of the combined use of whole genomic tools, transcriptomic analysis, and mouse and zebrafish models to investigate the function of a gene or a pathway is the ongoing work on the Carney Triad.

An important discovery in the last 3–4 years was that mice with neural crest–, heart-, and adrenal-specific knockouts (KO) of R1a or mice with other R1a defects develop lesions caused by proliferation of stem cell–like, tissue-specific pluripotential cells (TSPCs) in adult tissues such as the adult skeleton (Reference 3). We studied bone and the adrenal cortex. Given that various models of R1a deficiency appear to feature the growth of lesions derived from TSPCs, we are characterizing these cells in the adrenal and bone and creating laboratory conditions (i.e., culture systems) to propagate them *in vitro*, study their growth and proliferation, exploit their therapeutic potential, and/or identify molecules that affect the cells for targeting the related tumors in humans.

We continue to accrue patients under several clinical protocols, identify unique patients and families with rare phenotypes, and/or explore (mostly on a collaborative basis) various aspects of endocrine and related diseases. Paramount to these investigations is the availability of modern genetic tools such as copy number variation (CNV) analysis, comparative genomic hybridization (CGH), whole-exome sequencing (WES), and DNA sequencing (DSeq). As part of these clinical protocols, much clinical research is also being done, consisting mostly of observations of new associations, description of novel applications or modifications, and improvements in older diagnostic methods, tests, or imaging tools. This is a particularly

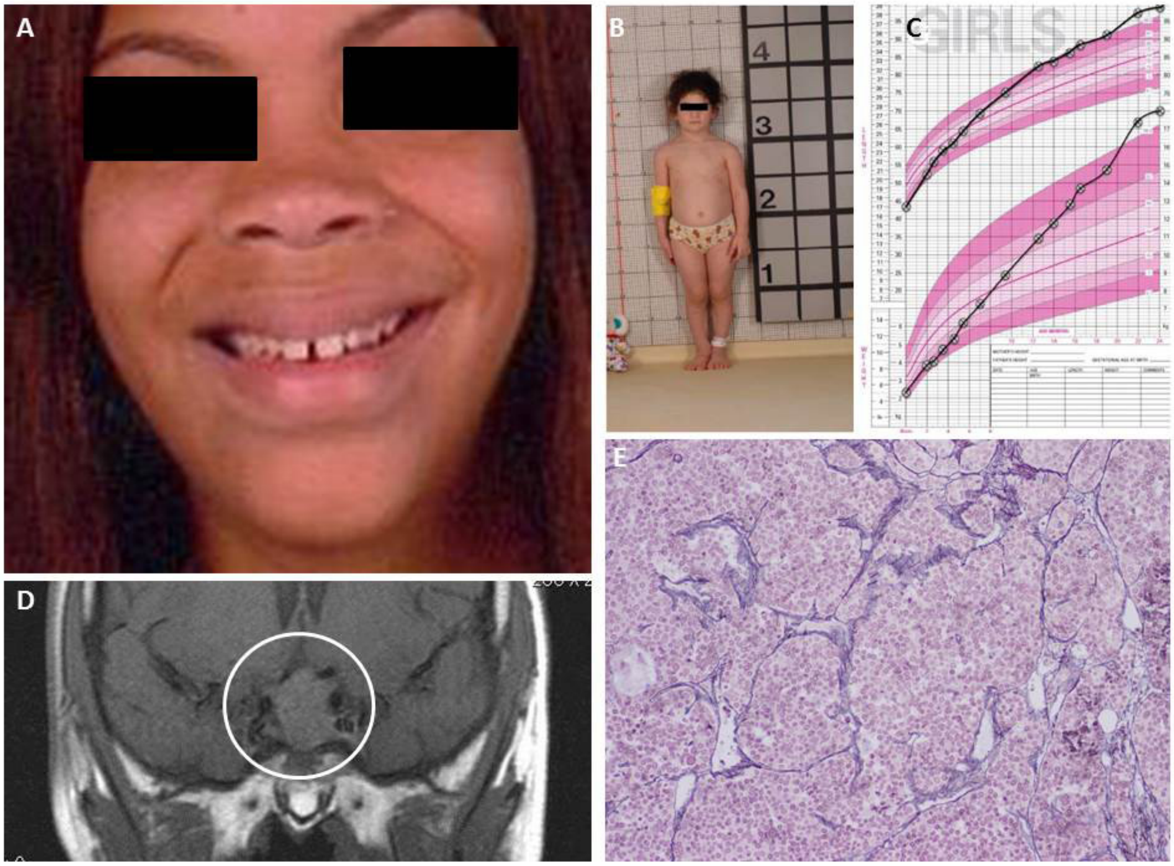

Figure 1. Clinical and molecular genetics of acromegaly: a 2015 study from the Stratakis Laboratory

A. Characteristics of acromegaly: coarse facial features, large lips and nose, with teeth separation, and jaw protrusion (prognanthism).
B. Patient who has familial gigantism (X-linked acrogigantism) at the age of 3 years.
C. Her growth chart up to 24 months of age shows the rapid acceleration in height.
D. GH-secreting macroadenoma extending to the suprasellar region. E. One of the best and most reliable histopathologic features is the lack of reticulin network within tumor and compression of reticulin fibers around the tumor in normal pituitary tissue. Large clusters of tumor cells can be seen without reticulin. For more information please see Trivellin et al. *N Engl J Med* 2014;371(25):2363-2374.

fruitful area of research, especially for our clinical fellows who matriculate at our laboratory during their two-year-long research time. The approach also leads to important new discoveries that may steer us into new directions.

One such discovery was the recent identification by our laboratory of the defect that explains the vast majority of cases of early pediatric overgrowth or gigantism (Reference 4). What regulates growth, puberty, and appetite in children and adults is poorly understood. We identified the gene *GPR101*, encoding a G protein–coupled receptor, which was overexpressed in patients with elevated growth hormone (GH). Patients with *GPR101* defects have a condition that we called X-LAG for X-linked acrogigantism caused by Xq26.3 genomic duplication and characterized by early-onset gigantism resulting from excess GPR101 function and consequently elevated GH.

Carney complex (CNC) genetics

We have collected families with CNC and related syndromes from several collaborating institutions worldwide. Through genetic linkage analysis, we identified loci harboring genes for CNC on chromosomes 2 (2p16) and 17 (17q22–24) and are currently searching for other possible loci for this genetically heterogeneous condition. With the application of state-of-the-art molecular cytogenetic techniques, we are investigating the participation of these currently identified genomic loci in the expression of the disease and have constructed a comprehensive genetic and physical map of the 2p16 chromosomal region for cloning the CNC–associated sequences from this region. Studies in cultured primary tumor cell lines (established from our patients) identified a region of genomic amplification in CNC tumors in the center of the map. The *PRKAR1A* gene on 17q22–24, the gene responsible for CNC in most cases of the disease, appears to undergo loss of heterozygosity in at least some CNC tumors. PRKAR1A is also the main regulatory subunit (subunit type 1-a) of PKA, a central signaling pathway for many cellular functions and hormonal responses. We have increased the number of CNC patients in genotype-phenotype correlation studies, which are expected to provide insight into the complex biochemical and molecular pathways regulated by PRKAR1A and PKA. We expect to identify new genes by ongoing genome-wide searches for patients and families who do not carry *PRKAR1A* mutations.

PRKAR1A, protein kinase A activity, and endocrine and other tumor development

We are investigating the functional and genetic consequences of *PRKAR1A* mutations in cell lines established from CNC patients and their tumors. We measure both cAMP and PKA activity in these cell lines, along with the expression of the other subunits of the PKA tetramer. In addition, we are seeking mutations of the *PRKAR1A* gene in sporadic endocrine and non-endocrine tumors (thyroid adenomas and carcinomas, adrenocortical adenomas and carcinomas, ovarian carcinomas, melanomas and other benign and malignant pigmented lesions, and myxomas in the heart and other sites)—mutations that would further establish the gene's role as a general tumor suppressor. Many investigators within the NIH and around the world provide specimens on a collaborative basis.

Prkar1a$^{+/-}$ and related animal models

In collaboration with Heiner Westphal, Lawrence Kirschner, while in our laboratory, developed a *Prkar1a* knockout mouse floxed by a lox-P system for the purpose of generating, first, a novel *Prkar1a*$^{+/-}$ and, second, knockouts of the *Prkar1a* gene in a tissue-specific manner after crossing the new mouse model with mice expressing the cre protein in the adrenal cortex, anterior lobe of the pituitary, and thyroid gland (Kirschner et al., *Cancer Res* 2005;65:4506). The heterozygote mouse develops several tumors reminiscent of the equivalent human disease. We have now developed new crosses that show protein kinase A subunit involvement in additional phenotypes (Reference 3), and Salpea et al, (2015, in press).

PRKAR1A, the cell cycle, and other signaling pathways

We work to identify *PRKAR1A*–interacting mitogenic and other growth-signaling pathways in cell lines expressing *PRKAR1A* constructs and/or mutations. Several genes that regulate PKA function and increase cAMP–dependent proliferation and related signals may be altered in the process of endocrine tumorigenesis initiated by a mutant *PRKAR1A*, a gene with important functions in the cell cycle and in chromosomal stability. Recently, we found an interaction with the mTOR pathway in both human and mouse cells with altered PKA function.

Phosphodiesterase (PDE) genes in endocrine and other tumors

In patients who did not exhibit CNC or have *PRKAR1A* mutations but presented with bilateral adrenal tumors similar to those in CNC, we found inactivating mutations of the *PDE11A* gene, which encodes phosphodiesterase-11A, an enzyme that regulates PKA in the normal physiologic state. Phosphodiesterase 11A is a member of a 22 gene–encoded family of proteins that break down cyclic nucleotides controlling PKA. PDE11A appears to act as a tumor suppressor such that tumors develop when its action is abolished. In what proved to be the first cases in which mutated PDE was observed in a genetic disorder predisposing to tumors, we found pediatric and adult patients with bilateral adrenal tumors. Recent data indicate that *PDE11A* sequence polymorphisms may be present in the general population. The finding that genetic alterations of such a major biochemical pathway may be associated with tumors in humans raises the reasonable hope that drugs that modify PKA and/or PDE activity may eventually be developed for use in both CNC patients and those with other, non-genetic, adrenal tumors—and perhaps other endocrine tumors.

After the identification of a patient with a *PDE8B* mutation and Cushing's syndrome, additional evidence emerged for yet another cAMP–specific PDE to be involved in endocrine conditions. We are currently studying *PDE8B*–modified animal models to learn more about PDE8B.

Genetic investigations into other adrenocortical diseases and related tumors

Through collaborations, we (1) apply general and pathway-specific microarrays to a variety of adrenocortical tumors, including single adenomas and MMAD, to identify genes with important functions in adrenal oncogenetics; (2) examine candidate genes for their roles in adrenocortical tumors and development; and (3) identify additional genes that play a role in inherited pituitary, adrenocortical, and related diseases.

Genetic investigations into pituitary tumors, X-LAG, other endocrine neoplasias, and related syndromes

In collaboration with several other investigators at the NIH and elsewhere, we are investigating the genetics of CNC– and adrenal-related endocrine tumors, including childhood pituitary tumors, related or unrelated to *PRKAR1A* mutations. As part of this work, we have identified novel genetic abnormalities.

We identified the gene *GPR101* (References 4, 5), encoding an orphan G protein–coupled receptor (GPCR) (Figure 1), which was overexpressed in patients with elevated growth hormone (GH) or gigantism. Patients with GPR101 defects have a condition that we called X-LAG, for X-linked acrogigantism, caused by Xq26.3 genomic duplication and characterized by early-onset gigantism resulting from excessive GPR101 function and consequent GH excess. To find additional patients with this disorder, we collaborated with a group at the University of Liège headed by Albert Beckers (Reference 5), but all the molecular work for the gene identification was carried out here at the NIH. We have found that the gene is expressed in areas of the brain that regulate growth and we identified a few small-molecule compounds that bind to GPR101 (unpublished). The data are now being used to identify more specific compounds that bind to GPR101.

Genetic investigations into the Carney Triad, other endocrine neoplasias, and related syndromes; hereditary paragangliomas and related conditions

As part of a collaboration with other investigators at the NIH and elsewhere (including an international consortium organized by our laboratory), we are studying the genetics of a rare syndrome that predisposes to adrenal and other tumors, the Carney Triad, and related conditions (associated with gastrointestinal stromal tumors, or GIST). In the course of our work, we identified a patient with a new syndrome, known as the paraganglioma and gastrointestinal stromal tumor syndrome (or the Carney-Stratakis syndrome), for which we found mutations in the genes encoding succinate dehydrogenase (SDH) subunits A, B, C, and D. In another patient, we found a novel germline mutation of the *PDFGRA* gene. Most recently, in collaboration with a group in Germany, we identified an epigenetic defect (methylation of the *SDHC* gene) that may be used diagnostically to identify patients with the Carney Triad.

Clinical investigations into the diagnosis and treatment of adrenal and pituitary tumors

Patients with adrenal tumors and other types of Cushing's syndrome (and occasionally other pituitary tumors) come to the NIH Clinical Center for diagnosis and treatment. Ongoing investigations focus on (1) the prevalence of ectopic hormone receptor expression in adrenal adenomas and PMAH/MMAD; (2) the diagnostic use of high-sensitivity magnetic resonance imaging for the earlier detection of pituitary tumors; and (3) the diagnosis, management, and post-operative care of children with Cushing's syndrome and other pituitary tumors.

Clinical and molecular investigations into other pediatric genetic syndromes

Mostly in collaboration with several other investigators at the NIH and elsewhere, we are conducting work on pediatric genetic syndromes seen in our clinics and wards.

ADDITIONAL FUNDING

» INSERM, Paris, France (Co-Principal Investigator): “Clinical and molecular genetics of Carney complex” 06/2003-present (480,000 Euros/year).

» Several small grants supporting staff members from France, Brazil, Greece, Austria, Germany, and elsewhere

PUBLICATIONS

1. Assié G, Libé R, Espiard S, Rizk-Rabin M, Guimier A, Luscap W, Barreau O, Lefèvre L, Sibony M, Guignat L, Rodriguez S, Perlemoine K, René-Corail F, Letourneur F, Trabulsi B, Poussier A, Chabbert-Buffet N, Borson-Chazot F, Groussin L, Bertagna X, Stratakis CA, Ragazzon B, Bertherat J. ARMC5 mutations in macronodular adrenal hyperplasia with Cushing's syndrome. *N Engl J Med* 2013; 369:2105–2114.

2. Beuschlein F, Fassnacht M, Assié G, Calebiro D, Stratakis CA, Osswald A, Ronchi CL, Wieland T, Sbiera S, Faucz F, Schaak K, Schmittfull A, Schwarzmayr T, Barreau O, Vezzosi D, Rizk-Rabbin M, Zabel U, Szarek E, Salpea P, Forlino A, Vetro A, Zuffardi O, Kisker C, Diener S, Meitinger T, Lohse MJ, Reincke M, Bertherat J, Strom TM, Allolio B. Constitutive activation of PRKACA in adrenal Cushing's syndrome. *N Engl J Med* 2014; 370:1019–1028.
3. Liu S, Saloustros E, Mertz EL, Tsang K, Starost MF, Salpea P, Faucz FR, Szarek E, Nesterova M, Leikin S, Stratakis CA. Haploinsufficiency for either one of the type-II regulatory subunits of protein kinase A improves the bone phenotype of Prkar1a$^{+/-}$ mice. *Hum Mol Genet* 2015; 24:6080–6092.
4. Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, Schernthaner-Reiter MH, Szarek E, Leal LF, Caberg JH, Castermans E, Villa C, Dimopoulos A, Chittiboina P, Xekouki P, Shah N, Metzger D, Lysy PA, Ferrante E, Strebkova N, Mazerkina N, Zatelli MC, Lodish M, Horvath A, de Alexandre RB, Manning AD, Levy I, Keil MF, de la Luz Sierra M, Palmeira L, Coppieters W, Georges M, Naves LA, Jamar M, Bours V, Wu TJ, Choong CS, Bertherat J, Chanson P, Kamenický P, Farrell WE, Barlier A, Quezado M, Bjelobaba I, Stojilkovic SS, Wess J, Costanzi S, Liu P, Lupski JR, Beckers A, Stratakis CA. Gigantism and acromegaly due to Xq26 microduplications and GPR101 defects. *N Engl J Med* 2014; 371:2363–2374.
5. Beckers A, Lodish MB, Trivellin G, Rostomyan L, Lee M, Faucz FR, Yuan B, Choong CS, Caberg JH, Verrua E, Naves LA, Cheetham TD, Young J, Lysy PA, Petrossians P, Cotterill A, Shah NS, Metzger D, Castermans E, Ambrosio MR, Villa C, Strebkova N, Mazerkina N, Gaillard S, Barra GB, Casulari LA, Neggers SJ, Salvatori R, Jaffrain-Rea ML, Zacharin M, Santamaria BL, Zacharieva S, Lim EM, Mantovani G, Zatelli MC, Collins MT, Bonneville JF, Quezado M, Chittiboina P, Oldfield EH, Bours V, Liu P, de Herder W, Pellegata N, Lupski JR, Daly AF, Stratakis CA. X-linked acrogigantism syndrome: clinical profile and therapeutic responses. *Endocr Relat Cancer* 2015; 22:353–367.

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The prevalence of overweight and obesity in children and adults has tripled during the past 40 years. The alarming rise in body weight has likely occurred because the current environment affords easy access to calorie-dense foods and requires less voluntary energy expenditure. However, such an environment leads to obesity only in those individuals whose body weight–regulatory systems are not able to control body adiposity with sufficient precision in our high calorie/low activity environment, which suggests there are subgroups in the U.S. with a uniquely high susceptibility to weight gain under the prevailing environmental conditions. Our primary goal is to elucidate the genetic underpinnings of the metabolic and behavioral endo-phenotypes that contribute to the development of obesity in children. Using our unique longitudinal cohort of children at risk for adult obesity, who have undergone intensive metabolic and behavioral phenotyping, we examine genetic and phenotypic factors predictive of progression to adult obesity in children who are in the 'pre-obese' state, allowing characterization of phenotypes unconfounded by the impact of obesity itself. Once they are identified as linked to obesity, we study intensively genetic variants that impair gene function. We expect that these approaches will improve our ability to predict which children are at greatest risk for obesity and its comorbid conditions and will lead to more targeted, etiology-based prevention and treatment strategies for pediatric obesity.

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Genetic factors important for childhood body weight regulation

To identify gene variants affecting body composition, we have been examining polymorphisms in genes involved in the leptin signaling pathway. Genes include the leptin receptor (*LEPR*), *FTO* (fat mass and obesity-associated gene), and those encoding proopiomelanocortin (*POMC*), the melanocortin 3 receptor (*MC3R*), the melanocortin 4 receptor (*MC4R*), and brain-derived neurotrophic factor (*BDNF*) (Reference 1). We are currently studying a variant MC3R that is associated with adiposity in children and appears to have functional significance for MC3R signal transduction. Children who were homozygous variant for both C17A and G241A polymorphisms have significantly greater fat mass and higher plasma levels of insulin and leptin than unaffected or heterozygous children and appear to eat more at laboratory test meals (Figure 1). *In vitro* studies subsequently found that signal transduction and protein expression were significantly lower for the double mutant MC3R. Our ongoing studies attempt to understand the mechanisms by which these sequence alterations may affect body weight. Transgenic 'knock-in' mice expressing the human wild-type and human double-mutant MC3R were therefore developed. Preliminary analyses suggest alterations in body composition consistent with our observations in humans, but show evidence of surprising metabolic health despite their obesity (Figure 2). We will continue to phenotype these mice intensively over the next two years. We have also initiated a clinical investigation of lipolysis and lipogenesis in humans with these polymorphisms.

We also investigated the BDNF-TrkB pathway in relation to body mass in children. In a cohort of 328 children, age 3–19 years, we found that obese children had significantly lower BDNF; BMI, BMI-Z, and body fat were all negatively associated with BDNF. The data suggest that some obese individuals with low serum BDNF for age may have mutations that alter BDNF function. We therefore assessed the role of BDNF haploinsufficiency as a cause of obesity in patients with syndromes attributable to deletions in the vicinity of 11p14.1, where the human *BDNF* gene is located. In 33 subjects with the WAGR (Wilms tumor, aniridia, genitourinary, and renal abnormalities) syndrome who had heterozygous 11p deletions, ranging in size from 1.0–26.5 Mb, 19 had regions of deletion that involved the *BDNF* gene ($BDNF^{+/-}$). Compared with those with intact *BDNF* ($BDNF^{+/+}$), $BDNF^{+/-}$ individuals had significantly greater body mass during childhood, starting at age two (Figure 3). A recent investigation examined common polymorphisms in the *BDNF* gene region. We examined human hypothalamic BDNF expression in association with 44 BDNF SNPs. We found that the minor C allele of rs12291063 is associated with significantly lower human ventromedial hypothalamic BDNF expression and greater adiposity in both adult and pediatric cohorts. We also demonstrated that the major T allele for rs12291063 binds to heterogeneous nuclear ribonucleoprotein D0B, a transcriptional regulator, knockdown of which disrupts transactivation by the T allele (Reference 2). Binding and transactivation functions are both disrupted by substitution of T with the minor C allele. The findings provide a rationale to pursue augmentation of BDNF expression and BDNF receptor signaling as targeted obesity treatments for individuals with the rs12291063 CC genotype.

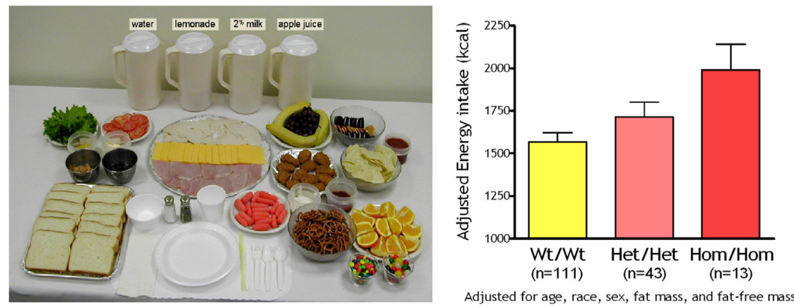

Figure 1. Energy intake studied by using free-access buffet meals of palatable foods

Children homozygous for two polymorphisms in the *MC3R* gene (Hom/Hom) consumed more at the buffet than heterozygotes (Het/Het) or those with wild-type *MC3R* (Wt/Wt).

Physiology, metabolism, and psychology of childhood body weight regulation

Our studies are directed at understanding the physiological, psychological, and metabolic factors that place children at risk for undue weight gain. As part of these studies, we examined how best to measure eating-related psychopathology, insulin sensitivity, changes in body composition, energy intake, and energy expenditure in children, and studied the short- and long-term stability of the components of metabolic syndrome. We found that leptin is an important predictor of weight gain in children: those with high leptin gain even more weight when followed longitudinally. We also documented that hyperinsulinemia is positively related to energy intake in non-diabetic, obese children, leading to treatment studies to reduce hyperinsulinemia (see below). We also examined the relationship between depressive symptomatology and insulin resistance in children and adolescents, finding strong associations both cross-sectionally and prospectively between depressive symptoms and insulin resistance independent of body weight. The associations suggest mechanisms whereby insulin resistance may contribute to excessive weight gain in children and have informed some of our treatment approaches to pediatric obesity (described below).

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors also are associated with adiposity in children and abnormalities in metabolism (Reference 3). We found that binge-eating behaviors may predict future weight gain in children at-risk for obesity: Children reporting binge-eating behaviors such as loss of control (LOC) over eating gained, on average, an additional 2.4 kg of weight per year compared with non-binge-eating children. Our data also suggest that children endorsing binge eating consume more energy during meals. Actual intake during buffet meals averaged 400 kcal more in children with binge eating, but despite their greater intake, such children reported shorter-lived satiety than children without binge-eating episodes. The ability to consume large quantities of palatable foods, especially when coupled with decreased subsequent satiety, may play a role in the greater weight gain found in binge-eating children. We demonstrated that, among a cohort of 506 lean and obese youth, youth with LOC eating had significantly higher serum leptin than those without LOC episodes, even after adjusting for adiposity and other relevant covariates. The data also suggest that interventions targeting disordered eating behaviors may be useful in preventing excessive fat gain in children prone to obesity and have led to ongoing trials of preventative strategies related to binge eating. Because binge eating appears to be a heritable trait, we also initiated studies to investigate potential genetic factors linked to LOC over eating. For example, we previously reported among 229 youth, aged 6–19 years, who were genotyped for *FTO* SNP rs9939609, subjects with at least one A allele (67.7%)

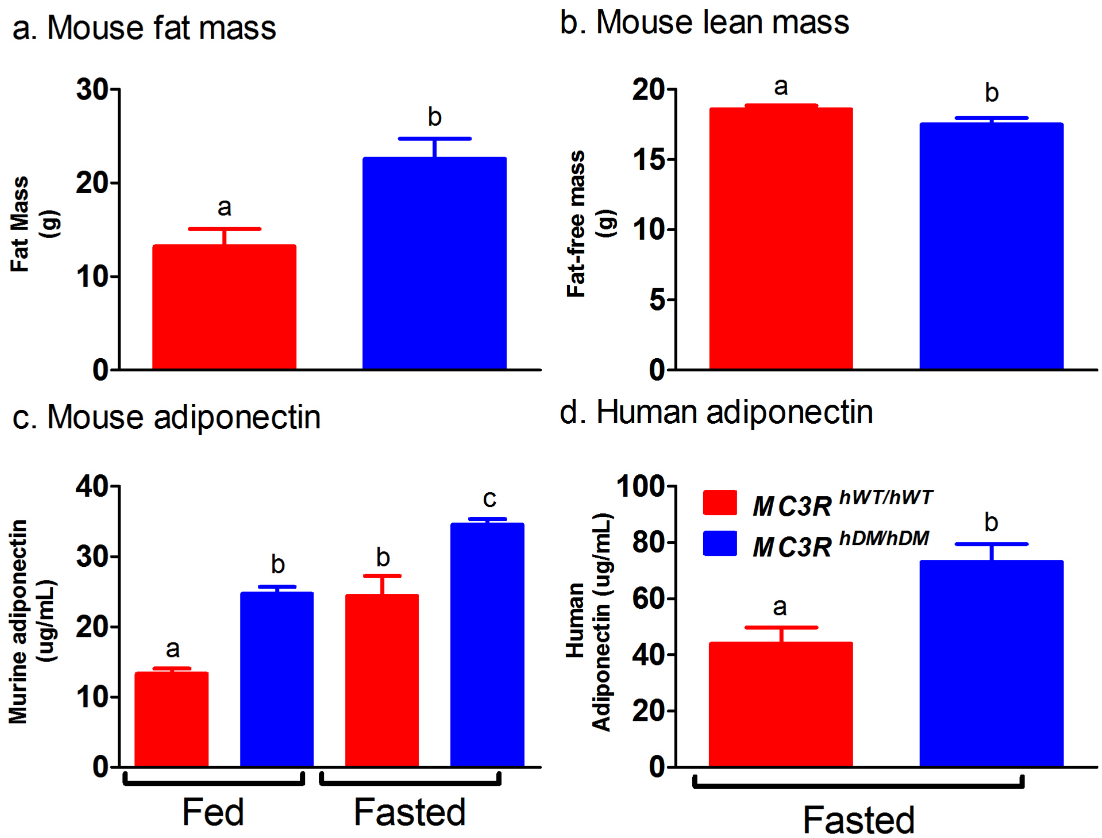

Figure 2. Studies of a human *MC3R* variant containing two naturally occurring polymorphisms
The variant is associated with pediatric-onset obesity. We found that mice whose *MC3R* was replaced by human versions of the gene were obese when they expressed the double-mutant gene ($MC3R^{hDM/hDM}$)—with greater fat mass (panel a) and lower fat-free mass (panel b), but surprisingly greater adiponectin concentrations (panel c) than mice with the normal human MC3R ($MC3R^{hWT/hWT}$). Humans with the double-mutant receptor also showed greater adiponectin (panel d).

had significantly greater BMI, BMI Z-scores, and fat mass. We also found preliminary evidence of a link between *FTO* SNP rs9939609 and eating in the absence of hunger.

We study normal weight children and adolescents, children who are already obese, and the non-obese children of obese parents, in order to determine the factors that are most important for development of the complications of obesity in youth. We examine body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, and genetic factors believed to regulate metabolic rate and body composition. Psychological and behavioral factors, such as propensity to engage in binge-eating behavior (Figure 4), are also studied. Children are being followed longitudinally into adulthood. In two protocols, we study actual food consumption of children during meals, to elucidate differences in the calorie and macronutrient content of meals and the circulating hormones related to hunger and satiety in those who either endorse binge-eating behaviors or report no such behaviors. We found that eating in the absence of physiological hunger is a replicable trait that appears linked to obesity. We hypothesize that differences in these factors will predict the development of obesity in the populations studied and thus may be of great importance in developing rational approaches for the prevention and treatment of obesity in the diverse US population.

Treatment of obesity and the co-morbid conditions associated with obesity

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in children and adults is urgently needed, yet current pharmacologic approaches are extremely limited for both children and adults (Yanovski SZ and Yanovski JA, *JAMA* 2014;311:74-86). In several clinical protocols, we examined approaches for the prevention and treatment of excessive body weight. We completed a pilot study demonstrating that severely obese adolescents can lose weight when enrolled in a comprehensive weight management program that includes the gastrointestinal lipase inhibitor orlistat as an adjunct to a behavioral modification program. We also completed a placebo-controlled randomized trial studying whether the weight loss of African American and Caucasian children and adolescents who have obesity-related comorbidities was improved by the use of orlistat 120 mg three times a day. Subjects participated in a 12-week weight reduction program. We compared body weight and body composition (by DXA and air displacement plethysmography), glucose homeostasis by frequently sampled intravenous glucose tolerance test (FSIGT), fasting lipids, pulse, and blood pressure before and after treatment. 200 adolescents, 65% female, 61% African American, mean age±SEM 14.6±0.10y, BMI 41.7±0.6 kg/m^2 (range 27-87 kg/m^2) were studied. 85.5% of subjects completed the trial. Adolescents treated with orlistat lost significantly more weight, BMI units, and fat mass. Although pulse and blood pressure decreased significantly during the trial, orlistat treatment did not significantly alter these variables. Similarly, HOMA-IR (homeostasis model assessment–insulin resistance), SI (insulin resistance) by FSIGT, apolipoprotein B, total and LDL–cholesterol, and triglycerides declined significantly in proportion to weight loss, but orlistat use was not associated with significant reductions in any of these obesity-related laboratory comorbidities. Both aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are used to measure liver function, unexpectedly increased significantly with orlistat treatment. We concluded that orlistat added to a behavioral program significantly improved weight loss over a 6-month interval, but had little impact on obesity-related co-morbid conditions in obese adolescents.

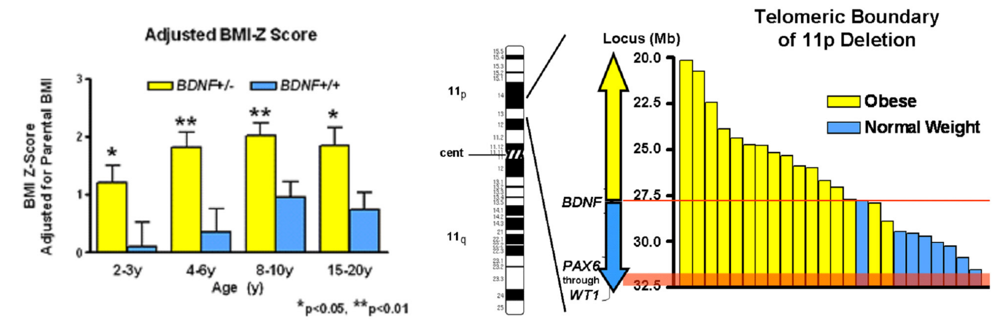

Figure 3.
Patients with WAGR syndrome who have haploinsufficiency for the brain-derived neurotrophic factor gene (*BDNF*) had a higher BMI standard deviation score (BMI Z-score) than children and adults with WAGR syndrome who retained two copies of the *BDNF* gene (left panel). Deletions that extended into exon 1 of *BDNF* were associated with 100% risk of childhood-onset obesity (right panel).

A second obesity treatment study examined the mechanism by which metformin may affect the body weight of younger children who have hyperinsulinemia and are therefore at risk for later development of type 2 diabetes. We conducted a single-center, 6-month, randomized, double-blind, placebo-controlled trial of the effects of metformin, 1000 mg twice a day, administered with meals, in severely obese children (6–12y) who manifested hyperinsulinemia and insulin resistance. Subjects participated in a monthly dietitian-administered weight reduction program. Body mass index and body composition (by air displacement plethysmography), glucose homeostasis (by HOMA-IR), and lipids were measured before and after 6-months' treatment. 100 obese children (60% female, 11% Hispanic, 3% Asian, 40% African American), mean age 10.2±1.5y, with mean BMI 34.6±6.6 kg/m^2 (range 23-58 kg/m^2) were enrolled between October, 2000, and April, 2007. Compared with placebo-treated children, the BMI, BMI Z-score, and body fat mass of those randomized to metformin declined to a significantly greater extent. Serum glucose and HOMA-IR also declined significantly more in metformin-treated than in placebo-treated children. Compared with placebo, metformin treatment also elicited significant reductions from baseline in the mean energy intake independently of changes in body composition. Metformin also significantly decreased ratings of hunger and increased ratings of fullness after the pre-meal load (Reference 4). The data suggest that lower perceived hunger resulting in diminished food intake are among the mechanisms by which metformin treatment reduces body weight in overweight children with hyperinsulinemia. We concluded that metformin, added to a monthly behavioral program, significantly improved weight loss and insulin resistance over a 6-month interval in severely obese, insulin-resistant children.

A third clinical trial examined the efficacy of two diets among Hispanic children and adolescents, where the prevalence of obesity and insulin resistance is considerably greater than in non-Hispanic white children. A low-glycemic load diet (LGD) has been proposed as an effective dietary intervention for pediatric obesity, but no published study had examined the effects of an LGD in obese Hispanic children. We compared the effects of an LGD and a low-fat diet (LFD) on body composition

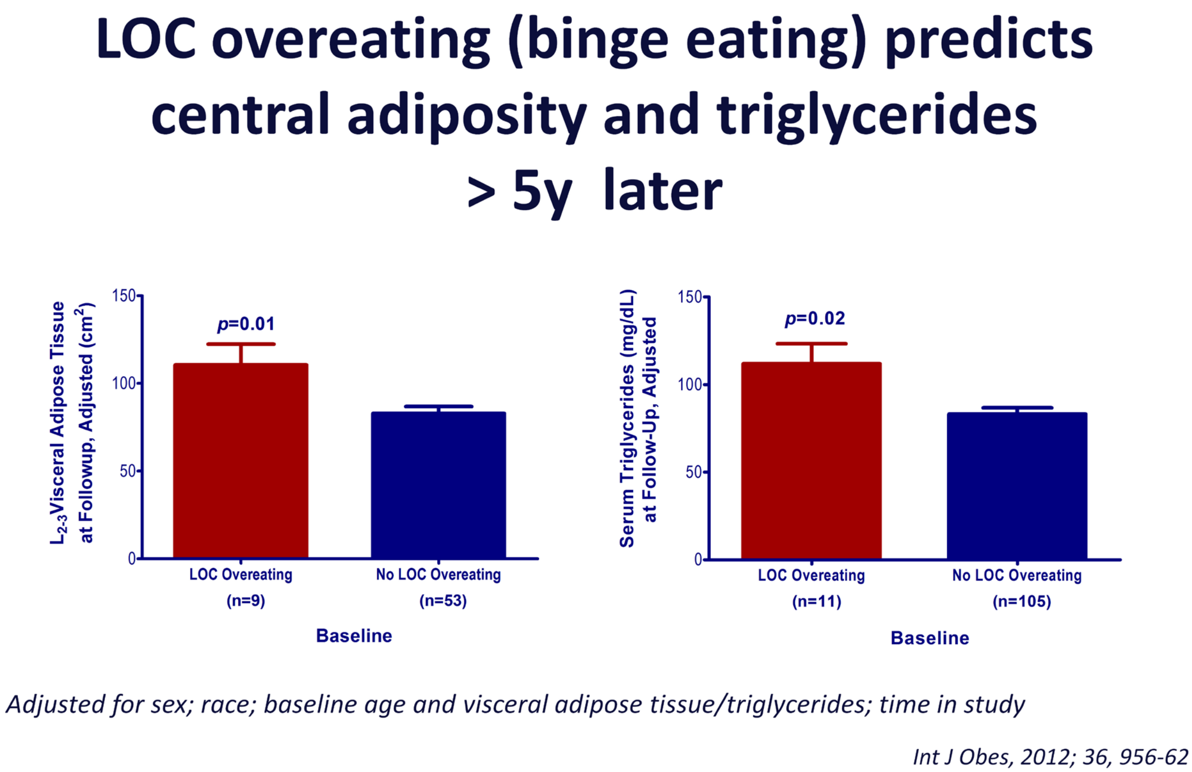

Figure 4.

On average (±SE), children who engage in binge eating at baseline have more visceral adipose tissue at L_{2-3} at follow-up than children who do not engage in binge eating at baseline, adjusting for sex, race, baseline age, baseline visceral adipose tissue at L_{2-3}, and time in study ($P = 0.01$).

On average (±SE), children who engage in binge eating at baseline have higher follow-up triglycerides than children who do not engage in binge eating at baseline, adjusting for sex, race, baseline age, body mass index (kg/m^2), baseline triglycerides, and time in study ($P = 0.02$).

and components of metabolic syndrome in obese Hispanic youth. Obese Hispanic children (7–15 years of age) were randomly assigned to consume an LGD or an LFD in a two-year intervention program. Body composition and laboratory assessments were obtained at baseline and 3, 12, and 24 months after intervention. In the 113 children who were randomly assigned, 79% of both groups completed three months of treatment and 58% of LGD and 55% of LFD subjects attended 24-month follow-up. Compared with the LFD, the LGD decreased the glycemic load per kilocalories of reported food intakes in participants at 3 months. Both groups had a significant reduction in BMI Z-score and improved waist circumference and systolic blood pressure at 3, 12, and 24 months after intervention. However, there were no significant differences between groups for changes in BMI, insulin resistance, or components of metabolic syndrome. We thus found no evidence that an LGD and an LFD differ in efficacy for the reduction of BMI or aspects of metabolic syndrome in obese Hispanic youth. Both diets reduced the BMI Z-score when prescribed in the context of a culturally adapted, comprehensive weight-reduction program.

A fourth recently completed study examined prevention of weight gain using interpersonal therapy in adolescents reporting loss of control eating behaviors (Reference 5). An additional ongoing study tests methods to reduce insulin resistance in adolescents at-risk for type 2 diabetes. This protocol completed accrual in 2014 and will complete follow-up in 2015.

A novel intervention to be studied in the next three years with a randomized-controlled trial involving the use of colchicine to ameliorate inflammation associated with obesity and thus improve its complications.

ADDITIONAL FUNDING

» NIH Clinical Center "Bench to Bedside" Award: Testing Neurobehavioral Endophenotypes of Loss of Control Eating, NICHD 2014–2015
» NICHD Division of Intramural Research Director's Investigator Award: Suppression of NLRP3 inflammasome activation to ameliorate obesity-associated metabolic abnormalities 2014–2015
» Office of Disease Prevention, NIH: Grant supplement to support the clinical protocol "Mood and Insulin Resistance in Adolescents at Risk for Diabetes" 2014-2015
» Zafgen Inc: Randomized, Double-Blind, Placebo Controlled, Phase 3 Trial of ZGN-440 (Subcutaneous Beloranib in Suspension, Zafgen, Inc.) in Obese Subjects with Prader-Willi Syndrome to Evaluate Total Body Weight, Food-related Behavior, and Safety over 6 Months. Funds an RCT testing a new methionine aminopeptidase inhibitor in patients with Prader-Willi Syndrome and obesity. 2014 – 2017

PUBLICATIONS

1. Hohenadel MG, Thearle MS, Grice BA, Huang H, Dai MH, Tao YX, Hunter LA, Palaguachi GI, Mou Z, Kim RC, Tsang MM, Haack K, Voruganti VS, Cole SA, Butte NF, Comuzzie AG, Muller YL, Baier LJ, Krakoff J, Knowler WC, Yanovski JA, Han JC. Brain-derived neurotrophic factor in human subjects with function-altering melanocortin-4 receptor variants. *Int J Obes (Lond)* 2014;2:56ra81.
2. Mou Z, Hyde TM, Lipska BK, Martinowich K, Wei P, Ong C, Hunter LA, Palaguachi GI, Morgun E, Teng R, Lai CF, Condarco TA, Demidowich AP, Krause AJ, Marshall LJ, Haack K, Voguganti S, Cole SA, Butte NF, Comuzzie AG, Nalls MA, Zonderman AB, Singleton AB, Evans MK, Martin B, Maudsley S, Tsao JW, Kleinman JE, Yanovski JA, Han JC. Human obesity associated with an intronic SNP in the brain-derived neurotrophic factor locus. *Cell Rep* 2015;13:1073–80.
3. Radin RM, Tanofsky-Kraff M, Shomaker LB, Kelly NR, Pickworth CK, Shank LM, Altschul AM, Brady SM, Demidowich AP, Yanovski SZ, Hubbard VS, Yanovski JA. Metabolic characteristics of youth with loss of control eating. *Eat Behav* 2015;19:86–89.
4. Adeyemo MA, McDuffie JR, Kozlosky M, Krakoff J, Calis KA, Brady SM, Yanovski JA. Effects of metformin on energy intake and satiety in obese children. *Diabetes Obes Metab* 2015;17:363–370.
5. Tanofsky-Kraff M, Shomaker LB, Wilfley DE, Young JF, Sbrocco T, Stephens M, Ranzenhofer LM, Elliott C, Brady S, Radin RM, Vannucci A, Bryant EJ, Osborn R, Berger SS, Olsen C, Kozlosky M, Reynolds JC, Yanovski JA. Targeted prevention of excess weight gain and eating disorders in high-risk adolescent girls: a randomized controlled trial. *Am J Clin Nutr* 2014;100:1010–1018.

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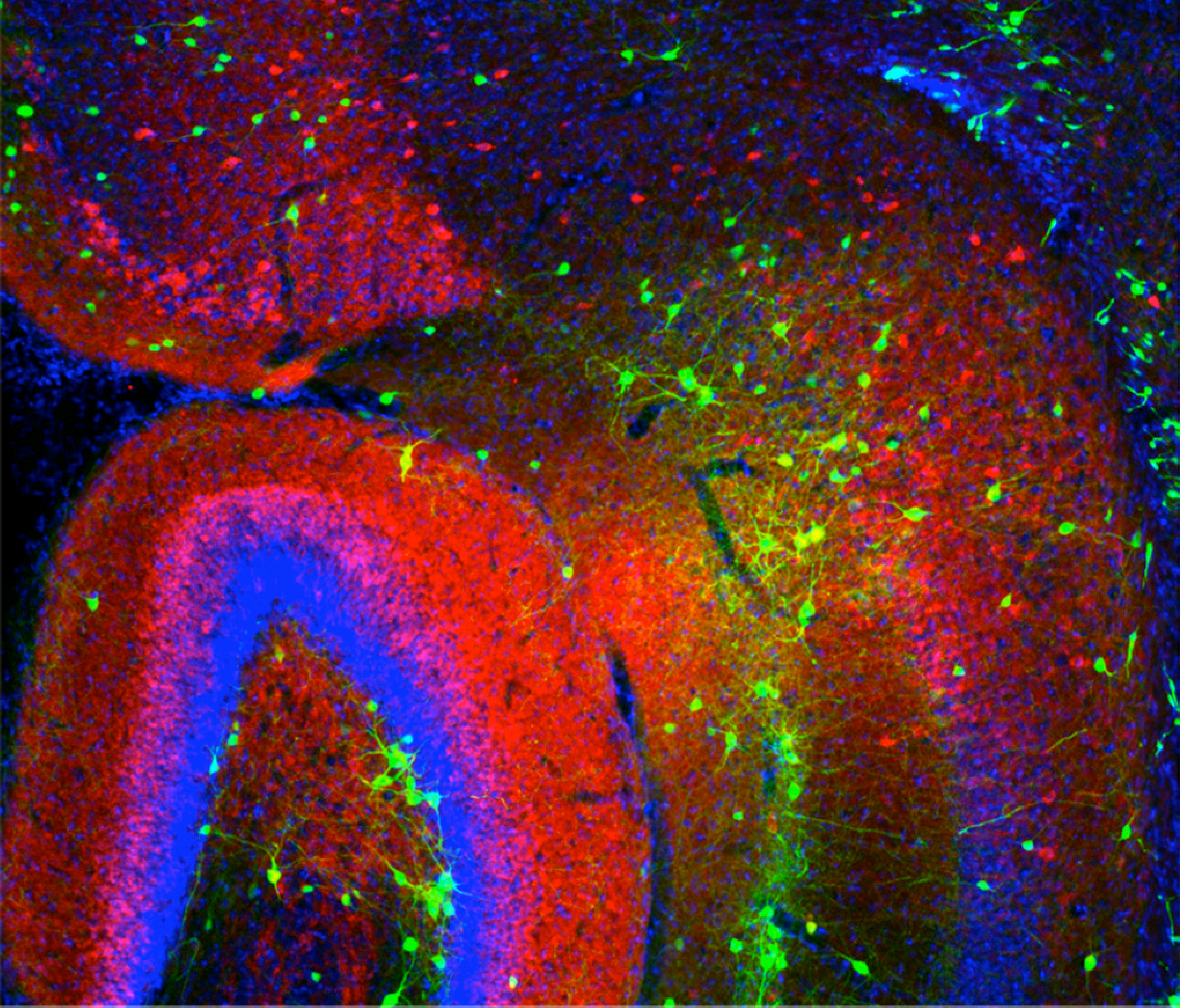

Program in Developmental Neuroscience

Director: Chris J. McBain, PhD

ABOUT THIS IMAGE

Interneurons in the GAD-65 GFP mouse: numerous subpopulations are present within a hippocampal section derived from a GAD-65 GFP transgenic mouse. The image was provided by Chris McBain, PhD, of the *Section on Cellular and Synaptic Physiology*.

Program *in* Developmental Neuroscience

The *Program in Developmental Neuroscience (PDN)* takes a comprehensive approach to the study of developmental neuroscience by using techniques of neurophysiology, molecular and cellular biology, crystallography, and imaging. Overall, the research focuses on the development, physiology, and pathophysiology of the mammalian central nervous system. Researchers study receptors, ion channels, and cellular and synaptic signaling mechanisms in preparations that range from isolated proteins and cells to highly ordered neural networks, in physiological and pathophysiological conditions observed in both wild-type and numerous transgenic animals.

The *Section on Molecular Signal Transduction*, headed by Tamás Balla, investigates the role of phosphoinositide-derived messengers in mediating the actions of hormones, growth factors, and neurotransmitters in mammalian cells.

The *Section on Molecular Neurophysiology and Biophysics*, headed by Dax Hoffman, continues to investigate the role of the voltage-gated potassium channel subunits in regulating dendritic excitability and synaptic integration of hippocampal neurons.

The *Section on Cellular Neurobiology*, headed by Y. Peng Loh, explores the mechanisms of intracellular trafficking and secretion of peptide hormones, neuropeptides, and neurotrophins in endocrine cells and neurons as well as the role of the prohormone-processing enzyme carboxypeptidase E in stress, neuroprotection, and tumorigenesis.

The *Section on Cellular and Synaptic Physiology*, headed by Chris McBain, investigates the development and regulation of cortical excitability, in particular glutamatergic and GABAergic synaptic transmission and plasticity in the hippocampal formation. The Section investigates mechanisms underlying the differential regulation of transmitter release at functionally divergent presynaptic terminals along a common axon, the roles of ionotropic and metabotropic glutamatergic and cholinergic receptors in controlling cell excitability, and bi-directional synaptic plasticity at both inhibitory and excitatory axon terminals.

The *Section on Cellular Signaling*, headed by Stanko Stojilkovic, investigates signaling pathways in pituitary cells.

The *Section on Sensory Coding and Neural Ensembles*, headed by Mark Stopfer, is interested in how the brain gathers and organizes sensory information to build transient and sometimes enduring internal representations of an animal's surroundings; the animal actively collects information, which is then processed and dramatically transformed in myriad ways. The Section's goal is to understand the mechanisms by which sensory information is collected, transformed, stabilized, and compared as it makes its way through the nervous system.

We investigate signal transduction pathways that mediate the actions of hormones, growth factors, and neurotransmitters in mammalian cells, with special emphasis on the role of phosphoinositide-derived messengers. Phosphoinositides constitute a small fraction of the cellular phospholipids but play critical roles in the regulation of many signaling protein complexes that assemble on the surface of cellular membranes and are intracellular lipid messengers controlling a variety of cellular functions. Phosphoinositides regulate protein kinases and GTP–binding proteins as well as membrane transporters, including ion channels, thereby controlling many cellular processes such as proliferation, apoptosis, metabolism, cell migration, and differentiation. We focus on the phosphatidylinositol 4 (PtdIns4)–kinases (PI4Ks), a family of enzymes that catalyze the first committed step in polyphosphoinositide synthesis. Current work aims to: (1) understand the function and regulation of several PI4Ks involved in the control of cellular signaling and trafficking pathways; (2) find specific inhibitors for the individual PI4Ks; (3) define the molecular basis of PtdIns4*P*–regulated pathways through identification of PtdIns4*P*–interacting molecules; (4) develop tools to analyze inositol lipid dynamics in live cells; and (5) determine the importance of the lipid-protein interactions in the activation of cellular responses by G protein–coupled receptors and receptor tyrosine kinases.

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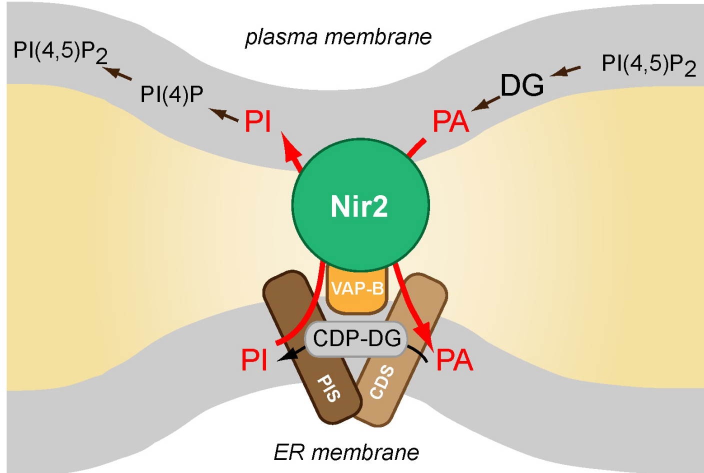
EFR3 proteins are critical for G protein–coupled receptor re-sensitization.

We showed previously that significant amounts of phosphatidylinositol 4-phosphate (PI4P) can be found in the plasma membrane (PM) and that these pools are generated by the PI4KA enzyme. We set out to determine the mechanism by which PI4KA acts at the PM. Endogenous PI4K is detected in the ER, and GFP–tagged PI4KA is found in the cytosol but cannot be detected in the PM, even with TIRF microscopy. Yeast studies from Scott Emr's lab at Cornell University showed that two yeast proteins, Efr3p and Ypp1p, are required for the yeast ortholog of PI4KA, Stt4p, to localize to the PM. We thus investigated the role of the homologs of these proteins (EFR3A and EFR3B, and TTC7A and TTC7B) in localizing PI4KA to the PM in mammalian cells. We found that EFR3 proteins are palmitoylated at their N-termini and are localized to the PM and that their RNAi–mediated knock-down interferes with signaling of the G protein–coupled angiotensin II (AngII) receptors that activate phospholipase C (PLC) enzymes. While our studies reached their conclusions, Pietro De Camilli's group at the Yale School of Medicine reported on the role of the TTC7 and EFR3 proteins in localizing PI4KA in the PM. Our results were in agreement with those reported in their publication but also revealed an additional role of the EFR3 proteins, namely their critical role in the resensitization of the AngII receptors. Without the EFR3 proteins, AngII receptors rapidly uncoupled from the Gq protein and became desensitized. Our results explained the remarkable phenotype of a *Drosophila* mutant (rolling blackout, or *rbo*), which carries a mutated *Efr3* gene and shows rapid desensitization of light-induced electrical responses in the eye. Whether the effects of *Erf3* knock-down on GPCR resensitization are related to EFR3's role in supporting PI4KA function in the PM is a question that we are currently investigating.

Schematics of Nir2 function in ER-PM contact sites

The Nir2 protein is a lipid transporter that moves phosphatidylinositol (PI) from the endoplasmic reticulum (ER) to the plasma membrane (PM) while transferring phosphatidic acid (PA) in the other direction. Nir2 translocates to the ER-PM contact sites when cells are stimulated via receptors that activate phospholipase C (PLC) enzymes to fulfill the protein's lipid exchange function. Therefore, Nir2 helps maintain plasma membrane lipid identity and signaling competence when phosphoinositides are rapidly consumed during receptor-mediated activation of PLC enzymes.



Identification of Nir2 as a phosphatidylinostol and phosphatidic acid lipid transfer protein critical for maintaining signaling competence from phospholipase C–activating receptors

Another important achievement of this year's research was the completion of a study that addressed the question of how structurally important lipids are exchanged between the endoplasmic reticulum (ER) and the plasma membrane (PM) during the actions of hormones and neurotransmitters. Our studies focused on the question of how phosphatidylinositol (PI), the precursor lipid of the regulatory phosphoinositides, is transported from the ER to other membranes. We tested all known PI transfer proteins (PITPs) by RNA–mediated knock-down and found that only Nir2 had a profound effect on phosphoinositide levels. Nir2 is a large PITP, first described in *Drosophila* as the *RdgB* mutant, which develops light-induced retinal degeneration. We found that Nir2–depleted cells' ability to supply the PM with PI was compromised, as was their PI synthesis, owing to a defect in phosphatidic acid (PA) transport from the PM to the ER. We also found that Nir2 was enriched in ER–PM contact sites after stimulation of phospholipase C (PLC) enzymes and that the Nir2 protein was anchored to the ER via interaction with VAP-A and VAP-B proteins. The studies answered a long-standing question of how the lipid products of PLC activation are recycled to maintain the signaling competence of cells under chronic stimulation. Given that some familial forms of amyotrophic lateral sclerosis (ALS or Lou-Gehrig's disease) are caused by mutations within the VAP-B protein, we also tested these mutants for their ability to interact with the Nir2 protein. We found that some disease-causing mutations largely interrupted the interaction between Nir2 and VAB-B, while others had more moderate effects. The studies may give us further clues regarding the molecular details of the processes that are critical for the pathology of ALS.

Analysis of the fatty acid preference of cytidine-diphosphate-diacylglycerol synthase (CDS) enzymes

Cytidine-diphosphate(CDP)-diacylglycerol(DG) synthase (CDS) enzymes catalyze the formation of CDP-DG from phosphatidic acid (PA) and cytosine triphosphate (CTP) and are found in a wide range of organisms, from prokaryotes to humans. The reaction is the first enzymatic step in generating phosphatidylinositol (PI), which is the precursor of phosphoinositides. Cells have multiple forms of CDS enzymes; CDS1 and CDS2 are multi-pass transmembrane proteins located in the ER, while the mitochondrial CDS, called Tam41 is a soluble protein associated with the inner mitochondrial membrane. In this set of studies, performed in collaboration with Richard Epand's group, we compared the human CDS1 and CDS2 enzymes' selective preference for PA with special fatty acid side-chain composition. The importance of this question lies in the fact that the fatty acid side chain composition of newly synthesized PA is dipalmitoyl, whereas those generated from hydrolysis of phosphoinositides following phospholipase C activation are 1-stearoyl, 2-arachidonyl. Therefore, the analysis was designed to investigate whether the two CDS enzymes show any preference for the PA that originates from receptor-mediated breakdown of phosphoinositides. The studies revealed that the CDS2 enzyme prefers the 1-stearoyl, 2-arachidonyl version of PA, while CDS1 can use any form of PA without distinction. Although both enzymes are located in the ER, the functional difference suggests a metabolic channeling of PA toward the various phospholipid metabolic pathways by the two CDS enzymes.

PUBLICATIONS

1. Hammond GR, Balla T. Polyphosphoinositide binding domains: key to inositol lipid biology. *Biochim Biophys Acta* 2015; 1851(6):746–758.
2. Bojjireddy N, Guzman-Hernandez ML, Reinhard NR, Jovic M, Balla T. EFR3s are palmitoylated plasma membrane proteins that control responsiveness to G-protein-coupled receptors. *J Cell Sci* 2015; 128:118–128.
3. D'Souza K, Kim YJ, Balla T, Epand RM. Distinct properties of the two isoforms of CDP-diacylglycerol synthase. *Biochemistry* 2014; 53:7358–7367.
4. Jeschke A, Zehethofer N, Lindner B, Krupp J, Schwudke D, Haneburger I, Jovic M, Backer JM, Balla T, Hilbi H, Haas A. Phosphatidylinositol 4-phosphate and phosphatidylinositol 3-phosphate regulate phagolysosome biogenesis. *Proc Natl Acad Sci USA* 2015; 112:4636–4641.
5. Kim YJ, Guzman-Hernandez ML, Wisniewski E, Balla T. Phosphatidylinositol-phosphatidic acid exchange by Nir2 at ER-PM contact sites maintains phosphoinositide signaling competence. *Dev Cell* 2015; 33:549–561.

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Potassium Channels and Dendritic Function in Hippocampal Pyramidal Neurons

The central nervous system underlies all our experiences, actions, emotions, knowledge, and memories. With billions of neurons each firing hundreds of times per second, the complexity of the brain is stunning. To pare down the task of understanding something so complex, our research approach calls for studying the workings of a single central neuron—the pyramidal neuron from the CA1 region of the hippocampus. The hippocampus is essential for long-term memory in humans and is among the first brain regions affected by epilepsy and Alzheimer's disease. To understand how the hippocampus stores and processes information, we focus on one of its principal cell types, the CA1 pyramidal neuron. Each pyramidal neuron in the CA1 region of the hippocampus receives tens of thousands of inputs onto its dendrites, and it is commonly thought that information is stored by altering the strength of individual synapses (synaptic plasticity). Recent evidence suggests that the regulation of synaptic surface expression of glutamate receptors can, in part, determine synaptic strength. However, the dendrites contain an abundance of ion channels that are involved in receiving, transforming, and relaying information in the dendrites, adding an additional layer of complexity to neuronal information processing.

We found that the A-type potassium channel subunit Kv4.2 is highly expressed in the dendritic regions of CA1 neurons in the hippocampus and, as one of the primary regulators of dendritic excitability, plays a pivotal role in information processing. Kv4.2 is targeted for modulation during the types of plasticity thought to underlie learning and memory. Moreover, we found that the functional expression level of Kv4.2 regulates the subtype expression of NMDA–type glutamate receptors, the predominant molecular devices controlling synaptic plasticity and memory. We are currently following up on these findings with more detailed investigations into the mechanisms of activity-dependent Kv4.2 regulation and its role in neuronal development. In addition, we are investigating the role of dendritic voltage-gated channels in CNS disorders, including autism-spectrum disorder and Alzheimer's disease.

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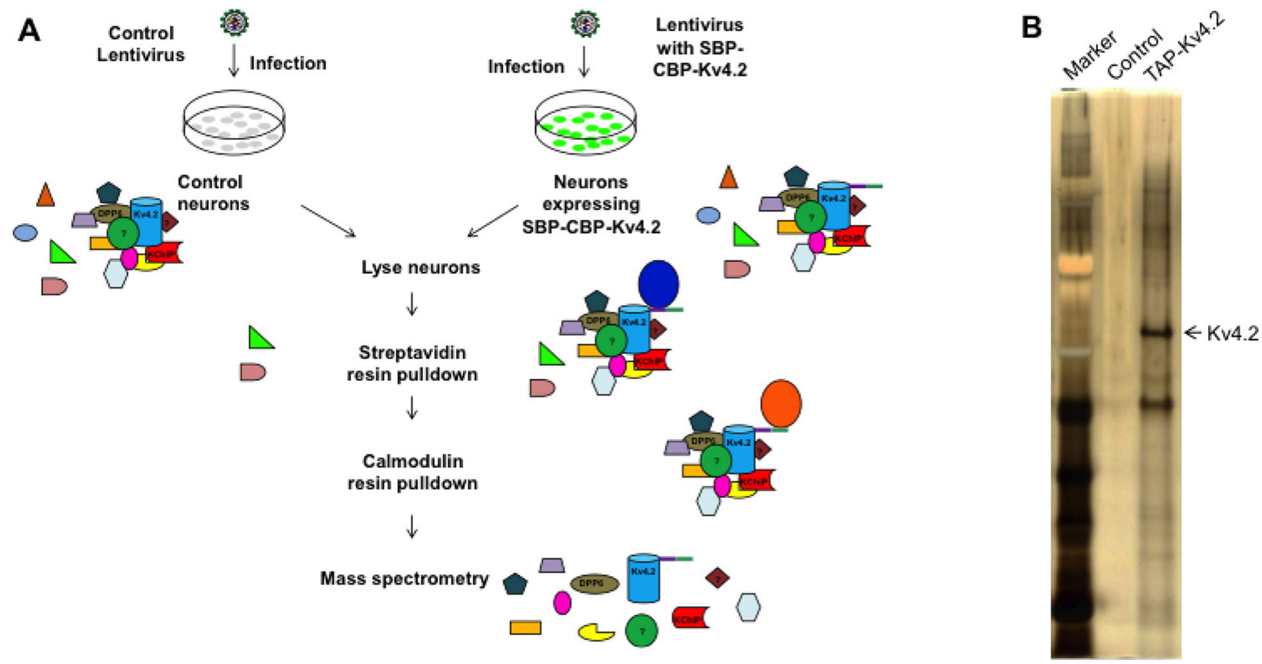
Role of voltage-gated ion channels in synaptic development and disease

Kv4.2 control of firing patterns in hippocampal CA1 pyramidal neurons

Although recent molecular cloning studies found that several families of voltage-gated K^+ channel genes are expressed in the mammalian brain, information about the relationship between the protein products of these genes and their various neuronal functions is still lacking. Our lab used a combination of molecular, electrophysiological, and imaging techniques to show that Kv4.2, an A-type voltage-gated potassium channel subunit, controls action potential (AP) half-width, frequency-dependent AP broadening, and dendritic AP propagation. More recently, we examined the role of A-type K^+ channels in regulating synaptic plasticity, neuronal development and disease.

Neuronal excitability in Alzheimer's disease

Alzheimer's disease (AD), the most common form of dementia, is



Tandem Affinity Purification (TAP) of Kv4.2

A. Diagram of TAP. B. Silver staining of Kv4.2 and its protein complex. Note that the control was very clean and TAP-Kv4.2 pulled down many components.

characterized by progressive neuronal loss, which eventually leads to death. Despite massive efforts over the last few decades, the etiology of AD is not well understood. A major challenge for AD research, and for the development of treatments, is that most AD patients are not diagnosed until neuronal function is irreversibly compromised. Therefore, it is crucial to identify neuronal changes at pre-clinical stages, which could provide a basis for early diagnosis and help identify novel therapeutic targets. Neuronal hyperexcitability occurs early in the pathogenesis of AD and contributes to network dysfunction in AD patients. Although the beta-amyloid (Aβ) hypothesis suggests that AD is caused by extracellular accumulation of insoluble Aβ plaques, mounting evidence suggests that synaptic and memory impairments are mediated by soluble Aβ. In collaboration with the Roberson lab, former postbaccalaureate fellow Ben Throesch, tested the hypothesis that Aβ–induced hyperexcitability originates in the dendrites. We found that dendrites, but not the somata, of hippocampal neurons were hyperexcitable in mice adult mice overexpressing Aβ. The dendritic hyperexcitability was associated with selective depletion of Kv4.2, a dendritic potassium channel important for the regulation of dendritic excitability and synaptic plasticity.

The role of DPP6 domain in its localization and function

Dipeptidyl peptidase–like protein 6 (DPP6) is an auxiliary subunit of the Kv4 family of voltage-gated K^+ channels known to enhance channel surface expression and potently accelerate their kinetics. DPP6 is a single-transmembrane protein, which is structurally remarkable for its large extracellular domain. Included in this domain is a cysteine-rich motif, the function of which is unknown. Lin Lin found that the cysteine-rich domain of DPP6 is required for the protein's export from the ER and expression on the cell surface. Disulfide bridges formed at C349/C356 and C465/C468 of the cysteine-rich domain are necessary for the enhancement of Kv4.2 channel surface expression but not for DPP6's interaction with Kv4.2 subunits. The short intracellular N-terminal and transmembrane domains of DPP6 associate with and accelerate the recovery from inactivation of Kv4.2, but the entire extracellular domain is necessary to enhance Kv4.2 surface expression and stabilization. Our findings show that the cysteine-rich domain of DPP6 plays an important role in protein folding of DPP6, which is required for transport of DPP6/Kv4.2 complexes out of the ER.

We showed recently that DPP6 regulates the formation and stability of dendritic filopodia during early neuronal development, which is independent of Kv4.2. To identify additional DPP6–binding proteins, Jiahhua Hu employed a TAP (tandem affinity purification) approach in order to isolate the DPP6 protein complex from hippocampal neurons (Figure 1). Mass-spectrometry analysis identified known proteins such as Kv4 family members and numerous novel synaptic proteins, which Jiahua Hu and Jung Park are currently examining.

Dendritic trafficking of voltage-gated calcium channels

We are currently investigating the expression and trafficking of the voltage-gated calcium channel Cav2.3. Cav2.3 is highly expressed in the dendrites of hippocampal and cortical neurons, where it is capable of generating large calcium spikes in response to both back-propagating action potentials and synaptic activity. Thus, alterations in Cav2.3 mRNA localization and translation could have a dramatic impact on cellular excitability and calcium signaling. Recent evidence suggests that Cav2.3 mRNA can be targeted by the Fragile-X mental retardation protein (FMRP), an mRNA–binding protein that regulates translation in dendritic spines. Loss of FMRP results in Fragile X syndrome, the most common form of inherited intellectual disability in humans. We are therefore investigating the possibility that FMRP can regulate translation of Cav2.3 and will determine whether this regulation underlies aspects of Fragile X syndrome.

Toward this goal, Ying Liu performed real time PCR on mRNA isolated from the hippocampi or cortex of wild-type and FMRP knockout (KO) male mice and examined the mRNA levels of several dendritic proteins. When comparing hippocampi from FMRP–KO with wild-type mice at 3- or 8-weeks of age, she found no significant difference in the mRNA levels of Cav2.3, Kv4.2, PSD-95, or HCN-1. She will further characterize this regulation by identifying FMRP–binding sites on Cav2.3.

In conjunction with these experiments, Ivan Trang is determining how FMRP affects Cav2.3 protein expression. From synaptoneurosomes isolated from mouse cortex, he found that, at three weeks of age, Cav2.3 protein levels are lower in FMRP–KO mice than in wild-type mice. In addition, primary neurons cultured from FMRP–KO mice exhibit lower surface Cav2.3 levels than do wild-type mice. Thus, loss of FMRP leads to a reduction in both synaptic and surface Cav2.3 protein. To determine how this might affect neuronal physiology, Erin Gray will record Cav2.3–mediated calcium currents as well as basic firing properties from wild-type and FMRP–KO neurons.

While FMRP has a clear role in regulating mRNA stability, recent evidence suggests that the protein may directly regulate the internalization and degradation of voltage-gated calcium channels. Given that little is known about the pathways that underlie Cav2.3 degradation, Erin Gray and former postbaccalaureate fellow Joshua Lee began experiments aimed at a better understanding of this process. In heterologous cells overexpressing Cav2.3, they showed that Cav2.3 undergoes activity-dependent ubiquitination and degradation by the proteasome. Gray has begun to further investigate a possible role for ubiquitin-mediated alterations in surface levels of Cav2.3 and plans to perform a variety of electrophysiological recordings to determine the physiological consequences of this regulation.

Regulation of local protein expression in a mouse model of Fragile X syndrome

Alterations in synaptic plasticity are implicated in several diseases involving cognitive dysfunction, including autism, mental retardation, Alzheimer's disease, and schizophrenia. Using a mouse model of FXS combining nascent imaging technology with electrophysiology, Jon Murphy seeks to understand how local protein synthesis in hippocampal dendrites is regulated by FMRP.

Co-regulation of HCN1 and Kv4.2

In CA1 pyramidal neuron dendrites, HCN channels, responsible for I_h current, and Kv4.2 channels, responsible for I_A current, are critical for signal processing and dendritic integration of synaptic inputs. Both channels show a similar pattern of distribution, with an increased density from the soma to the apical dendrite. Using hippocampal primary cultured neurons, Emilie Campanac studied the potential co-regulation of HCN1 and Kv4.2. Results so far point to a reciprocal regulation, with overexpression of Kv4.2 being associated with an increase in I_h current density without any change in sodium and calcium current, while overexpression of HCN1 leads to an increased in I_A current density. Pharmacological blockade of I_h with cesium caused a reduction in both current densities. Our data strongly suggest homeostatic regulation between I_A and I_h currents. We are currently investigating the molecular mechanism underlying this co-regulation.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Lin L, Long LK, Hatch MM, Hoffman DA. DPP6 domains responsible for its localization and function. *J Biol Chem* 2014; 289(46):32153–32165.
2. Hall AM, Throesch BT, Buckingham SC, Markwardt SJ, Peng Y, Wang Q, Hoffman DA, Roberson ED. Tau-dependent Kv4.2 depletion and dendritic hyperexcitability in a mouse model of Alzheimer's disease. *J Neurosci* 2015; 35(15):6221–6230.
3. Murase S, Lantz CL, Kim E, Gupta N, Higgins R, Stopfer M, Hoffman DA, Quinlan EM. Matrix metalloproteinase-9 regulates neuronal circuit development and excitability. *Mol Neurobiol* 2015; E-pub ahead of print.
4. Li W, Lee MH, Henderson L, Tyagi R, Bachani M, Steiner J, Campanac E, Hoffman DA, von Geldern G, Johnson K, Maric D, Morris HD, Lentz M, Pak K, Mammen A, Ostrow L, Rothstein J, Nath A. Human endogenous retrovirus-K contributes to motor neuron disease. *Sci Transl Med* 2015; 7(307):307ra153.

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Neurosecretory Proteins in Neuroprotection, Neurodevelopment, and Cancer

We study the cell biology of neuroendocrine cells and the function of neuropeptides and the neurotrophic factor Neurotrophic Factor-α1 (NF-α1) in health and disease. Our focus is three-fold, to: (1) investigate the mechanisms of biogenesis and intracellular trafficking of dense-core secretory granules containing neuropeptides and their processing enzymes; (2) investigate the role of serpinin, a novel chromogranin A–derived peptide discovered in our lab, in neural and cardiac function; and (3) determine the non-enzymatic neurotrophic role of carboxypeptidase E (CPE)/NF-α1 in neuronal function and cancer. Our work led to the discovery of novel molecular mechanisms of protein trafficking to the regulated secretory pathway (RSP) and identified players and mechanisms that control secretory granule biogenesis and transport in neuroendocrine cells. Recently, we found a new role for CPE/NF-α1 as a trophic factor that mediates neuroprotection, neurodevelopment, and anti-depression. We also identified a splice variant of CPE (CPE-deltaN) that drives metastasis in various cancer types. Using cell lines, primary cell cultures, mouse models, and human tumor specimens and sera, our studies have deepened the understanding of diseases related to neurodegeneration, memory, learning, depression, cardiac function, obesity, and metastasis in cancer.

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Mechanism of sorting and vesicle transport of pro-neuropeptides, neurotrophins, and their processing enzymes to the regulated secretory pathway for secretion

The intracellular sorting of pro-neuropeptides and neurotrophins to the RSP is essential for processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of pro-opiomelanocortin (POMC, pro-ACTH/endorphin), proinsulin, and brain-derived neurotrophic factor (BDNF) to the RSP. Our studies showed that these pro-proteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum (ER) to the trans-Golgi network (TGN). In the TGN, the pro-proteins are sorted into dense-core granules of the RSP for processing by prohormone convertases and CPE and then secreted. We showed that the sorting of prohormones to the RSP occurs by a receptor-mediated mechanism. Site-direct mutagenesis studies identified a 3-D consensus sorting motif consisting of two acidic residues found in POMC, proinsulin, and BDNF. We identified the transmembrane form of CPE as an RSP sorting receptor that is specific for the sorting signal of these proproteins.

We investigated the role of membrane CPE and secretogranin III (SgII) as sorting receptors for targeting POMC to the RSP. Using RNA interference (siRNA) to knock down SgIII or CPE expression in pituitary AtT20 cells, we demonstrated in both cases that POMC secretion via the constitutive secretory pathway was elevated. In double CPE-SgIII knock-down cells, elevated constitutive secretion of POMC and stimulated secretion of ACTH were perturbed. Thus, CPE mediates trafficking of POMC to the RSP, and SgIII may play a compensatory role for CPE in POMC sorting to the RSP.

Transport of vesicles containing hormone or BDNF to the plasma membrane for activity-dependent secretion is critical for endocrine function and synaptic plasticity. We showed that the cytoplasmic tail of a transmembrane form of CPE in hormone- or BDNF–containing dense-core secretory vesicles plays an important role in their transport to the vesicles' release site. In hippocampal neurons and primary pituitary and AtT20 cells, overexpression of the CPE tail inhibited the movement of BDNF– and POMC/CPE–containing vesicles to the processes, respectively. The CPE tail interacts with the microtubule-based motors dynactin and KIF1A/KIF3A to effect anterograde vesicle movement to the plasma membrane for secretion. CPE anchors POMC/ACTH and BDNF vesicles to the microtubule-based motor system for transport along the processes to the plasma membrane for activity-dependent secretion in endocrine cells and neurons.

Role of CPE/NF-α1 in neuroprotection, stress, and neurodevelopment

We showed that CPE knockout (KO) mice exhibit nervous system deficiencies. Morris water maze and object-preference tests indicate defects in learning and memory, and forced swim tests indicate depression. In 6- to 14-week-old CPE–KO mice, dendritic pruning was poor in cortical and hippocampal neurons, which could affect synaptogenesis. Electrophysiological measurements revealed a defect in the generation of long-term potentiation in hippocampal slices of the mice. A major cause for the defect was the loss of neurons in the CA3 region of the hippocampus of CPE–KO animals observed at four weeks of age and older when the animals are weaned. Hippocampal neurons in CA3 region are enriched in CPE and were normal at 3 weeks of age just before weaning. When weaning was delayed for a week, the degeneration was not observed. When given carbamezapine i.p. at two weeks of age, degeneration was prevented. The results suggest that the degeneration is correlated with possible epileptic-like neuronal firing during the stress of weaning and that CPE is important for the survival of CA3 neurons during that period. We then showed that, when CPE was applied externally to cultured hippocampal or cortical neurons, they were protected from apoptosis after inducing oxidative stress with hydrogen peroxide, indicating that CPE acts as an extracellular signaling molecule in neuroprotection. Its action involved activation of the ERK1/2 and the Akt signaling pathways, which then caused phosphorylation and translocation of the transcription factor Sp1 from the cytosol to the nucleus. This then led to enhanced transcription/translation of BCL2, a pro-survival mitochondrial protein, inhibition of caspase 3 activation and promotion of neuronal survival (Reference 1). Furthermore, we showed that this CPE–mediated neuroprotection pathway can be activated by rosiglitazone, a PPARg ligand, because the CPE promoter contains a PPARg binding site (Reference 2). Thus, CPE is a novel neuroprotective trophic factor, which we renamed Neurotrophic factor-alpha1 (NF-α1). We then demonstrated that CPE/NF-α1 has a neuroprotective role *in vivo*. During and after mild chronic restraint stress (CRS) for 1h/day for seven days, CPE/NF-α1 mRNA and protein levels, as well as those of the anti-apoptotic factor Bcl2, were significantly elevated in the hippocampus. *In situ* hybridization studies indicated especially elevated CPE/NF-α1 mRNA expression in the CA3 region and no gross neuronal cell death after mild CRS. Studies on primary hippocampal neurons in culture demonstrated elevated CPE and Bcl2 expression and a decline in Bax, a pro-apoptotic protein, after treatment with the synthetic glucocorticoid dexamethasone; the regulation was mediated by glucocorticoid binding to glucocorticoid-regulatory element (GRE) sites on the promoter of the *cpe* gene. The findings indicate that, during mild CRS, when glucocorticoid is released, CPE/NF-α1 and Bcl2 expression are coordinately up-regulated to mediate neuroprotection of hippocampal neurons. The importance of CPE as a neuroprotective agent was demonstrated by the absence of the increase in Bcl2 in the hippocampus of CPE–KO mice, leading to the degeneration of the CA3 neurons.

We also investigated the role of NF-α1 in preventing restraint stress–induced depression. Prolonged (6h/day for 21 days), but not short-term (1h/day for 7days), restraint stress reduced fibroblast growth factor 2 (FGF2) in the hippocampus, leading to depressive-like behavior in mice. We found that, after short-term restraint stress in mice, hippocampal NF-α1, FGF2, and doublecortin, a marker for immature neurons, rose, suggesting increased neurogenesis. Indeed, we showed that, in cultured hippocampal neurons, exogenous NF-α1 could raise FGF2 expression. After prolonged restraint stress, mice showed reduced NF-α1 and FGF2 levels. Moreover, NF-α1–KO mice exhibited severely reduced hippocampal FGF2 levels and immature neuron numbers in the subgranular zone. The mice displayed depressive-like behavior, which was rescued by FGF2 administration. Thus, NF-α1 prevents stress-induced depression by up-regulating hippocampal FGF2 expression, which leads to enhanced neurogenesis and anti-depressant activity (Reference 3). Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway (Reference 3) and has previously been shown to be effective in treating diabetic patients with bi-polar disorders.

Recently, we found that NF-α1 plays a role during embryonic development. NF-α1mRNA was expressed in mouse embryos as early as day E5.5, rising each day, peaking at E8.5, and falling slightly at E9.5. NF-α1 mRNA expression then declined sharply at E10.5–11.5 to below E5.5 levels and then rose sharply at E12.5, in parallel with the development of the endocrine system, and continued to increase into adulthood. *In situ* hybridization studies indicate that NF-α1 is expressed primarily in the forebrain and dorsal root ganglia in mouse embryos. To study neural stem cell proliferation, exogenous recombinant NF-α1 was added to E13.5 neocortex–derived neurospheres, which contain stem cells and neuroprogenitors. NF-α1 treatment reduced the number and size of the neurospheres formed, suggesting inhibition of proliferation and maintenance of the 'stemness' of the stem cells in the neurospheres. NF-α1 down-regulated the wnt pathway in the neurospheres, leading to reduced levels of β-catenin, a protein known to enhance proliferation, suggesting that NF-α1's inhibitory effect on proliferation is mediated by negatively regulating the wnt pathway. We carried out differentiation studies using neurospheres from seven-day cultures that were dissociated into single cells and cultured for an additional five days. We observed an increase in astrocytes in the presence of NF-α1 without alteration in the percentage of neuronal and oligodendrocyte populations. Interestingly, dissociated cells from neurospheres derived from NF-α1–KO mouse embryos showed fewer astrocytes but more neurons. *In vivo*, NF-α1–KO mouse cortex (E16.5) showed lower astrocyte numbers than in WT animals, confirming the *ex vivo* data. Our results suggest a novel role of NF-α1 as an extracellular signal to differentiate neural stem cells into astrocytes.

We also studied the role of NF-α1 in neurite outgrowth, which is key to the formation of synapses and the neural network during development. We found that NF-α1 prevented Wnt-3a inhibition of NGF–stimulated neurite outgrowth in PC12 cells and cortical neurons. Moreover, NF-α1 augmented Wnt-5a–mediated neurite outgrowth. Thus, by interplaying with NGF to prevent neurite outgrowth inhibited by Wnt-3a and augmenting neurite outgrowth mediated by Wnt-5a, NF-α1, could play an important role in regulating these positive and negative cues that are critical for neurodevelopment (Reference 4).

Serpinin, a chromogranin A–derived peptide, regulates secretory granule biogenesis, cell survival, cardiac function, and angiogenesis.

Our previous studies in pituitary AtT-20 cells provided evidence that an autocrine mechanism up-regulates large dense-core vesicle (LDCV) biogenesis to replenish LDCVs following stimulated exocytosis of the vesicles. We identified the autocrine signal as serpinin, a novel 26 amino-acid, chromogranin A (CgA)–derived peptide cleaved from the C-terminus of CgA. Serpinin is released in an activity-dependent manner from LDCVs and activates adenyl cyclase to raise cAMP levels and protein kinase A in the cell. This leads to translocation of the transcription factor sp1 from the cytoplasm into the nucleus and enhanced transcription of a protease inhibitor, protease nexin 1 (PN-1), which then inhibits granule protein degradation in the Golgi complex, stabilizing and raising granule protein levels in the Golgi and enhanced LDCV formation. We also identified modified forms of serpinin, pyroglutamyl-serpinin (pGlu-serpinin) and a C-terminally extended form, serpinin-RRG, in secretion medium of AtT20 cells and in rat heart tissue. pGlu-serpinin is synthesized and stored in secretory granules and secreted in an activity-dependent manner from AtT20 cells. We observed pGlu-serpinin immunostaining in nerve terminals of neurites in mouse brain, olfactory bulb, and retina, suggesting a role as a neurotransmitter or neuromodulator. Additionally, pGlu-serpinin exhibited neuroprotective activity against oxidative stress in AtT20 cells and against low K^+–induced apoptosis in rat cortical neurons. In collaboration with Bruno Tota, we found that pGlu-serpinin has positive inotropic activity in cardiac function, with no change in blood pressure and heart rate. pGlu-serpinin acts through a β1-adrenergic receptor/adenylate cyclase/cAMP/PKA pathway in the heart. pGlu-serpinin and other CgA–derived cardio-active peptides emerge as novel β-adrenergic inotropic and lusitropic modulators. Together, they can play a key role in the myocardium's orchestration of its complex response to sympatho-chromaffin stimulation. Additionally, we recently found that pGlu serpinin is a powerful cardioprotectant after ischemia.

Carboxypeptidase E/ CPE-deltaN in tumorigenesis and as a biomarker for predicting future metastasis

Our studies indicate an important role of the CPE gene in mediating tumor growth, survival, and metastasis. Recently, we described a novel splice isoform of CPE (CPE-deltaN) that is elevated in metastatic hepatocellular, colon, breast, head, and neck carcinoma cell lines. CPE-deltaN is translocated from the cytoplasm to the nucleus of metastatic cancer cells. Overexpression of CPE-deltaN in hepatocellular carcinoma (HCC) cells promoted their proliferation and migration. siRNA knockdown of CPE-deltaN expression in highly metastatic HCC cells inhibited their growth and metastasis in nude mice. CPE-deltaN promoted migration by up-regulating expression of the metastasis gene *Nedd9*, through interaction with histone deacetylase (HDAC) 1 or 2. The enhanced invasive phenotype of HCC cells stably transfected with CPE-deltaN was suppressed when Nedd9 was silenced by siRNA. Microarray studies of HCC cells overexpressing CPE-deltaN showed elevated expression of 27 genes associated with metastasis, including *Nedd9*, claudin 2 (*cldn2*), matrix metallopeptidase 1 (*mmp1*), and inositol 1,4,5-trisphosphate 3-kinase A (*itpka*), while 30 genes associated with tumor suppressor function, such as insulin-like growth factor binding protein 5 and 3 (*igfbp5* and *igfbp3*) were down-regulated.

CPE and CPE-deltaN, each play distinctive roles in tumor progression. In some cancer cell lines, overexpression of wild-type (WT) CPE has been demonstrated to enhance proliferation, e.g., glioma cell lines; however, in other cell lines, e.g., PANC-1, there was no effect of WT CPE on proliferation. Interestingly, wild-type full-length CPE secreted by neuroendocrine tumors negatively regulates the canonical wnt pathway and likely mediates the anti-metastatic effects observed when tumor cells are treated with WT CPE. *In vivo*, the interplay between CPE-deltaN and WT CPE could influence the metastatic potential of tumors.

In clinical studies, we showed that CPE/CPE-deltaN is a good prognostic biomarker for HCC and lung adenocarcinoma. Previously, we carried out a retrospective study on hepatocellular carcinoma patients and showed that CPE-deltaN is a powerful prognostic biomarker for predicting future recurrence (Reference 5). We have now carried out a prospective study to further evaluate the role of CPE-deltaN mRNA as a biomarker for predicting recurrence in 120 HCC patients from the Liver Network patients in Taiwan. We focused on Stage I and II patients, given that these patients generally have better prognosis, but the tumor recurrence rate is still high. Using the same methodology as we had published previously, we determined the Tumor/Normal (T/N) ratio of CPE-DN mRNA. The follow up time ranged from 9.0 months to 106 months. Our results demonstrated that the recurrence-free survival of HCC patients was significantly associated with CPE expression level (T/N greater than 2) for both stage I and II patients identical to that found in our previous retrospective study. CPE mRNA expression level in HCC can therefore be a useful biomarker clinically for predicting tumor recurrence in HCC patients who are in the early pathology stage and able to receive curative resection.

In collaboration with Y-Ching Wang, we carried out a prospective study to evaluate CPE-deltaN as a biomarker for predicting recurrence and death in patients with lung adenocarcinoma, the major sub-type of non-small cell lung carcinoma, which accounts for 85% of lung cancer. Eighty six patients were recruited and followed up for up to seven years post-resection of the tumor to determine recurrence and death. Kaplan-Meier survival analysis showed that patients with high CPE-deltaN copy numbers had a shorter time of disease-free survival and shorter time to death. In subgroup analyses, the association of disease-free survival time with CPE-deltaN copy number was particularly strong among stage I and II lung cancer patients. Thus CPE-deltaN mRNA is a potentially powerful biomarker for predicting recurrence and death at all histo-pathological stages in lung adenocarcinoma patients and is especially useful in identifying patients at high risk of recurrence at early stages I and II of the disease, which will facilitate treatment strategies after surgery.

We also successfully developed a blood assay using circulating exosomes to determine the CPE/CPE-deltaN biomarker levels and showed significant differences between pheochromocytoma and normal control patients. This assay will be very useful for screening patients for cancer.

PUBLICATIONS

1. Cheng Y, Cawley NX, Loh YP. Carboxypeptidase E/NF-a1: a new neurotrophic factor against oxidative stress-induced apoptotic cell death mediated by ERK and PI3-K/AKT pathways. *PLoS One* 2013; 8:e71578.
2. Thouennon E, Cheng Y, Falahatian V, Cawley NX, Loh YP. Rosiglitazone-activated PPARg induces neurotrophic factor-a1 transcription contributing to neuroprotection. *J Neurochem* 2015; 134:463–470.
3. Cheng Y, Rodriguiz RM, Murthy SRK, Senatorov V, Thouennon E, Cawley NX, Aryal D, Ahn S, Lecka-Czernik B, Wetsel WC, Loh YP. Neurotrophic factor-a1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone. *Mol Psychiatry* 2015; 20:744–754.
4. Selvaraj P, Huang JSW, Chen A, Skalka N, Rosin-Abesfeld R, Loh YP. Neurotrophic factor-a1 modulates NGF-induced neurite outgrowth through interaction with Wnt-3a and Wnt-5a in PC12 cells and cortical neurons. *Mol Cell Neurosci* 2015; 11:222–233.
5. Lee TK, Murthy SRK, Cawley NX, Dhanvantari S, Hewitt SM, Lou H, Lau T, Ma S, Huynh T, Wesley RA, Ng IO, Pacak K, Poon RT, Loh YP. An N-terminal truncated carboxypeptidase E splice isoform induces tumor growth and is a biomarker for predicting future metastasis in human cancers. *J Clin Invest* 2011; 121:880–892.

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Hippocampal Interneurons and Their Role in the Control of Network Excitability

Cortical and hippocampal local-circuit GABAergic inhibitory interneurons are 'tailor-made' to control Na^{+}- and Ca^{2+}-dependent action potential generation, regulate synaptic transmission and plasticity, and pace large-scale synchronous oscillatory activity. The axons of this diverse cell population make local, usually short-range projections (some subpopulations project their axons over considerable distances) and release the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) onto a variety of targets. A mounting appreciation of the roles played by interneurons in several mental health conditions such as epilepsy, stroke, Alzheimer's disease, and schizophrenia have placed this important cell type center stage in cortical circuit research. Our main objective is to understand the developmental programs that regulate their integration into cortical circuits and how both ionic and synaptic mechanisms regulate the activity of cortical neurons at the level of small, well defined networks. To this end, we use a variety of electrophysiological, immunohistochemical, molecular, and genetic approaches in both wild-type and transgenic animals. Over the past few years, we have continued our study of the differential mechanisms of glutamatergic and GABAergic synaptic transmission and plasticity within the hippocampal formation and the modulation of voltage- and ligand-gated channels expressed in inhibitory neurons. We also incorporate genetic approaches to unravel the embryogenesis and development of hippocampal interneurons and the circuits in which they are embedded. We are particularly interested in discovering the rules that dictate co-ordinated protein expression in nascent interneuron subpopulations as they migrate and integrate into the developing cortical circuit.

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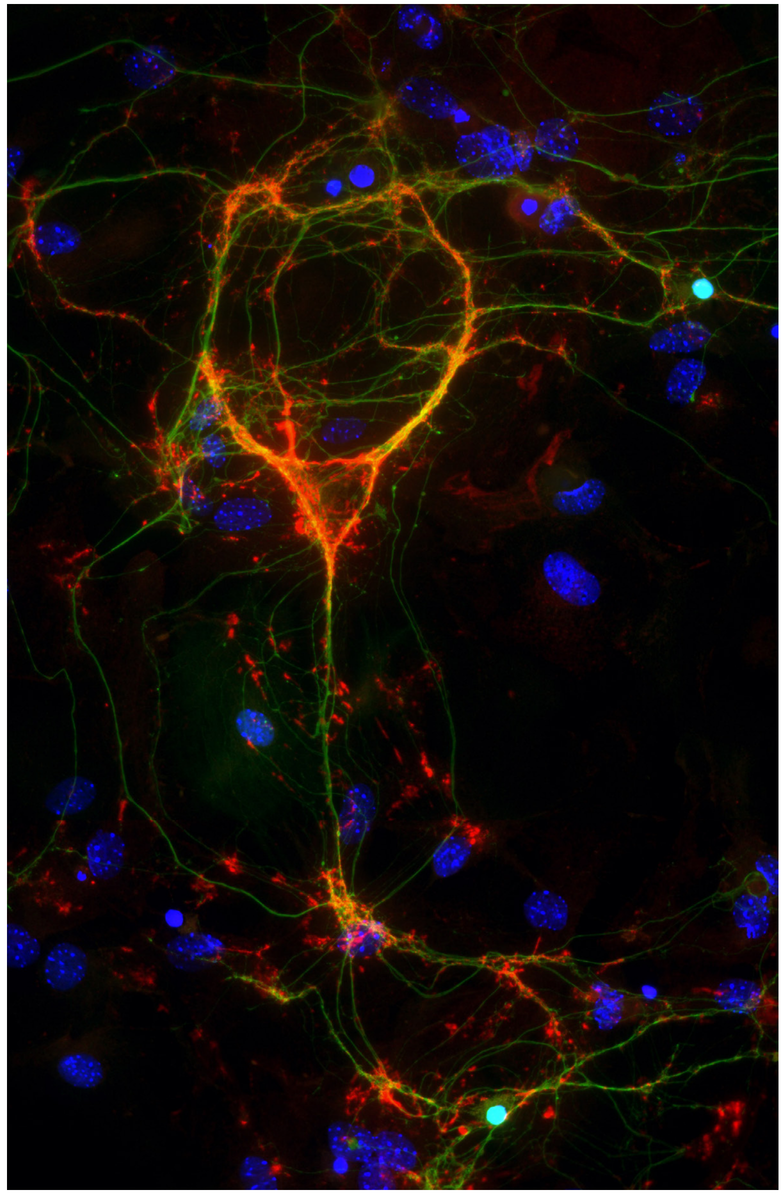
Pentraxins coordinate excitatory synapse maturation and circuit integration of parvalbumin interneurons.

Circuit computation requires precision in the timing, extent, and synchrony of principal cell (PC) firing that is largely enforced by parvalbumin-expressing, fast-spiking interneurons (PVFSIs). To reliably coordinate network activity, PVFSIs exhibit specialized synaptic and membrane properties that promote efficient afferent recruitment such as expression of high-conductance, rapidly gating AMPA receptors (AMPARs) that contain the GluA4 subunit. We found that PVFSIs upregulate GluA4 during the second postnatal week, coincident with increases in the AMPAR–clustering proteins NPTX2 and NPTXR. Moreover, GluA4 is dramatically reduced in $NPTX2^{-/-}/NPTXR^{-/-}$ mice, with consequent reductions in PVFSI AMPAR function. Early postnatal $NPTX2^{-/-}/NPTXR^{-/-}$ mice exhibit delayed circuit maturation with a prolonged critical period permissive for giant depolarizing potentials. Juvenile $NPTX2^{-/-}/NPTXR^{-/-}$ mice display reduced feedforward inhibition, yielding a circuit deficient in rhythmogenesis and prone to epileptic discharges. Our findings demonstrate an essential role for NPTX proteins in controlling network dynamics, highlighting potential therapeutic targets for disorders with inhibition/excitation imbalances such as schizophrenia.

Fast gamma oscillations are generated intrinsically in CA1 without the involvement of fast-spiking basket cells

Information processing in neuronal networks relies on the precise synchronization of ensembles of neurons, coordinated by the diverse family of inhibitory interneurons. Cortical interneurons can be usefully parsed by embryonic origin, with the vast majority arising from either the caudal or medial ganglionic eminences (CGE and MGE). We examined the activity of hippocampal interneurons during gamma oscillations in the mouse CA1 hippocampal region, using an *in vitro* model in which brief epochs of rhythmic activity were evoked by local application of kainate (KA). We found that this CA1 KA–evoked gamma oscillation was faster than that in the CA3 region and, crucially, did not appear to require the involvement of fast-spiking basket cells. In contrast to CA3, we also found that optogenetic inhibition of pyramidal cells in CA1 did not significantly affect the power of the oscillation, suggesting that excitation may not be essential for gamma genesis in this region. We found that MGE–derived interneurons were generally more active than CGE interneurons during CA1 gamma oscillations, although a group of CGE–derived interneurons, putative trilaminar cells, were strongly phase-locked with gamma oscillations and, together with MGE–derived axo-axonic and bistratified cells, provide attractive candidates for being the driver of this locally generated, predominantly interneuron-driven model of gamma oscillations.



GluA4–decorated parvalbumin-containing interneuron

A parvalbumin (*green*)-containing inhibitory interneuron stained for the glutamate receptor subunit GluA4 (*red*)

Neto auxiliary protein interactions regulate kainate and NMDA receptor subunit localization at mossy fiber-CA3 pyramidal cell synapses.

Neto1 and Neto2 auxiliary subunits co-assemble with NMDA receptors (NMDARs) and kainate receptors (KARs) to modulate the receptors' function. In the hippocampus, Neto1 enhances the amplitude and prolongs the kinetics of KAR–mediated currents at mossy fiber (MF)-CA3 pyramidal cell synapses. However, whether Neto1 trafficks KARs to synapses or simply alters channel properties is unresolved. Therefore, we performed post-embedding electron microscopy to investigate the localization of GluK2/3 subunits at MF-CA3 synapses in *Neto*-null mice. Postsynaptic GluK2/3 immunogold labeling was substantially reduced in *Neto*-null mice compared with wild types. Moreover, spontaneous KAR–mediated synaptic currents and metabotropic KAR signaling were absent from CA3 pyramidal cells of *Neto*-null mice. A similar loss of ionotropic and metabotropic KAR function was observed in *Neto1*–, but not *Neto2*–, single knock-out mice, specifically implicating Neto1 in regulating CA3 pyramidal cell KAR localization and function. Additional controversy pertains to the role of Neto proteins in modulating synaptic NMDARs. While immunogold labeling for GluN2A at MF-CA3 synapses was comparable between wild-type and *Neto*-null mice, labeling for postsynaptic GluN2B was robustly increased in such mice. Accordingly, NMDAR–mediated currents at MF-CA3 synapses exhibited increased sensitivity to a GluN2B–selective antagonist in *Neto*1 knockouts compared with wild types. Thus, despite preservation of the overall MF-CA3 synaptic NMDAR–mediated current, loss of Neto1 alters the NMDAR subunit composition. The results confirm that Neto protein interactions regulate synaptic localization of KAR and NMDAR subunits at MF-CA3 synapses, with implications for both ionotropic and metabotropic glutamatergic recruitment of the CA3 network.

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PUBLICATIONS

1. Matta JA, Pelkey KA, Craig MT, Chittajallu R, Jeffries BW, McBain CJ. Developmental origin dictates interneuron AMPA and NMDA receptor subunit composition and plasticity. *Nat Neurosci* 2013; 16:1032–1041.
2. Wyeth MS, Pelkey KA, Petralia RS, Salter MW, McInnes RR, McBain CJ. Neto auxiliary protein interactions regulate kainate and NMDA receptor subunit localization at mossy fiber-CA3 pyramidal cell synapses. *J Neurosci* 2014; 34:622–628.
3. Pelkey KA, Barksdale E, Craig MT, Yuan X-Q, Sukumaran M, Vargish GA, Mitchell RM, Wyeth MS, Petralia RS, Chittajallu R, Karlsson R-M, Cameron HA, Murata Y, Colonnese MT, Worley PF, McBain CJ. Pentraxins coordinate excitatory synapse maturation and circuit integration of parvalbumin interneurons. *Neuron* 2015; 85:1257–1272.
4. Craig MT, McBain CJ. Fast gamma oscillations are generated intrinsically in CA1 without the involvement of fast-spiking basket cells. *J Neurosci* 2015; 35:3616–3624.

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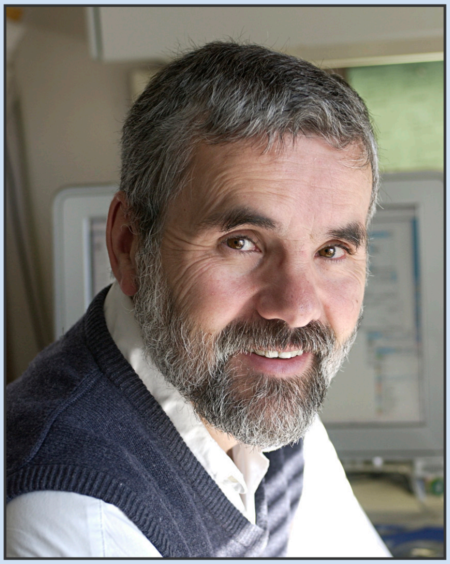
Using multidisciplinary and collaborative approaches, we investigate receptors and voltage- and ligand-gated channels, their activation by hormones and neurotransmitters, and their roles in intracellular signaling, gene expression, and hormone secretion in neuroendocrine cells. The work includes characterization of native and recombinant receptors and channels that were cloned from these cells. Currently, we are studying the calcium-mobilizing receptor-coupled gene network in non-transformed cells using RNA sequencing and qRT-PCR analysis of primary pituitary cells from developing and postpubertal mice and rats. We are also determining how the structural features of pituitary channels relate to the channels' functions and how plasma membrane receptors and the intracellular signaling milieu affect channel activity.



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Cell type–specific sexual dimorphism in pituitary gene expression during maturation

In rats, the postnatal period is divided into five developmental phases based on morphological and endocrine changes: (1) *neonatal* [the first seven postnatal days (PND)]; (2) *infantile* (from PND eight to 21); (3) *juvenile* (from PND 22 to 30 in females and from PND 22 to 35 in males); (4) *peripubertal* (from PND 31 to 40 in females and PND 36 to 60 in males); and (5) *adult* phase (from PND 41 in females and 61 in males). Sexual differentiation of rat pituitary functions has been extensively studied at the level of hormone production and release, leading to a better understanding of developmental phases and puberty. There have also been several attempts to characterize the sex-specific transcriptional activity in pituitary glands in developing animals using various methods. However, none of the studies analyzed the differentiation of gene expression for all five secretory cell types in both sexes during development.

In a recent study, we examined the expression of major anterior pituitary genes in five secretory cell types of embryonic and developing males and females with real-time quantitative reverse transcription–PCR, using pre-designed TaqMan Gene Expression Assays, which is a sensitive and reliable method to study gene expression in both male and female pituitaries *in vivo* and *in vitro*. Corticotrophs show comparable proopiomelanocortin (*Pomc*) profiles in both sexes, with the highest expression occurring during the infantile period. Somatotrophs and lactotrophs also exhibit no difference in growth hormone (*Gh*) and prolactin (*Prl*) profiles during embryonic-to-juvenile age but show the amplification of *Prl* expression in females and *Gh* expression in males during peripubertal and postpubertal ages. Gonadotrophs exhibit highly synchronized luteinizing hormone beta (*Lhb*), follicle-stimulating hormone beta (*Fshb*) gonadotroph/thyrotroph-specific alpha (*Cga*), and gonadotropin-releasing hormone receptor (*Gnrhr*) gene expression in both sexes, but the peak of expression occurs during the infantile period in females and at the end of the juvenile period in males. Thyrotrophs also show different developmental thyroid-stimulating hormone beta (*Tshb*) profiles, which are synchronized with the expression of gonadotroph genes in males but not in females. The results indicate the lack of influence of sex on *Pomc* expression and the presence of two patterns of sexual dimorphism in the expression of other pituitary

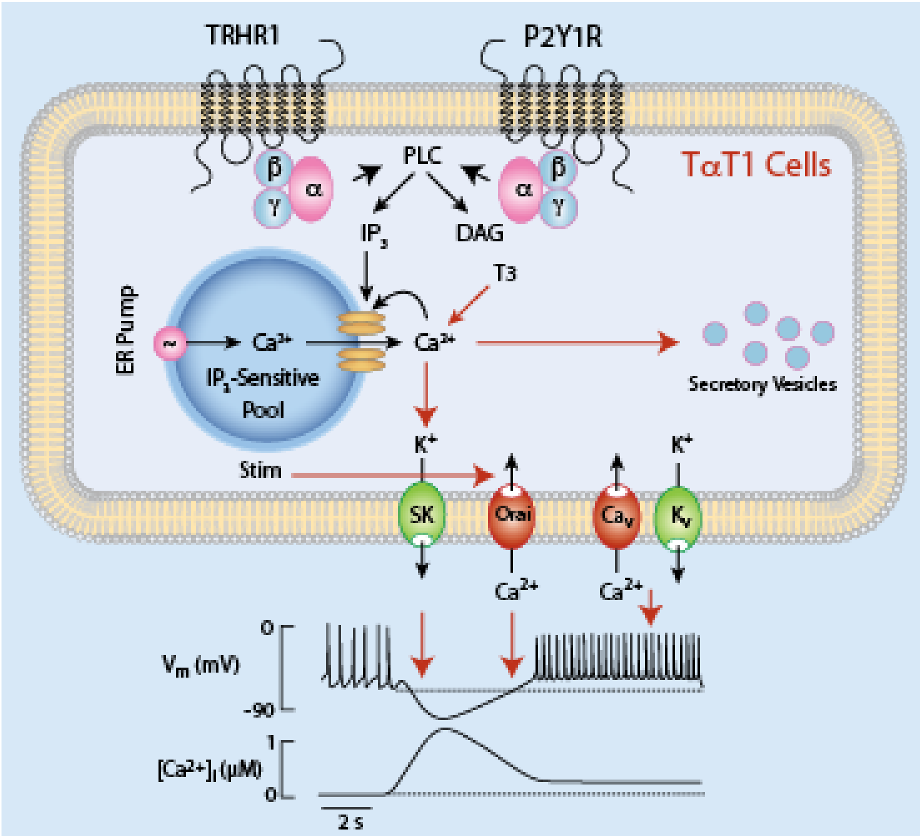

Figure 1. Schematic representation of calcium-signaling pathways in a thyrotroph cell line, mouse TalphaT1 cells
Cav, voltage-gated calcium channels; DAG, diacylglycerol; Kv, voltage-gated potassium channels; Nav, voltage-gated sodium channels; IP3, inositol 1,4,5-trisphosphate; Orai, store-operated calcium channels; SK, small calcium-controlled potassium channels; Stim, single-pass transmembrane protein gating Orai; T3, triiodothyronine.

genes: a time shift in the peak expression during postnatal development, most likely reflecting the perinatal sex-specific brain differentiation, and modulation of the amplitude of expression during late development, which is secondary to the establishment of the hypothalamic-pituitary-gonadal and -thyroid axes (Reference 1).

Signaling, gene expression, and secretion in pituitary thyrotrophs

At present, there is limited information about signaling pathways, gene expression, and hormone release in pituitary thyrotrophs, which in part reflects difficulties in purifying thyrotrophs and single-cell identification. Mellon and co-workers developed TalphaT1 cells, which represent a differentiated thyrotroph cell line and which could provide a useful model for studies on *Tshb* expression and other cellular functions. Recently, we characterized, for the first time, calcium-signaling pathways in TalphaT1 cells. Our electrophysiological experiments revealed that the cells are excitable and fire action potentials spontaneously and in response to application of thyrotropin-releasing hormone (TRH), the native hypothalamic agonist for thyrotrophs. Spontaneous electrical activity is coupled with small amplitude fluctuations in intracellular calcium, whereas TRH stimulates both calcium mobilization from intracellular pools and calcium influx. Non-receptor–mediated depletion of the intracellular pool also leads to a prominent facilitation of calcium influx. Both receptor- and non-receptor–stimulated calcium influx are substantially attenuated but not completely abolished by inhibition of voltage-gated calcium channels, suggesting that depletion of the intracellular calcium pool in these cells provides a signal for both voltage-independent and -dependent calcium influx, the latter by facilitating the pacemaking activity. We also found that the cells express purinergic P2Y1 receptors and that their activation by extracellular ATP mimics TRH action on calcium mobilization and influx (Figure 1). We also found that the thyroid hormone triiodothyronine prolongs duration of TRH–induced calcium spikes during a 30-minute exposure. The data indicate that TalphaT1 cells are capable of responding to feed-forward TRH signaling and intrapituitary ATP signaling with acute calcium mobilization and sustained calcium influx. Amplification of TRH–induced calcium signaling by triiodothyronine further suggests the existence of a pathway for positive feedback effects of thyroid hormones, probably in a non-genomic manner (Reference 2).

In a further study on this topic, we examined the *in vivo* and *in vitro* expression pattern of three genes that are operative in the thyrotroph subpopulation from postpubertal animals: *Cga*, *Tshb*, and that encoding the thyrotropin-releasing hormone receptor (*Trhr*). We consistently observed the robust and sex-specific expression of *Tshb* and C*ga* during development, whereas the expression of *Trhr* was lower, representing 1–2% of the expression of *Gapdh*, the housekeeping gene, whose expression did not significantly change during development or *in vitro* without or with TRH. *In vitro,* there was a relatively rapid down-regulation of the expression of the genes in both dispersed pituitary cells and pituitary fragments; *Tshb* expression was most dramatically affected. In parallel with mRNA measurements, the analysis of protein content showed a significant drop in the TSHB content in cultured pituitary cells. Under continuous TRH stimulation, there was a rapid but transient stimulation of *Tshb* expression in pituitary fragments lasting for about 6 hours. The inhibitory effect of triiodothyronine on *Tshb* expression was preserved in pituitary fragments as well as in freshly dispersed and cultured pituitary cells. However, TRH applied in different concentrations periodically and continuously to freshly dispersed or cultured pituitary cells was unable to stimulate *Tshb* expression. Single-cell calcium measurements and secretory studies with perifused pituitary cells showed that TRH receptors were functional at least in a fraction of thyrotrophs. The loss of stimulatory effect of TRH on *Tshb* expression in dispersed pituitary cells indicates that additional factors contribute to TRH induction of *Tshb* expression *in situ*. We speculate that connections among thyrotrophs and/or electrical or chemical interactions between thyrotrophs and other cellular networks are critical for thyrotroph function and the proper action of TRH on gene expression (Reference 3).

Dual action of antipsychotics on lactotroph function

For decades, hyperprolactinemia (HPRL) has been recognized as a common side effect of antipsychotic medications used in the treatment of patients with schizophrenia. Moreover, HPRL has clinical consequences on physical health in both short- and long-term treatments in youth and adults alike. Among atypical antipsychotics, risperidone (RIS), a dopamine (DA) D2 receptor higher affinity full antagonist, poses the greatest risk for marked and sustained HPRL. In fact, paliperidone (PAL), the 9-hydroxy main metabolite of RIS, is the main causative factor of HPRL. In contrast, aripiprazole (ARI) is a PRL–sparing drug, labeled as the first D2/D3 receptor partial agonist with a unique pharmacological profile. Others have suggested that the ‘atypicality’ of ARI is most likely combined with its actions on non-DA receptors. Yet, our understanding of the pathophysiology of antipsychotic-induced HPRL is incomplete. This includes the lack of mechanistic (basic) studies, which could help to distinguish the direct effects of PAL and ARI on human lactotrophs from those that might involve altered hypothalamic secretion of DA and other PRL–inhibiting and/or PRL–stimulating factors, as well as the contribution of receptors other than DA to the action of these drugs.

To address these questions, we studied the direct effects of PAL and ARI on lactotroph function, using a primary culture of female rat anterior pituitary cells as a model system. We used both static cultures and perifusion experiments to study the effects of the compounds on cAMP production and PRL secretion. In cells in static cultures, cAMP was measured intracellularly and extracellularly to account for a substantial cAMP release by cAMP transporters expressed in pituitary cells. DA inhibited spontaneous cAMP/calcium signaling and prolactin release. In the presence of DA, PAL rescued cAMP/calcium signaling and prolactin release in a concentration-dependent manner, whereas ARI was only partially effective. In the absence of DA, PAL stimulated cAMP/calcium signaling and prolactin release, whereas ARI inhibited signaling and secretion more potently but less effectively than DA. Forskolin-stimulated cAMP production was facilitated by PAL and inhibited by ARI, although the latter was not as effective as DA. None of the compounds affected prolactin transcript activity, intracellular prolactin accumulation, or growth hormone secretion. The data indicate that PAL has dual hyperprolactinemic actions in lactotrophs by (1) preserving the coupling of spontaneous electrical activity and prolactin secretion in the presence of DA and (2) inhibiting intrinsic D2 receptor activity in the absence of DA, leading to enhanced calcium signaling and secretion. In contrast, ARI acts on prolactin secretion by attenuating, but not abolishing, calcium-secretion coupling (Reference 4).

Use-dependent desensitization of pituitary purinergic P2X2 receptor channels

The purinergic P2X2 receptor (P2X2R) is an ATP–gated non-selective cation channel expressed in various tissues, including pituitary gonadotrophs, where it has important roles in the control of spontaneous and GnRH–stimulated electrical activity, calcium signaling, and gonadotropin secretion. In recent work, our group showed that P2X2R can dilate and desensitize simultaneously and that the desensitization process includes calcium-dependent and -independent mechanisms. During repetitive agonist application, with washout periods of 4–5 minutes, there is a progressive increase in the rates of calcium-dependent desensitization, a phenomenon termed use-dependent desensitization (UDD). Recently, we characterized the desensitization properties of recombinant P2X2R expressed in HEK293 cells. Using, rat, mouse, and human receptors, we showed that two processes contribute to receptor desensitization: bath calcium-independent and -dependent. Calcium-independent desensitization was minor and comparable during repetitive agonist application in cells expressing the full-size receptor, but was pronounced in cells expressing shorter versions of receptors, indicating a role of the C terminus in controlling receptor desensitization. UDD was substantial during initial agonist application and progressively increased during repetitive agonist application in ATP- and bath calcium-concentration–dependent manner. Experiments with substitution of bath sodium with NMDG, a large organic cation, indicated that, in contrast to receptor desensitization, receptor pore dilation was a calcium-independent process. A reduction in the driving force for calcium by changing the holding potential from −60 to +120 mV further indicated that calcium influx through the channel's pore at least partially accounts for UDD. Experiments with various receptor chimeras also indicated that the transmembrane and intracellular domains of P2X2R are required for development of UDD and that decrease in the amplitude of current slows receptor desensitization. Simultaneous calcium and current recording showed development of UDD without a rise in global intracellular calcium concentrations. Combined with experiments with clamping intrapipette concentrations of calcium at various levels, these experiments indicate that domain calcium is sufficient to establish UDD in experiments with whole-cell recordings (Reference 5).

PUBLICATIONS

1. Bjelobaba I, Janjic MM, Kucka M, Stojilkovic SS. Cell type-specific sexual dimorphism in rat pituitary gene expression during maturation. *Biol Reprod* 2015; 93:1–9.
2. Tomic M, Bargi-Souza P, Leiva-Salcedo E, Nunes MT, Stojilkovic SS. Calcium signaling properties of a thyrotroph cell line, mouse TaT1 cells. *Cell Calcium* 2015; 58:598–605.
3. Bargi-Souza P, Kucka M, Bjelobaba I, Tomic M, Janjic MM, Nunes, MT, Stojilkovic SS. Loss of basal and TRH-stimulated Tshb expression in dispersed pituitary cells. *Endocrinology* 2015; 156:242–254.
4. Kucka M, Tomic M, Bjelobaba I, Stojilkovic SS, Budimirovic DB. Paliperidone and aripiprazole differentially affect the strength of calcium-secretion coupling in female pituitary lactotrophs. *Sci Rep* 2015; 5:8902.
5. Coddou C, Yan Z, Stojilkovic SS. Role of domain calcium in purinergic P2X2 receptor channel desensitization. *Am J Physiol Cell Physiol* 2015; 308:C729–C736.

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Olfactory Coding and Decoding by Ensembles of Neurons

All animals need to know what is going on in the world around them. Brain mechanisms have thus evolved to gather and organize sensory information in order to build transient and sometimes enduring internal representations of the environment. Using relatively simple animals and focusing primarily on olfaction and gustation, we combine electrophysiological, anatomical, behavioral, computational, genetic, and other techniques to examine the ways in which intact neural circuits, driven by sensory stimuli, process information. Our work reveals basic mechanisms by which sensory information is transformed, stabilized, and compared, as it makes its way through the nervous system.

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A temporal channel for information in sparse sensory coding

Brain circuits encode sensory information into a variety of neural representations ranging from dense, time-varying patterns of spikes in overlapping sets of neurons to sparse spikes in a few selective neurons. Sparse codes are used by nearly all sensory systems, including vision, audition, somato-sensation, and olfaction, and are thought to be advantageous for distinguishing between similar stimuli and for learning associations. In dense codes, the timing of spikes has been shown to contain sensory information, but the role of timing in the relatively few spikes in sparse sensory codes is unclear.

We used the olfactory system of awake locusts to test whether the timing of spikes in Kenyon cells, a population of neurons that respond sparsely to odors, carries sensory information to, and influences, follower neurons. First, we characterized two major classes of direct followers of Kenyon cells in a brain area called the β-lobe. With paired intracellular and field-potential recordings made during odor presentations, we found that the followers portray odor identity in the temporal patterns of their spikes but not in the spike rate, the spike phase, or the identities of active neurons. We also found that subtly manipulating the relative timing of Kenyon cell spikes, using temporally and spatially structured electrical microstimulation, reliably altered the responses of the followers. Our results show that even the remarkably sparse spiking responses of Kenyon cells provide information through odor-specific variations in timing on the order of tens to hundreds of milliseconds and that the variations determine responses downstream.

Sparse coding in sensory areas has been viewed as an outcome of, rather than as a substrate for, temporal processing. Stimulus-specific variations in spike timing provide a useful channel to increase the coding capacity of neurons while retaining the benefits of sparseness. Our results establish the importance of spike timing in sparse sensory codes.

Functional analysis of the lateral horn, a higher olfactory center

Understanding how information is processed through a neural circuit requires characterizing its structures and functions. The lateral horn of the insect brain is a prominent area widely thought to play several important roles in olfaction.

These include maintaining the sparseness of responses to odors in Kenyon cells by means of feed-forward inhibition and encoding preferences for innately meaningful odors. However, relatively little was known about the structure and function of lateral horn neurons (LHNs), making it difficult to test these ideas. We surveyed more than 250 LHNs in locusts, using sharp-electrode recordings to test their responses to sensory stimuli, dye-fills to define their morphologies, and immunostaining to characterize their neurotransmitters.

We found a great diversity of LHNs, which we organized into ten highly distinct morphological classes, thus establishing a useful dataset. Surprisingly, we found no evidence to support a long-suspected role for these neurons in the feed-forward inhibition proposed to mediate olfactory response sparsening. Instead, through paired recording experiments and phase analyses of the odor-elicited responses of several types of neurons, we found that a different mechanism, feed-back inhibition from a newly identified giant GABAergic neuron (GGN), plays this role. Further, we found that all tested LHNs responded to all odors we tested, making it unlikely these neurons serve as “labelled” lines mediating specific, innate behavioral responses to specific odors. Instead, our results point to three other possible roles of LHNs: extracting general stimulus features such as odor intensity; mediating bilateral integration of sensory information; and integrating multimodal sensory stimuli. This work was recently published (Gupta N, Stopfer M, *Curr Biol* 2014;24:2247-2256).

Tradeoff between information format and capacity in the olfactory system

How does the nervous system 'decide' what format to use to encode information? The brain’s internal representation of the environment is built from patterns of neural activity bearing information about sensory stimuli. As this activity travels through the brain, a succession of neural circuits manipulates it into a series of coding formats, each thought to provide specific advantages for processing information. But each coding format has advantages and disadvantages. How the benefits of a given format are balanced against its costs is largely unknown.

One common coding format uses periodic inhibition to coordinate neural spiking into synchronous oscillations. Oscillatory synchrony has been proposed to offer several benefits, including enhancing the discriminability of sensory representations, and ‘binding’ diverse stimulus features into coherent percepts. We examined how neural oscillatory synchronization affects another measure of coding quality: the rate information is transmitted. We evaluated this potential tradeoff between coding format and information rate in the olfactory system of the locust. To test the possible tradeoffs imposed by synchrony, we needed a richly structured olfactory stimulus appropriate for the measurement of information properties. We decided to focus on stimulus timing because we could provide, with a synthetic odor plume, a broad sample of an environmentally meaningful stimulus space. Using artificial plumes based on the statistical structures of temporal variability measured outdoors in real odor plumes allowed us to calculate lower bound estimates of information in the temporal structure of an ethologically relevant stimulus. Thus, we delivered odorants as controlled, repeatable plumes while recording responses from populations of projection neurons as they transmitted information about the plume’s temporal structure. We evaluated the information content of neurons in terms of the mutual information rate between the temporal dynamics of the odorant stimulus and the neuronal response by finding the difference between the unconditional and stimulus-conditioned response entropies.

Surprisingly, our results showed that pharmacologically blocking synchronization by locally injecting picrotoxin led to a significant increase in information rate. Thus, the use of a synchronous coding scheme introduces a tradeoff: synchrony allows correlation coding and fine olfactory discrimination; however, by reducing the number of spikes and spike positions available for encoding information, it also reduces the ability of the system to rapidly transmit information about the stimulus. The inhibition-induced reduction in transmission capacity that we observed in the olfactory system is likely to occur in any neural circuit using periodic inhibition. Our results suggest reformatting to an oscillatory structure comes at a cost and represents a fundamental tradeoff between coding capacity and other aspects of format utility.

Spatiotemporal coding of individual chemicals by the gustatory system

Four of the five major sensory systems (vision, olfaction, somatosensation, and audition) are thought to be encoded by spatiotemporal patterns of neural activity. The only exception is gustation. Gustatory coding by the nervous system is thought to be relatively simple: every chemical (‘tastant’) is associated with one of a small number of basic tastes, and the presence of a basic taste, rather than the specific tastant, is represented by the brain. In mammals as well as insects, five basic tastes are usually recognized: sweet, salty, sour, bitter, and umami. The neural mechanism for representing basic tastes is unclear. The most widely accepted proposal is that, in both mammals and insects, gustatory information is carried through labelled lines: separate channels, from the periphery to sites deep in the brain, of cells sensitive to a single basic taste. An alternate proposal is that the basic tastes are represented by populations of cells, with each cell sensitive to multiple basic tastes.

Testing these ideas requires determining, point-to-point, how tastes are initially represented within the population of receptor cells and how this representation is transformed as it moves to higher order neurons. However, it has been very challenging to deliver precisely timed tastants while recording cellular activity from directly connected cells at successive layers of the gustatory system. Using a new moth preparation, we designed a stimulus and recording system that allowed us to fully characterize the timing of tastant delivery and the dynamics of the tastant-elicited responses of gustatory receptor neurons and their monosynaptically connected second order gustatory neurons, before, during, and after tastant delivery.

Surprisingly, we found no evidence consistent with a basic taste model of gustation. Instead, we found that the moth's gustatory system represents individual tastant chemicals as spatiotemporal patterns of activity distributed across the population of gustatory receptor neurons. Further, we found that the representations are transformed substantially as multiple types of gustatory receptor neurons converge broadly upon follower neurons. The results of our physiological and behavioral experiments suggest that the gustatory system encodes information not about basic taste categories, but rather about the identities of individual tastants. Further, this information is carried not by labelled lines, but rather by distributed, spatiotemporal activity, which is a fast and accurate code. The results provide a dramatically new view of taste processing.

An identified gustatory second-order neuron in the *Drosophila* brain

Little is known, in any species, about neural circuitry immediately following gustatory sensory neurons, which makes it difficult to know how gustatory information is processed by the brain. By genetically labeling and manipulating specific parts of the nervous system, we identified and characterized a bilateral pair of gustatory second-order neurons in *Drosophila*. Previous studies had already identified gustatory sensory neurons that relay information to distinct parts of the gnathal (subesophageal) ganglia. To identify candidate gustatory second-order neurons, we took an anatomical approach. We screened about 5,000 GAL4 driver strains for lines that label neural fibers innervating the gnathal ganglia. We then combined GRASP (GFP reconstitution across synaptic partners) with presynaptic labeling to visualize potential synaptic contacts between the dendrites of the candidate gustatory second-order neurons and the axonal terminals of Gr5a–expressing sensory neurons, which have been shown to respond to sucrose. Results of the GRASP analysis, followed by a single cell analysis by FLP-out recombination, identified a specific pair of neurons that contact Gr5a axon terminals in both brain hemispheres and send axonal arborizations to a distinct region within the gnathal ganglia. To characterize the input and output branches, respectively, we expressed the fluorescence-tagged acetylcholine receptor subunit (Dα7) and active-zone marker (Brp) in the gustatory second-order neurons.

We found that input sites of the gustatory second-order neurons overlaid GRASP–labeled synaptic contacts to Gr5a neurons, while presynaptic sites were broadly distributed throughout the neurons' arborizations. GRASP analysis and further tests with a new version of GRASP that labels active synapses suggested that the identified second-order neurons receive synaptic inputs from Gr5a–expressing sensory neurons, but not Gr66a–expressing sensory neurons, which respond to caffeine. The identified second-order neurons relay information from Gr5a–expressing sensory neurons to stereotypical regions in the gnathal ganglia. Our findings suggest an unexpected complexity for taste-information processing in the first relay of the gustatory system. We are presently following up on this work to identify additional second-order neurons and with optical imaging and intracellular electrophysiology experiments to characterize their functions and information-coding strategies.

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PUBLICATIONS

1. Reiter S, Campillo Rodriguez C, Sun K, Stopfer M. Spatiotemporal coding of individual chemicals by the gustatory system. *J Neurosci* 2015; 35:12309–12321.
2. Aldworth Z, Stopfer M. Tradeoff between information format and capacity in the olfactory system. *J Neurosci* 2015; 35:1521–1529.
3. Huston SJ, Stopfer M, Cassenaer S, Aldworth ZN, Laurent G. Neural encoding of odors during active sampling and in turbulent plumes. *Neuron* 2015; 88:1–16.
4. Miyazaki T, Lin TY, Ito K, Lee CH, Stopfer M. A gustatory second-order neuron that connects sucrose-sensitive primary

neurons and a distinct region of the gnathal ganglion in the Drosophila brain. *J Neurogenet* 2015; 29:144–55.
5. Kee T, Sanda P, Gupta N, Stopfer M, Bazhenov M. Feed-forward versus feedback inhibition in a basic olfactory circuit. *PLoS Comput Biol* 2015; 11:e1004531.

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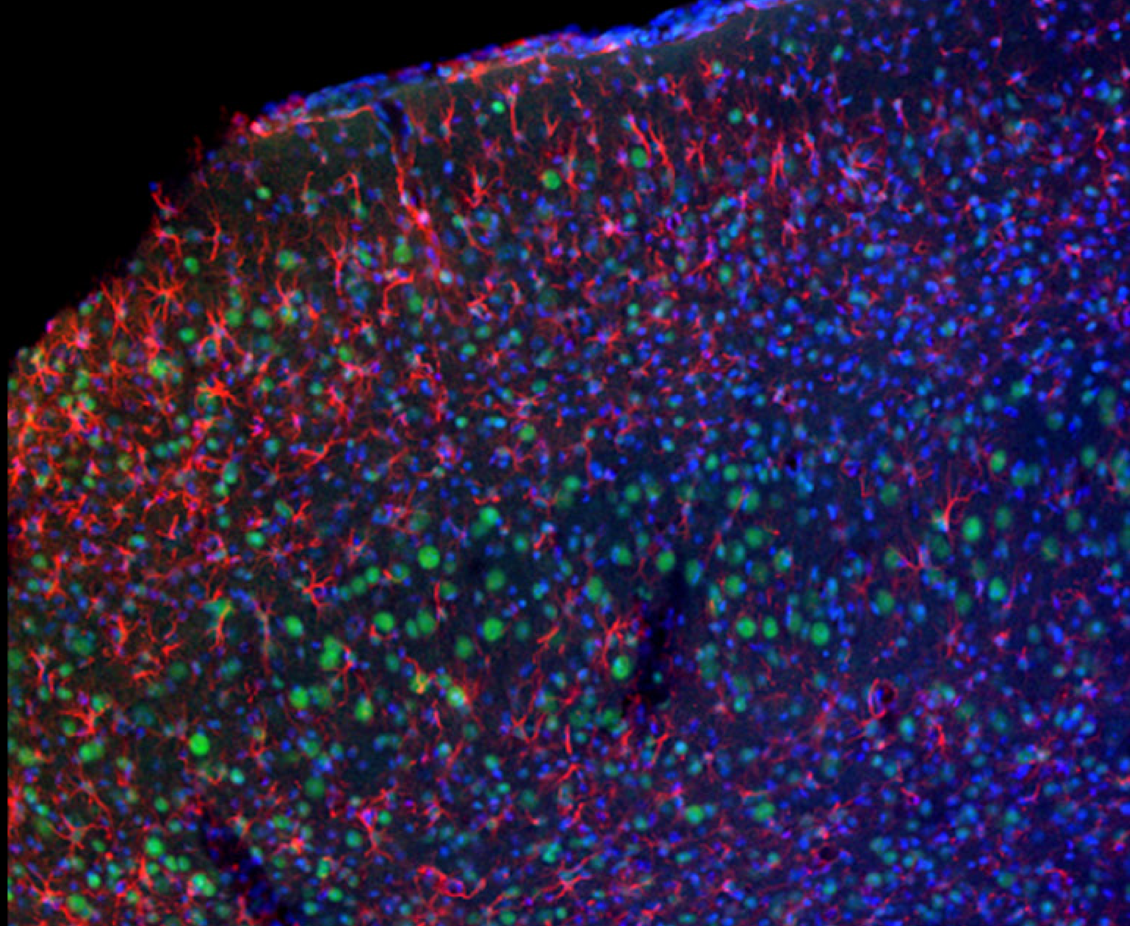
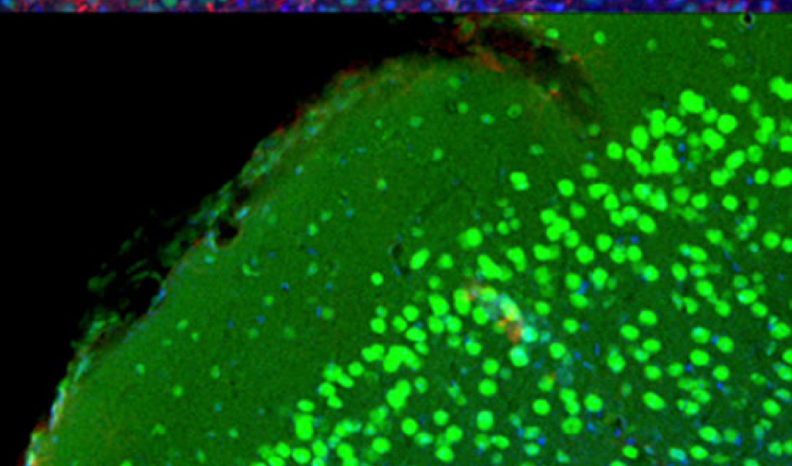
LaKO



Wild-type



Program on Genomics of Differentiation

Director: Brant Weinstein, PhD

ABOUT THIS IMAGE

Deletion of mouse La protein results in neurodegeneration and astrocyte invasion of the neocortex.

Genetic conditional knockout (KO) of the mouse gene encoding the La protein homolog of the human La autoantigen in excitatory neurons causes progressive neurodegeneration. La is seen in the green channel by immunofluorescence, the astrocyte marker, GFAP in the red channel and nuclei are in blue. The healthy control sibling cortex (wild-type) has no astrocyte reactivity and robust La expression, in contrast to the conditional LaKO mutant in which is observed widespread invasion by astrocytes. Additional analyses indicate subsequent phagocytosis of dying neurons (not shown). This study by Blewett et al. is an in depth examination of a phenomenon first reported by our group in 2014 by Gaidamakov et al (1). Image by Nathan Blewett, PhD, of the *Section on Molecular and Cell Biology*.

(1) Gaidamakov, S., Maximova, O.A., Chon, H., Blewett, N.H., Wang, H., Crawford, A.K., Crouch, R.J., Morse, H.C., 3rd, et al. (2014). Targeted deletion of the gene encoding the La protein SSB autoantigen in B cells or cerebral cortex causes extensive tissue loss. *Mol Cell Biol* 34:123-31.

Program *on* Genomics of Differentiation

The *Program in Genomics of Differentiation (PGD)* is a diverse and highly interactive program in cellular, molecular, and developmental biology research within the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), Division of Intramural Research (DIR). With 20 principal investigators, the PGD is the largest program in the NICHD DIR, encompassing several research areas, including developmental differentiation and patterning, chromatin dynamics and epigenetics, the immune system, the viral life cycle, DNA replication, gene regulation, and RNA metabolism. Program investigators perform research using a wide variety of models, including viruses, bacteria, mammalian cell culture, yeast, fruit flies, zebrafish, frogs, and mice. Vertebrate models are a major focus of the program. The zebrafish is used as a model for the analysis of embryonic development and organogenesis as well as for modeling certain human conditions. Using genetics, genomics, and high-resolution imaging techniques, PGD investigators study cell-cell signaling and cellular behavior in early embryogenesis, formation and morphogenesis of the vascular system, cellular specification in the developing nervous system, and cellular and molecular mechanisms underlying behavior. The mouse provides another important vertebrate model. PGD investigators employ advanced gene-targeting and transgenic technologies to study genes that control mouse development, transcriptional control in the early embryo, the role of Lim-homeobox genes and chromatin-binding proteins, mechanisms of genomic imprinting, regulation of immune cells, the development of the central and peripheral nervous systems, and the behavior of neural stem cells in the adult organism. In addition, the PGD generates mouse models of a diverse array of human genetic disorders.

Harold Burgess's *Unit on Behavioral Neurogenetics* studies the development and function of neural circuits required for behavioral control in larval zebrafish. Brainstem circuits, which control behavior in zebrafish larvae, represent the core of the movement control system in higher vertebrates and are impaired in numerous neurological disorders. The Section applies computational analysis to high-speed video recordings of larvae challenged with distinct sensory stimuli to determine the function of identified brainstem neurons in transgenic fish. The group generated a library of brain-specific enhancer trap lines and used these to analyze control of sensory responsiveness by environmental context and internal behavioral states such as arousal. The group is currently mapping neuronal circuitry that modulates the startle response to understand how genetic mutations in schizophrenic patients lead to defects in prepulse inhibition.

Mike Cashel's *Section on Molecular Regulation* discovered two guanine nucleotide analogs (ppGpp and pppGpp) many years ago, when these were called magic spots, which function as second messengers in bacteria and plants to couple global regulation of gene expression to nutrient availability. This year, the Section's research took three directions. The first was to mutate the ability of the animal kingdom "Mesh" hydrolase to degrade (p)ppApp but not (p)ppGpp in order to use it as a diagnostic tool to match bacterial hydrolase with the reverse specificity. Catalytic site mutants based on structures are promising. The second was to make a nucleotide test system in *Escherichia coli* for unusual nucleotides; studies with pGpp were initiated. The third was to exploit the Section's discovery that the omega subunit is part of the (p)ppGpp binding site for RNA polymerase. Genetic studies revealed that (p)ppGpp function is not only intertwined with chaperone functions but that the chaperone activities also appear to be secondary functions of GreA and DksA, which are RNAP accessory proteins involved in initiation, promoter recognition, and elongation arrest.

Ajay Chitnis and colleagues in the *Section on Neural Developmental Dynamics* are examining how the posterior lateral line system is built in the zebrafish nervous system. The lateral line is a mechanosensory system that consists of sensory organs called neuromasts, which are distributed in a stereotypic pattern over the surface of the zebrafish. Development of hair cells in the lateral line neuromasts is remarkably similar to that of hair cells in the human ear. Furthermore, the mechanisms that guide migration of the lateral line primordium, as it deposits neuromasts under the skin, are remarkably similar to those that determine migration of metastatic cancer cells. The goal of the laboratory is to define the gene-regulatory network that coordinates cell fate and morphogenesis in the lateral line system and to build computational models, based on these studies, to understand how this relatively simple sensory system in zebrafish builds itself.

David Clark and his colleagues in the *Section on Chromatin and Gene Expression* study the role of chromatin structure in gene activation. Gene activation must occur in the presence of nucleosomes, which are compact structures capable of blocking transcription at every step. To circumvent the chromatin block, eukaryotic cells possess chromatin-remodeling and histone-modifying complexes. Mutations in many of these complexes are strongly associated with cancer. The laboratory used Illumina paired-end sequencing to determine nucleosome positions genome-wide in remodeling mutants of the yeast *Saccharomyces cerevisiae* and found that the chromatin remodeling complex known as RSC plays a much more important role than the related SWI-SNF complex in organizing yeast chromatin. RSC affects the phasing of nucleosomes relative to the transcription start sites of genes and controls the width of the nucleosome-depleted region that is characteristic of gene promoters. Currently, the laboratory is studying interactions among the various chromatin-remodeling machines to determine their specific roles in chromatin organization.

Robert Crouch, who leads the *Section on Formation of RNA*, studies RNases H, enzymes that degrade RNA in RNA/DNA hybrids. Failure to degrade RNA in DNA can lead to loss of mitochondrial DNA, detrimental DNA recombination, severe neurological defects, and a general loss of genome integrity. Type I RNase H is structurally and functionally related to an essential RNase H of the HIV-AIDS virus and could be a target for HIV drug therapy. In humans, defective Type II RNase H can result in Aicardi-Goutières syndrome, an encephalopathy that mimics *in utero*–acquired viral infection. In eukaryotes, type II RNase H2 has two distinct activities: removal of single ribonucleotides misincorporated in DNA and of longer stretches of RNA/DNA hybrids, thereby helping to maintain genome stability. Studies on defective in RNase H2 in mice and yeast are providing insights into the contribution of this protein to genome integrity.

Igor Dawid, who heads the *Section for Developmental Biology*, and colleagues study early development in zebrafish and the frog *Xenopus*. Recent efforts focused on the formation of the neural crest, pineal transcriptome analysis, and the modulation of cell adhesion in the early embryo. In analyzing neural crest development, the group studied the role of the BTB–domain protein Kctd15. Kctd15 inhibits neural crest formation, and its action is believed to delimit the neural crest domain in the zebrafish embryo. Kctd15 inhibits transcription factor AP-2, an important neural crest regulator. The mechanism of this inhibition involves the specific binding of Kctd15 to the AP-2 activation domain. SUMO modification of Kctd15 was also studied, a modification that is not required for the inhibition of AP-2 by Kctd15 but may play a role in distinct developmental processes. In studying the RNA population of the pineal gland, the group noted a factor, Unc119c, that is differentially expressed in the pineal. Knock-down studies indicate that Unc119c is required in the pineal gland to guide the habenular commissure across the midline in the developing zebrafish brain. Another project showed that the transmembrane E3 ubiquitin ligase March8 is a modulator of cell-cell adhesion in the early frog and fish embryo.

Mel DePamphilis heads the *Section on Eukaryotic DNA Replication*, which studies the control of DNA replication and gene expression during mammalian development. The Section's current work focuses on three questions: how genome duplication is restricted to once per cell division in proliferating cells; how these mechanisms can be circumvented in order to selectively kill cancer cells; and how the mechanisms are circumvented during normal mammalian development to allow some cells to differentiate into viable, nonproliferating, polyploid cells. Recently, members of the Section discovered that one of the proteins (Geminin) involved in preventing DNA from re-replicating prior to mitosis is essential to prevent DNA re-replication and apoptosis in cells derived from human cancers, but not in cells derived from normal tissues. They are pursuing this discovery by investigating the effects of Geminin ablation in mice, by using high-throughput screening to identify all genes essential for restricting genome duplication in cancer cells to once per cell division, and by identifying small molecules that selectively target these genes. Such molecules would be useful in cancer chemotherapy as well as in research into the regulation of cell proliferation. To understand how normal cells escape the restrictions on genome duplication, the Section is investigating how trophoblast stem cells exit their mitotic cell cycle and differentiate into the polyploid giant cells essential for both implantation and placentation.

Judith Kassis, who heads the *Section on Gene Expression*, studies the mechanism of gene silencing by the Polycomb group genes (PcG). PcG proteins act as protein complexes that control gene expression by modifying chromatin structure; PcG proteins are therefore known as epigenetic regulators. The Section uses biochemical, molecular, genomic, and genetic methods to understand how PcG protein complexes regulate gene expression. In the fruit fly *Drosophila*, DNA elements called Polycomb response elements (PREs) are required for PcG protein recruitment. PREs are several hundred nucleotides in length and consist of binding sites for numerous proteins. Recent results from the Section indicate that PREs recruit PcG proteins using a complex and variable combination of DNA–binding proteins, allowing for specialization of PREs. In an effort to understand the role of PREs in the context of other regulatory DNA, the Section studies PRE activity at the PcG target gene complex *engrailed* and *invected (en/inv)*, co-regulated genes whose regulatory sequence extends over a 70kb region. There are at least four PREs in this gene complex, and work from the laboratory shows that they can behave differently in biological assays. Nevertheless, the PREs appear to have redundant activities in the endogenous locus. There is also apparent redundancy in the *en/inv* enhancers (DNA sequences that activate expression). Ongoing work in the laboratory seeks to understand the interplay between gene activation and PcG repression.

Jim Kennison, who heads the *Section on Drosophila Gene Regulation*, studies genomic organization and function in *Drosophila*. His group defined both *cis*-acting regulatory sequences and *trans*-acting factors required to maintain activation and/or repression of the *Sex combs reduced* homoeotic gene. Many of the *trans*-acting factors are components of chromatin-remodeling or histone-modification complexes that specify the transcriptional state of the homoeotic genes. Genetic elements about 70 kb apart in the *Sex combs reduced* gene must be in *cis* to maintain proper repression after embryogenesis. The elements appear to correspond to clusters of Polycomb group response elements (PREs). New *trans*-acting factors that act through the PREs were identified in genetic screens for recessive mutations that fail to maintain repression in somatic clones. The group also analyzed two regions that span about 1% of the *Drosophila* genome, identifying all genes required for zygotic viability or male fertility. The studies identified the first gene desert in *Drosophila*, a region of 55kb with many evolutionarily conserved DNA sequences, but with no apparent function under laboratory conditions. The studies also showed that an appreciable fraction (as much as 25%) of the *Drosophila* genome is dedicated to male fertility.

Paul Love's laboratory, the *Section on Cellular and Developmental Biology*, studies mammalian hematopoiesis. A main area of research centers on T lymphocyte development, particularly signal transduction molecules and pathways that regulate T cell maturation in the thymus. The group revealed a critical function for T cell antigen receptor (TCR) signaling in controlling key developmental events essential for normal T cell function and for the prevention of autoimmunity. The laboratory also recently described a previously unknown T cell–specific protein, Themis, which functions to sustain TCR signaling during T cell development. Additional studies aimed at gaining a better understanding of early events in hematopoiesis identified an essential function for the nuclear adapter LIM–domain protein-1 (Ldb1) in hematopoietic stem cell maintenance and erythropoiesis. Current work on Ldb1 includes studies examining a potential role for this protein in regulating self-renewal of T cell progenitors in the thymus and in the genesis of T cell Acute Lymphoblastic Leukemia (T-ALL), one of the most common childhood malignancies.

Todd Macfarlan's *Unit on Mammalian Epigenome Reprogramming* uses a multidisciplinary approach that combines mouse genetics, molecular biology, biochemistry, cell biology, multi-dimensional imaging, and next-generation sequencing approaches to explore mechanisms of gene regulation and epigenetic inheritance during embryo development. Furthermore, the group is taking advantage of genetically engineered embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, which can be differentiated into specific cell types, to model early cell-fate decisions. The cells can be grown in very large numbers to allow biochemical interrogation of the epigenome.

Richard Maraia, who heads the *Section of Molecular and Cell Biology*, studies RNA metabolism, with a continued interest in tRNA. Efforts focus on tRNA 3′-end formation by RNA polymerase III and the RNA 3′-end–stabilizing protein La. The Section's recent data suggest that La-related protein-4 (LARP4) binds to 3′ poly(A) and stabilizes mRNAs. Another focus of the Section's work is Tit1p, a tRNA anticodon loop–modification enzyme, and its effects on the specific activity of its substrate tRNAs in decoding during translation and its more global effects on the subsets of mRNAs with cognate codon bias. The studies led to a new view of the genetic-information potential of the redundancy component of the genetic code. The Section uses genetics coupled with genome-wide profiling and this-generation sequencing, cell and structural biology, and biochemistry in model systems that include yeast, mammalian cultured cells, and gene-altered mice.

Keiko Ozato heads the *Section on Molecular Genetics of Immunity,* which studies gene regulation in innate immunity, with an emphasis on the role of chromatin. Current research centers on three nuclear proteins, IRF8, BRD4, and histone H3.3. IRF8 is a DNA–binding transcription factor that directs the development of macrophages and dendritic cells, the cell type principally responsible for innate immunity. As such, IRF8 is critical for eliciting early anti-pathogen resistance. BRD4 binds to acetylated chromatin through its bromodomain, as well as to the elongation factor P-TEFb through the C-terminal domain, and controls transcription of many cellular and viral genes. The histone variant H3.3, also known as a replacement histone, is incorporated into chromatin in conjunction with transcription. There is mounting recognition of the significance of the replacement histones, given that it has become clear that nucleosomes are subject to destabilization and eviction during transcription and repair. However, the activity of H3.3 in transcription activation, particularly during immune responses, is not well understood. Studying interferon (IFN)–stimulated genes as a model, the group showed that IFN triggers the recruitment of BRD4 to IFN–stimulated genes and that the recruitment is the primary event initiating productive elongation. Subsequent work with this model revealed that transcription leads to a large-scale exchange of chromatin in the IFN–stimulated genes, replacing the standard histone H3 with the variant H3.3, which creates a lasting epigenetic mark on previously expressed IFN–stimulated genes. To further study the activity of these proteins *in vivo*, the group recently generated novel knock-in and conditional knockout mice for *Irf8*, *Brd4*, and *H3f3a/b* and began studying their activity in various immune cells.

The *Section on Genomic Imprinting*, led by Karl Pfeifer, examines the regulated expression and biological functions of a cluster of imprinted genes on the distal end of mouse chromosome 7. Imprinting is an unusual form of gene regulation in which expression of a gene depends on parental-specific epigenetic modifications of the chromosome. Imprinted loci are an excellent model system for studying how epigenetic mechanisms regulate development and how developmental processes establish the epigenome. The group identified and characterized a 2.4 kb element that organizes higher-order chromosomal structures and long-range DNA interactions across a 120 kb region. Current work focuses on the role of non-coding RNAs in regulating enhancer activity and in establishing the chromosomal structures specific to maternal and paternal chromosomes. The group also establishes mouse models for human diseases associated with genetic lesions in the region. Additional studies focus on conditional gain of function and conditional loss of function of the cardiac *calsequestrin* 2 gene as well as loss-of-function studies on *H19*, a gene encoding long noncoding RNA.

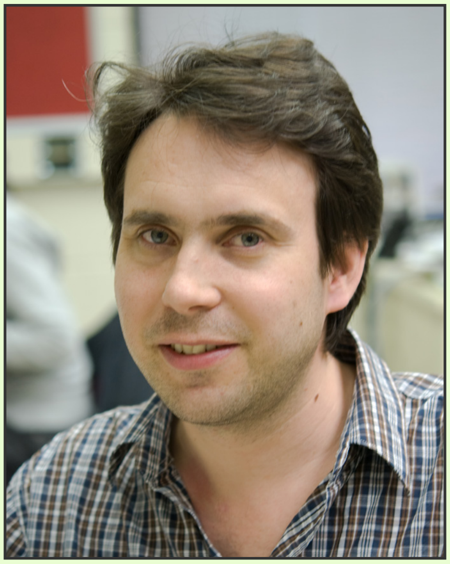
Tom Sargent, who heads the *Section on Vertebrate Development*, and colleagues study the development of the cranial neural crest (NC) in zebrafish. Previous contributions from this laboratory include describing the central role of the transcription factor TFAP2a in NC induction and the function of the homeodomain protein DLX3 in NC/neural/epidermal boundary formation in *Xenopus*. The group also investigated the function of pak4 (p21-activated kinase 4), a Rho-GTPase effector molecule that was identified earlier as an interaction partner for Inka; Inka is a novel cranial NC–regulatory factor initially identified by this laboratory. Experiments using antisense morpholinos (MOs) led to the conclusion that *pak4* is a maternal-effect gene that is required for multiple aspects of organogenesis, including formation of myeloid cells (macrophages and granulocytes) and morphogenesis of axial muscles. The group recently generated lines of zebrafish in which the pak4 gene was ablated using TALEN–based gene targeting. Surprisingly, homozygous *pak4* null fish appear to be normal and fertile, suggesting that, unlike in mammals, pak4 may be dispensable in zebrafish, and furthermore raising questions about the validity of MOs as primary tools for characterizing gene function. The laboratory currently employs a transgenic approach to express regulatory molecules in the cranial NC cells of live fish embryos, including lines expressing dominant negative, inducible versions of Dlx factors, driven specifically in the NC by a *sox10* promoter element. The lines are used to investigate the function of Dlx genes in zebrafish craniofacial development and to decode the gene-regulatory networks that control this process. A novel assay system based on transient expression of synthetic mRNAs in zebrafish embryos, followed by RNAseq analysis, is being developed as a tool for discovering downstream targets of transcription factors or other proteins of biomedical interest.

Brant Weinstein's *Section on Vertebrate Organogenesis* studies blood- and lymphatic-vessel formation during vertebrate embryogenesis. Vessel formation is of intense clinical interest, given the roles blood and lymphatic vessels play in cancer and ischemia. Using the zebrafish, the group developed a now widely used confocal microangiography method, compiled an atlas of the vasculature, developed numerous vascular-specific transgenic lines, and pioneered methods for high-resolution *in vivo* imaging of blood vessels. The group discovered a novel pathway of artery specification, a role for neuronal guidance factors in vascular patterning, and a mechanism for vascular tube formation *in vivo*. Weinstein and his colleagues also identified the lymphatic vascular system in zebrafish. Current studies use genetic screening, experimental analysis, and imaging to examine cues directing vascular patterning and morphogenesis, regulation of vascular integrity, assembly of the lymphatic system, and hematopoietic stem cell formation.

Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

Our goal is to understand how the nervous system selects appropriate motor responses, under diverse environmental contexts, in a way that best satisfies internal motivational objectives. We use zebrafish as a model because the larval brain exhibits the basic architecture of the vertebrate brain but is much less complex than the mammalian brain. Despite the relative simplicity of the nervous system, larvae have a sophisticated repertoire of sensory-guided and internally driven behaviors. In addition, the optical clarity of the embryo facilitates visualization of individual neurons and their manipulation with genetic techniques. Behavior in larvae is innate and therefore exhibits minimal variability between fish. Subtle alterations in behavior can therefore be robustly scored, making it possible to assess quickly the contribution of identified neurons to a variety of motor behaviors.

We focus on two aspects of behavioral regulation: the mechanisms by which sensory context regulates behavioral decisions; and pathways that sustain changes in behavioral state. In addition, we are developing a suite of genetic tools and behavioral assays to probe the nexus between neuronal function and behavior at single-cell resolution. The neuronal connections that allow the brain to integrate sensory and internal state information are established through genetic interactions during development. We aim to identify genes and neurons that are required for functional development of such connections. In vertebrates, neuronal circuits situated in the brainstem form the core of the locomotor control network and are responsible for balance, posture, motor control, and arousal. Accordingly, many neurological disorders stem from abnormal formation or function of brainstem circuits. Insights into the function of brainstem circuits in health and disease have come from genetic manipulation of neurons in zebrafish larvae in combination with computational analysis of behavior.



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Molecular identification of neurons that mediate prepulse inhibition

Startle responses are rapid reflexes that are triggered by sudden sensory stimuli and help animals to defend against and escape from potential threatening stimuli. In both fish and mammals, startle responses are initiated by giant reticulospinal neurons in the medulla, which receive short-latency sensory input from diverse sensory modalities. Although highly stereotyped, startle responses are nevertheless modulated by sensory context and behavioral state and are therefore an excellent system in which to understand how such information is integrated with behavioral selection.

In characterizing how larvae respond to sensory cues, we observed well coordinated escape responses in response to a brief electric field pulse (EFP). The reaction time for EFP responses was remarkably short, leading us to question whether they were initiated by peripheral sensory neurons, as we had suspected. To address this question, we first determined whether the central neurons that drive escape responses are active during EFPs. We expressed the genetically encoded fluorescent calcium reporter GCaMP in reticulospinal neurons in order to monitor firing. As previously described, vibrational stimuli

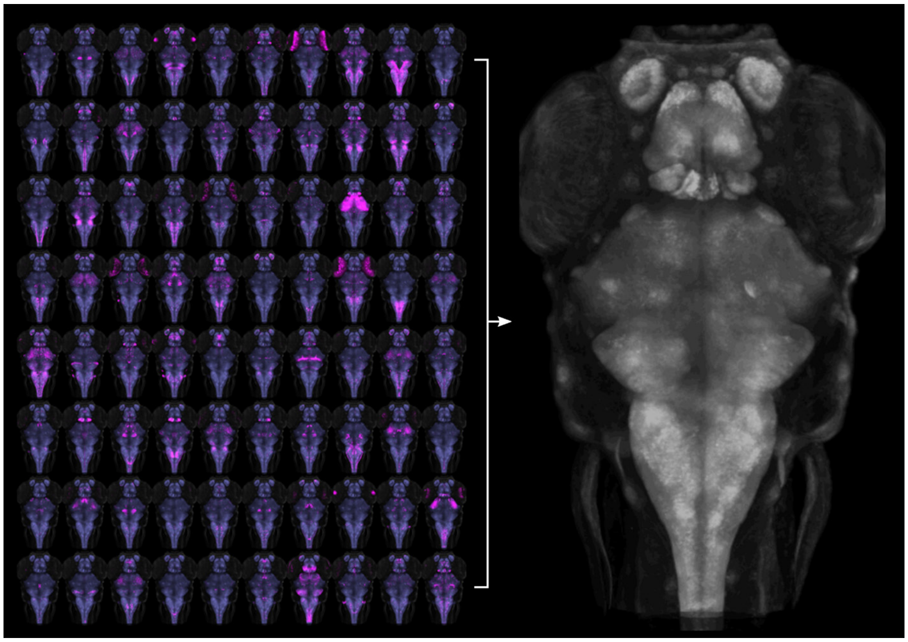

Figure 1. Registration of transgenic lines to a common reference brain
To aid functional neuroanatomical analysis in zebrafish, we scanned 80 *Gal4* transgenic lines (*left, magenta*) at single micron resolution, to characterize cellular level expression patterns. We then computationally registered brain scans to a common reference brain (*right*).

activated a broad network of reticulospinal neurons, including the Mauthner cell, the largest reticulospinal neuron in the larval brain. EFP stimuli also activated the Mauthner cell but failed to drive activity in other reticulospinal neurons. Ablation of the Mauthner cell completely abolished EFP responses, indicating that it is required for this behavior. Then, using genetic and pharmacological techniques, we isolated the Mauthner cell from synaptic input from other neurons, and found that it remained susceptible to activation by EFPs. We therefore proposed that EFPs bypass sensory cells and directly activate the Mauthner cell (Reference 1). This is likely because the Mauthner cell is a command neuron, which can drive an integrated behavioral response and has the largest diameter axon in the nervous system of the fish. Consistent with this, EFPs selectively triggered responses when larvae were aligned such that the longitudinal axis of the Mauthner axon was parallel to the field. Understanding the mechanism by which EFPs trigger escape responses now enables us to probe Mauthner cell excitability non-invasively so that we can measure how different sensory stimuli and behavioral states affect escape responsiveness (Reference 1).

In mammals, including humans, the startle response to a strong auditory stimulus can be inhibited by pre-exposure to a weak acoustic 'prepulse.' This form of startle modulation, termed prepulse inhibition, is diminished in several neurological conditions, including schizophrenia. The precise pattern of neuronal connections that enable prepulse inhibition is not known, yet understanding the mechanism of prepulse inhibition would permit rational investigation of how schizophrenia risk factors influence neuronal circuitry. Vibrational stimuli trigger rapid-escape swims in zebrafish that are mediated by giant reticulospinal neurons similar to the central neurons controlling startle responses in mammals. Escape swims are suppressed by pre-exposure to a prepulse, allowing us to apply the powerful suite of genetic tools in zebrafish to identify neurons that mediate prepulse inhibition.

To identify a transgenic zebrafish line that genetically labels neurons required for prepulse inhibition, we conducted a circuit-breaking screen. In this screen, we first generated a library of neuron-specific Gal4–enhancer trap lines marking distinct populations of neurons in the brain, then ablated the neurons in each enhancer trap line before testing for prepulse inhibition. We identified a transgenic line, *y252*, that labeled a discrete population of neurons in the hindbrain whose ablation or optogenetic inhibition led to dysregulation of prepulse inhibition. Dorsally located in the hindbrain, *y252* neurons sent ventral projections toward the lateral dendrite of the Mauthner cell, the command neuron for escape responses in fish. We analyzed expression of neurotransmitter markers in *y252* neurons and found that the neurons are located within one of four longitudinal columns of glutamatergic neurons extending through the rostro-caudal axis of the hindbrain. Moreover, blockade of NMDA receptors with MK801 phenocopied the effect of *y252* neuron ablation, confirming that glutamatergic signaling is a central part of the mechanism for prepulse inhibition. Next, we discovered that the neurons genetically labeled in *y252* are specified by Gsx1, a transcription factor previously implicated in differentiation of both excitatory and inhibitory interneurons in the spinal cord. Gsx1 was expressed in the mouse brainstem in the proliferative zone that gives rise to regions previously implicated

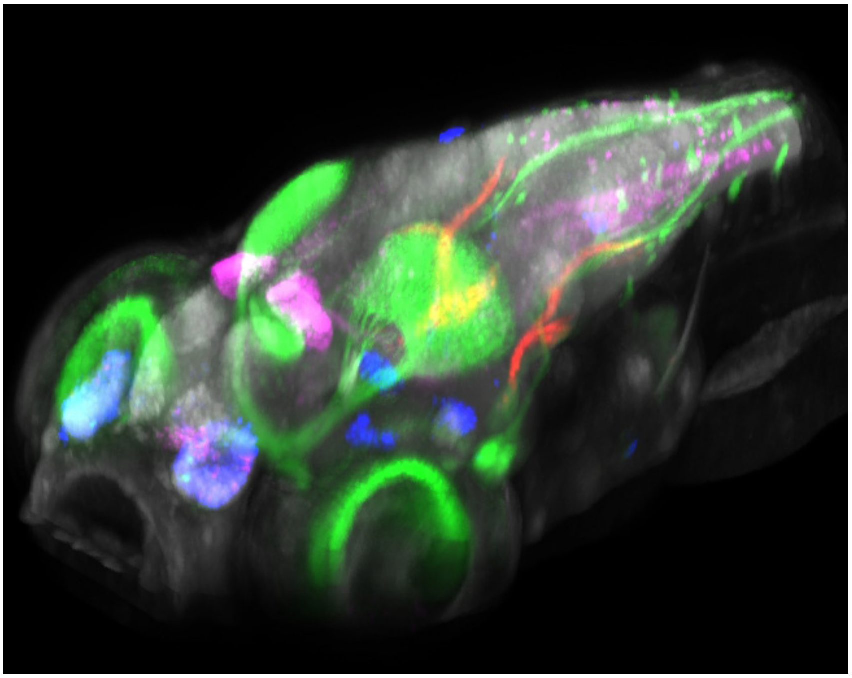

Figure 2. Brain browser software for visualizing expression of transgenes in larval zebrafish
3D projection view of a larval zebrafish, showing the expression of five transgenes: pan-neuronal Cerulean (*grey*), islet2b:GFP (*green*), and enhancer trap lines that label small populations of neurons (*magenta, blue, and red*). The lab imaged more than 100 transgenic lines at high resolution and computationally registered images to a common reference brain, so that expression patterns could be overlaid. The software allows transgenic lines that label selected brain regions to be quickly identified and permits prediction of transgenic lines that co-express in the same cell populations.

in prepulse inhibition. We obtained *Gsx1* knockout mice and performed behavioral testing. Knockout mice showed normal startle sensitivity but a strong reduction in prepulse inhibition. The results thus show that Gsx1 expression defines neurons that are required for prepulse inhibition across vertebrate species. Given that prepulse inhibition is abnormal in neuropsychiatric disorders with developmental origins, including schizophrenia and autism, our work will help identify and probe fundamental defects in circuitry abnormal in these conditions (Reference 2). We have now produced *gsx1*–mutant zebrafish, allowing us to analyze how defects in molecular signaling affect the development of prepulse inhibition circuitry.

Tools for analyzing neuronal circuits that control behavior

We previously described a new method for restricting transgene expression to the nervous system, by incorporating a neuronal-restrictive silencing element. Using this method, we constructed a library of 240 Gal4 enhancer-trap transgenic fish with unique and restricted patterns of neuronal expression. We have now performed high-resolution brain imaging on more than 300 larvae, including 80 of these lines and 29 other transgenic lines. Using software developed for human brain imaging, we aligned the brain scans to a common reference, so that the expression in any of these lines can be compared at cellular level resolution (Figure 1). We then wrote *brain browser* software, which allows users to navigate within the larval zebrafish brain and to identify a transgenic line that labels any selected region (Figure 2). This is a powerful resource for neurobiological studies, as the 109 lines included in the database together provide a way to transgenically target more than 70% of the larval brain. Additionally, the software enables users to identify multiple transgenic lines that target the same cells, which will be useful for future studies using intersectional transgenic methods for targeting small populations of neurons (Reference 3).

To complement these Gal4 lines that genetically target reporter genes to specific groups of neurons, we also built a set of UAS (upstream activation sequence) reporter lines. To optimize expression from our UAS lines, we analyzed DNA sequence features of genes that are highly expressed in zebrafish and developed an algorithm for optimizing transgene expression in zebrafish. We used the method to build strongly expressing UAS reporter lines to monitor and manipulate neurons and we also generated transgenic UAS lines for two novel genetic tools. First, through codon optimization and introduction of amino acid substitutions, we developed a significantly more active version of the bacterial nitroreductase gene (*epNTR*) that is used for cellular ablation (Reference 4). To complement this loss-of-function approach, we also tested overexpression of the voltage-gated sodium channel SCN5 in order to sensitize neurons to synaptic input. After SCN5 expression in motor neurons, larvae showed larger angle escape responses but normal startle sensitivity, and conversely, after SCN5 expression in the Mauthner cell, larvae showed normal escape kinematics but a reduced response threshold (References 1 and 5). Together, the methods enable us to interrogate neuronal cell function *in vivo* and assess their contribution to behavior. In addition, as many useful Gal4 lines

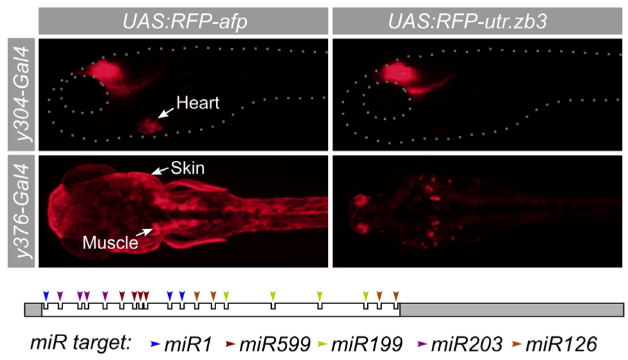

Figure 3. A synthetic 3′ untranslated region that suppresses expression outside the brain

Many *Gal4* transgenic lines show useful expression in brain regions coupled with non-neuronal expression, such as *y304* (heart) and *y376* (muscle and skin). To suppress expression outside the brain, we designed a novel 3′ untranslated region (*utr.zb3*) that contains microRNA target sites for microRNAs expression in muscle, skin, and brain. A UAS (upstream activating sequence) reporter that expresses TagRFPT coupled to the new 3′ untranslated region strongly reduces expression in these tissues (*right panels*).

show expression outside the nervous system, we developed a synthetic 3′ untranslated region incorporating microRNA binding sites that suppress reporter expression in heart, muscle, and skin (Figure 3) (Reference 3).

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PUBLICATIONS

1. Tabor K, Bergeron SA, Horstick EJ, Jordan DC, Aho V, Porkka-Heiskanen T, Haspel G, Burgess HA. Direct activation of the Mauthner cell by electric field pulses drives ultrarapid escape responses. *J Neurophysiol* 2014; 112:834–844.
2. Bergeron SA, Carrier N, Li GH, Ahn S, Burgess HA. Gsx1 expression defines neurons required for prepulse inhibition. *Mol Psychiatry* 2015; 20:974–985.
3. Marquart GD, Tabor KM, Brown M, Strykowski JL, Varshney GK, LaFave MC, Mueller T, Burgess SM, Higashijima I, Burgess HA. A 3D searchable database of transgenic zebrafish Gal4 and Cre lines for functional neuroanatomy studies. *Front Neural Circuits* 2015; 9:78.
4. Horstick EJ, Jordan DC, Bergeron SA, Tabor KM, Serpe M, Feldman B, Burgess HA. Increased functional protein expression using nucleotide sequence features enriched in highly expressed genes in zebrafish. *Nucleic Acids Res* 2015; 43:e48.
5. Horstick EJ, Tabor KM, Jordan DC, Burgess HA. Genetic silencing and selective activation using nitroreductase and TTX insensitive channels. *Methods Mol Biol: Zebrafish* 2015; in press.

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Global Regulation of Gene Expression by ppGpp

Our laboratory identified the structure and central regulatory features in ppGpp and pppGpp over 45 years ago, and we continue studies on these two nucleotide regulators of gene expression, which are analogs of GDP and GTP with a pyrophosphorylated ribose 3′-hydroxyl. The chemically unusual nucleotides are biologically ubiquitous and act as sensors of nutritional and environmental stress for bacteria, archea, plants, and arguably in animal cells. Sometimes called alarmones, they sense a bewildering variety of subtle changes in central metabolism and respond by triggering adaptive responses. Only two sensing mechanisms are well characterized; more are known but remain mechanistically elusive. The first mechanism that activates ppGpp synthetase senses even mild limitations of any charged tRNA species to keep up with the demands of protein synthesis, which serves to survey the metabolic availability of each amino acid, whether limited by *de novo* synthesis, by uptake, or by catabolism. The second known mechanism alters a conformational switch of a bifunctional hydrolase-synthetase enzyme when acylated or when nonacyl carrier protein (ACP) binds to the protein. The switch is thrown when lipids are limiting, which lowers rates of (p)ppGpp hydrolysis and activates (p)ppGpp synthesis leading to net accumulation. The circuit is thought to provide a broad sensor for changes in lipid, membrane physiology, energy metabolism, and possibly carbohydrate metabolism given that lipids are formed from assembly of two carbon units. Generally (p)ppGpp provokes very rapid physiological adjustments that circumvent the consequences of stress. This short-term adaptive cascade ultimately activates systems that ensure long-term survival.

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Regulation by (p)ppGpp is global.

(p)ppGpp can alter about one third of total genomic expression at the level of transcription by interacting directly with RNA polymerase in Gram-negative bacteria. Gram-positive bacteria also regulate transcription with ppGpp, but indirectly by limiting GTP availability. Specific effects are noted as well with enzymes of protein synthesis, DNA synthesis, and intermediary metabolism. Past research from many laboratories, including our own, has focused on binding of ppGpp and pppGpp probably to two sites on RNA polymerase and how binding leads to promoter-specific regulation. The regulatory mechanism is complex and aided by a protein called DksA, which acts in concert with ppGpp to modify the kinetics of promoter initiation rather than classical inducer/repressor DNA sequence recognition. Different patterns of global regulation by ppGpp have emerged in different bacteria, which share abilities to exploit ppGpp to meet various stresses. A generic model is that, during stress, (p)ppGpp inhibits processes associated with luxuriant growth, which become superfluous during starvation, and activates functions that overcome the stress.

Regulation by (p)ppGpp is important.

The research has considerable therapeutic potential and has gained recently in popularity. (p)ppGpp has emerged as necessary for almost all pathogens to survive sources of stress imposed by hosts during infection. Several studies have documented key roles played by (p)ppGpp for enhancing virulence and required for a viable ‘persistence’ or tolerance of antibiotic-sensitive pathogens to the presence of antibiotics that target active metabolism. The most epidemiologically significant among bacterial pathogens is *M. tuberculosis*, whose persistence in carriers is globally epidemiologically significant. Key roles for ppGpp have also become evident for pathogens whose toxins are co-induced with bacteriophage lysogens, such as diphtheria and shigellosis. These features have led to ongoing searches for antibiotics aimed at limiting ppGpp synthesis. In addition, genetic manipulation of ppGpp has provided methods for dissecting out stages of pathogenesis, as defined by their (p)ppGpp dependence.

Our work is enlarging its focus.

We are collaborating to develop systematic approaches to discover new members of the (p)nNpp nucleotide family. Proof of principle of this approach is outlined in a book chapter (Potrykus and Cashel, *Stress and Environmental Control of Gene Expression in Bacteria,* Wiley, 2015;in press). The first example discovered is pGpp, the 5′ GMP derivative (Reference 3). This year, we concluded that biological approaches appear superior to custom structural design efforts to alter substrate specificities for ribosyl-3′ pyrophosphate hydrolysis. A network of functional interactions deduced from synthetic lethal phenotypic behavior was reported last year to include ppGpp, chaperones, and the omega RNA polymerase subunit during normal unstressed growth. This network of interactors during normal growth has now been extended to include the master chaperone GroEsEl, as well as involvement of gene functions that counter oxidative stress.

Characterization of a new regulator, pGpp

The early literature provides fleeting observations of unusual (p)ppGpp-like nucleotides, such as pGpp, ppGp, ppApp, pppAp and A5′p(p_n)p5′Gpp, found in extracts of diverse bacteria when subjected to various sources of stress. We reported (Reference 3) that purified RelQ protein from the firmicute *Enterococcus faecalis* ($RelQ_{EF}$) catalyzes pyrophosphate transfer from ATP to the ribosyl-3′ hydroxyl of GMP without the need for additional supplements typically shown by RSH enzymes (ribosomes with an empty A site, mRNA, and codon-specified uncharged tRNA). The observation with $RelQ_{EF}$ argues against the possibility that pGpp is a degradation product of (p)ppGpp formed by a nudix-like (phosphohydrolase) enzyme. It is notable that the metabolic turnover of pGpp, ppGpp, or pppGpp has not been reported in firmicutes, so there is a formal possibility that pGpp accumulation is regulated at the level of turnover. The kinetic rate constants for pGpp synthesis reveal $RelQ_{EF}$ enzymatic efficiencies (K_{cat}/K_m) within a factor of two of those for forming ppGpp or pppGpp, yet far below activities typical for Rel_{EF}, the RSH protein of *E. faecalis.* Both chemical and enzymatic labilities of pure pGpp (by Rel_{EF}) are similar to those of (p)ppGpp. We compared regulatory activities of pGpp with those known for ppGpp and pppGpp including PRPP–dependent 6-hydroxypurine transport, GMP kinase, transcription initiation of rRNA chains from an *E. coli* promoter, and regulatory effects on (p)ppGpp synthetases. Generally, we found pGpp regulatory activity to be quantitatively but not qualitatively different from that of ppGpp and pppGpp. A central question remains as to whether pGpp has unique regulatory functions that would serve to explain the evolutionary conservation of the enzyme.

Rational approach to understanding the structural basis of (p)ppNpp substrate specificity differences

Last year, we used a synthetic biology approach to obtain enzymes with dramatically different ribosyl-3′pyrophospho-hydrolase specific activities as an aid to find novel nucleotides. We took a designer approach because protein structures are available for two hydrolases, one G-specific and the other active towards A- and G-nucleotides. While in our laboratory, Katarzyna Potrykus verified the observations that the Mesh hydrolase is able to hydrolyze (p)ppGpp and discovered it could also hydrolyze (p) ppApp. Salient structural differences were modeled by Tamara James, while in our laboratory, and predictions were made for changes that might restrict the ability of Mesh to hydrolyze either (p)ppApp or (p)ppGpp. A set of nine mutant proteins were expressed, purified, their catalytic properties assessed, and their effects on catalytic properties used to generate three more mutants. Kinetic constants for hydrolysis were measured by a new assay developed in our laboratory and performed by Nathan Thomas. He found several mutants that lowered the efficiency of catalysis (k_{cat}/k_m) relative to parental enzyme, but the effects applied equally for both substrates. Only two mutants were found to have a substrate preference: a five-fold higher efficiency for (p)ppApp for one and a three-fold preference for (p)ppGpp in the other. The changes may be useful for future modeling but are not strong enough for planned genetic approaches. Katarzyna Potrykus, now our collaborator in Gdansk, Poland, is pursuing these studies, exploring naturally occurring bacterial enzymes. The lack of specificity of the Mesh hydrolase may be sufficient for our purposes. Last year, we reported that expression of (p)ppA/Gpp Mesh hydrolase is toxic for cells unless (p) ppGpp is elevated. Given that (p)ppGpp is not essential for growth, an unknown Mesh substrate appears to be required for growth and may be spared by excess (p)ppGpp titrating out its hydrolysis. This behavior suggests that it is the hydrolase activity itself that is toxic rather than there being nonspecific effects of a toxic protein. Catalytically inactive Mesh proteins encountered among some members of our set of catalytic inactive missense mutants provide appropriate controls to rule out growth inhibition resulting from general protein toxicity.

Functional interaction network between ppGpp regulation, chaperones, the omega subunit of RNA polymerase and oxidative stress during rapid growth in rich media

Last year, we described synthetic lethal phenotypes for different combinations of deletion alleles: (1) the ppGpp synthetase gene (*relA*); (2) chaperones (*dnaK, dnaJ, tig, clpB*), and *rpoZ,* the RNA polymerase omega subunit. Functional interactions can be deduced if the phenotypes for combinations are more severe than the simple sum of the same deletions present singly. In this manner, it is observed that *relA*+, *dnaK,* or *dnaJ* enhance temperature sensitivity, i.e., show conditional synthetic lethality because one deletion functionally enhances the deficiency of the other. This is a plausible relation given that *dnaK* phenotypes are known to be suppressed by DksA, a known partner for ppGpp regulation. Similar interactions can be observed for triple deletions (*relA* and omega with either *tig* or *clpB*) or (*relA*, omega and *groEsEl*), further strengthening the links between ppGpp, chaperones, and the omega portion of the ppGpp–binding site of RNA polymerase. The work has been extended to include complementation assays, showing that transcription factor proteins (GreA and GreB) are able to complement the temperature sensitivity of a *dnaK* deletion. This is significant because GreA and GreB have superimposable protein structure with the DksA protein mentioned earlier, which acts both for ppGpp regulation and complementation of *dnaK.* Importantly, missense mutants of GreA, GreB, or DksA abolish their classical activities in transcription but do not abolish their ability to complement the defective chaperone phenotype of *dnaK.* This suggests that transcription factors have chaperone activities as ‘moonlight’ functions. The dksA mutant also complements the synthetic lethal triple mutant (*relA*, omega and *tig*), which appears to close the loop to include the three major channel factors, which are probably in the same functional network.

We made preliminary discoveries from gene libraries that complement *relA-dnaK* or *relA-dnaJ* synthetic lethality and therefore warrant further exploration. The functions of complementing genes predictably include those known for ppGpp regulation, chaperone, and transcription activity. Surprisingly, other complementing genes were found that mediate responses to oxidative stress, such as *oxyR* and catalases. We discovered over a decade ago that ppGpp regulates an alternative sigma factor (RpoS), which then activates catalases. The interconnections of these functions can be viewed as physiologically plausible, given that ppGpp functions to preclude damage from stress by adjustments to circumvent stress, while chaperone activities renature proteins denatured by stress conditions. However, the observations of synthetic lethality were made with cells growing luxuriantly in rich LB media under no apparent stress; nevertheless these functions appear to prevail as an operative functional stress network. This behavior suggests that there is a balance between these activities during normal growth, a balance that may even be necessary for normal growth. A few years ago, we published evidence that ppGpp is necessary and sufficient to control growth rate in rich as well as poor minimal media. Classically, this feature is usually interpreted in terms of ppGpp regulation of ribosome synthesis. Our current work suggests that other factors may be involved and opens approaches for discovering their identity.

PUBLICATIONS

1. Mechold U, Potrykus K, Murphy H, Murakami KS, Cashel M. Differential regulation by ppGpp versus pppGpp in Escherichia coli. *Nucleic Acids Res* 2013; 41:6175–6189.
2. Vinella D, Potrykus K, Murphy H, Cashel M. Effects on growth by changes of the balance between GreA, GreB and DksA suggest mutual competition and functional redundancy in Escherichia coli. *J Bacteriol* 2012; 194:261–273.
3. Gaca AO, Kudrin P, Colomer-Winter C, Beljantseva J, Liu K, Anderson B, Wang JD, Rejman D, Potrykus K, Cashel M, Hauryliuk V, Lemos JA. From (p)ppGpp to (pp)pGpp: characterization of regulatory effects of pGpp synthesized by the small alarmone synthetase of Enterococcus faecalis. *J Bacteriol* 2015; 197(18):2908–2919.

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Our current studies examine how the posterior lateral line system is built in the zebrafish nervous system. Our goal is to define the genetic regulatory network that coordinates cell fate and morphogenesis in the lateral line system and to build computational models that help us understand how this relatively simple and extremely accessible sensory system in zebrafish builds itself.

The lateral line is a mechanosensory system that detects water flow and consists of sensory organs called neuromasts, which are distributed in a stereotypic pattern over the surface of the zebrafish. Each neuromast has sensory hair cells at its center, which are surrounded by support cells that serve as progenitors for production of more hair cells during growth and regeneration of neuromasts. The development of this superficial sensory system in zebrafish can be easily observed in live embryos with transgenic lines expressing fluorescent proteins in specific subsets of cells of the lateral line system. In addition, a range of genetic and cellular manipulations can be used to investigate gene function.

The function of sensory hair cells in fish neuromasts is remarkably similar to that of hair cells in the vertebrate ear. Furthermore, the gene-regulatory network that determines specification of neuromast hair cells is very similar to the one specifying hair-cell fate in the human ear. Like the hair cells in our ears, neuromast hair cells can be damaged by exposure to drugs such as aminoglycosides, to copper ions, and to noise. However, unlike our ears, in which the loss of hair cells can be permanent, neuromast hair cells have a remarkable ability to regenerate. Hence, the lateral line system serves as an excellent model system for understanding development and for developing strategies to engineer regeneration of sensory hair cells.

The posterior lateral line system is initially established by the posterior lateral line (pLL) primordium, a cluster of about a 100 cells that migrate from the ear to the tip of the tail, periodically depositing neuromasts. Recent studies showed that the mechanisms that determine and guide collective migration and deposition of cells from the pLL primordium are remarkably similar to those that determine the collective migration of metastatic cancer cells. Hence, the lateral line system has also recently emerged as an excellent system for studying the biology of metastatic cancer cells.

Our expectation is that understanding the genetic regulatory network that coordinates cell fate in and morphogenesis of the zebrafish lateral line system will ultimately have a profound impact on translational studies that address a wide range of issues, including the development and regeneration of sensory systems and therapies directed at limiting the spread of cancer through metastasis.

Self-organization of the zebrafish lateral line primordium

Interactions between the Wnt, FGF (fibroblast growth factor), Notch, BMP (bone morphogenetic protein), and chemokine signalling pathways in the pLL primordium provide a framework for understanding how cell fate and morphogenesis are coordinated in this group of about a hundred cells, as they

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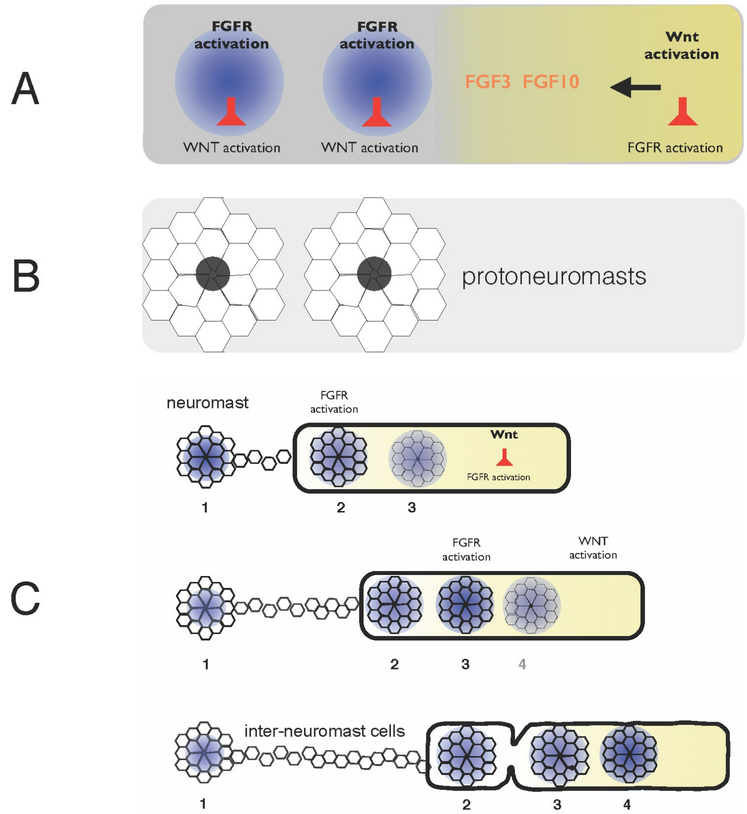

Self-organization of zebrafish lateral line primordium

A. FGFs secreted in response to Wnt activity in a leading zone (*yellow*) activate FGF receptors (FGFRs) in the trailing zone (*blue*). Wnt activity inhibits FGFR activation, and FGFR activation induces expression of a secreted factor that inhibits Wnt activity.

B. Activation of FGFRs coordinates formation of protoneuromasts, in which cells reorganize to form epithelial rosettes, and a central cell (*black*) is specified as a sensory hair-cell precursor.

C. Once about two neuromasts form, the primordium starts migrating. The Wnt zone (*yellow*) progressively shrinks as new protoneuromasts form progressively closer to the leading end. As the Wnt system shrinks, so does the primordium, and neuromasts and interneuromast cells are shed from the trailing end.

collectively migrate under the skin from the ear to the tip of the tail. We used a combination of experiment and computational modeling to define the mechanisms that determine both the periodic formation of neuromasts and their deposition along with inter-neuromast cells, as the pLL primordium migrates along a path defined by chemokine expression.

Wnt proteins initially activate Wnt-beta catenin in a broad leading zone of the primordium. Cells with Wnt activity respond by becoming a source of FGF ligands. At the same time, they express factors that prevent a response to these FGFs. As leading cells with relatively high levels of Wnt activity are prevented from responding to the FGFs, an FGF–responsive center is initially established at the trailing end of the pLL primordium, where Wnt activity is weakest. The activation of FGF receptors in these trailing cells coordinates formation of nascent neuromasts, where cells reorganize to form epithelial rosettes, and a central cell is specified as a sensory hair-cell progenitor. However, the FGF–responsive center also becomes the source of a secreted Wnt antagonist, which progressively restricts the domain of active Wnt activity to the smaller leading zone. The shrinking Wnt system allows the formation of another FGF signaling–dependent 'protoneuromast' in its wake. In this manner, as the Wnt system shrinks, new protoneuromasts are formed, progressively closer to its leading end. After about the first two protoneuromasts form, the primordium starts migrating toward the tail.

As the pLL primordium migrates, cells are deposited from its trailing end, and the pLL primordium progressively shrinks. Cells that were incorporated into protoneuromasts are deposited as neuromasts, while cells that were not effectively incorporated into epithelial rosettes are deposited as interneuromast cells. Interestingly, shrinkage of the pLL primordium correlates with shrinking of the Wnt system, and the length of the Wnt active zone is always roughly 60% of the length of the primordium. While the reason for this correlation remains unclear at this time, knowing the initial size of the Wnt system, and the rate at which it shrinks provides a way to predict the permitted length of the primordium at any point during the course of migration. Trailing cells in a position that exceeds the permitted length of the primordium slow down, stop migrating, and are shed from the trailing end. The rate at which cells leave the migrating primordium depends on the rate at which the Wnt system shrinks and on the cell proliferation rate, as addition of cells adds to the length of the primordium, and hence to the possibility that trailing cells will fall outside the permitted length of the migrating primordium.

To build an agent-based model of lateral line primordium neuromast formation and deposition, we used data on the initial size of the primordium, the rate of Wnt shrinking, the average speed of the primordium, and the fact that the primordium stops migrating and terminates after the Wnt system reaches a critical minimum size. The model now provides a fairly good prediction of the average distance the primordium is expected to migrate and the average number neuromasts it is expected to deposit before it terminates. Though close, the model still slightly overestimates the number of neuromasts deposited and underestimates the distance travelled. Current studies are aimed at identifying deficits in our understanding that might be contributing to the discrepancies.

PUBLICATIONS

1. Dalle Nogare D, Somers K, Rao S, Matsuda M, Reichman-Fried M, Raz E, Chitnis AB. Leading and trailing cells cooperate in collective migration of the zebrafish posterior lateral line primordium. *Development* 2014; 141(16):3188–3196.

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Chromatin Remodeling and Gene Activation

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Aberrant gene regulation is the basis of many disease states. Our main objective is to understand how genes are activated for transcription in the context of chromatin structure. Chromatin is not just a packaging system for DNA in eukaryotic cells but also participates in gene regulation. The structural subunit of chromatin is the nucleosome, which contains nearly two turns of DNA coiled around a central core histone octamer. Nucleosomes are generally quite regularly spaced along the DNA, like beads on a string. Gene regulation involves either attenuation of the inherently repressive properties of nucleosomes to facilitate gene expression or enhancement of those properties to ensure complete repression. These events are choreographed by DNA sequence–specific transcription factors (activators and repressors) and chromatin-remodeling complexes. The latter can be divided into two groups: histone- or DNA–modifying enzymes that implement the 'epigenetic code,' and ATP–dependent remodeling machines that move or displace nucleosomes. We are exploiting the new high-throughput technologies to obtain genome-wide maps of nucleosomes, chromatin-remodeling complexes and RNA polymerase II in budding yeast to determine what happens to nucleosomes on genes when they are activated. We find that transcription results in disruption and loss of some nucleosomes on the gene with re-positioning of the remaining nucleosomes. The current objectives of our yeast studies are to: (1) determine the roles of various chromatin-remodeling complexes (RSC, SWI/SNF, ISW1, ISW2, and CHD1) in chromatin organization and gene expression. They are important because genes encoding subunits of some of these enzymes are often mutated in various cancers and in some developmental diseases; (2) understand the dynamics of transcription by RNA polymerase II through chromatin *in vivo*, with an emphasis on potential regulation of the dissociation of polymerase after transcription.

In addition, through collaborations, we are now extending our studies of chromatin remodeling from yeast to mouse. Mammalian systems are much more complex and challenging than yeast. We are investigating the effects of dexamethasone on mouse cell chromatin in a collaboration with the Hager lab. We are also comparing the chromatin structures of neurons, oligodendrocytes, and astrocytes in a collaboration with the Fields lab.

Disruption of nucleosomes during heavy transcription

The histone octamer contains an H3-H4 tetramer and two H2A-H2B dimers. Gene activation is associated with chromatin disruption: a wider nucleosome-depleted region (NDR) at the promoter and reduced nucleosome occupancy over the coding region. We examined the nature of disrupted chromatin after induction, using MNase-seq to map nucleosomes and sub-nucleosomes and a refined high-resolution ChIP-seq method to map histones H4 and H2B and RNA polymerase II (Pol II) genome-wide. Over coding regions, induced genes show a differential loss of H2B relative to H4, which correlates with Pol II density and the appearance of sub-nucleosomes. After induction, Pol II is surprisingly low at the promoter, but accumulates on the gene and downstream of the termination site, implying that dissociation is slow. Thus, induction-dependent chromatin disruption reflects both eviction of H2A-H2B dimers from nucleosomes and the presence of queued Pol II elongation

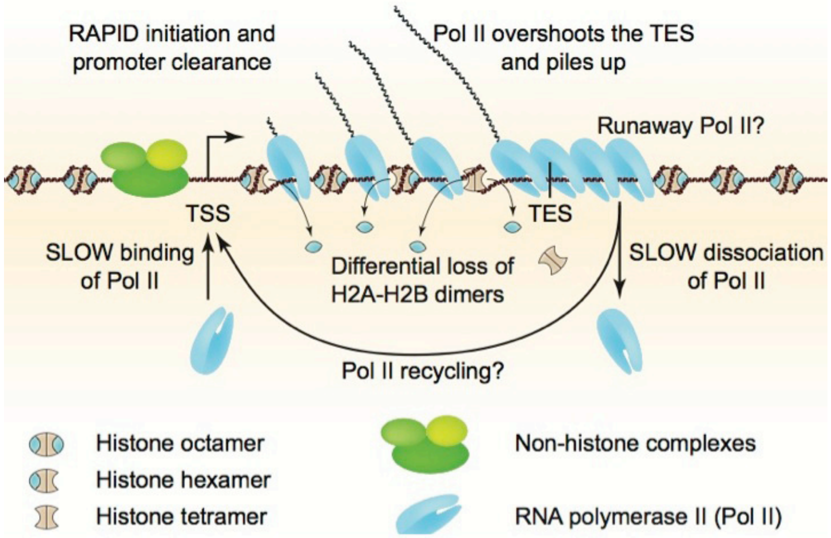

Figure 1. Chromatin structure of a heavily transcribed gene

Model: RNA polymerase II (Pol II) binds near the transcription start site (TSS) and clears the promoter rapidly. Pol II levels over the remainder of the gene are high and correlate with some nucleosome displacement and some loss of histone H2A-H2B dimers, resulting in sub-nucleosomal particles (tetrasomes and hexasomes). Pol II overshoots the transcript end site (TES) and accumulates downstream owing to slow dissociation from DNA, resulting in a queue of polymerases behind it. Slow dissociation might facilitate transfer of Pol II back to the promoter ('recycling'), thereby increasing the transcription rate.

complexes (Figure 1). We propose that slow Pol II dissociation after transcription is a major factor in chromatin disruption and that it may be of critical importance in gene regulation (Reference 1), a hypothesis that we are currently testing.

Novel nucleosomal particles containing core histones and linker DNA but no histone H1

Eukaryotic chromosomal DNA is assembled into regularly spaced nucleosomes, which play a central role in gene regulation by determining accessibility of control regions. The nucleosome contains about 147 bp of DNA wrapped approximately 1.7 times around the core histone octamer. The linker histone H1 binds both to the nucleosome, sealing the DNA coils, and to the linker DNA between nucleosomes, directing chromatin folding. Micrococcal nuclease (MNase) digests the linker to yield the chromatosome, containing H1 and about 160 bp, and then converts it to a core particle, containing about 147 bp and no H1. Sequencing of nucleosomal DNA obtained after MNase digestion (MNase-seq) generates genome-wide nucleosome maps that are important for understanding gene regulation. We developed an improved MNase-seq method involving simultaneous digestion with exonuclease III, which removes linker DNA. Remarkably, we discovered two novel intermediate particles containing 154 or 161 bp, corresponding to 7 bp protruding from one or both sides of the nucleosome core (Reference 2). The particles are detected in yeast lacking H1 and in H1–depleted mouse chromatin. They can be reconstituted *in vitro* using purified core histones and DNA. We propose that these "proto-chromatosomes" are fundamental chromatin subunits, which include the H1 binding site and influence nucleosome spacing independently of H1 (Figure 2).

The ISW1 and CHD1 ATP–dependent chromatin remodelers compete to set nucleosome spacing *in vivo*.

ATP–dependent chromatin remodeling machines play a central role in gene regulation by manipulating chromatin structure. Most genes have a nucleosome-depleted region at the promoter and an array of regularly spaced nucleosomes phased relative to the transcription start site. The three nucleosome spacing enzymes in yeast (CHD1, ISW1, and ISW2) catalyze the formation of arrays with different average spacing *in vitro* (around 160, 175, and 200 bp, respectively). We used MNase-seq to show that nucleosome spacing *in vivo* reflects competition between CHD1 and ISW1 to set the spacing on individual genes, such that CHD1 dominates genes with shorter spacing and ISW1 dominates genes with longer spacing. ISW2 plays a minor role limited to inactive genes. Active genes exhibit extreme spacing, both very short and very long. H1 binding increases with spacing, suggesting that arrays with shorter spacing, created by CHD1, may exclude H1 because the linker is too short for tight binding (Ocampo J, Chereji RV, Eriksson P, Clark DJ, manuscript submitted).

Genome-wide cooperation by HAT Gcn5, remodeler SWI/SNF, and chaperone Ydj1 in promoter nucleosome eviction and transcriptional activation

As mentioned above, gene activation generally involves nucleosome removal and/or nucleosome shifts in the promoter region.

The process of nucleosome assembly and disassembly at promoters involves histone chaperones, chromatin-remodeling complexes, and histone acetyltransferases. We investigated whether these co-factors function ubiquitously and what effect nucleosome eviction has on transcription genome-wide. We performed chromatin immunoprecipitation (ChIP-seq) experiments to detect histone H3 and Pol II in mutants lacking single or multiple co-factors (the SWI/SNF remodeling complex, the Gcn5 histone acetyltransferase, and the Ydj1 histone chaperone). We focused on about 200 genes regulated by the transcription activator Gcn4. After induction, 70 of these genes show significantly reduced H3 occupancy at their promoters. All three co-factors examined play a role in H3 eviction at most, but not all, Gcn4 target promoters, with Gcn5 playing the greatest role and Ydj1 the least. In fact, the three co-factors cooperate to various extents in H3 eviction at virtually all yeast promoters. Reduced H3 eviction in co-factor mutants is correlated with reduced Pol II occupancy on Gcn4–regulated genes and on the most highly expressed constitutive genes, but the relative Pol II levels at most genes are unaffected. Our observations indicate that promoter nucleosome eviction is important for heavy transcription of highly expressed genes, but that other steps in gene activation are more important for most other yeast genes (Qiu H, Hu C, Chereji R, Cole HA, Rawal Y, Clark DJ, Hinnebusch AG, manuscript submitted).

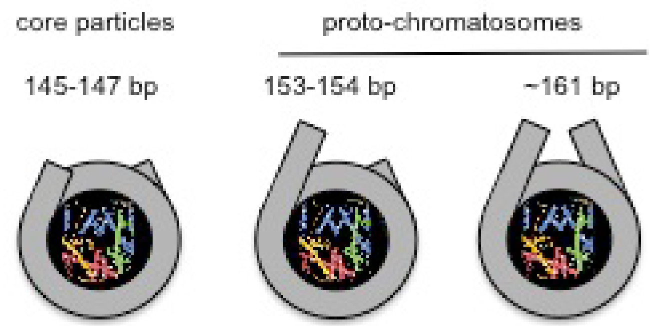

Figure 2. Proto-chromatosomes form the scaffold for linker histone binding.

Views of the nucleosome core particle (147 bp) and proto-chromatosomes from above, drawn approximately to scale, based on the nucleosome crystal structure. The final 10 bp on each side of the nucleosome core are almost straight, projecting a short distance out of the particle. Proto-chromatosomes are shown with an extra 7 bp on one side (154 bp) or both sides (161 bp) with a continuing straight trajectory. Linker histone (H1) binds to the 161-bp proto-chromatosome, interacting with the protruding DNA on both sides and with the central turn of DNA within the nucleosome.

Chromatin remodeling in neurons and oligodendroglia

The aim of this project, which is being conduction in collaboration with Douglas Fields and Philip Lee, is to determine the relationship between chromatin organization and gene expression in dorsal root ganglion neurons and oligodendroglial cells. Neuronal chromatin is remarkably atypical: the linker DNA between nucleosomes is much shorter than in other cell types, including oligodendroglial cells, and neurons are deficient in histone H1. The global organization of neuronal chromatin is therefore very different from other cells, but the significance of this organization is unknown. We are testing the relationship between global chromatin organization and transcription. We are mapping nucleosomes genome-wide in isolated mouse neurons and in oligodendroglial cells, using paired-end sequencing, and are measuring gene expression by RNAseq. We will correlate their chromatin structures with transcriptional activity. The next step is to address the roles of ATP–dependent chromatin remodelers in neuronal and oligodendroglial chromatin structure. The experiments should provide insight into why neuronal chromatin organization is so different from other cells, including glia.

PUBLICATIONS

1. Cole HA, Ocampo J, Iben JR, Chereji RV, Clark DJ. Heavy transcription of yeast genes correlates with differential loss of histone H2B relative to H4 and queued RNA polymerases. *Nucleic Acids Res* 2014; 42:12512–12522.
2. Cole HA, Cui F, Ocampo J, Burke TL, Nikitina T, Nagarajavel V, Kotomura N, Zhurkin V, Clark DJ. Novel nucleosomal particles containing core histones and linker DNA but no histone H1. *Nucleic Acids Res* 2016; 44:573–81.

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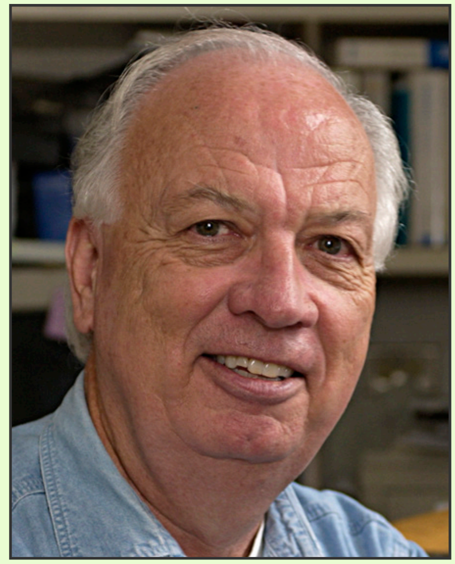
Physiological, Biochemical, and Molecular-Genetic Events Governing the Recognition and Resolution of RNA/DNA Hybrids

Damaged DNA is one of the leading causes of many human diseases and disorders. We study the formation and resolution of RNA/DNA hybrids, which occur during DNA and RNA synthesis. Such hybrid molecules may lead to increased DNA damage but may also play critical roles in normal cellular processes. We are interested in how RNA/DNA hybrids are resolved and in the role that ribonucleases H (RNases H) play in their elimination (Reference 1). Two classes of RNases H, Class I and Class II, are present in most organisms. Our studies have shown that mice deleted for the *Rnaseh1* gene arrest embryonic development at day 8.5 as a result of failure to amplify mitochondrial DNA. Others have found that the Aicardi-Goutières Syndrome (AGS), a severe neurological disorder with symptoms appearing at or soon after birth, can be caused by defective human RNase H2. We are examining mouse models of AGS to gain insight into the human disorder. We employ molecular-genetic and biochemical tools in yeast and mouse models in our research to understand the mechanisms and functions of RNases H and their associated substrates.



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There are several types of RNA/DNA hybrids structures formed and processed differently. Simple RNA/DNA hybrids consist of one strand of RNA paired with one strand of DNA. The HIV-AIDS reverse transcriptase (RT) forms such hybrids when copying its genomic RNA into DNA. The RT also has an RNase H domain that is structurally and functionally similar to the class I cellular RNase H and is necessary for several steps of viral DNA synthesis. R-loop hybrids have two separated DNA strands, with one hybridized to RNA while the other is in single-stranded form. These structures sometimes form during transcription and can lead to chromosomal breakage. However, they are also part of the normal recombination process of switching (recombination) from one form of immunoglobulin to another, resulting in different isoforms of antibodies. Another form of hybrid are single or multiple ribonucleotides incorporated into DNA during replication (Reference 2). The first two types of hybrid are substrates for class I and II RNases H. The third is uniquely recognized by type 2 RNases H.

Contrasts between Class I and Class II RNases H

Many of our investigations over the past few years have focused on RNase H1. RNase H1 recognizes the 2′-OH of four consecutive ribonucleotides while the DNA strand is distorted to fit into a pocket of the enzyme. Thus, the enzyme requires more than one ribonucleotide for cleavage of RNA in RNA/DNA hybrids. RNases H1 consist of a single polypeptide in both eukaryotes and prokaryotes. In contrast, RNase H2 is a complex of three distinct polypeptides in eukaryotes but is a single polypeptide in prokaryotes. The catalytic subunit of the heterotrimeric RNase H2 of eukaryotes is similar in its primary amino acid sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave a single ribonucleotide or the transition from the ribonucleotide in the case of RNA–primed DNA synthesis (e.g., rrrr*rD*DDD in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide).

Dual activities of RNase H2 and Aicardi-Goutières syndrome

Eukaryotic RNases H2 recognize and resolve RNA hybridized or covalently attached to DNA—two chemically distinct structures—using the same catalytic mechanism of hydrolysis. RNase H2 mutations that reduce catalytic activity, or fail to properly interact with *in vivo* substrates, cause Aicardi-Goutières syndrome (AGS). Mutations in seven genes are known to cause AGS, with more than 50% of AGS patients having mutations in any of the three subunits of RNase H2. We previously expressed (in *Escherichia coli)* and purified human RNases H2 with mutations corresponding to several of those seen in AGS patients. We found only one with significant loss of RNase H2 activity. Using the structure we determined, we can locate all known mutations in RNase H2 that cause AGS, and the mutation with low RNase H2 activity is located near the catalytic center of the enzyme. The wide distribution of the mutations suggests that modest changes in stability, interaction with other unknown proteins, and loss of catalysis can all cause AGS.

We are developing mouse models of AGS to clarify which defects are associated with each RNase H2 activity. Mice bearing the RNase H2A G37S mutation in homozygous form are perinatal lethal, i.e., either dead at birth or die within a few hours of birth. Mutations in another gene, *TREX1*, also cause AGS, and it has been shown that homozygous knockout (KO) mice are viable but die after a few weeks owing to a cardiomyopathy that can be prevented by either blocking an innate or adaptive immune response. In contrast, the G37S-mutant perinatal lethality and the fact that RNase H2 KO mice die early in embryogenesis suggest a more severe defect than that seen in *TREX1*-KO mice. We attempted to rescue the perinatal phenotype by eliminating one part of the innate immune pathway or by completely inactivating the adaptive immune response. These mice are no different than the innate or adaptive competent mice, suggesting the possibility of a different issue between the *TREX1*– and RNase H2–mutant mice. However, the expression of several interferon-stimulated genes (ISGs) is elevated in mouse embryonic fibroblasts (MEFs) derived from G37S homozygous embryos, supporting a role for innate immunity the AGS phenotype. Damaged DNA that finds its way to the cytoplasm can be sensed by the cGAS protein producing the small molecule cGAMP, which interacts with the Sting protein, an important protein for the DNA–sensing innate immune pathway. Mice that are homozygous for G37S and deleted for the *Sting* gene are mostly perinatal lethal but no longer exhibit increases in ISGs. Interestingly, a small fraction of the double G37S–*Sting* KO are viable, indicating only limited involvement of ISGs in perinatal lethality. Further studies are under way, which we expect will lead us to the cause of lethality.

To distinguish the defects that persistent RNA/DNA hybrids and single ribonucleotides joined to DNA caused *in vivo*, we modified RNase H2 to make an enzyme that could only cleave one type of substrate. Based on a rational design comparing the structures of RNases H2 and H3, we unlinked the two activities to yield an enzyme that processes RNA/DNA, but leaves single ribonucleoside monophosphates (rNMPs) attached to DNA. RNases H2 and H3 have similar 3D structures, but RNase H3 does not cleave single rNMPs in DNA. We first examined *in vitro* activities of our new RNase H2 mutant using, among others, the ribonucleotide excision repair (RER) assay recently developed by our collaborators Peter Burgers and Justin Sparks (Reference 2). The *in vitro* results show complete lack of removal of single ribonucleotides but only modest reduction in hydrolysis of RNA/DNA hybrids. *In vivo*, our RNase H2 mutant gave the signature 2–5 bp deletions in the yeast *CAN1* gene associated with incorporation of rNMP into DNA in the absence of RNase H2. The mutant enzyme, which we call RED (Ribonucleotide Excision Deficient) resolves RNA/DNA hybrids, which are substrates of both RNase H1 and RNase H2. However, our RNase H2^{RED} identified a unique set of hybrids that formed when homologous recombination is defective, owing to loss of SGS1 helicase, and that can only be processed by RNase H2, most likely because it has special access via contacts with other cell components. Thus, the synthetic defect observed in *sgs1D*, *rnh201D* strains results from problems associated with persistent R-loops. Given that our RNase H2 mutant enzyme resolves these R-loops, the synthetic defect is absent when the RNase H2^{RED} is present in a strain deleted for *SGS1* (Reference 3).

The RNaseh2a G37S mutation, which is found in a few AGS patients, results in limited activity *in vitro*. We investigated how the corresponding mutation in yeast RNase H2 (G42S) protein functions. *In vitro* it had very poor activity on RNA/DNA hybrids and on cleavage of single rNMPs in DNA. *In vivo* it was almost as defective as a deletion of an RNase H2 subunit for removing single rNMPs (measured by 2–5 bp deletions in the CAN1 gene), had limited activity in removing R-loops accessible to either RNase H2 or H1, yet displayed remarkably good activity when it interacted with other proteins involved in R-loops associated with DNA replication/repair (Reference 3).

RNase H2 activity is not required in the yeast *Saccharomyces cerevisiae*, but in mouse, and presumably in humans, Rnaseh2A-, 2B- or 2C-null mutations are lethal, with mouse embryonic development arresting at E8.5. The mouse strain carrying the *Rnaseh2a*–G37S described above lives longer than the *RNaseh2*-null mice and retains reduced RNA/DNA hybrid–degradative and RER activities. To examine an RNase H2^{RED} mouse strain, we recently developed mice that are defective in initiating removal of rNMPs in DNA and are examining the effects of this mutation on viability and for other possible defects.

Steric gate in pol eta limits incorporation of rNMPs during DNA replication.

DNA polymerases are able to limit incorporation of ribonucleotides into DNA by sterically limiting binding of the closely related ribonucleotide triphosphates (rNTPs). However, the cellular concentrations of rNTPs are substantially greater than dNTPs, and it is now clear that all three replicative DNA polymerases actually synthesize DNA containing rNMPs. Together with the Woodgate lab (Reference 4), we examined DNA polymerase eta (pol eta), the DNA polymerase important for repair of UV–damaged DNA. Based on other DNA pol steric-gate binding sites, we identified the residue Phe35 as a candidate amino acid limiting incorporation of rNMPs in DNA, for discrimination between rNTPs and dNTPs. Substitution of an Ala for Phe35 opened the active site, allowing incorporation rNMPs. Employing the mutation F35A in pol eta together with the mutated RNase H2^{RED}, we were able to show that rNMPs are incorporated into DNA generating 2–5 bp deletions in short repetitive sequences characteristically found in RNase H2-null mutations. The defect in RNase H2^{RED} permits us to conclude that the mutations are the result of single rNMPs in DNA but not of RNA/DNA hybrids because RNase H2^{RED} retains the ability to hydrolyze RNA of RNA/DNA hybrids. Interestingly, we detected another, new type of mutation in which a deletion of 1 basepair in a run of seven Ts is much more prevalent than the 2–5 bp deletions. In addition, the overall mutation frequency is lower in the pol eta wild-type strain without active RNase H2. We believe that this is a consequence of recognition of single rNMPs in DNA by the mismatch repair pathway, providing another opportunity to repair any mistakes during UV repair by pol eta.

Mitochondrial DNA and RNase H1 in the mouse

During embryonic development, RNase H1 is required for progress beyond day E8.5. A single transcript of the mouse *Rnaseh1* gene is translated to make two nearly identical proteins, one localizing to the nucleus and the other to the mitochondrion. We previously showed that the *Rnaseh1*–deleted embryos fail to amplify mtDNA (mitochondrial DNA), causing developmental arrest. Nuclear DNA replicated normally in *Rnase H1*–KO embryos. We are examining loss of the *Rnaseh1* gene during B-cell development to follow the process of RNase H1 depletion in a simpler system. Following conditional deletion of *Rnaseh1* at an early stage of B-cell development in mouse B-cells, we found that resting, naive B-cells are formed but that they are unable to become activated to class-switch to other isotypes (e.g., IgG), and that sera from these mice have a major deficit in antibodies. We are currently determining whether the loss of mitochondrial DNA is the explanation for the inability of the resting B-cells to be completely activated, as well as what changes in mRNA levels are different between the conditional KO and WT *Rnaseh1* genes.

J. Brad Holmes, a former graduate student in the NIH-Cambridge graduate student program co-mentored by myself and Ian Holt, examined the roles of RNase H1 in mitochondrial DNA replication by using, among other techniques, analysis of intermediates on two-dimensional gels. Our findings thus far indicate that elevated expression of RNase H1 in mitochondria alters mtDNA replication. In addition, data obtained by Holmes, supporting the existence of RNA/DNA hybrids as replication intermediates, indicate that RNase H1 is important for the removal of RNA primers of mtDNA replication. We concluded that the absence of RNase H1 activity results in accumulation of RNA from the Light Strand Promoter (LSP) fused to DNA, a result that clearly indicates that a transcript from LSP serves to initiate DNA replication of the "H" strand oriH (Reference 5).

ADDITIONAL FUNDING

» Scientific Director's Intramural Award

PUBLICATIONS

1. Cerritelli SM, Crouch RJ. Ribonuclease H: the enzymes from eukaryotes. *FEBS J* 2009; 276:1494–1505.
2. Sparks JL, Chon H, Cerritelli SM, Kunkel TA, Johansson E, Crouch RJ, Burgers PM. RNase H2-initiated ribonucleotide excision repair. *Mol Cell* 2012; 47:980–986.
3. Chon H, Spark JL, Rychlik M, Nowotny M, Burgers PM, Crouch RJ, Cerritelli SM. RNase H2 roles in genome integrity revealed by unlinking its activities. *Nucleic Acids Res* 2013; 41:3130–3143.
4. Donigan KA, Cerritelli SM, McDonald JP, Vaisman A, Crouch RJ, Woodgate R. Unlocking the steric gate of DNA polymerase η leads to increased genomic instability in Saccharomyces cerevisiae. *DNA Repair* 2015; 35:1–12.

5. Holmes JB, Akman G, Wood SR, Sakhuja K, Cerritelli SM, Moss C, Bowmaker MR, Jacobs HT, Crouch RJ, Holt IJ. Primer retention owing to the absence of RNase H1 is catastrophic for mitochondrial DNA replication. *Proc Natl Acad Sci USA* 2015; 112:9334–9339.

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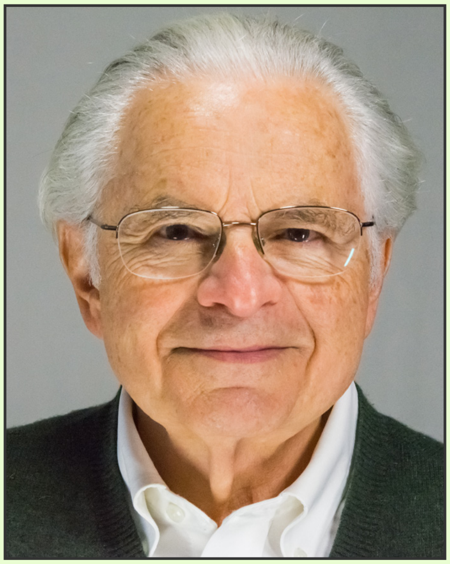
The laboratory uses the frog *Xenopus laevis* and the zebrafish *Danio rerio* as experimental systems in which to study molecular-genetic mechanisms of early vertebrate development. Recently, we focused on mechanisms of neural crest specification, axon guidance, cell-cell adhesion, and targeted gene disruption.



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The BTB domain protein Kctd15 is an inhibitor of neural crest formation and of the transcription factor AP-2.

Our laboratory has a long-standing interest in the formation of the neural crest (NC), a group of cells with stem-cell properties that arise at the dorsal neural tube and migrate to many locations in the embryo to give rise to a large number of varied differentiated derivatives. A recent focus has been the role of the BTB domain–containing protein Kctd15, which is capable of inhibiting NC formation. In pursuing the molecular mechanism of Kctd15 action, we found that Kctd15 regulates the activity of transcription factor AP-2. AP-2 is known to be a key factor in the induction and differentiation of NC cells. We found that AP-2 and Kctd15 can interact when co-expressed in cultured cells. Further, Kctd15 is a highly effective inhibitor of AP-2 activity in a reporter assay (Figure 1). In studying the mechanism of inhibition of AP-2 by Kctd15, we found that Kctd15 interacts with the activation domain of AP-2. We further analyzed this system using a fusion product between the Gal4 DNA–binding domain and the AP-2–activation domain. Within the latter domain, a conserved proline-rich motif proved critical for Kctd15 interaction: mutation of proline 59 to alanine (P59A) in the Gal4-AP-2 fusion resulted in a protein that was active but could not bind and was insensitive to inhibition by Kctd15 (Figure 2). This proline residue was also essential for Kctd15 sensitivity in the context of the full-length AP-2 molecule. Thus, we conclude that Kctd15 inhibits AP-2 by binding a to specific site in its activation domain (Reference 1).

Lnx2 ubiquitin ligase is essential for exocrine cell differentiation in the early zebrafish pancreas.

Pancreas development is of great interest as a prime example of organogenesis and because of the importance of understanding the development of insulin-producing cells and regulation of insulin synthesis. We found that the E3 ubiquitin ligases Lnx2a and Lnx2b have an important role in early pancreas differentiation in the zebrafish. While mammals have just one *Lnx2* gene, zebrafish have two closely related paralogs, *lnx2a* and *lnx2b*. In studying the gene that encodes Lnx2a, we found that it is expressed in the ventral pancreatic bud, in addition to being expressed in the nervous system (Figure 3). In the early zebrafish embryo, the ventral bud gives rise to exocrine cells, and the dorsal bud gives rise to the primary islet, which contains endocrine cells. In later development, ventral bud progenitors give rise to secondary islets and duct cells in addition to exocrine cells. We found that a splice morpholino (MO) that blocks *lnx2a* exon2/3 splicing inhibits exocrine marker expression while leaving endocrine marker expression unaffected (Figure 4). To test MO specificity, we used TALEN–mediated gene targeting to generate a frame shift mutation, *lnx2a*$^{\Delta 70}$, that does not produce Lnx2a protein. The mutant fish proved to be phenotypically wild type (WT), with normal exocrine gene expression (Figure 5A–E). Further analysis showed that the splice MO led to

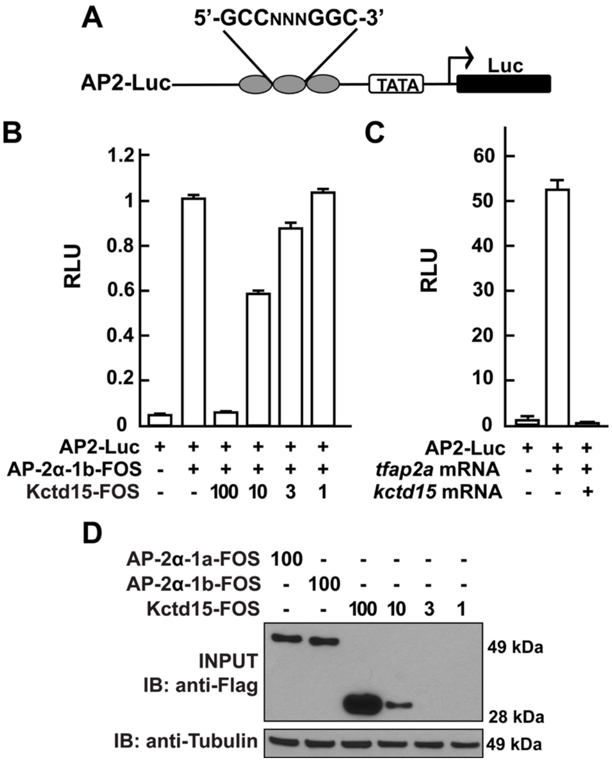


Figure 1. Kcdt15 represses AP-2 function.

A. AP2-Luc reporter, containing three AP-2 consensus sites driving expression of luciferase (Luc).
B. The reporter was strongly stimulated by zebrafish AP-2α and dramatically inhibited by zebrafish Kctd15 (levels indicated in ng). RLU, relative light units.
C. Reporter activity in zebrafish embryos.
D. Cells were transfected with FOS–tagged zebrafish AP-2α and Kctd15, and lysates were blotted to assay expression of both proteins. From Zarelli and Dawid, 2013 (Reference 1).

exon skipping, generating an mRNA that produces a protein shorter than the WT (Figure 5F–H). The protein was shown by mass spectrometry to be an N-truncated version of the WT protein that had lost the catalytically active RING domain. We wondered whether the truncated protein could act as an interfering factor and found this to be the case, as injection of RNA encoding the truncated protein into *lnx2a*$^{\Delta 70}$ embryos reproduced the exocrine deficiency phenotype (Figure 5I,J). The effect is the result of inhibition of Lnx2b function by the truncated Lnx2a, as indicated by the fact that injection of *lnx2b*-MO into *lnx2a*$^{\Delta 70}$ reproduced the exocrine deficiency phenotype (Figure 5D,E). Definitive genetic evidence for our interpretation of the mechanism of the *lnx2a*-splice MO phenotype came from a mutation, *lnx2a*$^{\Delta 329}$, that deletes the exon/intron boundary targeted by the splice MO. *lnx2a*$^{\Delta 329}$-mutant fish effectively reproduce the exocrine deficiency phenotype seen originally in *lnx2a*-splice MO–injected embryos (Figure 6).

The most widely studied role of the ubiquitin pathway and E3 ubiquitin ligases is the regulation of protein turnover. Numb is known as a target of mammalian Lnx proteins, and we confirmed that zebrafish Lnx2a can mediate the ubiquitination and degradation of Numb. Numb is a known inhibitor of the Notch signaling pathway, and Notch is an important factor in early pancreas formation. Thus we hypothesized that depletion of Lnx2a and Lnxb leads to stabilization of Numb when it normally should be turned over, resulting in inhibition of Notch and, through this, to a defect in pancreas cell differentiation. This view is strongly supported by the fact that a Numb-MO can rescue the loss of endocrine marker expression in *lnx2a*-splice MO–injected embryos and in *lnx2a*$^{\Delta 329}$-mutant fish (Figure 7).

Previous work by several laboratories may be summarized in our context by the following statements: (1) Notch signaling is required in pancreatic cell differentiation; (2) high Notch activity maintains precursor pools, lower activity allows differentiation, in certain cases preceded by proliferation; (3) cells that downregulate Notch cannot maintain their precursor status. Our results led us to the following model for the role of Lnx2 proteins in pancreas development in zebrafish. We suggest that Lnx2a, together with Lnx2b, destabilizes Numb in ventral bud–derived cells, allowing Notch activity, which may be required in the specification and expansion of precursor cells. Loss of Lnx2 activity in the *lnx2a*$^{\Delta 329}$ mutant or *lnx2a* morphant stabilizes Numb to inhibit Notch in cells where it is normally active, ultimately interfering with the normal developmental progression of these cells. We suggest that, in normal development, the regulation of Numb protein stability by Lnx2a/b is an important component of the system that controls the levels of Notch activity during pancreas formation and the assignment of cells to different pancreatic lineages (Reference 5).

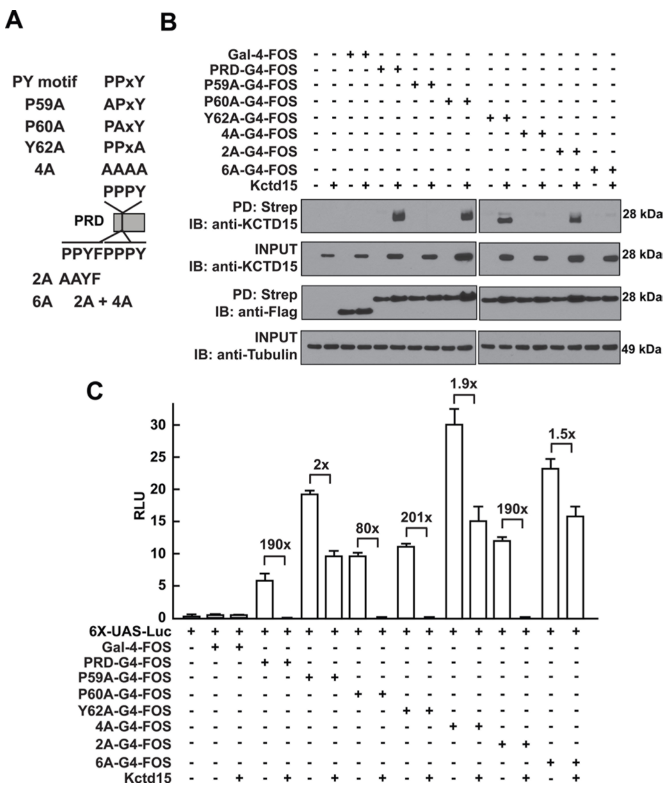


Figure 2. Critical role of P59 for Kctd15 interaction and inhibition of AP-2

A. Mutants in the conserved PPxY motif within the AP-2α proline-rich domain (PRD) and two additional mutants in the adjoining region are shown. PRDs were fused to the Gal4-DNA–binding domain and the FOS tag.

B. Kctd15 interaction depends on P59. WT and mutant fusion constructs were expressed in HEK293T cells alone or with Kctd15, and complexes were pulled down using a Strep-Tactin matrix. PRDs could bind to Kctd15 except in the constructs containing the P59A mutation (P59A, 4A and 6A). The constructs were expressed at similar levels.

C. PPxY mutants activate the UAS-Luc reporter two-to-five fold more highly than does WT PRD-Gal4. PRD-Gal4 WT and P60A, Y62A, and 2A were dramatically inhibited by zebrafish Kctd15, whereas P59A and mutants containing this change were inhibited two-fold or less. Figure from Zarelli and Dawid, 2013 (Reference 1).

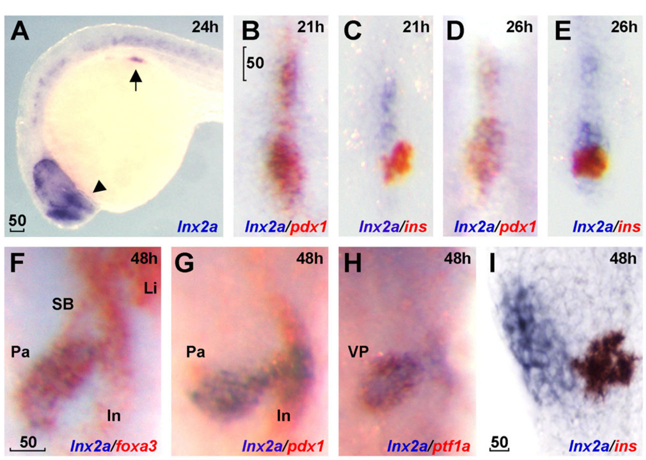
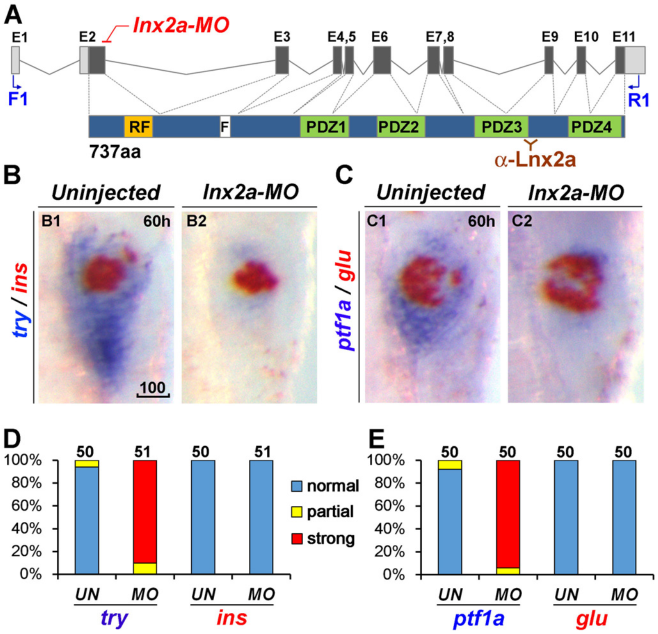


Figure 3. *lnx2a* is expressed in the ventral pancreas during early pancreas specification.

Lateral views (A and I), dorsal views (B–E), and ventral views (F–H). A. *lnx2a* transcripts (*blue*) are detected in regions of the endoderm (*arrow*), forebrain (*arrowhead*) and the spinal cord at 24 (hours post fertilization) (hpf). Expression of *pdx1* (B, D, and G), *ins* (C, E, and I) and *foxa3* (F) are in red. *lnx2a* expression is observed in the antero-ventral part of *pdx1*-positive pancreas precursors at 21 (B), and 26 (D) hpf, but excluded from b-cells (*ins*⁺) in the dorsal pancreas at 21 (C), 26 (E), and 48 (*I*) hpf. At 48 hpf, *lnx2a* expression is detected in the ventral pancreas but not in the intestine, swim bladder, or liver (F–H). Li, Liver; In, Intestine; Pa, Pancreas; SB, Swim bladder; VP, Ventral pancreas; Scale bar, 50μm.Dawid, 2013 (Reference 1).



◀ Figure 4. *lnx2a* knock-down causes defects in the exocrine pancreas.

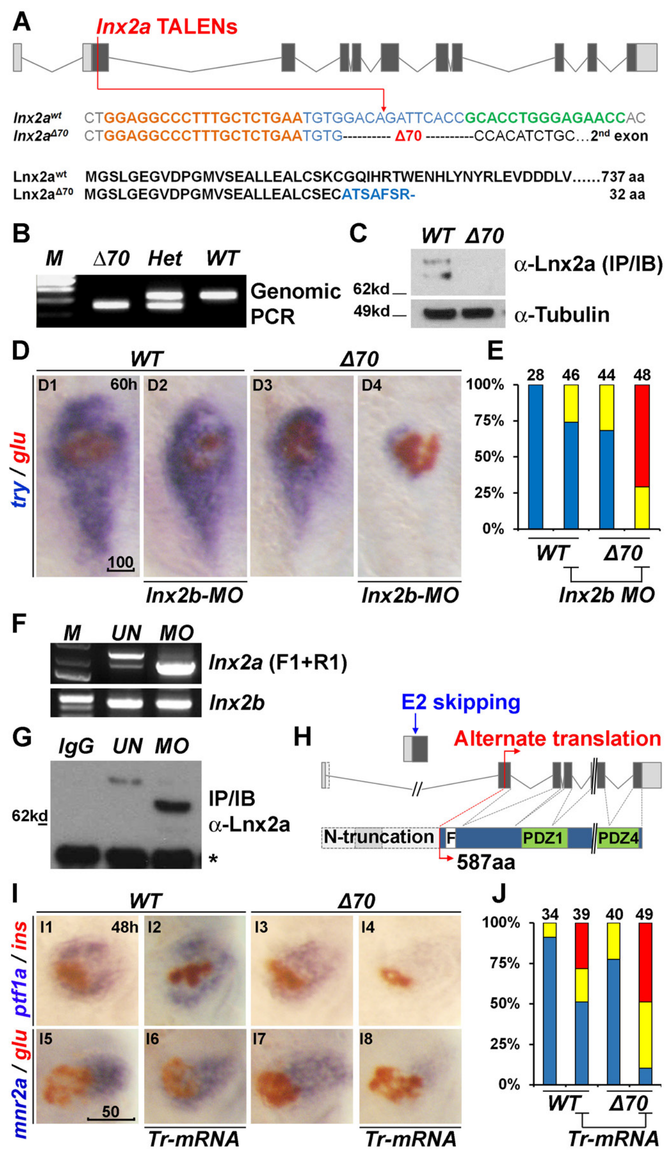
A. Schematic drawing of the *lnx2a* locus containing 11 exons and the Lnx2a protein (737 aa) containing a RING-finger domain (RF), the Numb binding NPAF motif (F), and four PDZ domains. The translation initiation site is located in exon 2. The *lnx2a-MO* targets the splice donor site of exon 2. Primers in exon 1 (F1) and exon 11 (R1) are shown, as is the epitope for the Lnx2a antibody.

B and C. The *lnx2a*-MO leads to inhibition of exocrine markers (*try, ptf1a*), but not endocrine markers (*ins, glu*) at 60 hpf.

D and E. Quantification of marker expression. UN, Uninjected; MO, *lnx2a-MO*; Scale bar, 100μm.

Figure 5. Generation of *lnx2a*$^{\Delta70}$ mutant using TALENs, and the functional redundancy of *lnx2* genes ▶

A. Schematic representation of the *lnx2a* locus and the TALEN target site in exon 2. TALEN targets (*left, orange* and *right, green*), and the *lnx2a*$^{\Delta70}$ mutation are shown. Next, the protein sequences of wild-type and the *lnx2a*$^{\Delta70}$ frameshift allele are shown. Genotype of the *lnx2a*$^{\Delta70}$ mutant was analyzed by genomic PCR (B) and immuno-blotting of Lnx2a protein (C). D. Marker gene (*try* and *glu*) expression shows little effect in *lnx2a*$^{\Delta70}$-null mutants or in *lnx2b-MO*–injected embryos. However, *lnx2b-MO* injection into *lnx2a*$^{\Delta70}$-mutant embryos shows suppression of exocrine markers. E. Quantification of defects. F–H. *lnx2a-MO* leads to production of N-truncated Lnx2a protein. F. RT-PCR using primers F1 and R1 (Figure 4A) followed by sequencing shows that *lnx2a-MO* injection results in exon 2 skipping; *lnx2b* expression was unchanged. G. Endogenous Lnx2a showed a smaller protein in *lnx2a-MO*–injected embryos. H. Schematic drawing of exon 2 skipping, alternate translation start site, and N-truncated protein in *lnx2a-MO*–injected embryos. I. N-truncated Lnx2a has interfering effect. *Tr-mRNA* was injected into the *WT* and *lnx2a*$^{\Delta70}$-mutant embryos. J. Quantification of pancreatic defects in (I). Scale bar, 100 mm (D1) and 50 mm (I5).



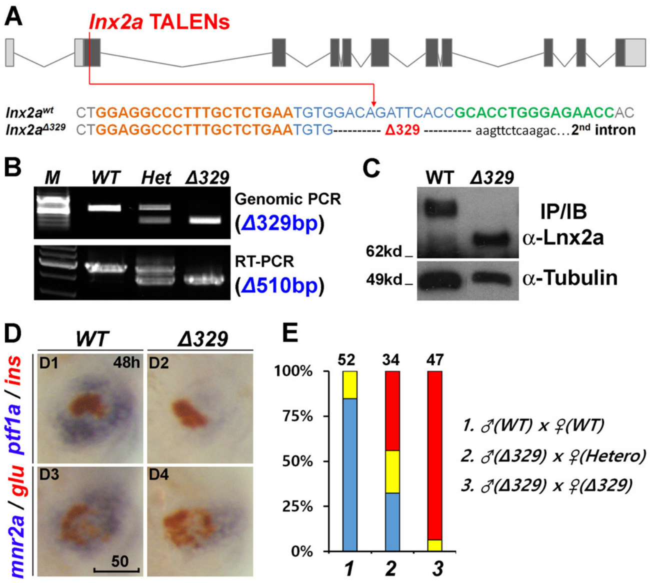


Figure 6. The $lnx2a^{\Delta 329}$ mutant recapitulates the morphant phenotype.

A. Sequence of the $lnx2a^{\Delta 329}$ mutation. The mutant has lost the exon 2 splice donor site. B and C. Genotyping of $lnx2a^{\Delta 329}$ by genomic PCR (Δ329), RT-PCR (Δ510 equaling exon 2) (B), and by Western blotting showing N-truncated protein (C). D. Phenotypic analysis of $lnx2a^{\Delta 329}$ mutants at 48 hpf. E. Quantification of D. Scale bar, 50mm.

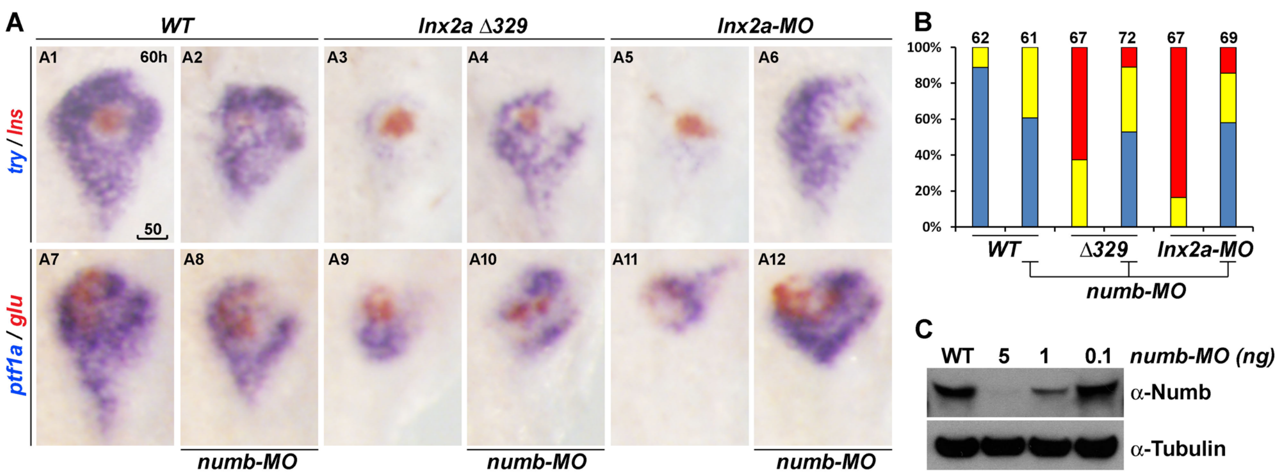


Figure 7. Ventral pancreas defects in $lnx2a^{\Delta 32}$-mutant and *lnx2a-MO*–injected embryos can be rescued by knock-down of Numb.

A. The translation-blocking MO for Numb was injected into WT, $lnx2a^{\Delta 329}$- and *lnx2a-MO*–injected embryos, and pancreatic phenotype was examined at 60 hpf.
B. Quantification of pancreatic defects by analysis of *ptf1a* expression. Exocrine pancreas defects were substantially rescued by knock-down of Numb; *numb-MO* had little effect in WT embryos.
C. Immuno-blotting of embryo extracts with Numb antibody shows the efficiency of the *numb-MO*. Scale bar, 50 mm.

PUBLICATIONS

1. Zarelli VE, Dawid IB. Inhibition of neural crest formation by Kctd15 involves regulation of transcription factor AP-2. *Proc Natl Acad Sci USA* 2013; 110:2870–2875.
2. Toyama R, Kim MH, Rebbert ML, Gonzales J, Burgess H, Dawid IB. Habenular commissure formation in zebrafish is regulated by the pineal gland-specific gene unc119c. *Dev Dyn* 2013; 242:1033–1042.
3. Kim MH, Rebbert ML, Ro H, Won M, Dawid IB. Cell adhesion in zebrafish embryos is modulated by March 8. *PLoS One* 2014; 9:e94873.
4. Komiya Y, Mandrekar N, Sato A, Dawid IB, Habas R. Custos controls ß-catenin to regulate head development during vertebrate embryogenesis. *Proc Natl Acad Sci USA* 2014; 111:13099–13104.
5. Won M, Ro H, Dawid IB. Lnx2 ubiquitin ligase is essential for exocrine cell differentiation in the early zebrafish pancreas. *Proc Natl Acad Sci USA* 2015; 112:12426–12431.

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Nothing is more fundamental to living organisms than the ability to reproduce. Each time a human cell divides, it must duplicate its genome, a problem of biblical proportions. A single fertilized human egg contains 2.1 meters of DNA. An adult of about 75 kg (165 lb) consists of about 29 trillion cells containing a total of about 60 trillion meters of DNA, a distance equal to 400 times the distance from Earth to sun. Not only must the genome be duplicated trillions of times during human development, but must be duplicated once and only once each time a cell divides (termed mitotic cell cycles). If we interfere with this process by artificially inducing cells to re-replicate their nuclear genome before cell division, the result is DNA damage, mitotic catastrophe, and programmed cell death (apoptosis). On rare occasions, specialized cells can duplicate their genome several times without undergoing cell division (termed endocycles), but when this occurs, it generally results in terminally differentiated polyploid cells that are viable but no longer proliferate. As we age, however, the ability to regulate genome duplication diminishes, resulting in genome instability, which allows genetic alterations that can result in promiscuous cell division—better known as cancer. For a comprehensive description of genome duplication in all forms of life, refer to *Genome Duplication* (Figure 2).

Our research program focuses on three questions. What are the mechanisms that restrict genome duplication to once per cell division? How are these mechanisms circumvented to allow developmentally programmed induction of polyploidy in terminally differentiated cells? How can we manipulate these mechanisms to destroy cancer cells selectively?

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Regulation of DNA replication in mammalian cells

Genome duplication begins when the six-subunit ORC (origin recognition complex) binds to specific chromosomal loci termed origins of bidirectional replication, which we and others mapped at specific sites in the genomes of flies and mammals. The sites are determined by both genetic and epigenetic features. The number and location of replication origins in the cells of multicellular organisms can change from an average of one in every 10 to 20 kb in the rapidly cleaving embryos of frogs, flies, and fish to one in every 50 to 300 kb in the differentiated cells of adult organisms. Developmental changes in origin density also occur during specific stages in animal development. Thus, metazoan genomes contain many potential replication origins but, during development, some of these sites are selectively activated while others are suppressed, a concept introduced many years ago as the "Jesuit Model," because many are called, but few are chosen (DePamphilis ML, *Ann Rev Biochem* 1993;62:29; *Curr Opin Cell Biol* 1993;5:434; *J Biol Chem* 1993;268:1).

ORC initiates assembly of prereplication complexes (preRCs) consisting of a DNA helicase loader [ORC(1–6) and replication factors Cdc6 and Cdt1] and the replicative DNA helicase [Mcm(2–7)]. Several years ago, we discovered that the behavior of ORC in mammalian cells differs significantly from that in single-cell eukaryotes such as yeast. In contrast to yeast, Orc1 associates weakly with the stable core complex ORC(2–5), and the ability of ORC to initiate

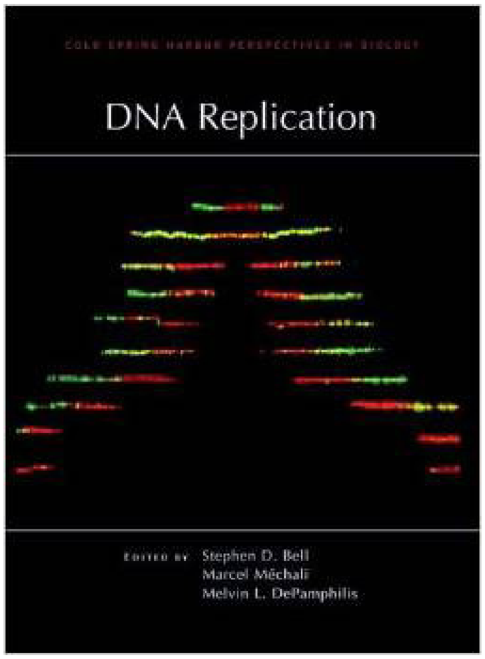


Figure 1. *DNA Replication* (Cold Spring Harbor Perspectives in Biology, 2013), edited by Marcel Mechali, Melvin L. DePamphilis, and Stephen D. Bell
The collection covers all aspects of DNA replication and its control across all domains of life. The contributors examine the molecular machinery involved in the assembly of replication origin complexes, the establishment of replication forks, unzipping of the double helix, priming of DNA synthesis, and elongation of daughter strands. Chromatin organization and dynamics, lagging-strand maturation, telomere replication, and mechanisms to handle errors and damage in DNA are also discussed.

DNA replication depends on this interaction. Moreover, it appears that the interaction of Orc1 with ORC(2–5) is one of the mechanisms that regulate when and where initiation events occur. We termed this concept the ORC cycle, and we and others have established its basic features (Figure 3). Cell cycle–dependent modifications of Orc1 regulate Orc1 activity, and Orc1 activity regulates ORC activity, which regulates initiation of DNA replication.

The ORC cycle is only one of six known mechanisms that can determine when and where DNA replication begins in human cells. Cdk2•CcnA (cyclin-dependent kinase-2•cyclin A) also suppresses Cdc6 (cell vision cycle-6) and Cdt1 (chromatin licensing and DNA replication factor-1) activities by phosphorylation, and Cdt1 is targeted by two ubiquitin ligases and by geminin, a Cdt1–specific protein inhibitor (Figure 4). However, not every pathway is active in all cell types. For example, cells derived from human cancers are dependent on geminin to prevent DNA re-replication, whereas cells derived from normal

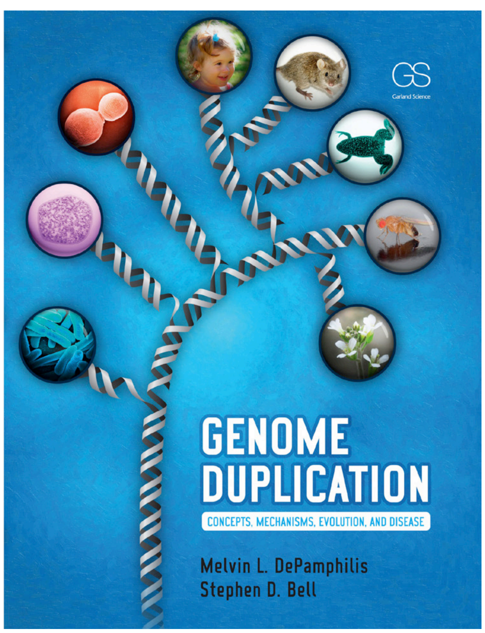
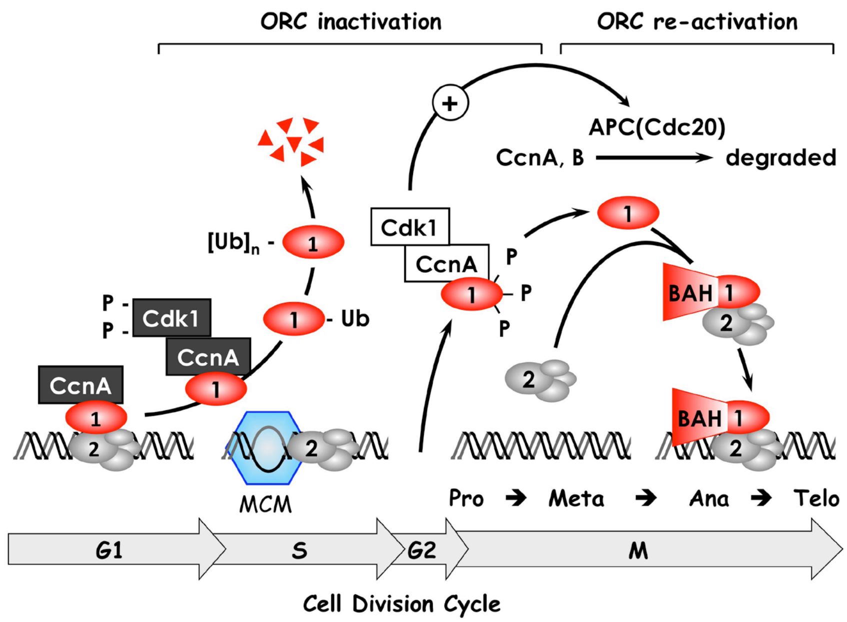


Figure 2. *Genome Duplication* (Garland Science, 2010) by Melvin L. DePamphilis and Stephen D. Bell
The book contains the first comprehensive description of the subject since Kornberg and Baker's book *DNA Replication*, second edition (W.H. Freeman, 1992). *Genome Duplication* describes the principles, concepts, and mechanisms common to genome duplication in all three domains of life: bacteria, archaea, and eukarya. In addition, it discusses the evolution of the DNA replication machinery and the impact of DNA replication on human disease.

Figure 3. The ORC cycle in mammalian cells (see Noguchi *et al., EMBO J* 2006;25:5372 and references therein)



ORC(1–6) is bound to chromatin during the G1 phase of the cell cycle, at which time it is part of a prereplication complex. When S phase begins, the association between Orc1 and chromatin-bound ORC(2–6) is destabilized by selective CDK–dependent phosphorylation and ubiquitination. Monoubiquitinated Orc1 is exported to the cytoplasm. The remaining ORC subunits are subsequently released from chromatin. Polyubiquitinated Orc1 is degraded by the 26S proteasome. Orc1 levels are restored during the G2-to-M transition, but Orc1 is hyperphosphorylated, an event that prevents ORC assembly. During the anaphase-to-G1 phase transition, Orc1 is dephosphorylated and, together with other ORC subunits, binds to chromatin, an event facilitated by the Orc1 BAH domain. If Orc1 is not associated with other ORC subunits, or if it is not phosphorylated or ubiquitinated, it induces apoptosis.

human tissues are dependent on both geminin and cyclin A–dependent CDK (cyclin-dependent kinase) activity.

A rare event in mammals, endoreduplication, by which the nuclear portion of the genome is duplicated one or more times (endocycles) without an intervening mitosis, is a common event among arthropods and plants. In mammals, it first occurs during peri-implantation development when cells within the trophectoderm (TE) of the blastocyst are deprived of the mitogenic factor FGF4 (fibroblast growth factor-4). Trophoblast stem (TS) cells then differentiate into the polyploid, viable, nonproliferating trophoblast giant (TG) cells required for embryo implantation and placentation (Figure 5). PreRC assembly requires the absence of both CDK activity and geminin, a condition that occurs in mitotic cell cycles during the anaphase-to-G1 phase transition. We discovered that this condition could be induced in TS cells by selective chemical inhibition of CDK1, the enzyme required for entry into mitosis, but not in embryonic stem (ES) cells, in which the consequence is apoptosis. Therefore, selective inhibition of CDK1 triggers endoreduplication only in cells programmed to differentiate into polyploid cells. Similarly, FGF4 deprivation of TS cells induced expression of two CDK–specific inhibitors: p57/Kip2 and p21/Cip1. One (p57) was essential for endoreduplication while the other (p21) appeared to facilitate p57 activity by suppressing expression of Chk1 (checkpoint kinase-1) and the mitotic inhibitor Emi1 (Figure 6). TS cells (+FGF4) express both the *p57* and *p21* genes, but the nonactivated form of Chk1 phosphorylates the p57 and p21 proteins, thereby targeting them for ubiquitin-dependent degradation. In TG cells, CHK1 is suppressed to allow p57 expression during G phase, CDK2 is required for DNA replication, and p57 is degraded during S phase to allow endocycles.

DNA replication and cell differentiation during pre-implantation development

Fertilization activates the first round of genome duplication, after which the fertilized egg cleaves into a two-cell embryo (Figure 5). In mice, the two-cell embryo then activates expression of about 300 genes that are required to continue development of the organism (termed zygotic gene activation). Initially, every cell (blastomere) produced by the cleavage events is 'totipotent,' that is, it is capable of giving rise to the entire organism. But, within five rounds of cell cleavage, a blastocyst appears, marking the beginning of cell differentiation. The blastocyst consists of a spherical monolayer of epithelial cells called the TE that gives rise to TS cells, TG cells, and eventually to the placenta. The TE layer encompasses a group of cells called the inner cell mass that gives rise to ES cells and eventually to the embryo.

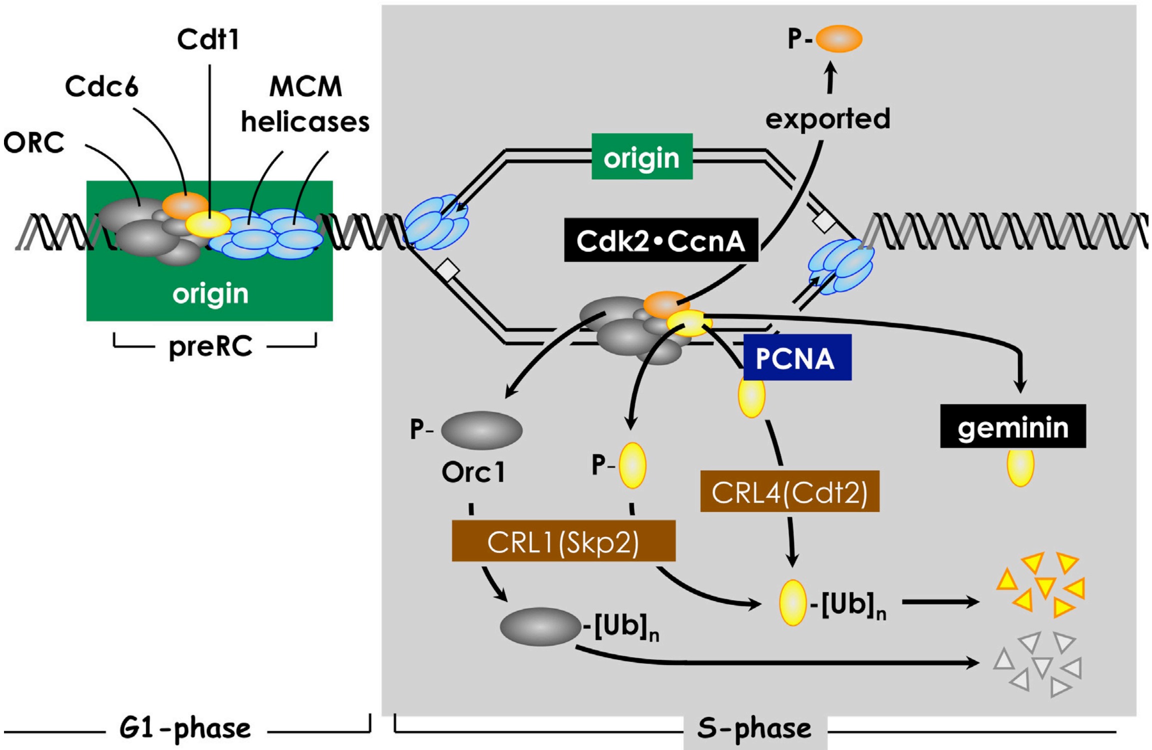
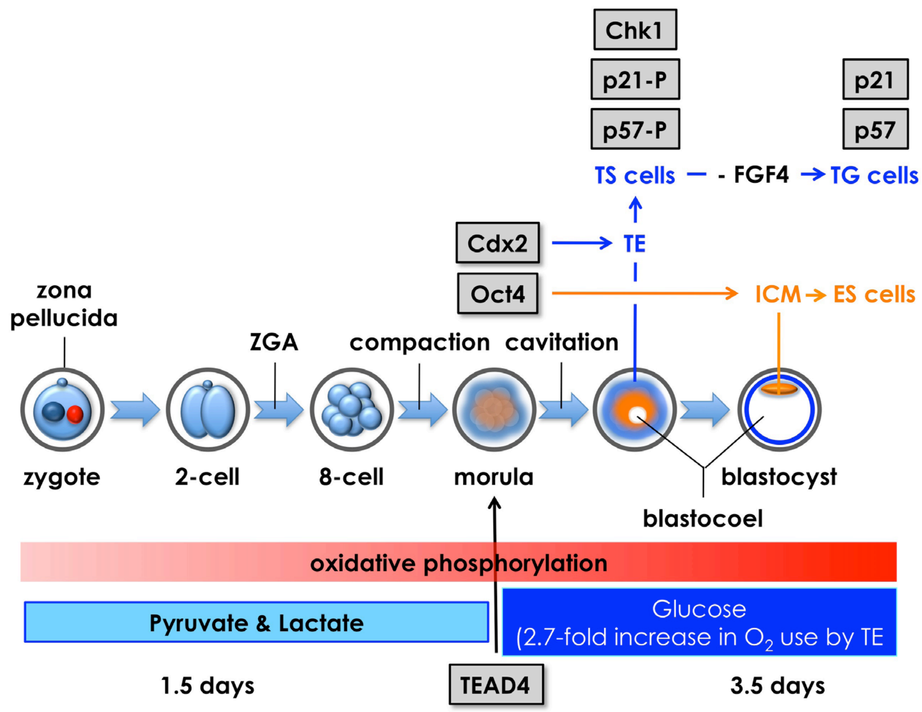


Figure 4. Several convergent pathways restrict genome duplication to once per cell division.
In human cells, activity of the replication factor Cdt1 is down-regulated in four ways. Free Cdt1 is phosphorylated by Cdk2 in association with Cyclin A (CcnA), thereby suppressing Cdt1 activity and converting Cdt1 into a substrate for the CRL1(Skp2) ubiquitin ligase. As replication forks pass through the origin, Cdt1 binds to the proliferating cell nuclear antigen (PCNA) clamp, which holds the replicative DNA polymerase onto the replication fork. The Cdt1•PCNA•chromatin form of Cdt1 is a substrate for the CRL4(Cdt2) ubiquitin ligase. Ubiquitinated Cdt1 is then degraded by the 26S proteasome. Finally, geminin binds to Cdt1 and inhibits its activity. In addition to inactivation of Cdt1, CcnA–dependent phosphorylation of Orc1 prevents it from binding to chromatin during mitosis, and CcnA–dependent phosphorylation of Cdc6 suppresses its activity and promotes its nuclear export.

Our goal for some years has been to determine whether the requirements for genome duplication in cultured cells were the same for cleavage-stage embryos, for the stage prior to cell differentiation, and for the transition from mitotic cell cycles to endocycles. To this end, we identified genes that we thought might be critical for pre-implantation development. The results have been fruitful, but surprising. We discovered that *Dkkl1*, a gene unique to mammals, is expressed specifically during implantation of the embryo and development of spermatocytes into sperm. Moreover, we showed that inactivation of *Dkkl1* in mice resulted in the production of sperm that are defective in fertilization. We discovered that *Tead2*, one of a highly conserved family of four transcription factors that share a common DNA–binding domain, is expressed from the two-cell embryo throughout pre-implantation development but, remarkably, is not required until after implantation and the start of nervous system formation. Mice lacking a functional *Tead2* gene have difficulty forming a neural tube. Failure to close the neural tube in mice is called exencephaly, which is related to anencephaly, the common human birth defect that can be prevented by folic acid. Finally, we discovered that *Tead2* and *Tead4* are the only *Tead* genes expressed in pre-implantation mouse embryos and that, in contrast to genetic inactivation of *Tead2*, genetic inactivation of *Tead4* results in the formation of a morphologically abnormal morula, the absence of TE–specific genes, and failure to produce a blastocoel. These and other results in the literature strongly suggested that *Tead4* is a master gene that sets in motion the first round of cell differentiation during mammalian development. Surprisingly, our subsequent research demonstrated otherwise.

Master genes trigger a sequence of events that specify cell fate. However, genes essential for maintaining a metabolic state that

Figure 5. Mouse pre-implantation development: zygotic gene activation (ZGA) begins at the two-cell stage.



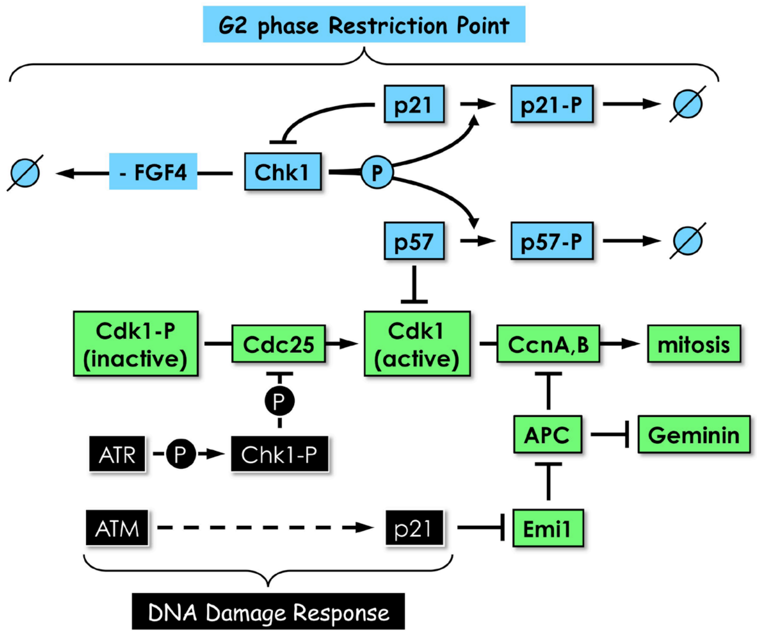
The eight totipotent blastomeres at the eight-cell stage compact into a morula, and transcription factors Tead4, Cdx2, and Oct4 mark the beginning of a chain of events that specifies the trophectoderm (TE) and inner cell mass (ICM). *Oct4* is the first gene whose expression is essential for maintaining blastomeres in a totipotent state, thereby producing the ICM and embryonic stem (ES) cells that differentiate into embryonic tissues. *Tead4* is the first gene whose expression is essential for differentiation of blastomeres into TE and trophoblast stem (TS) cells, which produce the placenta. In the absence of *Tead4* expression, all totipotent blastomeres produce OCT4 protein, revealing that the TEAD4 protein is required for *Cdx2* gene expression. However, TEAD4 is essential for blastocyst formation only under conditions that increase the rate of oxidative phosphorylation. Following compaction, pre-implantation embryos switch energy substrates from pyruvate and lactate to glucose in order to meet the increased energy demands of blastocoel formation, an event unique to the TE. Thus, *Tead4* does not specify the TE; rather, TEAD4 maintains energy homeostasis so that other genes can specify the TE.

allows other genes to specify cell fate may be mistaken for master genes. We validated the concept by showing that *Tead4*, the presumptive master gene for trophectoderm specification, is a homeostatic gene that is essential for blastocoel formation *in utero*, a prerequisite for embryo implantation and placentation. Requirement for *Tead4* is induced metabolically, not developmentally, and conditions that minimize oxidative stress allow embryos lacking TEAD4 to develop into blastocysts expressing genes required to produce functional trophectoderm. TEAD4, which is expressed at the eight-cell stage, is essential only under conditions that promote energy production by oxidative phosphorylation (Figure 5). Given that either inhibition of the mTOR pathway or an anti-oxidant alleviates the requirement, TEAD4 appears to regulate changes in redox potential that are expected to occur during the high levels of oxidative phosphorylation associated with blastocyst formation *in utero* (Kaneko and DePamphilis, *Development* 2013;140:3680-3689).

Geminin prevents trophoblast stem cells from exiting mitosis and differentiating into the nonproliferating, viable, giant cells essential for implantation and placentation.

The protein geminin is involved in both DNA replication and cell fate acquisition. Although it is essential for mammalian preimplantation development, its role remains unclear. In one study, ablation of the geminin gene (*Gmnn*) in mouse preimplantation embryos resulted in apoptosis, suggesting that geminin prevents DNA re-replication, whereas in another study it resulted in differentiation of blastomeres into TG cells, suggesting that geminin regulates trophoblast specification and differentiation. Other studies led to the conclusion that trophoblast differentiation into TG cells is regulated by FGF4 and that geminin is required to maintain endocycles. We showed that ablation of *Gmnn* in TS cells proliferating in the presence of FGF4 closely mimics the events triggered by FGF4 deprivation: arrest of cell proliferation, formation of giant cells, excessive DNA replication in the absence of DNA damage and apoptosis, and changes in gene expression that include loss of the checkpoint kinase Chk1 with up-regulation of p57 and p21. Moreover, FGF4 deprivation of TS cells reduces geminin to a

Figure 6. Proliferating trophoblast stem (TS) cells exhibit a G2 phase restriction point analogous to the G1 restriction point in proliferating cultured mammalian cells.



In the presence of the mitogen FGF4 and in the absence of DNA damage, Chk1, the same checkpoint kinase that is activated by DNA damage, phosphorylates both p57 and p21, thereby targeting them for ubiquitin-dependent degradation. In the absence of FGF4 and DNA damage, Chk1 expression is suppressed by an as yet unidentified mechanism. Both p57 and p21 proteins accumulate; p57 inhibits CDK1, and TS cells exit their mitotic cell cycle and differentiate into trophoblast giant (TG) cells; and p21 plays several roles, including suppressing Chk1 expression to maintain TG cell status and inhibiting Emi1 to activate the anaphase-promoting complex (APC), which promotes preRC assembly by targeting cyclins A and B and geminin for degradation. Both Chk1 and p21 are also components of the DNA–damage response that prevents mitosis until the problem is corrected; the features that distinguish their roles in the two regulatory mechanisms remain to be elucidated.

basal level that is required for maintaining endocycles in TG cells. Thus, geminin acts like a component of the FGF4 signal transduction pathway that governs trophoblast proliferation and differentiation, and the protein is required to maintain endocycles (de Renty, Kaneko and DePamphilis, *Dev Biol* 2014;387:49-63).

Geminin is essential to prevent DNA re-replication–dependent apoptosis in pluripotent cells, but not in differentiated cells.

Geminin is a dual-function protein unique to multicellular animals with roles in modulating gene expression and preventing DNA re-replication. We showed that geminin is essential at the beginning of mammalian development to prevent DNA re-replication in pluripotent cells, exemplified by ES cells, as they undergo self-renewal and differentiation. ES cells, embryonic fibroblasts, and immortalized fibroblasts were characterized before and after geminin was depleted either by gene ablation or siRNA. Depletion of geminin under conditions that promote either self-renewal or differentiation rapidly induced DNA re-replication, followed by DNA damage, then a DNA–damage response, and finally apoptosis. Once differentiation had occurred, geminin was no longer essential for viability, although it continued to contribute to prevent DNA re-replication–induced DNA damage. We detected no relationship between expression of geminin and genes associated with either pluripotency or differentiation. Thus, the primary role of geminin at the beginning of mammalian development is to prevent DNA re-replication–dependent apoptosis, a role previously believed essential only in cancer cells. The results suggest that regulation of gene expression by geminin occurs only after pluripotent cells differentiate into cells in which geminin is not essential for viability (Reference 1).

Cytoplasmic localization of p21 protects trophoblast giant cells from DNA damage–induced apoptosis.

Proliferating TS cells can differentiate into nonproliferating, but viable, TG cells that are resistant to DNA damage–induced apoptosis. Differentiation is associated with selective up-regulation of the Cip/Kip cyclin–dependent kinase inhibitors p57 and p21, while expression of p27 remains constant. Previous studies showed that p57 localizes to the nucleus in TG cells, where it is essential for endoreplication. We showed that p27 also remains localized to the nucleus during TS cell differentiation, where it complements the role of p57. Unexpectedly, p21 localized to the cytoplasm, where it was maintained throughout both the G- and S-phases of endocycles, and where it prevented DNA damage–induced apoptosis. This unusual status for a Cip/Kip protein was dependent on site-specific phosphorylation of p21 by the Akt1 kinase that is also up-regulated in TG cells. Although cytoplasmic p21 is widespread among cancer cells, among normal cells it has been observed only in monocytes. The fact that it also occurs in TG cells reveals that p57 and p21 serve nonredundant functions and suggests that the role of p21 in suppressing apoptosis is restricted to terminally differentiated cells (Reference 2).

And-1 coordinates with Claspin for efficient Chk1 activation in response to replication stress.

The replisome is important for DNA replication checkpoint activation, but how specific components of the replisome coordinate with the ATR kinase to activate Chk1 in human cells remains largely unknown. We demonstrated that And-1, a replisome component, acts together with ATR to activate Chk1. And-1 is phosphorylated at T826 by ATR following replication stress, and the phosphorylation is required for And-1 to accumulate at the damage sites, where And-1 promotes the interaction between Claspin and Chk1, thereby stimulating efficient Chk1 activation by ATR. Significantly, And-1 binds directly to ssDNA (single-strand DNA) and facilitates the association of Claspin with ssDNA. Furthermore, And-1 associates with replication forks and is required for the recovery of stalled forks. The studies establish a novel ATR–And-1 axis as an important regulator for efficient Chk1 activation and reveal a novel mechanism by which the replisome regulates the replication checkpoint and genomic stability (Reference 3).

PUBLICATIONS

1. Huang YY, Kaneko KJ, Pan H, DePamphilis ML. Geminin is essential to prevent DNA re-replication-dependent apoptosis in pluripotent cells, but not in differentiated cells. *Stem Cells* 2015; 33:3239–3253.
2. de Renty C, DePamphilis ML, Ullah Z. Cytoplasmic localization of p21 protects trophoblast giant cells from DNA damage induced apoptosis. *PLoS One* 2014; 9:e97434.
3. Hao J, de Renty C, Li Y, Xiao H, Kemp MG, Han Z, DePamphilis ML, Zhu W. And-1 coordinates with Claspin for efficient Chk1 activation in response to replication stress. *EMBO Journal* 2015; 34:2096–2110.
4. Kohn MJ, DePamphilis ML. Derivation of mouse trophoblast stem cells from preimplantation embryos. *NIH Intramural Pluripotent Stem Cell Protocol Book* 2014; 1:1–20.
5. Ullah Z, de Renty C, DePamphilis ML. Propagation and differentiation of mouse trophoblast stem cells. *NIH Intramural Pluripotent Stem Cell Protocol Book* 2014; 1:21–41.

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Control of Gene Expression during Development

During development and differentiation, genes become competent to be expressed or are stably silenced in an epigenetically heritable manner. The selective activation/repression of genes leads to differentiation of tissue types. Much evidence supports the model that modifications of histones in chromatin contribute substantially to determining whether a gene is expressed. Two groups of genes, the Polycomb group (*PcG*) and Trithorax group (*TrxG*), are important for inheritance of the silenced and active chromatin state, respectively. In *Drosophila*, regulatory elements called Polycomb group response elements (PREs) are required for the recruitment of chromatin-modifying PcG protein complexes. TrxG proteins may act through the same or overlapping *cis*-acting sequences. Our group aims to understand how PcG and TrxG proteins are recruited to DNA. Toward that end, one major project in the lab has been to determine all sequences and DNA–binding proteins required for PRE activity. In the *Drosophila* genome, there are hundreds of PREs that regulate a similar number of genes, and it was not known whether all PREs are alike. Our recent data showed that there is functional and architectural diversity among PREs, suggesting that PREs adapt to the environment of the gene they regulate. A second major project in the lab is to determine how the PREs of the *engrailed/invected* gene complex act to control these genes in their native location. Surprisingly, we found that not all PREs are required *in vivo*, suggesting a redundancy in PRE function. To understand the interplay between PREs and enhancers (sequences important for activation of gene expression), we completed an analysis of the regulatory DNA of the *engrailed/invected* gene complex. We found that regulatory sequences are spread throughout at least a 79kb region in that gene complex and that the same enhancers activate both *engrailed* and *invected* expression. The finding lays the groundwork for future studies aimed at understanding how distant regulatory sequences coordinately regulate gene activity. We also conducted a genetic screen to find genes that regulate PRE activity and found an interesting cohesin-Polycomb connection, a project that has now been moved into a collaborative endeavor in the mouse. The aim of these studies is to probe the regulation of gene expression more deeply so as to permit an understanding of how gene expression can malfunction and lead to developmental abnormalities and disease.

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Polycomb group response elements (PREs)

PcG proteins act in protein complexes that repress gene expression by modifying chromatin. The best studied PcG protein complexes are PRC1 and PRC2. PRC2 contains the histone methyltransferase Enhancer of Zeste, which tri-methylates lysine 27 on histone H3 (H3K27me3). The chromatin mark H3K27me3 is the signature of PRC2 function. At most well studied genes PRC2 acts with PRC1, which binds to H3K27me3, mono-ubiquitinates histone H2A at lysine 119, and inhibits chromatin remodeling. In *Drosophila*, PRC1 and PRC2 are recruited to the DNA by PREs (Reference 1). We are interested in determining how this occurs, and, to that end, we defined all the DNA sequences and are finding all DNA–binding proteins required for the activity of a single 181–bp PRE of the *Drosophila engrailed* gene (PRE2). Binding sites for seven different proteins are required for the activity of the PRE2 (Figure 1) (Reference 2). There are several binding sites for some of

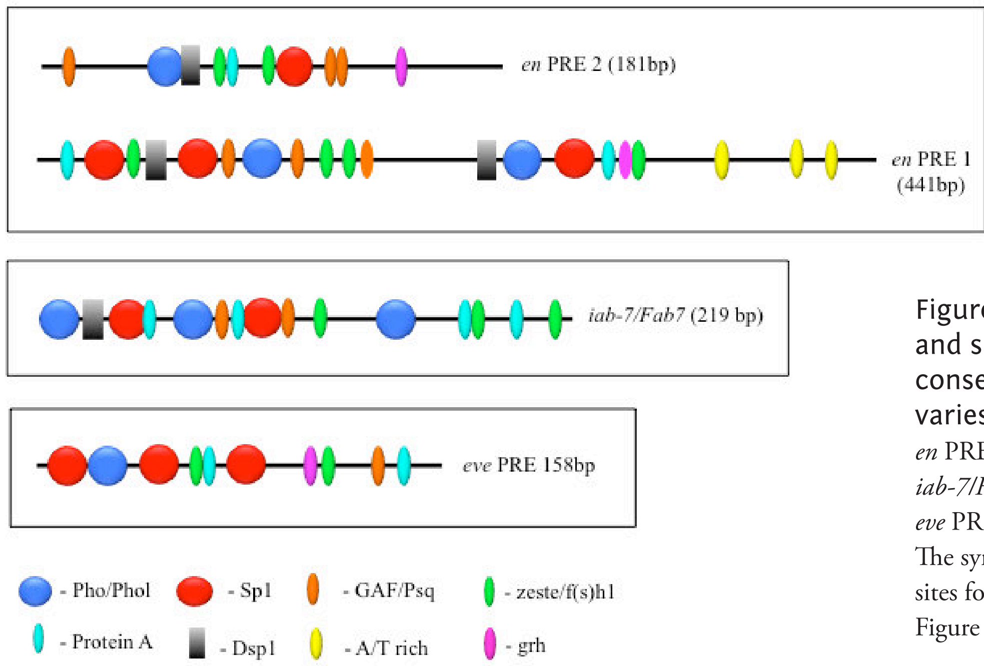

Figure 1. The order, number, and spatial arrangement of consensus-factor binding sites varies in different PREs.
en PRE1 & 2 are from the *engrailed* gene; *iab-7/Fab-7* PRE is from the *Abd-B* gene; *eve* PRE is from the *even-skipped* gene. The symbols represent consensus binding sites for the proteins indicated below. Figure reprinted from reference 2.

these proteins. Our laboratory identified four of the proteins: Pho (the first PRE–binding protein identified); the related protein Pho-like Spps; and a protein we call Protein A (manuscript in preparation). Genome-wide studies show that Pho and Spps bind to hundreds of PREs located throughout the *Drosophila* genome.

Studies designed to test the function of PREs in transgenes showed that PREs are largely interchangeable in some assays, with subtle activity differences. To determine how similar PREs are, we compared the binding-site arrangements and requirements in two closely linked *engrailed* PREs, PRE1 and PRE2, and compared them with two other PREs in the genome (Figure 1). All these PREs mediate transcriptional repression of the reporter gene *mini-white* in transgenic *Drosophila*, but the arrangement, number, and order of the binding sites vary dramatically among the different PREs. We tested the *engrailed* PREs in another reporter vector, one that gives β-galactosidase expression in embryos, larval salivary glands, and brains (Figure 2). In the vector, PRE1 but not PRE2 is able to repress expression in the anterior part of the embryo, an indication of PRE activity in this vector (Figure 2A). In contrast, both PREs are able to repress β-galactosidase expression (Figure 2B) and cause the deposition of the H3K27me3 repressive mark over the PRE and *lacZ* gene in salivary glands (Figure 3). However, only PRE1 is able to silence β-galactosidase expression in a subset of cells in the brain (Figure 2C). The data show that PREs are a diverse group of elements that share some but not all activities. Because PREs regulate many different genes, in different tissues and times of development, the differences may be important for the fine-tuning of PcG repression. It is also possible that different PREs recruit different PcG protein complexes. We are currently conducting experiments to test the hypothesis.

The role of PREs at the *en* gene

The *Drosophila engrailed (en)* gene encodes a homeodomain protein that plays an important role in the development of many parts of the embryo, including formation of the segments, nervous system, head, and gut. By specifying the posterior compartment of each imaginal disk, *en* also plays a significant role in the development of the adult. Accordingly, *en* is expressed in a highly specific and complex manner in the developing organism. The *en* gene exists in a gene complex with an adjacent gene, *invected* (*inv*); *inv* encodes a protein with a nearly identical homeodomain, and *en* and *inv* are co-regulated and express proteins with largely redundant functions.

The *en* and *inv* genes exist in a 113kb domain that is covered by the H3K27me3 chromatin mark. Within the *en/inv* domain there are four major PREs, strong peaks of PcG protein binding. One popular model posits that DNA–binding proteins bound to the PREs recruit PcG protein complexes and that PRC2 tri-methylates histone H3 throughout the domain until it comes to either an insulator or an actively transcribed gene. As discussed above, there are two PREs upstream of the *en*

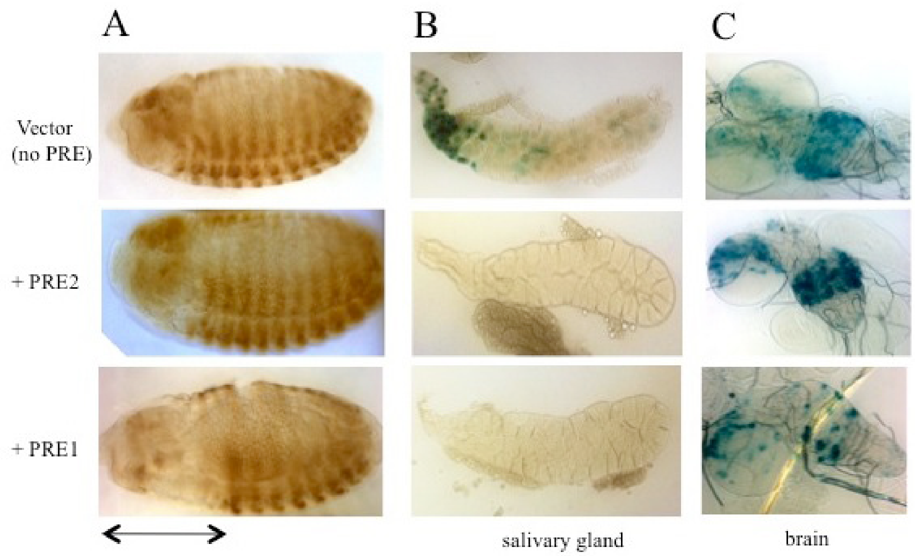

Figure 2. Activity of PRE1 and PRE2 in a transgene reporter construct in embryos and in larval salivary glands and brains

A. *Drosophila* embryos (anterior left, dorsal up) stained with antibody against β-galactosidase (β-gal) show that PRE1 but not PRE2 is able to repress expression of β-gal in the anterior part of the embryos (denoted by the double-headed arrow).
B. β-gal activity stain in salivary glands: β-gal is expressed from the vector alone (no PRE), but is repressed by either PRE1 or PRE2.
C. Expression in the larval brain is partially repressed by PRE1 but not by PRE2.

transcription unit, PRE1 and PRE2. Both PREs reside within a 1.5kb fragment located from –1.9kb to –400bp upstream of the major *en* transcription start site. There are also two major *inv* PREs, one located at the promoter and another about 6kb upstream of that. Our laboratory showed that all these PREs have the functional properties attributed to PREs in transgenic assays. To test their function at the intact *en/inv* domain, we set out to delete these PREs from the genome. Given that PREs work as repressive elements, the predicted phenotype of a PRE deletion is a gain-of-function ectopic expression phenotype. Unexpectedly, when we made a 1.5kb deletion removing PRE1 and PRE2, flies were viable and had a partial loss-of-function phenotype in the wing. Similarly, deletion of *inv* PREs yielded viable flies with no mis-expression of *en* or *inv*. Importantly, the H3K27me3 *en/inv* domain is not disrupted in either of these mutants. We hypothesize that *en* and *inv* PREs are redundant with each other, so that either is sufficient to recruit PRC2, which tri-methylates histone H3 throughout the *en/inv* domain.

In *Drosophila*, PREs are easily recognizable as discrete peaks of binding of PcG proteins in chromatin-immunoprecipitation experiments, but the H3K27me3 mark spreads throughout large regions. PcG proteins are conserved in mammals; however, PcG binding usually does not occur in sharp peaks, and PREs have been much harder to identify. We recently created a chromosome in which both the *en* and *inv* PREs are deleted. Surprisingly, the flies are viable, and preliminary results suggest that there is no mis-expression of *en* or *inv*. The question arises as to how PcG proteins are recruited to the *en/inv* domain in the absence of these PREs. We performed chromatin-immunoprecipitation followed by Next-Gen sequencing (ChIP-Seq) on the PcG proteins Pho and Polyhomeotic (Ph). The data showed that, in addition to the large Pho/Ph peaks at the known PREs, there are many smaller Pho/Ph peaks within the inv/en domain. Our data show that the peaks may also function as PREs. Thus, rather than a few PREs, there are many PREs controlling *inv/en* expression, and some of them may act in tissue-specific ways.

Increasing cohesin binding stability counteracts PcG silencing in *Drosophila*.

Cohesin consists of the proteins Smc1, Smc3, Rad21, and Stromalin (SA) and is important for sister chromatid cohesion and proper chromosome segregation during mitosis. In addition, cohesin and cohesin-associated proteins play an important role in regulating gene expression. In a recent study, others found that the cohesin subunits Smc1, Smc3, and Rad21 co-purify with the PcG protein Polycomb, suggesting that the protein complexes may physically interact at some loci. Wapl protein regulates binding of the cohesin complex to chromosomes during interphase and helps remove cohesin from chromosomes at mitosis. We isolated a dominant mutation in *wapl* (*wapl*^{AG}) in a screen for mutations that counteract silencing mediated by an *engrailed* PRE (Reference 3). *wapl*^{AG} hemizygotes die as pharate adults and have an extra sex combs phenotype characteristic

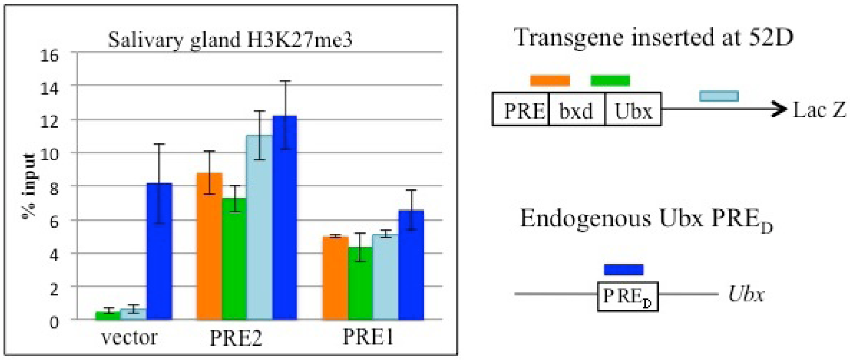

Figure 3. PRE1 and PRE2 lead to tri-methylation of histone H3 (H3K27me3) across the *LacZ* reporter gene in transgenic *Drosophila*.

Chromatin immunoprecipitation with anti-H3K27me3 antibodies on salivary glands from transgenic larvae with vector alone or with vector plus PRE1 or PRE2. A Ubx PRE is used as a positive control. Both PRE1 and PRE2 cause accumulation of the repressive H3K27me3 mark over the transgene.

of males with mutations in PcG genes (Figure 4). The *wapl* gene encodes two proteins, a long form and a short form. *wapl*^{AG} introduces a stop codon at amino acid 271 of the long form and produces a truncated protein. The expression of a transgene encoding the truncated Wapl-AG protein causes an extra-sex-comb phenotype similar to that seen in the *wapl*^{AG} mutant. Mutations in the cohesin-associated genes *Nipped-B* and *pds5* suppress and enhance *wapl*^{AG} phenotypes, respectively. A Pds5–Wapl complex (releasin) removes cohesin from DNA, while Nipped-B loads cohesin, suggesting that Wapl–AG might exert its effects through changes in cohesin binding. Consistent with this model, Wapl-AG was found to increase the stability of cohesin binding to polytene chromosomes. Our data suggest that increasing cohesin stability interferes with PcG silencing at genes that are co-regulated by cohesin and PcG proteins. In collaboration with Karl Pfeifer, we are making a conditional mutant in mouse *Wapl*. We will investigate whether mutations in mouse *Wapl* similarly disrupt PcG–regulated silencing at some loci. Genome-wide studies in *Drosophila* show that cohesin and PRC1 components co-localize at many locations throughout the genome (Reference 4). Functional studies suggest that cohesin binding may control the availability of PRC1 components for gene silencing (Reference 4). Our *Wapl* mouse mutant will provide a valuable reagent to test whether similar PRC1–cohesin interactions are important regulators of gene expression in mammals.

Enhancer-promoter communication

Enhancers are often located tens or even hundreds of kb away from their promoter, sometimes even closer to promoters of genes other than the one they activate. We showed that *en* enhancers can act over large distances, even skipping over other transcription units, choosing the *en* promoter over those of neighboring genes (Kwon *et al.*, *Development* 2009;136;3067). Such specificity is achieved in at least three ways. First, early-acting *en* stripe enhancers exhibit promoter specificity. Second, a proximal promoter-tethering element is required for the action of the imaginal disk enhancer(s). Our data point to two partially redundant promoter-tethering elements. Third, the long-distance action of *en* enhancers requires a combination of the *en* promoter and sequences within or closely linked to the promoter-proximal PREs. The data show that several mechanisms ensure proper enhancer-promoter specificity at the *Drosophila en* locus, providing one of the first detailed views of how promoter-enhancer specificity is achieved.

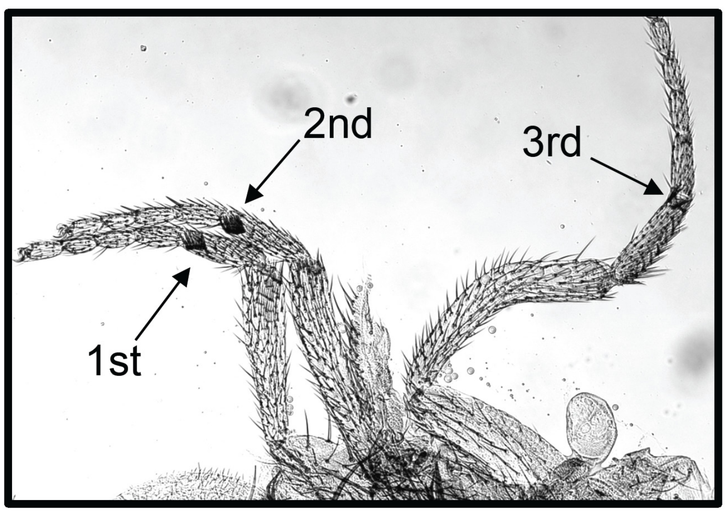

Figure 4. Wapl-AG causes extra sex comb teeth, the defining feature of PcG mutants.

This *wapl*^{AG} pharate adult male has sex comb teeth on all three legs (*arrows*). The second leg has 8 sex comb teeth, and the third leg has 2 sex comb teeth.

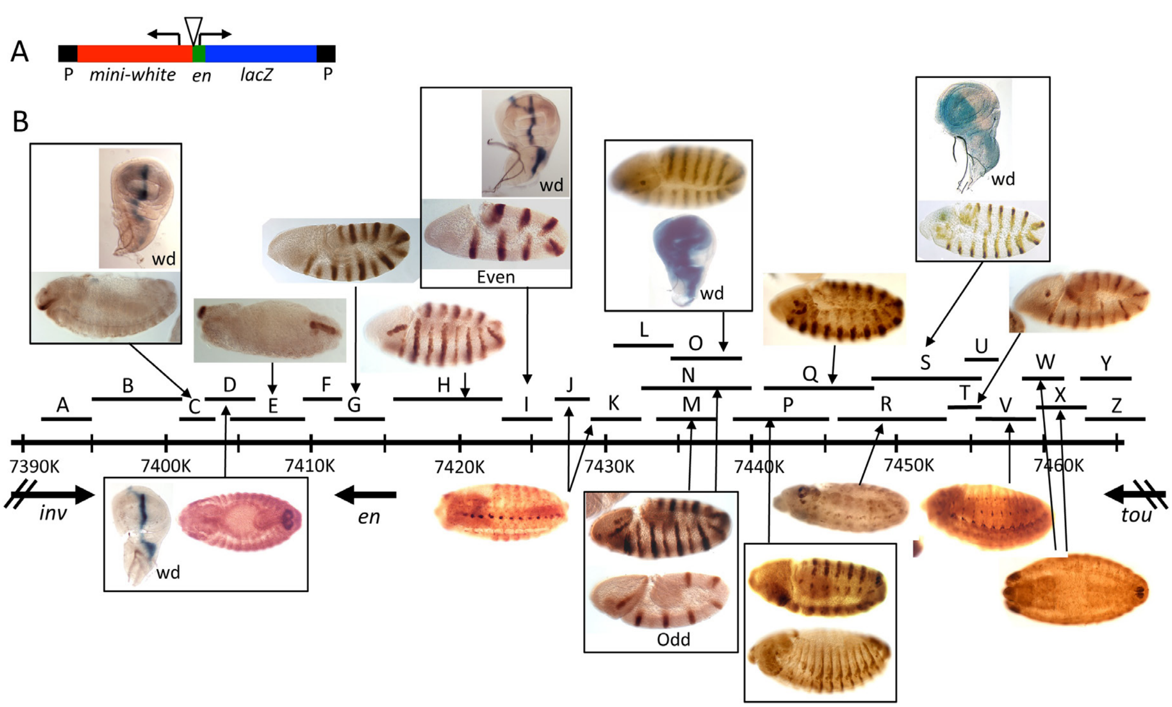

Figure 5. Enhancers of the *invected* and *engrailed* genes

A. P-element vector (*P[en]*), used to assay the function of *en* regulatory DNA, contains the *en* promoter, 396bp of upstream sequences, and an untranslated leader fusion between the *en* transcript and the *Adh-lacZ* reporter gene. *inv/en* DNA fragments were added to this vector at the location of the triangle. B. The extent of each fragment cloned into *P[en]* is shown as a black line with a letter above the *inv/en* genomic DNA map (indicated by a long black line with hatch marks at 10kb intervals; numbers are coordinates on chromosome 2R, genome release v5). Expression pattern in embryos or the wing imaginal disc (wd) are shown above or below the genomic DNA, with arrows pointing to the fragment(s) that generate the pattern (Reference 5).

As a follow-up to these studies, we located all the enhancers that regulate the transcription of *engrailed* (*en*) and the closely-linked co-regulated gene *invected* (*inv*) (Reference 5). Our dissection of *inv/en*–regulatory DNA showed that most enhancers are spread throughout a 62kb region. We used two types of constructs to analyze the function of this DNA: P-element–based reporter constructs with small pieces of DNA fused to the *en* promoter driving *lacZ* expression (Figure 5); and large constructs with HA–tagged *en* and *inv* inserted in the genome with the phiC31 system. In addition, we generated deletions of *inv* and *en* DNA *in situ* and assayed their effects on *inv/en* expression. Our results support and extend our knowledge of *inv/en* regulation. First, *inv* and *en* share regulatory DNA, most of which is flanking the *en* transcription unit. In support of this finding, a 79-kb *HA-en* transgene can rescue *inv en* double mutants to viable, fertile adults. In contrast, an 84-kb *HA-inv* transgene lacks most of the enhancers for *inv/en* expression. Second, there are multiple enhancers for *inv/en* stripes in embryos; some may be redundant but others play discrete roles at different stages of embryonic development. Finally, no small reporter construct gave expression in the posterior compartment of imaginal discs, a hallmark of *inv/en* expression. Robust expression of *HA-en* in the posterior compartment of imaginal discs is evident from the 79-kb *HA-en* transgene, while a 45-kb *HA-en* transgene gives weaker, variable imaginal disc expression. We suggest that the activity of the imaginal disc enhancer(s) is dependent on the chromatin structure of the *inv/en* domain. We are currently investigating the properties of the *inv/en* imaginal disc enhancer(s) using a variety of methods, including deleting them from the endogenous *inv/en* domain using Crisper/Cas9.

PUBLICATIONS

1. Kassis JA, Brown JL. Polycomb group response elements in Drosophila and vertebrates. *Adv Genet* 2013; 81:83–118.
2. Brown JL, Kassis JA. Architectural and functional diversity of polycomb group response elements in Drosophila. *Genetics* 2013; 195:433–441.

3. Cunningham MD, Gause M, Cheng Y, Noyes A, Dorsett D, Kennison JA, Kassis JA. Wapl antagonizes cohesin binding and promotes Polycomb group silencing in Drosophila. *Development* 2012; 139:4172–4179.
4. Dorsett D, Kassis JA. Check and balances between cohesin and Polycomb in gene silencing and transcription. *Curr Biol* 2014; 24:R535–R539.
5. Cheng Y, Brunner AL, Kremer S, DeVido SK, Stefaniuk CM, Kassis JA. Co-regulation of invected and engrailed by a complex array of regulatory sequences in Drosophila. *Dev Biol* 2014; 395:131–143.

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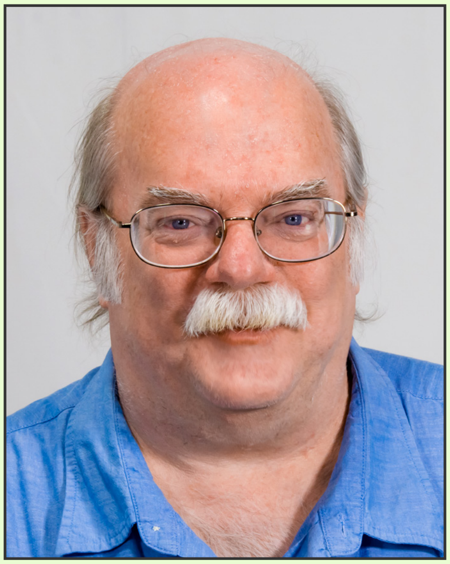

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Our goal is to understand how linear information encoded in genomic DNA functions to control cell fates during development. The *Drosophila* genome is about one twentieth the size of the human genome. However, despite its smaller size, most developmental genes and at least half of the disease- and cancer-causing genes in man are conserved in *Drosophila*, making *Drosophila* a particularly important model system for the study of human development and disease. One of the important groups of conserved developmental genes are the homeotic genes. In *Drosophila*, the homeotic genes specify cell identities at both the embryonic and adult stages. The genes encode homeodomain-containing transcription factors that control cell fates by regulating the transcription of downstream target genes. The homeotic genes are expressed in precise spatial patterns that are crucial for the proper determination of cell fate. Both loss of expression and ectopic expression in the wrong tissues lead to changes in cell fate. The changes provide powerful assays for identifying the *trans*-acting factors that regulate the homeotic genes and the *cis*-acting sequences through which they act. The *trans*-acting factors are also conserved between *Drosophila* and human and have important functions, not only in development but also in stem-cell maintenance and cancer.

Cis-acting sequences for transcriptional regulation of the *Sex combs reduced* (*Scr*) homeotic gene

Assays in transgenes in *Drosophila* previously identified *cis*-acting transcriptional regulatory elements from homeotic genes. The assays identified tissue-specific enhancer elements as well as *cis*-regulatory elements that are required for the maintenance of activation or repression throughout development. While these transgenic assays have been important in defining the structure of the *cis*-regulatory elements and identifying *trans*-acting factors that bind to them, their functions within the context of the endogenous genes remain poorly understood. We used a large number of existing chromosomal aberrations in the *Scr* homeotic gene to investigate the functions of the *cis*-acting elements within the endogenous gene. The chromosomal aberrations identified an imaginal leg enhancer about 35 kb upstream of the *Scr* promoter. The enhancer is not only able to activate transcription of the *Scr* promoter that is 35 kb distant but can also activate transcription of the *Scr* promoter on the homologous chromosome. Although the imaginal leg enhancer can activate transcription in all three pairs of legs, it is normally silenced in the second and third pairs of legs. The silencing requires the Polycomb-group proteins. We are currently attempting to identify the *cis*-regulatory DNA sequences in the *Scr* that are required for Polycomb-group silencing in the second and third legs. Characterization of the chromosomal rearrangements shown in Figure 1 also revealed that two genetic elements (proximal and distal MES [maintenance elements for silencing]), about 70 kb apart in the *Scr* gene, must be in *cis* to maintain proper repression. When not physically linked, the elements interact with elements on the homologous chromosome and cause derepression of its wild-type *Scr* gene. Using a transgenic assay, we identified at least five DNA fragments from the *Scr* gene that silence transcription from a reporter gene. The transcriptional silencers are clustered in the two regions whose interactions are required for the maintenance of silencing in the endogenous genes. We also use the silencer

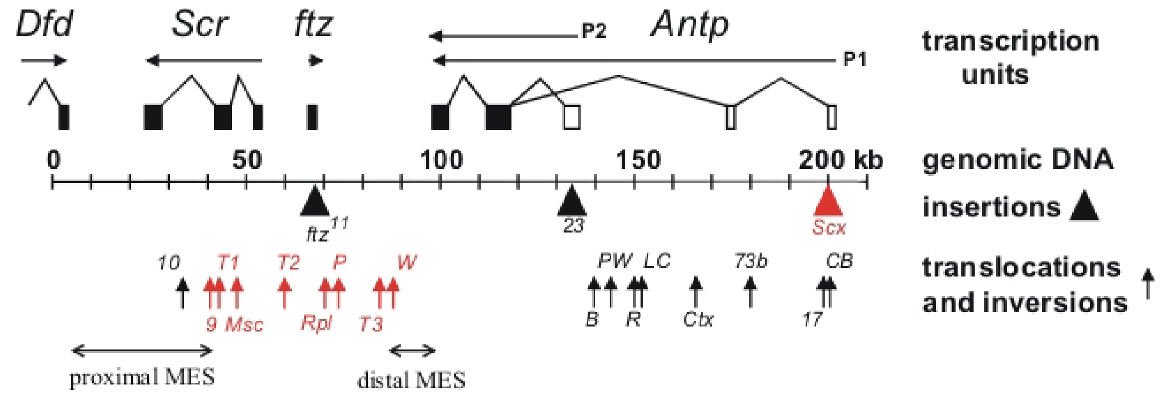


Figure 1. Chromosomal aberrations in the distal half of the *Antennapedia* complex
The transcription units are shown above the genomic DNA, while chromosomal aberrations are shown below (solid triangles indicate insertions of transposable elements and upward arrows indicate breakpoints of translocations and inversions). Chromosomal aberrations (*red*) interfere with silencing in the adult second and third legs. The regions that include the proximal and distal MES are indicated by horizontal arrows.

elements to screen for mutations in *trans*-acting silencing factors.

Trans-acting activators and repressors of homeotic genes

The initial domains of homeotic gene repression are set by the segmentation proteins, which also divide the embryo into segments. Genetic studies identified the trithorax group of genes that are required for expression or function (such as maintenance of transcriptional activation) of the homeotic genes. Maintenance of transcriptional repression requires the proteins encoded by the Polycomb-group genes. To identify new trithorax-group activators and Polycomb-group repressors, we screened for new mutations that mimicked the following phenotypes: loss of function or ectopic expression of the homeotic genes. We generated over 4,000 lethal mutants and, among those that die late in development, identified two dozen mutants with homeotic phenotypes. Some of the homeotic phenotypes are shown in Figure 2. The mutants identify genes required for expression or function of the homeotic genes.

We also use Polycomb-group response elements from the *Scr* gene to screen for recessive Polycomb-group mutations. Transgenes with a Polycomb-group response element and a reporter gene (the *Drosophila mini-white* gene) exhibit reporter gene expression in flies heterozygous for the transgene, but reporter gene expression is repressed in flies homozygous for the transgene. In flies homozygous for transgenes with the *mini-white* reporter gene silenced by the Polycomb-group response elements, we generate clones of cells in the eye that are homozygous for newly induced mutations, using the yeast FLP/FRT site-specific recombination system (Figure 3). Silencing mutations are detected by the appearance of pigmented spots in the white-eyed flies (cells that derepress the silenced *mini-white* reporter gene). Several examples of the new mutations recovered are shown in Figure 4. We screened about 98% of the genome and recovered almost 400 new silencing mutants. Almost 20% of the new mutants do not carry a new silencing mutation but bear chromosome aberrations that generate aneuploid cells after mitotic recombination. The aneuploid regions include the reporter transgene and they disrupt silencing by changing copy number. Although the mutants do not identify new genes, the phenomenon that we discovered will be very useful for detecting chromosomal aberrations in F1 mutant screens. Most of our mutants are not associated with large chromosomal aberrations and carry mutations in genes required for Polycomb-group transcriptional silencing. About half the mutations analyzed to date are in known Polycomb-group genes. The remaining mutations identify new genes required for silencing. For the new genes we identified, we are determining the corresponding transcription units, using a combination of meiotic recombination mapping and whole-genome sequencing. The new silencing genes include DNA–binding proteins, chromatin-remodeling factors, and insulator proteins.

Structure and function of the *Drosophila* genome

The *Drosophila melanogaster* genome has been intensely studied for over 100 years. Recently, sequencing of the majority of the

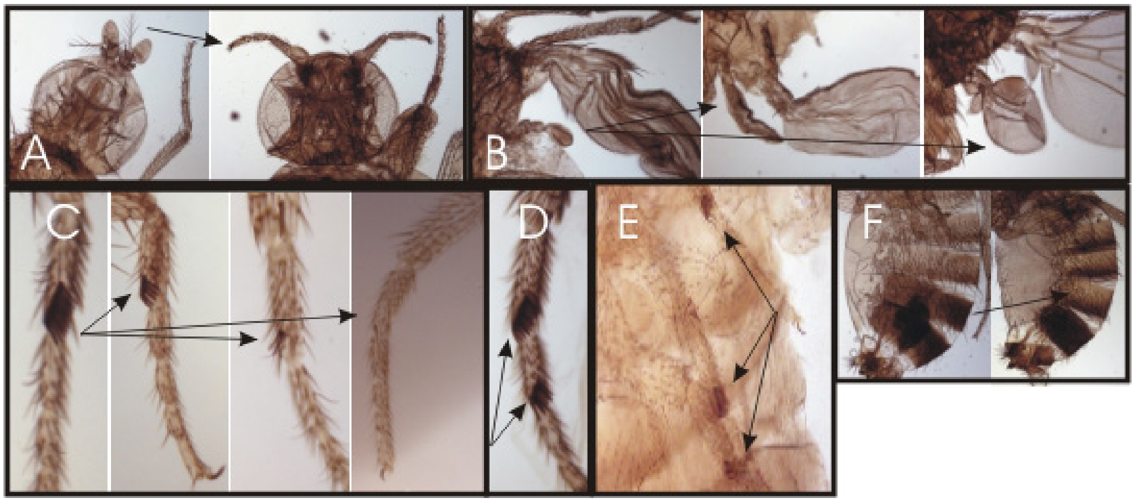
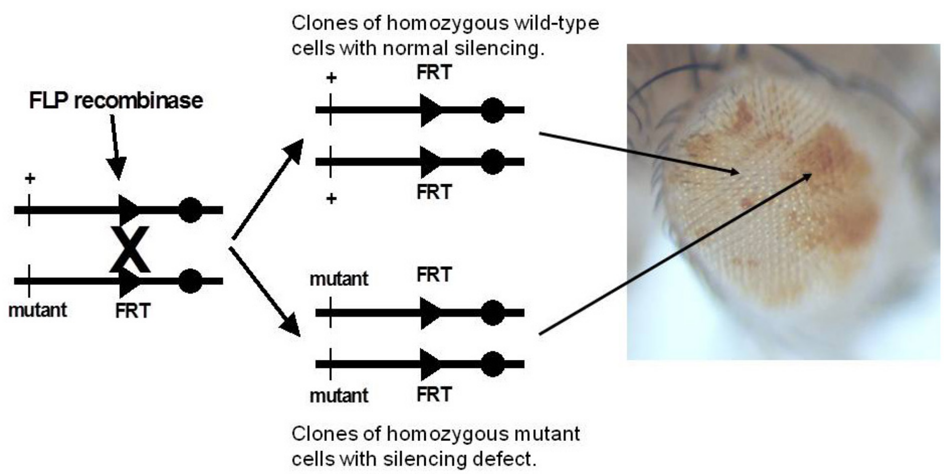


Figure 2. Homeotic phenotypes of new pharate-adult lethal mutants
A. Wild-type on the left and the transformation of aristae to distal leg on the right. B. Wild-type haltere on the left and transformations of anterior and posterior haltere to anterior and posterior wing in the middle and right, respectively. C. First legs from a wild-type male on the left and three different mutants with reduced sex combs on the right. D. Mutant male with sex combs on both the first and second tarsal segments. E. Mutant male with sex combs on all three pairs of legs. F. Abdominal segments from a wild-type male on the left; mutant male with transformation of the fifth abdominal segment to a more anterior identity on the right.

genomic DNA revealed much about the structure and organization of the genome. Despite those molecular advances, much remains to be discovered about the functions encoded within the genome. As part of a long-term project to understand the function and organization of the *Drosophila* genome, we set out to identify all genes essential for viability or male fertility in two regions of the genome that span almost 1 megabase of DNA (shown in Figures 5 and 6). We identified 10 gene clusters that appear to have arisen by tandem duplication. The clusters include 34 of the 137 predicted genes. We identified 47 genes essential for zygotic viability, including two of the pairs of tandemly duplicated genes. We identified the transcription

Figure 3. Genetic screen for new mutations that disrupt pairing-sensitive silencing
Flies homozygous for transposons carrying the *mini-white* reporter gene and a pairing-sensitive silencing element have white eyes. Clones of cells homozygous for newly induced mutants are generated using the yeast site-specific recombinase (FLP recombinase) and its target site (FRT). The clones of mutant cells are able to express the *mini-white* reporter gene and are pigmented (shown in the eye on the right of the figure).



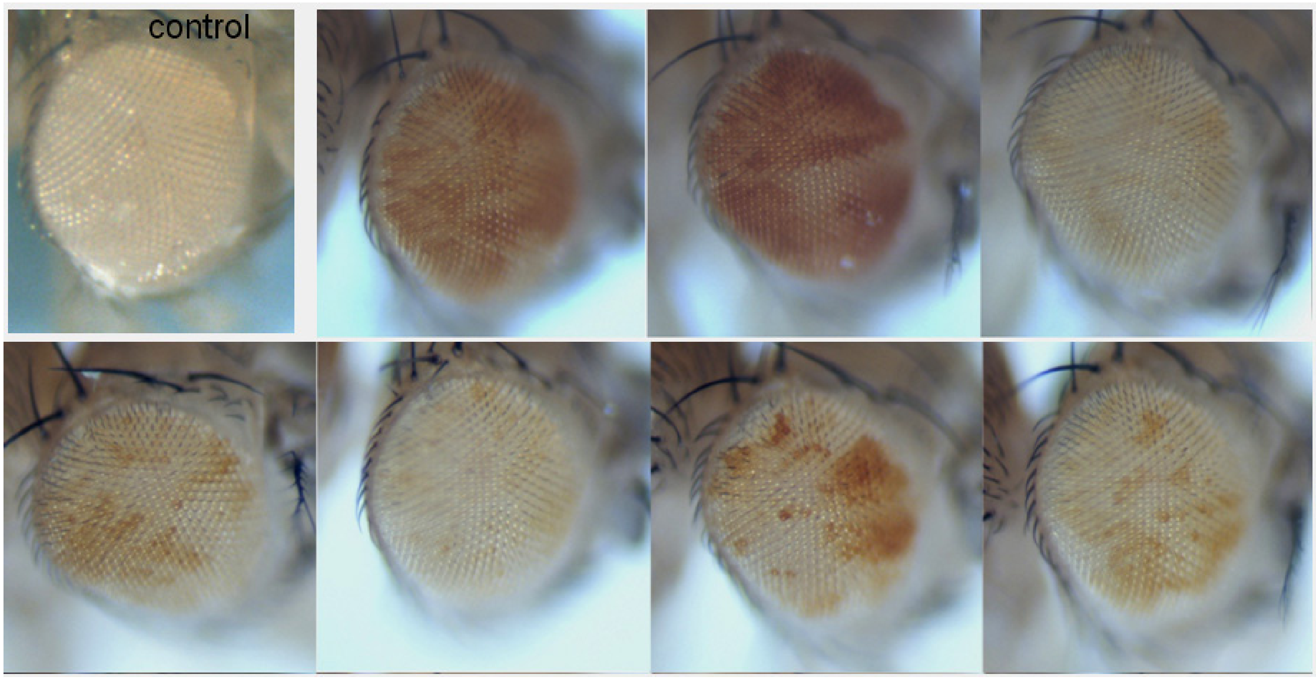


Figure 4. New mutations that disrupt pairing-sensitive silencing

The eye in the top left is the control with normal pairing-sensitive silencing. The remaining eyes are from flies with new mutations that disrupt pairing-sensitive silencing. The homozygous mutant cells are able to express the *mini-white* reporter gene in the transposon and are pigmented.

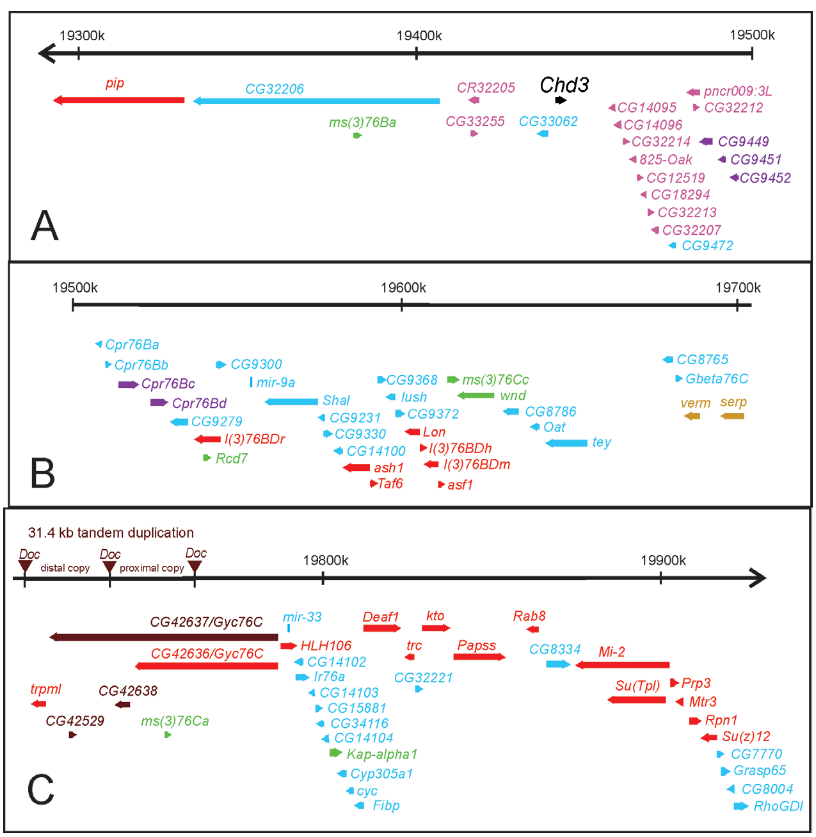


Figure 5. Molecular map of the genomic region deleted in *Df(3L) kto2* (polytene region 76B-D)

The approximately 640 kb of genomic DNA (from 3L: 19291k to 19926k, Release 5.23) is broken into three parts (A, B, and C) and is represented by the horizontal black arrows at the top of each part. The annotated transcription units are represented by colored thick horizontal arrows. Transcription units essential for viability are red and gold. Transcription units essential only for fertility are green. The clusters of transcription units encoding related proteins are purple, pink (the OAK cluster in 76B), and gold (the *verm* and *serp* genes essential for viability). The non-essential transcription unit *Chd3* is in black. All other transcription units are blue. A 31.4 kb tandem duplication (distal copy and proximal copy) flanked by *Doc* transposable elements in the sequenced *iso-1* strain (but not in other wild-type strains) is shown on the genomic DNA at the left of Panel C, with the *Doc* elements represented by inverted brown triangles.

units corresponding to all genes essential for viability. We also identified eight genes that are required only for fertility, most of which are male-specific in expression. The transcription units corresponding to all the genes required for fertility have been identified. With the exception of the *Antennapedia* and *bithorax* homeotic gene complexes, for no other regions of the *Drosophila* genome have transcription units essential for viability and fertility been as completely analyzed. We were also able to assess the progress of the *Drosophila* Gene Disruption Project. While the project has tagged about two-thirds of the annotated genes with transposon insertions, the insertions disrupt the function of only 45% of the genes. Our analysis of data from the modENCODE project suggests that 20% or more of the genome is expressed only in males, consistent with the observations that male-sterile mutations are recovered at almost 15% the frequency of lethal mutations. In contrast, only about 1-2% of the genome is expressed only in females.

Surprisingly, when we deleted a genomic region that spanned 55 kb, we found no effects on either viability or fertility. Although this nonessential region includes seven predicted genes, there is no evidence that the genes are expressed under any known conditions. The seven predicted genes are also not evolutionarily conserved. While there are no evolutionarily conserved open reading frames within this gene desert, the region has 48 DNA sequences of between 12 and 33 base pairs that are each identical in 12 different *Drosophila* species. The strong conservation indicates that the sequences must have some function that is beneficial to flies living outside the laboratory.

PUBLICATIONS

1. Monribot-Villanueva J, Juarez-Uribe RA, Palomera-Sanchez Z, Gutierrez-Aguiar L, Zurita M, Kennison JA, Vazquez M. TnaA, an SP-RING protein, interacts with Osa, a subunit of the chromatin remodeling complex BRAHMA and with the SUMO pathway in Drosophila melanogaster. *PLoS One* 2013; 8:e62251.
2. Lindsley DL, Roote J, Kennison JA. Anent the genomics of spermatogenesis in Drosophila melanogaster. *PLoS One* 2013; 8:e55915.

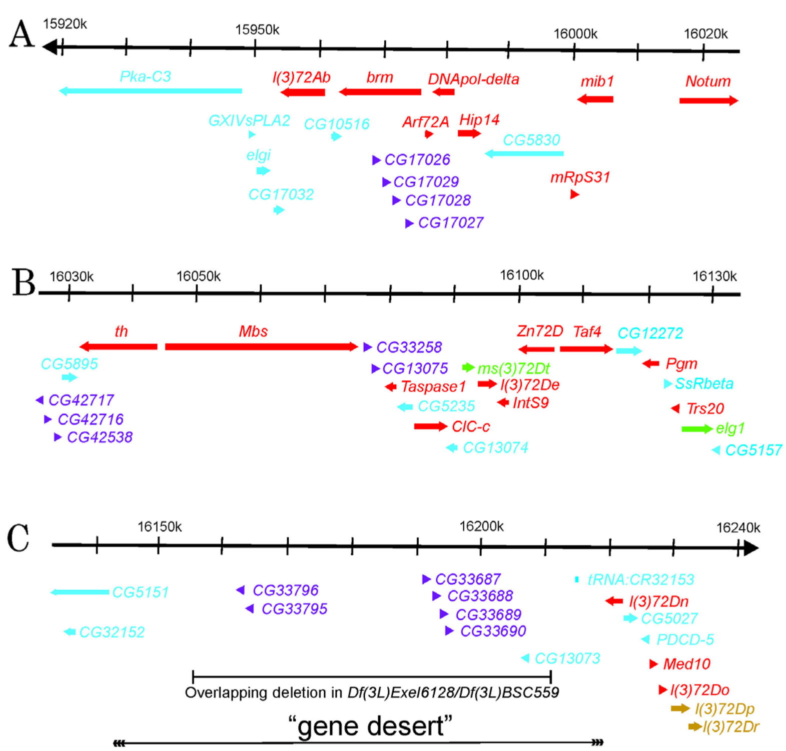


Figure 6. Molecular map of the genomic region deleted in *Df(3L)th102* (polytene region 72A-D)
The approximately 320 kb of genomic DNA (from 3L: 15918k to 16240k, Release 5.23) is broken into three parts (A, B, and C) and is represented by the horizontal black arrows at the top of each part. The annotated transcription units are represented by colored thick horizontal arrows. Transcription units essential for viability are red and gold. Transcription units essential only for fertility are green. The clusters of transcription units encoding related proteins are purple and gold [the *l(3)72Dp* and *l(3)72Dr* genes essential for viability]. All other transcription units are blue. The DNA missing in flies *trans*-heterozygous for the overlapping deletions *Df(3L)Exel6128* and *Df(3L)BSC559* and the putative 'gene desert' are indicated by the horizontal black bars at the bottom of C.

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Genes and Signals Regulating Mammalian Hematopoiesis

Our research focuses on the development of the mammalian hematopoietic system. Of particular interest is the characterization of signal-transduction molecules and pathways that regulate T cell maturation in the thymus. Current projects include the generation of transgenic and conditional deletion mutants to evaluate the importance of T cell antigen receptor signaling at specific stages of T cell development. We are also using microarray gene profiling to identify molecules that are important for thymocyte selection, a process that promotes the survival and further development of functional T cells and the death of auto-reactive T cells, thereby preventing autoimmunity.

A newer project involves analyzing the function of Themis, a T cell–specific signaling protein recently identified by our laboratory. Another recently initiated area of investigation focuses on hematopoietic stem cells (HSCs), which give rise to all blood cell lineages. We have begun to characterize the genes that are important for the generation and maintenance of HSCs and for their differentiation into specific hematopoietic cell types. The studies revealed a critical function for one protein (Ldb1) in controlling the self-renewal/differentiation cell-fate decision in both HSCs and erythroblasts by acting as a key component of multi-subunit DNA–binding complexes. Global (ChIP-seq) screening for Ldb1–complex DNA–binding sites identified many targets for Ldb1–mediated regulation of transcription in hematopoietic cells, demonstrating an important role for Ldb1 in hematopoietic gene regulation. Current work on Ldb1 includes an examination of a potential role for this protein in regulating self-renewal of T cell progenitors in the thymus and in the genesis of T cell Acute Lymphoblastic Leukemia (T-ALL), one of the most common childhood malignancies.

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T cell antigen receptor (TCR) signaling in thymocyte development

Much of our research focuses on the role of TCR signal transduction in thymocyte development. Signal transduction sequences, termed Immunoreceptor Tyrosine-based Activation Motifs or ITAMs, are contained within four distinct subunits of the multimeric TCR complex (CD3-zeta, CD3-gamma, CD3-delta, and CD3-epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement; their function is to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell–activation cascade. Though conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be partly regulated by distinct ITAMs. We previously generated CD3-zeta–deficient and CD3-epsilon–deficient mice by gene targeting. We genetically reconstituted the mice with transgenes encoding wild-type or signaling-deficient (ITAM–mutant) forms of CD3-zeta and CD3-epsilon and characterized the developmental and functional consequences of the alterations for TCR signaling. We found that TCR–ITAMs are functionally equivalent but act in concert to amplify TCR signals and that TCR signal amplification is critical for thymocyte selection, the process by which potentially useful immature T cells are instructed to survive and differentiate further (positive selection) and by which potentially auto-reactive cells, which may cause

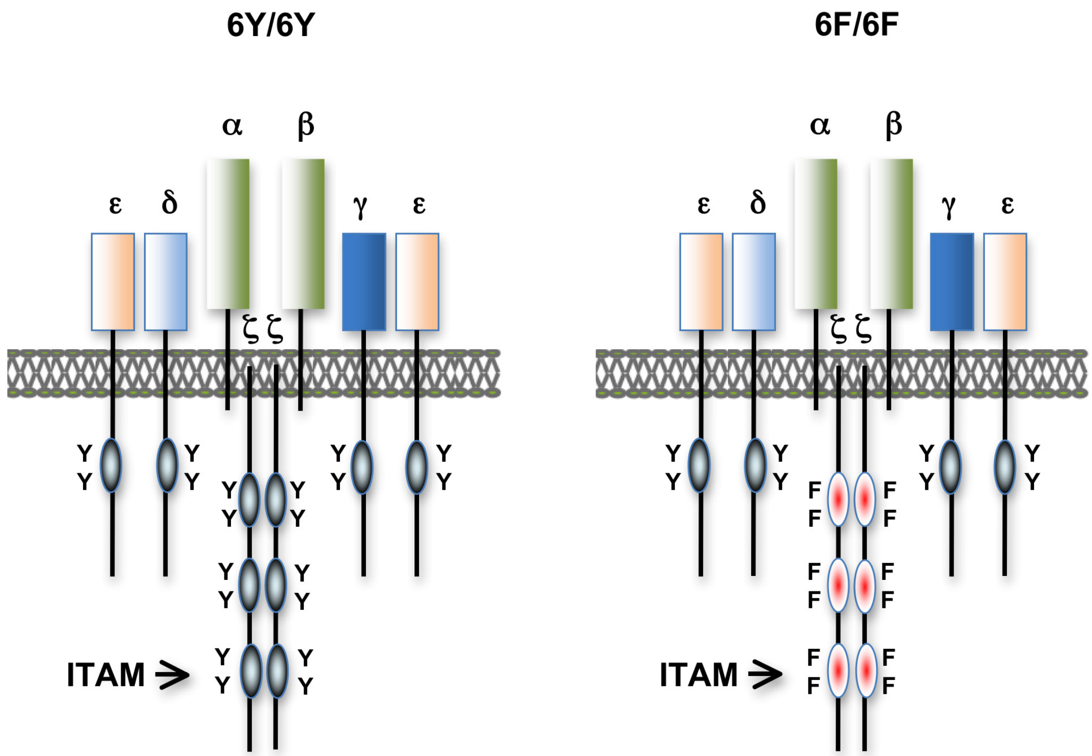

Figure 1. T cell antigen receptors expressed in 6Y/6Y and 6F/6F knock-in mice
Subunit composition of the T cell antigen receptors in 6Y/6Y and 6F/6F mice. 6Y/6Y mice express wild-type zeta chain dimers with functional ITAM signaling motifs that contain two tyrosine (Y) residues. 6F/6F mice express mutant zeta chain dimers in which the ITAM tyrosines have been changed to phenylalanine (F).

auto-immune disease, are deleted in the thymus (negative selection). Unexpectedly, we found that a complete complement of TCR–ITAMs is not required for most mature T cell effector functions. However, recent work showed a requirement for ITAM multiplicity for the generation of T follicular helper cells, which are required for optimal B cell antibody responses. One possible explanation for the relatively mild phenotype observed in the TCR ITAM–reduced mice is that ITAM–mediated signal amplification is not required for most mature T cell activation responses. Another is that, in ITAM–mutant mice, T cells exhibit normal functional responsiveness because of compensatory mechanisms imposed during development. To resolve this question, we recently generated a TCR–zeta chain conditional knockin mouse in which T cell development and selection can occur without attenuation of TCR signaling (i.e., in the presence of wild-type 3-ITAM "6Y" zeta chain), but in which mature, post-selection T cells may be induced to express TCRs containing signaling-defective (0-ITAM "6F") zeta chains in lieu of wild-type zeta chains (Figure 1). Thus, mature T cell signaling should not be influenced by potential compensatory mechanisms that operate during T cell maturation, and T cells in these mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. Experiments with the mice confirmed that the knockin zeta locus functions as predicted. We are currently using this model system to evaluate the role of ITAM multiplicity and ITAM–mediated signal amplification in T cell development, immune tolerance, and mature T cell function.

Identification and characterization of proteins important for TCR fine tuning and TCR signaling

We extended our analysis of TCR–signaling subunits to other molecules that participate in or influence the TCR–signaling response. The cell-surface protein CD5 negatively regulates TCR signaling and functions in thymocyte selection. Examination of CD5 expression during T cell development revealed that surface levels of CD5 are regulated by TCR signal intensity and by the affinity of the TCR for self-peptide ligands in the thymus that mediate selection. To determine whether the ability to regulate CD5 expression is important for thymocyte selection, we generated transgenic mice that constitutively express high

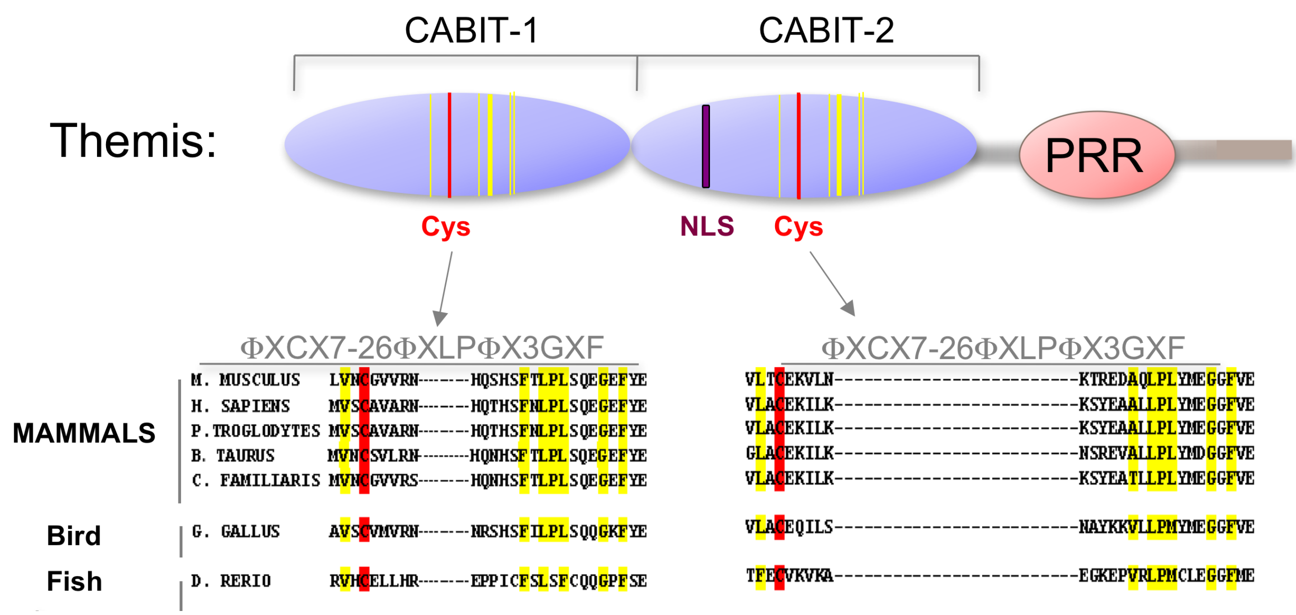

Figure 2. Themis is highly conserved in vertebrates.
Themis contains two novel CABIT domains, each with a conserved cysteine (*red*) and conserved flanking residues (*yellow*), a nuclear localization signal (NLS), and a Proline-Rich Region (PRR).

levels of CD5 throughout development. Overexpression of CD5 significantly impaired positive selection of some thymocytes (those that would normally express low levels of CD5) but not of others (those that would normally express high levels of CD5). The findings support a role for CD5 in modulating TCR signal transduction and thereby influencing the outcome of thymocyte selection. Current studies are centered on identifying the mechanism by which CD5 inhibits TCR signaling and whether the protein's regulated expression during development is important for preventing autoimmunity. The ability of individual thymocytes to regulate CD5 expression represents a mechanism for "fine tuning" the TCR signaling response during development so that the integrated signaling response can be adjusted to permit T cell functional competency without causing autoimmunity. Reasoning that, in addition to CD5, other molecules participate in TCR tuning, we initiated microarray-based screening for genes differentially expressed in developing T cells under conditions of high- or low-affinity TCR interactions. We identified several genes from this screen for further study and are validating their function as tuning molecules. Given that the molecules regulate TCR signaling, they represent potential autoimmune-disease susceptibility markers and potential targets for treatment of patients with autoimmune disease, similar to current 'checkpoint inhibitor' therapies that are based on blocking the function of the induced inhibitory molecules PD-1 and CTLA-4.

Identification and characterization of Themis, a novel protein required for T cell development

Using a subtractive cDNA library–screening approach, we recently identified Themis, a novel T cell–specific adapter protein (Figure 2). To investigate the function of Themis in T cell signaling and development, we generated Themis-knockdown cell lines, Themis knockout mice (conventional and conditional), and Themis-transgenic mice. Analysis of the effects of modulating Themis expression revealed a critical role for the protein in late T cell development. Current data indicate that Themis functions in the signaling pathway downstream of the T cell receptor (TCR), perhaps by integrating or sustaining TCR signaling. Our ongoing studies are focusing on elucidating the mechanism through which Themis participates in T cell signaling and regulates T cell development.

Role of Ldb1 transcription complexes in hematopoiesis and in T cell acute lymphoblastic leukemia

Lim domain binding protein-1 (Ldb1) is a ubiquitously expressed nuclear protein that contains a LIM–zinc finger protein

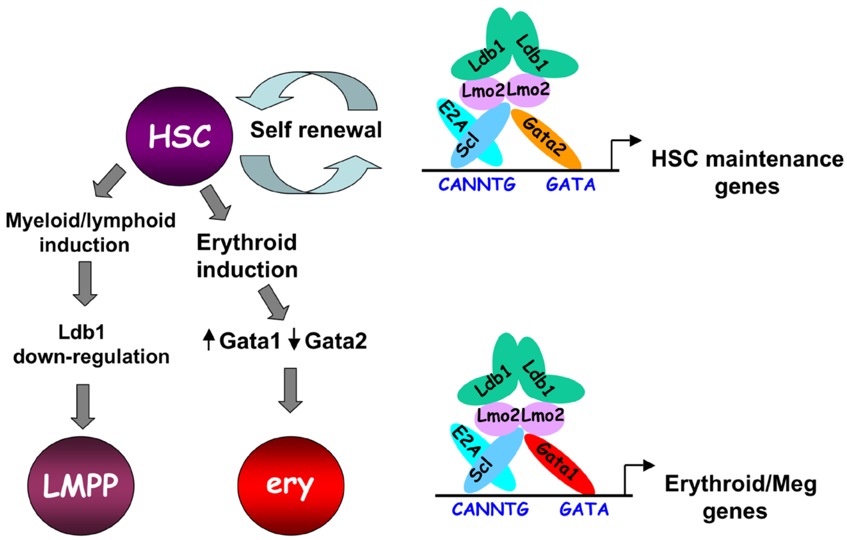

Figure 3. Model of Ldb1 function in the hematopoietic lineage

Ldb1 forms a multimeric DNA–binding complex in hematopoietic cells with the adapter Lmo2 and the transcription factors Scl and Gata1 or Gata2. In hematopoietic stem cells (HSCs), in which Gata2 is highly expressed, Ldb1-Lmo2-Scl-Gata2 complexes positively regulate expression of HSC maintenance genes. Differentiation of HSCs to the myeloid or lymphoid lineage (LMPP) is triggered by downregulation of Ldb1, whereas commitment to the erythroid lineage (ery) is triggered by induction of Gata1 and downregulation of Gata2, resulting in the formation of an Ldb1-Lmo2-Scl-Gata1 complex, which positively regulates expression of erythroid-specific genes.

interaction motif and a dimerization domain. In hematopoietic cells, Ldb1 functions by interacting with and/or recruiting specific partners (including the LIM–only protein Lmo2 and the transcription factors SCL/Tal1 and Gata1 or Gata2) to form multi-molecular transcription complexes (Figure 3). Within the hematopoietic lineage, expression of *Ldb1* is highest in progenitor cells, which include hematopoietic stem cells (HSCs). *Ldb1*–null (*Ldb1*$^{-/-}$) mice die between day 9 and 10 of gestation, preventing us from directly studying the impact of loss of Ldb1 on fetal or adult hematopoiesis. We investigated the role of Ldb1 in hematopoiesis by following the fate of *Ldb1*$^{-/-}$ embryonic stem cells (ESCs) in mouse blastocyst chimeras and by conditional, stage-specific deletion of *Ldb1*. Significantly, *Ldb1*$^{-/-}$ ES cells were capable of generating HSCs, which could give rise to both myeloid and lymphoid lineage cells; however, the number of *Ldb1*$^{-/-}$ HSCs gradually diminished at later stages of development. Following adoptive transfer of fetal liver hematopoietic progenitor cells, *Ldb1*$^{-/-}$ HSCs were rapidly lost, indicating a failure of self-renewal or survival. More recent data indicate that the loss of *Ldb1*$^{-/-}$ HSCs results from differentiation rather than cell death. Although expressed in ESCs, *Ldb1* expression is not required for ESC maintenance, indicating a selective requirement in adult stem cell populations. We performed a genome-wide screen for Ldb1–binding sites using ChIP-seq. Analysis of the ChIP-Seq data revealed that Ldb1 complexes bind at the promoter or regulatory sequences near a large number of genes known to be required for HSC maintenance. The data suggest that Ldb1 complexes function in a manner similar to Oct4/nanog/Sox2 in ES cells to regulate a core transcriptional network required for adult stem cell maintenance. Examination of the function of Ldb1 in lineages downstream of the HSC identified an essential function in the erythroid lineage but not in other myeloid cells or lymphoid cells. Interestingly, ChIP-Seq analysis of Ldb1 DNA–binding complexes demonstrated that, in HSCs, Ldb1 complexes contain Gata2 whereas, in erythroid progenitors, Ldb1 complexes contain Gata1 (which is highly expressed in the erythroid lineage). The results indicate that multimeric Ldb1 transcription complexes have distinct functions in the hematopoietic system depending on their subunit composition, with Gata2–containing complexes regulating expression of HSC–maintenance genes and Gata1 complexes regulating expression of erythroid-specific genes (Figure 3). Current studies are aimed at investigating how Ldb1 complexes regulate gene expression and the role of Ldb1 dimerization in mediating long-range promoter-enhancer interactions in hematopoietic cells. In addition, the lab is investigating a potential role for Ldb1 in regulating self-renewal of T cell progenitors in the thymus.

Acute lymphoblastic leukemias are the most common type of cancer in children. T cell acute lymphoblastic leukemia (T-ALL) results from oncogenic transformation of immature T cell progenitors (thymocytes). Mouse models of T-ALL have been generated, and one of the most informative is the Lmo2-transgenic (*Lmo2*-tg) mouse, which expresses high levels of the nuclear adapter Lmo2 in thymocytes. The model closely mimics a prevalent type of human T-ALL, which is associated with chromosomal mutations that result in increased expression of Lmo2. We recently reported that overexpression of Lmo2 in mouse thymocytes induces T-ALL at two distinct stages of development (an early 'ETP' stage and a later 'DN3' stage).

Notably, human T-ALLs can also occur at two similar stages of thymocyte maturation. The most immature forms of T-ALL in *Lmo2*-tg mice and in humans express high levels of the transcription factor *Hhex* and are designated Early T Progenitor (ETP) T-ALL, whereas later-stage tumors are *Hhex* low but express high levels of more mature markers of T cell development, including *Notch1*, *Dtx1*, *Ptcra*, and *Hes1*. Lmo2 functions as a subunit of multimeric Ldb1-nucleated DNA binding complexes. We found that normal ETP thymocyte progenitor cells express the same Ldb1 complex subunits that are present in HSCs and that ETPs exhibit HSC characteristics including self-renewal potential. ETPs in *Lmo2*-tg mice appear to be 'locked' into a pattern of perpetual self-renewal and are refractory to normal inductive signals that promote further differentiation. *Hhex* is a target of Ldb1 complexes in HSCs and ETPs, a result that strongly suggests that Ldb1 complexes are responsible for the aberrant self-renewal in *Lmo2*-tg mice that predisposes to oncogenesis. We hypothesize that Ldb1 complexes regulate self-renewal in ETPs as well as in HSCs. Lmo2 is normally down-regulated when thymocytes undergo T-lineage commitment, suggesting that extinguishing expression of Lmo2 (and by extension, Ldb1 complexes) is important for T cell differentiation and that failure to do so predisposes to oncogenesis via 'second-hit' transforming events.

In preliminary RNA-Seq gene expression experiments, we found that the RNA expression signatures of *Lmo2*-tg immature thymocytes and HSCs are very similar, consistent with the notion that Lmo2 overexpression 'freezes' cells in a stem cell self-renewal state. To determine whether Ldb1 complexes are in fact required for ETP self-renewal and to explore the genes regulated by these complexes, we will determine whether Ldb1 is required for *Lmo2*-tg–induced thymocyte self-renewal. These experiments will allow us to address several key questions, including whether, as predicted, Ldb1, and by extension Ldb1 complexes, regulate expression of genes that control a self-renewal genetic program in ETPs and whether Ldb1 complexes are necessary for the transcriptional/developmental effects of Lmo2. Importantly, we will also analyze the mice for T-ALL. T-ALL formation is highly penetrant in *Lmo2*-tg mice (virtually 100% of mice develop T-ALL by one year). We will investigate whether the timing or frequency of T-ALL is different if Ldb1 is deleted. We anticipate that these results will provide insights into the mechanisms controlling T-ALL oncogenesis in humans and may provide new therapeutic avenues for treatment of this devastating pediatric disease.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, Love PE, Peng W, Xue HH, Crotty S. LEF-1 and TCF-1 orchestrate TFH differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol* 2015; 16:980–990.
2. Hwang S, Palin AC, Li L, Song KD, Lee J, Herz J, Tubo N, Chu H, Pepper M, Lesourne R, Zvezdova E, Pinkhasov J, Jenkins MK, McGavern D, Love PE. TCR ITAM multiplicity is required for the generation of follicular helper T-cells. *Nat Commun* 2015; 6:6982.
3. Smith S, Tripathi R, Goodings C, Cleveland S, Mathias E, Hardaway JA, Elliott N, Yi Y, Chen X, Downing J, Mullighan C, Swing DA, Tessarollo L, Li L, Love P, Jenkins NA, Copeland NG, Thompson MA, Du Y, Davé UP. LIM domain only-2 (LMO2) induces T-cell leukemia by two distinct pathways. *PLoS One* 2014; 9:e85883.
4. Vacchio MS, Wang L, Bouladoux N, Carpenter AC, Xiong Y, Williams LC, Wohlfert E, Song KD, Belkaid Y, Love PE, Bosselut R. A ThPOK-LRF transcriptional node maintains the integrity and effector potential of post-thymic CD4+ T cells. *Nat Immunol* 2014; 15:947–956.
5. Yokoyama W, Kohsaka H, Kaneko K, Walters M, Takayasu A, Fukuda S, Miyabe C, Miyabe Y, Love PE, Nakamoto N, Kanai T, Watanabe-Imai K, Charvat TT, Penfold ME, Jaen J, Schall TJ, Harigai M, Miyasaka N, Nanki T. Abrogation of CC chemokine receptor 9 ameliorates collagen-induced arthritis of mice. *Arthritis Res Ther* 2014; 16:445.

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Embryonic development requires the coordinated activities of transcription factors that bind to DNA and the enzymatic machinery that alters the chromatin state of DNA to "dial" gene expression programs and achieve a prodigious diversity of transcriptional outputs. This includes the activation of genes necessary to carry out cellular functions in distinct cell types, but also suppression of parasitic mobile elements, including endogenous retroviruses (ERVs), that are a threat to genomic stability. Our objective is to understand the dichotomy between transcription factors and chromatin. On the one hand, transcription factors bind to DNA to guide the formation of specific heritable chromatin states (which we explore by studying the initiation of heterochromatin formation by the KRAB-ZFP protein family), but on the other, the chromatin state also imposes a layer of control on how transcription factors interact with DNA, effectively "gating" transcription factor occupancy (which we explore by studying the effect of chromatin state on transcription factors implicated in motor neuron development). The tools we use to explore these questions are the same. We apply cutting-edge genetic and synthetic biology approaches to mouse embryonic stem cell–based models of development, allowing us to perform biochemical and next-generation sequencing experiments, which require large cell numbers. These *in vitro* approaches complement *in vivo* studies with mutant and transgenic mice to give us a unique mechanistic understanding of transcriptional regulation in mammalian development. Such foundational studies will lay the framework for our long-term objectives: to be able to use transcription factors in a rational way for cellular programming such that we can build better models of human disease and open new avenues in regenerative medicine.

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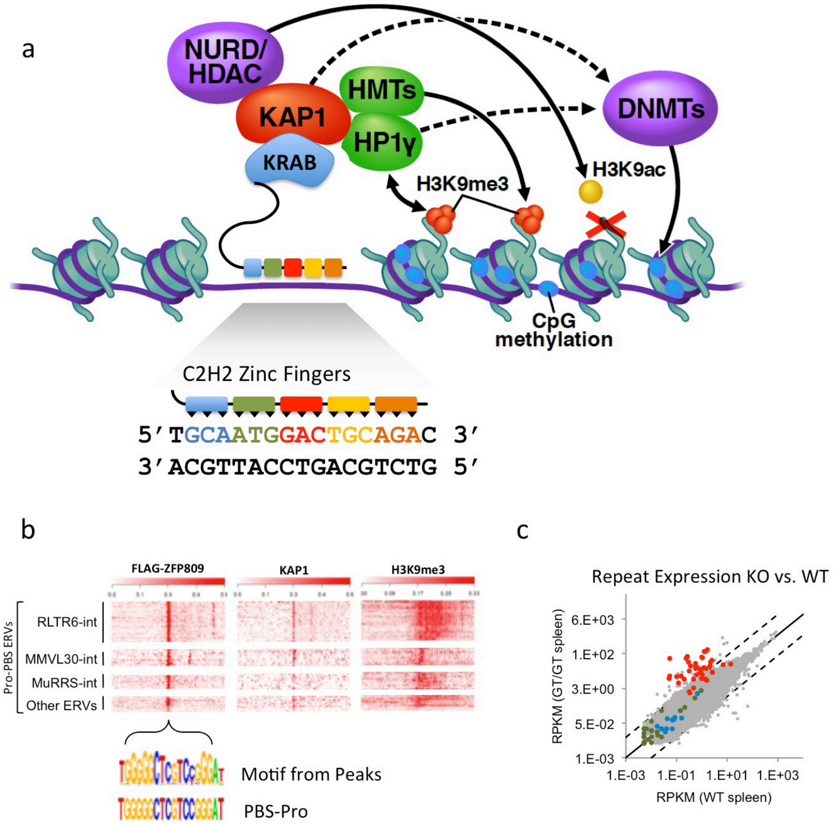
Regulation of endogenous retroviruses by KRAB-zinc finger proteins

Retroviruses pose a threat to human health by infecting somatic cells, but retroviruses have also been infecting our mammalian ancestors for millions of years, accumulating in the germ-line as ERVs that account for nearly 10% of our genomic DNA. The laboratory studies ERVs from two perspectives: (1) as parasites that must be kept in check by the host to prevent widespread viral activation; and 2) as symbionts that can be co-opted by the host for evolutionary advantage. Our objective is to understand how the host has adapted recognition machinery to establish stable epigenetic silencing of ERVs, how ERVs sometimes evade these silencing mechanisms, and how the evasive activities have led to host co-option of viral regulatory sequences that may have contributed to evolution of mammals. We hypothesize that the rapidly diversifying KRAB-ZFP family plays a critical role in the recognition and silencing of ERVs.

Krüppel-associated box zinc finger proteins (KRAB-ZFPs) have emerged as candidates that recognize ERVs. KRAB-ZFPs are rapidly evolving transcriptional repressors that emerged in tetrapods. They make up the largest family of transcription factors in mammals (estimated to be about 200–300 in mice and humans)[1]. Each species has its own unique repertoire of KRAB-ZFPs, with a small number shared with closely related species and a larger fraction specific to each species. Despite their abundance, little is known about



ZFP809 represses VL30^{Pro} LTR-retroelements.

a. Model of KRAB-ZFP protein function. KRAB-ZFPs consist of KRAB–repression domain that interacts with KAP1 to assemble a corepressor complex, establishing stable epigenetic silencing of targets. Tandem C2H2 zinc finger (ZF) domains mediate sequence-specific DNA interactions, with each ZF interacting with three nucleotides of target DNA.
b. Heat map plots of ChIP-seq data (with indicated antibodies) of Flag-ZFP809 peak regions. Peaks were centered on PBS^{Pro}–like motif and grouped according to repeat masker annotation.
c. RNA-seq differential expression analysis of all repeat masker annotated elements in Zfp809 wild-type (WT) vs. knockout (KO) spleen. Red dots indicate VL30^{Pro} elements, most of which are reactivated in KOs.

their physiological functions. KRAB-ZFPs consist of an N-terminal KRAB domain that binds to the co-repressor KAP1[2] and a variable number of C-terminal C2H2 zinc finger domains that mediate sequence-specific DNA binding. KAP1 directly interacts with the KRAB domain[3], which recruits the histone methyltransferase (HMT) SETDB1 and heterochromatin protein 1 (HP1) to initiate heterochromatic silencing[4,5]. Several lines of evidence point to a role for the KRAB-ZFP family in ERV silencing. First, the number of C2H2 zinc finger genes in mammals correlates with the number of ERVs[6]. Second, the KRAB-ZFP protein ZFP809 was isolated based on its ability to bind to the primer binding site for proline tRNA (PBS^{Pro}) of murine leukemia virus (MuLV)[7]. Third, deletion of the KRAB-ZFP co-repressors *Trim28* or *Setdb1* leads to activation of many ERVs[8,9]. Thus, we have begun a systematic interrogation of KRAB-ZFP function as a potential adaptive repression system against ERVs.

We focused on ZFP809 as a likely ERV–suppressing KRAB-ZFP owing to the fact that it was originally identified as part of a repression complex that recognizes infectious MuLV via direct binding to the 18 nt PBS^{Pro} sequence[7,10]. We hypothesized that ZFP809 might function *in vivo* to repress other ERVs that utilized the PBS^{Pro}. Using ChIP-seq of epitope-tagged ZFP809 in embryonic stem cells (ESCs) and embryonic carcinoma (EC) cells, we determined that ZFP809 bound to several sub-classes of ERV elements via the PBS^{Pro}. We generated *Zfp809* knockout (KO) mice to determine whether ZFP809 was required for the silencing of VL30^{Pro}, an endogenous retroviral element that contains PBS^{Pro}. We found that *Zfp809* KO tissues display high levels of VL30^{Pro} elements and that the targeted elements display an epigenetic shift from repressive epigenetic marks (H3K9me3 and CpG methylation) to active marks (H3K9Ac and CpG hypo-methylation). ZFP809–mediated repression extended to a handful of genes that contained adjacent VL30^{Pro} integrations. Furthermore, using a combination of conditional alleles and rescue experiments, we determined that ZFP809 activity is required in development to initiate silencing, but not in somatic cells to maintain silencing. The studies provided the first demonstration for the *in vivo* requirement of a KRAB-ZFP in the recognition and silencing of ERVs.

KRAB-ZFPs such as ZFP809 initiate ERV silencing by establishing the methylation of histone H3 on lysine 9 via the recruitment of the histone methylatransferase SETDB1. Mice have three histone H3 variants (H3.1, H3.2, and H3.3), and whether one or more of these variants was critical for ERV silencing had not been explored. Using ChIP-seq in primary mouse embryonic fibroblasts (PMEFs) and induced pluripotent stem cells (iPSCs) containing genetically tagged histone H3.3 genes, we found a strong enrichment of the variant histone H3.3 at ERVs co-occupied by KAP1, SETDB1, and H3K9me3, including VL30[Pro] elements recognized by ZFP809. Importantly, this enrichment was present only in pluripotent cells. We therefore explored the possibility that the deposition of histone H3.3 might be required for ERV silencing. To test this hypothesis, we used CRISPR/Cas9 to create a homozygous 'floxed' *Daxx* gene, which had previously been shown to be responsible for H3.3 deposition at telomeres in ESCs[11]. We found that acute loss of *Daxx* by Cre-mediated recombination in ESCs caused a complete loss of histone H3.3 at ERVs, but only very minor ERV reactivation. The data suggest that DAXX–dependent deposition of histone H3.3 is dispensable for ERV silencing. The finding is in conflict with a recent report that argued that some ERVs display reactivation in histone H3.3 KO ESCs, and that IAP elements, in particular, become transpositionally active[12]. Our re-analysis of this dataset challenges this central conclusion. We found that there is no correlation between ERVs marked by histone H3.3 and those showing reactivation in H3.3 KOs; furthermore, we demonstrated that the reported IAP 're-integrations' are not a result of retrotransposition, but were simply polymorphic IAP ERV elements mixed into the genetic background of the ESCs used for the study. Our data support a model in which histone H3.3 is deposited into ERVs in pluripotent cells, but that this deposition is not required for ERV silencing, which has been supported further in the recent literature[13,14].

[1] Urrutia R. KRAB-containing zinc-finger repressor proteins. *Genome Biol* 2003;4:231.

[2] Friedman JR et al. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev* 1996;10:2067–2078.

[3] Peng H et al. Biochemical analysis of the Kruppel-associated box (KRAB) transcriptional repression domain. *J Biol Chem* 2000;275:18000–18010.

[4] Ryan RF et al. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol Cell Biol* 1999;19:4366–4378.

[5] Schultz DC et al. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 2002;16:919–932.

[6] Thomas JH, Schneider S. Coevolution of retroelements and tandem zinc finger genes. *Genome Res* 2011;21:1800–1812.

[7] Wolf D, Goff SP. Embryonic stem cells use ZFP809 to silence retroviral DNAs. *Nature* 2009;458:1201–1204.

[8] Rowe HM et al. KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature* 2010;463:237–240.

[9] Matsui T et al. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 2010;464:927–931.

[10] Wolf D, Goff SP. TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. *Cell* 2007;131:46–57.

[11] Goldberg AD et al. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* 2010;140:678–691.

[12] Elsasser SJ et al. Histone H3.3 is required for endogenous retroviral element silencing in embryonic stem cells. *Nature* 2015;522:240–244.

[13] Jang CW et al. Histone H3.3 maintains genome integrity during mammalian development. *Genes Dev* 2015;29:1377-1392.

[14] Sadic D et al. Atrx promotes heterochromatin formation at retrotransposons. *EMBO Rep* 2015;16:836–850.

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PUBLICATIONS

1. Wolf G, Yang P, Füchtbauer A, Füchtbauer EM, Silva AM, Park C, Nielsen AL, Pedersen FS, Macfarlan TS. The KRAB zinc finger protein ZFP809 is required to initiate epigenetic silencing of endogenous retroviruses. *Genes Dev* 2015; 29:538–554.
2. Wang J, Telese F, Tan Y, Li W, Jin C, He X, Basnet H, Ma Q, Merkurjev D, Zhu X, Liu Z, Zhang J, Ohgi K, Taylor H, White RR, Macfarlan TS, Pfaff SL, Rosenfeld MG. LSD1n is a H4K20 demethylase regulating memory formation via transcriptional elongation control. *Nat Neurosci* 2015; 18:1256–1264.

3. Lee H, Kim M, Kim N, Macfarlan T, Pfaff SL, Mastick GS, Song MR. Slit and Semaphorin signaling governed by Islet transcription factors positions motor neuron somata within the neural tube. *Exp Neurol* 2015; 269:17–27.
4. Wolf G, Greenberg D, Macfarlan TS. Spotting the enemy within: targeted silencing of foreign DNA in mammalian genomes by the Kruppel-associated box zinc finger protein family. *Mobile DNA* 2015; 6:17.
5. Wolf G, Macfarlan TS. Revealing the complexity of retroviral repression. *Cell* 2015; 163:30–32.

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We are interested in how the biogenesis of and metabolism pathways for RNAs, especially tRNAs and certain mRNAs, intersect with pathways related to cell proliferation, growth, and development. We focus on the synthesis of tRNAs by RNA polymerase (RNAP, Pol) III, the early phases of their post-transcriptional handling by the RNA–binding protein La, and certain modifications that impact their translational function. The La protein is a target of autoantibodies prevalent in (and diagnostic of) patients with Sjögren's syndrome, systemic lupus, and neonatal lupus. La contains several nucleic acid–binding motifs as well as several subcellular trafficking signals and associates with non-coding and messenger RNAs to coordinate activities in the nucleus and cytoplasm. The La protein functions by protecting its small RNA ligands from exo-nucleolytic decay and also by serving as chaperone during folding. In addition to its major products (tRNAs and 5S rRNA), RNAP III synthesizes certain other non-coding RNAs. La-related protein-4 (LARP4) interacts with certain mRNAs and contributes to translational control and the cell's growth capacity. Somewhat similar to La, LARP4 interacts with the 3′ end regions of its target RNAs, but in this case directed to the poly(A) motif (Yang et al., 2011 *Mol Cell Biol* 31:542-556).

We focus on the tRNA–modification enzymes Trm1, which synthesizes dimethyl-guanosine-26 (m2,2G26) in several tRNAs, and tRNA isopentenyltransferase (TRIT1), which modifies tRNA by adding an isopentenyl group onto the adenine at position 37 of certain tRNA (i6A37) molecules. We are examining the effects of TRIT1 on tRNA activity in translation in a codon-specific manner (Lamichhane et al., *Mol Cell Biol* 2013;33:4900; Lamichhane et al., *Mol Cell Biol* 2013;33:2918), and during mammalian development, and how its deficiency leads to childhood metabolic disease. We are also investigating how differences in the copy number of tRNA genes, which we found does indeed vary among humans, can affect how the genetic code is deciphered. Tumor suppressors and oncogenes mediate deregulation of transcript production by Pol III, the RNA polymerase responsible for tRNA synthesis, thus contributing to increased capacity for proliferation of cancer cells.

We thus strive to understand the structure-function relationship and cell biology of La, TRIT1, and LARP4 and their contribution to growth and development. We use genetics, cell and structural biology, and biochemistry in model systems that include yeast, human tissue culture cells, and gene-altered mice.

Functions of the La antigen in RNA expression

Recent findings regarding nucleolar localization, cytoplasmic splicing, and retrograde transport indicate that the tRNA production pathway is more complex in its biochemistry, spatial organization, and sequential order than previously thought. By binding to UUU-3′OH, the La protein shields newly transcribed pre–tRNAs from 3′-end digestion and functions as a chaperone for misfolded or otherwise imperfect pre–tRNAs. Thus, it has become clear that La serves the tRNA pathway at several levels, including protection of pre–tRNAs from 3′ exonucleases; nuclear retention of pre–tRNAs, thereby

Figure 1. The fission yeast *Schizosaccharomyces pombe* as a model organism
Red-white colony differentiation by tRNA–mediated suppression

preventing premature export of pre–tRNAs; and promotion of a newly identified processing step distinct from 3′-end protection.

Studies in gene-altered mice revealed that La is required for cell survival in developing B cells of the immune system and in post-mitotic cells in the cerebral cortex in the developing brain (Gaidamakov et al., *Mol Cell Biol* 2014;34:123-131).

To study Pol III– and La-dependent tRNA biogenesis, we developed a red-white tRNA–sensitive reporter system in the fission yeast *Schizosaccharomyces pombe* (Figure 1), a yeast that generally appears more similar to the human organism than does *Saccharomyces cerevisiae* with respect to cell-cycle control, gene-promoter structure, and the complexity of pre–mRNA splicing. From sequence analysis of Pol III–transcribed genes, we predicted and then confirmed that Pol III termination–signal recognition in *S. pombe* would be more similar to human Pol III than it is for *S. cerevisiae* Pol III. Our system is based on tRNA–mediated suppression of a nonsense codon in ade6-704 and affords the benefits of fission yeast biology while lending itself to certain aspects of 'humanization.' We have been able to study the tRNA processing–associated function of the human La protein (hLa) because it is so highly conserved that it can replace the processing function of the *S. pombe* La protein Sla1p *in vivo*.

Briefly, we found that: (1) the human pattern of phosphorylation of hLa at the serine-366 target site by the protein kinase CK2 occurs faithfully in *S. pombe* and promotes tRNA production; (2) various conserved subcellular trafficking signals in La proteins can be positive or negative determinants of tRNA processing; (3) La can protect pre–tRNAs from the nuclear surveillance 3′ exonuclease Rrp6p; (4) the 3′ exonuclease that processes pre–tRNAs in the absence of Sla1p is distinct from Rrp6p; (5) Sla1p is limiting in *S. pombe* cells, and the extent to which it influences the use of alternative tRNA maturation pathways is balanced by the RNA 3′–5′ cleavage activity of the Pol III termination–associated Pol III subunit Rpc11p; and (6) La proteins use distinct RNA–binding surfaces, one on the La motif (LM) and the other on the RNA recognition motif-1 (RRM1), to promote different steps in tRNA maturation.

Results of our recent work suggest that La can use several surfaces, perhaps combinatorially, to engage various classes of RNAs, e.g., pre–tRNAs versus mRNAs, or to perform different functions (Huang *et al.*, *Nat Struct Mol Biol* 2006;13:611; Maraia and Bayfield, *Mol Cell* 2006;21:149). Consistent with this notion, some pre–tRNAs require only the UUU–3′OH binding activity while others depend on a second activity in addition to 3′-end protection that requires an intact RRM surface to promote a previously unknown step in tRNA maturation. One of our objectives is to identify cellular genes other than La that contribute to this 'second' activity. Toward this goal, we isolated and have begun to characterize *S. pombe* revertant mutants that overcome a defect in the second activity.

Activities of RNA polymerase III and associated factors

The RNA polymerase III (RNAP III, Pol III) enzyme consists of 17 subunits, several with strong homology to subunits of RNAPs I and II. In addition, the transcription factor TFIIIC, composed of six subunits, binds to the A- and B-box promoters and recruits TFIIIB to direct Pol III to the correct start site. Pol III complexes are highly stable and demonstrate great productivity in supporting many cycles of initiation, termination, and re-initiation. For example, each of the 5S rRNA genes in human cells must produce approximately 10^4 to 10^5 transcripts per cell division to provide sufficient 5S rRNA for ribosomes. While RNAPs I, II, and III are homologous, their properties are distinct in accordance with the unique functions related to the different types of gene they transcribe. Given that some mRNA genes can be hundreds of kilobase-pairs long, RNAP II must be highly processive and avoid premature termination. RNAP II terminates in response to complex termination/RNA–processing signals that require endo-nucleolytic cleavage of RNA upstream of the elongating polymerase. By contrast, formation of the UUU–3′OH terminus of nascent RNAP III transcripts appears to occur at the RNAP III active center. The dT(n) tracts at the ends of class III genes directly signal pausing and release by RNAP III such that termination and RNA 3′-end formation are coincident and efficient (Arimbasseri and Maraia, *Mol Cell Biol* 2013;33:1571).

Transcription termination delineates 3′ ends of gene transcripts, prevents otherwise runaway RNA polymerase (RNAP) from intruding into downstream genes and regulatory elements, and enables release of the RNAP for recycling. While other RNAPs require complex *cis* signals and/or accessory factors to accomplish these activities, eukaryotic RNAP III does so autonomously with high efficiency and precision at a simple oligo(dT) stretch of 5–6 bp. A basis for this high density *cis* information is that both the template and non-template strands of the RNAP III terminator carry distinct signals for different stages of termination. High-density *cis* information is a feature of the RNAP III system that is also reflected by dual functionalities of the tRNA promoters as both DNA and RNA elements. Furthermore, the TFIIF–like RNAP III subunit C37 is required for this function of the non-template strand signal. The results reveal the RNAP III terminator as an information-rich control element. While the template strand promotes destabilization via a weak oligo(rU:dA) hybrid, the non-template strand provides distinct sequence-specific destabilizing information through interactions with the C37 subunit (Reference 5).

La-related protein-4 (LARP4) in translation-coupled mRNA stabilization

Ubiquitous in eukaryotes, the La proteins are involved in two broad functions: (1) metabolism of a wide variety of precursor tRNAs and other small nuclear RNAs by association with the RNAs' common UUU-3′ OH–terminal elements; and (2) by unknown mechanisms, translation of specific subsets of mRNAs, such as those containing iron-response elements (IRES) and other motifs. The La-related protein LARP7/PIP7S exhibits a specialized UUU-3′ OH–related function in its specific interaction with 7SK snRNA. Another La-related protein, LARP4, is conserved in metazoa and, in accordance with experimental data we obtained, appears to be a translation factor. Unlike La and LARP7, LARP4 localizes to the cytoplasm, as demonstrated by immunofluorescence, and contains a highly conserved sequence similar to but a variant of the poly-A binding protein (PABP)–interaction motif-2 (PAM2) consensus found in other translation factors, including Paip1 and Paip2. PABP co-immunoprecipitates with Flag-LARP4 (F-LARP4) from human cells in an RNase–insensitive manner, while substitution of two key residues in the variant PAM2 consensus reduces PABP co-immunoprecipitation. F-LARP4 specifically co-immunoprecipitates with two other translation factors that we examined—elF4G and RACK1—although the interactions are sensitive to RNase. Antibodies to LARP4 showed that native endogenous LARP4 is cytoplasmic, co-immunoprecipitates with PABP in an RNase–insensitive manner, and co-sediments with the 40S subunit peak and polysomes; however, the peak shifts upon puromycin treatment to one indicating a smaller size than the 40S mRNP. Luciferase translation reporter assays in control and siRNA LARP4 knockdown cells provided evidence that LARP4 promotes general translation. The ability of LARP4 to stimulate translation of the luciferase reporter is correlated with its ability to stabilize mRNA levels. Indeed, actinomycin D studies show that LARP4 is an mRNA–stabilizing factor. Additional assays developed to better examine decay indicate a role for LARP4 in mRNA stability.

Fission yeast as a model system in which to study pathways of rapamycin sensitivity caused by defects in tRNA metabolism

The antitumor drug rapamycin inhibits the master growth regulator and signal integrator TOR, which coordinates ribosome biogenesis and protein-synthetic capacity with nutrient homeostasis and cell cycle progression. Rapamycin inhibits proliferation of the yeast *S. cerevisiae* and human cells whereas proliferation of the yeast *S. pombe* is resistant to rapamycin. We found that deletion of the *tit1* gene, which encodes tRNA isopentenyltransferase, causes *S. pombe* proliferation to become sensitive to rapamycin, with a 'wee' phenotype (smaller than normal cells as a result of premature entry into mitosis), suggesting a cell-cycle defect. The gene product of *tit1* is a homolog of *S. cerevisiae* MOD5, the human tumor suppressor TRIT1, and the *C. elegans* life-span gene product GRO-1, enzymes that isopentenylate N6-adenine-37 (i6A37) in the anticodon loop of a small subset of tRNAs. Anticodon loop modifications are known to affect codon-specific decoding activity. Indicating a requirement for i6A37 for optimal codon-specific translation efficiency, as well as defects in carbon metabolism related to respiration, *tit1*$^{\Delta}$ cells exhibit anti-suppression. Genome-wide analyses of gene-specific enrichment of codons cognate to i6A37–modified tRNAs identify genes involved in ribosome biogenesis, carbon/energy metabolism, and cell cycle genes, congruous with *tit1*$^{\Delta}$ phenotypes. We found that mRNAs enriched in codons cognate to i6A37–modified tRNAs are translated less efficiently than mRNAs with low content of the cognate codons. We determined that the Tit1p–modified tRNA Tyr exhibits about five-fold higher specific decoding activity during translation than the unmodified tRNA Tyr.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Yarham JW, Lamichhane TN, Pyle A, Mattijssen S, Baruffini E, Bruni F, Donnini C, Vassilev A, He L, Blakely EL, Griffin H, Santibanez-Koref M, Bindoff LA, Ferrero I, Chinnery PF, McFarland R, Maraia RJ, Taylor RW. Defective i6A37 modification of mitochondrial and cytosolic tRNAs results from pathogenic mutations in TRIT1 and its substrate tRNA. *PLoS Genet* 2014; 10:e1004424.
2. Maraia RJ, Iben JR. Different types of secondary information in the genetic code. *RNA* 2014; 20:977–984.
3. Arimbasseri AG, Kassavetis GA, Maraia RJ. Transcription. Comment on "Mechanism of eukaryotic RNA polymerase III transcription termination." *Science* 2014; 345:524.
4. Iben JR, Maraia RJ. tRNA gene copy number variation in humans. *Gene* 2014; 536:376–384.
5. Arimbasseri AG, Maraia RJ. Mechanism of transcription termination by RNA polymerase III utilizes a non-template strand sequence-specific signal element. *Mol Cell* 2015; 58:1124–1132.

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Macrophages and dendritic cells (DC) respond to pathogen stimuli by producing cytokines, including interferons (IFNs) and IL-1 (interleukin-1), IL-6, and TNF-alpha. While IFNs impart anti-viral and anti-microbial protection to the host, the latter cytokines are associated with inflammatory responses. IFNs are produced upon activation of IRF (interferon regulatory factor) family of transcription factors, while inflammatory cytokines are produced by the activation of NFκB. Our goal is to study the molecular pathways that direct the development and function of macrophages and DCs. To this end, we focus on the role of IRF8 in innate immunity. IRF8, a member of the IRF family, is expressed in macrophages, DCs, and microglia at high levels and is required for the production of both type I and type II IFNs. IRF8 is essential for mounting the first line of defense against various invading pathogens prior to the initiation of antigen-specific immune responses.

Transcriptionally active genes are embedded in chromatin that is dynamically exchanged, whereas silenced genes are surrounded by more stable chromatin. The chromatin environment contributes to the epigenetic states of given cells and influences transcriptional processes. We have long been working on BRD4, a chromatin-binding protein that affects transcription elongation. In this context, we are interested in histone H3.3, the variant histone that is associated with actively expressed genes. H3.3 has the unique property of being incorporated into nucleosomes and DNA only in actively transcribed genes. In contrast, H3.1, H3.2, and other standard core histones are incorporated into nucleosomes during DNA replication. For this strong association with transcription, H3.3 is implicated for epigenetic regulation. However, the process and its physiological significance of H3.3 deposition remains obscure. Our goal is to elucidate the activity of BRD4 and histone H3.3 in the context of epigenetic regulation of innate immunity.

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Dynamics of histone H3.3 deposition

H3.3 is similar to the canonical histone H3 (H3.1 and H3.2) in its structure, differing only in a few amino acids. Unlike the canonical histone H3, however, H3.3 (encoded by two genes in the human and mouse) is synthesized outside of the S phase and is incorporated into chromatin along with transcription. It is thought that H3.3 associates with the preexisting canonical histone H4 to form a new nucleosome, presumably along with the histone H2AZ and the preexisting H2B. H3.3 incorporation into chromatin requires specific chromatin assembly factors distinct from those involved in replication-coupled histone deposition. HIRA is a major H3.3–specific histone chaperone dedicated to transcription-coupled H3.3 incorporation. HIRA carries the WD40 domain and B domain, through which it interacts with three other subunits to act as a chromatin assembly factor. Two other H3.3–specific chaperones, ATRX and Daxx, are reported to be involved in H3.3 incorporation into telomeres and pericentric heterochromatin.

We previously studied transcription-coupled H3.3 incorporation in IFN–stimulated genes (ISGs) in mouse embryonic fibroblasts (MEFs). Conventional ChIP analysis demonstrated that H3.3 is incorporated into ISGs upon IFN stimulation throughout the gene body and transcription end

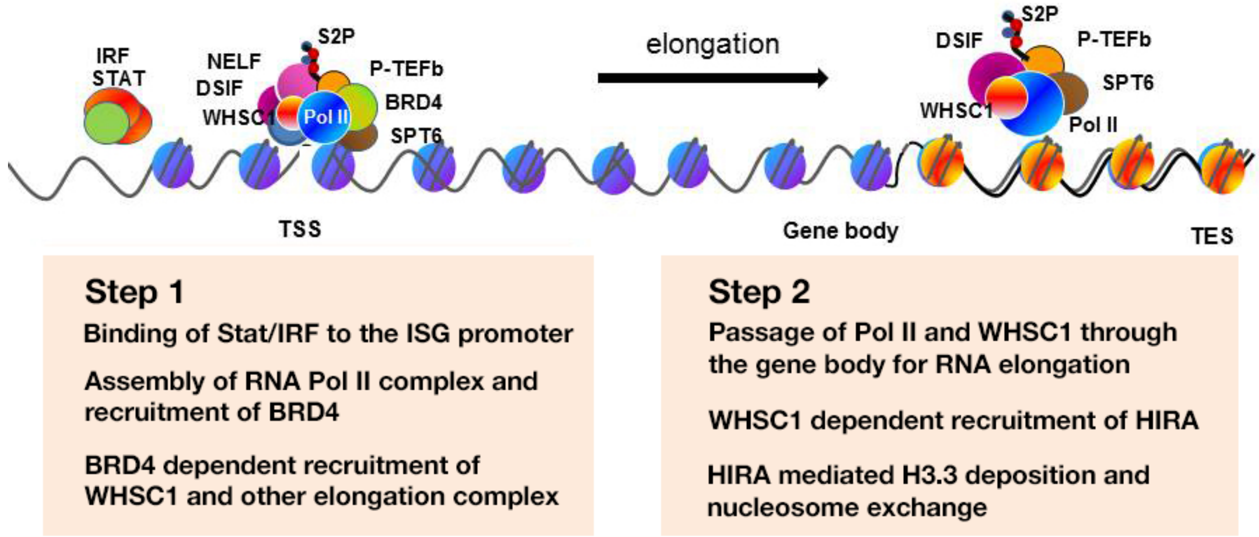


Figure 1. Elongation-coupled histone H3.3 deposition in IFN-stimulated genes

site (TES). Additional methylation of H3.3 at lysine 36 was induced after IFN stimulation, closely correlating with the H3.3 incorporation both in timing and spatial distribution. We discovered that WHSC1 (also known as NSD2) plays a significant role in H3.3 deposition and H3K36 methylation (mostly dimethylation), as evidenced by the absence of H3.3 deposition in Whsc1$^{-/-}$ MEFs. With additional data, we proposed a working model in which WHSC1 interacts with Brd4 and HIRA to prompt H3.3 deposition into ISGs.

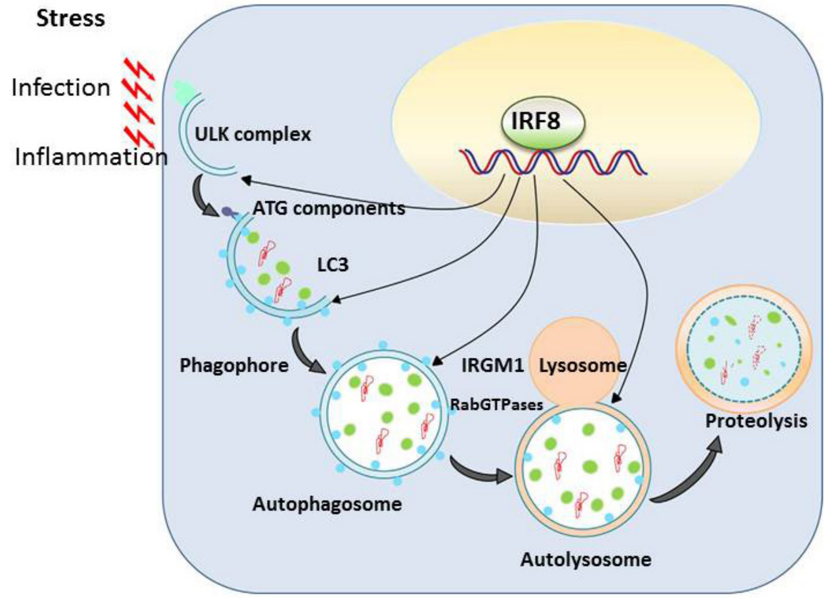
IFN stimulates H3.3 deposition in macrophages: analysis of H3.3-HA knock-in mice.

To study the activity of H3.3 *in vivo*, we generated mouse strains in which the endogenous H3.3 genes (H3.3A, *H3f3a* and H3.3B, *H3f3b*) are replaced by HA–tagged H3.3 genes (Figure 1). We confirmed that H3.3-HA are expressed at variable levels throughout adult tissues, including all immune cells of myeloid and lymphoid lineages. We performed ChIP-seq analysis to study genome-wide distribution of H3.3A and H3.3B in bone marrow–derived macrophages from these mice. Results revealed that H3.3 deposition was rapidly induced upon IFNg stimulation, which continued for 24 h to 48 h. IFNg is a potent macrophage activator, which drives macrophages to enhance their innate immune responses. Despite the marked increase in H3.3 deposition, the total levels of H3.3A and H3.3B were not altered during this period, indicating a shift in the overall H3.3 distribution in the genome. Supporting this view, our data indicate that H3.3A and H3.3B in the intergenic regions are mobilized to support deposition in stimulated genes, a view that is supported by the reduced amounts of H3.3 in intergenic regions upon IFN stimulation.

BRD4 supports elongation of the eRNA and the coding RNA.

BRD4 has two bromodomains through which it binds to the acetylated histones H3 and H4. BRD4 also interacts with P-TEFb, the elongation factor composed of Cyclin T and CDK9. The interaction relieves the 5′-paused RNA Polymerase II (Pol II) to trigger elongation. Recent studies from various laboratories showed that BRD4 promotes cancer cell growth, including several forms of leukemia, primarily by promoting C-MYC expression. Interestingly, pharmacological inhibitors for BET bromdomains are found to be effective anti-leukemia drugs in animal models. To further study the extent of BRD4 involvement in elongation, we performed RNA-seq and ChIP-seq analyses of chromatin-bound RNA in quiescent and serum–stimulated fibroblasts. We were particularly interested in the enhancer regions that carry epigenetic histone marks, such as H3K4me1 and H3K27ac, thus affecting expression of the coding genes. We showed that serum stimulation activates transcription of many cell growth–regulated genes in their coding region as well as the enhancer (e) sequences present either in upstream or downstream. Transcription of both coding and eRNAs were dependent on BRD4's interaction with acetylated histones, given that JQ1, a BRD4 small molecule inhibitor, blocked both RNAs. In addition, ChIP-seq data showed that BRD4 localized to the coding and enhancer regions overlapping with the localization of Pol II, supporting the view that BRD4 takes part in the elongation processes by virtue of its ability to bind to acetylated histones. To confirm the broad role of BRD4

Figure 2. IRF8 is a master regulator of autophagy in macrophages.
Various stresses, including pathogen infection and inflammation, trigger autophagy in macrophages, activating a set of autophagy genes responsible for the entire autophagic progression. IRF8 binds to the promoter/enhancer of these genes and directs their transcription, allowing replenishment of autophagy factors. IRF8 induction of autophagy genes is required for innate clearance of intracellular bacteria, such as *Listeria monocytogenes*.



in RNA transcription, nascent RNA was labeled with Br-UTP and immunoprecipitated by BRD4 antibody and then with BrdU antibody. Double immunoprecipitated RNA samples were analyzed by deep sequencing. We found that BRD4 is bound to newly synthesized eRNAs as well as to coding RNAs. We suggest a model wherein BRD4 directs elongation of eRNA and coding RNA (mRNA) (Figure 1). The process is guided by the distribution of acetylated histones, highlighting an epigenetic component of transcription.

The role of IRF8 in autophagy and protection against infectious pathogens

Autophagy is a conserved mechanism by which misfolded self-proteins and damaged organelles (such as mitochondria) are captured and degraded through the lysosomal pathway. Macrophages and DCs activate autophagy in response to various stresses such as infection and inflammation, presumably owing to oxidative stress. Autophagy is thus a critical cellular means by which macrophages and DCs eliminate intracellular infectious agents. Autophagy has been shown to play a major role in the elimination of mycobacteria, including *Mycobacterium tuberculosis*, and of *Salmonella*. In our previous microarray analysis, we noted that a number of autophagy genes are transcriptionally induced following exposure to Toll-like receptor ligands from bacterial/viral components. However, autophagy gene induction was completely absent in macrophages and DCs from IRF8 knockout (KO) mice. Subsequent analysis found that IRF8 activates transcription of more than 15 autophagy genes that are necessary for the entire steps of autophagy in response to various stress. Autophagy genes induced by IRF8 include those for initial activation, autophagosome formation, fusion with lysosomes, and proteolytic degradation of captured materials. By quantitative ChIP assay, we found that IRF8 bound to the enhancer/promoter of many of these genes. Furthermore, retroviral transfer of Irf8 cDNA rescued expression of autophagy genes, supporting the possibility that IRF8 promotes autophagic progression in macrophages and DCs as part of innate immune responses. Thus, macrophages from IRF8–KO mice failed to form autophagosomes, as verified by diminished LC3 (light-chain 3) expression and its conversion to the membrane-associated form. Instead, IRF8–KO cells accumulated a large intracellular aggregates that contained ubiquitin-conjugated proteins and the adaptor protein SQSTM1 (p62), which are likely to be harmful to normal cellular function. This led us to study the role of IRF8 in protection against *Listeria monocytogenes.* Like *M. tuberculosis*, the bacterium resides and grows within macrophages. We showed that, upon *Listeria* infection, autophagy genes are induced at very high levels, which were sustained throughout infection. This was entirely absent in macrophages from IRF8–KO mice. *Listeria* infection also led to high IRF8 induction in wild-type macrophages. We also found that the *Listeria* bacilli are captured by autophagosomes in macrophages, which colocalized with LC3. However, in IRF8–KO macrophages, as a result of the paucity of autophagosomes, the bacteria multiplied rapidly without being autophagically captured. To further support the essential role for IRF8, we showed that *Irf8* transfer results in partial rescue of autophagy during *Listeria* infection and in control of bacterial growth. Together, the results led us to propose a model in which IRF8 acts as a master regulator of autophagy and enhances host defense against invading pathogens (Figure 2).

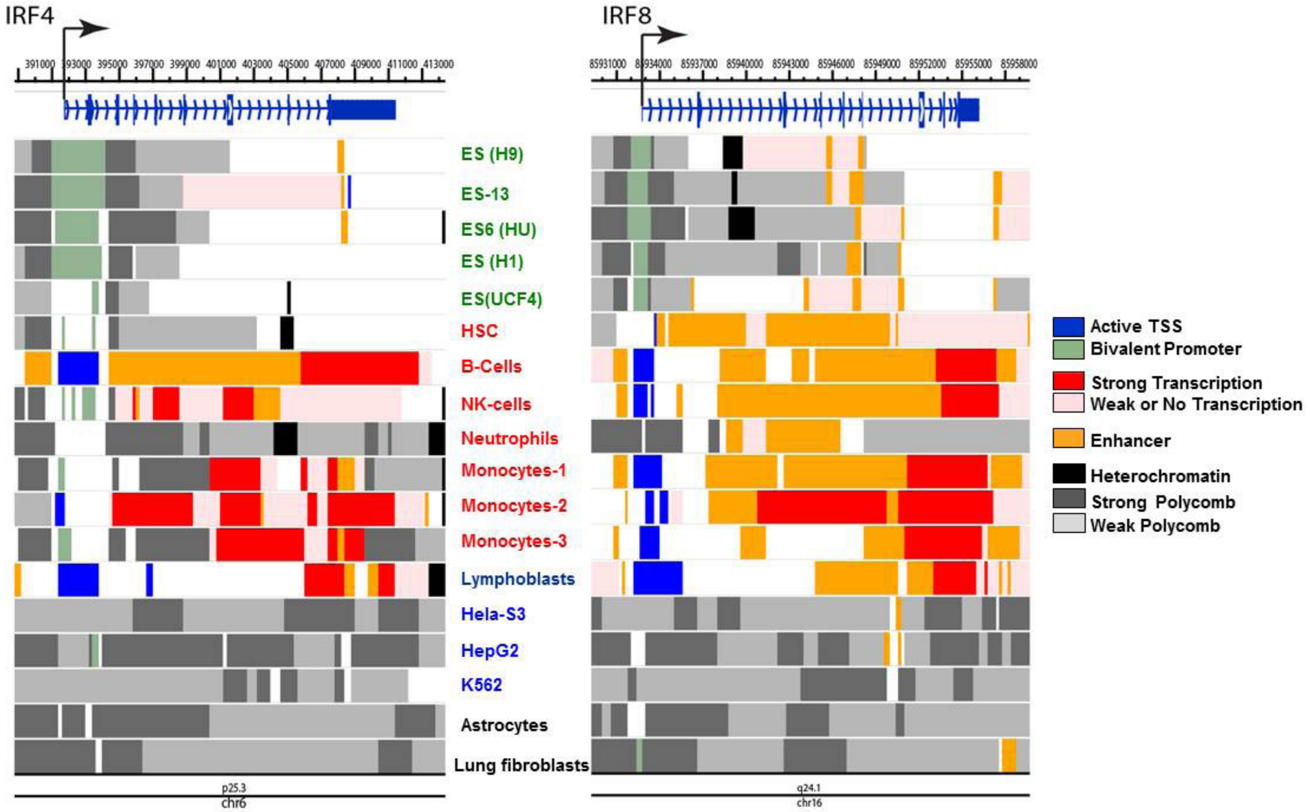


Figure 3. Chromatin landscape of the *IRF4* and *IRF8* genes in human cells
Histone modifications associated with specific epigenetic states were mined from the ENCODE database, which compiles various genome-wide analyses of human cells. Data were reorganized to visualize indicated chromatin states, using the Washington University Epigenome Browser (http://epigenomegateway.wustl.edu/browser/). Cells examined were ES cells (*green*), primary immune cells (*red*), and cancer cell lines (*blue*). Chromatin states were assessed by the indicating modifications: Active TSS (H3K3me3, H3K4me2, H3K27Ac, H3K9Ac); Bivalent promoter (H3K4me3, and H3K27me3); Strong promoter (H3K36me3, H4K20me1, H3K79me2); Weak or no transcription (H3K36me3, H4K20me1, H3K79me2); Enhancer (H3K4me1, H3K27Ac, H3K9Ac); Heterochromatin (H3K9me3); Polycomb repression (H3K27me3).

In a broad view, IRF8 is viewed as a primary (pioneer) factor that sets an epigenetic landscape in the cells of innate immunity. We can thus envisage that the *IRF8* gene itself assumes a chromatin signature that reflects its epigenetic status relevant to this function and lineage derivation. To this end, we mined the Encore database deposited for genome-wide chromatin marks and assembled data for various chromatin marks for the IRF4 and IRF8 loci from several human cells. The marks were then put together to show active enhancer, active transcription start site (TSS), bivalent promoter, heterochromatin and polycomb-based repression, etc. (Figure 3).

Reflecting the similarity of the two IRF factors in their expression profiles, IRF4 and IRF8 exhibit similar chromatin marks among different cell types: in ES cells, active chromatin marks are largely absent from both genes, although IRF8 shows curious enhancer marks in all five ES cells, which are lacking in IRF4. Further, IRF8 is characterized by a strong enhancer signature in hematopoietic stem cells (HSC) and weak transcription, whereas IRF4 lacks these signatures. However, both IRF4 and IRF8 show active marks in primary B cells, NK cells, and monocytes, consistent with their expression and activities in these cells. We noted that IRF4 and IRF8 show multiple repressive marks in the cell lines HeLa S2, HepG, fibroblasts, and the myeloid lineage cells K562, and do not carry active transcription signatures. The analyses reveal previously unrecognized epigenetic marks through which IRF4 and IRF8 exert their activity in a cell type–specific manner. Similar analyses showed a distinct similarity between IRF3 and IRF7 and between IRF1 and IRF2 in their epigenetic marks.

PUBLICATIONS

1. Kanno T, Kanno Y, LeRoy G, Campos E, Sun HW, Brooks SR, Vahedi G, Heightman TD, Garcia BA, Reinberg D, Siebenlist U, O'Shea JJ, Ozato K. BRD4 assists elongation of both coding and enhancer RNAs by interacting with acetylated histones. *Nat Struct Mol Biol* 2014; 12:47–57.
2. Gupta M, Shin DM, Ramakrishna L, Goussetis DJ, Platanias LC, Xiong H, Morse HC 3rd, Ozato K. IRF8 directs stress-induced autophagy in macrophages and promotes clearance of Listeria monocytogenes. *Nat Commun* 2015; 6:6379.
3. Mancino A, Termanini A, Barrozi I, Ghisletti S, Otsuni R, Prosperini E, Ozato K, Natoli G. A dual cis-regulatory code links IRF8 to constitutive and inducible gene expression in macrophages. *Genes Dev* 2015; 29:394–408.
4. Ayithan N, Bradfute SB, Anthony SM, Stuthman KS, Bavari S, Bray M, Ozato K. Virus-like particles activate type I interferon pathways to facilitate post-exposure protection against Ebola virus infection. *PLoS One* 2015; 10:e0118345.
5. Bradfute SB, Anthony SM, Stuthman KS, Ayithan N, Tailor P, Shaia CI, Bray M, Ozato K, Bavari S. Mechanisms of immunity in post-exposure vaccination against Ebola virus infection. *PLoS One* 2015; 10:e0118434.

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Molecular Genetics of an Imprinted Gene Cluster on Mouse Distal Chromosome 7

Genomic imprinting is an unusual form of gene regulation by which an allele's parental origin restricts allele expression. For example, almost all expression of the non-coding RNA tumor suppressor gene *H19* is from the maternal chromosome. In contrast, expression of the neighboring insulin-like growth factor 2 gene (*Igf2*) is from the paternal chromosome. Imprinted genes are not randomly scattered throughout the chromosome but rather are localized in discrete clusters. One cluster of imprinted genes is located on the distal end of mouse chromosome 7 (Figure 1). The syntenic region in humans (11p15.5) is highly conserved in gene organization and expression patterns. Mutations disrupting the normal patterns of imprinting at the human locus are associated with developmental disorders and many types of tumors including Wilms' tumor and rhabdosarcomas in children. In addition, inherited cardiac arrhythmia is associated with mutations in the maternal-specific *Kcnq1* gene. We use mouse models to address the molecular basis for allele-specific expression in this distal 7 cluster. We use imprinting as a tool to understand the fundamental features of epigenetic regulation of gene expression. We also generate mouse models for the several inherited disorders of humans, specifically models to study defects in cardiac repolarization associated with loss of *Kcnq1* function, and recently we characterized the phenotype associated with loss of *Calsequestrin2* gene function.

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Alternative long-range interactions between distal regulatory elements establish allele-specific expression at the *Igf2/H19* locus.

Our studies on the mechanisms of genomic imprinting focus on the *H19* and *Igf2* genes, which lie at one end of the distal 7 imprinted cluster (Figure 1). Paternally expressed *Igf2* lies about 80 kb upstream of the maternal-specific *H19* gene. Using cell-culture systems as well as transgene and knockout experiments *in vivo*, we identified the enhancer elements responsible for activation of the two genes. The elements are shared and are all located downstream of the *H19* gene (Figure 2).

Imprinting at the *Igf2/H19* locus is dependent upon the 2.4 kb *H19* Imprinting Control Region (H19 ICR), which lies between the two genes, just upstream of the *H19* promoter (Figure 2). On the maternal chromosome, binding of the CTCF protein, a transcriptional regulator, to the ICR establishes a transcriptional insulator that organizes the chromosome into loops. The loops favor *H19* expression but block interactions between the maternal *Igf2* promoters and the downstream shared enhancers, thus preventing maternal *Igf2* expression. Upon paternal inheritance, the CpG sites within the ICR are methylated, which prevents binding of the CTCF protein, so that a transcriptional insulator is not established. Thus, paternal *Igf2* promoters and the shared enhancers interact via DNA loops, and expression of paternal *Igf2* is facilitated. Altogether, we find that the fundamental role of the ICR is to organize the chromosomes into alternative 3-D configurations that promote or prevent expression of the *Igf2* and *H19* genes.

The H19 ICR is not only necessary but is also sufficient for genomic

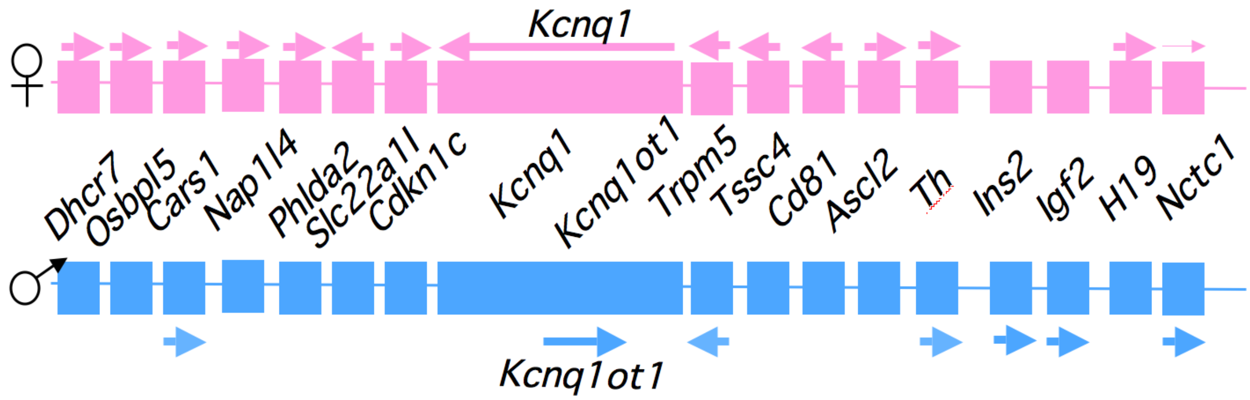


Figure 1. An imprinted domain on mouse distal chromosome 7
Maternal (*pink*) and paternal (*blue*) chromosomes are indicated. Horizontal arrows denote RNA transcription.

imprinting. To demonstrate this, we used knock-in experiments to insert the 2.4 kb element at heterologous loci and demonstrated its ability to imprint these regions. Further, analyses of the loci confirmed and extended the transcriptional model described above. Upon maternal inheritance, even ectopic ICR elements remain unmethylated, bind to the CTCF protein, and form transcriptional insulators. Paternally inherited ectopic ICRs become methylated, cannot bind to CTCF, and therefore promote alternative loop domains distinct from those organized on maternal chromosomes. Most curious was the finding that DNA methylation of ectopic ICRs is not acquired until relatively late in development, after the embryo implants into the uterus. In contrast, at the endogenous locus, ICR methylation occurs during spermatogenesis. The findings thus imply that DNA methylation is not the primary imprinting mark that distinguishes maternally from paternally inherited ICRs.

The *Nctc1* gene lies downstream of H19 and encodes a long non-coding RNA that is transcribed across the muscle enhancer element (ME in Figure 2), which is shared by *Igf2* and *H19*. *Nctc1* expression depends on this enhancer element. Concordantly, the shared enhancer interacts with the *Nctc1* promoter just as it interacts with the maternal *H19* and the paternal *Igf2* promoters. We showed that all three co-regulated promoters (*Igf2*, *H19*, and *Nctc1*) also physically interact with

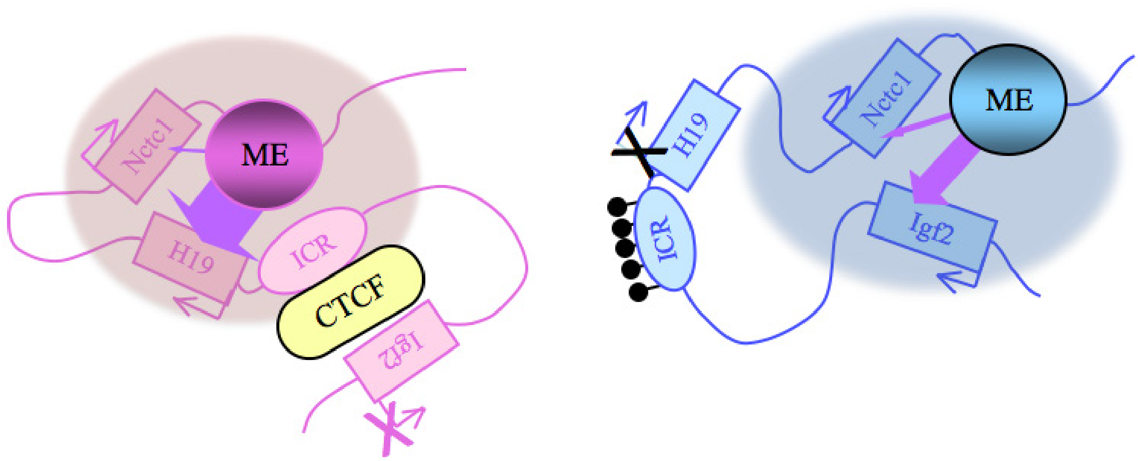


Figure 2. Distinct maternal and paternal chromosomal conformations at the distal 7 locus
Epigenetic modifications on the 2.4 kb ICR generate alternative 3D organizations across a large domain on paternal (*blue*) and maternal (*pink*) chromosomes and thereby regulate gene expression. ICR, imprinting control region; ME, muscle enhancer; filled lollipops, CpG methylation covering the paternal ICR.

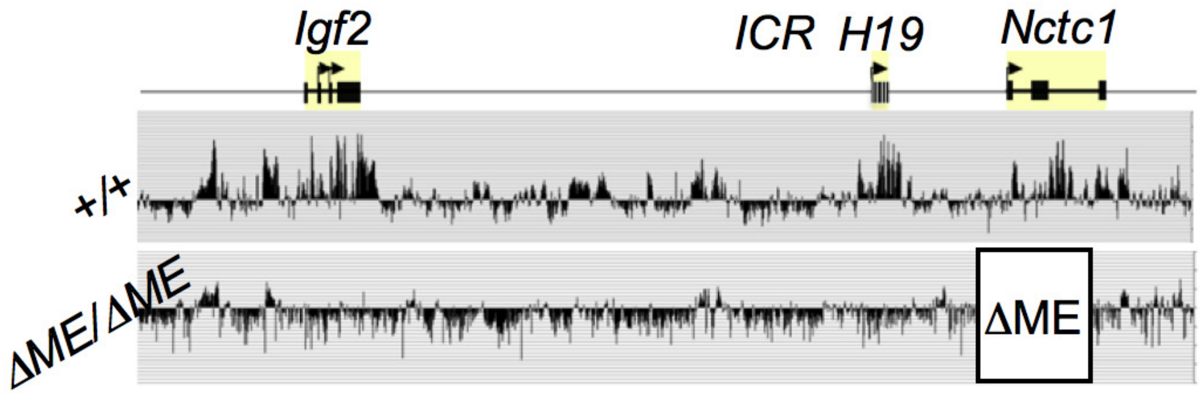


Figure 3. The shared muscle enhancer (ME) directs RNAP binding and RNA transcription across the entire 150 kb locus.

each other in a manner that depends on their interactions with the shared enhancer. Thus, enhancer interactions with one promoter do not preclude interactions with another promoter. Moreover, we demonstrated that these promoter-promoter interactions are regulatory, and they explain the developmentally regulated imprinting of *Nctc1* transcription. Taken together, our results demonstrate the importance of long-range enhancer-promoter and promoter-promoter interactions in physically organizing the genome and establishing the gene expression patterns that are crucial for normal mammalian development (References 1 and 2).

Molecular mechanisms for tissue-specific promoter activation by distal enhancers

Normal mammalian development is absolutely dependent on establishing the appropriate patterns of expression of thousands of developmentally regulated genes. Most often, development-specific expression depends on promoter activation by distal enhancer elements. The *Igf2/H19* locus is a highly useful model system for investigating mechanisms of enhancer activation. First, the biological significance of the model is clear, given that expression of these genes is so strictly regulated. Even two-fold changes in RNA levels are associated with developmental disorders and with cancer. Second, we already know much about the enhancers in this region and have established powerful genetic tools to investigate their function. *Igf2* and *H19* are co-expressed throughout embryonic development and depend on a series of tissue-specific enhancers that lie between 8 and more than 150 kb downstream of the *H19* promoter (or between 88 and more than 130 kb downstream of the *Igf2* promoters). The endodermal and muscle enhancers have been precisely defined, and we generated mouse strains carrying deletions that completely abrogate enhancer function. We also generated insulator insertion mutations that specifically block muscle enhancer activity. We used these strains to generate primary myoblast cell lines so that we can combine genetic, molecular, biochemical, and genomic analyses to understand the molecular bases for enhancer functions.

A long non-coding RNA is an essential element of the muscle enhancer (Reference 2).

Transient transfection analyses define a 300–bp element that is both necessary and sufficient for maximal enhancer activity. However, stable transfection and mouse mutations indicate that this core element is not sufficient for enhancer function in a chromosomal context. Instead, the *Nctc1* promoter element is also essential. (The *Nctc1* gene encodes a spliced, polyadenylated long non-coding RNA). The *Nctc1* RNA itself is not required (at least in trans). Instead mutational analysis demonstrates that it is *Nctc1* transcription through the core enhancer that is necessary for enhancer function. Curiously, the *Nctc1* promoter has chromatin features typical of both a classic enhancer and a classic peptide-encoding promoter. Several recent genomic studies also suggested a role for non-coding RNAs in gene regulation and enhancer function. We will use our model system to characterize the role of *Nctc1* transcription in establishing enhancer orientation, enhancer promoter specificity, and enhancer tissue specificity.

The muscle enhancer (ME) directs RNA polymerase II (RNAP) not only to its cognate promoters (i.e., to the H19 and Igf2 promoters) but also across the entire intergenic region.

We used ChIP-on-chip to analyze RNAP localization on chromatin prepared from wild-type and from enhancer-deletion

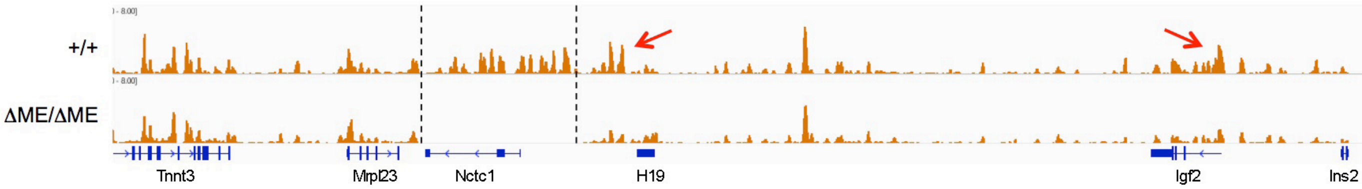


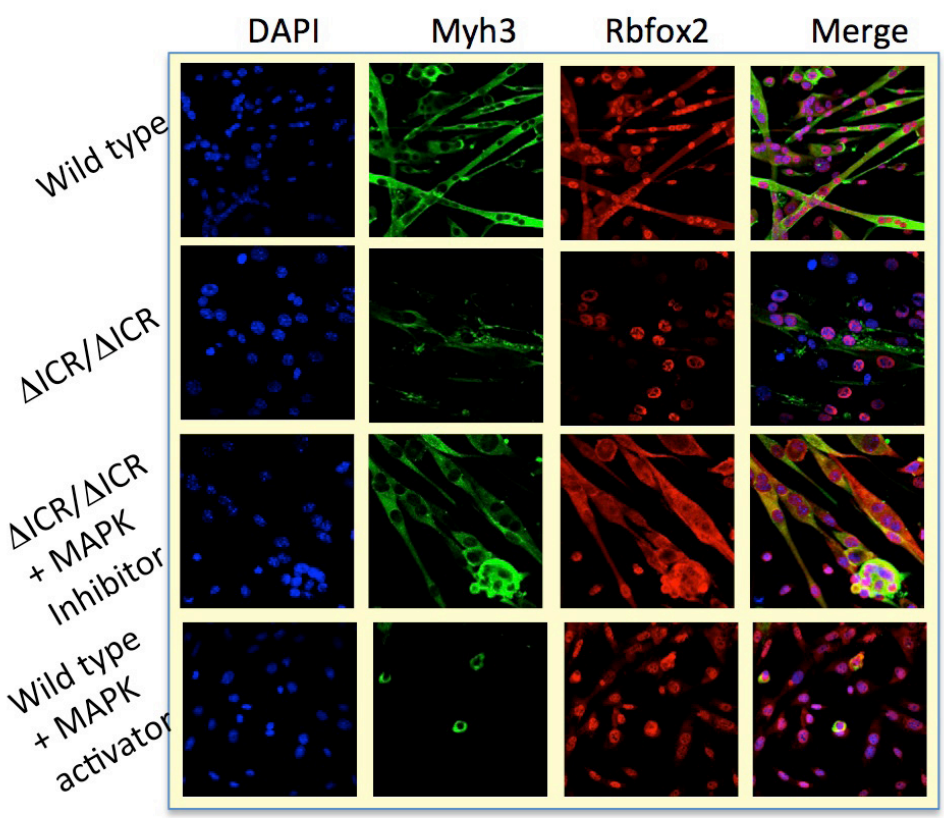
Figure 4. Chromatin patterns at the *Igf2/H19* locus are independent of enhancer activity.
Chromatin was isolated from wild-type and enhancer-deletion muscle cells using antibodies to H3K4me1 and analyzed by DNA sequencing.

(ΔME) cell lines (Figure 3). As expected, RNAP binding to the *H19* and *Igf2* promoters is entirely enhancer-dependent. Curiously, we also noted enhancer-dependent RNAP localization across the entire locus, including the large intergenic domain between the two genes. Furthermore, the RNAP binding is associated with RNA transcription. Thus, the enhancer regulates accessibility and RNAP binding not only at specific localized sites but across the entire domain. The results support a facilitated tracking model for enhancer activity.

RNAP binding at 'real' genes and across the intergenic regions is qualitatively different.

We used naturally occurring single nucleotide polymorphisms (SNPs) to investigate allelic differences in binding of RNAP and activation of gene expression in wild-type cells and in cells carrying enhancer deletions or insulator insertion mutations. RNAP binding across the *Igf2* and *H19* genes is both enhancer-dependent and insulator-sensitive; that is, a functional insulator located between an enhancer and its regulated gene prevents RNAP binding and likewise prevents RNA transcription. Across the intergenic regions, RNAP binding and RNA transcription are similarly enhancer-dependent (see above). However, intergenic RNAP binding and transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as

Figure 5. Muscle cell–differentiation defects in *Igf2/H19* Loss-of-Imprinting mice
Differentiation defects in Loss-of-Imprinting (ΔICR) myoblasts can be rescued by blocking MAP kinase 3 activity. Conversely, artificial activation of the MAPK activity in wild-type cells mimics the genetic defect.



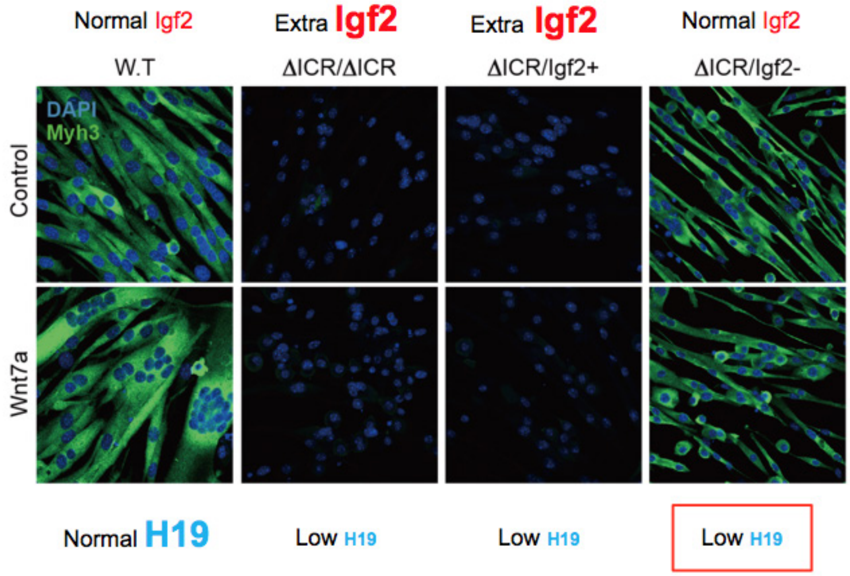


Figure 6. The long noncoding H19 RNA is required for normal myotube fusion and hypertrophy. Loss of Imprinting defects at the Igf2/H19 locus result in extra expression of Igf2 and defects in myotube differentiation: Compare W.T (wild type) with DICR/DICR and DICR/Igf2+ cells. Mutation of the paternal *Igf2* gene can restore normal Igf2 expression levels and thus restore normal differentiation. (See DICR/Igf2- cells). However, these cells still do not make the H19 long noncoding RNA, do not can fuse efficiently, and do not respond to Wnt7a signaling.

a physical block for RNAP progression, but rather they specifically interfere with certain RNAP states or activities.

The muscle enhancer regulates RNAP binding and RNA transcription but does not establish chromatin structures.

RNA transcription and also RNAP binding across the *Igf2*/*H19* domain are both entirely dependent upon the muscle enhancer. For example, levels of *H19* RNA are reduced more than 10,000-fold in muscle cells in which the enhancer has been deleted. To test the dependence of chromatin structure on enhancer activity, we performed ChiP-Seq on wild-type and on enhancer-deletion cell lines using antibodies to the histones H3K4me1, H3K43me3, and H3K36me3. Surprisingly, we saw no changes in the patterns of chromatin modification (Figure 4). Thus, a functional enhancer and active RNA transcription are not important for establishing chromatin structures at this locus.

Function of the *H19* and *Igf2* genes in muscle cell growth and differentiation

Misexpression of *H19* and *IGF2* is associated with several developmental diseases (including Beckwith-Wiedemann syndrome and Silver-Russell syndrome) and with several kinds of cancer, especially Wilms' tumor and rhabdosarcoma. In humans, misexpression is most often caused by loss of imprinting mutations that result in biallelic expression of *IGF2* and loss of expression of *H19*. We generated and characterized primary myoblast cell lines from mice carrying deletion of the *H19 imprinting control region* (ICR) that phenocopies the loss of imprinting expression phenotypes; that is, ICR–deletion mice make extra *Igf2* but no *H19*. Mice carrying this mutation do not develop rhabdosarcoma but show defects in their ability to respond to and to heal muscle injury. Moreover, primary myoblast lines derived from mutant mice are defective in their ability to differentiate *in vitro* (Figure 5) (Reference 3).

To understand the molecular basis for the differentiation phenotype, we performed RNA sequencing and identified several hundred genes whose expression levels are altered by the ICR deletion. GO (gene ontology) pathway analysis demonstrates that these differentially expressed genes were highly enriched in the MAP kinase signaling pathway. Of special note, expression of the *Mapk3* gene is increased almost 10-fold in mutant cell lines.

To determine the significance of the changes in *Mapk3*, we used drug inhibitors to block MAP kinase activity. In mutant cell lines, we can restore normal differentiation by blocking activation of the MAP kinase target MEK1. Similarly, treatments that activate MAP kinase in wild-type cells can mimic the ICR–deletion phenotype. The results suggest that *H19*/*Igf2* act through MAP kinase to regulate differentiation of myoblast cells.

To distinguish the roles for Igf2 over-expression and H19 under-expression, we analyzed additional mouse strains that restore H19 via a Bacterial Artificial chromosome transgene or that restore normal levels of Igf2 expression via a second mutation

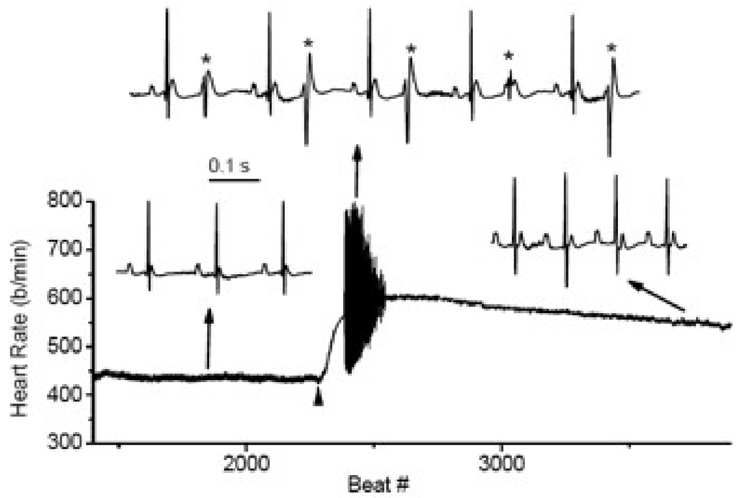


Figure 7. Cardiac arrhythmias in calsequestrin-2–deficient mice phenocopy the human disease.
Premature ventricular complexes (*) are induced by stress in *Casq2*–deficient but not in wild-type mice.

in the paternal *Igf2* gene. Analyses of cell lines from these mice demonstrate that extra Igf2 is the direct cause of failure to differentiate in Loss of Imprinting mutations but that H19 is essential for normal fusion and for muscle hypertrophy in response to Wnt pathways (Figure 6). Further molecular and genetic analyses will uncover the molecular pathways underlying these defects. Furthermore, to understand how the H19 long noncoding RNA can accomplish its functions, we used CRISPR technology to mutagenize conserved domains within the *H19* gene including sites that encode microRNAs and sites that bind to the let7 RNA.

Role of calsequestrin2 in regulating cardiac function

Mutations in the *CASQ2* gene, which encodes cardiac calsequestrin (CASQ2), are associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden death. The survival of individuals homozygous for loss-of-function mutations in *CASQ2* was surprising, given the central role of Ca^{2+} ions in excitation-contraction (EC) coupling and the presumed critical roles of CASQ2 in regulating Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytoplasm. To address this paradox, we generated a mouse model for loss of *Casq2* gene activity. Comprehensive analysis of cardiac function and structure yielded several important insights into CASQ2 function. First, CASQ2 is not essential to provide sufficient Ca^{2+} storage in the SR of the cardiomyocyte. Rather, a compensatory increase in SR volume and surface area in mutant mice appears to maintain normal Ca^{2+} storage capacity. Second, CASQ2 is not required for the rapid, triggered release of Ca^{2+} from the SR during cardiomyocyte contraction. Rather, the RyR receptor, an intracellular calcium ion channel, opens appropriately, resulting in normal, rapid flow of Ca^{2+} into the cytoplasm, thus allowing normal contraction of the cardiomyocyte. Third, CASQ2 is required for normal function of the RyR during cardiomyocyte relaxation. In the absence of CASQ2, significant Ca^{2+} leaks occur through the RyR and lead to premature contractions and cardiac arrhythmias (Figure 7). Fourth, CASQ2 function is required to maintain normal levels of the SR proteins junctin and triadin. We do not yet understand what role, if any, the compensatory changes in these two SR proteins play in modulating the loss of *Casq2* phenotype.

To address these issues and to model cardiac disorders associated with late-onset (not congenital) loss of CASQ2 activity, we established and are analyzing two new mouse models in which changes in *Casq2* gene structure are induced by tissue-specific transgenes activated by tamoxifen treatment. In the first model, an invested/null allele is restored to normal function by the addition of the drug. In the past year, we demonstrated the effectiveness of this model and noted that full Casq2 protein levels are restored within one week of treatment. In the second model, a functional gene is ablated by the addition of the drug. The *Casq2* gene and mRNAs are deleted from cardiac cells within four days of hormone treatment. Phenotypic analyses shows that the mice present a profoundly more defective heart than congenitally mutant mice; that is, arrhythmias are more frequent, of longer duration, and qualitatively much more severe. Our results indicate that the developmental program has flexibility in coping with the loss of Casq2 protein that the adult heart lacks. Our current efforts focus on finding the molecular basis for the coping mechanisms as a means to better understand cardiac development and to perhaps identify clues as to how to handle Casq2 deficiency, clues that may be of therapeutic value.

ADDITIONAL FUNDING

» NICHD Director's Award

PUBLICATIONS

1. Eun B, Sampley ML, Good AL, Gebert CM, Pfeifer K. Promoter cross-talk via a shared enhancer explains paternally biased expression of Nctc1 at the Igf2/H19/Nctc1 locus. *Nucleic Acids Res* 2013; 41:817–826.
2. Eun B, Sampley ML, Van Winkle MT, Good AL, Kachman MM, Pfeifer K. The Igf2/H19 muscle enhancer is an active transcriptional complex. *Nucleic Acids Res* 2013; 41:8126–8134.
3. Dey BK, Pfeifer K, Duta, A. The H19 long non-coding RNA gives rise to microRNAs miR-675-3p and -5p to promote skeletal muscle differentiation and regeneration. *Genes Dev* 2014; 28:491–501.
4. Leikina E, Melikov K, Sanyal S, Verma SK, Eun B, Gebert CM, Pfeifer K, Lizunov V, Kozlov M, Chernomordik LV. Extracellular annexins and dynamin are important for sequential steps in myoblast fusion. *J Cell Biol* 2013; 200:109–123.
5. Xia J, Varudkar N, Baker C, Abukenda I, Martinez C, Natarajan A, Grinberg A, Pfeifer K, Ebert SN. Targeting of the enhanced green fluorescent protein reporter to adrenergic cells in mice. *Mol Biotechnol* 2013; 54:350–360.

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The lab focuses on mechanisms regulating the differentiation of cranial neural crest cells that give rise to the bone and cartilage of the vertebrate jaw, neurocranium, and other structures of the face and head. Our approach is to manipulate transcriptional control mechanisms in the intact zebrafish embryo using gain and loss of function strategies, with the aim of identifying the regulatory networks that control craniofacial development. Disruption of these developmental programs is the most common source of birth defects in humans, the detection, prevention, and treatment of which is a central aspect of NICHD's mission. Equally important, understanding the regulation of gene expression in this complex embryonic tissue represents a challenging and fascinating problem in basic molecular and developmental biology.

The origin of the lab's neural crest research was our discovery in 2003 that, in the frog *Xenopus*, the transcription factor TFAP2a is both necessary and sufficient to trigger the conversion of cells at the neural plate border from neural to neural crest identity. We went on to show that TFAP2a mediates the transcriptional response to bone morphogenetic protein (BMP) signaling in neural crest induction. The lab also carried out pioneering research on the homeodomain factor Dlx3, performing the first mouse knock-out of the gene encoding this factor and demonstrating the factor's function in the development of mammalian epidermis and placenta. We were also the first to demonstrate the phylogenetic conservation of *Dlx* gene–regulatory elements by transferring *Xenopus Dlx* enhancers into the mouse genome, showing that enhancers were conserved between these two distantly related vertebrates, including those active in tissues such as hair follicle and mammary gland that have no counterpart in amphibians. We also investigated the role of Dlx3 and other members of this family in establishing the boundary of the neural crest in *Xenopus*. The Dlx family remains our current focus of research.

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Allisan Aquilina-Beck, BA, *Technician (part time)*

Dlx gene function in cranial neural crest development

In the previous year, the lab completed-gain-of function experiments based on over-expression of Dlx factors by injecting synthetic mRNA into fertilized eggs. Injected embryos were harvested early, at the beginning of gastrulation, and RNA isolated and subjected to RNAseq analysis. The hypothesis is that, because early embryonic cells are pluripotent, over-expressing a given transcription factor will result in precocious activation of cognate target genes, revealing potential downstream components of the regulatory network based on the factor injected (i.e., a Dlx factor). This was confirmed by the strong activation of several genes already known to be downstream of *Dlx* genes. We also confirmed numerous additional candidate targets. The data are being integrated with genomics studies on mouse *Dlx* mutants by our collaborating lab headed by Maria Morasso.

Most recently, we focused on loss-of-function experiments. Following reports from numerous labs, including our own, indicating that "knock down" analysis of gene function using antisense morpholinos frequently yielded results that were artifacts or otherwise at variance with chromosomally based gene inactivation strategies, we set out to apply the CRISPR/Cas targeting approach to the Dlx family in zebrafish. The objective of this project is to identify and characterize embryonic craniofacial phenotypes resulting from loss of individual *Dlx* genes or combinations thereof. The targeting is being conducted in a line of fish carrying a GFP fluorescent marker expressed in cranial cartilage, enabling high-resolution imaging of affected tissues using confocal microscopy. Until the writing of this report, we had identified in G0 founders individual mutations in all six major *Dlx* genes, as well as fish in which pairs of linked *Dlx* genes had been deleted (or inverted) in tandem. Out-crossing to GFP reporter individuals was initiated and is expected to be completed shortly, followed by in-crossing to yield *Dlx* nulls. We anticipate that these studies will reveal some of the earliest events that lead to Dlx-dependent craniofacial dysmorphology, with significant impact on the diagnosis of and ultimately on developing therapy for affected human fetal and neonatal patients.

PUBLICATIONS

1. Law SHW, Sargent TD. The serine-threonine protein kinase PAK4 is dispensable in zebrafish: identification of a morpholino-generated pseudophenotype. *PLoS One* 2014; E100268.

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Development of the Vertebrate Circulatory System

The overall objective of this project is to understand how the elaborate networks of blood and lymphatic vessels arise during vertebrate embryogenesis. Blood vessels supply every tissue and organ with oxygen, nutrients, and cellular and humoral factors. Lymphatic vessels drain fluids and macromolecules from the interstitial spaces of tissues, returning them to the blood circulation, and play an important role in immune responses. Our studies on the formation of blood and lymphatic vessels are engendering intense clinical interest because of the roles both types of vessel play in cancer and ischemia. The zebrafish (*Danio rerio*) is a small tropical freshwater fish that possesses a unique combination of features that make it particularly suitable for studying vessel formation. Zebrafish are genetically tractable vertebrates with externally developing, optically clear embryos, which are readily available for observation and experimental manipulation. Such features make the fish highly advantageous for studying vascular development, permitting observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific defects.

Current studies use genetic screening, experimental analysis, and imaging to examine cues directing vascular patterning and morphogenesis, regulation of vascular integrity, assembly of the lymphatic system, and hematopoietic stem cell formation.

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Developing tools for experimental analysis of vascular development in the zebrafish

The development of new tools to facilitate vascular studies in the zebrafish is an important ongoing aim of our work. Previously, we (1) established a micro-angiographic method for imaging patent blood vessels in the zebrafish and used the method to compile a comprehensive staged atlas of the vascular anatomy of the developing fish (**http://zfish.nichd.nih.gov**); (2) generated a variety of transgenic zebrafish lines expressing various fluorescent proteins within vascular or lymphatic endothelial cells, making it possible for us to visualize vessel formation in intact, living embryos; and (3) developed methodologies for long-term multiphoton confocal time-lapse imaging of vascular development in transgenic fish. Recent technical advances have greatly facilitated the generation of new transgenic lines, and we are currently developing many new lines useful for *in vivo* vascular imaging as well as for *in vivo* endothelial-specific functional manipulation of signaling pathways involved in vascular specification, patterning, and morphogenesis. Furthermore, we are taking advantage of recently developed CRISPR (clustered regularly interspaced short palindromic repeats) gene-editing technology to easily generate germline mutations in any zebrafish gene of interest. We are also in the process of generating a detailed staged anatomical atlas of the developing lymphatic system in the zebrafish comparable to the atlas of the circulatory system that we had published previously.

Genetic analysis of vascular development

We use forward-genetic approaches to identify and characterize new zebrafish mutants that affect the formation of the developing vasculature. Using transgenic zebrafish expressing green fluorescent protein (GFP) in blood

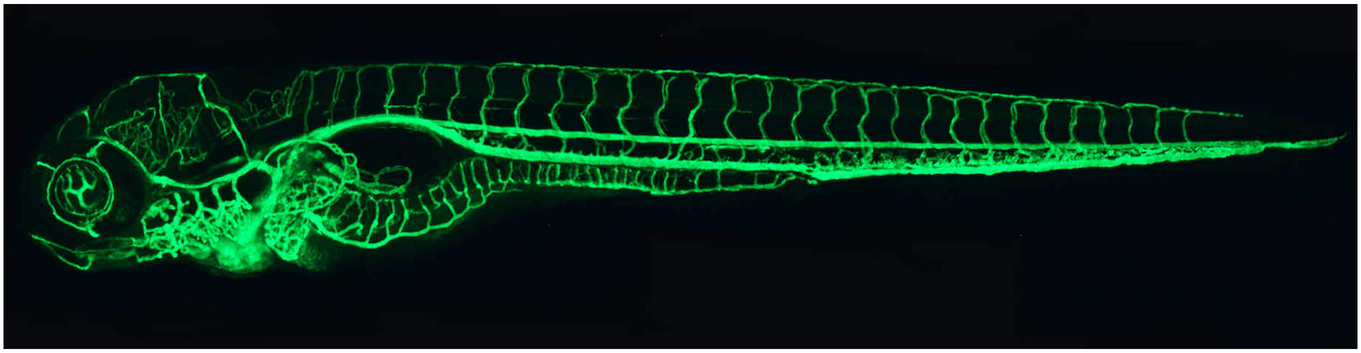

Figure 1. The zebrafish vascular system
Confocal microangiogram of the vascular system of a 4-1/2-day-old zebrafish larva labeled by injecting fluorescent microspheres. The transparency of zebrafish larvae makes it possible to use high-resolution optical imaging methods to visualize the entire vasculature in exquisite detail.

vessels (Figure 1) or lymphatic vessels, we are carrying out ongoing large-scale genetic screens for mutants induced by *N*-ethyl-*N*-nitrosourea (ENU). We already identified hundreds of new vascular mutants with phenotypes that include loss of most vessels or subsets of vessels, increased sprouting/branching, and vessel mis-patterning. We recently carried out a new genetic screen to identify hemorrhagic stroke susceptibility genes. We are currently using lymphatic-specific transgenic lines to perform screens for mutants specifically affecting the development of lymphatic vessels. We are pursuing the molecular cloning of the defective genes from all our new mutants, using next-generation whole-exome sequencing. To facilitate identification of the causative mutations (as opposed to naturally occurring polymorphisms) in our next-generation sequencing (NGS) data, we generated a large database of naturally occurring SNP (single nucleotide polymorphism) variants in three commonly used laboratory zebrafish strains. We also developed a web-based tool ("SNPfisher") to facilitate querying and manipulating the database. Whole-exome sequencing combined with our SNP identification tools has already made it possibly to rapidly identify the causative mutations in some of our more recently identified mutants. The identification of additional defective genes from our mutants should result in further new insights into the molecular mechanisms underlying vascular development and vascular integrity. Together, our ongoing mutant screens continue to yield a rich harvest of novel vascular mutants and genes, bringing to light new pathways that are critical during vascular development and vascular disease.

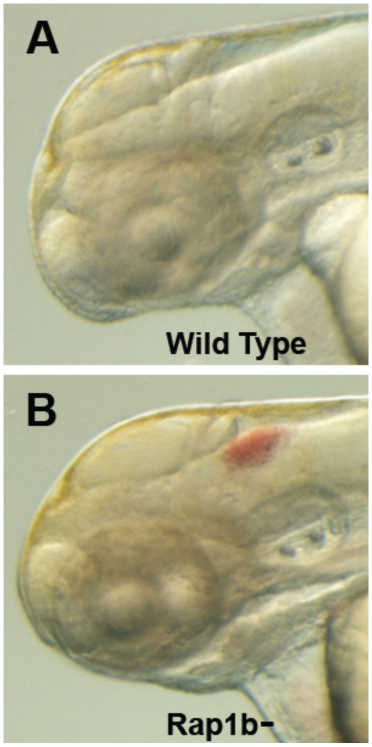

Figure 2. Intracranial hemorrhage (ICH) in the developing zebrafish
The clarity of zebrafish larvae also makes it straightforward to screen for animals with intracranial hemorrhage, as is evident in comparing lateral views of a 2-day-old wild-type larva (A) with a hemorrhage-prone larva deficient in *rap1b* (B).

Analysis of vascular morphogenesis and integrity

Proper morphogenesis of vascular tubes and the maintenance of their integrity is of critical importance to human health. Malformation or rupture of vessels is the basis for stroke, the third leading cause of death and the most common cause of disability in developed nations. Intracerebral hemorrhage (ICH) accounts for 10 percent of stroke and is a particularly severe form of the disease, with disproportionately high rates of death and long-term disability. Therapeutic tools are still very limited, and prevention remains the most important way to reduce morbidity and mortality. In previous studies, we used high-resolution time-lapse two-photon imaging to examine vessel morphogenesis in living zebrafish, showing that the formation and intra- and intercellular fusion of endothelial vacuoles drive vascular lumen formation *in vivo*. We are currently examining the formation and maintenance of vascular endothelial cell-cell junctions, which are particularly important in stroke. To begin dissecting the molecular-regulatory mechanisms controlling endothelial junction formation, we examined a variety of genes required for vascular morphogenesis and vascular integrity, including the *ccm*, *pak2a*, and *rap1b* genes (see Figure 2 for the effects of *rap1b* disruption). We are also developing transgenic lines that permit us to visualize the dynamics of endothelial cell-cell junctions and intracellular cytoskeletal structures in order to examine their role in the cellular rearrangements that occur during vascular sprouting and growth and vascular tube formation. As noted above, we also carried out a genetic screen for genes that increase susceptibility to ICH. The screen identified many new mutants that elevate the propensity for ICH. Whole-exome NGS of the mutants is leading to the identification of novel genetic modifiers of the onset and severity of hemorrhagic stroke and, potentially, to the development of new therapeutic targets for the treatment and prevention of human ICH.

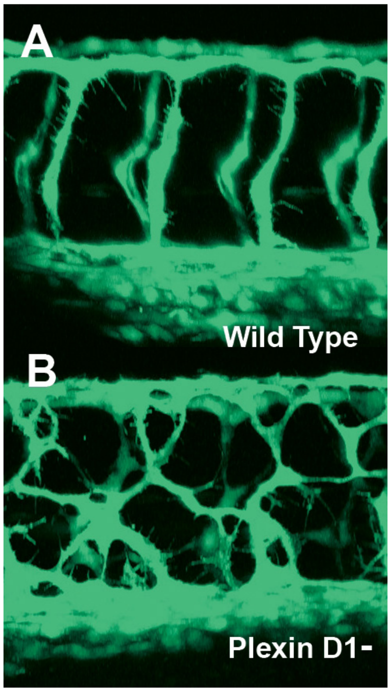

Figure 3. Mispatterned trunk vessels in larvae lacking the vascular semaphorin receptor plexin D1
Confocal imaging of trunk vessels in a 2½-day-old wild-type (A) and a plexin D1–deficient (B) larva, showing loss of proper patterning of the trunk vessels caused by inability to receive semaphorin repulsive guidance signals.

Analysis of vascular patterning

We used multiphoton time-lapse imaging to characterize patterns of vessel assembly throughout the developing zebrafish. Our ongoing studies aim to understand how the patterns arise and what cues guide vascular network assembly during development. We previously demonstrated that known neuronal guidance factors play an important, previously unknown role in vascular guidance and vascular patterning, showing that semaphorin signaling is an essential determinant of trunk blood vessel patterning (Figure 3). More recently, we also showed that chemokine signaling orchestrates the assembly and patterning of the developing lymphatic vasculature of the trunk. Current studies are elucidating the role of additional factors that guide the patterning of developing blood and lymphatic vascular networks *in vivo*, both in the trunk and in vascular beds in the eye, aortic arches, hindbrain, and other anatomical locales.

Analysis of lymphatic development

The lymphatic system has become the subject of great interest in recent years because of the recognition of its important role in normal and pathological processes, but progress in understanding the origins and early development of the system has been hampered by difficulties in observing lymphatic cells *in vivo* and performing defined genetic and experimental manipulation of the lymphatic system in currently available model organisms. We recently demonstrated that the zebrafish possesses a lymphatic system that shares many of the morphological, molecular, and functional characteristics of the lymphatic vessels found in other vertebrates, thus providing a powerful new model for imaging and studying lymphatic development. As we continue to examine the origins and assembly of the lymphatic system of the zebrafish, we are developing new transgenic tools for imaging the development of the lymphatic system and for forward-genetic screening for lymphatic mutants. Our genetic analysis has already identified several novel genes involved in lymphatic development and patterning. We are also studying the roles of several different genes required for specification, assembly, or patterning of the lymphatic endothelium, including a role for chemokine signaling in guidance and patterning of lymphatic vessel assembly in the developing larval trunk. Our ongoing studies will thus provide new insights into the molecular regulation of lymphatic development.

Epigenetic regulation of hematopoietic stem and progenitor cell emergence

We recently discovered a novel mechanism for epigenetic regulation of hematopoietic stem and progenitor cell (HSPC) specification. HSPCs emerge from the ventral wall of the dorsal aorta in all vertebrates. We found that a gene encoding a DNA methyltransferase (*dnmt*) is expressed specifically in the ventral aortic endothelium, which gives rise to HSPCs. The gene functions downstream from a previously described genetic pathway for specification of HSPCs to promote the long-term maintenance of hematopoietic cell fate. Loss of the *dnmt* gene *in vivo* results in loss of HSPCs, while early ectopic overexpression of the *dnmt* gene is sufficient to induce ectopic hematopoietic gene expression. We are currently continuing to study the role of DNA methylation in HSPC formation.

PUBLICATIONS

1. Butler M, Iben JR, Marsdan KC, Epstein JA, Granato M, Weinstein BM. SNPfisher: a tool for probing genetic variation in laboratory zebrafish. *Development* 2015; 142:1542–1545.
2. Kallakuri S, Yu JA, Li J, Li Y, Weinstein BM, Nicoli S, Sun Z. Endothelial cilia are essential for developmental vascular integrity in zebrafish. *J Am Soc Nephrol* 2015; 26:864–875.
3. Yu JA, Castranova D, Pham VN, Weinstein BM. Single cell analysis of endothelial morphogenesis in vivo. *Development* 2015; 142:2951–2961.
4. Ulrich F, Carretero-Ortega J, Menéndez J, Sun B, Lancaster E, Pershad V, Trzaska S, Véliz E, Narvaez C, Kamei M, Prendergast A, Kidd KR, Shaw KM, Castranova DA, Pham VN, Lo BD, Martin BL, Raible DW, Weinstein BM, Torres-Vázquez J. Reck enables cerebrovascular development by promoting canonical Wnt signaling. *Development* 2015; 143(6):1055.
5. Gore AV, Athans B, Iben JR, Johnson K, Russanova V, Castranova D, Pham VN, Iben JR, Butler MG, Williams-Simons L, Nichols JT, Bresciani E, Feldman B, Bresciani E, Kimmel C, Liu PP, Weinstein BM. Epigenetic regulation of hematopoiesis by DNA methylation. *eLife* 2016; 5:e11813.

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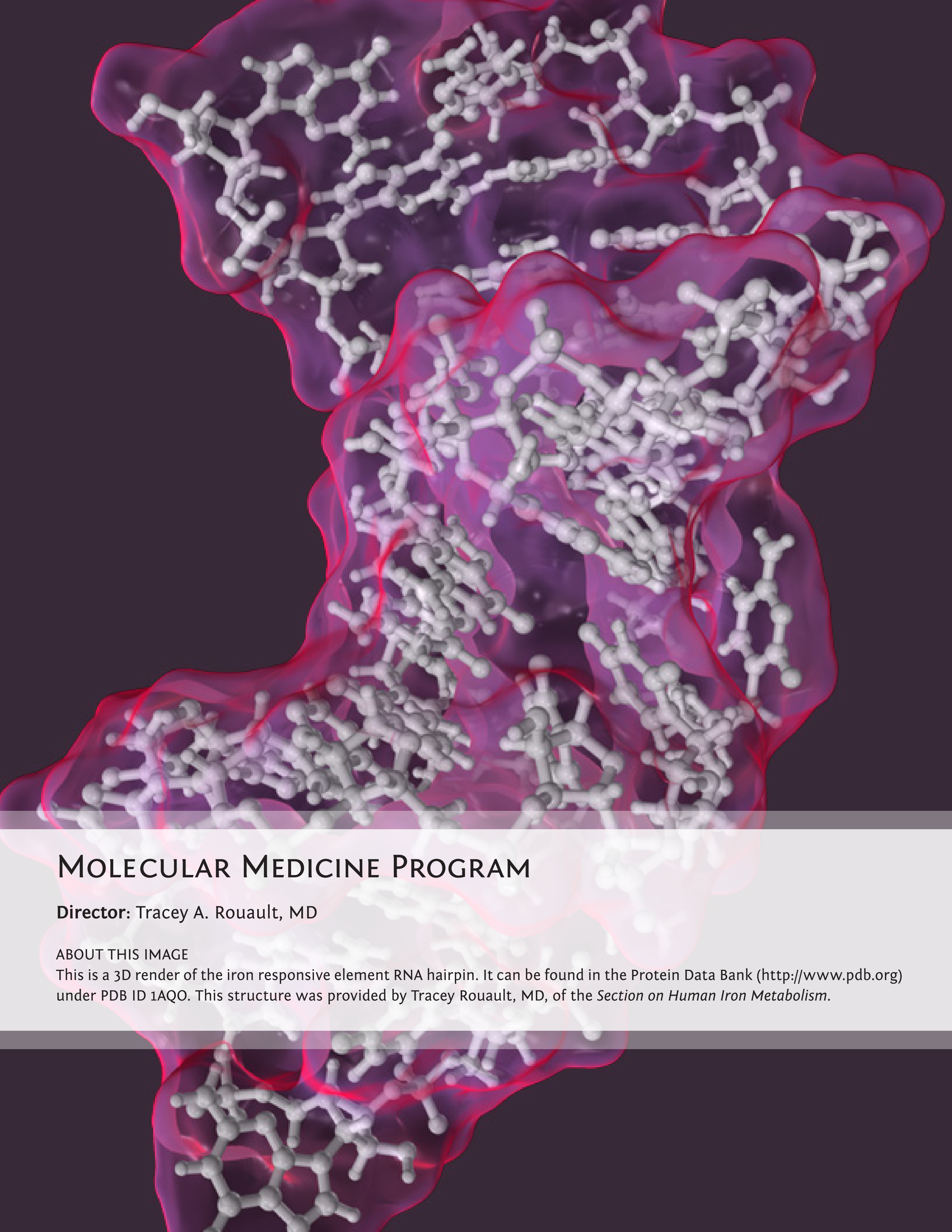

Molecular Medicine Program

Director: Tracey A. Rouault, MD

ABOUT THIS IMAGE

This is a 3D render of the iron responsive element RNA hairpin. It can be found in the Protein Data Bank (http://www.pdb.org) under PDB ID 1AQO. This structure was provided by Tracey Rouault, MD, of the *Section on Human Iron Metabolism*.

Molecular Medicine Program

The *Molecular Medicine Program (MMP)* strives to identify causes and pathophysiology of rare human diseases and evaluate novel treatment approaches though preclinical testing of new therapies and by performing clinical trials. Metabolic disorders that affect hematopoiesis and cause neurodegeneration are common themes. The MMP seeks to apply cutting-edge basic science methods to understand normal and abnormal metabolism and improve health by identifying promising potential solutions, including gene therapy, which can be developed and tested in humans.

Group picture of the Molecular Medicine Program
Members of the Section on Human Iron Metabolism and the Section on Translational Neuroscience gathered for a picture.

The *Section on Translational Neuroscience*, headed by Stephen Kaler, investigates the genetic causes of inherited copper transport diseases and how the responsible genes participate in neurologic processes. The laboratory seeks to dissect and understand disease mechanisms and to use the knowledge gained to improve health through rational treatments, including gene therapy. Kaler identified the molecular basis for occipital horn syndrome and, with international collaborators, delineated the causes of several other conditions affecting copper homeostasis, including ATP7A (a copper pump)–related isolated distal motor neuron degeneration, and unique syndromes caused by mutations in SLC33A1 (an acetylCoA transporter), CCS (a copper chaperone), and AP1S1 (adapter protein complex 1 sigma subunit). The Section pioneered early identification of infants at risk for Menkes disease, using neurochemical plasma measurements, and developed a predictive test for responsiveness to copper treatment of this illness based on residual copper transport activity by certain mutant *ATP7A* alleles in a yeast complementation assay. The Section recently rescued a lethal mouse model of Menkes disease by brain-directed adeno-associated virus (AAV)–mediated gene addition. Extension of the latter proof-of-concept investigations, in tandem with preclinical toxicology studies, will pave the way for a first-in-human gene therapy trial for Menkes disease patients with complete loss-of-function *ATP7A*–gene defects. The Section is also working to distinguish the mechanisms responsible for normal copper transport activity from those of intracellular trafficking of ATP7A and of ATP7B, a closely related copper-transporting ATPase.

The *Section on Human Iron Metabolism*, headed by Tracey Rouault, studies mammalian iron metabolism using mouse models and tissue culture. Rouault previously identified and characterized two major cytosolic iron-regulatory proteins (IRPs 1 and 2). Targeted deletion of IRP2 in mice revealed that misregulation of iron metabolism resulting from loss of IRP2 causes functional iron deficiency, erythropoietic protoporphyria, anemia, and neurodegeneration, which adversely affect motor neurons in particular. The Section has also focused for many years on mammalian iron-sulfur cluster assembly, initially because of its relevance to IRP1 regulation. IRP1–deficient mice develop polycythemia and pulmonary hypertension because of translational derepression of HIF2 alpha (alpha subunit of the hypoxia-inducible factor HIF2). Researchers in the Section characterized numerous mammalian genes involved in iron-sulfur cluster synthesis and developed *in vitro* and *in vivo* methods to assess iron-sulfur cluster biogenesis. The Section's discoveries may promote understanding and treatment of neurodegenerative diseases, including Friedreich's ataxia, and hematologic disorders such as refractory anemias and erythropoietic protoporphyria. The Section discovered that the use of Tempol, a stable nitroxide, prevents neurodegeneration in a mouse deficient in IRP2. Using newly developed antisense technology and genetic engineering of stem cells derived from patients with genetic diseases, the Section is pursuing studies to elucidate the pathophysiology and develop treatments for three diseases caused by defects in iron-sulfur cluster biogenesis, including Friedreich's ataxia, ICSU (iron-sulfur cluster assembly enzyme) deficiency myopathy, and GLRX5 (glutaredoxin 5) sideroblastic anemia, as well as mutations in the *NFU* (iron-sulfur cluster scaffold) gene. The Section is also studying cancers caused by mutations in succinate dehydrogenase subunit B and fumarate hydratase, with emphasis on understanding metabolic remodeling, including changes in iron metabolism that accompany the switch to aerobic glycolysis in cancer. In the past several years, investigators in the Section discovered an important mechanism by which iron-sulfur clusters are delivered to specific recipient proteins. Using the succinate dehydrogenase B subunit (SDHB), they discovered that the HSC20 co-chaperone directly binds to SDHB to include it an iron-sulfur cluster transfer complex. They identified a small peptide motif, LYR, which binds to the C-terminus of SDHB at several points. The LYR motif also is likely to be critical in aiding respiratory chain assembly factors to facilitate correct ligation of iron-sulfur clusters in complexes I–III. Investigators envision that discovery of this motif will lead to discovery of many more mammalian iron sulfur proteins than are now recognized, perhaps changing our understanding of numerous metabolic pathways and the redox state of normal cells.

The Section of Translational Neuroscience strives to dissect and understand mechanisms of human neurometabolic disease and to use the knowledge gained to develop new treatments, including gene therapy, for difficult illnesses. Core values including integrity, humility, hard work with purpose, moving forward rapidly, concern for patients and their families, and mutual support among laboratory members guide the Section's efforts. In addition to molecular genetics, we employ model organisms (mouse, zebrafish, yeast) and cellular, biochemical, and biophysical approaches, and we conduct clinical trials. Preclinical work in the laboratory currently focuses on viral gene therapy in mouse models of Menkes disease and lysosomal storage disease. On the basic neuroscience side, we pursue the molecular mechanisms responsible for certain forms of motor neuron degeneration.

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Adeno-associated viral (AAV) gene therapy for neurometabolic diseases

Brain-directed intracerebroventricular (ICV) recombinant adeno-associated virus serotype 5 (rAAV5) gene therapy in *mo-br* male mice, a mouse model of Menkes disease, resulted in rescue from early lethality by efficient transduction of choroid plexus (CP) epithelia. Rescued animals manifested elevation of brain copper concentrations and improved activity of dopamine-beta-hydroxylase, a copper-dependent enzyme. CP tissues are highly vascularized neuroectoderm-derived structures that project into the ventricles of the brain. Besides creating the blood–CSF (cerebrospinal fluid) barrier, the polarized epithelia of the CP produce CSF by transporting water, ions, and proteins into the ventricles from the blood. We hypothesized that lysosomal storage diseases, a different category of neurometabolic diseases, would benefit from a CP–targeted gene therapy approach, given that recombinant rAAV transduction results in sustained episomal transgene expression and that CP epithelia have a negligible turnover rate. Furthermore, lysosomal enzyme secreted into CSF should reach the entire brain, with delivery enhanced by cross-correction.

To further refine the *mo-br* rescue and plan a path forward to human application, we are currently pursuing an approach that combines brain-directed ICV administration of rAAV9 or rAAVrh10, more potent AAV serotypes than rAAV5, with subcutaneous injections of copper histidinate, the compound and mode of administration we employ in our Menkes disease clinical trial. Results suggest improved survival and performance quality using rAAV9 and subcutaneous copper and thus augur well for future FDA IND (investigational new drug) approval, which will permit a first-in-human clinical trial of this approach in Menkes disease patients with complete loss-of-function mutations in the ATP7A copper transporter.

Choroid plexus–targeted gene therapy may be especially relevant to gene therapy of lysosomal storage diseases (LSDs) that impact the CNS. Intrathecal delivery (by injecting enzyme into the cerebrospinal fluid during a spinal tap) of recombinant lysosomal enzymes has been successful in ameliorating LSDs in some animal studies and in human clinical trials. However, a major drawback to this approach is the need for repeated (e.g., monthly) intrathecal injections owing to the short half-lives of recombinant enzymes. An alternative strategy is to remodel CP epithelial cells with an AAV vector containing the cDNA for the enzyme of interest. Given the extremely low turnover rate of CP epithelia, the approach could generate a permanent source of enzyme production for secretion into the CSF and penetration into cerebral and cerebellar structures. For the project supported by our 2014 NIH U01 Award, entitled "Choroid plexus-directed gene therapy for Alpha-mannosidosis," in collaboration with John Wolfe, we will use both mouse and cat models of alpha-mannosidosis to evaluate choroid plexus transduction by several rAAV vectors as well as post-treatment alpha-mannosidase concentration and distribution in brain. Studies in the mouse model (obtained by NICHD through a Material-CRADA [Cooperative Research and Development Agreement] with the University of Kiel, Germany) will require less virus and the mice will be easier to breed. The cat model (housed at the University of Pennsylvania) features a gyrencephalic brain more similar to the human brain. Thus, the study of these two models will be complementary. In a related study, we are collaborating with Patricia Dickson to compare the efficiency of CP–mediated lysosomal enzyme production with intrathecal enzyme replacement in a mouse models of mucopolysaccharidosis type 3B (Sanfilippo syndrome).

Disease mechanisms that underlie ATP7A–related distal motor neuron degeneration

The P-type ATPase ATP7A regulates cellular copper homeostasis by its activity at the *trans*-Golgi network (TGN) and plasma membrane (PM), with its location normally governed by intracellular copper concentration. In addition to causing Menkes disease, defects in ATP7A may lead to the disease variants occipital horn syndrome and ATP7A–related distal motor neuropathy, a newly discovered adult-onset condition for which the precise pathophysiology has been obscure. We characterized the two ATP7A motor-neuropathy mutations (T994I, P1386S) and identified molecular mechanisms for abnormal intracellular trafficking. In the patients' fibroblasts, total internal reflection fluorescence (TIRF) microscopy indicated a shift in steady-state equilibrium of $ATP7A^{T994I}$ and $ATP7A^{P1386S}$, with excess PM accumulation. Transfection of 293T cells and NSC-34 motor neurons with the mutant alleles tagged with Venus fluorescent protein also showed enhanced PM localization and delayed endocytic retrieval of the mutant alleles to the TGN.

Immunoprecipitation assays revealed an abnormal interaction between $ATP7A^{T994I}$ and p97/VCP (valosin-containing protein), a protein that normally associates with the endocytic trafficking proteins clathrin and early endosomal autoantigen 1 (EEA1) and which is mutated in two autosomal dominant forms of motor neuron disease: amyotrophic lateral sclerosis and inclusion body myopathy with early-onset Paget disease and fronto-temporal dementia. Small-interfering RNA (siRNA) knockdown of p97/VCP corrected $ATP7A^{T994I}$ mislocalization. VCP did not interact significantly with $ATP7A^{P1386S}$, the other mutant allele associated with the motor-neuropathy phenotype. However, flow cytometry documented that non-permeabilized $ATP7A^{P1386S}$ fibroblasts bound to a carboxyl-terminal ATP7A antibody, a finding consistent with partially destabilized insertion of the eighth transmembrane helix and relocation of the di-leucine endocytic retrieval signal from the cytosolic to the extracellular face of the PM. The findings illuminated mechanisms underlying ATP7A–related distal motor neuropathy, established a common link between genetically distinct forms of motor neuron disease, clarified the normal process of ATP7A endocytosis, and highlighted the possible functional role of ATP7A in the peripheral nervous system.

We recently extended our studies on this topic to elucidate the specific association of adaptor protein complexes 1 and 2 (AP-1, AP-2) with normal trafficking of ATP7A. We are also investigating the MEDNIK syndrome (caused by mutations in an AP-1 subunit) with animal models (mouse, zebrafish) and clinical studies of affected patients. The role of post-translational modifications, such as acetylation and palmitoylation, are among other topics under active investigation. We hope that these studies will help resolve unanswered questions concerning the molecular mechanisms of altered copper ATPase intracellular trafficking.

Clinical protocols

1. Principal Investigator, 90-CH-0149: Early copper histidine treatment in Menkes disease: relationship of molecular defects to neurodevelopmental outcomes
2. Associate Investigator, 02-CH-0023: Studies of pediatric patients with metabolic or other genetic disorders
3. Principal Investigator, 09-CH-0059: Molecular bases of response to copper treatment in Menkes disease, related phenotypes, and unexplained copper deficiency
4. Principal Investigator, 14-CH-0106: Clinical Biomarkers in Alpha-mannosidosis
5. Associate Investigator, Partnership for Research on Ebola Virus in Liberia PREVAIL III (15-I-N122); Monrovia, Liberia
6. Sub-Investigator, Partnership for Research on Ebola Virus in Liberia PREVAIL I (15-I-N071); Monrovia, Liberia
7. Associate Investigator; Phase II Study of AAV9-GAA Gene Transfer in Pompe Disease (NHLBI U01 Award, Co-PIs: B. Byrne/A. Arai)

Patents filed

- » Patent 4239-81164-01: Identification of subjects likely to benefit from copper treatment. International Filing Date: 06 October, 2008
- » Patent 62/244,594: Codon-optimized reduced-size ATP7A cDNA and uses for treatment of copper transport disorders. Filing date: 21 October, 2015

ADDITIONAL FUNDING

- » 2015 NIH Bench-to-Bedside Award (Kaler/Petris/Feldman)
- » U01-CH-079066-01. Choroid plexus-directed gene therapy for alpha-mannosidosis
- » U01-HL121842-01A1. Phase II Study of AAV9-GAA Gene Transfer in Pompe Disease

PUBLICATIONS

1. Haddad MR, Donsante A, Zerfas P, Kaler SG. Fetal mouse brain-directed AAV gene therapy results in rapid, robust, and persistent transduction of choroid plexus epithelia. *Mol Ther Nucleic Acids* 2013; 2:101–108.
2. Kaler SG. Neurodevelopment and brain growth in classic Menkes disease is influenced by age and symptomatology at initiation of copper treatment. *J Trace Elem Med Biol* 2014; 28:427–430.
3. Bandmann O, Weiss KH, Kaler SG. Wilson's disease and other neurological copper disorders. *Lancet Neurol* 2015; 14:103–113.
4. Choi EY, Patel K, Haddad MR, Yi L, Kaler SG. Duplication of ATP7A exons 1-7 neither impairs gene expression nor causes a Menkes disease phenotype. *JIMD Rep* 2015; 20:57–63.
5. Ling Y, Kaler SG. Direct interactions of adaptor protein complexes 1 and 2 with the copper transporter ATP7A mediate its anterograde and retrograde trafficking. *Hum Mol Genet* 2015; 24:2411–2425.

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Our goal is to understand how mammals regulate intracellular and systemic iron metabolism. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) regulate the expression of numerous proteins of iron metabolism. In iron-depleted cells, the proteins bind to RNA stem-loops in transcripts known as iron-responsive elements (IRE). IRP binding stabilizes the mRNA that encodes the transferrin receptor and represses the translation of transcripts that contain IREs near the 5′ end of the ferritin H and L chains. IRP1 is an iron-sulfur protein that functions as an aconitase in iron-replete cells. IRP2 is homologous to IRP1 but undergoes iron-dependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration—all likely the result of functional iron deficiency. Biochemically and with expression arrays, we have studied the mechanisms that lead to anemia and neurodegeneration in $IRP2^{-/-}$ mice. We are using our mouse model of neurodegeneration to identify compounds that can prevent neurodegeneration; for example, we found that the antioxidant Tempol works by activating the latent IRE–binding activity of IRP1. We are evaluating the possibility that loss of IRP2 in humans causes mild refractory anemia and adult-onset neurodegeneration, which is characterized by limb weakness and might be diagnosed as amyotrophic lateral sclerosis. Given that mitochondrial energy production is required to maintain axonal integrity and that motor neurons have the longest and most vulnerable axons, we hypothesize that mitochondrial dysfunction resulting from iron deficiency causes axonal degeneration. We discovered that deficiency in IRP1 causes polycythemia and pulmonary hypertension owing to translational derepression of HIF2α through the IRE–IRP system. Our discovery introduces a new level of physiological regulation of erythropoiesis and provides a model for early pulmonary hypertension.

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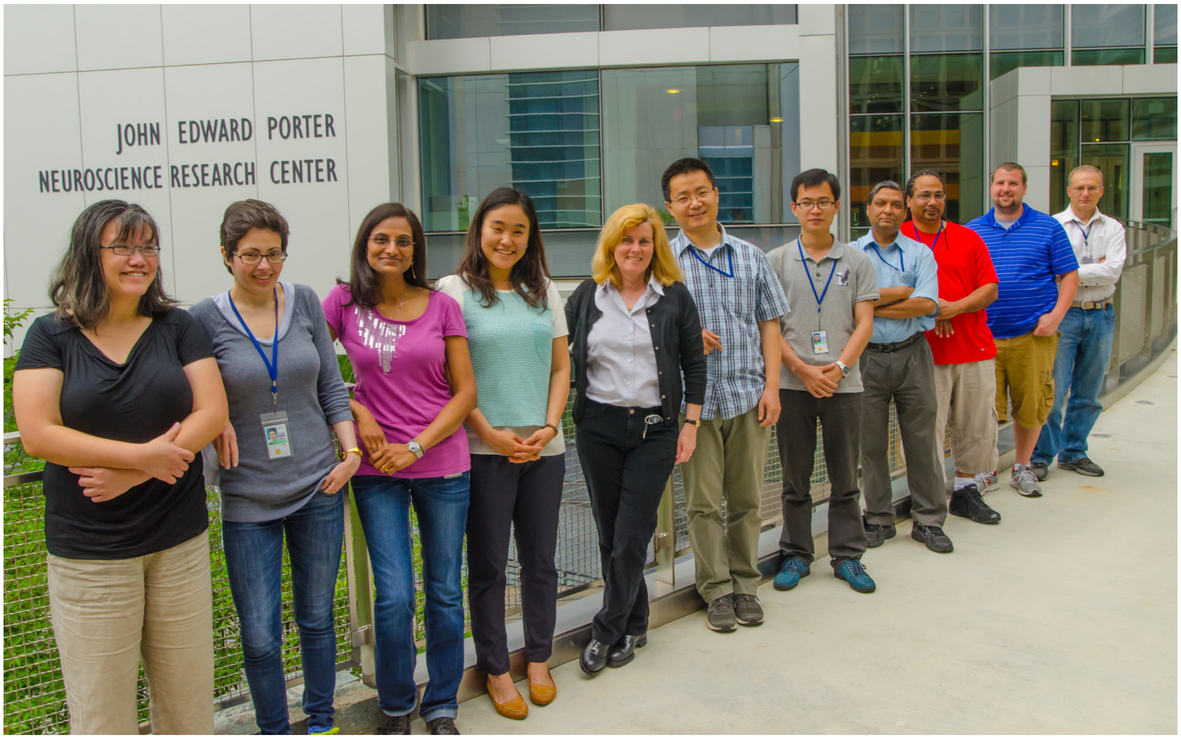
The molecular basis for regulation of intracellular iron metabolism in mammals

In previous years, our laboratory identified and characterized the *cis* and *trans* elements mediating iron-dependent alterations in the abundance of ferritin and the transferrin receptor. IREs are RNA stem-loops found in the 5′ end of ferritin mRNA and the 3′ end of transferrin receptor mRNA. We cloned, expressed, and characterized two essential iron-sensing proteins, IRP1 and IRP2. IRPs bind to IREs when iron levels are depleted, resulting in either inhibition of translation of ferritin mRNA and of other transcripts that contain an IRE in the 5′-untranslated regions (UTR) or stabilization of the transferrin receptor mRNA and possibly other transcripts that contain IREs in the 3′ UTR. The IRE–binding activity of IRP1 depends on the presence of an iron-sulfur cluster (see "Mammalian iron-sulfur cluster biogenesis" below). IRP2 also binds to IREs in iron-depleted cells but, unlike IRP1, is degraded in iron-replete cells, where it is selectively ubiquitinated and then degraded by the proteasome.

To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we initially observed no abnormalities in iron metabolism associated with



Members of the Section on Human Iron Metabolism

loss of IRP1 function. IRP2$^{-/-}$ mice develop a progressive neurologic syndrome characterized by gait abnormalities and axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. IRP2$^{-/-}$ animals develop iron-insufficiency anemia and erythropoietic protoporphyria. In animals that lack IRP1, IRP2 compensates for loss of IRP1's regulatory activity in most cell types, but we discovered several cell types and accompanying phenotypes in which IRP2 expression cannot be sufficiently increased to compensate. Animals that lack both IRP1 and IRP2 die as early embryos. The adult-onset neurodegeneration of adult IRP2$^{-/-}$ mice is exacerbated when one copy of IRP1 is also deleted. IRP2$^{-/-}$ mice offer a unique example of spontaneous adult-onset, slowly progressive neurodegeneration; analyses of gene expression and iron status at various stages of disease are ongoing. Dietary supplementation with the stable nitroxide Tempol prevents neurodegeneration; the treatment appears to work by recruiting IRE–binding activity of IRP1. We found that motor neurons were the most adversely affected neurons in IRP2$^{-/-}$ mice and that neuronal degeneration accounted for the gait abnormalities.

We discovered a form of the iron exporter ferroportin that lacks an IRE at its 5′ end and is important in permitting iron to cross the duodenal mucosa in iron-deficient animals and in preventing developing erythroid cells from consuming excessive amounts of iron in iron-deficient animals. In addition, we recently discovered that loss of IRP1 causes polycythemia and pulmonary hypertension through derepression of hypoxia-inducible factor 2-α (HIF-2α) translation in renal interstitial through the IRE–IRP system.

We also elucidated the pathophysiology of intravascular hemolysis and hyposplenism in animals that lack heme oxygenase 1. Their tissue macrophages die because they cannot metabolize heme after phagocytosis of red cells. To mitigate or reverse disease, we performed bone marrow transplants from wild-type animals to supply animals with functional macrophages; the bone marrow transplants were successful. We thus aim to correct the heme oxygenase defect in hematopoietic stem cells, using CRISPR technology, and to fully correct heme-oxygenase deficiency in the mice, experiments that would pave the way to treating heme oxygenase 1–deficient human patients, an underdiagnosed rare patient group.

Mammalian iron-sulfur cluster biogenesis

Our goals in studying mammalian iron-sulfur biogenesis are to understand how iron-sulfur prosthetic groups are assembled and delivered to target proteins in the various compartments of mammalian cells, including mitochondria, cytosol, and nucleus. In addition, we seek to understand the role of iron-sulfur cluster assembly in the regulation of mitochondrial iron homeostasis and the pathogenesis of diseases such as Friedreich's ataxia and sideroblastic anemia, which are both characterized by incorrect regulation of mitochondrial iron homeostasis.

IRP1 is an iron-sulfur protein related to mitochondrial aconitase, which is a citric acid cycle enzyme that functions as a cytosolic aconitase in iron-replete cells. Regulation of RNA–binding activity of IRP1 involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound to a form that loses both iron and aconitase activity. The [4Fe-4S]–containing protein does not bind to IREs. Controlled degradation of the iron-sulfur cluster and mutagenesis reveal that the physiologically relevant form of the RNA–binding protein in iron-depleted cells is an apoprotein. The status of the cluster appears to determine whether IRP1 binds to RNA. We identified numerous mammalian enzymes of iron-sulfur cluster assembly that are homologous to those encoded by the *nifs*, *iscu*, and *nifu* genes, which are implicated in bacterial iron-sulfur cluster assembly, and we observed that mutations in several iron-sulfur cluster biogenesis proteins cause disease. Loss of frataxin causes Friedreich's ataxia, which is characterized by progressive compromise of balance and cardiac function. In a cohort of patients of Swedish descent, we found that loss of ISCU causes skeletal myopathy. To explain the tissue specificity of ISCU myopathy, we studied myoblasts and other patient-derived tissue samples and cell lines. We discovered that many factors contribute to insufficiency of the iron-sulfur scaffold protein ISCU in skeletal muscle, including more pronounced abnormal splicing and unusual sensitivity of ISCU to degradation upon exposure to oxidative stress. Thus, oxidative stress may impair the ability of tissues to repair damaged iron-sulfur clusters by directly damaging a key component of the biogenesis machinery. A splicing abnormality of glutaredoxin 5 was found to be associated with sideroblastic anemia in one patient. In the affected tissues, mitochondrial iron overload is a feature common to all three diseases. In collaborative work, we discovered mutations of two new iron-sulfur cluster assembly proteins, NFU1 and BOLA3, which are required to provide iron-sulfur clusters to lipoate-dependent enzymes. We identified a tripeptide motif, LYR, in apoproteins that are recipients of nascent iron-sulfur clusters. The co-chaperone HSC2 binds to HSPA9, its partner HSP70–type chaperone, and that chaperone complex binds to ISCU that contains a nascent iron-sulfur cluster and two recipient proteins. We identified several direct iron-sulfur–recipient proteins in a yeast two-hybrid assay using HSC20 as bait. By studying one known iron-sulfur recipient, succinate dehydrogenase subunit B, we discovered that multiple LYR motifs of the SDHB primary sequence engage the iron-sulfur transfer apparatus by binding to the C-terminus of HSC20, facilitating delivery of the three iron-sulfur clusters of succinate dehydrogenase subunit B. We further discovered that the assembly factor SDHAF1 also engages the iron sulfur cluster transfer complex to facilitate transfer of iron sulfur clusters to SDHB. The discovery of the LYR motif will aid in identification of unknown iron-sulfur proteins, which are likely much more common in mammalian cells than has been previously appreciated.

Using expression arrays, we analyzed the mechanisms by which compromised mitochondrial iron-sulfur cluster biogenesis leads to mitochondrial iron overload. We postulate that regulation of mitochondrial iron homeostasis depends on intact synthesis of an iron-sulfur cluster–regulatory protein. Once this pathway is better understood, insights may lead to treatments for several rare diseases.

We are pursuing the use of anti-sense therapy as a treatment for ISCU myopathy, and we have been able to correct the causal splicing defect in patient myoblasts using stable anti-sense RNAs that were manufactured by high-quality techniques suitable for use in patients.

PUBLICATIONS

1. Kovtunovych G, Ghosh MC, Ollivierre W, Weitzel RP, Eckhaus MA, Tisdale JF, Yachie A, Rouault TA. Wild-type macrophages reverse disease in heme oxygenase 1-deficient mice. *Blood* 2014; 124:1522–1530.
2. Maio N, Singh A, Uhrigshardt H, Saxena N, Tong WH, Rouault TA. Iron-sulfur cluster biogenesis in mammalian cells: new insights into the molecular mechanisms of cluster delivery. *Cell Metab* 2014; 19:445–457.
3. Rouault TA. Mammalian iron-sulphur proteins: novel insights into biogenesis and function. *Nat Rev Mol Cell Biol* 2015; 16:45–55.
4. Ghosh MC, Zhang L, Rouault TA. Iron misregulation and neurodegenerative disease in mouse models that lack iron regulatory proteins. *Neurobiol Dis* 2015; 81:66–75.
5. Rouault TA. Iron-sulfur proteins hiding in plain sight. *Nat Chem Biol* 2015; 11:442–445.

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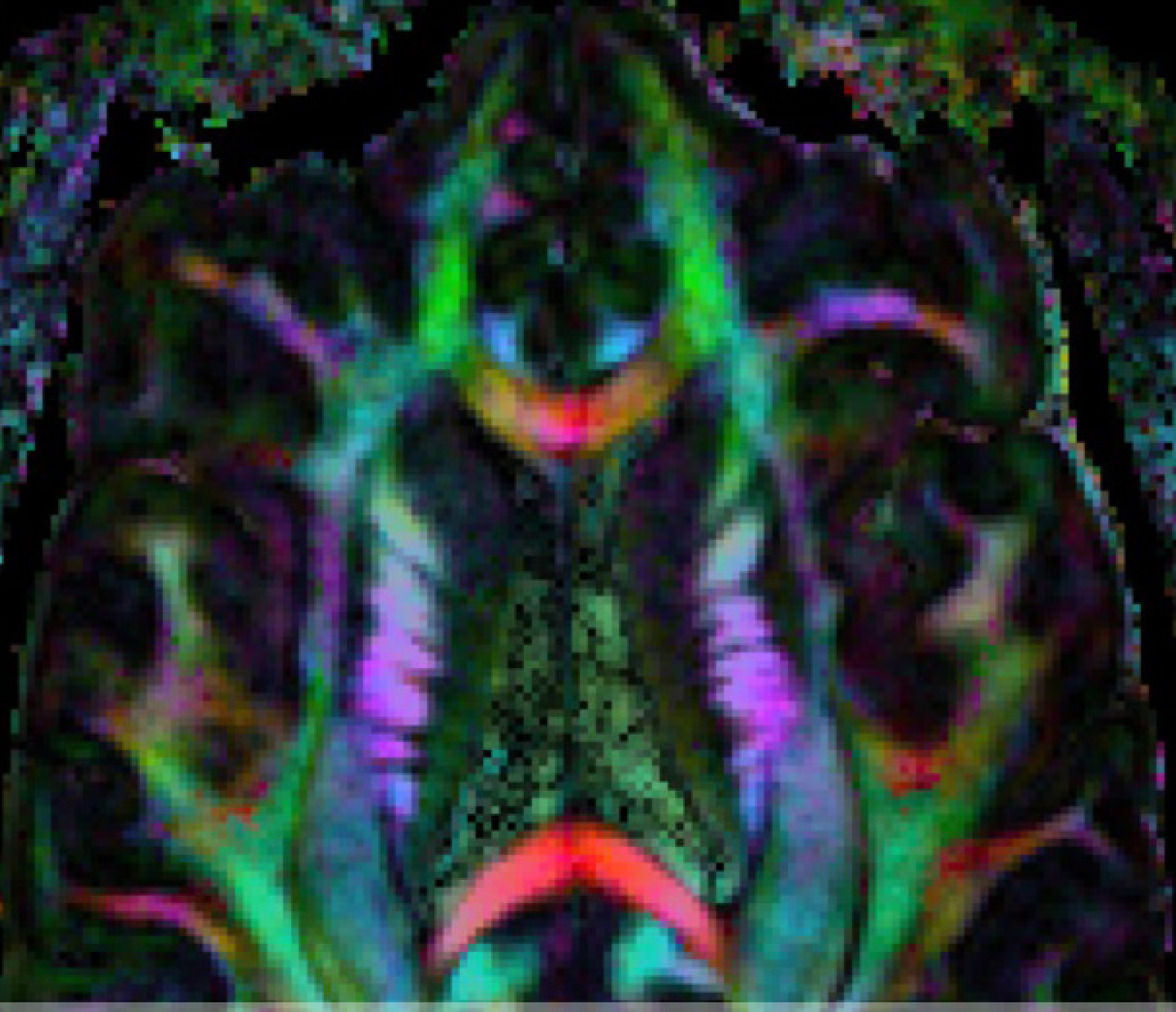
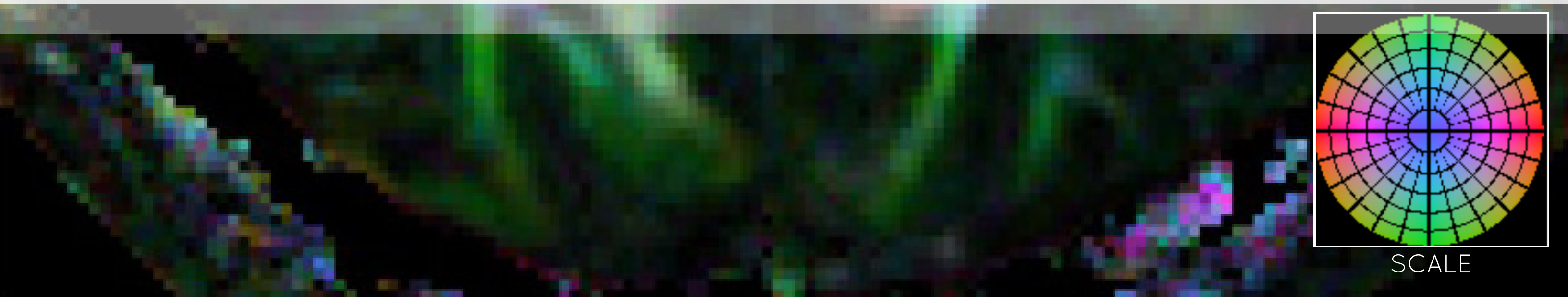


Program in Pediatric Imaging and Tissue Sciences

Director: Peter J. Basser, PhD

ABOUT THIS IMAGE

High resolution Directionally Encoded Color map of white matter fiber orientation obtained from Diffusion Tensor MRI data in two axial slices of the brain at the level of the PONS and in a more cranial slice in the brain. This image was provided by Carlo Pierpaoli, PhD, of the *Section on Tissue Biophysics and Biomimetics*.



Program *in* Pediatric Imaging and Tissue Sciences

The *Program on Pediatric Imaging and Tissue Sciences (PPITS)* was created in January, 2010, to address critical, unmet needs in Pediatric Radiology, particularly Pediatric Neuroradiology. The unique Program sponsors a broad range of basic, applied, and translationally oriented research aimed at improving the assessment of normal development and at screening, diagnosis, and prognosis of diseases, disorders, or disabilities common in the pediatric population. To this end, PPITS scientists invent, develop, and apply non-invasive imaging methods and modalities to produce quantitative imaging biomarkers that can sensitively and selectively measure key features of target tissues or organs. To achieve such translational goals, PPITS supports and performs a wide array of basic and applied research in tissue sciences, which aim to identify and characterize potentially salient quantitative biomarkers, as well as in the physical, mathematical, and imaging sciences to provide a conceptual framework for measuring such biomarkers.

The *Section on Tissue Biophysics and Biomimetics (STBB)*, headed by Peter Basser, strives to understand fundamental relationships between functional properties of soft tissues and their structure *in vivo*, in "engineered" tissue constructs, and in tissue analogs (e.g., polymer gels). Structure/function relationships are studied in an integrative fashion, primarily by probing key interactions and processes over a wide range of length and time scales, as well as by developing and studying relevant biological, mathematical, physical, and computational models and model systems that illuminate such relationships. Achievements include the development of a method based on anomalous X-ray scattering to measure the ion distribution around charged biopolymer molecules and construction of a tissue micro-osmometer that permits continuous monitoring of water uptake of small specimens. The STBB also developed an experimental method to map the elastic properties of tissues and cells at a micron scale. These activities support and buttress STBB's initiatives to invent, develop, and translate novel quantitative *in vivo* methods for imaging tissues and organs, in particular, new quantitative MRI methodologies to probe tissue microstructure and architectural organization in the brain and, increasingly, in other soft tissues. Recent examples include non-invasive MRI methods to measure and map the diameter distribution of axons within white matter pathways and to parcellate the cerebral cortex *in vivo*, based on local microstructural features. The STBB is involved in several pre-clinical, clinical, and translational studies intended to migrate promising quantitative imaging biomarkers from "bench to bedside."

The *Section on Analytical and Functional Biophotonics (SAFB)*, headed by Amir Gandjbakhche, devises quantitative biophotonics imaging technologies and methodologies, translating benchtop studies to the bedside. Research in the Section explores endogenous (scattering and absorption) and exogenous (using fluorescence probes) optical contrast mechanisms to characterize abnormal development and function in tissues. The SAFB is using near infrared spectroscopy and imaging to assess biomarkers for a wide range of brain development abnormalities and injuries, specifically, but not limited to, cognitive and behavioral disorders. The Section is also involved in clinical and preclinical studies aimed at characterizing the growth and development of various abnormal tissues and monitoring the efficacy of treatment, using optical methods such as fluorescence and multi-spectral imaging.

We strive to understand fundamental relationships between function and structure in living tissues, 'engineered' tissue constructs, and tissue analogs. Specifically, we are interested in how microstructure, hierarchical organization, composition, and material properties of tissues affect their biological function or dysfunction. We investigate biological and physical model systems at relevant hierarchical length and time scales, performing physical measurements in tandem with developing mathematical and computational models to explain salient features of these systems. Experimentally, we use water molecules to probe both equilibrium and dynamic interactions among tissue constituents from nanometers to centimeters and from microseconds to lifetimes. To determine the equilibrium osmo-mechanical properties of well defined model systems, we vary water content or ionic composition systematically. To probe tissue structure and dynamics, we employ atomic force microscopy (AFM), small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), static light scattering (SLS), dynamic light scattering (DLS), and one and two-dimensional nuclear magnetic resonance (NMR) relaxometry. We develop and use mathematical models to help us understand how observed changes in tissue microstructure and physical properties affect essential transport processes (e.g., of mass, charge, and momentum). The most direct and powerful noninvasive *in vivo* method for characterizing transport processes in tissues is magnetic resonance imaging (MRI), which we use to follow microstructural changes in development, degeneration, aging, and trauma. A goal of our basic tissue sciences research is to translate our bench-based quantitative methodologies, and the understanding we glean from them, to the bedside.

In vivo MRI histology

We aim to develop novel next-generation *in vivo* MRI methods to better understand brain structure and organization in normal and abnormal development, disease, degeneration, and trauma. The most mature technology that we invented and developed is Diffusion Tensor MRI (DTI), by which we measure a diffusion tensor of water, D, voxel-by-voxel within an imaging volume. Information derived from this quantity includes white matter fiber–tract orientation, the mean-squared distance that water molecules diffuse in each direction, the orientationally averaged mean diffusivity, and other scalar (invariant) quantities that are intrinsic to the tissue and independent of the experiment. The parameters behave like quantitative histological 'stains' even though they are obtained from endogenous tissue water non-invasively and are 'developed' without exogenous contrast agents or dyes. The bulk or orientationally averaged diffusivity is the most successful DTI parameter proposed to date and is widely used to identify ischemic regions in the brain during acute stroke, as well as for many other indications. Our measures of diffusion anisotropy (e.g., the "Fractional Anisotropy" or FA) are universally used to follow changes in normally and abnormally developing white matter, including dysmyelination and demyelination. Our group also pioneered the use of fiber direction–encoded color (DEC) maps to display the orientation of the main association, projection, and commissural white matter pathways in the brain. To assess anatomical connectivity among various cortical brain areas, we also proposed and developed DTI "Streamline" Tractography.

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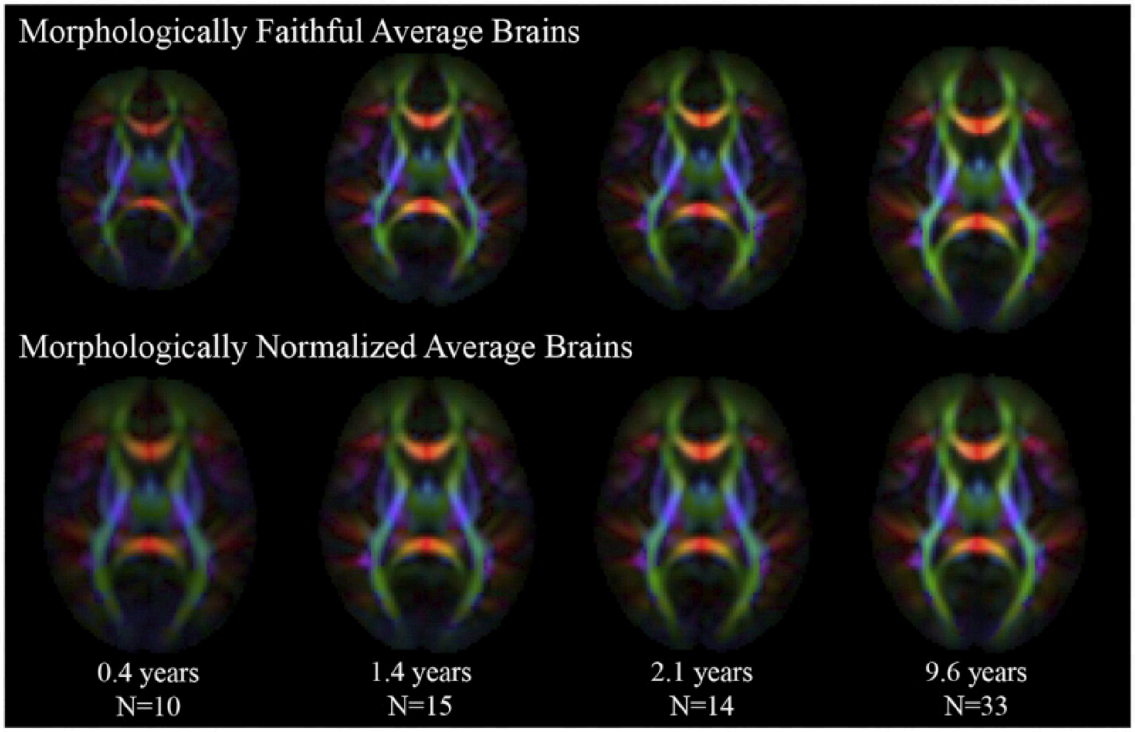

Figure 1. Brain white matter development in healthy pediatric subjects

Diffusion Tensor Imaging (DTI) directionally-encoded color (DEC) maps computed from age-specific average brains. Registration was performed using a novel diffeomorphic transformation framework. Two types of DTI templates are now available for download (**https://ndar.nih.gov/edit_collection.html?id=1151**) from the database: morphologically faithful templates, which represent the average morphology of the subjects included in each age group (top row), and morphologically normalized templates in which the average brain is further warped to the morphology of the 18–20 year old group (bottom row).

More recently, we invented and developed more advanced *in vivo* MR methods to measure fine-microstructural features of axons and fascicles, which previously could only be measured optically using laborious *ex vivo* histological methods. We are developing efficient means for performing "k and q-space MRI" in the living brain, the most recent of which is "Mean Apparent Propagator" (MAP) MRI. The approach can detect subtle microstructural and architectural features in both gray and white matter at a finer spatial resolution than can DTI and also subsumes DTI, as well as providing a bevy of new *in vivo* quantitative 'stains' to measure and map. We can also use MAP-MRI to characterize features of 'anomalous' or fractal diffusion that we recently observed in the neuropil, which consists of a patchwork of interwoven dendrites and axons and of neuroglial cells in the gray matter of the central nervous system. We also developed a family of diffusion MRI methods to enable us to 'drill down into the voxel' and measure features such as average axon diameter (AAD) and axon diameter distribution (ADD) within large white-matter fascicles, dubbing the methods CHARMED and *AxCaliber* MRI, respectively. After careful validation studies, we reported the first *in vivo* measurement of ADDs within the rodent corpus callosum. The ADD is important neurophysiologically and developmentally given that axon diameter determines conduction velocity and therefore affects the rate of information transfer along white matter pathways among various brain areas. We developed mathematical models to explain the observed ADDs in different fascicles, suggesting that they represent a balance between maximizing information flow while minimizing metabolic demands. We also developed novel multiple pulsed-field gradient (mPFG) methods and demonstrated their feasibility for use *in vivo* on conventional clinical MRI scanners as a further means to extract quantitative features to measure and map in the nervous system. The methods provide an independent measurement of mean axon diameter and other features of cell size and shape.

Although gray matter appears featureless in DTI maps, its microstructure and architecture are rich and varied, not only along the brain's cortical surface, but also within and among its different cortical layers. We have been developing several noninvasive, *in vivo* methods to measure unique features of cortical gray matter microstructure and architecture that are currently invisible

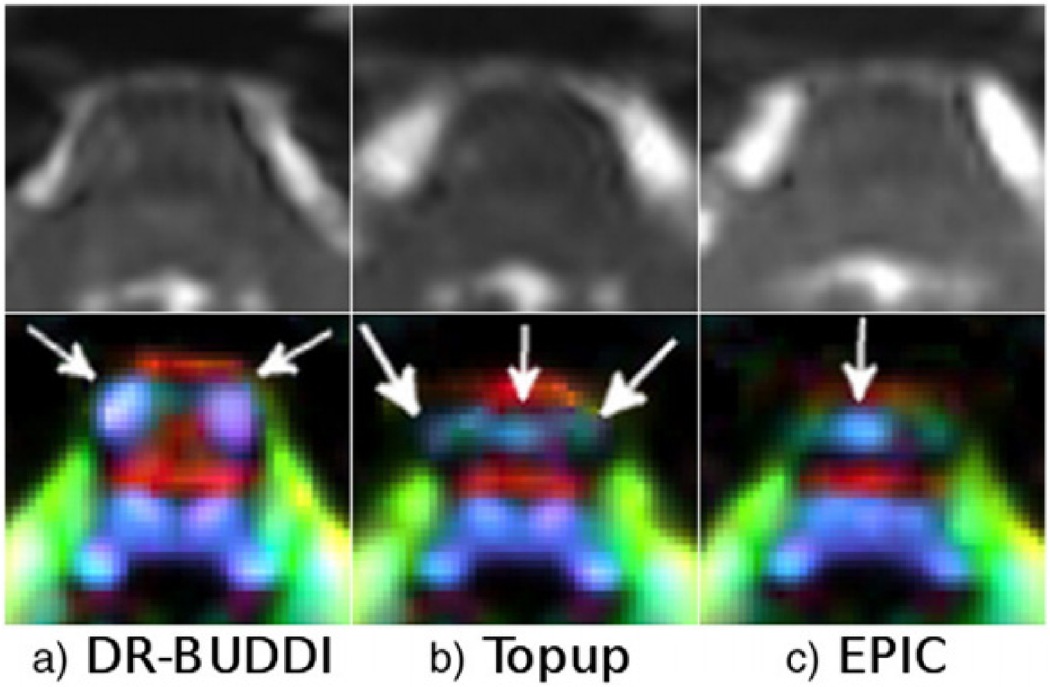

Figure 2. Comparison of tensor-based registration methods

Dr. BUDDI, our group's new method for correcting distortion caused by the diffusion weighted Echo-Planar Imaging (EPI) acquisition, is juxtaposed with two other widely used methods for correcting these artifacts. While T_2-weighted images (top row) look fairly similar in the pons, there are significant artifacts in the white matter architecture within this structure (bottom row) when employing two widely used distortion-correction schemes. *Topup* shows three sensory pathways rather than two; *EPIC* causes the two known sensory pathways to coalesce into one. *Dr. BUDDI* preserves the two known neuroanatomical structures in this important brain region and generally out-performs other methods available to address this important imaging artifact. *Dr. BUBBI* is one of the methods we are developing to advance the field of quantitative imaging.

in conventional MRI. One goal is to 'parcellate' or segment the cerebral cortex *in vivo* into its approximately 500 distinct cytoarchitechtonic or Brodmann-like areas. To this end, we are developing advanced MRI sequences to probe correlations among microscopic displacements of water molecules in the neuropil as well as sophisticated mathematical models to infer distinguishing microstructural and morphological features of gray matter that can aid us in the parcellating the cortex. We are continuing to develop such methods, which will permit us to follow normal and abnormal development, and aid in the diagnosis of various diseases and disorders affecting the cerebral cortex, noninvasively and *in vivo*, and to provide information to help neurosurgeons plan operations and interventions.

Quantitative pediatric MRI

MRI is considered safer for noninvasively scanning pediatric subjects than X-ray–based methods, such as computed tomography (CT). However, clinical MRI still lacks the quantitative character of CT data. Clinical MRI relies upon the acquisition of 'weighted images,' whose contrast is affected by many factors, some intrinsic to the tissue and some dependent on the details of the experiment and experimental design. The diagnostic utility of conventional MRI for many neurological disorders is unquestionable. However, the scope of conventional MRI applications is limited to revealing either gross morphological or focal abnormalities, which result in regional differences in signal intensities within a given tissue. To detect pathology, conventional MRI relies on differences in contrast between areas that are presumed 'affected' and those presumed 'normal,' rendering it intrinsically insensitive to subtle global changes that may affect the entire tissue or organ. Clinical MRI also lacks biological specificity. Although quantification *per se* does not assure improved specificity, it is nonetheless necessary for developing imaging 'biomarkers.' In particular, the MRI assessment of normal brain development and developmental disorders has benefiting greatly from 'quantitative' clinical MRI techniques, in which one obtains maps of meaningful physical quantities or chemical variables that can be measured in physical units and compared among and between tissue regions, in both longitudinal and cross-sectional studies. Quantitative MRI methods, such as DTI, also increase sensitivity, providing a basis for monitoring subtle changes that occur during the progression or remission of disease by comparing measurements in a single subject against normative values acquired in a healthy population. Quantitative MRI methods should also enhance our ability to perform comparative effectiveness research for a variety of diagnosis and therapy assessments and provide the tools to perform precision imaging studies.

Our group has been carrying out several clinical studies that utilize novel quantitative MRI acquisition and analysis methods, whose aim is to improve accuracy and reproducibility in diagnosis and to detect and follow normal and abnormal development. The studies include the following:

1) The NIH Study of Normal Brain Development, jointly sponsored by four NIH Institutes (NICHD, NIMH, NINDS, and NIDA), was a multi-center effort to advance our understanding of normal brain development in typical healthy children and in adolescents. The Brain Development Cooperative Group (**http://www.brain-child.org/brain_group.html**), created by this mechanism, is still active, publishing numerous papers each year, primarily by mining this rich data set. Structural MRIs and the results of standardized neuropsychological tests performed on this population are also available to researchers outside the consortium. Our role in this interdisciplinary project was to serve as the DTI Data-Processing Center (DPC). While we have now processed all admissible DTI data and uploaded them to a database accessible to all interested investigators, we continue to analyze the data, developing age-specific DTI atlases of normal brain development. We also publicly released various versions of software that we developed for this project (and related documentation), which can be downloaded from **http://www.tortoisedti.org**. We continue to support and update the software. We are continuing to use our advanced DTI–processing pipeline to produce high-quality normative data from the project, and we make them publicly available through the National Database for Autism Research (NDAR; **ndar.nih.gov**).

2) In collaboration with Susan Swedo, we study autistic subjects using DTI and quantitative relaxometry methods. Several MRI studies reported abnormal features in the autistic brain, but no clear MRI 'biomarker' of autism exists. The aim of the study is to use robust quantitative metrics to identify potential anatomical abnormalities in the autistic brain and to find candidate imaging biomarkers for the disorder (an earlier study was described in Walker et al., *Biol Psychiatry* 2012;72:1043).

3) In collaboration with Katherine Warren, we acquire quantitative MRI data in children with pontine gliomas to identify MRI prognostic factors. With John Park, we are scanning subjects with supratentorial gliomas to distinguish between recurrence and radiation necrosis.

4) In collaboration with Filippo Arrigoni (**http://www.emedea.it/english_medea/**), we use multimodal MR imaging (DTI, fMRI, and quantitative relaxometry) to evaluate cerebral reorganization caused by various rehabilitation protocols in children with cerebral palsy and traumatic brain injury (TBI). We collected diffusion MRI data on subjects affected by the pure form of hereditary spastic paraparesis, as well as those with additional cognitive impairment. There are remarkable neuroanatomic differences between the two groups.

5) We are working under the auspices of the Center for Neuroscience and Regenerative Medicine (CNRM), **http://www.usuhs.mil/cnrm/**, to investigate potential plasticity changes after rehabilitation in military personnel affected by TBI and post-traumatic stress disorder (PTSD).

6) In collaboration with Sharon Juliano, we received grant support from the Congressionally Directed Medical Research Program (CDMRP) to investigate TBI in the ferret, using advanced MRI methods, particularly MAP-MRI, developed within STBB, combined with histopathological techniques provided by Juliano's laboratory. We are currently acquiring MRI data.

Our involvement in TBI research is expanding, because it is of high relevance to the mission of NICHD and because it has become an acute problem in our young military men and women. DTI provides essential information for the diagnosis of TBI and has the potential to be developed into an important tool for the assessment of potential structural damage in PTSD. For clinical applications, however, reliable imaging protocols are needed. Part of our work is to develop a robust DTI data–processing pipeline in order to improve the accuracy and reproducibility of DTI findings for CNRM investigators and for the larger clinical and scientific community involved in TBI research. To this end, we are adding new modules to our existing state-of-the-art DTI data–processing pipeline as well as tools to permit calibration of DTI experiments, using our novel polymer-

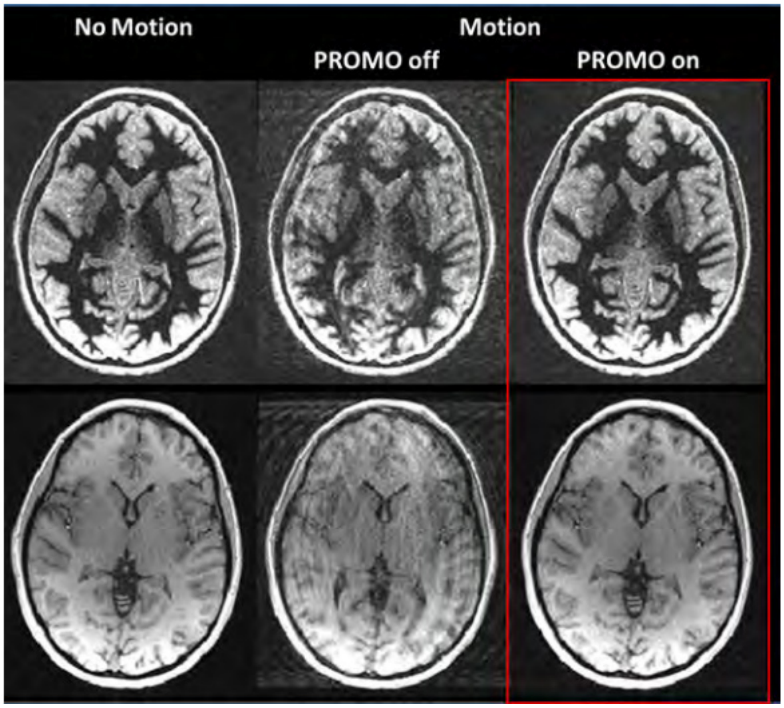

Figure 3. Prospective Motion Correction could improve pediatric image data quality. Prospective Motion Correction (PROMO)–enabled MP2RAGE MRI methods we are developing successfully correct for subject motion in the scanner. Without PROMO, subject motion generates significant artifacts that degrade spatial accuracy and tissue contrasts. Clearly, the method has promise in scanning pediatric subjects, who often have difficulty remaining still in the scanner.

based diffusion MRI phantom that we developed and are disseminating to a number of clinical sites.

Looking ahead, to permit analysis of novel MRI data such as those described above, as well as to develop new clinical and biological applications of quantitative MRI, we need to create a mathematical, statistical, and image sciences–based infrastructure. To date, we have developed algorithms that generate a continuous, smooth approximation of the discrete, noisy, measured DTI field data so as to reduce noise and allow us to follow fiber tracts more reliably. We proposed a novel Gaussian distribution for *tensor*-valued random variables that we used in designing optimal DTI experiments and interpreting their results. In tandem, we developed nonparametric empirical (e.g., Bootstrap) methods for determining the statistical distribution of DTI–derived quantities in order to study, for example, the inherent variability and reliability of white-matter fiber-tract trajectories. Such parametric and nonparametric statistical methods will enable us to apply powerful hypothesis tests to assess statistical significance in a wide range of important biological and clinical questions that are currently being addressed using *ad hoc* statistical methods. We are also developing novel methods to register or warp different brain volumes, and to generate group-average data or atlases from subject populations. Recently, our group has been developing methods for studying the reproducibility and reliability of different tractography methods, given that, increasingly, they are being used to assess anatomical connections between different brain regions *in vivo*. In the area of artifact remediation and correction, we pioneered methods to correct for subject motion, the distortion caused by induced eddy-currents, echo-planar imaging (EPI) distortion. However, much work remains to be done in order to address and remedy MRI artifacts to permit one to draw statistically significant inferences from clinical DTI data, obtained in longitudinal and multi-center studies but particularly in single subject studies.

Biopolymer physics: water-ion-biopolymer interactions

Molecular self-organization is ubiquitous in biology. Examples include DNA nanostructure formation and protein folding. Self-assembly of aberrant proteins into nanofibers is associated with neurodegenerative conditions, such as Creutzfeldt-Jakob, Alzheimer's, and Pick's disease. One objective of this research is to better understand self-assembly of biomolecules from a physical perspective by studying, primarily in polynucleic acids and proteins, particularly the interactions between water, ions, and the biopolymer.

By combining macroscopic osmotic swelling pressure measurements and small-angle scattering measurements, we developed a multi-scale experimental approach to study the structure (morphology) and thermodynamic interactions in biopolymer assemblies as a function of the length scale (spatial resolution). Swelling pressure measurements probe the system at large (macroscopic) length scales, providing information on its overall thermodynamic response, while SANS and SAXS allow us to investigate biopolymers at the atomic and molecular levels. Measurements of physical properties, such as molecular conformation and osmotic pressure, as a function of changes in environmental conditions, such as ion concentration, ion valance, pH, and temperature, all help us uncover which length scales govern the system's key macroscopic thermodynamic properties. The information cannot be obtained by other techniques.

Divalent cations, particularly calcium ions, are ubiquitous in the biological milieu. However, existing theories of polyelectrolyte solutions and gels do not adequately explain or account for their effects on, or interactions with, charged macromolecules. In pilot studies, we used our new non-destructive procedures to determine the effect of calcium ions on the physical properties of hyaluronic acid (HA) and DNA gels. The gel systems mimic certain essential features of the extracellular matrix (ECM) and have potential as scaffolds for tissue-regeneration applications. We investigated the binding mechanism in glucose sensors made from smart zwitterionic hydrogels containing boronic acid moieties. Our combined SANS and osmotic pressure measurements provided a thermodynamic explanation for the enhanced selectivity of the gels for glucose over fructose. This class of material is important for the development of implantable continuous glucose sensors intended for use in managing Type I and Type II diabetes.

We also developed a method to control the size, compactness, and stability of DNA nano-particles by mediating their interactions with ions. We quantified the effects of salt, pH, and temperature on the stability of the nano-particles and on their biological activity. Such polyplexes are pathogen-like particles, 70–300 nm in size, with shapes resembling the spherical viruses that evolved naturally to deliver nucleic acids to cells. The nano-particles contain pDNA encapsulated by synthetic polymers bearing surface sugar residues, which are recognized by "M" cells and dendritic cells as pathogens. The biological activity of such nano-medicines depends on the competing requirements of sufficient stability to escape endosomal degradation after cellular uptake and retention of biological activity upon arrival in the nucleus, where the nano-particle releases the pDNA to achieve gene expression. Such knowledge is essential to design and control properties of DNA–based nano-medicines. DNA–based vaccines are promising in several disease indications and have potential applications in the treatment of infectious diseases, cancers, and allergies. We studied nano-structures formed from small gelator molecules via enzyme-assisted self-assembly inside live cells. The gelator molecule consists of a self-assembly motif and an enzyme (phosphatase) substrate (tyrosine phosphorous ester). After the enzymatic reaction removes the phosphorous group, intermolecular interactions facilitate the self-assembly of the molecules, leading them to form nano-fibers. The approach offers a new way to control cell behavior. For example, intracellular formation of molecular assemblies could be used to selectively inhibit the growth of cells that over-express certain enzymes. Developing molecular self-assembly–based approaches and exploring their potential applications in biomedicine (e.g., intracellular drug delivery) may ultimately lead to new ways to regulate cell functions.

Functional properties of extracellular matrix

Our goal is to understand and quantify the interactions among the major macromolecular components of ECM that give rise to their key functional properties. Specifically, we are studying interactions among collagen, proteoglycans (PG), water, and ions, which govern macroscopic biomechanical and transport properties, using cartilage as a model system. The biomechanical behavior of cartilage and other ECMs reflects both biochemical and microstructural changes occurring during development, disease, degeneration, and aging. Understanding the basis of key functional properties of cartilage requires an array of experimental techniques that probe a wide range of relevant length and time scales. Knowledge of the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential for predicting its load-bearing and lubricating abilities, which are mainly governed by osmotic and electrostatic forces. The knowledge can aid in tissue-engineering or regenerative-medicine strategies to grow, repair, and reintegrate replacement cartilage. To gain a self-consistent physical picture of tissue structure/function relationships, we measure various physical/chemical properties of tissues and tissue analogs at different length- and time-scales using a variety of complementary static and dynamic experimental techniques, e.g., osmometry, SANS, SAXS, neutron spin-echo (NSE), SLS, DLS, AFM, and fluorescence correlation spectroscopy (FCS).

Controlled tissue hydration provides a direct means of determining the patency and load-bearing ability of cartilage. Previously, we used controlled hydration to measure physical/chemical properties of the collagen network and of proteoglycans. We developed and built a novel tissue micro-osmometer to perform swelling pressure measurements in a practical, rapid, and automated manner. The instrument measures minute amounts of water vapor absorbed by small tissue samples (less than 1 microgram) as a function of the water activity (vapor pressure). A quartz crystal detects the water uptake of the tissue specimen. The high sensitivity of the quartz crystal's resonance frequency to small changes in the amount of adsorbed water allows us to measure mass uptake precisely. We use osmotic pressure measurements to determine the contributions of individual components of cartilage ECM (e.g., aggrecan, hyaluronic acid [HA], and collagen) to the total tissue-swelling pressure. Our measurements on aggrecan–HA systems revealed that, in the physiological concentration range, the osmotic modulus of the aggrecan–HA complex exceeds that of random assemblies of aggrecan bottlebrushes, providing direct evidence that the aggrecan–HA complex could increase the load-bearing ability of cartilage. In addition, we demonstrated that aggrecan–HA assemblies behave like microgels, contributing to improved dimensional stability and tissue-lubricating ability. We also found that aggrecan is highly insensitive to changes in the ionic environment, particularly to Ca^{2+} ions. The latter result is consistent aggrecan's role as a calcium ion reservoir mediating calcium metabolism in cartilage and bone.

We also developed a novel method for mapping the local elastic properties of cartilage (and other heterogeneous tissues) using AFM. Many impediments that previously hindered AFM's use to probe biological samples were addressed. The technique utilizes the precise scanning capabilities of AFM to generate compliance 'maps,' from which the relevant elastic properties can be extracted. We combined AFM with tissue micro-osmometry to produce elastic and osmotic modulus maps of cartilage. The results clearly show that the spatial variation of the osmotic modulus is similar to that of the elastic (shear) modulus but that the modulus's numerical value is significantly greater. Knowledge of the osmotic modulus is particularly important because it determines the tissue's resistance to external compressive loads.

We have begun applying such critical tissue-sciences understanding of structure/function relationships of components of ECM to develop and design novel MR imaging methods with the aim of inferring ECM patency and functional properties non-invasively and *in vivo*. This extremely challenging goal is aided by our receipt of a DIR Director's Award to investigate this proposed line of research. Specifically, we are developing a family of imaging methods directed at measuring key compositional and structural features of cartilage ECM, which we can use to estimate functional properties of the tissue with the aid of a biophysical modeling framework.

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PUBLICATIONS

1. Thomas C, Ye FQ, Irfanoglu MO, Modi P, Saleem KS, Leopold DA, Pierpaoli C. Anatomical accuracy of brain connections derived from diffusion MRI tractography is inherently limited. *Proc Natl Acad Sci USA* 2014; 111(46):16574–16579.
2. Chandran PL, Dimitriadis EK, Lisziewicz J, Speransky V, Horkay F. DNA nanoparticles with core–shell morphology. *Soft Matter* 2014; 10(38):7653–7660.
3. Benjamini D, Basser PJ. Joint radius-length distribution as a measure of anisotropic pore eccentricity: an experimental and analytical framework. *J Chem Phys* 2014; 141(21):214202.
4. Sadeghi N, Nayak A, Walker L, Irfanoglu MO, Albert P, Pierpaoli C, Brain Development Cooperative Group. Analysis of the contribution of experimental bias, experimental noise, and inter-subject biological variability on the assessment of developmental trajectories in diffusion MRI studies of the brain. *NeuroImage* 2015; 109:480–492.
5. Bai R, Cloninger A, Czaja W, Basser PJ. Efficient 2D MRI relaxometry using compressed sensing. *J Magn Reson* 2015; 255:88–99.

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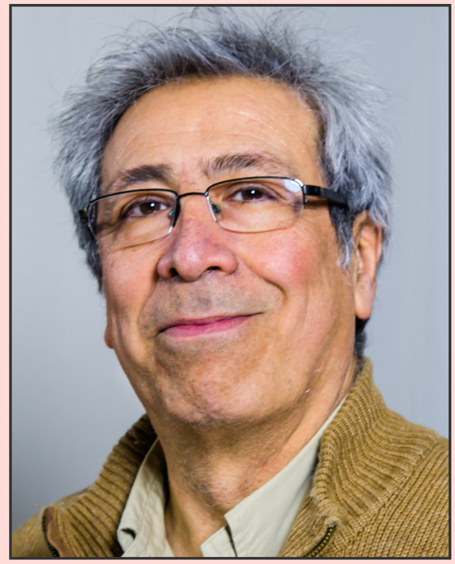
Quantitative Biophotonics for Tissue Characterization and Function

Our general goal is to devise quantitative methodologies and associated instrumentation to bring technology from the bench to bedside. We select our projects in the framework of the mission of the NICHD, focusing on childhood and adolescent disorders, as well as abnormal developments of cells such as occur in cancer. We operate in the so-called 4B research mode: at the Blackboard, one models the methodology; at the Bench, one designs the prototype to be brought to the Bedside; at this stage one goes Back to the Blackboard for improving the imaging system.



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Structural and functional brain imaging

Functional near-infrared spectroscopy (fNIRS) is an emerging non-invasive imaging technique to assess the brain function. The technique is also portable and therefore applicable in studies of children and toddlers, especially those with neurodevelopmental disorders. fNIRS measurements are based on the local changes in cerebral hemodynamic levels (oxy-hemoglobin and deoxy-hemoglobin) associated with brain activity. Given the low optical absorption of biological tissues at NIR wavelengths (700–900 nm), NIR light can penetrate deep enough to probe cortical regions up to 1–3 cm deep. Furthermore, the NIR absorption spectrum of the tissue is sensitive to changes in the concentration of major tissue chromophores such as hemoglobin, so that measurements of temporal variations of backscattered light can capture functionally evoked changes in the outermost cortex and can be used to assess the brain function. However, there is a need to address the changes in NIRS signal related to underlying physiological processes in the brain such as cerebral autoregulation (CA). In short, the mechanism of CA maintains blood flow over the range of arterial blood pressure and, owing to the high metabolic demand of neurons, becomes a vital process for a brain function. Devising a novel method of data processing to enrich informational content of measured characteristics from fNIRS is therefore crucial for further studies of brain function and development.

In one of our current studies, we use frequency analysis of oscillatory patterns from fNIRS to formulate a new metric of variations in local oxygen saturation. To characterize this metric, we introduce an Oxygen Variability Index (OV Index) and analyze two frequency bands (less than 0.1 Hz and 0.2–0.3 Hz). We then examined the relationship between OV Index in the two bands and age in a group of normally developing children. We hypothesize that the OV index, measured with the non-invasive and patient-friendly fNIRS modality, will reveal differences based on chronological age as seen in the extant literature on cerebral hemodynamics in children. We first investigated the relationship between age and OV Index, for frequencies of less than 0.1 Hz, across the Go, No-Go, and rest conditions and found a significant quadratic relationship for frequencies of less than 0.1 Hz, across task. We investigated both linear and nonlinear trends for the respiration frequencies (0.2–0.3 Hz) and found no significant change of OV index with age. The OV index values were higher for frequencies of less than 0.1 Hz, for GO/No-Go Task conditions when compared with rest. The finding is in line with those showing increases in efficiency of CA during completion of cognitive tasks (Panerai

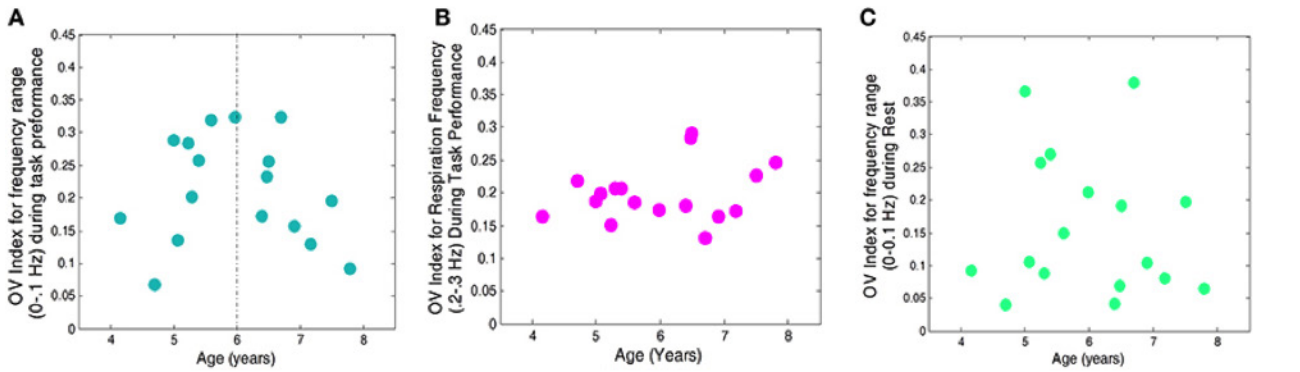

Figure 1. OV index vs. age for 17 subjects

A. Frequencies related to autoregulation (<0.1 Hz) during the task (collapsed across No-Go and Go conditions). B. Frequencies related to respiration (0.2–0.3 Hz) during the task. C. Frequencies related to autoregulation (<0.1 Hz) during rest.

et al., *Am J Physiol Heart Circ Physiol* 2005;289:H1202). Moreover, we did not find a similar trend of change with OV index during the rest when compared with the task (Figures 1 and 2).

We also explored potential prefrontal hemodynamic biomarkers to characterize subjects with Traumatic Brain Injury (TBI) by introducing a novel task-related hemodynamic response detection followed by a heuristic search for an optimum set of hemodynamic features. The hemodynamic response from a group of 31 healthy controls and 30 chronic TBI subjects were recorded as they performed a complexity task (i.e., number of events). To identify the hemodynamic signals that show task-related hemodynamic activity, trials with negatively correlated HbO and HbR and HbO larger than HbR were considered for analysis. To identify the optimum hemodynamic features, unlike common single-feature analyses for studying TBI and healthy subjects, we evaluated all possible combinations of multiple hemodynamic features to compare the TBI and healthy populations. Eleven hemodynamic features were extracted from oxygenated hemoglobin (HbO) to determine the optimum set of biomarkers. We investigated the effectiveness of the extracted features in distinguishing TBI from healthy subjects by utilizing a machine learning classification algorithm to score all the possible combinations of features according to their predictive power. The identified optimum feature elements resulted in classification accuracy, sensitivity, and specificity of 85%, 85%, and 84%, respectively. The sensitivity value of 85% suggests that TBI subjects have been characterized for the identified biomarkers with reasonable accuracy. We conducted a spatio-temporal classification to identify regions within the prefrontal cortex (PFC) that contribute to distinguishing TBI from healthy subjects. As expected, Brodmann area (BA) 10 within the PFC was isolated as the region in which healthy subjects (unlike TBI subjects) showed major hemodynamic activity in response to the High Complexity task. Overall, our results indicate that identified temporal and spatio-temporal features from PFCs hemodynamic activity are promising biomarkers in classifying subjects with TBI (Figure 3).

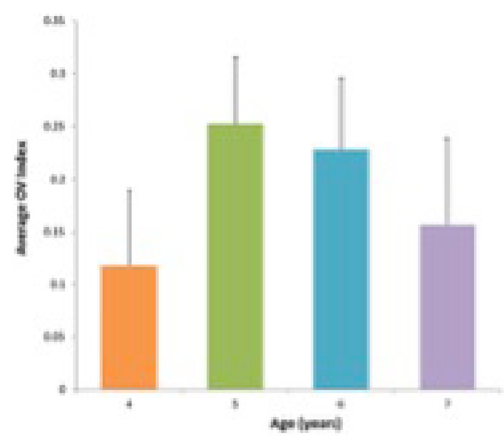

Figure 2. Average value for OV index across age groups corresponding to the frequency band <0.1 Hz during performance of the task

The OV index increases with age between 4 and 6 years and is followed by subsequent decline with age.

Multispectral imaging

Facial redness is one of the earliest described clinical features of Cushing's syndrome (CS). We are continuing a study that is aimed at quantifying the changes of facial plethora in CS as an early assessment of cure. CS patients are recruited for optical imaging sessions, before and after surgery, with a follow-up sessions six months and one year after surgery. To date, 51 patients with CS (30 females) have been enrolled. Among these, 38 patients had CD due to ACTH–secreting pituitary tumors,

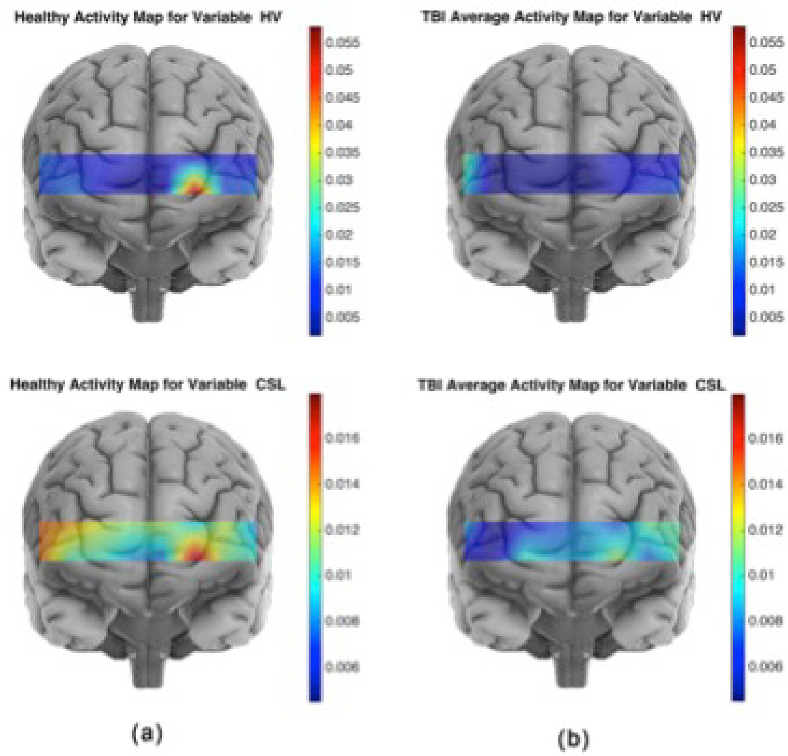

Figure 3. Average activity maps for the CSL and HV features for the healthy (a) and TBI (b) subjects

The activity map for a spatio-temporal feature associated to a population is obtained by averaging every subject's (from the corresponding population) spatio-temporal feature set. For the TBI population, the larger HV values are located at multiple locations with largest on the right hemisphere, whereas for the healthy population the largest HV is concentrated on the left hemisphere of the Brodmann area 10 (BA 10). Furthermore, on average healthy subjects show larger HV values for the HbO signal, which indicates that oxygenation signal has shown higher variation in the healthy subjects. The HbO signal in response to the High Complexity task for the healthy subjects shows larger variation and is spatially less diffuse than for the TBI subjects. Larger CSL values correspond to a faster rate of oxygenation consumption. Considering the activity map for healthy subjects, largest CSL values cover the left frontopolar of the BA 10. A comparison of healthy and TBI subjects' CSL activity map reveals that healthy subjects have shown higher oxygenation consumption rate in response to the High complexity task at all the sites of fNIRS data collection.

five had ACTH–independent adrenocortical tumors, and two had an ectopic ACTH–secreting pulmonary carcinoid. Six patients with CD were excluded from the study—two due to severe facial acne, two to fever on the day of imaging and the last two owing to cortisol injection on imaging day. Three of the patients required two consecutive surgeries, as the initial trans-sphenoidal surgery was unsuccessful. We performed non-invasive multi-spectral near-infrared imaging on the right cheek of the patients before and two days or up to two weeks after surgery. Patients were defined as cured by postoperative measurements of plasma cortisol of less than 3 mcg/dl and/or adrenocortical insufficiency, for which they received replacement. Clinical data, obtained from the 45 patients indicate that a reduction in facial plethora after surgery, as evidenced by a decrease in blood volume fraction, is well correlated with cure of CS. The results were published (Reference 1) (Figure 4).

We are also pursuing Kaposi Sarcoma (KS) studies in ongoing clinical trials under four different NCI protocols and therapeutic agents. The goal is to further evaluate diffuse multispectral imaging as a potential supplement to existing response assessment in KS, providing an early non-invasive marker of treatment efficacy. In our preliminary results, multi-spectral images of KS skin lesions were taken over the course of treatment, and blood volume and oxygenation concentration maps were obtained through Principal Component Analysis (PCA) of the data. We compared corresponding images with clinical and pathological assessment, provided by conventional means. Supporting our hypothesis that successful treatment would reduce the blood volume in the lesions, the normalized standard deviation for blood volume decreased in each of the eight patients whose lesion responded to treatment, while the normalized standard deviation for blood volume rose in two patients whose lesion did not respond to therapy. These initial results confirm that concentrations of oxygenated hemoglobin in the tumor can be a quantitative marker of tumor response to the therapy.

To map more biological chromophores, we are working on designing a new multi-spectral system that has a 12–wavelength imaging capability.

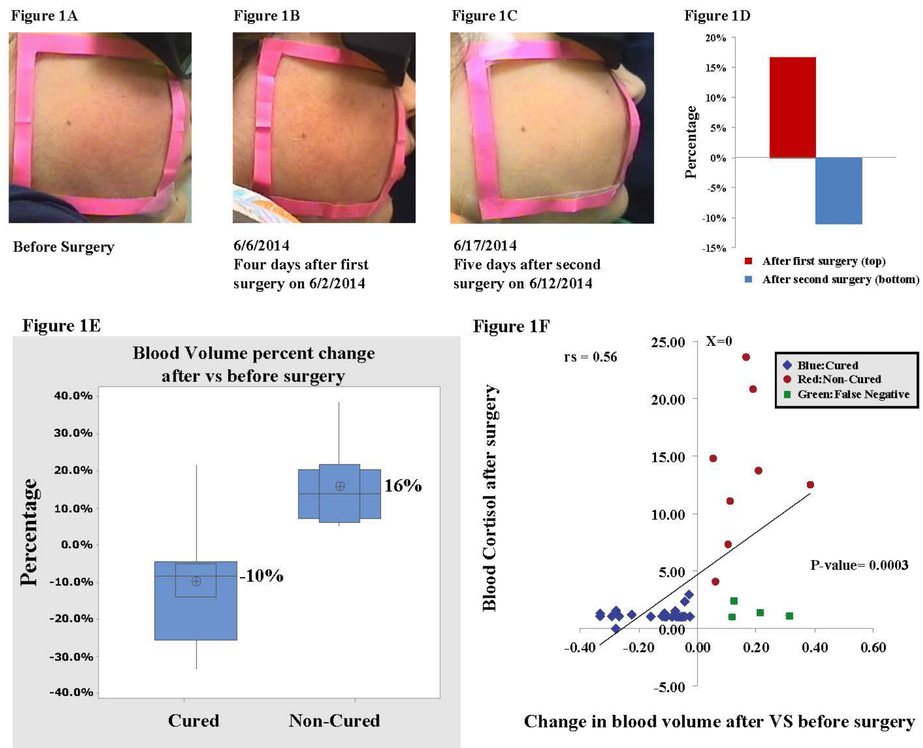

Figure 4.

A–C: Facial plethora in a patient with CD before TSS, after the first TSS (non-cured), and after the second TSS (cured)

D. The blood volume fraction percentage change of the patient's left cheek in panels A–C at different imaging sessions.

E. Boxplot of mean percentage change indicating change in blood volume after surgery, compared to before surgery, in each of the cured and noncured Cushing's patients. The distribution shows median confidence interval box and interquartile range.

F. Change in blood volume from pre- to postsurgery vs. blood cortisol level after surgery in 34 patients undergoing 36 surgical treatments for CS.

Fluorescence methods in pre-clinical studies of HER2-positive breast cancer and of basal cell carcinoma expressing BerEP4

HER2–specific fluorescently labeled probes

Detection of specific oncogenic biomarkers is an important factor in choosing the proper targeted cancer therapy. Current cancer therapy is focused on drugs that selectively attack the cancer-causing biomarkers, inactivate molecular mechanisms responsible for cell malignancy, and deliver a toxin specifically to the malignant cells. The most prominent example of this approach is the use of the monoclonal antibody (mAb) Trastuzumab antibody–drug conjugate T-DM1 to treat human epidermal growth factor receptor 2 (HER2)–positive cancers. Elevated HER2 expression is associated with increased proliferation and survival of cancer cells, thereby contributing to poor therapy outcomes. Given that the efficacy of the mAb depends on overexpression of its target on tumor cells, development of techniques to assess receptor expression is extremely important for monitoring the efficacy of therapy and optimizing treatment. Current clinical evaluation of HER2 expression is based on immunohistochemistry (IHC) or fluorescence *in situ* hybridization (FISH) staining of biopsied tissue. Both are *ex vivo* techniques and, owing to tumor heterogeneity, may deliver false-positive or false-negative results and are therefore unreliable for monitoring therapy. By contrast, molecular imaging using HER2–specific fluorescently labeled probes allows assessment of the status of HER2 receptors *in vivo* and the following of treatment in real time. After injection, probes such as the HER2–specific Affibody concentrate in the tumor. Probe accumulation at tumor cells can occur in parallel with the delivery of some HER2–specific drugs, such as Trastuzumab, to the malignant tumor. As a result, HER2–specific Affibody fluorescent probes can be used to monitor HER2 expression without interfering with the treatment itself, making this probe suitable for an image-and-treat paradigm.

Besides the information that can be extracted from the serial imaging of fluorescence intensity at different time points, additional information about tumor characteristics can be extracted from lifetime fluorescence measurements, including binding affinity of the probe to cancer cells and environmental conditions (e.g., pH), based on local variations in lifetimes for specially designed probes at a given site.

We studied the potential of *in vivo* fluorescence lifetime imaging to monitor the efficacy of treatment, in particular the feasibility of fluorescence lifetime imaging to monitor *in vivo* expression of the HER2 receptor in a breast carcinoma (mouse model) during the course of treatment. We observed a considerable difference between the fluorescence lifetime of HER2–specific optical probes at the tumor and a contralateral site before and seven days after the last treatment with 17-DMAG (an HSP90 inhibitor), when the tumor regrew to almost its pretreatment volume. However, soon after the therapy (12 hours), when the effect of drug on HER2 degradation is maximal, the difference decreased significantly. Based on our previous findings on the relationship between fluorescence lifetime and binding of a HER2–specific probe to corresponding receptors, we believe that the differences between the fluorescence lifetimes at the tumor and contralateral site, observed for mice with BT-474 xenografts after treatment with 17-DMAG, result from the strong downregulation of HER2 receptors soon after the therapy (i.e., less binding sites for the HER2–specific probe) and corresponding changes in the fraction of bound to total fluorescent probes in the tumor. Immediately after treatment, the fraction of bound to total fluorophores inside the tumor changed considerably, resulting in a noticeable increase in the average fluorescence lifetime. Subsequent tumor and HER2 expression recovery a week later caused gradual restoration of the original level of the binding ratio of HER2–targeting probe in the tumor and a corresponding return to pre-treatment values of the fluorescence lifetime. The results reveal that fluorescence lifetime imaging, based on evaluating the fraction of the bound and unbound fluorophores inside the tumor, can be used as an alternative *in vivo* imaging approach to characterize tumors, separate high from low HER2–expression tumors, and monitor the efficacy of targeted therapies (Figure 5).

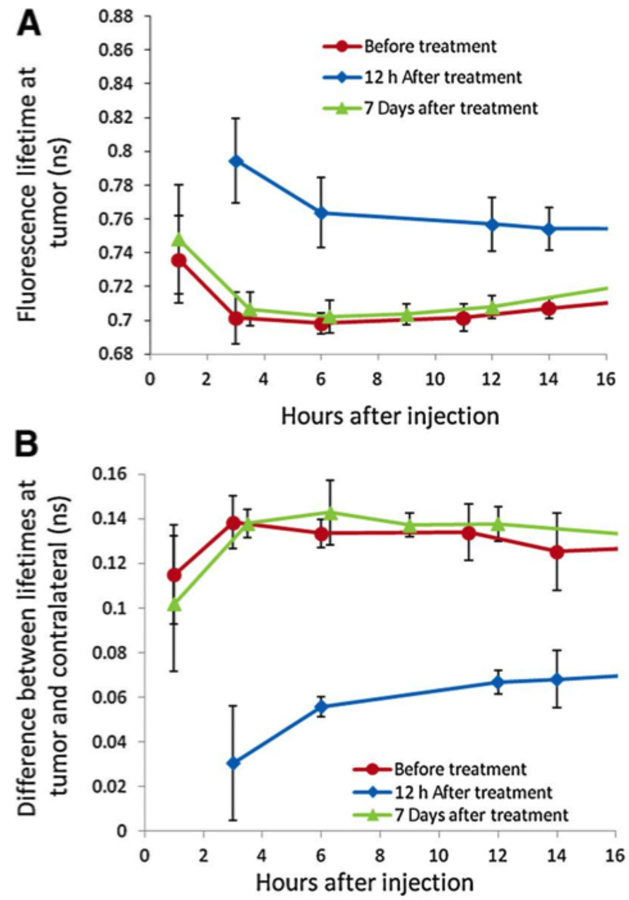

Figure 5. Fluorescence lifetimes at the tumor

A. The average fluorescence lifetime at the tumor site for five mice at different time points after injection of ABD-HER2–specific Affibody probe; before, 12 hours, and one week after the last treatment with 17-DMAG.
B. The difference between the fluorescence lifetimes at the tumor and contralateral site for the same mice as in panel A.

Molecular biomarkers targeting basal cell carcinoma (BCC) towards development of theranostics to diagnose, treat, and follow up BCC

In collaboration with Alan Halpern, we conducted a pilot study to determine the affinity and selectivity of BerEp4 antibody conjugated with fluorescence probe and to assess its possible use in designing theranostic probes for BCC. BCC appears macroscopically and microscopically similar to many other skin lesions, which makes differential diagnosis difficult. Based on initial cell culture results, BerEP4 appears to be a promising biomarker for molecular imaging of BCC and can be used in conjunction with *in vivo* near-infrared fluorescence imaging. To prepare BerEP4 for eventual theranostic use, we examined the feasibility of a combined macro-/micro-optical approach to imaging BCC with various histologies.

With an annual incidence of 3.5 million per year in U.S., BCC is the most common cancer in humans. Although, the rate of metastasis is very rare, it warrants treatment owing to local destruction and associated morbidities and even mortalities if left untreated. The current standard of care has changed little in the past 100 years. Like any other skin cancer, BCC is diagnosed by biopsy, followed by histopathology evaluation. Once diagnosed, the lesion is surgically removed to achieve cure. Such a process is time consuming, highly trained labor–intensive, invasive (biopsy and surgery), and very costly. The cost of diagnosis and treatment (simple or Mohs micrographic surgery) in U.S. alone is estimated to be 7–10 billion dollars. EpCam is an ideal biomarker to target BCC because it is expressed in more than 95% of human BCC. More importantly, healthy stratified

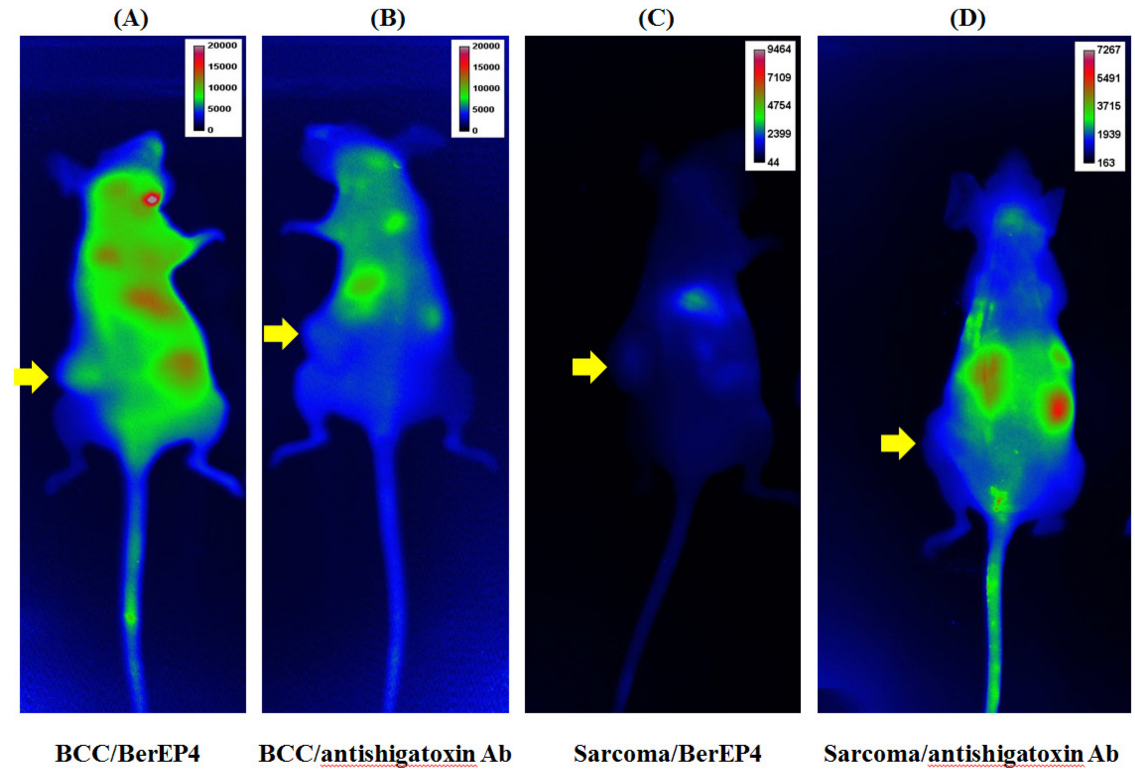

Figure 6. A and B: BCC (EpCAM–expressing xenograft mice model). C and D; sarcoma (EpCAM–negative xenograft mice model) labeled with BerEP4 antibody (+ve probe) or anti-shigatoxin antibody (-ve probe)

As illustrated, only BCC mice (A) (+ve biomarker) labeled with BerEP4 Ab (+ve probe) shows the presence of fluorescence-conjugated antibody at the site of the tumor, 6 hours after tail vein injection, which is consistent with a persisting *in vivo* affinity and selectivity of our molecular probe (BerEP4 Ab) to detect presence of EpCAM expression on tumor cells. Alexa-750 was used as the fluorescent chromophore conjugated with antibody (BerEP4 and anti-shigatoxin) for all experiments. The yellow arrows point to the location of the xenograft tumors in mice.

squamous cell epithelium expresses negligible amount of EpCAM, and benign lesions such as actinic keratosis and seborrheic keratosis do not express this biomarker.

During an *in vitro* phase, we showed specificity and selectivity of BerEP4 antibody to target EpCAM on live cells. In the subsequent *in vivo* phase, using xenograft mouse models, we reproduced persisting *in vivo* specificity and selectivity of BerEP4 antibody to detect EpCAM–expressing xenograft tumors, when injected systemically via tail veins (Figure 6). Moving forward, we plan to take the following steps: (1) modify our antibody to a smaller molecule—a stable aptamer or single strand antibody—to facilitate topical deliver (instead of systemic) for skin cancer; (2) repeat the *in vivo* experiment to show reproducible results with modified and smaller probe; this phase would encompass initial systemic injection of the probe followed by experiments to test ways to best achieve topical delivery, including micro-injection; (3) once *in vivo* non-invasive targeting of BCC is achieved via topical introduction, we plan to further engineer our diagnostic probe to be photosensitized to achieve photodynamic treatment at the time of diagnosis.

PUBLICATIONS

1. Afshari A, Ardeshirpour Y, Lodish MB, Gourgari E, Sinaii N, Keil M, Belyavskaya E, Lyssikatos C, Chowdhry FA, Chernomordik V, Anderson AA, Mazzuchi TA, Gandjbakhche A, Stratakis CA. Facial plethora: modern technology for quantifying an ancient clinical sign and its use in Cushing syndrome. *J Clin Endocrinol Metab* 2015; 3928–3933.

2. Karamzadeh N, Ardeshirpour Y, Kellman M, Chowdhry F, Anderson A, Chorlian D, Wegman E, Gandjbakhche A. Relative brain signature: a population-based feature extraction procedure to identify functional biomarkers in the brain of alcoholics. *Brain Behav* 2015; e00335.
3. Amyot F, Arciniegas DB, Brazaitis MP, Curley KC, Diaz-Arrastia R, Gandjbakhche A, Herscovitch P, Hinds SR 2nd, Manley GT, Pacifico A, Razumovsky A, Riley J, Salzer W, Shih R, Smirniotopoulos JG, Stocker D. A review of the effectiveness of neuroimaging modalities for the detection of traumatic brain injury. *J Neurotrauma* 2015; 32:1693–721.
4. Dasgeb B, Smirnov A, Ardeshirpour Y, Gandjbakhche A, Halpern A, Knutson JR, Mehregan D, Sackett DL. Multi-scale BerEp4 molecular imaging of microtumor phantom towards theranostics for basal cell carcinoma. *Mol Imaging* 2014; 13:1–9.
5. Anderson AA, Smith E, Chernomordik V, Ardeshirpour Y, Chowdhry F, Thurm A, Black D, Matthews D, Rennert O, Gandjbakhche AH. Prefrontal cortex hemodynamics and age: a pilot study using functional near infrared spectroscopy in children. *Front Neurosci* 2014; 8:393.

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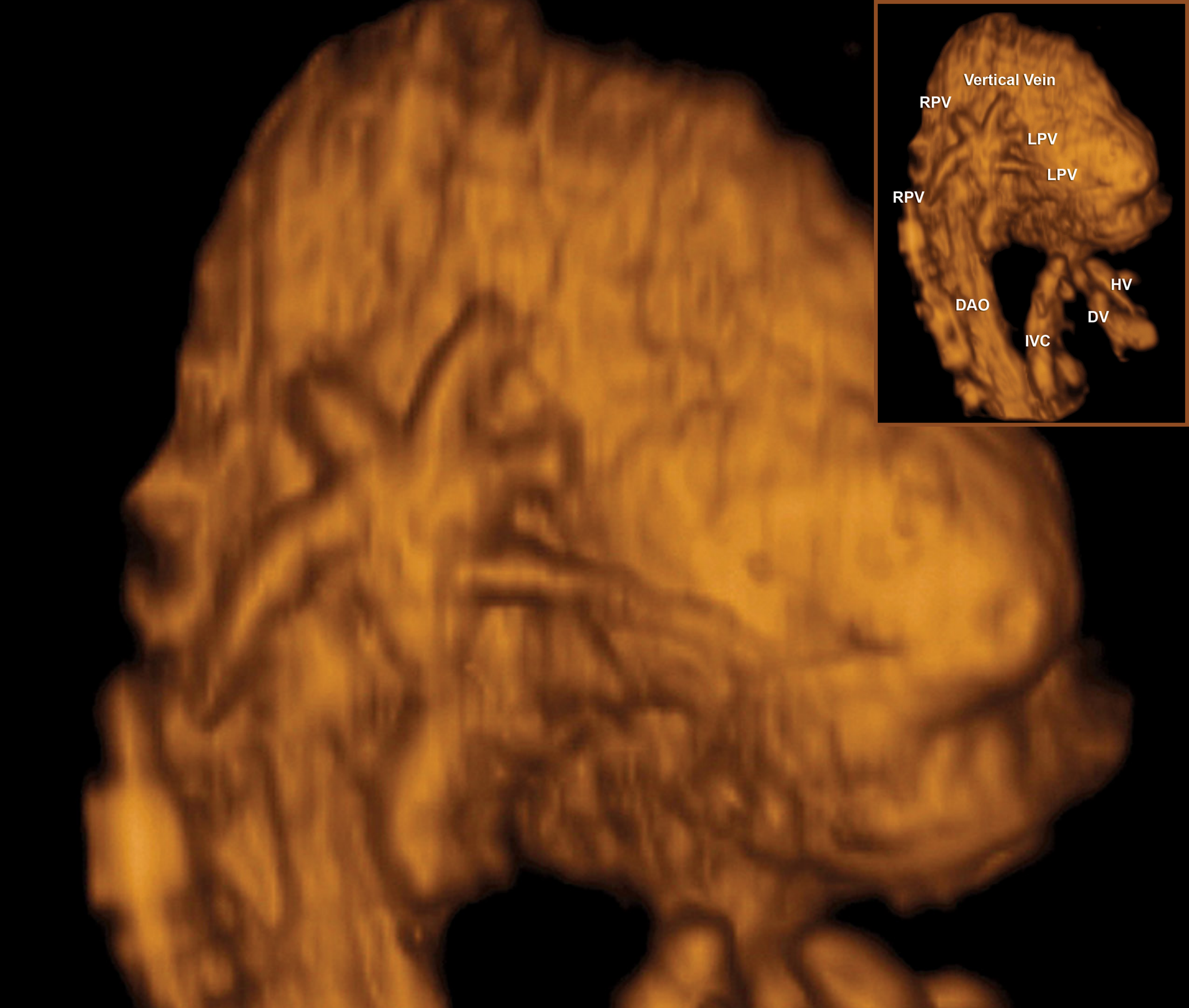

Program in Perinatal Research and Obstetrics

Director: Roberto Romero, MD

ABOUT THIS IMAGE
Oblique view from a left posterior aspect of the heart. B-flow imaging of a fetus with tetralogy of fallot with pulmonary atresia and total anomalous pulmonary venous return. A confluence of pulmonary veins, behind the right atrium and above the coronary sinus, resembles a “star fish.” LPV (left pulmonary vein), RPV (right pulmonary vein), DAO (descending aorta), IVC (inferior vena cava), DV (ductus venosus), and HV (hepatic vein). The image was provided by Roberto Romero, MD.

Program *in* Perinatal Research and Obstetrics

The *Program in Perinatal Research and Obstetrics (PPRO)* conducts clinical and laboratory research on maternal and fetal diseases responsible for excessive infant mortality in the United States. The Program focuses on the mechanisms of disease responsible for premature labor and delivery, with particular emphasis on the role of subclinical intrauterine infection and inflammation. The prenatal diagnosis of congenital anomalies is also a major area of interest.

The *Perinatology Research Branch* was created by Public Law (1993) to address the causes of the excessive infant mortality in the United States. The Branch, now called the Program in Perinatal Research and Obstetrics, uses a multidisciplinary approach to study complications of pregnancy, which include the disciplines of Maternal-Fetal Medicine, Neonatology, Placental Pathology, Perinatal Epidemiology, and High-Dimensional Biology (genomics, proteomics, metabolomics, and computational biology), as well as Immunology, Microbiology, and Nanomedicine. The Program is housed in Detroit, Michigan, on the campus of Wayne State University and at the Detroit Medical Center. The location is appropriate given the high perinatal and infant mortality in the city, as well as the contribution of ethnic social disparities to adverse pregnancy outcome. The PPRO provides state-of-the-art prenatal care to women enrolled in NICHD protocols and has made major contributions to the diagnosis of congenital anomalies and the understanding of mechanisms of disease in premature labor/delivery and preeclampsia.

Preterm birth is the leading cause of perinatal mortality and morbidity worldwide. The cost of prematurity in the U.S. alone is $26 billion per year. The Perinatology Research Branch proposed that preterm parturition is a syndrome caused by multiple pathologic processes (Preterm Labor: One Syndrome, Many Causes; Science, 2014). The PPRO established that a sonographic short cervix is a risk factor for preterm delivery and that the administration of vaginal progesterone can reduce the rate of preterm birth in these patients by 45%. This year, the PPRO reported the public health implications of universal cervical assessment in the midtrimester and treatment with vaginal progesterone. The approach is at least as cost-effective as, if not more than, other screening strategies during pregnancy (e.g., screening for group B streptococcus, asymptomatic bacteriuria, etc.). The PPRO calculated that the number of patients needed to be screened to prevent one preterm delivery (with cervical length and vaginal progesterone) is 125, which compares favorably with other interventions in obstetrics (magnesium sulfate to prevent eclampsia, aspirin to prevent recurrent preeclampsia, magnesium sulfate to prevent cerebral palsy, etc.).

One of the areas of emphasis has been the study of intra-amniotic infection and inflammation in spontaneous preterm birth, the only proven cause of spontaneous preterm labor. This year, the PPRO reported that a rapid point-of-care test for the cytokine interleukin-6 (IL-6) can be employed for the rapid diagnosis of intra-amniotic inflammation (within 20 minutes), which brings discoveries made by the PPRO a step closer to clinical application. Moreover, the PPRO reported that a new combination of antimicrobial agents can reduce the risk of adverse pregnancy outcome and histologic inflammation in the context of preterm premature rupture of membranes (PROM). Given that one third of all preterm deliveries are related to physician-initiated birth due to preeclampsia or intrauterine growth restriction, the PPRO also identified biomarkers for the prediction of indicated preterm delivery in mothers carrying small-for-gestational age fetuses.

The second cause of infant mortality in the U.S. is congenital anomalies. Therefore, the prenatal diagnosis of congenital anomalies is a major area of interest of the PPRO. Among anomalies, congenital heart disease is the leading organ-specific birth defect, as well as the leading cause of infant mortality from congenital malformations. The detection of these disorders in the U.S. is suboptimal, and nearly half of newborns affected by congenital heart disease are not diagnosed before birth. Screening for fetal congenital heart disease requires technological developments to improve examination of the fetal heart. The PPRO developed fetal intelligent navigation echocardiography (FINE) as a method to extract and display diagnostic planes recommended by professional societies for the screening of congenital heart disease. This year, members of the PPRO reported that the collection of STIC volumes (datasets of the heart) is possible in 75% of cases and can therefore improve the screening for congenital heart disease.

Clinical chorioamnionitis is the most frequent infection diagnosed in labor and delivery units around the world and affects 5–12% of all pregnant women. The condition is a major risk factor for maternal death, post-operative wound infections, neonatal sepsis, and meconium aspiration syndrome. Last year, the PPRO reported, for the first time, the use of cultivation and molecular microbiologic techniques to identify the most frequent organisms involved in these infections (*Gardnerella vaginalis*, *Ureaplasma* spp.). This year, the PPRO characterized the nature of the intra-amniotic inflammatory response, the maternal and fetal cytokine profile, placental pathology, and the accuracy of the diagnostic criteria for this disorder. It is now clear that the clinical criteria routinely used for diagnosis have limited accuracy (about 50%). Importantly, a subset of patients diagnosed as having clinical chorioamnionitis do not have any evidence of infection or inflammation, which results in overdiagnosis and unnecessary administration of antibiotics in the perinatal period, which can alter the microbiota and have long-term consequences (increased risk of obesity, diabetes, etc.). An important line of investigation of the PPRO is to improve the accuracy of the diagnosis of clinical chorioamnionitis to optimize the outcome of mothers and newborns.

The Human Placenta Project is a major initiative of NICHD. One of the stated goals of the Project has been to identify new experimental systems to study placental function, and specifically, the development of a "placenta-on-a-chip". The most important function of the placenta is the exchange of exogenous and endogenous substances, which permit an adequate supply of oxygen and nutrients, excretion of fetal metabolic waste, and protection against potentially harmful agents (xenobiotics, bacteria, viruses, parasites). Studies of placental transport are difficult to perform in humans, are time-consuming, and always carry the risk of fetal exposure. This year, the PPRO reported a new approach to model placental transport that combines microfluidics and microfabrication technologies with the culture of placental-derived human cells to recapitulate the organ-specific architecture and physiologic microenvironment of the placental barrier. The PPRO developed a "placenta-on-a-chip" microdevice that permits perfusion of co-cultured human trophoblast and human umbilical endothelial veins on a thin extracellular matrix membrane. Members of the PPRO also tested the physiologic function of the microengineered placental barrier by measuring glucose transport across the trophoblast endothelial interface over time. The "placenta-on-a-chip" model has the potential to serve as a low-cost experimental platform with a broad range of applications. Such a biomimetic model may also enable the quantitative analysis of placental transport of small molecules and biologics for the development and screening of new therapeutic modalities.

Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. The Perinatology Research Branch proposed that preterm parturition is a syndrome caused by multiple pathologic processes, one of which is an untimely decline in progesterone action, manifested by "silent" shortening of the uterine cervix. Previous work conducted by our Program showed that asymptomatic women who have a cervical length less than 15mm in the second trimester of pregnancy have a 50% likelihood of delivering an early preterm neonate. However, prediction of preterm birth needs to be accompanied by a strategy to reduce the frequency of this complication. In a previous randomized clinical trial of vaginal progesterone vs. placebo in women with a short cervix, we reported that treatment was associated with a 45% reduction in the rate of preterm birth (less than 33 weeks of gestation) and a decrease in the rate of respiratory distress syndrome, the most common complication of prematurity. This year, we conducted studies to examine the relative merits of vaginal progesterone vs. cervical cerclage in patients with a short cervix and a prior history of preterm birth and found that medical treatment is as effective as the surgical approach. We also identified a novel pathologic finding associated with preterm labor without infection: chronic chorioamnionitis, which is likely attributable to maternal anti-fetal rejection, and we described a novel form of systemic fetal inflammation.

The Program also studies other great obstetrical syndromes that account for the high rate of infant mortality in the United States, including preeclampsia, fetal growth restriction, fetal death, clinical chorioamnionitis, and meconium aspiration syndrome.

Congenital anomalies continue to be a leading cause of perinatal mortality in the United States. Congenital heart disease is the leading organ-specific birth defect, as well as the leading cause of infant mortality from congenital malformations. The lack of prenatal identification of congenital heart defects can have adverse consequences for the neonate. More than half the infants affected with congenital heart disease are born to mothers without any previously known risk factors, which provides the impetus to perform a comprehensive screening examination of the fetal heart in all pregnancies. Yet, the prenatal diagnosis of congenital heart disease remains a challenge, as the sensitivity of ultrasound has ranged from 15–39%. Examination of the fetal heart is time-consuming, requiring expertise and skill. Therefore, the examination frequently does not include all the standard recommended cardiac views. Spatiotemporal image correlation (STIC) technology allows the acquisition of a volume dataset from the fetal heart and displays a cine loop of a complete single cardiac cycle in motion. A growing body of evidence suggests that 4-D sonography with STIC facilitates examination of the fetal heart. However, extracting and displaying the recommended diagnostic planes from a volume dataset that can be dissected in many ways (i.e., planes) requires an in-depth knowledge of anatomy and is difficult and operator-dependent. After several years of work, we developed a novel method for visualizing standard fetal echocardiography views from volume datasets obtained with STIC and application of "intelligent navigation" technology. We have also explored fetal

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brain neuroconnectivity using magnetic resonance imaging.

Development of Placenta-on-a-Chip, a novel platform to study the biology of the human placenta

NICHD launched the Human Placenta Project and affirmed that the development of new experimental methods to test placental function is a priority for the Institute and specifically called for using the "organ-on-a-chip" technology to develop a "placenta-on-a-chip."

The most important function of the placenta is the exchange of endogenous and exogenous substances, which permits an adequate supply of oxygen and nutrients, excretion of fetal metabolic waste, and protection against potentially harmful agents, such as xenobiotics, bacteria, viruses, and parasites. Prior studies on placental transport have used a wide range of experimental systems, including *in vivo* animal models, *ex vivo* placental perfusion systems, and *in vitro* cell cultures. In some cases, placental transfer has been studied in humans for frequently used therapeutic agents, such as antibiotics and hormones. However, such studies are difficult to perform, time-consuming, and always carry the risk of fetal exposure.

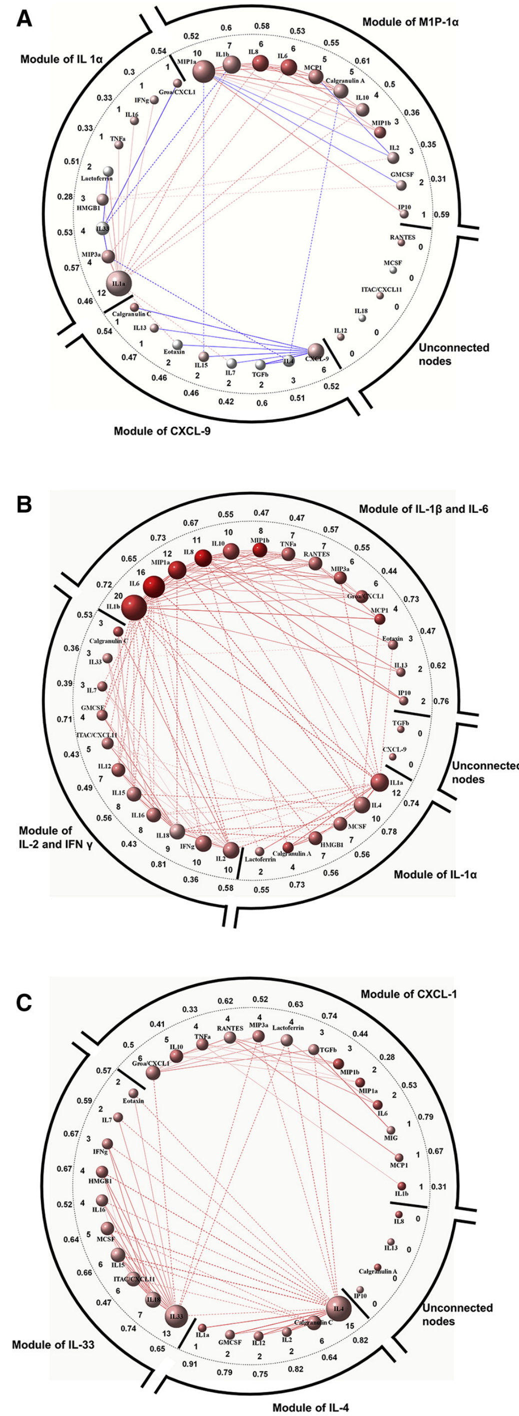
This year, we used a new bioengineering approach to model placental transport that combines micro-fluidics and micro-fabrication technologies with the culture of placenta-derived human cells to recapitulate the organ-specific architecture and physiological micro-environment critical to placental barrier function. Specifically, we developed a Placenta-on-a-Chip micro-device that enabled the compartmentalized perfusion co-culture of human trophoblasts (JEG-3) and human umbilical vein endothelial cells (HUVECs) on a thin extracellular matrix membrane (ECM) to create a physiological placental barrier *in vitro*. We also tested the physiological function of the micro-engineered placental barrier by measuring glucose transport across the trophoblast-endothelial interface over time. Our micro-fluidic cell culture system provided a tightly controlled fluidic environment conducive to the proliferation and maintenance of JEG-3 trophoblasts and HUVECs on the ECM scaffold. Prolonged culture in this model produced confluent cellular monolayers on the intervening membrane that together formed the placental barrier. The *in vivo*–like micro-architecture was also critical for creating a physiologically relevant effective barrier to glucose transport. Our Placenta-on-a-Chip model has the potential to serve as a low-cost experimental platform with a broad range of applications. This biomimetic model may also permit the quantitative analysis of placental transport of small molecules and biologics for the development and screening of new therapeutic modalities. The micro-engineering approach demonstrated in this study could also be leveraged to recapitulate key pathological features of various placental disorders to develop new types of *in vitro* human disease models.

Evidence of perturbations of the cytokine network in preterm labor

Intra-amniotic infection/inflammation is the only mechanism of disease with persuasive evidence of causality for spontaneous preterm labor/delivery. Previous studies on the behavior of cytokines in preterm labor were based largely on the analysis of the behavior of each protein independently. Emerging evidence indicates that the study of biologic networks can provide insight into the pathobiology of disease and improve biomarker discovery. The goal of this study was to characterize the network of inflammatory-related proteins in the amniotic fluid in patients with preterm labor.

Patients with preterm labor and intact membranes who had microbial-associated intra-amniotic inflammation had a higher amniotic fluid inflammatory-related protein concentration correlation than those without intra-amniotic inflammation (113 perturbed correlations). Interleukin-1beta (IL-1β), IL-6, macrophage inflammatory protein-1α, and IL-1α were the most connected nodes (highest degree) in this differential correlation network. Patients with sterile intra-amniotic inflammation had correlation patterns of inflammatory-related proteins that were both increased and decreased when compared with those without intra-amniotic inflammation (50 perturbed correlations). IL-1α, macrophage inflammatory protein-1α, and IL-1β were the most connected nodes in this differential correlation network. There were more coordinated inflammatory-related protein concentrations in the amniotic fluid of women with microbial-associated intra-amniotic inflammation than in those with sterile intra-amniotic inflammation (60 perturbed correlations), with IL-4 and IL-33 having the largest number of perturbed correlations (see Figure).

We reported, for the first time, an analysis of the inflammatory-related protein network in spontaneous preterm labor. Patients with preterm labor who had microbial-associated intra-amniotic inflammation had more coordinated amniotic fluid inflammatory-related proteins than either those with sterile intra-amniotic inflammation or those without intra-amniotic



Network Medicine to Understand Preterm Labor: the Intra-amniotic Inflammatory Network in Spontaneous Preterm Labor with and without Inflammation and Infection (collaboration between the PPRO and the Section on Intercellular Interactions, Program in Physical Biology)

Each node (*sphere*) represents one of the 33 analytes, with a link (*line*) between two nodes that represent a significantly perturbed correlation. The node color represents the direction of concentration change (*red*, increased; *blue*, decreased; *white*, no change in the first group compared with the second/reference group of the comparison). The color of links gives the direction of correlation change (*red*, increased correlation; *blue*, decreased correlation); the type of line denotes the nature of the link (*solid line*, within module link; *dashed line*, cross-module link). Thick radial lines separate the modules and the set of unconnected nodes. The numbers inside/outside the dotted black circle represent the node degree/average absolute difference in correlations.

A. Network of perturbed inflammatory-related protein concentration correlations between sterile intra-amniotic inflammation and no intra-amniotic inflammation.
B. Network of perturbed inflammatory-related protein concentration correlations between microbial-associated intra-amniotic inflammation and no intra-amniotic inflammation.
C. Network of perturbed inflammatory-related protein concentration correlations between microbial-associated intra-amniotic inflammation and sterile intra-amniotic inflammation.

inflammation. The correlations were also stronger in patients with sterile intra-amniotic inflammation than in those without intra-amniotic inflammation. The findings could be of value in the development of biomarkers of preterm labor.

A point-of-care test for interleukin-6 in amniotic fluid

Preterm premature rupture of membranes (PROM) accounts for 30-40% of spontaneous deliveries at less than 37 weeks of gestation and is a major cause of perinatal morbidity and mortality. Amniotic fluid (AF) interleukin-6 (IL-6) concentrations can identify patients with intra-amniotic inflammation, at risk of impending preterm delivery and adverse pregnancy outcome. The conventional method to determine IL-6 concentrations in AF is an enzyme-linked immunosorbent assay (ELISA). However, the, technique is not available in clinical settings, and the results may take several days to become available. A lateral flow-based immunoassay, or point-of-care test, has been developed to address these challenges. We conducted a study to compare the performance of AF IL-6 determined by the point-of-care test (POCT) with that determined by ELISA for the identification of intra-amniotic inflammation and for assessing risk of spontaneous preterm delivery in patients with preterm PROM. The study included 56 women with singleton pregnancies who presented with preterm PROM. The findings were, first, a positive POCT for AF IL-6 concentrations had 97% sensitivity and 96% specificity for the identification of intra-amniotic inflammation, as defined by ELISA; and second, results of the POCT were equivalent to those determined by ELISA in identifying patients with microbial invasion of amniotic cavity, as well as those with acute inflammatory lesions of the placenta. The findings suggest that a POCT for AF concentrations of IL-6 can be used in place of ELISA to identify intra-amniotic inflammation in women with preterm PROM. Results can be available within 20 minutes, which makes it possible to quickly implement interventions designed to treat intra-amniotic inflammation and improve pregnancy outcome.

Antibiotic treatment of patients with preterm prelabor rupture of membranes

PROM affects 10% of all pregnant women, and preterm PROM occurs in 1% of all gestations. PROM in the preterm gestation accounts for 30% of all preterm births. The administration of antibiotics to patients with preterm PROM has become the standard of practice and is effective in increasing the duration of the latency period and reducing the rate of clinical chorioamnionitis and neonatal sepsis. Studies reported by our Branch provided evidence that antibiotics administration do not eradicate subclinical intra-amniotic infection in patients with preterm PROM or prevent subsequent infection. This led us to conduct a retrospective study of the outcome of pregnancy in patients given conventional antimicrobial vs. a new combination of antibiotics effective against ureaplasma species and anaerobic bacteria. We compared perinatal outcomes in 314 patients with PROM at less than 34 weeks receiving antimicrobial regimen 1 (ampicillin and/or cephalosporins; n=195) with regimen 2 (ceftriaxone, clarithromycin, and metronidazole; n=119). The outcomes of preterm PROM treated with standard antibiotic administration vs. a new combination proved that the new combination, consisting of ceftriaxone, clarithromycin, and metronidazole, prolonged the latency period, reduced acute histologic chorioamnionitis and funisitis and improved neonatal outcomes in patients with preterm PROM. The studies call for a reexamination of the current clinical practice and suggest that alternative antimicrobial agents may be more effective in this common complication of pregnancy.

Elastography to assess biophysical properties of the human cervix and the risk of preterm delivery

A sonographically identified short cervix is the most powerful predictor of spontaneous preterm birth, the leading cause of perinatal morbidity and mortality worldwide. However, only half of women with a sonographic short cervix have a preterm delivery; therefore, to avoid unnecessary treatment, there is a need to identify those women with a short cervix not at risk for preterm delivery.

Throughout gestation, the cervix undergoes dynamic changes in tissue composition characterized by dynamic remodeling of the collagen network and an increasing concentration of glycosaminoglycans and water content in the extracellular matrix, which change the biophysical properties of the organ. Elastography is a sonographic technique for estimating tissue displacement or deformation when oscillatory compression is applied. Tissue displacement or strain can be tracked, using Doppler techniques or cross-correlation analysis, and converted to an elastic modulus as an indirect estimation of tissue stiffness. We proposed that elastography examination of the uterine cervix may help identify patients with a short cervix who may be at low risk for preterm delivery.

We used quasi-static elastography to estimate cervical strain in 545 pregnant women with singleton gestations from 11 to 28 weeks of gestation. Cervical strain was evaluated in one sagittal plane, and in cross-sectional planes of both the internal cervical os and external cervical os. Women with strain values in the 3rd or 4th quartiles at the internal cervical os had a higher risk of spontaneous preterm delivery at 34 weeks or less and less than 37 weeks of gestation than women with the lowest quartile strain values. Even after adjusting for gestational age and a short cervix, women with strain values in the 3rd quartile maintained a significantly elevated risk for spontaneous preterm delivery, while those with highest quartile strain values had a marginally increased risk (compared with women with lowest quartile strain values). This is the first study describing a relationship between cervical strain and preterm delivery at 34 weeks or less and magnitudes of association between cervical strain and preterm delivery adjusted for both gestational age at examination and the presence of a short cervix. The study lays the groundwork for further investigation on the clinical benefit of elastography in combination with cervical length measurement to identify women at risk for spontaneous preterm delivery.

Biomarkers to predict induced preterm delivery

Inducted or induced preterm deliveries account for 30% of all preterm births and occur because of maternal and/or fetal indications such as preeclampsia or intrauterine growth restriction. Small-for-gestational-age (SGA) fetuses could be constitutionally small or be the result of intrauterine malnutrition. The differential diagnosis between these two conditions has been a challenge. This year, we reported a study that determined whether maternal plasma concentrations of specific biomarkers (angiogenic and anti-angiogenic factors) can predict which mothers diagnosed with suspected SGA will develop preeclampsia or require an indicated early preterm delivery (at 34 weeks of gestation or less), and whether risk assessment performance is improved using these proteins in addition to clinical factors and Doppler parameters. The study included 314 women with singleton pregnancies diagnosed with suspected SGA (estimated fetal weight less than the 10th percentile) between 24 and 34 weeks of gestation. We found that the biomarkers measured in maternal blood between 24–34 weeks of gestation can indeed identify the majority of mothers diagnosed with suspected SGA who subsequently develop preeclampsia or those who require preterm delivery at 34 weeks or less of gestation. Moreover, the addition of these biomarkers to other clinical parameters improved the prediction of preterm delivery.

Prevention of recurrent fetal death with the use of a statin

Massive perivillous fibrin deposition in the placenta and the related maternal floor infarction are serious conditions associated with recurrent complications including fetal death and severe fetal growth restriction. There is no method to evaluate the risk of adverse outcomes in subsequent pregnancies, or effective prevention. Recent observations reported by our Branch discovered that maternal floor infarction is characterized by an imbalance in angiogenic/anti-angiogenic factors in early pregnancy. Statins can increase the production of angiogenic factors and inhibit anti-angiogenic forces. We reported for the first time the use of statins to prevent recurrent fetal death. Abnormalities in the anti-angiogenic factor sVEGFR-1 and soluble endoglin were detected early in the index pregnancy, and treatment with pravastatin corrected the abnormalities. Treatment resulted in a live-birth infant near term who had normal biometric parameters and developmental milestones at the age of 2. This is the first reported successful use of pravastatin to reverse an angiogenic/anti-angiogenic imbalance and prevent fetal death. Whether other interventions can reverse the anti-angiogenic state associated with massive perivillous fibrin deposition in the placenta remains to be established.

Fetal cardiac examination using Fetal Intelligent Navigation Echocardiography (FINE)

Congenital heart disease is the most common group of malformations affecting both fetuses and newborn infants. Up to 90% of cases of cardiac defects occur in pregnancies without high risk features, which provides the impetus to perform a comprehensive screening examination of the fetal heart in all women to maximize the detection of heart defects. Yet, even when more than 90% of women in the population undergo a prenatal ultrasound examination, studies report a low sensitivity (22.5%–52.8%) in the detection of congenital heart disease, which has been attributed to issues related to operator-dependent factors. Some of the limitations of conventional two-dimensional ultrasound could be addressed by technological advances designed to reduce operator dependency. Four-dimensional sonography with spatiotemporal image correlation (STIC) allows acquisition of volume datasets of the fetal heart, and displays a cine loop of a complete single cardiac cycle in motion. Such sonographic volumes allow cardiac planes to be extracted and displayed in any orientation; however, the process requires an in-depth knowledge of anatomy and is difficult, operator-dependent, and time-consuming.

Recently, we developed and reported a novel method known as Fetal Intelligent Navigation Echocardiography (FINE), which interrogates STIC volume datasets using intelligent navigation technology. The method allows the automatic display of nine standard fetal echocardiography views required to diagnose most cardiac defects, can simplify examination of the fetal heart, and reduce operator dependency. This year, we conducted a study to prospectively evaluate the performance of the FINE method applied to STIC volume datasets of the normal fetal heart acquired between 19 and 30 weeks of gestation. One or more STIC volumes were successfully obtained in 72.5% (150/207) of women undergoing ultrasound examination. Approximately 96% (n=351) of STIC volumes evaluated by STICLoop were determined to be appropriate. Nine fetal echocardiography views were generated by the FINE method using a combination of diagnostic planes and/or VIS-Assistance® (Virtual Intelligent Sonographer Assistance) in 98–100% of cases. For each STIC volume dataset, 86% of volumes demonstrated either 8 or all 9 echocardiography views (via diagnostic planes), while 98% of volumes demonstrated all 9 echocardiography views (via VIS-Assistance®). For each STIC volume dataset, the success rate of obtaining four views (four chamber, left ventricular outflow tract, short-axis view of great vessels/right ventricular outflow tract, abdomen/stomach) was 93% and 100%, using diagnostic planes and VIS-Assistance®, respectively. The findings thus suggest that FINE could be implemented for fetal cardiac anomaly screening during routine prenatal care.

PUBLICATIONS

1. Romero R, Grivel JC, Tarca AL, Chaemsaithong P, Xu Z, Fitzgerald W, Hassan SS, Chaiworapongsa T, Margolis L. Evidence of perturbations of the cytokine network in preterm labor. *Am J Obstet Gynecol* 2015; 213:836.e1–836.e18.
2. Garcia M, Yeo L, Romero R, Haggerty D, Giardina I, Hassan SS, Chaiworapongsa T, Hernandez-Andrade E. Prospective evaluation of the fetal heart using Fetal Intelligent Navigation Echocardiography (FINE). *Ultrasound Obstet Gynecol* 2016; 47:450–9.
3. Kim CJ, Romero R, Chaemsaithong P, Kim JS. Chronic inflammation of the placenta: definition, classification, pathogenesis, and clinical significance. *Am J Obstet Gynecol* 2015; 213(4Supp):S53–69.
4. Lee JS, Romero R, Han YM, Kim HC, Kim CJ, Hong JS, Huh D. Placenta-on-a-chip: a novel platform to study the biology of the human placenta. *J Matern Fetal Neonatal Med* 2016; 29:1046-54.
5. Chaiworapongsa T, Romero R, Korzeniewski SJ, Chaemsaithong P, Hernandez-Andrade E, Segars JH, DeCherney AH, McCoy MC, Kim CJ, Yeo L, Hassan SS. Pravastatin to prevent recurrent fetal death in massive perivillous fibrin deposition of the placenta (MPFD). *J Matern Fetal Neonatal Med* 2016; 29:855-62.

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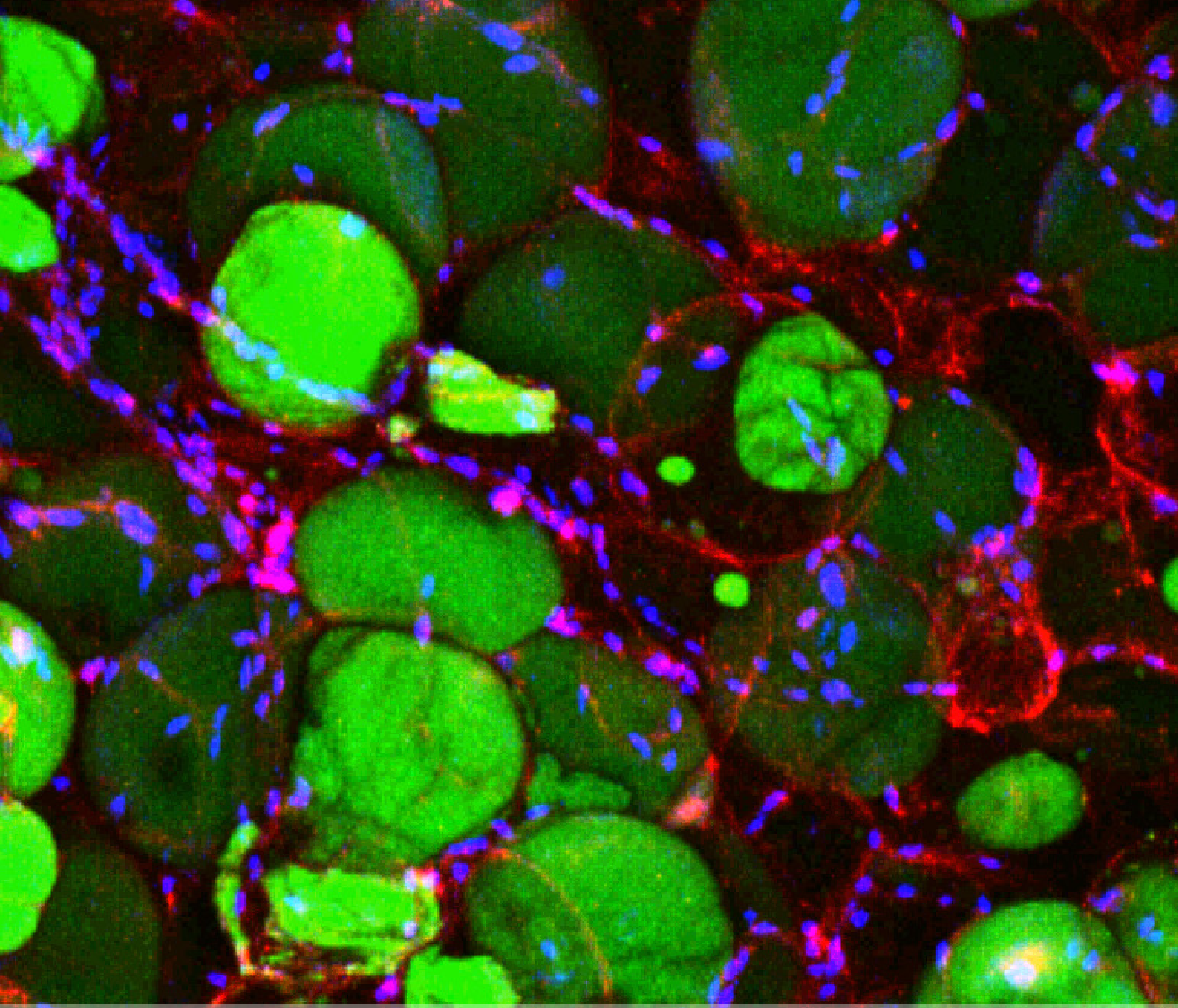

Program in Physical Biology

Director: Joshua Zimmerberg, MD, PhD

ABOUT THIS IMAGE

This is a 3D reconstruction of a confocal z-stack of adipose tissue. Glucose transporter-4 is labeled with Alexa-594 Anti- body (red). Lipid droplets are labeled with BODIPY 493/503 (green). Nuclei are stained with DAPI (blue). This image was acquired in collaboration with Dr. Karin Stenkula (NIDDK), and submitted by Vladimir A. Lizunov, PhD, of the *Section on Membrane and Cellular Biophysics*.

PROGRAM *in* PHYSICAL BIOLOGY

Human embryonic development, on which the future child's health depends, is a complex phenomenon within the female starting with egg-spermatozoa fusion. In each individual, a plethora of molecular recognition events mediate the development of an immune system to defend against pathogens, a musculo-skeletal system to maintain the body, and flexible networks of molecular expression to manage environmental stress. Traditionally, studies on these processes have been divided among biochemistry, cell biology, virology, toxicology, etc. However Nature does not know these artificial divisions, and new understandings are emerging from the interface between mathematically minded physical scientists and biomedical researchers. The *Program in Physical Biology*, led by JOSHUA ZIMMERBERG, is a unique scientific body that approaches human development in normal life and pathology as an integral process and which encompass first-class cell biologists, physical chemists, biophysicists, virologists, and immunologists, who not only successfully train post-docs and students within their own fields in work on their own projects but also collaborate widely, building and uniquely promulgating multidisciplinary approaches to the most important biomedical problems in the framework of the NICHD mission. This year marks the retirement of our dear friend and colleague, Donald Rau, to whom we extend our very best wishes.

The *Section on Molecular Transport*, led by SERGEY BEZRUKOV, advances biophysical methods as tools to understand molecular interactions, notably by studying, in the context of human development, disease, and pharmacological intervention, the interactions of beta-barrel membrane channels with drugs and cytosolic proteins, as regulated by upstream signaling. Using single-molecule functional approaches, one project aims to unveil the physical mechanisms regulating the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane in cell proliferation, reprogrammed cancer metabolism, kinase-regulated cell signaling, cytoprotection, and neurodegeneration. The past year's work on the VDAC focused on the effect of alpha-synuclein, an intrinsically disordered protein known to be involved in mitochondrial dysfunction in neurodegeneration. The group showed that alpha-synuclein reversibly blocks (and is able to translocated through) the VDAC, the major transport pore of the mitochondrial outer membrane, which suggests that alpha-synuclein participates in the regulation of normal mitochondrial respiration, in synuclein-induced mitochondrial dysfunction, or both. The results thus point to a plausible molecular mechanism for the protein's (patho-)physiological function. Work on the physical theory of facilitated transport concentrated on bulk-mediated surface diffusion to support experimental findings pointing to the importance of the membrane-binding step in the interaction of cytosolic proteins with beta-barrel channels.

The long-term goal of the *Section on Membrane Biology*, led by LEONID CHERNOMORDIK, is to understand how proteins drive membrane rearrangements in important cell biology processes. Whereas each kind of protein has its individual personality, membrane lipid bilayers have rather general properties manifested by their resistance to disruption and bending. Analysis of molecular mechanisms underlying important and diverse membrane rearrangements will clarify the generality of emerging mechanistic insights and likely bring about new strategies for treating diseases involving cell invasion by enveloped viruses, intracellular trafficking, and intercellular fusion. In recent studies, in parallel with ongoing studies on the cell-to-cell fusion stage during development and on regeneration of muscles and bones, the Section focused on the mechanisms of cell entry by cell-permeable cationic peptides.

During the last year, the *Section of Intercellular Interactions*, led by LEONID MARGOLIS, pursued the following aims. The first was to study HIV-1 infectivity in human tissue by analyzing individual virions' spikes, which mediate virus-cell fusion; it was found that viruses exhibit little mosaicism—on the majority of virions either all spikes are functional or are all defective; such an all-or-nothing viral strategy is likely to aid immune evasion by subverting the focus of humoral responses to generate multiple non-neutralizing antibodies at no cost to infectious virions. The second aim was to apply the flow virometry technology developed for HIV-1 analysis to other viruses, in particular to dengue virus; analysis of individual viral particles permitted the Section to distinguish immature from mosaic virions by the presence of the prM protein on the viral surface. The third aim was to investigate pathogen-triggered changes in the cytokine network of the amniotic fluid of women in preterm labor, according to the presence or absence of intra-amniotic inflammation and microorganisms in the amniotic cavity. The study showed that the cytokine network connectivity with microbial-associated intra-amniotic inflammation is denser and more coordinated than in women with sterile inflammation or without intra-amniotic inflammation. This new network analysis provides a deeper insight into the pathophysiological mechanisms of intra-amniotic infection/inflammation in preterm labor and helps identify potentially relevant modules of cytokines that correspond to distinct disease pathways. A similar network analysis can now be applied to other pathogens. The fourth aim was to perform a broad analysis of the biomedical literature and our unpublished results to determine the possible role of immuno-activation in human disease. Immuno-activation appears to be a common denominator or general mechanism of pathogenesis and may explain similarities in pathology among otherwise unrelated human diseases. Identification of general mechanisms of immuno-activation may lead to the development of new therapeutic strategies applicable to many diseases, even before detailed knowledge of their specific etiology and pathogenesis is available.

The *Section on Cell Biophysics*, led by Ralph Nossal, studies physical and physical-chemical mechanisms underlying cell behavior, for which the Section develops and applies mathematical and computational methodologies and uses biochemical and cell-biological techniques. Projects currently include: (1) elaborating a mathematical model to understand the physical basis of coated vesicle biogenesis during receptor-mediated endocytosis, focusing on how the size dependence of nanoparticle uptake relies on mesoscopic cell mechanics; (2) developing quantitative measures of eukaryotic cell chemotaxis; (3) exploring how the physiological behavior of neural tissues and other cell elements is mediated by temperature-linked phase changes in the lipid bilayer of the cell membrane; and (4) understanding how certain small molecules interact with microtubules to act as antimitotic agents, and how microtubule arrays function in mitosis to produce accurate segregation of chromosomes. The Section also develops new experimental modalities to characterize these and related phenomena, with a particular interest in the ways in which cellular activities are coordinated in space and time.

The *Section on Macromolecular Recognition and Assembly*, headed by Donald Rau, focuses on the nature of forces, structure, and dynamics of biologically important assemblies. The group showed that measured forces differ from those predicted by current theories and interpreted the observed forces to indicate the dominant contribution of water-structuring energetics. To investigate the role of water in binding, the group measures and correlates changes in binding energies and hydration that accompany recognition reactions of biologically important macromolecules, particularly sequence-specific DNA–protein complexes. By investigating differences in water sequestered by complexes of sequence-specific DNA–binding proteins bound to different DNA sequences, members of the Section correlated binding energy and water incorporated with the energy necessary to remove hydrating water from complexes. The emphasis on water permits a different approach to recognition reactions than standard practice. The Section also continued its work on sperm nuclear DNA packing and on the effect of temperature on the packing transitions in viruses, which facilitate infection. The Section discovered a novel, temperature-mediated transition in DNA packing for both the bacteriophage lambda and human herpes simplex virus type 1 (HSV-1). Both X-ray scattering and cryoEM reconstruction indicated a transition in DNA packing that occurs at about 30°C and is characterized by a substantial increase in the amount of disordered DNA in the center of the capsid.

The *Section on Membrane and Cellular Biophysics*, led by Joshua Zimmerberg, studies membranes, viruses, organelles, cells, and tissues in order to understand the molecular organization of cellular membranes, the physico-chemical mechanisms of membrane remodeling, and the molecular anatomy of tissues, which will lead to deeper insights into viral, parasitic, metabolic, developmental, and neoplastic diseases. The Section aims to use the expertise and techniques it perfected over the years to address several biological problems that have in common the underlying regulation or disturbance of protein/lipid interactions. During the past year, the Section (1) performed long-term time-lapse microscopy of a novel fluorescent protein construct that reports the expression of the transcription factors OCT4, SOX2, and NANOG; (2) invented a new chamber for high-resolution, time-lapse, optical microscopy with precise control of shear forces over a time intervals, forces associated with blast shock waves known to create traumatic brain injury in the field during exposure to explosive blasts; (3) achieved direct chemical detection of the lipids around domains of influenza hemagglutinin on the plasma membrane of fibroblasts; and (4) tested the idea that the response to insulin would be gradually diminished as the metabolic health of the individual diminished; instead a biphasic response was found.

We explore the physical mechanisms that are responsible for the functioning and regulation of mitochondrial and bacterial beta-barrel channels under normal and pathological conditions. These membrane proteins, which form large water-filled pores, are the gateways for metabolite exchange between various cellular compartments and cells. Because they are also recognized as multifunctional membrane receptors and components of many toxins, beta-barrel channels are also promising as novel drug targets. Our main strategy is to reconstitute the proteins into planar lipid membranes, which allows us to study channels at the single-molecule level by high-resolution recording of ionic currents. Combining physical theory of channel-facilitated transport with experiments on channel reconstitution, we seek to develop new approaches to the treatment of various diseases for which regulation of transport through ion channels plays a key role.

To understand the general principles involved in channel regulation at the molecular level, we work with a variety of proteins and channel-forming peptides. Among them are VDAC (the Voltage-Dependent Anion Channel from the outer membrane of mitochondria), alpha-Hemolysin (a toxin from *Staphylococcus aureus*), translocation pores of *Bacillus anthracis* (PA63), *Clostridium botulinum* (C2IIa) and *Clostridium perfringens* (Ib) binary toxins, Epsilon toxin (from *Clostridium perfringens*), OmpF (a general bacterial porin from *Escherichia coli*), LamB (a sugar-specific bacterial porin from *Escherichia coli*), OprF (porin from *Pseudomonas aeruginosa*), Syringomycin E (a lipopeptide toxin from *Pseudomonas syringae*), and the bacterial peptide TisB involved in persister cell biofilm formation. We also use Gramicidin A (a linear pentadecapeptide from *Bacillus brevis*) as a molecular sensor of membrane mechanical properties. To study the channel-forming proteins under precisely controlled conditions, we first isolate them from the host organisms, purify them, and then reconstitute them into planar lipid bilayers with precisely controlled physical properties. This allows us to explore channel interactions with the lipid membrane when modified by volatile anesthetics, with cytosolic proteins such as tubulin and alpha-synuclein, and with newly synthesized drugs such as blockers of the translocation pores of bacterial toxins. By learning the physics, chemistry, and physiology of channel functioning, we strive to determine how to design new agents and strategies that effectively correct the deviant interactions associated with disease.

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Alpha-synuclein interaction with VDAC as a mechanism of mitochondrial regulation and toxicity in Parkinson disease

Emerging evidence establishes the critical role of mitochondria in the pathogenesis of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease. Dysfunction of mitochondrial enzyme complexes, impaired oxidative phosphorylation, increased production of reactive oxygen species, mitochondrial outer membrane permeabilization, enhanced apoptosis, and morphological alterations of mitochondria have been associated with these pathologies. Neurons are especially sensitive to mitochondrial dysfunction because of their high demand for energy and the characteristic subcellular distribution of their mitochondria. Participation of the small, intrinsically disordered protein alpha-synuclein (α-syn) in PD pathogenesis has been well documented. Although recent research demonstrated the involvement of α-syn in mitochondrial dysfunction in neurodegeneration and suggested direct interaction of α-syn with mitochondria, the molecular mechanism(s) of α-syn toxicity and its effect on neuronal mitochondria remain sketchy. Using the channel reconstitution technique, we found that, at nanomolar concentrations, α-syn reversibly blocks VDAC, the major transport pore of the mitochondrial outer membrane, a pore that controls most of the metabolite fluxes in and out of the mitochondria. It is clear that any restriction of metabolite exchange through VDAC may lead to disturbance of the mitochondrial energetic function and therefore to cell metabolism dysfunction. Moreover, through the detailed analysis of the blockage kinetics, we came to the conclusion that α-syn is able to translocate through the channel. Implication of VDAC in regulation of α-syn entrance into mitochondria may be of an immediate importance for the understanding of mitochondrial dysfunctions in PD. Indeed, after reaching the intermembrane space, α-syn may target complexes of the mitochondrial respiratory chain in the inner mitochondrial membrane. Thus, our results suggest that, depending on the physiological conditions, α-syn interaction with VDAC could be involved in regulation of normal mitochondrial respiration, in α-syn–induced mitochondrial dysfunction, or both. Supporting our *in vitro* experiments, a yeast model of PD showed that α-syn toxicity in yeast depends on VDAC, demonstrating α-syn interaction with VDAC in living cells. The functional interactions between VDAC and α-syn, revealed by our study, point toward the long sought-after physiological and pathophysiological roles for monomeric α-syn in PD and in other α-synucleinopathies and, for the first time, suggest a pathway for α-syn across the mitochondrial outer membrane. We believe that these results can reconcile several previous conflicting observations of various α-syn effects on mitochondrial bioenergetics.

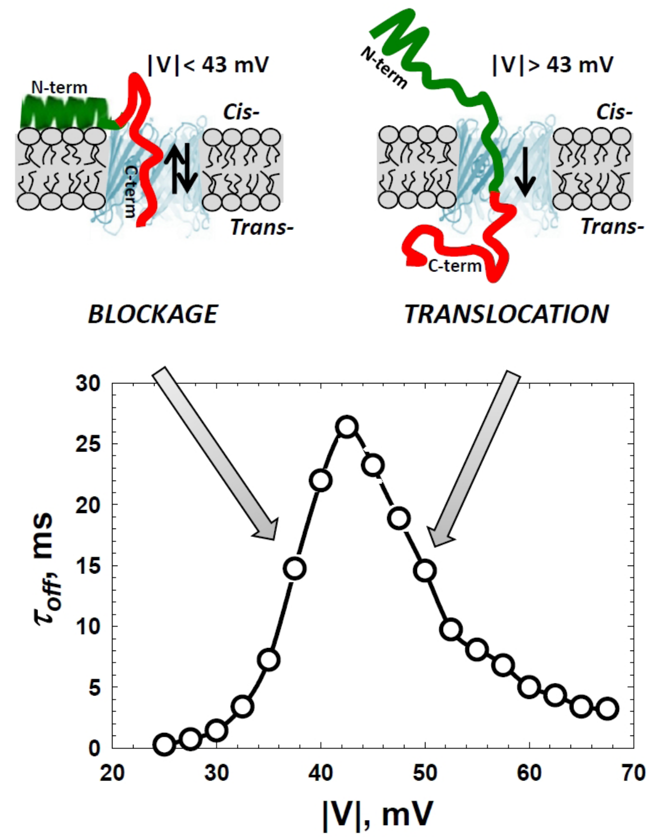

Figure 1. Alpha-synuclein interaction with VDAC

The cross-over in the voltage dependence of the residence time of alpha-synuclein in the VDAC pore separates regimes of reversible blockage and protein translocation through the pore. Depending on the amplitude of the applied voltage, it either reversibly blocks or translocates through the pore. At relatively low voltages, upon the capture by the channel, the field pulls on the negatively-charged C-terminus (*red*) with a force that is not enough to detach the N-terminal part (*green*) from the membrane surface. This results in a reversible capture of the C-terminus and in an exponential increase of its residence time in the pore with the voltage. Voltages above a certain threshold (around 45 mV) are high enough to induce detachment of the N-terminal part and to pull the whole peptide chain through the pore. In this translocation regime, the increasing field threads protein through the pore faster, which results in the lower residence time (Reference 1).

Complexity of voltage gating of beta-barrel channels

The functional role of voltage gating—voltage-sensitive transitions of VDAC between its open and closed states—is to regulate both ATP delivery to the cytosol and ADP access to the electron transport chain complexes in the mitochondrial inner membrane. According to this line of reasoning, VDAC closure limits mitochondrial oxidative phosphorylation whereas VDAC opening favors it. VDAC gating is remarkably complex. Nevertheless, reports on its reproducible dependence on such environmental conditions as, among others, pH, osmotic pressure, presence of poly-anions, and membrane lipid content necessitate some means of its empirical quantitative characterization. Channel-conformation sensitivity to the applied transmembrane voltage manifests itself in conductance hysteresis, which is observed when the voltage is periodically varied with time. The objective of our study was to examine VDAC conductance hysteresis in a wide frequency range in an attempt to reconcile the recognized complexity of VDAC gating with the simplicity of its well accepted characterization in terms of a two-state equilibrium model for the opening branches of the hysteresis curves. Although this phenomenon has been used in studies of VDAC gating for nearly four decades, full hysteresis curves have never been reported, because the focus was solely on the opening branches of the hysteresis loops. We studied the hysteretic response of a multichannel VDAC system in a planar bilayer to a triangular voltage ramp, the frequency of which was varied over nearly three orders of magnitude, from 0.5 mHz to 0.2 Hz. We found that, in this wide frequency range, the area encircled by the hysteresis curves changes by less than a factor of three, suggesting broad distribution of the characteristic times and strongly non-equilibrium behavior. At the same time, we observed quasi-equilibrium two-state behavior for the hysteresis branches corresponding to VDAC opening. This enables calculation of the usual equilibrium gating parameters, gating charge, and voltage of equi-partitioning, which were found to be almost insensitive to the ramp frequency. To rationalize this peculiarity, we hypothesize that, during voltage-induced closure and opening, the system explores different regions of the complex free energy landscape and, in the opening branch, follows quasi-equilibrium paths.

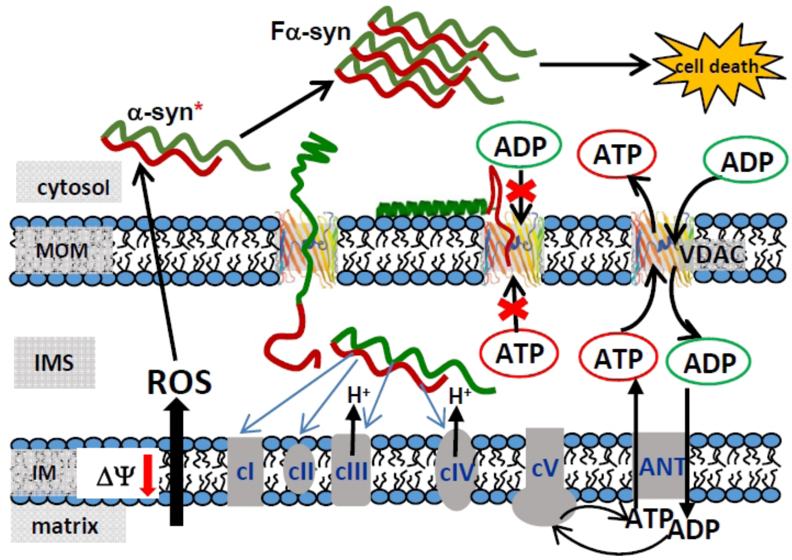

Figure 2. A model of mitochondrial outer membrane permeability regulation and α-syn–induced cytotoxicity

The model is based on alpha-synuclein (α-syn) interaction with and translocation through VDAC. VDAC blockage by α-syn disrupts ATP/ADP exchange between mitochondria and the cytosol, thus distorting the substrate balance for the adenine-nucleotide translocator (ANT) located in the inner membrane (IM). This leads to depletion of ATP-synthase (cV) with ADP, reduced mitochondrial potential, and impaired oxidative phosphorylation. By crossing the mitochondrial outer membrane (MOM) through VDAC into the intermembrane space (IMS), α-syn is able to directly target complexes of the electron transport chain (cI, cII, cIII, and cIV) in the IM. This could lead to mitochondrial dysfunction characterized by enhanced production of the reactive oxygen species (ROS). In turn, ROS induces monomeric α-syn oxidation in the cytosol, causing α-syn oligomerization and consequent amplification of fibrillar α-syn (Fa-syn) neurotoxicity, eventually resulting in cell death (Reference 1).

Physical theory of transport

This year, we focused on the problems of (1) bulk-mediated surface diffusion and (2) biased diffusion in three-dimensional comb-like structures. Bulk-mediated surface diffusion is attracting much attention in both theory and experiment owing to its well recognized relevance to a wide variety of processes in nature and technology. This kind of diffusion characterizes the motion of particles on a surface that is in a contact with the bulk in a way that allows for the dynamic partitioning of the particles between the surface-bound and bulk states. As a result, particles intermittently diffuse on the surface and in the bulk, allowing for an accelerated propagation of the particles over the surface when bulk diffusivity is significantly larger than its surface counterpart. We were motivated by our recent studies of the interaction between certain cytosolic proteins and their membrane-embedded targets, which demonstrated that it involves bulk-mediated diffusion over the membrane surface as an essential step. We developed a new approach to the problem, which reduces its solution to that of a two-state problem of the particle transitions between the surface and the bulk layer, focusing on the cumulative residence times spent by the particle in the two states. The times are random variables, the sum of which is equal to the total observation time. The advantage of the approach is that it allows for a simple exact analytical solution for the double Laplace transform of the conditional probability density of the cumulative residence time spent on the surface by the particle for the observation time. The solution was used to find the Laplace transform of the particle mean square displacement and to analyze the peculiarities of its time behavior over the entire range of time. In the case of biased diffusion in three-dimensional comb-like structures, we developed a formalism that allowed us to derive analytical expressions for the Laplace transforms of the first two moments of the particle displacement along the main tube axis. Through these one can find the time dependencies of the two moments for arbitrary values of both the drift velocity and the dead-end length, including

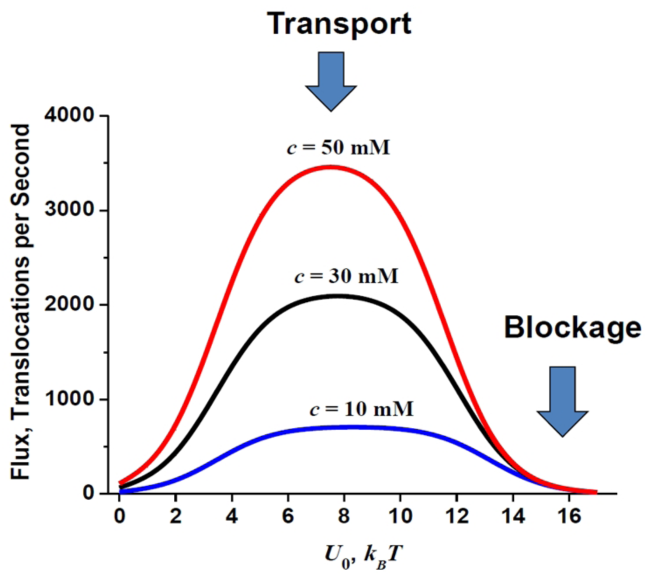

Figure 3. Optimal transport versus channel blockage
The flux through the channel depends on the strength of channel-particle interactions in a non-monotonic way. For the cylindrical channel geometry of 5 nm length and 0.2 nm radius and the range of the particle concentrations specified in the figure, the depth of the rectangular well that optimizes the transport is around 6 to 10 k_BT per molecule. Blockage of the channel, which is often a mechanism of channel regulation in nature, is achieved at higher well depths (Reference 5).

the limiting case of infinitely long dead ends, where the unbiased diffusion becomes anomalous at sufficiently long times. The results give us a set of rigorous tools for quantitative analysis of experimental findings.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Rostovtseva TK, Gurnev PA, Protchenko O, Hoogerheide DP, Yap TL, Philpott CC, Lee JC, Bezrukov SM. Alpha-synuclein shows high-affinity interaction with voltage-dependent anion channel suggesting mechanisms of mitochondrial regulation and toxicity in Parkinson disease. *J Biol Chem* 2015; 290:18467–18477.
2. Rappaport SM, Teijido O, Hoogerheide DP, Rostovtseva TK, Berezhkovskii AM, Bezrukov SM. Conductance hysteresis in the voltage-dependent anion channel. *Eur Biophys J* 2015; 44:465–472.
3. Berezhkovskii AM, Dagdug L, Bezrukov SM. Biased diffusion in three-dimensional comb-like structures. *J Chem Phys* 2015; 142:134101.
4. Berezhkovskii AM, Dagdug L, Bezrukov SM. A new approach to the problem of bulk-mediated surface diffusion. *J Chem Phys* 2015; 143:084103.
5. Berezhkovskii AM, Bezrukov SM. On the applicability of entropy potentials in transport problems. *Eur Phys J Spec Top* 2014; 223:3063–3077.

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Membrane Rearrangements in Cell Entry by Cationic Peptides and in Cell-Cell Fusion

Diverse membrane remodeling reactions are tightly controlled by protein machinery but are also dependent on the lipid composition of membranes. Whereas each kind of protein has its own individual characteristics, membrane lipid bilayers have rather general properties manifested by their resistance to disruption and bending. Our long-term goal is to understand how proteins break and reseal membrane lipid bilayers in important cell biology processes, such as membrane fusion and crossing of cell membranes by water-soluble drugs on their way to intracellular targets. We expect that the analysis of the molecular mechanisms of different membrane rearrangements will clarify the generality of emerging mechanistic insights. Better understanding of these mechanisms will bring about new ways of controlling them and lead to new strategies for treating diseases involving cell invasion by enveloped viruses, intracellular trafficking, and intercellular fusion. In addition to our on-going work on myoblast fusion and osteoclast fusion, in recent studies we focused on the mechanisms underlying cell entry by cell-penetrating cationic peptides. We identified a highly efficient intracellular calcium–regulated pathway of cationic peptide entry into the cytosol and nucleus of adherent cells. Specific mechanisms by which cationic peptides activate intracellular signaling pathways and then cross the plasma membrane remain to be clarified. A better understanding of these mechanisms may enhance our knowledge of the properties of plasma membrane as well as guide development of future drug delivery vehicles.

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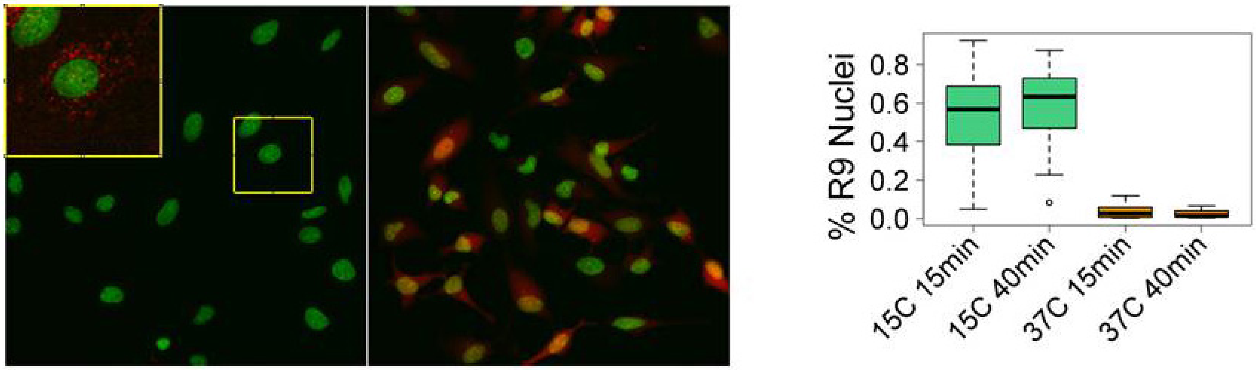
Efficient entry of the cell-penetrating peptide nona-arginine into adherent cells involves a transient increase in intracellular calcium.

Macromolecular drugs such as polynucleotides, their mimics (peptide nucleic acids and phosphorodiamidate morpholino oligomers), bioactive peptides, and full-length proteins have great potential in the treatment of various diseases. Rapid development of such drugs is greatly facilitated by progress in biotechnology and by better understanding of the molecular mechanisms of pathogenesis. However, delivery of macromolecular drugs to their intracellular targets, which often reside in the cell cytosol or nucleus, remains challenging, because, along the way, several permeability barriers must be overcome, including a cell membrane that is generally impermeable to most macromolecules. Cationic cell-permeable peptides (CCPPs) are a promising delivery vehicle for a wide range of potential macromolecular drugs and have been successfully used to deliver various macromolecular cargo molecules both *in vitro* and *in vivo*. Over the past two decades, significant progress has been made in identifying and designing new CCPPs. In contrast, a clear understanding of the mechanisms by which CCPPs reach their targets in the cytosol and nucleus is still lacking. Most importantly, we still do not know how highly charged CCPPs and their cargo cross cell membranes (plasma membrane or endosomal membrane), which are presumed to present a non-permeable barrier, enter the cytosol and nucleus. This greatly hampers rational design of new CCPPs.

In earlier studies, we and others showed that, at 37°C, various pathways of



Temperature drop induces efficient entry of Rhodamine-labeled R9 peptide (Rh-R9) into HeLa cells.

A. Cells were incubated with 2mM of Rh-R9 (*red*) for 15 min at 37°C or 15°C, washed, and imaged immediately without fixation. Nuclei are labeled with Hoechst (*green*). Inset shows an enlargement of the highlighted area with Rh signal amplified to demonstrate endocytic entry.

B. Fraction of cells with Rh-labeled nuclei after 15 min or 40 min incubation at 15°C or 37°C. Boxplots show the median as central line, 1st and 3rd quartiles as edges of the box, range as whiskers, and outliers as symbols.

endocytosis are involved in CCPPs' entry into cells. It has been suggested that endosome acidification and/or changes in lipid composition of the endosome upon maturation play an important role in CCPP escape. Regardless of the mechanism, the efficiency of endosomal escape is very low, with most of the peptide (and associated cargo) trapped in endosomes and then degraded. In search for more efficient ways of drug delivery into the cytosol and nucleus, we unexpectedly found that the rapid transfer of cells into cold medium (15°C) in the presence of the prototypical CCPP R9 (1–2μM) dramatically boosts the efficiency of R9 entry into the cytosol and nucleus, detected as nuclear labeling by rhodamine-tagged R9 (Figure 1). The amount of the peptide entering an individual cell at 15°C within 15 min was at least 50 times higher than the amount of the peptide entering a cell within 40 min at 37°C by endocytosis. Similarly, efficient entry of CCPPs into cytosol and nucleus can be induced by high concentrations of the peptide (10μM or more at 37°C). Importantly, such efficient entry is limited to the cationic peptide and is not accompanied by the entry of concomitantly added small membrane-impermeable dyes such as calcein or SYTOX Green. Intriguingly, whether induced by temperature drop or by a high CCPP concentration, we could detect peptide entry into cytosol and nucleus only in a sub-population of cells, suggesting an active role of the cell in the entry process. Indeed, both entry pathways were inhibited by depletion of intracellular ATP. We also observed an intriguing dependence of both pathways on a rise in intracellular Ca^{2+} concentration. In particular, addition of high concentrations of the peptide to the cells induced repetitive transient increases in intracellular Ca^{2+} concentration. Interestingly, we observed a significant variation in cell response to the peptide, with repetitive spikes of intracellular Ca^{2+} noted in some but not all cells within the same observation field. Importantly, there was a significant correlation between CCPP entry and appearance of Ca^{2+} spikes. Inhibition of both pathways of peptide entry by a chelator of intracellular Ca^{2+} further confirmed the role of the intracellular Ca^{2+} and suggested involvement of intracellular signaling pathways in the process. Using chemical inhibitors, we also demonstrated that both entry of Ca^{2+} from extracellular medium and release of Ca^{2+} from intracellular stores play an important role in the mechanism of CCPP entry.

Taken together, our data indicate that interactions of CCPPs with cells activate intracellular signaling cascades that result in significant changes in plasma membrane permeability for highly cationic peptides. Importantly, we demonstrated that cells do not act as mere targets for drug delivery but may actively interact with the added drug conjugate. Clearly, the side effects of the drug on cell metabolism should be taken into account in designing future CCPPs but may also be useful. For example, manipulation of intracellular calcium levels with drugs could be used to promote entry of the CCPPs. Indeed, we demonstrated that flulfenamic acid (a member of the anthranilic acid derivatives class of nonsteroidal anti-inflammatory drugs) induces entry of R9 at 37°C at a peptide concentration (2μM) that does not induce an increase in the intracellular calcium level and at which no entry of the peptide into cytosol/nucleus is observed when peptide is added alone. These 'proof of principle' experiments underscore the importance of our discovery for development of new drug delivery vehicles and protocols.

Modulation of plasma membrane permeability for highly cationic peptides by intracellular calcium signaling is an important new phenomenon that deserves further investigation. Of the many important questions that await an answer, we highlight just a few, such as how interactions of the CCPP with plasma membrane induce increase in intracellular Ca^{2+} concentration. It is possible that cell surface–bound cationic peptides electrostatically modulate activity of proximal plasma-membrane cation channels. Indeed, we previously reported that binding of R9 to negatively charged lipids strongly modulates conductance of the gramicidin A channel reconstituted into black lipid membranes. Alternatively, direct interaction of the peptide with cell-surface receptors may lead to their activation and to the opening of associated Ca^{2+} channels. A better understanding of this first step should provide guidance for the design of more efficient CCPPs but can also provide valuable insight into the cellular interactions of other important cationic peptide, defensins being one example. The specifics of calcium signaling pathways that lead to an increase in plasma membrane permeability to CCPPs is another largely unexplored but very important area. In our work, using phosphatidylserine-binding domain of lactadherin, we demonstrated that Ca^{2+}-dependent cell-surface exposure of phosphatidylserine, a lipid normally residing only in the inner leaflet of the plasma membrane, is involved both in temperature- and high concentration–induced entry of R9. However, the precise mechanistic role of the phosphatidylserine cell-surface exposure in R9 entry remains to be clarified. We expect that better delineation of the specific activation sequences involved in Ca^{2+}-dependent peptide entry will suggest new, more specific, and efficient approaches to induce CCPP entry. The mechanism of plasma membrane permeability to cationic peptide remains elusive, in particular, whether it is mediated through changes in lipid composition (perhaps surface exposure of phosphatidylserine is one of them) or involves some protein channels or transporters. Addressing this question is important not only for the drug delivery field but more broadly for understanding the transport properties of the plasma membrane.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Uygur B, Abramo K, Leikina E, Vary C, Liaw L, Wu WS. SLUG is a direct transcriptional repressor of PTEN tumor suppressor. *Prostate* 2015; 75:907–916.
2. Kozlov MM, Chernomordik LV. Membrane tension and membrane fusion. *Curr Opin Struct Biol* 2015; 33:61–67.
3. Melikov K, Hara A, Yamoah K, Zaitseva E, Zaitsev E, Chernomordik LV. Efficient entry of cell-penetrating peptide nona-arginine into adherent cells involves a transient increase in intracellular calcium. *Biochem J* 2015; 471:221–230.
4. Chernomordik LV, Kozlov MM. Myoblast fusion: playing hard to get. *Dev Cell* 2015; 32:529–530.
5. Zicari S, Arakelyan A, Fitzgerald W, Zaitseva E, Chernomordik LV, Margolis L, Grivel J-Ch. Evaluation of the maturation of individual dengue virions with flow virometry. *Virology* 2016; 88:20-7.

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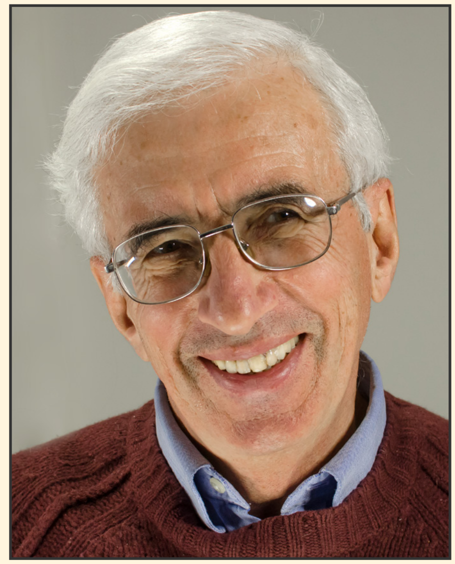
Pathogenesis of HIV-1 and its Copathogens in Human Tissues

The general goal of the Section of Intercellular Interactions is to understand the mechanisms of pathogenesis of various pathogens in human tissues, including the human immunodeficiency virus (HIV). Given that the critical events of this pathogenesis occur in tissues, and thus difficult to study *in vivo*, for our study we used a system of human tissues *ex vivo*, developed in our Section and now adopted by many investigators, to study viral infections and to test antivirals.

During the last year, we focused on three aims, to: (1) investigate pathogens' interactions mediated by cytokine network; (2) study HIV-1 infectivity in human tissue by analyzing individual virions' individual spikes, that mediate virus-cell fusion; (3) extend our flow virometry technology developed for HIV-1 analysis to other viruses, in particular to Dengue virus; and (4) investigate whether the immunoactivation that we demonstrated to be the consequence of HIV-1 infection plays a role in other human diseases. The research conducted during the last year provided new insights into general mechanisms of the interaction of human pathogens with the infected host, leading to new concepts in therapeutic strategies.



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Mosaics of spikes on HIV-1: analysis of individual Envs on individual HIV-1 virions

Owing to its high mutation rate, HIV remains one of the most variable viruses, in particular in the envelope encoded by *env*. As a result, such quick changes allow the virus to evade host immune responses that target predominantly viral surface proteins. The HIV envelope plays a critical role in HIV infection. Functional HIV envelope protein (Env) or spike is a non-covalently linked trimer of heterodimers, consisting of three surface homodimers of gp120 and trans-membrane gp41 subunits (stumps). The correct conformation of Env is critical for the virus to bind to cell receptors (CD4) and coreceptors (CCR5/CXCR4) and to undergo the complex conformational changes required for plasma membrane fusion and viral entry. Malfunctioning of this machinery renders virions incapable of binding/fusing with cells. The malfunctioning may be mediated by incorporation of dysfunctional forms of Env into the virion membrane or by the instability of incorporated functional Env. Indeed, a significant proportion of virions within any viral population are thought to be non-infectious. Several ways in which the HIV Env can be dysfunctional include uncleaved Env, dimeric or monomeric Env, or gp41 stumps. Each HIV-1 virion carries 7–14 spikes and, in principle, it is possible that on a given virion all spikes are either defective or are all functional, rendering the former virion defective and the latter virion infectious. Alternatively, virions may carry both functional and non-functional Envs in different conformations, as is currently thought. Understanding the extent of this mosaicism is important both for understanding of the basic mechanisms of HIV infection and for the development of new therapeutic and prevention strategies, in particular vaccines. To answer this question it is necessary to analyze the conformation of individual Env proteins on individual virions. To date, however, the majority of the biochemical and immunochemical analysis of HIV surface proteins is performed in bulk so that the diversity of individual virions is lost. Recently, we developed a nanoparticle-based technique called flow virometry, which permits the analysis of antigenicity on individual viral particles. We applied flow virometry to investigate the functional and non-functional conformations of Envs on individual virions, using a panel of antibodies that discriminate between various gp120 conformations. Individual virions were immuno-captured with 15–nm magnetic nanoparticles and stained with antibodies recognizing different conformations of Envs. The resulting complexes were separated from non-bound fluorescent antibodies and their aggregates in a magnetic field and analyzed on a regular commercial flow cytometer.

We found that viruses exhibit little mosaicism: on the majority of virions either all the spikes are functional or all are defective. Only a small subfraction of the virions were mosaic with virions carrying both functional and non-functional Envs. Contribution of this minor fraction in HIV infection of human tissue *ex vivo* appears to be small. The results of our study suggest that spikes may not be formed independently, and our approach can be used to describe mosaicism of HIV-1 in plasma of individual patients, thus determining individual treatment.

This all-or-nothing viral strategy is likely to aid immune evasion by subverting the focus of humoral responses to generate multiple non-neutralizing antibodies at no cost to infectious virions. Therefore, only the induction of antibodies that target functional Envs, and thus target predominantly infectious viruses, appears to be critical for the development of effective prophylactic strategies.

Maturity of individual dengue virions

The dengue virus (DENV) is a positive single-stranded RNA–positive virus that belongs to the family of *Flaviviridae*, genus Flavivirus. The diameter of the virus varies between 50–60nm, depending on the maturation state. The virus is enveloped with a lipid membrane, and carries on its surface 180 copies of the envelope (E) glycoprotein, which is responsible for cell attachment and fusion to the plasma membrane, and 180 copies of the structural membrane (M) protein. Viral maturation of DENV is determined by the cleavage of the prM precursor into M protein and pr peptide. Although based on bulk analysis, it is possible to evaluate the presence of prM on DENV virions and thus to determine the average degree of maturation of a viral preparation. Only the analysis of individual viral particles would allow us to distinguish immature from mosaic virions, and thus carry prM, or determine whether there is a fraction of fully mature viruses that do not carry prM. We performed a flow virometry analysis. We collected DENV virions from supernatant of infected BHK-21 and LoVo cells and labeled them with the fluorescent lipidic dye DiI. The labeled viral preparations were purified from floating dye in a discontinuous Optiprep density gradient then filtered through 0.22µm filters. To identify DENV virions among other membrane particles produced by BHK-21 and LoVo cells, we captured them with fluorescently labeled Zenon Alexa Fluor 488 3H5-1-MNPs, specific for the E protein of DENV that is present on all DENV viral envelopes. The MNPs are magnetic nanoparticles with a biopolymer coating consisting of a monolayer of oleic acid and a monolayer of amphiphilic polymer that contains carboxyl groups and can be coupled to amine-containing proteins via a two-step carbodiimide process. We coupled them with 3H5-1 antibodies and then labeled them with Zenon Alexa Fluor 488. We defined a DENV virus as a particle that is labeled with DiI and positive for 3H5-1. After the Optiprep purification gradient, all the membrane particles isolated in our viral preparations carried E protein, thus representing DENV particles. To be able to distinguish between mature and immature/partially mature virions, we used another antibody, 2H2, that is specifically against the prM protein. To validate our technology, we compared maturation of DENV produced in BHK-21 and LoVo cells. The latter are furin-deficient and should thus produce more immature virions, given that furin is required for prM cleavage and therefore for the maturation process. For our flow virometry study, DiI–labeled virus produced from both cell types was stained with 2H2 Alexa Fluor 647 antibodies, then captured by Zenon Alexa Fluor 488 3H5-1-MNPs. In a direct flow analysis of this preparation it would be difficult to distinguish virions from antibodies (or their aggregates) by size or by fluorescence making it impossible to attribute any detected fluorescent signal to labeled viruses or to free-floating antibodies occupying the cytometer interrogation chamber. Therefore, it was critical to separate them physically before the flow analysis. Towards this goal, we ran the preparation in magnetic column, which allowed us to exploit the magnetic properties of our MNPs so that our viral particles captured by 3H5-1-MNPs were retained in the column while free-floating antibodies were washed away during the washing step. Such a separation removes free antibody almost entirely.

We found that, in DENV produced in BHK-21 cells, prM is present on about 45% of DENV particles, while in DENV produced in LoVo cells about 85% of DENV virions carry uncleaved prM on their surface, indicating their non-fully mature state; the difference was statistically highly significant. The existence of mosaic flaviviruses had been documented in earlier electron microscopy studies that revealed co-existing smooth and spiky elements on viral surface. The specificity of DENV capture was evident from the comparison of the number of complexes captured with MNPs coupled to specific 3H5-1 antibodies with those captured with MNPs coupled to non-specific isotype control MNPs.

Flow virometry, earlier applied to the analysis of single HIV virions and used more recently for the analysis of single DENV virions, appears to be a universal method for the analysis of heterogeneity of different viruses, which allows quantification of virions carrying particular surface proteins. Their physical separation will allow us to correlate the antigenic composition of virions with their biological functions.

Cytokines as mediators of immunoactivation by human pathogens.

The change in the cytokine network in response to a pathogen invasion is common for various infections. Previous studies on cytokine concentrations and cytokine networks in spontaneous labor at term and preterm labor have been based on data derived from bioassays for these molecules and from specific individual immunoassays. Given that biological functions are the expression of integrated and interdependent networks of cells and molecules, the study of biological networks, rather than individual cells/molecules, is considered necessary to improve the understanding of the pathophysiology of disease. The objective of this study was to characterize the behavior of the cytokine network in the amniotic fluid of women in preterm labor, according to the presence/absence of intra-amniotic inflammation and microorganisms in the amniotic cavity. Earlier, we had performed such an analysis for HIV infection. We extended and further developed such an analysis to the intra-amniotic infection/inflammation that may lead to spontaneous preterm labor/delivery. We found that: (1) patients with preterm labor and intact membranes who had microbial-associated intra-amniotic inflammation had a higher amniotic fluid cytokine concentration correlation than those without intra-amniotic inflammation. IL-1β, IL-6, MIP-1α, and IL-1α were highly connected nodes (highest degree) in this differential correlation network; (2) patients with sterile intra-amniotic inflammation had correlation patterns of inflammatory-related proteins that were both increased and decreased when compared with those without intra-amniotic inflammation. IL-1α, MIP-1α, and IL-1β were the most connected nodes in this differential correlation network; and (3) there were more coordinated inflammatory-related protein concentrations in the amniotic fluid of women with microbial-associated intra-amniotic inflammation than in those with sterile intra-amniotic inflammation. IL-4 and IL-33 had the largest number of perturbed correlations with other inflammatory-related proteins in the differential correlation network. The observations provide evidence that the cytokine network behaves differently in women with preterm labor according to the presence or absence of intra-amniotic inflammation and/or microorganisms.

In general, our observations show that the cytokine network connectivity with microbial-associated intra-amniotic inflammation is denser and more coordinated than in women with sterile inflammation or without intra-amniotic inflammation. Our network analysis provides deeper insight into understanding the pathophysiological mechanisms of intra-amniotic infection/inflammation in preterm labor, as well as identifying potentially relevant modules of cytokines that correspond to distinct disease pathways in preterm labor. Also, such an approach may help to minimize the number of individual cytokines measured in order to characterize pathologic states, given that some elements of the network may have key roles in the regulation of the entire network/modules. A similar network analysis can now be applied to other pathogens. In particular, we studied the cytokine perturbation in cohorts of HIV–infected individuals receiving antiretroviral therapy. We found that a particular cytokine pattern associated with coinfection with human cytomegalovirus may predispose these individuals to syphilis acquisition.

Immunoactivation as a common pattern for various human diseases

We undertook a broad analysis of the biomedical literature and reviewed our own published results to determine the possible role of immunoactivation in human disease. It appears that many human diseases, including general aging, have one common driving mechanism, an inappropriate and dysregulated chronic immunoactivation. The analysis led us to conclude that immunoactivation can become dysregulated and promote the pathogenesis of diverse diseases with both known and unknown etiologies. Immunoactivation appears to be a common denominator or general mechanism of pathogenesis and may explain the association and similarities in pathology among otherwise unrelated human diseases. For example, immunoactivation may link rheumatoid arthritis, type 2 diabetes, or end-stage renal disease to atherosclerosis. It is possible that immunoactivation caused by irritation of the airway by cigarette smoke may explain why smoking is a common risk factor not only in cancer of the lung, where the products of smoking are deposited, but also in many apparently unrelated pathologies: cardiovascular diseases, preeclampsia, type 2 diabetes, and others. Also, the well known link of stress to cardiovascular disease seems to involve immunoactivation, as suggested earlier, and was evidenced by a recently described stress-induced activation of haemopoetic stem cells and release of high numbers of neutrophils, monocytes, and lymphocytes into blood. Not only the links between apparently unrelated diseases may be explained in the framework of immunoactivation but also similarities in the pathogenic details. For example, as mentioned above, induction of angiogenesis is typical for solid tumors. Similarly, abnormal angiogenesis, albeit of leaky vessels, is the main feature of the wet form of age-related macular degeneration (AMD). In an apparent recognition of this similarity, the same angiogenesis inhibitors are currently used in treatment of AMD and of some solid tumors.

The idea that immunoactivation contributes to pathogenesis and disease progression (the "immunoactivation hypothesis") is not new. At different times, it was introduced in various fields of medicine. However, in most cases the concept of damaging immunoactivation remained confined to the field of research where it was formulated. Now, it is becoming clear that the immunoactivation hypothesis is applicable generally. Thus, immunoactivation, which evolved as a system of host defense against pathogens, can become dysregulated and promote the pathogenesis of diverse diseases with both known and unknown etiologies and even be an important factor in general aging. Identification of general mechanisms of immunoactivation may lead to the development of new therapeutic strategies applicable to many diseases, even before detailed knowledge of their specific etiology and pathogenesis may be available.

ADDITIONAL FUNDING

» Intramural-to-Russia (I-to-R) Program

PUBLICATIONS

1. Arakelyan A, Ivanova O, Vasilieva E, Grivel J-C, Margolis L. Antigenic composition of single nano-sized extra-cellular vesicles. *Nanomedicine* 2015; 11:489–498.
2. Vanpouille C, Lisco A, Grivel JC, Bassit LC, Kauffman RC, Sanchez J, Schinazi RF, Lederman MM, Rodriguez B, Margolis L. Valacyclovir decreases plasma HIV-1 RNA in HSV-2 seronegative individuals: a randomized placebo-controlled crossover trial. *Clin Infect Dis* 2015; 60:1708–1714.
3. Gianella S, Smith DM, Daar ES, Dube MP, Lisco A, Vanpouille C, Margolis L, Haubrich RH, Morris SR. Cytomegalovirus replication predicts syphilis acquisition among HIV-1 infected men who have sex with men. *PLoS One* 2015; 10:e0130410.
4. Margolis L. Immunoactivation at the crossroads of human disease. *Am J Med* 2015; 128:652–656.
5. Romero R, Grivel J-C, Tarca AL, Chaemsaithong P, Xu Z, Fitzgerald W, Hassan S, Chaiworapongsa T, Margolis L. Evidence of perturbations of the cytokine network in preterm labor. *Am J Obstet Gynecol* 2015; 213:836.e1–836.e18.

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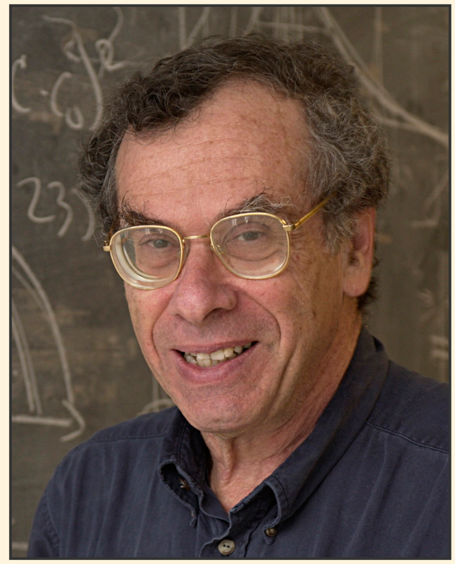
Cell Biophysics

Our investigations focus on how cell behavior is linked to underlying physical mechanisms, for which we develop and apply methodologies based on mathematical and physical principles. Projects currently include: (1) elaborating a physical model to understand the dependence on mesoscopic mechanical parameters of eukaryotic cell uptake of viruses and nanoscopic drug-delivery particles via receptor-mediated endocytosis; 2) understanding the nature and consequences of temperature-linked phase transitions in biological cell membranes, particularly those occurring near the temperature at which the cells grow and are maintained; (3) optimizing the use of *in vitro* assays for amoeboid cell chemotaxis by assessing how such tests depend on cell sensitivity to chemotaxins, motility parameters of the putative chemotactic cells, and assay geometry; and (4) understanding how certain small molecules interact with microtubules and thereby act as antimitotic agents, and how microtubule arrays function in mitosis to produce accurate segregation of chromosomes. We have a particular interest in the ways cellular activities are coordinated in space and time.



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Complex systems biophysics

For the past several years, we have been developing physical models to better understand the biogenesis of coated vesicles involved in clathrin-mediated endocytosis (CME) and other intracellular transport processes. CME is the principal pathway for the regulation of receptors and internalization of certain nutrients and signaling molecules at the plasma membrane of eukaryotic cells. Defects in CME can lead to metabolic disorders, aberrant signaling related to various cancers, and neurological disorders. A second major research area involves using published neurophysiological data to develop a deeper basic understanding of the biophysical behavior of cell membranes at the normal (growth or gestation) temperatures of organisms (see below). Additionally, in the recent past we have collaborated on the development of a mathematical model to explore how the difference in the average number of parasites (merozoites) released from malaria-infected blood cells can affect outcomes of therapy.

The early stage of receptor-mediated endocytosis involves the formation of transient structures known as clathrin-coated pits (CCPs). The process depends on the detailed energetics of protein binding and associated membrane transformations. The CCPs either mature into clathrin-coated vesicles (CCVs) or regress and vanish from the cell surface. During CCP formation, clathrin and several other proteins assemble to form a coat on the cytoplasmic side of the outer cell membrane. We previously developed a simple physical model for CCP dynamics and carried out Monte Carlo simulations to investigate the time development of CCP growth. Recently, we extended that model to investigate the role CME plays in the uptake of viruses and nanoparticles (NPs). Understanding how nano-sized particles interact with cell receptors and the clathrin coat is important when designing NPs for biomedical purposes as well as for developing strategies to inhibit cellular entry of viruses. Heretofore, theoretical models on this subject did not take clathrin coat assembly into explicit consideration. Thus, we developed a framework to study the endocytosis of single NPs that focuses on protein coat assembly and derived a simple analytical formula for the mean internalization time of NPs, defined as the average time between the binding of a NP to the plasma membrane and its entry into the cell. We studied the dependence of this quantity on coat parameters as well as NP size. There appears to be an optimal size at which cellular uptake is highest. Various published results indicate that the sizes appear to be independent of the type of cell, nanoparticle, or ligand. Moreover, experiments indicate that there is a maximum size beyond which uptake via clathrin-mediated endocytosis does not occur. We were able to show that such observations are consequences of the energetics and kinetics of protein coat formation during CCP production and predict how the efficacy of clathrin-mediated endocytosis depends on the mechanical properties of coat and membrane components (Reference 1 and manuscript in preparation).

We also investigated putative temperature-dependent lipid phase transitions occurring in higher eukaryotes. It is well established that microorganisms adjust the lipid composition of their membranes in response to changes in the temperature at which they are grown. Moreover, investigations carried out over many years demonstrated that whole lipid extracts from various higher organisms, as well as from microorganisms, exhibit singular properties at their growth temperatures. We investigated previously published data concerning the temperature dependence of the electrophysiological responses of cells obtained from representative animals (e.g., frog, squid, rat), searching for unusual features occurring at the gestation/growth temperature, T_g, of these animals. Special emphasis was given to the giant axons of the temperate squids *Loligo forbesi* and *Loligo pealei*, for which the temperature dependencies of the resting potentials and action potentials exhibit behaviors that strongly suggest the onset of a membrane state change at T_g, mirroring, in the case of squids, anomalies seen in the physical properties of films formed of whole lipid extracts obtained from those organisms. Based on approximations to the classic Hodgkin-Huxley equations, analysis of axonal responses indicates that observed changes in these electrophysiological characteristics most likely reflect reversible molecular couplings between voltage-switchable ion channels and surrounding lipids in the plasma membrane, which can affect the probability of channel opening. The change in electrophysiological properties with increasing temperature for these animals yields activation energies close to values noted for other putative lipid bilayer–linked kinetic processes that we have examined, as well as being seen in the diffusion of exogenous probes in lipid extracts (e.g., Jinet al., *Biochemistry* 1999;38:13275). These and other observations substantiate the view that, to first order, the lipid bilayer acts as a universal solvent for the embedded, integral proteins found in the plasma membrane.

Tubulin polymers and cytoskeletal organization

We continued our study of the properties of microtubules (MT), of drugs that alter these properties, and of the biology of MT arrays such as the mitotic spindle. In the course of this work, it was necessary to revisit the biophysical methods used for monitoring MT–targeting drug effects on cells exposed to the drugs. In addition, we addressed the mechanism that might underlie the potency of the drugs in treating patient tumors. We had previously presented evidence that this mechanism is not based on inhibition of mitosis, as often thought.

Microtubule-targeting drugs, and other chemotherapy agents, cause an increase in cellular reactive oxygen species (ROS) as a direct or ancillary effect of their intracellular action. Some such increases are known to be the result of effects on mitochondria, while others are attributable to other, often unknown, mechanisms. We pursued oxidative damage in cells by developing a fluorescent "tag" molecule that binds to and 'lights up' sites of oxidative damage inside cells, in particular carbonyl residues. We built on our previous use of "click-chemistry" to design a cell-permeable fluorophore that reacts with oxidized molecules. The molecules often contain carbonyl groups as a result of oxidation, and our click chemistry approach pairs a fluorophore with a hydrazine moiety to react spontaneously and specifically with the carbonyl groups. The result is the covalent attachment of a fluorophore to the sites of oxidation in the cell, which can then be visualized in the fluorescence microscope or identified biochemically following cell lysis and chromatography. We have now produced several variations on this oxidation sensor, with different fluorescence properties. We are currently attempting to make this approach work using fluorophores with longer excitation wavelengths than the current UV– or near UV–requiring molecule (Reference 2).

We also developed new fluorescent probe molecules that, in fluorescence microscopy, allow significant improvements in resolution. The probes utilize two wavelengths of light in a structured excitation beam to illuminate a sample and produce an image that has spatial resolution that is smaller than the limit set by the diffraction of light. The improved spatial resolution is achieved with this dye because it controls quenching of fluorescence emission by a "doughnut" of one wavelength of light, which effectively narrows the central, unquenched region to produce fluorescence emission due to the second wavelength of light from a spot smaller than the diffraction limit. The procedure is called Super-resolution via Transiently Activated Quencher, or STAQ. An advantage of the method, in addition to the improvement in spatial resolution, is the fact that the light power required for the procedure is much lower than the level required for other super-resolution methods. We are continuing to improve the dye and its applications (Reference 3).

MT–targeting drugs induce arrest of mitosis in rapidly dividing cells but also alter MT function during the 95 percent or more of the time that a cell is not in mitosis. The latter functions are likely to be the reason that such drugs are effective chemotherapy agents against many human cancers, given that mitosis is rare in tumors; and given that mitosis is not the main target of these drugs in tumors and that the drugs are effective against tumors, the target must be something else and present throughout the cell cycle, not only during mitosis. A candidate for the target is intracellular signaling and trafficking, which rely on microtubules to provide directional specificity. This may also underlie the well documented increase in clinical effectiveness observed when these agents are combined with DNA–damaging drugs. We hypothesized that the increased effectiveness occurs because the cells normally respond to DNA damage by recruiting DNA–repair enzymes from the cytoplasm to the nucleus. Such movement relies on microtubules to direct the traffic of the enzymes to the nucleus. Therefore, damaging the MT array with MT–targeting drugs blunts the cells' ability to repair DNA damage, resulting in increased cell death. We tested the hypothesis with several cell lines and several combinations of DNA–damaging agents and MT–targeting agents, and the results appear to confirm our hypothesis. We are now extending our results to trafficking of other cancer-related proteins, such as oncogene proteins (Reference 4).

Biophysical methods and models

Our goal is to develop new methodologies to characterize the physical attributes and responsiveness of biological materials. Our work typically involves such techniques as mathematical modeling and simulation, polymer gel technology, and Fourier transform analysis of fluctuating optical signals. The work is directed towards a deeper understanding of the physical basis of quantitative measurement techniques and expanding the mathematical theory underlying related data analysis.

Thus we continued a project to provide the basis for quantitative assessment of the chemotactic response of neutrophils and other amoeboid cells. Migration of cells along gradients of effector molecules is necessary during an immune response and is involved in tissue development and cancer metastasis. The experimental assessment of chemotaxis is thus of great interest but is difficult to measure. Chemotaxis is frequently inferred by determining how many cells cross a boundary in a chemotaxis assay, for example how many cells crawl into the filter in the filter-migration assay or how many cells crawl into a defined region in the under agarose assay or agarose spot assay. The major limitation of these approaches is that such motion is not necessarily directed by the chemo-attractant gradient. To determine how reliably methods based on boundary crossing can indicate chemotactic motion of cells, we used information about the gradient sensitivity of neutrophils and MDA-MB-231 breast cancer cells to model how much gradient sensing increases the rate of boundary crossing relative to a random motility control, and over what duration. As part of this effort, we showed that neutrophils can sense chemo-attractant gradients generated in the under agarose and agarose spot assays for 1 to 2 hours. We also determined the chemo-attractant profile in the filter migration assay for filters of low porosity, and inferred that, in the filter assay, a neutrophil would be able to reliably perceive a gradient for about 10 hours. In contrast, chemotaxis of MDA-MB-231 cells, and cells with similar sensitivity to gradients, cannot be reliably measured by counting the cells in the agarose spot and under agarose assays. Moreover, although measurement of chemotaxis of these cells using the filter assay can be accomplished, doing so requires stringent controls. (Reference 5, and manuscript in preparation.)

A further activity involved the development of a method to track and quantify the movements of HIV viruses through dense mucus. We analyzed images obtained by time-resolved fluorescence confocal microscopy (t-FCM) and examined the motion of fluorescently labeled, inactivated HIV viruses (about 100 nm) that were added to samples of crude, untreated cervical mucus. After delineating the pixel locations of the fluorescent peaks associated with the viruses, we tracked the center of mass of their centroids over consecutive frames. To assess the randomness of the motion of each virus, we calculated changes of its statistical mean-squared displacement (MSD). As compared with our previous report (Boukari et al., *Biomacromolecules* 2009;10:2482), we doubled the observation time (about 34 second), which provided additional insight into the overall behavior of the viruses. Half the tracked viruses appeared significantly constrained, with their MSDs being very weakly dependent on time. The others showed relative mobility with MSDs that are proportional to $t^a + v^2t^2$ over a time range t, depicting a combination of anomalous diffusion (a about 0–0.4) and/or slow, flow-like behavior. The MSD data reveal plateaus attributable to possible stalling and caging of the viruses during their motion, providing quantitative information that can guide the development of physical theories to deal with the way the heterogeneity and internal stresses of the mucus affect the movement of embedded nanoparticles.

PUBLICATIONS

1. Banerjee A, Berezhkovskii A, Nossal R. Efficiency of cellular uptake of nanoparticles via receptor-mediated endocytosis. *arXiv* 2014; 14117348.
2. Mukherjee K, Chio TI, Sackett DL, Bane SL. Detection of oxidative stress-induced carbonylation in live mammalian cells. *Free Radic Biol Med* 2015; 84:11–21.
3. Rosales T, Sackett DL, Xu J, Shi ZD, Xu B, Li H, Kaur G, Frohart E, Shenoy N, Cheal SM, Wu H, Dulcey AE, Hu Y, Li C, Lane K, Griffiths GL, Knutson JR. STAQ: a route toward low power, multicolor nanoscopy. *Microsc Res Tech* 2015; 78:343–355.
4. Poruchynsky MS, Komlodi-Pasztor E, Trostel S, Wilkerson J, Regairaz M, Pommier Y, Zhang X, Kumar Maity T, Robey R, Burotto M, Sackett D, Guha U, Fojo AT. Microtubule-targeting agents augment the toxicity of DNA-damaging agents by disrupting intracellular trafficking of DNA repair proteins. *Proc Natl Acad Sci USA* 2015; 112:1571–1576.
5. Szatmary AC, Stuelten CH, Nossal R. Improving the design of the agarose spot assay for eukaryotic cell chemotaxis. *RSC Adv* 2014; 4:57343–57349.

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The Regulation or Disturbance of Protein/Lipid Interactions in Influenza, Malaria, Diabetes, Muscular Dystrophy, Brain Trauma, and Obesity

Eukaryotic life must create the many shapes and sizes of the system of internal membranes and organelles that inhabit the variety of cells in nature. The membranes must remodel so that cells can secrete signaling macromolecules, express surface transporters, import macromolecular cargo, store energy, repair a damaged plasmalemma, and deal with infectious agents such as viruses and parasites. Such basic membrane mechanisms must be highly regulated and highly organized in various hierarchies in space and time to allow the organism to thrive despite environmental challenges, genetic instability, an unpredictable food supply, and physical trauma. We are using our expertise and the techniques we have perfected over the years to address several different biological problems that nevertheless share the underlying regulation or disturbance of protein/lipid interactions. Our overall goal is to determine the physico-chemical mechanisms of membrane remodeling in cells and to understand the mechanisms of cellular secretion and endocytosis at physical, biophysical, and chemical levels, including the concentration and diffusion of key vesicular components prior to and after fusion or fission.

This year, we focused on four topics: (1) the discovery that adipose cells from human subjects with varying degrees of metabolic syndrome (insulin resistance) were either refractory or sensitive to insulin *in vitro*, depending upon their metabolic status; (2) the formation and disintegration of a layer of protein made up of the internal scaffold of the influenza virus; (3) the colocalization of different lipids with the hemagglutinin of the influenza virus, showing no special 'raft' composition of clusters of hemagglutinin; and (4) the introduction of a new lentiviral reporter for cancer stem cells.

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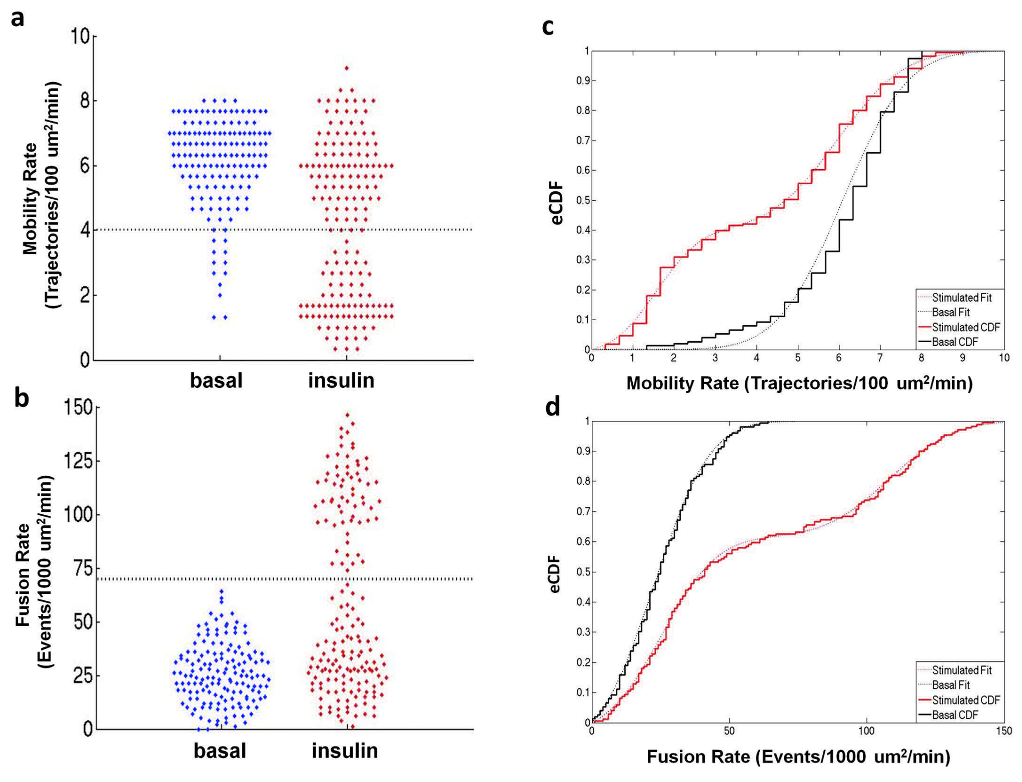
Adipose cell biopsies from insulin-resistant individuals reveal refractory fat cells.

While intercellular communication processes are frequently characterized by switch-like transitions, the endocrine system, including the adipose tissue response to insulin, has been characterized by graded responses. We found, however, that individual cells from adipose tissue biopsies are best described by a switch-like transition between the basal and insulin-stimulated states for the trafficking of the glucose transporter GLUT4. Two statistically defined populations best describe the observed cellular heterogeneity, representing the fractions of refractive and responsive adipose cells. Furthermore, subjects exhibiting high systemic insulin sensitivity indices (SI) have high fractions of responsive adipose cells *in vitro*, while subjects exhibiting decreasing SI have increasing fractions of refractory cells *in vitro*. Thus, a two-component model best describes the relationship between cellular refractory fraction and subject SI. Given that isolated cells exhibit such different response characteristics in the presence of constant culture conditions and milieu, we suggest that a physiological switching mechanism at the adipose cellular level ultimately drives systemic SI.

The matrix protein from influenza A forms and disintegrates at different pH, providing tension for membrane fusion during infection.

Influenza virus is taken up from a pH–neutral extracellular milieu into an endosome, whose contents then acidify, causing changes in the viral matrix protein (M1) that coats the inner monolayer of the viral lipid envelope. At a pH of around 6, M1 interacts with the viral ribonuclear protein (RNP) in a putative priming stage; at this stage, the interactions of the M1 scaffold coating the lipid envelope are intact. The M1 coat disintegrates as acidification continues to pH around 5 to clear a physical path for the viral genome to transit from the viral interior to cytoplasm. We investigated the physico-chemical mechanism of M1's pH–dependent disintegration. In pH–neutral media, the adsorption of M1 protein on the lipid bilayer was electrostatic in nature and reversible. The interaction energy of M1 molecules to each other in M1 dimers was about ten-fold weaker than M1 to lipid bilayer. Acidification drives conformational changes in M1 molecules owing to changes in M1 charge, leading to alterations in their electrostatic interactions. Lowering the pH from 7.1 to 6.0 did not disturb the M1 layer; lowering it further partially desorbed M1 as a result of increased repulsion between M1 monomers still adhering to the membrane. Lipid vesicles coated with M1 demonstrated pH–dependent rupture of vesicle membrane, presumably owing to the tension generated by this repulsive force. Thus, the disruption of the vesicles coincident with M1 protein scaffold disintegration at pH 5 likely stretches the lipid membrane to the point of rupture, promoting fusion pore widening for RNP release.



Bee Swarm plot of single cell mobility and fusion rate for all subjects

Hemagglutinin, the spike protein of influenza, clusters in the plasma membrane without increased cholesterol and sphingolipids.

It is hypothesized that the clusters of the influenza envelope protein hemagglutinin within the plasma membrane are enriched with cholesterol and sphingolipids. We directly tested this hypothesis by using high-resolution secondary ion mass spectrometry to image the distributions of antibody-labeled hemagglutinin and isotope-labeled cholesterol and sphingolipids in the plasma membranes of fibroblast cells that stably express hemagglutinin. We found that the hemagglutinin clusters were neither enriched with cholesterol nor did they colocalize with sphingolipid domains. Thus, hemagglutinin clustering and localization in the plasma membrane is controlled neither by cohesive interactions between hemagglutinin and liquid-ordered domains enriched with cholesterol and sphingolipids nor from specific binding interactions between hemagglutinin, cholesterol, and/or the majority of sphingolipid species in the plasma membrane.

Direct observation of cancer stem cells

Many tumors are hierarchically organized with a minority cell population that has stem-like properties and enhanced ability to initiate tumorigenesis and drive therapeutic relapse. Such cancer stem cells (CSCs) are typically identified by complex combinations of cell-surface markers that differ among tumor types. We developed a flexible lentiviral-based reporter system that allows direct visualization of CSCs based on functional properties. The reporter responds to the core stem-cell transcription factors OCT4 and SOX2, with further selectivity and kinetic resolution coming from use of a proteasome-targeting degron (degradation signal, i.e., sequence of amino acids determining the starting place of degradation). Cancer cells marked by this reporter have the expected properties of self-renewal, generation of heterogeneous offspring, high tumor- and metastasis-initiating activity, and resistance to chemotherapeutics. With this approach, the spatial distribution of CSCs can be assessed in settings that retain microenvironmental and structural cues, and CSC plasticity and response to therapeutics can be monitored in real time.

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» Jain Foundation Award
» NICHD Director's Award (Co-Principal Investigator with Jack Yanovski)

PUBLICATIONS

1. Batishchev OV, Shilova LA, Kachala MV, Tashkin VY, Sokolov VS, Fedorova NV, Baratova LA, Knyazev DG, Zimmerberg J, Chizmadzhev YA. pH-Dependent formation and disintegration of the influenza A virus protein scaffold to provide tension for membrane fusion. *J Virol* 2015; 90:575-85.
2. Wilson RL, Frisz JF, Klitzing HA, Zimmerberg J, Weber PK, Kraft ML. Hemagglutinin clusters in the plasma membrane are not enriched with cholesterol and sphingolipids. *Biophys J* 2015; 108:1652–1659.
3. Lizunov VA, Stenkula KG, Blank PS, Troy A, Lee J-P, Skarulis MC, Cushman SW, Zimmerberg J. Human adipose cells in vitro are either refractory or responsive to insulin, reflecting host metabolic state. *PloS One* 2015; 10:e0119291.
4. Tang B, Raviv A, Esposito D, Flanders KC, Daniel C, Nghiem BT, Garfield S, Lim L, Mannan P, Robles AI, Smith WI Jr, Zimmerberg J, Ravin R, Wakefield LM. A flexible reporter system for direct observation and isolation of cancer stem cells. *Stem Cell Reports* 2015; 4:155–169.

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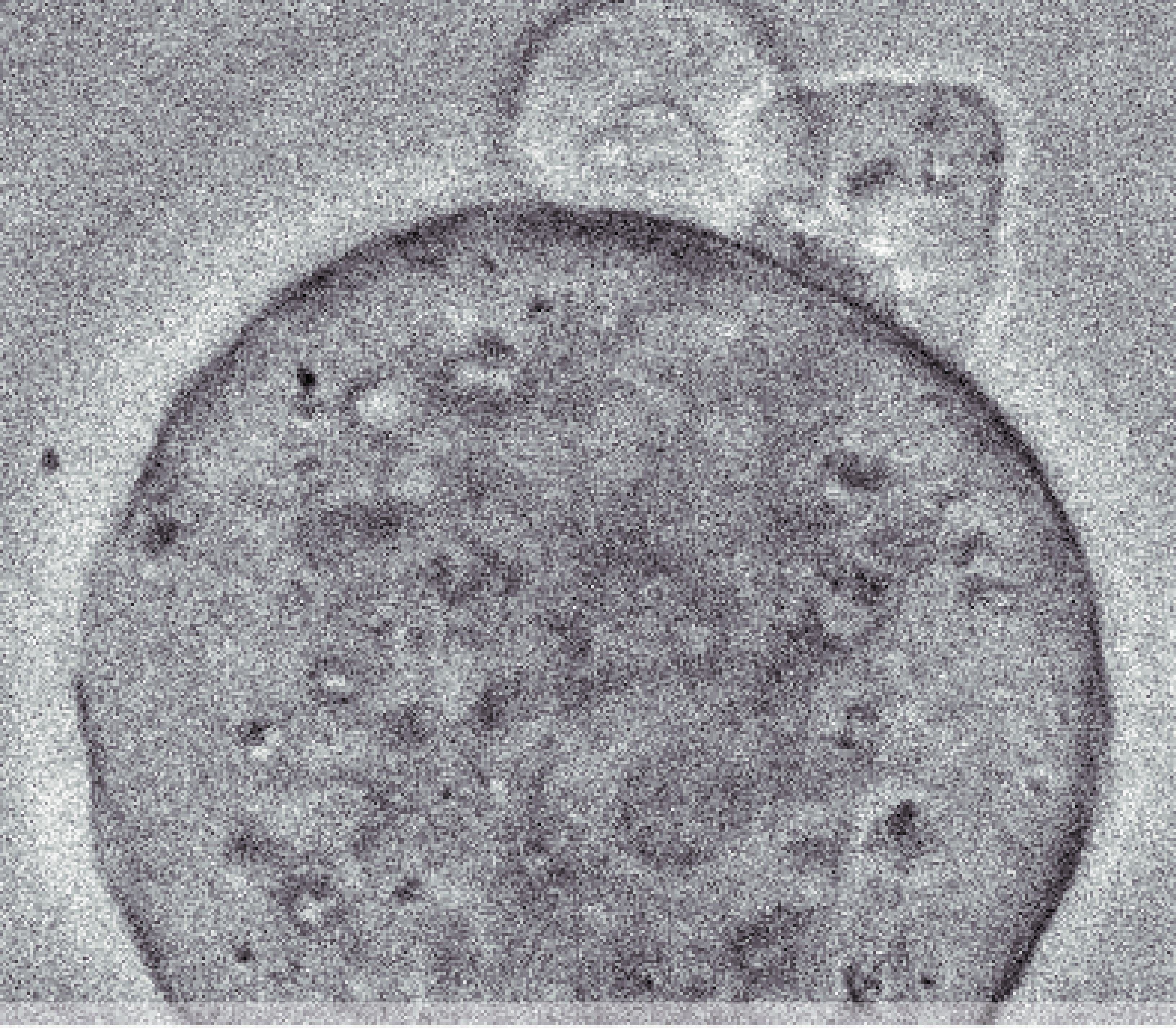


Program in Reproductive and Adult Endocrinology

Director: Alan DeCherney, MD

ABOUT THIS IMAGE
A mouse zygote in the two-pronuclear stage.

PROGRAM *in* REPRODUCTIVE AND ADULT ENDOCRINOLOGY

The *Program in Reproductive and Adult Endocrinology (PRAE)* conducts biomedical research and training as well as clinical activities in the area of reproductive endocrinology and adult endocrinology. Physician-scientists from all Sections and Units of the Program admit patients to NIH's Clinical Research Center for research-directed, approved clinical protocols and see patients in the outpatient setting for protocol evaluation, follow-up, and consultations. The PRAE trains fellows in Adult Endocrinology as well as Reproductive Endocrinology and Infertility (REI), the former approved by the Accreditation Council for Graduate Medical Education (ACGME) and the latter by the American Board of Obstetrics and Gynecology. The Program takes three REI fellows (one in the military track) and two medical endocrine fellows per year. The medical endocrinology training program (The NIH Inter-Institute Endocrinology Fellowship Program) is a joint effort with the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the National Institute of Dental and Craniofacial Research (NIDCR). The PRAE collaborates closely with the Pediatric Endocrine Fellows, and all three fellowships include rotations on the adult endocrine unit and joint conferences. Elective time has been added to allow the fellows the opportunity to obtain advanced degrees (e.g., an MPH, Duke University Program in Clinical Research).

LYNNETTE NIEMAN leads the *Section on Reproductive Endocrinology*, which currently conducts a clinical and basic research program on cortisol physiology and pathophysiology. Previous studies with the antiprogestin CDB-2914 formed the basis for FDA approval of the agent as a morning-after pill in 2010; it was submitted to the EMA (European Medicines Agency) in 2012 for approval for the treatment of symptomatic leiomyoma. Nieman's research interest in both adrenal and reproductive steroids dates to her initial work, in 1983, with the antiprogestin and antiglucocorticoid agent mifepristone. Since that time, her group has helped to develop diagnostic tests and their interpretation for the differential diagnosis of Cushing's syndrome, including salivary and midnight cortisol, 8 mg dexamethasone suppression, the CRH stimulation test, the dexamethasone–CRH test, inferior petrosal sinus sampling, and high-dose Octreoscan and FDG-PET imaging. The work continues with current studies on F-DOPA PET and the use of mifepristone for the treatment of ectopic ACTH secretion. A database of Cushing's syndrome patients has been constructed. Current studies explore aspects of glucocorticoid action and will continue. Nieman's research has shifted away from the study of fibroids.

The *Section on Medical Neuroendocrinology,* led by KAREL PACAK, was established in 2001 to develop novel approaches for the diagnosis, localization, and treatment of pheochromocytoma (PHEO) and to search for new molecular and genetic markers for the etiology of this tumor. Initial studies focused on the development of novel methods and criteria to diagnose and localize pheochromocytoma. Current studies focus mainly on how to further characterize the tumors, using functional imaging approaches, identifying new molecular and genetic mechanisms of tumorigenesis, and introducing new treatment options for malignant pheochromocytoma. Currently, the Section has the world's largest population of patients with malignant and succinate dehydrogenase subunit B–related pheochromocytomas. To assist in its research and objectives, the Section also established a unique PHEO tumor tissue and blood bank. The Section's current goal is to introduce new algorithms for diagnosis and localization of PHEO in order to: (i) explain the molecular basis for different clinical presentations and establish the pathways of tumorigenesis (focusing on genotype-phenotype correlations in various pheochromocytomas, using gene expression and proteomic profiling); (ii) search for new molecular and genetic markers for diagnosis and treatment of malignant pheochromocytoma (using microarray and proteomic approaches and evaluating new chemotherapeutic compounds that target identified proteins and pathways); (iii) facilitate new and improved collaborations and interdisciplinary studies and support a coordinated approach to basic and clinical research, which should lead to an improved understanding of the biology of pheochromocytoma and more rapid advances in treatment for patients with malignant tumors.

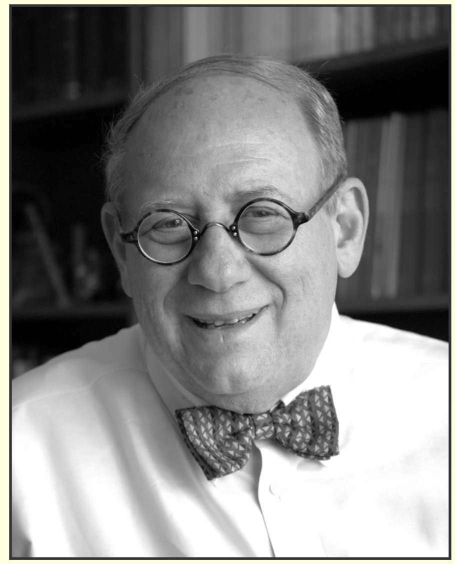
The *Unit on Reproductive Regenerative Medicine* is led by ERIN FORAN WOLFF. Wolff is a 2010 PRAE REI Fellowship graduate and was selected by a formally constituted NICHD Assistant Clinical Investigator Search Committee in a competitive process. For her independent translational research, Wolff is working with the National Heart, Lung, and Blood Institute (NHLBI) under John Tisdale and with NICHD under ALAN DECHERNEY. Her work aims to characterize the effects of stem cell therapies on the reproductive tract, with the long-term goal of developing cellular therapy applications for gynecologic conditions. Currently, the only option for women suffering from idiopathic premature ovarian failure/primary ovarian insufficiency or from ovarian failure resulting from chemotherapy or radiation is donor eggs from younger women. Recently, several new stem cell advances have been made that could be used in future to treat women with premature ovarian aging. Until recently, it was thought that female ovarian germline stem cells did not exist. However, such cells have now been isolated in both a murine and human models, using magnetic beads and FACS with antibodies against the germ line marker Ddx4, cultured ex vivo with mitotic expansion, and transplanted in murine and human xenograft models. Adult germline stem cells were able to enter into meiosis after transplantation back into a donor ovary, where they gave rise to offspring in mice. The goal of this project is to translate the technical advances in stem cell research into ways to help women with reproductive disorders. However, significant differences in reproductive physiology exist between rodents and primates (e.g., menstrual cycles and hemachorial placentation), which make it essential to perform proof-of-concept studies in a non-human primate to determine the viability of the approach for future human therapy.

The NIH Inter-Institute Endocrinology Fellowship Program provides a comprehensive training experience that involves many of the NIH clinical branches working in endocrinology, as well as the Georgetown University Hospital, Washington Hospital Center, and Walter Reed Army Medical Center, all in the Washington area. The basic and clinical endocrine research facilities at the NIH are among the most extensive and highly regarded in the world. The fellowship is thus ideal for physicians who seek a broad education in both research and clinical endocrinology. Clinical training occurs largely in the first year. The remaining two years are spent primarily conducting laboratory or clinical research under the direction of a senior investigator in one of the several endocrinology branches of the NIH. During this research period, active clinical experience continues through a weekly continuity outpatient clinic, consult service, and participation in clinical conferences.

Fellows are responsible for five to ten patients at any one time on the inpatient service of the NIH. Under the supervision of the endocrine faculty, the trainee has total responsibility for all aspects of the patients' care. Fellows make daily rounds, discuss patients with the attending physicians, and participate in management decisions related to both patient care and clinical investigation. Although all patients are admitted under peer-reviewed research protocols, there are many other aspects of diagnosis and patient care that fall completely under the discretion of the endocrine fellows. In addition, fellows on the endocrine service serve as consultants to the rest of the Clinical Center, where patients are not selected with regard to endocrine problems. Thus, the fellows gain experience with several common problems of endocrine disease that may occur in any general medical ward. Clinical research activities include programs in all the areas of endocrine and metabolic diseases. Study design, outcome measures, statistical analysis, and ethical and regulatory issues are stressed.

There are major gaps in knowledge regarding the etiologic mechanisms, psychosocial effects, natural history, and medical and psychosocial management of disorders of the hypothalamic-pituitary-ovarian axis in women. An international research consortium and disease registry formed under the guidance of an umbrella organization would provide a pathway to comprehensively increase basic and clinical knowledge about these conditions. Such a consortium and patient registry also would provide clinical samples and clinical data with a goal toward defining the specific pathogenic mechanisms. An international collaborative approach that combines the structure of a patient registry with the principles of integrative care and community-based participatory research is needed to advance the field of primary ovarian insufficiency. The program is in the early phases of organizing such an international effort using primary ovarian insufficiency as the focus.

Spontaneous POI affects 1 in 100 women by the age of 40. With no apparent cause, the ovaries of affected women do not function normally. They stop regularly releasing eggs and produce low levels of reproductive hormones, including estradiol (a type of estrogen) and testosterone (a predominantly male hormone that is also produced by women but in smaller amounts than by men). Women with POI have hot flashes, fertility problems, and irregular or no menstrual cycles. They also have reduced bone mineral density, which can lead to osteoporosis and bone fractures. Hormone replacement therapy (HRT) regimens have been well studied and optimized to improve bone health in postmenopausal women. But there has been limited research on the effects of these therapies in younger women. We found that HRT restores bone mineral density to normal in young women with primary ovarian insufficiency (POI). The findings provide important treatment information for women with POI and their physicians.



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Normalization of bone density by HRT in women with POI

A team led by Vaishali B. Popat and Lawrence M. Nelson tested the effects of HRT on bone health in young women with POI. The trial was carried out at the NIH Clinical Center in Bethesda, Maryland. The team enrolled 145 women with POI between the ages of 18 and 42. The women were randomly assigned to two groups. One received an estradiol patch, progestin pills, and a testosterone patch. The other received an estradiol patch, progestin pills, and an inactive placebo patch. The researchers used bone density scans of the hip and lower spine to measure the effects of the regimens. For comparison, the scientists also measured bone mineral density in an untreated group of 70 women with normal ovarian function.

Both hormone treatment regimens led to increases in bone mineral density at three years. When the study began, women with POI had significantly lower hip and spine bone mineral density levels than those in the control group. By the study's end, bone density measures in both treatment groups had increased to the same level as in the women without POI. The group receiving a testosterone patch did not gain further benefits over those with a placebo patch. Studies with a greater number of women would be needed to learn whether testosterone replacement might benefit women with POI, the researchers noted. The study showed that not only could hormone treatment reduce the rate at which women with POI lose bone mineral density, but it could actually restore bone density to normal levels.

Lack of effect of testosterone treatment on mood disorders in POI

Women with primary ovarian insufficiency (POI) display low androgen levels, which could contribute to the mood and behavioral symptoms observed in this condition. We examined the effects of physiologic testosterone therapy added to standard estrogen/progestin therapy on quality of life, self-esteem, and mood in women with POI. One hundred twenty-eight women with 46,XX spontaneous POI participated in a 12-month randomized, placebo-controlled, parallel-design investigation of the efficacy of testosterone augmentation of estrogen/progestin therapy. Quality of life, self-esteem, and mood symptoms were evaluated with standardized rating scales and a structured clinical interview. Differences in outcome measures between the testosterone and placebo treatments were analyzed by Wilcoxon rank sum tests.

No differences in baseline characteristics, including serum hormone levels, were found. Baseline mean (SD) Center for Epidemiologic Studies Depression Scale scores were 10.7 (8.6) and 9.2 (7.8) for testosterone and placebo, respectively. After 12 months of treatment, measures of quality of life, self-esteem, and mood symptoms did not differ between treatment groups. Serum testosterone levels achieved physiologic levels in the testosterone group and were significantly higher than in the placebo group. Baseline testosterone levels were not associated with either adverse or beneficial clinical effects.

We conclude that a 150-microgram testosterone patch achieves physiologic hormone levels in women with POI. However, our findings suggest that augmentation of standard estrogen/progestin therapy with physiologic testosterone therapy in young women with POI neither aggravates nor improves baseline reports of quality of life or self-esteem and had minimal effects on mood. Other mechanisms might play a role in the altered mood accompanying this disorder.

PUBLICATIONS

1. Popat VB, Calis KA, Kalantaridou SN, Vanderhoof VH, Koziol D, Troendle JF, Reynolds JC, Nelson LM. Bone mineral density in young women with primary ovarian insufficiency: results of a three-year randomized controlled trial of physiological transdermal estradiol and testosterone replacement. *J Clin Endocrinol Metab* 2014; 99(9):3418–3426.
2. Guerrieri GM, Martinez PE, Klug SP, Haq NA, Vanderhoof VH, Koziol DE, Popat VB, Kalantaridou SN, Calis KA, Rubinow DR, Schmidt PJ, Nelson LM. Effects of physiologic testosterone therapy on quality of life, self-esteem, and mood in women with primary ovarian insufficiency. *Menopause* 2014; 21:952–961.
3. Falorni A, Minarelli V, Eads CM, Joachim CM, Persani L, Rossetti R, Yurttas Beim P, Pellegrini VA, Schnatz PF, Rafique S, Kissell K, Calis KA, Popat V, Nelson LM. A clinical research integration special program (CRISP) for young women with primary ovarian insufficiency. *Panminerva Med* 2014; 56:245–261.
4. Joachim CM, Eads CM, Persani L, Yurttas Beim P, Nelson LM. An open letter to the primary ovarian insufficiency community. *Minerva Ginecol* 2014; 66:519–520.
5. Gordon CM, Kanaoka T, Nelson LM. Update on primary ovarian insufficiency in adolescents. *Curr Opin Pediatr* 2015; 26:511–519.

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Investigation of Adrenal Gland Disorders and Disorders of Female Reproduction

Over the past decade, we have made important contributions to the differential diagnosis of hypercortisolism. We established the corticotropin releasing hormone (CRH) test and inferior petrosal sinus sampling (IPS) as major diagnostic tools for the identification of pituitary adenomas causing Cushing's syndrome. However, the detection of Cushing's syndrome remains difficult, as does the localization of ectopic ACTH–producing tumors. We also evaluate endometrial function and the pathophysiology and potential new treatments for fibroids in women. The reproductive disorder is common, poorly understood, and lacks optimal medical treatments. In addition, we study the effect of gonadal steroids on mood disorders, particularly those surrounding parturition and menopause.

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Adrenal gland disorders

Identification of a pituitary corticotropinoma is essential for the cure of Cushing's disease. Inferior petrosal sinus sampling (IPSS) is considered the gold-standard test to distinguish Cushing's disease (CD) from ectopic ACTH syndrome (EAS). Anomalous venous drainage, abnormal venous anatomy, and lack of expertise can lead to false-negative IPSS results and thereby misclassification of patients with ACTH–dependent Cushing's syndrome. We participated in the development of guidelines for the treatment of Cushing's syndrome. The guidelines recommend surgical resection of the causative lesion. If that is not possible, secondary treatment options must be individualized to each person's unique characteristics.

We also examined the utility of measuring aldosterone during the Cortrosyn(™) stimulation test to distinguish primary from secondary adrenal insufficiency. A Cortrosyn(™)-stimulated aldosterone level of 5 ng/dl (0.14 nmol/l) had 88% sensitivity and positive predictive value and 89.7% specificity and negative predictive value for distinguishing primary adrenal insufficiency (PAI) from secondary adrenal insufficiency (SAI). Spot urine sodium levels showed a strong correlation with peak aldosterone levels ($r = -0.55$, $P = 0.02$, $n = 18$) in the SAI but not PAI or HV groups. Posture did not have a significant effect on results. Thus, measurement of aldosterone during this test can help identify the cause of adrenal insufficiency, but the influence of sodium intake must be taken into consideration.

Disorders of female reproduction

Perimenopause is accompanied by an increased risk of new and recurrent depression. The coincidence of declining ovarian function with the onset of depression led to the inference that 'withdrawal' from physiologic estradiol levels underpinned depression in perimenopause. To our knowledge, this is the first controlled systematic study to directly test the estrogen withdrawal theory of perimenopausal depression (PMD). We evaluated initial open-label treatment with estradiol followed by randomized, double-blind, placebo-controlled, parallel-design evaluation of continued estradiol treatment at an outpatient research facility at the National Institutes of Health Clinical Center. An intent-to-treat analysis was performed between October, 2003, and July, 2012. Participants included asymptomatic postmenopausal women with past PMD responsive to hormone therapy (n = 26) and asymptomatic postmenopausal women with no history of depression (n = 30), matched for age, body mass index, and reproductive status, who served as controls. After three weeks of open-label administration of transdermal estradiol (100 µg/d), participants were randomized to a parallel design to receive either estradiol (100 µg/d; 27 participants) or matched placebo skin patches (29 participants) for three additional weeks under double-blind conditions. We obtained Center for Epidemiologic Studies-Depression Scale and 17-item Hamilton Depression Rating Scale (completed by raters blind to diagnosis and randomization status), self-administered visual analog symptom ratings, and blood hormone levels at weekly clinic visits. None of the women reported depressive symptoms during open-label use of estradiol. Women with past PMD who were crossed over from estradiol to placebo experienced a significant increase in depression symptom severity demonstrated using the Center for Epidemiologic Studies-Depression Scale and 17-item Hamilton Depression Rating Scale, with mean [SD] scores increasing significantly from estradiol (i.e., 2.4 [2.0] and 3.0 [2.5]) to placebo (8.8 [4.9] and 6.6 [4.5], respectively). Women with past PMD who continued estradiol therapy and all women in the control group remained asymptomatic. Women in both groups had similar hot-flush severity and plasma estradiol levels during use of placebo. In women with past PMD that was previously responsive to hormone therapy, the recurrence of depressive symptoms during blinded hormone withdrawal suggests that normal changes in ovarian estradiol secretion can trigger an abnormal behavioral state in susceptible women. Women with a history of PMD should be alert to the risk of recurrent depression when discontinuing hormone therapy.

Changes in neurosteroid levels during the luteal phase of the menstrual cycle may precipitate affective symptoms. To test the hypothesis, we stabilized neurosteroid levels by administering the 5α-reductase inhibitor dutasteride to block conversion of progesterone to its neurosteroid metabolite allopregnanolone in women with premenstrual dysphoric disorder (PMDD) and in asymptomatic control women. Sixteen women with prospectively confirmed PMDD and 16 control women participated in one of two separate randomized, double-blind, placebo-controlled, cross-over trials, each lasting three menstrual cycles. After one menstrual cycle of single-blind placebo, participants were randomized to receive, for the next two menstrual cycles, either double-blind placebo or dutasteride (low-dose 0.5 mg/day in the first eight PMDD and eight control women or high-dose 2.5 mg/day in the second group of women). All women completed the daily rating form (DRF) and were evaluated in clinic during the follicular and luteal phases of each menstrual cycle. Main outcome measures were the DRF symptoms of irritability, sadness, and anxiety. In the low-dose group, no significant effect of dutasteride on PMDD symptoms was observed compared with placebo (i.e., symptom cyclicity maintained), and plasma allopregnanolone levels increased in women with PMDD from follicular to the luteal phases, suggesting the absence of effect of the low-dose dutasteride on 5α-reductase. In contrast, the high-dose group experienced a statistically significant reduction in several core PMDD symptoms (i.e., irritability, sadness, anxiety, food cravings, and bloating) on dutasteride compared with placebo. Dutasteride had no effect on mood in controls. Stabilization of allopregnanolone levels from the follicular to the luteal phase of the menstrual cycle by blocking the conversion of progesterone to its 5α-reduced neurosteroid metabolite mitigates symptoms in PMDD. The data provide preliminary support for the pathophysiologic relevance of neurosteroids in this condition.

ADDITIONAL FUNDING

» Some of these projects received external funding from two cooperative research and development agreements (CRADAs) executed with HRA-Pharma, Paris France, under ongoing CRADAs.

PUBLICATIONS

1. Martinez PE, Rubinow DR, Nieman LK, Koziol DE, Morrow AL, Schiller CE, Cintron D, Thompson KD, Khine KK, Schmidt PJ. 5α-Reductase Inhibition Prevents the Luteal Phase Increase in Plasma Allopregnanolone Levels and Mitigates Symptoms in Women with Premenstrual Dysphoric Disorder. *Neuropsychopharmacol* 2015; 41:1093–102.
2. Schmidt PJ, Ben Dor R, Martinez PE, Guerrieri GM, Harsh VL, Thompson K, Koziol DE, Nieman LK, Rubinow DR. Effects of estradiol withdrawal on mood in women with past perimenopausal depression: a randomized clinical trial. *JAMA Psychiatry* 2015; 72:714–726.
3. Nieman LK, Biller BM, Findling JW, Murad MH, Newell-Price J, Savage MO, Tabarin A. Treatment of Cushing's syndrome: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2015; 100:2807–2831.
4. Lacroix A, Feelders RA, Stratakis CA, Nieman LK. Cushing's syndrome. *Lancet* 2015; 386:913–927.
5. Abraham SB, Abel BS, Sinaii N, Saverino E, Wade M, Nieman LK. Primary vs secondary adrenal insufficiency: ACTH-stimulated aldosterone diagnostic cut-off values by tandem mass spectrometry. *Clin Endocrinol (Oxf)* 2015; 83:308–314.

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Diagnosis, Localization, Pathophysiology, and Molecular Biology of Pheochromocytoma and Paraganglioma

We conduct patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma and paraganglioma. Projects include both translational research—applying basic science knowledge to clinical diagnosis, pathophysiology, and treatment—and 'reverse translation research,' by which clinical findings lead to new concepts for pursuit by basic researchers in the laboratory. Our goals are to (1) establish new and improved methods and strategies for novel diagnostic and localization approaches to pheochromocytoma/paraganglioma; (2) explain the molecular and cellular basis for varying clinical presentations of pheochromocytomas/paragangliomas and establish the pathways of tumorigenesis; (3) search for new molecular and genetic markers for diagnosis and treatment of metastatic pheochromocytoma/paraganglioma; (4) introduce new therapeutic options for malignant/metastatic pheochromocytoma/paraganglioma; and (5) facilitate new and improved collaborations and interdisciplinary studies. To achieve these goals, we enter into multidisciplinary collaborations with investigators from several NIH Institutes and outside medical centers. We link a patient-oriented component with two bench-level components. The patient-oriented component (medical neuroendocrinology) is the driving force for our hypotheses and discoveries. The two bench-level components (tumor pathogenesis/genetics and chemistry; biomarkers) emphasize, first, technologies of basic research tailored for pathway and target discovery and, second, the further development of discoveries into clinical applications.

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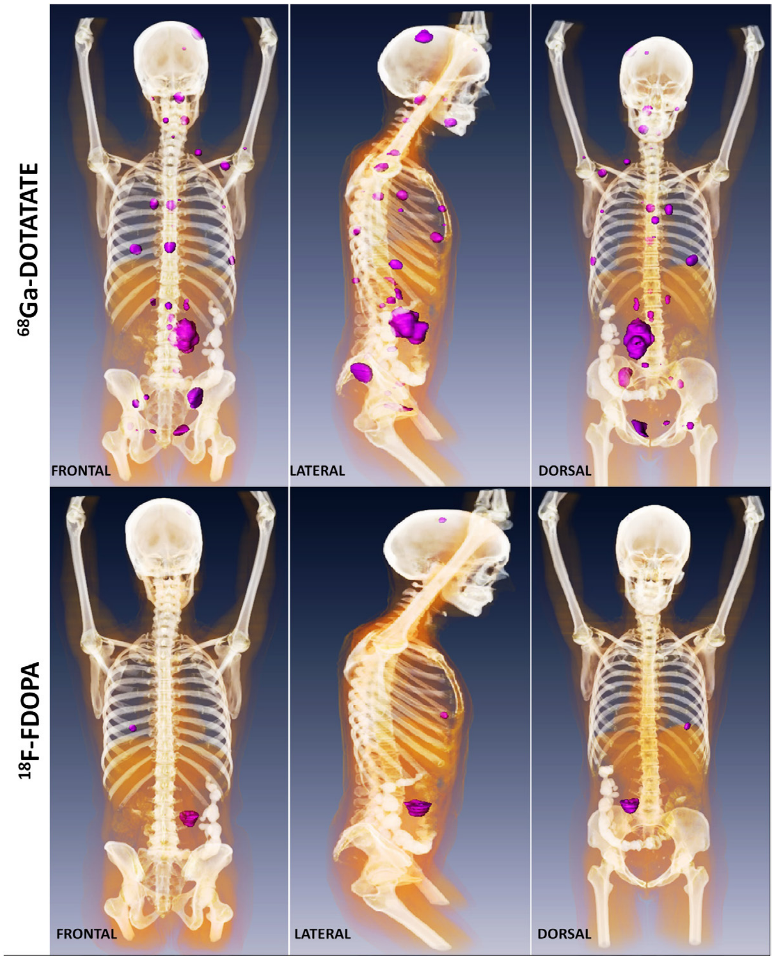
Clinical, biochemical, and metabolomic aspects of pheochromocytoma and paraganglioma

Testing for mutations in *SDHB*, the gene encoding a subunit of the mitochondrial enzyme succinate dehdrogenase (SDHx), is recommended in all patients with metastatic pheochromocytoma/paraganglioma (PHEO/PGL), but may not be required when metastatic disease is accompanied by adrenaline production. A retrospective cohort study aimed to establish the prevalence of *SDHB* mutations among patients with metastatic PHEO/PGL characterized by production of adrenaline compared with those without production of adrenaline, and to establish genotype-phenotype features of metastatic PHEOs/PGLs according to underlying gene mutations. 205 patients (114 males) age (range 9 to 86 years) at the diagnosis of metastatic PHEO/PGL, with and without adrenaline production, were tested for the presence of *SDHB* mutations or deletions. Twenty-three of the 205 patients (11%) had disease characterized by production of adrenaline, as defined by increased plasma concentrations of metanephrine greater than 5% of the combined increase of both normetanephrine and metanephrine. None of the 23 patients had *SDHB* mutations. Of the other 182 patients with no tumoral adrenaline production, 51% had *SDHB* mutations. Metastases in the bone were 36% to 41% more prevalent among patients with *SDHB* mutations or extra-adrenal primary tumors than those without mutations or with adrenal primary tumors. Liver metastases were 81% more prevalent among patients with adrenal than extra-adrenal primary tumors. *SDHB* mutation testing has no utility among patients with adrenaline-producing metastatic PHEOs/PGLs, but is indicated in other patients with metastatic disease. Our study also reveals novel associations of metastatic spread with primary tumor location and the presence of *SDHB* mutations.

Mutations of SDHx genes increase susceptibility to development of PHEOs/PGLs, with particularly high rates of malignancy associated with *SDHB* mutations. We assessed whether altered succinate dehydrogenase product-precursor relationships, manifested by differences in tumor ratios of succinate to fumarate or other metabolites, might aid in identifying and stratifying patients with SDHx mutations. PHEO/PGL tumor specimens from 233 patients, including 45 with SDHx mutations, were provided from eight tertiary referral centers for mass-spectrometric analyses of Krebs cycle metabolites. Diagnostic performance of the succinate:fumarate ratio was used for the identification of pathogenic SDHx mutations. SDH–deficient PHEOs/PGLs were characterized by 25-fold higher succinate and 80% lower fumarate, *cis*-aconitate, and isocitrate tissue levels than PHEOs/PGLs without SDHx mutations. Receiver-operating characteristic curves for the use of succinate to fumarate or to *cis*-aconitate and isocitrate ratios to identify SDHx mutations indicated areas under the curves of 0.94 to 0.96; an optimal cut-off of 97.7 for the succinate:fumarate ratio provided a diagnostic sensitivity of 93% at a specificity of 97% to identify SDHx–mutated PHEOs/PGLs. Succinate:fumarate ratios were higher in both *SDHB*–mutated and metastatic tumors than in those due to *SDHD/C* mutations or without metastases. Mass spectrometric-based measurements of ratios of succinate:fumarate and other metabolites in PHEOs/PGLs offer a useful method to identify patients for testing of SDHx mutations, with additional utility to quantitatively assess functionality of mutations and metabolic factors responsible for malignant risk.



Metastatic paraganglioma detected by ^{68}Ga-DOTATATE PET/CT and ^{18}F-FDOPA PET/CT

Detection of metastatic paraganglioma with the novel imaging modality ^{68}Ga-DOTATATE PET/CT compared with ^{18}F-FDOPA PET/CT (frontal, lateral, and dorsal views). Authors: Papadakis GZ, Bagci U, Millo CM, Janssen I, Patronas NJ, Stratakis CA, Pacak K

The present study investigated the impact of genetic alterations on metabolic networks in PGLs. Homogenates of 32 sporadic PGLs and 48 PGLs from patients with mutations in genes encoding SDHB, SDHD, SDHAF-2, von Hippel–Lindau tumor suppressor (VHL), RET, and NF-1 were subjected to proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy at 500 MHz for untargeted and HPLC tandem mass spectrometry for targeted metabolite profiling. ^{1}H-NMR spectroscopy identified 28 metabolites in PGLs, of which 12 showed genotype-specific differences. Part of these results were published earlier and reported significantly low complex II activity and significantly low ATP/ADP/AMP content in SDH–related PGLs compared with sporadics and PGLs of other genotypes. Extending these results, we observed significantly lower levels of *N*-acetylaspartic acid (NAA) in SDH tumors and creatine in VHL tumors than in sporadics and other genotypes. A positive correlation was observed between NAA and ATP/ADP/AMP content and NAA and complex II activity of PGLs. Targeted purine analysis in PHEOs/PGLs showed significantly lower adenine in cluster 1 than in cluster 2 tumors (see next section), while significantly lower levels of guanosine and hypoxanthine were observed in RET tumors than in SDH tumors. Principal component analysis (PCA) of metabolites could distinguish PGLs of different genotypes. The present study gives a comprehensive picture of alterations in energy metabolism in SDH– and VHL–related PGLs and establishes the inter-relationship between energy metabolism and amino acid and purine metabolism in PGLs.

One hundred and six patients with *SDHB* mutation–related PHEO/PGL were included in this retrospective study. We analyzed the recorded largest diameters, locations, and patient ages at initial diagnosis of *SDHB*–related primary tumors in the context of time-to-metastasis and patient survival. First, the development of metastatic disease in patients with primary tumors measuring 4.5 cm or greater was significantly earlier than in patients with smaller tumors. Second, patients with primary tumors larger than 5.5 cm also had worse overall survival rates than patients with smaller tumors. Third, age at initial diagnosis was found to be an independent predictor of patient survival. Fourth, we did not observe a significant difference in survival based on the specific *SDHB* mutations or patient sex. Receiver-operating-characteristic curves established 4.5 cm as the best value to dichotomize the primary SDHB–related PHEO/PGL in order to evaluate the development of metastatic disease and 5.5 cm as the best value for survival prediction. Subsequently, we found the size of the primary tumor to be an age-independent predictor of patient survival and metastases development in PGL. In both PHEO and PGL, age at diagnosis was found to be a size-independent predictor of patient survival. We found no significant difference in metastases development or patient survival between males and females or among specific *SDHB* mutations. The data further extend and support previous recommendations that carriers with *SDHB* mutations must undergo early and regular evaluations to detect PHEO/PGL in order to achieve the best clinical outcome.

Hereditary pheochromocytoma and paraganglioma

Many solid tumors, including PHEO and PGL, are characterized by a (pseudo)hypoxic signature. (Pseudo)hypoxia has been shown to promote both tumor progression and resistance to therapy. The major mediators of the transcriptional hypoxic response are hypoxia-inducible factors (HIFs) (Reference 1). High levels of HIFs lead to transcription of hypoxia-responsive genes, which are involved in tumorigenesis. PHEOs and PGLs are catecholamine-producing tumors arising from sympathetic- or parasympathetic-derived chromaffin tissue. In recent years, substantial progress has been made in understanding the metabolic disturbances present in PHEO and PGL, especially as a result of the identification of some disease-susceptibility genes. To date, nineteen PHEO and PGL susceptibility genes have been identified. Based on the main transcription signatures of the mutated genes, PHEOs and PGLs were divided into two clusters: pseudohypoxic cluster 1 and pseudohypoxic cluster 2 that is rich in kinase receptor signaling and protein translation pathways. Although the two clusters appear to show distinct signaling pathways, recent data suggest that both clusters are interconnected by HIF signaling as the important driver in their tumorigenesis, and mutations in most PHEO– and PGL–susceptibility genes seem to affect HIFα regulation and its downstream signaling pathways. HIF signaling appears to play an important role in the development and growth of PHEOs and PGLs, which could suggest new therapeutic approaches for the treatment of these tumors

Previously, no *HIF2A* mutations had been identified in any cancer. First, we reported two novel somatic gain-of-function *HIF2A* mutations in two patients, one presenting with multiple PGLs and a second with both multiple PGLs and multiple duodenal somatostatinoma, both associated with polycythemia (Reference 2). Both mutations showed increased HIF2alpha activity and protein half-life. While germline mutations of HIF2α regulators, including VHL, EGLN1, SDHB, SDHC, and SDHD, had been reported in PHEOs/PGLs, this was the first report of a somatic gain-of-function mutation in HIF2α. Subsequently, we investigated two additional unrelated patients and found them to present with the same disease cluster (Jochmanova et al., *J Natl Cancer Inst* 2013;105:1270-1283). Recently, we described new ocular findings in the patients, findings that indicate the existence of a new syndrome with multiple PGLs and somatostatinomas associated with polycythemia (Pacak-Zhuang syndrome). The new syndrome results from somatic gain-of-function *HIF2A* mutations, which cause an upregulation of hypoxia-related genes, including that encoding erythropoietin (*EPO*) and genes important in cancer biology.

We investigated the genetic/pathogenetic factors associated with a new clinical entity in patients presenting with PHEO/PGL and polycythemia. Two patients without hypoxia-inducible factor 2α (*HIF2A*) mutations, who presented with similar clinical manifestations, were analyzed for other gene mutations, including prolyl hydroxylase (*PHD*) mutations. We found, for the first time, a germ-line mutation in *PHD1* in one patient and a novel germ-line *PHD2* mutation in a second patient. Both mutants resulted in reduced protein stability with substantial quantitative protein loss and thus compromised catalytic activities. Given the unique association of patients' polycythemia with borderline or mildly elevated erythropoietin (EPO) levels, we also performed an *in vitro* sensitivity assay of erythroid progenitors to EPO and for EPO receptor (EPOR) expression. The results show inappropriate hypersensitivity of erythroid progenitors to EPO in these patients, indicating increased EPOR expression/activity. In addition, the study indicates that HIF dysregulation resulting from *PHD* mutations plays an important role in the pathogenesis of these tumors and associated polycythemia. The *PHD1* mutation appears to be a new member contributing to the genetic landscape of this novel clinical entity. Our results support the existence of a specific PHD1– and PHD2–

associated PHEO/PGL–polycythemia disorder.

Imaging of various pheochromocytomas and paragangliomas

The aim of this pilot study was to determine whether metabolic tumor volume (MTV) and total lesion glycolysis (TLG) could serve as predictors of biochemical remission and pharmacotherapy-free interval in patients with metastatic PHEO/PGL. Patients with metastatic PHEOs/PGLs have a high rate of biochemical recurrence, which can be associated with increased cardiovascular morbidity. Predictors of biochemical response are needed to guide and select patients who may benefit from therapy. We calculated whole body MTV and TLG from preoperative ^{18}F-FDG PET/CT scans and analyzed them as markers of biochemical response and pharmacotherapy-free interval. Seventeen patients underwent a total of 19 procedures, with a median follow-up time of 26.4 months. Whole body MTV of patients with biochemical recurrence (n = 13, mean 73.8 mL) was higher than those who had a biochemical response (n = 6, mean 14.7 mL). Patients with low MTV (less than 37.2 mL) had a significantly improved durable partial biochemical response, and a statistical trend for complete biochemical remission and pharmacotherapy-free interval. In eight patients with metastatic disease outside the abdomen, four had less than 35% of their disease burden outside the abdomen, and these patients had a significantly more durable partial biochemical response than did patients with greater than 35% of their disease burden outside the abdomen. Whole body MTV and TLG represents novel and valuable predictors of biochemical response for patients with metastatic PHEOs/PGLs. A larger prospective study should be performed to validate these findings.

SDHx–related PHEOs/PGLs are characterized by compromised oxidative phosphorylation and a pseudohypoxic response, which mediates an increase in aerobic glycolysis, also known as the Warburg effect. We explored the hypothesis that increased uptake of ^{18}F-FDG in SDHx-related PHEOs/PGLs is reflective of increased glycolytic activity and correlated with expression of different proteins involved in glucose uptake and metabolism through the glycolytic pathway. We investigated twenty-seven PHEOs/PGLs collected from patients with hereditary mutations in *SDHB* (n = 2), *SDHD* (n = 3), *RET* (n = 5), *NF-1* (neurofibromatosis type 1) (n = 1), and *MAX* (myc-associated factor X) (n = 1) as well as sporadic patients (n = 15). Preoperative ^{18}F-FDG PET/CT studies were analyzed; mean and maximum standardized uptake values (SUVs) in manually drawn regions of interest were calculated. We examined the expression of proteins involved in glucose uptake (glucose transporters types 1 and 3 [GLUT-1 and -3, respectively]), phosphorylation (hexokinases 1, 2, and 3 [HK-1, -2, and -3, respectively]), glycolysis (monocarboxylate transporter type 4 [MCT-4]), and angiogenesis (vascular endothelial growth factor [VEGF], CD34) in paraffin-embedded tumor tissues using immunohistochemical staining with peroxidase-catalyzed polymerization of diaminobenzidine as a read-out. The expression was correlated with corresponding SUVs. Both maximum and mean SUVs for SDHx–related tumors were significantly higher than those for sporadic and other hereditary tumors. The expression of HK-2 and HK-3 was significantly higher in SDHx–related PHEOs/PGLs than in sporadic PHEOs/PGLs. The expression of HK-2 and VEGF was significantly higher in SDHx–related PHEOs/PGLs than in other hereditary PHEOs/PGLs. No statistical differences in the expression were observed for GLUT-1, GLUT-3, and MCT-4. The percentage anti-CD34 staining and mean vessel perimeter were significantly higher in SDHx–related PHEOs/PGLs than in sporadic tumors. Mean SUVs significantly correlated with the expression of HK-2, HK-3, VEGF, and MCT-4. The activation of aerobic glycolysis in SDHx–related PHEOs/PGLs is associated with increased ^{18}F-FDG accumulation owing to accelerated glucose phosphorylation by hexokinases rather than increased expression of glucose transporters.

Therapeutic aspects of pheochromocytoma and paraganglioma

Multi-modality therapy is used in treating malignant PHEO/PGL; however, few data exist on the role of external beam radiation therapy (EBRT). In a retrospective review, we assessed response to EBRT in malignant PHEOs or PGLs. We studied records of patients treated at the NIH who received EBRT between 1990 and 2012. Patients were assessed for symptomatic control, biochemical response, local and distant control by response evaluation criteria in solid tumors or stable disease on imaging reports, toxicity by radiation therapy oncology group (RTOG) criteria, and survival. There were 47 patients treated who received EBRT to lesions either of the abdomen (n = 3), central nervous system (n = 4), or bone (n = 40). Lesions were treated with 3D conformal EBRT to a mean dose of 31.8 Gy in 3.3 Gy fractions, or fractionated stereotactic radiosurgery to 21.9 Gy in 13.6 Gy fractions. Patients experienced acute (n = 15) and late (n = 2) RTOG toxicities. Symptomatic control was achieved in 81.1% of lesions. Stable radiographic response was achieved in 86.7% of lesions with progression in 13%. Distant progression was observed overall in 75% of patients and average survival was 52.4 months. In conclusion, malignant PHEO/PGL often do not respond well to current systemic therapies. In these cases, EBRT can be considered in patients with symptomatic, localized disease progression.

To date, malignant PHEOs/PGLs cannot be effectively cured, and thus novel treatment strategies are urgently needed. Lovastatin has been shown to effectively induce apoptosis in mouse PHEO cells (MPC) and the more aggressive mouse tumor tissue–derived cells (MTT), which was accompanied by reduced phosphorylation of mitogen-activated kinase (MAPK) pathway players. The MAPK pathway plays a role in numerous aggressive tumors and has been associated with a subgroup of PHEOs/PGLs, including K-RAS-, RET-, and NF1-mutated tumors. Our aim was to establish whether MAPK signaling plays a role in aggressive, succinate dehydrogenase (SDH) B mutation–derived PHEOs/PGLs. Expression profiling and western blot analysis indicated that specific aspects of MAPK signaling are active in SDHB PHEOs/PGLs, suggesting that inhibition by statin treatment could be beneficial. Moreover, we aimed to assess whether the anti-proliferative effect of lovastatin on MPC and MTT differed from that exerted by fluvastatin, simvastatin, atorvastatin, pravastatin, or rosuvastatin. Simvastatin and fluvastatin reduced cell proliferation most effectively, and the more aggressive MTT cells appeared more sensitive in this respect. Inhibition of MAPK1 and/or MAPK3 phosphorylation following treatment with fluvastatin, simvastatin, or lovastatin was confirmed by western blot. Increased levels of CASP-3 and PARP cleavage confirmed induction of apoptosis following the treatment. At a concentration low enough not to affect cell proliferation, spontaneous migration of MPC and MTT was significantly inhibited within 24 hours of treatment. In conclusion, lipophilic statins may present a promising therapeutic option for treatment of aggressive human PGLs by inducing apoptosis and inhibiting tumor spread.

Currently, there are no reliably effective therapeutic options for metastatic PHEO/PGL. Moreover, there are no therapies that may prevent the onset or progression of tumors in patients with SDHB, which are associated with very aggressive tumors. Therefore, we tested the approved and well-tolerated drugs lovastatin and 13-*cis*-retinoic acid (13cRA) *in vitro* in an aggressive pheochromocytoma (PCC) mouse cell line, mouse tumor tissue-derived (MTT) cells, and *in vivo* in a PCC allograft nude mouse model, in therapeutically relevant doses. Treatment was started 24 hours before tumor cell injection and continued for 30 more days. We measured tumor sizes from the outside by caliper and sizes of viable tumor mass by bioluminescence imaging. Lovastatin showed antiproliferative effects *in vitro* and led to significantly smaller tumor sizes *in vivo* than with vehicle treatment. 13cRA promoted tumor cell growth *in vitro* and led to significantly larger viable tumor mass and significantly faster increase of viable tumor mass *in vivo* over time than with vehicle, lovastatin, or combination treatment. However, when combined with lovastatin, 13cRA enhanced the antiproliferative effect of lovastatin *in vivo*. Combination-treated mice showed the slowest tumor growth of all groups with significantly slower tumor growth than in vehicle-treated mice and significantly smaller tumor sizes. Moreover, the combination-treated group displayed the smallest size of viable tumor mass and the slowest increase in viable tumor mass over time of all groups, with a significant difference compared with the vehicle- and 13cRA-treated group. The combination-treated tumors showed the highest extent of necrosis, lowest median microvessel density, and highest expression of α-smooth muscle actin. The combination of high microvessel density and low α-smooth muscle actin is a predictor of poor prognosis in other tumor entities. Therefore, the drug combination may be a well-tolerated novel therapeutic or preventive option for malignant PHEO/PGL.

Animal model of pheochromocytoma and cell culture studies

The lack of sensitive animal models of PHEO has hindered the study of this tumor and *in vivo* evaluation of antitumor agents. Previously, we generated two sensitive luciferase models using bioluminescent PHEO cells: an experimental metastasis model to monitor tumor spreading; and a subcutaneous model to monitor tumor growth and spontaneous metastasis. The models offer a platform for sensitive, non-invasive, and real-time monitoring of PHEO primary growth and metastatic burden to follow the course of tumor progression and for testing relevant antitumor treatments in metastatic PHEO. Currently, we are testing several new drugs on this animal model.

PUBLICATIONS

1. Zhuang Z, Yang C, Lorenzo F, Merino M, Fojo T, Kebebew E, Popovic V, Stratakis CA, Prchal JT, Pacak K. Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia. *N Engl J Med* 2012; 367:922–930.
2. Pacak K, Jochmanova I, Prodanov T, Yang C, Merino MJ, Fojo T, Prchal JT, Tischler AS, Lechan RM, Zhuang Z. New syndrome of paraganglioma and somatostatinoma associated with polycythemia. *J Clin Oncol* 2013; 31:1690–1698.
3. Schovanek J, Martucci V, Wesley R, Fojo T, Del Rivero J, Huynh T, Adams K, Kebebew E, Frysak Z, Stratakis CA, Pacak K. The size of the primary tumor and age at initial diagnosis are independent predictors of the metastatic behavior and survival of patients with SDHB-related pheochromocytoma and paraganglioma: a retrospective cohort study. *BMC Cancer* 2014; 14:523.
4. Fliedner SM, Engel T, Lendvai NK, Shankavaram U, Nölting S, Wesley R, Elkahloun AG, Ungefroren H, Oldoerp A,

Lampert G, Lehnert H, Timmers H, Pacak K. Anti-cancer potential of MAPK pathway inhibition in paragangliomas-effect of different statins on mouse pheochromocytoma cells. *PloS One* 2014; 9:e97712.
5. Janssen I, Blanchet EM, Adams K, Chen CC, Millo CM, Herscovitch P, Taïeb D, Kebebew E, Lehnert H, Fojo AT, Pacak K. Superiority of [68Ga]-DOTATATE PET/CT to other functional imaging modalities in the localization of SDHB-associated metastatic pheochromocytoma and paraganglioma. *Clin Cancer Res* 2015; 21:3888–3895.

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The long-term goal of our laboratory is to develop regenerative medicine approaches for reproductive disorders. We conduct basic stem-cell, translational non-human primate, and clinical research on reproductive conditions. Our projects are designed to complement basic stem-cell research with our ongoing cutting-edge assisted-reproductive medicine research. Using this integrated approach, we aim to pioneer the next breakthroughs in reproductive medicine.

The role of T-regulatory cells in the endometrium

While investigating hematopoietic stem cell transplantation in non-human primate (NHP) models, we became interested in the role of T-regulatory cells (Treg) in stem-cell engraftment for both bone marrow transplants and endometrial engraftment. In parallel with ongoing Treg cell experiments in the lab, we began considering the involvement of Treg cells for both future reproductive tract stem-cell transplant therapies (such as embryonic stem cell–derived therapies) and embryonic engraftment in the uterus in pregnancy. However, little was known about the role of Treg cells in the endometrium. Maternal immune tolerance to fetal engraftment is critical for the establishment of pregnancy, but the mechanisms permitting such a semi-allograft are not completely understood. Given that Treg cells are known to promote tolerance to foreign antigens, we hypothesized that Treg cells could be one way the maternal immune system is able to permit implantation of the semi-allogeneic embryo. To study this, we utilized a conditional knockdown model of Treg cells, in which a transgenic mouse harbors a diphtheria toxin (DT) receptor–eGFP fusion protein under the control of the FoxP3 locus: the 'DEpletion of REGulatory T cells' (DEREG) mouse model. In women, we are studying Treg cells in healthy volunteers, as well as in women with infertility, to determine whether abnormalities of Treg cell numbers or function could explain forms of infertility such as recurrent implantation failure and recurrent miscarriage.

Ovary-derived stem cells transplant in a Rhesus macaque model

In adult non-human primates (NHPs), we are testing the ability of ovary-derived stem cells (OSCs) to undergo meiosis and give rise to oocytes. To this end, we isolate OSCs, culture them *in vitro*, label them, and transplant them into the ovaries of a recipient NHP to test for meiotic potential. We are studying this in both a healthy autologous transplant model as well as in an ovarian injury model of NHPs that have been previously irradiated. In humans, we are studying OSCs in healthy women as well as in women with ovarian failure and insufficiency, to determine whether OSCs are involved in the infertility associated with these disorders. However, significant controversy exists regarding the stem-cell markers used to isolate OSCs. A separate aim of this project is to better characterize OSC markers.

Female germline differentiation of induced pluripotent stem cells (iPSCs) from the Rhesus macaque

While male germ cell differentiation from embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) has been achieved for some time,

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female germline differentiation has typically been more difficult to accomplish. However, in 2012, viable oocyte differentiation from mouse iPSC was reported for the first time by Hayashi et al., and the oocytes could be fertilized normally and give rise to offspring. In ongoing work in our lab, we are utilizing an iPSC approach to generating oocytes in NHPs. The clinical significance of this approach is that it would allow women with premature ovarian insufficiency to have their own genetic offspring. As a first step toward human trials, we adapted the Hayashi mouse protocol to NHPs by testing several *in vitro* conditions. Our optimized protocol resulted in primordial germ cell–like cell (PGCLC) differentiation. Future work will focus on differentiating the iPSC–derived PGCLCs into gametes by inducing meiosis and formation of oocytes.

Fertility preservation for women undergoing gonadotoxic therapies

For women undergoing gonadotoxic therapies, future fertility is often one of the greatest concerns. At the NIH, investigators have made remarkable strides in treated benign and malignant conditions such as sickle cell disease and cancer. Unfortunately, while these breakthroughs cure young women of their disease, patients are often rendered infertile. Historically, the most effective form of fertility preservation was to freeze fertilized embryos prior to gonadotoxic treatment, using routine assisted reproductive technologies such as IVF. However, this is prohibited at the NIH because embryos would be created under research protocols. Historically, only fertilized embryos could be frozen and thawed successfully, but oocytes (the largest cell in the human body) were too sensitive to the cytoplasmic ice crystal formation that occurs with traditional freezing methods. Newer methods of oocyte freezing (i.e., vitrification) were developed that dramatically improved the success of oocyte freezing. The approaches are now considered to have similar success rates as routine IVF. Given these advances, we are now able to offer fertility preservation to patients at the NIH by freezing oocytes. Cryo-preserved oocytes are given to patients, and they are stored at private storage facilities until the patients have completed treatments and are ready to start a family. We are studying patients with rare conditions (e.g., sickle cell disease) undergoing the fertility preservation cycles to develop the safest and most effective IVF protocols suited to their underlying disease.

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PUBLICATIONS

1. Hernandez SF, Vahidi NA, Park S, Weitzel RP, Tisdale J, Rueda BR, Wolff EF. Characterization of extracellular DDX4- or Ddx4-positive ovarian cells. *Nat Med* 2015; 21(10):1114–1116.
2. Koh C, Canini L, Dahari H, Zhao X, Uprichard SL, Haynes-Williams V, Winters MA, Subramanya G, Cooper S, Pinto P, Wolff EF, Bishop R, Thanda Han MA, Cotler SJ, Kleiner DE, Idilman R, Keskin O, Yurdaydin C, Glenn JS, Heller T. Treatment of chronic delta hepatitis with Lonafarnib. *Lancet Infect Dis* 2015; 15(10):1167–1174.
3. Wolff EF, Mutlu L, Elsworth JD, Redmond E, Taylor HS. Endometrial stem cell transplantation in MPTP-exposed primates: an alternative cell source for treatment of Parkinson's disease. *J Cell Mol Med* 2015; 19(1):249–256.
4. Wolff EF, Uchida N, Donahue RE, Metzger ME, Libfraind LL, Hill MJ, Tisdale JT. Peripheral blood stem cell transplants do not result in endometrial stromal engraftment. *Fertil Steril* 2013; 99:526–532.
5. Hill MJ, Cooper JC, Levy G, Alford C, Richter KS, DeCherney AH, Katz C, Levens ED, Wolff EF. Ovarian reserve and subsequent ART outcomes following methotrexate therapy for ectopic pregnancy and pregnancy of unknown location. *Fertil Steril* 2014; 101(2):413–419.

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Child and Family Development Across the First Decades of Life

The Child and Family Research Section (CFRS) was established with the broad aim of investigating the ways in which human development is affected by variations in the conditions under which humans are reared. We investigate dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in humans across the first three decades of life. Our research goals are to describe, analyze, and assess (1) the capabilities and proclivities of developing children and youth, including their physiological functioning, perceptual and cognitive abilities, emotional and social growth, and interactional styles; (2) the nature and consequences of interactions within the family and the social world for offspring and parents; (3) the consequences for development of exposure to areas of childhood vulnerability (to illness, to accidents, in risk taking); and (4) influences on development of children's exposure to and interactions with natural and designed environments. Research topics concern the origins, status, and development of psychological constructs, structures, functions, and processes across the first three decades of life; effects of child characteristics, activities, and vulnerabilities on parents; and the meaning of variations in parenting and in the family across different socio-demographic and cultural groups as well as across variations in health conditions in the child. Laboratory and home-based studies employ a variety of approaches, including psycho-physiological recordings, behavioral observations, standardized assessments, rating scales, interviews, and demographic/census records in both longitudinal and cross-sectional designs. Socio-demographic comparisons under investigation include, for example, family socio-economic status, maternal age and employment status, parenthood status (adoption, birth), child parity, and daycare experience. Our research program also investigates the developmental sequelae of cancer in infancy; children's understanding of and coping with medical experiences; parental depression and child development; development following preterm birth; the deaf culture; and behavior problems in adolescence. In addition to the United States, cultural study sites include Argentina, Belgium, Brazil, Cameroon, Chile, England, France, Israel, Italy, Japan, Kenya, Peru, and the Republic of South Korea; in all places, we pursue intra-cultural as well as cross-cultural comparisons.

To meet this multifaceted charge, we pursue two integrated multi-age, multi-variate, multi-cultural research programs that are supplemented with a variety of ancillary investigations. The research programs represent an en bloc effort. The first program is a prospective longitudinal study designed to explore multiple aspects of child development in the context of major socio-demographic comparisons. The second program broadens the perspectives of the first to encompass cultural influences on development within the same basic longitudinal framework. Our ultimate aims are to promote aware, fit, and motivated children who will grow into knowledgeable, healthy, and happy adults.

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The child, the parent, and the family across the first two+ decades

Two independent prospective longitudinal studies that cumulatively spanned the age interval from 4 years to 14 years used multi-wave designs to investigate developmental associations between language and behavioral adjustment (internalizing and externalizing behavior problems). The primary questions addressed by our developmental analyses were: What are the adolescent language outcomes for young children with different levels of behavioral adjustment? What are the adolescent behavioral adjustment outcomes for young children with different levels of language skills? Two secondary questions were presumptive to these primary questions: Are language and internalizing and externalizing behavioral adjustment stable individual-difference constructs from childhood to adolescence? What are the interconnections between language and these two forms of behavioral adjustment at different time points from childhood to adolescence? Altogether, 224 children, their mothers, and teachers provided data. We used series of nested path analysis models to determine the most parsimonious and plausible paths among the three constructs over and above stability in each across age and their covariation at each age. In both studies, children with poorer language skills in early childhood had more internalizing behavior problems in later childhood and in early adolescence. These developmental paths between language and behavioral adjustment held after taking into consideration children's nonverbal intellectual functioning, maternal verbal intelligence, education, parenting knowledge, and social desirability bias, as well as family socioeconomic status, and they applied equally to girls and boys. A unique longitudinal relation obtains when one intrapersonal characteristic influences another over time apart from temporal stability in each and their concurrent covariation. Such developmental paths are conservative and robust and imply that one characteristic shapes another in more than a transient way. In two independent prospective longitudinal multi-method, multi-informant converging analyses, we reported such pathways between language and internalizing and externalizing behavior problems from early childhood to early adolescence.

Among a community sample of families (N = 128), the study examined how family members' shared and unique perspectives of family dysfunction relate to dyad members' shared views of dyad adjustment within adolescent-mother, adolescent-father, and mother-father dyads. Independent of a family's family perspective (shared perspective of family dysfunction), the adolescent's unique perspective was associated with lower security and higher conflict with both mother and father, the father's unique perspective was associated with lower security and higher conflict with the adolescent as well as lower marital quality with mother, and the mother's unique perspective was associated with lower marital quality with the father. Moreover, for adolescent-parent dyads, compared with the parent's unique perspective, the adolescent's unique perspective was more strongly associated with dyad adjustment. The findings indicate that both shared and unique views of the family system—the adolescent's unique view in particular—independently relate to the health of family subsystems. They also suggest that research as well as therapeutic interventions that focus on only the shared view of the family may miss important elements of family dysfunction.

Child development and parenting in multicultural perspective

Using nationally representative samples of 45,964 2- to 9-year-old children and their primary caregivers in 17 developing countries, we sought to understand relations between children's cognitive, language, sensory, and motor disabilities and caregivers' use of discipline and violence. Primary caregivers reported on their child's disabilities and whether they or anyone in their household had used nonviolent discipline, psychological aggression, or physical violence toward the target child and whether they believed that using corporal punishment is necessary. Logistic regression analyses supported the hypothesis that children with disabilities are treated more harshly than children without disabilities. The findings suggest that policies and interventions are needed to work toward the United Nations' goals of ensuring that children with disabilities are protected from abuse and violence.

The vast majority of infants are born in poor countries, but most of our knowledge about infants and children has emerged from high-income countries. In 2003, Tomlinson and Swartz conducted a survey of articles on infancy between 1996 and 2001 from major international journals and reported that a meager 5% of articles emanated from parts of the world other than North America, Europe, or Australasia. We conducted a similar review of articles on infancy published between 2002 and 2012 to assess whether the status of cross-national research has changed in the subsequent decade. Results indicate that, despite slight improvements in research output from the rest of world, only 2.3% of articles published in those 11 years included data from low- and middle-income countries—where 90% of the world's infants live. The discrepancies indicate that progress is still needed to bridge the 10/90 gap in infant mental health research. Cross-national collaboration is urgently required to ensure expansion of research production in low-resource settings.

The Convention on the Rights of the Child has prompted countries to protect children from abuse and exploitation. Exposure to domestic violence and corporal punishment are risk factors in children's development. We investigated how women's attitudes about domestic violence are related to attitudes about corporal punishment, how their attitudes are related to harsh behaviors toward children, and whether country-wide norms regarding domestic violence and corporal punishment are related to psychological aggression and physical violence toward children. Data were drawn from the Multiple Indicator Cluster Survey, a nationally representative and internationally comparable household survey developed by UNICEF. Measures of domestic violence and discipline were completed by 85,999 female caregivers of children between the ages of 2 and 14 years from families in 25 low- and middle-income countries. Mothers who believed that husbands were justified in hitting their wives were more likely to believe that corporal punishment is necessary to rear children. Mothers who believed that husbands were justified in hitting their wives and that corporal punishment is necessary to rear children were more likely to report that their child had experienced psychological aggression and physical violence. Country-wide norms regarding the acceptability of husbands hitting wives and the advisability of corporal punishment moderated the links between mothers' attitudes and their behaviors toward children. Pediatricians can address parents' psychological aggression and physical violence toward children by discussing parents' attitudes and behaviors within a framework that incorporates social norms regarding the acceptability of domestic violence and corporal punishment.

PUBLICATIONS

1. Bornstein MH, Putnick DL, Cote LR, Haynes OMH, Suwalsky JTD. Mother-infant contingent vocalizations in 11 countries. *Psychol Sci* 2015; 26:1272–1284.
2. Bornstein MH, Putnick DL, Gartstein MA, Hahn C-S, Auestad N, O'Connor DL. Infant temperament: stability of by age, gender, birth order, term status, and socioeconomic status. *Child Dev* 2015; 86:844–863.
3. Bornstein MH, Putnick DL, Lansford JE, Deater-Deckard K, Bradley RH. A developmental analysis of caregiving modalities across infancy in 38 low- and middle-income countries. *Child Dev* 2015; 86:1571–1587.
4. Jager J, Yuen CX, Putnick DL, Hendricks C, Bornstein MH. Adolescent-peer relationships, separation and detachment from parents, and internalizing and externalizing behaviors: linkages and interactions. *J Early Adolesc* 2015; 35:511–537.
5. Karasik LB, Tamis-LeMonda CS, Adolph KE, Bornstein MH. Places and postures: a cross-cultural comparison of sitting in 5-month-olds. *J Cross Cult Psychol* 2015; 46:1023–1038.

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Neuregulin–ErbB Signaling in Neuronal Development and Psychiatric Disorders

Failure of cortical microcircuits to properly regulate excitatory\inhibitory (E\I) balance is a key feature in the etiology of several developmental psychiatric disorders and neurological diseases, such as schizophrenia, autism, ADHD, and epilepsy. E\I balance is important for the synchronization of the firing pattern of local neuron ensembles, and its dysregulation can degrade cognitive functions and, in extreme cases, result in epileptiform activity. Network activity, in particular oscillatory activity in the gamma-frequency range (30–80 Hz), is altered in psychiatric disorders and may account for their cognitive and behavioral symptoms. We are interested in how Neuregulin (NRG) and its receptor ErbB4, which are both genetically linked to psychiatric disorders, function in an activity-dependent fashion (i.e., experience) in the developing brain to regulate synaptic plasticity, neuronal network activity, and, in rodents, behaviors that model features of psychiatric disorders. We identified a functional interaction between NRG/ErbB4, GABAergic, and dopamine signaling in GABAergic interneurons that is critical for understanding how NRG can regulate E\I balance and synchronous activity in neuronal networks, processes that are important for cognitive functions altered in psychiatric disorders.

Our earlier studies demonstrated that, in the hippocampus and neocortex, expression of ErbB4, the major NRG neuronal receptor, is restricted to GABAergic interneurons—particularly the parvalbumin-positive (Pv^+) fast-spiking interneurons that are critical for modulating gamma oscillation induction and strength (i.e., power). Using genetically targeted mouse mutant models, we went on to show that NRG–ErbB4 signaling regulates synaptic plasticity, neuronal network activity, and behaviors associated with psychiatric disorders. More recently, our group has been investigating other aspects of NRG expression throughout the brain, its processing in response to neuronal activity, and its function in distinct neuronal populations of the developing and maturing nervous system. To achieve these goals, we are using a combination of techniques, including electrophysiological recordings in acute brain slices prepared from normal and genetically altered mice, multi-electrode field recordings from brains of freely moving rats, reverse-microdialysis neurochemistry, confocal fluorescence microscopy in fixed and live tissue, proteomics analyses, and behavioral testing. The ultimate goal of this multi-disciplinary approach is to generate holistic models to investigate the developmental impact of genes that modulate E\I balance and neuronal network activity and that consequently affect behaviors and cognitive functions altered in psychiatric disorders.

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Unprocessed NRG2 accumulates at endoplasmic reticulum–plasma membrane (ER-PM) junctions, where it is protected from alpha-secretase processing.

The cellular and molecular processes that promote the conversion of NRG ligands from inactive pro-forms to signaling-competent ligands that can engage ErbB4 receptors to mediate their aforementioned biological effects in the developing and maturing brain remain mostly unknown. To address this major unresolved question, we investigated the role of Neuregulin 2

(NRG2), a Neuregulin isotype that is prominently expressed in the developing postnatal and adult CNS. Using a novel double-labeling *in situ* hybridization technique (RNAScope) and newly generated monoclonal antibodies, we found that, in the rodent hippocampus, NRG2 mRNA and protein are highly expressed in ErbB4–positive GABAergic interneurons, suggesting that NRG2 can engage in autocrine ErbB4 signaling (Figure 1). Interestingly, we found no evidence of NRG2 protein in axons but instead found that unprocessed proNRG2 accumulates at large somato-dendritic puncta on the plasma membrane. Immunogold electron-microscopy of NRG2 in dissociated hippocampal neurons, performed in collaboration with Susan Cheng, revealed that the puncta are found atop of subsurface cisterns (SSCs)—sites of close endoplasmic reticulum–plasma membrane (ER–PM) apposition (Figure 1). Based on these observations, we hypothesized that pro-NRG2 clustering at SSCs serves to protect the protein from constitutive processing by extracellular sheddases.

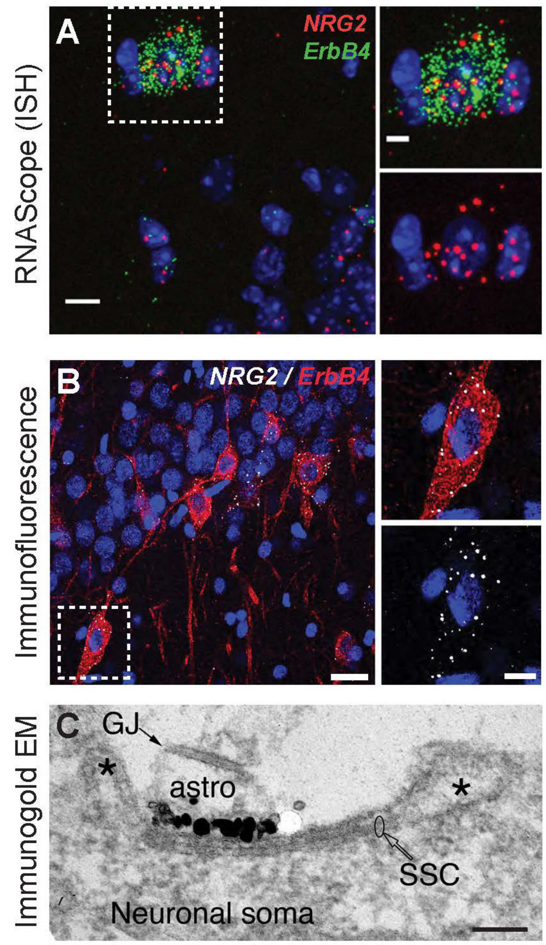


Figure 1. Neuregulin-2 is expressed in ErbB4-positive GABAergic interneurons and accumulates at subsurface cisterns.
A. Double fluorescence ISH of NRG2/Gad67 and NRG2/ErbB4 in the mouse *stratum oriens* of CA1. B. NRG2 immunoreactivity in ErbB4^{+} interneurons in rat CA1 *strata pyramidale* and *radiatum*. C. Silver-enhanced immunogold EM of a DIV 35 hippocampal neuron with a patch of concentrated label for NRG2 at the plasma membrane atop intracellular membrane stacks characteristic of SSCs.

NMDA receptor function on cortical interneurons promotes proNRG2 processing and, in turn, NRG2 signaling via ErbB4 downregulates NMDAR function on interneurons.

Previous studies reported that clustering of potassium channel Kv2.1 at SSCs is regulated by neuronal activity, in particular via activation of the glutamate receptor NMDAR (*N*-methyl-D-aspartate receptor). Interestingly, using co-immunofluorescence experiments, we found that proNRG2 puncta reside inside the doughnut-shaped Kv2.1 clusters. Treatment of neuronal cultures with glutamate or NMDA rapidly causes the dispersal of both proteins from the SSC and the processing of proNRG2 by extracellular alpha-secretases to release signaling-competent NRG2.

What could be the function of released NRG2 from GABAergic interneurons, which are known to express the ErbB4 receptor? In collaboration with Sanford Markey's group, we used ErbB4 immunoprecipitation from the soluble fraction of metabolically active synaptosomes followed by LC/MS/MS (liquid chromatography coupled with tandem mass spectrometry) to characterize the ErbB4 proteome. Using this approach, we identified the NMDAR GluN2B subunit as an ErbB4–interacting protein following NRG treatment of synaptosomes. The interaction was confirmed in cultured hippocampal neurons, where NRG2 treatment was shown to enhance the internalization of GluN2B–containing, but not GluN2A–containing, NMDARs. Consistent with this observation, we found that NRG2 also caused a dramatic reduction of whole-cell NMDAR currents in dissociated hippocampal ErbB4–positive interneurons, but not in ErbB4–negative glutamatergic neurons. Using whole-cell voltage-clamp recordings in acute medial prefrontal cortical slices, we found that NRG2 selectively reduced NMDAR synaptic currents (EPSCs), and not AMPAR EPSCs, at glutamatergic synapses onto GABAergic interneurons (Figure 2). The results are consistent with the idea that the bidirectlional signaling between NRG2/ErbB4 and NMDAR activity can play a major role in modulating the activity of GABAergic neurons and cortical E/I balance.

Neuregulin-2 ablation results in dopamine dysregulation and severe behavioral phenotypes relevant to psychiatric disorders.

While the neurophysiological and behavioral phenotypes of *Nrg1*–mutant mice have been investigated extensively, little is

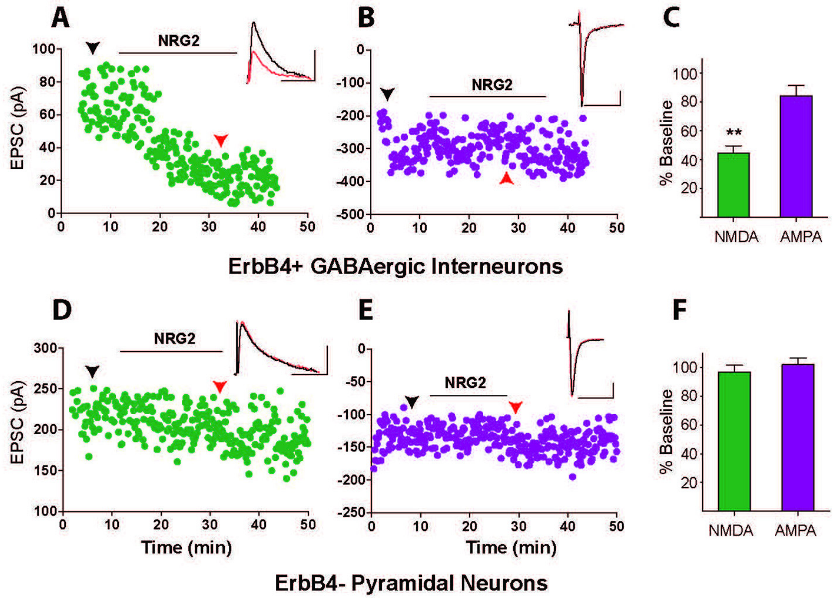


Figure 2. Neuregulin-2 selectively reduces NMDAR currents, but not AMPAR currents, in GABAergic interneurons.
A and B. Representative scatter plots of NMDAR– and AMPAR–mediated evoked EPSCs recorded from an ErbB4–positive GABAergic interneuron and (D and E) recorded from a pyramidal neuron. C and F. Summary graphs of NRG2 effects on NMDA and AMPA eEPSCs. Data normalized to baseline.

known about the function of NRG2 (see above), the closest NRG1 homolog. We found that NRG2 expression in the adult rodent brain does not fully overlap with NRG1 and is more extensive than originally reported, including expression in the striatum and medial prefrontal cortex (mPFC). We therefore generated *Nrg2* knockout (KO) mice to study the homolog's function. *Nrg2* KO mice have higher extracellular dopamine levels in the dorsal striatum but lower levels in the mPFC, similar to schizophrenia subjects. *Nrg2* KOs also performed abnormally in a battery of behavioral tasks relevant to psychiatric disorders. The mutant mice exhibit hyperactivity in the open field, hypersensitivity to amphetamine, deficits in prepulse inhibition, reduced anxiety-like behavior in the elevated plus-maze, and antisocial behavior. Acute administration of clozapine (1mg/kg) increased the concentration of mPFC dopamine and improved performance in the T-maze alteration reward task (Figure 3). We also demonstrated that NMDA receptor synaptic currents in *Nrg2* KOs are augmented at hippocampal glutamatergic synapses and more sensitive to ifenprodil, indicating an increased contribution of GluN2B-containing NMDARs. Our findings reveal a novel role for NRG2 in the modulation of behaviors relevant to the core symptoms of schizophrenia (Yan et al., *Mol Psychiatry* 2015;in review).

Neuregulin 3 (NRG3) is a dual-pass transmembrane protein targeted to the axons in an activity-dependent process.

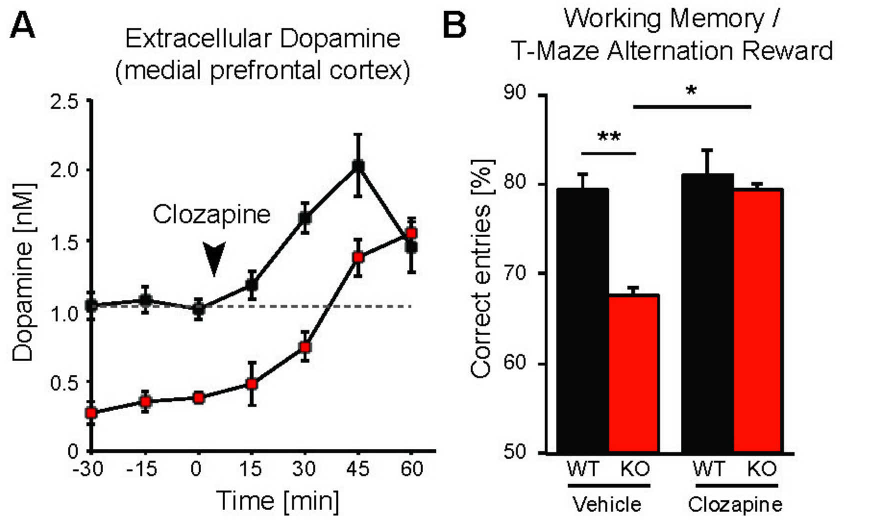
NRGs are encoded by different genes in the brain (*NRG1-3*), which are transcribed from different promoters and alternatively spliced to generate a large variety of isoforms. In contrast to NRG1, little is known about the cellular expression and functions of NRG2 and NRG3 in the brain. Our study, funded by an NICHD Director's Award, aimed to determine the cellular and subcellular distribution pattern of NRG3 and its function in neurons. Using a novel triple-fluorescence *in situ* hybridization technique (RNAScope), we found that NRG3 transcripts are widely expressed in the adult mouse brain and relatively more abundant in V-Glut1–positive excitatory cells than in GAD1–positive inhibitory interneurons. Contrary to prior studies reporting NRG3 as a single-pass type I membrane protein, our analysis revealed that NRG3 is a dual-pass transmembrane-domain (TMD) protein. Interestingly, the secondary structure and axonal distribution of NRG3 is extremely similar to the CRD (cysteine-rich domain, type III) variant of NRG1 (CRD-NRG1) but differs significantly from other NRG1 variants (type I and II) and NRG2 that accumulate at somatodendritic compartments (Ahmed et al., manuscript in preparation).

Mesocortical and nigrostriatal DA function in mice with targeted mutations of ErbB4 in parvalbumin-positive (Pv$^+$) and tyrosine hydroxylase–positive (TH$^+$) neurons

Dysfunctional NRG–ErbB4 signaling in the hippocampus, pre-frontal cortex (PFC), and striatum may contribute to alterations in dopamine (DA) function associated with several schizophrenia symptoms. Because we had shown that NRG1 acutely increases extracellular DA levels to regulate LTP and gamma oscillations, and that ErbB4 expression is confined to GABAergic interneurons (cortex) and TH$^+$ mesocortical DA, we are investigating the relative role NRG/ErbB4 signaling in

Figure 3. Administration of antipsychotic clozapine restores dopamine levels in pre-frontal cortex of Neuregulin-2 knockout mice and improves performance on a cognitive task.

A. Extracellular dopamine levels in the mPFC of *Nrg2* knockout (KO) mice and wild-type (WT) littermates before and after clozapine injection (*arrowhead*). B. Poorer performance by *Nrg2* KO mice in a T-maze reward alternation task than by WT littermates (*left*) can be restored by clozapine treatment (*right*).



the two different neuronal populations. To this end, we are measuring how NRG signaling affects extracellular DA levels in the PFC, hippocampus, and striatum in mice harboring targeted mutations of the receptor in either Pv^+ or TH^+ neurons. We also are comparing their behaviors to begin unraveling the role of Neregulin-ErbB4 signaling in Pv^+ interneurons vs. TH^+ terminals in regulating schizophrenia-associated behaviors (Skirzewski et al., manuscript in preparation). In collaboration with Alon Shamir and Idit Golani, we analyzed the effects of developmentally disrupting the NRG-ErbB signaling pathway on dopamine balance and behaviors. Alteration of ErbB signaling during adolescence were found to affect the dopaminergic system and to coincide in the adult with deficits in learning and hedonic behaviors. The results suggest a possible role of the NRG–ErbB pathway in the development of cognitive skills.

Functional analysis of the extra-synaptic ErbB4 receptor proteome using proteomics approach; identifying an interaction with GABA-A receptors

Most studies on NRG–ErbB4 signaling focused on the receptor that accumulates at glutamatergic post-synaptic densities (PSDs). However, little research has been performed on the pool of extra-synaptic ErbB4 receptors that may constitute up to 80% of the total receptor population in cortical and hippocampal interneurons. In collaboration with Sanford Markey's group, our laboratory used ErbB4 immunoprecipitation from the soluble fraction of metabolically active synaptosomes followed by LC/MS/MS to characterize the ErbB4 proteome. Using this approach, we identified the GABA-A receptor α1 subunit (GABAR α1) as an ErbB4–interacting protein. Consistent with this observation, we found that GABAR α1 receptors are highly expressed in ErbB4–positive fast-spiking Pv^+ in the hippocampus and that *Erbb4*–knockout mice have reduced GABAR α1 levels. Remarkably, we found that this novel ErbB4 signaling pathway, which suppresses postsynaptic GABAR currents on GABAergic interneurons, acts independently of ErbB4s canonical receptor tyrosine kinase (RTK) activity. While the effects of NRG on GABAR α1 internalization do not require RTK activity, the ErbB4 protein is necessary for clathrin-mediated endocytosis and reduction of GABA receptor IPSCs (inhibitory postsynaptic currents).

Dopamine regulation of prefrontal cortical gamma oscillations in freely moving rats

Mounting evidence suggests that gamma oscillations are atypically high at baseline in disorders that affect attention such as schizophrenia and ADHD. A lower evoked gamma oscillatory power in these subjects than in healthy controls may constitute an endophenotype associated with risk for developing the psychiatric disorders. Based on these observations, we hypothesized that drugs targeting either the D4 or ErbB4 receptors may have therapeutic potential in treating cognitive deficits in patients with schizophrenia. To examine abnormal oscillatory patterns and connect them with the firing patterns of single neurons, we, in collaboration with Judith Walters, are using multi-electrode recordings from the medial prefrontal cortex and dorsomedial thalamus of rats acutely treated with ketamine to analyze the effects of D4– and ErbB4–targeting drugs on gamma oscillations in this mouse model with “face validity” for schizophrenia (Figure 4).

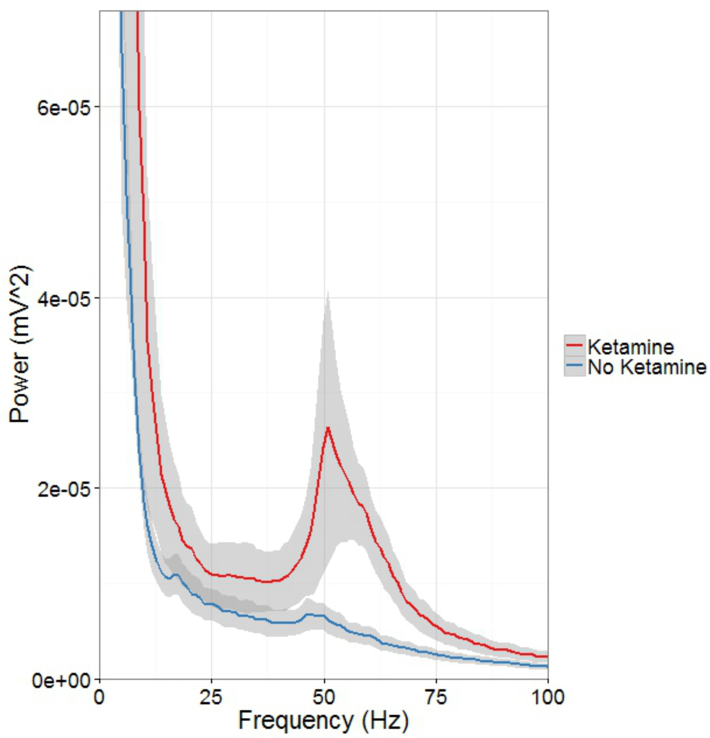


Figure 4. Ketamine raises gamma (40-65Hz) frequency power in the medial prefrontal cortex (mPFC) during controlled movement.
Multi-electrode recordings in the mPFC revealed an over three-fold increase in gamma (40–65Hz) frequency power while rats walked counter-clockwise at 9–10 revolutions per minute on a circular treadmill. Blue traces come from an epoch 30 minutes before ketamine administration, and the red trace comes from epochs 15 minutes after ketamine administration.

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» NICHD DIR Director's Investigator Awards (RRC# D-14-07)
» Bench-to-Bedside Award

PUBLICATIONS

1. Vullhorst D, Mitchell RM, Keating C, Roychowdhury S, Karavanova I, Tao-Cheng JH, Buonanno A. A negative feedback loop controls NMDA receptor function in cortical interneurons via neuregulin 2/ErbB4 signalling. *Nat Commun* 2015;6:7222.
2. Golani I, Tadmor H, Buonanno A, Kremer I, Shamir A. Disruption of the ErbB signaling in adolescence increases striatal dopamine levels and affects learning and hedonic-like behavior in the adult mouse. *Eur Neuropsychopharmacol* 2014;24:1808–1018.
3. Furth KE, Mastwal S, Wang KH, Buonanno A, Vullhorst D. Dopamine, cognitive function, and gamma oscillations: role of D4 receptors. *Front Cell Neurosci* 2013;7:102 (1–19).
4. Penzes P, Buonanno A, Passafaro M, Sala C, Sweet RA. Developmental vulnerability of synapses and circuits associated with neuropsychiatric disorders. *J Neurochem* 2013;126:165–182.
5. Mitchell RM, Janssen MJ, Karavanova I, Vullhorst D, Furth K, Makuskey A, Markey S, Buonanno A. ErbB4 receptor reduces synaptic GABAA currents independently of its tyrosine kinase activity. *Proc Natl Acad Sci USA* 2013;110:19603–19608.

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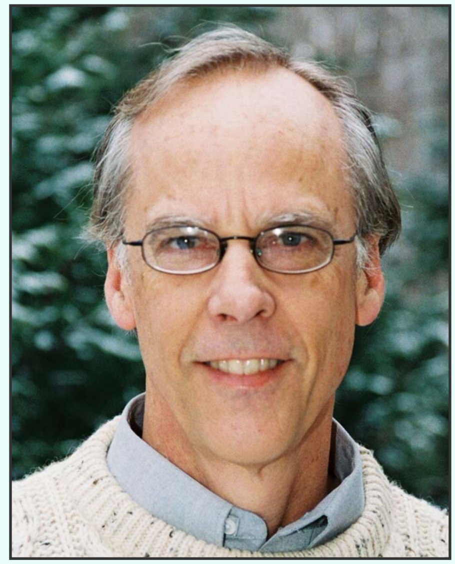
Healthy brain and cognitive development in children is central to the mission of NICHD. Unlike the brains of most animals, the human brain continues to develop postnatally, through adolescence and into early adulthood. The prolonged postnatal period of brain development allows environmental experiences to influence brain structure and function, rather than having brain function specified entirely by genes. Activity-dependent plasticity also compensates for developmental defects and brain injury. Our research is concerned with understanding the molecular and cellular mechanisms by which functional activity in the brain regulates development of the nervous system during late stages of fetal development and early postnatal life. We are especially interested in investigating novel mechanisms of activity-dependent nervous system plasticity that are particularly relevant to the period of childhood and those that operate beyond the synapse and beyond the neuron doctrine. Our work has three main areas of emphasis: myelination and neuron-glia interactions; cellular mechanisms of learning; and gene regulation by neuronal firing.

Traditionally, the field of activity-dependent nervous system development has focused on synapses, and we continue to explore synaptic plasticity; however, our research is also advancing understanding of how non-neuronal brain cells (glia) sense neural impulse activity and how activity-dependent regulation of glia contributes to development, plasticity, and the cellular mechanisms of learning. A major emphasis of our current research is to understand how myelin (white matter in the brain) is regulated by functional activity. By changing conduction velocity, activity-dependent myelination may be a non-synaptic form of plasticity regulating nervous system function by optimizing the speed and synchrony of information transmission through neural networks. Our studies identified several cellular and molecular mechanisms for activity-dependent myelination, and the findings have important implications for normal brain development, learning and cognition, and psychiatric disorders. Our research showing that myelination of axons by glia (oligodendrocytes and Schwann cells) is regulated by impulse activity provides evidence for a new form of nervous system plasticity and learning that would be particularly important in child development, given that myelination proceeds throughout childhood and adolescence. The mechanisms we identified suggest that environmental experience may alter myelin formation in an activity-dependent manner, thereby improving function based on experience.

Learning is perhaps the most important function of childhood. Our research is delineating the molecular mechanisms that convert short-term memory into long-term memory. While we continue our long-standing research on synaptic plasticity, our laboratory is actively exploring new mechanisms of nervous system plasticity during learning that extend beyond the neuron doctrine, such as neurons firing antidromically and the release of neurotransmitters along axons. We are investigating how gene expression necessary for long-term memory is controlled and how intrinsic activity in the brain (oscillations and neuronal firing) forms memories. Our recent research showed that neurons in the hippocampus fire backwards (antidromically) during sharp-wave ripple complexes, which are most frequent during slow-wave sleep, and that the firing



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reduces the strength of all synapses on that neuron (AP-LTD).

Information in the nervous system is encoded in the temporal pattern of action potential firing. If functional experiences produce lasting effects on brain development and plasticity, specific genes must be regulated by specific patterns of impulse firing. We verified the hypothesis and are determining how different patterns of neural impulses regulate specific genes controlling development and plasticity of the nervous system and how impulse activity affects neurons and glia.

Regulation of myelination by neural impulse activity

Myelin, the multilayered membrane of insulation wrapped around nerve fibers (axons) by glial cells (oligodendrocytes), is essential for nervous system function, increasing conduction velocity at least 50-fold. Myelination is an essential part of brain development. The processes controlling myelination of appropriate axons are not well understood. Myelination begins in late fetal life and continues throughout childhood and adolescence, but myelination of some brain regions is not complete until an individual's early twenties. Our research shows that neurotransmitters that are released along axons firing action potentials activate receptors on myelinating glia (Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system) as well as astrocytes and other cells, which in turn release growth factors or cytokines that regulate development of myelinating glia.

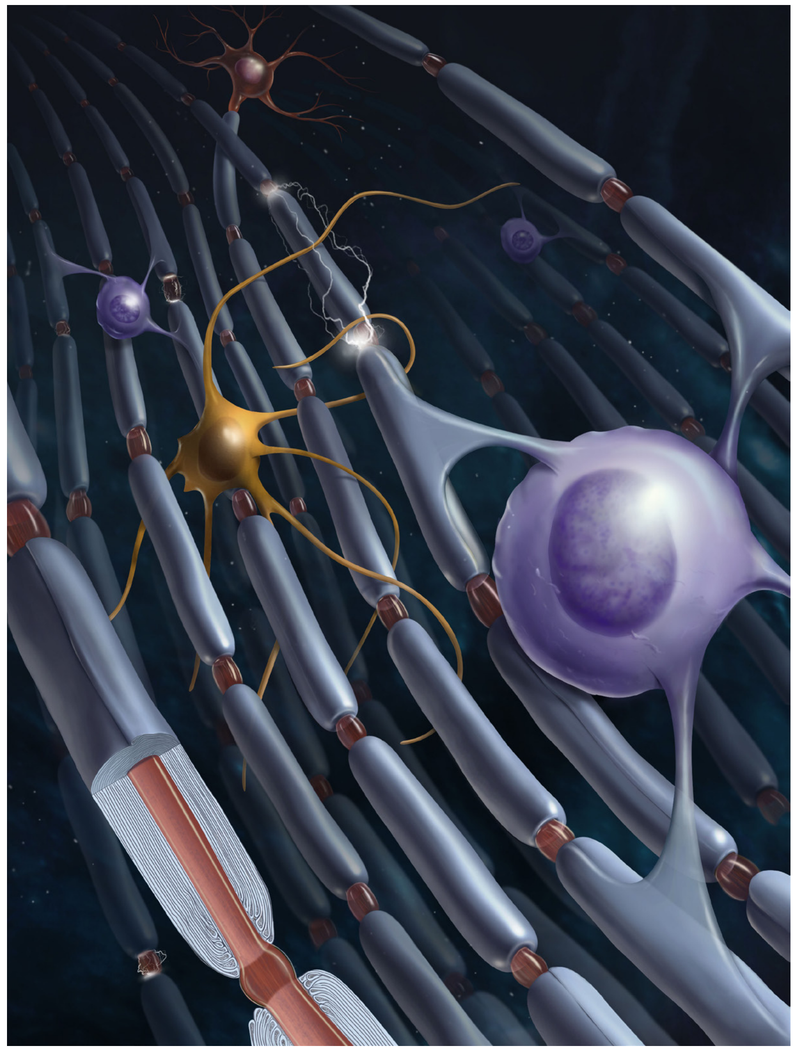

Figure 1. Myelin

Induction of myelination by action potentials

In addition to establishing the effects of impulse activity on proliferation and development of myelinating glia, we determined that release of the neurotransmitter glutamate from vesicles along axons promotes the initial events in myelin induction, including stimulating the formation of cholesterol-rich signaling domains between oligodendrocytes and axons and increasing the local synthesis of myelin basic protein, the major protein in the myelin sheath, through Fyn kinase–dependent signaling. Axon-oligodendrocyte signaling would thus promote myelination of electrically active axons to regulate neural development and neural function according to environmental experience. The findings are also relevant to such demyelinating disorders as multiple sclerosis and to remyelination after axon injury.

Synaptic and nonsynaptic transmission in myelination

The surprising discovery of synapses formed on glial progenitors—oligodendrocyte progenitor cells (OPCs, also called NG2 cells)—has remained enigmatic for over a decade. The cells mature to form myelin insulation on axons, and a leading hypothesis is that these synapses may stimulate myelination selectively on electrically active axons, which would have significant effects on neural function, given that myelin increases the speed of impulse transmission. Using an *in vitro* system, we found that oligodendrocytes preferentially myelinate electrically active axons but that synapses from axons onto myelin-forming oligodendroglial cells are not required. Instead, vesicular release at non-synaptic axo-glial junctions induces myelination. Axon release of neurotransmitter from vesicles that accumulate in axon varicosities induces a local rise in cytoplasmic calcium in glial cell processes at such nonsynaptic functional junctions, and this signaling stimulates local translation of myelin basic protein to initiate myelination. Preferential myelin induction on electrically active axons would have profound effects on circuit function by elevating conduction velocity and would thus provide another mechanism of plasticity complementing synaptic plasticity. These new findings may also have implications for disease, including psychiatric illness and impaired remyelination after conduction block in multiple sclerosis and other demyelinating disorders.

We also found that other signaling molecules released from axons, notably ATP, stimulate differentiation of oligodendrocytes and increase myelination. In collaboration with colleagues in Italy, we found that GPR-17, a newly discovered membrane receptor on oligodendrocyte progenitor cells, regulates oligodendrocyte differentiation. The release of neuronal messengers outside synapses has broad biological implications, particularly with regard to communication between axons and glia. We identified a mechanism for nonsynaptic, nonvesicular release of ATP from axons through volume-activated anion channels (VAACs), which are activated by microscopic axon swelling during action potential firing. The studies combine imaging of single photons to measure ATP release in a luciferin/luciferase assay with imaging of intrinsic optical signals, intracellular calcium, time-lapse video, and confocal microscopy. Microscopic axon swelling accompanying electrical depolarization of axons activates the VAACs to release ATP. Such nonvesicular, nonsynaptic communication may mediate various activity-dependent interactions between axons and nervous system cells under normal conditions, during development, and in disease.

Myelin in activity-dependent plasticity

Although the significance of myelin has been traditionally viewed in terms of conduction failure and spike-time arrival in determining synaptic function and plasticity, we are exploring how myelination affects the frequency, phase, and amplitude coupling of oscillations in the brain, as well as the propagation of brain waves. Abnormalities in brain waves and synchrony are associated with many psychiatric and developmental conditions, including, among others, schizophrenia, epilepsy, dyslexia, and autism.

Gulf War Illness

After decades of research there is still no understanding of how a large group of Gulf War veterans became chronically ill with Gulf War Illness. It is believed that exposure to low levels of sarin nerve gas and combinations of organophosphate insecticides, which impair synaptic function, may be responsible. Our recent discovery that glutamatergic transmission between axons and oligodendrocytes triggers myelination led us to propose that impairments in myelination due to disrupted neurotransmission from axons to oligodendrocytes may be an underlying cause of Gulf War Illness. Funded in part by a grant from the Department of Defense, we are investigating this hypothesis as part of an international consortium of researchers studying Gulf War Illness, headed by Kimberly Sullivan.

Myelin damage in children exposed to pesticides

Children are uniquely vulnerable to uptake of pesticides, which act chemically through the same mechanism as nerve gas. Exposure, through ingestion and inhalation, is higher than for adults because of their greater intake of food and fluids per pound of body weight. In 2003, FDA monitoring determined that 49% of fruit, 29% of vegetables, and 26% of grain products produced in the United States have pesticide residue. The concentrations of pesticides in imported fruits and vegetables are higher, and 5–7% of imported foods with pesticide residue have levels of contamination above the legal tolerance limit for consumption. Fetal development is compromised by parental and maternal exposure to organophosphate pesticides. The neurodevelopmental effects are well documented; they include developmental delay, lower IQ, and ADHD. Our research is thus exploring the possible involvement of myelin damage in the adverse developmental effects of pesticides on children.

Synaptic plasticity

To maintain the general level of neural impulse activity within normal limits, homeostatic mechanisms are required to control the formation and maintenance of synaptic connections during development and learning. How genes controlling these processes are coordinately regulated during homeostatic synaptic plasticity is unknown. Micro RNAs (miRNAs) exert regulatory control over mRNA stability and protein translation and may contribute to local activity-dependent post-transcriptional control of synapse-associated mRNAs. Using a bioinformatics screen to search for sequence motifs enriched in the 3′ UTR of mRNAs that are rapidly destabilized after increasing impulse activity in hippocampal neurons, we identified a developmentally and activity-regulated miRNA (miR-485) and showed that it controls dendritic spine number and synapse formation in an activity-dependent, homeostatic manner. Many plasticity-associated genes contain predicted miR-485–binding sites, including ones for the presynaptic protein SV2A. We found that miR-485 reduces SV2A abundance and negatively regulates dendritic spine density, clustering of the post-synaptic density protein (PSD-95), and surface expression of the glutamate receptor GluR2. Overexpression of miR-485 reduces spontaneous synaptic responses and transmitter release, as measured by miniature excitatory postsynaptic current analysis and staining with the membrane dye FM 1-43. The findings demonstrate that miRNAs participate in homeostatic synaptic plasticity, with possible implications for neurological disorders such as Huntington's and Alzheimer's disease, in which miR-485 has been found to be dysregulated.

Regulation of gene expression by action-potential firing patterns

To determine how gene expression in neurons and glia is regulated by impulse firing, we stimulate nerve cells to fire impulses in differing patterns by delivering electrical stimulation through platinum electrodes in specially designed cell culture dishes. After stimulation, we measured mRNA and protein expression by gene arrays, quantitative RT-PCR (reverse transcriptase–polymerase chain reaction), Western blot, and immunocytochemistry. The results confirm our hypothesis that precise patterns of impulse activity can increase or reduce expression of specific genes (in neurons and glia). The experiments are revealing signaling and gene-regulatory networks that respond selectively to appropriate temporal patterns of action potential firing. Temporal aspects of intracellular calcium signaling are particularly important in regulating gene expression according to neural impulse firing patterns in normal and pathological conditions. Our findings thus provide a deeper understanding of how nervous system development and plasticity are regulated by information coded in the temporal pattern of impulse firing in the brain. The findings are also relevant to chronic pain as well as to the regulation of nervous system development and myelination by functional activity.

In addition to investigating how electrical activity in neurons regulates gene transcription and translation into proteins in neurons and glia, we are exploring epigenetic mechanisms that modulate mRNA stability during synaptic activity and regulate networks of genes important for neural plasticity and development. Through a systematic microarray analysis together with bioinformatics techniques, we identified mRNA transcripts that are rapidly destabilized by synaptic activity in hippocampal neurons, and then identified sequence motifs in the 3′ UTRs of these transcripts, motifs enriched selectively in transcripts that are destabilized rapidly by synaptic activity. The sequences corresponded to several microRNA–binding sites. The miRNAs identified include miR-326-3p/miR-330-5p, miR-485-5p, miR666-3p, and miR-761 and are predicted to regulate networks of genes important for plasticity and nervous system development. We showed that miR-485, for example, controls neurite outgrowth, tau (a protein that stabilizes microtubules) expression, and axon development. This epigenetic mechanism contributes to the process of refining synaptic connections during development and synaptic connections associated with learning and memory.

In collaboration with David Clark, we are investigating chromatin structure and remodeling in neurons and glia. This research is supported in part by a Director's Investigator Award jointly to Drs. Clark and Fields.

Hippocampal synaptic plasticity

In contrast to sensory-evoked stimulation, intrinsic activity in the brain often operates in non-traditional modes. We demonstrated how non-traditional modes of neuronal firing in the hippocampus during high-frequency oscillations affect synaptic plasticity in the process of memory consolidation during slow-wave sleep. During slow-wave sleep and periods of quiet wakefulness, CA1 neurons in the hippocampus fire backwards (antidromically) during brief high-frequency oscillations called sharp-wave ripple complexes. The action potential is initiated in the distal axon and propagates back into the cell body and dendrites. Our studies show that such antidromic firing reduces the strength of all synaptic inputs to the neuron (action potential–induced long-term depression [AP–LTD]) and that the synapses then become sensitized to strengthening by subsequent sensory input. The process of globally reducing synaptic strength participates in the formation of transiently stable functional assemblies of neurons and is necessary for incorporating new information together with existing memories to form a schema, or coherent memory, combining multiple sensations and temporal sequence into a cognitive framework. A newly recognized form of synaptic plasticity, AP–LTD may contribute to memory consolidation by sharpening the specificity of subsequent synaptic input and promoting the incorporation of novel information.

It is widely appreciated that there are two types of memory: short-term and long-term. It has been known for decades that gene expression is necessary to convert short-term into long-term memory, but it is not known how signals reach the nucleus to initiate this process or which genes make memories permanent. Long-term potentiation (LTP) and long-term depression (LTD) are two widely studied forms of synaptic plasticity that can be recorded electrophysiologically in the hippocampus and are believed to represent a cellular basis for memory. We use cDNA microarrays to investigate the signaling pathways, genes, and proteins involved in LTP and LTD. The work is contributing to a better understanding of how regulatory networks are controlled by the appropriate patterns of impulses, leading to different forms of synaptic plasticity, and is identifying new molecular mechanisms regulating synaptic strength.

ADDITIONAL FUNDING

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» Director's Award

PUBLICATIONS

1. Wake H, Ortiz FC, Woo DH, Lee PR, Angulo MC, Fields RD. Nonsynaptic junctions on myelinating glia promote preferential myelination of electrically active axons. *Nat Commun* 2015; 6:7844.
2. Fields RD, Woo DH, Basser PJ. Glial regulation of the neuronal connectome through local and long-distant communication. *Neuron* 2015; 86:374–386.
3. Pajevic S, Basser P, Fields RD. Role of myelin plasticity in oscillations and synchrony of neuronal activity. *Neuroscience* 2014; 276:135–147.
4. Cohen JE, Lee PR, Fields RD. Systematic identification of 3'-UTR regulatory elements in activity-dependent mRNA stability in hippocampal neurons. *Philos Trans R Soc Lond B Biol Sci* 2014; 369:1652.
5. Fields RD, Araque A, Johansen-Berg H, Lim S-S, Lynch G, Nave K-A, Nedergaard M, Perez R, Sejnowski T, Wake H. Glial biology in learning and cognition. *Neuroscientist* 2014; 20:426–431.

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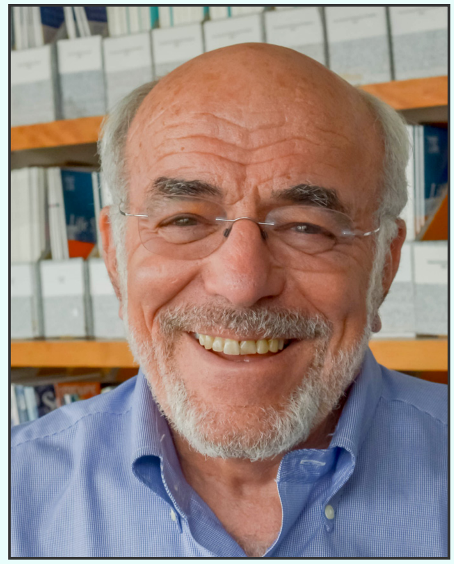
Pineal Gland, Chronobiology, Neurotranscriptomics and Neuroepigenetics

Among the most important advances made by the Section are discoveries that resulted in the recognition of the suprachiasmatic nucleus as the site of the master oscillator controlling circadian biology in vertebrates; and the identifcation of the enzyme that serves to control melatonin production, the 'timezyme' arylalkylamine N-acetyltransferase (AANAT). The work has broad implications for vertebrate biology and is of special interest to clinical scientists studying human diseases related to circadian rhythms, including endocrine pathologies, sleep and mood disorders, and deficiencies in alertness. Other areas studied include the characterization of the transcriptome of the vertebrate pineal gland and the regulation of daily and developmental changes in the transcriptome of the pineal gland and of other tissues.



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Cong Fu, PhD, *Guest Researcher*

The 'timezyme' arylalkylamine N-acetyltransferase

The penultimate enzyme in the melatonin synthesis pathway is AANAT (Figure 1). We found that the enzyme is critical to the control of the rhythm in melatonin synthesis. In all species examined to date, the large increase in melatonin synthesis at night is a highly conserved feature of vertebrate physiology.

The transcriptional mechanisms that control expression of the *Aanat* gene in rodents and the chicken include interactions of cyclic AMP–response elements in the promoter region; other response elements in the promoter, including the E-box and photoreceptor conserved elements, appear to determine the marked tissue-specific pattern of expression of the gene, which is limited to the pineal gland and retina. We determined that, in rodents, expression of *Aanat* rises about 100-fold at night; however, this feature of regulation is not highly conserved. For example, it was discovered that there is little or no nocturnal increase in *Aanat* expression in ungulates or nonhuman primates, in contrast to rodents, birds, and fish. Thus, transcriptional control mechanisms are not important for the regulation of melatonin synthesis in all vertebrates.

Whereas transcriptional regulation is not highly conserved, post-translational control is and is mediated by cyclic AMP and involves phosphorylation of AANAT at C- and N-terminal phosphorylation sites by cyclic AMP–dependent protein kinase. This leads to formation of a reversible complex with 14-3-3 protein, in which the enzyme is stabilized and activated, as indicated by structural, *in vivo*, and *in vitro* studies. When cyclic AMP levels fall, AANAT is dephosphorylated and destroyed by the proteasome, causing enzyme activity and melatonin production to fall in parallel, with a halving time of about 3 to 4 minutes.

The form of AANAT found in the pineal gland evolved very rapidly approximately 500 million years ago from an enzyme dedicated to detoxification; this ancestor lacked the regulatory and catalytic features that are characteristic of the regulation and production of melatonin in the pineal gland. The evolutionary change—termed neo-functionalization—coincided with the appearance of the pineal gland and lateral eyes. The findings support the hypothesis that lateral eyes and the pineal gland evolved from a common primitive photodetector, which was facilitated by a primitive form of AANAT, which served to enhance photodetection by removing toxic arylalkylamines. The elevated activity at night is likely to have enhanced the ability to detect low levels of light. Subsequently, one of the downstream products of acetylation, melatonin, became a unique and valuable signal of night time—a time-keeping signal—which promoted the independent evolution of eyes, dedicated to image detection, and the pineal gland, dedicated to melatonin production and time keeping.

Neuroepigenetics: global control of daily changes in pineal gene expression are regulated by an adrenergic/cyclic AMP mechanism.

Transcriptome profiling

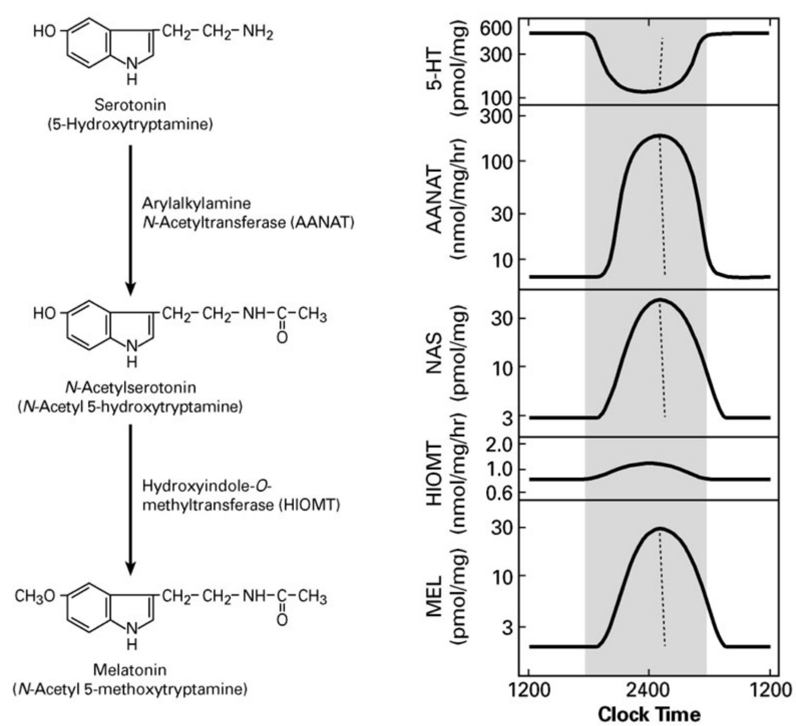
Several projects aimed to obtain a global picture of differences in gene expression that occur on a night/day basis and to identify genes that are highly enriched in the pineal gland. In collaborative work, we characterized pineal gene expression in the zebrafish, rat, mouse, chicken, and rhesus monkey as a function of time of day and of development. We used transcriptome profiling to analyze the pineal gland, by employing the most advanced profiling methods, which now include RNA sequencing, an effort that resulted in the identification of a set of genes that are highly expressed in the pineal gland. Some of the genes are expressed at high levels during the day and night; in other cases there are large changes in expression. The changes are seen in thousands of genes; of special interest are those with 10 to 100-fold changes in transcript abundance. Studies in the rat indicate that these are under neural control (Figures 2 and 3). This line of investigation has led to a rapid increase in knowledge of the biochemical profile, conserved across species, of the pineal gland. It is also pointing to new transcriptional pathways controlled by previously unrecognized transcription factors. Analysis of the transcription factors and the promoters of genes that are upregulated at night or highly expressed in the pineal gland will lead to the construction of a regulatory network that describes the cascade of transcription factors underlying the control of pineal gene expression. Our results permit a full-scale biochemical and physiological analysis of the control of genes in the pineal gland. The RNA sequencing work on the pineal transcriptome that clearly revealed a broad effect of neural/cyclic AMP regulation of the pineal gland (Figure 3) led to the creation of a new title for this line of investigation: neurotranscriptomics.

Long noncoding RNAs

RNA sequencing identified a set of highly tissue-specific and rhythmically expressed non-coding RNAs. The transcripts range in size from approximately 200 bp to over 10,000 bp and vary in their genomic location from genetic deserts to being transcribed from introns of known genes. In at least one case, a long noncoding RNA overlaps a known gene and, in another, the long noncoding RNA is transcribed from the strand of DNA that is opposite to that encoding a known gene. The

Figure 1. Daily rhythm in indole metabolism in the pineal gland

The daily rhythm in circulating melatonin production reflects the increased production in the pineal gland, as depicted here. During the day, melatonin production is low owing to low levels of AANAT. At night, AANAT activity rises, resulting in an increase in *N*-acetylserotonin. The increase in *N*-acetylserotonin raises melatonin production by a mass action effect; the level of the last enzyme in melatonin synthesis is constant. Changes in melatonin synthesis cause parallel changes in melatonin release. Circulating melatonin is rapidly destroyed in the liver, which allows changes in production and release to be immediately reflected in changes in circulating melatonin.



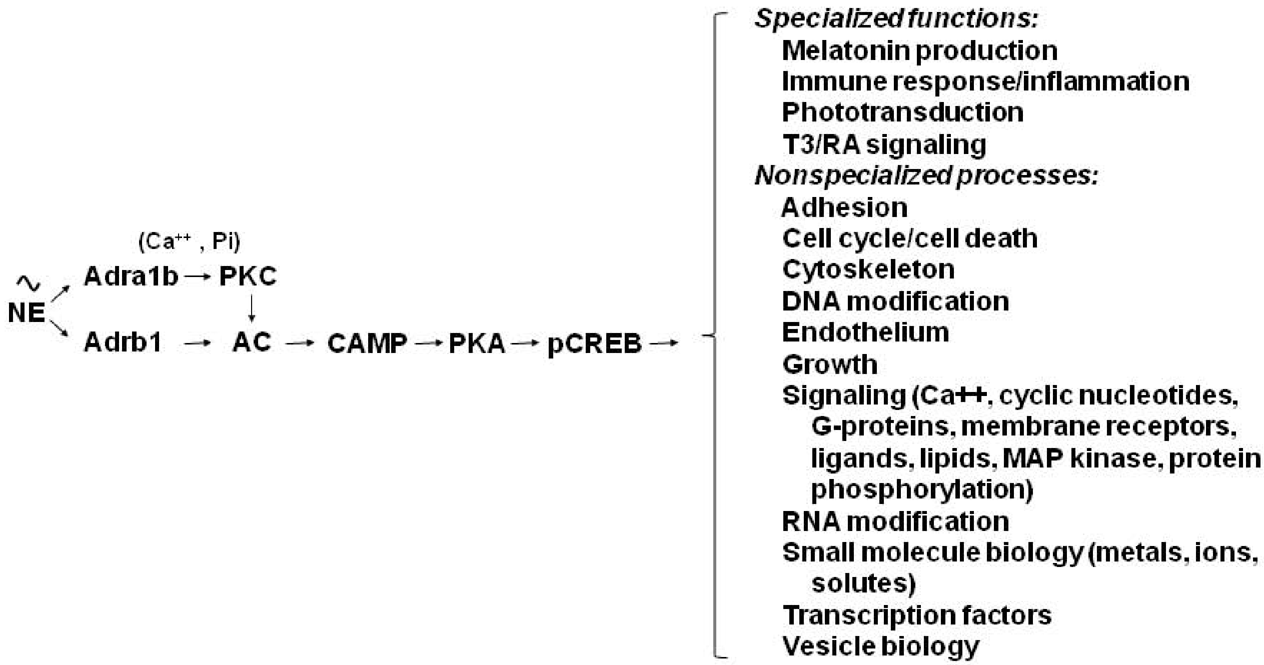


Figure 2. Neural regulation of gene expression in the rat pineal gland

Sympathetic nerves release norepinephrine (NE), which binds to beta1-adrenergic receptors (Adrb1) and to alpha$_{1b}$-adrenergic receptors (Adra1b). NE acts through the Adrb1 receptor to partially activate adenylate cyclase (AC) and through Adra1b to potentiate activation of the Adrb1 receptor. The interaction involves Adra1b–dependent activation of Ca^{2+} and phosphatidylinositol (Pi) activation of protein kinase C (PKC), which enhances Adrb1 stimulation of AC. The resulting increase in cyclic AMP (CAMP) leads to activation of protein kinase A (PKA), which phosphorylates cyclic AMP response element–binding protein (CREB). The consequence of this is a global shift in gene expression. The transcription factor profile unique to this tissue establishes which genes are regulated by CREB phosphorylation.

expression of these interesting molecules is regulated by the same neural system that controls coding transcripts. The transcripts may function in biological regulation through interaction with DNA or proteins.

MicroRNAs (miRNAs)

miRNAs play a broad range of roles in biological regulation. We profiled rat pineal miRNAs for the first time and evaluated their importance by focusing on melatonin synthesis, the main function of the pineal gland. Massively parallel sequencing and related methods revealed that the miRNA population is dominated by a small group of miRNAs as follows: about 75% is accounted for by 15 miRNAs; miR-182 represents 28%. In addition to miR-182, miR-183 and miR-96 are also highly enriched in the pineal gland, a distinctive pattern that is also found in the retina. The work also identified previously unrecognized miRNAs and other small noncoding RNAs. Pineal miRNAs do not exhibit a marked night/day differences in abundance with few exceptions (e.g., two-fold night/day differences in the abundance of miR-96 and miR-182), in sharp contrast to the dynamic 24-hour pattern that characterizes the pineal transcriptome. During development, the abundance of miRNAs most enriched in the pineal gland increases; however, there is a marked decline in at least one, miR-483. Based on the following observations, miR-483 is a likely regulator of melatonin synthesis: it inhibits the melatonin synthesis of pinealocytes in culture; it acts via predicted binding sites in the 3′ UTR of *Aanat* mRNA; and it exhibits the reverse developmental profile to that of *Aanat* transcripts. Additionally, a miR-483–targeted antagonist increased melatonin synthesis in neonatal pinealocytes. The observations support the hypothesis that miR-483 suppresses *Aanat* mRNA levels during development and that the developmental decline in miR-483 abundance promotes melatonin synthesis.

New element in biological timing

Studies of the zebrafish pineal gland identified a new element in chronobiology, the rhythmic gene *camk1gb* (encoding

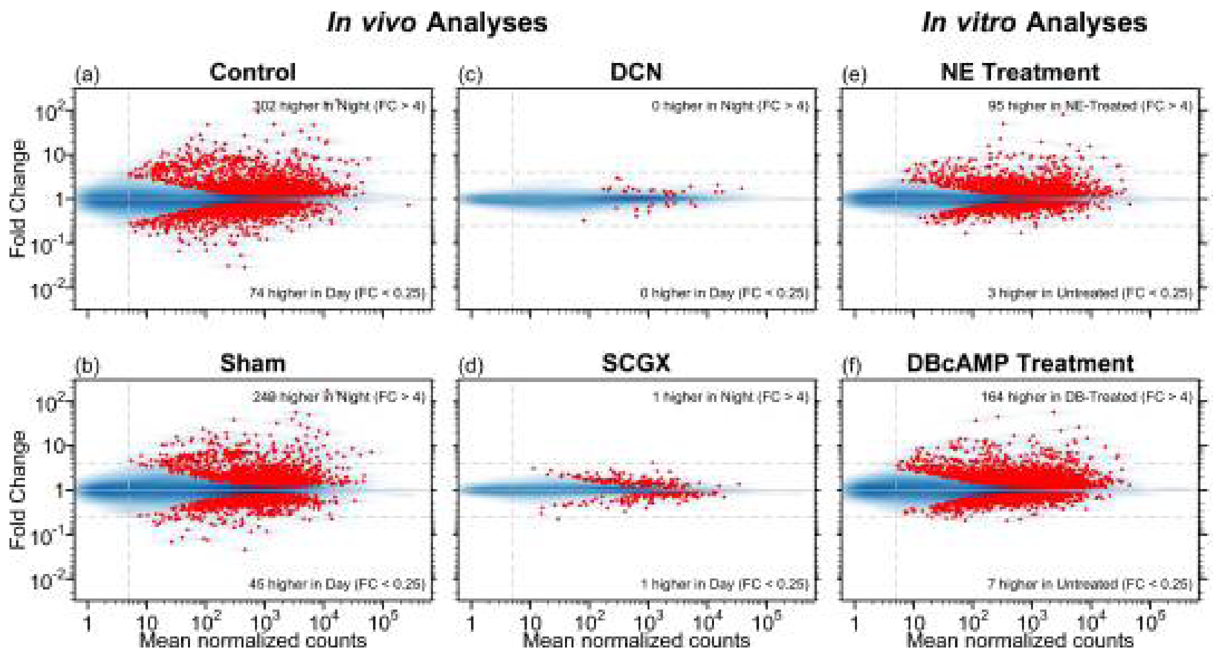


Figure 3. MA plots for six differential expression analyses

The MA plots [visual representation of two-channel DNA microarray gene expression data that has been transformed onto the M (log ratios) and A (mean average) scale] display the mean normalized read-pair counts (x-axis) versus the estimated fold change (y-axis), on a log-log scale for four *in vivo* analyses and two *in vitro* analyses. The blue shading indicates the density of genes, and each red point represents a gene with statistically significant differential expression. Dashed horizontal lines mark four-fold changes in both directions, dashed vertical line indicates minimum abundance threshold for the statistical tests. The four *in vivo* analyses compared night and day time points in adult rats for the following groups: (a) no surgery (Control); (b) neonatal sham surgery (Sham); (c) neonatal superior cervical ganglia decentralization (DCN); (d) neonatal superior cervical ganglionectomy (SCGX). The two *in vitro* analyses compared treated/untreated pineal glands: (e) norepinephrine-treated (NE) vs. untreated; and (f) dibutyryl-cyclic-AMP–treated (DBcAMP) vs. untreated (Reference 1).

calcium/calmodulin-dependent protein kinase 1Gb), which is driven by the biological clock and links the clock with locomotor activity. This is important to an understanding of circadian control mechanisms in vertebrates.

Impact of RNA sequencing

The Section has been successful in promoting the use of RNA sequencing in programs not involved in pineal research, including work with other investigators at the NIH and elsewhere, such as studies on pain pathways, the hypothalamus, and the pituitary gland. In the latter case, RNA sequencing and our subsequent analysis revealed that the hypothalamic peptide GnRH induces a greater than 600-fold increase in expression of dentin matrix protein-1 (Kucka M et al., *Mol Endocrinol* 2013;27:1840-1850). The finding provided the foundation for a large body of work establishing that the response took place in the gonadotroph, that it was mediated by the GnRH receptor, and that it developed following puberty. We also found that the response is less than 5% as robust in males as in females and that expression of the gene was observed *in vivo* only during ovulation. Dentin matrix protein-1 had not previously appeared in the neuroendocrine literature, underscoring the potential impact that application of RNA sequencing can have on our understanding of biological mechanisms.

We stimulated the use of RNA sequencing within NIH through the establishment of an RNA interest group; it brings together a broad range of NIH scientists to discuss each other's work and to evaluate new technologies related to RNA sequencing.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Hartley SW, Coon SL, Savastano LE, Mullikin JC, NISC Comparative Sequencing Program, Fu C, Klein DC. Neurotranscriptomics: the effects of neonatal stimulus deprivation on the rat pineal transcriptome. *PLoS One* 2015; 10:e0137548.
2. Falcón J, Coon SL, Besseau L, Cazaméa-Catalan D, Fuentès M, Magnanou E, Paulin C-H, Boeuf G, Sauzet S, Jorgensen EH, Mazan S, Wolf Y, Koonin EV, Steinbach PJ, Hyodo S, Klein, DC. Drastic neofunctionalization associated with evolution of the “timezyme” AANAT 500 million years ago. *Proc Natl Acad Sci USA* 2014; 111:314–319.
3. Yamazaki F, Kim HH, Lau P, Hwang CK, Iuvone PM, Klein D, Clokie SJ. pY RNA1-s2: a highly retina-enriched small RNA that selectively binds to Matrin 3 (Matr3). *PLoS One* 2014; 9:e88217.
4. Yamazaki F, Møller M, Fu C, Clokie SJ, Zykovich A, Coon SL, Klein DC, Rath MF. The Lhx9 homeobox gene controls pineal gland development and prevents postnatal hydrocephalus. *Brain Struct Funct* 2015; 220(3):1497–1509.
5. Klein DC. Pineal gland and melatonin in endocrinology. *Endocrinology: Adult and Pediatric 7th Edition. Eds: Jameson JL et al., Elsevier, St. Louis* 2015; 312–322.

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Extracellular Matrix Disorders: Molecular Mechanisms and Treatment Targets

The extracellular matrix (ECM) is involved in a wide variety of disorders, ranging from rare genetic abnormalities of skeletal development (skeletal dysplasias) to such common ailments as osteoporosis, fibrosis, and cancer. Our interest in ECM biology began with studies on basic principles relating the helical structure of collagen and DNA to their interactions and biological function. Over the years, the focus of our research shifted to collagens, which are the most abundant ECM molecules, and then to ECM disorders and the development of novel treatments for these disorders. We gradually phased out the DNA studies and concentrated on ECM pathology in cancer, fibrosis, osteogenesis imperfecta, Ehlers-Danlos syndrome, chondrodysplasias, osteoporosis, and other diseases. Together with other NICHD and extramural clinical scientists, we strive to improve our knowledge of the molecular mechanisms underlying those diseases. We hope to use the knowledge gained through our studies for diagnostics, characterization, and treatment, bringing our expertise in physical biochemistry and theory to clinical research and practice.

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Procollagen folding and its role in bone disorders

Collagens are triple-helical proteins forming structural scaffolds that hold together bone, cartilage, skin, and other tissues. In bone, they are produced by osteoblasts, the cells responsible for synthesis and mineralization of new bone material. By far the most abundant proteins in all vertebrates, collagens account for up to 30% of all proteins synthesized by osteoblasts. Yet, folding of their procollagen precursors within the endoplasmic reticulum (ER) presents an extraordinary challenge for cells. We discovered that the equilibrium state of type I procollagen in the absence of chaperones is a random coil. Folding of the human procollagen triple helix in an ER–like environment is favorable below 35°C but not at normal body temperature. At this temperature, natively folded collagen molecules become stable only after incorporation into fibers. When not incorporated, individual molecules denature within several hours and become susceptible to rapid proteolytic degradation. To overcome such intrinsic instability, cells must use specialized chaperones to fold the triple helix. It is not completely clear which chaperones are involved in this process, how they function, and how cells recognize properly folded procollagen and handle it when misfolded. Because of massive procollagen synthesis, osteoblast malfunction caused by procollagen misfolding might be an important factor in bone pathologies. For instance, osteogenesis imperfecta (OI) appears to be primarily a procollagen misfolding disease (References 1 and 2). Moreover, an inability of aging osteoblasts to handle normal procollagen folding load might also contribute to common osteoporosis. Understanding procollagen folding and the cellular response to its misfolding is therefore important both from fundamental and practical perspectives.

The most common mutations affecting procollagen folding are Gly substitutions in the obligatory $(\text{Gly-X-Y})_n$ sequence of the triple helix. Such substitutions in type I collagen are responsible for approximately 80% of severe OI cases. The substitutions' effects on procollagen folding are determined by the location and identity of the substituting residue. Our studies of collagens from OI patients with over 50 different Gly substitutions revealed several structural regions within the triple helix where mutations might be responsible for distinct OI phenotypes. For example, the first 85–90 amino acids at the N-terminal end of the triple helix form an "N-anchor" domain with higher than average triple helix stability. Gly substitutions within this region disrupt the whole N-anchor, preventing normal cleavage of the adjacent N-propeptide. Incorporation of collagen with uncleaved N-propeptides into fibrils leads to hyperextensibility and joint laxity more characteristic of the Ehlers-Danlos syndrome (EDS). α1(I) Gly substitutions within another region, which surrounds the collagenase cleavage site, might be lethal because the sequence of this region prevents efficient renucleation of normal C-to-N-terminus helix folding, once the mutation is encountered.

At present, we are focusing on understanding how cells respond to misfolding of procollagen with different Gly substitutions. We hypothesize that unconventional folding requirements produce an unconventional cell stress response to accumulation of misfolded procollagen. In collaboration with Peter Byers, we found that Gly substitutions cause slower folding as well as misfolding of procollagen, its accumulation in the ER of cultured patient fibroblasts, ER dilation, and activation of a cell stress response reminiscent of the "ER Overload" associated with misfolding of serpin-family proteins in serpinopathies. The observations provided an important clue to OI pathophysiology and highlighted autophagy as a potentially important therapeutic target.

To further pursue molecular mechanisms of the cell stress response and its targeting, we are investigating a mouse OI model with a Gly610 to Cys substitution in the triple helical region of the α2(I) chain. The G610C mouse mimics the mutation found in a large group of related patients. Our study of cultured fibroblasts and osteoblasts as well as tissues in this model revealed misfolding and accumulation of mutant molecules in the ER similar to human patient fibroblasts. Increased phosphorylation of EIF2α indicated the presence of cell stress response, yet we found no evidence of conventional unfolded protein response (UPR) signaling. We found that misfolded procollagen molecules are degraded by lysosomes via autophagy, rather than by proteasomes via ER–associated degradation. Osteoblasts adapt to the misfolding of mutant molecules and eliminate them by enhancing the autophagy. However, the adaptation occurs at the cost of abnormal cell function, e.g., blunted response to stimulation of collagen production by TGF-β and abnormal Wnt signaling. Abnormal function of the latter pathways likely contributes to the abnormal osteoblast differentiation and maturation observed in G610C animals. For instance, the animals exhibit delayed bone mineralization during early development, and their cultured bone marrow stromal cells (BMSCs) form fewer colonies capable of producing mineralized matrix. As an adaptation to deficient osteoblast maturation, G610C mice generate more osteoblastic cells. However, each mutant cell produces much less bone than its normal counterpart. The combination of increased bone formation surfaces with reduced bone formation rate at these surfaces disrupts normal bone modeling, causing, e.g., long-term entrapment of poorly organized woven bone between layers of lamellar bone. The resulting disruption of the cortical bone matrix structure leads to more brittle bones, which have a heightened susceptibility to fracture upon high energy impact despite normal cortical thickness and slightly increased cortical bone mineral density.

Development of novel OI treatments

We hypothesize that, by enhancing autophagy, it might be possible to prevent excessive accumulation of misfolded procollagen molecules in the cell, reducing cell stress and thereby improving osteoblast differentiation and function. As an initial approach to testing the hypothesis, we placed G610C mice on a low protein diet from 8 to 17 weeks of age, which partially normalized mineralization of newly deposited cortical bone and improved BMSC differentiation in culture. However, the positive effects were counterbalanced by reduced animal growth and less deposition of new bone. Overall, our preliminary data suggest that autophagy enhancement might be a useful therapeutic strategy, but a continuous low protein diet is not likely to be a suitable approach, particularly in pediatric OI patients.

Surprisingly, BMSCs appeared to "remember" the animal's diet for up to several weeks at identical cell culture conditions. Apparently, the diet produced some epigenetic changes, e.g., resulting in enhanced autophagy even after cell removal from animals and prolonged culture, suggesting that an intermittent low protein diet might be useful for alleviating osteoblast cell stress without reducing bone deposition. However, diet affects multiple physiological functions. While the dietary treatment is easy to implement, the mechanisms underlying its effects are difficult to interpret, and even an intermittent low-protein diet may have a variety of unintended long-term consequences. It is therefore important to understand how bone formation and underlying osteoblast differentiation and function are affected by more specifically targeted autophagy manipulation that has no, or at least fewer, off-target effects.

To address this question, we generated G610C mice, in which autophagy can be repressed or activated by conditional targeted knock-out or overexpression of the *Atg5* gene in cells of osteoblast lineage. In an ongoing study of these animals, we found that knock-out of both *Atg5* alleles in mature osteoblasts significantly worsens the bone phenotype. This initial observation supports the importance of autophagy in the animal adaptation to mutant procollagen misfolding, but validation of autophagy as a useful therapeutic target requires testing the effects of its osteoblast-specific enhancement. We are currently completing animal breeding for these experiments, which we hope will provide a better understanding of the role of autophagy in normal function and pathology of osteoblasts as well as identify novel targets and therapeutic approaches to OI and other collagen-misfolding disorders.

Translational studies of patients with novel or unusual OI and EDS mutations

Abnormal collagen biosynthesis and malfunction of osteoblasts are also important factors in OI caused by other collagen mutations as well as by mutations in other proteins. Over the last several years, we assisted several clinical research groups in characterizing collagen biosynthesis and folding in fibroblasts from patients with newly discovered recessive forms of OI and closely related skeletal dysplasia caused by mutations in cartilage-associated protein (CRTAP), prolyl-3-hydrohylase (P3H1), cyclophilin B (CYPB), FKBP65, WNT1, and TRICB. In particular, our collaboration with Joan Marini suggested that the CRTAP/P3H1/CYPB complex functions as a procollagen chaperone. A deficiency in any of the three proteins delays procollagen folding, although their exact role in procollagen folding remains unclear (Reference 3). More surprisingly, we found no detectable changes in the procollagen folding rate in cultured fibroblasts from patients with *FKBP65* mutations. Our data suggest that FKBP65 may affect post-translational modification of procollagen and deposition of collagen matrix by a different mechanism. It remains unclear why some *FKBP65* mutations cause severe OI with joint contractures (Bruck syndrome) while others cause joint contractures without pronounced OI (Kuskokwim syndrome) or OI without pronounced joint contractures.

More recently, in collaboration with Joan Marini, we investigated collagen and ECM biosynthesis abnormalities caused by deficiencies in the transmembrane protein TRICB in fibroblasts from Bedouin OI patients in Israel and Saudi Arabia. The study revealed unusual underhydroxylation of lysine residues in collagen chains, in contrast to overhydroxylation of lysine caused by most other OI mutations, as well as secretion of 30–40% of procollagen molecules with abnormal conformation and reduced thermal stability. Given that TRICB is involved in Ca^{2+} release from the ER and that its absence causes abnormal Ca^{2+} concentrations and flux in patient cells, OI in these patients may be caused by dysregulation of several collagen-specific chaperones and modifying enzymes in the ER through Ca^{2+} modulation. However, TRICB may also have a more direct chaperone function in procollagen folding. We also assisted Paul Coucke's laboratory in characterization of procollagen biosynthesis abnormalities in a complex lethal osteochondrodysplasia caused by defective TAPT1 protein (Reference 4).

Most single amino acid substitutions in X and Y positions of the repeating Gly-X-Y collagen sequence are considered to be neutral variants. Several Arg-to-Cys substitutions were recently reported, but it remained unclear whether the resulting OI and EDS were caused by aberrant disulfide bonds formed in the ECM by the Cys residue, which is not present in normal type I collagen, or by abnormal biosynthesis of mutant molecules. In collaboration with Peter Byers, we analyzed collagen-metabolism pathology caused by substitutions of the same Y-position Arg780 in the α1(I) chain to Leu and Cys. The study revealed that the loss of Y-position Arg causes abnormal procollagen folding by reducing triple helix stability and eliminating important HSP47–binding sites, resulting in accumulation of misfolded procollagen in the ER and ER dilation similar to the effects of Gly substitutions. These effects are likely responsible for the bone fragility caused by Y- but not X-position Arg substitutions, while aberrant Cys disulfide bonds likely contribute to EDS in patients with either Y- or X-position substitutions.

Extracellular matrix pathology in tumors and fibrosis

Another important advance from our work of the past several years was the characterization of a collagenase-resistant, homotrimeric isoform of type I collagen and its potential role in cancer, fibrosis, and other disorders. The normal isoform of type I collagen is a heterotrimer of two α1(I) chains and one α2(I) chain. Homotrimers of three α1(I) chains are produced in some fetal tissues, carcinomas, fibrotic tissues, as well as in rare forms of OI and EDS associated with α2(I) chain deficiency. We found the homotrimers to be at least 5–10 times more resistant to cleavage by all mammalian collagenases than the heterotrimers, and we determined the molecular mechanism of this resistance. Our studies suggested that cancer cells might utilize the collagen isoform to build collagenase-resistant tracks, thus supporting invasion through stroma of lower resistance.

We also collaborated with Constantine Stratakis's lab to investigate bone tumors caused by defects in protein kinase A (PKA), a key enzyme in the cAMP signaling pathway. Initially, we investigated synthesis of type I collagen homotrimers. However, over the last three years, the focus of the study has shifted to abnormal differentiation of osteoblastic cells and deposition of bone within these tumors (Reference 5). We found that knockouts of various PKA subunits cause not only abnormal organization and mineralization of bone matrix but also novel bone structures that had not been previously reported. For instance, we observed free-standing cylindrical bone spicules with osteon-like organization of lamellae and osteocytes but an inverted mineralization pattern, a highly mineralized central core, and decreasing mineralization away from the central core. Currently, we are assisting the Stratakis lab in characterizing abnormal osteoblast maturation, the role of an abnormal inflammatory response, and effects of anti-inflammatory drug treatments in these animals. Improved understanding of bone tumors caused by PKA deficiencies may not only clarify the role of cAMP signaling but also suggest new approaches to therapeutic manipulation of bone formation in skeletal dysplasias.

Multi-modal micro-spectroscopic imaging and mapping of tissues

Label-free micro-spectroscopic infrared and Raman imaging of tissues and cell cultures provides important information about the chemical composition, organization, and biological reactions inaccessible by traditional histology. However, applications of these techniques were severely restricted by light-path instabilities in thin hydrated specimens under physiological conditions. We resolved the problem by designing specimen chambers with precise thermo-mechanical stabilization for high-definition (HD) infrared imaging and Raman micro-spectroscopy, achieving spectral reproducibility up to two orders of magnitude better than in leading commercial instruments. The HD technology was essential for analysis of abnormal collagen matrix deposition by CRTAP– and FKBP65–deficient cells. It has enabled us to assist NIBIB scientists in characterizing a functionalized carbon-nanotube approach to the delivery of anticancer agents into cells that overexpress hyaluronate receptors and is crucial for our current studies of bone structure and mineralization in the mouse models of OI and PKA deficiencies described above.

The power of the technology is best illustrated by our studies of ECM structure and composition effects on the function of cartilage in a mouse model of diastrophic dysplasia (DTD) caused by mutations in the SLC26A2 sulfate transporter, deficient sulfate uptake by chondrocytes, and resulting under-sulfation of glycosaminoglycans in cartilage matrix. In collaboration with Antonella Forlino and Antonio Rossi, we found that the deficiency results in under-sulfation of chondroitin and disorientation of collagen fibers, disrupting a thin protective layer at the articular surface and causing subsequent cartilage degradation. We investigated the relationship between chondroitin under-sulfation and the rate of its synthesis across the growing epiphyseal cartilage and built a mathematical model for the sulfation pathway, predicting treatment targets for sulfation-related chondrodysplasias and genes that might contribute to the juvenile idiopathic arthritis recently associated with single nucleotide polymorphisms in the SLC26A2 transporter.

We are extending the technology by combining imaging of bone and cartilage ECM composition and structure with biomechanical measurements at the same length scales. The mechanical properties of bone and cartilage should depend on the deformation length scale because of the heterogeneous microscopic structure and the presence of different macroscopic regions and zones in these tissues. Nevertheless, biomechanical studies are rarely accompanied by mapping of tissue composition and structure. To address the problem, we are collaborating with Peter Basser and Emilios Dimitriadis on mapping cartilage elasticity by force microscopy at length scales appropriate for examining the material properties of the ECM and on combining it with our multimodal imaging technology.

While the ECM plays a key role in normal development and pathology of all tissues, most studies focus on expression of its components rather than its overall organization. Our multimodal imaging technology is helping to close this gap in *in vitro* studies of tissue sections and cell cultures. To translate these advances into clinical practice, we established a new collaboration with Peter Basser on utilizing the technology to calibrate and test newer methods for noninvasive *in vivo* ECM studies by the solid state magnetic resonance imaging (MRI) that is being developed in his laboratory.

Currently, in collaboration with Peter Basser and Roberto Romero, we are adapting HD infrared and Raman technologies for quantitative imaging of structural organization, composition and mechanical properties of ECM in the normal and pathological human placenta. Our initial measurements have already identified several previously unreported features of placental ECM: (1) highly anisotropic organization of fibrin and collagen, which may depend on and affect the blood flow and determine the tissue strength; (2) deposition of cholesteryl ester aggregates in fibrin fibrinoids from maternal blood; and (3) formation of collagen "fibroids" and high collagen content in matrix fibrinoids. In addition to identifying novel features of placental ECM organization, we are utilizing the HD infrared and Raman imaging to guide detection of ECM abnormalities in the same sample by single-sided (SS-NMR) and magnetization exchange (MEX-MRI) NMR/MRI methods, for subsequent application in noninvasive real-time imaging of human placenta pathology.

ADDITIONAL FUNDING

» NICHD Director's Award (2014): Targeting Autophagy of Misfolded Molecules in Collagen-Related Dysplasias
» NICHD Director's Human Placenta Award (2015): Imaging Extracellular Matrix in Human Placenta

PUBLICATIONS

1. Makareeva E, Leikin S. Collagen structure, folding and function. *Osteogenesis Imperfecta: A translational approach to brittle bone disease. Eds: Shapiro et al., Academic Press* 2014; 71–84.
2. Mirigian LS, Makareeva E, Leikin S. Pulse-chase analysis of procollagen biosynthesis by azidohomoalanine labeling. *Connect Tissue Res* 2014; 55:403–410.
3. Cabral WA, Perdivara I, Weis M, Terajima M, Blissett AR, Chang W, Perosky JE, Makareeva EN, Mertz EL, Leikin S, Tomer KB, Kozloff KM, Eyre DR, Yamauchi M, Marini JC. Abnormal type I collagen post-translational modification and crosslinking in a cyclophilin B KO mouse model of recessive osteogenesis imperfecta. *PLoS Genetics* 2014; 10:e1004465.
4. Symoens S, Barnes AM, Gistelinck C, Malfait F, Guillemyn B, Steyaert W, Syx D, D'Hondt S, Biervliet M, De Backer J, Witten EP, Leikin S, Makareeva E, Gillessen-Kaesbach G, Huysseune A, Vleminckx K, Willaert A, De Paepe A, Marini JC, Coucke PJ. Genetic defects in TAPT1 disrupt ciliogenesis and cause a complex lethal osteochondrodysplasia. *Am J Hum Genet* 2015; 97:521–534.
5. Liu S, Saloustros E, Mertz EL, Tsang K, Starost MF, Salpea P, Faucz FR, Szarek E, Nesterova M, Leikin S, Stratakis CA. Haploinsufficiency for either one of the type-II regulatory subunits of protein kinase A improves the bone phenotype of Prkar1a$^{+/-}$ mice. *Hum Mol Genet* 2015; 24:6080–6092.

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The Molecular Mechanics of Eukaryotic Translation Initiation

The goal of our research group is to elucidate the molecular mechanisms underlying the initiation phase of protein synthesis in eukaryotic organisms. We use the yeast *Saccharomyces cerevisiae* as a model system and employ a range of approaches—from genetics to biochemistry to structural biology—in collaboration with Alan Hinnebusch's and Tom Dever's labs here at NICHD and several other research groups around the world.

Eukaryotic translation initiation is a key control point in the regulation of gene expression. It begins when an initiator methionyl tRNA (Met-tRNAi) is loaded onto the small (40S) ribosomal subunit. Met-tRNAi binds to the 40S subunit as a ternary complex (TC) with the GTP–bound form of the initiation factor eIF2. Three other factors, eIF1, eIF1A, and eIF3, also bind to the 40S subunit and promote the loading of the TC. The resulting 43S pre-initiation complex (PIC) is then loaded onto the 5′-end of an mRNA with the aid of eIF3 and the eIF4 group of factors: the RNA helicase eIF4A; the 5′-7-methylguanosine cap-binding protein eIF4E; the scaffolding protein eIF4G; and the 40S subunit– and RNA–binding protein eIF4B. Both eIF4A and eIF4E bind to eIF4G and form the eIF4F complex. Once loaded onto the mRNA, the 43S PIC is thought to scan the mRNA in search of an AUG start codon. The process is ATP–dependent and likely requires multiple RNA helicases, including the DEAD–box protein Ded1p. Recognition of the start site begins with base pairing between the anticodon of tRNAi and the AUG codon. Base pairing then triggers downstream events that commit the PIC to continuing initiation from that point on the mRNA. These events include ejection of eIF1 from its binding site on the 40S subunit, movement of the C-terminal tail (CTT) of eIF1A, and release of phosphate from eIF2, which converts eIF2 to its GDP–bound state. In addition, the initiator tRNA moves from a position that is not fully engaged in the ribosomal P site [termed $P_{(OUT)}$] to one that is [$P_{(IN)}$], and the PIC as a whole converts from an open conformation that is conducive to scanning to a closed one that is not. At this stage, eIF2•GDP dissociates from the PIC, and eIF1A and a second GTPase factor, eIF5B, coordinate joining of the large ribosomal subunit to form the 80S initiation complex. In a process that appears to result in conformational reorganization of the complex, eIF5B hydrolyzes GTP and then dissociates along with eIF1A.

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Dissecting the molecular mechanics of eukaryotic translation initiation

We made considerable progress this year in our studies of the mechanism of mRNA recruitment to the eukaryotic ribosome and the regulation of start codon recognition.

We analyzed a battery of mutant versions of the large, heteromultimeric translation initiation factor eIF3 for effects on key steps in the initiation pathway *in vitro*. The studies shed light on the functions of domains of the factor in binding to the ribosomal pre-initiation complex (PIC), enhancement of initiator tRNA binding and mRNA recruitment. In particular, the data indicate that the a subunit of eIF3 plays an important role in stabilizing mRNA binding in the exit channel of the ribosome, while the i and g subunits functionally interact in the entry channel with the cap-binding and RNA helicase complex eIF4F. We are currently starting work on one of two manuscripts describing these studies.

The work described above was significantly aided by the recent cryo-EM reconstructions of two new states of the yeast PIC that we collaborated on with Venki Ramakrishnan's and Alan Hinnebusch's groups. The structures, which were recently published in *Molecular Cell* (Reference 2), show the positions of the eIF3 subunits as well as the beta subunit of eIF2.

We also made significant progress in our studies on the roles of the RNA helicases eIF4A and Ded1 in promoting recruitment of mRNA to the PIC. We found that the two helicases perform distinct functions and that the ATPase activity of eIF4A is stimulated by interaction with components of the PIC. The latter result suggests that eIF4A does not function entirely on the mRNA but may work as part of an initial encounter complex between the PIC and the mRNA.

We also extended our studies of the regulation of the fidelity of start codon recognition using a range of yeast genomic approaches. We identified several kinases that, when knocked out, reduce or abrogate the regulation of start codon fidelity. We also used ribosome profiling to identify instances of regulation of start-codon recognition on specific mRNAs. We are preparing a manuscript describing this novel mode of post-transcriptional gene regulation.

ADDITIONAL FUNDING

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PUBLICATIONS

1. Mitchell SF, Lorsch JR. Protein affinity purification using intein/chitin binding protein tags. *Methods Enzymol* 2015; 559:111–125.
2. Llácer JL, Hussain T, Marler L, Aitken CE, Thakur A, Lorsch JR, Hinnebusch AG, Ramakrishnan V. Conformational differences between open and closed states of the eukaryotic translation initiation complex. *Mol Cell* 2015; 59:399–412.

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In an integrated program of laboratory and clinical investigation, the Bone and Extracellular Matrix Branch (BEMB) studies the molecular biology of the heritable connective tissue disorders osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS). Our objective is to elucidate the mechanisms by which the primary gene defect causes skeletal fragility and other connective tissue symptoms and then to apply this knowledge to the treatment of children with these conditions. Recently, we identified the long-sought cause of recessive OI. Discoveries of defects in collagen modification generated a new paradigm for collagen-related disorders of matrix. We established that structural defects in collagen cause dominant OI while deficiency of proteins that interact with OI for folding, post-translational modification, or processing cause recessive OI. Our challenge now is to understand the cellular and biochemical mechanisms of recessive OI. We also generated a knockin murine model for OI with a classical collagen mutation as well as a murine model for recessive type IX OI and are using these models to study disease pathogenesis and the skeletal matrix of OI, the effects of pharmacological therapies, and approaches to gene therapy. Our clinical studies involve children with types II and IV OI, who form a longitudinal study group enrolled in age-appropriate clinical protocols for the treatment of their condition.

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Mechanism of rare forms of osteogenesis imperfecta (OI)

The endoplasmic reticulum (ER)–resident 3-hydroxylation complex is responsible for the 3-hydroxylation of type I collagen α1(I) chains. Deficiency of components of the collagen P3H (prolyl 3-hydroxylase) complex causes recessive OI. Thus, deficiency in CRTAP (collagen-associated protein) causes type VII OI, while deficiency in P3H1 causes type VIII OI. Types VII and VIII OI are severe to lethal bone dysplasias that are indistinguishable clinically because these two components are mutually protective in the complex. They share characteristics that are distinct from dominant OI; affected individuals have white or light sclerae, small/normal rather than enlarged head circumferences, and rhizomelia of long bones. We are now investigating the subcellular localization of CRTAP. The protein has a transmembrane sequence at the amino end. Its tethering to the ER membrane could be important for the coordination of collagen chain folding and modification.

The third member of the complex, Cyclophilin B (CyPB), encoded by *PPIB*, is an ER–resident peptidyl-prolyl *cis-trans* isomerase (PPIase) that functions both independently and as a component of the collagen prolyl 3-hydroxylation complex. CyPB is proposed to be the major PPIase catalyzing the rate-limiting step in collagen folding. Previously, the BEMB characterized the first patient with deficiency of *PPIB*, which causes recessively inherited Type IX OI. Our group generated a *Ppib* knock-out (KO) mouse model that recapitulates the Type IX OI phenotype, including growth deficiency and brittle bones (Reference 2). Collagen from KO mouse tissue and cells is nearly lacking 3-hydroxylation. We investigated the role of CyPB in collagen biosynthesis and found that intracellular collagen folding occurs more slowly in CyPB-null cells, supporting its role in the rate-limiting step of folding. However, treatment of KO cells with the cyclophilin inhibitor cyclosporin A caused further delay in folding, providing support for the existence of another collagen PPIase. We also extended the reported role of CyPB in supporting collagen lysyl hydroxylase (LH1) activity. Analysis of bone and osteoblast type I collagen revealed site-specific alterations in helical lysine hydroxylation, in particular significantly reduced hydroxylation of the helical crosslinking residue K87. The alteration directly affects both the extent and type of collagen intermolecular crosslinks that form in bone tissue. Our studies demonstrated novel consequences of the effect of CyPB on collagen hydroxylation, glycosylation, crosslinking, and fibrillogenesis, showing that CyPB not only functions to regulate collagen folding and 3-hydroxylation in the ER but also indirectly regulates bone development and mechanical properties. A collaboration with Ehud Cohen brought an additional important novel finding for CyPB that extended its foldase role to presenilin 1 in Alzheimer's disease. Reduced quantities of active properly folded presenilin were observed in brains of CyPB KO mice, supporting the emerging mechanism that Alzheimer's disease can arise from altered presenilin folding and function. Thus, ER chaperones may be targets for the development of therapies for neurodegenerative disorders.

OI type V is caused by a recurrent dominant mutation (c.-14C→T) in *IFITM5*, which encodes BRIL, a bone-restricted interferon-induced transmembrane protein–like protein most strongly expressed in osteoblasts, which plays a role in mineralization. Patients with type V OI have distinctive clinical manifestations with overactive bone mineralization and mesh-like lamellation on bone histology. We identified eight patients with the recurrent type V OI mutation and used cultured osteoblasts from these patients to study the mechanism of this type of OI at the bone cell level. We demonstrated that the mutant Bril transcripts and protein were stable and expressed at levels comparable to control. Both early and late markers of osteoblast differentiation are elevated in type V OI osteoblasts, including the osteoblast differentiation factor Runx2, alkaline phosphatase, bone sialoprotein, osteopontin, and osteocalcin. Mineralization by osteoblasts, assayed by alizarin red staining, was also elevated in type V OI osteoblasts. However, this occurs despite the seemingly paradoxical reduction in transcripts for type I collagen in mid to late differentiation, which leads to a concomitant reduction in cross-linked collagen in matrix and an altered appearance of fibrils deposited in culture, which have a patchy rather than a network appearance. These studies demonstrated that a gain-of-function mechanism underlies type V OI and establish its collagen-related defect.

Recessive null mutations in *SERPINF1*, which encodes pigment epithelium–derived factor (PEDF), cause OI type VI. PEDF is already well-known as a potent anti-angiogenic factor. Type VI OI patients have no serum PEDF, elevated alkaline phosphatase (ALPL) as children, and bone histology with broad unmineralized osteoid and fish-scale pattern. At first, types V and VI OI appear to be unconnected, caused by different genes, with distinct phenotypes and histology. However, we identified a patient with severe OI whose phenotype is most like type VI OI. Her osteoblasts displayed minimal secretion of PEDF, but her serum PEDF was normal. *SERPINF1* sequences were normal despite bone histomorphometry typical of type VI OI, and elevated childhood serum ALPL. To identify the mutation, exome sequencing on the proband, parents, and an unaffected sibling surprisingly yielded a *de novo* mutation in *IFITM5* in one allele of the proband, causing a p.S40L substitution in the BRIL intracellular domain. *IFITM5* transcript and BRIL protein level were normal in proband fibroblasts and osteoblasts. *SERPINF1* expression and PEDF secretion were reduced in proband osteoblasts. In contrast, osteoblasts from a typical case of type V OI have elevated *SERPINF1* expression and PEDF secretion during osteoblast differentiation. Together, the data suggest that BRIL and PEDF have a relationship that connects the genes for types V and VI OI and their roles in bone mineralization (Reference 1). We are extending this investigation using the murine model for type VI OI. Our current studies focus on delineating the pathway connecting type V and VI OI in osteoblasts and the relationship of the angiogenic effect of PEDF to its effect on osteoblasts and bone tissue.

Studies of a very rare defect in *TAPT1*, which encodes the highly conserved transmembrane anterior posterior transformation 1 protein, have been conducted in collaboration with the Belgian laboratory studying OI, led by Paul Coucke and Anne De Paepe. Defects in this gene cause a complex lethal osteochondrodysplasia that overlaps with the findings in a ciliopathy and skeletal dysplasia. Two large pedigrees with several pregnancies that were lethal in the perinatal period as a result of skeletal undermineralization and fractures, as well as neurological abnormalities such as ventriculomegaly and hypoplastic cerebellum and abnormalities of lungs and kidney, were investigated. A homogygous acceptor splice site variant in *TAPT1* in one family, and a homozygous missense change in a highly conserved region of *TAPT1* in the second family, were identified. Both mutations affect the second extracellular/luminal loop of this transmembrane protein. In human fibroblasts, TAPT1 was shown to localize to the centrosome and ciliary basal body region in control cells. In cells from family 1, the primary cilium did not form, while in the missense mutation the structure of the primary cilium was disturbed. Proband cells also have disturbed collagen biochemistry. Collagen folds more slowly in these cells and the trafficking of secreted proteins through the Golgi was disturbed, with the Golgi more disperse and distended. Knockdown of *tapt1b* in zebrafish induced severe craniofacial cartilage manifestations and delayed ossification, consistent with the human phenotype (Reference 5).

C-propeptide cleavage site mutations increase bone mineralization.

Type I procollagen is processed to mature collagen by the removal of both N- and C-terminal propeptides. The C-propeptide is cleaved at the Ala-Asp peptide bond between the telopeptide and the C-propeptide of each chain by C-proteinase/BMP-1. Probands with substitutions at the four cleavage site residues have been identified. Our investigations of two of those probands has identified a High Bone Mass form of OI. These individuals have elevated bone density DEXA z-scores and patchy unmineralized osteoid on bone histology. The processing of the C-propeptide from collagen secreted by proband cells is delayed. In collaboration with Adele Boskey, we found that Fourier transform infrared spectroscopy (FTIR) demonstrated a higher mineral/matrix ratio in both trabecular and cortical bone of each patient than in either age-matched normal or classical OI controls, as well as marked maturation of collagen cross-links. We extended the investigation of mineralization with BMDD (bone mineral density distribution) and BEI (backscattered electron imaging) to show that, in the α2(I) cleavage site mutation, the bone had a uniformly higher mineral density, while in the α1(I) mutation, the average mineral density was typical of classical OI but markedly more heterogeneous, with areas of very high and low bone density.

To investigate the role of the C-propeptide in bone mineralization and developmental progression, we developing a knock-in murine model with a COL1A1 cleavage site mutation. Bone collagen fibrils showed a "barbed-wire" appearance consistent with the presence of the pC-collagen that was detected in extracts of bone from mutant mice, and with impaired collagen processing *in vitro*. Impaired C-propeptide processing affects skeletal size and biomechanics. The mice are smaller than wild-type litter mates. Their femora have extreme brittleness on mechanical testing, as well as reduced fracture load. We are currently investigating bone mineralization in these mice.

Insight from the Brtl mouse model for OI

The Brtl mouse model for OI, generated by the BEMB, is a knock-in mouse that contains a Gly349Cys substitution in the α1(I) chain. Brtl was modeled on a type IV OI child and accurately reproduces features of type IV OI. Brtl has provided important insights into both potential OI treatments and the mechanism of OI. First, we conducted a treatment trial of the bisphosphonate alendronate in Brtl and wild-type (WT) littermates. We found that bone density, bone volume, and trabecular number improved with treatment, as did load-to-fracture. However, detrimental side effects such as retained mineralized cartilage, reduced material properties, and altered osteoblast morphology occurred with treatment. The results reinforce the conclusion of the pediatric trial to limit the duration of bisphosphonate treatment. Recently, we also collaborated with Kenneth Kozloff's group to investigate a potential anabolic therapy, sclerostin antibody (Scl-Ab), which stimulates osteoblasts via the canonical wnt pathway. Scl-Ab stimulated bone formation in young Brtl mice and increased bone mass and load-to-fracture. In treatment with Scl-Ab, there was no detrimental change in Brtl bone material properties. Nano-indentation studies indicating unchanged mineralization showed that the hypermineralization of bisphosphonate treatment did not occur. In addition, Scl-AB was successfully anabolic in adult Brtl mice, and may be a therapy for adult patients who have fewer treatment options.

A third therapeutic trial involving Brtl approached allele-specific silencing of the *col1a1* mutation, undertaken in collaboration with Antonella Forlino. Specific small interfering RNA (siRNA) were evaluated *ex vivo* in Brtl fibroblasts for their effect on collagen transcripts and protein. A preferential reduction in mutant transcripts by about half was associated with a 40% decrease in mutant collagen chain. Further testing of siRNA delivered by lentivirus might allow treatment of OI patients by autologous transplantation.

Brtl also provided important information about the cytoskeletal organization in OI osteoblasts and their potential role in the phenotypic variability of OI. Abnormal cytoskeletal organization was demonstrated to occur only in lethal pups. Comparison of lethal and surviving Brtl pups' skin/bone and bone/skin hybrid networks highlighted three proteins involved in cytoskeletal organization: vimentin, stathmin, and coffin-1. The alterations were shown to affect osteoblast proliferation, collagen deposition, integrin, and TGF-beta signaling. Furthermore, aberrant cytoskeletal assembly was detected in fibroblasts obtained from lethal, but not non-lethal, OI patients with an identical glycine substitution. The data open the possibility of cytoskeletal elements as novel treatment targets for OI.

Two basic insights have emerged from Brtl studies. The hyper-mineralization of OI bone was previously thought to be a passive process. Altered levels for osteocyte transcripts involved in bone mineralization, such as Dmp1 and Sost1, demonstrated the presence of an actively directed component. Second, the osteoclast is important to the OI phenotype, with elevated numbers and TRAP (tartrate-resistant acid phosphatase) staining of osteoclasts and precursors. Co-culture experiments with Brtl and wild-type (WT) mesenchymal stem cells (MSCs) and osteoclast precursors yielded elevated osteoclast numbers from WT or Brtl precursors grown with Brtl MSCs, but not with WT MSCs. The results indicate that an osteoblast product is necessary and sufficient for elevated osteoclast numbers and could provide an important target for treatment of OI.

Natural history and bisphosphonate treatment of children with types III and IV OI

We recently brought the cardiopulmonary aspects of our natural history study on types III and IV OI to publication in collaboration with translational murine studies of our collaborator, Martin Hrabe de Angelis. The longitudinal evaluations were completed in 23 children with type III OI and 23 children with type IV OI, who had serial pulmonary function tests every 1–2 years. Comparison of their results with size-matched children showed a significant decline over time in pulmonary function, including lung volumes and flow rates. The decline was worse in the 36 children with scoliosis (average curve 25 degrees) but also occurred in 20 participants without scoliosis, who had declining function with restrictive disease, suggesting that the pulmonary dysfunction of OI is attributable to a primary defect in lung related to structurally abnormal collagen. The studies are important because pulmonary issues are the most prevalent cause of morbidity and mortality in OI, and affected individuals should now seek anticipatory evaluation and treatment.

Our randomized controlled trial of bisphosphonate in children with types III and IV OI was the first randomized trial in the United States and one of four worldwide. Our trial examined both direct skeletal and secondary gains reported in uncontrolled trials. For skeletal outcomes, we found increased BMD Z-scores and improved vertebral area and compressions. We noted that vertebral BMD improvement tapered off after two years treatment. Our treatment group did not experience fewer long-bone fractures, coinciding with the lack of improvement or equivocal improvement in fractures in other controlled trials. The BEMB controlled trial did not support the secondary gains claimed in observational trials, including improvement in ambulation level, lower-extremity strength, or alleviation of pain, suggesting these were placebo effects in observational trials. Our current recommendation is treatment for 2–3 years, with subsequent follow-up of bone status. We are now engaged in a dose-comparison trial, comparing the dose from our first trial with a lower dose, achieved by increasing the cycle interval at the same dose/kg/cycle. Given the decade-long half-life and side effects of bisphosphonate on normal as well as dysplastic bone, including osteonecrosis of the jaw (ONJ), bone healing, bone modeling, and decline in the quality of the bone material, it is important to determine the lowest cumulative dose that will provide vertebral benefits. Preliminary analysis indicates that OI children obtain benefits from lower pamidronate doses that are comparable to the benefits from higher doses.

OI Mutation Consortium

The BEMB assembled and leads an international consortium of connective tissue laboratories for the compilation and analysis of a database of mutations in type I collagen that cause OI. The Consortium Database assembled double the previously available number of collagen mutations, and when the first analysis of the database was published in 2007, it listed over 830 mutations, including 682 glycine substitutions and 150 splice-site defects. Genotype-phenotype modeling revealed distinct functions for each alpha chain of type I collagen, including the occurrence of exclusively lethal mutations in the Major Ligand Binding Regions (MLBR) of the α1(I) chain on the collagen monomer and the overlapping of the regularly spaced clusters of lethal mutations along the α2(I) chain with the proteoglycan binding sites on the collagen fibril. The modeling for α2(I) supports the Regional Model for mutation that was first proposed by the BEMB over 15 years ago and now correctly predicts 86% of clinical outcomes. The Consortium Database has provided the basis for our collaborators James San Antonio and Joseph Orgel to model functional domains in terms of the cell and matrix interactions of the collagen fibril. The Consortium Database now contains over 1,570 mutations from nine international laboratories. An upcoming analysis of this database will also examine the effects of interchain salt bridges and re-nucleation residues C-terminal to the substituted glycine.

ADDITIONAL FUNDING

» NICHD Director's Award

PUBLICATIONS

1. Farber CR, Reich A, Barnes AM, Becerra P, Rauch F, Cabral WA, Bae A, Quinlan A, Glorieux FH, Clemens TL, Marini JC. A novel IFITM5 mutation in severe atypical osteogenesis imperfect type VI impairs osteoblast production of PEDF.

J Bone Miner Res 2014; 29:1402–1411.
2. Cabral WA, Perdivara I, Weis MA, Terajima M, Blissett AR, Chang W, Makareeva EN, Mertz E, Leikin S, Tomer KB, Eyre DR, Yamauchi M, Marini JC. Abnormal type I collagen post-translational modification and crosslinking in a cyclophilin B KO mouse model of recessive osteogenesis imperfecta. *PLoS Genetics* 2014; 10(6):e1004465.
3. Reich A, Bae AS, Barnes AM, Cabral WA, Hinek A, Stimee J, Hill SC, Chitayat D, Marini JC. Type V primary osteoblasts display increased mineralization despite decreased COL1A1 expression. *Metabolism* 2015; 100(2):E325–332.
4. Forlino A, Marini JC. Osteogenesis Imperfecta: new forms reveal novel gene functions in bone development and unexpected protein-protein interactions. *Lancet, Invited Seminar. Online* 2015; in press.
5. Symoens S, Barnes AM, Gistelinck C, Malfait F, Guillemyn B, Steyaert W, Syx D, D'hondt S, Biervliet M, DeBacker J, Witten EP, Leikin S, Makareeva E, Gillessen-Kaesbach G, Huysseune A, Vleminskx K, Willaert A, DePaepe A, Marini JC, Coucke PJ. Genetic defects in TAPT1 disrupt ciliogenesis and cause a complex lethal Osteochondrodysplasia. *Am J Hum Genet* 2015; 97:521–534.

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Structural Biology of Glutamate Receptor Ion Channels

Ionotropic glutamate receptors (iGluRs) are membrane proteins that act as molecular pores permeable to sodium and calcium ions. iGluRs mediate rapid signal transmission at the majority of excitatory synapses in the mammalian nervous system, converting the pre-synaptic action potential–triggered release of glutamate into a depolarizing post-synaptic potential. The seven iGluR gene families in humans encode 18 subunits, which assemble to form three major functional families named after the ligands that were first used to identify iGluR subtypes in the late 1970s: AMPA, kainate, and NMDA. Given the essential role of iGluRs in normal brain function and development, and mounting evidence that dysfunction of iGluR activity mediates several neurological and psychiatric diseases as well as damage during stroke, we devote substantial effort to analyzing iGluR function at the molecular level. Atomic-resolution structures solved by protein crystallization and X-ray diffraction or by single-molecule cryo-EM provide a framework for designing electro-physiological and biochemical experiments aimed at defining the mechanisms underlying ligand recognition, the gating of ion channel activity, and the action of allosteric modulators. Information derived from these experiments will permit the development of subtype-selective antagonists and allosteric modulators with novel therapeutic applications and reveal the inner workings of a complicated protein machine that plays a critical role in brain function.

Key issues in the field include obtaining structures for iGluRs trapped in their three major conformational states, i.e., the resting, activated, and desensitized states, and obtaining insight into the energy landscapes connecting the states. Also of interest are the evolutionary relationships connecting iGluRs in different species and how structurally related chemosensors bind to a wide range of small molecules.

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Structural studies on full-length AMPA and kainate receptors

Our initial studies on the glutamate receptor GluK2 were limited by poor partitioning of GluK2 into the holes of holey carbon support grids, a common problem that frequently limits structural determination by single-particle cryo-electron microscopy (cryo-EM). This was overcome by depositing, on gold-coated carbon grids, a self-assembled monolayer whose surface properties were controlled by chemical modification. The procedure allowed partitioning of ionotropic glutamate receptors into the holes, thereby enabling higher resolution structural analysis than by using single-particle cryo-EM methods. Using the approach, in collaboration with the Subramaniam lab, we solved a series of structures for iGluRs trapped in different conformational states, using high-affinity ligands and allosteric modulators to determine how glutamate receptor ion channels accommodate the structural changes necessary for activation and desensitization. For the AMPA receptor GluA2, we solved structures in an antagonist-bound resting state, in a glutamate-bound active state trapped with the allosteric modulator LY451646, and in the desensitized state trapped by the high-affinity full-agonist quisqualic acid. Comparison of the closed- and active-state density maps reveals ligand-binding domain (LBD) 'clamshell' closure, as seen for isolated LBD dimers, that produces about 7 Å vertical contraction of the ATD-LBD assembly, measured as a downward movement at the top of the ATD (amino-terminal domain) tetramer, as well as unanticipated movements in the LBD, in which the dimer pairs rotate about an axis offset from the local axis of two-fold symmetry. This generates a cork screw–like motion, in which the four LBDs expand and likely unwind the iris of the M3 helix (transmembrane inner helix)–bundle closed state.

Analysis of cryo-electron microscopic images for the GluA2–desensitized state revealed evidence of substantial conformational heterogeneity at the ATD layer, precluding determination of a single desensitized-state 3D structure. Three-dimensional classification of the images enabled separation of three dominant classes at nominal resolutions of 21 Å, 23 Å, and 26 Å, with variable degrees of displacement between ATD dimers compared with the closed and active states. In all three classes, the LBD layer separates into four lobes of density, with different degrees of separation between the proximal and distal LBD subunits, strikingly different from the 'dimer-of-dimers' structure found in the closed and active states, but reminiscent of the GluK2 structure determined by tomography.

The GluK2–desensitized state was solved to a resolution of 7.6 Å and was adequate to unambiguously show that, in the desensitized state, the ion channel adopts a closed conformation in which the M3 helices form a crossed-bundle assembly with the pre-M1 helices (transmembrane outer helix) wrapped around the outside of the channel, similar to that seen for the antagonist-bound closed state, and revealed for the first time how the LBDs rearrange to permit the ion channel to close, even though the individual subunits retain a glutamate-bound closed-cleft active conformation. Desensitization occurs because, in the LBD assembly, the distal subunits swing clockwise by 125° in the horizontal plane, while the proximal subunits rotate by 13°. In the vertical plane, the distal and proximal subunits tilt 11° and 6° away from the global axis of symmetry. As a result of these movements, in the GluK2–desensitized state the LBD layer resembles an inverted pyramid, in which the four subunits are arranged with quasi four-fold symmetry, strikingly different from the two-fold symmetric dimer-of-dimers assembly in the active state. Overall, the results provide a detailed glimpse into the overall gating cycle of glutamate receptors, an evaluation of the similarities and differences in conformational changes observed in AMPA– and kainate-receptor families, and a molecular mechanism for the marked LBD movements that occur during the receptor gating cycle.

Drosophila larval neuromuscular junction glutamate receptors

The *Drosophila* larval neuromuscular junction (NMJ), at which glutamate acts as the excitatory neurotransmitter, is a widely used model for genetic analysis of synapse function and development. Despite decades of study, the inability to reconstitute NMJ glutamate receptor function using heterologous expression systems has complicated the analysis of receptor function, such that it is difficult to resolve the molecular basis for compound phenotypes observed in mutant flies. In a collaboration with Mihaela Serpe, we found that the auxiliary subunit *Drosophila* Neto functions as an essential component required for the function of NMJ glutamate receptors in heterologous expression systems, in accord with the results of genetic analysis that revealed paralysis in Neto knockouts. In combination with a crystallographic analysis of the GluRIIB ligand-binding domain, we used this system to characterize the subunit dependence of assembly, channel block, and ligand selectivity for *Drosophila* NMJ glutamate receptors.

We found that Neto weakly modulates, but is not required for, cell-surface expression of *Drosophila* iGluRs. Instead Neto profoundly increases receptor activation by glutamate. However, even with Neto, efficient receptor cell-surface expression and function requires coexpression of four different iGluR subunits, such that responses for GluRIIA/E and GluRIIA/C/E were on average still only 2–3% of the amplitude of those recorded for GluRIIA/C/D/E. Current-voltage plots for glutamate responses revealed pronounced biphasic rectification, as a result of channel block by cytoplasmic polyamines, consistent with the absence of mRNA editing for *Drosophila* iGluRs and the presence of a glutamine residue at the Q/R site of the pore loop. Argiotoxin (ATX) produced block of responses to glutamate at −60 mV for both GluRIIA/C/D/E and GluRIIB/C/D/E but with much faster recovery from block for GluRIIB/C/D/E. We then tested for activation by AMPA, kainate, and *N*-methyl-¿-aspartate (NMDA), the canonical ligands used to classify vertebrate iGluRs, and found that none of these produced functional responses, with only glutamate and quisqualate acting as agonists. The structural basis for this was revealed by the crystal structure of the GluRIIB ligand-binding domain, which revealed glutamate trapped in a cavity of volume 208 Å^3 together with three water molecules. Within domain 1 of the GluRIIB LBD structure, in the loop between β-strand 7 and α-helix D, the side chain of Asp509 forms a hydrogen bond with the hydroxyl group of Tyr481, a conserved aromatic residue that caps the entrance to the ligand-binding cavity, sealing it from extracellular solvent. Stacked above Tyr481, the side chain of Arg429 forms a cation pi interaction with the aromatic ring, further stabilizing the conformation of Tyr481. Amino-acid sequence alignments reveal that Asp509 is conserved in all *Drosophila* NMJ iGluRs, while in all vertebrate AMPA and kainate receptor subunits there is a proline at this position; likewise cation pi stacking by Arg429 is unique to GluRIIA, GluRIIB, and GluRIIC, because vertebrate AMPA and kainate receptor subunits have an isoleucine residue at this position. Consequently, AMPA, as a result of the different conformation of the isoxazazole group, is unable to bind to GluRIIB because the ligand's 5-methyl group makes steric clashes with Asp509 and Asn736. Likewise, although the ligand α-carboxyl, α-amino and γ-carboxyl groups of kainate are isosteric with those of glutamate, the isopropenyl group makes steric clashes with the Asp509 and Tyr481 side chains, thus explaining the unique ligand-binding properties of *Drosophila* larval neuromuscular junction iGluRs.

Structural studies on ctenophore glutamate receptors

Recent genome projects for ctenophores (commonly known as comb jellies) revealed the presence of numerous ionotropic glutamate receptors (iGluRs) in *Mnemiopsis leidyi* (ML) and *Pleurobrachia bachei* (Pb), which are among our earliest metazoan ancestors, perhaps evolving even before sponges and placazoans. Sequence alignments and phylogenetic analysis show that ctenophore iGluRs form a distinct clade from the well characterized AMPA, kainate, and NMDA iGluR subtypes found in vertebrates. Although annotated as glutamate and kainate receptors, crystal structures of the ML032222a and PbiGluR3 ligand-binding domains (LBDs) at resolutions of 1.21 and 1.5 Å reveal endogenous glycine in the binding pocket, while ligand-binding assays show that glycine binds with nM affinity; biochemical assays and structural analysis establish that glutamate is occluded from the binding cavity. Further analysis reveals ctenophore-specific features, such as an interdomain Arg-Glu salt bridge present only in subunits that bind to glycine, but also a conserved disulfide in loop 1 of the LBD that is found in vertebrate NMDA but not AMPA or kainate receptors. In electrophysiological experiments, we found that ML032222a forms homomeric glycine-activated ion channels, while ML05909a forms functional homomeric receptors that are activated by glutamate and for which glycine acts as a weak partial agonist. Because the affinity of ML05909a for glycine is greater than that for glutamate, and because the efficacy of glycine is so low, glycine acts as a functional glutamate antagonist, analogous to the partial agonist action of HA-966 on the GluN1 subunit of vertebrate NMDA receptors.

Could ML032222a, PbiGluR3, and related ctenophore iGluRs be relatives of NMDA receptor subunits that bind to glycine? Was binding of glycine a common feature of primitive iGluRs which subsequently evolved to bind to glutamate with high affinity? A surprising structural feature in all ctenophore iGluRs, which is revealed by our structural analysis, is a conserved disulfide bond in loop 1 that is found only in NMDA receptor subunits, including those from the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, but not in AMPA or kainate receptors. Did the ctenophore iGluRs acquire this signature after splitting from the last common ancestor of other animal families, or was this feature present in a primordial glutamate receptor that subsequently evolved to give rise to different iGluR clades? Our results highlight the difficulty of classifying the ligand-binding and functional properties of newly discovered iGluRs identified by genome sequencing projects, and suggest that attempts to classify iGluRs from invertebrate species into classes using the scheme developed for vertebrate AMPA, kainate, and NMDA receptors is not reliable and should be avoided.

PUBLICATIONS

1. Alberstein R, Grey R, Zimmet A, Simmons DK, Mayer ML. Glycine activated ion channel subunits encoded by ctenophore glutamate receptor genes. *Proc Natl Acad Sci USA* 2015; 112:E6048–57.
2. Han TH, Dharkar P, Mayer ML, Serpe M. Functional reconstitution of Drosophila melanogaster NMJ glutamate receptors. *Proc Natl Acad Sci USA* 2015; 112:6182–6187.
3. Meyerson JR, Kumar J, Chittori S, Rao P, Pierson J, Bartesaghi A, Mayer ML, Subramaniam S. Structural mechanism of glutamate receptor activation and desensitization. *Nature* 2014; 514:328–334.
4. Schauder DM, Kuybeda O, Zhang J, Klymko K, Bartesaghi A, Borgnia MJ, Mayer ML, Subramaniam S. Glutamate receptor desensitization is mediated by changes in quaternary structure of the ligand binding domain. *Proc Natl Acad Sci USA* 2013; 110:5921–5926.
5. Yao Y, Belcher J, Berger AJ, Mayer ML, Lau AY. Conformational analysis of NMDA receptor GluN1, GluN2, and GluN3 ligand-binding domains reveals subtype-specific characteristics. *Structure* 2013; 21:1788–1799.

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Autism spectrum disorder (ASD) normally exhibits onset of symptoms before three years of age and is characterized by severe impairment in reciprocal socialization and communication skills and by repetitive or restrictive behaviors. It is a heterogeneous condition of multiple etiologies; no precise clinical assessment tools currently allow accurate definition of the many variants, nor are there biological markers to distinguish the variants. A rise in the number of children identified with ASDs, from five to 72 cases per 10,000 children in the USA and Europe, and the absence of definitive forms of therapy have given rise to mounting public concern. Our studies focus on the relationship between transcriptional networks formed during normal human brain development and genes that have been associated with ASDs. The work highlights the temporal and spatial (brain region) relationship between gene pathways and human brain development. Additional studies utilize neuronal cultures derived from induced pluripotent stem cells obtained from patients with ASD for studies on gene expression and neurophysiological properties.

The human diseases of premature aging in humans are characterized by early onset of aging phenotypes that are now known to be caused by mutations in various genes. Werner's syndrome (WS) is an adult progeroid syndrome caused by mutations in the Werner syndrome, RecQ helicase-like (*WRN*) gene. The WRN protein has been implicated in a variety of biochemical processes, including DNA replication, repair, recombination, telomere maintenance, and transcription. Loss of WRN results in genomic instability and dysfunctional telomeres. Skin fibroblasts from WS patients demonstrate reduced replication potential and accelerated senescence in culture, possibly a result of dysfunctional telomeres.

We also study the biological role of RNA–binding proteins in cell fate decision. The decision between self-renewal and differentiation determines the fate of pluripotent cells in the development of an organism. Our current knowledge of the cellular control and maintenance of pluripotency is derived mainly from studies on the action of specific transcription factors (for example, Oct4, Nanog, and Sox2) that control the expression of their downstream target genes. The availability, stability, and efficiency of translation of RNA transcripts of pluripotency-related genes represent further levels of regulation in the context of cell-fate decision. We are interested in the role of RNA–binding proteins in the maintenance of pluripotency. We use embryonic stem cells and embryonal carcinoma cells as model systems in which to study the biological functions of RNA–binding proteins (Lin28a and Pumilio/Nanos families) that are preferentially expressed in pluripotent cells. We are also interested in the regulatory mechanism that controls the cell type–specific expression patterns of these gene products.

It is apparent that, in addition to protein-coding transcripts, the mammalian genome expresses a large amount of non–protein-coding RNA transcripts. Among them, several long non–protein-coding RNAs (lncRNAs) and many microRNAs (miRNAs) have been found to regulate cellular physiology by tuning the expression of other genes at transcriptional and translational levels, respectively. In mammalian cells, alterations in the expression levels of individual protein-coding transcripts by epigenetic modifiers, which promote either histone acetylation or DNA demethylation, have been documented. However, the global change in the full transcriptome (comprising protein-coding RNA transcripts and their non–protein-coding counterparts) under the influence of such modifiers has never been analyzed systematically.

Autism research

Improved strategies for early identification of specific phenotypic characteristics of and biological markers (e.g., electrophysiological changes) for ASD would improve the effectiveness of treatment. The invasiveness of collecting primary neuronal tissue from patients could be circumvented by using induced pluripotent stem cells (iPSCs) that subsequently become neuronally differentiated. The successful reprogramming of human fibroblasts into an ES cell–like (iPSC) state in the Yamanaka laboratory (Takahashi et al., *Cell* 2007;131:861) and subsequent derivation of iPSCs from patients with ALS, Parkinson disease, and other disorders into cultured neural cells has served as the proof of principle. The breakthroughs made it possible for us to generate a cell-culture model of ASD by applying the technology for subsequent neural differentiation. We thus established fibroblast cultures from patients (subjects with autism) and non-affected controls; the cells were reprogrammed into an ES cell–like state. We clone the reprogrammed cell colonies, propagate them, and induce them to differentiate *in vitro* into neuronal cultures. Based on our underlying assumption that synaptic transmission is aberrant in autism, the patient-specific neuronal cultures are used for neuronal network analysis using the photo-conductive stimulation system described by Gutiérrez et al. (*Eur J Neurosci* 2009;30:2042). Briefly, spontaneous or pulse-stimulated activity of networks is measured by fluorescent optical techniques and the structural basis of the patterns analyzed by fractal dimension analysis. We have the capacity to characterize the arrangement and complexity of the networks' axonal architecture. We confirmed the methodology with hippocampal cultures of a rat model carrying the neuroligin mutation *R471C-NL3* (identified in a subgroup of patients with ASD). We are attempting to evaluate membrane excitation and signal transduction in neural cells derived from patients with idiopathic autism.

We produced induced pluripotent cell lines (iPs) from four male children with idiopathic autism, four normal male siblings of these patients, four unaffected controls, and four patients with tuberous sclerosis; multiple clones of iP cultures from each group were established as well as neural progenitor cells and mature neural cultures from the cell lines. We developed a computer algorithm that quantitatively measures connectivity and synchronicity of activation. We are currently demonstrating proof of principle using neural cultures from hippocampal cultures derived from the mouse model of Rett syndrome. Studies are also under way using normal human neuronal cultures.

ASDs have a significant hereditary component, but the identified genetic loci are heterogeneous and complex. Consequently, there is a gap in our understanding of how diverse genomic aberrations can all result in the clinical ASD phenotype. Gene expression studies from autism brain tissue demonstrated aberrantly expressed protein-coding genes that may converge into common molecular pathways, potentially reconciling the strong heritability and shared clinical phenotypes with the disorder's genomic heterogeneity. Regulation of gene expression is extremely complex and governed by many mechanisms, including noncoding RNAs. However, no study of ASD brain tissue has assessed changes in regulatory lncRNAs, which represent a large proportion of the human transcriptome and actively modulate mRNA expression. To determine whether aberrant expression of lncRNAs plays a role in the molecular pathogenesis of ASD, we profiled, by microarray, over 33,000 annotated lncRNAs and 30,000 mRNA transcripts from postmortem brain tissue of autistic and control prefrontal cortex and cerebellum. We detected over 200 differentially expressed lncRNAs in ASD, which were enriched for genomic regions containing genes related to neurodevelopment and psychiatric disease. Additionally, comparison of differences in expression of mRNAs between the prefrontal cortex and cerebellum within individual donors revealed that ASD brains had more transcriptional homogeneity. Moreover, this was also true of the lncRNA transcriptome (1, 2, 3, 4). Our results suggest that future investigation of lncRNA expression in the autistic brain may further elucidate the molecular pathogenesis of the disorder.

Premature aging syndromes

Previous studies on the pathogenesis of WS were limited to skin fibroblasts or virus-transformed lymphocytes. Animal models of the mutant WRN helicase cannot accurately recapitulate the WS phenotype observed in humans. Reprogramming of WS cells to iPSCs may provide a cell model for the study of the pathogenesis, especially for the differentiation of WS embryonic and adult stem cells into specific, affected cell types such as skin, bone, and muscle. To establish an iPSC model of WS, we reprogrammed patient fibroblasts with the transcriptional factors OCT4, SOX2, KLF4, and c-MYC. The efficiency of reprogramming for WS was much lower than for normal fibroblasts. Given that WS fibroblasts quickly reach senescence (in less than 20 population doublings), many cells failed to form colonies. To increase the efficiency of reprogramming, we tested several inhibitors, including vitamin C, the histone deacetylase (HDAC) inhibitor valproic acid, the MAP kinase inhibitor SB203580, and a cocktail of HDAC and TGF-β RI kinase inhibitors as well as small molecules reported by Sheng Ding's group at the Gladstone Institute of Cardiovascular Disease, University of California San Francisco. Our preliminary data indicate that optimal efficiency was achieved with the HDAC and TGF-β RI kinase inhibitors; using these inhibitors, we observed the appearance of some colonies after 30 days of induction. The colonies could be expanded and expressed markers of embryonic stem cells (NANOG, OCT4, SOX2, SSEA3, SSEA4, TRA1-60, TRA1-81). By forming embryoid bodies in serum-containing medium, WS iPSCs could spontaneously differentiate into cells expressing markers of endoderm (α-fetoprotein), mesoderm (smooth muscle actin), and ectoderm (nestin).

Studies of transcription factors regulating cell renewal/proliferation

Epigenetic regulation of global transcriptome output

We have begun to examine the effect of epigenetic modifiers on transcriptome output in mammalian cells. Our preliminary analysis in several mouse cell lines indicated that the expression of specific lncRNAs is affected by the histone acetylation level. We are expanding the analysis to examine the effect of various epigenetic modifiers on full transcriptome output. From this study, we expect to identify subsets of non–protein-coding RNA transcripts that are commonly or distinctively regulated by different epigenetic modifiers, and we plan to investigate their roles in the homeostasis of the cells.

PUBLICATIONS

1. Ziats MN, Grosvenor L, Rennert OM. Functional genomics of human brain development and implications for autism spectrum. *Transl Psychiatry* 2015; 5:e665.
2. Mahfouz A, Ziats MN, Rennert OM, Lelieveldt BP, Reinders MJ. Shared pathways among autism candidate genes determined by co-expression network analysis of the developing human brain transcriptome. *J Mol Neurosci* 2015; 57(4):580–594.
3. Ziats MN, Edmonson C, Rennert OM. The autistic brain in the context of normal neurodevelopment. *Front Neuroanat* 2015; 9:115.
4. Rennert O, Lee TL. Epigenetics dynamics in development and disease. *Int J Biochem Cell Biol* 2015; 67:44.
5. Tu J, Ng SH, Shui Luk AC, Liao J, Jiang X, Feng B, Lun Mak KK, Rennert OM, Chan WY, Lee TL. MicroRNA-29b/Tet1 regulatory axis epigenetically modulates mesendoderm differentiation in mouse embryonic stem cells. *Nucleic Acids Res* 2015; 43(16):7805–7822.

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Genetic and Environmental Determinants of Primate Biobehavioral Development

We investigate primate behavioral and biological development through comparative longitudinal studies of rhesus monkeys (*Macaca mulatta*) and other primates. Our primary goals are to characterize different distinctive biobehavioral phenotypes in our rhesus monkey colony, to determine how genetic and environmental factors interact to shape the developmental trajectories of each phenotype, and to assess the long-term behavioral, biological, epigenetic, and health consequences for monkeys from various genetic backgrounds when they are reared in different physical and social environments. Another major program investigates how rhesus monkeys and other nonhuman primate species, born and raised under different laboratory conditions, adapt to placement in environments that contain specific physical and social features of their species' natural habitat. Adaptation is assessed by examining behavioral repertoires and by monitoring a variety of physiological systems in the subjects, yielding broad-based indices of relative physical and psychological health and well-being. The responses of subjects to experimental manipulation of selected features of their respective environments are also assessed in similar fashion. Whenever possible, we collect field data for appropriate comparisons. A major current focus is to investigate face-to-face interactions between mothers and infants during their initial days and weeks of life and to characterize the imitative capabilities of newborn infants and patterns of brain activity associated with imitative behavior. A second major focus is the study of cognitive and social behavioral development in capuchin monkeys (*Cebus apella*).

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Genetic and Environmental Determinants of Primate Biobehavioral Development

As in previous years, a major focus of this project were detailed longitudinal studies of the behavioral and biological consequences of differential early social rearing, most notably comparing rhesus monkey infants reared by their biological mothers in pens containing adult males and other mothers with same-age infants for their first 6–7 months of life (MR) with monkeys separated from their mothers at birth, hand-reared in the lab's neonatal nursery for their first month and then raised in small groups of same-age peers for the next six months (PR), or housed in individual cages containing an inanimate surrogate mother and given two hours of daily interaction with similarly reared peers (SPR). At 7–8 months of age, MR, PR, and SPR infants are all moved into one large pen, where they live together until puberty. Thus, the differential social rearing occurs only for the first 7–8 months; thereafter MR, PR, and SPR share the same physical and social environment. We previously demonstrated that PR monkeys cling more, play less, tend to be more impulsive and aggressive, and exhibit much greater behavioral and biological disruption during and immediately following short-term social separation at six months of age than do MR monkeys. They also exhibit deficits in serotonin metabolism (as indexed by chronically low values of cerebrospinal fluid [CSF] 5-HIAA, the major serotonin metabolite), as do SPR monkeys. Additionally, they have significantly lower levels of 5-HTT (serotonin transporter) binding throughout many brain regions than do MR subjects. Many of these differences between MR and PR monkeys persist throughout the childhood years in the absence of experimental interventions. More recently, we published data extending these rearing condition differences to include patterns of brain lateralization, cortisol concentration in hair (a measure of chronic hypothalamic-pituitary-adrenal [HPA] activity), and measures of brain structure and function, as assessed by structural MRI and positron emission tomography (PET), respectively. Additional differences in measures of social dominance status, maternal competence, telomere length, and physical health during childhood, adolescence, and adulthood were also documented. However, more recent studies indicated that many of these rearing condition differences in behavioral, biological, and health outcomes appear to be largely reversible following specific social interventions.

Another major focus of recent research for this project was to characterize interactions between differential early social rearing and polymorphisms in several candidate genes (G X E interactions), most notably the 5-HTTLPR polymorphic region of the *5-HTT* gene. During the past two years, we expanded the range of outcomes for which G×E interactions involving the 5-HTTLPR polymorphism and early rearing condition differences appear, including social play and behavioral reactions to a variety of social stressors, and in epigenetic regulation of brain activity. In addition, we recently reported significant G x E interactions between early MR vs. PR rearing and polymorphisms for several other candidate genes including: *DRD1*, which encodes the dopamine receptor D1; *NPY,* which encodes neuropeptide Y; *OPRM1*, which encodes the mu opioid receptor 1; *BDNF*, which encodes brain-derived neurotrophic factor; *NOS-1*, which encodes nitric oxide synthase 1 neuronal; and a single-nucleotide polymorphism (SNP) in the glucocorticoid gene, with outcome measures including play behavior, social buffering, behavioral and HPA reaction to an unfamiliar conspecific, naloxone treatment, alcohol consumption, and plasma BDNF concentrations. In virtually every case a similar pattern was observed, i.e., the less efficient (from a transcriptional point of view) allele was associated with a negative outcome among PR–reared monkeys but a neutral or, in some cases, even an optimal outcome for MR–reared subjects carrying that same less efficient allele, suggesting an overall buffering effect of MR rearing for individuals carrying these so-called risk alleles.

Additionally, we recently published the results of two sets of studies investigating the effects of differences in early social rearing (MR vs. SPR) on genome-wide patterns of mRNA expression in leukocytes, and on methylation patterns in prefrontal cortex (PFC) and in T-cell lymphocytes. Our research involving mRNA expression, carried out in collaboration with Steven Cole and James Heckman, examined expression patterns in differentially reared 4-month-old infants. In all, 521 different genes were significantly more expressed in MR infants than in SPR infants, whereas the reverse was the case for another 717 genes. In general, SPR–reared infants showed enhanced expression in genes involved in inflammation, T-lymphocyte activation and cell proliferation, and suppression of antiviral and antibacterial responses, a pattern curiously also seen in leukocyte expression in adult humans who perceive themselves as socially isolated. Since that initial study, we completed a prospective longitudinal study in which differentially reared subjects were sampled at 14 days, 30 days, 6–7 months, and every three months thereafter until they reached puberty. Data analyzed to date revealed that the above rearing-condition differences in genome-wide patterns of mRNA expression in leukocytes persist throughout development in the absence of any changes in the social environment but change dramatically whenever the social environment is altered during the juvenile years.

The other set of studies, carried out in collaboration with Moshe Szyf and his lab, involved genome-wide analyses of methylation patterns in differentially reared monkeys when they were adults. The initial study compared such patterns in PFC tissue and T-cell lymphocytes obtained from 8-year-old monkeys differentially reared for their initial 6–7 weeks of life and thereafter maintained under identical conditions until adulthood. The analyses revealed that (a) more than 4,400 genes were differentially methylated in both PFC and lymphocytes; (b) although there was considerable tissue specificity, approximately 25% of the affected genes were identical in both PFC and lymphocytes, and (c) in both PFC and lymphocytes, methylated promoters tended to cluster both by chromosomal region and gene function. This past year, we completed a prospective longitudinal study of genome-wide methylation patterns in lymphocytes, collecting samples from exactly the same MR and SPR monkeys at exactly the same time points as in the aforementioned longitudinal study of mRNA expression. Preliminary finding suggest that, at least in lymphocytes, extensive effects of rearing conditions are present within the first month of life but can at least in part be significantly minimized and/or re-directed subsequently following a social environmental intervention utilizing "foster" grandparents.

In another collaboration with the Szyf lab, we examined the epigenetic consequences of high vs. low ranking in established social groups of adult female monkeys and in offspring whose relative social dominance status matched that of their mothers. It appeared that the cross-generational transmission of social status was mediated, at least in part, by the placenta, in that the genome-wide pattern of methylation in tissues collected from placentas immediately after birth differed dramatically between offspring of high- and low-ranking females; not only did the order of magnitude of these differences match that of the above-mentioned early social rearing condition differences, but many of the same genes were involved, suggesting the existence of a subset of “early adversity” genes, i.e., genes sensitive to a range of different early life adversities.

Mothers interact emotionally with their newborns through exaggerated facial expressions and mutual gaze, a capacity that has long been considered uniquely human. We previously initiated a research program on early face-to-face interactions in rhesus monkeys after we serendipitously discovered that very young rhesus monkey infants did, in fact, engage in extensive face-to-face interactions with their mothers, but only during the first month of life. This past year, we further characterized face-to-face interactions between mothers and their newborn infants in a naturalistic setting. We found large individual variability in rates of maternal/infant face-to-face interactions, in that mothers who had only one or two infants engaged in mutual gazing/lip-smacking in the first 30 days of life significantly more than mothers who had had three or more infants, whereas the more experienced mothers let their infants out of arms’ reach significantly more in the first 30 days of life than newer mothers. Overall, mothers tended to engage in more face-to-face interactions with their male infants.

We also discovered that, during their first week, some (but not all) infants could accurately match certain facial gestures produced by a human experimenter, even after a delay. For those infants who could imitate in this fashion, the capability was evident on the first postnatal day. We have since investigated brain activity during periods of imitation using scalp electrodes to record EEG activity and found a distinctive EEG signature involving significant suppression of mu rhythm activity at low frequencies in frontal and parietal brain regions exclusively during periods of imitation. We reported that this pattern of EEG activity intensified through that first week and was significantly stronger in mother-reared than in nursery-reared neonates. The findings demonstrate similarities between infant human and infant monkey EEG during periods of imitation.

We also demonstrated, using eye-tracking technology, that week-old infants readily respond to a computer-generated dynamic monkey avatar, and that those infants who imitate tend to focus on different aspects of the avatar’s face (eyes and mouth) compared with those that do not imitate (mouth only). We also compared neonatal imitation abilities in mother-reared and nursery-reared monkeys, focusing on day 3 performance only. We reported that, even though nursery-reared (NR) infants show an imitation effect when tested over the first week, they do not exhibit imitation specifically on day 3. In contrast, MR monkeys responded to facial gestures with more gestures themselves, consistent with our previous EEG findings that MR infants show larger mu suppression than NR infants when viewing facial gestures.

Given the potential impact of neonatal imitation on infants’ social, cognitive, and emotional development, we devised one intervention whereby NR infants either received additional facial gesturing from a human caretaker, received additional handling (but did not see facial gestures), or remained in standard nursery rearing. We found that only the group that had received facial gesturing showed improved performance on the standard neonatal imitation task on day 7 as well as greater sensitivity to facial identity of others in a standardized stranger task. Infants from the facial gesturing group also showed increased preference for a social video at day 30 and again at day 40, had better memory for social stimuli when tested at day 60, and had higher levels of social contact with peers from day 40 to day 60 than did infants in the handling and standard rearing groups.

A second intervention designed to increase infants’ social perception and social sensitivity looked at the effects of oxytocin on infants’ social interactions. NR infants were nebulized with either oxytocin or saline and then tested in an imitation recognition task. We reported increased time spent looking at faces following oxytocin, but not saline, treatment. Salivary assays confirmed increased levels of oxytocin, and infants also showed increased affiliative gesturing towards a human experimenter following oxytocin administration.

We further explored infants’ facial processing strategies by presenting them with various faces and facial configurations on a remote eye tracker. Rhesus macaque infants generally prefer faces with normally arranged features over faces with linearly arranged features, suggesting a special sensitivity to faces and face-like stimuli. The preferences are particularly strong for faces of conspecifics, which suggests a genetic predisposition towards rhesus faces in particular. We also reported that neonatal imitators, but not non-imitators, exhibit particular sensitivities towards the eye region, which may indicate that neonatal imitation and differential social sensitivity are intricately linked.

A project begun last year involved the analysis of mothers’ milk in rhesus monkeys with respect to parity and early life history (i.e., rearing condition). In collaboration with Katie Hinde, we collected milk samples from mothers over the first 30 days of their infant's life and analyzed the samples for cortisol content and nutrient composition. Similar to Hinde's studies of human mothers' milk in older infants, we found that parity predicted milk yield volume (MYE) in the first month of life. Our findings also indicated that mothers with higher hair cortisol during pregnancy had a higher MYE in the first 30 days of life. Additionally, we found that cortisol levels in mothers' milk predicted infant cognitive functioning and social behavior later in life. Infants who ingested milk with higher cortisol content were less impulsive in a cognitive task but also initiated social behaviors with peers less frequently.

We used hair cortisol as a measure of chronic HPA activity in three additional studies completed this past year. First, hair cortisol levels shortly after birth, presumably reflecting prenatal HPA activity from mid-gestation onward, predicted cognitive performance capabilities and infant temperament in the first postnatal months. Second, changes in hair cortisol concentrations during the juvenile years predicted differences in social dominance status among adult female monkeys.

A third project centered on the incidence of alopecia and related physiological processes. We had previously observed that many females undergo severe hair loss during pregnancy, only to regain full hair growth in the two months postpartum. In collaboration with Melinda Novak and Jerrold Meyer, we examined the role of chronic HPA axis activity, as assessed by hair cortisol concentrations in alopecia. Our early results indicate that overall concentrations change across pregnancy and that monkeys that exhibit the greatest amount of hair loss have higher hair cortisol concentrations than those that do not.

We continued our research program on personality and facial characteristics with our capuchin monkeys, focusing on five personality dimensions (Assertiveness, Openness, Neuroticism, Sociability, and Attentiveness), and found that the monkeys' facial width-to-height ratio, as well as their face width/lower face height, are positively and significantly associated with Assertiveness. A lower face width/face height ratio was also associated with neuroticism. This past year, we also provided some of our capuchins with stone tools and observed for the first time in our colony spontaneous use of those tools to crack open walnuts. Nut-cracking has been observed in a few isolated wild populations of this species but is clearly far from universal. We plan to study its pattern of propagation in our captive colony, especially among juvenile and adolescent group members.

PUBLICATIONS

1. Dettmer, AM, Rosenberg KL, Suomi SJ, Meyer JS, Novak MA. Associations between parity, hair hormone profiles during pregnancy and lactation, and infant development in rhesus monkeys (Macaca mulatta). *PLoS One* 2015; 10:e0131692.
2. Dettmer AM, Woodward RA, Suomi SJ. Reproductive consequences of a matrilineal overthrow in rhesus monkeys. *Am J Primatol* 2015; 77:346–352.
3. Paukner A, Simpson EA, Ferrari PF, Mrozek T, Suomi SJ. Neonatal imitation predicts how infants engage with faces. *Dev Sci* 2015; 17:833–840.
4. Sclafani V, Paukner A, Suomi SJ, Ferrari PF. Imitation promotes affiliation in infant macaques at risk for impaired social behaviors. *Dev Sci* 2015; 18:614–621.
5. Vanderwert RE, Simpson EA, Paukner A, Suomi SJ, Fox NA, Ferrari PF. Early social experience affects neural activity to affiliative facial gestures in newborn nonhuman primates. *Dev Neurosci* 2015; 37:243–252.

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Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells

Under optimal conditions, the fidelity of DNA replication is extremely high. Indeed, it is estimated that, on average, only one error occurs for every 10 billion bases replicated. However, given that living organisms are continually subjected to a variety of endogenous and exogenous DNA–damaging agents, optimal conditions rarely prevail *in vivo*. While all organisms have evolved elaborate repair pathways to deal with such damage, the pathways rarely operate with 100% efficiency. Thus, persisting DNA lesions are replicated, but with much lower fidelity than in undamaged DNA. Our aim is to understand the molecular mechanisms by which mutations are introduced into damaged DNA. The process, commonly referred to as translesion DNA synthesis (TLS), is facilitated by one or more members of the Y-family of DNA polymerases, which are conserved from bacteria to humans. Based on phylogenetic relationships, Y-family polymerases may be broadly classified into five subfamilies: DinB-like (polIV/pol kappa–like) proteins are ubiquitous and found in all domains of life; in contrast, the Rev1-like, Rad30A (pol eta)–like, and Rad30B (pol iota)–like polymerases are found only in eukaryotes, and the UmuC (polV)–like polymerases only in prokaryotes. We continue to investigate TLS in all three domains of life: bacteria, archaea, and eukaryotes.

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Prokaryotic DNA repair and mutagenesis

Most damage-induced mutagenesis in *Escherichia coli* is dependent upon the UmuD′C protein complex, which comprises DNA polymerase V (pol V). Pol V has intrinsically weak catalytic activity, but is dramatically stimulated by interactions with ATP and the bacterial recombinase RecA to form a functional complex called the DNA/pol V mutasome (pol V Mut). In a collaborative study with Michael Cox and Myron Goodman, we recently identified the protein-protein interface between UmuC and RecA that is critical for pol V activation (Reference 1). This was achieved by using mutant RecA proteins, a range of *in vitro* and *in vivo* approaches, and a photo-activated unnatural amino acid at RecA N113.

In particular, we identified a RecA surface defined by residues 112–117 that either directly interacts with, or is in very close proximity to, amino acid residues on two distinct surfaces of the UmuC subunit of pol V. One surface is uniquely prominent in the active pol V Mut. Several conformational states are populated in the inactive and active complexes of RecA with pol V. The RecA D112R and RecA D112R N113R double mutant proteins exhibit successively reduced capacity for pol V activation. The double mutant RecA is specifically defective in the ATP–binding step of the activation pathway. Unlike the classic non-mutable RecA S117F (*recA1730*), the RecA D112R N113R variant exhibits no defect in filament formation on DNA and promotes all other RecA activities efficiently. An important pol V activation surface of RecA protein is thus centered in a region encompassing amino acid residues 112, 113, and 117, a surface exposed at the 3′-proximal end of a RecA filament. The same RecA surface is not utilized in the RecA activation of the homologous and highly mutagenic RumA′$_2$B polymerase encoded by the integrating-conjugative element (ICE) R391, indicating a lack of structural conservation between the two systems. The RecA D112R N113R protein therefore represents a new separation of function mutant, proficient in all RecA functions except SOS mutagenesis.

We extended our studies on the regulation of pol V through a collaboration with Antoine van Oijen and Andrew Robinson. In particular, we focused on the hitherto under-appreciated spatial regulation imposed on pol V in *Escherichia coli* (Reference 2). Spatial regulation is often encountered as a component of multi-tiered regulatory systems in eukaryotes, where processes are readily segregated by organelle boundaries. Well characterized examples of spatial regulation are much less common in bacteria. Given its mutagenic potential, pol V activity has previously been shown to be controlled by means of an elaborate regulatory system at both the transcriptional and post-translational levels. However, by using single-molecule fluorescence microscopy to visualize UmuC inside living cells in space and time, we demonstrated that pol V is also subject to a novel form of spatial regulation. After an initial delay (about 45 min) post UV irradiation, UmuC is synthesized, but is not immediately activated. Instead, it is sequestered at the inner cell membrane. The release of UmuC into the cytosol requires cleavage of UmuD to UmuD′ mediated by the nucleoprotein filament RecA* (active RecA filaments on single-stranded DNA). Classic SOS damage response mutants either block [*umuD*(K97A)] or constitutively stimulate [*recA*(E38K)] UmuC release from the membrane. Furthermore, foci of mutagenically active pol V Mut (UmuD′$_2$C-RecA-ATP) formed in the cytosol after UV irradiation do not co-localize with pol III replisomes, suggesting the capacity to promote translesion DNA synthesis at lesions skipped over by DNA polymerase III. In effect, at least three molecular mechanisms limit the amount of time that pol V has to access DNA: (1) transcriptional and post-translational regulation that initially keep the intracellular levels of pol V to a minimum; (2) spatial regulation via transient sequestration of UmuC at the membrane, which further delays pol V activation; and (3) the hydrolytic activity of a recently discovered pol V Mut ATPase function that limits active polymerase time on the chromosomal template.

Eukaryotic DNA repair and mutagenesis

Our studies on the eukaryotic TLS polymerases focused on *Saccharomyces cerevisiae* polymerase eta (pol eta) and human polymerase iota (pol iota). Pol eta is best characterized for its ability to perform accurate and efficient TLS through cyclobutane pyrimidine dimers (CPDs). To ensure accurate bypass, the polymerase is not only required to select the correct base but also discriminate between NTPs and dNTPs. Most DNA polymerases have a conserved ‘steric gate’ residue, which functions to prevent incorporation of NMPs during DNA synthesis. In our collaborative study with Susana Cerritelli and Robert Crouch, we demonstrated that the Phe35 residue of *S. cerevisiae* pol eta functions as a steric gate to limit the use of ribonucleotides during polymerization both *in vitro* and *in vivo* (Reference 3). Unlike the related pol iota enzyme, wild-type pol eta does not readily incorporate NMPs *in vitro*. In contrast, a pol eta F35A mutant incorporates NMPs on both damaged and undamaged DNA *in vitro* with a high degree of base selectivity. An *S. cerevisiae* strain expressing pol eta F35A (*rad30*-F35A) that is also deficient for nucleotide excision repair (*rad1*delta) and the TLS polymerase pol zeta (*rev3*delta) are extremely sensitive to UV light. The sensitivity is attributable, in part, to RNase H2 activity, as an isogenic *rnh201*delta strain is roughly 50-fold more UV–resistant than its *RNH201*$^+$ counterpart. Interestingly the *rad1*delta *rev3*delta *rad30*-F35A *rnh201*delta strain exhibits a significant increase in the extent of spontaneous mutagenesis with a spectrum dominated by 1 bp deletions at runs of template thymine nucleotides. We hypothesized that the increased mutagenesis is the result of adenine ribonucleotide (rA) incorporation at these sites and that the short poly rA tract is subsequently repaired in an error-prone manner by a novel repair pathway that is specifically targeted to polyribonucleotide tracks. These data indicate that, under certain conditions, pol eta can compete with the cell’s replicases and gain access to undamaged genomic DNA. Such observations are consistent with a role for pol eta in replicating common fragile sites (CFS) in human cells.

Our studies on the human TLS polymerases focused on how they are regulated by post-translational modification. Indeed, both human pol eta and pol iota were previously shown to be monoubiquitinated *in vivo* (Reference 4). Pol eta was shown to be ubiquitinated at one primary site. When this site is unavailable, three nearby lysines may become ubiquitinated. In contrast, mass-spectrometry analysis of monoubiquitinated pol iota revealed that it is ubiquitinated at over 27 unique sites (Reference 5). Many of these sites are located in different functional domains of the protein, including the catalytic polymerase domain, the PCNA–interacting region, the Rev1–interacting region, as well as its Ubiquitin Binding Motifs, UBM1 and UBM2. Pol iota monoubiquitination remains unchanged after cells are exposed to DNA–damaging agents such as UV light (generating UV photoproducts), ethyl methanesulfonate (generating alkylation damage), mitomycin C (generating interstrand crosslinks), or potassium bromate (generating direct oxidative DNA damage). However, when exposed to naphthoquinones, such as menadione and plumbagin, which cause indirect oxidative damage through mitochondrial dysfunction, pol iota becomes transiently polyubiquitinated via K11– and K48–linked chains of ubiquitin and subsequently targeted for degradation. Polyubiquitination does not occur as a direct result of the perturbation of the redox cycle, as no polyubiquitination was observed after treatment with rotenone or antimycin A, which inhibit mitochondrial electron transport. Interestingly, polyubiquitination was observed after the inhibition of the lysine acetyltransferase KATB3/p300. We hypothesize that the formation of polyubiquitination chains attached to pol iota occurs via an interplay between lysine acetylation and ubiquitination of ubiquitin itself at K11 and K48 rather than oxidative damage per se.

ADDITIONAL FUNDING

» NIH Director's Challenge Award
» U01HD085531-01: Replisome dynamics in M. tuberculosis: linking persistence to genetic resistance

PUBLICATIONS

1. Gruber AJ, Erdem AL, Sabat G, Karata K, Jaszczur MM, Vo DD, Olsen TM, Woodgate R, Goodman MF, Cox MM. A RecA protein surface required for activation of DNA polymerase V. *PLoS Genet* 2015; 11(3):e1005066.
2. Robinson A, McDonald JP, Caldas VE, Patel M, Wood EA, Punter CM, Ghodke H, Cox MM, Woodgate R, Goodman MF, van Oijen AM. Regulation of mutagenic DNA polymerase V activation in space and time. *PLoS Genet* 2015; 11(8):e1005482.
3. Donigan KA, Cerritelli SM, McDonald JP, Vaisman A, Crouch RJ, Woodgate R. Unlocking the steric gate of DNA polymerase eta leads to increased genomic instability in Saccharomyces cerevisiae. *DNA Repair* 2015; 35:1–12.
4. McIntyre J, McLenigan MP, Frank EG, Dai X, Yang W, Wang Y, Woodgate R. Posttranslational regulation of human DNA polymerase iota. *J Biol Chem* 2015; 290:27332–44.
5. McIntyre J, Woodgate R. Regulation of translesion DNA synthesis: posttranslational modification of lysine residues in key proteins. *DNA Repair* 2015; 29:166–179.

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Administrative Management Branch

The Administrative Management Branch (AMB) in the Division of Intramural Research (DIR), NICHD, assists in the planning and managing of a variety of administrative management projects. The AMB provides administrative oversight for adherence to rules and regulations and expertise in administrative services to assure that the NICHD continues to move forward in its mission. The AMB staff are a key component and resource to the Scientific Director for the management and overall planning for the DIR.

The senior leadership within the AMB works directly with the Scientific Director and Deputy Scientific Director, particularly on strategic planning and administrative oversight, and plays a key role in maximizing the resources available to the DIR. The leadership provides guidance in all aspects of administration, represents the DIR at various NICHD programs and NIH–wide committees as well as focus groups concerned with administration.

The AMB administrative staff provide professional, technical, and administrative support in functional areas that further the mission of the DIR. The functional areas include, among others, budget and financial management, human resources, visas, travel, simplified acquisition, contract management and project officer support, safety and security, building and facilities management, timekeeping, program planning and evaluation, and general administrative services.

The AMB staff serve as a liaison between the laboratories/branches that they support and the many other entities at the NIH, such as the Office of the Scientific Director, NICHD; the Office of the Director, NICHD; the Office of Intramural Research, OD; the Fogarty International Center; Human Resources offices; the Office of Research Services including the Division of International Services; the Office of Research Facilities; and the Technology Transfer Branch.

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The Research Animal Medicine Branch (RAMB) is responsible for directing the program of laboratory animal care and use for NICHD's Division of Intramural Research (DIR). The program includes animal facilities managed by NICHD as the lead institute, NICHD animals housed in other facilities, and all activities involving NICHD DIR–owned animals. The Branch (1) provides primary veterinary care; (2) advises the NICHD Scientific Director on animal care and use practices; (3) assures that animal use within DIR is in accordance with applicable regulatory standards; (4) coordinates intramural animal use, including appropriate animal model selection, support requirements, and Animal Study Proposal review; (5) advises scientific staff on comparative medicine, Animal Study Proposal design, disease interference, and on other factors that may complicate or invalidate research results; (6) implements and coordinates animal health monitoring; (7) coordinates quarantine for incoming animals of unknown health status to prevent the introduction of agents pathogenic to humans or animals; (8) coordinates a central ordering program for the NICHD; (9) provides administrative management of the NICHD Animal Care and Use Committee (ACUC); and (10) interfaces with organizations and institutions concerned with the ethical and humane care and use of animals in research.

NICHD manages three Shared Animal Facilities (SAF) as the Lead Institute: the Bldg 6B rodent SAF, the NIH Animal Center (NIHAC) Non-Human Primate SAF in Poolesville, MD, and the Bldg 6 Shared Zebrafish Facility.

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Tannia S. Clark, DVM, *Facility Veterinarian, 6B Shared Animal Facility/6 Shared ZF Facility*
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Lauren Pandolfo, BS, MS, *Aquatics Facility Manager*
Klara Post, MS, *Animal Care and Use Committee Coordinator*

RAMB supports animal use research in the NICHD Division of Intramural Research.

The RAMB operates and manages the Building 6B Shared Animal Facility (SAF), the NIH Animal Center (NIHAC) SAF, Suite 6C127 of the Ambulatory Care Research Facility (ACRF) Animal Facility, and the NICHD aquatics facilities. The DIR Animal Program and ACUC have oversight over these facilities regarding animal use as well as over NICHD and NIA animals in the Building 49 SAF, the Porter Neurosciences Research Center (PNRC) SAF, the 10A Central Animal Facility (CAF), and in CAFs managed by the Division of Veterinary Resources (DVR) including Building 14F. The RAMB has contractual oversight over an aquatic animal husbandry task that includes husbandry and research support for NICHD, NHGRI, NHLBI, and NCI.

The Building 6B SAF supports the animal research activities of four Institutes (NICHD, NIAID, NEI, and NIAMS) in a restricted-access disease-free rodent facility; it also includes a room for *Xenopus* and a zebrafish facility.

The NIHAC SAF supports the animal research activities of three Institutes (NICHD, NIMH, and NCI). This facility houses nonhuman primates and rodents. Animal holding areas include indoor housing, indoor/outdoor runs, seasonal outdoor housing, and a five-acre free-ranging primate field station that is located adjacent to permanent structures.

Suite 6C127 of the ACRF Animal Facility supports the animal research activities of two Institutes (NICHD and NIA). The facility occupies 121 m^2

(1,299 ft^2) in Building 10 and has five animal rooms and two procedure rooms and provides care and housing for rodent and aquatic species. It is a restricted-access conventional facility and is operated under contract.

The Bldg 6 Shared Zebrafish Facility (SZF) supports NICHD and NHGRI with 15,000 2-liter tanks; the total capacity of the SZF is approximately 330,000 zebrafish.

As part of the NIH, the RAMB participates in the formulation of policies and procedures that impact the care and use of laboratory animals throughout the country. In 2011, the RAMB led the effort for triennial re-certification by the Association for the Assessment and Accreditation of Laboratory Care, International (AAALACi). The RAMB and various animal-user investigators have been active contributors to the NIH Animal Research Advisory Committee's (ARAC) efforts to adopt the new "Guide to Care and Use of Laboratory Animals," which is a primary-source set of guidelines used by the AAALACi and the NIH Office of Animal Welfare.

ADDITIONAL FUNDING

» In addition to direct funding by the Intramural Research Programs of NICHD, the RAMB is also funded by facility users from other NIH Institutes and Centers.

PUBLICATIONS

1. Strykowski JL, Schech JM. Effectiveness of recommended euthanasia methods in larval zebrafish (Danio rerio). *J Am Assoc Lab Anim Sci* 2015;54:81–84.

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NICHD Biomedical Mass Spectrometry Core Facility

The NICHD Biomedical Mass Spectrometry Core Facility was created under the auspices of the Office of the Scientific Director to provide high-end mass-spectrometric services to scientists within the NICHD Division of Intramural Research (DIR). Particular focus has been in the areas of proteomics, biomarker discovery, protein characterization, and detection of post-translational modifications. The Facility also performs quantitative analyses of small bio-molecules, including lipids and steroids. In addition, the facility develops and modifies methods for the isolation and detection of biomolecules by mass spectrometry, as well as novel methods for data analysis. The Facility is located in the 9D corridor of Building 10 on the NIH campus.

Peter S. Backlund, PhD, *Staff Scientist, Acting Director*
Vince Pozsgay, PhD, *Staff Scientist*
Nancy E. Vieira, MS, *Senior Research Assistant*
Alfred L. Yergey, PhD, *Scientist Emeritus*

Mode of operation

The Facility is available to all labs within the DIR, provided that existing resources are distributed equally among investigators requesting services. The philosophy of the Facility is to ensure that its instruments obtain only reliable, high-quality data and that its clients receive only statistically meaningful analyses. The Facility's staff are available for consultation on both project design and data interpretation. Before the start of a project, staff members meet with the Principal Investigator (PI) and other scientists involved in each study to discuss experimental goals and data requirements. The Facility has an internationally recognized capability in characterization of proteins and peptides by mass spectrometry, including: (1) identification of proteins isolated by electrophoresis; (2) confirming molecular weights of recombinant or synthetic proteins and peptides; (3) determining sites of specific post-translational modifications including phosphorylation, glutamylation, AMPylation, and disulfide bond formation; (4) quantification of specific post-translational modifications; and (5) sequencing peptides *de novo*. In addition, the Facility has extensive experience and skill in the identification and quantification of small endogenous molecules including phospholipids, steroids, and sugars. In this latter area, the capability is primarily in quantification of endogenous levels of particular molecules and their metabolites.

Instrumentation

The facility currently has four mass spectrometers in use for specific areas of analysis.

ABI 4800 MALDI TOF/TOF

The state-of-the-art high-performance MALDI TOF (matrix-assisted laser desorption/ionization) TOF/(time-of-flight) instrument can be operated in both positive- and negative-ion modes and is used for peptide identification in peptide mixtures without chromatographic separation. Methodology is also available to perform off-line liquid chromatography (LC) separation and sample spotting. Additional uses include relative peptide quantification for iTRAQ (isobaric tags for relative and absolute quantitation)–labeled peptides and sequence determination through *de novo* sequencing techniques for unusual peptides not present in gene-based protein databases.

Agilent 6560 Ion Mobility-qTOF

The state-of-the art instrument was delivered and installed in early 2014. It couples an ion-mobility-drift cell with a high-resolution qTOF mass spectrometer. Ion mobility spectrometry (IMS) provides an added dimension of sample separation that is orthogonal to both chromatography and mass spectrometry. The instrument is currently being used to determine collision cross-section measurements of ions for small molecules and intermolecular complexes and well as separation and analysis of complex mixtures of lipids and peptides.

Agilent 6460 LC-ESI QqQ (Triple Quad)
The instrument is currently being used for small-molecule analysis and quantification, principally for steroid profiling, and determination of amino acid and glycolytic pathway metabolites.

ABI Voyager MALDI TOF
The instrument is used for the analysis of protein mixtures and to verify molecular weights of intact proteins. It is also available for general use after a prospective user has undergone appropriate training.

Facility usage
The Mass Spectrometry Facility currently serves between 10–15 sections within the institute, representing about 40 projects, as well as two PIs of sister institutes on the NIH campus and collaborations with five outside institutions.

Major projects
Protein quantification in tissues and cerebral spinal fluid (CSF) using isobaric tags
Protein quantification is an important aspect of proteome characterization and is crucial to understanding biological mechanisms and human diseases. Discovery-based or un-targeted studies have often used covalent tagging strategies (i.e., iTRAQ®, TMT™) for which reporter ion signals collected in the tandem MS experiment are used for quantification. However, it has been difficult to establish the relative changes in reporter ion signals that are required to detect significant changes at the protein level. We studied the behavior of iTRAQ 8-plex chemistry using MALDI-TOF/TOF instrumentation. To better understand the behavior of the reporter ions, we evaluated the use of within-spectra normalization, which we termed 'row-normalization.' When applied to replicate protein mixtures of equal concentration, the reporter ion ratios were found to have a normal Gaussian distribution around the expected ratio of 0.125. Therefore, the width of the distribution can be used to establish a confidence level for a given reporter ion ratio.

We developed a method to compare relative levels of specific proteins in the cerebrospinal fluid (CSF) from patients, using the 8-plex iTRAQ labeling process to mass-tag peptides generated from proteins present in each sample. The method will maintain individual patient information and provide the ability to compare results across multiple iTRAQ experiments. Initially, we used the *Npc1* knock-out mouse model to determine the analytical variation of the method. The method is now being applied to CSF from patients with Smith-Lemli-Opitz syndrome (SLOS) or Niemann-Pick disease, type C1 (NPC-1), in order to profile protein changes and identify biochemical alterations correlated with disease progression and treatments. One family of proteins elevated in CSF from SLOS patients was the granin protein family. Examples include Chromogranin A, Secretogranin 3, Neurosecretory protein VGF, and ProSAAS. These secretory granule proteins are involved in the neuroendocrine secretory pathway and are stored and released with neuropeptides and hormones via a regulated exocytosis mechanism.

Gel-based proteomic studies in human genetic disorders
In collaboration with NICHD DIR clinical groups, several studies were performed by the facility to measure changes in protein expression in several animal models of human genetic disorders or in tissues from patients with the genetic disorders. Changes in protein expression were quantified by the intensity of spot staining for proteins separated by two-dimensional gel electrophoresis (2D-GE). Differentially expressed proteins were then identified by in-gel digestion and peptide analysis by MS/MS fragmentation. Studies using this approach were previously completed by this lab for two mouse models of human diseases: (1) Niemann-Pick Disease-Type C1 (NPC1); and (2) the tumor aggressiveness of pheochromocytomas/paragangliomas. Currently, we are using the approach to identify changes in protein expression in adrenal gland tissue from patients with a variety of adrenal-gland disorders, for which the genetic defect may not yet be determined.

Identification of amyloidosis-associated proteins in clinical samples
We developed a non-targeted approach to identify amyloidosis-associated proteins. The method was used to identify leukocyte cell–derived chemotaxin-2 (LECT2) as a component of amyloid plaques in adrenal tissue. Although the patient's adrenal tissue was positive for amyloid by Congo Red staining, specific immunostaining for proteins commonly known to form amyloid

plaques were all negative. In our non-targeted method, plaque proteins from disease tissue were extracted and separated by 1D SDS/PAGE. After digestion of the gel bands, we identified serum-amyloid P-component and LECT2 as components of the plaques. LECT2 has been reported in amyloid plaques in kidney tissue, but this is the first observation for this protein causing amyloidosis in adrenal tissue. The high accumulation of LECT2 in adrenal amyloid plaques from this patient was confirmed by Western blots using a specific anti-LECT2 antibody. The method demonstrates the advantage of using a non-targeted approach to detect novel proteins involved in amyloidosis.

Ion mobility mass spectrometry for detection of isobaric lipids and ion complexes

The Agilent Model 6560 Ion Mobility Q-TOF LC/MS instrument combines an ion mobility drift cell in front of a high resolution Q-TOF mass spectrometer. Implementing ion mobility spectrometry (IMS) prior to mass analysis adds a dimension of separation to sample analysis that is orthogonal to both chromatography and mass spectrometry. Given that IMS operates on a millisecond time scale, the device offers the ability to perform separations of complex mixtures at a much higher rate than is possible with liquid chromatography. In addition, IMS separations are associated with collision cross section (CCS) of ions (CCS is essentially a 'shape' parameter of ions in the gas phase), so that molecules of identical molecular weights can be separated on the basis of their CCS, which has implications for separations of isobaric biomolecules, including numerous steroids, lipids, and peptides. IMS also offers the ability to study intermolecular complexes and determine their stoichiometry. One of the first studies to be undertaken was to investigate beta-cyclodextrin–cholesterol complexes; preliminary results of the measurements suggest formation of a trimeric complex that incorporates calcium ions as a bridge. In addition, pH–dependent changes in cyclodextrin conformations have been suggested by the observation of two distinct drift times of isobaric species and by an indication of changes in CCS attributable to two distinct conformations of the ions. We have begun molecular modeling studies of the cyclodextrin molecules to explain the two conformational states.

Quantitation of plasma melatonin (5-methoxy-*N*-acetyltryptamine) and *N*-acetyltryptamine

A multiple-reaction-monitoring (MRM)–based assay was developed in the Facility to quantify *N*-acetyltryptamine and melatonin in plasma. *N*-acetyltryptamine is a melatonin-receptor mixed agonist/antagonist. The assay provided the first evidence for the presence of *N*-acetyltryptamine in plasma from human, rats, and rhesus monkeys. The liquid chromatography/tandem mass spectrometric method employs deuterated internal standards to quantitate *N*-acetyltryptamine and melatonin. *N*-acetyltryptamine was detected in daytime plasma from human volunteers, rhesus macaques, and rats. Twenty-four-hour studies of rhesus macaque plasma revealed that *N*-acetyltryptamine increases at night to concentrations that exceed those of melatonin. The findings establish the physiological presence of *N*-acetyltryptamine in the circulation and support the hypothesis that this tryptophan metabolite plays a significant physiological role as an endocrine or paracrine chronobiotic though actions mediated by the melatonin receptor.

Mass spectrometry–based profiling of urinary steroids

Current approaches to the analysis of urinary steroids typically employ either immunoassay- or mass spectrometry–based technologies. Immunoassay-based methods often lack specificity owing to cross-reactivity with other steroids, and targeted LC-MS/MS is limited to the analysis of pre-determined analytes. We therefore developed a new LC-MS/MS approach to urinary steroid profiling that enables us to detect the steroids that have truly changed in a patient cohort without prior knowledge of the steroids' identity (i.e., untargeted metabolomics of steroids). Initially, the studies detected, in polycystic ovary syndrome (PCOS) patients, elevated levels of an unknown compound consistent with an androgenic steroid. We were then able to identify the unknown as a mixture of androsterone-sulfate and etiocholanolone-sulfate. The approach is being further extended to additional PCOS patients and to studies on patients with congenital adrenal hyperplasia (CAH). In a more targeted assay to quantitate specific steroids, we developed an MRM (multiple reaction monitoring) assay for quantitation of 5-α-pregnane-3α,17-α-diol-20-one (pdiol) and its 5-β stereoisomer, 17-α-hydroxypregnanolone (5-β-pdiol). Pdiol is an intermediate in the 'backdoor pathway' from 17OH progesterone to dihydrotestosterone. Using this assay in a study of CAH patients, urinary levels of both pdiol and 5-β-pDiol were directly correlated with the serum levels of androstenedione. In addition, we have begun to develop a product ion spectrum database of known steroids to improve our ability to identify novel steroids.

Community outreach

The Mass Spectrometry Facility is committed to promoting mass-spectrometric aspects of proteomics and other mass-spectrometric analyses in NICHD's DIR. We make serious efforts to educate investigators on the benefits and pitfalls of the techniques used in the Facility. In particular, we provide coaching on the principles of appropriate methods for sample isolation and staining of gels. We also support an NIH–wide seminar series featuring internationally known experts in proteomics.

In parallel, the staff of the Facility have developed collaborations with other Institutes to promote exchange of information and to bring new mass-spectrometric techniques to NICHD. In addition, Peter Backlund is the moderator of the NIH Mass Spectrometry Interest Group.

PUBLICATIONS

1. Debono M, Mallappa A, Gounden V, Nella AA, Harrison RF, Crutchfield CA, Backlund PS, Soldin SJ, Ross RJ, Merke DP. Hormonal circadian rhythms in patients with congenital adrenal hyperplasia: identifying optimal monitoring times and novel disease biomarkers. *Eur J Endocrinol* 2015; 173:727–737.
2. Cologna SM, Crutchfield CA, Searle BC, Blank PS, Toth CL, Ely AM, Picache JA, Backlund PS, Wassif CA, Porter FD, Yergey AL. An efficient approach to evaluate reporter ion behavior from MALDI-MS/MS data for quantification studies using isobaric tags. *J Proteome Res* 2015; 14:4169–4178.
3. Pu J, Schindler C, Jia R, Jarnik M, Backlund P, Bonifacino JS. BORC, a multisubunit complex that regulates lysosome positioning. *Dev Cell* 2015; 33:176–188.
4. Rauschecker ML, Cologna SM, Xekouki P, Nilubol N, Shamburek RD, Merino M, Backlund PS, Yergey AL, Kebebew E, Balow JE, Stratakis CA, Abraham SB. Clinical Case Report: LECT2-associated adrenal amyloidosis. *AACE Clinical Case Reports* 2015; 1:e59–e67.
5. Dolinska MB, Kovaleva E, Backlund P, Wingfield PT, Brooks, BP, Sergeev YV. Albinism causing mutations in recombinant human tyrosinase alter intrinsic enzymatic activity. *PLoS One* 2014; 9:e84494.

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The NICHD Zebrafish Core; Primary Germ Layer Formation

The NICHD Zebrafish Core was established in May 2012. The goal of the Core is to provide its clients with consultation, access to equipment and reagents, and service in the area of zebrafish genetics. NICHD investigators as well as investigators from other NIH institutes and from outside the NIH are its clientele. The oversight committee for the Core comprises Thomas Sargent (Chair), Harold Burgess, Ajay Chitnis, Igor Dawid, and Brant Weinstein. The Core's activities consist of (1) oversight and support of client-specific projects, (2) maintenance and improvement of equipment and infrastructure, (3) improvement of operational procedures, and (4) service and educational outreach. Feldman's research on primary germ layer formation is also making contributions to the field of Developmental Biology.

Benjamin Feldman, PhD, *Staff Scientist and Director of the NICHD Zebrafish Core*

Oversight and support of client-specific projects

In the past year, we engaged in research projects with seven labs: four from NICHD, one from NCI, one from NHLBI, and one from the Children's National Medical Center.

Zebrafish model of the human pediatric disease Smith-Lemli-Opitz syndrome (SLOS)

SLOS is an autosomal recessive, multiple malformation syndrome with pediatric onset. It is characterized by intellectual disability and aberrant behavior. Several zebrafish lines are being investigated that carry mutant alleles of *dhcr7*, the zebrafish ortholog of *DHCR7,* the human gene. The lines were established through the Core *via* TALEN genome engineering two years ago. In the interim, our efforts focused on defining phenotypes associated with the mutant alleles affecting metabolism, morphology, viability, fertility, and behavior. During the past year, we conducted experiments to determine which phenotypes were modulated by environmental and metabolic interventions. This project is ongoing.

Function of zebrafish orthologs to human genes implicated in childhood gigantism

Gigantism arises as a result of excess growth hormone (GH) secretion during childhood, before the growth plates close. Two years ago, the zebrafish ortholog of a human gene implicated as a driver of gigantism was cloned and its effects (when transiently overexpressed) on zebrafish morphology and growth were investigated. We recently completed a description of this gene's native expression in zebrafish. To understand whether chronic misexpression of this gene can drive hypertophic growth in zebrafish, we built a construct that expresses the gene in response to Gal4 and established zebrafish lines carrying the construct. We plan to cross these lines with lines that express Gal4 in the pituitary, hypothalamus, or throughout the central nervous system (CNS). We have developed novel software and zebrafish holding systems to facilitate longitudinal growth measurements. The project in ongoing.

Function of zebrafish orthologs of human genes implicated in familial adrenal hyperplasia

The following novel approach was initiated two years ago. To understand the molecular function of the human gene *ARMC5*, implicated as a tumor suppressor for adrenal hyperplasia, global RNA sequencing was performed on zebrafish embryos in which the zebrafish ortholog to this gene, *armc5*, was either downregulated by antisense morpholino oligonucleotide injection, upregulated by RNA injection, or unperturbed. The sequencing was outsourced. Identification and validation of targets and pathways affected by both up- and down-regulation are ongoing. We obtained a genetic *armc5* mutant generated by the Sanger TILLING project and bred it to homozygosity. The above studies will include validation in these mutants.

RNA expression constructs were obtained and antisense probes generated for *in situ* RNA stains of genes whose expression marks the zebrafish inter-renal primordium, a tissue that is the functional equivalent to the human adrenal gland. We also performed *in situ* staining of zebrafish *armc5* RNA and initiated protocol optimization for co-localization studies to determine whether *armc5* is expressed in the inter-renal primordium. The project is ongoing.

Modeling copper deficiency–associated distal motoneuropathy

The Menkes gene on Xq13.3 encodes ATP7A, a P-type cation-transporting ATPase localized to the plasma membrane and the trans-Golgi network (TGN) and critical for proper intracellular copper distribution. Two ATP7A missense mutations, T994I and P1386S, cause a milder syndrome than Menkes disease but is still debilitating to children and young adults.

Two years ago, we began a project to clarify the structure-function relationship of ATP7A and motor neuron defects. From outside sources we obtained a zebrafish line carrying mutations in the orthologous *atp7a* gene and a transgenic line that expresses GFP in motor neurons and crossed them in anticipation of experiments that will search for motor neuron defects in *atp7a* mutants, which were recently documented. Last year, RNA expression constructs were also built in anticipation of structure-function assays that will compare the ability of synthetic RNA from wild-type and mutant alleles to rescue motor neuron defects as well as other defects in *atp7a*-null embryos. The project was awarded a Bench-to-Bedside grant.

Assessing human metastatic cell behaviors in a whole-body (zebrafish embryo) microenvironment

The goal of this project is twofold: to determine the trophic range of certain metastatic melanoma and breast carcinoma cell lines and to document cellular dynamics during early tumor formation from metastatic cells that have seeded into new microenvironments. Two years ago, we optimized delivery of cells into the anterior CNS of embryos (for tumor formation studies) and the delivery of cells into the circulatory system (for trophism studies). Last year, we collected a substantial amount of trophism data. We also imported optically transparent and immuno-compromised zebrafish lines, which will permit the observation of tumor behaviors for longer periods of time. The project is ongoing.

Finding neuroprotective drugs to mitigate hyperammonemia, a consequence of urea cycle defects and liver failure

Exposure of the brain to high levels of ammonia causes neuro-cognitive deficits, intellectual disabilities, coma, and death. Two years ago, the Core developed a strategy to use zebrafish embryos to identify small molecules capable of diminishing the effects of hyperammonemia. The protocol was then substantially improved with the addition of an automated system for quantifying the spontaneous movement of experimental embryos. Last year, we screened a library of hundreds of small molecules with known safety profiles for humans and identified several promising candidates for follow-up validation studies in zebrafish and other animal models. The project is ongoing.

Other projects

During the past year, the Core assisted the Lippincott-Schwartz laboratory (NICHD) with the acquisition of transgenic fish lines that label various cells of the immune system, in preparation for their own project that will examine the intersection of regeneration and immunity. Core resources were also used by the Waterman laboratory (NHLBI) to examine how altering the level of selected cytoskeletal proteins affects cellular behaviors.

Maintenance and improvement of equipment and infrastructure

All equipment has been maintained in good order. The Core also acquired the following: a state-of-art automated whole-mount *in situ* hybridization machine; a new camera and microscope adaptor components to establish a new photomicroscopy station; a portable UV-illumination system and microscope blackout hood to visualize fluorescent zebrafish lines in the lab and as an educational outreach activity. A new generation of wild-type (WT) zebrafish was introduced and new generations were established for other mutant and transgenic lines frequently needed for Core projects and/or educational outreach. The Core also added several new lines of fish its collection, including transparent, colored (Glo-fish), and immuno-compromised lines.

Improvement in operational procedures

The 'do-it-yourself and pay-as-you-go' model of facilitated, supervised research is working well; additional services must be limited due to the small size of the Core's staff (Feldman only). These can include monthly meetings with Feldman, the PI, and all relevant trainees; RNA syntheses upon request; zebrafish line preservation via sperm freezing and line recovery via *in vitro* fertilization. This past year, Feldman adapted optimized approaches for Crispr/Cas9 mutagenesis and is providing quality-controlled reagents, protocols, and training for Crispr/Cas9 mutagenesis to users of the Core. Feldman is also now offering to generate F0 carriers of mutations upon request for a limited number of genes per year.

Service and educational outreach

Service

Feldman led the team responsible for writing the new NICHD Mission Statement, as part of the major reorganization that took place last year.

Educational Outreach

Feldman helped orchestrate 2015 'Take Your Child to Work Day' events at the Central Aquatics Facility and received a group award for his previous year's similar activity. He demonstrated principles of embryology and gene expression to children and families, using zebrafish, at the Rockville Science day in 2015. He hosted a high-school student from The School Without Walls in Washington, DC, together with her mentor, for a one-week 'Environmentor' project (a National Council for Science and the Environment activity), and the project was ranked in first place. Feldman also provided consultation and zebrafish embryos on several occasions to Bill Wallace and his students from Georgetown Day School in Washington, DC, enabling them to complete and prepare posters summarizing several science projects.

Primary germ layer formation

Feldman's overall research goal in independent work is to elucidate molecular and cellular events that control the formation of mesoderm and/or mesendoderm in zebrafish, with an eye towards general principles that are relevant to other species, particularly humans. A year ago, Feldman completed an extensive chapter on mesoderm formation for a textbook that was published in 2015. Other manuscripts based on earlier work at NHGRI are still in preparation. Data from one of these earlier projects was included in a paper from Harold Burgess's lab (Reference 2).

ADDITIONAL FUNDING

» $16,400 in supplemental funding was obtained in FY15 as follows: $3,900 in fee-for-use charges from NICHD laboratories, $2,500 in fee-for-use charges from other NIH laboratories, and $10,000 as the first of three installments of a Bench-to-Bedside award.

PUBLICATIONS

1. Feldman B. Taking the middle road: vertebrate mesoderm formation and the blastula-gastrula transition. *Principles of Developmental Genetics, 2nd Edition. Ed: Moore S. Academic Press* 2014; 203–236.
2. Horstick EJ, Jordan DC, Bergeron SA, Tabor KM, Serpe M, Feldman B, Burgess HA. Increased functional protein expression using nucleotide sequence features enriched in highly expressed genes in zebrafish. *Nucleic Acids Res* 2015; 43:e48.

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Molecular Genomics Core Facility

With the goal of understanding genetic changes and mechanisms underlying human diseases, the Molecular Genomics Core Facility supports NICHD investigators by providing deep sequencing and project data analysis.

Forbes D. Porter, MD, PhD, *Director, Molecular Genomics Core Facility*
Steven L. Coon, PhD, *Staff Scientist*
James R. Iben, PhD, *Senior Research Fellow*
Tianwei Li, PhD, *Staff Scientist*

Next-Generation sequencing and bioinformatics support

The Molecular Genomics Core (MGC) provides DNA and RNA sequencing services for genomic and genetic research to investigators within the NICHD. The MGC is currently operating with four sequencing machines. Two are high-capacity, production-scale machines: an Illumina HiSeq 2500, and an Applied Biosystems SOLiD 5500xl. The two others, an Illumina MiSeq and an Ion Torrent Personal Genomics Machine, are smaller, faster machines, which can generate longer sequence reads. This array of sequencers provides a suite of scales and capabilities. Our sequencing services include whole exome, targeted exome, and gene-specific DNA sequencing, as well as whole transcriptome sequencing (RNA-Seq), microRNA sequencing, microbiome sequencing, bisulfite sequencing (DNA methylome), ChIP-Seq, and ribosomal profiling. The MGC provides significant primary data processing and downstream bioinformatic support and can assist in designing experiments or sequencing strategies (for example, optimization of targeted exome design). During the past year, the MGL provided sequencing for 14 projects across the full spectrum of sequencing types; the projects involved 12 NICHD Principal Investigators. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support for 15 NICHD investigators and one NICHD/NHGRI collaboration. Our mission is to offer accurate and innovative sequencing and bioinformatic tools to facilitate research into the diagnosis, counseling, and treatment of hereditary disorders.

PUBLICATIONS

1. Wassif CA, Cross JL, Iben J, Sanchez-Pulido L, Cougnoux A, Platt FM, Ory DS, Ponting CP, Bailey-Wilson JE, Biesecker LG, Porter FD. High incidence of unrecognized visceral/neurological late-onset Niemann-Pick disease, type C1, predicted by analysis of massively parallel sequencing data sets. *Genet Med* 2015; 18(1):41–8.
2. Hartley SW, Coon SL, Savastano LE, Mullikin JC, NISC Comparative Sequencing Program, Fu C, Klein DC. Neurotranscriptomics: The effects of neonatal stimulus deprivation on the rat pineal transcriptome. *PLoS ONE* 2015;10(9):e0137548.
3. Hickey A, Esnault C, Majumdar A, Chatterjee AG, Iben JR, McQueen PG, Yang AX, Mizuguchi T, Grewal SI, Levin HL. Single nucleotide specific targeting of the Tf1 retrotransposon promoted by the DNA-binding protein Sap1 of Schizosaccharomyces pombe. *Genetics* 2015; 201(3):905–924.
4. Zhang J, Hung GC, Lei H, Li T, Li B, Tsai S, Lo SC. Draft genome sequence of Pantoea sp. strain MBLJ3, isolated in a laboratory environmental control study. *Genome Announc* 2015;3(1):e01595–14.
5. Singh P, Plumb M, Ferris A, Iben J, Wu X, Fadel H, Poeschla E, Hughes S, Kvaratskhelia M, Levin, HL. LEDGF/p75 interacts with mRNA splicing

factors and targets HIV-1 integration to highly spliced genes. *Genes Dev* 2015; 29:2287–97.

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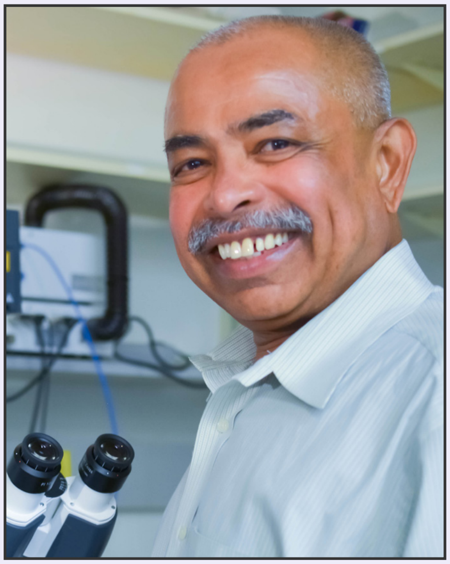
The Microscopy and Imaging Core Facility

The mission of the NICHD Microscopy and Imaging Core (MIC) is to provide state-of-the-art light microscopy and electron microscopy services to all NICHD scientists. The Core is designed as a multi-user facility where investigators can, with a minimum of time and effort, prepare, image, and analyze their samples. Since the move to the Porter Neuroscience Research Center (PNRC II), building 35-A, in February, 2014, the MIC has been functioning smoothly in the new location. The PNRC facility is effective, efficient, and accessible to all MIC's clients. The MIC is staffed by three full-time microscopists working under the supervision of James Russell. Vincent Schram oversees the light-microscopy operations and IT infrastructure, Lynne Holtzclaw supports light microscopy and immunohistochemistry, and Louis (Chip) Dye manages the electron microscopy component of the facility.

Within the PNRC II, the MIC is expected to provide microscopy and imaging resources to eight other Institutes (NINDS, NIMH, NIDCD, NIDA, NIA, NEI, NIAAA, and NIDCR) in addition to NICHD's Division of Intramural Research (DIR). Since the move to PNRC, MIC has totally abandoned its fee-for-service policy, and services are now free of charge.



James T. Russell, DVM, PhD, *Director, Microscopy and Imaging Core Facility*
Vincent Schram, PhD, *Staff Scientist*
Louis (Chip) Dye, BS, *Staff Scientist*
Lynne A. Holtzclaw, BS, *Senior Research Assistant (Biologist)*

Mode of operation

The equipment and staff of the MIC are available to everyone within the NICHD DIR, as well as to all occupants of the PNRC. The philosophy of the MIC is to ensure that only reliable, high-quality data are recorded on its instruments. For every new project, MIC staff meet with the Principal Investigator and the postdoctoral scientists involved in the study to discuss details of experimental design, the background of the project, and its imaging goals and the most appropriate techniques and instrumentation are determined. Users are asked to sign a document outlining the policies to be followed when using the Core equipment. Positive feedback from long-term users suggests that this high level of interaction greatly improves the quality of data obtained and efficiency of each imaging project. The facility is accessible 24/7 using the NIH ID system, and users can reserve time on each instrument by using an online calendar. The MIC also offers periodic training courses on the principles of and detailed techniques in microscopy and specimen preparation techniques. Periodic invited lectures and new equipment demonstrations offered by microscope manufacturers are also offered.

Light microscopy

The light microscopy arm of the MIC operates in four different areas:

Equipment maintenance and upgrade

The MIC operates several confocal laser scanning microscopes optimized for various applications: a Zeiss LSM 780 inverted for high-resolution confocal microscopy of both live and fixed fluorescently labelled cells and tissues; a Zeiss 510 inverted for high-resolution confocal imaging of fixed specimens; during 2015, the aging LSM 510 point-scanning confocal microscope was replaced by a new-generation point-scanning confocal, the LSM 710 for two-photon imaging of live tissue sections and live animals. During the summer of 2013, the MIC acquired a Nikon inverted microscope platform with all the required accessories to implement a high-resolution, ultra-fast imaging platform using spinning disc confocal scanning, and a high sensitivity camera. This high-end instrument is also capable of providing Total Internal Reflection (TIRF) microscopy as well as direct Stochastic Optical Reconstruction Microscopy or dSTORM. A sub-resolution microscope with a home-built Photo-Activation Localization Microscope (PALM) is also available at the MIC. In addition, two high-end wide-field fluorescence microscopes are available, as is a fluorescence stereo microscope.

Live-cell, tissue, and live-animal imaging are supported with temperature, CO_2, and humidity control and heated perfusion on most microscopes. Instrument downtime is kept to a minimum by providing full-time support to end users (by phone and pager). For problems that require extensive repairs, most instruments are covered by manufacturers' service contracts and are usually serviced within a few days.

User training and support

After counseling on specimen preparation and staining, each user receives hands-on training on the light microscope required for the project. The training covers the principles of fluorescence microscopy, confocal imaging, and optimum operation of the hardware platform and is followed by periodic refreshers at the user's request or when the MIC's staff feel that the equipment is not being used optimally. When necessary, hands-on training in sample preparation is provided in the MIC's labs so that postdoctoral fellows are adequately trained in optimal sample preparation. Individual training in tissue handling and sample preparation is offered by MIC staff upon request.

Image analysis

The MIC operates a data-analysis center with three high-end workstations and image-processing software environments (Metamorph, Volocity, Imaris, and Zeiss AIM, Image J, and Fiji). At the user's request, we provide training and support for each software package and, when required, custom macros and high-throughput image analysis solutions. The facility offers extensive data-storage services with an enterprise-level file server and a data-backup system. The infrastructure is used to safeguard images and move data from the facility to each user's location on the NIH campus.

Methods development

The MIC develops methods for novel microscopy and image-processing techniques when the need arises. For example, a single-molecule imaging platform was implemented, and Vincent Schram developed software required for imaging and analysis. Similar methods-development efforts will be undertaken according to Institute needs and availability of funds. The MIC actively entertains such requests.

Electron microscopy

Because of the complex nature of electron microscopy (EM) sample preparation, the EM component of the MIC (MIC-EM) functions differently, and all sample preparation and microscopy are carried out by the staff microscopist. This is necessary because, to achieve optimal image quality, the procedures involved are critical and exacting.

Sample processing

Typically, all EM processing (fixation, embedding, tissue sectioning, and staining) is undertaken in-house by Chip Dye. Lynne Holtzclaw is also now proficient in EM sample-preparation techniques and assists Chip Dye in this effort. The MIC maintains and operates a fully equipped EM laboratory with a LKB Pyramitome, a Leica CM3050-S cryostat, and a Reichert Ultracut-E ultramicrotome. By providing consistent and controlled incubation parameters, the PELCO Biowave Pro programmable incubator (Ted Pella, Inc.) has been instrumental in improving the preservation of ultrastructure and the quality of immuno-labeling.

Imaging

EM imaging is done mostly by the microscopist, except in cases where the user has the necessary inclination and training. Imaging is carried out on a JEOL JEM 1400 series electron microscope. The instrument has become the work-horse for transmission electron microscopy within the MIC. It offers two new imaging methodologies: cryo-EM and tomography. The main advantages of cryo-EM are that it preserves a high level of immuno-reactivity and allows specimens to be imaged in a near-native state. Cryo-EM improves the quality of studies that rely on immuno-gold labeling. Tomography provides three-dimensional imaging of structures at the EM level. We are exploring the possibility of expanding the EM component by including specimen preparation equipment for high-pressure freezing and freeze substitution. The techniques should provide better immune detection at EM–level resolutions with well-preserved cellular architectures.

Methods development

Chip Dye recently set up the required techniques for EM immuno-histochemistry and dual immuno-labeling to simultaneously image two separate antigens at EM resolution; he is using LUXFilm EM grids, which provide a view of the entire specimen and are crucial for imaging large structures, tracing features, searching for special details, and tomography imaging. The MIC also implemented a digital archive of all EM images and parameters, which is available online to investigators. Dye is expected to incorporate cryo-EM techniques and tomography in his repertoire.

Ancillary support

Given that the NICHD's Division of Intramural Research laboratories are scattered over the NIH campus, the MIC provides all necessary resources and facilities, such as tissue-culture space, 5% and 10% CO_2 incubators, animal-holding and preparative space, and vibratomes for live and fixed tissues. Lynne Holtzclaw provides outstanding technical expertise in advanced cell and tissue-sample preparation for immuno-histochemistry experiments. The recently completed move to PNRC facilities requires us to provide microscopy support to all occupants of the PNRC, which has somewhat increased our user base.

Facility usage

The MIC currently serves over 100 scientists associated with 41 NICHD principal investigators (PIs) and three PIs of sister Institutes within the campus. On any given week, approximately 10 separate users spend half a day or more on the MIC's microscopes. Since its creation in 2004, usage has resulted in more than 120 publications, many of which were co-authored by Core personnel. For a full list of publications made possible through use of the MIC's facilities, go to **https://science.nichd.nih.gov/confluence/display/mic/Publications**.

2014 and 2015 publications emanating from work carried out in the MIC facility

Bojjireddy N, Botyanszki J, Hammond G, Creech D, Peterson R, Kemp DC, Snead M, Brown R, Morrison A, Wilson S, Harrison S, Moore C, Balla T. Pharmacological and genetic targeting of the PI4KA enzyme reveals its important role in maintaining plasma membrane phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate levels. *J Biol Chem* 2014;289:6120-6132.

Farber CR, Reich A, Barnes AM, Becerra P, Rauch F, Cabral WA, Bae A, Quinlan A, Glorieux FH, Clemens TL, Marini JC. A novel IFITM5 mutation in severe atypical osteogenesis imperfecta type VI impairs osteoblast production of pigment epithelium-derived factor. *J Bone Miner Res* 2014;29:1402-1411.

Guzmán-Hernández ML, Potter G, Egervári K, Kiss JZ, Balla T. Secretion of VEGF-165 has unique characteristics, including shedding from the plasma membrane. *Mol Biol Cell* 2014;25:1061-1072.

Hammond GR, Machner MP, Balla T. A novel probe for phosphatidylinositol 4-phosphate reveals multiple pools beyond the Golgi. *J Cell Biol* 2014;205:113-126.

Jovic M, Kean MJ, Dubankova A, Boura E, Gingras AC, Brill JA, Balla T. Endosomal sorting of VAMP3 is regulated by PI4K2A. *J Cell Sci* 2014;127:3745-3756.

Lin L, Long LK, Hatch MM, Hoffman DA. DPP6 domains responsible for its localization and function. *J Biol Chem* 2014;289:32153-32165.

Swift MR, Pham VN, Castranova D, Bell K, Poole RJ, Weinstein BM. SoxF factors and Notch regulate nr2f2 gene expression during venous differentiation in zebrafish. *Dev Biol* 2014;390:116-125.

Wang H, Kane AW, Lee C, Ahn S. Gli3 repressor controls cell fates and cell adhesion for proper establishment of neurogenic niche. *Cell Rep* 2014;8:1093-1104.

Chandra G, Bagh MB, Peng S, Saha A, Sarkar C, Moralle M, Zhang Z, Mukherjee AB. Cln1 gene disruption in mice reveals a common pathogenic link between two of the most lethal childhood neurodegenerative lysosomal storage disorders. *Hum Mol Genet* 2015;24:5416-5432.

Cheng Y, Rodriguiz RM, Murthy SRK, Senatorov V, Thouennon E, Cawley NX, Aryal DK, Ahn S, Lecka-Czernik B, Wetsel WC, Loh YP. Neurotrophic factor-a1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone. *Mol Psychiatry* 2015;20:744-54.

Choi EY, Patel K, Haddad MR, Yi L, Holmes C, Goldstein DS, Dutra A, Pak E, Kaler SG. Tandem duplication of exons 1-7 neither impairs ATP7A expression nor causes a Menkes disease phenotype. *JIMD Rep* 2015;20:57-63.

Kim YJ, Guzman-Hernandez ML, Wisniewski E, Balla T. Phosphatidylinositol-phosphatidic acid exchange by Nir2 at ER-PM contact sites maintains phosphoinositide signaling competence. *Dev Cell* 2015;33:549-561.

London E, Wassif CA, Horvath A, Tatsi C, Angelousi A, Karageorgiadis AS, Porter FD, Stratakis CA. Cholesterol biosynthesis and trafficking in cortisol-producing lesions of the adrenal cortex. *J Clin Endocrinol Metab* 2015;100:3660-3667.

Pelkey KA, Barksdale E, Craig MT, Yuan X, Sukumaran M, Vargish GA, Mitchell RM, Wyeth MS, Petralia RS, Chittajallu R, Karlsson RM, Cameron HA, Murata Y, Colonnese MT, Worley PF, McBain CJ. Pentraxins coordinate excitatory synapse maturation and circuit integration of parvalbumin interneurons. *Neuron* 2015;85:1257-1272.

Vullhorst D, Mitchell RM, Keating C, Roychowdhury S, Karavanova I, Tao-Cheng JH, Buonanno A. 2015. A negative feedback loop controls NMDA receptor function in cortical interneurons via neuregulin 2/ErbB4 signalling. *Nat Commun* 2015;6:7222.

Xie Y, Zhou B, Lin MY, Wang S, Foust KD, Sheng ZH. Endolysosomal deficits augment mitochondria pathology in spinal motor neurons of asymptomatic fALS mice. *Neuron* 2015;87:355-370.

Yi L, Kaler SG. Direct interactions of adaptor protein complexes 1 and 2 with the copper transporter ATP7A mediate its anterograde and retrograde trafficking. *Hum Mol Genet* 2015;24:2411-2425.

Community outreach

The MIC is committed to promoting light and electron microscopy in the PNRC as well as for NICHD's Division of Intramural Research. We are making efforts to educate investigators on the benefits and pitfalls of advanced imaging techniques. Our initiatives include: (1) coaching users on the principles of confocal microscopy, during training and by publishing comprehensive operating protocols for each microscope; (2) on-campus demonstrations of new instruments and software by vendors such as Zeiss, Olympus, Photometrics, Nikon, and Perkin-Elmer; and (3) on-site assistance to investigators in their laboratories operating their own imaging equipment to optimize the quality of the data recorded. Furthermore, the MIC's website (**http://mic.nichd.nih.gov**) is an important resource for tutorials and protocols for both fixed and live cell microscopy.

In addition, during 2011, 2013, and 2015, we conducted educational outreach programs. The goal was to educate individual NICHD DIR senior scientists and post-doctoral scientists on microscopy techniques from the ground up. Instruction included lectures as well as hands-on experimental work organized within the MIC's facilities. More than 50 participants took part in a month-long series of lessons and lab work. The program was very well received by our user-base both at senior level and by postdoctoral scientists. We plan to offer this education curriculum whenever there is demand, and plan to query the community of their wishes. In addition, we have offered individual training when such requests are received; Ms. Holtzclaw provides such training in our laboratories.

Parallel to these efforts, the staff have continued to collaborate with other Institutes to promote an exchange of information and bring new imaging technologies to this Institute. Ongoing collaborations include: imaging of live animals (NINDS, NIMH); and correlative light and electron microscopy in the same specimen. We are also initiating discussions on implementing some form of light-sheet microscopy within the MIC.

NICHD has contributed to a jointly funded Stimulated Emission Depletion (STED) microscopy platform, which is hosted by the NHLBI Imaging core facility headed by Chris Combs.

PUBLICATIONS

1. Subramanian J, Dye L, Morozov A. Rap1 signaling prevents L type calcium channel dependent neurotransmitter release. *J Neurosci* 2013;33:7245–7252.

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Our team provides informatics and research services to Intramural investigators at NIH in four key areas: clinical informatics, bioinformatics, biovisualization web services, and core IT support.

David Songco, *Head, Unit on Computer Support Services*
Marco Crosby, *Program Engineer*
John Czapary, BS, *Laboratory Technical Manager*
Michelle Duverneau, *IT Specialist*

Clinical informatics

During the past year, the Unit on Computer Support Services (UCSS) continued to support and develop applications related to clinical and translational medicine, including the Clinical Trials Database (CTDB) project. Such informatics tools allow researchers to design, collect, and report clinical observations related to natural history and interval-based studies. The total number of protocols and research projects supported by the CTDB team increased to approximately 410 for 14 NIH institutes, with an expansion of research questions to over 180,000. Our software development group completed three releases that included features for the electronic regulatory binder, machine data upload, and bio-repository improvement. We supported the Clinical Trial Survey System (CTSS), an application for patient surveys, used for 136 protocols. The Clinical Datamart was expanded to include vitals, medications, and ECG/EKG datamarts and upgraded to support new protocols.

Biological visualization web services

The UCSS team provides DIR laboratories with scientific communications and media services, including publication support, website support, audio visuals production, medical arts, and print media. We participated in the Science in 3D meeting hosted by NIAID and helped launch the NIH 3D Printing Exchange and their participation in the White House Maker Fair. We continued contributing to the virtual collection of biomedical 3D printing files by producing 34 videos and video tutorials, which serve as a public resource for biomedical scientists and educators. UCSS presented on 3D printing and graphics to the Graduate Partnership Program, the “3 Blind Mice” (the NIH visually impaired user group), and visiting students. We designed and produced 3D representations of Cajal Illustrations for an exhibit in the Porter Neuroscience building and provided input and support to the NIH Library’s Technology Sandbox in their effort to provide direct 3D printing capabilities to all NIH staff.

The web activities of the DIR web services program include: laboratory websites, annual report, and internet applications. A recent feature was added to collect and submit data to the Intramural Research Program website, building on the annual report on-line data collection system. The application was also enhanced to include the Fellows’ Annual Progress Report, a unified means of tracking and mentoring intramural trainees along with easing the re-appointment process. The UCSS continued to maintain approximately 50 public DIR websites and began developing graphics for a redesign effort. We created medical illustrations for several publications by NICHD scientists. In addition, we provided photography and graphic, web, and media production for the DIR Annual Fellows and PI (principal investigator) Retreats, the NIH Research Festival, Science in 3D, and the NICHD Exchange series. The team continued support for the NICHD Connection, a monthly newsletter for Intramural research fellows and also expanded the Science NICHD wiki (**http://science.nichd.nih.gov**) to over 168 projects. The Science wiki allows PIs to create blogs and share documents/data in a secure manner. It played an integral part in intramural reorganization and was a platform for conducting the scientific review by the Board of Scientific Counselors. As part of this project, the group collaborated with the NIH Library, the NLM, the NINDS, and the NIH Office of the Director.

Bioinformatics

The bioinformatics team assisted the Molecular Genomics Laboratory with data collection and analysis for highthroughput sequencing. The scientific informatics group also develops research tools to assist investigators with genomic data management and analysis. TileMapper was developed as a comprehensive web-based tool for mapping various genomic annotation features to the tiling microarray data derived from transcriptome, ChIPchip, or MeDIP studies. The data are visualized in a tabulated format, which permits flexible processing, and further analyzed by downstream pipelines to relate the data and perform interactions analysis. TileMapper accepts transcribed fragments (transfrag) information in Browser Extensible Display (BED) format generated from Affymetrix Integrated Genome Browser (IGB) or downloaded from the UCSC (University of California Santa Cruz) server. The SAGEWorks application was maintained and transitioned to a new server to leverage the robust public databases available in combination with the SAGE (Serial Analysis of Gene Expression) data to accelerate gene and pathway discovery. This workspace accommodates any SAGE data and provides storage of large datasets. With built-in navigation/search toolsets and automated updates, SAGEWorks permits the scientific community to minimize the redundancy of manual manipulation. A high-performance computing (HPC) cluster was set up to assist with genomics computational requirements. The Genomics workbench Galaxy was installed and configured on the HPC to take advantage of the parallel processing capabilities. The UCSS also continues to provide and manage dozens of Terabytes of storage to support genomic research.

Core IT services

The Unit continued to expand its services to the Division of Intramural Research (DIR) community in core IT areas.

Network and desktop services

The unit supports reliable, secure, and efficient information technology solutions. This includes acquisition, maintenance, and support for licensed software used by the DIR research community e.g., EndNote/Reference Manager, Bookends, GraphPad Prism, PyMol, network services (e-mail, data backups, VPN, helix, PDAs, wireless configurations); and crossplatform desktop, server, and application hosting in the Rockledge Data Center. We host software licenses for computation, 3D imaging, and sequencing e.g., Amira, ArrayStar and QSeq, Autodesk Maya, DNASTAR Lasergene Core Suite, MathWorks MATLAB, MolSoft, and SeqMan NGen. This year, MATLAB Distributed Computing Server, software and hardware (128GB, 16 Worker), was added. Hosting these licenses permits users to leverage their research with additional tools available on Helix and Biowulf. We also assist users in identifying, researching, and purchasing custom hardware configurations to match research instrument requirements.

Data-recovery services

Core data-recovery tools were implemented for all media, hard drive, SSD, and flash etc., including RAID 0 and 5 recovery tools. Since 2005, the Unit has recovered over a Terabyte of research data from failed drives and media, at a minimum of $2500 savings per instance to the DIR research budget.

Custom software development for scientific and administrative support

This aspect of support includes software applications for DIR services. For example, we continued to enhance the Manuscript Tracking System (MTrac), a web-based application that automates the clearance and approval process for manuscripts in the DIR. The system now includes a web service feature to connect to NLM PubMed and File Transfer Process (FTP) connections to PubMed Central to allow PIs to comply with NIH's Public Access policies. The team completed three production releases, including users' support for all DIR programs. UCSS also continued the requirements process for an automated travel log system to streamline travel requests. The UCSS deployed the Cost Tracker module for the DIR, enabling the capture, organization, and reporting of various expenses on a per-protocol basis. The UCSS worked closely with the Office of the Clinical Director to develop a model for protocol cost vs. effectiveness.

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PUBLICATIONS

1. Huser V, Sastry C, Breymaier M, Idriss A, Cimino JJ. Standardizing data exchange for clinical research protocols and case report forms: an assessment of the suitability of the Clinical Data Interchange Standards Consortium (CDISC) Operational Data Model (ODM). *J Biomed Inform* 2015; 57:88–99.

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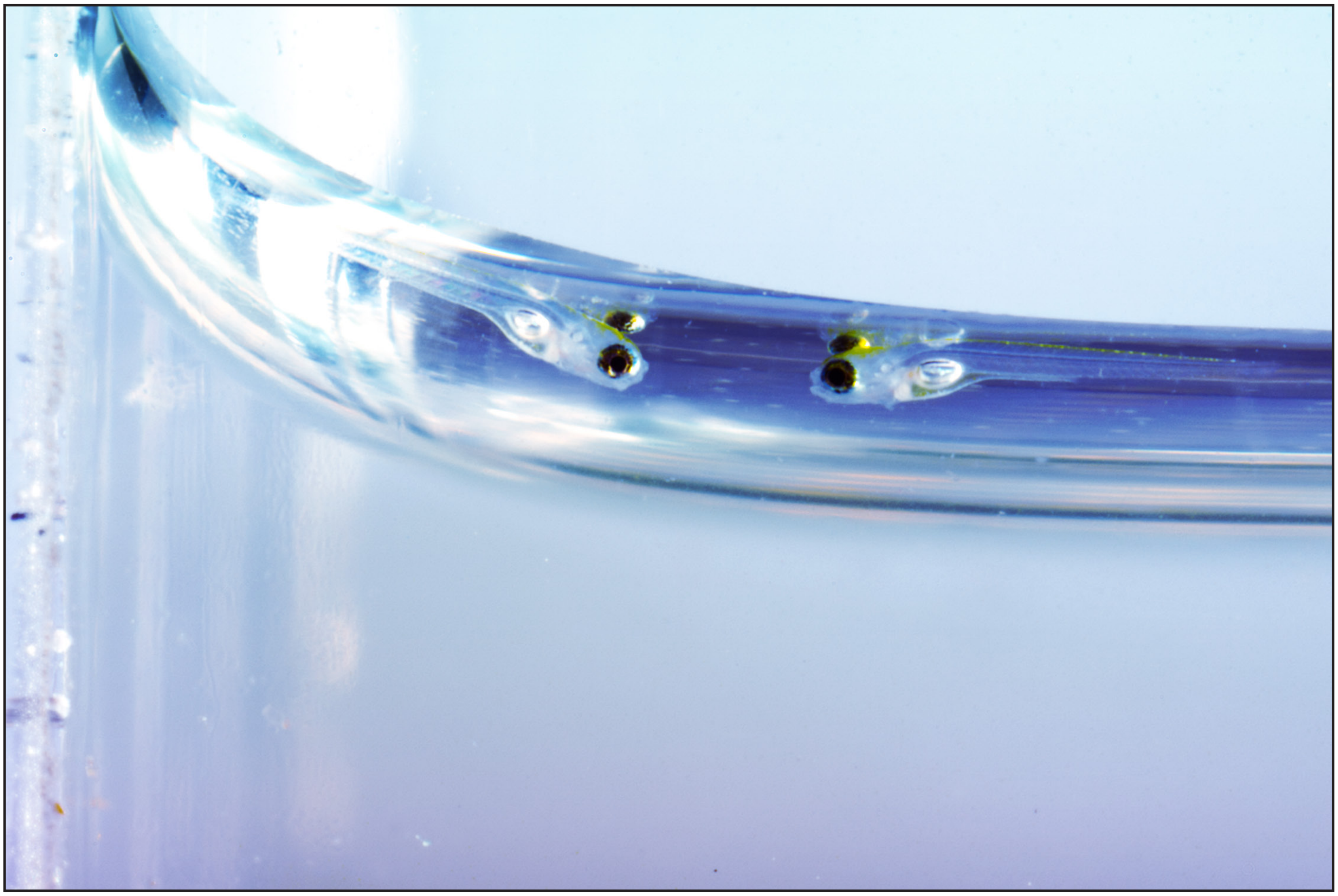
About the Cover Images



Larval zebrafish are optically clear, allowing researchers to observe the nervous system as it develops, and monitor the activity of neurons during behavior. The Burgess laboratory uses zebrafish to define the neuronal circuits that control behavior, and identify the genes which contribute to the development of these circuits. Pictured here, 6 day-old zebrafish swim near the water's surface.

Photo by Jeremy Swan in collaboration with Kathryn Tabor, PhD, of the Burgess lab.