2017

NICHD Division *of* Intramural Research

ANNUAL REPORT

Eunice Kennedy Shriver National Institute
of Child Health and Human Development

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Board *of* Scientific Counselors

** nominee*

Scott A. Rivkees, MD, *Chair* 7/1/15 – 6/30/20 Pediatric Endocrinology	Professor, Nemours Eminent Scholar and Chair Department of Pediatrics, University of Florida Physician in Chief, Shands Hospital for Children
Kate G. Ackerman, MD* 7/1/16 – 6/30/21 Developmental Biology, Genetics, & Pediatrics	Associate Professor, Departments of Pediatrics and Biomedical Genetics University of Rochester Medical Center School of Medicine and Dentistry
Vanessa Auld, PhD* 7/1/17 – 6/30/22 Cell & Developmental Biology	Professor, Department of Zoology University of British Columbia
Elizabeth Bonney, MD, MPH* 7/1/17 – 6/30/22 Immuniobiology, Molecular Biology, Obstetrics & Gynecology	Professor, Director of Research Division Department of Obstetrics, Gynecology, and Reproductive Sciences University of Vermont College of Medicine
Jeanne Brooks-Gunn, PhD 7/1/13 – 6/30/18 Developmental Psychology & Behavioral Science	Virginia and Leonard Marx Professor of Child Development Teachers College and College of Physicians and Surgeons Columbia University
Serdar E. Bulun, MD* 7/1/16 – 6/30/21 Obstetrics & Gynecology	John J. Sciarra Professor and Chair, Department of Obstetrics and Gynecology Northwestern University Feinberg School of Medicine Chief, Division of Obstetrics and Gynecology-Reproductive Biology Research Prentice Women's Hospital
Frances E. Jensen, MD* 7/1/15 – 6/30/20 Neuroscience	Professor and Chair, Neurology Department Perelman School of Medicine, University of Pennsylvania

Kojo A. Mensa-Wilmot, PhD* 7/1/16 – 6/30/21 Cellular Biology	Professor and Head, Department of Cellular Biology University of Georgia
Antonios G. Mikos, PhD 7/1/13 – 6/30/18 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
Yoel Sadovsky, MD 7/1/14 – 6/30/19 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
Susan S. Taylor, PhD 7/1/14 – 6/30/19 Structural Biology & Molecular Biochemistry	Professor of Chemistry and Biochemistry Professor of Pharmacology University of California, San Diego
Eric Vilain, MD, PhD 7/1/15 – 6/30/20 Molecular & Human Genetics	A. James Clark Distinguished Professor of Molecular Genetics and Director, Center for Genetic Medicine Research Children's Research Institute, Children's National Medical Center
Martha M. Werler, DSc* 7/1/17 – 6/30/22 Epidemiology	Professor and Chair, Department of Epidemiology Boston University School of Public Health

Message *from the* Scientific Director

Our 2017 annual report of the Division of Intramural Research (DIR) for the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development is now available to you electronically, either on the web and on your cell phones or tablets, at: *http://annualreport.nichd.nih.gov*

We invite you to look through the report site, 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 comprise a broad array of basic, translational, and clinical researchers. Our work is reflected in our 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 and working with a number of other animal models, from fruit flies to rats and mice, and supported by a wide array of core services, including imaging, proteomics and molecular genomics. Each investigator participates in at least one, and typically more, affinity groups. These affinity groups are team-based and future-oriented—to build on thematic interests while responding to rapidly shifting scientific priorities as new knowledge is uncovered.

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 Research 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 committed to training the next generation of scientists and physician scientists; they include tenure-track investigators who have recently joined us and accomplished investigators who continue to forge new scientific paths. Link to their reports on the web to learn about their work in 2017. I also invite you to reach out to me with your ideas and proposals for collaborative initiatives we may undertake together, at *stratakc@mail.nih.gov*.

Our drive and purpose, on behalf of the American public and the international community, is to strive to

uncover fundamental answers to our existence, whether the basic science that underpins life or the complexities of human health and disease. This is our privilege and responsibility to our chosen professions.

Sincerely yours,

Constantine A. Stratakis, MD, D(med)Sci
Scientific Director, NICHD, NIH

Office *of the* Scientific Director

The Office of the Scientific Director (OSD) is responsible for programmatic oversight, review, and administration of the Division of Intramural Research (DIR), NICHD. The Office has several key functions including:

- Serving our staff, investigators, administrators, trainees, and contractors to meet their needs and ensure that we are all working together to serve the mission of NICHD.
- Planning, coordinating, and directing the basic and clinical scientific research programs conducted within the DIR, all with the advice of the Board of Scientific Counselors (BSC) and in consultation with the Institute's Director and other offices.
- Providing oversight of scientific organization, setting of overarching research priorities, and allocation of resources.
- Coordinating the recruitment and review of principal investigators and other key staff.
- Providing oversight of the scientific mentoring and training activities. Ensuring that we provide state-of-the-art training in basic, translational, and clinical research for the next generation of scientific and clinical leaders is a high priority.
- Encouraging the implementation of new technologies, application of new techniques and treatments through clinical trials, and promoting research collaboration.
- Developing and maintaining the necessary scientific capability in the DIR to foster and guide an effective research and clinical program.
- Ensuring compliance with safety, ethics, and other legal and policy requirements.
- Providing clearance of manuscripts and other publications and ensuring the deposition of data and published manuscripts in appropriate public databases (e.g. *PubMedCentral*, *Clinicaltrials.gov*).
- Serving in an advisory role to the Institute Director and other senior leadership of the Institute and other institutes, offices, and centers across NIH.
- Actively participating in the governance of the NIH Intramural Research Program (IRP).
- Contributing to the development and support of trans-NIH initiatives.
- Work closely with other intramural research programs across NIH to support scientific and other efforts across institutes.

To maintain the highest quality of research, principal investigators and other key staff of the DIR are evaluated by the BSC which meets biannually, the first Friday of December and June of each year. The BSC reviews site visit reports, evaluations, and all other activities of the OSD. Each NICHD investigator is reviewed at least once every 4 years as

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

Sara K. King, *Chief of Staff*

Jessica Rigby, *Administrative Support Specialist*

Jeanne Jones, *Scientific Program Analyst*

per the NIH policy (NIH Sourcebook Process for Reviewing NIH Intramural Science, *https://oir.nih.gov/sourcebook/processes-reviewing-nih-intramural-science*).

This Board is made up of accomplished senior extramural researchers to ensure the most effective use of public dollars toward high-quality, high-impact research. Membership of the BSC is listed at (*https://annualreport.nichd.nih.gov/bsc.html*).

Annually, the Scientific Director reports on the activities of the OSD, the reviews of our investigators and their accomplishments, and all BSC recommendations to NICHD's National Advisory Child Health and Human Development (NACHHD) Council (*https://www.nichd.nih.gov/about/advisory/nachhd/Pages/index.aspx*).

Office *of* Education

The Office of Education, Division of Intramural Research, NICHD, was established in 2004 to support the needs of intramural trainees at all levels. We offer academic support programs, career counseling, and individualized opportunities to a population averaging 250 trainees, including postdoctoral, visiting, and research fellows; clinical fellows and medical students; graduate students; and postbaccalaureate fellows.

Goals and Objectives

To meet the training needs of intramural scientists, fellows, and students at all levels, the Office of Education recruits and develops academic support programs; contributes to mentoring, evaluation, and career guidance; and creates of new training initiatives. Our professional development workshops focus on various topics, including public speaking, job interviewing, leadership and management, and scientific writing and publishing. Additional areas of involvement include programming for career exploration, networking among fellows and alumni, grantsmanship, and the enhancement of fellows' competitiveness for research awards, as well as support of new tenure-track investigators.

Notable accomplishments of the past year

The office developed and manages an online Annual Progress Review for fellows, launched in 2016, to track scientific and career development and progress. As part of investigators' assessments of mentoring, the fellows' reports are also provided for the BSC site visits. The database of NICHD alumni from 2008 to the present continues to be updated, and we compiled a valuable list of organizations that accept grant applications from NIH intramural fellows, through both NIH and non-NIH funding mechanisms. Twenty-nine NICHD fellows were recipients of the 2018 FARE (Fellows Award for Research Excellence) awards. In addition to the Fellows Intramural Grants Supplement (FIGS) continuing to recognize and stimulate grant applications among fellows, we launched a new competitive internal funding opportunity for NICHD postdoctoral and clinical fellows: our Intramural Research Fellowships (IRFs). Its ultimate goal is to promote grant writing and enhance fellows' awareness of various components for an NIH grant.

For our annual three-minute talk (TmT) training and awards program, we once again partnered with NHGRI and NIDCR; TmT emphasizes the importance to scientists of communicating their research and its significance to improving human health. TmT participants completed several oral presentation workshops, received one-on-

Yvette R. Pittman, PhD, *Acting Director, Office of Education*

Carol Carnahan, *Program Coordinator*

one professional coaching, and were judged by internal and external panels. Dr. Arup Chakraborty in the laboratory of Dr. Melvin DePamphilis received the third-place award. The Office of Education continued our Graduate Student Talks initiative, established in 2014; it provides the Institute's graduate students with experience in presenting their thesis research to a non-specialist scientific audience.

We established a new research ethics training plan for intramural fellows, providing eight hours of training within a fellow's initial two years, in fulfillment of Responsible Conduct of Research requirements. The Institute also established an exchange program with INSERM (the Institut National de la Santé et de la Recherche Médicale) in France, which provides a unique opportunity for U.S. and French scientists to obtain postdoctoral training with French and U.S. mentors, respectively. The Fellows Recruitment Incentive Award (FRIA) continues to support investigators who recruit postdocs from populations traditionally under-represented in science. This year, the award was given to Dr. Mark Stopfer, who recruited Dr. Alejandra Boronat Garcia. The alumni of our NICHD Scholars program, in its seventh year, has expanded to ten individuals; our two new alumni started medical school at the University of Kansas and University of North Carolina at Chapel Hill, two graduated from medical school and one from an MD/PhD program in May 2017, and two new scholars joined the program. The Scholars program focuses on "developing talent" and supporting trainees' academic and career progression. We offered a three-week summer training course for college teaching and curriculum development associated with University of Maryland. Postdoctoral fellows were also given the opportunity to organize and teach our annual course for postbaccalaureate trainees, which entered its twelfth year. The Office of Education is fully committed and actively involved in graduate and professional school advising and career counseling for all of our fellows. The 13th annual meeting of fellows took place at the Smithsonian Museum of the American Indian and featured keynote speakers Dr. Peter Agre, 2003 Nobel Laureate in Chemistry, and Dr. Adam Ruben, who shared his perspective on science careers using comedy. Each spring, this retreat includes presentations by fellows and a poster presentation by each attendee and is held for about 100 people to address scientific developments and careers. The program is developed and run by a fellows' steering committee. In September 2017, the Division of Intramural Research gave its tenth Mentor of the Year awards to Tamás Balla, MD, PhD (investigator) and Amber Stratman, PhD (fellow). Lastly, the *NICHD Connection* monthly newsletter continues its focus on mentoring, careers, and academic programs for young scientists, publishing its 90th issue in December 2017 and reaching all members of the intramural division and alumni.

Contact

For further information, contact *pittmanyv@mail.nih.gov* or Carol Carnahan (*carnahac@mail.nih.gov*).

Office *of the* Clinical Director, NICHD

The NICHD intramural clinical research program currently includes 86 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 32 NICHD principal investigators and 161 associate investigators. The NICHD clinical protocol portfolio spans the spectrum from Natural History to therapeutic trials. Eleven protocols involve an investigational drug or device. Four 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 Karim Calis, PharmD, MPH, and has twelve members and two 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 it 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.

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*

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DuShon Hutchinson, *Patient Care Coordinator*

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Loc Trinh, *Research Chemical Engineer*

Alan DeCherney, MD, *Senior Investigator*

Kisha Jenkins, BS, RN, *Clinical Nurse*

Karim Calis, PharmD, MPH, *Clinical Investigator*

Frank Pucino, PharmD, *Volunteer*

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* or *https://dir.nichd.nih.gov/dirweb/clinicaltrials.html*). 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*.

Bone and Matrix Biology in Development and Disease

» 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*.

» Screening and diagnosis on patients with suspected connective tissue disorders. Patients and their families receive comprehensive evaluations, counseling, and risk assessment. Patients may be referred to **DR. JOAN MARINI** at *oidoc@helix.nih.gov*.

Developmental Endocrine Oncology and Genetics

» 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*.

» 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.

» Research investigating the causes, complications, and treatment of Primary Aldosteronism. Patients may be referred to **DR. ANDREW DEMIDOWICH** at *andrew.demidowich@nih.gov* or **MR. CHARALAMPOS LYSSIKATOS** at *charalampos.lyssikatos@nih.gov* or 301-496-6633.

» Studies into how genetics and inflammation play a role in the development of obesity and type 2 diabetes. Patients may be referred to **DR. ANDREW DEMIDOWICH** at *andrew.demidowich@nih.gov* or **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-435-8201.

Maternal–Fetal Medicine, Imaging, and Behavioral Development

» Studies with healthy subjects to test and calibrate non-invasive optical imaging technology for functional brain imaging. The study is important to investigate the NIRS imaging system to explore techniques that will potentially improve the feasibility and reliability of the system according to the needs of the population whom existing imaging systems are unsuitable for. Functional near infrared spectroscopy

(fNIRS) is an emerging non-invasive imaging technique to assess brain function. fNIRS measurements are based on the local changes in cerebral hemodynamic levels (oxy-hemoglobin and deoxy-hemoglobin) associated with brain activity. Due to neuro-vascular coupling, local changes in oxyhemoglobin and deoxyhemoglobin levels can serve as an indirect measure of brain activity. To probe changes in Oxy- and Deoxy-hemoglobin concentrations in the cortex that are caused by brain activity, different tasks such as the n-back test will be administered to quantify spatial and temporal brain activity. Subjects may be referred to **DR. AMIR GANDJBAKHCHE** at *amir@helix.nih.gov*.

» 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*.

» *Biological Markers for the Prediction of the "great obstetrical syndromes":* A Longitudinal Study: This is a prospective cohort study of biomarkers in the great obstetrical syndromes to examine the natural history of normal pregnancy and the most frequent pregnancy complications. The goal is to develop sensitive, specific, and parsimonious predictive models to identify the patients at risk for developing complications of pregnancy using a combination of clinical and biological markers (biochemical and biophysical). For more information on the study, please contact **DR. ROBERTO ROMERO** at *romeror@mail.nih.gov*.

» *Normal and Abnormal Fetal Anatomy using Three- and Four-Dimensional Ultrasound and Magnetic Resonance Imaging:* In this study, we use state-of-the-art sonographic and MRI sequencing techniques to evaluate normal anatomy and function of the human fetus, cardiovascular system, neuroconnectivity, and placental hemodynamics. Imaging of the fetus and intrauterine environment is a powerful tool to assess fetal anatomy, growth, pathology, cardiovascular disorders, and neuropathology, and remains the essential tool to evaluate whether a fetus has a congenital anomaly. For more information on the study, please contact **DR. ROBERTO ROMERO** at *romeror@mail.nih.gov*.

» *Establishment of a Clinical Perinatal Database and Bank of Biological Materials:* This is an observational study that allows examination of materials from the mother (maternal blood, vaginal fluid, etc.) and umbilical cord blood. Placentas are collected after delivery. For more information on the study, please contact **DR. ROBERTO ROMERO** at *romeror@mail.nih.gov*.

Metal Biology and Molecular Medicine

» 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*.

Pediatric Endocrinology, Metabolism, and Genetics

» Studies on 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.

- 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. PADMA VEERARAGHAVAN** at 301-451-0399.
- Clinical and genetic studies of patients with disorders of puberty and reproduction, including early and late entry into puberty, and congenital central hypogonadism, including isolated GnRH deficiency. Patients may be referred to **DR. ANGELA DELANEY** at *delaneya@mail.nih.gov*.
- 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* or **MS. NICOLE FARHAT** at 301-594-1765.
- Study of individuals with CLN3, or Juvenile Neuronal Ceroid-Lipofuscinosis (Juvenile Batten Disease) and their family members. Interested participants may be referred to **DR. AN NGOC DANG DO** at *an.dangdo@nih.gov* or **MS. KISHA JENKINS** at 301-594-2005.
- Studies of patients with genetic disorders related to an abnormal function of the creatine transporter gene causing creatine transport deficiency (CTD). Patients may be referred to **MS. DYLAN HAMMOND** at 301-827-1048 or to **DR. SIMONA BIANCONI** at *simona.bianconi@nih.gov*.
- Studies to identify novel genetic causes of idiopathic growth disorders using exome sequencing. Subjects will include children and adults with either short stature or tall stature without a known cause. Patients may be referred to **DR. JEFFREY BARON** at *baronj@cc1.nichd.nih.gov* or **DR. YOUN HEE JEE** at *jeeyh@mail.nih.gov*.

Physical Biology and Medicine

- Studies on patients with genetic disorders related to fragile sarcolemma muscular dystrophy. This includes Limb-Girdle Muscular Dystrophy type (LGMD) 2B-F, I, L, Myoshi Myopathy (MM), Becker Muscular Dystrophy (BMD), Myoshi Muscular Dystrophy -3 (MMD3). Patients may be referred to **DR. JOSHUA ZIMMERBERG** at *zimmerbj@mail.nih.gov* or **MS. HANG WATERS** at *watershn@mail.nih.gov*.

Reproductive Endocrinology and Gynecology

- Research on reproductive disorders affecting the endometrium (such as recurrent implantation failure) using endometrial biopsy. Patients can contact **DR. ALAN DECHERNEY** at *decherna@mail.nih.gov* or 301-594-5494.
- Research on reproductive function in sickle cell disease. Patients can contact **DR. ALAN DECHERNEY** at *decherna@mail.nih.gov* or 301-594-5494.

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 18–month clinical rotations 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 (DMC) in Detroit, MI. The program is approved for seven positions, two of which are funded by the Perinatology Research Branch (PRB), which is also housed at the DMC, and the remainder by the DMC itself. The Fellowship emphasizes clinical, translational, and basic research (18 months are dedicated to research). Ideal candidates for the Program are well trained individuals from a university program who wish to pursue a career in academic medicine, thrive in a rigorous and challenging environment, and are goal-oriented and self-motivated.

The Fellowship stresses a multi-disciplinary approach to the complications of pregnancy. There is a strong emphasis on prenatal diagnosis of congenital anomalies with ultrasound, and 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 at the PRB in the fields of parturition, reproductive immunology, placental pathology and biology, biomarker discovery, and systems biology in reproduction. Alternative opportunities are available at the C.S. Mott Center of Wayne State University.

The PRB's primary areas of interest 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: *http://www.med.wayne.edu/prb/*. The website also contains information about the faculty and their publications and awards. The Director of the Fellowship Program is Dr. Lami Yeo, and the Associate Director is Dr. Roberto Romero, Chief of the PRB. The Program is sponsored by the PRB, the DMC, and Wayne State University. Fellows are employees of the DMC, and program oversight is with the Office of Graduate Medical Education of the DMC.

Medical or Pediatric Genetics Training Program

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 National Institute of Child Health and Human Development (NICHD). Training in genetics is offered through NICHD'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 NICHD 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 the NIH leadership to training initiatives in translational research.

The program is headed by:
Dr. Constantine A. Stratakis, MD, D(med)Sci
Scientific Director, NICHD, NIH
Email: *stratakc@mail.nih.gov*

and

Dr. Forbes D. Porter, MD, PhD
Clinical Director, NICHD, NIH
fdporter@mail.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), the National Institute of Child Health and Human Development (NICHD), and the National Institute of Dental and Craniofacial Research (NIDCR) 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 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 disease. Study design, outcome measures, statistical analysis, and ethical and regulatory issues are stressed.

Ranganath Muniyappa, MD, PhD, *Director, Inter-Institute Endocrine Training Program; Diabetes, Endocrinology, and Obesity Branch, NIDDK*

Michael T. Collins, MD, *Director, Career Development and Research Oversight, NIDCR Senior Investigator, CSDB, NIDCR*

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The IETP provides a comprehensive training experience that involves not only the NIH clinical branches working in endocrinology but also the Georgetown University Hospital, Washington Hospital Center, and Walter Reed Medical Center. 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.

Publications

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3. Considine B, Kamilaris CDC, Bailey UV, Bauer FA, Lassman MN. Coronary vasospasm and bowel ischemia in a patient with metastatic gastrointestinal carcinoid. *Conn Med* 2016 80:463-466.

Collaborators

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

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.

The following URL provides more detailed information about the program: http://pe.nichd.nih.gov

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 five months at the NIH clinical research center, five months at Children's National Health Systems (CNHS), one month at The Johns Hopkins University Hospital, Baltimore, MD, and one month at Walter Reed National Military Medical Center in Bethesda, MD. 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, among 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

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Pediatric Endocrinology Fellows and Faculty, 2017

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 a 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 choose academic careers following graduation.

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 a three-year clinical fellowship in Reproductive Endocrinology and Infertility, which is accredited by the Accreditation Council for Graduate Medical Education (ACGME). 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 60 physicians in reproductive endocrinology. 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 is served by 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 civilian and military 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.

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Lauren A. Bishop, MD, *Clinical Fellow*

Matthew T. Connell, DO, *Clinical Fellow*

Nicole Doyle, MD, PhD, *Clinical Fellow*

Michael B. Evans, DO, *Clinical Fellow*

Katherine Green, MD, *Clinical Fellow*

Terrence D. Lewis, MD, PhD, *Clinical Fellow*

Carter Monique Owen, MD, *Combined Reproductive Endocrinology Infertility-Medical Genetics Clinical Fellow*

Toral Parikh, MD, *Clinical Fellow*

Justin Pilgrim, DO, *Clinical Fellow*

Jessica R. Zolton, DO, *Clinical Fellow*

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 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 at the Shady Grove Fertility Center. Experience in the Assisted Reproductive Technologies (ART) is provided by rotation in the newly renovated, state-of-the-art Walter Reed Bethesda ART facility and in the Shady Grove Fertility Center. Fellows also obtain 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 first-year fellows, which take place from July to September of the first year. The introductory seminars provide a historical perspective and basic understanding of the practice of Reproductive Endocrinology. In a weekly NIH teaching rounds conference, fellows review and discuss challenging cases with faculty and fellows. In addition, all faculty and fellows of all years are expected to attend the weekly Pre-operative and Fellows' conferences. In addition, fellows attend weekly research conferences sponsored by the NICHD and present updates on thesis work at the weekly "Research in Progress Conference." Core Accreditation Council for Graduate Medical Education (ACGME) training objectives are covered in special NIH grand rounds and by courses at NIH or Walter Reed in Bethesda. NIH Endocrine Grand Rounds provide 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, during which management of patients pursing ART is discussed and outcomes are reviewed. In addition to larger groups, mentors of individual laboratories to which the fellow is affiliated generally meet on a weekly basis to review research progress. 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

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3. 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 6:36273643.
4. Falk M, DeCherney A, Kahn P. Mitochondrial replacement techniques—implications for the clinical community. *N Engl J Med* 2016 374:1103-1106.
5. Plowden T, Schisterman E, Sjaarda L, Zarek S, Perkins N, Silver R, Galai N, DeCherney AH, Mumford S. Subclinical hypothyroidism and thyroid autoimmunity are not associated with fecundity, pregnancy loss, or live birth. *J Clin Endocrinol Metab* 2016 101:2358-2365.

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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 ensure 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.

Francie Kitzmiller, *Chief, Administrative Management Branch*

Becky Preston, *Deputy Branch Chief*

Valerie Leftwood, *Deputy Branch Chief*

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, Office of the Director; 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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Michelle Hudson, *Administrative Officer – Team Leader*
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Bonnie Lancey, *Administrative Officer*
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Research Animal Management Branch

The Research Animal Management 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 animals owned by the NICHD DIR. The Branch's activities include the following: (1) providing primary veterinary care; (2) advising the NICHD Scientific Director on animal care and use practices; (3) insuring that animal use within DIR is in accordance with applicable regulatory standards; (4) coordinating intramural animal use, including appropriate animal model selection, support requirements, and Animal Study Proposal review; (5) advising scientific staff on comparative medicine, Animal Study Proposal design, disease interference, and on other factors that may complicate or invalidate research results; (6) implementing and coordinating animal health monitoring; (7) coordinating quarantine for incoming animals of unknown health status to prevent the introduction of agents pathogenic to humans or animals; (8) coordinating a central animal ordering program for the NICHD; (9) providing administrative management of the NICHD Animal Care and Use Committee (ACUC); and (10) interfacing with organizations and institutions concerned with the ethical and humane care and use of animals in research.

Joseph M. Schech, DVM, *Head, Research Animal Management Branch*

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Lauren Pandolfo, 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 with regard to animal use as well as over NICHD and NIA (National Institute on Aging) animals in the Porter Neurosciences Research Center (PNRC) SAF and in the Building 10A Central Animal Facility (CAF). 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 three Institutes (NICHD, NEI, and NIAMS) in a restricted-access,

disease-free rodent facility; it also includes a room for *Xenopus*.

The NIHAC SAF supports the animal research activities of the NICHD. This facility houses nonhuman primates. Animal holding areas include indoor housing and indoor/outdoor runs.

Suite 6C127 of the ACRF Animal Facility supports the animal research activities of the NICHD. 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 Building 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.

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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 biomolecules, 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 8S corridor of Building 10 on the NIH campus. The Mass Spectrometry Facility currently serves both clinical and basic research laboratories within the NICHD intramural research program. As resources permit, we also collaborate with principal investigators (PIs) of other institutes within NIH and with other outside institutions.

The Facility is committed to promoting mass-spectrometric aspects of proteomics and other mass-spectrometric analyses in NICHD's DIR. We make substantial efforts to educate investigators on the benefits and pitfalls of the techniques used in the Facility. In particular, we provide advice and protocols for appropriate methods of sample isolation that are compatible with analysis by mass spectrometry. 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 the NICHD. In addition, Peter Backlund is the moderator of the *NIH Mass Spectrometry Interest Group*.

Peter S. Backlund, PhD, *Staff Scientist, Acting Director*
Vince Pozsgay, PhD, *Staff Scientist*
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 PI and other scientists involved in each study to discuss experimental goals and data requirements. The Facility has an internationally recognized capability in the characterization of proteins and peptides by mass spectrometry, including: (1) identification of proteins isolated by electrophoresis; (2) confirmation of molecular weights of recombinant or synthetic proteins and peptides; (3) determination of sites of specific post-translational modifications, including phosphorylation, glutamylation, AMPylation, and disulfide bond formation; (4) quantification of specific post-translational modifications; and (5) sequencing of 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.

SimulTOF 300 MALDI TOF/TOF: The state-of-the-art high-performance MALDI (matrix-assisted laser desorption/ionization) TOF/TOF (time-of-flight/time-of-flight) instrument can be operated in either positive- or negative-ion modes. The instrument is most often 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 couples a one-meter ion-mobility-drift cell with a high-resolution qTOF mass spectrometer. Ion-mobility spectrometry (IMS) prior to mass analysis provides an added dimension of sample separation that is orthogonal to both chromatography and mass spectrometry. The instrument is currently used to determine collision cross-section measurements of ions for small molecules and intermolecular complexes and for separation and analysis of complex mixtures of lipids and peptides.

Agilent 6495 LC-ESI QqQ (Triple Quad): The instrument is coupled to an Infinity 1290 UPLC system with either an ESI or APCI ion source, and is currently used for small-molecule analysis and quantification, principally for steroid profiling and the analysis 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.

Major projects

ION–MOBILITY MASS SPECTROMETRY FOR DETECTION OF ISOBARIC BIOMOLECULES AND ION COMPLEXES

Given that ion-mobility spectrometry (IMS) operates on a millisecond time scale, the technique performs separations of complex mixtures much faster than is possible with liquid chromatography (LC). In addition, IMS separations are associated with the 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 but with different

structures can be separated on the basis of their CCS. This has great potential for separating isobaric biomolecules, including numerous steroids, lipids, and peptides. In addition to the separation of structural isomers, IMS also offers the ability to study intermolecular complexes in the gas phase to determine conformational changes and stoichiometry. One of the first studies we undertook with the Core Facility's Agilent Model 6560 Ion Mobility Q-TOF LC/MS instrument was to investigate beta-cyclodextrin–cholesterol complexes in the presence of different monovalent and divalent cations. The measured CCS of different ions could be attributed to two distinct conformations of the ions, and we have begun molecular modeling studies to independently explain the different conformational states. More recently, this instrument has been used to detect structural differences between two commercial preparations of hydroxypropyl-modified beta-cyclodextrins (Reference 1). One of these preparations is currently being used in clinical trials to treat Neimann-Pick disease type C1 patients.

We are currently using ion-mobility/mass spectrometry to analyze complex mixtures of phospholipids extracted from mouse tissues in order to analyze branched-chain fatty acid incorporation into phosphatidylcholine (PC) in animals fed a diet supplemented with phytol, a saturated C20 branched-chain alcohol. Phytol is metabolized to phytanic acid, which can be incorporated into phospholipids and triglycerides. Muscle-tissue lipids were extracted and then analyzed by ion-mobility/mass spectrometry. The muscle PC species profiles under the phytol and control diet were similar, except that some additional species were detected in the phytol-diet muscle. We tentatively identified the two most abundant novel species as PC 20:0-16:0 and PC 20:0-22:6. The drift times for these novel species are consistent with the molecules containing the branched phytanoyl fatty acyl group. The separation of phospholipids by ion mobility also makes it possible to quantitate complex mixtures of PC species without the longer time period required for LC separation of these mixtures, shortening run times from 60 to 3 minutes of IMS separation.

QUANTITATION OF PLASMA MELATONIN (5-METHOXY-*N*-ACETYLTRYPTAMINE) AND *N*-ACETYLTRYPTAMINE

We developed a multiple reaction monitoring (MRM)–based assay to quantify *N*-acetyltryptamine and melatonin in plasma. *N*-acetyltryptamine is a melatonin-receptor mixed agonist/antagonist. The assay provided the first evidence for endogenous *N*-acetyltryptamine in the daytime plasma from human volunteers, rhesus monkey, and rats. The mass-spectrometric method employs deuterated internal standards to quantitate *N*-acetyltryptamine and melatonin. Twenty-four-hour studies of rhesus macaque plasma revealed increases in *N*-acetyltryptamine at night to concentrations that exceed those of melatonin. We also used the technique to measure these compounds in tissues known to be involved in melatonin biosynthesis, and *N*-acetyltryptamine was present in both pineal and retinal tissue from rhesus macaques. 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 chrono-biotic though actions mediated by the melatonin receptor (Reference 2).

MASS SPECTROMETRY–BASED PROFILING AND QUANTIFICATION OF SERUM AND URINARY STEROIDS

We previously developed an MRM–based mass-spectrometry method to quantify several androgenic steroids in urine and applied the method to studies of polycystic ovary syndrome (PCOS) patients and patients with congenital adrenal hyperplasia (CAH). The assay was used to quantify 5-alpha-pregnane-3-alpha,17-alpha-diol-20-one (known also as pdiol) and its 5-beta stereoisomer, 17-alpha-hydroxypregnanolone (known also as 5-β-pdiol); pdiol is an intermediate in the 'backdoor pathway' from

17OH progesterone to dihydrotestosterone. In a study of CAH patients, we found urinary levels of both pdiol and 5-β-pdiol to be directly correlated with the serum levels of androstenedione (Reference 3). The assay also measures etiocholanolone, androsterone, and testosterone. More recently, we also developed an MRM–based assay for quantification of glucocorticoids in serum, including cortisol, cortisone, 11-deoxycortisol, and corticosterone.

In addition to the targeted assays, we also developed a novel un-targeted LC-MS/MS approach to profile urinary steroids, which permits detection of steroids that have changed in a patient cohort without prior knowledge of the steroids' identity (i.e., untargeted metabolomics of steroids). Initial studies in PCOS patients detected 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. In order to expand this approach, it would be helpful to compare observed unknown peaks with a database of known steroid standards, and we are currently developing a spectral database that would be compatible with this type of spectral search.

IMPROVED METHOD FOR PROTEIN IDENTIFICATION BY ANALYSIS OF BOTH MS AND MS/MS SPECTRAL DATA

We developed an improved method for protein identifications based on the combined analysis of MS and MS/MS spectral data collected from tryptic digests of proteins in gel bands using MALDI TOF-TOF instrumentation. The method uses theoretical peptide masses and the measurement errors observed in the matched MS spectra to confirm protein identifications obtained from a first-pass MS/MS database search. The method makes use of the mass accuracy of the MS1–level spectral data that heretofore were ignored by most peptide database search engines. We developed a probability model to analyze the distribution of mass errors of peptide matches in the MS1 spectrum and to thus provide a confidence level to the additional peptide matches. The additional matches are independent of the MS/MS database search identifications and provide additional corroboration to identifications from MS/MS–based scores that are otherwise considered to be of only moderate quality. Straightforward and easily applicable to current proteomic analyses, this 'ProteinProcessor' provides a robust and invaluable addition to current protein identification tools (Reference 4).

Publications

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Collaborators

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The NICHD Zebrafish Core Facility

The NICHD Zebrafish Core was established in May 2012. The goal of the Facility is to provide its clients with consultation, access to equipment and reagents, and service in the area of zebrafish genetics. To maximize the efficiency of our services, we implemented project management software. 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 Harold Burgess, Ajay Chitnis, Igor Dawid, and Brant Weinstein. The Core's activities consist of (1) oversight and support of client-specific projects, (2) introduction and troubleshooting of new methodologies with promising application in zebrafish and research in the field of developmental biology, (3) maintenance and improvement of equipment and infrastructure, and (4) service and educational outreach.

Benjamin Feldman, PhD, *Staff Scientist and Director of the NICHD Zebrafish Core*

ChonHwa Tsai-Morris, PhD, *Staff Scientist and Assistant Director of the NICHD Zebrafish Core*

Oversight and support of client-specific projects

Over 2016-17, Feldman engaged in research projects with six labs: three from NICHD, one from NIEHS, one from NINR, and one from the Children's National Medical Center.

Porter Lab (NICHD): genetic dissection and creation of human disease models of sterol metabolism. Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive, multiple malformation syndrome with pediatric onset characterized by intellectual disability and aberrant behavior. Phenotypic characterization is ongoing of zebrafish carrying mutant alleles of *dhcr7*, the zebrafish ortholog to the human SLOS gene *DHCR7*, which were generated with support from the Core in previous years. The Core also used Crispr-Cas9 technology to create additional genetic mutant lines for the Porter lab in genes with roles in other steps in cholesterol metabolism. Phenotypic characterization by the Porter lab is ongoing.

Stratakis Lab (NICHD): function of zebrafish orthologs to human genes implicated in disorders of the pituitary-adrenal axis. (1) Gigantism is the result of excess growth hormone (GH) secretion during childhood, before the growth plates close. Since 2012, the Core has supported this lab's investigation of the zebrafish ortholog to a human gene implicated as a driver of gigantism. Feldman is co-author on a

FIGURE 1. Screen shot of tracking software for measuring swimming zebrafish

paper (Reference 2) that included a description of this gene's developmental expression in zebrafish. This year, the Stratakis lab also began to test the effect on growth and development of zebrafish in which this gene is chronically overexpressed in tissue-specific or ubiquitous locations, using the Gal4/UAS transgene system. Last year, the Core used Crispr-Cas9 methods to generate, for the Stratakis lab, zebrafish carrying loss-of-function mutations in the above gene and three other zebrafish orthologs to genes implicated human growth anomalies. Characterization of the resulting phenotypes is ongoing. Since 2012, the Core has also supported this lab's investigation into the function of two zebrafish orthologs to human adrenal hyperplasia– and Cushing's disease–associated genes. Over previous years, the Core helped Stratakis's lab generate and acquire, respectively, zebrafish carrying loss-of-function mutation for each of these orthologs. Phenotypic characterization found notable effects on juvenile growth in the case of one gene and on early embryogenesis in the case of the other, and these efforts will continue. This year, we generated mutants for six new genes whose human orthologs are implicated in adrenal hyperplasia and/or Cushing's disease, and we plan phenotypic characterization of these mutations.

Kaler Lab (NICHD): modeling copper deficiency–associated distal motoneuropathy. The Menkes gene encodes ATP7A, a copper-binding ATPase localized to the plasma membrane and the trans-Golgi network (TGN); ATP7A is critical for proper intracellular copper distribution. Complete loss of ATP7A causes Menkes' disease, a severe human disease, leading to childhood death without early intervention with copper therapy. Two *ATP7A* missense mutations cause a milder syndrome than Menkes' disease, a distal motoneuropathy that is nevertheless debilitating for children and young adults. Since 2013, the Core has supported a project to clarify the structure-function relationship of ATP7A with motor neuron defects from the perspective of these missense mutations. Over previous years, the Core supported the Kaler lab's work to visualize and compare motor neuron growth during embryogenesis of wild-type zebrafish embryos and embryos homozygous for null mutations in their *ATP7A* ortholog *atp7a*, work that will continue. In parallel with these studies, Feldman

and Tsai-Morris are making a concerted effort to establish genome-editing technology in the Core, using CRISPR-Cas9 technology in combination with donor DNA and by comparing two general approaches: double-stranded donor DNA for homologous recombination–based genome editing and single-stranded DNA for homology-directed, repair-based genome editing. The initial goal is to induce formation of zebrafish *atp7a* point mutations cognate to one of the human *ATP7A* motoneuropathy alleles.

Blackshear Lab (NIEHS): assessing functions of a zinc-finger protein gene family in zebrafish hematopoiesis. The Blackshear lab is interested in dissecting the connections between a family of zinc-finger proteins and blood development. They contracted the NICHD Zebrafish Core to create null mutations in each of the seven zebrafish orthologs to this family, to determine the viability or fertility of such mutants and, if viable, to provide blood samples from mutants for the Blackshear lab to analyze. This year, we created null alleles for six out of the seven genes. Phenotypic analysis has commenced, with no aberrations in homozygous null fish yet identified for three of the seven genes that have been tested.

Meilleur Lab (NINR): ability of small molecules to mitigate myopathy in zebrafish ryr1b *mutants*. For this new project, we helped Katy Meilleur and colleagues formulate a plan to test candidate drugs they are currently identifying for their ability to potentially ameliorate muscle defects seen in zebrafish mutants that carry mutations in the gene *ryr1b*; mutations in the gene's human counterpart are implicated in various myopathies. We have acquired larvae carrying the *ryr1b* mutation from an outside source and are currently raising them to adulthood.

Tuchman Lab (Children's National Medical Center): neuroprotective drugs to mitigate hyperammonemia, a consequence of urea cycle defects and liver failure. Exposure of the brain to high ammonia levels causes neurocognitive deficits, intellectual disabilities, coma, and death. Since 2012, the Core has helped this lab use zebrafish embryos to identify small molecules capable of diminishing the effects of hyperammonemia. Over previous years, a library of hundreds of small molecules with known safety profiles for humans was screened, and several promising candidates were identified for follow-up validation studies in zebrafish and other animal models. Last year, the Core supported the Tuchman lab to increase the throughput of this screen, bolstered by additional personnel from the Tuchman lab and the Core's implementation of NICHDs massive embryo production systems (MEPS; see below) as a source for embryos, enabling them to complete a screen of an additional 10,000 compounds last year. Additional candidate compounds were thus identified and secondary screens and dose-response studies on lead compounds will continue.

Basic gene knockouts

The Core continues to produce new mutant lines using CRISPR/Cas9 technology. We optimized the process so successfully in 2015–16 that, last year, we were able to offer the creation of at least two novel CRISPR/Cas9 frame-shifting alleles per gene on a fee-for-service basis. Using this mechanism, we have been contracted to create knockouts for 15 distinct genes for three labs: seven for the Blackshear lab (NIEHS), six for the Stratakis lab and two for the Porter lab. We generated carriers of at least two distinct frame-shifting alleles for 10 of these and one frame-shifting allele for another. We identified gene-specific CRISPR/Cas9 components (i.e., gRNAs) that mutagenize with sufficient efficiency for four of the five remaining genes, and we used these to produce likely carriers of multiple alleles that are currently being raised. We are still performing tests to identify a suitable gRNA for the fifteenth gene.

Precise genome editing

We have also continued to troubleshoot methods for precise genome editing. Our approach, which combines co-injection of "rescue" templates along with the CRISPR/Cas9 components, is discussed above in the context of our support for the Kaler lab. Furthermore, to help ensure that our precise genome editing efforts reflect state-of-the-art practices and insights, we have established or reinforced contact and dialogue with the laboratories of Raman Sood (NHGRI Zebrafish Core), Shawn Burgess (NHGRI), David Grunwald (University of Utah), and Darius Balciunas (Temple University).

Steady source of zebrafish embryos

As part of the Central Aquatic Facility, NICHD has two large and two small mass embryo production systems (MEPS) distributed between two of our procedure rooms. Initial optimization by the Research Animal Management Branch (RAMB) and Charles River staff already had one of the two larger MEPS running on a continuous basis, with one embryo collection per week. However, NICHD research through the NICHD Zebrafish Core and through other facilities requires embryos more frequently. For small-molecule screens, at least 1500 embryos per experimental day are required. For microinjection experiments, two morning waves of at least 500 synchronously fertilized embryos are required. To achieve these goals, working together with RAMB and Charles River, we successfully adjusted husbandry, feeding and embryo collection methods and timing to enable the collection of two synchronous waves of a sufficient quantity of freshly fertilized embryos on three days per week. More recently, we reduced the workload for staff and the production burden for the fish by having only one collection day per week for each of two MEPS. Over time, we will assess yields and fecundity associated with this reduced collection schedule in 2017–2018. In addition to the EK strain we have been using, we will introduce a second wild-type zebrafish strain, TAB5, that is better suited for precise genome editing projects, so that one MEPS will house each strain.

Robust software and hardware for longitudinal growth studies

An increasingly common phenotypic characterization of genetic variants in zebrafish is the measurement of their sizes and weights and how certain genetic conditions can alter these parameters over time. i.e., change their growth. Indeed, the use of the Core's macro-photography lens and milligram balance to measure and weigh various genetic models has risen strikingly since the Core's inception in 2012. Of relevance to the NICHD Zebrafish Core's projects, growth defects have been found in some of the Stratakis and Porter lab's genetic zebrafish models. Traditional approaches to size measurement and zebrafish husbandry present three challenges that we are trying to overcome: (1) they are labor-intensive; (2) they require life-threatening immobilization of the fish; and (3) there is no practical strategy for re-identifying the individual fish for longitudinal measurements over time. In 2014, the Core contracted Viewpoint Life Sciences to write software for their behavior tracking systems designed to measure birds-eye view lengths and surface areas of free-swimming zebrafish embryos, larvae, juveniles, and adults. A promising package was installed on the three Viewpoint behavioral tracking units owned by NHGRI and shared by NICHD. Last year, the software was delivered and tested, and we found that it can recapitulate manual measurements with an overall accuracy of about 90%. We will work with Viewpoint to reformulate the software for greater accuracy. Last year, we also worked with R&D Aquatics to develop a system for rearing individual zebrafish for the entirety of their approximately two months of larval and juvenile growth. We confirmed 100% survival for these first two months of life in an initial trial of wild-type fish. We plan to combine this hardware and software to enable the generation and comparison of growth curves for individual wild-type and mutant zebrafish using a minimum of investigator effort.

Additional Funding

- NICHD Customers: $14,250 in fee-for-use charges
- Non-NICHD Customers: $17,280 in fee-for-use charges

Publications

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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 next-generation deep sequencing and project data analysis.

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Director, Molecular Genomics Core Facility
Steven L. Coon, PhD, *Staff Scientist*
James R. Iben, PhD, *Staff Scientist*
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 three sequencing machines. Most of our work is conducted on our high-capacity, production-scale machine: an Illumina HiSeq 2500. The two other sequencers, an Illumina MiSeq and an Ion Torrent Personal Genomics Machine, are smaller, faster machines, which can generate longer sequence reads. Our recently acquired cBot liquid handler will allow even higher through-put on the Illumina HiSeq. This array of sequencers provides a suite of scales and capabilities. Our sequencing services include whole genome, 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.

This year, the MGC acquired a 10X Genomics Chromium Single Cell Controller. The Chromium converts a suspension of single cells into cDNA libraries that are barcoded by cell of origin. The cDNAs can then be converted to sequenceable libraries and run on our Illumina HiSeq 2500 to generate cell-specific transcriptomes.

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 MGC provided sequencing for 54 projects across the full spectrum of sequencing types; the projects involved 21 NICHD Principal Investigators from eight Affinity Groups. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support for 12 NICHD investigators across seven Affinity Groups. Our mission is to offer accurate and innovative sequencing and bioinformatic tools to

facilitate research into the diagnosis, counseling, and treatment of hereditary disorders, and to support basic research, which promotes understanding of human health and development.

Publications

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NICHD Microscopy and Imaging Core Facility

The mission of the NICHD Microscopy and Imaging Core (MIC) is to provide service in three different areas: (1) wide-field and confocal light microscopy, (2) transmission electron microscopy (EM), and (3) sample preparation for light and electron microscopy studies.

Vincent Schram is the point person for light microscopy and image analysis and is working in close collaboration with his NINDS counterpart, Carolyn Smith, the director of the Light Imaging Facility, NINDS, also in the Porter Neuroscience building. This mode of operation is beneficial to the community as it provides extended support hours, wider expertise, and access to more equipment than each Institute can afford on its own. The EM branch of the facility is staffed by Chip Dye. Lynne Holtzclaw is in charge of sample preparation. Both report directly to Vincent Schram, who serves as executive director under the management of Chris McBain. Tamás Balla acts as scientific advisor for the facility.

Vincent Schram, PhD, *Staff Scientist*

Louis (Chip) Dye, BS, *Research Assistant*

Lynne A. Holtzclaw, BS, *Research Assistant*

Mode of operation

Located on the ground floor of building 35A, the facility is accessible 24/7, and users can reserve time on each microscope by using an online calendar (https://next.cirklo.org/nichd/). The MIC is available free of charge to all NICHD investigators and, resources allowing, to anyone within the Porter building.

For light microscopy, sample preparation and image acquisition are typically handled by end users, with support and training provided by Lynne Holtzclaw (specimen preparation) and Vincent Schram (imaging acquisition and data analysis). For electron microscopy (EM), most of the sample processing and imaging is done in-house by Chip Dye to ensure consistent and reproducible conditions.

Light microscopy

The facility operates four modern confocal microscopes, each optimized for specific applications: (1) a Zeiss LSM 710 inverted for high-resolution confocal imaging of fixed specimen and live cells; (2) a Zeiss LSM 780 for challenging specimens that require both high resolution and high sensitivity; (3) a Nikon Spinning Disk/Total Internal Reflection Fluorescence (TIRF) hybrid microscope for high-

speed confocal imaging or selective recording of membrane-bound events in live cells; (4) a Zeiss LSM 880 2-photon confocal for thick specimen and live animal microscopy, which the facility recently aquired. Several conventional (wide-field) light microscopes provide imaging modalities such as transmission (visible stains), large-scale tiling of tissue slices, high-speed phase contrast, and differential interference contrast (DIC). High-end computer workstations with imaging software (Zeiss Zen, Nikon Element, Bitplane Imaris, SVI Hyugens, Metamorph, and ImageJ) are also available.

After an initial orientation during which the project is discussed and the best approach is decided upon, users receive hands-on training on the equipment and/or software best suited to their goals, followed by continuous support when required. Additional training and support is offered to the community in different ways: (1) on-site assistance and training on equipment owned by individual investigators; (2) a yearly workshop on microscopy covering light and electron microscopy, image analysis, and sample processing, organized by the MIC; (3) FAES courses on microscopy, which MIC staff volunteer to teach; (4) frequent campus-wide demonstrations of new instruments and software by vendors in the field, which the MIC organizes.

The MIC has a total of 210 registered users in 65 laboratories. At 7,582 instrument hours, overall usage has grown slightly since last fiscal year, although this figure includes wide-field microscopes, which are now seeing significant usage. Half of the total usage is by NICHD investigators, most of them within the Porter building. The MIC used an additional 21% of resources for training and pilot projects, and other Institutes, predominantly NINDS, used the remaining 28%. All three confocal microscopes were used equally at an average of 43 hours per week. To alleviate this high usage, the facility is acquiring two additional point scanning instruments.

Electron microscopy

The electron microscopy branch of the facility processes specimens from start to finish: fixation, embedding, cutting, ultra-fine sectioning, staining, and imaging on the JEOL 1400 transmission electron microscope. Because of the labor involved, the volume is necessarily smaller than the light microscopy branch, where end users do their own processing. In the past 12 months, Chip Dye processed a total of 85 samples: 70 from NICHD investigators, nine from MIC internal test projects, and six from other Institutes.

Thanks to a gift from Joshua Zimmerberg, the facility received an AMT Biosprint 29 camera with a wider field of view, enhanced resolution, and slightly lower pixel size. The JEOL 1400 electron microscope is now on the facility's calendar, and trained investigators have direct access to the instrument. After the MIC, Joshua Zimmerberg's (NICHD) and Ling-Gang Wu's (NINDS) groups are the major users.

Tissue preparation

Lynne Holtzclaw continues to provide sample processing training and services to the facility's users, both for light and electron microscopy applications. She spends a significant amount of time training users in various techniques such as rodent perfusion, cryopreservation, cryosectioning, immunofluorescence, and tissue clearing. During the past year, she trained staff, processed samples and acquired images for Tamás Balla, Jeffrey Baron, Peter Basser, Sergei Bezrukov, Juan Bonifacino, Robert Crouch, Mary Dasso, Douglas Fields, Dax Hoffman, David Klein, Mary Lilly, Peng Loh, Chris McBain, Anil Mukherjee, Keiko Ozato, Mario Penzo (NIMH), Forbes Porter, Stanko Stojilkovic, Mark Stopfer, and Constantine Stratakis.

At the same time, she is pursuing a collaborative endeavor with David Klein on rat pineal glands to characterize the cell types in which three genes of interest, identified by single-cell RNA sequencing, are located. The project includes perfusion, cryosectioning of pineal glands, immunofluorescence, and imaging.

Collaborators

- Carolyn L. Smith, PhD, *Light Imaging Facility, NINDS, Bethesda, MD*
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Research Informatics Support for NICHD's Division of Intramural Research

The Computer Support Services Core (CSSC) Facility provides informatics and research services to intramural investigators at NICHD in four key areas: clinical informatics, bioinformatics, biovisualization web services, and core IT support.

During the past year, the team initiated a new hardware upgrade of all its infrastructure by increasing storage capacity and computing resources and by providing a 'failover' backup operational mode.

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Patricia Pullen, *Contractor*
Jeremy Swan, *Contractor*
Nichole Swan, *Contractor*
George Tran, *Contractor*
Loc Vu, *Contractor*

Clinical informatics

The CSSC 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 530 for 16 NIH institutes, with an expansion of research questions to over 200,000. The software development group completed one release that included features for the repeating-questions group, auto-fill searchable textbox, questions-option upload, quality-assurance query improvement, and various performance enhancements. We also supported the Clinical Trial Survey System (CTSS), an application for patient self-reporting, now used for 158 protocols. The Clinical Datamart, which provides lab results, vitals, medications, and ECG/EKG datamarts, was upgraded to support new protocols. The Clinical Datamart now also provides reporting for audits and edited information from the CTDB. The database and migration team worked on approximately 600 reports during the year and migrated a large amount of data for NHLBI, an institute that had previously chosen MetaData Rave as a Clinical Research Tool.

Biological visualization web services

The CSSC team provides the laboratories of NICHD's Division of Intramural Research (DIR) with scientific communication and consulting and media services, including publication support, website and content management support, audio/visual production, image-processing services and consulting, bio-medical graphics, 3D

printing, Virtual Reality support, and print media pre-production. This year, we were invited to co-lead the *Virtual and Augmented Reality Interest Group (VARIG)*.

The web activities of the DIR web services program include: DIR and Division of Intramural Population Health Research (DIPHR) laboratory websites, administrative support sites, the annual report, and internet applications. The CSSC continued to maintain approximately 139 public DIR websites, while reducing the number and footprint of web properties, as well as implementing a redesign effort by adapting 60 websites into a content-management system under a standardized design template; we also facilitated a data feed for updating IRP website.

The bioviz team created many illustrations for publications and journal covers on behalf of NICHD scientists. We provided research video support, recording audio/video for the Adult and Pediatric Endocrinology Training Programs, DIPHR's anniversary celebration, and NICHD's Three Minute Talks. In addition, we provided photography, design, and printing support for the DIR Annual Fellows and PI Retreats. For the IRP Research Festival, we expanded the activities of the previous year, coordinating with the NIH Library, NINDS, NLM, and NHGRI to provide eight virtual reality (VR) headsets to demonstrate how VR is being used, or could be used around the NIH, and helping to produce and print booth materials. The team continued graphic design support for and authored several articles for *The NICHD Connection*, a monthly newsletter for Intramural research fellows. Continued support of the *Science@NICHD wiki* allows principal investigators (PIs) to maintain a customized web presence for external audiences. We also support an intranet for knowledge management, collaboration within the DIR and with other NIH labs, the sharing of lab protocols and scientific data, and the recruitment of fellows.

Bioinformatics

The CSSC works with the scientific community in NICHD's DIR to create and manage web applications supporting research publications and the dissemination of information within the community. For example, the CSSC runs several applications that facilitate sharing and searching of genomic data from mutations to gene expression. The bioinformatics team assisted the Molecular Genomics Core in the maintenance of related applications. 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 parallel processing capabilities. The CSSC also continues to provide and manage dozens of terabytes of storage to support genomic research.

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. Improvements were also made to the Fellows Annual Progress Report for the DIR. This module helps facilitate a strong relationship between mentor and fellow while granting the Office of Education invaluable feedback on the training program. Significant changes were made to the division's Training Tracking module to improve staff registration and safety training compliance. The team completed multiple production releases, including user support for all DIR programs. The CSSC completed a new Cost Tracker release, an application that permits capturing, organizing, and reporting of various expenses on a per-protocol basis. The CSSC continues to work closely with the Office of the Clinical Director to improve protocol cost vs. effectiveness and a protocol-cost estimator module.

Core IT Services

The CSSC continued to expand its services to the DIR community in core IT areas.

NETWORK AND DESKTOP SERVICES

We support reliable, secure, and efficient information technology solutions, which 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 (email, data backups, VPN, helix, PDAs, and wireless configurations); and cross-platform 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 the high-performance computing systems Helix and Biowulf. We also assist users in identifying, researching, and purchasing custom hardware configurations to match research instrument requirements.

DATA-RECOVERY SERVICES

We implemented core data-recovery tools for all media, hard drive, solid state drive (SSD), and flash, etc., including RAID 0 and 5 recovery tools. Since 2005, the Core 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.

Additional Funding

- The Clinical Trials Database (CTDB) project receives funding from other NIH Intramural Institute or Center programs, including NINR, NIMH, NIDCR, NIEHS, NIAMS, NINDS, CC, NHLBI, NIDCD, NHGRI, NCCIH, and NIDDK.

Collaborators

- Karel Pacak, MD, PhD, DSc, *Section on Medical Neuroendocrinology, NICHD, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Clinical Director, NICHD, Bethesda, MD*
- Peter Schmidt, MD, *Behavioral Endocrinology Branch, NIMH, Bethesda, MD*
- Steven Stanhope, PhD, *University of Delaware, Newark, DE*
- Constantine Stratakis, MD, D(med)Sci, *Scientific Director, Division of Intramural Research, NICHD, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

Contact

For more information, email *songcod@mail.nih.gov*.

Affinity Groups

Scientists and physicians in the NICHD Division of Intramural Research (DIR) are organized into 13 affinity groups (AGs). Each AG is an intellectual hub for a group of investigators, creating a forum to share ideas and collaborate around common themes in support of the DIR mission.

The AGs serve as catalysts for new initiatives. Each investigator has a primary affiliation with an AG most closely aligned with his or her scientific interests. Secondary affiliations allow for communication across specialties in support of translational research and new collaborations.

Each AG has its own mission statement, shared research goals and objectives, and resources. Collectively, the AGs contribute to recruitment, mentoring, and the annual DIR scientific retreat.

Aquatic Models of Human Development

The **Aquatic Models of Human Development Group** uses zebrafish as a model vertebrate organism to study human development. We exploit the advantages of the zebrafish model for genetic screens, genomic manipulation, microscopic imaging, and cell and behavioral biology to gain insights into mechanisms controlling human development and defects therein that can result in disease. By maintaining a high level of expertise and resources, the group lowers the barrier to entry for other researchers in the intramural program for use of zebrafish as a research tool for investigating biological processes *in vivo*. The five PIs and the Zebrafish Core Facility, along with approximately 30 postdocs, doctoral trainees and technical staff, all focusing on zebrafish as a model system and all working in the Building 6 complex, create a critical mass of talent unsurpassed at NIH.

The group specializes in the generation of induced and engineered mutations and transgenic zebrafish lines that are used to study *in vivo* the function and integration of regulatory factors and pathways that are critical to the development, health, and survival of vertebrates, including humans. Extensive formal collaborations and informal interactions between group members promote the exchange of reagents and technical information to ensure that NICHD researchers remain at the cutting edge of zebrafish research worldwide.

Harold Burgess	Igor Dawid	Ben Feldman (Core)	Brant Weinstein
Ajay Chitnis	Katie Drerup	Tom Sargent	

Basic Mechanisms of Genome Regulation

The mission of the **Basic Mechanisms of Genome Regulation (BMGR) Group** is to do basic research into the molecular mechanisms of fundamental processes ubiquitous to all cells. Such research inevitably results in new knowledge that impacts our understanding of both health and disease. The members of the BMGR group have a strong history of producing knowledge-changing advances in several fundamental processes essential to life: DNA replication, DNA repair, nucleotide metabolism, RNA biogenesis and metabolism, chromatin-mediated control of gene expression, and genome integrity. Simply put, the BMGR group is a unique collection of world experts in processes involving DNA and RNA metabolism and the consequences of alterations of these processes to cells and organisms. Recent advances from work by BMGR members, as well as others,

has revealed that although these processes are ubiquitous, defects in them are often manifested as specific health disorders with distinctive deficiencies in development and with tissue-specificity, or in cancer. Inherent to the mission is to increase understanding of how natural genetic diversity in a population contributes to these fundamental processes in ways that affect disease and to apply such knowledge so that specific strategies for improving health can be developed.

The vision is to elucidate new knowledge about fundamental processes that will promote the discovery of novel strategies for treatment and prevention alternatives across a multitude of diseases that share involvement of altered nucleic acid metabolism, gene expression, and genome integrity. Through basic research that incorporates modern advances in biochemistry, genetics, and genomics, the BMGR group will continue to generate new knowledge relevant to the fundamental processes essential to growth, development, and health.

The BMGR group vision includes the promotion of collaborations and communication that support its mission. This group has the ability to discover fundamental aspects of growth and development from multifaceted perspectives and disciplines and to investigate how disturbances in one process can affect another. Because our interests are not principally focused on any particular disorder, they extend beyond the tissue-specific gene expression aspects of animal development and provide unique perspectives into growth, development, and disease. Indeed, different defects in a single process can be manifested as different diseases. Given the NIH's penchant for high-risk endeavors, the BMGR group and the many collaborations among its member foster a greater depth and breadth of fundamental discovery than would exist in its absence.

Mike Cashel
David Clark
Bob Crouch
Melvin DePamphilis
Richard Maraia
Roger Woodgate

Bone and Matrix Biology in Development and Disease

Matrix biology is central to the NICHD mission because it is intrinsic to the formation and reshaping of tissues before and after birth. This group aims to elucidate the mechanisms by which primary gene defects cause skeletal fragility and other matrix disorders and to apply this knowledge to the treatment of affected children. This goal is pursued with a close relationship between basic, translational, and clinical science.

The current primary focus of the group is on translational studies of skeletal dysplasias and related bone disorders. We have identified and characterized molecular mechanisms of several novel skeletal development disorders in this spectrum. In addition to building on these successes and expanding our knowledge of mechanisms and pathology of skeletal development, we are developing novel approaches to therapeutic intervention, which will be translated to clinical trials. We are expanding our research to extracellular matrix (ECM) development and pathology in other tissues and organs. We also anticipate expanding our research program toward studies of ECM disorders in placenta, cartilage, growth plate, and other tissues and organs that are involved in fetal health, prematurity, and early child growth and development.

Sergey Leikin
Joan Marini

Cell and Structural Biology

The **Cell and Structural Biology Group (CSBG)** conducts basic research on the molecular mechanisms that underlie fundamental cellular processes, such as organelle biogenesis and function, regulation of cell metabolism by small RNAs, small proteins, post-translational modifications and developmental programs, host-pathogen interactions, and the structure, traffic, and function of signaling proteins in the central nervous system. Knowledge gained from these studies is applied to the elucidation of the pathogenesis of various diseases, including neurodevelopmental, behavioral, and neurodegenerative disorders, metabolic disorders, and infectious diseases.

The CSBG has outstanding facilities for microscopic imaging of cell structure and function and for recombinant protein production in multiple prokaryotic and eukaryotic expression systems for X-ray crystallographic and biochemical analyses. In addition, the group has expertise in generating targeted mutations in rodents and in the anatomical, neurochemical, electrophysiological, and behavioral analyses of the mutant animals.

Anirban Banerjee
Juan Bonifacino
Andres Buonanno
Mary Lilly
Jennifer Lippincott-Schwartz
Matthias Machner
Gisela Storz

Cell Regulation and Development

The mission and vision of the **Cell Regulation and Development Group** is to increase fundamental knowledge of basic molecular mechanisms of cell biology and animal development and to enhance understanding of how dysregulation of these processes contributes to human disability and disease.

By combining expertise in the genetics of model organisms (including transgenic and null mouse models), cell biology, biochemistry, molecular biology, biophysics, and enzymology, members of the group advance individual research objectives by regularly providing insights and advice to one another and through collaborations enabling synergy in research methods and experimental approaches. These interactions have engendered the development of novel technologies and strategies that will facilitate future discoveries in the areas of molecular mechanisms of synaptic circuit assembly and function, the mechanisms and regulation of protein synthesis and transcriptional activation, the functions and regulation of GRTH/DDX25 in spermatogenesis, the role of signaling pathways in transcriptional control of LH and Prolactin receptors, the identification of molecular markers for diagnosing and treating prostate diseases, technologies based on transposable elements and deep sequencing for genome-wide profiles of gene function, the role of transposable elements in reorganizing the host genome in response to stress, and the mechanisms governing adult organ formation during postembryonic vertebrate development.

Mary Dasso
Tom Dever
Maria Dufau
Alan Hinnebusch
Chi-Hon Lee
Henry Levin
Jon Lorsch
Ida Owens
Mihaela Serpe
Yun-Bo Shi

Developmental Endocrine Oncology and Genetics

The mission and vision of the **Developmental Endocrine Oncology and Genetics Group** is to 1) establish new and improved methods, strategies, technologies, and algorithms for the diagnosis, localization, and management of various endocrine tumors, 2) explain the molecular basis for different clinical presentations and establish pathways of tumorigenesis for these tumors, 3) search for new molecular, genetic, proteomic, and metabolomic markers for the development of better diagnosis and localization and seek novel targets for the treatment of metastatic endocrine tumors and biomarkers for predicting responses to therapies, 4) facilitate the implementation of newly available diagnostic techniques and treatment options, including the initiation of new clinical trials, 5) facilitate new and improved intramural, national, and international collaborations, interdisciplinary studies, and team approaches, and 6) facilitate the establishment of national and international databases/networks and train physician-scientists in endocrine tumors.

Peter Backlund (Core)
Maya Lodish (Training)
Karel Pacak
Stanko Stojilkovic
Constantine Stratakis

Genetics and Epigenetics of Development

The mission of the **Genetics and Epigenetics of Development Group** is to research the genetic and epigenetic mechanisms that control reproduction, embryogenesis, and organ system development. Our vision is to apply the knowledge gained to enhance human health.

Judy Kassis
Jim Kennison
Paul Love
Todd Macfarlan
Keiko Ozato
Karl Pfeifer

Maternal–Fetal Medicine, Imaging, and Behavioral Development

The mission of the **Maternal-Fetal Medicine, Imaging, and Behavioral Development Affinity Group** is to understand what determines behavior and behavioral changes during development using nonhuman primate models and normative and clinical populations.

Quantitative Imaging and Tissue Sciences (Basser) invents, develops, and translates methods in novel *in vivo* microstructural and functional MRI to measure salient changes in the developing brain and to assess diseases and disorders of the brain. Novel quantitative imaging biomarkers critical to this work are also useful in the neurosciences for understanding connectivity and brain network dynamics.

Child and Family Research (Bornstein) investigates dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in people across the first three decades of life. Overall research goals are to describe, analyze, and assess the capabilities and proclivities of developing human beings, including their genetic characteristics, physiological functioning, perceptual and cognitive abilities, emotional, social, and interactional styles, as well as the nature and consequences for children and parents of family development, and children's exposure to and interactions with their physical surroundings. The researchers use experimental, longitudinal, and cross-sectional, as well as intra-cultural and cross-cultural, research designs.

Analytical and Functional Biophotonics (Gandjbakhche) uses multi-disciplinary approaches to devise functional imaging technologies and methodologies for translating benchtop studies to the bedside. For example, near infrared spectroscopy and electroencephalogram are used to assess biomarkers for a wide range of brain development abnormalities and injuries, specifically, but not limited to, cognitive and behavioral disorders in children and traumatic brain injury. The laboratory explores endogenous (scattering and absorption) and exogenous (using fluorescence probes) optical contrast mechanisms for characterizing abnormal development and function in tissues such as the placenta. They also are involved in clinical and preclinical studies aimed at characterizing growth and development of various abnormal tissues and monitoring the efficacy of their treatment using photonics methods, such as fluorescence life time and multi spectral imaging.

Intercellular Interactions (Margolis) studies viral and non-viral pathogenesis in the context of human tissues. The laboratory developed a system of *ex vivo* human tissues that preserves their cytoarchitecture and important in vivo functions, and it studies lymphoid, cervico-vaginal, and placenta tissues to investigate mechanisms of cell-cell, cell-pathogen, and cell-extracellular vesicles interaction under normal as well as disease conditions.

Perinatology Research (Romero) investigates normal pregnancy and its most frequent complications such as preterm labor, preterm prelabor rupture of membranes, preeclampsia, fetal growth disorders, and fetal death—conditions which account for the excessive rate of infant mortality in the United States. The Laboratory conducts clinical and translational research and develops diagnostic, predictive, therapeutic, and preventative methods to reduce adverse pregnancy outcomes.

Comparative Behavioral Genetics (Suomi) employs multidisciplinary approaches to study behavioral, social-emotional, cognitive, biological, and epigenetic development in rhesus monkeys and other primates, including humans, across the lifespan. The laboratory collects longitudinal data representing multiple levels of analysis in both naturalistic and experimental physical and social environments to generate models of developmental processes from prenatal, perinatal, infant, juvenile, and adolescent periods into early and late adulthood, across generations, to facilitate subsequent comparative analyses.

Peter Basser
Marc Bornstein
Amir Gandjbakhche
Leonid Margolis
Roberto Romero
Stephen Suomi

Metals Biology and Molecular Medicine

The mission and vision of the **Metals Biology and Molecular Medicine Group** is to continue our current research in the areas of metal biology and implications for pathophysiology and treatment of human diseases, including gene therapy. There is much synergy to be gained by comparing mechanisms for maintenance of iron and copper homeostasis.

Stephen Kaler
Claire Le Pichon
Tracey Rouault

Neurosciences

Understanding the structure and function of the nervous system is a prerequisite for predicting and treating neuropathologies. Our group uses a variety of preparations, including animal models and human tissue, and a variety of techniques to study the biology of development and function of the nervous system and underlying basic biological processes in both health and disease.

Tamás Balla
Douglas Fields
Dax Hoffman
Y. Peng Loh
Chris McBain
Tim Petros
Mark Stopfer

Pediatric Endocrinology, Metabolism, and Genetics

The mission of the **Pediatric Endocrinology, Metabolism, and Genetics Group** is to advance our understanding of endocrine, genetic, and metabolic disorders that impair human development, causing disease and disability. The research in this group encompasses basic, translational, and clinical science to elucidate the etiology of these diseases and to develop new diagnostic and therapeutic approaches.

Jeff Baron
Janice Chou
Anil Mukherjee
Forbes Porter
Jack Yanovski

Physical Biology and Medicine

Human 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 musculoskeletal system to maintain the body, and flexible networks of molecular expression to manage environmental stress. Traditionally, studies of these processes are divided into biochemistry, cell biology, virology, toxicology, etc. However, nature does not know these artificial divisions, and new understandings emerge from the crucible that interfaces mathematically minded physical scientists with biomedical researchers. The **Physical Biology and Medicine Group** is a unique scientific body that approaches human development in normal life and pathology as an integral process and encompasses first-class cell biologists, physical chemists, biophysicists, virologists, and immunologists who not only successfully train postdocs and students within their own fields, but widely collaborate, building and uniquely promulgating multidisciplinary approaches to the most important biomedical problems in the framework of the NICHD mission.

By choosing carefully which biophysical projects really answer the fundamental problems that limit advancement in medicine, we as a group will suffuse these identified problems with our basic wisdom of biophysics. This wisdom includes a deep knowledge of polymer physics, membrane biology and virology, inter- and intracellular communication, the theory of transporters and diffusion of domains, the physics of channel permeation and protein conformational change, the physical chemistry of membrane hydration forces and recognition, the physiology of cell signaling and receptor activation, lipid/protein interactions, and the physiology of secretion, viral infection, parasite invasion, fertilization, adipose transporter trafficking and insulin signaling, and developmental cell fusion. Our strategy is to sharpen our techniques and power

of observation to prove our discoveries, including proteomic architecture of signaling complexes and cellular structures, TIRF microscopy, long-term live-cell time-lapse imaging of tissue and cultured cells, confocal and two-photon scanning microscopy, electrophysiology, physical theories to devise experimental tests of hypotheses, cryoelectron microscopy, and lipidomic analyses.

We are now in a position to tackle the developmental changes seen in obesity and genetic disorders, the pathophysiology of influenza, dengue, and HIV viral infection and assembly, a 3D immunohistochemical microscopy of normal and compromised placenta at super-resolution levels, the use of human brain culture in dissociated and slice culture to study traumatic brain injury and glioblastomal neoplasia, membrane domain organization, regulation of mitochondrial metabolism by cytosolic proteins, and muscle molecular ultrastructure. We currently anticipate testing if hydrogen bond theory can explain hydration repulsion of DNA and membranes, developing a deeper understanding of mechanical, electrical, and chemical interactions in complex, multicomponent cell communities, achieving the conceptual integration of cutting-edge immunological information and assays for cell-cell communication in the onset of tissue pathology and discovering the role and mechanisms of microRNA vehicles in serum.

Sergey Bezrukov
Leonid Chernomordik
Alexander Sodt
Joshua Zimmerberg

Reproductive Endocrinology and Gynecology

The **Reproductive Endocrinology and Gynecology Group** consists of the primary NICHD investigators who carry out research and clinical care in women's health. Our mission is to conduct innovative translational research and provide direct patient care in reproductive endocrinology and gynecology. Consultative services are provided to all NIH institutes.

Alan DeCherney (Training)

Phosphoinositide Messengers in Cellular Signaling and Trafficking

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, which assemble on the surface of cellular membranes and control a variety of cellular functions. Phosphoinositides regulate protein kinases and GTP–binding proteins as well as membrane transporters, including ion channels, thereby influencing 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 phosphatidylinositol 4-phosphate (PtdIns4*P*)–regulated pathways by identifying 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.

Tamás Balla, MD, PhD, *Head, Section on Molecular Signal Transduction*

Yeun Ju Kim, PhD, *Staff Scientist*

Alejandro Alvarez-Prats, PhD, *Postdoctoral Fellow*

Takashi Baba, PhD, *Postdoctoral Fellow*

Joshua Pemberton, PhD, *Postdoctoral Fellow*

Nivedita Sengupta, PhD, *Postdoctoral Fellow*

Mira Sohn, PhD, *Postdoctoral Fellow*

Dániel Tóth, MD, PhD, *Postdoctoral Fellow*

Ljubisa Vitkovic, PhD, *Special Volunteer*

Elisa Arthofer, MS, *Graduate Student*

Molecular anatomy of the activation of the ER–resident Ca^{2+}–regulatory protein STIM1

We studied the molecular events that control the entry of Ca^{2+} into mammalian cells. Ca^{2+} is one of the most important signaling molecules in the cell, and its concentration is kept at very low levels (about 10^{-7} M) in the cytoplasm. Cells elevate Ca^{2+} from this low level to trigger a variety of cellular pathways by releasing Ca^{2+} from intracellular storage organelles such as the endoplasmic reticulum (ER) or by opening specific gates at the plasma membrane (PM) for Ca^{2+} entry from outside the cell. An important Ca^{2+} entry pathway, called Store-Operated Calcium Entry (SOCE), consists of the PM Orai1 channels, which are activated by ER–localized STIM1 molecules that respond to depletion of the luminal Ca^{2+} content of the ER. Mutations in Orai1 and STIM1 that cause various diseases, most prominently

severe immune deficiencies, have been detected in humans. Upon ER Ca^{2+} store depletion, STIM1 molecules cluster and activate the PM Orai1 channels via their SOAR (Stim1-Orai-Activation Region) domain. It is not well understood how the information on ER luminal Ca^{2+} decrease is transmitted to the cytoplasmic part of STIM1 through the single transmembrane segment. A key step in STIM1 activation is the release of its SOAR domain from an intramolecular clamp formed with the part of the STIM1 molecule that immediately follows the transmembrane region in the cytosolic side; this membrane-adjacent region is called the coiled-coil (CC1) region because of its predicted secondary structure. We used STIM1 molecules that were truncated immediately after the CC1 domain to show that they are capable of capturing or releasing the isolated the SOAR domain, depending on luminal ER Ca^{2+} concentrations. Using this limited STIM1 construct, we analyzed the very early molecular events that control the intramolecular clamp formed between the CC1 and SOAR domains. We found that STIM1 molecules form constitutive dimers even in the resting state and that their CC1 domain can also bind to the SOAR of another STIM1 molecule in *trans*. We developed a unique approach to oligomerize STIM1 molecules by targeting multiples of a small protein module, called FRB, to the ER lumen. These FRB multimers could oligomerize STIM1 molecules engineered by adding of a small protein module (called FKBP12) to their ER luminal side after adding rapamycin, which induces binding of FRBs to FKBP modules. Such artificial oligomerization, however, failed to liberate the SOAR domain or activate STIM1 molecules, whereas decreasing ER luminal Ca^{2+} was still capable of activation. The data suggested that oligomerization alone cannot trigger STIM1 activation. We propose that the release of SOAR from the STIM1–CC1 interaction is controlled by changes in the orientation of the two CC1 domains in STIM1 dimers upon Ca^{2+} unbinding. The importance of these studies is that they shed new light on the STIM1 activation process and could thus help us better understand how these molecules work and how the process is affected by mutations that cause human diseases due to SOCE dysfunction. The studies can also identify new ways to pharmacologically manipulate this process for the benefit of patients suffering from either hyperactivation or defective activation of the pathway.

The role of phosphatidylinositol 4-kinases during phagocytosis

A study performed in collaboration with the group of Sergio Grinstein made use of our recently developed tool to visualize phosphatidylinositol 4-phosphate (PI4P), a small regulatory lipid, in living phagocytic cells. Grinstein's group has been studying the molecular events that govern the various steps during phagocytosis in macrophages. Phagocytosis is one of the most ancient defense mechanisms against foreign organisms (bacteria or cells), and it also plays critical roles in clearing cellular debris following cell death or tissue damage. Phosphoinositide lipids play an important role in all steps of phagocytosis, but changes in PI4P and their importance have not been explored because of the lack of appropriate tools to visualize the lipid during the process.

Using our recently developed PI4P reporter, the Grinstein group showed that PI4P undergoes a biphasic change during phagocytosis, a change that is localized to the phagocytic cup membrane during its maturation. PI4P, which is present in the plasma membrane (PM) before engagement of the target particle, is transiently enriched in the phagosomal cup. After the phagosome seals, i.e., it closes around the particle, PI4P levels rapidly drop as a result of the hydrolytic activity by Sac2, a PI4P phosphatase, and phospholipase C, the enzyme that hydrolyzes PI4P and $PI(4,5)P_2$. PI4P disappearance coincides with the emergence of phagosomal PtdIns3P (PI3P), another inositol lipid that only differs from PI4P in the position of the phosphate on the inositol ring and which is formed by a different enzyme. Conversely, the disappearance of PI3P, which signals the transition from early to late phagosomes, is accompanied by resurgence of PI4P,

which is associated with the recruitment of phosphatidylinositol 4-kinase 2A (PI4K2A). This secondary appearance of PI4P can be prevented by silencing PI4K2A or by eliminating PI4P by a recruitable form of Sac1, another PI4P 4-phosphatase, using the FRB–FKBP heterodimerization system (a system that is based on the rapamycin-induced heterodimerization of the FKBP12 protein and the small FKBP12–interaction region of the mTOR protein, called FRB). Importantly, the secondary accumulation of PI4P was found to be necessary for proper phagosomal acidification. The results demonstrated the complex dynamics of PI4P during phagocytosis and suggested that this phosphoinositide plays important roles during the maturation of the phagosome. The significance of these studies is that they highlighted a hitherto unrecognized and important role of PI4P in phagocytosis. A better understanding of the molecular players in the phagocytic process will enhance our ability to counter the processes that parasitic bacteria developed to evade degradation by lysosomes.

The role of inositol lipids in membrane recruitment of Ras proteins

Ras proteins are small globular proteins functioning as molecular switches that change conformation upon GTP binding, a conformation that is distinct from from the GDP–bound forms. Ras protein mutations are some of the most common causes in cancer and hence have attracted much attention. Membrane attachment of Ras proteins is critical to their signaling function, and it is their C-termini that contain localization signals providing for membrane attachment. Of the three forms of Ras proteins (N-, H-, and K-Ras), K-Ras has a polybasic sequence preceding its C-terminal farnesyl lipid modification. In contrast, N-Ras has a palmitoyl residue in addition to the C-terminal prenyl modification. It has been known that the polybasic residue of K-Ras is critical for its membrane interaction via binding to phosphoinositides. In collaboration with Péter Várnai's group, we investigated how the rapid depletion of PM PPIns can regulate the intracellular distribution of the K- and H-Ras proteins. Várnai's group created a BRET sensor for monitoring the movement of PM–bound Ras proteins and followed them after they are released from the PM. The analysis showed that K-Ras or its isolated targeting sequence moves to either the ER or the Golgi upon the rapid depletion of PM phosphoinositides (PPIns) caused by receptor stimulation. No similar translocation was found with the N-Ras proteins. Furthermore, PM PPIns depletion significantly attenuated the proliferation of cells expressing constitutively active K- but not H-Ras mutants, highlighting the different functional role of the PPIns–dependent PM anchoring in K-Ras and H-Ras signaling. The importance of these studies is that they help us understand the molecular determinants of Ras–membrane interactions and that they showed that Golgi-associated K-Ras proteins are unable to elicit a full proliferative effect.

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- NSERC Postdoctoral Fellowship supporting Dr. Joshua Pemberton
- JSPS-NIH Fellowship supporting Dr. Takashi Baba

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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. The biomedical relevance of membrane proteins is underscored by the fact that more than 50% of drug targets are membrane proteins. We are interested in the structural basis of the function of several integral membrane protein families. We combine high-resolution structural techniques such as X-ray crystallography with functional analyses using a range of biochemical and biophysical techniques. In addition to solving high-resolution structures and using them to guide functional experiments, we are also working on discovering novel small molecule probes for our target proteins using a combination of computational and experimental approaches. Not only are our target proteins critically important to a range of physiological processes, they have been linked to several human diseases, most notably neuropsychiatric diseases, various forms of cancer, and protoporphyria. Through our biochemical and biophysical studies, we are providing the first ever detailed molecular mechanisms of their functions that, in turn, are leading to novel hypotheses that can be tested experimentally. Through our small-molecule discovery program, we hope to develop new tools to investigate the cellular functions of our target proteins as well as to permit the development of novel therapies for human diseases.

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Molecular mechanism of post-translational protein lipidation by DHHC palmitoyltransferases

Covalent modification of proteins by attachment of fatty acids to internal cysteines through acyl linkages constitutes one of the most pervasive and physiologically important post-translational modifications. These are referred to collectively as protein palmitoylation, or more generally, protein *S*-acylation. The repertoire of palmitoylated proteins has expanded rapidly in recent years, with hundreds of new members being added to the cellular "palmitoylome." The physico-chemical effect of palmitoylation is to

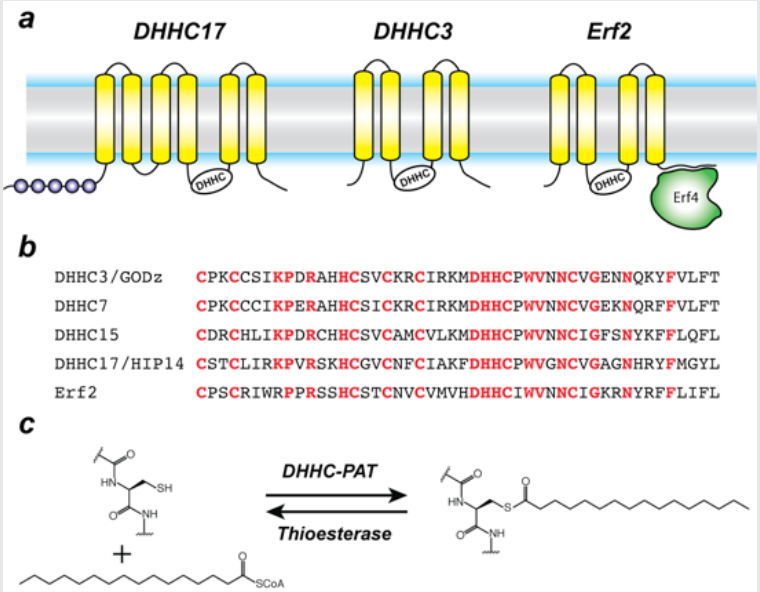

FIGURE 1. Organization and properties of DHHC palmitoyltransferases

a) The organization of three different DHHC-PATs are shown schematically. The spheres indicate protein-protein interaction domains. Erf2 associates with a cytoplasmic subunit, Erf4, to form the active enzyme.

b) The DHHC-CRD region of a few representative DHHC-PAT's are aligned. The conserved amino acids are shown in red.

c) Reaction catalyzed by DHHC-PATs; the reverse reaction is catalyzed by acylprotein thioesterases (APT).

alter the local hydrophobicity of the substrate protein. The thioester bond makes *S*-acylation unique in that it is a labile moiety and can be cleaved, in the cellular context, by thioesterase enzymes (Figure 1). Thus *S*-acylation is one of the few dynamic post-translational modifications. The physiological effects of *S*-acylation are diverse and have critical cellular importance; Ras, a small GTPase that is critical for cellular growth and differentiation and mutated in about one-third of all human cancers, is palmitoylated at the Golgi and is subsequently targeted to the plasma membrane by vesicular transport. Palmitoylated Ras localizes to cholesterol-rich domains on the plasma membrane. However, it subsequently gets depalmitoylated by the thioesterase APT1 and dissociates from the plasma membrane and redistributes on endomembranes including the Golgi. This dynamic recycling of Ras is critical for its function.

Protein *S*-acylation is catalyzed by a large group of enzymes known as DHHC-palmitoylacyltransferase (DHHC-PAT), so named because they contain a signature D-H-H-C motif in a cysteine-rich domain in an intracellular loop (Figure 1). These are low-abundance polytopic integral membrane proteins localized at a variety of cellular compartments. Yeast has seven DHHC-PATs and mammals have up to 24 putative DHHC-PATs encoded in their genome. Beyond the shared DHHC domain, DHHC-PATs vary considerably; some possess ankyrin repeats, a few have six transmembrane helices instead of four and at least one forms a functional heterodimer with a cytoplasmic auxiliary subunit (Figure. 1). To date, there are no reported consensus sequences for palmitoylation. A specific DHHC-PAT can palmitoylate multiple substrates, and conversely a given substrate can be palmitoylated by multiple DHHC-PATs. This redundancy has been one of the most intriguing aspects of DHHC-PATs and makes it difficult to assign substrates by overexpression/knockout strategies, given that in the absence of one enzyme, others can take over. However, this does not necessarily reflect the true enzyme-substrate relationship. The situation has been even more confounded by the lack of specific inhibitors of DHHC-PATs. Even though 2-bromopalmitate is widely used as a global inhibitor of DHHC-PATs, it has been shown that it broadly targets other proteins involved in lipid metabolism.

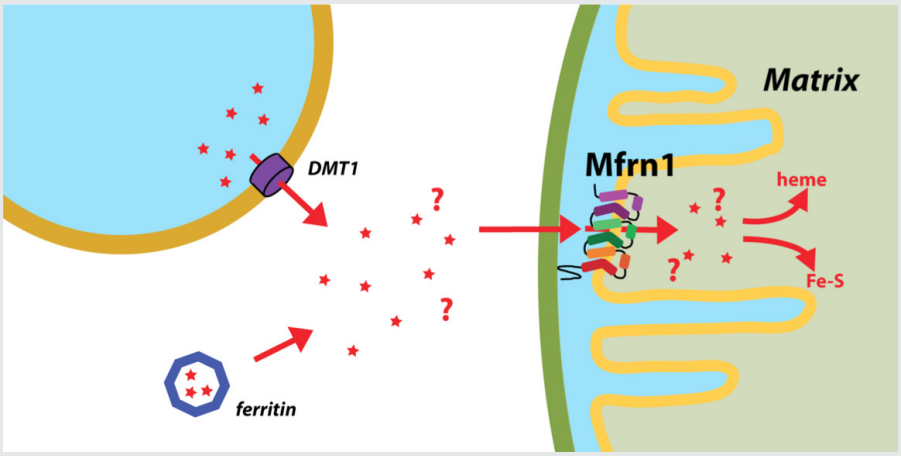

FIGURE 2. Mitoferrin and mitochondrial iron transport

Iron is transported out of endosomes into the cytoplasm by Divalent Metal Ion Transporter 1 and is subsequently directed to Mitoferrin by an unknown mechanism. Mitoferrins are the only known major transporters into mitochondria of the iron that is subsequently utilized for important processes such as heme and Fe-S cluster biosynthesis.

Besides its broad importance in cell biology, palmitoylation has been linked to several diseases, most notably neuropsychiatric disorders such as Huntington's disease and various forms of cancer. Very recently, it was shown that DHHC20 palmitoylates epidermal growth factor (EGFR) and is thus a potential therapeutic target for a broad range of cancers. However, despite their importance across a broad spectrum of biological pathways and their biomedical importance, very little is known about the molecular mechanism of DHHC palmitoyltransferases. There is nothing known about their structural organization or how they interact with substrates and the fatty acyl CoA that serves as the acyl donor.

In the first breakthrough in this field, we solved a high-resolution crystal structure of the complex between the substrate recognition domain of human DHHC17 and a fragment of Snap25b, one of the canonical substrates of DHHC17. Through structure-guided mutagenesis, we discovered key residues in DHHC17 that are critically important for interaction with Snap25b. We further extended our finding by showing that the same residues are also crucial for the interaction of DHHC17 with Huntingtin, one of its most relevant substrates. We are currently studying other members of this intriguing family of enzymes to obtain a detailed molecular understanding of how they function.

Molecular mechanism of iron transport into mitochondria

The importance of iron in biology cannot quite be overstated. In higher organisms, mitochondria are the 'hotspot' for the cell biology of iron because this is where Fe-S clusters are biosynthesized and iron is inserted into heme. Mitochondrial iron homeostasis plays a critical role in cellular iron homeostasis and in the overall physiology of the cell. In vertebrates, the only known major transporters of iron into mitochondria are mitoferrin-1 and mitoferrin-2, two homologous members of a large group of mitochondrial transporters known as the Mitochondrial Carrier family (Figure 2). Mitoferrin-1 (Mfrn1) is expressed mainly in erythroid cells while mitoferrin-2 is expressed ubiquitously. Knockout of Mfrn1 is embryonically lethal, reflecting the importance of mitoferrins in vertebrate physiology.

Mfrn1 and Mfrn2 were discovered more than 10 years ago. However, the proposed iron transport activity of either transporter has not yet been demonstrated using an *in vitro* functional reconstitution assay. Also no

report about their interaction with iron or other related metal ions exists, most likely because heterologous overexpression and purification of mitoferrins have not yet been reported in literature. If mitoferrins do indeed transport iron, it is important to know how selective they are for iron over other similar metal ions, what residues in mitoferrin-1 and -2 are important for their function, and whether they even bind iron. These fundamental questions about mitoferrin function have, to date, not been addressed.

We have now carried out heterologous purification and *in vitro* functional reconstitution and mutational dissection of a vertebrate Mfrn1. This is the first demonstration that Mfrn1 can indeed transport iron. We showed that Mfrn1 is a promiscuous metal ion transporter in that it also transports other first-row transition metal ions. Through mutagenesis, we discovered candidate residues that are important for metal ion transport by Mfrn1 and those that could be involved in forming metal-ion binding sites during transport.

Our studies provide the first biochemical insights into Mfrn function and form the starting point for future high-resolution structural studies of Mfrn function. Our transport assay and the purification strategy will lead to more detailed biochemical and biophysical experiments into the mechanistic basis of iron transport by Mfrn1 and Mfrn2. Our *in vitro* proteoliposome-reconstituted iron and copper transport assay is likely to be used for studying other iron and copper transporters and, considering the importance of iron and copper transport in biology, these assays will be important tools for biochemical dissection of the transporters of these metal ions.

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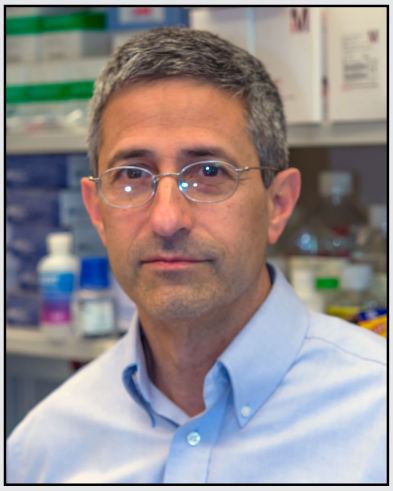
Regulation of Childhood Growth

We investigate the cellular and molecular mechanisms governing childhood growth and development. We focus particularly on growth at the growth plate, which drives bone elongation and therefore determines height. One goal of this work is to gain insight into the many human genetic disorders that cause childhood growth failure or overgrowth. A second goal is to develop new treatments for children with severe growth disorders.

The growth plate is a thin layer of cartilage found near the ends of juvenile bones. In the growth plates new cartilage is produced through chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis, and this newly formed cartilage is then remodeled into bone. The process, termed endochondral ossification, results in bone elongation, which causes children to grow in height (linear growth). Consequently, mutations in genes that regulate growth plate chondrogenesis cause abnormal bone growth and short stature in children (Reference 1). Depending on the severity and nature of the genetic abnormality, the phenotype can range from chondrodysplasias with short, malformed bones, to severe, often disproportionate, short stature, to mild proportionate short stature (Reference 1). If the genetic defect affects tissues other than the growth plate cartilage, the child may present with a more complex syndrome, which includes other clinical abnormalities (Reference 1).

Numerous gene defects that affect the growth plate and thereby cause childhood growth disorders have been identified. However, for many children who are brought to medical attention for linear growth disorders, clinical, laboratory, and genetic evaluation fail to identify the underlying etiology. Genome-wide association studies, which we helped analyze, and molecular-biological studies of growth plate biology suggest that there are hundreds of genes that control linear growth. Therefore, it is likely that many genetic causes of linear growth disorders remain to be discovered.

To discover new genetic causes of childhood growth disorders, we study families with monogenic growth disorders using powerful genetic approaches, including SNP arrays to detect large deletions, duplications, mosaicism, and uniparental disomy, combined with exome sequencing to detect single nucleotide variants and small



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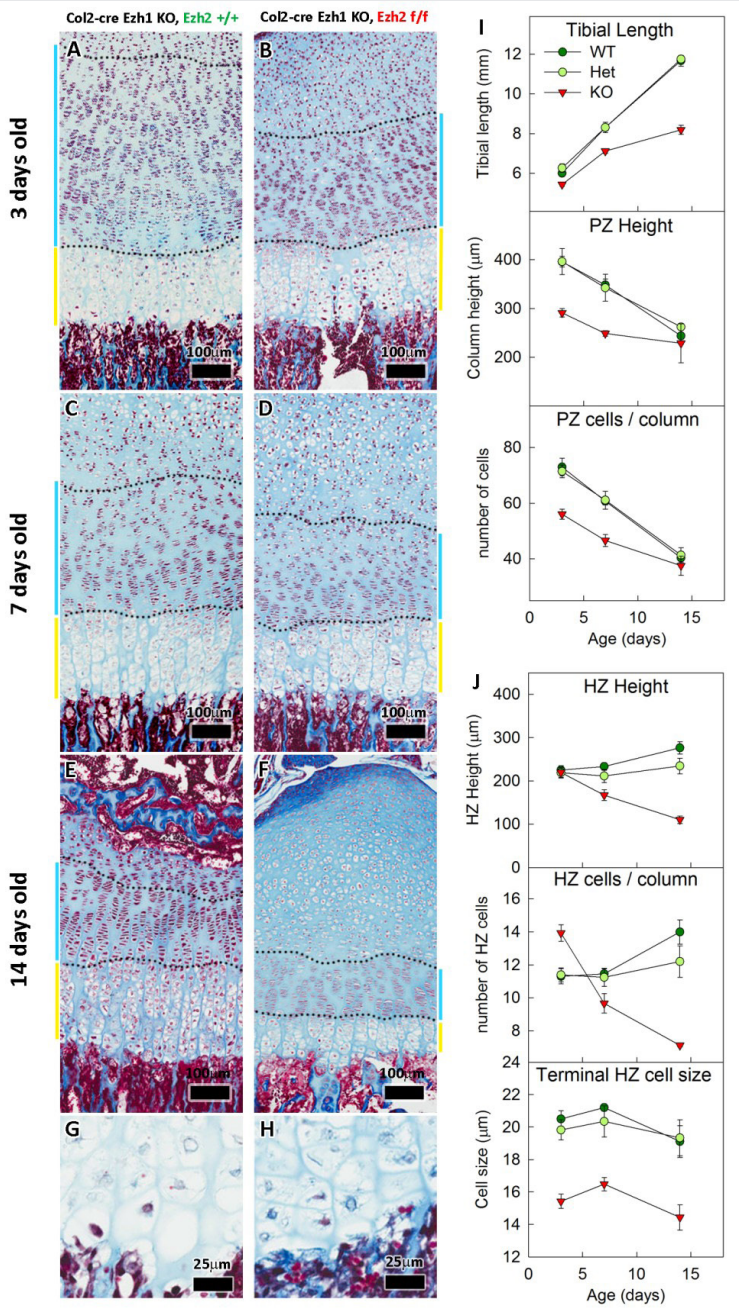

FIGURE 1. Effects of Ezh1/2 deficiency on growth plate

A-F: Histological sections of proximal tibias.

G–H: Higher magnification of hypertrophic chondrocytes at 3 days old.

I–J: Quantitative histological measurements

insertions/deletions in coding regions and splice sites.

This analysis led to our identification of heterozygous mutations in the gene *ACAN* that cause autosomal dominant short stature with advanced bone age and premature osteoarthritis. *ACAN* encodes the proteoglycan aggrecan, which is an important component of cartilage extracellular matrix, including that of the growth plate. We then participated in a multi-center collaboration to study several families with this disorder to define the clinical phenotype (Reference 2). We identified 103 individuals from 20 families with

heterozygous *ACAN* mutations. The phenotype was found to include disproportionate short stature and a history of early growth cessation. The condition was frequently associated with early-onset osteoarthritis and inter-vertebral disc disease. Most children exhibited an advanced bone age. Growth hormone therapy appeared to modestly increase growth velocity. In all subjects the disorder was inherited in an autosomal dominant fashion, thus presenting as familial short stature. However, in a subsequent study, we found that non-familial short stature can be caused by *de novo ACAN* mutations (Reference 3). We also recently reported that heterozygous mutations in *ACAN* can present with a bone age less than chronological age (Reference 3). The findings expand the known phenotypic spectrum of heterozygous *ACAN* mutations and indicate that this diagnosis should be considered in children without a family history of short stature and children without accelerated skeletal maturation.

Similar approaches were used to identify biallelic mutations in the *BRF1* gene in a family with impaired postnatal linear growth, markedly delayed bone age, dysmorphic facies, cognitive impairment, and central nervous system anomalies (Reference 4). *BRF1* encodes the RNA polymerase III transcription initiation factor 90 kDa subunit. Expression of *BRF1* in yeast confirmed that the mutations affect protein function. The findings supported a previous report showing that biallelic mutations in *BRF1* cause cerebellar-facial-dental syndrome. Our findings also help define the growth phenotype, indicating that the linear growth failure can become clinically evident before neurological abnormalities and that a severely delayed bone age may serve as a diagnostic clue.

In a boy with tall stature, advanced bone age, and mild dysmorphic features, exome sequencing identified a *de novo* missense in *EZH2*, a gene involved in maintaining the transcriptional repressive state of genes. The findings were consistent with previous reports that heterozygous mutations in *EZH2* cause Weaver syndrome, which is characterized by tall stature, advanced bone age, characteristic facies, and variable intellectual disability. Similarly, genome-wide associated studies have implicated *EZH2* in the control of height. Thus, the data indicate that *EZH2* regulates skeletal growth. *EZH2* encodes a histone methyltransferase that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27), which serves as an epigenetic signal for chromatin condensation and transcriptional repression. To investigate the mechanisms by which this epigenetic mark affects skeletal growth, we created a mouse lacking both *Ezh1* and *Ezh2* in cartilage (Reference 5). The combined loss severely impaired skeletal growth. Both principal processes underlying growth plate chondrogenesis, chondrocyte proliferation and hypertrophy, were compromised. The decrease in chondrocyte proliferation was attributable in part to derepression of the cyclin-dependent kinase inhibitors Ink4a/b, while ineffective chondrocyte hypertrophy was the result of suppression of IGF signaling by the increased expression of IGF–binding proteins. Collectively, our findings reveal a critical role for H3K27 methylation in the regulation of chondrocyte proliferation and hypertrophy in the growth plate, which are the central determinants of skeletal growth.

We also investigated the role of bone-morphogenetic proteins in the regulation of the growth plate and articular cartilage. Articular and growth plate cartilage both arise from condensations of mesenchymal cells, but ultimately develop important histological and functional differences. Each is composed of three layers: the superficial zone, the mid and deep zones of articular cartilage, and the resting, proliferative, and hypertrophic zones of growth plate cartilage. A gradient in expression of *BMP*–related genes has been observed across growth plate cartilage, likely playing a role in zonal differentiation. To investigate the presence of a similar expression gradient in articular cartilage, we used laser capture microdissection (LCM)

to separate murine growth plate and articular cartilage from the proximal tibia into their six constituent zones and used a solution hybridization assay with color-coded probes to quantify mRNAs for 30 different *BMP*–related genes in each zone. *In situ* hybridization and immunohistochemistry were then used to confirm spatial expression patterns. We found evidence that BMP signaling gradients exist across both growth plate and articular cartilage and that the gradients contribute to the spatial differentiation of chondrocytes in the postnatal endochondral skeleton.

Additional Funding

- 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 Julian Lui (2015, concluded): "Cartilage-Targeted IGF-I for Treatment of Growth Disorders"
- Pediatric Endocrine Society Clinical Scholar Award to Youn Hee Jee (2016, ongoing): "The role of PSD-93 in the initiation of puberty and in the etiology of pubertal delay"
- NICHD Division of Intramural Research Director's Award Project (2016-2018, ongoing): The role of DLG2/PSD-93 in the initiation of puberty and the impact of mutations on NMDA receptor signaling and pubertal disorders
- NIH U01 award: 1U01HD086838-01A1 (2017-2021, ongoing): "Genetic Diagnosis of Childhood Growth Disorders"

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Quantitative Imaging and Tissue Sciences

In our tissue sciences research, we strive to understand fundamental relationships between function and structure in living tissues, using '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 various length and time scales, performing physical measurements in tandem with developing physical/mathematical models to explain their functional properties and behavior. Experimentally, we use water 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 and diffusometry. A goal of our basic tissue sciences research is to develop tools that can be translated from bench-based quantitative methodologies to the bedside.

Our tissue sciences activities dovetail with our basic and applied research in quantitative imaging that is intended to generate measurements and maps of intrinsic physical quantities, including diffusivities, relaxivities, or exchange rates, rather than qualitative stains and images conventionally used in neuro-radiology. Our quantitative imaging group uses knowledge of physics, engineering, applied mathematics, imaging and computer sciences, and insights gleaned from our tissue sciences research to discover and develop novel imaging biomarkers that sensitively and specifically detect changes in tissue composition, microstructure, or microdynamics. The ultimate translational goal of developing such biomarkers is to assess normal and abnormal development, diagnose childhood diseases and disorders, and characterize degeneration and trauma. Primarily, we use MRI as our imaging modality of choice because it is well suited for many NICHD mission–critical applications; it is non-invasive, non-ionizing, requires in most cases no exogenous contrast agents or dyes, and is deemed safe for use with fetuses and children

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(continued)

in both clinical and research settings.

A technical objective has been to transform clinical MRI scanners into scientific instruments capable of producing reproducible, highly accurate, and precise imaging data to measure and map useful imaging quantities for various applications, including single scans, longitudinal and multi-site studies, personalized medicine, genotype/phenotype studies, and for populating imaging databases with high-quality normative data.

- Okan Irfanoglu, PhD, *Contractor funded by the Henry Jackson Foundation-Center for Neuroscience and Regenerative Medicine*
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In vivo MRI histology

We aim to develop novel next-generation *in vivo* MRI methods to better understand brain structure and function in normal and abnormal development, disease, degeneration, and trauma. The most mature technology that we invented, developed, and translated is Diffusion Tensor MRI (DTI), by which we measure *D*, a diffusion tensor of water, 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 intrinsic scalar (invariant) quantities. These imaging parameters behave like non-invasive quantitative histological 'stains' obtained by probing endogenous tissue water *in vivo* without requiring exogenous contrast agents or dyes. The bulk or orientationally averaged apparent diffusion coefficient (mean ADC) is the most successful and widely used DTI parameter to identify ischemic regions in the brain during acute stroke and to follow response to therapy in tumors. Our measures of diffusion anisotropy (e.g., 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 and deep brain gray matter areas, we also proposed and developed DTI "Streamline" Tractography.

More recently, we invented and developed a family of advanced *in vivo* diffusion MR methods to measure fine-scale microstructural features of axons and fascicles, which otherwise could only be measured using laborious *ex vivo* histological methods. We have been developing efficient means for performing "k and q-space MRI" in the living brain, such as "Mean Apparent Propagator" (MAP) MRI. This approach detects subtle microstructural and architectural features in both gray and white matter at micron-scale resolution, several orders of magnitude smaller than the MRI voxel. It also subsumes DTI, as well as providing a bevy of new *in vivo* quantitative 'stains' to measure and map. We also developed a family of diffusion MRI methods 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 them 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 functionally important given that axon diameter

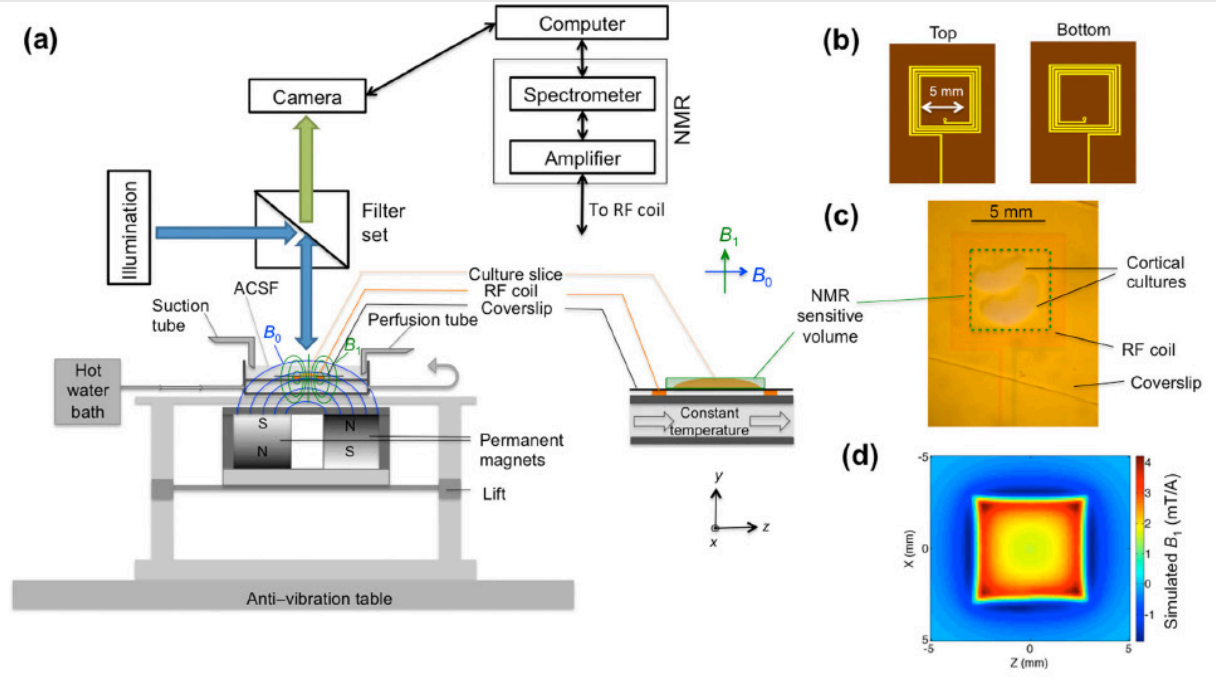

FIGURE 1. Novel instrument for simultaneous functional MR and calcium imaging studies

Schematic diagram of a novel instrument for simultaneous functional MR and calcium imaging studies of organotypic cultured brain tissue.
(a) Schematic diagram of the simultaneous MR and fluorescence imaging test bed (*left*) and an enlargement of the components near the organotypic cultured tissue (*right*), which is immersed in artificial cerebral spinal fluid (ACSF).
(b) Top and bottom layers of the two-layer multi-turn radio-frequency (RF) surface coil.
(c) Real image of the coil with the cortical culture mounted under approximately 0.63Å magnification.
(d) Simulated two-dimensional B1 field distribution at y = 0.2 mm in the x–z plane. This device is allowing us to test existing fMRI methods without cardiovascular, respiratory, or other artifacts and confounds. It also provided a test-bed for us to develop new fMRI approaches.

helps determine conduction velocity and therefore the rate at which information flows along white matter pathways as well as delays or latencies between and among different brain areas. We then developed a companion mathematical theory to explain the observed ADDs in different fascicles, suggesting that they represent a trade-off between information flow and 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 central nervous system (CNS). The methods can also provide an independent measurement of the AAD and other features of cell size and shape.

Although brain gray matter appears featureless in DTI maps, its microstructure and architecture are rich and varied throughout the brain, not only along the brain's cortical surface, but also within and among its various cortical layers and within deep gray matter regions. To target this tissue, we have been developing several noninvasive, *in vivo* methods to measure unique features of cortical gray matter microstructure and

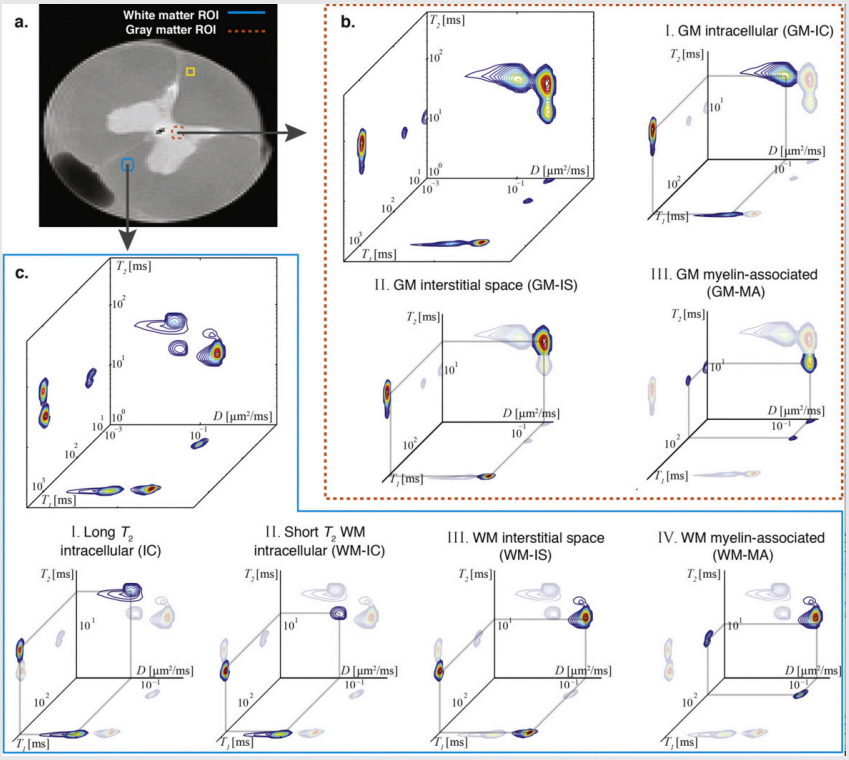

FIGURE 2. Two-dimensional relaxometry MRI of ferret spinal cord

Spectra from gray matter (GM) and white matter (WM) regions of interest (ROIs).(a) A high-resolution proton density MR image is shown with the locations of representative ROIs: WM (*blue box*) and GM (*red box*) ROIs. After voxel-wise analysis, the spectra in each of the GM and WM ROIs were averaged and are presented in (b) and (c), respectively. The different peaks are assigned to microscopic neuro-anatomical components according to their respective diffusion (D), T_1, and T_2 values. Assignments of different spectra to cellular and tissue components can be made using histological images obtained from the same slice. GM: I. Intracellular (GM-IC); II. GM interstitial space (GM-IS); and III. GM myelin-associated (GM-MA). WM: I. Long T_2 intracellular (IC); II. Short T_2 intracellular (WM-IC); III. WM interstitial space (WM-IS); and IV. WM myelin-associated (WM-MA).

architecture that are currently invisible in conventional MRI. One goal is to 'parcellate' or segment the cerebral cortex *in vivo* into its approximately 500 distinct cyto-architechtonic 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. Within the past year, we pioneered and developed several promising two-dimensional MRI relaxometry, exchange, and diffusometry methods, which we plan to use to study water mobility and exchange in gray matter. We believe these will have promise in identifying inflammation and redistribution of tissue water in brain parenchyma.

In general, we are continuing to develop translationally oriented methods to follow normal and abnormal development, aid in the diagnosis of various diseases and disorders of the brain, noninvasively and *in vivo*, and provide information to help neurosurgeons plan operations and interventions.

Quantitative pediatric MRI

MRI is considered safer than X-ray–based methods, such as computed tomography (CT), for scanning infants and children. However, clinical MRI data 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 features 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 ensure improved specificity, it is nonetheless necessary for developing robust and reliable imaging 'biomarkers.' In particular, MRI assessment of normal brain development and developmental disorders has benefiting greatly from the introduction of '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 different 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, e.g., during the progression or remission of disease, by comparing measurements in a single subject with normative values acquired in a healthy population. Quantitative MRI methods should also aid in precision imaging studies, whereby MRI phenotypic data can be meaningfully linked to a subject's genotype.

Our group has been carrying out several clinical studies that utilize novel quantitative MRI acquisition and analysis methods and whose aim is to improve accuracy and reproducibility in diagnosis and to detect and follow normal and abnormal development. These studies include the following:

1) The NIH Study of Normal Brain Development, jointly sponsored by a consortium of 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 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 these rich data. Tandem structural MRIs and standardized neuropsychological tests performed on this population are also available to researchers outside the consortium. Our role in this interdisciplinary project was 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 mine and analyze the data, having recently developed age-specific DTI atlases of normal brain development. We also publicly released various versions of our software package, TORTOISE, which 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 this advanced DTI–processing pipeline to produce high-quality normative data from the project, which we make publicly available through the National Database for Autism Research (NDAR; *http://ndar.nih.gov*).

2) In collaboration with Susan Swedo, we studied autistic subjects using DTI and quantitative MRI relaxometry methods. While several MRI studies reported abnormal features in the autistic brain, 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 this disorder.

3) In collaboration with Katherine Warren, we acquired quantitative MRI data in children with pontine gliomas to identify MRI prognostic factors. With John Park we scanned subjects with supratentorial gliomas to distinguish recurrence from radiation necrosis.

4) In collaboration with Filippo Arrigoni, we use multi-modal MR imaging (DTI, fMRI, and quantitative relaxometry) to evaluate cerebral reorganization caused by various rehabilitation protocols in children with

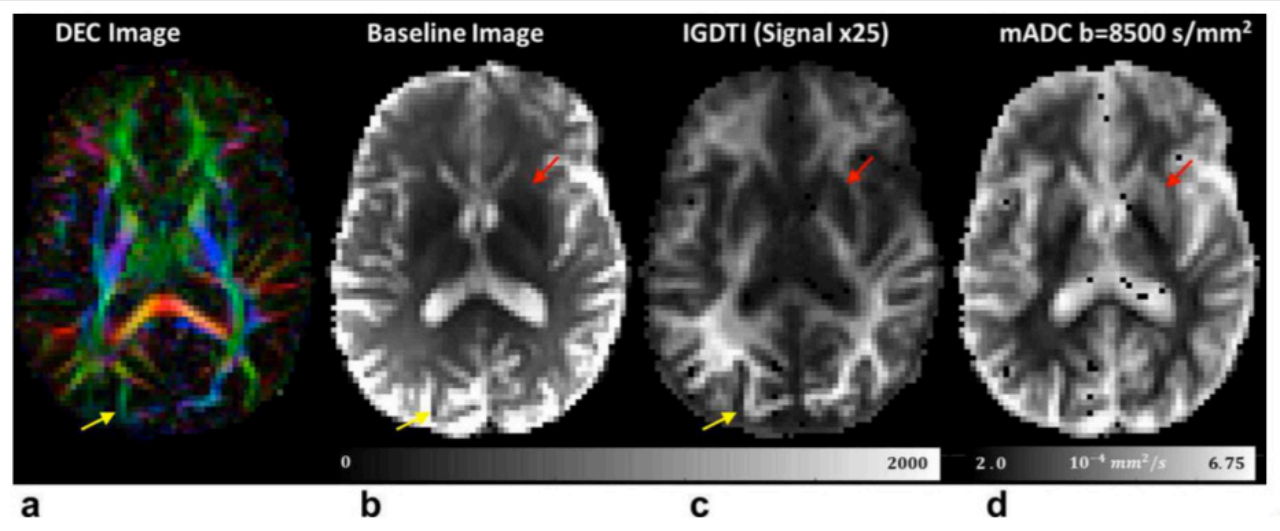

FIGURE 3. New rapid isotropically weighted MRI method for fetal and pediatric MRI applications

Isotropically weighted generalized diffusion tensor imaging (IGDTI) provides a rapid means of obtaining diffusion MRI data that is intrinsic and insensitive to diffusion anisotropy. We proposed a new experimental design and analysis framework to measure orientationally averaged diffusion-weighted images (DWIs), which removes the modulation in image intensity attributable to anisotropic structures, such as brain white matter. The approach is expected to have significant applications in neuro-radiology, particularly for fetal and pediatric imaging, where subject movement can be a significant confound.

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 neuro-anatomic differences between the two groups.

5) We are continuing to work under the auspices of the Center for Neuroscience and Regenerative Medicine (CNRM), DoD, to investigate potential plasticity changes after rehabilitation in military personnel affected by TBI or post-traumatic stress disorder (PTSD). We are also exploring the potential clinical utility of MAP-MRI to provide quantitative imaging biomarkers that are more sensitive and specific than those provided by DTI, to determine whether they could be used to detect and assess mild TBI (mTBI).

6) We received grant support from the Congressionally Directed Medical Research Program (CDMRP) to investigate, in collaboration with Sharon Juliano, a ferret model of mTBI, using advanced MRI methods, particularly MAP-MRI, combined with histopathological techniques provided by Juliano's laboratory. We are currently acquiring MRI data.

7) In collaboration with the Veteran's Administration, DoD, and the Chronic Effects of Neurotrauma Consortium (CENC), we are enabling the acquisition of multi-site DTI data from various clinical centers in the VA network.

Our involvement in TBI research, particularly in detecting mTBI, has continued to expand, because it is of high relevance to the NICHD mission, it is an acute problem in the pediatric population, and it afflicts

men and women in the military. 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 polyvinyl pyrrolidone (PVP) polymer–based diffusion MRI phantom that we developed, patented, and 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 non-parametric empirical (e.g., Bootstrap) methods to determine the statistical distribution of DTI–derived quantities in order to study, for example, the inherent variability and reliability of computed white-matter fiber-tract trajectories. Such parametric and non-parametric statistical methods enable us to apply powerful hypothesis tests to assess the statistical significance of findings in a wide range of important biological and clinical applications that are currently being tested 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 various subject populations. Recently, our group has been developing methods for studying the reproducibility and reliability of different tractography methods, given their widespread use 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 and for artifacts caused by induced eddy-current and 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, particularly single-subject studies.

Biopolymer physics: water–ion–biopolymer interactions

A major focus of this basic research project is to understand the effect of ion-water-biopolymer interactions. These are ubiquitous in biology, ranging from the movement of water and ions across channels, to the self-assembly of aberrant proteins into nanofibers in neuro-degenerative processes, such as Creutzfeldt-Jakob disease, Alzheimer's disease, and chronic traumatic encephalopathy (CTE). Despite their importance and prevalence, however, little is understood about these interactions from a physics perspective.

To address this dearth in our understanding, we developed a multi-scale experimental framework to study these interactions by combining macroscopic techniques (e.g., osmotic swelling pressure measurements, mechanical measurements) with higher-resolution scattering methods (e.g., SANS and SAXS). Swelling pressure measurements provide information on the overall thermodynamic response, while SANS and SAXS allow us to investigate biopolymers at the molecular and supramolecular levels and to quantify the effect of ion concentration, ion valance, pH, and temperature on the structure and macroscopic (thermodynamic) properties of the tissue. We also apply computational techniques to model water-ion-biopolymer interactions in these systems.

A major success of this basic polymer physics approach has been to exploit our understanding of water-polymer interactions to tailor the properties of diffusion MRI phantoms so as to calibrate MRI scanners, specifically to assure quality of the imaging data and to assess scanner performance on an on-going basis. Our recently issued U.S. Patent for a "Phantom for diffusion MRI imaging" is allowing, for the first time, quantitative diffusion MRI studies to be performed. The polymer polyvinylpyrrolidone (PVP) has ideal properties for this demanding application. It is chemically and thermally stable, has a long shelf life, is safe and non-toxic, can be shipped from site to site, and has stable diffusion and relaxation properties. Michael Boss, NIST has incorporated our PVP polymer in NIST's own diffusion MRI standard. The technology is also being promulgated commercially e.g., by *http://hpd-online.com/diffusion-phantom.php*.

Functional properties of extracellular matrix (ECM)

Our goal is to understand and quantify the interactions among the major macromolecular components of ECM that give rise to its unique functional properties. ECM is present in every tissue and performs a key role in determining normal and abnormal organ function. Specifically, we are studying interactions among the primary ECM components, namely collagen, proteoglycans (PG), water, and ions, which govern ECM's macroscopic biomechanical and transport properties, using cartilage as a model system. The biomechanical behavior of cartilage and other ECMs reflects biochemical and microstructural changes occurring during development, disease, degeneration, and aging. Understanding the basis of important functional properties of cartilage, particularly its load-bearing and lubricating abilities, requires an array of experimental techniques that probe a wide range of relevant length and time scales. Understanding the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential for predicting these properties, which are mainly governed by osmotic and electrostatic forces. This knowledge can inform tissue-engineering or regenerative-medicine strategies to grow, repair, and reintegrate replacement cartilage. To obtain a self-consistent physical picture of tissue structure/function relationships, we measure various physical/chemical properties of ECM 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 viability and load-bearing ability of cartilage. Previously, we designed and built a tissue micro-osmometer to perform high-precision swelling pressure measurements on small tissue samples (less than 1 microgram) as a function of the water activity (vapor pressure). We make osmotic pressure measurements to determine how the individual components of cartilage ECM (e.g., aggrecan, hyaluronic acid [HA], and collagen) contribute to the total load-bearing capacity of the tissue. We demonstrated that aggrecan–HA aggregates self-assemble into microgels, contributing to improved dimensional stability and the tissue's lubricating ability. We also found that aggrecan is highly insensitive to changes in the ionic environment, particularly to the concentration of calcium ions, which is critically important in maintaining the tissue's mechanical integrity in high Ca^{2+} environments and allowing aggrecan to serve as a calcium ion reservoir in cartilage and bone.

We recently developed a new biomimetic model of cartilage ECM consisting of a dispersion of polyacrylic acid (PAA) microgel particles embedded within a polyvinyl alcohol (PVA) gel matrix. In this system, PAA mimics the behavior of proteoglycan assemblies while PVA mimics the role of the collagen network. The PVA/PAA biomimetic model system reproduces not only the shape of the cartilage swelling pressure curves, but also the numerical values reported for healthy and osteoarthritic human cartilage samples. Systematic

studies made on model composite hydrogels is expected to provide invaluable insights into the effects of various factors (matrix stiffness, swelling pressure, fixed charge density, synovial fluid composition, etc.) on the macroscopic mechanical/swelling properties, and ultimately the load-bearing ability of cartilage. Similar systematic studies cannot be obtained from measurements made on biological tissues because their composition and physical properties cannot be independently and systematically varied as they can in these synthetic model polymer composites.

The resistance of tissue to external loads is determined by its osmotic modulus. Therefore, maps of the osmotic modulus are particularly useful for characterizing the load-bearing properties of cartilage. We developed a method that utilizes the precise scanning capabilities of the AFM to generate compliance maps, from which relevant elastic properties can be extracted. We then combined AFM with tissue micro-osmometry to generate elastic and osmotic modulus maps of cartilage.

We have begun translating this critical tissue-science understanding of the structure/function relationships of components of ECM to develop and design novel non-invasive MR imaging methods with the aim of inferring ECM composition, patency, and functional properties *in vivo*. Our goal is to use MRI for early diagnosis of cartilage and other ECM diseases, as well as to provide a means for following normal and abnormal development of the ECM. This challenging project entails making 'invisible' components of ECM, (e.g., collagen and PGs) 'visible' and then using our understanding of biopolymer interactions to predict tissue load-bearing properties. One major obstacle is that water molecules bound to immobile species (e.g., collagen) cannot be visualized with conventional MRI approach. However, magnetization transfer (MT) MRI (as well as other methods) make it possible to detect these bound protons indirectly by transferring their magnetization to the free water surrounding them. It also makes it possible to estimate the collagen content in tissue. In a pilot study, we applied the new MT MRI method to determine the concentration and distribution of the main macromolecular constituents in bovine femoral-head cartilage samples. The results obtained by the MT MRI method were qualitatively consistent with those obtained by histological techniques, such as high-definition infrared (HDIR) spectroscopy. Our work was aided by the previous receipt of a DIR Director's Award to investigate this proposed line of research, along with our collaborators Sergey Leikin and Edward Mertz.

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Biophysics of Large Membrane Channels

Healthy cell functioning and development require both precise compartmentalization and effective communication between cells and cell organelles. It is critically important for the systems to regulate metabolic transport, one of these being membrane channels. Deviant alterations in these complex dynamic structures often cause a shift from health to disease. Keeping this in mind, we study "large" beta-barrel channels of eukaryotes and bacteria, which are responsible for metabolite fluxes. With regard to their structure and mechanisms of selectivity and regulation, the channels are fundamentally different from the conventional ion channels studied by neurophysiology.

Specifically, we seek to understand the physical principles of their functioning under normal and pathological conditions. Among many wet-lab approaches, such as fluorescence correlation spectroscopy and confocal microscopy, our hallmark method is reconstitution of channel-forming proteins into planar lipid membranes that allows us to study them at the single-molecule level. Findings obtained in these experiments are rationalized within a framework of a physical theory of channel-facilitated transport that brings the knowledge necessary for intelligent drug design and other strategies to effectively correct aberrant interactions associated with disease.

To grasp the general principles of beta-barrel channel functioning and regulation, we work with a broad variety of proteins and peptides that form channels, including VDAC (Voltage-Dependent Anion Channel from the outer membrane of mitochondria), alpha-Hemolysin (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 (general bacterial porin from *Escherichia coli*), LamB (sugar-specific bacterial porin from *Escherichia coli*), OprF (porin from *Pseudomonas aeruginosa*), MspA (major outer-membrane porin from *Mycobacterium smegmatis*), Alamethicin (amphiphilic peptide toxin from *Trichoderma viride*), Syringomycin E (lipopeptide toxin from *Pseudomonas syringae*), and the bacterial peptide TisB involved in persister cell formation. We also use Gramicidin A (linear pentadecapeptide from *Bacillus brevis*) as a molecular sensor of membrane mechanical properties. With the goal of studying the

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channel-forming proteins under controlled conditions, we first isolate them from the host organisms, purify them, and then reconstitute them into planar lipid bilayers, the model membrane system with precisely defined physical properties. This allows us to observe channels interacting with the lipid membrane as modified by volatile anesthetics, by cytosolic proteins such as tubulin and alpha-synuclein, and by newly synthesized drugs that act as blockers of the translocation pores of bacterial toxins. Our motivation is that learning the molecular mechanisms of channel functioning is vital for the development of new approaches to treatment of various diseases, for which regulation of transport through ion channels plays the key role.

Structural features and lipid–binding domain of tubulin on biomimetic mitochondrial membranes

Tubulin has emerged as a highly unexpected component of mitochondrial membranes involved in regulation of membrane permeability through its inhibition of VDAC. The discovery of this functional role has reawakened interest in the nature of the tubulin-membrane interaction to answer a new question: how tubulin, a cytosolic protein known for its role in microtubule structure and dynamics, targets mitochondrial membranes. This year, using a combination of five biophysical methods—surface plasmon resonance, electrochemical impedance spectroscopy, bilayer overtone analysis, neutron reflectometry, and molecular dynamics simulations—we studied binding of tubulin to biomimetic membranes of different lipid compositions. We found that tubulin distinguishes between lamellar and nonlamellar lipids through a highly conserved amphipathic binding motif (Figure 1). Specifically, alpha-tubulin targets cell and organelle membranes by sensing lipid-packing defects, with broad consequences for both normal cellular function

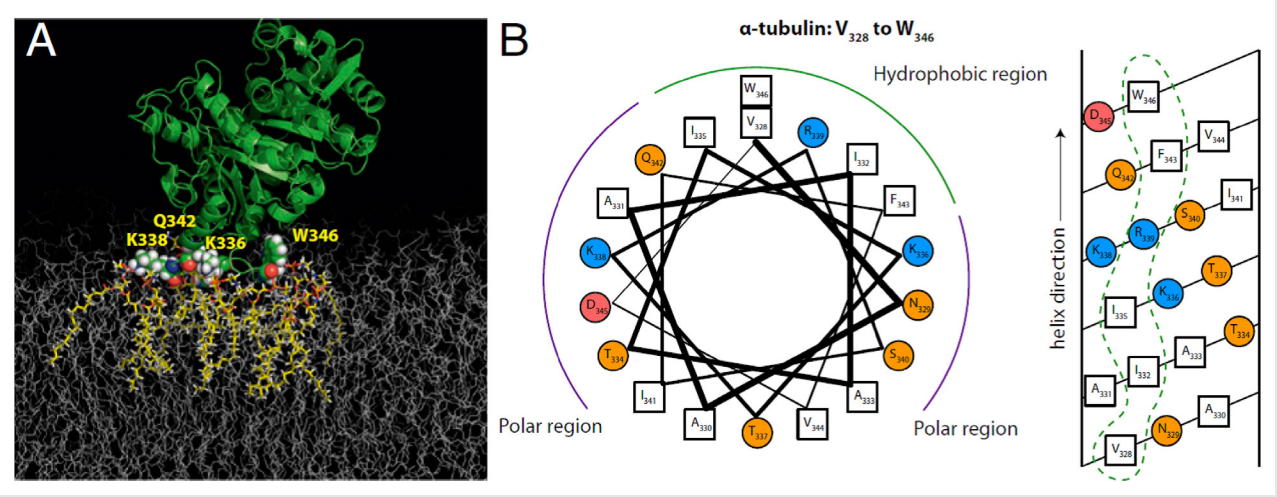

FIGURE 1. Tubulin binding residues identified by molecular dynamics (MD) simulations (Reference 1)

The proposed binding residues in alpha-tubulin (shown as van der Waals spheres) on the DOPE (dioleyl phosphatidylethanolamine) membrane (A). The binding residues were in direct contact with lipids (highlighted in yellow) for the entire duration of the simulation (about 1 ms). Helical wheel (*left*) and helical net (*right*) diagrams for the putative amphipathic binding region at the alpha end of the tubulin heterodimer (B). Hydrophobic residues are denoted by open squares, polar uncharged residues by orange circles, and positively and negatively charged residues by blue and red circles, respectively. Dashed loops indicate membrane-binding surfaces.

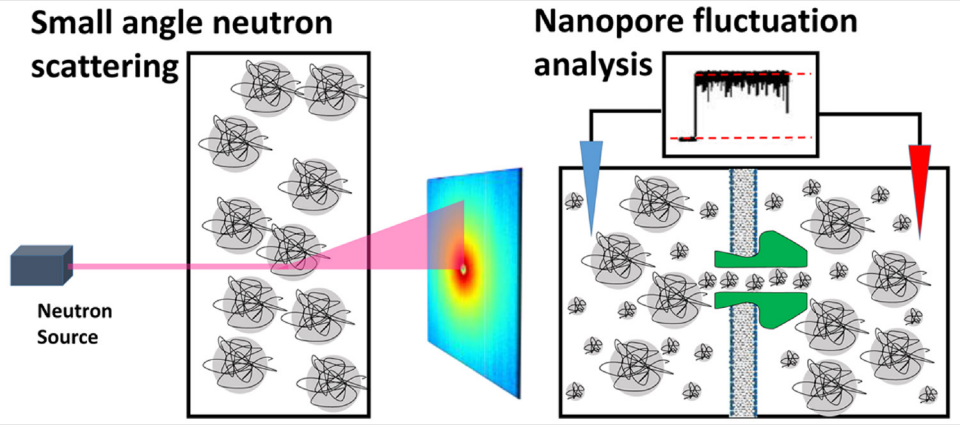

FIGURE 2. Cartoons of small-angle neutron scattering from bulk solutions (*left*) and nanopore conductance fluctuation analysis (*right*) (Reference 2)

These methods were used to probe structural and dynamic features of poly(ethylene glycol) in water/salt solutions in the dilute and semidilute regimes.

and disease. We showed that tubulin belongs to the group of peripherally bound amphitropic proteins, the subfamily of peripheral membrane proteins that interact directly with the lipid membrane rather than with intrinsic membrane proteins and are therefore strongly influenced by lipid composition. Several diseases, such as atherosclerosis, type II diabetes, and lysosomal storage disorders, are associated with defective distribution of intracellular lipids. Although involvement of amphitropic proteins in various cell functions has been unequivocally established, the mechanisms of their interaction with cellular membranes are only beginning to be understood because their weak reversible binding to the membranes creates obvious experimental difficulties in assessing binding conformations and kinetics.

Partitioning of water-soluble polymers into beta-barrel channels in their functional states

Understanding polymer partitioning into nanoscale cavities of differing natures is important for many technological applications that include, but are not limited to, analytical chromatography, separation techniques, and purification methods. It is also critical in the qualitative interpretation and quantitative analysis of molecular interactions and biological regulation in the crowded cellular environment. This necessitates model studies with polymer solutions explored in both dilute and semidilute regimes. Recently, we studied polymer partitioning from semidilute water-based solutions of poly(ethylene glycol) (PEG) mixtures into a number of membrane-spanning beta-barrel channels of various origins, and the results were rationalized within the earlier formulated “polymers-pushing-polymers” model of nanopore partitioning. The model is based on the assumption that the larger component of the polymer mixture, being preferentially excluded from the channel nanopore, pushes the smaller component into the nanopore, thus representing forced polymer redistribution between the bulk and the channel. This year, we explored polymer mixtures by using two different methods, small-angle neutron scattering and nanopore conductance fluctuation analysis (Figure 2), to quantify the larger polymer parameters in the bulk and the degree of its partitioning in the pore, respectively. We first showed that the reduction of the PEG 3400 characteristic size with its increasing concentration in the bulk is statistically significant but small. We then demonstrated that partitioning of the larger polymer in the nanopore is negligible if its relative weight fraction is kept under 50%, in excellent agreement with the major assumptions of the model mentioned above. In our opinion, demonstration of this selective, “forced” partitioning of the small polymers into protein cavities to a

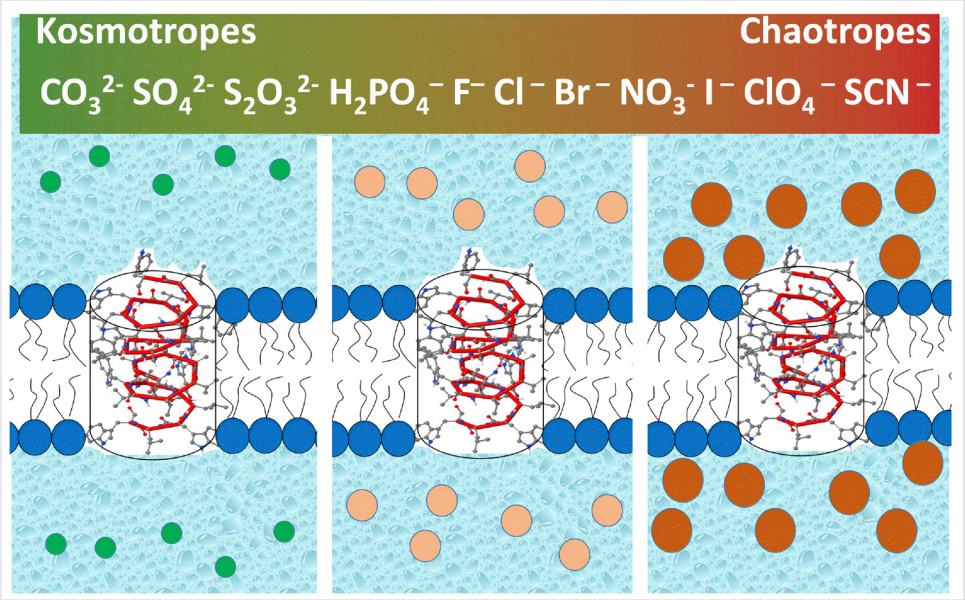

FIGURE 3. Gramicidin A pore in a lipid membrane bathed by anions of different polarizability (Reference 3)

Both the conformational dynamics and conductance of this purely cation-selective peptide pore are regulated by anions in correlation with their position in the Hofmeister series by preferential depletion of kosmotropic anions (or accumulation of chaotropic anions) at the membrane-water interface.

significant excess of their bulk concentration is a step forward to a better qualitative and quantitative understanding of molecular crowding effects. Furthermore, it represents a new type of regulation of macromolecular transport, which may be of practical importance in the design and directed delivery of water-soluble drugs.

Ion specificity of the Hofmeister ranking

Ion specificity and, in particular, the distinctive effects of anions in salt-induced protein precipitation have been known since the 1880s, when Franz Hofmeister established the ranking of anions in their ability to precipitate egg yolk protein from water solutions. Experimental and theoretical studies have given a detailed empirical picture of the phenomenon, but the nature of the ionic interactions with the surfaces leading to the Hofmeister effect is still under debate. The only consensus is that it cannot be explained by standard theories of electrolytes. For example, bromide is unique in that its salts were recognized as a drug to treat epilepsy a couple of dozen years before Hofmeister's studies and they are still in use to treat specific types of refractory seizures in children, but the mechanism of their action remains elusive. Although the Hofmeister ranking of salts has been a frequent target of biological studies including channel-facilitated membrane transport, this year we decided to take advantage of arguably the simplest ion transport model of modern biophysics—the channel formed by a linear pentadecapeptide, gramicidin A. Counter-intuitively, we found that conductance of this perfectly cation-selective channel increases about two-fold in the "Hofmeister anion series" H_2PO_4<Cl=Br=NO_3<ClO_4<SCN. Channel dissociation kinetics show even stronger dependence, with the dwell time increasing about 20-fold. While the conductance can be quantitatively explained by the changes in membrane surface potential owing to exclusion of kosmotropes from (or accumulation of chaotropes at) the surface (Figure 3), the kinetics proved to be more difficult to treat. We estimated the effects of changes in the energetics at the bilayer surfaces on the channel dwell time, concluding that the change would have to be greater than typically observed for the Hofmeister effect outside the context of the lipid bilayer. We believe that our results are of importance for further progress in

understanding ion specificity, which manifests itself in many physicochemical and biological phenomena, including more than century-old medical applications.

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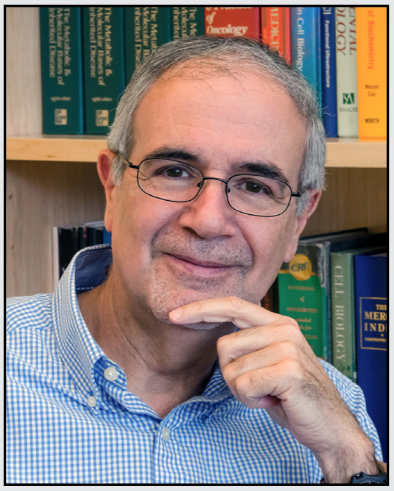
Protein Sorting in the Endosomal–Lysosomal System

We investigate the molecular mechanisms by which transmembrane proteins (referred to as cargo) are sorted to different compartments of the endo-membrane 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., in epithelial cells and neurons). Transport of cargo between the compartments is mediated by carrier vesicles or tubules, which 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 various 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 this research to the elucidation of protein trafficking diseases such as the Hermansky-Pudlak syndrome (HPS), a pigmentation and bleeding disorder, and the MEDNIK syndrome, a neuro-cutaneous disorder. In addition, we study how the molecular mechanisms of protein transport are exploited by intracellular pathogens such as HIV-1.



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Function of the BORC complex in the regulation of lysosome movement

The multiple functions of lysosomes are critically dependent on their ability to move bidirectionally along microtubules between the center and periphery of the cell. Centrifugal and centripetal movement of lysosomes is mediated by kinesin and dynein motors, respectively. We recently discovered a multi-subunit complex named BORC, which recruits the small GTPase Arl8 to lysosomes to promote their kinesin-dependent movement toward the cell periphery. We showed that

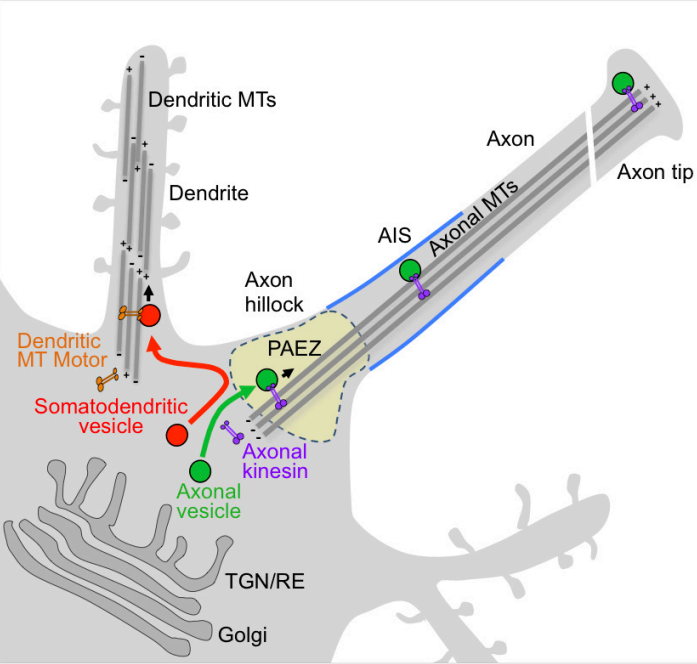

FIGURE 1. Sorting of somato-dendritic and axonal vesicles at the pre-axonal exclusion zone (PAEZ)

BORC and Arl8 function upstream of two structurally distinct kinesin types: kinesin-1 (KIF5B) and kinesin-3 (KIF1B and KIF1A). Remarkably, KIF5B and KIF1B/KIF1A move lysosomes along different microtubule tracks. The findings thus established BORC as a master regulator of lysosome positioning through coupling to different kinesins and microtubule tracks.

The BORC complex coordinates encounter and fusion of lysosomes with autophagosomes.

Whereas the mechanisms involved in autophagosome formation have been extensively studied for the past two decades, those responsible for autophagosome-lysosome fusion have only recently begun to garner attention. We recently found that BORC is also required for efficient autophagosome-lysosome fusion. Knock-out (KO) of BORC subunits impairs both the encounter and fusion of autophagosomes with lysosomes. Reduced encounters result from an inability of lysosomes to move toward the peripheral cytoplasm, where many autophagosomes are formed. BORC KO also reduces the recruitment of the HOPS (homotypic fusion and vacuole protein sorting) tethering complex to lysosomes and assembly of a trans–SNARE (SNARE proteins mediate vesicle fusion) complex involved in autophagosome-lysosome fusion. Through these dual roles, BORC integrates the kinesin-dependent movement of lysosomes toward autophagosomes with HOPS–dependent autophagosome-lysosome fusion.

A Ragulator–BORC interaction controls lysosome positioning in response to amino acid availability.

Lysosomes play key roles in the cellular response to amino acid availability. Depletion of amino acids from the medium turns off a signaling pathway involving the Ragulator complex and the Rag GTPases, causing release of the inactive mTORC1 serine/threonine kinase from the lysosomal membrane. Decreased phosphorylation of mTORC1 substrates inhibits protein synthesis while activating autophagy. Amino acid depletion also causes clustering of lysosomes in the juxtanuclear area of the cell, but the mechanisms responsible for this phenomenon are poorly understood. This past year, we showed that Ragulator directly interacts with BORC, the multisubunit complex previously found to promote lysosome dispersal through coupling to the small GTPase Arl8 and the kinesins KIF1B and KIF5B (see above). Interaction with Ragulator exerts a negative regulatory effect on BORC that is independent of mTORC1 activity. Amino acid depletion strengthens this interaction, explaining the redistribution of lysosomes to the juxtanuclear area. The findings demonstrate that amino acid availability controls lysosome positioning through Ragulator-dependent, but mTORC1–independent, modulation of BORC.

Segregation in the Golgi complex precedes export of endo-lysosomal proteins in distinct transport carriers.

Biosynthetic sorting of newly synthesized transmembrane cargos to endosomes and lysosomes is thought to occur at the TGN through recognition of sorting signals in the cytosolic tails of the cargos by adaptor proteins, leading to cargo packaging into coated vesicles destined for the endo-lysosomal system. This past year, we obtained evidence for a different mechanism, by which two sets of endo-lysosomal proteins undergo early segregation to distinct domains of the Golgi complex by virtue of the proteins' luminal and transmembrane domains. Proteins in one Golgi domain exit into predominantly vesicular carriers by interaction of sorting signals with adaptor proteins, but proteins in the other domain exit into predominantly tubular carriers shared with plasma membrane proteins, independently of signal-adaptor interactions. The findings demonstrate that sorting of endo-lysosomal proteins begins at an earlier stage than, and involves mechanisms that differ from, those described by classical models.

Polarized organelle segregation in neurons by differential interactions with microtubule motors

Polarized sorting of newly synthesized proteins to the somato-dendritic and axonal domains of neurons occurs by selective incorporation into distinct populations of vesicular transport carriers. We recently found that segregation of these carriers to their corresponding neuronal compartments occurs at a region in the axon hillock named the pre-axonal exclusion zone (PAEZ) though differential coupling to different microtubule motors. We also discovered a chain of interactors including Rab5, the FHF complex, and dynein-dynactin, which retrieve somatodendritic proteins from the axon, thus contributing to their somatodendritic distribution at steady state. We further demonstrated that an ensemble composed of BORC, Arl8, SKIP, and kinesin-1 specifically drives lysosome transport into the axon, and not the dendrites, in cultured rat hippocampal neurons. We also found that the transport is essential for the maintenance of axonal growth-cone dynamics and autophagosome turnover. The findings illustrate how a general mechanism for lysosome dispersal in non-neuronal cells is adapted to drive polarized transport in neurons and, furthermore, emphasize the importance of this mechanism for critical axonal processes.

A role for AP-1 in sorting presenilin-2 to late endosomes and lysosomes

In collaboration with Wim Annaert, we demonstrated a role for the AP-1 complex in the sorting of a novel form of gamma-secretase to late endosomes and lysosomes. Gamma-secretases are a family of intramembrane-cleaving proteases involved in the pathogenesis of Alzheimer's disease. We identified a sorting motif in the cytosolic tail of the presenilin-2 (PSEN2) subunit of gamma-secretase that targets this enzyme to late endosomes and lysosomes. The motif is recognized in a phosphorylation-dependent manner by AP-1. PSEN2 selectively cleaves late endosomal/lysosomal-localized substrates and generates a prominent pool of intracellular amyloid-beta peptide. The findings reveal potentially important roles for lysosome-generated amyloid-beta peptide in Alzheimer's disease.

Mechanism of cargo recognition by the retromer complex

Retromer is a multi-protein complex that recycles transmembrane cargo from endosomes to the TGN and the plasma membrane. Defects in retromer impair various cellular processes and underlie some forms of Alzheimer's disease and Parkinson's disease. Although retromer was discovered over 15 years ago, the mechanisms for cargo recognition and recruitment to endosomes had remained elusive. X-ray–crystallographic, biochemical, and cellular studies conducted in collaboration with Aitor Hierro showed that

cargos bind to a cooperative assembly of the VPS26 and VPS35 subunits of retromer in complex with the sorting nexin SNX3.

Identification of TSSC1 as a novel component of the endosomal retrieval machinery

Endosomes function as a hub for multiple protein sorting events, including retrograde transport to the TGN and recycling to the plasma membrane, processes that are mediated by tubular-vesicular carriers that bud from early endosomes and fuse with a corresponding acceptor compartment. We previously investigated the role of two multisubunit tethering complexes named GARP and EARP, which participate in SNARE–dependent fusion of endosome-derived carriers with the TGN and recycling endosomes, respectively. We have now discovered that a previously uncharacterized WD40/beta-propeller protein named TSSC1 is a specific interactor of both GARP and EARP and a novel component of the endosomal retrieval machinery. Interference with TSSC1 impairs both retrograde transport to the TGN and retrieval to the plasma membrane. The findings contribute to an understanding of the pathogenesis of progressive cerebello-cerebral atrophy type 2, a neurodegenerative disorder caused by mutations in the shared Vps53 subunit of GARP and EARP.

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Child and Family Development Across the First Three Decades

The Child and Family Research (CFR) Section investigates dispositional, experiential, and environmental factors that contribute to physical, mental, emotional, and social development in human beings across the first three decades of life. The research goals of the CFR 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; and (3) influences on development of children's exposure to and interactions with the natural and designed environments.

The CFR pursues two integrated multi-age, multi-informant, multi-variate, and multi-cultural research programs that are supplemented by a variety of ancillary investigations. The research programs represent an en bloc effort. The first includes a prospective longitudinal study designed to explore several aspects of child development in the context of major socio-demographic comparisons. As a part of this program, the CFR carries out investigations into developmental neuroscience (cardiac function and EEG in psychological development; eye-tracking, perception, and cognition; and categorization) and behavioral pediatrics (developmental sequelae of cancer in infancy; children's understanding and coping with medical experiences; parental depression, preterm birth, deaf culture and child development; and behavior problems in adolescence), addressing questions at the interface of child development, biology, and health.

The second CFR program broadens the perspectives of the first to encompass cultural influences on development within the same basic longitudinal framework. Cultural study sites include Argentina, Belgium, Brazil, Cameroon, Chile, England, France, Israel, Italy, Japan, Kenya, Peru, and the Republic of South Korea, as well as the United States; in all places, intra-cultural as well as cross-cultural comparisons are pursued. In this effort, the CFR collaborates with the Parenting Across Cultures project, which studies 8- to-16-year-olds and their families longitudinally in 11 cultural groups in nine countries and makes use of the UNICEF Multiple Indicator Cluster

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Survey of about 50 low- and middle-income countries globally. Overall, CFR 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 and activities on parents; and the meaning of variations in parenting and in the family across a wide variety of socio-demographic and cultural groups. The ultimate aims of both CFR research programs are to promote aware, fit, and motivated children who will, it is hoped, eventually grow into knowledgeable, healthy, happy, and productive adults.

The child, the parent, and the family across the first three decades

Children's and adolescents' cognitive abilities, social adaptation, and externalizing behaviors are broadly associated with each other at the bivariate level; however, the direction, ordering, and uniqueness of these associations had yet to be identified. Developmental cascade models are particularly well suited first to discern unique pathways among psychological domains and second to model stability in and covariation among constructs, allowing for conservative tests of longitudinal associations. The study identified specific cascade effects among children's cognitive abilities, social adaptation, and externalizing behaviors over a 10-year period beginning in preschool and extending into adolescence. Children (46.2% female) and mothers (N = 351 families) provided data when children were 4, 10, and 14 years old. Cascade effects highlighted significant stability in these domains. Unique longitudinal associations were identified between (1) cognitive abilities at age 10 and social adaptation at age 14, (2) social adaptation at age 4 and externalizing behavior at age 10, and (3) externalizing behavior at age 10 and social adaptation at age 14. The findings suggest that children's social adaptation in preschool and externalizing behavior in middle childhood may be ideal intervention targets to enhance adolescent wellbeing.

Parenting has strong instrumental connotations and is widely believed to contribute in central ways to the course and outcome of child development and adjustment by regulating the majority of child-environment interactions and helping to shape children's adaptation. Insofar as parenting practices embody or are motivated by parenting cognitions, cognitions are thought to generate and give meaning to practices and mediate their effectiveness. It is therefore often assumed that caregiving cognitions engender caregiving practices and, ultimately, children's development and adjustment. In a large-scale (N = 317) prospective 8-year longitudinal multi-age, multi-domain, multi-variate, multi-source study, we tested a conservative 3-term model linking parenting cognitions in toddlerhood to parenting practices in preschool to classroom externalizing behavior in middle childhood, controlling for earlier parenting practices and child externalizing behavior. Mothers who were more knowledgeable, satisfied, and attributed successes in their parenting to themselves when their toddlers were 20 months of age engaged in increased supportive parenting two years later when their children were 4 years of age, and six years after that their 10-year-olds were rated by teachers as having fewer classroom externalizing behavior problems. This developmental cascade of a "standard model" of parenting applied equally to families with girls and boys, and the cascade from parenting attributions to supportive parenting to child externalizing behavior obtained. Conceptualizing socialization in terms of cascades helps identify points of effective intervention.

Many adolescents display risk behaviors that may persist into adulthood and contribute to an enormous public health and social financial burden. Young people may engage in adverse, aggressive, and otherwise reckless behaviors, including driving without a seatbelt and drug and alcohol use, which can threaten their own and others' physical health and safety. Moreover, adolescents and young adults have the highest

age-specific diagnosis rates for many sexually transmitted diseases compared with other age groups. Given the possible co-occurrence of different risk behaviors, a better understanding of health risk among adolescents requires a more holistic approach, by which a broader range of risk behaviors are considered simultaneously. We identified four latent profiles based on risk levels (Low, Modest, Medium and High) of safety and violence, sexual behavior, alcohol use, and marijuana and other drug use for 229 adolescents at 18 years and 23 years. Some adolescents maintained their latent profile membership over time, but more transitioned between risk profiles. Adolescents with more depressive symptoms had a higher probability of developing into the High Risk versus Low and Modest Risk profiles at 23 years. Adolescents in the High, Low, and Modest Risk profiles at 18 years developed more depressive symptoms in young adulthood compared with Medium Risk adolescents. Further, we found reciprocal associations between a High Risk profile and depressive symptoms over time. The findings suggest that mental health and behavioral risks are intertwined and that targeting one of the two aspects may be effective in treating the other.

Child development and parenting in multicultural perspective

Researchers report a neurobiological basis for human mothers' responses to infant cries. The extent to which human caregivers are biologically programmed to respond to their infants' cries remains unclear. To shed light on this question, together with colleagues we observed the behavior of 684 mothers averaging 27 years of age with infants approximately 5 months old in 11 countries and found that, across all countries, mothers preferentially responded to their infants' crying by picking up and holding and talking to the infant. Based on these observations, the authors hypothesized that infant crying would elicit common responses in the brains of new mothers from different cultures. We then conducted fMRI experiments involving 43 American mothers, average age 33 years, with 3.5 month-old infants, and 44 Chinese mothers, average age 30.5 years, with 7.6 month-old infants. In both sets of mothers, the sound of infants' cries activated the supplementary motor area associated with the intention to move and speak, Broca's area and the superior temporal regions associated with processing speech and complex sounds, and midbrain and striatal regions associated with caregiving. The results suggest a neurobiological and evolutionary basis for the way human mothers respond to infant cries.

As survival rates following a preterm birth have risen as a result of improvements in obstetrics and neonatology, preterm birth has emerged as a risk factor for poor development in an increasing proportion of the population. Language skills have been regularly reported to be impaired in children born very preterm. However, language findings are somewhat less consistent for children born moderate-to-late preterm. This study investigated whether children born very preterm, moderate-to-late preterm, and term (37–41 weeks) differ in their average level and individual-difference stability in language performance over time. Samples of 204 very preterm, 276 moderate-late preterm, and 268 term children were given language assessments at 5 months, 20 months, 4, 6, and 8 years of age. Very preterm children consistently performed worse than term-born children, and moderate-late preterm children scored in between. Language performance was stable from 5 months through 8 years in all gestation groups combined, and stability increased between each succeeding testing wave. The study's findings have several implications for pediatricians and parents. Pediatricians and parents should be made aware that preterm-born children, even those born moderate-late preterm, are at risk for delayed language abilities compared with term children. By 20 months of age, children who are performing poorly relative to their peers are likely to continue to perform poorly at later ages; stability in language performance appears to strengthen over time. Finally, very preterm children appear to be at the greatest risk for problems with language development.

Through regular checkups, pediatricians have the opportunity to connect children who have lagging language skills with critical services.

Most studies of the effects of parental religiousness on parenting and child development focus on a particular religion or cultural group, which limits generalizations that can be made about the effects of parental religiousness on family life. We assessed associations among parental religiousness, parenting, and children's adjustment in a three-year longitudinal investigation of 1198 families from 9 countries. We included four religions (Catholicism, Protestantism, Buddhism, Islam) plus unaffiliated parents, two positive (efficacy and warmth) and two negative (control and rejection) parenting practices, and two positive (social competence and school performance) and two negative (internalizing and externalizing) child outcomes. Parents' greater religiousness had both positive and negative associations with parenting and child adjustment. Greater parent religiousness when children were eight was associated with higher parental efficacy at nine and, in turn, children's better social competence and school performance and fewer child internalizing and externalizing problems at 10. However, greater parent religiousness at eight was also associated with more parental control at nine, which in turn was associated with more child internalizing and externalizing problems at 10. Parental warmth and rejection had inconsistent relations with parental religiousness and child outcomes depending on the informant. With a few exceptions, similar patterns of results held for all four religions and the unaffiliated, nine sites, mothers and fathers, girls and boys, and controlling for demographic covariates.

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Neuregulin–ErbB Processing and 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 to synchronize 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 many psychiatric disorders and may account for their cognitive and behavioral symptoms. We are interested in how Neuregulin and its receptor ErbB4, which are both genetically linked to psychiatric disorders, function in rodents in an activity-dependent fashion (i.e., experience) in the developing brain to regulate synaptic plasticity, neuronal network activity, and behaviors that model features of many psychiatric disorders. We identified functional interactions between the Neuregulin/ErbB4, dopaminergic, and glutamatergic signaling pathways in GABAergic interneurons that are critical for understanding how Neuregulins regulate neuronal plasticity, E/I balance, and synchronous activity in neuronal networks.

Our earlier studies demonstrated that in the hippocampus and neocortex expression of ErbB4, the major Neuregulin neuronal receptor, is restricted to GABAergic interneurons. ErbB4 levels are especially high in a subtype of GABAergic neurons, known as parvalbumin-positive (Pv^{+}) fast-spiking interneurons, that are necessary for the generation and modulating of gamma oscillations. Using genetically targeted mouse mutant models, we went on to show that Neuregulin–ErbB4 signaling regulates synaptic plasticity, neuronal network activity (i.e., gamma oscillations), and behaviors associated with psychiatric disorders. Our group more recently investigated other aspects of Neuregulin brain expression: the subcellular expression and trafficking of different Neuregulin isotypes, their post-translational processing in response to neuronal activity, and their functions in the developing and maturing nervous system. To achieve these goals, we use 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-

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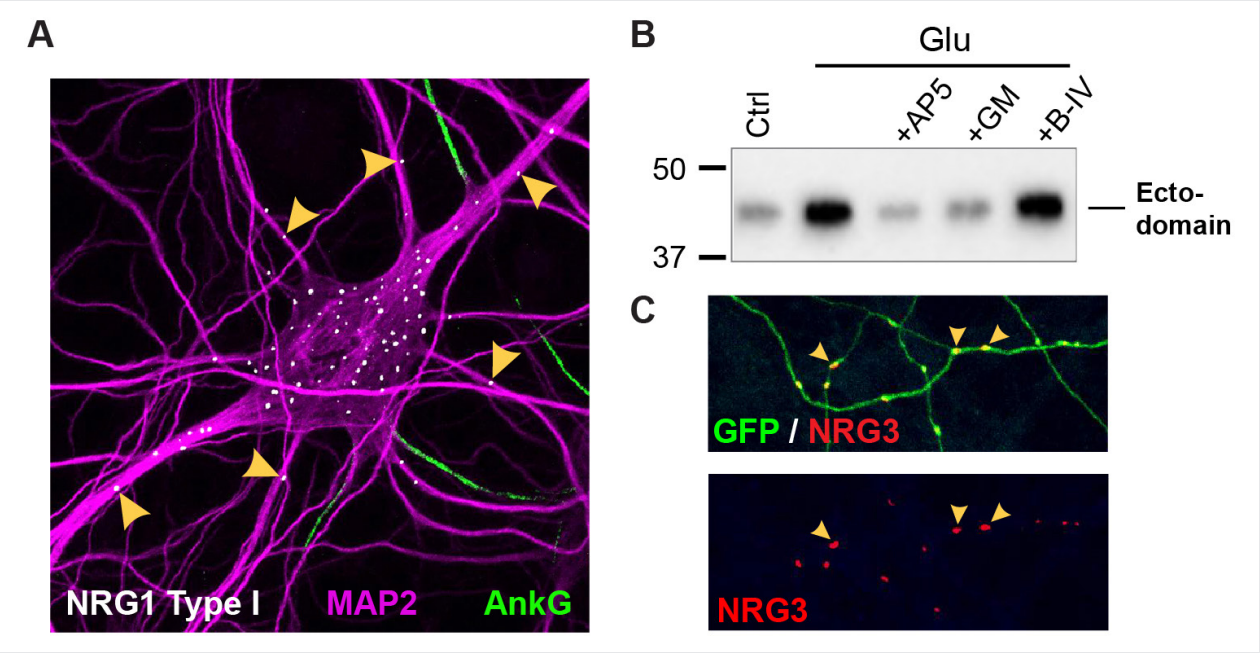


FIGURE 1. Differential trafficking of NRG isoforms in transfected hippocampal neurons

A. NRG1 type I accumulates as puncta on soma and proximal MAP2–positive dendrites, while axons (proximal segments identified by Ankyrin G) and more distal dendrites are negative.

B. Stimulation of neurons with 20 μM glutamate (Glu) promotes NRG1 ectodomain shedding. The effect is blocked by NMDA receptor (AP5) or metalloprotease (GM6001) inhibition.

C. NRG3 puncta accumulate on GFP–filled axons, away from cell bodies.

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 many psychiatric disorders.

Subcellular distribution and functions of single transmembrane (TM) Neuregulins in central neurons

Numerous Neuregulins (NRGs) are generated through the use of four different genes (NRG1–NRG4), promoters (NRG1: types -I, -II and -III), and alternative splicing, but the functional significance of this evolutionarily conserved diversity remains poorly understood. 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 numerous biological effects in the developing and maturing brain remain mostly unknown. As discussed here and further below, our recent studies reveal that NRGs can be categorized by their distinct membrane topologies, which impart fundamentally different subcellular trafficking properties. NRGs whose pro-forms contain a single transmembrane domain, such as NRG1 (types I and II) and NRG2, target to the cell body plasma membrane, where they accumulate at specialized contact

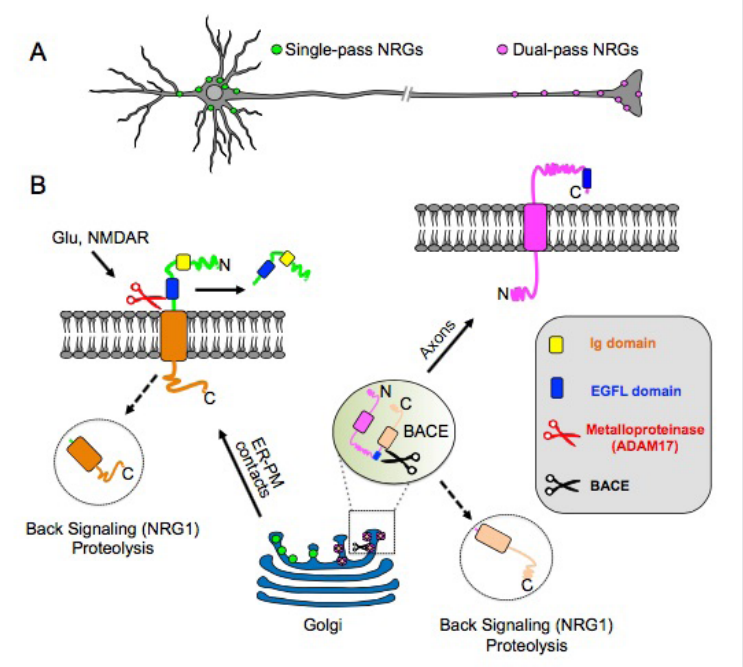


FIGURE 2. Transmembrane (TM) topology of unprocessed pro–NRGs determines their neuronal subcellular targeting and processing in central neurons.

A. A schematic presentation, based on our findings (Reference 1), that depicts single TM NRGs accumulating at somatic subsurface cistern and at proximal dendrites to mediate NRG\ErbB4 signaling, whereas dual TM NRGs accumulate on axons and their terminals to drive long-range signaling.

B. Single TM NRGs are trafficked through the secretory pathway as unprocessed pro-forms and accumulate at subsurface cisternae (SSC) sites, where NMDAR activity promotes ectodomain shedding. By contrast, unprocessed dual TM NRGs traffic via the Golgi and are cleaved by BACE (beta-secretase) to release the amino-terminal region, which is transported into axons in an anterograde manner. Both single– and dual–TM NRGs are processed by gamma-secretase to release carboxyl-terminal proteolytic fragments, which can "back-signal" into the nucleus to regulate transcription.

sites with the underlying endoplasmic reticulum. We began our studies by analyzing NRG2, an 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 and ErbB4 can engage in autocrine signaling in this neuron type. Interestingly, we found no evidence of NRG2 protein in axons or distal dendrites; instead, we found that unprocessed proNRG2 accumulates as large somato-dendritic puncta on the neuronal soma and proximal dendrites (Reference 2). Our more recent studies on the other single TM NRGs (NRG1 types I and II) demonstrate a similar subcellular distribution. Moreover, we found that the ectodomains of single TM NRGs are cleaved by sheddases in an activity-dependent manner to signal in paracrine and autocrine fashion, and that this activity is mediated by NMDA receptor activation on cortical interneurons to promote proNRG2 shedding and subsequent ErbB4 receptor signaling (Figure 1 A,B). In turn, the activation of ErbB4 promotes its association with NMDARs and their internalization. In this fashion, there is bidirectional signaling between NRG/ErbB4 and NMDAR that can function as a homeostatic mechanism to regulate interneuron excitability (References 1,2).

Subcellular distribution of dual transmembrane Neuregulins in central neurons

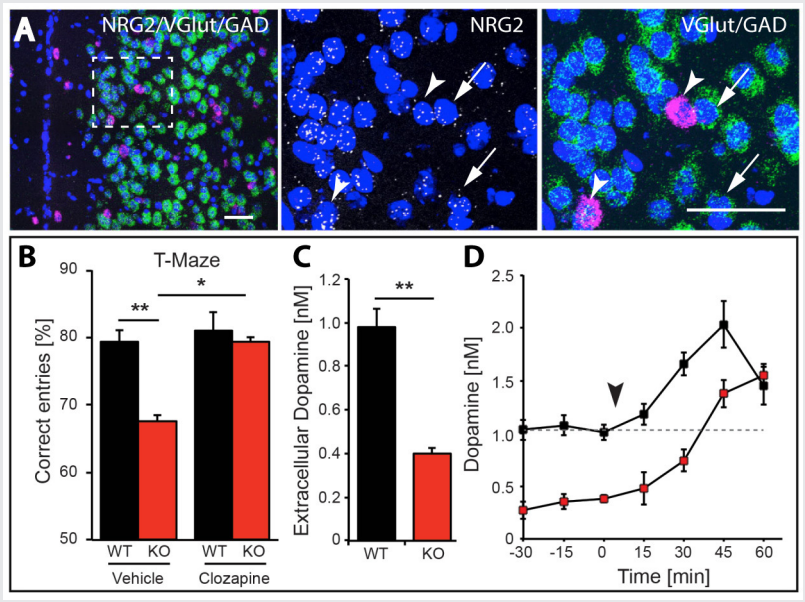
By contrast to single TM NRGs, we found that dual TM NRGs, such as CRD (cysteine-rich domain)–NRG1 (type III) and NRG3, for which we recently reported its unexpected dual TM topology (Reference 1), are targeted to axons where they signal in juxtacrine mode (Figure 1C). Moreover, unlike single TM NRGs, processing of dual TM NRGs is constitutive and independent of NMDA receptor activity. Interestingly, processing is a

FIGURE 3. NRG2 null mice exhibit working memory deficits and reduced dopamine levels that are restored by clozapine.

A. Expression of NRG2 in the prefontal cortex (PFC) analyzed by triple *in situ* hybridization. NRG2 transcripts (*white*) are expressed in both glutamatergic (*green*) and GABAergic (*magenta*) neurons.

B. Poor performance by NRG2 knockout (KO) mice in a T-maze reward alternation task, as compared with WT littermates (*left*), can be restored by administration of the antipsychotic clozapine (*right*).

C. Reduced extracellular dopamine levels in the mPFC of NRG2 KO mice.



D. Extracellular dopamine levels in the mPFC of NRG2 KO mice rise after clozapine injection (*arrowhead*) at a time that coincides with improved performance on the T-maze.

prerequisite for axonal accumulation of dual TM NRGs; treatments that interfere with processing cause intracellular accumulation of their pro-forms. Taken together, our recent findings about the relationships between transmembrane topology, modes of proteolytic processing, subcellular distribution, and signaling modality suggest that single- and dual-pass NRGs regulate neuronal functions in fundamentally different ways (Figure 1C,D). This work was supported by a Directors Investigator Award.

Neuregulin-2 knockout mice exhibit dopamine dysregulation and severe behavioral phenotypes with relevance to psychiatric disorders.

We found that NRG2 expression in the adult rodent brain does not 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 NRG2's function (Figure 2). NRG2 KOs have higher extracellular dopamine levels in the dorsal striatum but lower levels in the mPFC than wild-type mice—a pattern with similarities to dopamine dysbalance in schizophrenia. Like ErbB4 KO mice, NRG2 KOs performed abnormally in a battery of behavioral tasks relevant to psychiatric disorders. NRG2 KOs exhibit hyperactivity in a novelty-induced open field, deficits in prepulse inhibition, hypersensitivity to amphetamine, antisocial behaviors, reduced anxiety-like behavior in the elevated plus maze and deficits in the T-maze alteration reward test—a task that is dependent on hippocampal and mPFC function. Acute administration of clozapine rapidly increased extracellular dopamine levels in the mPFC and improved alternation T-maze performance (Figure 2D). Similar to mice treated chronically with *N*-methyl-D-aspartate receptor (NMDAR) antagonists, we demonstrated that NMDAR synaptic currents in NRG2 KOs are augmented at hippocampal glutamatergic synapses and exhibit augmented sensitivy to ifenprodil, indicating an elevated contribution of GluN2B–containing NMDARs. Our findings reveal a novel role for NRG2 in the modulation of behaviors with relevance to psychiatric disorders (Reference 3).

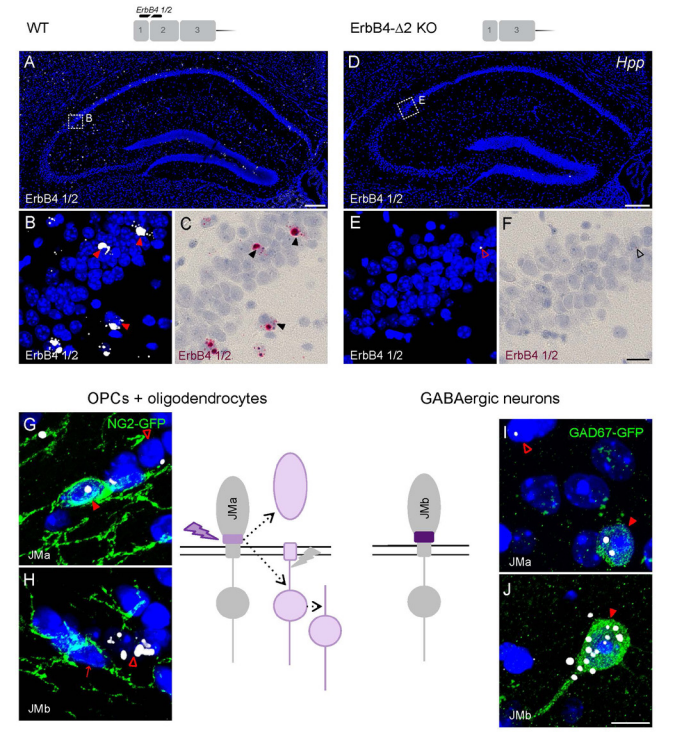


FIGURE 4. Oligodendrocytes and neurons express distinct ErbB4 JM transcripts.

The sensitivity and specificity of single-pair probe *in situ* hybridization was demonstrated by the presence of signals in sparse GABAergic hippocampal neurons of wild-type (WT) mice hybridized with a probe corresponding to exon 2 of the ErbB4 receptor gene (A–C), and by the absence of signal in sections prepared from ErbB4 knockout mice that lack exon 2 (D–F). Oligodendrocyte precursors cells (OPCs) and mature oligodendrocytes express ErbB4 JMa isoforms, which are susceptible to shedding and can back-signaling (G,H), whereas GABAergic neurons express the cleavage-resistant JMb ErbB4 receptor (I,J).

Analysis of ErbB4 function in mice harboring targeted mutations in GABAergic and dopaminergic 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. Given that we had shown that NRG1 acutely increases extracellular DA levels to regulate long-term potentiation (LTP) and gamma oscillations, and that ErbB4 receptor expression is confined to GABAergic interneurons (cortex) and TH$^+$ mesocortical DAergic neurons, we used genetic, biochemical, and behavioral approaches to measure DA function in the hippocampus, PFC, and striatum in mice harboring targeted mutations of ErbB4 in either PV$^+$ or TH$^+$ neurons. Interestingly, we have found that, in contrast to GABAergic neurons, ErbB4 is highly expressed on the axons of DA neurons, suggesting that NRG/ErbB4 signaling may directly regulate the presynaptic function of these neurons. We found NRG regulates the increase in extracellular DA levels, at least in part, by regulating DA transporter (DAT) function. In contrast to mice harboring CNS–wide or GABAergic–restricted mutation of ErbB4, which show sensory-motor gating deficits and increases in motor activity, mice with ablation of ErbB4 in TH$^+$ neurons only manifest behavioral deficits in cognitive-related tasks, such as performance on the T-maze, Y-maze, and Barnes maze. Our findings suggest that direct effects of NRG/ErbB4 signaling in GABAergic and DAergic neurons in combination regulate cortical circuits and DA homeostasis to affect numerous behaviors relevant to schizophrenia (Reference 4).

A novel exon–specific *in situ* hybridization approach uncovers a new pattern of ErbB4 isoform expression in brain neurons and oligodendrocytes.

ErbB4 receptor isoforms are generated by the alternatively splicing of exons that encode either one of the two juxtamembrane domains (JMa/JMb) and either one of the cytoplasmic domains (CYT-1/CYT-2), which impart different stability and downstream signaling modes to the receptor. Importantly, the

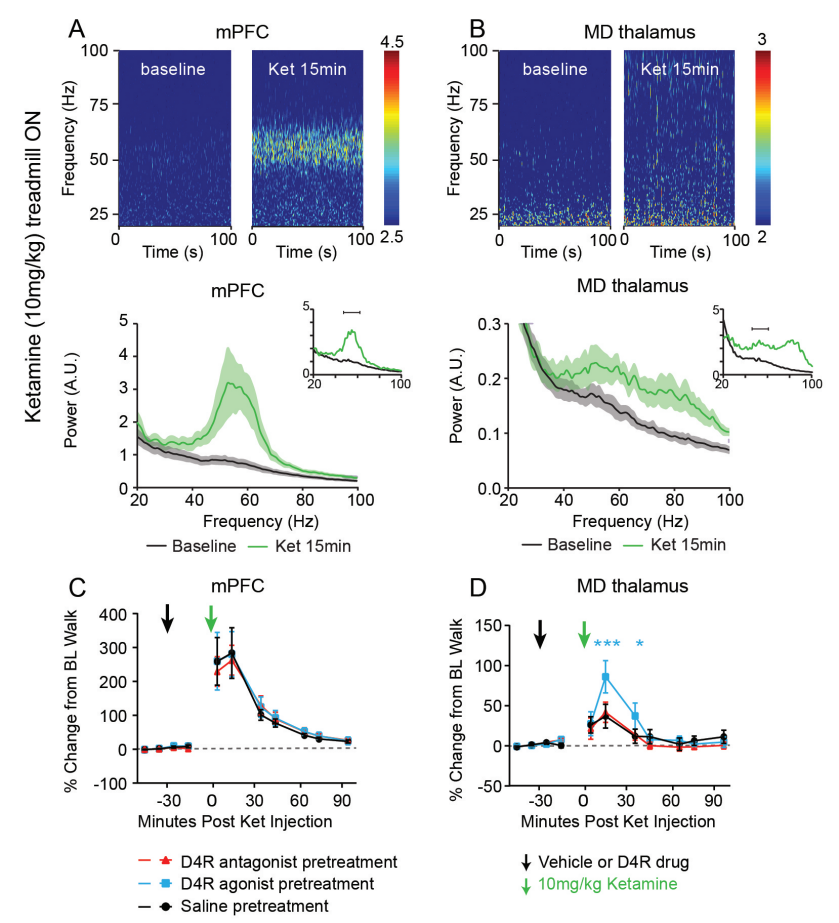


FIGURE 5. Ketamine-induced gamma oscillations in the medial prefrontal cortex (mPFC) and medial thalamus (MD) of walking rats, and regulation by D4R agonist

Multi-electrode recordings of local field potentials in the (A) mPFC and (B) MD reveal a dramatic increase in gamma frequency (40–70Hz) power in walking rats following ketamine injection (*green*), relative to baseline (BL) walking by rats injected with saline (*black*). C,D. Effects of D4R drugs on ketamine-induced gamma power. Administration of saline or a D4R antagonist (*red*) had no effect on ketamine-induced gamma power in either the (C) mPFC or the (D) MD, whereas the D4R agonist A-412997 (*cyan*) selectively increased gamma power in the MD.

ratios of JMa/JMb– and Cyt-1/Cyt-2–containing ErbB4 receptor isoforms are specifically augmented in the postmortem prefrontal cortex of individuals with schizophrenia. In order to study expression of these four alternatively spliced ErbB4 variants at a single-cell level, we implemented a novel highly sensitive fluorescent/colorimetric *in situ* hybridization approach that uses single-pair oligonucleotide probes (about 50bp) targeting exon/exon splice junctions. By comparing ErbB4 hybridization on sections from wild-type (WT) with ErbB4 knockout mice (missing exon 2), we initially demonstrated that single-pair probes provide the sensitivity and specificity to visualize and quantify the expression of ErbB4 isoforms (Figure 3A–F). Our analyses then identified brain regions of differential ErbB4 isoform expression conserved from mouse to humans. Using transgenic mice that drive the GFP reporter selectively in either neurons or glial cells, we went on to demonstrate that expression of distinct ErB4 isoforms differs between neurons and cells of the oligodendrocyte lineage. Interestingly, GABAergic interneurons mostly express the ErbB4 JMb isoform, whereas oligodendrocytes predominantly or exclusively express JMa receptor variants (Fig. 3G–J). Whereas the original reports attributed the changes of ErbB4 isoforms in postmortem brain to the result of altered expression in GABAergic neurons, our findings suggest that the increased JMa/JMb ratio observed in the postmortem brains of schizophrenia subjects could result from expression of ErbB4 JMa isoforms in cortical oligodendrocytes or their progenitors.

Effects of ketamine on cortical gamma oscillations and the role of dopamine receptors

Mounting evidence suggests that gamma oscillations are atypically high at baseline in disorders that affect attention, such as schizophrenia and ADHD. Ketamine, an antagonist of the NMDAR, has profound effects on gamma oscillation power, and it phenocopies schizophrenia by eliciting psychoto-mimetic symptoms and affecting cognitive functions in healthy individuals. In collaboration with Judith Walters' lab, we used multi-electrode recordings from the medial prefrontal cortex (mPFC) and mediodorsal thalamus (MD) of rats acutely treated with ketamine, which serves as a rodent model with "face validity" for schizophrenia, to study the drug's effects on spiking and gamma local field potentials in the mPFC and MD of freely moving rats. We found that ketamine raises gamma local field potentials and frequencies in both brain areas, but does not increase thalamocortical synchronization. Based on our prior *in vitro* studies, showing that a "cross-talk" between dopamine D4 (D4R) and ErbB4 receptors regulate gamma oscillation power in acute hippocampal slices, we investigated whether and how D4R–targeting drugs regulate gamma oscillations. We found that a D4R agonist (A-412997) increased ketamine-induced gamma power that was blocked by a D4R–selective antagonist (L-745870) in both mPFC and MD, but that neither drug altered ketamine-induced gamma power or frequency in the mPFC. Interestingly, in the MD, the D4R agonist increased the power of ketamine-induced gamma oscillations (Reference 5). Experiments are in progress to evaluate the effects of drugs targeting dopamine D4R receptors on attention and impulsivity behaviors in mice, using the five-choice serial-reaction time task (5CSRTT).

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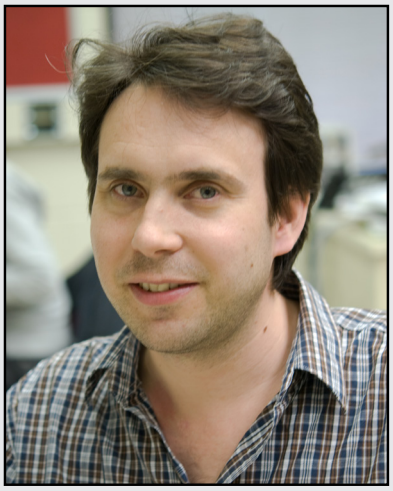
Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

Our goal is to understand how, under diverse environmental contexts, the nervous system selects appropriate behavioral actions in a way that best satisfies internal motivational objectives. We use the zebrafish as a model because, in the larval stage, its brain exhibits the basic architecture of the vertebrate brain but is much less complex than the mammalian brain. Despite the relative simplicity of their nervous system, larvae have a sophisticated repertoire of sensory-guided and internally driven behaviors. Furthermore, 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 quickly assess 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 (Reference 1) and pathways that sustain changes in behavioral state (References 2,3). 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 (References 4,5). 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 the 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 larval 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 that help animals defend against or escape from potentially threatening stimuli. In both fish and mammals,

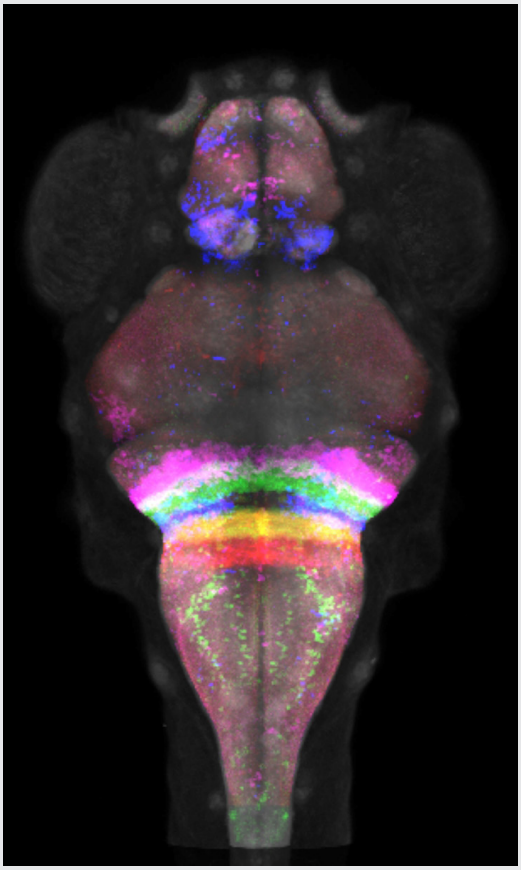

FIGURE 1. Intersectional interrogation of hindbrain circuitry using rhombomere-specific Cre lines

We developed a library of Cre lines that can be used in intersectional genetic experiments. Visualized here are confocal images of five Cre lines after co-registration to our atlas of transgene expression. Together, the lines partition the hindbrain into its rhombomeres 2–6, enabling neurons in each rhombomere to be functionally addressed.

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 for behavioral choice. 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 (PPI), is diminished in several neurological conditions. Vibrational stimuli trigger rapid-escape swims in zebrafish, which are mediated by giant reticulospinal neurons, in a manner 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 available in zebrafish to identify neurons that mediate prepulse inhibition.

To identify a transgenic zebrafish line that genetically labels neurons required for PPI, we screened a library of neuron-specific Gal4–enhancer trap lines marking distinct populations of neurons in the brain, by ablating the neurons in each enhancer trap line before testing for PPI. The transgenic line *y252* labels a discrete population of neurons in the hindbrain whose ablation or opto-genetic inhibition eliminates PPI. The neurons genetically labeled in *y252* are specified by the transcription factor *Gsx1*. We found that *Gsx1*–knockout mice showed a strong reduction in PPI, suggesting that a conserved circuit involving *Gsx1*–specified neurons mediates PPI across vertebrate species (Reference 1). To identify the precise subset of *Gsx1* neurons that mediate PPI, we then used volumetric calcium imaging to simultaneously visualize the activity in thousands of *Gsx1* neurons during a PPI paradigm. At the same time, we recorded tail movement responses to startle stimuli, with and without prepulse exposure. The method enabled us to locate a specific region that contains *Gsx1* neurons whose activity correlates strongly with behavioral PPI. To demonstrate that such neurons are causally related to PPI, we established an intersectional genetic method to ablate subgroups of *Gsx1* neurons by developing a library of Cre lines (Figure 1) and a UAS vector that requires both Gal4 and Cre expression in the same cell for reporter expression (UAS:KillSwitch). Ablation of *Gsx1* neurons in the PPI–active zone eliminated PPI. We next visualized the morphology and projections of these neurons in order to reveal how they connect to the

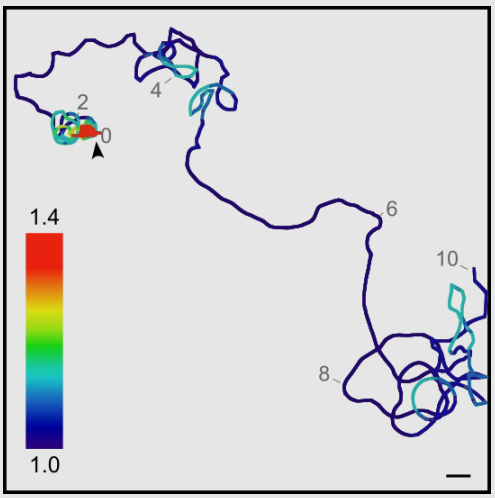

FIGURE 2. Local search behavior in larval zebrafish: swim trajectory of a single larva over a 10-minute period

The color scale indicates the fractal dimension within 30-second windows. Note that for the first two minutes, larvae swim in an area-restricted pattern, which then transitions to an outward-swim profile.

core startle circuit. For this, we developed a new method that exploits the low efficiency of B3 recombinase in zebrafish to achieve stochastic labeling of isolated neurons in cells that co-express Gal4 and Cre recombinase. *Gsx1* neurons that mediate PPI project to the lateral dendrite of the Mauthner cell, the command neuron for startle responses in fish, enabling them to gate auditory information. Given that PPI is abnormal in neuropsychiatric disorders with developmental origins, including schizophrenia and autism, our work will help identify and probe fundamental defects in circuitry that are abnormal in these conditions.

Neural mechanisms for behavioral state control

Over the course of the day, motivational goals change in response to both internal and external cues. At any given moment, an individual's current behavioral state strongly influences decisions on how to interact with the environment. A major question in neuroscience is to identify the neural systems that maintain short-term behavioral states and to determine how they interact with central mechanisms for behavioral choice (Reference 2). In zebrafish, loss of illumination triggers a short-term behavioral state in which larvae show heightened locomotor activity. We previously demonstrated that light-sensitive neurons in the hypothalamus trigger this state of hyperactivity. However, the experiments were performed on larvae confined to small shallow chambers, making it difficult to characterize the underlying behavioral state. By recording larval movement in large-volume arenas, we found that the response to loss of illumination is partly light-search behavior. For the first two minutes in the dark, larval swim patterns are similar to a behavior characterized as area-restricted search across species, with high fractal dimension and repeated turning behavior (Figure 2). Larvae quickly locate and swim toward a light-spot if activated during this period. If no light-spot is found, the locomotor profile transitions to a remote-search behavior, in which larvae efficiently locate illuminated regions that are not visible in the original environment. Using a G0 cas9/crispr screen, we found that the transition between these two behavioral states requires the activity of *otpa* neurons, including *opn4a*–expressing deep brain photoreceptors (Reference 3).

Tools for decoding neuronal circuits

A unique feature of brain imaging in zebrafish is the ability to visualize the total architecture of the brain while simultaneously recording the position and morphology of every constituent labeled neuron. Brain

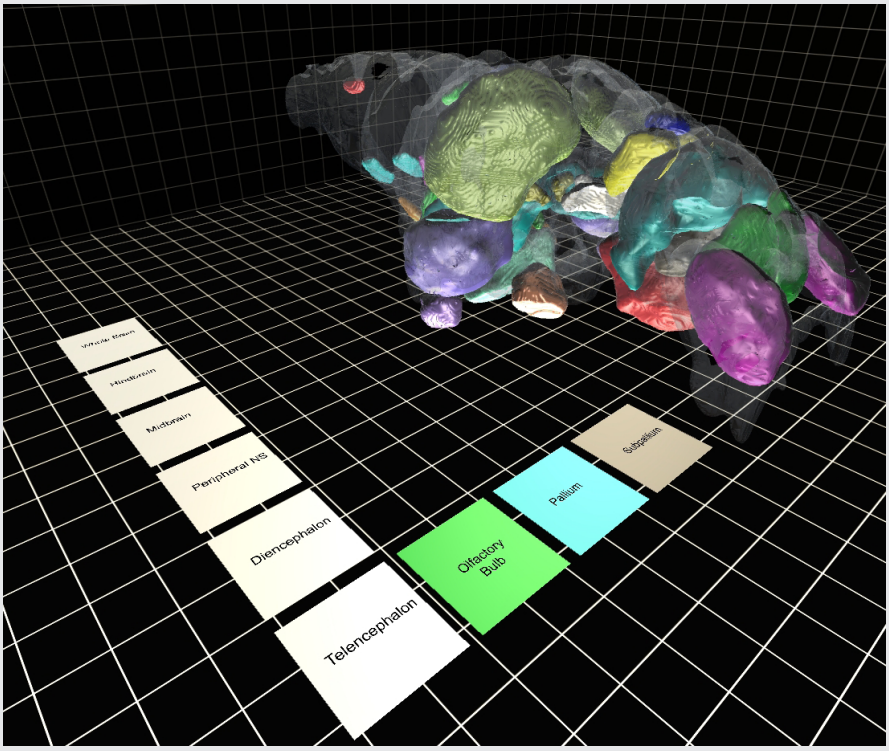

FIGURE 3. Virtual-reality zebrafish brain atlas

We developed a virtual-reality brain atlas for larval zebrafish. Using an inexpensive Cardboard viewer, different brain regions can be selected, enabling the neuro-anatomical structure of the larval brain to be visualized in three dimensions.

registration techniques enable data from several individuals to be quantitatively compared, so that experiments can systematically address the functional contributions of neurons across the entire brain. However, a challenge for deformable brain registration in larval zebrafish is to achieve highly precise global registration, without severely distorting the shape of individual cells. We calibrated parameters for the symmetric diffeomorphic normalization algorithm in ANTs (Advanced Normalization Tools) and found that it was possible to align larval zebrafish brains to a precision of around 1 cell diameter without sacrificing cell morphology. Using this method, we co-aligned more than 250 transgene expression patterns, derived from more than 1000 brain scans, to a single reference brain. Our Zebrafish Brain Browser (ZBB) software allows the expression in any of these lines to be compared at cellular-level resolution. Users can select a small region of the larval zebrafish brain and identity transgenic lines that express *Gal4* or *Cre* in the corresponding region. Together with new tools that allow selective visualization or ablation of neurons that express both *Gal4* and *Cre*, the ZBB atlas enables us to target highly specific neurons within the larval brain for visualizing activity or altering function, helping us and others decode their role within neuronal circuits (Reference 4). Moreover, multi-channel registration with ANTs allowed us to integrate our database of transgene expression patterns with Z-Brain, an independent zebrafish brain atlas that contains additional gene-expression patterns and neuro-anatomical annotations (Reference 5). To improve 3D visualization, we also developed tools for virtual reality projection of the zebrafish brain (Figure 3).

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Global Regulation of Gene Expression by ppGpp

The goal of our research continues to be to learn how bacteria use noncanonical nucleotides as regulatory signals, which they do in two ways. First, they sense sources of nutritional and physical stress that limit growth and then they either adjust host gene expression to counteract stress or become metabolically dormant until the stress has passed. The first example to be discovered was a cyclic nucleotide, 3'-5' cAMP, which senses glucose limitation. We have a very long-standing research interest in sensing and response functions of noncyclic analogs of GTP and GDP that contain pyrophosphate residues on their ribosyl 3' hydroxyl. These are pppGpp and ppGpp, respectively, abbreviated as (p)ppGpp (the extra phosphate termed the 5' gamma phosphate). The (p)ppGpp nucleotides are ubiquitous in bacteria and plant plastids, and their regulatory scope is broad. Changes in gene expression affect about one third of bacterial and chloroplast genomes. The (p)ppGpp nucleotides sense and respond to both physical, nutritional, and pathogenic sources of stress. In bacteria these regulatory systems are fundamentally relevant to pathogenesis because the mechanisms of host defenses and bacterial antibiotics that are designed to eliminate invading bacteria trigger counteractive (p)ppGpp responses. Evidence for the role of (p)ppGpp in pathogenicity is pervasive, both with respect to antibiotic resistance and the reduction in host carrier states. The current crisis occasioned by the lack of new antibiotics coupled with emergence of multidrug-resistant pathogens has led to widespread interest in (p)ppGpp. Our lab is, however, too small to develop new antibiotics. Instead, we continue to study how structural features of (p)ppGpp contribute to its function, to discover new basic regulatory responses to (p)ppGpp, such as the role found for ppGpp in coupled transcription DNA repair, and to explore comparative studies with (p)ppApp, a new analog. Our specific objectives are to learn what regulatory effects are altered by changing individual residues of pppGpp, to pursue our discovery that (p)pGpp is necessary and sufficient for initiation of bacterial DNA synthesis, and to determine whether (p)ppGpp alters transcription during lytic T4 bacteriophage infection.

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Regulatory effects of changing individual residues of (p)ppGpp

Most bacteria accumulate both ppGpp and pppGpp but the relative amounts differ in systematic ways. For example, Gram-negative

bacteria such as *E. coli* accumulate both pppGpp and ppGpp in roughly equal proportions during the first few minutes of amino acid starvation, but thereafter the level of ppGpp greatly exceeds that of pppGpp. When the same bacteria are starved for a glucose or other carbon sources, ppGpp levels greatly exceed those of pppGpp. For Gram-positive bacteria the opposite happens, i.e., more pppGpp accumulates than ppGpp. Regulation is different for this class and responds instead to GTP limitation caused by ppGpp. The question arises as to whether the 5' gamma phosphate alters regulatory activities, i.e., whether ppGpp regulates differently than does pppGpp. We have genetic constructs of *E. coli* cell lines that accumulate either ppGpp or pppGpp, but not both. In either case, accumulation can be varied artificially but not as a consequence of stress. These studies revealed that ppGpp is a more potent inhibitor of growth and of other regulatory events *in vivo*. We thus found ppGpp to be a more potent regulator *in vitro* for initiation of rRNA transcripts, which is how ppGpp regulates growth rate. RNA polymerase has two ppGpp–binding sites, and our structural studies revealed that both ppGpp and pppGpp bound to the same site (site 1) on RNA polymerase, a site that is distant from the catalytic center; however, binding constants were not obtained. Other labs found that ppGpp also binds at a second site near the catalytic center; however, pppGpp was not studied. So far, in *E. coli*, we conclude that ppGpp is a more potent regulator than pppGpp, i.e., the 5'-gamma phosphate matters.

Studies on pGpp allow us to test whether the 5'-beta phosphate in ppGpp also matters. We collaborated in the discovery that a (p)ppGpp synthetase (RelQ) from the Gram-positive *Enterococcus faecalis* can also use GMP as a substrate to make pGpp *in vitro*. Comparative studies of several *in vitro* reactions regulated by (p)ppGpp, including *E. coli* RNA polymerase, reveal that pGpp has relatively little regulatory activity. This allows us to come to a conclusion relating structure to function: altering ppGpp (the GDP derivative) by either adding a 5'-gamma phosphate (pppGpp, the GTP derivative) or by removing its 5'-beta phosphate (pGpp, the GMP derivative) compromises its regulatory potency.

COMPARISON OF PPPGPP WITH (P)PPAPP, A NEW NUCLEOTIDE REGULATOR

Initial studies that led to a focus on (p)ppApp have been presented in our earlier annual reports. Our interest in (p)ppApp arose because of the discovery by Katarzyna Potrykus, while a postdoctoral fellow in our lab, that a (p)ppGpp hydrolase called MESH, present in animals, could hydrolyze (p)ppApp as well as (p)ppGpp. Potrykus returned to Gdansk, and her laboratory discovered a strain with a MESH–like enzyme that not only hydrolyzes (p)ppApp but also lacks the archetypical (p)ppGpp hydrolase activity. The discovery suggests that (p)ppApp is biologically significant. The group also has evidence that the regulatory interactions of (p)ppApp and (p)ppGpp are distinct. Evidently, exchanging the G nuclease in ppGpp for A also makes a difference.

(p)ppGpp plays a necessary and sufficient role in initiation of DNA replication.

Our interest in regulatory events related to glucose starvation led to the discovery that (p)ppGpp plays an important role in regulating the initiation of bacterial DNA synthesis. We first found that increasing basal (p)ppGpp slows growth, which is correlated with an increase in the levels of acetyl phosphate (Ac~P). We also learned that Agnieska Szalewska (also a former postdoctoral fellow) made an important discovery, which provided a clue to our observations. She found that a mutant of a gene (*dnaA*46), necessary for DNA initiation, was reactivated by deleting genes necessary for Ac~P formation, genes that are also related to ppGpp. The DnaA protein works to start DNA replication by forming multimers attached to each other in a DNA sequence–dependent manner at the chromosomal start region, called ori. This first step in the process

requires ATP in order to phosphorylate a specific amino acid (lysine 178) in the ATP–binding site of the DnaA protein. A recent report convincingly showed that acetylation of the crucial K178 can occur, and when it does, it is associated with reversible inhibition of DnaA oligomerization at chromosomal origins. Increased Ac~P concentrations can acetylate lysine 178 either non-enzymatically or enzymatically. We initially thought that non-enzymatic acetylation linked Ac~P levels to ppGpp, but we now have genetic evidence that DnaA activity is regulated instead by enzymatic acylation and enzymatic deacylation. We can show that elevated ppGpp exerts its inhibitory effects on initiation even in mutants that cannot form Ac~P. This suggests that elevated (p)ppGpp is sufficient for inhibition of chromosomal DNA initiation. Conversely, cells completely deficient in ppGpp do not inhibit DNA synthesis initiation despite slow rates of growth, suggesting that (p)ppGpp is also necessary for DNA replication inhibition. However, the observation that (p)ppGpp is necessary and sufficient does not reveal details of the mechanism by which this occurs. We are currently pursuing this question.

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Fusion Mediated by HIV-1 Env and by HAP2 Gamete Protein Fusogens

Disparate membrane remodeling reactions are tightly controlled by protein machinery but are also dependent on the lipid composition of the membranes. Whereas each kind of protein has an individual personality, 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 the crossing of cell membranes by water-soluble drugs on their way to intracellular targets. We expect that the analysis of the molecular mechanisms of various membrane rearrangements will clarify the generality of emerging mechanistic insights. Better understanding of these mechanisms will bring about new ways to control 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 the fusion stages in the formation of multi-nucleated myotubes and osteoclasts, where even the identities of proteins involved remain to be clarified, in two recent studies we focused on the HIV-1–mediated fusion stage and on characterization of the gamete fusogen HAP2.

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The fusion stage of HIV-1 entry depends on virus-induced cell-surface exposure of phosphatidylserine.

Human Immunodeficiency virus 1 (HIV-1), the causative agent of AIDS, delivers its RNA into cells by fusing the viral envelope with the cell membrane. The fusion process is mediated by the viral envelope glycoprotein Env, a trimer of heterodimers consisting of gp120 and gp41 subunits. Fusion is initiated by gp120 interactions with the CD4 receptor and one of the two G protein–coupled receptors (GPCR) coreceptors, CCR5 and CXCR4 at the surface of target cells. Several studies, particularly those on resting primary CD4 T cells, have suggested that efficient Env–mediated fusion and infection also depends on intracellular signaling. Specifically, Ca^{2+} signaling is triggered by engagement of the coreceptors with gp120. However, the role of signaling in HIV-1 fusion and infection remains controversial and appears to be dependent on cell type and activation status.

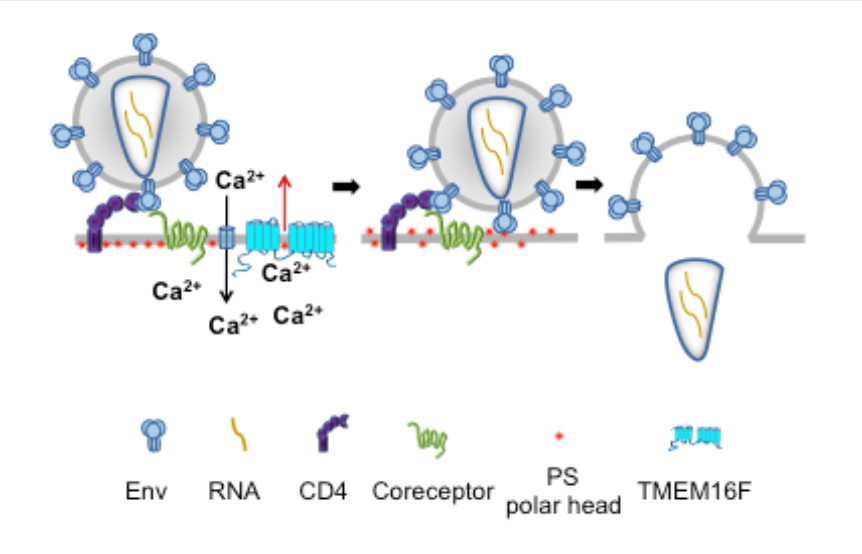

FIGURE 1. The fusion stage of HIV-1 entry depends on virus-induced cell-surface exposure of phosphatidylserine.

Our data (Reference 2) indicate that binding of HIV to a cell triggers phosphatidylserine (PS) exposure at the cell surface. This non-apoptotic PS exposure depends on gp120–coreceptor interactions, Ca^{2+} signaling, and TMEM16F scramblase. Suppression of PS exposure inhibits Env restructuring, viral fusion, and infection.

In collaboration with Leonid Margolis and Gregory Melikian, we recently reported that HIV-1 binding to its receptors induces non-apoptotic exposure of phosphatidylserine (PS) at the surface of the target cell and that externalized PS strongly promotes Env–mediated membrane fusion and HIV-1 infection (Figure 1 and Reference 2). Specific interactions between the gp120 subunit of Env and coreceptors triggered Ca^{2+} signaling–dependent transmembrane protein 16 (TMEM16F)–mediated PS externalization. Blocking externalized PS with PS–binding proteins or suppressing TMEM16F function inhibited Env–mediated fusion at a stage that follows the formation of pre-fusion Env–CD4–coreceptor complexes and precedes gp41 restructuring, which brings about hemifusion and fusion. Exogenous PS (exoPS) added to the outer leaflet of the plasma membrane (PM) promoted fusion, and the extent of this promotion increased for the target cells with lower levels of coreceptor expression and upon reduction of the number of fusion-competent Envs. We also found that both single-round infection with HIV-1 Env pseudoviruses and replicative infection with live virus, including HIV-1 infection of human lymphoid tissue *ex vivo,* depend on PS externalization in the target cells. The dependence is conserved between X4–tropic viruses and R5–tropic viruses, including the physiologically relevant, high CD4–requiring, non-macrophage-tropic R5 virus JR-CSF.

Our findings suggest that cell-surface PS acts as an important cofactor that promotes the fusogenic restructuring of pre-fusion Env–CD4–coreceptor complexes. A similar promotion of Env–mediated fusion by PS and another anionic lipid, phosphatidylglycerol, suggests the importance of electrostatic interactions rather than specific interactions with the polar head group of PS. We propose that PS at the surface of the target cell lowers the minimal number of coreceptor molecules that need to be engaged by each Env trimer to initiate gp41 refolding. An especially strong fusion promotion by exoPS for target cells with a relatively low density of accessible CCR5 (C-C chemokine receptor type 5) may reflect a stronger dependence of their fusion on gp120 trimer–coreceptor complexes with fewer than three coreceptors. However, we found that fusogenic restructuring of Env depends on cell-surface PS, even for target cells with exceptionally high levels of CCR5 expression, such as TZM-bl cells (HeLa–derived cells expressing CD4 and CCR5 [10^5 CCR5/cell] and often used in HIV research). The dependence of HIV-1 fusion on surface PS and, by extension, on

the signaling triggered by Env–coreceptor interactions was much stronger for the JC10 cells (HeLa–derived cells expressing CD4 and relatively low numbers of CCR5 [about 2×10^3 CCR5/cell], approaching those characteristic for resting peripheral blood lymphocytes [about 600 CCR5/cell]). This finding suggests that PS signaling is essential in physiologically relevant conditions. The uncovered link between HIV-1 infection and PS externalization identifies a bi-directional signaling pathway, in which the classic outside-in signaling through coreceptor triggers, via increase in intracellular Ca^{2+}, inside-out PS externalization mediated by TMEM16F. Given that disrupting the PS externalization pathway suppressed HIV-1 infection, this pathway may present a new target for anti-HIV-1 drugs.

Identification of HAP2 as a gamete protein fusogen

Although all forms of sexual reproduction depend on cell-cell fusion, the machinery mediating sperm-egg fusion has yet to be identified. While HAP2 proteins have been implicated as potential gamete fusogens in *Arabidopsis, Chlamydomonas, Tetrahymena, Dictyostelium*, and *Plasmodium*, it was unknown whether the proteins directly mediate gamete fusion, act as an accessory molecule that regulates the activity of an as-yet-unidentified "real" protein fusogen, or are involved in pre-fusion stages, such as signaling or tight adhesion. In our recent study (Reference 1), conducted in collaboration with Benjamin Podbilewicz and Pablo Aguilar, we showed that *Arabidopsis* HAP2 expression in mammalian cells is sufficient to promote cell-cell hemifusion and fusion. Intriguingly, structural modeling of the HAP2 protein family suggests a striking similarity between HAP2 and the *C. elegans* fusogens EFF-1 and AFF-1, characterized earlier by us and others, and class II viral fusogens such as dengue protein E and Semliki Forrest virus protein E1. Despite the structural similarity with viral fusogens that merge two membranes when expressed in only one of them, HAP2–mediated fusion (like EFF-1 and AFF-1 fusions) requires the presence of the protein in both fusing membranes. We found earlier that HAP2 expressed in only one of the membranes does not mediate even hemifusion, an early fusion stage that precedes the opening and expansion of a fusion pore. This bilateral requirement can be bypassed by replacing HAP2 with EFF-1 in one of the fusing cells, suggesting that trans HAP2–HAP2 interactions can be substituted by trans HAP2–EFF-1 interactions. Within a month after our paper appeared, two more independent studies, including X-ray crystallographic demonstration of striking similarity between *Chlamydomonas* HAP2, protein E of dengue virus and ZIKV, and EFF-1, further substantiated the conclusion that HAP2 is indeed the first gamete fusogen.

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- Office of Aids Research Award, 2017, 2018
- NICHD Director's Awards, 2016, 2017

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Building the Zebrafish Lateral Line System

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 the production of more hair cells during the growth and regeneration of neuromasts. The development of this superficial sensory system in zebrafish can be easily observed in live embryos carrying 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 loud sounds. However, unlike our ears, in which the loss of hair cells can be permanent, zebrafish 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 140 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.

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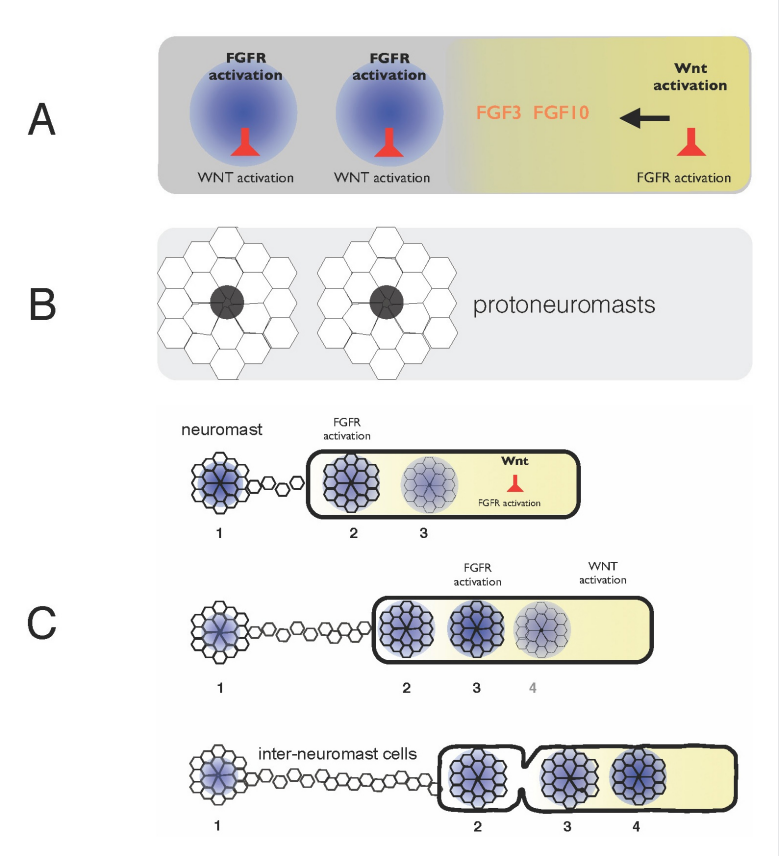

FIGURE 1. Self-organization of zebrafish lateral line primordium

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 ever 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.

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 (a signaling protein), FGF (fibroblast growth factor), Notch (a transmembrane receptor), BMP (bone morphogenetic protein), and chemokine signaling 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 collectively migrate under the skin from the ear to the tip of the tail. We combined an experimental approach with 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.

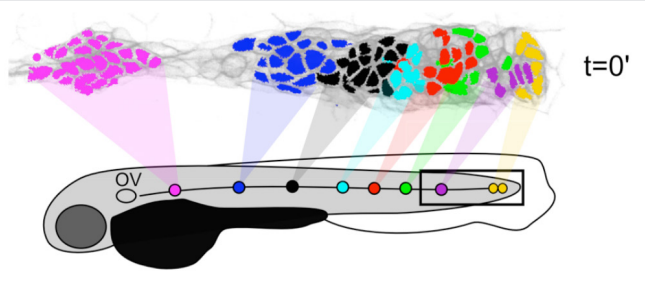

FIGURE 2. Neuromasts are formed by the local proliferation of cells along the length of the primordium.

Filled circles represent sequentially deposited neuromasts. Corresponding colors of cells in the PLLp at time t=0' show which populations underwent local expansion to give rise to each of these neuromasts.

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 the 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. 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. We used this framework to build a computational model that effectively predicts how far the pLL primordium will migrate, how many neuromasts it will deposit and the length, on average, of the spacing between deposited neuromasts. A comparison of data from both wild-type embryos and *lef1* mutant embryos, in which the proliferation is half that of wild-type embryos and migration speed progressively decreases, showed that the computational model uses a few simple parameters to make fairly accurate predictions about the pattern of neuromast deposition by the migrating primordium.

While the framework described above provides a broad description of the early self-organization of the PLLp system, many questions remain. In recent years, we have focused on the following questions: (1) the

precise lineage of cells in the migrating primordium and how cells in different locations contribute to the sequential formation of neuromasts and to specific fates within deposited neuromasts; (2) the mechanisms that determine the dynamics of Wnt-FGF signaling in the pLL primordium, including those that determine its initial polarization, progressive shrinking of the leading Wnt system, and sequential formation of trailing FGF signaling centers in its wake; (3) the signaling mechanisms that determine collective migration of the pLL primordium and how these signaling systems are linked to the mechanics of migration; (4) what the behavior of individual cells within the primordium reveals about the mechanisms of collective migration and cell-cell communication; (5) whether what has been learned from these studies will allow us to build computational models and a theoretical framework that helps reconstruct how specific interactions between cells and their environment result in the coordination of morphogenesis, cell fate, and migration of the PLLp and, importantly, whether failure of some of our models to recapitulate specific phenomena helps identify gaps in our understanding and define questions for future examination.

Characterizing cell migration, proliferation, lineage, and fate specification in the migrating PLLp at single-cell resolution

Many basic aspects of the biology of the PLLp, including proliferation patterns, lineage relationships, and the dynamics of neuromast deposition remain incompletely characterized. To unambiguously resolve such questions, we conducted time-lapse imaging of the PLLp, first at low resolution for some embryos, and then again for three independent embryos at a resolution that allowed us to track and catalog every single cell in the PLLp from early in migration until termination (approximately 24 hours). From this dataset, we constructed digital models of the PLLp. We could query these models to define the lineages of specific cells or subsets of cells, such as those that form specific neuromasts or that adopt specific fates within the neuromasts. Previous studies had suggested that the leading zone with Wnt activity maintains a proliferative population of progenitor cells that divide to produce a subset of daughter cells that move to a trailing zone, where, under the influence of FGF signaling, they differentiate to sequentially form neuromasts. However, our analysis revealed that proliferation in the PLLp is largely unpatterned and not especially high in the leading zone. Furthermore, protoneuromasts are not formed in the trailing zone from a self-renewing population of progenitors in a leading zone. Instead, they are formed by the local proliferation of cells along the length of the primordium in response to FGF signaling, as the Wnt signaling system, which locally inhibits protoneuromast formation, is progressively restricted to a smaller leading zone of the PLLp. The fate of deposited cells, within neuromasts, as central sensory hair cell progenitors, surrounding support cells, or as interneuromast cells between deposited neuromasts, is not determined by any obvious stereotyped lineages. Instead, their fate is determined, somewhat stochastically, as a function of a cell's distance from the center of a maturing protoneuromast. This observation is consistent with the notion that a cell's fate is determined in the PLLp by its level of exposure to active FGF signaling; the central cell in the protoneuromast, most likely specified earlier as a sensory hair cell progenitor by its exposure to the highest level of FGF signaling and via lateral inhibition mediated by Notch, becomes a source of FGF10. We suggest that proximity to this new central source of FGFs subsequently determines the level of FGF signaling and the eventual fate of surrounding cells in the maturing protoneuromast.

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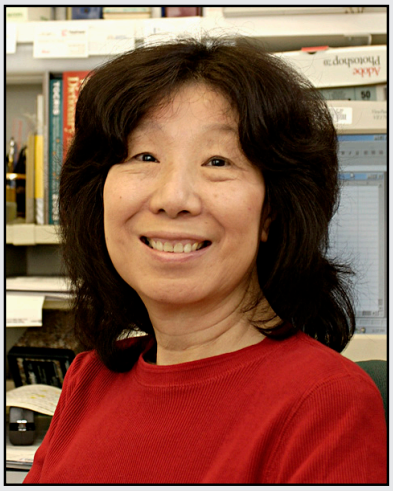
Molecular Genetics of Heritable Human Disorders

We conduct research to delineate the pathophysiology and develop novel therapies for the three subtypes of type I glycogen storage disease (GSD-I): GSD-Ia, GSD-Ib, and GSD-Irs (GSD-I related syndrome). GSD-Ia is caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC), GSD-Ib is by a deficiency in the glucose-6-phosphate (G6P) transporter (G6PT or SLC37A4), and GSD-Irs, also known as severe congenital neutropenia syndrome type 4, by a deficiency in G6Pase-β. G6Pase-α and G6Pase-β 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-α complex maintains interprandial glucose homeostasis while the G6PT/G6Pase-β complex maintains energy homeostasis and functionality of neutrophils. GSD-Ia and GSD-Ib patients manifest a common metabolic phenotype of impaired glucose homeostasis not shared by GSD-Irs. GSD-Ib and GSD-Irs patients manifest a common myeloid phenotype of neutropenia and myeloid dysfunction not shared by GSD-Ia. Inactivation of G6PT or G6Pase-β in neutrophils leads to enhanced apoptosis, which underlies neutropenia in GSD-Ib and GSD-Irs. A deficiency in either G6PT or G6Pase-β in neutrophils prevents recycling of glucose from the ER to the cytoplasm, leading to the impaired energy homeostasis that underlies neutrophil dysfunction in GSD-Ib and GSD-Irs. There is no cure for GSD-Ia, GSD-Ib, or GSD-Irs. Animal models of the three disorders are available and are being exploited to both delineate the disease more precisely and develop new treatment approaches, including gene therapy. We recently generated several efficacious G6Pase-α–expressing recombinant adeno-associated virus (rAAV) vectors and provided a proof-of-principle gene therapy in murine GSD-Ia that is safe, efficacious, and appropriate for entering clinical trials. Working with our commercial and clinical collaborators, we are expecting to initiate phase I/II clinical trials for human GSD-Ia in 2018/2019.



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Molecular mechanism preventing hepatocellular adenoma and carcinoma (HCA/HCC) in GSD-Ia mice receiving gene therapy

The predominant subtypes of HCA in GSD-Ia are inflammatory HCA (IHCA, 52%) and β-catenin–mutated HCA (bHCA, 28%). We previously

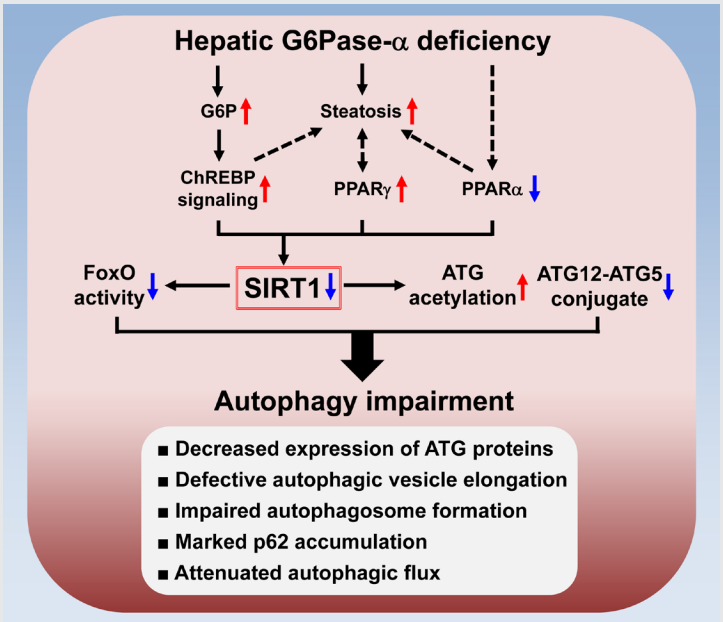

FIGURE 1. The mechanism underlying autophagy impairment in hepatic G6Pase-α deficiency

Hepatic G6Pase-α deficiency leads to metabolic alterations including G6P accumulation and suppressed expression of PPARα, a master regulator of fatty acid β-oxidation. The G6P–mediated activation of ChREBP signaling induces lipogenesis, leading to hepatic steatosis, which increases the expression of PPARγ, another lipogenic factor. Moreover, aberrant PPARγ overexpression aggravates hepatic steatosis. The net outcome is downregulation of hepatic SIRT1 signaling. Impaired SIRT1 signaling increases ATG acetylation and reduces ATG12–ATG5 conjugation along with downregulation of FoxO signaling, which induces autophagy genes. Accordingly, hepatic G6Pase-α deficiency–mediated autophagy impairment is characterized by reduced expression of ATG proteins, defective autophagic vesicle elongation, impaired autophagosome formation, marked accumulation of the p62 adaptor protein (implicated in selective autophagy), and attenuated autophagic flux.

showed that non–tumor bearing (NT), rAAV–treated GSD-Ia mice (AAV-NT mice) expressing a wide range (0.9–63%) of normal hepatic G6Pase-α activity maintain glucose homeostasis and display physiologic features mimicking animals living under calorie restriction. We showed that in AAV-NT mice, the signaling pathways of the calorie restriction mediators AMPK and SIRT1 were activated, leading to inhibition of the activity of STAT3 and NFκB, pro-inflammatory and cancer-promoting transcription factors. SIRT1 also inhibits cancer metastasis by increasing the expression of E-cadherin, a tumor suppressor, and reducing the expression of mesenchymal markers. Consistently, in AAV-NT mice, hepatic levels of active STAT3 and of the p65 subunit of nuclear factor κB (NFκB) were reduced, as were expression of mesenchymal markers, STAT3 targets, NFκB targets, and β-catenin targets. AAV-NT mice also expressed elevated levels of E-cadherin and fibroblast growth factor 21 (FGF21), targets of SIRT1, and β-klotho, which can act as a tumor suppressor. Importantly, treating AAV-NT mice with a SIRT1 inhibitor markedly reversed many of the observed anti-inflammatory/anti-tumorigenic signaling pathways. In summary, activation of hepatic AMPK/SIRT1 and FGF21/β-klotho signaling pathways combined with down-regulation of STAT3/NFκB–mediated inflammatory and tumorigenic signaling pathways can explain the absence of hepatic tumors in AAV-NT mice.

Hepatic G6Pase- activity is required to prevent HCA/HCC in GSD-Ia.

The hallmarks of GSD-Ia are impaired glucose homeostasis and long-term risk of hepatocellular adenoma and carcinoma (HCA/HCC). We previously developed a G6Pase-α–expressing rAAV vector, rAAV-G6PC, and showed that rAAV-G6PC–treated *G6pc*$^{-/-}$ mice expressing 3–63% of normal hepatic G6Pase-α activity (AAV mice) maintain glucose homeostasis and do not develop HCA/HCC. However, the threshold of hepatic G6Pase-α activity required to prevent tumor formation remained unknown. To increase the efficacy of

the gene transfer vector, we constructed rAAV-co-G6PC, a rAAV vector expressing a codon-optimized (co) G6Pase-α and showed that rAAV-co-G6PC was more efficacious than rAAV-G6PC in directing hepatic G6Pase-α expression. Over an 88-week study, we showed that both rAAV-G6PC– and rAAV-co-G6PC–treated *G6pc*$^{-/-}$ mice expressing 3–33% of normal hepatic G6Pase-α activity maintained glucose homeostasis, lacked HCA/HCC, and were protected against age-related obesity and insulin resistance. Of the eleven rAAV-G6PC/rAAV-co-G6PC–treated *G6pc*$^{-/-}$ mice harboring 0.9–2.4% of normal hepatic G6Pase-α activity (AAV-low mice), three expressing 0.9–1.3% of normal hepatic G6Pase-α activity developed HCA/HCC, while eight did not (AAV-low-NT). We also showed that the AAV-low-NT mice exhibited a phenotype indistinguishable from that of AAV mice expressing 3% or more of normal hepatic G6Pase-α activity. The results establish the threshold of hepatic G6Pase-α activity required to prevent HCA/HCC and show that GSD-Ia mice harboring less than 2% of normal hepatic G6Pase-α activity are at risk for tumor development.

Downregulation of SIRT1 signaling underlies hepatic autophagy impairment in GSD–Ia.

The most severe long-term complication in GSD-Ia is HCA/HCC of unknown etiology. The global *G6pc*$^{-/-}$ mice die early, well before HCA/HCC can develop, making studies on the mechanism of HCA/HCC in these mice difficult. We therefore generated liver-specific *G6pc* knock-out (L-*G6pc*$^{-/-}$) mice, which survive to adulthood and develop HCA. A recent report showed that G6Pase-α deficiency causes impairment in autophagy, a recycling process important for cellular metabolism. However, the underlying mechanism is unclear. We showed that liver-specific knockout of G6Pase-α led to downregulation of SIRT1 signaling, which activates autophagy via deacetylation of autophagy-related (ATG) proteins, and of the FoxO family of transcriptional factors, which trans-activate autophagy genes. Consistently, defective autophagy in G6Pase-α–deficient liver was characterized by attenuated expressions of autophagy components, increased acetylation of ATG5 and ATG7, decreased conjugation of ATG5 and ATG12, and reduced autophagic flux. We further showed that hepatic G6Pase-α deficiency resulted in activation of ChREBP, a lipogenic transcription factor, increased expression of PPAR-γ, a lipid regulator, and suppressed expression of PPAR-α, a master regulator of fatty acid β-oxidation, all contributing to hepatic steatosis and downregulation of SIRT1 expression. An adenovirus vector–mediated increase in hepatic SIRT1 expression corrected autophagy defects but failed to rectify metabolic abnormalities associated with G6Pase-α deficiency. Importantly, rAAV vector–mediated restoration of hepatic G6Pase-α expression corrected metabolic abnormalities, restored SIRT1-FoxO signaling, and normalized defective autophagy (see Figure). Taken together, the data show that hepatic G6Pase-α deficiency–mediated down-regulation of SIRT1 signaling underlies defective hepatic autophagy in GSD-Ia.

Liver–directed gene therapy for murine GSD–Ib

The *G6pt*$^{-/-}$ mice manifest both metabolic and myeloid dysfunction characteristic of human GSD-Ib. When left untreated, the *G6pt*$^{-/-}$ mice rarely survive weaning, reflecting the juvenile lethality seen in human GSD-Ib patients. Studies have shown that the choice of transgene promoter can impact targeting efficiency, tissue-specific expression, and the level of immune response or tolerance to the therapy. We therefore examined the safety and efficacy of liver-directed gene therapy in *G6pt*$^{-/-}$ mice using rAAV-GPE-G6PT and rAAV-miGT-G6PT, two G6PT–expressing rAAV8 vectors directed by the human *G6PC* and *G6PT* promoter/enhancer, respectively. Both vectors corrected hepatic G6PT deficiency in murine GSD-Ib but the *G6PC* promoter/enhancer was more efficacious. Over a 78-week study, we showed that *G6pt*$^{-/-}$ mice expressing 3–62% of normal hepatic G6PT activity exhibited a normalized liver phenotype. Two of the 12 mice expressing less than 6% of normal hepatic G6PT activity developed HCA. All treated mice were leaner and more sensitive to insulin than wild-

type mice. Mice expressing 3–22% of normal hepatic G6PT activity exhibited higher insulin sensitivity than mice expressing 44–62%. The levels of insulin sensitivity correlated with the magnitude of hepatic ChREBP signaling activation. In summary, we established the threshold of hepatic G6PT activity required to prevent tumor formation and showed that mice expressing 3–62% of normal hepatic G6PT activity maintained glucose homeostasis and were protected against age-related obesity and insulin resistance.

GSD-Ib neutrophils exhibit impaired cell adhesion and migration.

GSD-Ib, caused by a deficiency in G6PT, is characterized by impaired glucose homeostasis, myeloid dysfunction, and long-term risk of HCA. Neutrophils play an essential role in the defense against invading pathogens. The recruitment of neutrophils towards the inflammation sites in response to inflammatory stimuli is a tightly regulated process involving rolling, adhesion, and transmigration. We investigated the role of G6PT in neutrophil adhesion and migration using *in vivo* and *in vitro* models. We showed that *G6pt*$^{-/-}$ (GSD-Ib) mice manifested severe neutropenia in both blood and bone marrow, and that treating *G6pt*$^{-/-}$ mice with granulocyte colony–stimulating factor (G-CSF) corrected neutropenia. However, upon thioglycolate challenge, neutrophils from both untreated and G-CSF–treated *G6pt*$^{-/-}$ mice exhibited lowered ability to migrate to the peritoneal cavity. *In vitro* migration and cell adhesion of G6PT–deficient neutrophils were also significantly impaired. Defects in cell migration were not the result of enhanced apoptosis or altered fMLP receptor expression. Remarkably, the expression of the β2 integrins CD11a and CD11b, which are critical for cell adhesion, was greatly reduced in G6PT–deficient neutrophils. The study suggests that deficiencies in G6PT cause impairment in neutrophil migration and adhesion via aberrant expression of β2 integrins; our finding should facilitate the development of novel therapies for GSD-Ib.

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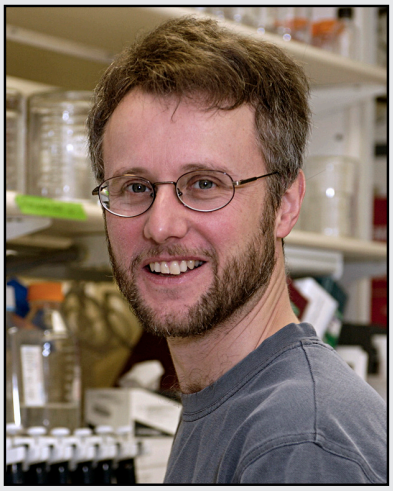
Chromatin Remodeling and Gene Activation

Our basic goal is to understand how chromatin structure influences gene regulation. Chromatin is generally repressive in nature but its structure is manipulated by cells in a regulated way to determine which genes are potentially transcriptionally active and which genes remain repressed in a given cell type. The regulation depends on interactions between DNA sequence–specific transcription factors, chromatin enzymes, and chromatin.

The structural subunit of chromatin is the nucleosome core, which contains about 147 bp of DNA wrapped approximately 1.7 times around a central histone octamer composed of two molecules each of the four core histones (H2A, H2B, H3, and H4). Generally, nucleosomes are regularly spaced along the DNA, like beads on a string. At physiological salt concentrations, the beads-on-a-string structure folds spontaneously to form a fiber of about 30 nm width, assisted by the linker histone (H1), which binds to the nucleosome core and to the linker DNA. Thus, collectively, the histones control DNA accessibility.

Gene activation involves the recruitment of a set of factors to a promoter in response to appropriate signals, ultimately resulting in the formation of an initiation complex by RNA polymerase II (Pol II) and transcription. These events occur in the presence of nucleosomes, which are compact structures capable of blocking transcription at every step. To circumvent and regulate the chromatin block, eukaryotic cells possess dedicated enzymes, including ATP–dependent chromatin-remodeling machines, histone-modifying complexes and histone chaperones. The remodeling machines use ATP to move nucleosomes along or off DNA (e.g., the SWI/SNF, RSC, CHD, and ISWI complexes), or to exchange histone variants between nucleosomes (e.g., the SWR complex). The histone-modifying complexes contain enzymes that modify the histones post-translationally to alter their DNA–binding properties and to mark them for recognition by other complexes, which have activating or repressive roles (the "histone code" hypothesis). Histone-modifying enzymes include histone acetylases (HATs), deacetylases (HDACs), methylases, and kinases. Histone chaperones mediate histone transfer reactions that occur during transcription and DNA replication (e.g., Asf1 and the CAF-1 complex). These enzymes,



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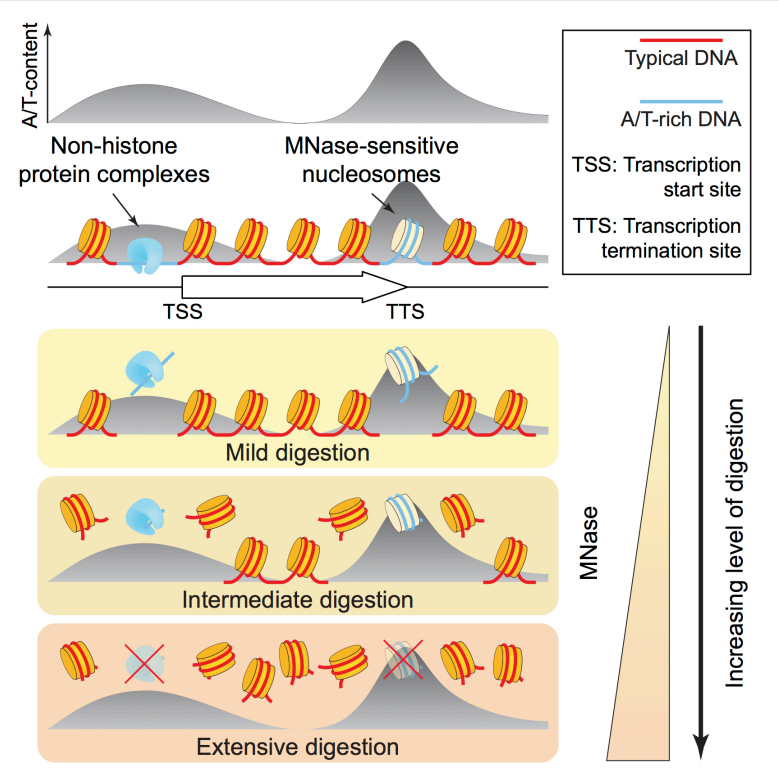

FIGURE 1. MNase–sensitive non-histone barrier complexes at promoters and MNase–sensitive nucleosomes containing AT–rich DNA at 3'-untranslated regions

Products of MNase digestion of chromatin: non-histone complexes (shown in blue) are located at promoters (at nucleosome-depleted regions, "NDRs") and act as barriers to nucleosome formation (Reference 1). MNase: micrococcal nuclease. TSS: transcription start site. TTS: transcription termination site (i.e., the poly-A addition site). Yellow discs are canonical nucleosomes.

together with DNA–methylating and de-methylating enzymes, are central to epigenetics.

Many human diseases have been linked to chromatin-remodeling enzymes and epigenetic modifications. For example, mutations in the hSNF5 subunit of the SWI/SNF complex are strongly linked to pediatric rhabdoid tumors. The CHD class of ATP–dependent remodelers has also been linked to cancer and to autism. Cancer therapies and drugs aimed at epigenetic targets are being tested. Recent studies have revealed a correlation between a linker histone variant and tumor heterogeneity. A full understanding of the functions of chromatin structure, enzymes, and modifications is therefore vital.

Non–histone barrier complexes occupy nucleosome–depleted promoters.

Our aim is to dissect chromatin-remodeling mechanisms *in vivo* and elucidate their contributions to gene regulation. Our current efforts are focused on elucidating the contributions of the various ATP–dependent chromatin-remodeling complexes to chromatin organization *in vivo*. Most of our work involves the use of budding yeast *Saccharomyces cerevisiae* as a model organism, but we are also involved in some mouse chromatin studies. During the past year, we made significant progress towards understanding the nature of promoter chromatin, more specifically, on the question of what occupies the nucleosome-depleted region that is characteristic of most yeast promoters and many mouse and human promoters. Our study (Reference 1) is summarized below.

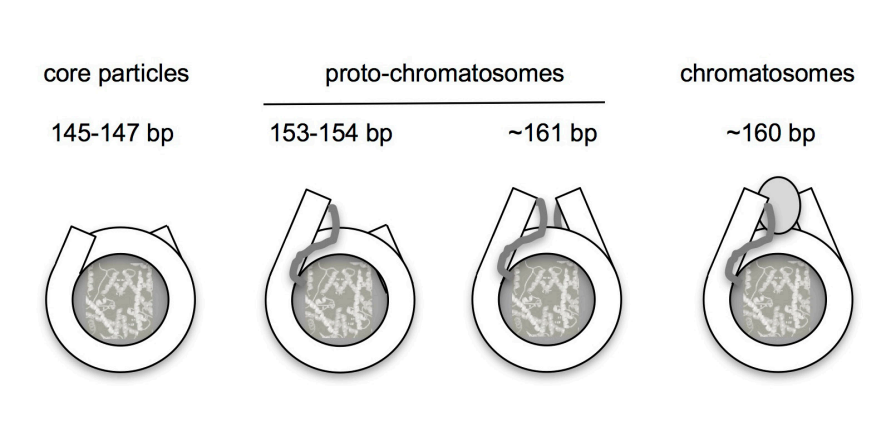

FIGURE 2. Structural relationship between nucleosome core particles, proto-chromatosomes, and chromatosomes

Views from above, drawn approximately to scale, based on the nucleosome 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. We propose that the fundamental particle is the 161-bp proto-chromatosome, which has both extensions. We suggest that each extension is protected by a specific but unidentified core histone–DNA contact, most likely a histone tail (as indicated), to form the H1–binding site. The chromatosome is formed when H1 binds (shown here as a light gray oval in a symmetrical location, although this is controversial). From Reference 3.

Most genes in yeast and in higher organisms have a characteristic chromatin organization, in which the promoter, just upstream of the transcription start site (TSS), is depleted of nucleosomes (the nucleosome-depleted region or NDR). The NDR is flanked by well positioned nucleosomes in phased arrays with a characteristic spacing. These observations are based primarily on experiments using micrococcal nuclease (MNase), which digests linker DNA rapidly but nucleosome core DNA much more slowly. A central question in our work is the nature of the NDR, whether it is protein-free or occupied by a large non-histone complex. An important factor is the DNA sequence itself: poly(dA) sequences are commonly found in promoters (though they are not universal) and exclude nucleosomes to some extent *in vitro*, an effect that is, however, relatively weak. Recently, several labs reported that many promoter NDRs are not actually nucleosome-free but occupied by easily digested, unstable "fragile nucleosomes." However, other labs reported high-resolution mapping by ChIP and tiling microarray that provide little evidence for histones at yeast promoters. Micrococcal nuclease (MNase)–sensitive nucleosomes have also been reported in higher eukaryotes.

We addressed the important issue of the nature of the NDR in yeast (Reference 1). We confirmed that an MNase–sensitive complex is present at yeast promoters. The critical question is whether histones are present at NDRs, as predicted if fragile nucleosomes are formed at NDRs. We used two different approaches to detect histones H4 and H2B: MNase-ChIP-seq, which involves immuno-precipitation (IP) of nucleosomes from MNase digests; and standard ChIP-seq using sonication, which does not depend on the use of MNase. Both sets of data show that NDRs are strongly depleted of histones. Although we found no evidence of fragile nucleosomes at promoters, we did detect MNase–sensitive nucleosomes elsewhere in the genome. However, they have high A/T content, suggesting that MNase sensitivity does not indicate structural instability but a preference of MNase for A/T–rich DNA—simply, that A/T–rich nucleosomes are digested faster than G/C–rich nucleosomes. We confirmed our conclusions by analyzing ChIP-exo, chemical mapping, and ATAC-seq data from other labs. By analogy with stable RNA polymerase III transcription complexes at tRNA genes, which are composed of TFIIIB and TFIIIC, we propose that RNA polymerase II promoters are

occupied by similar stable transcription complexes (Figure 1). Currently, we are endeavoring to identify the MNase–sensitive complexes that occupy promoter NDRs.

Chromatin organization in trypanosomes

The compaction of DNA in chromatin in eukaryotes allowed the expansion of genome size and coincided with significant evolutionary diversification. However, chromatin generally represses DNA function, and mechanisms co-evolved to regulate chromatin structure and its impact on DNA. This included the selection of specific nucleosome positions to modulate accessibility to the DNA. *Trypanosoma brucei*, a member of the Excavates supergroup, falls into an ancient evolutionary branch of eukaryotes and provides valuable insight into the organization of chromatin in early genomes.

In a collaboration with the Patterton and Rundenko labs, we mapped nucleosome positions in both the bloodstream and the procyclic forms of *T. brucei*, using MNase-seq (Reference 2). We found that chromatin organization in *T. brucei* is quite different from that of other eukaryotes, namely the RNA polymerase II initiation regions in *T. brucei* do not exhibit pronounced nucleosome depletion and show little evidence for defined –1 and +1 nucleosomes. In contrast, a relatively well positioned nucleosome is formed on the splice acceptor sites within the polycistronic transcription units. The RNA polyadenylation sites are depleted of nucleosomes, with a single well positioned nucleosome present immediately downstream of the predicted sites. The regions flanking the silent variant surface glycoprotein (VSG) gene cassettes show extensive nucleosomal arrays, which may repress cryptic transcription initiation. The silent VSG genes themselves exhibit a less regular nucleosomal pattern in both bloodstream and procyclic-form trypanosomes. DNA replication origins, when present within silent VSG gene cassettes, have a distinct nucleosomal organization when compared with replication origins in other chromosomal core regions.

The proto-chromatosome

As described above, eukaryotic DNA is packaged into regularly spaced nucleosomes, resembling beads on a string, with each bead containing about 147 bp wrapped around a core histone octamer. Linker histone (H1) binds to the linker DNA to drive chromatin folding. Micrococcal nuclease (MNase) digestion studies reveal two mono-nucleosomal intermediates: the core particle (about 147 bp) and the chromatosome (about 160 bp; a core particle with additional DNA protected by H1). We recently developed an improved method for mapping nucleosomes, using exonuclease III to remove residual linker (MNase-Exo-seq). We discovered two new intermediate particles corresponding to core particles with about 7 bp of linker protruding from one side (about 154 bp) or both sides (about 161 bp), which are formed in the absence of H1. In a review article (Reference 3), we proposed that these “proto-chromatosomes” are stabilized by core histone–DNA contacts in the linker, about 7 bp from the nucleosome boundaries (Figure 2). The contacts may determine the topography of the H1–binding site, facilitating the binding of linker histone and chromatin folding.

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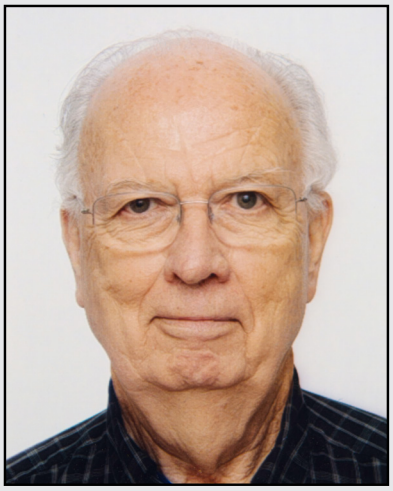
Physiological, Biochemical, and Molecular-Genetic Events Governing the Recognition and Resolution of RNA/DNA Hybrids

Damaged DNA is a 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. Two classes of RNases H, Class I and Class II, are present in most organisms.

Human patients with mutations in *RNASEH1* exhibit a typical mitochondrial muscular phenotype (Reference 1). Our studies were the first to show that RNase H1 is essential for maintenance of mitochondrial DNA. Mice deleted for the *Rnaseh1* gene arrest embryonic development at day 8.5 as a result of failure to amplify mitochondrial DNA (Reference 2). Aicardi-Goutières syndrome (AGS), a severe neurological disorder with symptoms appearing at or soon after birth, can be caused by defective human RNase H2 (Reference 3). We are examining mouse models of AGS to gain insight into the human disorder. To understand the mechanisms, functions, substrates, and basic molecular genetics of RNases H, we employ molecular-genetic and biochemical tools in yeast and mouse models.



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Contrasts between Class I and Class II RNases H

Many of our investigations over the past few years 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. In both eukaryotes and prokaryotes, RNases H1 consist of a single polypeptide. In contrast, RNase H2 is a complex of three distinct polypeptides in eukaryotes but a single polypeptide in prokaryotes. The catalytic subunit of the hetero-trimeric RNase H2 of eukaryotes is similar in its primary amino-acid sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave both RNA/DNA hybrids and a single ribonucleotide (Reference 4) or the transition from the ribonucleotide in the case of RNA–primed DNA synthesis (e.g., rrrr*r*DDD in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide).

Several types of RNA/DNA hybrid structures are formed, which are 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 (three-stranded nucleic acid structures) have two separated DNA strands, with one hybridized to RNA while the other is in a single-stranded form. These structures sometimes form during transcription and can lead to chromosomal breakage. However, they are also part of the normal 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 4). 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.

Dual activities of RNase H2; 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 for 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, one having significant loss of RNase H2 activity, *RNASEH2A-G37S* (G37S). Using the 3D structure of the human enzyme we had determined, we could locate all known mutations in RNase H2 that cause AGS. 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. A mutation near the catalytic center of G37S found in some AGS patients results in low RNase H2 activity for both embedded ribonucleotides in DNA and RNA/DNA hybrids (Reference 3). We are developing mouse models of AGS to clarify which defects are associated with each RNase H2 activity.

Mice bearing the G37S mutation in homozygous form are perinatal lethal, i.e., either dead at birth or die within a few hours of birth (Reference 3). 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. Viability of these mice is no different from that of the innate or adaptive competent mice. It is possible that there are additional defects in G37S mice that are directly related to viability, not innate immunity. 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 *cGAS* or *Sting* genes 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 (Reference 3). 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*, Hyongi Chon, a former postdoctoral fellow, rationally designed a modified RNase H2 to make an enzyme unable to cleave single ribonucleotides embedded in DNA but which retained RNA/DNA hydrolytic activity. The mutant enzyme, which we call RED (Ribonucleotide Excision Deficient) resolves RNA/DNA hybrids, which are substrates of both RNase H1 and RNase H2. Unlike the mouse and human RNases H2, RNase H2 activity is not required in the yeast *Saccharomyces cerevisiae*. Employing the ease of genetic mutation studies in in yeast, we demonstrated that yeast producing the RNase $H2^{RED}$ enzyme acted *in vivo* leaving embedded ribonucleotides (rNMPs) in DNA but was potent in removal of RNA in RNA/DNA hybrids.

Embryonic lethality of mice *Rnaseh2b*–KO stains has been attributed to accumulation of rNMPs in DNA, but lethality could be the result of loss of RNA/DNA hydrolysis or a combination of both rNMP and RNA/DNA hydrolysis defects. To distinguish among the possible causes of embryonic lethality, we generated a mouse that produces the RNase $H2^{RED}$ enzyme. Mouse embryo fibroblasts (MEFs) derived from *Rnaseh2*^{RED} mice have the same high level of rNMPs as seen in *Rnaseh2b–KO* MEFs. Interestingly, the *Rnaseh2*^{RED} mice die around the same time as the *Rnaseh2b-KO* mice. Therefore, lethality of the Knockout and RED RNase H2 mouse strains led to embryonic death. *Rnaseh2a*$^{G37S/RED}$ embryos also arrest at approximately the same as *Rnaseh2a*$^{RED/RED}$ embryos because of better association of RNase $H2^{RED}$ than RNase $H2^{G37S}$ to substrate with embedded rNMPs. The result is important because some RNase H2-AGS patients have similar compound heterozygous mutations in which there may be a dominant mutated enzyme.

Mitochondrial DNA, class switch recombination, and RNase H1

During embryonic development, RNase H1 is required for progress beyond day E8.5 (References 1,2). 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 *Rnaseh1*–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 carry out class switch recombination (CSR) 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 differ between the conditional KO and WT *Rnaseh1* genes.

CSR occurs when B cells are stimulated with cytokines for growth and isotype switching. R-loops have been known to be present at the sites to be recombined. Transcription at the two sites required for recombination forms R-loops. Activation-induced cytidine deaminase (AID) initiates breakage by deamination of cytidines followed by recombinational events. AID also creates somatic hypermutations adding to the repertoire of possible antibodies. We collaborated with Frederic Chedin using DNA RNA immuno-precipitation sequence (DRIP-seq) to detect R-loop formation in the absence of RNase H1. In resting cells there are few R-loops at the site for CSR. However, there is an increase after stimulation, indicating the start of CSR but, owing to the absence of mtDNA function, the process is not completed. Current evidence indicates that RNA of the R-loops is removed by the RNA exosome but not by RNase H. We found that expression of very high levels of the nuclear form of RNase H1 removes R-loops (Reference 5). Frequency of CSR is not increased but AID

gains access to the transcribed DNA strand, creating more somatic hypermutations (SHM)—apparently more efficiently than the RNA exosome. These findings emphasize the importance of transcription to form R-loops as the most important limitation on frequency of CSR. Stated another way; without transcription-generated R-loops AID can neither generate SHM nor CSR. The findings also support a model in which removal of RNA from the R-loops leaves the two DNA strands as single strands, possibly because of the formation of stable DNA structures such as G4 quadruplexes, and they are relevant to stable R-loops present at many promoter and 3'-UTR portions of genes. We are interested in determining how the R-loops are protected from normal levels of RNase H1 and whether this also occurs in CSR, where there appears to be insufficient RNase H1.

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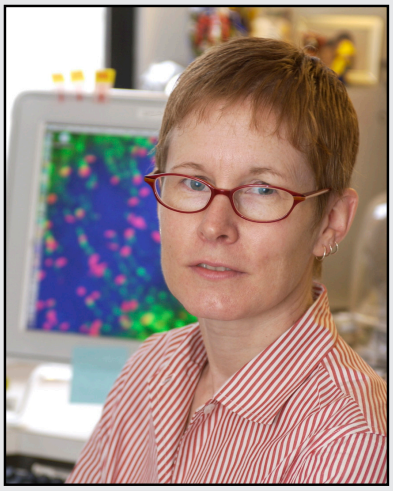
Mechanisms of Nuclear Genome Organization and Maintenance

We are interested in mechanisms of genome maintenance and organization. During interphase, chromosomes are surrounded by the nuclear envelope (NE), separating the nuclear and cytoplasmic compartments of the cell. Exchange of all molecules between the nucleus and the rest of the cell occurs through nuclear pore complexes (NPCs; Figure 1), which are embedded in the NE. NPCs are not only conduits for nucleo-cytoplasmic trafficking, they also promote many aspects of interphase nuclear function, including gene expression and heterochromatin organization. As mammalian cells divide, the NE breaks down and chromosomes condense to allow their partitioning into daughter cells. Remarkably, NPC proteins (nucleoporins) and other components of the nuclear transport machinery have distinct and important mitotic roles that assure the accurate segregation of chromosomes.

Our focus is to elucidate the role of nucleoporins, NPC–associated proteins (e.g., the SUMO pathway, spindle checkpoint proteins), and other components of the nuclear transport machinery (e.g., the Ran pathway) throughout the cell cycle. We are interested both in how they maintain nuclear organization during interphase and how they promote chromosome segregation. To address both aspects, we have adapted CRISPR–based degron strategies to the study of these proteins, that is, we tagged the proteins with auxin-induced degrons (AID), which cause them to be specifically destroyed upon the addition of auxin, a plant hormone. The ultimate goals of our studies are to understand how these pathways enable correct genome organization and accurate chromosome segregation, as well as to discover how their functions are coordinated with each other and with other aspects of cell physiology.

Targeting nucleo–cytoplasmic transport proteins for selective degradation

NPCs consist of multiple copies of roughly 30 proteins (nucleoporins), which form a series of stable sub-complexes. Many nucleoporins play additional roles during mitosis, and some sub-complexes localize to kinetochores, where they facilitate chromosome segregation. Understanding the activities of individual nucleoporins in each of these contexts has been complicated by their multi-faceted nature:



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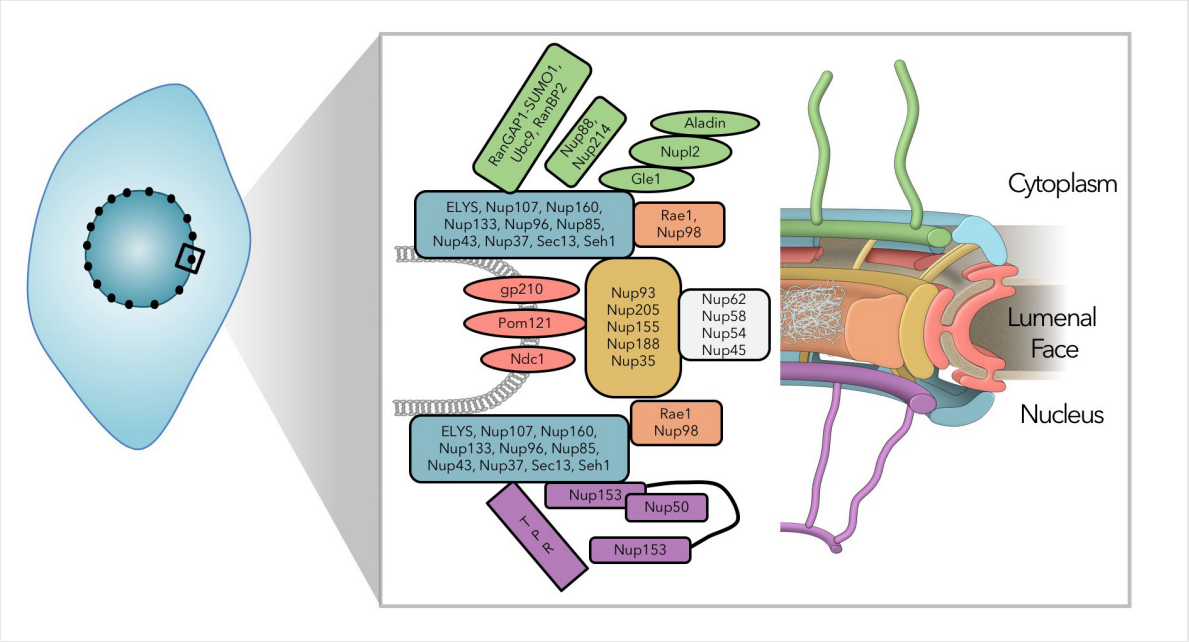


FIGURE 1. The nuclear pore complex is a large, multi-functional structure.

Schematic representation of the nuclear pore complex (NPC): NPCs are embedded in the nuclear envelopes of all eukaryotic cells, where they are not only conduits for nucleocytoplasmic trafficking, but they also promote many aspects of interphase nuclear function, including gene expression and heterochromatin organization. Around thirty proteins have been identified as nucleoporins through proteomic analysis of NPCs (*left*), whose corresponding distribution is indicated by color in the structural representation (*right*).

for example, RNAi–mediated depletion of nucleoporins in mammalian cells causes phenotypic defects, including altered gene expression and abnormal mitotic progression. However, the abundance and unusual stability of these proteins requires an extended interval for their depletion, so that many phenotypes could be indirect consequences of disrupted nuclear trafficking as the number and quality of NPCs is decreased.

To better address the role of individual nucleoporins, we adapted auxin-induced degron (AID) strategies for selective and rapid degradation of individual proteins (Figure 2). We are using CRISPR/Cas9 to construct cell lines in which sequences encoding AIDs are inserted into both alleles of targeted genes of human tissue-culture cells that stably express the Transport Inhibitor Response 1 (TIR1) protein. TIR1 acts as a subunit of the SCF ubiquitin ligase complex, so that the AID–tagged fusion proteins undergo rapid, selective degradation upon addition of the plant hormone auxin. In general, we also add a fluorescent tag to the targeted proteins, allowing the degradation to be monitored visually as well as biochemically.

We have been successful in developing lines that allow conditional depletions of nucleoporins associated with the cytoplasmic and nuclear faces of the NPC, as well as nucleoporins that reside within the central domain of the NPC. We are examining the function of these nucleoporins in interphase and mitosis, as discussed below. We are continuing our efforts to make lines for conditional depletion of all other nucleoporins as well. Importantly, we understand that these cell lines can be used to address the role of

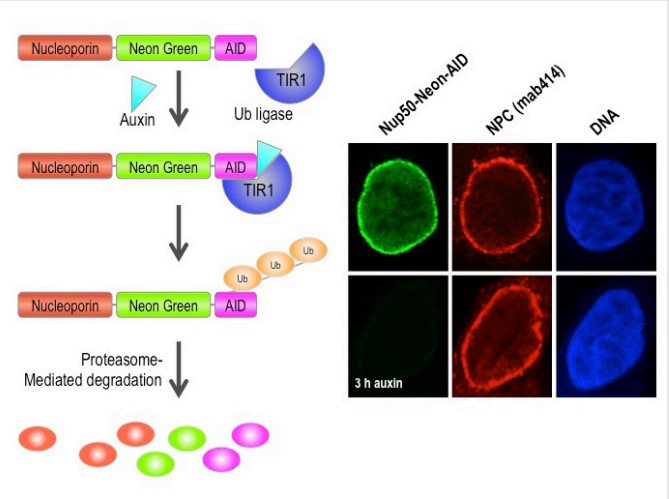


FIGURE 2. Auxin-induced degradation of AID–tagged nucleoporins

Cells expressing the TIR1 protein recognize proteins tagged with auxin-induced degron (AID) domains upon the addition of the plant hormone auxin. This leads to their rapid conjugation with ubiquitin (Ub) and destruction. We took advantage of this system by homozygously targeting endogenous nucleoporin genes with the AID tag and a fluorescent marker (*neon green*) in TIR1–expressing DLD1 cells. As shown on the right for the nucleoporin Nup50, we observe rapid and uniform degradation after auxin addition (*left panels*). Note that the nuclear pore is not generally disrupted, as indicated by staining with an antibody that recognizes a family of nucleoporins (mab414, *middle panels*).

NPCs in far more aspects of biology than we can realistically address—for example, the role of nucleoporins during development and during viral infection. We are therefore collaborating extensively with other groups who have developed approaches to address these important questions.

The role of nucleoporins in interphase nuclear organization and function

Besides nuclear-cytoplasmic transport, NPCs are implicated in the maintenance of nuclear architecture, the organization of interphase chromatin, mRNA export, and transcription regulation. The involvement of the NPC in these various processes offers a rich variety of possible mechanisms for biological regulation via nucleoporins and for coordination among cellular functions. However, these possibilities remain largely unexplored for technical reasons because the abundance and stability of nucleoporins makes them difficult to analyze using standard methods, such as RNAi. Moreover, extended depletion times are associated with the loss of non-targeted nucleoporins and with defects in NPC assembly, leading to further complications in interpretation of resultant phenotypes. The rapid degradation of nucleoporins using AID-tagged cell lines has proven able to circumvent a number of these issues, and we are currently using them to examine three major aspects of interphase nuclear structure and function.

First, we are examining the role of individual nucleoporins within the NPC structure, testing previously described requirements for individual nucleoporins in the stable assembly of others. We find that the results are distinct depending upon whether we examine the stability of exiting NPC structures or the post-mitotic re-assembly of NPCs.

Second, we are examining the role of individual nucleoporins' different nuclear trafficking pathways. We are currently assaying their contributions to nuclear protein import, protein export, and RNA export. We find distinct sensitivities of these pathways to the loss of individual nucleoporins, supporting the idea that the transport pathways have different NPC structural requirements.

Third, we are examining gene expression by RNA-seq with and without auxin, to observe the consequences

of acute and prolonged nucleoporin depletion. Our results suggest that different nucleoporins have distinct roles in gene expression.

Defining which individual nucleoporins contribute to each of these processes will allow better design of future experiments to examine the role of those selected proteins in the regulation particularly of cellular pathways.

Mitotic roles of nuclear pore complex proteins

We have a long-standing interest in the process of chromosome segregation. Defects in chromosome segregation lead to aneuploidy, a condition in which cells possess an abnormal number of chromosomes. Several common birth defects, such as Down's syndrome, result from aneuploidy arising during meiotic cell divisions, and aneuploidy arising from mitotic divisions is a hallmark of many types of solid tumors.

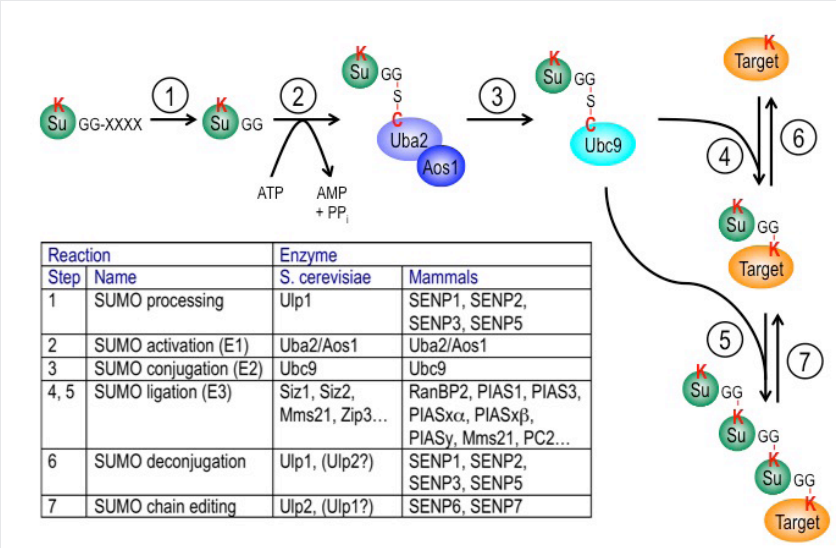
Kinetochores are proteinaceous structures that assemble at the centromere of each sister chromatid during mitosis and serve as sites of spindle microtubule attachment. The relationship between NPCs and mitotic kinetochores is surprisingly intimate but poorly understood. During interphase, many kinetochore proteins stably bind to NPCs (e.g., Mad1, Mad2, Mps1). After mitotic NPC disassembly in mammalian cells, many nucleoporins associate with mitotic spindles and kinetochores, where they play important roles in chromosome segregation.

As discussed above, the multifunctional nature of nucleoporins in controlling in interphase nuclear organization, and nuclear trafficking made it difficult to precisely study their mitotic role using previously available techniques, such as RNAi–based depletion strategies, without disrupting other cell functions. The construction of AID–tagged cell lines allows us to selectively degrade nucleoporins at or near the point of mitotic entry, so that we can circumvent the requirement for these proteins during interphase. We are currently analyzing the roles of individual nucleoporins and NPC–associated protein in kinetochore function, mitotic progression, and spindle assembly.

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. In cells, the conversion of Ran-GDP to Ran-GTP 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 correlated with post-translational modifications of RCC1, and the underlying molecular mechanism has not been reported.

FIGURE 3. The SUMO pathway



Reaction		Enzyme	
Step	Name	S. cerevisiae	Mammals
1	SUMO processing	Ulp1	SENP1, SENP2, SENP3, SENP5
2	SUMO activation (E1)	Uba2/Aos1	Uba2/Aos1
3	SUMO conjugation (E2)	Ubc9	Ubc9
4, 5	SUMO ligation (E3)	Siz1, Siz2, Mms21, Zip3…	RanBP2, PIAS1, PIAS3, PIASxα, PIASxβ, PIASy, Mms21, PC2…
6	SUMO deconjugation	Ulp1, (Ulp2?)	SENP1, SENP2, SENP3, SENP5
7	SUMO chain editing	Ulp2, (Ulp1?)	SENP6, SENP7

SUMO proteins are post-translationally processed (step 1). Processed SUMO polypeptides possess a C-terminal diglycine motif, which is activated to form an ATP–dependent thioester linkage with the SUMO E1 enzyme, the Aos1/Uba2 heterodimer (step 2). The activated SUMO is transferred to thioester linkage on a conserved cysteine of the SUMO E2 enzyme Ubc9 (step 3). Finally, the activated SUMO becomes covalently linked through an isopeptide bond to lysine residues within cellular target proteins, a reaction that is typically promoted by SUMO ligases (E3 enzymes) acting in conjunction with Ubc9 (step 4). For some substrates, additional SUMOs can be added to form SUMO chains that can act as a signal for proteolytic degradation (step 5). Both mono-SUMOylation (step 6) and poly-SUMOylation (step 7) can be reversed by a family of SUMO–specific proteases that are also major catalysts of post-translational SUMO processing, called Ulps (Ubiquitin-like protein proteases) in yeast and SENPs (Sentrin-specific protease) in vertebrates. The inserted table provides the names of proteins involved in each of these steps in budding yeast and human cells. Note that many of these enzymes (Ubc9, RanBP2, Ulp1, SENP1, SENP2) associate to the nuclear pore complex.

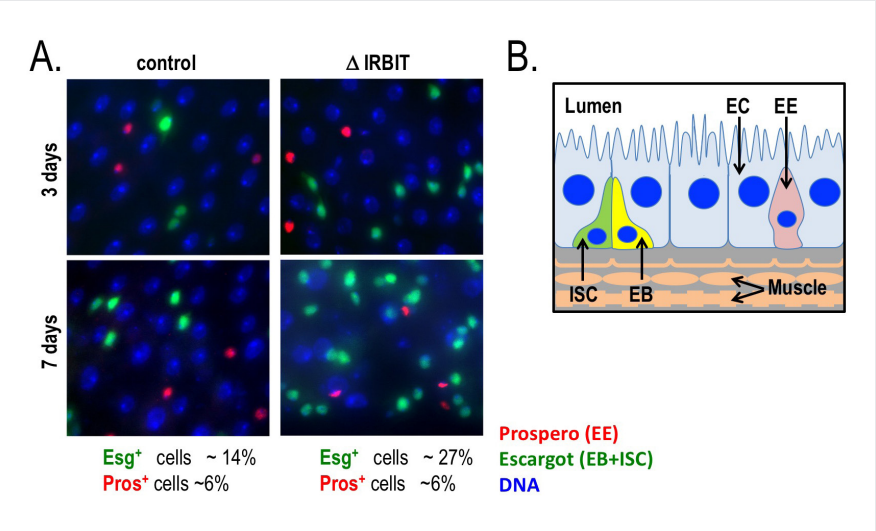
RanBP1 is a highly conserved Ran-GTP–binding protein, which acts as co-activator of RanGAP1 and can form a heterotrimeric complex with Ran and RCC1 *in vitro*. We found that RCC1 not associated with chromosomes during mitosis is sequestered and inhibited in RCC1/Ran/RanBP1 heterotrimeric complexes and 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. We are currently extending these findings to analyze whether RanBP1 plays an analogous role in mammalian cells during mitosis.

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 similar to ubiquitination (Figure 3). 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, 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

FIGURE 4. Loss of IRBIT disrupts tissue homeostasis in the *Drosophila* midgut.

A. Epithelia from control (left) and IRBIT$^{-/-}$ (*right*) flies at three (*top*) or seven (*bottom*) days after eclosure. The guts are stained with DNA dye Hoechst 33258 (*blue*), and antibodies against Prospero (EE cells) and Escargot (EB and ISC cells). We observe a rapid and progressive increase in the fraction of Escargot-positive cells in the IRBIT$^{-/-}$ flies over time. In conjunction with additional experiments, this accumulation is indicative of accumulation of undifferentiated enteroblast progenitor cells. B. Schematic of epithelium within the *Drosophila* midgut. EC: enterocyte; ISC: intestinal stem cell; EB: enteroblast; EE: enteroendocrine cell.



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, which 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, is encoded by an essential gene, and 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. We are currently analyzing AID–tagged alleles of both SENP1 and SENP2 to assess their roles in both interphase (nuclear trafficking and gene expression) and during mitosis (kinetochore function and mitotic progression), as well as their dependence upon individual nucleoporins for their targeting to the interphase NPC.

The role of the IRBIT protein in tissue homeostasis

We recently reported a conserved role for the IRBIT protein (IP3-receptor-binding protein released with inositol 1,4,5-trisphosphate) in inhibiting ribonucleotide reductase (RNR), an enzyme that produces

deoxynucleotide triphosphates (dNTPs) within the cell for DNA synthesis. We further found that mammalian tissue-culture cells show altered cell-cycle progression and potentially disrupted genome stability in the absence of IRBIT. In the same report, we showed that this mechanism is conserved between humans and flies (*Drosophila melanogaster*), so, in collaboration with Mihaela Serpe and Brian Oliver, we chose to use flies as a model organism to understand the role of this mechanism in development and tissue homeostasis.

In situ hybridization shows IRBIT expression in regions destined to become the midgut during embryogenesis, and IRBIT is expressed highly in the adult midgut. The *Drosophila* midgut has a tubular structure and is surrounded by visceral muscles. The adult midgut possesses a monolayered epithelium that is composed of four distinct cell types (Figure 4B): intestinal stem cells (ISCs), undifferentiated progenitor cells called enteroblasts (EBs), specialized absorptive enterocytes (ECs), and secretory enteroendocrine cells (EEs). The midgut is maintained through division of ISCs, giving rise to EBs, which in turn differentiate into EEs. Nutrients are absorbed from the lumen of the gut, which also contains a complex microbiota; the midgut acts both as a niche for commensal microbes and as the first line of defense for against microbial pathogens. Like the intestine of vertebrates, the epithelium of the midgut has a remarkable regenerative capacity, which has been extensively exploited for the study of stem cell–driven tissue self-renewal, as well as tissue homeostasis during aging.

We examined IRBIT's potential role in the midgut by generating an IRBIT null fly ($IRBIT^{-/-}$) (Figure 4A). The midguts of one-day-old wild-type and $IRBIT^{-/-}$ flies were essentially indistinguishable at the tissue-architecture level. However, we observed a rapid loss of tissue homeostasis in the $IRBIT^{-/-}$ flies, with a progressive increase in relative numbers of undifferentiated enteroblast progenitor cells and tissue dysplasia. $IRBIT^{-/-}$ flies also show fewer cell-cell contacts when stained for junctional proteins in the posterior midgut epithelium and altered gene expression patterns reminiscent of changes associate with inflammation and aging. The phenotypes are fully rescued through expression full-length IRBIT, and further experiments suggested that altered dNTP pools are likely to contribute to the $IRBIT^{-/-}$ phenotypes.

Together, our data suggest that IRBIT plays a key role in tissue homeostasis in the fly midgut by antagonizing RNR in nucleotide metabolism. We are currently examining the interplay of IRBIT with inflammatory pathways and microbiota changes that are characteristic features of aging in flies.

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- NICHD DIR Director's Investigator Award
- Developing Talent Scholar Award (Carlos Echeverria)

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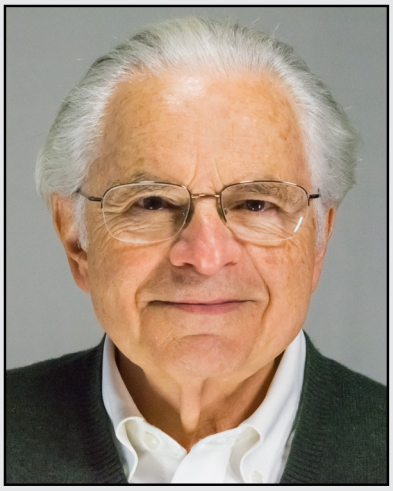
Molecular Genetics of Embryogenesis in Zebrafish and Xenopus

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 the mechanisms and role of protein stability in regulating early pancreas development and on the function of the BTB domain–containing protein Kctd15 in development. The BTB domain is a protein-protein interaction domain that is part of many proteins. Understanding its function in various contexts will help elucidate the mechanisms of protein interactions.



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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 was 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 the transcription factor AP-2. AP-2 is known to play a key role 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). The 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 to a specific site in its activation domain (Reference 1).

Recently, we tested for Kctd15 function by overexpressing this

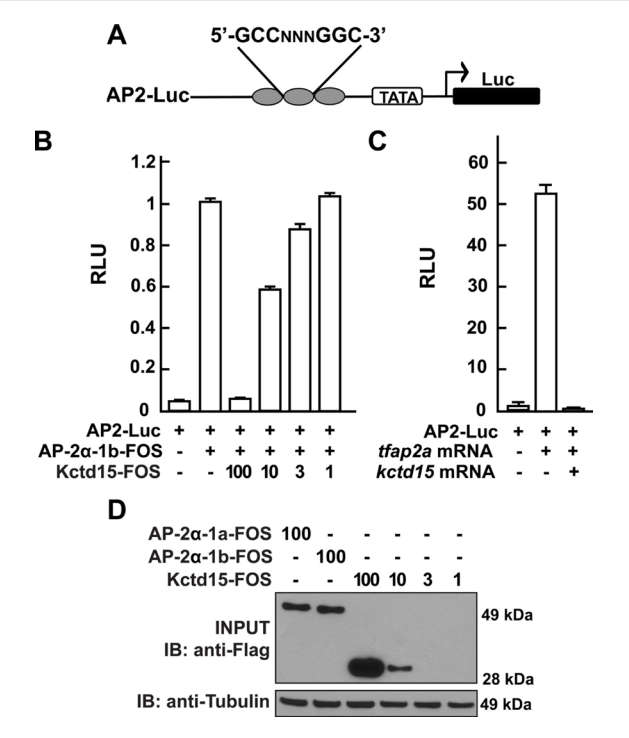

FIGURE 1. Kcdt15 represses AP-2 function.

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 Reference 1.

protein in animal explants (animal caps) from *Xenopus* embryos, followed by microarray analysis of the transcriptome of control and overexpressing explants. We confirmed that Kctd15 inhibits several markers of the neural crest, and we reported on additional genes affected by overexpression of this factor (Reference 5).

In addition, we generated frameshift mutations in both *kctd15* paralogs in zebrafish, using TALENs restriction enzymes for gene editing. Mutants in both *kctd15a* and *kctd15b* are viable and fertile but show slow growth and several additional phenotypes, which are currently under study.

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 play an important role in early pancreas differentiation in the zebrafish. While mammals have only 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 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*^{delta70}, that does not produce Lnx2a protein. The mutant fish proved to be phenotypically wild type (WT), with normal

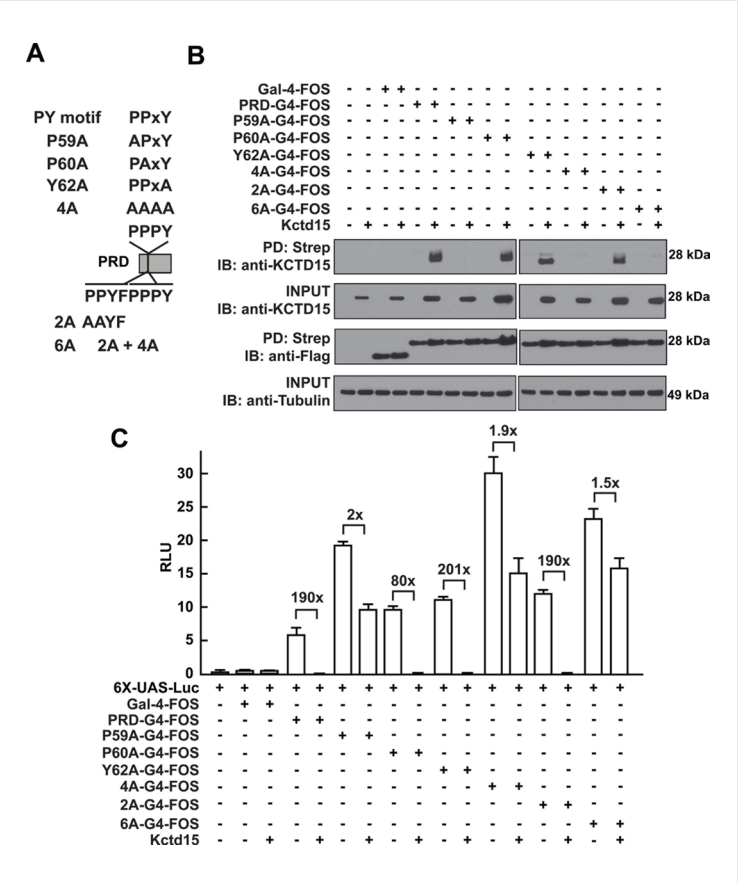

FIGURE 2. Critical role of P59 for Kctd15 interaction and inhibition of AP-2

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 proline 59 (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 (tyrosine 62 to alanine), and 2A were dramatically inhibited by zebrafish Kctd15, whereas P59A and mutants containing this change were inhibited two-fold or less. Figure from Reference 1.

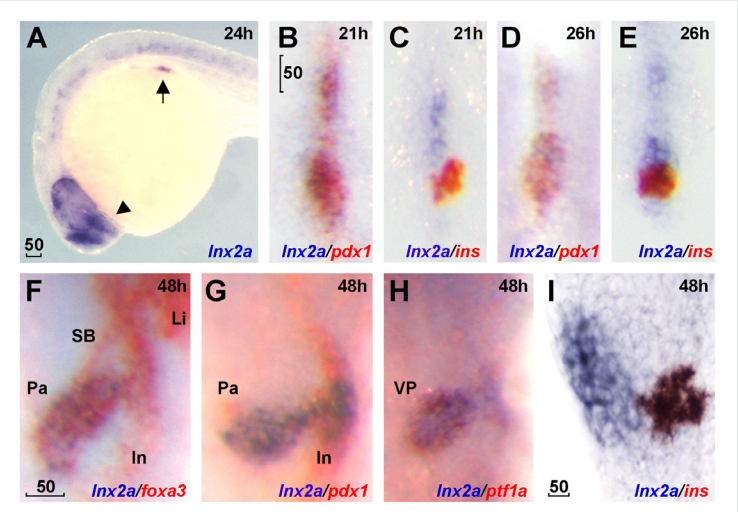

FIGURE 3. *lnx2a* is expressed in the ventral pancreas during early pancreas specification.

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 β-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.

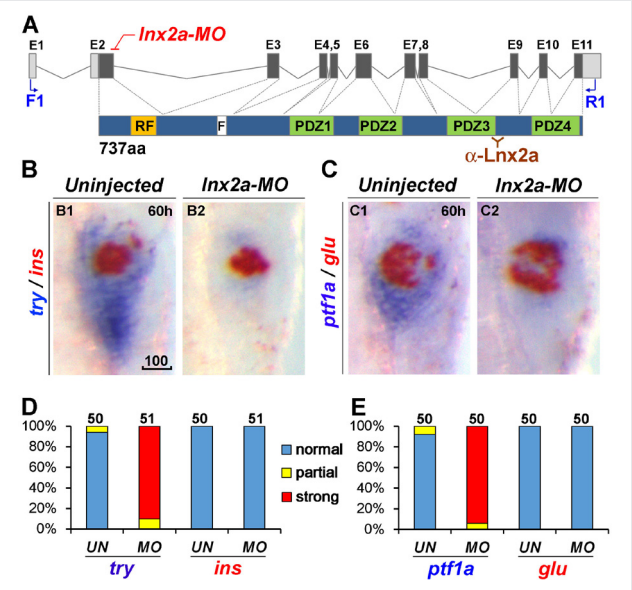

FIGURE 4. *lnx2a* knock-down causes defects in the exocrine pancreas.

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

D–E. Quantification of marker expression. UN, uninjected; MO, *lnx2a-MO*; scale bar, 100μm.

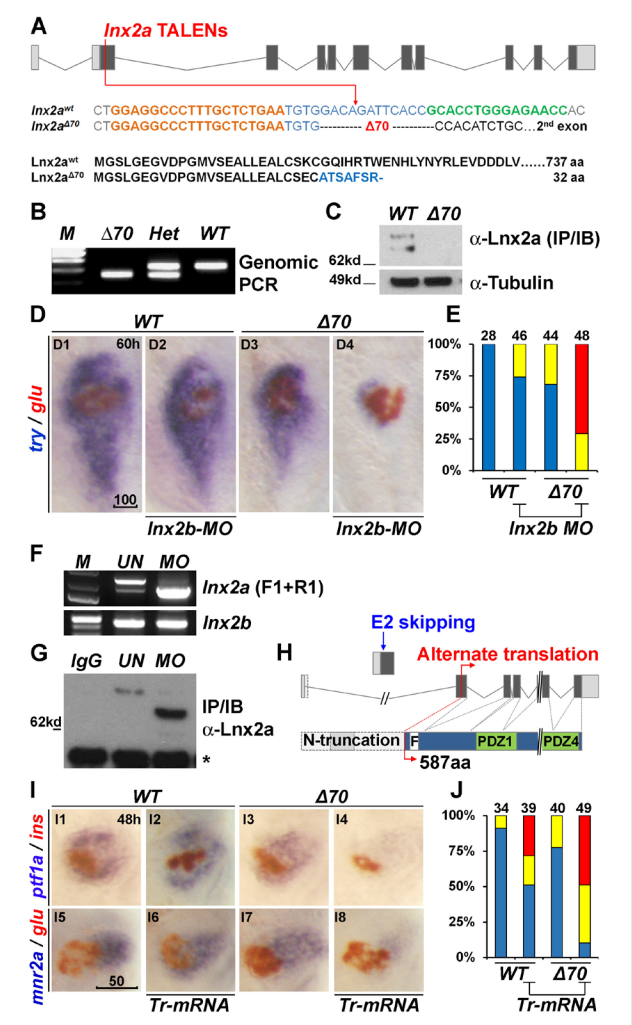

FIGURE 5. Generation of *lnx2a*$^{\Delta 70}$ mutant using TALENs, and the functional redundancy of *lnx2* genes

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*$^{\Delta 70}$ mutation are shown. Next, the protein sequences of wild-type and the *lnx2a*$^{\Delta 70}$ frameshift allele are shown. Genotype of the *lnx2a*$^{\Delta 70}$ mutant was analyzed by genomic PCR (B) and immunoblotting of Lnx2a protein (C).

D. Marker gene (*try* and *glu*) expression shows little effect in *lnx2a*$^{\Delta 70}$–null mutants or in *lnx2b-MO*–injected embryos. However, *lnx2b-MO* injection into *lnx2a*$^{\Delta 70}$–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 an interfering effect; *Tr-mRNA* was injected into the *WT* and *lnx2a*$^{\Delta 70}$–mutant embryos.

J. Quantification of pancreatic defects in (I). Scale bars: 100 mm (D1) and 50 mm (I5).

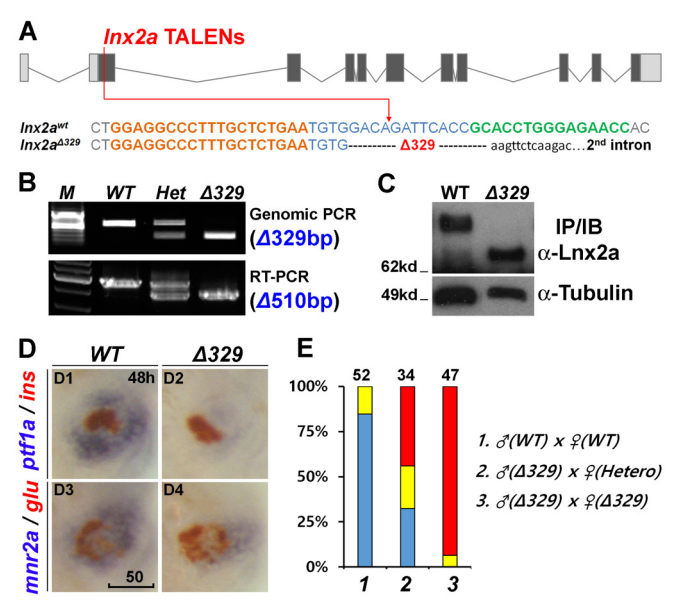

FIGURE 6. The *lnx2a*$^{\Delta 329}$ mutant recapitulates the morphant phenotype.

A. Sequence of the *lnx2a*$^{\Delta 329}$ mutation. The mutant has lost the exon 2 splice donor site.

B–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.

exocrine gene expression (Figure 5A–E). Further analysis showed that the splice MO led to exon skipping, generating an mRNA that produces a protein shorter than the WT (Figure 5F–H). Mass spectrometry showed the protein 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*^{delta70} embryos reproduced the exocrine deficiency phenotype (Figure 5I and 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*^{delta70} reproduced the exocrine deficiency phenotype (Figure 5D and E). Definitive genetic evidence for our interpretation of the mechanism of the *lnx2a*-splice MO phenotype came from a mutation, *lnx2a*^{delta329}, that deletes the exon/intron boundary targeted by the splice MO. *lnx2a*^{delta329}–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 in 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. We thus 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 thereby in 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*^{delta329}–mutant fish (Figure 7).

Previous work by several laboratories may be summarized in our context as follows: (1) Notch signaling is required in pancreatic cell differentiation; (2) high Notch activity maintains precursor pools, while 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

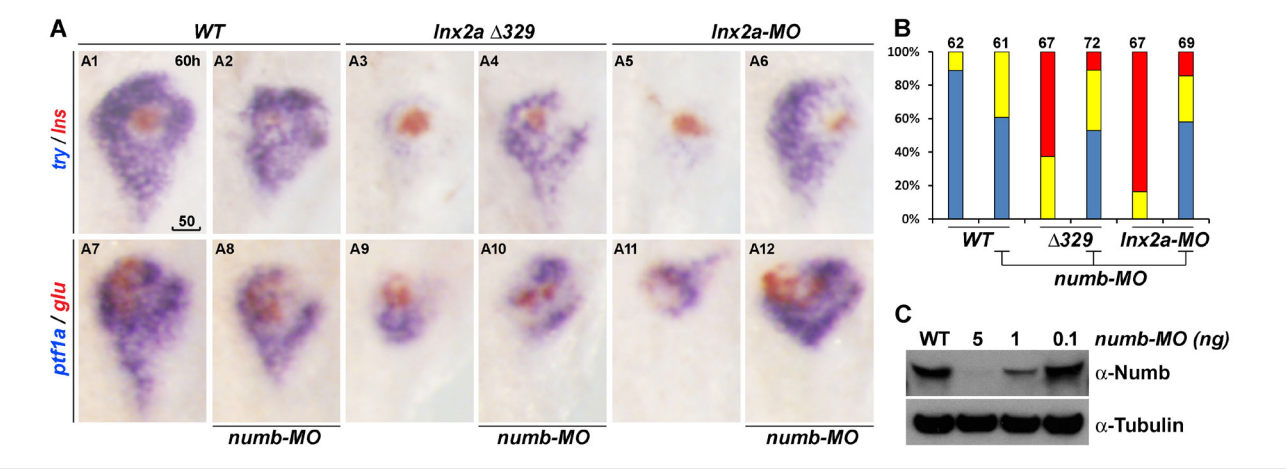

FIGURE 7. Ventral pancreas defects in *lnx2a*$^{\Delta 329}$–mutant and *lnx2a-MO*–injected embryos can be rescued by knock-down of Numb.

A. The translation-blocking MO for Numb was injected into WT, *lnx2a*$^{\Delta 329}$–mutant, 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.

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 for the specification and expansion of precursor cells. Loss of Lnx2 activity in the *lnx2a*$^{\text{delta329}}$ 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 4).

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Regulation of Mammalian Cell Proliferation and Differentiation

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 it 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, which 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 References 4,5,6.)

Our research program focuses on three questions: the nature of the mechanisms that restrict genome duplication to once per cell division; how these mechanisms are circumvented to allow developmentally programmed induction of polyploidy in terminally differentiated cells; and how we could manipulate these mechanisms to destroy cancer cells selectively.

Links between DNA replication, stem cells, and cancer

Cancers can be categorized into two groups: those whose frequency increases with age, and those resulting from errors during mammalian development. The first group is linked to DNA replication through the accumulation of genetic mutations that occur during proliferation of developmentally acquired stem cells that give rise to and maintain tissues and organs. These mutations, which result from DNA replication errors as well as environmental insults, fall into

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two categories: cancer-driver mutations that initiate carcinogenesis, and genome-destabilizing mutations that promote aneuploidy through excess genome duplication and chromatid mis-segregation. Increased genome instability results in accelerated clonal evolution, leading to the appearance of more aggressive clones with increased drug resistance. The second group of cancers, termed germ cell neoplasia, result from the mislocation of pluripotent stem cells during early development. During normal development, pluripotent stem cells that originate in early embryos give rise to all cell lineages in the embryo and adult, but when they mislocate to ectopic sites they produce tumors. Remarkably, pluripotent stem cells, like many cancer cells, depend on the geminin protein to prevent excess DNA replication from triggering DNA damage–dependent apoptosis. This link between the control of DNA replication during early development and germ cell neoplasia reveals geminin as a potential chemotherapeutic target in the eradication of cancer progenitor cells.

Genome duplication: the heartbeat of developing organisms

The mechanism that duplicates the nuclear genome during the trillions of cell divisions required to develop from zygote to adult is the same throughout the eukarya, but the mechanisms that determine where, when, and how much nuclear genome duplication occurs regulate development and differ among the eukarya. They allow organisms to change the rate of cell proliferation during development, to activate zygotic gene expression independently of DNA replication, and to restrict nuclear DNA replication to once per cell division. They allow specialized cells to exit their mitotic cell cycle and differentiate into polyploid cells, and in some cases, to amplify the number of copies of specific genes. It is genome duplication that drives evolution, by virtue of the errors that inevitably occur when the same process is repeated trillions of times. They are, unfortunately, the same errors that produce age-related genetic disorders such as cancer.

Genome duplication at the beginning of mammalian development

The basic mechanism for replicating DNA has been conserved throughout evolution, even though the magnitude of the problem became monumental. A human cell contains 670 times the DNA in an *E. coli* cell, and human development requires trillions of cell divisions that produce about 37 billion miles of DNA. But instead of increasing the speed of replication forks to compensate for increasing genome size and organism complexity, evolution simply increased the number of replication origins, which allowed mammals to regulate initiation of DNA replication during cell proliferation without interfering with the ever-changing demands of gene expression during cell differentiation. Moreover, it allowed developing tissues to complete genome duplication before beginning mitosis and to restrict genome duplication to once per cell division. However, to overproduce gene products during development, some cells are allowed to differentiate into nonproliferating polyploid cells. We are interested in the mechanisms that make these events possible. Ironically, aberrations in the mechanisms are linked to cancer. In fact, the pluripotent cells produced during preimplantation development not only share characteristics of cancer cells, but they can also initiate cancer.

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

Unique to multicellular animals, geminin is a dual-function protein 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 embryonic stem cells as they undergo self-renewal and differentiation. Embryonic stem 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 preventing DNA re-replication–induced DNA damage. We detected no relationship between the 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.

Geminin is essential for pluripotent cell viability during teratoma formation, but not for differentiated cell viability during teratoma expansion.

Pluripotent embryonic stem cells (ESCs) are unusual in that geminin has been reported to be essential either to prevent differentiation by maintaining expression of pluripotency genes or to prevent DNA re-replication–dependent apoptosis. To distinguish between these two incompatible hypotheses, immune-compromised mice were inoculated subcutaneously with ESCs harboring conditional *Gmnn* alleles alone or together with a tamoxifen-dependent Cre recombinase gene. Mice were then injected with tamoxifen at various times during which the ESCs proliferated and differentiated into a teratoma. For comparison, the same ESCs were cultured *in vitro* in the presence of monohydroxytamoxifen. The results revealed that geminin is encoded by a haplosufficient gene that is essential for ESC viability before the cells differentiate into a teratoma, but once a teratoma is established, the differentiated cells can continue to proliferate in the absence of *Gmnn* alleles, geminin protein, or pluripotent stem cells. Thus, differentiated cells did not require geminin for efficient proliferation within the context of a solid tissue, although they did when teratoma cells were cultured *in vitro*. The results provide proof-of-principle that preventing geminin function could prevent malignancy in tumors derived from pluripotent cells by selectively eliminating the progenitor cells with little harm to normal cells.

Identification of genes that are essential to restrict genome duplication to once per cell division

Nuclear genome duplication is normally restricted to once per cell division, but aberrant events that allow excess DNA replication (EDR) promote genomic instability and aneuploidy, both of which are characteristics of cancer development. We provided the first comprehensive identification of genes that are essential to restrict genome duplication to once per cell division. An siRNA library of 21,584 human genes was screened for those that prevent EDR in cancer cells with undetectable chromosomal instability. We validated candidates by testing multiple siRNAs and chemical inhibitors on both $TP53^{+}$ (also known as p53) and $TP53^{-}$ cells to reveal the relevance of this ubiquitous tumor suppressor to preventing EDR, and in the presence of an apoptosis inhibitor to reveal the full extent of EDR. The results revealed 42 genes that prevented either DNA re-replication or unscheduled endoreplication. They all participate in one or more of eight cell-cycle events. Seventeen of them had not been identified previously in this capacity. Remarkably, 14 of the 42 genes have been shown to prevent aneuploidy in mice. Moreover, suppressing a gene that prevents EDR increased the ability of the chemotherapeutic drug Paclitaxel to induce EDR, suggesting new opportunities for synthetic lethalities in the treatment of human cancers.

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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 in yeast and human cells to dissect the structure-function properties of translation factors, elucidate mechanisms that control protein synthesis, and characterize how mutations in the protein synthesis apparatus cause human disease. Of special interest are 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. We are characterizing eIF2gamma mutations that are associated with the MEHMO syndrome, a novel X-linked intellectual disability syndrome, and we are investigating the function of the translation factor eIF5A with a focus on its ability to stimulate the peptidyl transferase activity of the ribosome and facilitate the reactivity of poor substrates such as proline. We are also examining the role of the hypusine modification on eIF5A and the role of this factor in gene-specific translational control mechanisms.

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Analysis of eIF2gamma mutations that link intellectual disability with impaired translation initiation

Protein synthesis plays a critical role in learning and memory in model systems, and our studies have linked a human X-linked intellectual disability (XLID) syndrome to altered function of eIF2. In previous studies conducted in collaboration with Lina Basel-Vanagaite and Guntram Borck, we described a human XLID syndrome characterized by intellectual disability and microcephaly. The patients carry a mutation in the *EIF2S3* gene, which encodes eIF2gamma, and genetic and biochemical studies revealed that the mutation disrupts eIF2 complex integrity and translation start-codon selection. Over the past year, working in collaboration with Vera Kalscheuer, Daniela Gasperikova, and Clesson Turner, we characterized two additional mutations in eIF2gamma found

in patients exhibiting intellectual (mental) disability, epilepsy, hypogonadism and hypogenitalism, microcephaly, and obesity. Based on this constellation of phenotypes, the disease has been termed the MEHMO syndrome, and we now conclude that the syndrome is caused by mutations in *EIF2S3*. Our studies on a yeast model of these newly described MEHMO syndrome mutations in eIF2gamma reveal impaired eIF2 function, altered translational control of specific mRNAs, and reduced stringency of translation start-site selection (Reference 1). Consistent with these properties, the Integrated Stress Response, a translational regulatory response typically associated with eIF2alpha phosphorylation, is induced in patient cells. The findings directly link intellectual disability with impaired translation initiation and provide a mechanistic basis for the MEHMO syndrome resulting from partial loss of eIF2 function (Reference 1). Based on our studies, we propose that more severe *EIF2S3* mutations cause the full MEHMO phenotype, while less deleterious mutations cause a milder form of the syndrome with only a subset of symptoms.

Molecular analysis of eIF2alpha phosphorylation, dephosphorylation, and viral regulation

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 (endoplasmic reticulum) 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 (GCN4 is a transcription factor) 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 and elucidated an ordered mechanism of PKR activation by which catalytic domain dimerization triggers auto-phosphorylation, which in turn is required for specific eIF2alpha substrate recognition.

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. We showed that a novel N-terminal extension on yeast eIF2gamma binds to yeast PP1 (GLC7) and targets GLC7 to dephosphorylate eIF2alpha. Moreover, we reconstituted human GADD34 function in yeast cells and mapped a novel eIF2alpha–binding motif to the C-terminus of GADD34 (Reference 2). Interestingly, the eIF2alpha–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 eIF2alpha dephosphorylation. Taken together, our data demonstrate that GADD34 and its viral orthologs direct specific dephosphorylation of eIF2alpha by interacting with both PP1 and eIF2alpha through independent binding motifs (Reference 2).

The eIF2alpha kinase PKR is part of the cellular antiviral defense mechanism. When expressed in yeast, human PKR phosphorylates eIF2alpha, resulting in inhibition of protein synthesis and yeast cell growth.

To subvert the antiviral defense mediated by PKR, viruses produce inhibitors of the kinase. We are studying the inhibition of PKR by the poxviral double-stranded RNA–binding protein E3L, and we are currently characterizing mutations in PKR that confer resistance to E3L inhibition. In a related project, we characterized the insect baculovirus PK2 protein, an eIF2alpha kinase inhibitor that structurally mimics the C-terminal lobe of a protein kinase domain. Together with collaborators Frank Sicheri and Susuma Katsuma, we revealed that PK2 targets an insect HRI–like kinase through an unusual lobe-swapping mechanism to generate a nonfunctional pseudo-kinase complex.

Molecular analysis of the hypusine–containing protein eIF5A

The translation factor eIF5A, the sole protein containing the unusual amino acid hypusine [N^{e}-(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 depends on the hypusine modification. Given that eIF5A is a structural homolog of the bacterial protein EF-P, we proposed that eIF5A/EF-P is a universally conserved translation elongation factor.

Using *in vivo* reporter assays, we showed that eIF5A in yeast, like bacterial EF-P, stimulates the synthesis of proteins containing runs of three or more consecutive proline residues. Consistent with these *in vivo* findings, we showed that eIF5A was critical for the synthesis of polyproline peptides in reconstituted yeast *in vitro* translation assays, and, using directed hydroxyl radical probing experiments, we mapped eIF5A binding to near the E site of the ribosome. Thus, we proposed that eIF5A, like its bacterial ortholog EF-P, stimulates the peptidyl-transferase activity of the ribosome and facilitates the reactivity of poor substrates such as proline.

Over the past year, in collaboration with Rachel Green, we reported that eIF5A functions globally to promote both translation elongation and termination. Moreover, exploiting our *in vitro* reconstituted assay system, we used mis-acylated tRNAs to show that the imino acid proline but not tRNA(Pro) imposes the requirement for eIF5A. In addition, we found that the more flexible proline analog azetidine-2-carboxylic acid relaxes the eIF5A requirement for peptide synthesis. We also found that eIF5A could functionally substitute for polyamines to stimulate general protein synthesis (Reference 3).

Over the last year, and working with the X-ray crystallographer Marat Yusupov, we obtained a 3.25 Å–resolution crystal structure of eIF5A bound to the yeast 80S ribosome (Reference 4). The structure reveals interactions between eIF5A and conserved ribosomal proteins and rRNA bases. Moreover, eIF5A occupies the E site of the ribosome, with the hypusine residue projecting toward the acceptor stem of the P-site tRNA. Our studies reveal a previously unseen conformation of an eIF5A–ribosome complex, suggest a function for eIF5A and its hypusine residue in repositioning the peptidyl-tRNA in the P site to alleviate stalling, and highlight a possible functional link between conformational changes of the ribosome during protein synthesis and eIF5A–ribosome association (Reference 4). In related studies, we reported the structure of a diproline–tRNA analog bound to the ribosome, revealing that proline affects nascent peptide positioning in the ribosome exit tunnel.

Taken together, our studies support a model in which eIF5A and its hypusine residue function to reposition the acceptor arm of polyprolyl–tRNA in the P site to alleviate stalling and that the body of eIF5A functions like polyamines to enhance general protein synthesis. In ongoing studies, we have linked eIF5A to the regulation

of polyamine metabolism in mammalian cells. Synthesis of the antizyme inhibitor AZIN1, a positive regulator of polyamine synthesis, is inhibited by polyamines. We found that translational control of AZIN1 synthesis relies on polyamine inhibition of eIF5A function. Thus, eIF5A functions generally in protein synthesis, and modulation of eIF5A function by polyamines can be exploited to regulate specific mRNA translation.

Analysis of the role of eEF2 and its diphthamide modification in translation elongation and CrPV IRES translation

We are also studying the translation elongation factor eEF2. Like its bacterial ortholog EF-G, eEF2 promotes translocation of tRNAs and mRNA from the A site to the P site on the ribosome following peptide bond formation. In all eukaryotes and archaea, a conserved histidine residue at the tip of eEF2 is post-translationally modified to diphthamide through the action of seven non-essential proteins. The function of diphthamide and rationale for its evolutionary conservation are not well understood, and to date the only known function of diphthamide is to serve as a substrate for inactivation by diphtheria toxin. To gain insights into the role of eEF2 and diphthamide, we examined peptide synthesis in a reconstituted yeast *in vitro* translation system using unmodified eEF2 or eEF2 containing the diphthamide modification. Using the canonical initiation pathway to direct the synthesis of the peptide Met-Phe-Lys revealed no distinction between unmodified eEF2 and eEF2 with the diphthamide modification. In contrast, synthesis of the same peptide directed by the novel cricket paralysis virus (CrPV) internal ribosome entry site (IRES), which byasses canonical translation initiation and instead requires novel eEF2-directed pseudo-translocation reactions prior to peptide bond formation, was sensitive to the loss of diphthamide. As the pseudo-translocation steps are the main distinguishing feature of the CrPV IRES system, we propose that the precise phasing of pseudo-translocation, in which a tRNA–mimicking RNA element from the virus is translocated through the ribosome, is dependent on the diphthamide modification on eEF2.

Consistent with this interpretation, using electron cryomicroscopy, our collaborators in Venki Ramakrishnan's lab in Cambridge revealed that eEF2 interacts with the CrPV–IRES on the ribosome and stabilizes the IRES in a conformation reminiscent of a hybrid tRNA state. Interestingly, diphthamide appears to interact directly with the tRNA–mimicking element of the CrPV IRES, perhaps to facilitate its precise translocation in the ribosome and to break decoding interactions between conserved rRNA bases and the IRES. Thus, our studies provide the first evidence that diphthamide plays a role in protein synthesis, and we propose that diphthamide functions to disrupt the decoding interactions of rRNA in the A site and to maintain codon-anticodon interactions as the A-site tRNA is translocated to the P site (Reference 5).

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Regulation of Retrograde Axonal Transport in Developing and Mature Neural Circuits

The overarching theme of our lab is to determine the mechanistic regulation of retrograde transport in neurons and the functional ramifications of disrupting this process. Intracellular transport is critical in all cells of the body to form and maintain functional organ systems. Neurons are particularly reliant on this process, however, owing to their large size and high metabolic demand. Axons, the primary projection from the neuronal cell body to distant targets, can extend great distances from the cell body, where most proteins are produced. For example, axons that make up the sciatic nerve in humans can extend approximately a meter from the cell body to innervate the distal appendages. This makes the relative axon length to cell body diameter ratio approximately 100,000:1. In order to form this large and complicated structure, proteins and organelles must be continually shipped from the cell body through axons. This intracellular movement, termed axonal transport, utilizes molecular motors that move on cytoskeletal tracks to deliver cargo to the correct sub-cellular location. While anterograde (axon-terminal directed) transport utilizes a superfamily of motor proteins, retrograde (cell-body directed) transport utilizes a single motor protein complex called cytoplasmic dynein (Figure 1). We use genetics, biochemistry, and *in vivo* imaging in zebrafish to identify cargo-specific regulators of retrograde axonal transport. Subsequently, we can use these tools to determine how disruptions in cargo retrograde movement impact the stability and function of the neural circuit *in vivo*. Perhaps not surprisingly, disruptions in axonal transport have been correlated with numerous developmental and degenerative diseases of the nervous system, including Charcot-Marie-Tooth disease, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and hereditary spastic paraplegia, to name a few. However, whether axonal transport abnormalities are causal in disease progression is still largely unknown. Our combination of approaches, which rely on the unique advantages of the zebrafish system, will permit us to answer long-standing questions regarding the relationship between axonal transport regulation and disease pathology.

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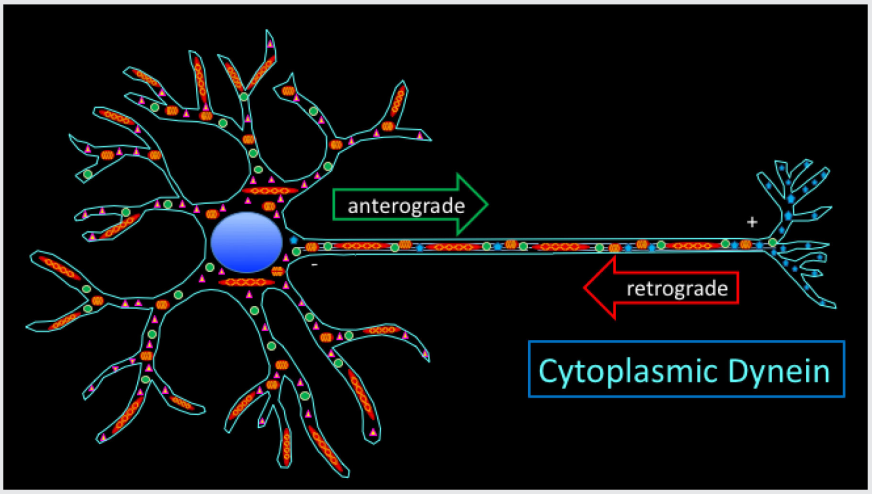
Mitochondrial transport, health, and function in axons

Mitochondrial transport is necessary to properly position this organelle in axons. Correct mitochondrial localization in axons is

FIGURE 1. Axonal transport and the neuron



Axonal transport is essential to move cargos, including proteins and organelles, between the neuronal cell body and axon terminals. While members of the kinesin superfamily of molecular motors move cargoes towards microtubule plus ends in axon terminals, a single motor protein complex, cytoplasmic dynein, is the primary motor required for retrograde cargo transport in axons. As the complicated subcellular architecture of neurons is required for their function, regulated movement of intracellular components to the proper location is essential for the formation and maintenance of functional neural circuits.

critical for energy production near sites of high metabolic demand, for calcium homeostasis to regulate neuronal activity, and to regulate axonal branching in certain contexts. Various model systems have revealed several mediators of mitochondrial movement. Anterograde mitochondrial transport requires the Kinesin-1 molecular motor in association with several other proteins, including Miro and Milton. Interestingly, loss of Miro or Milton, the two best characterized adaptors for mitochondrial transport, eliminates all mitochondrial movement. Therefore, how this organelle is selectively transported in the retrograde direction is still unclear.

In a forward genetic screen, we identified a novel zebrafish mutant strain with selective loss of retrograde mitochondrial transport. The causative mutation in this line causes loss of a protein known to associate with the dynein motor Actr10 (Figure 2). In *actr10* mutants, anterograde mitochondrial transport is intact but retrograde mitochondrial transport frequency is dramatically reduced, leading to accumulation of mitochondria in *actr10* mutant axon terminals. Using mitochondrial fractionation, we demonstrated that the loss of retrograde transport frequency is attributable to loss of mitochondria-dynein interaction in the absence of Actr10. We are currently using the *actr10* mutant as a tool to determine the impact of mitochondrial retrograde transport disruption on mitochondrial health and function. Additionally, we are pursuing the impact of mitochondrial accumulation on the health and function of the axon. Together, the work will define the molecular mechanisms of retrograde mitochondrial transport and provide insights into how loss of this specific cellular activity impacts the organelle, the cell, and the neural circuit *in vivo*.

Screening for novel regulators of cargo-specific retrograde axonal transport

Several pieces of evidence substantiate the importance of retrograde axonal transport for axon health and function. First, mutations in dynein and dynein-associated proteins are correlated with neurological disease. Second, retrograde transport of signaling endosomes is essential for the extension and maintenance of long axons. Third, abnormal localization of various cargoes, including mitochondria, correlates with neuronal

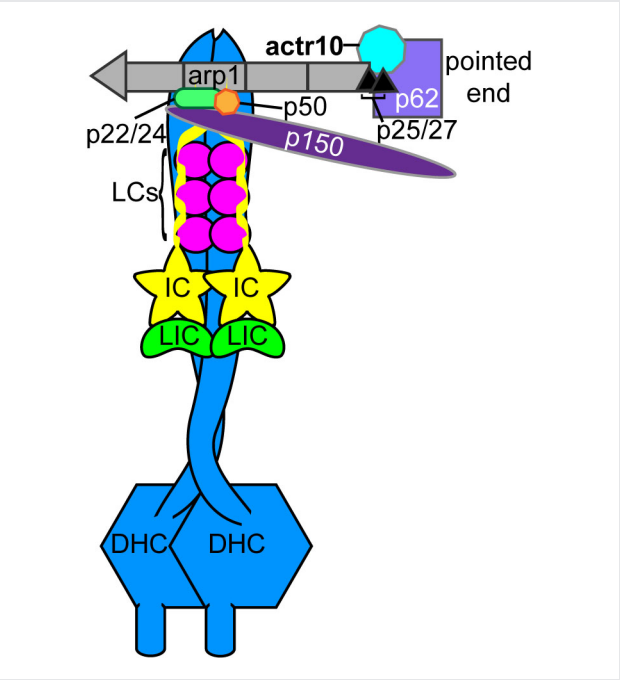


FIGURE 2. The dynein-dynactin complex

The core dynein motor is composed of heavy chains (HCs) with ATPase domains and a microtubule interaction domain. In addition, light chains (LCs), light intermediate chains (LICs), and intermediate chains (ICs) make up this complex. Dynein is bound to the accessory complex dynactin through interaction of p150 with the IC tails. Actr10, a component of the dynactin pointed end complex, is lost in the mutant line with disrupted mitochondrial retrograde transport.

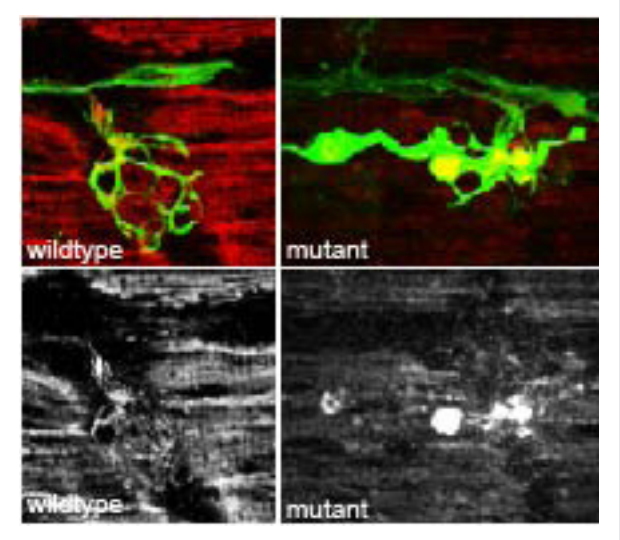


FIGURE 3. Cargo accumulation resulting from disrupted retrograde transport is associated with axon terminal swellings.

Axon terminal in a wild-type zebrafish larvae at 4 days post-fertilization (dpf; *left*). Axons are marked by the *neurod:egfp* transgene (*green*). Cytochrome C staining marks mitochondria in the axon terminal (*red on top, white on bottom*). An *actr10* mutant at 4 dpf has axon terminal swellings with mitochondrial accumulation resulting from disrupted retrograde mitochondrial transport.

disease. Despite the importance of the process, little is known about how various cargoes attach to and then are transported by the retrograde motor protein complex. We are using forward and reverse genetics in zebrafish to identify mediators of dynein-specific retrograde transport in axons.

Using a double transgenic line in a three-generation, forward genetic screen, we are identifying recessive mutant strains with axon abnormalities characteristic of disruptions in retrograde axonal transport. Our previous work and that of others showed that these phenotypes include axon terminal swellings such as those observed in *actr10* mutants (Figure 3). After identifying the strain, we use RNA–sequencing

approaches to identify the causal mutation. To date, we have identified three lines, which all have mutations in dynein-associated proteins. At present, we are using immuno-labeling approaches and *in vivo* imaging techniques we previously developed to determine whether these strains have deficits in the retrograde transport of specific cargoes. With these mutant strains we can first identify the proteins involved in the retrograde transport of particular cargoes and then use the strains as tools to determine how the specific disruptions affect the function of the axon.

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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 progression 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 relevance of PRL, estradiol and its receptor (liganded or un-liganded), epidermal growth factor (EGF), the EGF receptors ERRBB1/EGFR and ERRB2/HER2 in the up-regulation of the PRLR, and their mechanistic commonalities for definition of PRL/PRLR–induced progression and metastasis of breast tumors and their role in persistent invasiveness in certain states refractory to adjuvant endocrine therapies. 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, which 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 LH signaling, which regulate cyclic ovarian changes and testicular function through its presence in the membrane of granulosa-luteal cells and Leydig cells, respectively. Human *LHR* gene transcription is controlled by complex and diverse networks, in which coordination and interactions between regulatory effectors are essential for silencing/activation of *LHR* expression. The proximal promoter site for the transcription factor Sp1 recruits histone (H) deacetylases (HDAC) and the Sin3A (a transcriptional regulatory protein) corepressor complex, which contribute to the silencing of *LHR* transcription. Site-specific acetylation/methylation–induced phosphatase release serves as an on switch for Sp1

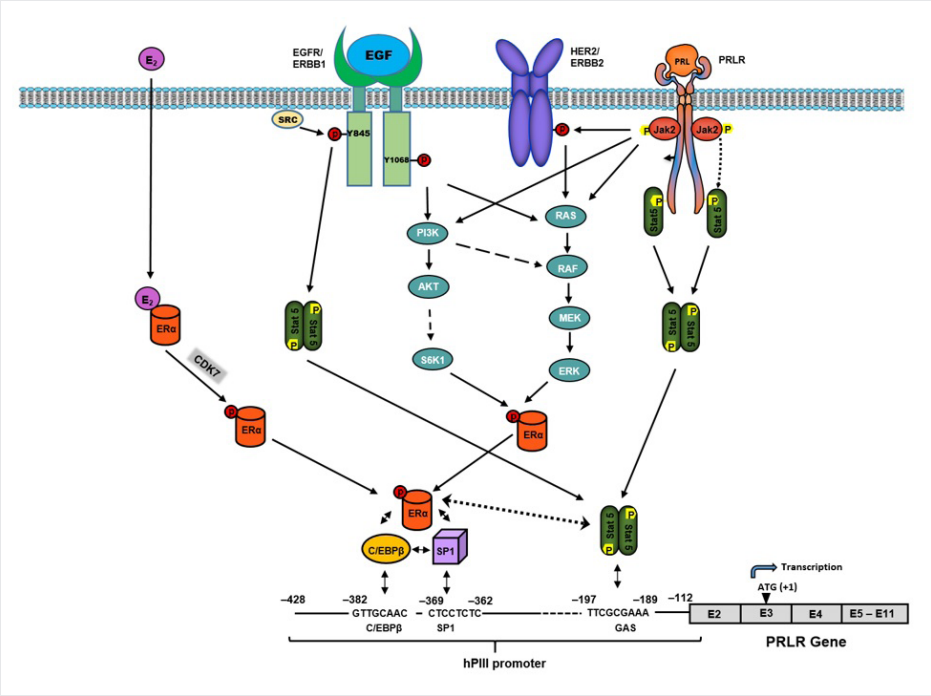

FIGURE 1. Figure 1. Mechanisms of the up-regulation of hPRLR induced by E2/ERa, EGF/ERBB1, and PRL/PRLR.

Essential requirements for transcriptional activation of PRLR: phosphorylation of ERα (liganded or unliganded) and STAT5, which binds to its site (GAS) at the hPIII promoter and stabilizes the complex pERα/C/EBPβ/Sp1 by association with non-DNA–bound pERα, a requirement for recruitment of TFIIB and Pol II and PRLR transcriptional activation. Presented schematically are the modalities of PRLR up-regulation via E2/ERα with PRL/PRLR requirement: PRL/PRLR/JAK2/STAT5 per se; or through HER2 transactivation by JAK2 in the absence of E2. Also, EGF functional effects via its cognate receptor ERBB1, independent of PRL/PRLR, and E2 on the activation of intrinsic receptor tyrosine kinase and signal transduction pathways participating in STAT5 and ERα phosphorylation/activation, essential for PRLR gene transcription through hPIII promoter. Inhibition of ligand (E2)-dependent pERα (S118) by the specific covalent CDK7 inhibitor THZ1 causes abolition of ERα/C/EBPβ/SP1 complex formation at the promoter and of PRLR transcription/expression. The use of this inhibitor singly or in combination with other inhibitory approaches targeting receptor function (PRLR, ERα, HER2, ERBB1) and/or their signaling pathways could effectively ablate transcription of PRLR and its contribution to breast cancer (References 1,3,4).

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, on H3 acetylation, and on HDAC/Sin3A release. Positive Cofactor 4 (PC4) has an important role in the assembly of the preinitiation complex (PIC) in trichostatin A (TSA)–mediated *LHR* transcription. 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. Further MS/MS studies revealed association of PC4 with the H3.3 variant acetylated at several Lys residues. The presence of these modifications was further confirmed by site-specific H3 antibodies in western blots. ChiP/reChiP analysis showed an increased recruitment of complexes of PC4/acetylated H3 at these sites to the LHR promoter upon TSA stimulation. Immunoprecipitation studies of cells transfected with PC4-Flag by Flag antibody demonstrated interaction of PC4 with H3.3 induced by TSA, using H3.3–specific antibody, and the presence of the complex PC4-H3.3 at the LHR promoter was demonstrated by reChiP.

Depletion of endogenous PC4 or H3.3 by siRNA caused marked reduction of TSA–induced formation of the complex, its recruitment to the promoter and of transcriptional activation of the *LHR* gene. Taken together, the findings indicate a critical role of PC4 association with acetylated H3.3 in TSA–induced Sp1–activated LHR transcription. The role of these interactions on chromatin structure and their participation in the assembly of the PIC and transcriptional activation are under investigation.

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 Leydig cells (LC) and meiotic germ cells and is essential for the completion of spermatogenesis. Males lacking GRTH are sterile owing to azoospermia resulting from failure of round spermatids to elongate. Besides its intrinsic RNA helicase activity, our studies demonstrated the essential participation of GRTH–mediated nuclear export/transport of mRNAs in the structural integrity of the Chromatoid Body (storage/processing of mRNAs), and of mRNAs transit/association to actively translating polyribosomes, where GRTH may regulate translational initiation of genes. GRTH is regulated by LH through the androgen (A)/androgen receptor (AR) at the transcriptional level in LCs (direct), with impact on hCG–induced steroidogenesis while not affecting basal circulating levels (of testosterone) in mice, and in germ cells (indirectly via AR in Sertoli cells); in germ cells GRTH's expression is both cell- and stage-specific. More recently, transgenic (tg) mouse models carrying *GRTH* 5'–flanking regions GFP provided *in vivo* systems that permitted differential elucidation of regions in the *GRTH* gene that direct its expression (upstream; –6.4/–3.6kb) in germ cells (pachytene spermatocytes and round spermatids) and downstream in LCs and GRTH's autocrine regulation by A/AR in LC and paracrine regulation in germ cells.

A functional binding site for the germ cell–specific transcription factor Germ Cell Nuclear Factor (GCNF), which is present in round spermatids (RS) and spermatocytes, resides in the distal region, –5270/–5252, of the GRTH gene, and we identified its paracrine regulation by A/AR, which is cell-specific and occurs exclusively in round spermatids (RS). The studies also provided evidence for the actions of androgen on GCNF cell-specific regulation of *GRTH* gene transcription/expression in RS. Moreover, GRTH associates with GCNF mRNA, and exerts of negative autocrine regulation of GCNF expression by reducing mRNA stability. Our *in vivo* and *in vitro* models link androgen's actions to germ cells through GCNF as an androgen-regulated transfactor that controls transcription of the GRTH gene. These important findings provided a connection of androgen action to two relevant germ-cell genes essential for the progress of spermatogenesis and established their regulatory inter-relationship (Reference 2).

Our early studies revealed that a missense mutation of R to H at amino acid 242 of GRTH found in 5.8% of patients with non-obstructive azoospermia, when express in COS1 cells, causes loss of the 61 KDa cytoplasmatic phospho-species with preservation of the nuclear 56 KDa non-phospho form. The finding provided an avenue to elucidate the function of phospho–GRTH in spermatogenesis. We generated a humanized mutant *GRTH* knock-in (KI) mouse. Very recent studies revealed that mutant KI mice are sterile with marked reduction in the size of the testes, which lack sperm, and with arrest at step 8 of round spermatids and complete loss of the phospho–GRTH species but with preservation of the non-phospho form. Furthermore, we demonstrated the requisite presence of the phospho–GRTH form to the integral functional structure of the chromatoid body. This mouse model will permit us to discern the biological and biochemical impact of the phospho-species in GRTH function and provide major novel insights for developing a male contraceptive.

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 plays an important role in the etiology and progression of breast cancer, tumoral growth, and chemo-resistance. PRLR expression is controlled at the transcriptional level by several promoters, one generic promoter (PIII), which lacks an estrogen response element (ERE) and is preferentially utilized, and five human-specific promoters (hPN1–hPN5), which we defined and characterized in our laboratory. Each promoter directs transcription/expression of a specific non-coding exon 1, a common non-coding exon 2, and coding exons (E3–E11). Complex formation of phosphorylated estrogen receptor alpha (ERa) homodimer (non-DNA bound) liganded or unliganded with the transcription factor Sp1 and dimers of the transcription factor C/EBPβ, bound to their sites at the PIII promoter, is required for basal (constitutive ERa homodimers) and estradiol (E2) or non-E2–induced transcriptional activation/expression of the human *PRLR* gene.

In tumoral breast, PRL causes cell proliferation by activating its cognate receptor. Exacerbation of PRL's actions in breast cancer, resulting from elevated receptor expression, can explain resistance to estrogen inhibitors in breast cancer. Using MCF7 breast cancer estrogen-responsive (ERa^+)/oncogene HER2–positive ($HER2^+$) cells, we demonstrated that, in the absence of E2, exogenous/endogenous PRL upregulated *PRLR* transcription/expression, with essential participation of ERa and of the JAK2/STAT5, mitogen-activated protein kinase (MAPK), and PI3K pathways. This occurs by interaction of phosphorylated ERa (generated by PRL/PRLR/JAK2), associated with Sp1 and C/EBPβ, with STAT5A and STAT5B bound to a nucleotide sequence, known as a GAS element, in the PIII promoter. We also found that ERRB/HER2, which is expressed in 10% of breast tumors, phosphorylated and activated by JAK2 via PRL/PRLR, induces ERa phosphorylation. Such cross-talk activation of ERBB2/HER2 signaling was identified as an alternate route for the PRLR increase, which is abolished by mutation of the GAS site (Stat5 DNA–recognition motif), by Stat5 siRNA, or by an ER antagonist (ICI). This indicates that ERa participates in PRLR transcription via PRL/PRLR/Stat5. PRL/PRLR induces phosphorylation of ERa through the JAK2/PI3K/MAPK/ERK– and HER2–activated pathways (Reference 1). Increased recruitment of phospho-ERa 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 by the up-regulation of *PRLR* transcription/expression via the Stat5/ERa activation loop, with requisite participation of signaling mechanisms. 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.

Paracrine inputs have an active role in breast tumor development, progression, and metastasis. Stromal fibroblasts secrete epidermal growth factor (EGF), which, through its receptor EGFR/ERRB1 present in breast tumors cells, activates signaling pathways, which in turn trigger requisite transcription factors and coactivators that can affect the proliferation of breast tumor cells. Other studies have addressed 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. We showed, in MCF7 cells, marked activation of PRLR gene transcription/expression by exogenous EGF through EGFR1/ERBB1, independent of PRL/PRLR/JAK2 or E2, with essential involvement of the MAPK, ER1/2, and PI3K-AKT signaling

pathways (Reference 3) for the recruitment of requisite phosphorylated transcription factors (ERa and STAT5b) to the PRLR PIII promoter. These pathways are mediated/activated by EGFR tyrosine kinase through p-tyrosines at 1068 and 1086 of the EGFR and by the tyrosine kinase cSRC–dependent phosphorylation of EGFR at tyrosine 845 for ERa and STAT5b phosphorylation, respectively. Apart from its independence of E2 and the activator requirements (PRL vs. EGF), there are important commonalities (prerequisite for ERa and STAT5) in the mechanism of PRLR transcription/expression (see below). Moreover, the studies revealed that STAT 5 interaction with ERa is essential for PRLR up-regulation. Our findings provide mechanistic avenues whereby, upon resistance to hormonal therapy, an increase of PRLR could promote progression and metastasis in breast cancer.

Further investigation revisiting our initial early findings on E2–induced upregulation of the PRLR, taking in to account our recent findings summarized above, have revealed the existence of at least three distinct up-regulatory modalities of PRLR in breast cancer cells relevant to breast cancer resistance and invasiveness. These relate to different inductors: (1) by endogenous PRL in the absence of E2; (2) by EGF in the absence of E2 and PRL; and (3) by E2 with PRL requirement. All these modalities of PRLR (1,2,3) transcription/expression, which participate in the upregulation of the PRLR, revealed commonalities. These are the central role of ERa phosphorylation via JAK2/PI3K/MAPK/ERK or JAK2/HER pathways induced by PRL/PRLR (1), or by EGF/ERBB1 signaling (2), or by CDK7 kinase induced by E2 (3). Another attribute they share is the requisite phosphorylation/activation of Stat5 (pStat5) by PRL/PRLR/JAK2 (1 & 3) and by EGF receptor tyrosine kinase induced by EGF (2). Also of relevance is the requisite interaction between pERa liganded or unliganded (associated with Sp1 and C/EBPb) and pStat5 bound to a GAS element at the PIII PRLR promoter. This interaction is required for productive stabilization of the ERa-Sp1-C/EBPb complex, which is essential for activation of PRLR promoter and transcription/expression of the PRLR receptor. With regard to modality 3, we demonstrated that the specific CDK7 kinase inhibitor (THZ1) significantly attenuates E2–induced phosphorylation at S118 and abrogates E2–induced PRLR transcription/expression. Moreover, the E2–induced cell migration was inhibited by PRL siRNA and THZ1, indicating its dependence on PRL/PRLR and CDK7, respectively. Our findings provide novel mechanistic insights into how the increase of PRLR in cancer maximizes the actions of endogenous/exogenous PRL that, upon resistance to hormonal therapy, could promote progression and metastasis in breast cancer (Figure 1). THZ1 treatment, singly or in combination with other inhibitory approaches targeting receptor function (PRLR, ERa, HER2, ERBB1) and/or the various signaling pathways (MAPK, PI3K, c-SRC, ERBB1 tyrosine kinase), with impact on ERa and Stat5 phosphorylation, could effectively ablate transcription/expression of PRLR fueled by tumor PRL and thus reverse or reduce resistance in breast cancer and, generally, its deleterious contribution to breast cancer.

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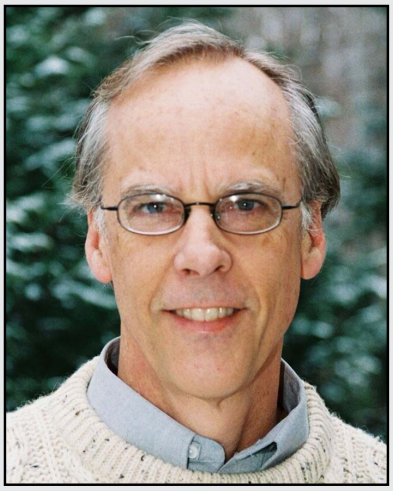
Nervous System Development and Plasticity

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 novel mechanisms of activity-dependent nervous system plasticity that are particularly relevant to the period of childhood and of 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 our 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



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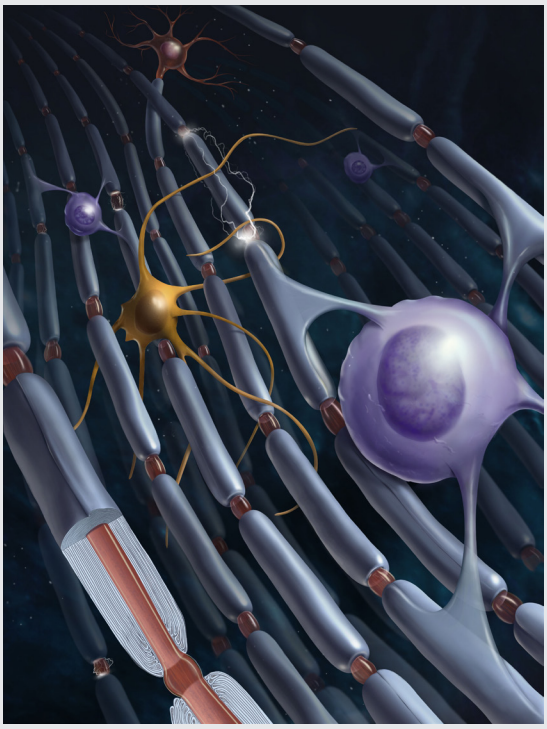

FIGURE 1. Myelin

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 (backwards) 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 antidromically during sharp-wave ripple complexes, which are most frequent during slow-wave sleep, and that the firing reduces the strength of all synapses on that neuron (action potential–induced long-term depression or 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, but 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 on astrocytes and other cells, which in turn release growth factors or cytokines that regulate development of myelinating glia.

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. As mentioned above, preferential myelin induction on electrically active axons would have profound effects on circuit function by increasing 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.

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 neuro-developmental 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

It is widely appreciated that there are two types of memory: short-term and long-term, and that sleep has a critical role in memory consolidation. 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 control strengthening and weakening of synapses in association with learning. 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 electrophysiology and 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.

Homeostatic controls are required to prevent a neural network from becoming incapacitated, and sleep is thought to be an important period for LTD and dendritic remodeling in memory consolidation. Although most studies of learning and memory use sensory stimulation, intrinsic activity in the brain, in particular oscillatory firing at different characteristic frequencies, is critical for information processing and learning. The brain's intrinsic activity includes many non-traditional modes of neuronal firing. In the hippocampus, high-frequency oscillations affect synaptic plasticity in the process of memory consolidation during slow-wave sleep and periods of quiet wakefulness. During these periods, neural sequences learned while encoding sensory information are replayed, and CA1 neurons of the hippocampus fire antidromically in brief high-frequency oscillations called sharp-wave ripple complexes. Action potential firing during sharp-wave ripple complexes 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 (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.

The neurotrophin brain-derived neurotrophic factor (BDNF) has been implicated in many forms of synaptic plasticity, nervous system development, and nervous system disorders. Our research shows that a rapid and persistent down-regulation of different mRNA transcripts of the *BDNF* gene accompanies AP-LTD. Moreover, AP-LTD is abolished in mice with the *Bdnf* gene knocked out in CA1 hippocampal neurons. Given the well established role of this neurotrophin in increasing synaptic strength and promoting synaptogenesis and dendritic complexity, the rapid reduction of *BDNF* mRNA during AP-LTD could contribute to reducing synaptic strength during AP-LTD and could promote synaptic remodeling.

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), RNAseq, microarray, 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 collaboration with David Clark, we are investigating chromatin structure and remodeling in neurons and glia.
This research is supported in part by a NICHD DIR Director's award jointly to David Clark and Douglas Fields.

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- NICHD DIR Director's award

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Quantitative Biophotonics for Tissue Characterization and Function

Our general goal is to devise quantitative methodologies and associated instrumentation to bring technologies 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: (1) at the Blackboard—modeling the methodologies; (2) at the Bench—designing the prototypes to be brought to the Bedside; (3) at the Bedside—piloting clinical protocols; and (4) Back to the Blackboard—improving the systems/methodologies.

Structural and functional brain imaging

Functional near-infrared spectroscopy (fNIRS) is an emerging, non-invasive imaging technique to assess the brain function. Because the technique is non-invasive and portable it is applicable to studies of children and toddlers, especially those with neuro-developmental disorders. fNIRS measurements are based on the local changes in cerebral hemodynamic levels (oxy-hemoglobin and deoxy-hemoglobin) associated with brain activity and function similar to BOLD fMRI. Near-infrared (NIR) light can penetrate enough to probe the cortical regions up to 1–3 cm deep. fNIRS is portable, low cost, and tolerant of patient movement. We address the changes in NIRS signal in relation to underlying physiological processes in the brain, such as cerebral autoregulation, and by using the technique to study cortical hemodynamics based on individual differences and development. 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 pilot studies, we evaluated the utility of fNIRS for measuring cerebral hemodynamics in the prefrontal cortex (PFC) in toddlers between 18–36 months of age. We also analyzed the correlation between fNIRS measures and the Composite Developmental Quotient (Composite-DQ) in toddlers with typical development and in those at risk (AR) for developmental delay, during a resting period. The analysis includes assessment of laterality index (percentage difference between brain activation in left and right prefrontal cortex), and the oxygenation variability (OV) index,

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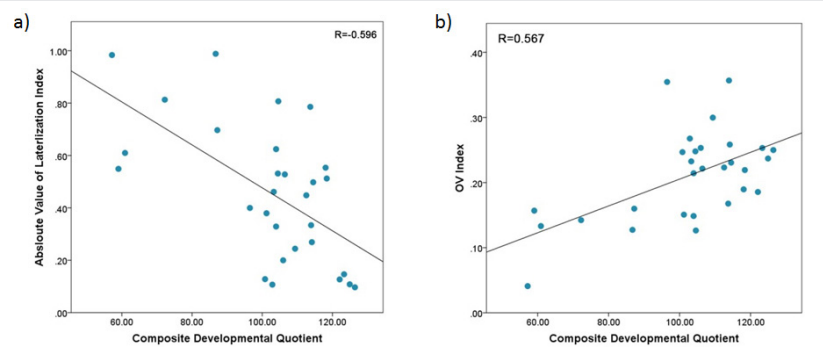


FIGURE 1.

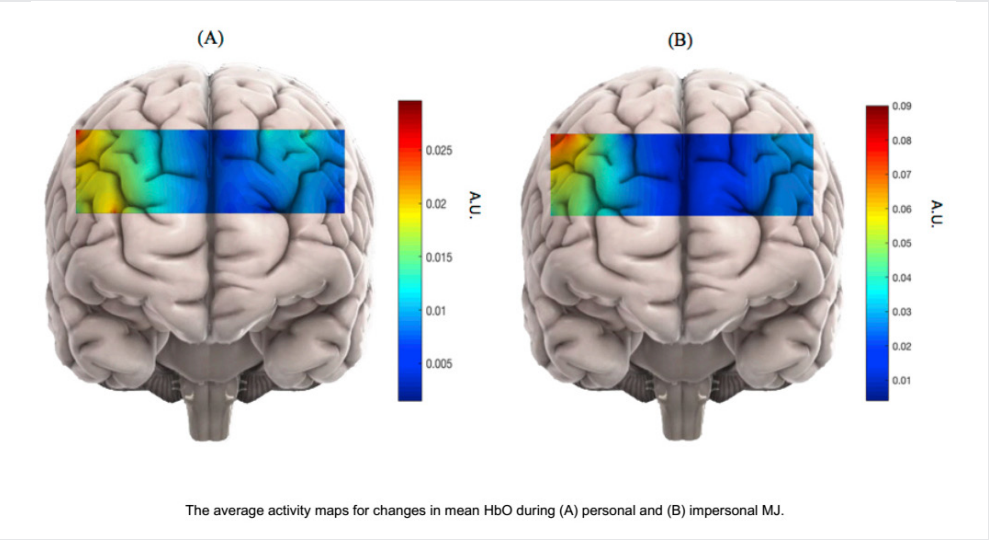
A. Correlation between Absolute values of Laterlization Index and Composite-Developmental Quotient (DQ) indicating increased differences between left and right prefrontal cortex (PFC) activation in subjects with lower Composite-DQ.

B. Correlation between oxygenation variability (OV) index values combined across Left and Right prefrontal cortex and Composite-DQ across all toddlers exhibiting trend of higher OV index in toddlers with higher Composite-DQ.

which quantifies the variability in oxygen saturation at frequencies attributed to cerebral autoregulation for each child. Cerebral autoregulation maintains the blood flow over the range of arterial blood pressure and is vital for brain function and development. The Composite-DQ was calculated based on the average of non-verbal and verbal developmental quotients. We found that toddlers with a lower Composite-DQ (AR) exhibited significantly more rightward activation. Moreover, toddlers with a lower Composite-DQ showed a greater discrepancy between left and right hemisphere activity, with a significant negative correlation between Composite-DQ and the absolute value of the laterality index (Figure 1a). In addition, toddlers with lower developmental scores showed a significantly lower OV index with those with higher scores (Figure 1b). While this study suggests that some features of prefrontal hemodynamics may vary in toddlers at risk for developmental delays due to early language delay, more research in this age group is required to clarify the specificity of these differences. These preliminary findings show the feasibility of using fNIRS in typical toddlers and those with delayed development and, in doing so, support future studies in larger samples.

We further examined the effects of individual differences on the underlying neural process of working memory (WM) tasks. Using fNIRS, we administered a visual and auditory n-back task to examine activation in the PFC, while considering the influences of age, task performance, and learning strategy (VARK score) in adults. Results showed age-related activation increase in the left and right medial PFC. Aside from age differences, high performance (HP) subjects (accuracy greater than 90%) showed lower activation than normal performance subjects. After accounting for learning style, we found a correlation between visual and aural VARK score and the level of activation in the PFC. Subjects with higher visual VARK scores displayed lower activation during auditory stimuli in the left dorsolateral PFC (DLPFC), while those with higher visual scores exhibited higher activation during auditory stimuli in bilateral DLPFC. Auditory HP subjects had higher visual VARK scores, indicating an effect of learning style on task performance and activation. The results of this study show that learning style and task performance can influence PFC activation, with applications toward and implications for neurological learning style and populations with deficits in auditory or visual processing.

Using fNIRS, we also study antisocial personality disorder (ASPD), which is characterized by a violation of the rights of others and lack of conformity to social norms. ASPD is prevalent in incarcerated populations and often goes undiagnosed. This poses a high cost on society, which indicates the critical need for early



The average activity maps for changes in mean HbO during (A) personal and (B) impersonal MJ.

FIGURE 2.

detection of ASPD to implement treatments. While ASPD is traditionally diagnosed through psychiatric evaluation in accordance with the symptoms outlined in DSMV, ASPD patients can be extremely manipulative, resulting in controversial diagnoses by subjective measures. However, understanding the neural basis behind ASPD can greatly enhance traditional diagnostic methods. Scientists have also found correlations between psychopathic personality traits and responses to moral judgment (MJ) tasks. Our study would be the first neuroimaging study to implement the MJ task with personality assessments of psychopathic traits in a cost-effective manner and a patient-friendly environment. We will utilize fNIRS to measure brain activation by monitoring changes of oxygenated hemoglobin in the brain similar to fMRI-BOLD. The task used in our study of MJ is based on a series of questions that examine personal versus impersonal dilemmas, defined as emotionally salient scenarios versus more distant ones. We hypothesized that the brain exhibits distinguishable hemodynamic patterns for each category. We will also investigate the correlation between these patterns and psychopathic traits. Using the hemodynamic responses of typical subjects, we will analyze the fNIRS data using a non-linear classification method called cubic SVM. We specifically chose SVM because it determines the separating hyperplane (high dimensional analog to the plane separating the two groups) only from signals located close to the interface between personal and impersonal hemodynamic responses. This should confirm our hypothesis of distinguishable hemodynamic patterns by category and suggests that it is possible to classify degrees of psychopathy based on neural activity. Consequently, we offer a novel approach to provide functional biomarker for ASPD using fNIRS, combined with advanced machine-learning techniques. In the future, we plan to apply our method to incarcerated populations to assess the degree of ASPD (Figure 2).

Multispectral imaging

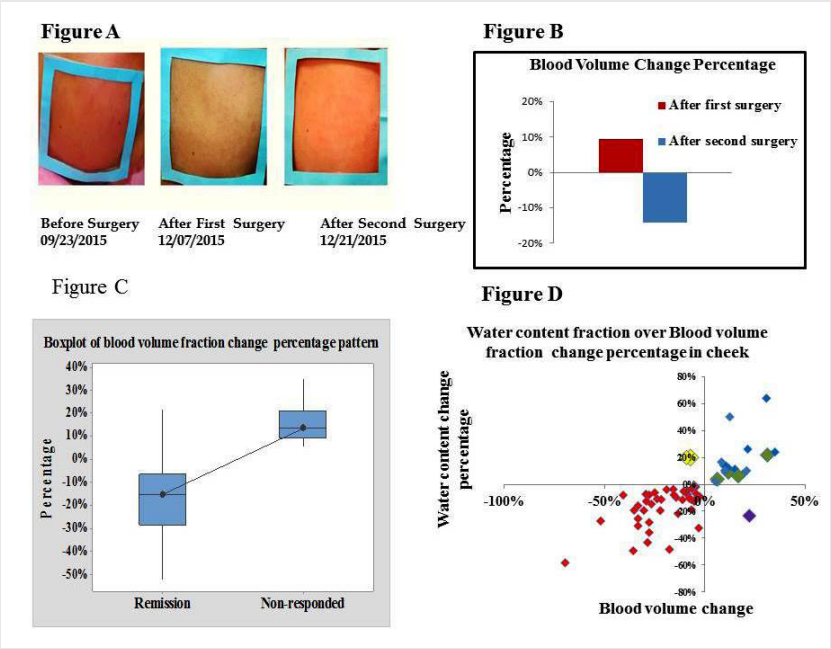
Facial plethora is one of the earliest described clinical features of Cushing's syndrome (CS). In collaboration with the Stratakis group, we continued to quantify changes of facial plethora in CS as an early assessment of cure. Cushing patients are recruited for optical imaging, before and after surgery, with follow-up sessions at six months and two years after surgery. We were able to reconstruct biological parameters of 73 subjects. Non-invasive, multi-spectral, near-infrared imaging was performed on the right cheek of a patient before

FIGURE 3.

A. Facial plethora in a patient with Cushing's disease (CD) before trans-sphenoidal surgery (TSS), after the first TSS (non-cured), and after the second TSS (cured).

B. The blood volume fraction percentage change of the patient's right cheek associated with Figure A at different imaging sessions underscores the advantage of the method over visual inspection.

C. Boxplot of medium percentage change indicating change in blood volume after surgery, compared with before surgery, in each of the remission and non-responding groups of CD patients. The distribution shows median confidence interval box and interquartile range.



D. Correlation of percentage change in blood volume and water content fractions of right cheek side of 57 CD patients who received surgical treatment.

and two to 14 days after surgery. Patients were defined as cured by post-operative measurements of plasma cortisol less than 3 mcg/dl and/or adrenocortical insufficiency, for which they received replacement. Clinical data obtained from 64 patients indicate that a reduction in facial plethora after surgery, as evidenced by a lowered blood volume fraction, is well correlated with cure of CS (Reference 6). Our recent findings show that, as a new biomarker, water content fraction could also quantify facial plethora. Using this information, we were able to distinguish between cured and non-cured CS patients within a short time after surgical treatment. The findings for both blood volume and water content fractions correlated significantly, but it appears that blood volume is better associated with clinical evaluations; the number of false-negatives in water content analysis is slightly higher. We ran our methodology and analytic algorithm for first and second post-surgery follow up. We processed data for 22 imaged patients from their first follow up (3 to 6 months after surgery) and ten patients in their second follow up (6 months after the first follow up). So far, all have been identified clinically as in remission by their last visit. The results confirmed our hypothesis that the blood volume fraction in the region of interest for patients who were assessed as cured, based on significant drop in their cortisol level after the surgery, was lower than before surgical treatment.

We are pursuing Kaposi Sarcoma (KS) studies in three ongoing NCI clinical trials. After processing more patients under a new classification and with the results of supporting the capability of our novel technology as a robust device, the goal is to further evaluate diffuse multispectral imaging in a relatively large sample as a potential supplement to existing response assessment in KS. In our preliminary experiments, multi-spectral images of KS skin lesions were made over the course of treatment. Blood volume and oxygenation concentration maps were obtained through Principal Component Analysis (PCA). We compared

FIGURE 4.

Wearable NIRS device and its schematics for placental oxygenation measurement. The device can be easily applied to different abdominal regions, and near-infrared light from the device will penetrate to different tissue depths.

corresponding images with conventional clinical and pathological assessment. In agreement with our hypothesis that successful treatment would reduce the blood volume in the lesions, the normalized standard deviation of blood volume decreased in each of the eight patients whose lesion responded to treatment, while it increased in two patients whose lesion did not respond to therapy. These initial results confirm that concentrations of oxygenated hemoglobin in the tumor can be used as a quantitative marker of tumor response to therapy.

Fluorescence methods in pre-clinical studies of HER2–positive breast cancer and of basal cell carcinoma expressing BerEP4

HER2–SPECIFIC FLUORESCENTLY LABELED PROBES

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 contra-lateral site before and seven days after the last treatment with 17-DMAG (a heat shock protein 90 [HSP90] inhibitor), when the tumor regrew to almost its pretreatment volume. However, soon after the therapy (12 hours), when the effect of the drug on HER2 degradation is maximal, the difference was significantly smaller. 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.

MOLECULAR BIOMARKERS FOR BASAL CELL CARCINOMA (BCC) FOR USE IN ITS DIAGNOSIS, TREATMENT, AND FOLLOW UP

We conducted a pilot study to determine the affinity and selectivity of the BerEp4 antibody conjugated with a fluorescence probe and to assess its possible use in designing theranostic probes for BCC. Based on initial cell culture results, BerEP4 appears to be a promising biomarker for molecular imaging of BCC and can be used in

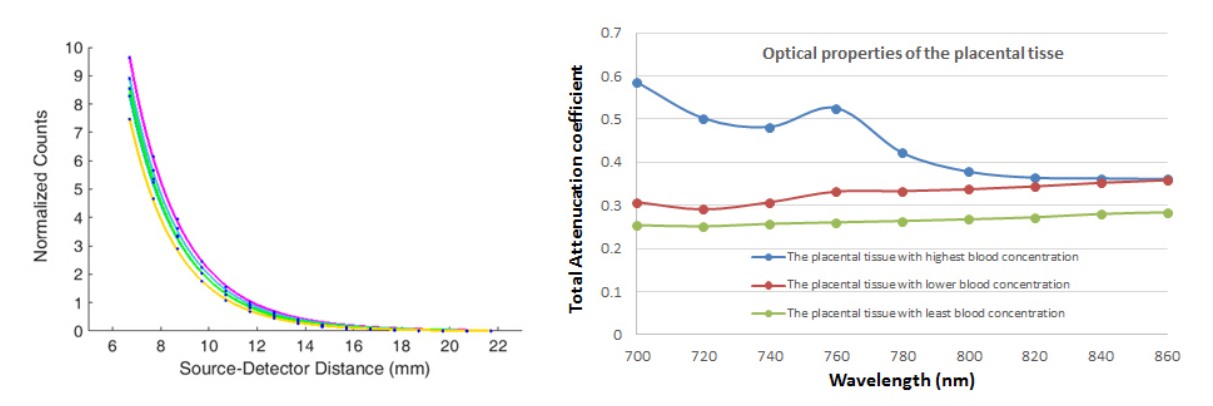


FIGURE 5.

Example of fitted curves based on photon counts as function of source-detector distance for several wavelengths that yield the values of total attenuation coefficients, which contain information about scattering and absorption of the tissue. Right: Total attenuation coefficient as a function of wavelength for the placental tissues with various blood content measured by the system.

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. During an *in vitro* phase, we showed specificity and selectivity of the BerEP4 antibody to target EpCAM on live cells. EpCAM is an ideal biomarker to target BCC because it is expressed in more than 95% of human BCC. In the subsequent *in vivo* phase, using xenograft mouse models, we reproduced persisting *in vivo* specificity and selectivity of the BerEP4 antibody to detect EpCAM–expressing xenograft tumors, when injected systemically via tail veins. Moving forward, we plan to: (1) modify our antibody to a smaller molecule—a stable aptamer or single-strand antibody—to facilitate topical delivery skin cancer; (2) repeat the *in vivo* experiment to show reproducible results with a modified and smaller probe; this phase would encompass initial systemic injection of the probe followed by experiments to test how best to achieve topical delivery, including micro-injection; (3) once *in vivo* non-invasive targeting of BCC is achieved via topical introduction, to further engineer our diagnostic probe to be photo-sensitized to achieve photodynamic treatment at the time of diagnosis.

Real-time oximetry of the anterior placenta using near-infrared spectroscopy

Monitoring placental oxygenation is critical to ensure a healthy pregnancy outcome. Problems with utero-placental perfusion have been associated with preeclampsia and intrauterine growth restriction and can lead to fetal hypoxia and cerebral palsy. Currently, there are no patient-friendly devices to measure the oxygenation of the anterior placenta. Therefore, it would be important to have a quantitative understanding of placental oxygenation to detect any abnormality compared to normal placental function. Near-infrared spectroscopy (NIRS) is a technique that can address these challenges with its wearable, wireless capability, which is convenient for dynamic monitoring. We intend to first find the baseline for the normal vs. abnormal pregnancies and to standardize the oxygenation data across pregnancies and then to correlate the oxygenation data with the pregnancy outcomes. To achieve these aims, we designed a non-invasive

and wearable device with wireless capability to allow continuous measurement of the oxygenation of the anterior placenta in a subject-friendly environment. This compact system can be positioned at different abdominal locations for localized measurement of oxygenation (Figure 4). The NIRS device uses the light in near-infrared region at two wavelengths (760 and 850 nm). The light enters the tissue at the location of source and the back-scattered light, which is sensitive to changes in oxy- and deoxy-hemoglobin, is detected at the detector sites. The device consists of two detectors and three sources, which permits probing different tissue depth. This would provide us with information regarding different tissue types and to distinguish between maternal and placental tissue.

We are also investigating the efficiency of the device to accurately distinguish between the oxygenation of the maternal and the placental tissue. Given that the light passes through several tissue compartments, we developed the multi-layer model that includes the effect of tissue types based on optical properties of skin (melanin concentration), fat (BMI), uterus, and placental tissue. This model is essential, given that calculation of the oxygenation requires prior knowledge of the optical properties of the given tissues, such as scattering and absorption. To determine the optical properties of the placenta, we use an in-house photodiode array unit combined with the laser system. The attenuation coefficient (as a function of scattering and absorption coefficient) for several near-infrared wavelengths is calculated based on the reflection curve (photon count as a function of source-detector distance) from the placental tissues with and without blood (Figure 5). Using Monte Carlo simulation along with our multi-layer model, we developed a system that includes parameters such as skin color, BMI, and uterus thickness into the calculation of oxygenation index.

In collaboration with Roberto Romero, Sonia Hassan, and Shad Deering, we plan to test our device through pilot studies to recruit pregnant subjects. The aim is to find a baseline of placental oxygenation in normal pregnancy. This involves refining our data analysis software by incorporating anatomical localization and standardizing it across pregnancy. We expect to thereby provide earlier detection of pregnancy complications, which can improve both maternal and fetal health.

Additional Funding

- Bench to Bedside Award 345 (2016): “Mirror neuron network dysfunction as an early biomarker of neurodevelopment” (Ongoing)
- Human Placenta Project-NICHD (2016) (Ongoing)

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Transcriptional and Translational Regulatory Mechanisms in Nutrient Control of Gene Expression

We study the fundamental mechanisms involved in the assembly and function of translation initiation complexes for protein synthesis, using yeast as a model system to exploit its powerful combination of genetics and biochemistry. The translation initiation pathway produces an 80S ribosome bound to mRNA with methionyl initiator tRNA (tRNAi) base-paired to the AUG start codon. The tRNAi is recruited to the small (40S) subunit in a ternary complex (TC) with GTP–bound eIF2 to produce the 43S preinitiation complex (PIC) in a reaction stimulated by eIFs 1, 1A, 3, and 5. The 43S PIC attaches to the 5' end of mRNA, facilitated by cap-binding complex eIF4F (comprising eIF4E, eIF4G, and the RNA helicase eIF4A) and poly(A)-binding protein (PABP) bound to the poly(A) tail, and scans the 5' untranslated region (UTR) for the AUG start codon. Scanning is promoted by eIFs 1 and 1A, which induce an open conformation of the 40S and rapid TC binding in a conformation suitable for scanning successive triplets entering the ribosomal P site (P-out), and by eIF4F and other RNA helicases, such as Ded1, that remove secondary structure in the 5' UTR. AUG recognition evokes tighter binding of the TC in the P-in state and irreversible GTP hydrolysis by eIF2, dependent on the GTPase–activating protein (GAP) eIF5, releasing eIF2-GDP from the PIC with tRNAi remaining in the P site. Joining of the 60S subunit produces the 80S initiation complex ready for protein synthesis. Our current aims in this research area are to (1) elucidate functions of eIF1, eIF1A, eIF2, and 40S proteins in TC recruitment and start codon recognition; (2) identify distinct functions of RNA helicases eIF4A (and its cofactors eIF4G/eIF4B), Ded1, and Dbp1 in mRNA activation, 48S PIC assembly, and scanning *in vivo*; (3) uncover the mechanisms of translational repression by the repressors Scd6, Pat1, Dhh1, and Khd1; (4) elucidate possible functions of yeast orthologs of eIF2A and eIF2D in eIF2–independent initiation of translation in stress conditions; (5) elucidate the *in vivo* functions of Rli1/ABCE1 (translation initiation factor/ATP-binding cassette E1, a ribonuclease inhibitor) and of yeast orthologs of eIF2D and the MCT-1/DENR complex (a translational enhancer) in ribosome recycling at stop codons *in vivo*.

We also analyze the regulation of amino acid–biosynthetic genes in budding yeast as a means of dissecting fundamental mechanisms of transcriptional control of gene expression. Transcription of

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these genes is coordinately induced by the activator Gcn4 during amino acid limitation owing to induction of Gcn4 at the translational level. The eviction of nucleosomes that occlude promoter DNA sequences and block access by RNA polymerase is thought to be a rate-limiting step for transcriptional activation. Previous studies implicated certain histone chaperones, ATP–dependent chromatin-remodeling complexes, or histone acetyltransferase complexes in eviction of promoter nucleosomes at certain yeast genes, but it was unclear whether these co-factors function at Gcn4 target genes. Our aim is to elucidate the full set of co-factors that participate in promoter nucleosome eviction at Gcn4 target genes, their involvement in this process genome-wide, and the transcriptional consequences of defective nucleosome eviction. Functional cooperation among the chromatin-remodeling complexes SWI/SNF, RSC, and Ino80 complex is the focus of current studies.

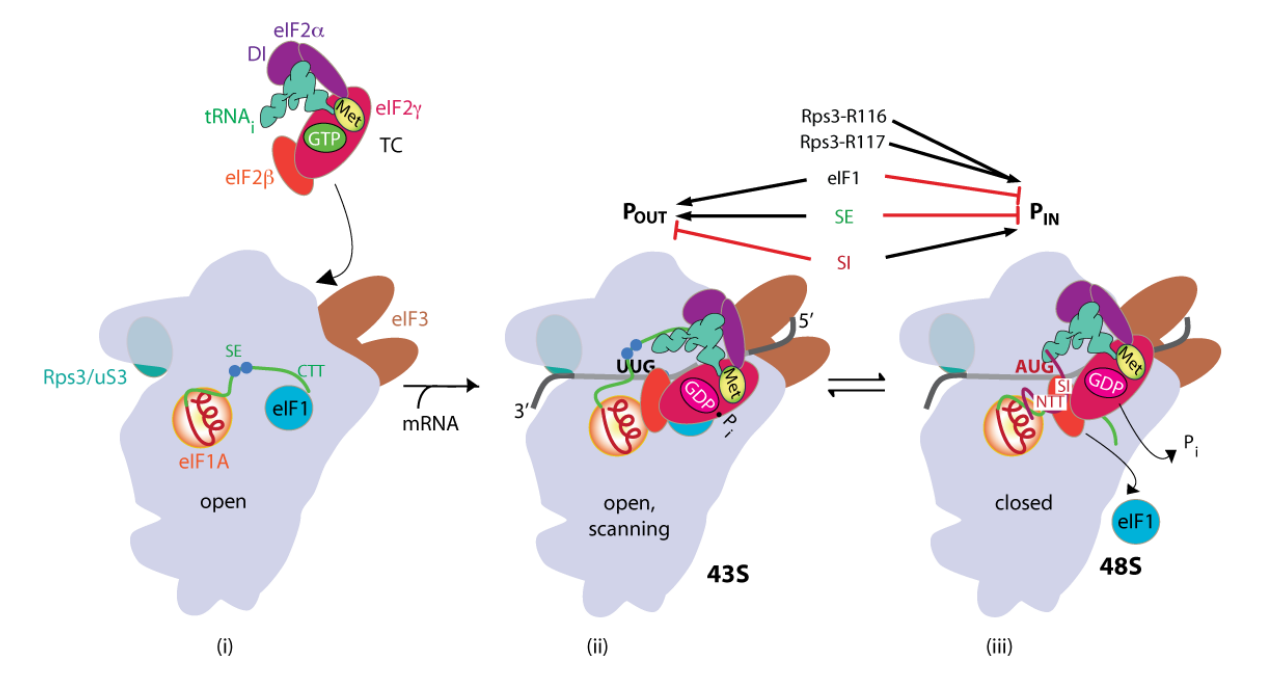

FIGURE 1. Rps3/uS3 plays a critical role in promoting mRNA binding at the 40S entry site and in stabilizing the preinitiation complex at the start codon.

A model describing known conformational rearrangements of the PIC during scanning and start codon recognition. (i) eIF1 and the scanning enhancers (SEs) in the C-terminal tail (CTT) of eIF1A stabilize an open conformation of the 40S subunit to which TC rapidly binds. Rps3 (uS3) is located on the solvent-exposed surface of the 40S near the entry channel; the bulk of eIF3 binds on the solvent-exposed surface with a prominent domain at the mRNA exit channel; (ii) The 43S PIC in the open conformation scans the mRNA for the start codon with Met-tRNA$_i^{Met}$ bound in the P$_{OUT}$ state. eIF2 can hydrolyze GTP to GDP•P$_i$, but release of P$_i$ is blocked. (iii) On AUG recognition, Met-tRNA$_i^{Met}$ moves from the P$_{OUT}$ to the P$_{IN}$ state, clashing with eIF1 and the CTT of eIF1A, provoking displacement of the eIF1A CTT from the P site, dissociation of eIF1 from the 40S subunit, and P$_i$ release from eIF2. The N-terminal tail (NTT) of eIF1A, harboring scanning inhibitor (SI) elements, adopts a defined conformation and interacts with the codon:anticodon helix. (*Above*) Arrows summarize that eIF1 and the eIF1A SE elements promote P$_{OUT}$ and impede transition to P$_{IN}$ state, whereas the scanning inhibitor (SI) element in the NTT of eIF1A stabilizes the P$_{IN}$ state. (*Below*) In contact with mRNA at the entry channel, uS3/Rps3 residues R116/R117 stabilize the P$_{IN}$ state and also promote PIC interaction with mRNA at the entry channel, augmenting the role of eIF3 in PIC-mRNA interactions at the exit channel.

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

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 tRNAi binding (P-in state), attendant displacement of the eIF1A-CTT (C-terminal tail) from the P site, and dissociation of eIF1 from the 40S subunit. In collaboration with Venki Ramakrishnan's and Jon Lorsch's groups, 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 partial yeast 48S PICs in open and closed conformations (py48S-open and py48-closed). The structures yield valuable information about conformational changes in the transition from scanning to AUG selection. The py48S-open complex, 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, 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. Formed with mRNA(AUG), py48S-closed 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 N-terminal tail (NTT) interacts with the AUG:anticodon duplex, consistent with its role in stabilizing P-in. As a prelude to eIF1 dissociation from the 40S subunit, eIF1 is repositioned on the 40S and deformed to prevent a clash with tRNAi. Both py48S–open and –closed complexes reveal eIF2beta and portions of the eIF3 complex. The eIF3 trimeric subcomplex eIF3b-CTD/eIF3i/eIF3g-NTT resides on the subunit-interface surface of the 40S and appears to lock mRNA into the 40S binding cleft. Exclusively in py48S-open, eIF2beta interacts with tRNAi and segments of eIF1 and eIF1A, which should stabilize binding of TC and eIF1 to the scanning PIC prior to AUG recognition. Indeed, we showed that mutations at the eIF2beta:eIF1 interface increase aberrant recognition of UUG codons *in vivo*—the expected consequence of shifting the system from the open to closed conformations—thus establishing the physiological relevance of the py48S-open and py48S-closed structures. Recently, we showed that contacts between eIF1A-NTT residues and the AUG:anticodon duplex in the P site stabilize the closed conformation of the PIC, promoting stable tRNAi binding to reconstituted PICs formed with mRNA containing a near-cognate UUG start codon, and increasing utilization of poor initiation sites *in vivo*, including near-cognate start codons and AUG codons with poor surrounding sequence context. Some of these substitutions are recurrent mutations in human uveal melanomas. We also demonstrated that a clash between loop-2 of eIF1 and the D-loop of tRNAi, predicted to impede rearrangement of the PIC to the closed conformation without full accommodation of tRNAi in the P site at an AUG codon, is an important determinant of stringent selection of AUG triplets in favorable context as initiation codons.

eIF4B and DEAD-box RNA helicases Ded1 and eIF4A preferentially stimulate translation of long mRNAs with structured 5 UTRs and low closed-loop potential but with weak dependence on eIF4G.

The RNA helicases eIF4A and Ded1 are believed to resolve mRNA secondary structures that impede ribosome attachment to the mRNA and scanning to the start codon, but whether they perform distinct functions *in vivo* was poorly understood. We compared the effects of mutations in Ded1 or eIF4A on translational efficiencies (TEs) by ribosome profiling. Despite similar reductions in bulk translation, inactivation of Ded1 substantially reduced the relative TEs of over 600 mRNAs, whereas inactivation of eIF4A similarly affected fewer than 40 mRNAs. Ded1–dependent mRNAs show greater than average 5' UTR length and propensity for secondary structure, implicating Ded1 in scanning though structured 5' UTRs. Thus, it appears that Ded1 is critically required for scanning through secondary structures in 5' UTRs,

while eIF4A promotes a step of initiation common to nearly all mRNAs, such as ribosome attachment. Ribosome profiling of a mutant lacking eIF4B showed that eliminating eIF4B preferentially impacts mRNAs with long structured 5' UTRs and reduces the relative TEs of many more genes than does inactivation of eIF4A. The findings support an eIF4A–independent role for eIF4B in addition to its known function as eIF4A cofactor. Mutations in eIF4B, eIF4A, and Ded1 also preferentially impair translation of longer mRNAs in a manner mitigated by the ability to form closed-loop mRNPs via eIF4F-Pab1 association, suggesting cooperation between closed-loop assembly and eIF4B/helicase functions in stimulating initiation. Recently, we determined that the Ded1 paralog Dbp1 functionally cooperates with Ded1 in promoting translation of mRNAs with long, structure-prone 5' UTRs *in vivo.* We identified genes in which the role of Ded1 is evident only in cells lacking Dbp1, and others in which Dbp1 function is unveiled by inactivation of Ded1. Moreover, Dbp1 overexpression can rescue translation of many Ded1–hyperdependent mRNAs. Interestingly, a small group of mRNAs with structurogenic sequences in coding regions are uniquely dependent on Dbp1.

Contacts of ribosomal uS3/Rps3 with mRNA at the 40S entry channel cooperate with PIC:mRNA interactions at the exit channel, requiring eIF3a to stabilize the closed conformation of the PIC at the start codon.

Collaborative work with the Lorsch laboratory, using the yeast reconstituted translation initiation system, identified distinct molecular functions for different domains/subunits of the 5-subunit eIF3 complex. Mutations throughout eIF3 were found to disrupt its interaction with the 43S PIC and diminish eIF3's ability to accelerate PIC recruitment to a native yeast mRNA. Alterations to the C-terminal domain (CTD) of the a-subunit and the eIF3b/i/g heterotrimeric module significantly slowed mRNA recruitment, and mutations within eIF3b/i/g destabilized binding of the eIF2.GTP.Met-tRNAi ternary complex to the PIC. Using model mRNAs lacking contacts with the 40S entry or exit channels, the eIF3a N-terminal domain was implicated in stabilizing PIC interactions with mRNA specifically at the 40S exit channel, while the eIF3a CTD plays an ancillary role at the entry channel. These functions are redundant, as defects at either channel can be rescued by filling the other channel with mRNA. The 40S ribosomal protein uS3 (yeast Rps3) makes direct contacts with the mRNA at the entry channel, and we showed that these contacts, involving two conserved arginine residues, are required for PIC–mRNA interactions at the entry channel. In the absence of these Rps3 arginines, PIC interactions with mRNA at the exit channel, mediated by the eIF3a–NTD, become essential for stable assembly of 43S-mRNA complexes *in vitro.* Substitutions of these Rps3 arginines also destabilize tRNAi binding to PICs assembled with mRNA harboring a UUG start codon *in vitro*, and they reduce initiation at UUG codons and a poor-context AUG codon *in vivo.* The results indicate that Rps3–mRNA interactions at the entry channel cooperate with mRNA interactions at the exit channel involving the eIF3a–NTD to promote 43S PIC binding to mRNA, and that PIC–mRNA interactions contribute to the overall stability of tRNAi binding to the P site of the closed conformation of the PIC and to the ability to utilize suboptimal initiation codons.

Interface between 40S exit channel protein uS7/Rps5 and eIF2 modulates start codon recognition *in vivo.*

Structures of yeast PICs have revealed that the β-hairpin of 40S ribosomal protein uS7/Rps5 is located in the 40S exit channel in proximity to mRNA nucleotides immediately 5' of the start codon. Our previous analysis of substitutions in Rps5 had implicated Arg-225 and amino acids in the β-hairpin, particularly Glu-144, in stabilizing the closed conformation of the PIC *in vitro*, and revealed a requirement for these residues in enabling utilization of near-cognate (UUG) or AUG start codons in poor context *in vivo.* Comparison of yeast PICs in open or closed conformations further suggested that remodeling of the interface between Rps5

and domain 1 of eIF2α, one of the components of the eIF2-GTP-Met-tRNAi ternary complex, occurs in the transition from open to closed states. We showed that Rps5 substitutions disrupting eIF2α contacts, favored in the open complex, increase initiation at suboptimal initiation sites (UUG codons and poor-context AUG codons) *in vivo*, and that one such substitution, Rps5-S223D, stabilizes tRNAi binding to PICs reconstituted with mRNA harboring a UUG start codon *in vitro*, thus indicating inappropriate rearrangement to the closed conformation of the PIC at suboptimal start codons. Conversely, Rps5-D215 substitutions, perturbing Rps5-eIF2α interaction favored in the closed state, confer the opposite defects of suppressing utilization of poor initiation sites *in vivo* and (for D215L) of destabilizing tRNAi binding to reconstituted PICs. The results indicate that remodeling of the Rps5/eIF2α interface stabilizes first the open and then the closed conformation of the PIC to regulate the accuracy of start codon selection *in vivo*.

Genome-wide cooperation by histone acetyl transferase Gcn5, remodelers SWI/SNF and RSC, and chaperone Ydj1 in promoter nucleosome eviction and transcriptional activation

We investigated the mechanism of promoter nucleosome eviction during gene activation by analyzing the effects of mutations in one or more chromatin remodeling complexes, histone acetyltransferase (HAT) complexes, or histone chaperone on eviction of histone H3 at the large cohort of genes induced by transcription factor Gcn4 in response to amino acid starvation. 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 intervening nucleosome-depleted region (NDR) at a large fraction of the Gcn4 transcriptome; and that these three cofactors function similarly at the majority of yeast genes. Surprisingly, defective H3 eviction in co-factor mutants was coupled with reduced transcription (Pol II densities measured by Rpb3 ChIP-Seq) only for a subset of genes, which included the induced Gcn4 transcriptome and the most highly expressed constitutively expressed yeast genes. In fact, the most weakly expressed genes displayed higher relative transcription levels than other genes in response to global attenuation of nucleosome eviction in the mutants. The results 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, whereas some other aspect of transcriptional activation is more generally rate-limiting for transcription at the majority of genes in amino acid–deprived yeast. More recently, we uncovered extensive cooperation between SWI/SNF and a second chromatin remodeling complex, RSC, in evicting nucleosomes from different locations in the promoter, and in repositioning the –1 and +1 nucleosomes to produce wider NDRs, which are more highly depleted of nucleosomes, during transcriptional activation. We also found that SWI/SNF functions on par with RSC at the most highly transcribed subset of constitutively expressed genes, suggesting general cooperation by these remodelers in achieving maximum transcription rates *in vivo.*

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Molecular Nature and Functional Role of Dendritic Voltage-Gated Ion Channels

The central nervous system (CNS) 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. In addition, we have begun to investigate the role of dendritic voltage-gated potassium and calcium channels in neuronal development and developmental disorders.

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

CALCIUM REGULATION OF TRANSIENT K^+ CURRENTS

Proteomic and subcellular localization studies suggest that Cav2.3–

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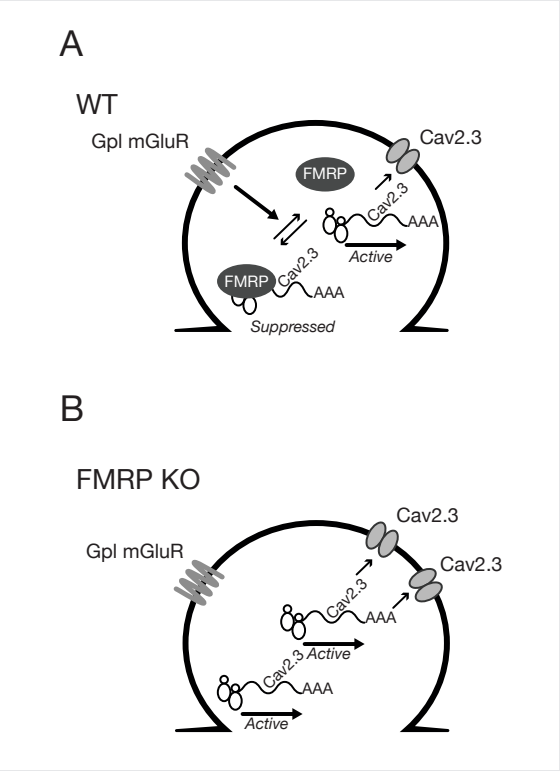

FIGURE 1. Disruption of Gpl mGluR–dependent Cav2.3 translation in a mouse model of Fragile X syndrome

Working model of FMRP's regulation of Cav2.3 translation.

A. In WT mice, FMRP binds to Cav2.3 mRNA and functions to suppress its translation in dendrites under basal conditions. Activation of Gpl mGluRs relieves FMRP–dependent suppression to allow translation of Cav2.3, and expression of Cav2.3 may be necessary for mGluR-LTD.

B. In the FMRP KO, loss of FMRP–mediated translational suppression of Cav2.3 leads to elevated steady-state levels of Cav2.3 in the plasma membrane. Gpl mGluR stimulation can no longer regulate Cav2.3 translation in the absence of FMRP.

containing voltage gated calcium channels could be a potential calcium source for a modulatory effect on Kv4.2–mediated A-type K currents (I_A) in CA1 hippocampal neurons. Jakob Gutzmann compared wild-type with Cav2.3 knock-out neurons and saw a significant reduction in somatic A-type potassium current in the mutant. We are now analyzing IA from somatic and dendritic attached patch recordings in order to investigate the potential influence of a loss of Cav2.3 on the distinct functional gradient that I_A shows along the apical dendrites of wild-type (WT) CA1 pyramidal neurons. To better understand the interplay between calcium and potassium channels that shape CA1 pyramidal cell electrical behavior, we are continuing to characterize Cav2.3 knockout animals.

ISOMERASE REGULATION OF POTASSIUM CHANNEL TRAFFICKING AND FUNCTION

To identify Kv4.2–binding proteins, Jiahua Hu employed a tandem affinity purification approach (TAP) to isolate the Kv4.2 protein complex from hippocampal neurons. Mass-spectrometry (MS) analysis identified known proteins such as KChIP family members and DPP6/10. The TAP–MS assay also identified an isomerase as a binding partner of Kv4.2. The binding was confirmed by brain co-immunoprecipitation, co-expression in HEK293T cells (a tissue-culture cell line), and peptide pull down *in vitro*. The isomerase binds to a specific Kv4.2 site, and the association is regulated by neuronal activity and seizure. To determine whether and how the isomerase regulates the trafficking of Kv4.2, we generated bungarotoxin binding site–tagged Kv4.2 at the second extracellular loop for visualizing Kv4.2 in live neurons. In biochemical and electrophysiological assays, the bungarotoxin binding site–tagged Kv4.2 showed similar channel properties to WT Kv4.2. The isomerizing activity may also regulate Kv4.2 binding to its auxiliary subunits. These data suggested that the isomerase plays a role in regulating Kv4.2 function. To further study the physiological function of isomerase and Kv4.2 channel, we generated a knockin mouse in which the isomerase binding site is specifically abolished using Crispr-Cas9 techniques. The mice are viable and appear normal. We are now working on learning- and memory-related behaviors, including novel objective recognition, Morris Water Maze, and fear conditioning.

DPP6 DELETION LEADS TO MEMORY IMPAIRMENTS IN MICE.

DPP6 plays an important role as an auxiliary subunit of Kv4, and the *DPP6* gene has been associated with neuro-developmental disorders in human. We found that DPP6 deletion leads to behavioral impairments in recognition, spatial learning, and memory, with lower body and brain weights, in adult mice. In addition, we found synaptic structure deficits in neuronal synapses in the hippocampal CA1 region. Using live-image and co-culture assay in neurons, we found that DPP6 induces synapse formation and regulates stabilization.

LOSS OF REGULATED CAV2.3 EXPRESSION IN A MOUSE MODEL OF FRAGILE X SYNDROME

Fragile X syndrome (FXS) is a severe form of intellectual disability that arises from the loss of the fragile X mental retardation protein (FMRP), an mRNA–binding protein that regulates translation downstream of group I metabotropic glutamate receptors (GpI mGluRs). Loss of FMRP leads to enhanced calcium spiking and neuronal excitability. Former postdoctoral fellow Erin Gray thus sought to explore the possibility that FMRP regulates expression of the dendritic voltage-gated calcium channel Cav2.3.

In our initial studies, we showed that loss of FMRP in mice alters Cav2.3 mRNA levels in both the cortex and hippocampus. Considering that FMRP is an mRNA–binding protein, we performed an RNA immunoprecipitation (RIP) assay and found that immunoprecipitation of FMRP from brain tissue also pulls down Cav2.3 mRNA. The results suggest that FMRP directly binds to Cav2.3 mRNA to regulate its abundance in neurons. FMRP–dependent regulation of Cav2.3 mRNA appears to impact Cav2.3 channel expression, as our previous data showed that FMRP knockout (KO) mice exhibit enhanced expression of Cav2.3 in cortical regions and in the hippocampus. The increase in Cav2.3 expression impacts neuronal physiology; Cav2.3 currents are enhanced in cultured hippocampal neurons isolated from FMRP KO mice compared with wild-type animals. Thus, it appears that FMRP binding normally represses Cav2.3 translation under basal conditions and that loss of FMRP leads to an increase of Cav2.3 protein in the membrane.

We are also investigating the possibility that repression of Cav2.3 expression by FMRP can be regulated by upstream activity of GpI mGluRs. In support of this idea, our previous data showed that stimulation of GpI mGluRs enhances local synaptic translation and expression of Cav2.3 in WT neurons but not in neurons lacking FMRP. To determine whether Cav2.3 expression downstream of GpI mGluR activation plays a role in synaptic plasticity, we induced long-term synaptic depression by stimulating GpI mGluRs (mGluR-LTD) in hippocampal slices from WT and Cav2.3 KO mice. Strikingly, we found that hippocampal slices from Cav2.3 KO mice lacked mGluR-LTD, demonstrating the importance of Cav2.3 in mGluR–dependent synaptic plasticity. Thus, FMRP serves as a key translational regulator of Cav2.3 expression under basal conditions and following activity, and this may be critical for mGluR–dependent forms of plasticity. Loss of regulated Cav2.3 expression could underlie the neuronal hyperactivity and aberrant calcium spiking in FMRP KO mice and contribute to FXS, potentially serving as a novel target for future therapeutic strategies (Figure).

Ying Liu showed that Cav2.3 mRNA levels were altered in FMRP KO neurons and that Cav2.3 protein levels were significantly enhanced in the FMRP KO mice. To determine whether Cav2.3 mRNA is one of the targets of FMRP, we performed RNA immunoprecipitation. Our data showed that FMRP interacts with Cav2.3 mRNA in transfected HEK293 cells and in mouse cortex and hippocampus. Our results suggest that FMRP binds to Cav2.3 mRNA directly or indirectly to repress Cav2.3 translation and thus regulates neuronal excitability.

In related work, Jon Murphy is examining whether the dendritic FMRP regulates mRNA trafficking and

protein expression of CaV2.3 and KV4.2 in dendrites of hippocampal neurons, using the FMRP KO mouse. Recent progress has centered primarily on analysis of mRNA localization and regulation of total protein translation in neuronal dendrites of WT and FMRP KO mouse neurons. Using fluorescence *in situ* hybridization to detect mRNAs for CaV2.3 and CaMKII (a serine/threonine-specific kinase regulated by Ca^{2+}) in neurons, we have found that CaMKII mRNA, a known dendritically synthesized protein, has increased abundance throughout the dendritic arbor of FMRP KO mice. FMRP is known to inhibit CaMKII translation through direct binding of CaMKII mRNA, suggesting that either CaMKII mRNA is more highly transcribed in FMRP KO mice, it is no longer sequestered in mRNA–protein complexes where *in situ* hybridization is inhibited, or both. Conversely, CaV2.3 mRNA signals are low throughout dendrites in both WT and FMRP KO neurons. Studies of Kv4.2 mRNA localization in WT and FMRP KO neurons are ongoing.

CELLULAR AND MOLECULAR PHENOTYPES ASSOCIATED WITH IDIOPATHIC AUTISM IN IPSC–DERIVED NEURONS

Idiopathic autism is an early onset neuro-developmental disorder that may result from genetic variation in several genes. We generated induced pluripotent stem cells (iPSCs) from three patients with intrinsic autism as well as from controls; the autistic iPSCs were subsequently differentiated into electrophysiologically active neurons comparable to control cells. Autistic iPSC–derived neurons displayed significant reductions in both Na^+ and fast K^+ voltage-gated currents but not in Ca^{2+} and slow K^+ voltage-gated currents. The defects accounted for the observation that autistic iPSC–derived neurons reach faster action potential saturation and are slightly more excitable than controls. We found that the amplitude and frequency of spontaneous excitatory post-synaptic currents from autistic neurons were significantly lower than in controls, indicating defects in excitatory synaptic transmission.

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Viral Gene Therapy for Inherited Neurometabolic Disorders

The Section of Translational Neuroscience strives to dissect and understand mechanisms of human neurometabolic disease and to use the knowledge gained to develop rational remedies, including gene therapy. Core values that guide the Section's efforts include integrity, humility, hard work with purpose, moving forward rapidly, concern for patients and their families, and mutual support among laboratory members. 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, utilizing murine and zebrafish models, as well as mouse primary motor neuron cultures.

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ATP7A–related copper transport disorders

The P-type ATPase ATP7A is a transmembrane protein with a distinctive intracellular trafficking itinerary essential for proper human copper transport. Mutations in *ATP7A* lead to Menkes disease, occipital horn syndrome (OHS), or an isolated adult-onset distal motor neuropathy (DMN), phenotypes that encompass variable neurologic disability and lifespan. The past several years have witnessed identification of additional human disorders involving gene products that normally coordinate with ATP7A and which, when mutated, can impair copper metabolism.

Through mutagenesis and immunoprecipitation studies, we recently identified a cryptic ubiquitin regulatory X (UBX) domain in the third luminal loop of ATP7A (between transmembrane segments 5 and 6) that becomes exposed through the conformational effects of a DMN–causing mutation, T994I, and binds to the multi-functional ATPase p97/VCP. Confocal imaging, total internal reflection fluorescence (TIRF) microscopy, and live-cell permeabilization experiments suggest that the abnormal interaction occurs at the plasma membrane of cells. The UBX domains contain an 80–amino acid ubiquitin-like sequence, including a conserved FP (Phe-Pro) dipeptide implicated in p97/VCP binding. The motif in ATP7A (F971/P972) is 22 residues

proximal to T994I, and mutation of F971/P972 to alanine residues dramatically diminishes the ATP7A–T994I interaction with p97/VCP. Mutations in *p97/VCP* itself cause several other inherited motor neuron diseases, confirming a link between this abnormal interaction and motor neuron degeneration.

Mutations in the acetyl-coA transporter gene *SLC33A1* cause Huppke-Brendel syndrome, a complex autosomal recessive phenotype that features congenital cataracts, hearing loss, profound neuro-developmental delay, and death during infancy or early childhood. The presence of low serum copper and ceruloplasmin and cerebellar atrophy similar to Menkes disease in affected patients implies possible effects on ATP7A. SLC33A1 normally mediates N-terminal acetylation, a reversible post-translational modification of numerous proteins, including at least six other ATPases. We employed tandem mass spectroscopy to document acetylation of ATP7A, used CRISPR/Cas9 to knock out *SCL33A1* in HEK293T cells, and studied ATP7A traffic in response to copper stimulation after over-expression of a Venus-tagged ATP7A construct. In contrast to normal HEK293T cells, ATP7A in *SLC33A1* knockout cells fails to traffic from the *trans*-Golgi compartment to the plasma membrane upon high copper loading. Cultured fibroblasts from three affected patients also show defective endogenous ATP7A trafficking after copper loading. The findings help explain the clinical and biochemical phenotypes of this condition and suggest that acetylation of ATP7A is involved in copper-responsive ATP7A trafficking.

For classic Menkes disease, the impact of existing and developing treatments, including viral gene therapy, would be enhanced by newborn screening (NBS), given that brain degeneration in affected infants typically begins by 6 to 8 weeks of age and leads to death by three years. The distinctively abnormal neurochemical metabolites in Menkes newborns are not amenable to detection in standard high volume NBS platforms owing to the need for an alumina extraction step. Interest in the application of genomic technologies to NBS has rised in recent years based on proof-of-concept studies involving DNA sequence analysis from dried newborn blood spots. In a blinded pilot study of 25 affected Menkes patients, over 95% of *ATP7A* mutations found by conventional mutation detection methods were detected accurately and efficiently from dried blood spots on NBS cards, auguring well for a future population-based application. These advances in early detection and ATP7A gene replacement have the potential to transform the natural history of Menkes disease by circumventing the largest current barriers to good health in affected patients.

Viral gene therapy for lysosomal storage diseases

Choroid plexus (CP)–targeted gene therapy represents a promising new approach to the treatment of lysosomal storage diseases (LSDs) that impact the central nervous system (CNS). Intrathecal delivery, by injecting enzyme into the cerebrospinal fluid (CSF) 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 adeno-associated virus (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" and conducted in collaboration with John Wolfe, we are using mouse and cat models to evaluate choroid plexus transduction by several recombinant AAV (rAAV) vectors as well as post-treatment alpha-mannosidase concentration and distribution in brain. Studies in the mouse model 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 are complementary.

In a related clinical natural history study, we evaluated six human subjects with alpha-mannosidosis, ranging in age from 11 to 38, at the NIH Clinical Center. In addition to newly appreciated brain magnetic resonance spectroscopy (MRS) findings, we identified distinctive biochemical and proteomic biomarkers in urine and the CSF, which provide invaluable benchmarks for assessing response to treatment in a planned future first-in-human pilot gene therapy trial.

Maternal and child health issues in survivors of the West Africa Ebola epidemic

In collaboration with NIAID, the Section participated in the Trans-NIH response to the 2014–2016 Ebola epidemic in Liberia, including a vaccine clinical trial (PREVAIL-1) and natural history study of survivors (PREVAIL-3). In contrast to AAV gene therapy, in which the brain's immunoprivileged status is advantageous, the Ebola filovirus poses neuro-cognitive and other health risks in survivors of the acute infection owing to immune sanctuary sites within the CNS.

While pregnancy during acute Ebola virus disease (EVD) was almost invariably associated with fetal loss, little is understood about the antenatal courses and pregnancy outcomes in female EVD survivors who conceived after recovery. In a retrospective cohort of 70 EVD survivors who conceived after recovery, the rate of adverse pregnancy outcomes suggested that EVD also engenders reproductive health risks after clinical disease has been resolved, especially when pregnancy occurs within two months of recovery (Reference 1). However, a recently completed prospective evaluation of 94 additional pregnancy outcomes in EVD survivors who conceived 14–24 months after discharge from an Ebola Treatment Unit suggests no maternal-infant transmission of Ebola when pregnancy occurs this long after recovery. The findings have important public health implications for EVD survivors of childbearing age and their health care providers.

Clinical research protocols

1. Principal Investigator, 90-CH-0149: Early copper histidine treatment in Menkes disease: relationship of molecular defects to neurodevelopmental outcomes
2. Principal Investigator, 09-CH-0059: Molecular bases of response to copper treatment in Menkes disease, related phenotypes, and unexplained copper deficiency
3. Principal Investigator, 14-CH-0106: Clinical biomarkers in alpha-mannosidosis
4. Associate Investigator, Partnership for Research on Ebola Virus in Liberia PREVAIL III (15-I-N122); Monrovia, Liberia
5. Sub-Investigator, Partnership for Research on Ebola Virus in Liberia PREVAIL I (15-I-N071); Monrovia, Liberia
6. 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

PCT/US2016/058124: Codon-optimized reduced-size ATP7A cDNA and uses for treatment of copper transport disorders. Filing date: October 21, 2016.

Additional Funding

- 2015 NIH Bench-to-Bedside Award (Kaler/Petris/Feldman). Mechanisms and treatment of motor neuron disease associated with copper metabolism defects
- 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
- 2016 NIH Bench-to-Bedside Award (Kaler/Dickson). Phenotypic effects of gene therapy to the choroid plexus epithelium for Sanfilippo B
- CRADA with Cyprium Therapeutics, Inc. New York, NY

Publications

1. Fallah MP, Skrip LA, Dahn BT, Nyenswah TG, Flumo H, Glayweon M, Lorseh TL, Kaler SG, Higgs ES, Galvani AP. Pregnancy outcomes in Liberian women who conceived after recovery from Ebola virus disease. *Lancet Glob Health* 2016 4:678-679.
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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 in which 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. PREs are made up of binding sites for several DNA–binding proteins. During the past year, our lab showed that the zinc-finger protein Combgap contributes to PcG recruitment at a subset of PREs. In addition, our recent genome-wide studies document the combinatorial nature and the redundancy of PcG recruitment in *Drosophila*.

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* (*en/inv*) gene complex. We found that regulatory sequences are spread throughout a region of at least 79kb 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

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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.

Polycomb group response elements (PREs)

PcG proteins act in protein complexes that repress gene expression by modifying chromatin (Reference 1). 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 and inhibits chromatin remodeling. In *Drosophila*, PRC1 and PRC2 are recruited to the DNA by PREs (Reference 2). 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). We found that binding sites for seven different proteins are required for the activity of the PRE2 (Figure 1). There are several binding sites for some of these proteins. Our laboratory identified four PRE–DNA binding proteins, Pho, Phol, Spps, and, most recently, Combgap (Reference 3). The Combgap protein has 10 zinc fingers and recognizes the sequence GTGTGT.

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

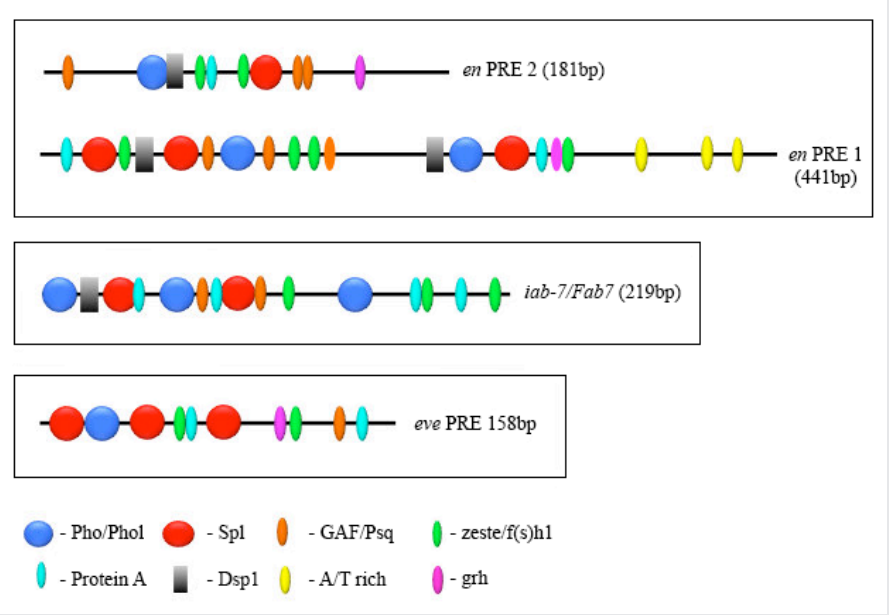

FIGURE 1. The order, number, and spatial arrangement of consensus-factor binding sites varies in different PREs.

en PRE1 and 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 Brown JL, Kassis JA. *Genetics* 2013;195:433.

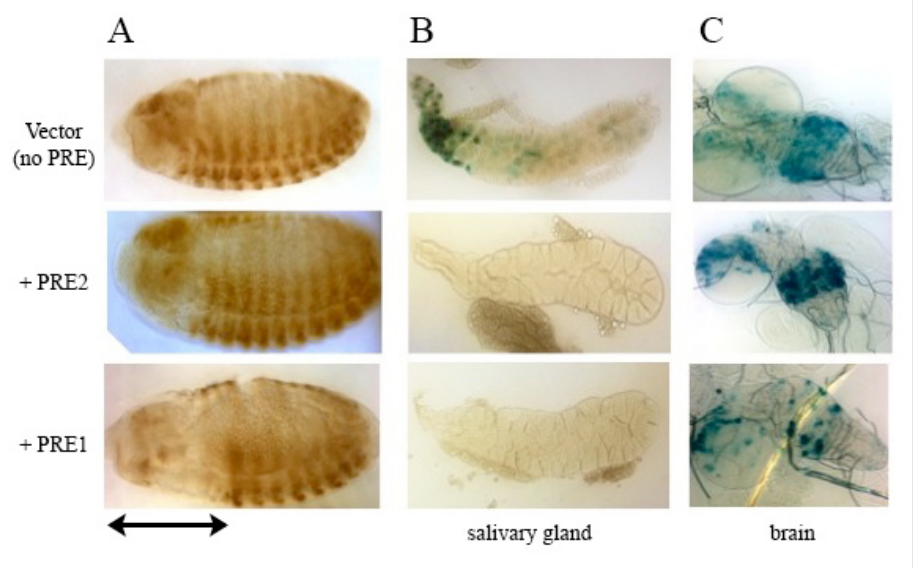

FIGURE 2. Activity of PRE1 and PRE2 in a transgene reporter construct in embryos and in larval salivary glands and brains

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.

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. Our recent genome-wide data support the hypothesis that there are many different types of PREs and much resiliency in PcG recruitment and activity.

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 *invected* (*inv*), an adjacent gene; *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 (Reference 4). 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

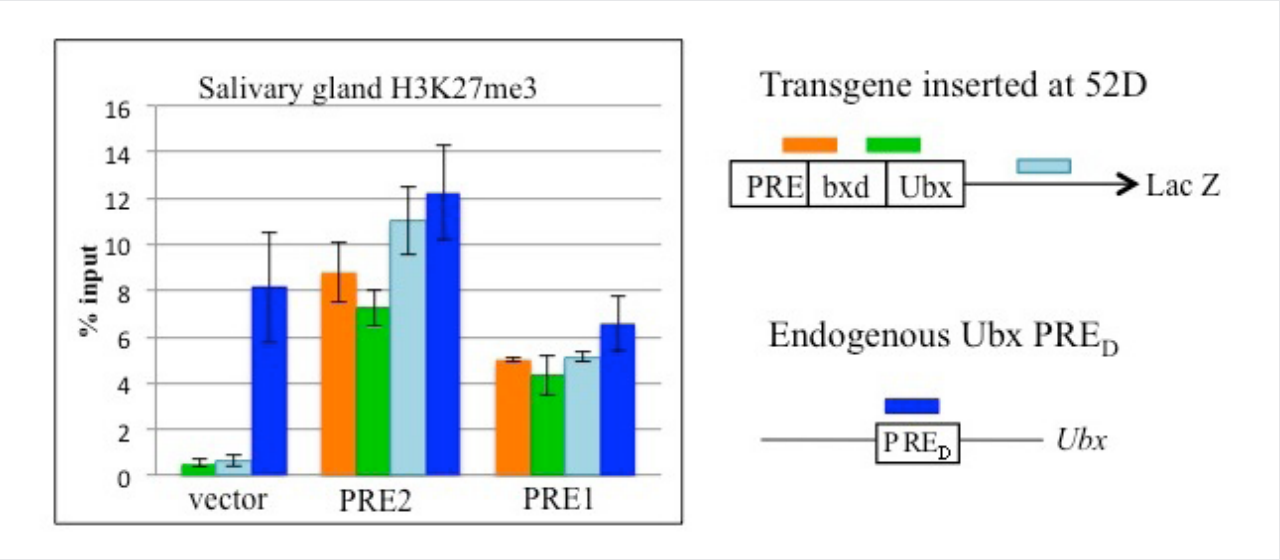

FIGURE 3. PRE1 and PRE2 lead to tri-methylation of histone H3 (H3K27me3) across the *LacZ* reporter gene in transgenic *Drosophila*.

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

tri-methylates histone H3 throughout the domain until PRC2 comes to either an insulator or an actively transcribed gene. As discussed above, there are two PREs upstream of the *en* 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.

In *Drosophila*, PREs are easily recognizable in chromatin immuno-precipitation experiments as discrete peaks of PcG protein binding, 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 (Reference 4). Surprisingly, the flies are viable, and there is no mis-expression of *en* or *inv* in embryos or larvae. 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. We found that the peaks may

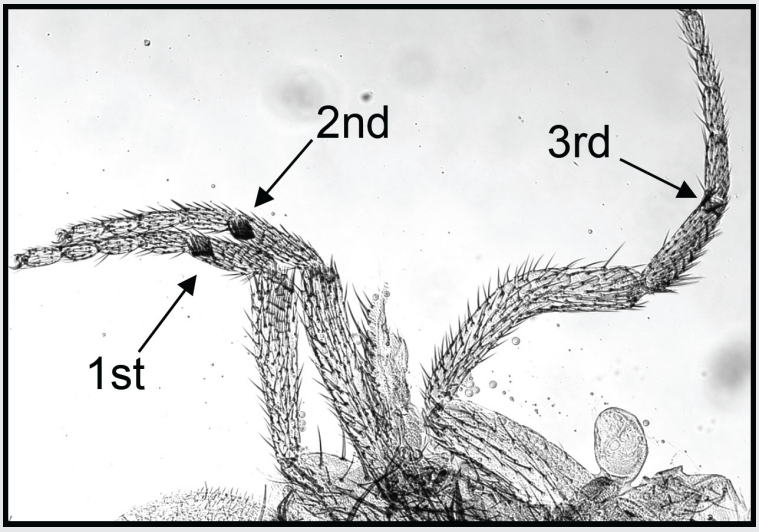

FIGURE 4. Wapl-AG causes extra sex comb teeth, the defining feature of PcG mutants.

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

also function as PREs. Thus, rather than a few PREs, there are many PREs controlling *inv/en* expression, and some may act in tissue-specific ways (Reference 4).

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 5). *wapl*^{AG} hemizygotes die as pharate adults and have the extra sex combs phenotype characteristic 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* respectively suppress and enhance *wapl*^{AG} phenotypes. 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 5). Functional studies suggest that cohesin binding may control the availability of PRC1 components for gene silencing (Reference 6). Our *Wapl* mouse mutant will provide a valuable reagent to test

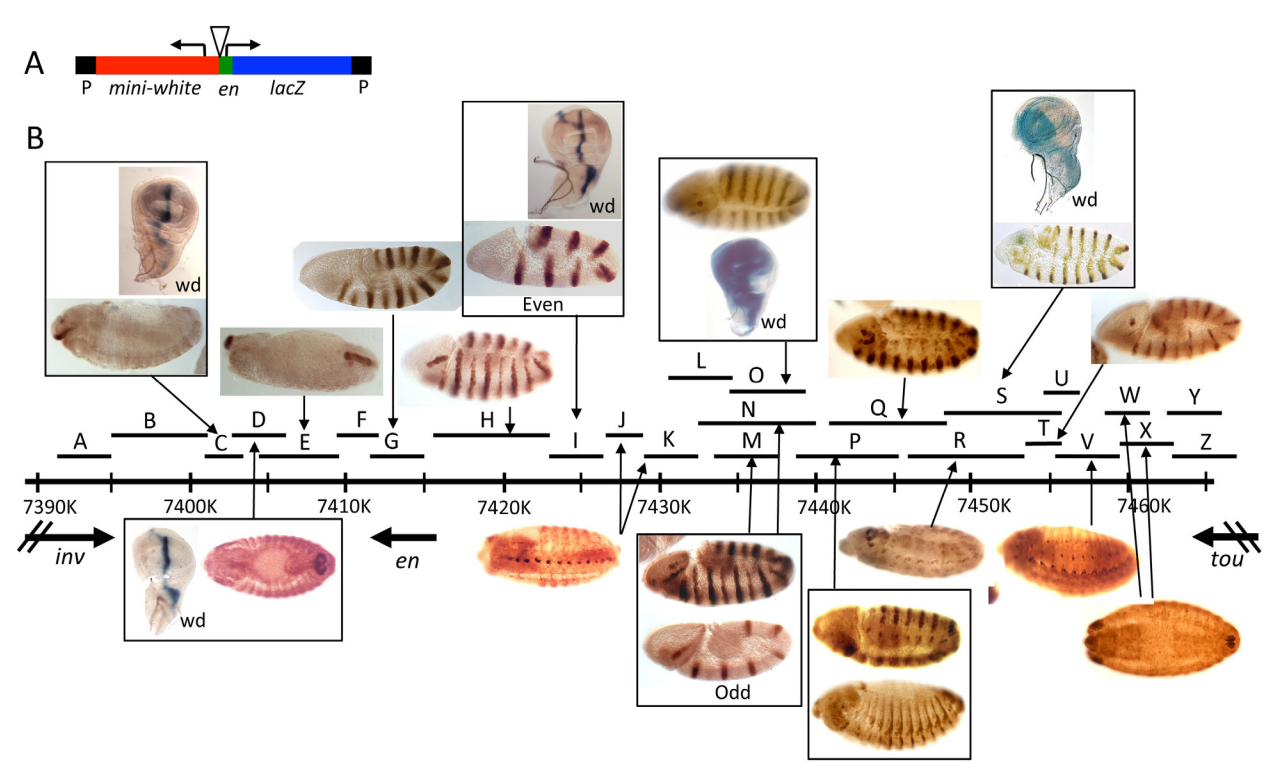

FIGURE 5. Enhancers of the *invected* and *engrailed* genes

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(s) the pattern (Reference 6).

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. Several years ago, 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. 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.

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 7). 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 integrase. 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 into 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 crispr/cas9.

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2. Kassis JA, Brown JL. Polycomb group response elements in Drosophila and vertebrates. *Adv Genet* 2013 81:83-118.
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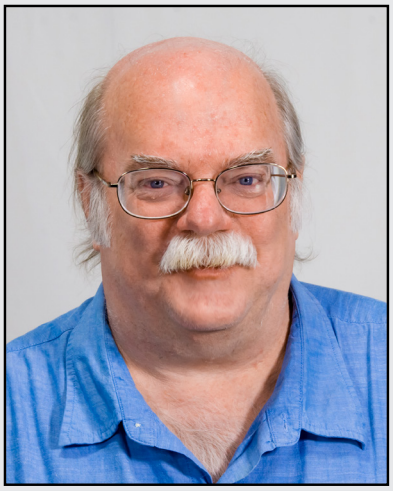
Genomics of Development in *Drosophila melanogaster*



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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* an excellent 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 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

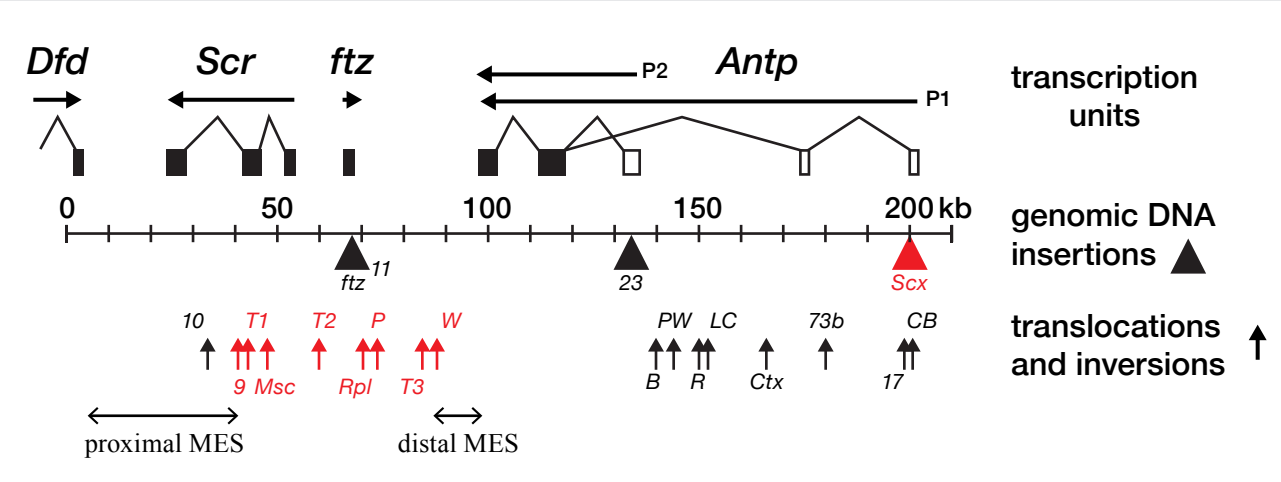


FIGURE 1. Chromosomal aberrations in the distal half of the *Antennapedia* complex

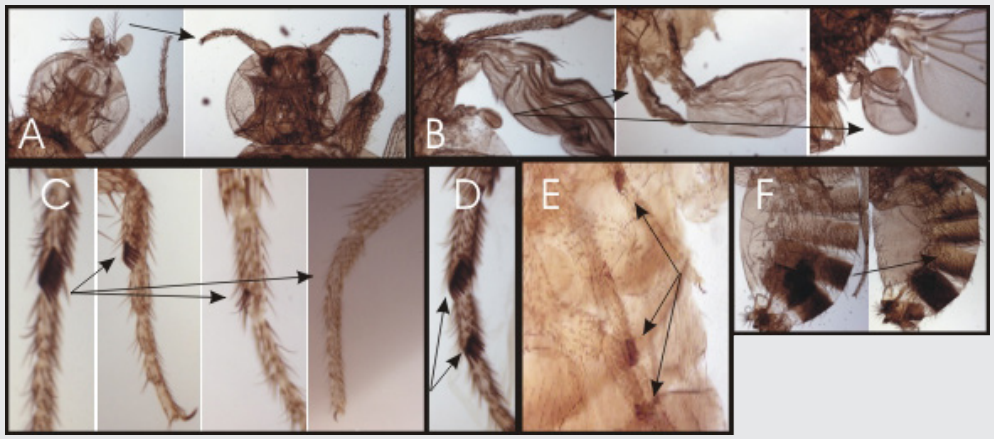
The transcription units are shown above the genomic DNA, while chromosomal aberrations are shown below (solid triangles indicate insertions of transposable elements and upward arrows indicate breakpoints of translocations and inversions). Chromosomal aberrations (*red*) interfere with silencing in the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

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 used the Crisper/Cas9 methodology to generate chromosomes lacking one, two, or three of the silencing elements. Silencing is only disrupted when multiple elements are deleted.

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 mimic 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

FIGURE 2. Homeotic phenotypes of new pharate-adult lethal mutants



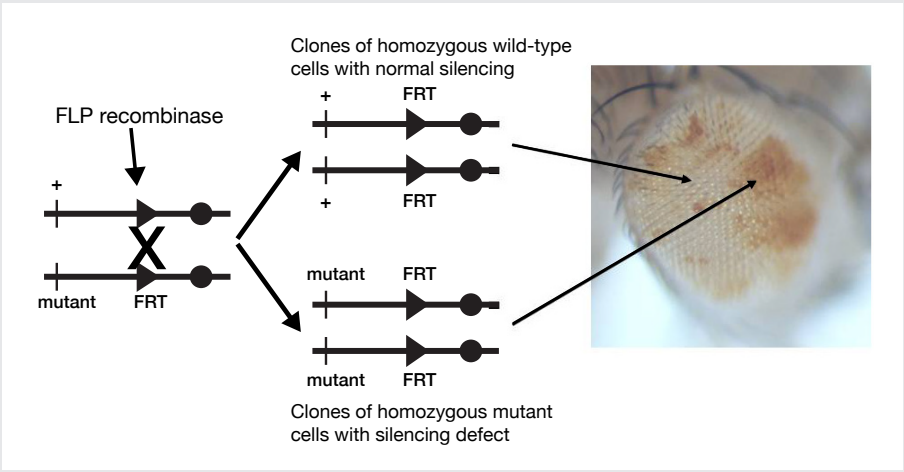
A) Wild-type on the left and the transformation of aristae to distal leg on the right.
B) Wild-type haltere on the left and transformations of anterior and posterior haltere to anterior and posterior wing in the middle and right, respectively.
C) First legs from a wild-type male on the left and three 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.

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). We screened about 98% of the genome and recovered 342 new silencing mutants. About one third 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. The remaining two thirds of our mutants are not associated with large chromosomal aberrations and carry mutations in genes required for Polycomb-group transcriptional silencing. Seventy-seven mutations are in 14 known Polycomb-group genes (*Pc*, *ph-d*, *ph-p*, *Psc*, *Su(z)2*, *E(z)*, *Su(z)12*, *esc*, *Sfmbt*, *Asx*, *calypso*, *Pcl*, *Scm*, and *crm*). The remaining mutations identify 61 additional genes required for silencing. For 44 of the silencing genes, we identified the corresponding transcription units using a combination of meiotic-recombination mapping and whole-genome sequencing. The new silencing genes encode DNA–binding proteins (*Spps*, *ftz-f1*, *grh*, *Dsp1*, *cut*, *Nf-YB*, *CG17829*, and *ocm*), chromatin-remodeling factors (*Mi-2*, *Iswi*, and *CG16908*), chromatin-modifying and chromatin-associated factors (*gpp*, *Tip60*, *CG9293*, *CG43736*, and *ptip*), insulator proteins, and two aminoacyl-tRNA synthetases (*ThrRS* and *AsnRS*).

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).



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 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. In addition to transcriptional regulation exerted through *cis*-regulatory elements by *trans*-acting factors, gene regulation can also be influenced by its location in the genome. Regulation of gene expression at the chromosomal level is a common phenomenon, and several examples have been extensively characterized, such as X chromosome inactivation in female mammals and hyper-transcription of X chromosome genes in male *Drosophila*. Less well-known examples are silencing or loss of paternally inherited chromosomes in male coccids and the unusual chromosomal behavior at multiple stages in the life cycle of the fungus fly *Sciara coprophila*. It was the investigations into chromosome behavior in *Sciara* that first used the term “imprinting” to describe the ability of some organisms to recognize the parental origin of their genetic material. An important tool in discovering or investigating many examples of chromosomal regulation has been the production of translocations, which exchange portions of chromosomes. We are investigating another example of chromosomal regulation that affects spermatogenesis in both *Drosophila* and mammals. Translocations between sex chromosomes and autosomes disrupt male fertility. The basis for the disruption is still not well understood. About two-thirds of X-autosome translocations in *Drosophila* are male-sterile, a sterility that is dominant and male-specific. The X chromosome breakpoints of the male-fertile X-autosome translocations are not random; the non-random pattern led investigators 50 years ago to propose a model in which the X chromosome is precociously inactivated during spermatogenesis and X-autosome translocations disrupt this precocious X inactivation. The major roadblock to testing this model is that only one mutant strain with a male-sterile X-autosome translocation is currently available, as all other X-autosome mutant strains were lost decades ago. We have generated a new collection of 80 X-autosome translocations to investigate the molecular basis of the dominant male sterility caused by translocations.

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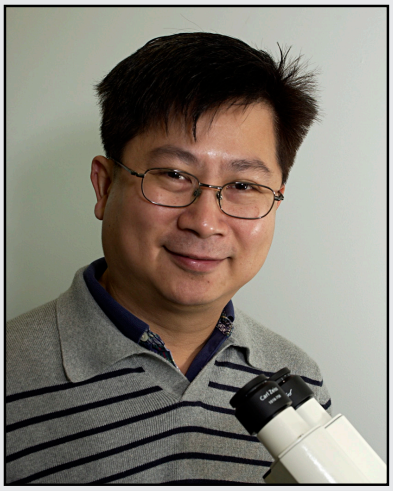
Assembly and Function of *Drosophila* Chromatic Circuits

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. With 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 the neurons' 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

Visual animals utilize spectral information in two ways: true color vision, which differentiates spectral compositions largely independent of brightness, allows animals to recognize objects and register and retrieve color memory; innate spectral preference, which depends on intensity but not learning, often reflects individual species' specific ecological needs. Using a combination of genetic, histological, electrophysiological, imaging, and behavioral approaches, our group studies how visual circuits process chromatic information to guide behaviors in *Drosophila*. Our strategy is (1) to identify key neuronal types and map their synaptic connections, (2) to examine functional requirement of identified neurons for color vision and spectral preference behaviors, and (3) to determine the synaptic mechanisms that transform visual signals at different processing stages.

Using molecular genetics, histology, and serial-EM reconstruction, we mapped the synaptic circuits of the chromatic photoreceptors R7s and R8s and their synaptic targets, the Tm and Dm neurons, in the peripheral visual system, as well as their downstream targets in the higher visual system, the lobula. The medulla projection (Tm) neurons (Tm5a/b/c, Tm9, and Tm20), which are analogous

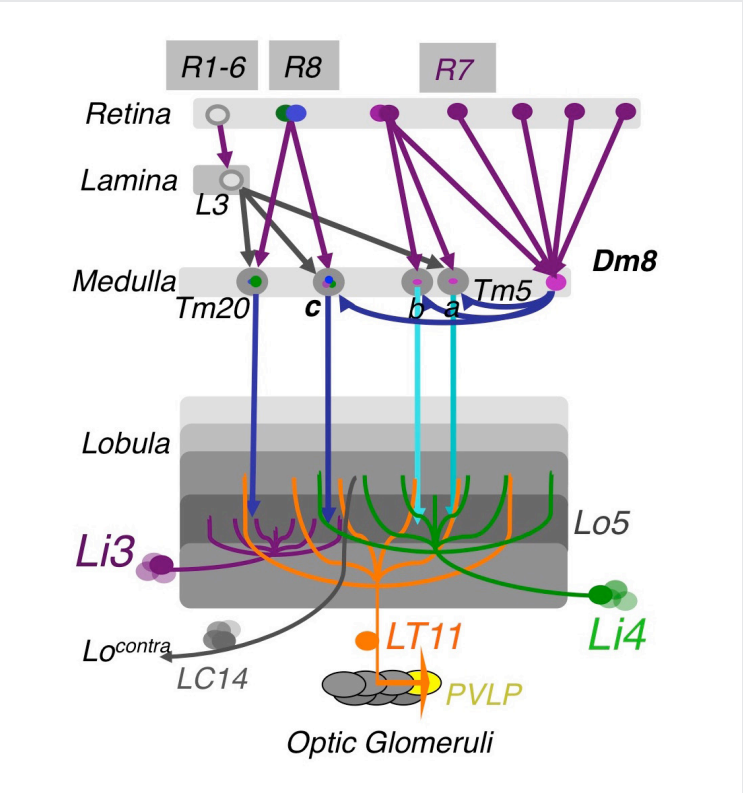

FIGURE 1. The chromatic circuit in *Drosophila*

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 vertebrate retinal ganglion cells, relay photoreceptors to higher visual centers, while the Dm (Dm8) neurons provide an indirect pathway by relaying photoreceptors to Tm neurons. To probe the synaptic connections between these neurons, we developed several versions of GRASP (GFP reconstitution across synaptic partners) methods to map active synapses. Using these methods, we found that the chromatic photoreceptors R7 (UV-sensing) and R8 (blue/green-sensing) provide inputs to a subset of first-order interneurons. Tm9/20/5c and Tm5a/b receive direct synaptic inputs from the retinotopic R8s and R7s, respectively, consistent with their functions in processing single visual pixel information. In contrast, the amacrine neuron Dm8 pools inputs from 14 R7s and provides input for Tm5c. Based on histology, we found that the Tm5a/b/c and Tm20 neurons relay photoreceptor signals to the lobula, the higher visual center, which, in insects, has been implicated in processing and relaying color information to the central brain. Using the GRASP method and a series of lobula neuron drivers, we identified the lobula neurons that receive direct synaptic inputs from the chromatic Tm neurons Tm5a/b/c and Tm20. We found four types of lobula neurons 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 Lo4-6, the entire lobula layers, and projects an axon to optic glomeruli in the central brain. Each Li4 extends dendrites to cover Lo5, about 60% of the lobula layer, while each of Li3's dendrites cover about 15% of lobular layers 5 and 6. To confirm synaptic connections observed at the light-microscopic level, we developed a two-tag EM double-labeling technique that highlights both presynaptic and postsynaptic terminals in the same preparation for EM analysis. By combining two orthogonal expression systems and two different peroxidases, HRP (horse radish peroxidase) and APX (ascorbate peroxidase), we expressed

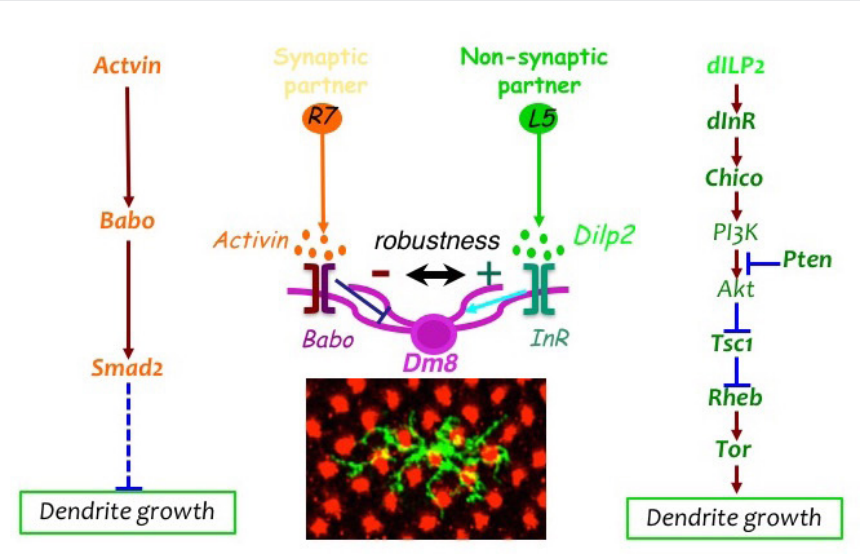

FIGURE 2. The afferent-derived factors, Activin and insulin-like peptide, antagonistically regulate the size of dendritic field of the amacrine neuron Dm8.

Two afferent-derived factors, Activin and *Drosophila* insulin-like peptide2 (Dilp2), signal on the first-order interneuron Dm8 to control the sizes of its dendritic field. Activin is provided by R7 photoreceptors, the synaptic partners of Dm8, while Dilp2 is provided by lamina neurons (LNs), non-synaptic partners of Dm8. Babo, Baboon (Activin receptor); InR, insulin receptor.

HA–tagged mitochondrion-targeted APX in Tm5c and membrane-tethered HRP in their postsynaptic neurons LT11, thereby confirming appositions among synaptic profiles. Our anatomical study revealed that the lobula neurons integrate multiple chromatic inputs from Tm neurons over a large receptive field, suggesting that color vision is of low spatial resolution.

To identify neurons that are involved in innate spectral preference, we used genetic methods to systematically inactivate different first-order interneurons and then examined their behavioral consequences. We 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. Using behavioral assays, we further found that inactivating Tm5c, one of Dm8's synaptic targets, abolished UV preference, establishing Tm5c as the key downstream target for spectral preference. Using single-cell transcript profiling and immunohistochemistry methods, we found that both Dm8 and Tm5c express the vesicular glutamate transporter (VGlut) and that Tm5c expresses four kainite-type glutamate receptors (Clumsy, DKaiR1C, DKaiR1C, and CG11155). RNAi-knockdown of these receptors in Tm5c, which prevents Tm5c from receiving Dm8 inputs, abolished UV preference, indicating that these receptors are functionally required in Tm5c for UV preference behaviors. Thus, the R7s→Dm8→Tm5c connections constitute a hard-wired glutamatergic circuit for detecting dim UV light. To determine the functional properties of these CNS glutamate receptors, we reconstituted DKaiR1D in oocytes and determined its electrophysiological properties. We found that DKaiR1D forms a rapidly activating and desensitizing homotetramer receptor that is glutamate-gated and calcium-permeable. Notably, DKaiR1D is inhibited by NMDA and AP5, the agonist and antagonist of vertebrate NMDA receptors, indicating that insect glutamate channels have a very different pharmacology from that of vertebrates. Two-photon calcium imaging of Dm8 and Tm5c expressing the calcium indicator GCaMP6f revealed that the activity of both neurons is suppressed by full-field UV light illumination. The light responses of Dm8 depend on R7 activity and Dm8's expression of the histamine chloride channel ORT, indicating that Dm8 receives inhibitory signals from photoreceptors via ORT and conveys sign-inverted signals to Tm5c via kainate receptors.

In contrast to UV preference, color vision in *Drosophila* is mediated by multiple and partially redundant pathways. To identify color-vision circuits, we developed a novel aversive operant conditioning assay to test animals' ability for intensity-independent color discrimination. We successfully trained 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 the color entrainment requires the function of the narrow-spectrum photoreceptors R7s and/or R8s. Furthermore, inactivating four types of first-order interneurons, Tm5a/b/c and Tm20, abolishes color learning but inactivating different subsets of these neurons is insufficient to block color learning. In summary, our results suggest that true color vision is mediated by parallel pathways with redundant functions. The apparent redundancy in learned color discrimination is in sharp contrast to innate spectral preference, which is mediated by the single pathway R7s→Dm8→Tm5c.

Dendritic development of *Drosophila* optic lobe neurons

For brains to be wired correctly requires axons and dendrites to be routed to appropriate regions, such as layers and columns, in order for correct synaptic connections to be formed during development. Many neuropsychiatric disorders, such as Down syndrome, Fragile X syndrome and Rett syndrome, have development origins and exhibit defects in dendritic morphology, such as changes in branching numbers and patterns. Dendritic defects could cause neuronal connectivity defects, which likely underline neurological and cognitive deficits. It remains unclear, however, how genetic disorders lead to dendritic patterning defects during development, which in turn lead to erroneous connections and functional deficits in adults.

Our group uses *Drosophila* optic lobe neurons as a model to study dendritic development and neural circuit assembly in the central nervous system. Similar to 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. Furthermore, fly visual neurons have unique advantages. First, the medulla neurons extend dendrites to form synapses in a lattice-like structure. Second, specific subtypes of medulla neurons can be specifically labelled and their genetics manipulated at the single-cell resolution. Third, the synaptic circuits have been characterized at the ultrastructural level and can be analyzed at the light-microscopic level. Forth, functional deficits can be fully characterized using behavioral assays and functional imaging assays. We have further developed several novel techniques to generate high-resolution images, to standardize and compare dendritic patterns, and to visualize synaptic connections at the light- and electron-microscopic levels, thus facilitating phenotypic analyses.

We carried out two genetic screens to identify molecular determinants that control dendritic patterning of Tm20 and Dm8 neurons. We focused on four types of dendritic developmental defects in: (1) the initiation of main dendritic branches; (2) the dendritic projection directions; (3) the layer-specific targeting of dendrites; (4) dendritic field sizes. From the genetic screens, we identified adhesion receptors, morphogen receptors, signaling molecules, and cytoskeletal regulators that are cell-autonomously required in Tm20 and/or Dm8 neurons for proper dendritic development. The RNAi-screen identified families of cadherins and cadherin-like receptors that are required for proper initiation of dendritic branches and receptive field sizes. N-cadherin, the classical cadherin, is required cell-autonomously in Tm20 neurons for layer-specific initiation of main dendritic branching points. Unlike wild-type Tm20 neurons, which extended most dendritic

branches from one or two primary branching nodes located in the medulla M3 layer, *Ncad*–mutant Tm20 neurons initiated the main dendritic branches in the M2 layers. The layer shift of the main branching nodes in *Ncad*–mutant Tm20 is further compounded with an alteration of layer-specific targeting of their dendritic arbors and their planar projection directions. Interestingly, the total dendritic length was unaffected. This suggests that the *Ncad* mutation specifically affects the initiation of primary dendritic branches rather than branch trimming.

The loss-of-function mosaic screen identified two pathways that regulate the sizes of dendritic trees. We previously demonstrated that the TGF-beta/Activin signaling pathway negatively controls the sizes of the dendritic fields of Tm20 and Dm8 neurons. Mutant Tm20 neurons 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 revealed that *baboon* and *smad2* mutations significantly reduced dendritic termination frequency but not branching frequency. Using a modified GRASP method we developed, we found that the expanded dendritic tree of a mutant Tm20 neuron forms aberrant synaptic contacts with several neighboring R8 photoreceptors. RNAi knock-down of Activin in R7 and R7 further showed that Activin derived from photoreceptors R7 and R8 acts at short ranges on R7's and R8's respective synaptic targets, Dm8 and Tm20. Recently, we found that the insulin signaling pathway positively regulates the dendritic tree size of Dm8. Mutant Dm8 neurons lacking insulin receptor or the downstream signaling components TOR (target of rapamycin) or Rheb have a small dendritic tree. On the other hand, mutant Dm8 neurons lacking the negative regulators of the insulin signaling pathway, Pten (phosphatase and tensin homolog) or TSC1 (tuberous sclerosis 1), have an abnormally expanded dendritic tree, as compared with the wild-type. Mis-regulation of TOR signaling, collectively called mTORopathies, has been implicated in several focal malformations of cortical development (MCD) subtypes associated with epilepsy and dendritic morphological defects. However, the dissection of TOR signaling pathways in complex mammalian nervous systems has been difficult. The Dm8 and Tm20 dendritic development system we developed thus provides a swift way to dissect the complex phenotypes of the TOR pathway and to determine the cell-autonomous functions of TOR signaling at the single cell resolution.

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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 DNA studies and concentrated on ECM pathology in cancer, fibrosis, osteogenesis imperfecta (OI), Ehlers-Danlos syndrome (EDS), 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 of many tissues and organs. Type I is the most common collagen. It is by far the most abundant protein in all vertebrates and the main structural protein of extracellular matrix (ECM) in bone, skin, and other tissues. Its procollagen precursor is assembled from two pro-alpha1(I) and one pro-alpha2(I) chains, folded in the endoplasmic reticulum (ER), trafficked through the Golgi apparatus, secreted, and then converted into a 300-nm-long triple helix of mature collagen by cleavage of C- and N-propeptides. Osteoblasts (cells responsible for making bone) produce and secrete the massive amounts of type I procollagen needed to build the skeleton, presenting a unique challenge for protein quality control and trafficking. Not only does an osteoblast secrete enough procollagen to fill an entire cell volume in just a day, but procollagen is one of the most difficult proteins to fold. We discovered that the conformation of natively folded human procollagen is less thermodynamically favorable than the unfolded one above 35°C. To fold procollagen at body temperature, cells use

specialized ER chaperones to stabilize the native conformation. Outside the cell, the native conformation is stabilized after procollagen is converted to collagen and incorporated into collagen fibrils. Unincorporated molecules denature within several hours of secretion and become susceptible to rapid proteolytic degradation. Up to 10–5% of procollagen is misfolded even under normal conditions, necessitating activation of cell stress–response pathways responsible for degradation of misfolded molecules and forcing an active osteoblast to always function in a high-stress mode. Our findings indicate that one of key factors in bone pathology is osteoblast malfunction resulting from excessive cell stress, which is often caused by increased procollagen misfolding, inability of the cell to handle the normal load of misfolded procollagen, or both (Reference 1).

The most common hereditary cause of increased procollagen misfolding is a Gly substitution anywhere in the obligatory (Gly-X-Y)n sequence that distinguishes all collagens. Such substitutions in type I collagen are responsible for over 80% of severe OI cases. Similar substitutions in other collagens cause EDS and a variety of other syndromes. Our studies of OI patients with over 50 different Gly substitutions revealed several structural regions within the collagen where these 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, mutations within which prevent normal N-propeptide cleavage. Incorporation of molecules with uncleaved N-propeptides into collagen fibrils leads to hyperextensibility and joint laxity more characteristic of EDS.

Bone pathology associated with excessive procollagen misfolding of nonhereditary origin is likely to be more prevalent than OI. Indeed, our data suggest that such misfolding should occur upon changes in the osteoblast ER environment associated with aging, environmental factors, inflammation, etc. It is likely to contribute to age-related osteoporosis, bone loss during cancer treatment, and many other common ailments. However, almost nothing is known about this pathophysiology mechanism because procollagen folding and consequences of its misfolding for the cell remain poorly understood.

Cell biology of procollagen misfolding

Based on all these findings, our current research is focused primarily on the cell biology of procollagen misfolding. In one approach, we are collaborating with Jennifer Lippincott-Schwartz on using live-cell imaging to investigate the synthesis, folding, trafficking, and degradation of fluorescently tagged procollagen in osteoblasts. Live-cell imaging of osteoblasts transiently transfected with fluorescent procollagen chains revealed completely unexpected features of procollagen quality control and trafficking. We observed sorting of normally folded and misfolded procollagen molecules at COPII (a coatomer protein, i.e., a vesicle coat protein that transports proteins from the rough ER to the Golgi apparatus) ER exit sites (ERES). Normally folded procollagen was loaded into giant (up to 500 nm) Golgi-bound transport vesicles. Contrary to widely held beliefs, the vesicles did not have a COPII coat and did not contain HSP47, which is a collagen-specific ER chaperone that preferentially binds to natively folded procollagen to assist in its folding and loading into ERES. Apparently HSP47 was removed from procollagen upon its entry into ERES. Misfolded procollagen was retained at ERES, resulting in a COPII–dependent modification of ERES membrane by autophagic machinery and subsequent lysosomal engulfment and degradation. The findings delineate a novel, COPII–dependent, non-conventional micro-autophagy-like pathway for recycling ERES–loaded cargo.

To validate their physiological significance and further build on these findings, we are expanding our tool chest by exploiting emerging gene editing technologies. We have already created an osteoblast cell line in which

endogenous proα2(I) is fluorescently tagged and which contains Flp–recombinase target sites for manipulating the tag, e.g., changing the fluorescence color or completely replacing it. Presently, we are introducing additional Flp-recombinase target sites into the same gene in order to manipulate almost the entire gene sequence. The strategy will enable us to perform live-cell imaging of endogenous rather than transiently transfected procollagen with a variety of OI mutations. It can then be used to generate mouse models and to study other proteins. Importantly, the discoveries we have already made may have wide implications beyond procollagen and ECM biology. For instance, COPII coat involvement in regulating autophagic degradation as well as cargo rerouting from the secretory to degradative pathway at ERES are likely to be general rather than collagen-specific phenomena. The hypothesis is currently under investigation in our and several collaborating laboratories. From clinical and translational perspectives, our findings may explain at least some of the pathology in patients with COPII mutations by deficient autophagic degradation of difficult-to-fold proteins, which is another line of investigation we and our collaborators hope to pursue in the future.

In another approach, we are investigating cell-stress response to procollagen with a Gly610 to Cys substitution in the triple helical region of proα2(I) in a mouse model of OI. The G610C mouse model mimics the Gly610 to Cys mutation found in a large group of patients from an Old Order Amish community in Pennsylvania. Our study of cultured fibroblasts and osteoblasts as well as of tissues in this model revealed misfolding and accumulation of mutant molecules in the ER. Elevated phosphorylation of the translation initiation factor EIF2a indicated the presence of cell stress, although 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 procollagen misfolding by enhancing autophagy and thereby reducing excessive accumulation of misfolded procollagen in the ER. Such an adaptation prevents cell death but is not sufficient to prevent abnormal cell function. The osteoblasts produce less and lower-quality bone matrix. Their abnormal differentiation into osteocytes appears to affect matrix mineralization as well. To compensate for reduced bone synthesis by each cell, G610C mice generate more osteoblasts. However, the combination of the resulting increase in 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 disruption of the cortical bone matrix structure leads to more brittle bones, which have an increased susceptibility to fracture upon high energy impact despite normal cortical thickness and slightly higher cortical bone mineral density. (References 1, 2)

Development of novel OI treatments

We hypothesize that bone pathology associated with procollagen misfolding may be at least partially reversed by targeting the cell stress response to misfolded procollagen accumulation in the ER, thereby improving osteoblast function. Although this would not eliminate potentially detrimental effects of secreted mutant collagen in OI bone, pharmacological treatment of osteoblast malfunction is a more realistic short-term approach to OI than suppression of dominant negative OI mutations by gene therapy or bone marrow transplantation. Moreover, the same approach is likely to be a better long-term strategy for treatment of osteoblast malfunction caused by procollagen misfolding in cases that do not involve pathogenic mutations.

In pursuing this strategy, we are currently targeting procollagen autophagy. Provided that misfolded procollagen accumulation in the ER is indeed involved in osteoblast malfunction, the simplest way to prevent such accumulation is to enhance the natural ability of the cell to remove and degrade the misfolded

molecules via autophagy. Our preliminary study of a low-protein diet's (LPD) effect on G610C mice provided encouraging evidence of improved osteoblast function as well as bone matrix quality and mineralization. However, in addition to enhancing autophagy, a LPD also causes cell stress associated with nutrient deprivation and alters animal growth. The latter and other confounding effects made the LPD study difficult to interpret (Reference 2). We are considering an intermittent LPD as a potential component of the eventual treatment strategy, but it may still have a variety of unintended consequences. To unequivocally validate autophagy as a target before embarking on optimizing dietary and pharmacological treatments, we are currently pursuing an approach based on altering the expression of *Atg5*, a key gene involved in the regulation of autophagosome formation.

Our experiments confirmed that reduced autophagy increases OI severity. In heterozygous G610C animals, with reduced *Atg5* expression in all tissues, we observed close to 50% perinatal lethality compared with wild-type littermates, in contrast to negligible/undetectable perinatal lethality of heterozygous G610C mice with normal *Atg5* expression. Conditional *Atg5* knockout in mature osteoblasts resulted in dramatically increased bone malformations. Preliminary experiments pointed to even more severe bone defects associated with conditional *Atg5* knockout in osteoblast precursors. To complete the study, we are presently examining *Atg5* gain-of-function effects, e.g., by switching from low to normal/high *Atg5* expression and by conditional targeted *Atg5* overexpression.

Translational studies on patients with novel or unusual OI and EDS mutations

Abnormal collagen biosynthesis and malfunction of osteoblasts are also important factors in OI that is caused by other collagen mutations as well as by mutations in other proteins. Over the past 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 dysplasias caused by mutations in cartilage-associated protein (CRTAP), prolyl-3-hydrohylase (P3H1), cyclophilin B (CYPB), the collagen-binding molecular chaperone FKBP65, the signaling protein WNT1, the ER–membrane ion channel TRICB, Golgi-membrane metalloprotease S2P, and the transmembrane anterior posterior transformation protein 1 (TAPT1). 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. 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. Our study of TRICB–deficient cells revealed abnormal conformation and reduced thermal stability of type I procollagen, suggesting dysregulation of collagen chaperones in the ER or direct involvement of TRICB in procollagen folding. Our experiments indicated that pathogenic effects of mutations in the transmembrane protein TAPT1 and in site-2 metalloprotease (S2P) might not be directly related to disruptions in synthesis, folding, or trafficking of procollagen chains (Reference 3).

More recently, in collaboration with Carsten Bonnemann, we investigated collagen biosynthesis abnormalities caused by mutations in collagen prolyl-4-hydroxylase 1 (P4H1), which result in complex

developmental abnormalities involving bones and other connective tissues (Reference 4). As expected, we found that patient skin fibroblasts secreted procollagen with significantly reduced thermal stability. However, we found no abnormalities in the procollagen folding or secretion rates and no evidence of misfolded procollagen accumulation in the cell. The latter findings are extremely surprising given that mutant P4H1 results in significantly reduced proline 4-hydroxylation, which is believed to be important for procollagen triple helix folding. We hypothesize that the folding rate is normalized through some compensatory action of procollagen chaperones. Consistently, our ongoing experiments point to abnormal composition of ER chaperones, but much remains to be done before we understand how patient fibroblasts manage to fold and secrete a normal amount of procollagen despite the P4H1 deficiency and how this deficiency causes connective tissue pathology.

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 alpha1(I) chains and one alpha2(I) chain. Homotrimers of three alpha1(I) chains are produced in some fetal tissues, carcinomas, fibrotic tissues, as well as in rare forms of OI and EDS associated with alpha2(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' 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 3–5 years, the focus of the study has shifted to abnormal differentiation of osteoblastic cells and deposition of bone within these tumors. 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 an 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 (Reference 5). 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 with leading commercial instruments. The HD technology was essential for the 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 of composition effects on the function of cartilage in a mouse model of diastrophic dysplasia (DTD), an autosomal recessive dysplasia that affects cartilage and bone development and is 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 we 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 gene encoding 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 Basser on using 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.

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The Neuronal Stress Response in Neurodegenerative Disease and Pain

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The launching point for our research is the dual leucine zipper kinase (DLK, also known as Map3k12), which is a critical regulator of the neuronal stress response to injury. Although the DLK stress response has been more extensively studied in the context of neuronal development as well as acute physical injury, such as nerve crush, we found that it also becomes activated in mouse models of neurodegenerative disease. DLK signaling directs a transcriptional program allowing the neuron to respond to the insult, which can manifest as degeneration as well as regeneration, depending on the context. We study DLK activation in mouse models of neurodegeneration as well as in acute surgical models, such as sciatic nerve injury. Our overall goal is to better understand (1) the mechanisms of DLK activation in disease, (2) the factors influencing degenerative versus regenerative outcomes, and (3) how the DLK pathway correlates with other pathways of cellular stress.

We recently showed that DLK/JNK signaling becomes activated in several animal models of neurodegenerative disease, and that deleting DLK or inhibiting it is protective and can delay, and even in some cases reverse, disease progression (Reference 1). The mouse models examined in this study included the SOD1(G93A) model of amyotrophic lateral sclerosis (ALS), and two mouse lines that model aspects of Alzheimer's disease (AD), a PS2APP model and the Tau(P301L) model. We also correlated these findings with evidence of DLK/JNK signaling in human tissue of ALS and AD patients. Although these diseases are characterized by very distinct genetic and pathological features, loss of DLK signaling appeared to protect neurons in both types of models. This work strongly suggested that DLK signaling is a pathway common to at least several distinct neurodegenerative diseases and can potentially act as an integrator of neuronal stress signaling.

Previous work had shown that DLK is a neuronally enriched kinase and an upstream regulator of the well studied JNK (c-Jun N-terminal kinase) signaling pathway. The function of DLK is critical for healthy developmental neurodegeneration as well as for an appropriate response to acute nerve injury. Given that the DLK response appears to be a feature common to so many different contexts and diseases, we aim to understand the mechanism(s) by which DLK activation

occurs, especially in pathological settings of chronic disease. We also seek a better understanding of the events downstream of DLK. For example, DLK signaling directs the transcriptional activation of genes associated with both degeneration and regeneration. We wish to determine what determines the ultimate outcome for the neuron, whether this response varies from cell to cell, or within a given neuron over time, whether DLK is a true integrator of cellular stress, and how it correlates with these other stress pathways.

Additional Funding

- DDIR Innovation Award

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The Biological Impact and Function of Transposable Elements

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 show clearly that integration occurs into specific types of sequences and that the targeting patterns vary depending on the specific 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. It is therefore essential to understand the mechanisms that position such integration. Our current work adopts methods of high-throughput sequencing to study dense integration patterns of model elements such as the long terminal repeat (LTR) retrotransposon Tf1 of *Schizosaccharomyces pombe.* This model element allows us to study integration mechanisms using highly informative techniques of yeast genetics (Reference 1). As an example, we generated an expression technique that tags each integration with a highly specific serial number. With this method, we sequenced 500,000 independent integration events. The improvements we made in sequencing technology are general and allowed us to generate dense profiles of HIV-1 integration. Our analyses of these datasets has greatly improved our understanding of integration and the mechanisms that select insertion sites.

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

Our initial use of deep sequencing revealed that Tf1 integration favors the promoters of RNA polymerase II (RNA pol II)–transcribed genes. In particular, the promoters of stress-response genes are strong targets. As DNA sequencing methods improved, it became possible to map a million integration events of Tf1 within *S. pombe.* A significant shortcoming of these dense maps of integration is the inability to measure repeated insertions at specific nucleotide

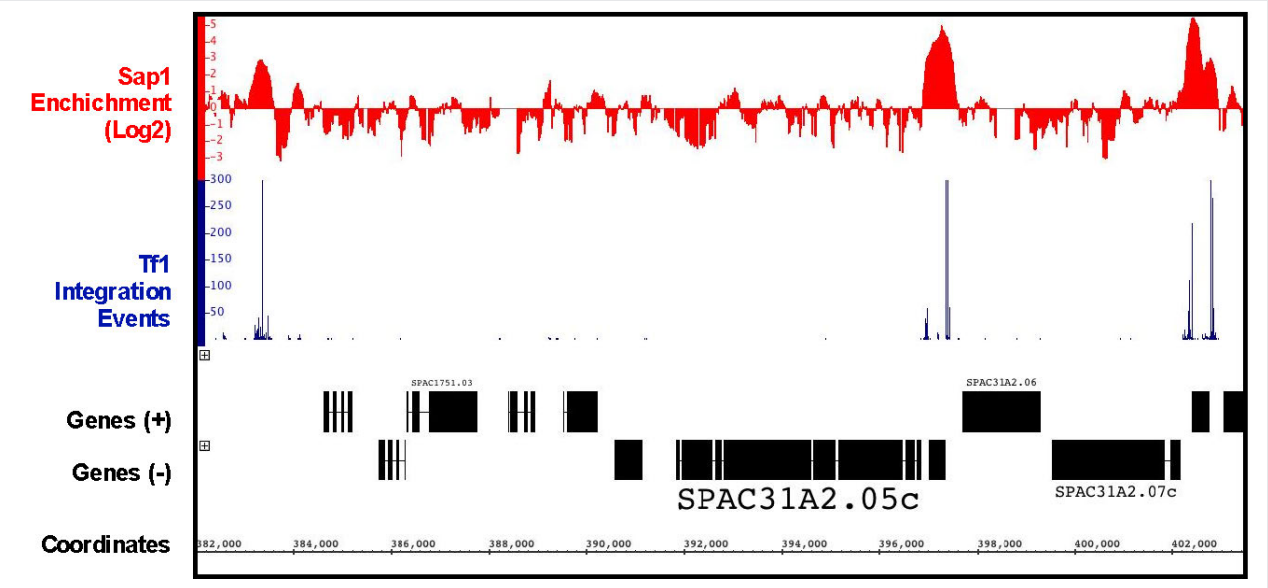

FIGURE 1. Serial number integration data correlates with the position of Sap1 enrichment from ChIP-seq data.

A representative segment of chromosome 1 is shown.

positions. This is because we and others discard duplicate sequence reads to avoid PCR–generated distortion. We addressed the problem by including a random eight-nucleotide serial number in the LTR of Tf1. With this method we can count the number of independent insertions at single nucleotide positions. While the serial number system identified specific sequence locations with high integration efficiency, sequence itself did not account for the selection of promoters. We had tested the transcription factors known to activate stress-response promoters and found that 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. 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 lower 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-target integration. Indeed, serial number sequencing of integration in cells with the *sap1-1* mutation showed position changes in 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. If Sap1 were directly responsible for positioning Tf1 integration, we would

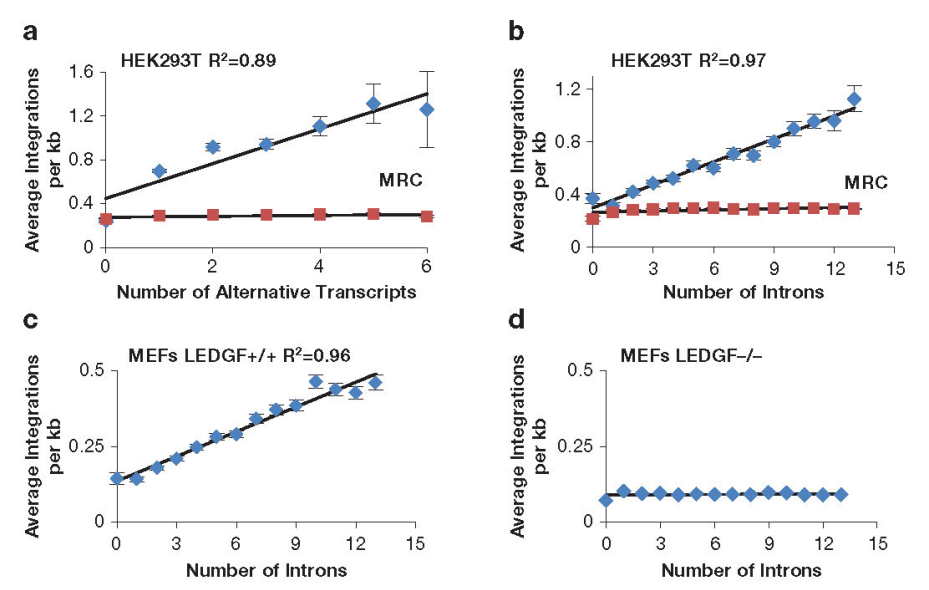

FIGURE 2. Integration density in transcription units correlates with amounts of splicing.

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 host factor LEDGF (C and D). MEFs, mouse embryonic fibroblasts; MRC, Matched Random Control.

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.

Host factors that promote retrotransposon integration are similar in distantly related eukaryotes.

Retroviruses and LTR retrotransposons have distinct patterns of integration sites. The oncogenic potential of retrovirus-based vectors used in gene therapy is dependent on the selection of integration sites associated with promoters. The LTR-retrotransposon Tf1 of *Schizosaccharomyces pombe* is studied as a model for oncogenic retroviruses because it integrates into the promoters of stress-response genes. Although integrases (INs) encoded by retroviruses and LTR retrotransposons are responsible for catalyzing the insertion of cDNA into the host genome, distinct host factors are required for the efficiency and specificity of integration. Our finding that Sap1 is located at positions of integration but does not interact with integrase suggested that other host factors are required for integration. We tested this hypothesis with a genome-wide screen of host factors that promote Tf1 integration. By combining an assay for transposition with a genetic assay that measures cDNA present in the nucleus, we could identify factors that

contribute to integration. We used this assay to test a collection of 3,004 *S. pombe* strains with single gene deletions (Reference 3). Using these screens and immunoblot measures of Tf1 proteins, we identified a total of 61 genes that promote integration. The candidate integration factors participate in a range of processes including nuclear transport, transcription, mRNA processing, vesicle transport, chromatin structure, and DNA repair. Two candidates, Rhp18 and the NineTeen complex, were tested in two-hybrid assays and were found to interact with Tf1 IN. Surprisingly, a number of pathways we identified were found previously to promote integration of the LTR retrotransposons Ty1 and Ty3 in *Saccharomyces cerevisiae,* indicating that the contribution of host factors to integration is common among distantly related organisms. The DNA repair factors are of particular interest because they may identify the pathways that repair the single-stranded gaps opposite integration sites of LTR retroelements.

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 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 the retrotransposon 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 that 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 the Gag retroviral gene 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 the transcription factor 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 the retrotransposon Tf1 is expressed, it integrates into promoters of RNA Pol II–transcribed genes and, in many cases, this increases transcription of adjacent genes. This 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 insertions that improve survival of environmental stress. We tested this hypothesis by studying the fitness of cells with genomic insertions of Tf1 when exposed to stress. Diverse cultures containing Tf1 integrated at 42,000 positions were grown competitively in cobalt. The proportion of cells with Tf1 at 141 positions greatly increased, 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 identify mechanisms that mitigate the toxicity of cobalt.

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

The promise of immunotherapy of cancer using 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 integrase (IN) interacts with the host factor LEDGF/p75 (a chromatin-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 tissue culture 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,D). 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 Matthew Plumb and Mamuka Kvaratskhelia used tandem mass-spectrometry (MS-MS) to identify cellular proteins from nuclear extracts of HEK293T cells that interacted with GST-LEDGF/p75 (LEDGF/p75 tagged with glutathione *S*-transferase). 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 *PSIP1,* the gene encoding LEDGF/p75. Analysis of 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 in large numbers of transcription units. These 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.

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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 the following questions, which are relevant to the development of all animal oocytes: the nature of strategies the oocyte uses to protect itself against inappropriate DNA replication; how the oocyte inhibits mitotic activity before meiotic maturation and the full growth and development of the egg; and how 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 and metabolic program of the developing ovarian cyst.

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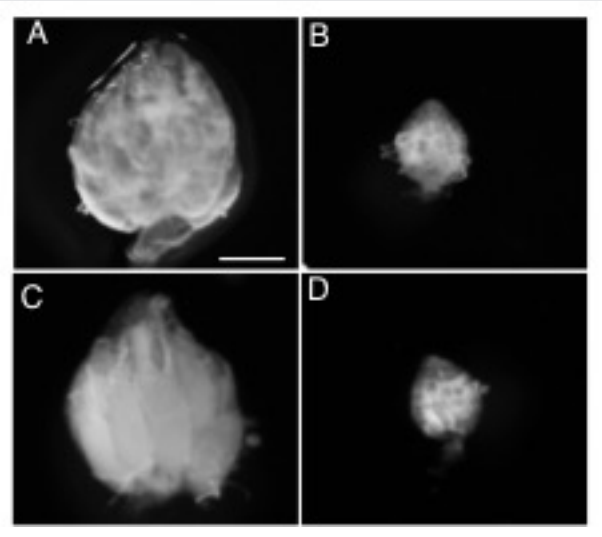

FIGURE 1. *seh1* RNAi depletion and co-depletion phenotypes

Representative images of (A) wild-type (WT) (large ovary), (B) nanos>*seh1*^{RNAi} (small ovary), (C) nanos>*seh1*^{RNAi}, nprl2RNAi (large ovary), (D) nanos>*seh1*^{RNAi}, mCherryRNAi (small ovary). Note that co-depletion of *nprl2* rescues the *seh1*^{RNAi} phenotype while co-depletion of *mCherry* fails to rescue the *seh1*^{RNAi} phenotype.

The 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, which responds to several upstream signals, including nutrient availability, energy status, and growth factors. In the past year, we defined the role of the GATOR complex in the regulation of metabolic homeostasis, autophagic flux, endomembrane dynamics, and oocyte development. Our findings define *Drosophila* as an excellent model for the genetic dissection of the GATOR complex and its role in preventing the developmental defects and pathologies associated with deregulated TORC1 activity. Additionally, they clearly establish a conserved role for the GATOR complex in the regulation of meiotic progression and gametogenesis.

The GATOR complex comprises two sub-complexes. The GATOR1 complex inhibits TORC1 activity in response to amino acid starvation by preventing the targeting of TORC1 to lysosomes, where the complex is activated by the GTP–binding protein Rheb. GATOR1 is a trimeric protein complex, consisting of the proteins Nprl2, Nprl3, and Iml1. Recent evidence from yeast and mammals indicates that the components of the GATOR1 complex function as GTPase–activating proteins (GAP) that inhibit TORC1 activity by inactivating the Rag GTPases, which are required for TORC1 lysosomal recruitment. Notably, *Nprl2* and *Iml1* are tumor suppressor genes while mutations in *Iml1*, known as *DEPDC5* in mammals, are the leading cause of hereditary epilepsy.

The GATOR2 complex comprises five proteins: Seh1, Sec13, Mio, Wdr24, and Wdr59. Our work, as well as that of others, found that the GATOR2 complex activates TORC1 by opposing the TORC1–inhibitory activity of GATOR1. Intriguingly, computational analysis indicates that Mio and Seh1, as well as several other members of the GATOR2 complex, have structural features consistent with coatomer proteins and membrane-tethering complexes. In line with the structural similarity to proteins that influence membrane curvature, we showed that three components of the GATOR2 complex, Mio, Seh1, and Sea2, localize to the outer surface of lysosomes, the site of TORC1 regulation. However, how GATOR2 inhibits GATOR1 activity, thus allowing for the robust activation of TORC1, remains unknown. Additionally, the role of the GATOR1 and GATOR2 complexes in both the development and physiology of multicellular animals remains poorly defined. Over the last year, we used molecular, genetic, and cell-biological approaches to define the role of the GATOR complex in the regulation of *Drosophila* development and physiology.

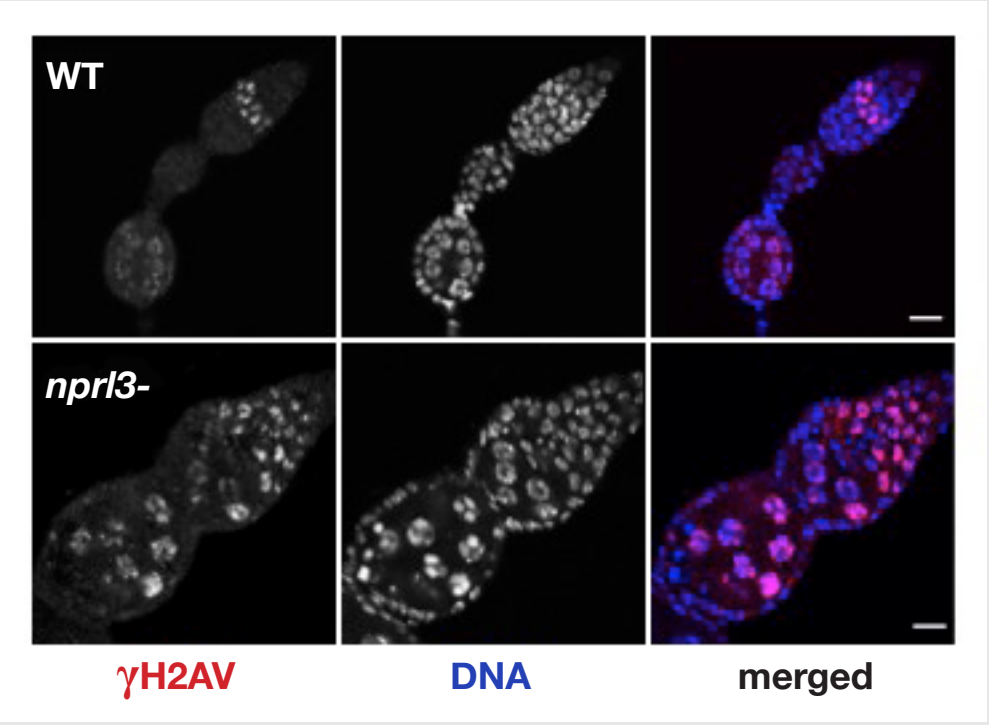

FIGURE 2. *nprl3*–mutant ovarian cysts have elevated levels of DNA damage.

Note that WT ovarian cysts rapidly repair meiotic double-stranded breaks, as indicated by the loss of gamma-H2Av in the early germarium. In contrast, *nprl3* mutants retain high levels of gamma-H2Av into late stages of oogenesis.

A genetic screen for new regulators of the GATOR complex

Mutations in the GATOR2 components *mio* and *seh1* cause the constitutive activation of the GATOR1 complex in the female germ line, resulting in permanent inhibition of TORC1 activity and a block to oocyte growth and development. We determined that germline RNAi depletions of any of the GATOR1 components in the *mio* and *seh1* mutant backgrounds relieve this permanent TORC1 inhibition and rescue the *mio* and *seh1* ovarian phenotypes. Over the last year, we used this epistatic relationship to conduct a high-throughput RNAi–based screen to identify upstream regulators and downstream effectors of the GATOR1 complex during oogenesis. First, we determined that expressing a short hairpin RNA against the *seh1* transcript, using the *nanos-Gal4* germline-specific driver, recapitulates the *seh1* mutant ovarian phenotype. Moreover, co-depleting *nprl2*, *nprl3*, and *iml1* dramatically rescues the *seh1*^{RNAi} ovarian phenotype (Figure 1). In order to identify additional genes that, when co-depleted with *seh1,* rescue the *seh1*^{RNAi} ovarian phenotypes, we used RNAi lines from the Transgeneic RNAi Project (TRiP) that have been optimized for germline expression. This co-depletion screen identified an array of new GATOR–interacting genes. Importantly, as anticipated, the screen identified several known regulators of TORC1, including Tsc1, Tsc2, and PTEN. Additionally, we identified many genes that function in translation, transcription, and the DNA–damage response that had not previously been implicated in metabolic regulation. These newly identified GATOR–interacting genes will provide a framework for our future studies on the regulation and function of the GATOR complex during oogenesis.

The GATOR1 complex promotes genomic stability during the early meiotic cycle.

Meiosis must accomplish two seemingly incompatible goals. First, it must faithfully copy and distribute genetic material to the next generation. Second, it must promote genomic diversity through meiotic

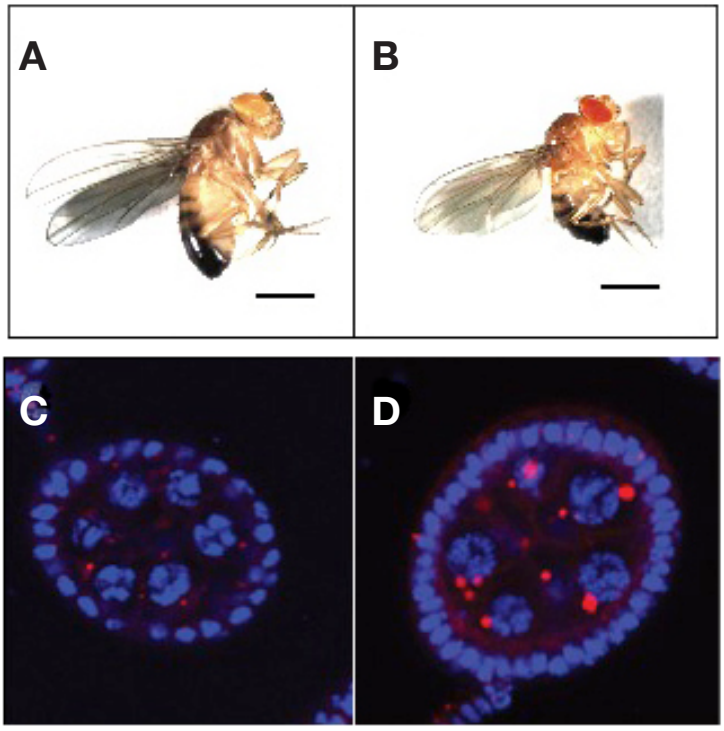

FIGURE 3. The GATOR2 component Wdr24 regulates growth and autophagy.

Representative images of (A) WT and (B) *wdr24*^{1}–mutant adult males. *wdr24*^{1} males are notably smaller than WT males. Size bar is 100 µm. Representative images of (C) WT and (D) *wdr24*^{1} egg chambers from well fed females stained with DAPI to mark DNA (*blue*) and LysoTracker (*red*) to mark autolysosomes. Note that *wdr24*^{1} egg chambers accumulate large numbers of autolysosome-like structures under nutrient-replete conditions.

recombination, a process initiated by the production of DNA double-stranded breaks. In our previous work, we demonstrated that, in *Drosophila* females, the timely entry into meiosis requires the down-regulation of TORC1 by the GATOR1 complex. However, precisely why a nutrient sensor has a retained an essential role in the regulation of the early meiotic cycle in a metazoan remained unclear. One model posits that, in addition to promoting meiotic entry, the GATOR1 complex might facilitate other events of the early meiotic cycle such as the highly conserved events of meiotic recombination. The controlled generation of DNA double-stranded breaks by the Spo11 enzyme initiates meiotic recombination in early prophase of meiosis I.

We determined that the GATOR1 complex promotes genomic stability in the presence of meiotic double-stranded breaks in both *Drosophila melanogaster* and mouse. Specifically, *GATOR1*–mutant oocytes exhibit a dramatic increase in the steady-state number of double-stranded breaks (Figure 2). Moreover, in contrast to wild-type oocytes that repair their double-stranded breaks prior to oocyte growth, GATOR1–mutant oocytes retain double-stranded breaks into late states of oogenesis. In GATOR1–mutant ovarian cysts, the increase in unrepaired DNA double-stranded breaks is accompanied by an increase in p53 activity, as monitored by a p53 reporter construct. p53 is a highly-conserved transcription factor that mediates a response to genotoxic stress. Strikingly, inhibiting the generation of meiotic double-stranded breaks strongly suppressed p53 activity in the *nprl3*$^{-/-}$–mutant background. From these data, we conclude that the GATOR1 complex is required to maintain genomic stability in the presence of meiotic double-stranded breaks and is thus essential for oocytes to safely complete meiotic recombination. Finally, in line with a conserved function for the GATOR1 complex in mammals, we showed that the GATOR1 component DEPCD5/Iml1 regulates meiotic double-stranded breaks in

mouse spermatocytes. Taken together, our data support a model in which the GATOR1 complex has retained a conserved role in the early meiotic cycle and early gametogenesis in metazoans.

The GATOR2 component Wdr24 has both TORC1–dependent and –independent functions.

While the GATOR1 complex has been implicated in a wide array of human pathologies, including cancer and hereditary forms of epilepsy, the *in vivo* relevance of the GATOR2 complex remains poorly understood in metazoans. We defined the *in vivo* role of the GATOR2 component Wdr24 in *Drosophila*. Using a combination of genetic, biochemical, and cell-biological techniques, we demonstrated that Wdr24 has both TORC1–dependent and TORC1–independent functions in the regulation of cellular metabolism. Through the characterization of a null allele, we found that Wdr24 is a critical effector of the GATOR2 complex required for the robust activation of TORC1 and cellular growth in a broad array of *Drosophila* tissues (Figure 3). Additionally, epistasis analysis between *wdr24* and genes that encode components of the GATOR1 complex revealed that Wdr24 has a second critical function: the TORC1–independent regulation of lysosome dynamics and autophagic flux. Notably, we found that two additional members of the GATOR2 complex, Mio and Seh1, also play a TORC1–independent role in the regulation of lysosome function. The results represent a surprising and previously unrecognized function of GATOR2 complex components in the regulation of lysosome structure and function. Consistent with our findings in *Drosophila*, through the characterization of a *wdr24*$^{-/-}$ knockout HeLa cell line, we determined that Wdr24 promotes lysosome acidification and autophagic flux in mammalian cells. Taken together, our data support a model in which Wdr24 is a key effector of the GATOR2 complex, required for both TORC1 activation and the TORC1–independent regulation of lysosomes. Moreover, our data raise the interesting possibility that the GATOR2 complex regulates GATOR1 through the control of lysosome structure and/or function.

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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 support 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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ER structure and dynamics visualized with increased spatio-temporal resolution

The endoplasmic reticulum (ER) consists of interconnected tubules and flattened sheets that extend from the nuclear envelope to the periphery of the cell, impacting every cellular compartment through

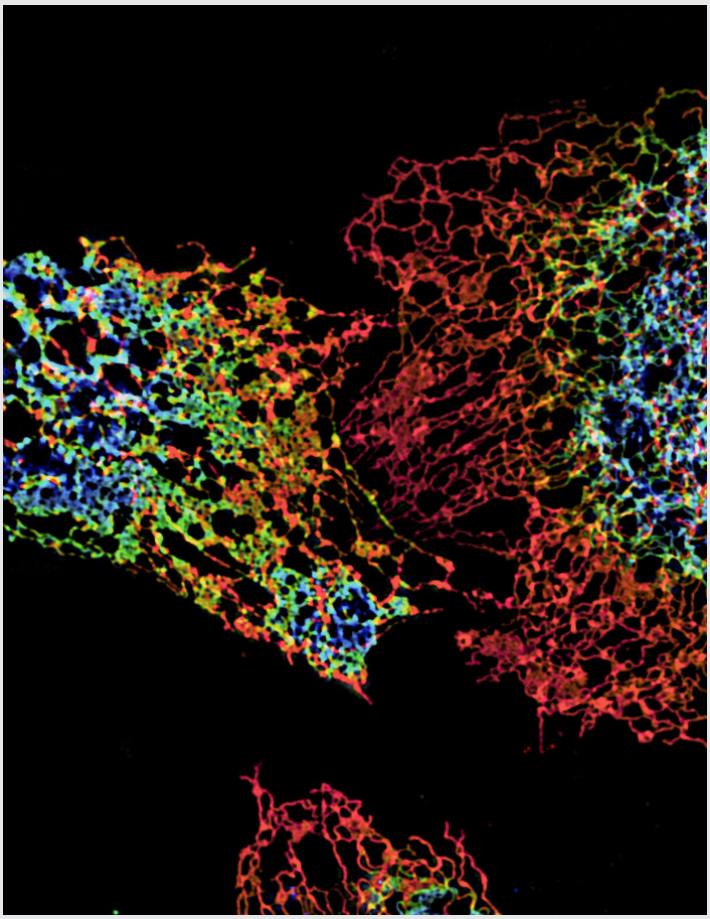

FIGURE 1. ER structure visualized using structured illumination microscopy (SIM)

Structure of peripheral endoplasmic reticulum (ER) in three cells labeled with an ER protein marker visualized by three-dimensional SIM. The color coding represents different z heights of the ER.

its contacts and functional interactions. Mutations in proteins that regulate the shape of the ER lead to various neurological disorders. We employed five different super-resolution technologies with complementary strengths and weaknesses in spatial and temporal capabilities to study the fine morphology and dynamics of the peripheral ER. A high-speed variation of structured illumination microscopy (SIM) allowed ER dynamics to be visualized at unprecedented speeds and resolution. Three-dimensional SIM (3D-SIM) and Airyscan imaging allowed comparison of the fine distributions of different ER–shaping proteins. Lattice light sheet point accumulation for imaging in nanoscale topography (LLS-PAINT) and focused ion-beam scanning electron microscopy (FIB-SEM) permitted 3D characterization of various ER structures. Using these approaches, we observed that many ER structures previously proposed to be flat membrane sheets are instead densely packed tubular arrays, which we call ER matrices. The matrices were extremely compact, with spaces between tubules below the resolving power of most super-resolution methodologies. We also discovered that ER tubules and junctions undergo fast oscillations, rapidly interconverting from tight to loose arrays. The oscillations of tubules and junctions were energy-dependent and allowed the ER to interconvert between tight and loose tubule networks. Our finding of dense tubular matrices in areas previously thought of as flat sheets provides a new model for maintaining and generating ER structure. In this model, ER matrices would sequester excess membrane proteins and lipids, and their dynamic interconversion into loose tubule arrays would permit the ER to rapidly extend its shape to reach the cell periphery, for example during cell locomotion.

ER trapping reveals that Golgi enzymes continually revisit the ER through a recycling pathway that controls Golgi organization.

The Golgi apparatus is the major processing and sorting station at the crossroads of the secretory pathway, containing specialized sorting and transport machinery, which either drive secretory trafficking or filter

out selected membrane and protein components for return to the ER for continued use. How the Golgi maintains its structure and function amidst this ongoing bi-directional membrane trafficking has been the subject of a long-standing debate. In particular, it is unclear whether Golgi enzymes remain localized within the Golgi or constitutively cycle through the ER.

To address this question, we used a rapamycin trapping assay to test whether Golgi enzymes become trapped in the ER upon expression of both an ER–trapping protein and Golgi enzyme bait. We found that, within four hours of rapamycin treatment, Ii-FKBP12 (i.e., the ER trapper) trapped nearly all Golgi-localized FKBP-rapamycin binding domain (FRB)–Golgi enzyme (i.e., the Golgi bait) in the ER. Direct redistribution from the Golgi to the ER during rapamycin treatment occurred because selective photoactivation of the FRB–tagged, photoactivatable Golgi enzyme in the Golgi resulted in the signal shifting to the ER. In the ER, fluorescent forms of FRB–Golgi enzyme and Ii-FKBP12 underwent FRET (fluorescence energy transfer), indicating direct binding upon rapamycin-induced redistribution. By contrast, use of Ii-FRB, which we showed is an inefficient ER trap, resulted in minimal redistribution of FKBP12–tagged Golgi enzymes during rapamycin treatment. These data demonstrated that Golgi enzymes constitutively cycle through the ER. Using our trapping scheme, we further identified roles of the protein Rab6a and the phospholipase iPLA $_2$ in Golgi enzyme recycling and showed that retrograde transport of Golgi membrane is necessary for Golgi dispersal during microtubule depolymerization and mitosis.

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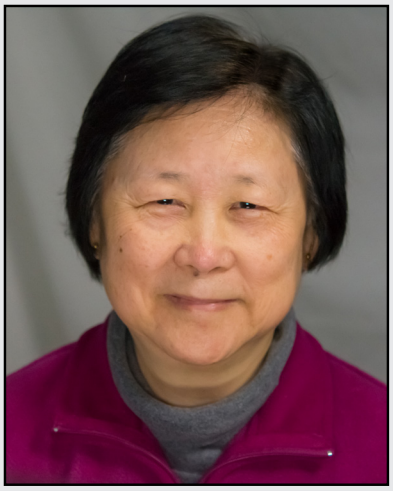
Neurosecretory Proteins in Neuroprotection, Neurodevelopment, and Cancer

We study the cell biology of neuroendocrine cells and the function of neuropeptides and the neurotrophic factor carboxypeptidase E (CPE)/Neurotrophic Factor-alpha1 (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 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 cloned a 40kD N-terminal truncated isoform of CPE/NF-α1 (CPE-deltaN) from embryonic mouse brain and hepatocellular carcinoma cancer cells 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 neurodegenerative diseases, memory, learning, depression, cardiac function, obesity, and metastasis in cancer.



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Mechanism of sorting, transport, and regulated secretion of neuroproteins

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, also known as 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 carboxypeptidase E (CPE) and then secreted. We showed that the sorting of prohormones to the RSP occurs by a

receptor-mediated mechanism. Site-directed 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 also investigated the role of secretogranin III (SgIII) as a surrogate sorting receptor for membrane CPE in 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; 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. Overexpression of the CPE tail inhibited the movement of BDNF– and POMC/CPE–containing vesicles to the processes in hippocampal neurons and pituitary cells, respectively. The transmembrane CPE tails on the POMC/ACTH and BDNF vesicles anchor these organelles, which interact with dynactin and the microtubule-based motors KIF1A/KIF3A to effect anterograde vesicle movement to the plasma membrane. Recently, in collaboration with Josh Park, we showed that another player, snapin, binds directly to the cytoplasmic tail of CPE and connects to the microtubule motor complex, consisting of dynactin and kinesin-2, to mediate the post-Golgi transport of POMC/ACTH vesicles to the process terminals of AtT20 cells for activity-dependent secretion. Our study has thus uncovered a novel complex for secretory vesicle transport in neuroendocrine cells.

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 enhancing LDCV formation. We also identified modified forms of serpinin, pyroglutamyl-serpinin (pGlu-serpinin), and serpinin-RRG, a C-terminally extended form, in the 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 found that pGlu serpinin is a powerful cardio-protectant after ischemia. The mechanism involved the activation of the reperfusion injury salvage kinase (RISK) pathway. In collaboration with Angelo Corti, we showed that serpin-RRG had anti-angiogenic activity.

Role of CPE/NF-1 in neuroprotection and anti-depression during stress

We generated a CPE/NF-α1 knock-out (KO) mouse to study the function of CPE *in vivo.* We found that this KO mouse exhibited obesity, infertility, and diabetes, as well as learning and memory deficits and depressive-like behavior. Interestingly, a null mutation in the gene encoding CPE/NF-α1 was recently identified in a female who has clinical features such as obesity, type 2 diabetes, learning disabilities, and hypogonadotrophic hypogonadism, similar to the *Cpe*-KO mouse, indicating the importance of CPE. Using the *Cpe*-KO mice as a model in which to study its nervous system deficiencies, as well as Morris water maze and object-preference tests, we showed defects in learning and memory and, in the forced swim test, depressive-like behavior. Analysis of the brain of 6- to 14-week-old *Cpe*–KO mice revealed poor dendritic pruning in cortical and hippocampal neurons, which could affect synaptogenesis. Electrophysiological measurements showed a defect in the generation of long-term potentiation in hippocampal slices. A major cause of the defects is the loss of neurons in the CA3 region of the hippocampus. Hippocampal neurons in CA3 region are enriched in CPE and were normal at three weeks of age just before weaning, indicating that the defect was not developmental. The degeneration is likely caused by epileptic-like neuronal firing, releasing large amounts of glutamate during weaning stress. Hence, CPE/NF-α1 is important for the survival of the CA3 neurons. We then showed that CPE/NF-α1, either overexpressed or applied externally to cultured hippocampal or cortical neurons, protected these neurons from apoptosis induced by oxidative stress with hydrogen peroxide. Moreover, a non-enzymatically active form of CPE/NF-α1 had the same effect, indicating that its action is independent of enzymatic activity. We propose that CPE/NF-α1 acts extracellularly as a signaling molecule by binding to a receptor to mediate neuroprotection. To this end, we demonstrated that ^{125}I CPE/NF-α1 bound to HT22 cells, an immortalized hippocampal neuronal cell line, in a saturable manner, and that the binding was specifically displaced by non-iodinated CPE/NF-α1 but not by bovine serum albumin, suggesting the existence of a receptor. We are currently screening a G protein–coupled receptor (GPCR) library for binding activity to CPE to try to identify a receptor.

The mechanism of action of CPE/NF-α1 in neuro-protection involves the activation of the ERK1/2 and the Akt signaling pathways, which then leads to enhanced transcription/translation of a pro-survival mitochondrial protein, Bcl2, inhibition of caspase 3 activation, and promotion of neuronal survival (Reference 1). Furthermore, this CPE/NF-α1–mediated neuroprotection pathway is activated by rosiglitazone, a PPARg ligand, which binds to PPARg binding sites in the CPE promoter. Examination of the pathway during stress *in vivo* revealed that, after mild chronic restraint stress (CRS) for 1h/day for seven days, mice showed significantly elevated levels of CPE/NF-α1 mRNA and protein, as well as of the anti-apoptosis protein Bcl2, in the hippocampus. *In situ* hybridization studies indicated especially elevated CPE/NF-α1 mRNA levels in the CA3 region and no gross neuronal cell death after mild CRS. Furthermore, primary hippocampal neurons in culture showed elevated CPE/NF-α1 and Bcl2 expression and a decline in Bax, a pro-apoptotic protein, after treatment with the synthetic glucocorticoid dexamethasone. This up-regulation was mediated by glucocorticoid binding to glucocorticoid-regulatory element (GRE) sites on the promoter of the *Cpe* gene. Thus, 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 an increase in Bcl2 in the hippocampus of *Cpe*-KO mice after CRS, leading to the degeneration of the CA3 neurons (Reference 2).

The relevance of CPE/NF-α1 in neuroprotection in humans was indicated by our studies on a mutation of the *CPE* gene found in an Alzheimer's disease (AD) patient (Reference 3). Our search in the GeneBank EST database identified a sequence entry from the cortex of an AD patient that had three adenosine inserts in the *CPE* gene, thereby introducing nine amino acids, including two glutamines, into the mutant protein, herein called CPE-QQ. Expression of CPE-QQ in Neuro2a cells indicates that it is not secreted. Co-expression of wild-type (WT) CPE and CPE-QQ in Neuro2a cells resulted in degradation of both forms of the protein and reduction in secretion of WT CPE. Immunocytochemical studies show that CPE-QQ stains in the perinuclear region of the cells and co-stains with Calnexin, an ER marker, consistent with localization of the mutant in the ER. Moreover, many cells appear rounded, indicating that they are unhealthy cells that might be undergoing ER stress, unlike the cells expressing WT, which show staining in the cell body and neurites. Overexpression of CPE-QQ in rat primary hippocampal neurons resulted in increased levels of the ER stress marker CHOP, reduced levels of pro-survival protein Bcl-2, and increased neuronal cell death, indicating that CPE-QQ induces cell death through ER stress and down-regulation of Bcl-2 expression. We then generated transgenic mice overexpressing CPE-QQ and showed that, at 50 weeks but not at 11 weeks, the animals exhibited memory deficits compared with WT mice, but that their spatial learning ability was unimpaired. The CPE-QQ mice were neither obese nor diabetic. However, they showed significantly fewer neurites in the CA3 region, the dentate gyrus of the hippocampus, and the medial prefrontal cortex, indicative of neurodegeneration. Moreover, they exhibited reduced neurogenesis in the subgranular zone and hyperphosphorylation of tau at ser^{395}, a hallmark of AD. The studies substantiated a neuroprotective role of CPE/NFα-1 in humans and identified a mutation in the *CPE* gene that can cause the neurodegeneration linked to AD and perhaps other forms of dementia.

Stress also induces depression. Huda Akil's group (University of Michigan) reported that fibroblast growth factor 2 (FGF2) is an anti-depressant. We found that prolonged (6h/day for 21 days) restraint stress reduced CPE/NFα-1 and FGF2 in the hippocampus of mice and induced depressive-like behavior. However, after short-term restraint stress, mice did not show depressive-like behavior despite elevated corticosterone levels indicative of stress. Moreover, hippocampal CPE/NFα-1, FGF2, and doublecortin, a marker for neurogenesis, were elevated in these mice, suggesting that the anti-depressive effects of CPE/NF-α1 are mediated through increased neurogenesis. Indeed, we found that exogenously applied CPE/NF-α1 could up-regulate FGF2 mRNA and protein expression in cultured hippocampal neurons, indicating that CPE/NF-α1 regulates FGF2 expression. CPE/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, CPE/NF-α1 prevents stress-induced depression by up-regulating hippocampal FGF2 expression, which leads to enhanced neurogenesis and anti-depressive activity (Reference 4). Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway (Reference 4). Roziglitazone has previously been shown to be effective in treating diabetic patients with bi-polar disorders.

CPE/NF−1 inhibits proliferation and induces differentiation of embryonic stem cells.

CPE/NFa-1mRNA is expressed in mouse embryos as early as day E5.5 and increases sharply at E12.5, in parallel with the development of the endocrine system, and continues to increase into adulthood. *In*

situ hybridization studies indicate that CPE/NF-α1 is expressed primarily in the forebrain in mouse embryos, suggesting a role of CPE/NF-α1 in neurodevelopment. We therefore began studying neural stem cell proliferation. Exogenous recombinant CPE/NF-α1 was added to E13.5 neocortex-derived neurospheres, which contain stem cells and neuroprogenitors. CPE/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. CPE/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 CPE/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 CPE/NF-α1 without alteration in the percentage of neuronal and oligodendrocyte populations. Interestingly, dissociated cells from neurospheres derived from *Cpe/Nf-α1*–KO mouse embryos showed fewer astrocytes but more neurons. *In vivo*, *Cpe/Nf-α1*–KO mouse cortex (at P1, the time of astrocytogenesis) showed 49% fewer astrocyte numbers than in WT animals, confirming the *ex vivo* data. Our results suggest a novel role for CPE/NF-α1 as an extracellular signal to inhibit proliferation and induce differentiation of neural stem cells into astrocytes, thus playing an important role in neurodevelopment (Reference 4).

Neurite outgrowth is key to the formation of synapses and the neural network during development. We found that CPE/NF-α1 prevented Wnt-3a inhibition of nerve growth factor (NGF)–stimulated neurite outgrowth in PC12 cells and cortical neurons. Moreover, CPE/NF-α1 augmented Wnt-5a–mediated neurite outgrowth. Thus, the interplay between NGF preventing neurite outgrowth, which is inhibited by Wnt-3a, and augmenting neurite outgrowth, which is mediated by Wnt-5a and CPE/NF-α1, could play an important role in regulating these positive and negative cues, which are critical for neurodevelopment.

Carboxypeptidase E/CPE-deltaN in tumorigenesis

Our studies indicate an important role of the CPE gene in mediating tumor growth, survival, and metastasis. We identified and cloned a novel splice isoform CPE (CPE-deltaN) mRNA, which encodes a 40kD N-terminal truncated protein 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. In nude mice, siRNA knockdown of CPE-deltaN expression in highly metastatic HCC cells inhibited their growth and metastasis. We found that 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. Recently, we showed that Panc-1 cells, a pancreatic cell line stably transfected with CPE-deltaN, exhibited enhanced proliferation and increased NEDD9 expression. Interestingly, WT CPE protein is poorly or not expressed in many epithelial cancer cell lines such as HCC and pancreatic cancer, but highly expressed in glioma cell lines and shown to promote proliferation, though not invasion. When transfected into Panc-1 cells at a comparable protein level to CPE-deltaN, WT CPE promoted proliferation, but had no effect on invasion. Thus, CPE-deltaN plays a dominant role in tumor growth and metastasis.

We carried out a prospective study to evaluate the role of CPE/CPE-deltaN mRNA as a biomarker for predicting recurrence in 120 HCC patients from the Liver Network patients in Taiwan. The study focused on Stage I and II patients, given that these patients generally have better prognosis, but whose 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/CPE-deltaN mRNA. The follow-up time ranged from 9 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 (Reference 7). The CPE/CPE mRNA expression level in HCC could therefore be a useful clinical biomarker for predicting tumor recurrence in HCC patients who are in an early pathology stage and able to receive curative resection.

Using circulating exosomes in humans, we are also developing a blood assay to determine the CPE/CPE-deltaN biomarker levels in cancer compared with normal controls. We found elevated levels of CPE/CPE-deltaN mRNA in secreted exosomes of different types of cancer cell lines. The CPE/CPE deltaN mRNA content in the exosomes was correlated with the metastatic potential of the cell lines. Thus, measuring CPE/CPE-deltaN mRNA in a human blood assay using exosomes could offer a non-invasive method for the diagnosis and assessment of treatment efficacy of cancer patients.

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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 several 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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The molecular mechanics of eukaryotic translation initiation

We completed our studies of the mechanism of action of the DEAD-box ATPase eIF4A in promoting mRNA recruitment to the eukaryotic translation pre-initiation complex (PIC). Our work showed that eIF4A stimulates recruitment of mRNAs to the PIC regardless of their degree of secondary structure. The result indicates a role for the factor beyond unwinding structures in the 5'-untranslated regions (5'-UTRs) of mRNAs. We also showed that the PIC, and the eIF3i and eIF3j subunits of eIF3 in particular, stimulate the ATPase activity of eIF4A. In addition, our data showed that mRNA structure on the 3'-side of the start codon inhibits mRNA recruitment in a manner relieved by eIF4A's ATPase activity, and that structure in the 5'-UTR and 3' to the start codon synergistically inhibit mRNA recruitment. Taken together, the results suggest a model whereby eIF4A acts to disrupt the overall structure of an mRNA rather than specific, stable secondary structures in the 5'-UTR. In addition, our data suggest that eIF4A plays a role in loading all mRNAs into the entry channel of the PIC, perhaps by modulating the conformation of the 40S ribosomal subunit. A manuscript describing this work is currently under revision.

We are also working on a manuscript describing our genome-wide analysis of the effects of temperature on the utilization of the cognate and near-cognate start codons of upstream open reading frames (uORFs) in yeast. Our data show that several mRNAs have uORFs that are differentially translated depending on temperature, although there is no global change in fidelity of start codon recognition. Translation of some uORFs is activated at higher growth temperatures, while translation of others is inhibited. The same dichotomy also holds true at reduced growth temperatures. The organization of the uORF within the 5'-UTR appears to exert an influence on this temperature-dependent regulation of translation.

We completed a collaborative project with Alan Hinnebusch to study the role of the small ribosomal subunit protein Rps3/uS3. Our results indicated that two arginine residues in the protein make stabilizing contacts with mRNA in the entry channel of the 40S subunit, augmenting a similar role played by eIF3 (Reference 2).

We also completed a collaborative project with Alan Hinnebusch, Venki Ramakrishnan, and Adesh Saini to elucidate the structural and mechanistic basis for the role of the *N*-terminal domain of eIF5 in start codon recognition. A manuscript is currently in preparation describing this study.

We are beginning work on a manuscript about the mechanism of action of the DEAD-box RNA-dependent ATPase Ded1. The work shows that Ded1's mechanism of action is distinct on different mRNAs and provides support for the proposal that the factor plays an important role in unwinding long, structured 5'-UTRs to promote mRNA loading onto the PIC.

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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 the potential role of 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 signaling in thymocyte development

Much of our research focuses on the role of T cell antigen receptor (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,

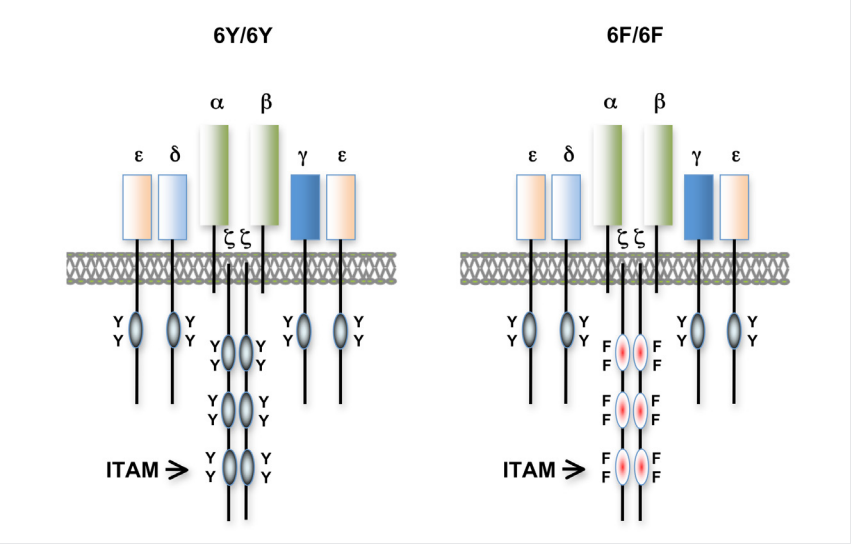


FIGURE 1. T cell antigen receptors expressed in 6Y/6Y and 6F/6F knock-in mice

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).

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 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 (such as regulated expression of other signaling molecules) 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 a 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

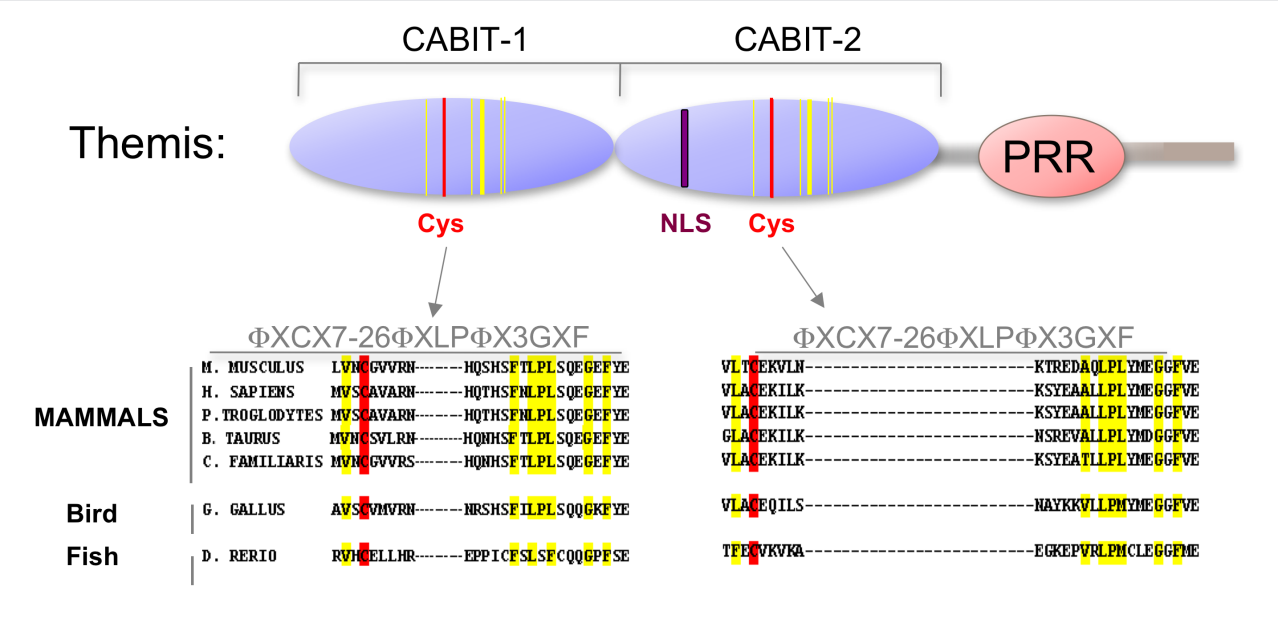


FIGURE 2. Themis is highly conserved in vertebrates.

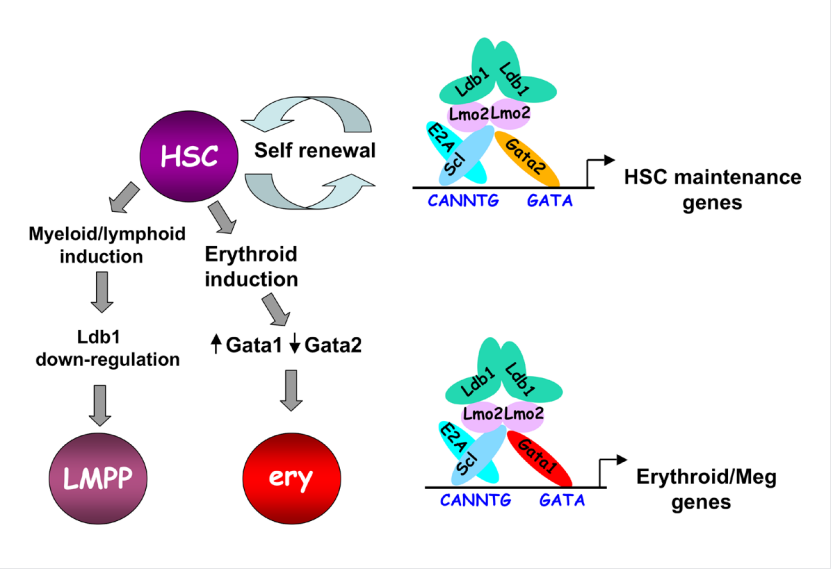
Themis contains two novel CABIT domains, each with a conserved cysteine (*red*) and conserved flanking residues (*yellow*), a nuclear localization signal (NLS), and a proline-rich region (PRR).

mediating specific, mature T cell responses. Experiments with the mice confirmed that the knockin zeta locus functions as predicted.

In the past year, we conducted an extensive evaluation of T cell effector responses in 6F/6F and control, 6Y/6Y mice. Consistent with our previous data, we found that 'general' T cell responses such as cytokine production and proliferation were not significantly impaired in 6F/6F mice. However, the TCR repertoire was skewed in 6F/6F mice, as assessed by TCRValpha usage. (We stained for the spectrum of TCR alpha chain usage on mature T cells with different Valpha–specific antibodies and found that the relative percentage of each Valpha was different in the 6Y/6Y and 6F/6F mice.) Despite this, we detected no obvious 'gaps' in the antigen-reactive TCR repertoire in 6F/6F mice (i.e., 6F/6F mice were able to mount T cell responses to a variety of antigens and pathogens and contained similar numbers of naive antigen-specific T cells, as assessed by tetramer enrichment). The generation of memory T cell subsets was also unimpaired in 6F/6F mice. However, 6F/6F mice exhibited a significant impairment in the generation of follicular helper T cells (TFH). The fact that the same defect was observed in 6F/6F:B6CD45.1 bone marrow chimeras confirmed that it was intrinsic to T cells and not secondary to the elevated numbers of suppressor T cells (Tregs) in 6F/6F mice. After we had established that 6Y/6Y and 6F/6F mice contain similar numbers of naive 2W peptide (an immunogenic peptide)–reactive CD4 T cells by tetramer enrichment, we observed the same TFH defect in mice infected with recombinant *Listeria monocytogenes* (Lm) that express a 2W peptide-OVA fusion protein (Lm-OVA), finally confirming that the defect was specifically attributable to generation of TFH cells and not a paucity of antigen-specific progenitors in 6F/6F mice. We are now 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.

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.



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 levels of CD5 throughout development. Over-expression 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 determining whether the protein's regulated expression during development is important for preventing autoimmunity. For that purpose, we generated a conditional CD5 deletion mouse in which CD5 expression can be removed before, during, or after T cell development. 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, now known as Themis1, a novel T cell–specific adapter protein (Figure 2). To investigate the function of Themis1 in T cell signaling and development, we generated Themis1-knockdown cell lines: Themis1 knockout mice (conventional and conditional), and Themis1-transgenic mice. Analysis of the effects of modulating Themis1 expression revealed a critical role for the protein in late T cell development. We obtained the following results: (1) The Themis1 paralog Themis2, which is expressed in B cells, can substitute for Themis1 in T cell development; we found that the ability of the B cell–specific family Themis member Themis2 was equivalent to that of Themis1 to restore normal T cell development in *Themis1*$^{-/-}$ mice, thus demonstrating functional redundancy of Themis1 and Themis2. (2) We generated retroviruses encoding domain-deletion mutants of Themis1, infected *Themis1*$^{-/-}$ bone marrow progenitors, and made bone marrow chimeras to determine which regions of Themis1 are important for *in vivo* function; we found that the Themis1 proline-rich sequence (PRS), which mediates binding to the signaling protein Grb2, was required for *in vivo* function, as assessed by rescue of the developmental block in *Themis1*$^{-/-}$ thymocytes, but that the CABIT (cysteine-containing, all beta in Themis)–domain cysteines are not essential. (3) We generated *Themis2*$^{-/-}$ mice and began a collaboration with Richard Cornall to characterize the mice; our results identified an important role for Themis2 in facilitating B cell activation by low avidity, but not high avidity, B cell receptor (BCR)–antigen interactions; Themis2 was required to elicit normal Ca^{2+} and signaling via the Erk pathway in response to low-avidity interactions and was necessary for positive selection of B1 cells and germinal center B cells by self and foreign antigens; we detected Themis2 in complexes with the signaling proteins Grb2, Lyn, and PLCγ2 and found that it was required for normal tyrosine phosphorylation of Lyn and PLC-gamma2; this subtle but clear phenotype of *Themis2*$^{-/-}$ mice was not detected in a previous and less extensive study of *Themis2*$^{-/-}$, which concluded that loss of Themis2 has no effect on B cell development or function.

Our findings show that the impact of loss of Themis1 and Themis2 on T and B cell development, respectively, is strikingly similar. In each case, the main effect is on positive selection, which is controlled by low-avidity antigen-receptor interactions. This, together with the ability of Themis2 to rescue T cell development in *Themis1*$^{-/-}$ mice, indicates that Themis1 and Themis2 perform similar functions in T and B cells.

In the past year, we focused on determining the molecular function of Themis1. Themis1, Themis2, and a large family of related metazoan proteins contain a novel globular domain of unknown function called the CABIT module (see above and Figure 2). Using cell transfection, biochemical, and protein assay techniques, we determined that CABIT modules bind to the catalytic domain of a key hematopoietic protein tyrosine phosphatase, SHP-1. In the presence of reactive oxygen species (ROS), which are generated in activated T cells, Themis1, via its CABIT module, promoted oxidation of the SHP-1 catalytic cysteine and therefore inactivated SHP-1. The CABIT modules from all five mammalian Themis-family proteins also inhibited SHP-1, indicating that this activity was common to the CABIT module. Given that SHP-1 is an inhibitory phosphatase that functions to dampen TCR signaling by de-phosphorylating multiple targets including protein tyrosine kinases, the finding established an activating function for Themis1 in cell signaling through its ability to bind to and inhibit SHP-1. Interestingly, Themis1 is highly expressed in developing thymocytes at the stage at which they undergo positive selection. It had been known for years that thymocytes were more sensitive to TCR stimulation than mature T cells, but the reason for this sensitivity was unknown. The function of Themis1, together with its high expression in thymocytes, provides an explanation for

the sensitivity of thymocytes to TCR signaling. We confirmed that the primary role of Themis1 is to inhibit SHP-1 by showing that deletion of the gene encoding SHP-1 rescues T cell development in Themis1$^{-/-}$ mice. In addition to identifying the function of CABIT modules, our results provide insight into the role of other CABIT–containing proteins in metazoans (which number in the hundreds). Our ongoing studies are focusing on further characterization of CABIT proteins and determining their role in development and their possible involvement in human disease.

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–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*$^{-/-}$ ESCs 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, transcription factors that are all essential to maintain the pluripotent ESC phenotype, 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 myeloid cells or lymphoid cells. Interestingly, ChIP-Seq analysis of Ldb1 DNA–binding complexes demonstrated that, in HSCs, Ldb1 complexes contain the transcription factor 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 aim to determine how Ldb1 complexes regulate gene expression and the role of Ldb1 dimerization in mediating long-range promoter-enhancer interactions in hematopoietic cells. In addition, we are 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 low in Hhex but express high levels of more mature markers of T cell development, including Notch1, Dtx1, Ptcra, and Hes1. Lmo2 functions as a subunit of the multimeric Ldb1–nucleated DNA–binding complexes described above. 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 recently determined that Ldb1 is required for *Lmo2*-tg–induced thymocyte self-renewal and T-ALL induction. Currently, we are addressing 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. 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

- Dr. Amy Palin is applying for an NIH K99 award.
- NICHD Director's Award

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Evolving Mechanisms of Transcriptional Control in Mammals

The "epigenome" refers to the heritable chemical changes in DNA and histone proteins that can be passed on through cell division and potentially across generations. Dramatic reprogramming of the epigenome begins at conception and continues throughout embryonic and postnatal development, underlying the gene-expression changes that drive the generation of diverse cell types that make up complex multicellular organisms. By exploring transcription factors that recognize parasitic mobile DNA elements to initiate stable heterochromatic silencing, we study how the epigenetic state of mammals is initially established in early development. We also study how one large family of these parasitic elements, the endogenous retroviruses (ERVs), play an important role in the development and evolution of new traits in mammals by rewiring gene expression networks. We also explore how the epigenetic state of a cell works in conjunction with combinatorial codes of transcription factors to generate the huge diversity of cell types required for mammalian development.

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 about 10% of our genomic DNA. The laboratory studies ERVs from two perspectives: first as parasites that must be kept in check by the host to prevent widespread viral activation and second, as symbionts that can be co-opted by the host for evolutionary advantage. Our long-term objective is to understand how the host has adapted recognition machinery to establish epigenetic silencing of ERVs, how ERVs sometimes evade these silencing mechanisms, and how this "arms race" between ERVs and the host have led to co-option of viral regulatory sequences that may have contributed to the evolution of mammals. We hypothesize that the rapidly diversifying KRAB-ZFP family (see below) plays a critical role in the recognition and silencing of ERVs and nearby genes, and we are taking systematic genetic and biochemical approaches to explore this function.

Kruppel-associated box zinc finger proteins (KRAB-ZFPs) have emerged as candidates that recognize ERVs. KRAB-ZFPs are rapidly evolving transcriptional repressors that emerged in a common

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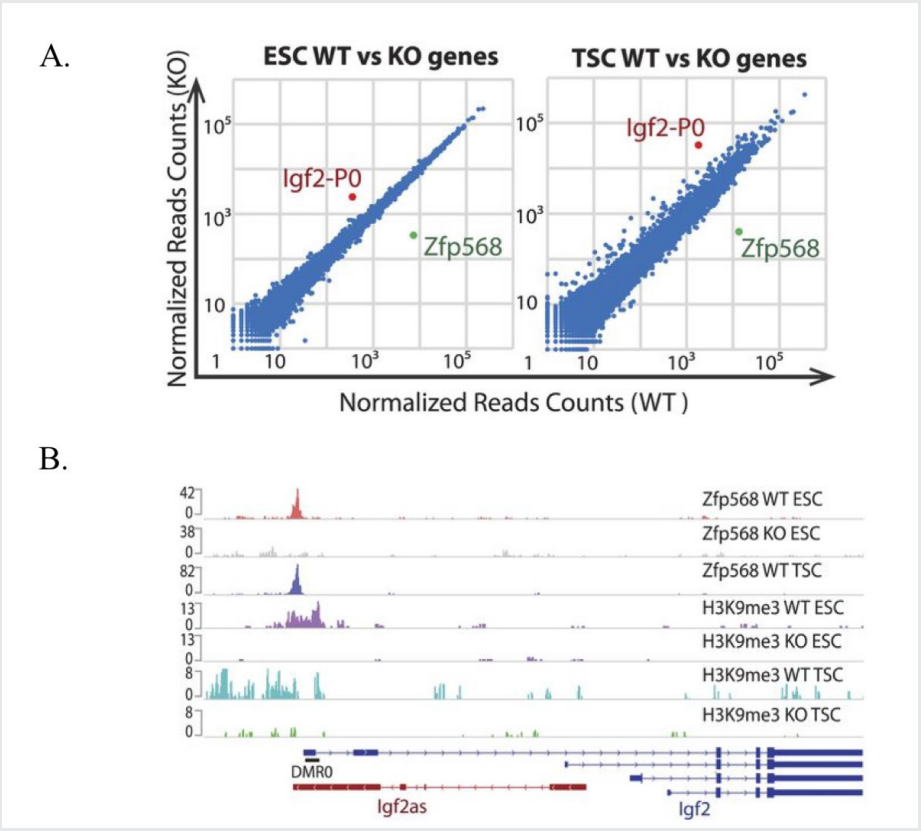

FIGURE 1. ZFP568 directly represses the Igf2-P0 transcript.

A. Scatter plots of gene expression in *Zfp568* WT and KO ESCs and TSCs, as determined by RNA-seq.

B. ZFP568 and H3K9me3 ChIP-seq signals at the *Igf2* locus in *Zfp568* WT and KO ESCs and TSCs. DMR0 is a differentially methylated region overlapping exon 1 of the *Igf2-P0* transcript. *Igf2as* is the *Igf2* antisense transcript.

ancestor of coelacanth, birds, and tetrapods. They make up the largest family of transcription factors in mammals (estimated to be several hundred in mice and humans). 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 their physiological functions. KRAB-ZFPs consist of an N-terminal KRAB domain that binds to the co-repressor KAP1 and a variable number of C-terminal C2H2 zinc finger domains that mediate sequence-specific DNA binding. KAP1 directly interacts with the KRAB domain, which recruits the histone methyltransferase (HMT) SETDB1 and heterochromatin protein 1 (HP1) to initiate heterochromatic silencing. 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. Second, the KRAB-ZFP protein ZFP809 was isolated based on its ability to bind to the primer binding site for proline tRNA (PBSPro) of murine leukemia virus (MuLV). Third, deletion of the KRAB-ZFP co-repressors *Trim28* or *Setdb1* leads to activation of many ERVs. We have thus begun a systematic interrogation of KRAB-ZFP function as a potential adaptive repression system against ERVs.

Given that it was originally identified as part of a repression complex that recognizes infectious MuLV via direct binding to the 18 nt Primer Binding Site for Proline (PBSpro) sequence, we focused on ZFP809 as a likely ERV–suppressing KRAB-ZFP. We hypothesized that ZFP809 might function *in vivo* to repress other ERVs that utilized the PBSpro. 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 PBSpro. We generated *Zfp809* knockout mice to determine whether ZFP809 was required for VL30pro silencing. We found that *Zfp809* knockout tissues displayed high levels of VL30pro 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 VL30pro integrations. Furthermore, using a combination of conditional alleles and rescue experiments, we determined that ZFP809 activity was required in development to initiate silencing, but not in somatic cells to maintain silencing. The studies thus provided the first demonstration for the *in vivo* requirement of a KRAB-ZFP in the recognition and silencing of ERVs.

As a follow-up to our studies on ZFP809, we have begun a systematic analysis of KRAB-ZFPs using a medium-throughput ChIP-seq screen and functional genomics of KRAB-ZFP clusters and individual KRAB-ZFP genes. Our ChIP-seq data demonstrate that the majority of recently evolved KRAB-ZFP genes interact with and repress distinct and partially overlapping ERV targets. This is supported by a recent knockout mouse line lacking about 17 KRAB-ZFPs (generated with CRISPR/Cas9 engineering) that displays an ERV reactivation phenotype.

Like ZFP809, KRAB-ZFPs initiate ERV silencing by establishing methylation of histone H3 lysine 9 via the recruitment of the histone methyltransferase SETDB1. Mice have three histone H3 variants (H3.1, H3.2, and H3.3), and we set out to explore whether one or more of these variants is critical for ERV silencing. Using ChIP-seq in primary mouse embryonic fibroblasts and induced pluripotent stem cells 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 VL30Pro 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 is required for ERV silencing. To test this hypothesis, we used CRISPR/Cas9 to create a homozygous "floxed" *Daxx* gene, given that DAXX had previously been shown to be responsible for H3.3 deposition at telomeres in ESCs. We found that acute loss of *Daxx* by Cre-mediated recombination in ESCs caused a complete loss of histone H3.3 at ERVs, but had little effect on ERV reactivation in contrast to deletion of *Setdb1*, which leads to massive ERV reactivation. These data suggest that DAXX–dependent deposition of histone H3.3 is dispensable for ERV silencing, a finding that is in conflict with a recent report arguing that some ERVs display reactivation in histone H3.3 KO ESCs, and that IAP elements, in particular, become retro-transpositionally active. 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 the result of retrotransposition, but were simply polymorphic IAP elements mixed into the genetic background of the ESCs used for the study. The authors of the study have acknowledged this error in their interpretation. Our data support a model in which histone H3.3 is deposited into ERVs and other KRAB-ZFP target genes in pluripotent cells but that this deposition is not required for ERV silencing, a model that has been supported further in the recent literature.

Although our data show that many KRAB-ZFPs repress ERVs, we also found that more ancient KRAB-ZFPs, which emerged in a human/mouse common ancestor, do not bind to and repress ERVs. One of these KRAB-ZFPs, ZFP568, plays an important role in silencing a key developmental gene that may have played a critical role in the onset of viviparity in mammals. Using ChIP-seq and biochemical assays, we determined that ZFP568 is a direct repressor of a placental-specific isoform of the Igf2 gene called *Igf2-P0*. Insulin-like growth factor 2 (Igf2) is the major fetal growth hormone in mammals. We demonstrated that loss of

Zfp568, which causes gastrulation failure, or mutation of the ZFP568 binding site at the *Igf2-P0* promoter causes inappropriate *Igf2-P0* activation. We also showed that this lethality could be rescued by deletion of *Igf2*. These data highlight the exquisite selectivity by which members of the KRAB-ZFP family repress their targets and identify an additional layer of transcriptional control of a key growth factor regulating fetal and placental development.

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Deciphering Microbial Virulence Mechanisms

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 had recently undergone surgery or under-water birth, and children who are immune-compromised. We are committed to an 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 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 killing the host cell and infecting neighboring cells.

Virulence of *L. pneumophila* relies on the activity of close to 300 proteins, or effectors, that are delivered into the host cytosol by a specialized translocation apparatus called Dot/Icm type IV secretion system (T4SS). *L. pneumophila* mutants with a non-functional type IV secretion system are degraded by macrophages, underscoring the importance of the translocated effectors for host cell manipulation and bacterial virulence.

Our main research objective is to obtain 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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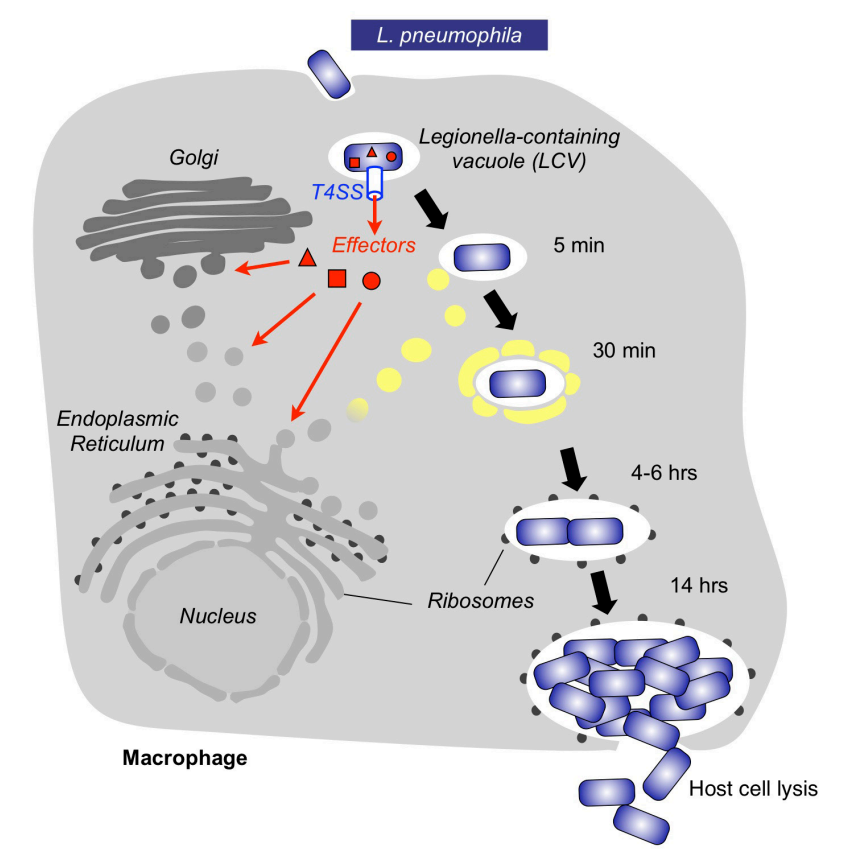

FIGURE 1. Intracellular replication cycle of *Legionella pneumophila*

Upon uptake by a macrophages, *L. pneumophila* delivers a large number of effector proteins (*red*) through the Dot/Icm type IV secretion system (T4SS) into the host cytosol. The effectors manipulate signaling and trafficking pathways in order to establish conditions favorable for *L. pneumophila* growth. Eventually, the host cell is lyzed, and *L. pneumophila* bacteria infect neighboring cells.

A bacterial E3 ligase relic hijacks host–cell ubiquitination.

Bacterial pathogens often target conserved host pathways by encoding proteins that are molecular mimics of cellular enzymes, thus tricking the host cell into surrendering its resources to the bacteria. We discovered that *L. pneumophila* uses such a strategy to exploit ubiquitination, a conserved post-translational modification that is mediated by a family of enzymes called E3 ubiquitin ligases. *L. pneumophila* encodes its own molecular mimics of E3 ligases, including the effector protein RavN, thereby subverting the ubiquitin pathway for its own benefit during infection. By testing truncated RavN variants in an *in vitro* reconstitution assay, we found that the E3 ligase activity of RavN is located within its N-terminal region. Using protein crystallography, we revealed that the fold of RavN shows only residual resemblance to conventional eukaryotic E3s. The N-terminal region of RavN displays a U-box–like domain, a structural motif that mediates the interaction with E2 ubiquitin–conjugating enzymes. In RavN, the U-box lacks the central alpha helix commonly found in other U-box domains, indicating that RavN is an E3 ligase relic that has undergone significant evolutionary alteration. Yet its mode of interaction with E2 enzymes, host proteins that are important for the ubiquitin transfer reaction, has been preserved throughout evolution, and substitution of amino acid residues within the predicted E2 binding interface render RavN inactive.

Identification of catalytic activities in *Legionella* effectors

Using RavN as a model for an *in silico* analysis, we discovered several additional E3 ligase mimics within

the effector repertoire of *L. pneumophila* that, similar to RavN, lack significant homology to known E3s but, nonetheless, catalyze the ubiquitination reaction. Our findings support the hypothesis that E3 ligases have been a vital part of the virulence program of *L. pneumophila* and that these effectors, despite having undergone extensive evolutionary changes, retain features that are critical for their biological function, including the ability to hijack factors that are part of the host ubiquitination machinery. The findings indicate that ubiquitination is more extensively exploited during infection by *L. pneumophila* than previously thought and that interference with this post-translational modification could constitute a novel therapeutic strategy to antagonize infections by *L. pneumophila* and related pathogens.

'Smarter' drugs that selectively target pathogenic bacteria

Most classical antibiotics kill bacteria or inhibit their growth by targeting key steps in their physiology. This approach, while effective in the past, has led to the rapid emergence of multidrug-resistant strains that have become insensitive to the microbicidal or microbiostatic activity of existing antibiotics. In addition, recent insight into the complexity of the human microbiome and its importance for human health has raised additional concerns about the excessive use of antibiotics and their collateral effect on the commensal microbiota. Thus, there is an urgent need for the development of 'smarter' therapeutics that discriminate between pathogens and commensals by selectively targeting virulence mechanisms of bacteria.

Given their essential role in virulence, the *L. pneumophila* T4SS and its translocated effectors represent compelling targets for the development of novel therapeutic agents. In collaboration with the National Center for the Advancement of Translational Sciences (NCATS), we screened over 18,000 bioactive compounds from various product libraries for their ability to target *Legionella* virulence factors. Candidate compounds that emerged from this screen underwent multiple rounds of rigorous validation and testing in order to select the most efficient molecules. Ultimately, we identified a small group of compounds that protected human macrophages from intracellular replication of *L. pneumophila*, making them ideal candidates for an in-depth analysis of their therapeutic potential. At the same time, these compounds are being analyzed in detail for their mechanism of action and for their efficacy in treating infections caused by other pathogens.

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RNA Metabolism in Cell Biology, Growth, and Development

We are interested in the biogenesis and metabolism pathways for tRNAs and certain mRNAs and how these intersect with pathways related to cell proliferation, growth, and development. We focus on the synthesis of tRNAs by RNA polymerase (RNAP) III, as well as the early phases of their post-transcriptional handling by the RNA–binding protein La, and certain modifications that impact their function in the translation of mRNAs into protein by the ribosome. The La protein has been conserved throughout eukaryotic evolution, from single-cell intracellular parasites, yeast, ancient animal cells, plants, and all higher eukaryotes. In some people, the La protein becomes a target of autoantibodies, such as in (and is diagnostic of) patients with Sjögren's syndrome, systemic lupus erythematosus (SLE), and neonatal lupus. Critical to its normal function, the La protein contains several nucleic acid–binding motifs as well as numerous subcellular trafficking elements and it associates with noncoding (nc) RNA as well as with mRNAs to coordinate activities in the nucleus and cytoplasm. In the nucleus, La binds to the 3' oligo(U) motif common to all RNAP III transcripts and functions by protecting its RNA ligands, principally the nascent precursor tRNAs, from 3' exonucleolytic decay and by serving as a chaperone to prevent their misfolding. In addition to the major products of RNAP III, which are the tRNAs, it also synthesizes 5S rRNA and certain other ncRNAs. We also study La–related protein-4 (LARP4), which is predominantly cytoplasmic and interacts with the 3' poly(A) region of certain mRNAs and contributes their 3' end metabolism and translational control (Reference 1).

We strive to understand the structure-function relationship and the cell and molecular biology of the La protein, LARP4, the tRNA isopentenyltransferase TRIT1, and the tRNA–modification enzyme Trm1 and their contributions 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.

Recent data from our lab suggest that the levels of cytoplasmic tRNA may uniquely regulate the translation of LARP4 mRNA, which in turn promotes stabilization of mRNAs encoding ribosomal proteins (Reference 1). Tumor suppressors and oncogenes mediate

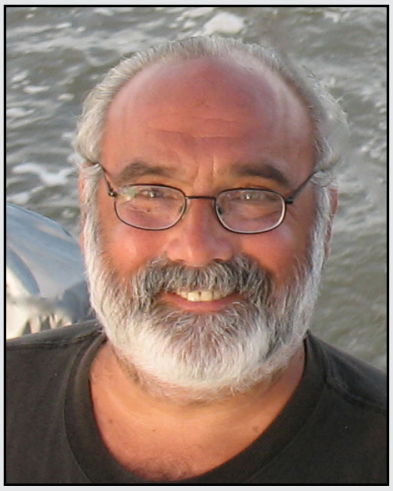

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FIGURE 1. The fission yeast *Schizosaccharomyces pombe* as a model organism

Red-white colony differentiation by tRNA–mediated suppression

deregulation of transcript production by RNAP III, the RNAP responsible for tRNA synthesis, thus contributing to increased capacity for proliferation of cancer cells. However, tRNA levels alone do not account for their differential nor regulated activity. tRNAs are the most heavily and the most diversely modified molecules in cells. We also study some of the key modifications that affect their translational activity.

We also focus on other aspects of tRNA biogenesis and function, such as Trm1, which synthesizes dimethyl-guanosine-26 (m2,2G26) in several tRNAs, and TRIT1, which adds an isopentenyl group onto Adenine-37 in the anticodon loop of certain tRNA (i6A37) molecules. We are examining the effects of TRIT1 on tRNA activity in translation in a codon-specific manner, as well as during mammalian development, using *trit1*-gene-altered mice as a model system, to try to understand how its deficiency leads to childhood mitochondrial dysfunction and metabolic disease. The studies are complemented by investigating how differences in the copy numbers of tRNA genes, which in humans is greater than 600 interspersed on all of the chromosomes of which many reside in clusters and which indeed vary (at the cluster and individual tRNA gene loci) among humans, can affect how the genetic code is deciphered via use of secondary information in the genetic code.

A model that emerges from integration of our studies whereby differential expression of tRNAs in a tissue- and temporal- specific manner, together with their differential modifications, controls mRNA decoding in a codon enrichment-specific hierarchical manner, which determines the translational output of mRNA transcriptomes of the corresponding cell types and tissues during growth and development, as well as in health and disease states. Shifts or perturbations in the levels of specific tRNAs or subsets/clusters thereof and/or their modifications can affect shifts in the hierarchical translation of mRNAs. In terms of genetics, perturbations in a tRNA's expression and/or activity can be considered as a potential modifier of a conventional disease-associated allele. Therefore, a vision for the future of genetics and medicine is that variances among the tRNAomes of individuals should be considered as potential sources contributing to disease penetrance, especially to multifactoral and multigene disorders.

Activities of RNA polymerase III (RNAP III) and associated factors

The RNAP 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 RNAP III to the correct start site. RNAP 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.

Transcription termination delineates 3' ends of gene transcripts, prevents otherwise runaway 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.

Control of the differential abundance or activity of tRNAs can be an important determinant of gene regulation. RNAP III synthesizes all tRNAs in eukaryotes, and its derepression is associated with cancer. Maf1 is a conserved general repressor of RNAP III under the control of TOR (target of rapamycin), which acts to integrate transcriptional output and protein-synthetic demand toward metabolic economy. We used tRNA-HydroSeq to document that little change occurred in the relative levels of different tRNAs in *maf1Δ* cells. By contrast, the efficiency of *N*2,*N*2-dimethyl G26 (m(2)2G26) modification on certain tRNAs was reduced in response to *maf1* deletion and associated with anti-suppression, which was validated by other methods. Over-expression of Trm1, which produces m(2)2G26, reversed *maf1* anti-suppression. The model that emerges is that competition by elevated tRNA levels in *maf1Δ* cells leads to m(2)2G26 hypo-modification resulting from limiting Trm1, thus reducing the activity of suppressor tRNASerUCA and accounting for anti-suppression. Consistent with this, RNAP III mutations associated with hypo-myelinating leukodystrophy reduce tRNA transcription, increase m(2)2G26 efficiency, and reverse anti-suppression. Extending this more broadly, a reduction in tRNA synthesis by treatment with rapamycin leads to increased m(2)2G26 modification, and this response is conserved among highly divergent yeasts and human cells. We published this work in 2015.

The ability of RNAP III to efficiently recycle from termination to reinitiation is critical for abundant tRNA production during cellular proliferation, development, and cancer. We used two tRNA–mediated suppression systems to screen for Rpc1 (subunit of RNAP III) mutants with gain- and loss- of termination phenotypes in *S. pombe*. We mapped 122 point mutation mutants to a recently solved 3.9 Å structure of the yeast RNAP III elongation complex (EC); they cluster in the active center bridge helix and trigger loop, as well as in the pore and funnel formation, the latter indicating involvement in termination of the RNA–cleavage domain of the C11 subunit. Biochemical kinetic and genetic data indicate that mutants with the RT (readthrough)

phenotype synthesize more RNA than wild-type cells, and surprisingly more than can be accounted for by their increased elongation rate. Importantly, similar mutations in spontaneous cancer suggest this as an unforeseen mechanism of RNAP III activation in disease.

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 these RNAs' common UUU-3' OH–transcription termination elements; and (2) translation of specific subsets of mRNAs, such as those containing 5' IRES motifs. LARP4 emerged later in eukaryotic evolution and is conserved in vertebrates as an mRNA–associated cytoplasmic translation factor. We showed that the two RNA–binding motifs of LARP4, which work together as a 'La module,' exhibit preferential binding to poly(A) and are flanked on each side by a different motif that independently interacts with the poly(A)–binding protein (PABP). LARP4 is controlled at the level of mRNA stability by the protein tristetraproline (TTP) and is regulated in mammalian cells through TTP by the tumor necrosis factor alpha (TNFa). LARP4 controls mRNA metabolism/homeostasis and translation.

Fission yeast as a model system for the study of tRNA metabolism and function in translation

About 22 ago, we began developing and have since been refining and advancing a tRNA–mediated suppression system in fission yeast (*Schizosaccharomyces pombe)* that provides a red-white phenotypic real-time assay *in vivo*. In fission yeast, the human La protein can replace the tRNA processing/maturation function of Sla1p, the fission yeast La protein. Moreover, in fission yeast, human La is faithfully phosphorylated on Ser-366 by protein kinase CKII, the same enzyme that phosphorylates Ser-366 in human cells, and the phosphorylation promotes pre-tRNA processing. We use this system to study other aspects of tRNA biogenesis, including transcription by RNAP III, post-transcriptional processing, and tRNA modifications by the conserved enzymes that produce tRNA isopentenyl-adenosine-37 and dimethyl-guanosine-26. 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 *Saccharomyces cerevisiae* and of 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 (a tRNA dimethylallyltransferase), the human tumor suppressor TRIT1, and the *Caenorhabditis 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.

tRNAs, codon use, and mRNA metabolism in growth and development

A major interest is in deciphering what we refer to as "secondary information" in the genetic code. This information derives from mRNAs' choice of synonymous codons that encode the same amino acid. This can produce a layer of information beyond providing the amino acid sequence of a protein; i.e., in addition to providing the template for the sequence of a protein, the use of certain synonymous codons can also produce additional biochemical effects, which we refer to as "secondary information." The effects can be related to ribosome pausing, which can affect protein folding, or alterations in the stability of the mRNA. Other types of secondary information can also be encoded in the use of synonymous codons; for example, sets of mRNAs that share similar patterns of synonymous codon bias are similarly sensitive to tRNAs with the same anticodon modification and exhibit similar patterns of efficiency of translation elongation. The components of the secondary information system are the tRNA pool, the tRNA–modification enzymes, and the codon bias distribution among the mRNAs. We recently found that synonymous codon use by the human LARP4 mRNA is a key determinant of the control of the expression levels of its mRNA and protein, and that increases in otherwise limiting tRNAs that are cognate to these codons increases LARP4 production. This in turn activates LARP4 to promote a net increase in the poly(A) tail length of heterologous mRNAs, including those that encode ribosomal protein subunits (Reference 1). This may be relevant because ribosome production is regulated during growth and development, and the potential circuit involving LARP4 control by tRNA could be an important point of control.

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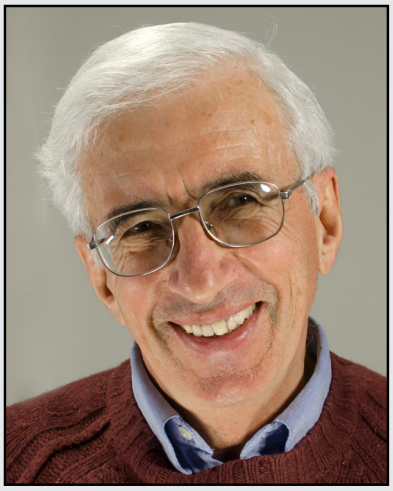
Pathogenesis of HIV-1 and Its Copathogens in Human Tissues

The general goal of the Section on Intercellular Interactions is to understand the mechanisms of pathogenesis and of the transmission of human pathogens, in particular of the human immunodeficiency virus (HIV). This year, we continued our studies on viral pathogenesis using our nanotechnology "flow virometry," developed in our laboratory for the analysis of individual virions and extracellular vesicles (EVs). We studied the distribution of the envelope glycoprotein (Env) in functional and non-functional conformations on the surface of individual HIV-1 virions. Such distribution determines the infectivity of the viral preparation. Also, we analyzed viral proteins on individual EVs released by HIV–infected cells and studied their roles in HIV pathogenesis. We also continued to study defense mechanisms against HIV infection.

During the past year, we (1) analyzed the functionality of Env on individual HIV virions;(2) identified EVs carrying HIV Env and investigated their role in HIV infection of human tissues *ex vivo*; (3) determined the mechanisms of inhibitory activity of an anti-cytomegalovirus (CMV) drug against HIV; and (4) investigated the mechanisms of lactobacillus-mediated prevention of HIV vaginal transmission.



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Env conformation on individual HIV virions

HIV Env plays a major role in HIV infection, given that the correct trimeric conformation of Env is critical for the virus to bind to cell receptors and co-receptors and fuse with the plasma membrane. Dysfunctional forms of Env render virions incapable of binding to/fusion with cells. Each HIV-1 virion carries 10–14 Env spikes, and in principle it is possible that, on a given virion, all spikes are either defective or functional, rendering the former virion defective and the latter virion infectious. Alternatively, virions may carry both functional and non-functional Envs in various conformations. Determining which of these possibilities exists is important both for understanding the basic mechanisms of HIV infection and for the development of new therapeutic and prevention strategies. In this project, we analyzed the distribution of different forms of Env on single HIV-1 virions, using flow virometry. Specifically, individual HIV virions were captured with 15-nm magnetic nanoparticles decorated with ('capture') antibodies that recognize different conformations of Env.

The results obtained from staining of individual virions with mixtures of antibodies that recognize trimeric ("functional") and "defective" conformations of Env indicate that only a minor fraction of virions carry both trimeric and defective Envs, while most virions carry either exclusively trimeric or exclusively defective Envs. Accordingly, depletion of virions that carry defective Envs only mildly reduced the infection of human lymphoid tissues. In conclusion, using flow virometry, we demonstrated that most HIV virions do not appear to be mosaic but rather to carry either only functional or only defective Envs.

The observed lack of Env mosaicism for the majority of infectious virions suggests that this all-or-nothing viral strategy likely aids immune evasion by subverting the focus of humoral responses to generate multiple non-neutralizing antibodies at no cost to infectious virions. In contrast, induction of antibodies that target functional Env and thus target predominantly infectious viruses appears to be critical for the development of effective prophylactic strategies.

Extracellular vesicles containing HIV env facilitate HIV infection.

Various cells *in vivo* and *in vitro* release extracellular vesicles (EVs), many of which are generated along pathways similar to the ones used by retroviruses, in particular HIV. Consequently, EVs are of the same size and physical properties as this virus and it is therefore almost impossible to separate such EVs from HIV virions. We overcame some of these problems by segregating EVs through the glycoprotein CD45 and/or acetylcholinesterase (AChE), two proteins that are not incorporated into HIV membranes and thus can be used to distinguish EVs from HIV virions. To capture and further identify these EVs, we applied our flow virometry nanotechnology (see #1).

In the present work, we addressed two questions regarding EVs released by HIV-infected cells: first, whether these EVs carry viral Envs, and second, whether EVs affect HIV infection. Using the tetraspanin CD81, which is shared by EVs and HIV, we captured both HIV virions and EVs with magnetic nanoparticles coupled with anti-CD81 antibodies and then identified EVs by the presence of either CD45 or AChE. When we stained our preparation with fluorescent anti-Env antibodies, approximately 50% of the events were positive both for EV markers and for Env. The results were similar whether we used CD45 or AChE for identification of EVs or whether we used prototypical CXCR4 or CCR5 HIV viral preparations. Thus, EVs released by HIV–infected cells carry HIV Env.

The question rises as to whether these EVs, which appear to be a part of HIV preparations, affect viral infection. We addressed this question by inoculating human lymphoid tissue *ex vivo* with a viral suspension depleted of specific EVs. Depletion of viral preparations of EVs, in particular of those that carry Env, reduced viral infection of human lymphoid tissue *ex vivo*. The reduction occurred because of EV depletion rather than concomitant depletion of viruses; the amount of p24 or HIV genomic RNA depleted by CD45 magnetic nanoparticles (MNPs) was negligible and did not differ from the depletion of p24 or HIV RNA by isotype control MNPs, that also served as a control for tissue infection.

Thus, our study indicates that HIV–infected cells release not only virions but also EVs and that some EVs carry viral Env, making them indistinguishable not only physically but also semantically from virions, in particular from those that are defective and are not capable of replication. EVs that carry Env identified in our work appear to facilitate HIV infection and may therefore constitute a new therapeutic target for antiviral strategy.

Mechanisms of HIV suppression by anti-CMV drug

Cytomegalovirus (CMV) is a common HIV-1 copathogen. Given that CMV infection is an important contributor to immune activation, the driving force of HIV disease, CMV–suppressive strategies have been investigated. Recent studies showed that valganciclovir, a common anti-CMV drug, is beneficial to CMV/HIV–coinfected individuals by reducing HIV viral load. The anti-HIV effect of this anti-herpetic drug was considered to be indirect and was ascribed to decreasing immunoactivation caused by CMV infection. However, we also investigated whether there was a direct effect of the drug on HIV infection. Towards this goal, we used ganciclovir (GCV), the active form of valganciclovir, and tested the effect of GCV on HIV replication. We treated tonsillar and cervico-vaginal tissues *ex vivo* with GCV and then inoculated tissues with HIV. On average, we found that GCV suppressed replication of HIV-1 in these tissues by 85–90%.

We deciphered the mechanism of this suppression. GCV is a synthetic purine nucleoside analog of guanine, which must undergo triphosphorylation to become active, with the initial monophosphorylation catalyzed by herpesvirus (HHV)–encoded kinase rather than by cellular kinases. HHVs appear to be necessary to inhibit HIV-1, as GCV did not inhibit HIV-1 in MT-4 cell cultures, which are free of endogenous HHVs. Moreover, we showed that the EC_{50} of GCV for HIV-1 was approximately 5 μM, whether human tissues were exogenously co-infected with CMV or not. Thus, it seems that kinases expressed by endogenous HHVs present in human tissues activate GCV by adding the first phosphate. The anti-HIV activity of the GCV occurs at clinically relevant concentrations. Indeed, although GCV penetration efficiency and drug clearance were unknown for *ex-vivo* tissues, the calculated EC_{50}s were in the range of what has been reported *in vivo*. Using an exogenous template reverse transcriptase (RT) assay, we showed that GCV-monophosphate inhibits HIV-1 RT by acting as a delayed chain terminator.

In conclusion, our results suggest that an anti-CMV strategy using valganciclovir in HIV-1–infected individuals may reduce HIV-1 viral load directly by inhibiting HIV-1 RT. Future trials should evaluate the relative contributions both of indirect mechanisms of HIV-1 suppression mediated by CMV reduction and of the direct suppression of HIV-1 RT by phosphorylated GCV.

Lactobacillus–mediated prevention of HIV–1 vaginal transmission

The vaginal microbiota of healthy reproductive-age women is generally dominated by *Lactobacillus* species that are involved in maintaining vaginal homeostasis and have been reported to protect against vaginal transmission of HIV. However, the exact mechanism of HIV inhibition by vaginal lactobacilli remains to be fully elucidated. We studied the protective mechanisms of lactobacilli against HIV-1 infection in the context of human cervico-vaginal and lymphoid tissues *ex vivo*. To address these effects in the context of human tissues, we first colonized them *ex vivo* with different strains of *Lactobacillus* that were isolated from vaginal swabs of healthy premenopausal women. Lactobacilli colonized and grew in human tissues *ex vivo* to densities comparable with those observed in vaginal specimens. To investigate whether lactobacilli release suppressive factors that inhibit HIV-1 replication, we applied bacteria-conditioned culture medium to human tissues *ex vivo* and infected tissues with HIV. We found that HIV-1 replication was significantly suppressed when human tissues were cultured in *Lactobacillus*-conditioned medium in both human cervico-vaginal and tonsillar tissues. Although such a medium may contain multiple inhibitory factors, we first focused on two, pH and lactic acid, whose roles in suppressing HIV infection were hypothesized in earlier studies. The pH of conditioned medium of all tested lactobacilli ranged from 6.3 to 6.9. Although acidification may be directly responsible for HIV-1 inhibition, no HIV-1 suppression was observed when the pH was adjusted to

6.9 with HCl in control experiments, which suggested that other factors beyond lowered pH may also be important for HIV-1 inhibition by lactobacilli. One of these factors may be the major *Lactobacillus* metabolite, namely lactic acid. We found a correlation between the concentration of lactic acid in the *Lactobacillus*-conditioned medium and its ability to suppress HIV-1 infection in human tissues *ex vivo.* Addition of lactic acid isomers D and L to tissue culture medium at the concentration that corresponded to their amount released by lactobacilli resulted in HIV-1 inhibition. We found that the L-isomer rather than the D-isomer was predominantly responsible for HIV-1 inhibition. The results thus indicate that lactic acid, in particular its L-isomer, inhibits HIV-1 independently of lowering of the pH.

Moreover, we investigated whether *Lactobacillus* could have a direct virucidal effect on HIV-1. We incubated an HIV-1 preparation in *Lactobacillus*-conditioned medium and then tested for HIV-1 infectivity in human tissue culture. We found that incubation of HIV-1 in *Lactobacillus*–conditioned medium significantly suppressed viral infectivity in cervico-vaginal tissues. We also investigated whether a direct interaction of lactobacilli with HIV may affect the virus and found that virions adhere to lactobacilli. Thus, lactobacilli can directly inactivate HIV-1 virions and also serve as a sink decreasing the amount of free virions.

In summary, in *ex vivo* systems we identified several mechanisms by which lactobacilli inhibit HIV infection. Extrapolated to *in vivo* conditions, the mechanisms may explain the protective effect of vaginal *Lactobacillus* on HIV infection. Further studies are needed to evaluate the potential of altering the spectra of the vaginal microbiota as effective strategies to enhance vaginal health. Human tissues *ex vivo* may serve as a test system for these strategies.

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Genetic Disorders of Bone and Extracellular Matrix

In an integrated program of laboratory and clinical investigation, we study the molecular biology of the heritable connective tissue disorders known as osteogenesis imperfecta (OI). 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 several genes among the long-sought causes of recessive OI. Discoveries of defects in collagen modification have 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 knock-in murine model for OI with a classical collagen mutation as well as a murine model for recessive type IX OI and X-linked type XVIII 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 III 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 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 investigated their osteoblasts in culture. 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 was also elevated in

type V OI osteoblasts, which occurs despite the seemingly paradoxical reduction in transcripts for type I collagen in mid to late differentiation. The studies demonstrated that a gain-of-function mechanism underlies type V OI and establish its collagen-related defect. We completed collaborative experiments with the labs of Nadja Fratzl-Zelman and Frank Rauch, focusing on the mineralization of bone tissue from patients with type V OI. qBEI of bone from untreated patients was used to determine BMDD (bone mineral density distribution) and lacunar density. As in collagen-based OI and consistent with the elevated mineralization seen in type V OI osteoblasts, the bone tissue mineralization was also significantly increased. The osteocyte lacunar density is elevated in association with immature mesh-like bone structure.

Recessive null mutations in *SERPINF1*, which encodes pigment epithelium–derived factor (PEDF), cause OI type VI. PEDF is 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 a fish-scale pattern. At first, types V and VI OI appear unconnected. However, we identified a patient with severe atypical type VI OI, whose osteoblasts displayed minimal secretion of PEDF, but whose *SERPINF1* sequences were normal despite typical type VI OI bone histology. Surprisingly, exome sequencing on this proband and family members yielded a *de novo* mutation in *IFITM5* in one proband allele, causing a p.S40L substitution in the BRIL intracellular domain. The *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.

The endoplasmic reticulum (ER)–resident procollagen 3-hydroxylation complex is responsible for the 3-hydroxylation of type I collagen alpha1(I) chains. Deficiency of components of the collagen P3H (prolyl 3-hydroxylase) complex causes recessive OI. Thus, deficiency in CRTAP (cartilage-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 clinically nearly indistinguishable because the two proteins are mutually protective.

For type VIII OI, we investigated bone and osteoblasts with collaborators Nadja Fratzl-Zelman and Cathleen Raggio. Collagen has near-absent 3-hydroxylation from both bone and dermis, demonstrating that P3H1 is the unique enzyme responsible for collagen 3-hydroxylation. Bone histomorphometry revealed reduced cortical width, very thin trabecular with patches of increased osteoid, although the overall osteoid surface was normal. Quantitative backscattered electron imaging (qBEI) showed increased mineralization of cortical and trabecular bone, as is typical of other OI types. However, the proportion of bone with low mineralization was higher in Type VIII bone than type VII, consistent with patchy osteoid in type VIII but not type VII.

The third member of the complex, Cyclophilin B (CyPB), encoded by *PPIB*, is an ER–resident peptidyl-prolyl *cis-trans* isomerase (PPIase), which functions both independently and as a component of the collagen prolyl 3-hydroxylation complex. CyPB is the major PPIase catalyzing collagen folding. We 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. Collagen from KO mouse tissue and cells is nearly lacking 3-hydroxylation. Intracellular collagen folding occurs more slowly in CyPB-null cells, supporting the enzyme's role as 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 a further collagen PPIase. We found that CyPB supports collagen lysyl hydroxylase (LH1) activity, demonstrating significantly reduced hydroxylation of the helical crosslinking residue K87, which directly affects both the extent and type of collagen intermolecular crosslinks in bone tissue. However, CyPB deficiency results in increased hydroxylation at telopeptide crosslinking sites in tendon, with moderate increase in glycosylation. CyPB interacts with all three LHs and has a site-specific impact on tissue organization. For example, the type I collagen in dentin of KO mice is under-hydroxylated in a different pattern than in bone, resulting in dentin defects. Also, in the brains of KO mice, absence of the chaperone CyPB increases aggregation of the prior protein PrP. Thus, post-translational modification if critically important in connective tissue formation.

Recessive type XIV OI is a moderately severe bone dysplasia caused by null mutations in *TMEM38B*, which encodes TRIC-B. TRIC-B forms a monovalent cation channel in the ER membrane that is thought to counterbalance IP_3R (inositol triphosphate receptor)–mediated calcium release from the ER to the cytoplasm. Using fibroblasts and osteoblasts from three independent probands, we found that TRIC-B was undetectable in their cells. Although TRIC-B is not a calcium channel, together with our collaborators Yoshi Yamada and Josh Zimmerberg, we showed that absence of TRIC-B results in reduced calcium flux from the ER and abnormal store-operated calcium entry, although ER steady-state calcium is normal. As expected, the disturbed calcium flux causes ER stress along the PERK pathway (involved in the unfolded protein response) and increased BiP, a regulator of ER stress. Disruption of intracellular calcium dynamics also alters the expression and activity of several collagen-modifying enzymes and chaperones in the ER. As a result, lysyl hydroxylation of the collagen helix by LH1 is reduced by 30% even though LH1 levels are increased and, conversely, hydroxylation of the telopeptide lysine by LH2 is increased, despite reduced levels of its PPIase FKBP65. Procollagen chain assembly is also delayed, likely through sequestration of protein disulfide isomerase (PDI) by calreticulin. A substantial proportion of the resulting misfolded collagen is retained in the cells, and any secreted lower-stability forms are not incorporated into matrix. These data support a role for TRIC-B in calcium homeostasis and directly connect *TMEM38B* defects to the collagen-related paradigm of OI. Other ER pathways are likely also disrupted by abnormal calcium flux in these cells, and redundancy for potassium and calcium flux likely modulates the impact of these defects on skeletal development.

In collaboration with Vorasuk Shotelersuk and Cecilia Giunta, we identified a new OI causative gene on the X-chromosome. This is the first type of OI with X-linked inheritance, and it causes a moderate to severe bone dysplasia with pre- and postnatal fractures of ribs and long bone, bowing of long bones, low bone density, kyphoscoliosis and pectal deformities, and short stature. The affected individuals have missense mutations in *MBTPS2*, which encodes the protein S2P. S2P is a transmembrane protein in the Golgi and is a critical component of regulated membrane proteolysis (RIP). In RIP, regulatory proteins are transported from the ER membrane to the Golgi in times of cell stress or sterol depletion, where they are sequentially cleaved by S1P/S2P to release activated N-terminal fragments that enter the nucleus and activate gene transcription. Mutant S2P protein is stable but has impaired RIP functioning, with deficient cleavage of the ER–stress transducers OASIS, ATF6, and SREBP. Furthermore, hydroxylation of the collagen residue K87 is reduced by half in proband bone, consistent with reduced LH in proband osteoblasts. Reduced collagen crosslinks presumably undermine bone strength. Also, proband osteoblasts have broadly defective differentiation. The mutations in *MBTPS2* demonstrate that RIP plays a fundamental role in bone development as well as in cholesterol metabolism.

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 any of the four cleavage site residues have a High Bone Mass form of OI, first reported by our lab in collaboration with Katarina Lindahl's group. The patients have elevated bone density DEXA Z-scores and, on bone histology, patchy unmineralized osteoid. The processing of the C-propeptide from collagen secreted by proband cells is delayed. We investigated mineralization with BMDD to show that, in the alpha2(I) cleavage site mutation, the bone had a uniformly higher mineral density, while in the alpha1(I) mutation, the average mineral density was markedly heterogeneous, with areas of either very high or low bone density.

To investigate the role of the C-propeptide in bone mineralization and developmental progression, we developed 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 exhibit extreme brittleness on mechanical testing, as well as reduced fracture load. BMDD measurement on femora from 2, 6, and 12-month-old mice show significantly increased mineralization compared with wild-type (WT), which continues to increase in HBM (high bone mass) mice even after WT mineralization plateaus at 6 months. Serum markers of bone remodeling, PINP and TRAP, are significantly increased in HBM mice. Osteocyte density is reduced but lacunar area is increased. We are currently investigating osteoblasts, osteocytes and osteoclast in HBM mice.

Insights from the Brtl mouse model for OI

The Brtl mouse model for OI, generated by the Marini lab, is a knock-in mouse that contains a Gly349Cys substitution in the alpha1(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. In a treatment trial of the bisphosphonate alendronate in Brtl and wild-type (WT) littermates, 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.

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. Treatment with Scl-Ab caused no detrimental change in Brtl bone material properties. Nano-indentation studies indicating unchanged mineralization showed that the hyper-mineralization 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. Because Scl-AB is a short-acting drug, we recently investigated sequential Scl-AB/bisphosphonate treatment. The study showed that administration of a single dose of bisphosphonate after Scl-AB cessation preserved anabolic gains from the Scl-AB treatment.

A third therapeutic trial involving Brtl approached allele-specific silencing of the *col1a1* mutation, undertaken

in collaboration with Antonella Forlino. Specific small interfering RNAs (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.

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. The data open the possibility that cytoskeletal elements may be 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, however, 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 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 published the cardiopulmonary aspects of our natural history study on types III and IV OI. Longitudinal evaluations were completed in 23 children with type III OI and 23 children with type IV OI, who had pulmonary function tests every 1–2 years. Compared with size-matched children, our patients 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 the lung related to structurally abnormal collagen. The studies are important because pulmonary issues are the most prevalent cause of morbidity and mortality in OI. Affected individuals should seek anticipatory evaluation and treatment.

Currently, OI–specific growth curves are not available, despite the fact that short stature is one of the cardinal features of OI. We assembled our longitudinal growth data on 100 children with type III and IV OI to generate sex- and type-specific growth curves for OI. The data show that gender and OI type have significant effects on height in OI, but not the collagen chain in which the causative mutation is located. Boys are taller than girls and type IV OI boys and girls are taller than type III. Weight differs by OI type, but not by gender or mutant collagen chain. Interestingly, head circumference does not differ by gender, OI type, or collagen mutation. Imposition of OI height curves on standard CDC curves reveals an overlapping of type III and IV percentiles and the absence of a growth spurt in type III OI. Standard growth curves for OI will be of great value to primary caregivers and families and will provide a baseline for treatment trials.

Our randomized controlled trial of bisphosphonate in children with types III and IV OI was the first

randomized bisphosphonate trial for OI in the United States. It examined direct skeletal and secondary gains reported in uncontrolled trials. For skeletal outcomes, we found increased BMD (bone mineral density) Z-scores and improved vertebral geometry. 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 claims for improvement in ambulation level, lower-extremity strength, or alleviation of pain, suggesting these were placebo effects in observational trials. Our current recommendation is for 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 decline in the quality of bone material, it is important to determine the lowest cumulative dose that will provide vertebral benefits. Preliminary analysis indicates that OI children obtain comparable benefits from lower and higher pamidronate 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. 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 alpha1(I) chain on the collagen monomer and the overlapping of the regularly spaced clusters of lethal mutations along the alpha2(I) chain with the proteoglycan binding sites on the collagen fibril. The modeling for alpha2(I) supports the Regional Model first proposed by the BEMB 20 years ago, which now correctly predicts 86% of clinical outcomes. The Consortium Database has provided the basis for others to model functional domains for interaction of fibrils with cells and matrix. The Consortium Database now contains over 1,570 mutations from nine international laboratories.

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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 on 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 coordinated protein expression in nascent interneuron subpopulations as they migrate and integrate into the developing cortical circuit.

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Afferent specific role of NMDA receptors for the circuit integration of hippocampal neurogliaform cells.

Appropriate integration of GABAergic interneurons into nascent cortical circuits is critical for ensuring normal information processing within the brain. Network and cognitive deficits associated with neurological disorders, such as schizophrenia, that result from NMDA receptor (NMDAR) hypofunction have been mainly attributed to dysfunction of parvalbumin-expressing interneurons that

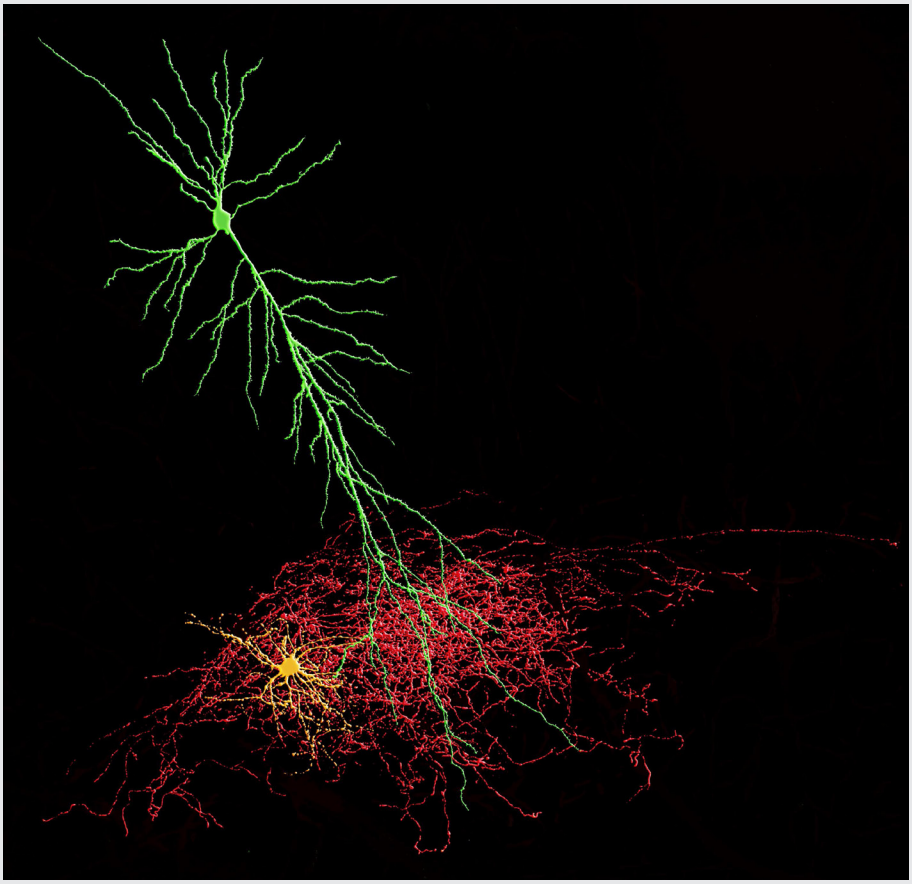

FIGURE 1. Neurogliaform-pyramidal cell connection

The axon of a neurogliaform inhibitory interneuron (cell body and dendrites shown in *yellow*, axon in *red*) forms a dense spider-web plexus (the cell was originally termed the arachniform cell by the Spanish classic anatomist Ramon y Cajal over a century ago) that wraps around the distal dendrites of a glutamatergic pyramidal cell (shown in *green*) in the mammalian hippocampus. Cells were recorded using patch-clamp electrophysiological techniques, filled with neurobiotin and recovered using Imaris software.

paradoxically express low levels of synaptic NMDARs. We revealed that, throughout postnatal development, thalamic and entorhinal cortical inputs onto hippocampal neurogliaform cells are characterized by a large NMDAR–mediated component. This NMDAR signaling is prerequisite for developmental programs ultimately responsible for the appropriate long-range glutamate receptor (AMPAR)–mediated recruitment of neurogliaform cells. In contrast, AMPAR–mediated input at local Schaffer-collateral synapses on neurogliaform cells remains normal following NMDAR ablation. These afferent specific deficits potentially impact neurogliaform cell–mediated inhibition within the hippocampus, and our findings reveal circuit loci implicating this relatively understudied interneuron subtype in the etiology of neuro-developmental disorders characterized by NMDAR hypofunction. Proper brain function depends on the correct assembly of excitatory and inhibitory neurons into neural circuits. We showed that, during early postnatal development in mice, NMDAR signaling via activity of long-range synaptic inputs onto neurogliaform cells is required for the cells' appropriate integration into the hippocampal circuitry.

Neto auxiliary subunits regulate interneuron somatodendritic and presynaptic kainate receptors to control network inhibition.

Although Netos are considered auxiliary subunits critical for kainate receptor (KAR) function, direct evidence for their regulation of native KARs is limited. Because Neto KAR regulation is specific for the KAR–subunit

GluK/Neto isoform, such regulation must be determined in cell type–specific contexts. We demonstrate Neto1/2 expression in somatostatin (SOM)-, cholecystokinin/cannabinoid receptor 1 (CCK/CB1)–, and parvalbumin (PV)-containing interneurons. KAR–mediated excitation of these interneurons is contingent upon Neto1 because kainate yields comparable effects in Neto2 knockouts and wild-types but fails to excite interneurons or recruit inhibition in Neto1 knockouts. In contrast, presynaptic KARs in CCK/CB1 interneurons are dually regulated by both Neto1 and Neto2. Neto association promotes tonic presynaptic KAR activation, dampening CCK/CB1 interneuron output, and the loss of this brake in Neto mutants profoundly increases CCK/CB1 interneuron–mediated inhibition. Our results confirm that Neto1 regulates endogenous somatodendritic KARs in diverse interneurons and demonstrate Neto regulation of presynaptic KARs in mature inhibitory presynaptic terminals.

Additional Funding

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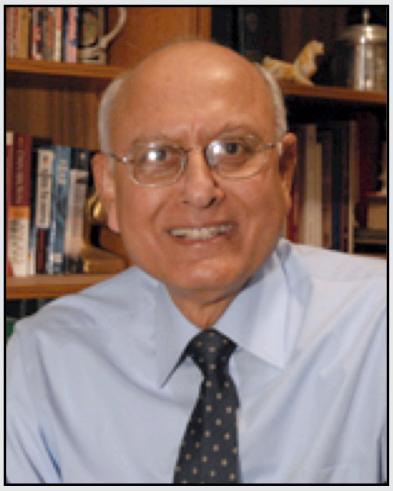
Childhood Neuro-degenerative Lysosomal Storage Disorders

We conduct both laboratory and clinical investigations into a group of the most common childhood neuro-degenerative lysosomal storage disorders (LSDs), called neuronal ceroid lipofuscinoses (NCLs) and commonly known as Batten disease. The diseases affect mostly children. There is no effective treatment for any of the NCLs. Mutations in over 13 different genes (called the *CLNs*) underlie various types of NCLs. Among these genes, *CLN1*, *CLN2*, *CLN10*, and *CLN13* encode soluble lysosomal enzymes; *CLN4* and *CLN14* encode peripherally associated cytoplasmic proteins; *CLN5* encodes a soluble lysosomal protein; *CLN11* encodes progranulin, a protein in the secretory pathway; and several transmembrane proteins with varying subcellular localizations are encoded by *CLN3*, *CLN6*, *CLN7*, *CLN8*, and *CLN12.*

Currently, our research focuses on understanding the molecular mechanisms of pathogenesis underlying infantile NCL (INCL: *CLN1*-disease), juvenile NCL (JNCL: *CLN3*-disease), and congenital NCL (CNCL: *CLN10*-disease). Interestingly, all 14 NCL types share some common pathologic features such as intracellular accumulation of autofluorescent material, epileptic seizures, progressive psychomotor decline resulting predominantly from loss of cortical neurons in the cerebrum, neuro-inflammatory findings, visual impairment resulting from retinal degeneration, and shortened lifespan. We first started investigating the INCL (*CLN1*-disease), which is caused by mutations in the *CLN1* gene encoding a lysosomal depalmitoylating enzyme, palmitoyl-protein thioesterase-1 (PPT1). Numerous proteins in the body, especially in the brain, undergo post-translational modification called *S*-palmitoylation (also called *S*-acylation). In this process, a long-chain fatty acid is attached to specific cysteine residues in polypeptides via thioester linkage. While *S*-palmitoylation plays important roles in membrane anchorage of soluble proteins, protein-protein interaction, and protein stability, these proteins must also be depalmitoylated for recycling or degradation in lysosome. PPT1 catalyzes the cleavage of thioester linkage *S*-palmitoylated proteins. This is important because *S*-palmitoylated proteins are refractory to degradation by lysosomal hydrolases, and PPT1 deficiency leads to lysosomal accumulation of these lipidated proteins (constituents of ceroid), leading to the pathogenesis of INCL. Children afflicted with INCL are normal at birth but, by 11 to 18 months of age, they exhibit signs of psychomotor retardation. By 2 years of age, they are



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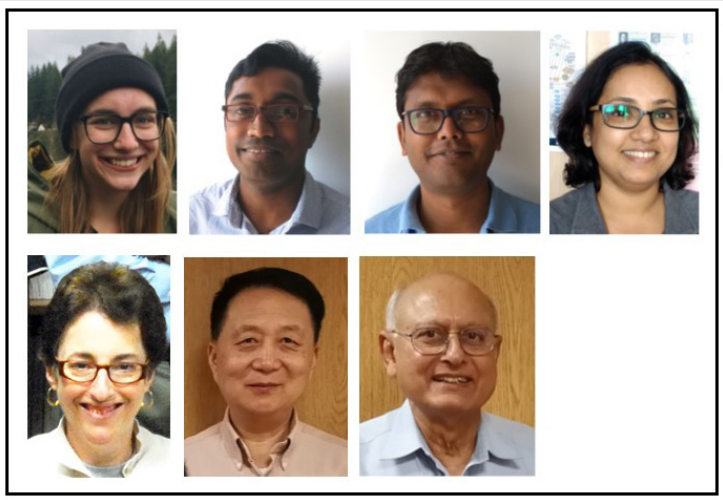

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Lower left to right: Sondra Levin, Gary Zhang, and Anil Mukherjee.

completely blind owing to retinal degeneration and, by age 4, they manifest no brain activity and remain in a vegetative state for 6 to 8 more years before eventual death. Such grim outcomes underscore the urgent need for the development of rational and effective therapeutic strategies not only for INCL but also for all NCLs.

The aim of our clinical studies is to apply the knowledge gained from laboratory investigations to develop novel therapeutic strategies for Batten disease. The results of our earlier investigations on INCL (CLN1-disease) led to a bench-to-bedside clinical trial (Reference 5). Using *Cln1*-knockout (*Cln1*$^{-/-}$) mice, which recapitulate virtually all clinical and pathological features of INCL, we discovered that PPT1 deficiency causes endoplasmic reticulum (ER) and oxidative stress, which at least in part causes neuronal death by apoptosis. During the past several years, we also delineated a mechanism by which PPT1 deficiency disrupts the recycling of the synaptic vesicle (SV) proteins, which are essential for regenerating fresh SVs to replenish the SV pool size at the nerve terminals to maintain uninterrupted neurotransmission. We also discovered that ER and oxidative stress contribute to neuronal apoptosis and neuro-inflammation in INCL. Further, we found that PPT1 deficiency causes misrouting of the V0a1 subunit of v-ATPase (the proton pump on lysosomal membrane), which regulates lysosomal acidic pH, causing elevated pH, which adversely affects lysosomal degradative function (Reference 1).

We also developed a non-invasive methods, using MRI and MRS (magnetic resonance spectroscopy), to evaluate the progression of neuro-degeneration in *Ppt1*$^{-/-}$ mice. These methods permit repeated evaluations of potential therapeutic agents in treated animals. Application of the methods in our clinical trial with INCL also allowed us to evaluate the progressive decline in brain volume and neuro-degeneration (Reference 2). 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 *Cln1*$^{-/-}$ as well as in *Cln1*–knock-in (KI) mice generated in our laboratory, which carry the most common nonsense mutation found in the INCL patient population in the US. We also discovered that the blood-brain barrier is disrupted in *Ppt1*$^{-/-}$ mice and that the pathology is ameliorated by treatment with resveratrol, which has anti-oxidant properties. More recently, we discovered that a nucleophilic small molecule with antioxidant properties, *N*-(*tert*-butyl) hydroxylamine (NtBuHA), ameliorates the neurological abnormalities in *Cln1*$^{-/-}$ mice and extends their

lifespan (Reference 4). These and related studies provide insight into the complex mechanisms of heritable disorders of neuro-degeneration like INCL (CLN1-disease) and identify several potential therapeutic targets. Our results suggest that thioesterase-mimetic small molecules such as NtBuHA are potential therapeutic targets for INCL. More recently, we discovered that cathepsin D (CD) deficiency in lysosomes is a common pathogenic link between INCL (CLN1 disease) and congenital NCL (CNCL) or CLN10 disease. Our ongoing laboratory and clinical investigations are attempting to advance our knowledge of *CLN1*, *CLN3*, and *CLN10* diseases. Our long-term plans are to apply the new findings arising from our laboratory studies to discover the pathogenic links among various NCLs and to develop novel therapeutic strategies not only for *CLN1* disease but also for *CLN3* and *CLN10* diseases.

Dysregulation of lysosomal acidification in the INCL mouse model

In eukaryotic organisms, the lysosome is the primary organelle for intracellular digestion. It contains more than 50 hydrolases, which require an acidic pH for optimal degradative function. Thus, lysosomal acidification is of fundamental importance in the degradation of macromolecules of intra- and extra-cellular origin that are delivered to the lysosome. Moreover, it has been reported that dysregulation of lysosomal acidification contributes to pathogenesis in virtually all lysosomal storage disorders (LSDs), including several NCLs. Furthermore, defective regulation of lysosomal pH has also been reported in common neurodegenerative diseases such as Alzheimer's and Parkinson's disease. However, despite intense

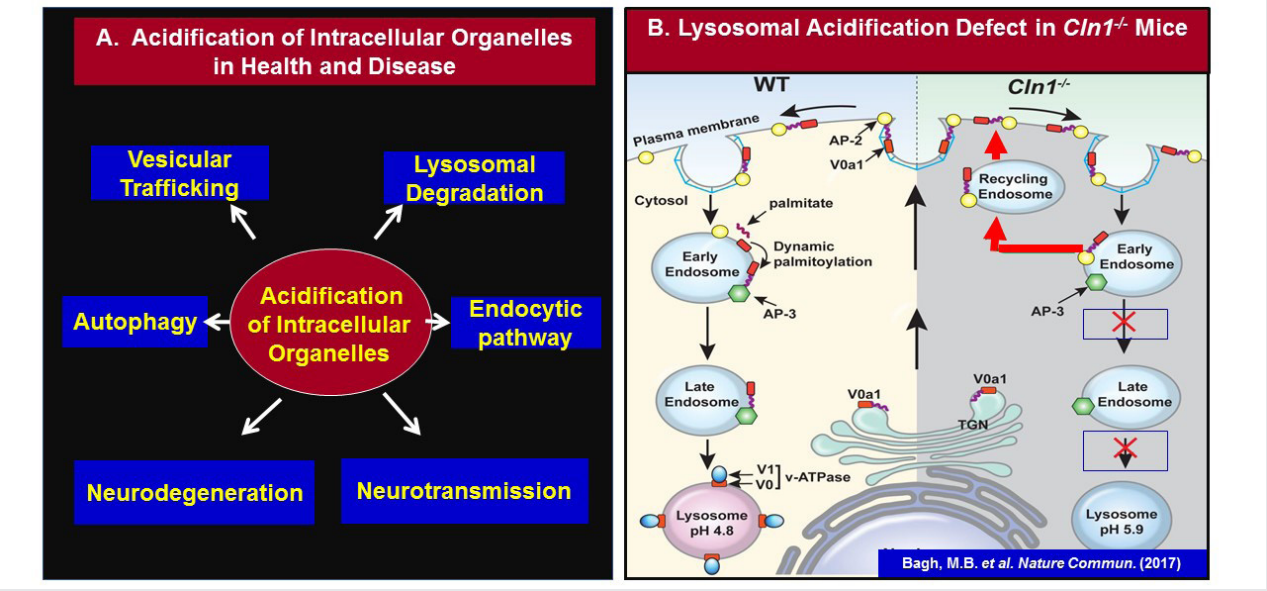

FIGURE 2. A mechanism for the disruption of lysosomal acidification in INCL

A. Importance of acidification of intracellular organelles in health and disease
B. Schematic representation of endosomal sorting and trafficking of V0a1 in WT (left panel) and in *Cln1*$^{-/-}$ (right panel) mice. V0a1 fails to dissociate from AP-2 in *Cln1*$^{-/-}$ cells, preventing it from interacting with AP-3, required for its transport from the sorting endosome to the late endosomal/lysosomal membrane. Consequently, the V0a1–AP-2 complex is misrouted via recycling endosome to the plasma membrane. This defect impairs lysosomal v-ATPase activity, thereby dysregulating lysosomal acidification in neurons of *Cln1*$^{-/-}$ mice, which mimic INCL.

studies, the mechanism(s) underlying the lysosomal acidification defect remains largely unclear. Lysosomal acidification is regulated by vacuolar ATPase (v-ATPase), a multisubunit protein complex composed of the cytosolic V1 sector and the lysosomal membrane–anchored V0-sector. Reversible assembly of V1/V0 sectors on the lysosomal membrane maintains functionally active v-ATPase, the proton pump of the cell

In the mammalian genome, 23 genes encode palmitoyl-acyl-transferases (PATs), which are evolutionarily conserved, cysteine-rich proteins containing Asp-His-His-Cys (DHHC) in the active site. In contrast, there are four thioesterases that have been characterized thus far. Two of these are cytosolic (acyl-protein thioesterase-1 [Apt1] and Apt2) and two (palmitoyl-protein thioesterase-1 [PPT1] and PPT2) are localized to the lysosome. Dynamic palmitoylation (palmitoylation-depalmitoylation), requiring coordinated action of both the DHHC-PATs and PPTs, maintains steady-state membrane localization and the function of numerous important proteins, especially in the brain. By catalyzing depalmitoylation, thioesterases also facilitate recycling or degradation of *S*-palmitoylated proteins (constituents of ceroid) by lysosomal hydrolases.

We tested the hypothesis that one or more subunits of v-ATPase may requires *S*-palmitoylation for endosomal sorting, trafficking, and reversible assembly of V1/V0 on the lysosomal membrane, which is essential for regulating lysosomal pH, and that Ppt1 deficiency disrupts v-ATPase activity, impairing its proton transport function, thereby dysregulating acidification of lysosomal lumen. Our results show that the lysosomal membrane–anchored V0a1 subunit of v-ATPase undergoes *S*-palmitoylation, which is required for its sorting and trafficking to the lysosomal membrane. The process appears to be defective in Ppt1–deficient *Cln1*$^{-/-}$ mice. Notably, we demonstrated that treatment of these mice with the thioesterase (Ppt1)–mimetic small molecule NtBuHA restores v-ATPase activity and rescues the defective lysosomal acidification phenotype.

Evaluation of INCL (CLN1) disease progression by magnetic resonance spectroscopy

As stated in the introduction, the accumulation of ceroid is a characteristic pathological finding in all NCLs. Although the components and ultrastructure of the storage material vary across the different types of NCLs, electron-microscopic analyses of the brain and other tissues from INCL patients show characteristic granular osmiophilic deposits (GRODs). Previously, studies using cell cultures derived from INCL patients demonstrated that phosphocysteamine suppresses apoptosis and depletes intra-lysosomal ceroid deposits. *N*-acetylcysteine, a potent antioxidant, has also been reported to have beneficial effects in other neurodegenerative diseases. Because INCL is a rare disease (1 in over 100,000 births) with a short life expectancy, recruitment of a large study group is impractical. This consideration, together with promising results from cell-culture experiments, led to a “compassionate use” type of experimental design in which all patients received treatment with cysteamine bitartrate (Cystagon) and *N*-acetylcysteine (Mucomyst), without a control group, with the intention of comparing to the natural history with future treatment interventions, using quantifiable measures. These included behavioral and developmental assessments, EEG, ERG, MRI–derived brain volume measurements, and quantification of intra-lysosomal ceroid deposits. Towards the end of the study's recruitment period, quantitative magnetic resonance spectroscopy (MRS) was added to the quantifiable measures. An overview of the study results was published (Reference 5), and details the quantitative MRS findings are listed below.

The evaluation of any treatment benefits of cysteamine bitartrate and *N*-acetylcysteine included quantitative measurement of brain metabolite levels using 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 the partial volume of cerebrospinal fluid. We compared INCL patients with a reference group 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 levels were initially elevated and fell during the follow-up period, whereas in the cerebellum and brainstem (affected later) choline and myo-inositol levels 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.

Common pathogenic link between INCL (CLN1-disease) and CNCL (CLN10-disease)

The lysosome is the major degradative organelle responsible for disposing of the damaged macromolecules and organelles brought into the cell from external and internal sources. It has been reported that impaired lysosomal degradative capability leads to pathogenesis of many neurodegenerative disorders, including LSDs. Neurodegeneration is a manifestation in the majority of the more than 60 LSDs. Moreover, impaired lysosomal degradative capability has been reported in several late-onset neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease. Cathepsin D (CD) is a major lysosomal aspartic protease essential for degradation of proteins delivered to the lysosome. 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, until now, remained poorly understood.

Whereas inactivating mutations in the *CLN1* gene, encoding palmitoyl-protein thioesterase-1 (PPT1), cause INCL, mutations in the *CLN10/CTSD* gene, encoding CD, underlie CNCL (CLN10-disease). We sought to determine whether there is a pathogenic link between INCL and CNCL. The synthesis of CD occurs in the 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), which generate, respectively, 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. Given that CD deficiency underlies CNCL, we propose that CD deficiency in the lysosome is indeed a common pathogenic link between INCL and CNCL.

Non-invasive brain volume measurements in INCL patients by magnetic resonance spectroscopy

Evaluation of the benefits from a treatment using a combination of cysteamine bitartrate (Cystagon) and *N*-acetylcysteine (Mucomyst) were carried out using serial measurements of patients' brain volumes with MR imaging (MRI). Ten patients with infantile neuronal ceroid lipofuscinosis participating in a treatment/follow-up study underwent brain MR imaging that included high-resolution, T1-weighted images. After manual placement of a mask delineating the surface of the brain, a maximum-likelihood classifier was applied to determine total brain volume, further subdivided as cerebrum, cerebellum, brain stem, and thalamus. Patients' brain volumes were compared with those of a healthy population. Major subdivisions of the brain followed similar trajectories with different timing. The cerebrum demonstrated early, rapid volume loss and may never have been normal postnatally. The thalamus dropped out of the normal range at around six months of age, the cerebellum around two years of age, and the brain stem around three years of age. Rapid cerebral volume loss was expected on the basis of previous qualitative reports. Because our study did not include a non-treatment arm and because progression of brain volumes in infantile neuronal ceroid lipofuscinosis has not been previously quantified, we could not determine whether our intervention had a beneficial effect on brain volumes. However, the level of quantitative detail in this study allows it to serve as a reference for evaluation of future therapeutic interventions.

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

UDP–glucuronosyltransferase (UGT) isozymes–distributed primarily in the liver, kidney, 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, dihydrotestosterone, and dietary aromatic-like therapeutics into 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/PKCepsilon–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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Pro- and anti-apoptotic sequences control human prostate DHT–metabolizing UGT-2B15 and UGT-2B17

Similar to each of the six of 19 UGTs examined, UGT-2B17 requires PKCalpha–mediated phosphorylation signaling at position 172, which enables catalysis of an unspecified number of substrates. Moreover, mass spectrometry confirmed that UGT-2B17 has the triple-phosphorylated sequence threonine/tyrosine/serine (TYS) at positions 98–100. Notably, analyses of anti-PKCalpha–immunocomplexed wild-type (wt) UGT-2B17 and its 4-phosphorylated anti-PKCepsilon–immunocomplexes of UGT-2B17 show a dense smearing pattern, except in the case of its Y99F (tyrosine→phenylalanine) mutant or following expression of

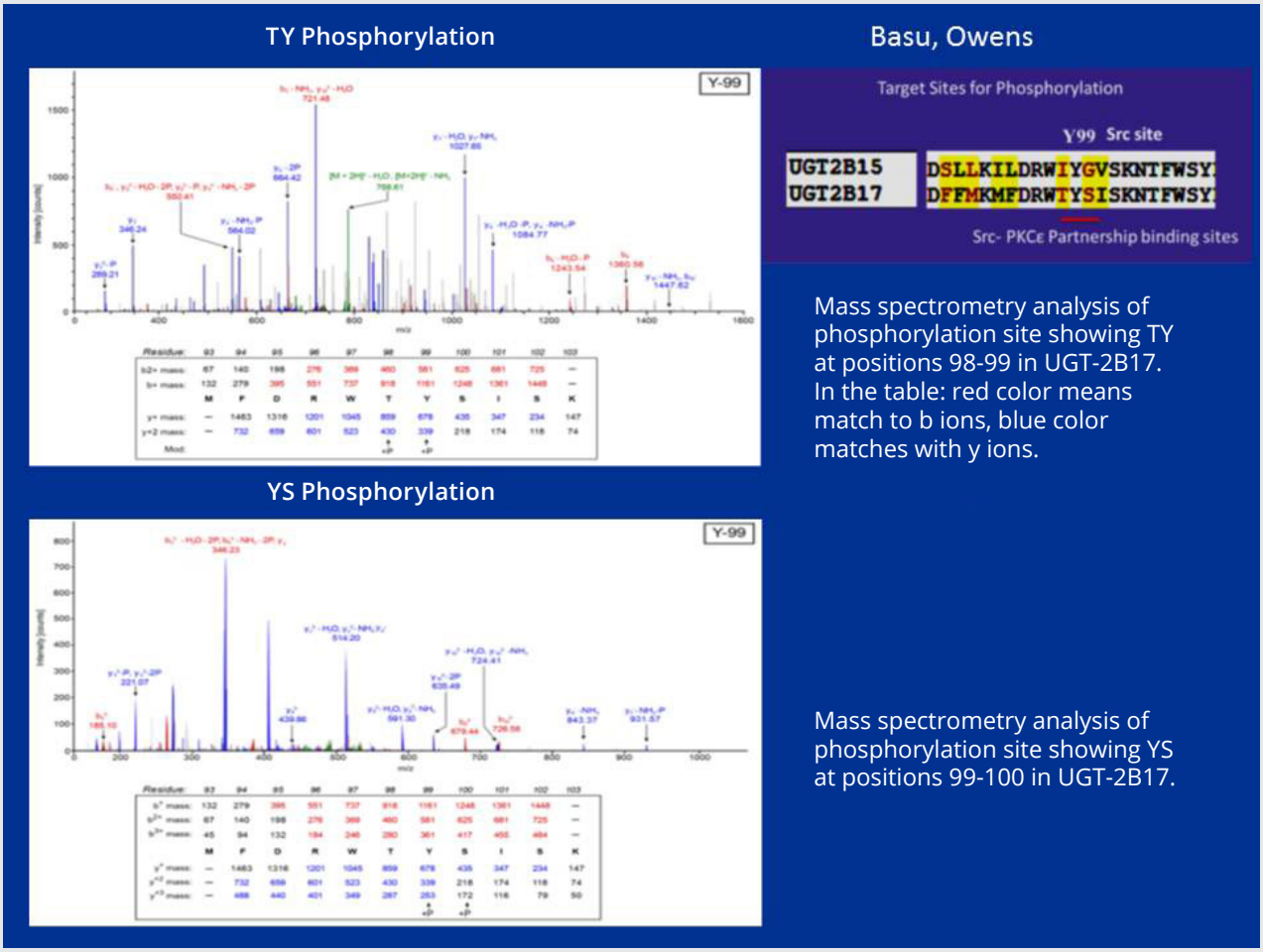


FIGURE 1. Mass-spectrometric analysis of TpYpSp phosphorylation of UGT-2B17

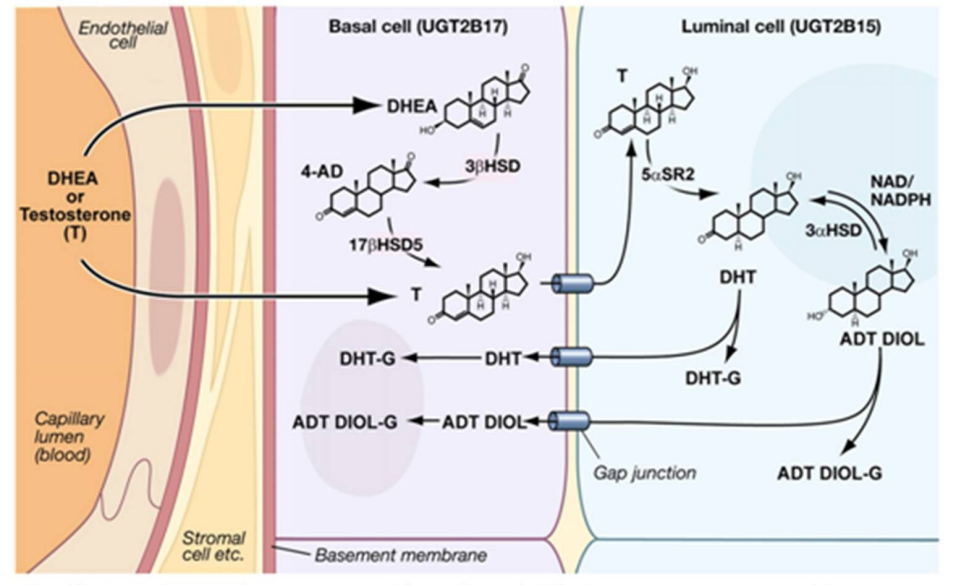
Phosphorylation of UGT-2B17 at 98–100 (TYS—threonine/tyrosine/serine) residues

constructs in Src-kinase–free cells. The smearing is also consistent with robust signaling surrounding the Src/PKCepsilon–partnership phosphorylation site. Following UGT-2B17 expression in $Src^{-/-}$ compared with $Src^{+/-}$ cells, glucuronidation of dihydrotestosterone (DHT) and its 3α-androstane-5α,17β-diol (ADT-diol) metabolite indicates that Src inhibits the glucuronidation by around 50%, which necessarily concomitantly elevates anti-apoptotic DHT levels. Following the exchange of isoleucine/tyrosine/glycine (IYG) in wild-type (wt) UGT-2B15(IYG) and TYS in wt UGT-2B17(TYS) at their comparable positions of 98 through 100 and subsequent transfection into COS-1 cells, UGT-2B17(IYG) generated 10-fold greater activation *in cellulo* of caspases 8/3 than did wt UGT-2B15, while mutant UGT-2B15(TYS) suppressed activation of caspases 8/3 more than 50% compared with UGT-2B15 levels. The evidence thus indicates that the triple-phosphorylated TpYpSp site on UGT-2B17 creates a signaling site involving Src and PKCepsilon that is anti-apoptotic, while the Src–specific binding/phosphorylation site at position 98–100 in UGT-2B15 is pro-apoptotic. The evidence also indicates that serine 172 is phosphorylated to carry out signaling-mediated catalysis, as shown for some seven out of 19 other human UGTs.

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

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

Image taken from Reference 5.

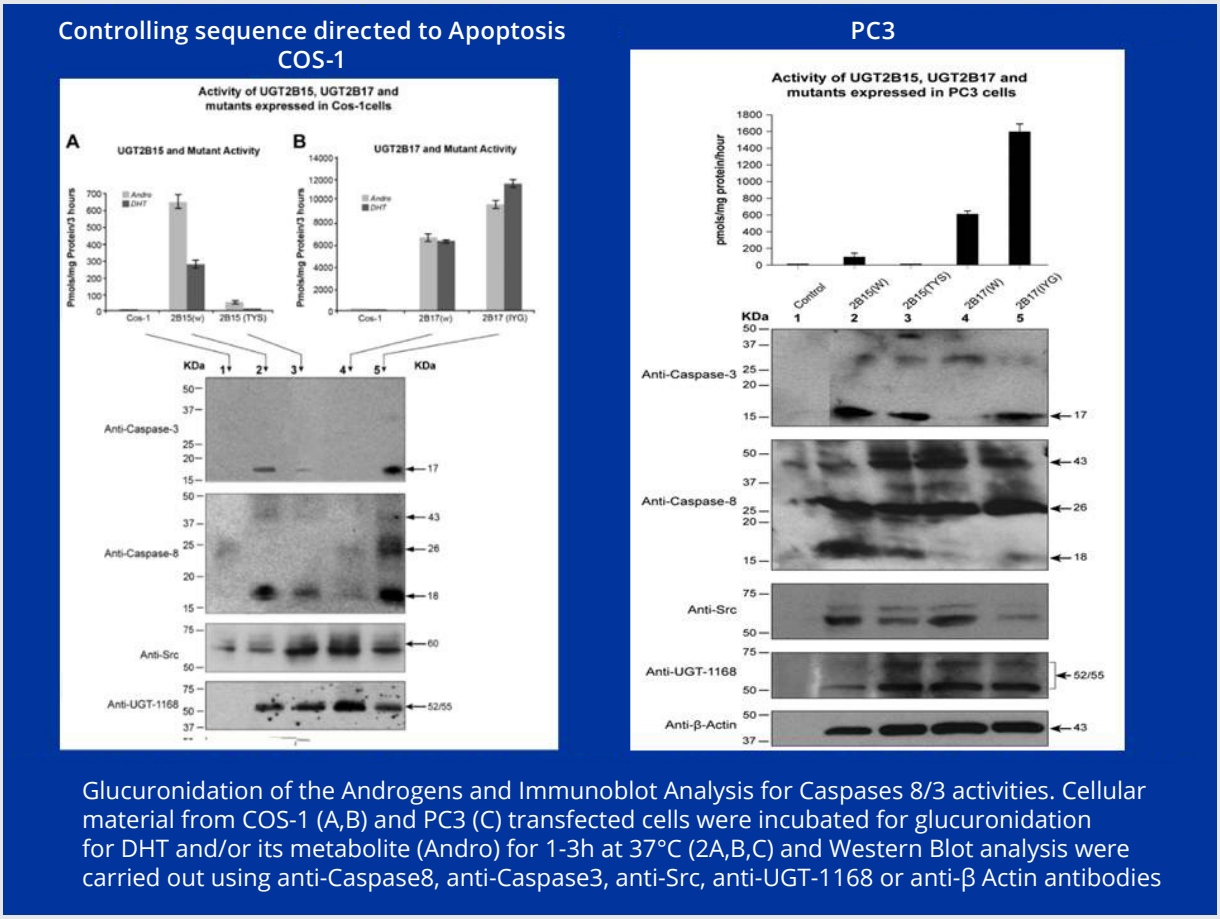


Whereas prostate biochemists discovered that, by occupying the androgen receptor, DHT plays a vital role in supporting the synthesis of the more than 200 secretory proteins necessary for sperm transport fluid, we established that the critically important UGT-2B15 is required for the luminal-cell conversion of DHT to a water-soluble DHT–glucuronide and for the overall maintenance of the luminal cell. More recently, prostate biochemists established that basal cell–distributed UGT-2B17 metabolizes DHT at 10 to 20-fold higher rates than does UGT-2B15. While the exact role of UGT-2B17 in the prostate basal cell remains unknown, studies indicate that the isozyme functions primarily in a supporting role to 'house' intermediate stem cells that contain both cytokeratin cell-surface markers for both luminal and basal cells in a 'Basal Cell Compartment.' Thus, the robust activity of UGT-2B17 is thought to play a role in protecting both newly generated luminal and basal cells for prostate continuity.

While the human prostate luminal cell–distributed UGT-2B15 supports regulated phosphorylation for two different functions, at least one phosphate per UGT-2B15 isozyme undergoes ongoing phosphate signaling at a non-fixed active site, which enables catalysis of an unspecified number of substrates, according to 6 of the 19 human UGTs examined.

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 respectively metabolized 4-hydroxyestrone with 90M K_m and



Glucuronidation of the Androgens and Immunoblot Analysis for Caspases 8/3 activities. Cellular material from COS-1 (A,B) and PC3 (C) transfected cells were incubated for glucuronidation for DHT and/or its metabolite (Andro) for 1-3h at 37°C (2A,B,C) and Western Blot analysis were carried out using anti-Caspase8, anti-Caspase3, anti-Src, anti-UGT-1168 or anti-β Actin antibodies

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

430M K_m. 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 the 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.

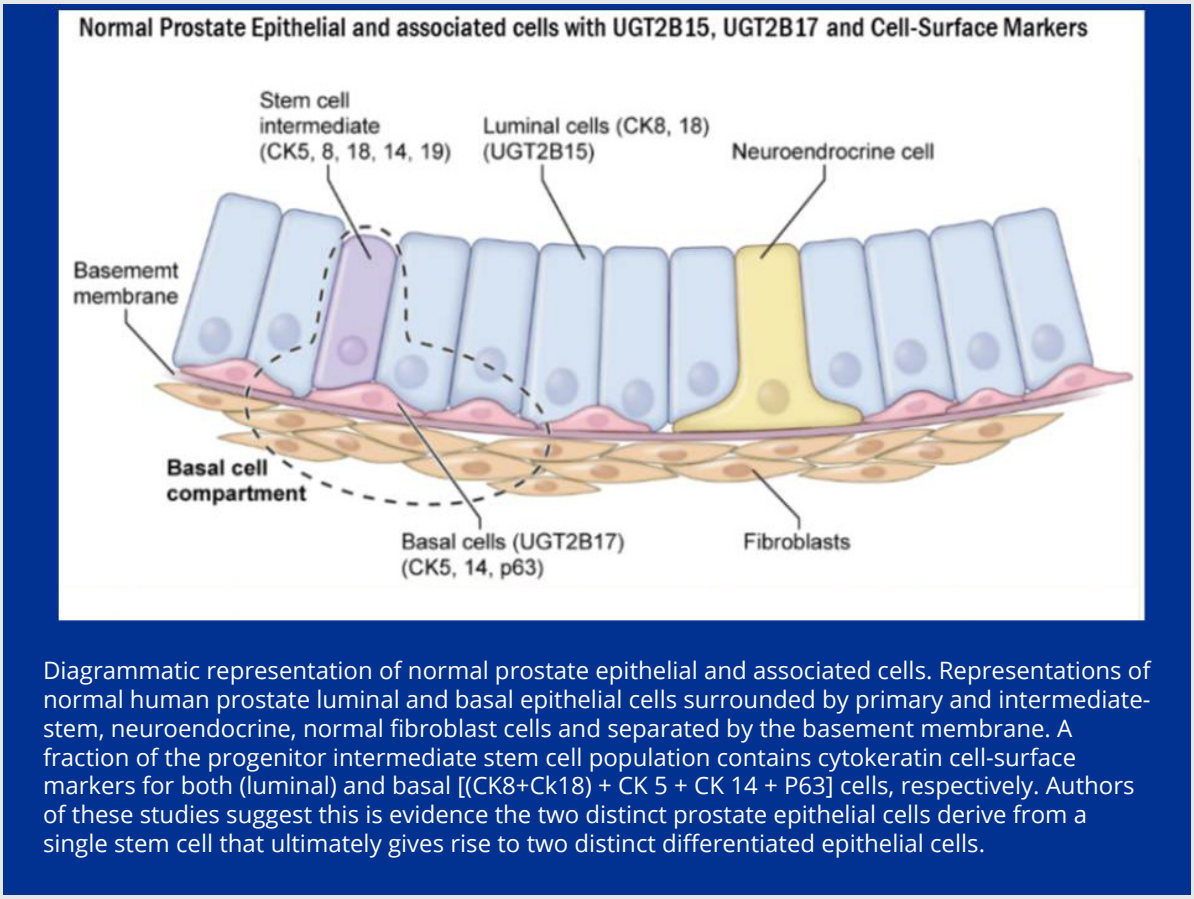


FIGURE 4. Diagrammatic representation of normal prostate epithelial and associated cells

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

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Gene Regulation in Innate Immunity

Macrophages and related cells recognize incoming pathogens and produce cytokines, such as interferons (IFNs) and IL-1/IL-6/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 the IRF (interferon regulatory factor) family of transcription factors, while inflammatory cytokines are produced by activation of the transcription factor NFκB. Our goal is to study the molecular pathways that direct the development and function of macrophages and other myeloid cells. To this end, we focus on the role of IRF8 in innate immunity. IRF8, a member of the IRF family, is expressed in macrophages, dendritic cells (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 adaptive 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 bromodomain protein that binds to acetylated histones and promotes active transcription. BRD4 is involved in the dynamic chromatin exchange that takes place in highly transcribed genes, an exchange that requires a special histone called H3.3. As a result of the association with transcription, H3.3 is implicated in epigenetic control of gene expression patterns. Our goal is to elucidate the activity of BRD4 and histone H3.3 in innate immunity.

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IRF8 confers innate protection against mycobacterium infection.

IRF8 is a transcription factor important for host defense against a variety of pathogens—from viruses to bacteria. IRF8 has been reported to play a pivotal role in protection against *Mycobacterium tuberculosis* (TB). Recent exome sequencing efforts at NIH revealed IRF8 mutations likely associated with susceptibility to another mycobacterium, the so called non-tuberculosis mycobacterium (NTM). The majority of patients infected with NTM suffer from chronic

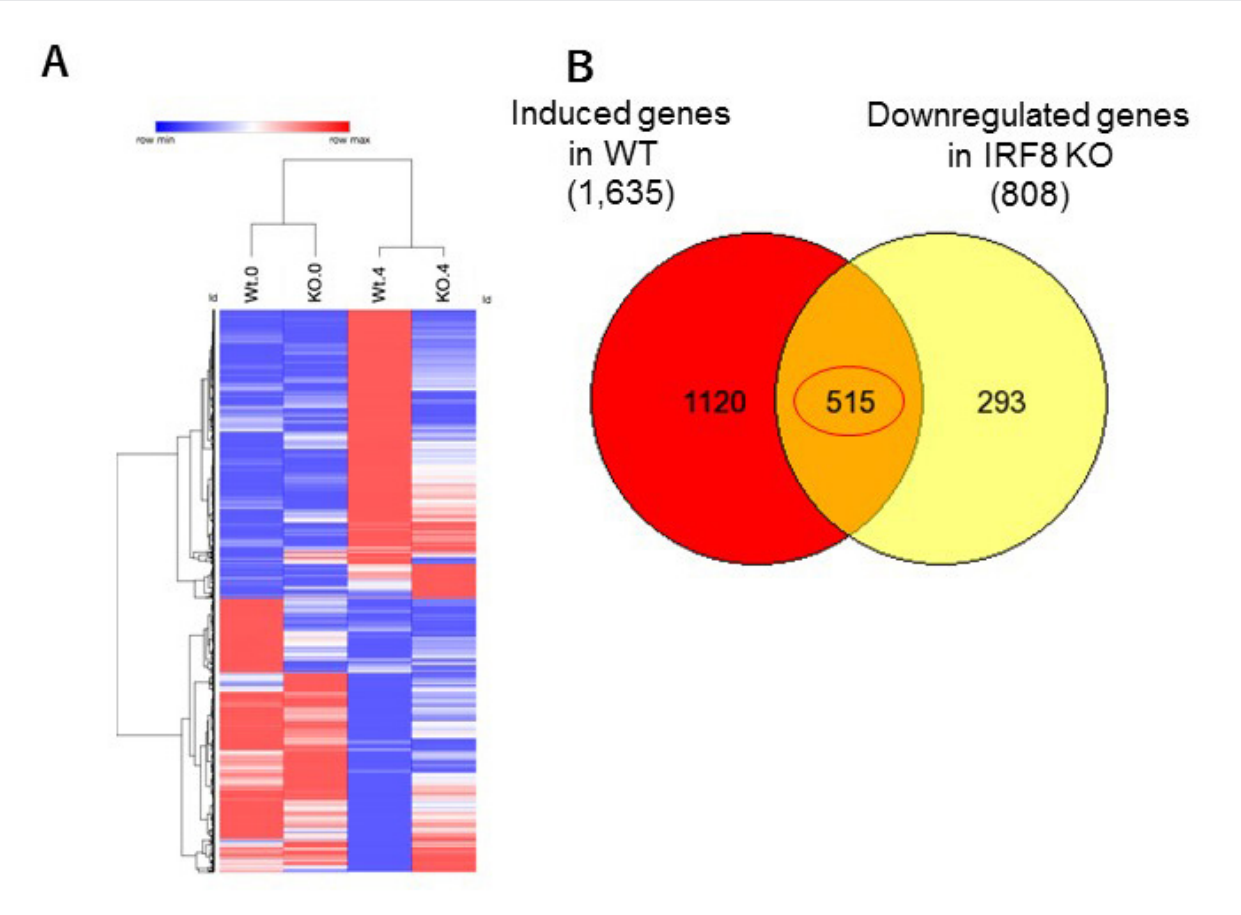


FIGURE 1. IRF8 KO macrophages are defective in innate response to mycobacterium infection.

RNA-seq analysis was performed for WT and IRF8 KO macrophages infected with NTM for 4 days.

Left: Hierarchical clustering of genes up- or down-regulated after NTM infection. The levels of RNA expression are shown by color gradients (References 1,2,3,4).

Right: Venn diagram showing the number of genes induced by NTM infection, but that are downregulated in IRF8 KO macrophages. KO macrophages failed to induce more than 30% of NTM response genes, including those well known for anti-microbial defense.

pulmonary distress, although there are also cases of widespread, disseminated infection. Similar to TB, NTM resides and replicates within macrophages for extended periods. There are relatively few antibiotics/drugs that are effective against NTM. While BCGs have been used for a vaccine, their efficacy is not established. Moreover, the mechanism of innate immunity against mycobacteria is poorly understood. These unsolved problems pose a public health threat, as the incidence of NTM infection is rising in developed countries.
In collaboration with Steven Holland and Katrin Mayer-Barber, we are investigating the role of IRF8 in the protection against NTM. As an *in vitro* approach, bone marrow-derived macrophages from wild type (WT) and *Irf8* knockout (KO) mice were infected with NTM (*M. avis* complex), and bacterial growth was measured by CFU (colony-forming unit) assay after seven days of incubation. We found that bacterial counts were more

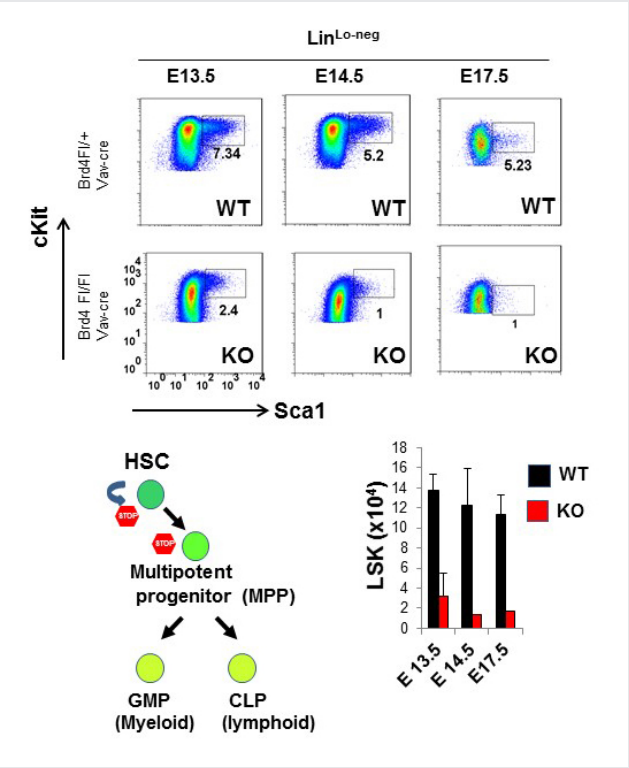


FIGURE 2. Hematopoietic stem cells depend on BRD4 for the self-renewal and progenitor differentiation.

Upper panel: Hematopoietic stem cells in fetal liver from WT and Brd4 KO embryos were identified at indicated days by flowcytometry.

Lower panel: (*left*) diagram showing the stages of HSC development. BRD4 is required for the earliest stage of hematopoiesis; (*right*) the total number of HSCs in WT and Brd4 KO embryos.

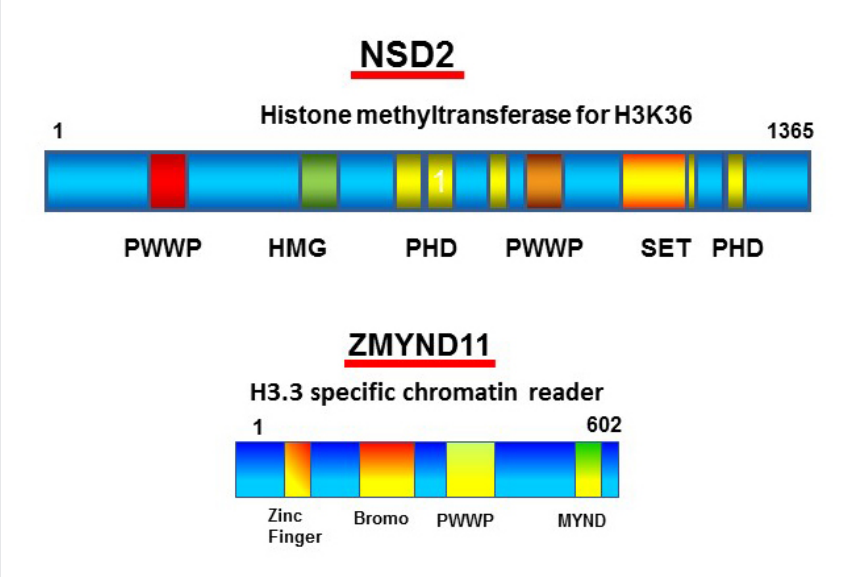
than 10 times higher in *Irf8* KO macrophages than in WT cells and that interferon gamma, known to facilitate innate resistance, was not effective in *Irf8* KO macrophages. RNA-seq analysis found that a series of genes were induced sequentially after NTM infection in WT macrophages. Some of early genes included *IL-1* and *TNF*, known to be important for resistance against TB. Thus, roughly one-third of 1,500 genes induced by NTM infection in WT macrophages were not induced in *Irf8* KO macrophages, including several autopagy-related genes and those important for lysosomal function. Our results point to the role of autophagy lysosomal pathway in the establishment of anti-mycobacterium resistance. The idea that this pathway is critical for anti-mycobacterium innate immunity is supported first by reports that the *Atg5* KO mouse, defective in autophagy, is more susceptible to TB infection and second by our previous demonstration that many autophagy genes are targets of IRF8. Consistent with this thinking, our immunostaining analysis revealed that NTM ingested within macrophages are captured by autophagosomes. In WT cells these processes can be visualized by LC3 puncta, a fluorescent autophagosome marker. However, *Irf8* KO macrophages, where LC3 expression is low, failed to form these punctate structures and did not associate with NTM.

Hematopoietic stem cells depend on BRD4 for their maintenance and progenitor differentiation.

BRD4 is a bromodomain protein of the BET (bromodomain and extraterminal domain) family, which this laboratory has been studying for many years. BRD4 is expressed at high levels in most, if not all cells, and is necessary for very early embryonic development. Thus, conventionally created Brd4 knockout (KO) mice are embryonic lethal. BRD4 is a so-called “chromatin reader” owing to its binding to acetylated histones. It also recruits the transcription elongation factor P-TEFb, thus facilitating transcriptional elongation. Moreover, BRD4 has a critical role in forming super-enhancers. Super-enhancers are long stretches of regulatory DNAs densely occupied by transcription factors and chromatin regulators. They direct strong transcription of select genes and thus help define cellular and lineage identity. In the past several years, research on BRD4 has seen a dramatic upturn owing to the development of small-molecule inhibitors that inhibit binding

FIGURE 3. NSD2 guides interferon-induced H3.3 deposition and regulates transcription by recruiting the chromatin reader ZMYND.

Schematic diagram of NSD2 and ZMYND. NSD2 is a histone methyltranferase that is involved in dimethylation of H3K36 through the SET domain. We found that interferon-induced H3.3 deposition requires other domains of NSD2; thus H3.3 deposition is abrogated in *Nsd2* KO cells. ZMYND was also recruited to transcribed genes in a NSD2– and H3.3–dependent manner. Our results provide mechanistic insight into the chromatin-marking event and its immediate impact on transcription.



of acetyl-histones to the BET family proteins. These inhibitors, affecting mostly the BET protein BRD4, antagonize cancer growth, particularly leukemia and lymphoma. Furthermore, BET inhibitors have been shown to inhibit inflammatory responses related to cardiovascular and autoimmune diseases. These reports implicate BRD4 in various disease processes and offer new therapeutic possibilities for several difficult-to-treat illnesses; indeed, clinical trials are being conducted for leukemia and inflammation. However, these developments present new issues stemming from the dearth of our understanding of the precise role of BRD4 in health and disease and of the mechanism of BRD4 action. Studies on inhibitors have inherent limitations due to uncertainty regarding their specificity, modality of action, and long-term consequences. For example, the impact of BET inhibitors on normal hematopoietic cells is not well understood, posing potential problems when treating blood cancers such as leukemia/lymphoma. BET inhibitor treatment may compromise the activity and maintenance of hematopoietic stem cells and may weaken the ability to combat infection, which is also relevant to treating inflammation, given that macrophages are the main effector of both inflammation and host defense.

We thus sought to gain a fuller understanding of BRD4's activity in normal hematopoiesis and during inflammatory and innate immune responses. We studied *Brd4* conditional knockout mice, focusing on hematopoiesis and macrophage responses. First, we tested mice in which *Brd4* is deleted in early hematopoiesis by using the Vav-Cre technique. We showed that *Brd4* KO mice die during fetal development owing to severe defects in the expansion of hematopoietic stem cells (HSC) and in the development of hematopoietic progenitor cells. As a consequence, *Brd4* KO embryos fail to develop immune cells of all lineages, including lymphocytes and myeloid cells, which are important for innate and adaptive immunity. We also found that BRD4 is essential for the proliferation of macrophages, based on LysM-Cre–dependent deletion of *Brd4* (LysM-Cre selectively targets macrophages and neutrophils); the resultant *Brd4* KO mice failed to start IL-4–dependent peritoneal macrophage expansion. These results strongly point to a central role of BRD4 in immune cell expansion, required for maintaining immunity.

We investigated genome-wide distribution of BRD4 in macrophages in a resting condition and after LPS stimulation. LPS is a pathogen component that rapidly induces inflammatory genes and interferon-stimulated genes important for protection against pathogens. We found that BRD4 broadly occupies genic and intergenic regions. Within the genic region, BRD4 binding peaked at the transcription start site (TSS), although binding was detected over the 5' promoter and within the coding regions. BRD4 binding over the genic regions markedly increased after LPS stimulation, indicating that BRD4 moves rapidly over the genome, presumably to accommodate a rapid alteration of histone acetylation. Furthermore, BRD4 displayed dense clustering over distant regulatory regions that represented super-enhancers. BRD4 clusters coincided with the H3K27 chromatin mark, which denotes super-enhancers as well as RNA polymerase II clustering. BRD4–containing super-enhancers localize to genes important for basic macrophage phenotypes and innate immune responses. Because it has been proposed that BRD4 is central for generation of super-enhancers, we were interested to know whether *Brd4* KO macrophages possessed super-enhancers.

NSD2 guides interferon–induced H3.3 deposition and regulates transcription by recruiting the chromatin reader ZMYND.

Interferons (IFN) rapidly stimulate transcription of many IFN–stimulated genes (ISGs). We previously showed that ISG transcription is coupled with H3K36 trimethylation and deposition of the histone variant H3.3, creating stable epigenetic marks on ISGs. NSD2 (WHSC1, MMSET1) is a histone methyltransferase that methylates H3K36. We showed that global distribution and IFN–induced deposition of H3.3 was abrogated in mouse embryonic fibroblasts from *Nsd2* KO mice, with only minor changes in the H3K36me3 distribution pattern. This led to aberrant ISG induction, in that a fraction of ISGs were constitutively expressed in *Nsd2* KO cells even prior to IFN stimulation. Further, more than half the ISGs (about 200) were induced more rapidly and at higher levels in KO cells than in wild-type cells. ZMYND11 is a “chromatin reader” that was recently reported to recognize H3.3 and shown to repress transcription of some genes. We demonstrated that ZMYND11 is recruited to ISGs upon IFN stimulation and that recruitment is inhibited in *Nsd2* KO cells. Consistent with these data, exaggerated ISG expression was observed in *ZMYND*11 KO cells. Our results reveal that ISG induction is internally coupled to NSD2-ZMYND11–dependent transcriptional suppression, which restrains transcriptional overdrive. ZMYND11–mediated repression may be linked to epigenetic memory for ISGs.

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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 (PHEO) and paraganglioma (PGL), collectively abbreviated as PPGLs. PPGLs are rare, neuroendocrine tumors derived from adrenal or extra-adrenal chromaffin cells, respectively. Metastases are discovered in 3–36% of patients at the time of diagnosis. Currently, only suboptimal treatment options exist. Therefore, new therapeutic compounds targeting metastatic PPGLs are urgently needed. Our 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 PPGL; (2) explain the molecular and cellular basis for varying clinical presentations of PPGLs and establish the pathways of tumorigenesis; (3) search for new molecular and genetic/epigenetic markers for diagnosis and treatment of metastatic PPGL; (4) introduce new therapeutic options for malignant/metastatic PPGL; 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 aspects of pheochromocytoma and paraganglioma

Childhood PPGLs are often hereditary and may present with different characteristics compared with adults. Hereditary PPGLs can be separated into cluster 1 and cluster 2 tumors, which result from to mutations impacting hypoxia and kinase receptor signaling pathways, respectively. The objective was to identify differences

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Valerie Valdez, MD, *Volunteer*

in presentation of PPGLs between children and adults. The study included 748 patients with PPGLs, including 95 with a first presentation during childhood. Genetic testing was available in 611 patients. Other data included locations of primary tumors, presence of recurrent or metastatic disease, and plasma concentrations of metanephrines and 3-methoxytyramine. Children showed significantly higher prevalence than adults of hereditary (80.4% vs. 52.6%), extra-adrenal (66.3% vs. 35.1%), multifocal (32.6% vs. 13.5%), metastatic (49.5% vs. 29.1%), and recurrent (29.5% vs. 14.2%) PPGLs. Tumors resulting from to cluster 1 mutations were significantly more prevalent among children than among adults (76.1% vs. 39.3%), and this paralleled a significantly higher prevalence of noradrenergic tumors, characterized by relative lack of increased plasma metanephrine, in children than in adults (93.2% vs 57.3%). We concluded that the higher prevalence of hereditary, extra-adrenal, multifocal, and metastatic PPGLs in children than in adults represents interrelated features that, in part, reflect the lower age of disease presentation of noradrenergic cluster 1 than adrenergic cluster 2 tumors. The differences in disease presentation are important to consider in children at risk for PPGLs due to a known mutation or previous history of tumor.

We then performed a retrospective study of 55 patients diagnosed at or before 21 years of age with PPGLs, with analysis of data on genetic testing and multimodal imaging. Eighty percent of patients (n = 44/55) had a germline mutation. The majority were found to have mutations either in the von Hippel-Lindau tumor suppressor (*VHL*) gene (38%) or in the *SDHB* (encoding succinate dehydrogenase complex iron sulfur subunit B) gene (25%). PHEO was present in 67% (n = 37/55) of patients and was bilateral in 51% (n = 19/37). The majority of patients with bilateral PHEOs had VHL (79%). Abdominal PGLs was present in 22% (n = 12/55), head and neck PGLs in 11% (n = 6/55), and thoracic PGLs in 2 of 55 patients. For PGLs, SDHx (succinate dehydrogenase) accounted for 72% (n = 13/18) of mutations. The rate of malignancy was 16% (n = 9/55), 56% of whom had *SDHB* mutations. In two-thirds of patients, functional imaging identified either extra-adrenal PGLs and/or metastatic disease. We concluded that the majority of pediatric patients with PPGLs have detectable germline mutations. Therefore, we strongly suggest that all pediatric patients with PPGLs undergo genetic testing and imaging to detect extra-adrenal paragangliomas and metastatic disease to guide treatment and follow-up.

In our review paper (Reference 1), we discussed a new molecular biology–based taxonomy that has been proposed for PPGL. Data from the Cancer Genome Atlas revealed clinically relevant prognostic and predictive biomarkers and stratified PPGLs into three main clusters. Each subgroup has a distinct molecular-biochemical imaging signature. Concurrently, new methods for biochemical analysis, functional imaging, and medical therapies have also become available. The research community now strives to match the cluster biomarkers with the most superior intervention. The concept of precision medicine has been long awaited and holds great promise for improved care. We reviewed the current and future PPGL classifications with a focus on hereditary syndromes. We discussed the current strengths and shortcomings of precision medicine, and suggested a condensed manual for diagnosis and treatment of both adult and pediatric PPGL patients. We also considered the future direction of this field, with a particular focus on how advanced molecular characterization of PPGL can improve a patient's outcome, including cures, and ultimately disease prevention.

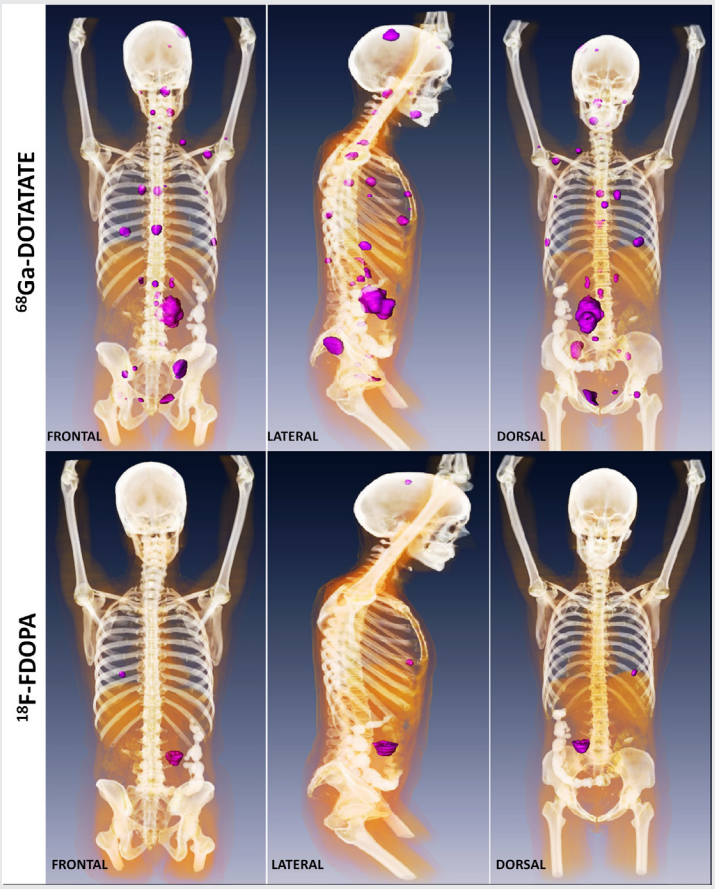

FIGURE 1. Metastatic paraganglioma detected by ^{68}Ga-DOTATATE PET/CT and ^{18}F-FDOPA PET/CT

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)

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Hereditary pheochromocytoma and paraganglioma

Succinate dehydrogenase B (SDHB)–associated PHEOs are associated with a higher risk of tumor aggressiveness and malignancy. The aim of the present study was to evaluate first the frequency of germline *SDHB* mutations in apparently sporadic patients with PHEO who undergo preoperative genetic testing and, second, the ability to predict pathogenic mutations. From 2012 to 2016, 82 patients underwent a PHEO surgical resection. Sixteen were operated in the context of hereditary PHEO and were excluded from analysis. Among the 66 remaining cases, 48 were pre-operatively screened for an *SDHB* mutation. In addition to imaging studies with specific radiopharmaceuticals (^{123}I-MIBG or ^{18}F-FDOPA) for exclusion of multifocality/metastases, 36 patients underwent ^{18}F-FDG PET/CT. From the 48 genetically screened patients, genetic testing found a germline *SDHB* variant in two (4.2%) cases: a variant of unknown significance, exon 1, c.14T>G (p.Val5Gly), and a most likely pathogenic mutation, exon 5, c.440A>G (p.Tyr147Cys), according to *in silico* analysis. Structural and functional analyses of the protein predicted that the p.Tyr147Cys mutation was pathogenic. Both tumors exhibited moderate ^{18}F-FDG PET uptake with similar uptake patterns to non–*SDHB* mutated PHEOs. The two patients underwent total laparoscopic adrenalectomies. Of the remaining patients, 44 underwent adrenalectomy laparoscopically and two by laparotomy. Pathology analysis of the tumors from patients bearing two germline *SDHB* variants revealed a typical PHEO. *Ex vivo* analyses (metabolomics, SDHB immunohistochemistry, and loss of heterozygosity analysis) allowed a reclassification of the two *SDHB* variants as probably non-pathogenic variants. The study illustrated that SDHx mutational analysis can be

misleading, even if structural and functional analyses are done. Surgeons should be aware of the difficulty of classifying new *SDHB* variants prior to including *SDHB* mutation status into a tailored surgical management strategy of a patient.

In another study, we reported a comprehensive molecular characterization of PPGLs. Multi-platform integration revealed that PPGLs are driven by diverse alterations affecting multiple genes and pathways. Pathogenic germline mutations occurred in eight PPGL susceptibility genes. We identified *CSDE1*, a gene encoding the RNA–binding protein cold shock domain–containing protein E1, as a somatically mutated driver gene, complementing four known drivers (*HRAS*, encoding a GTPase that is a protein kinase activator, *RET*, a gene encoding a receptor tyrosine kinase, *EPAS1*, a gene encoding a transcription factor involved in body response to oxygen levels, and *NF1*, a gene encoding a negative regulator of the ras signal transduction pathway). We also discovered fusion genes in PPGLs, involving *MAML3* (encoding a transcriptional coactivator for NOTCH proteins), *BRAF* (encoding the serine/threonine-protein kinase B-raf), *NGFR* (encoding a low-affinity receptor), and *NF1*. Integrated analysis classified PPGLs into four molecularly defined groups: a kinase signaling subtype, a pseudohypoxia subtype, a Wnt–altered subtype driven by *MAML3* and *CSDE1*, and a cortical admixture subtype. Correlates of metastatic PPGLs included the *MAML3* fusion gene. This integrated molecular characterization provides a comprehensive foundation for developing PPGL precision medicine.

In recent years, familial pPHEO with germline mutations in the *MAX* (MYC associated factor X) gene has been reported in a few cases. We investigated a 25-year-old patient with multiple PHEOs associated with a nonsense germline *MAX* mutation. Preoperative ^{18}F-FDOPA PET/CT revealed bilateral adrenal involvement with multiple tumors. In addition, both adrenal glands were found to have diffuse or nodular adrenal medullary hyperplasia (AMH), a histopathological feature previously described as a precursor of MEN2– and SDHB–related PHEOs but not of MAX. After bilateral adrenalectomy, different paraffin-embedded and frozen samples were analyzed for allelic imbalances of the *MAX* gene using allelic quantification by pyrosequencing. The expression of the protein MAX was studied by immunohistochemistry. All PHEOs, but also nodular AMH, exhibited a loss of the normal allele. By contrast, the diffuse AMH did not show loss of heterozygosity. Nevertheless, immunohistochemistry demonstrated loss of protein MAX expression in all samples including diffuse hyperplasia, suggesting a causative role of *MAX* mutation for both PHEOs and AMH. The case shows that both nodular and diffuse AMH belong to the spectrum of MAX–related disease. The data support the possible continuum between nodular AMH and PHEO, expanding the qualification of micro-PHEO to nodular AMH.

Imaging of pheochromocytomas and paragangliomas

Pheochromocytoma/paraganglioma (PPGL) syndromes associated with polycythemia have previously been described in association with mutations in the von Hippel-Lindau gene. Recently, mutations in the prolyl hydroxylase gene (*PHD*) 1 and 2 and in the hypoxia-inducible factor 2α (*HIF2A*) were also found to be associated with multiple and recurrent PPGL. Such patients also presented with PPGL and polycythemia, and later on, some presented with duodenal somatostatinoma. In additional patients presenting with PPGL and polycythemia, no further mutations have been discovered. Because the functional imaging signature of patients with PPGL-polycythemia syndromes is still unknown, and because these tumors (in most patients) are multiple, recurrent, and metastatic, the goal of our study was to assess the optimal imaging approach using four different PET radio-pharmaceuticals and CT/MRI in these patients. Fourteen patients (10 women, 4 men) with confirmed PPGL and polycythemia prospectively underwent imaging by: ^{68}Ga-DOTATATE, in 13 patients; ^{18}F-FDG, in 13 patients; ^{18}F-fluorodihydroxyphenylalanine (^{18}F-FDOPA), in all 14 patients; ^{18}F-fluorodopamine

(^{18}F-FDA), in 11 patients; and CT/MRI, in all 14 patients. Detection rates of PPGL lesions were compared between all imaging studies and stratified between the underlying mutations. ^{18}F-FDOPA and ^{18}F-FDA PET/CT showed similar combined lesion-based detection rates of 98.7% (95% confidence interval [CI], 92.7%–99.8%) and 98.3% (95% CI, 90.9%–99.7%), respectively. The detection rates for ^{68}Ga-DOTATATE (35.3%; 95% CI, 25.0%–47.2%), ^{18}F-FDG (42.3; 95% CI, 29.9%–55.8%), and CT/MRI (60.3%; 95% CI, 48.8%–70.7%) were significantly lower, irrespective of the mutation status. We concluded that ^{18}F-FDOPA and ^{18}F-FDA are superior to ^{18}F-FDG, ^{68}Ga-DOTATATE, and CT/MRI and should be the radio-pharmaceuticals of choice in this rare group of patients.

From 2007 to 2015, 175 patients with non-metastatic PPGL were evaluated by ^{18}F-FDOPA PET/CT for initial diagnosis/staging and follow-up. ^{18}F-FDOPA PET/CT was considered as falsely negative for at least one lesion in 10/126 (8%) patients (two sporadic, six SDHD, two SDHB PPGLs). The mRNA and protein expression levels of the LAT amino-acid transporters and a subunit of LAT1, CD98hc, were evaluated in samples with different genetic backgrounds and imaging phenotypes. The qRT-PCR (quantitative real-time PCR) and immunohistochemical analyses were performed in 14 and 16 tumor samples, respectively. The SDHx–mutated samples exhibited a significant decrease in mRNA expression of LAT3 when compared with sporadic PPGLs. There was also a statistical trend toward reduced CD98hc and LAT4 levels in SDHx vs. sporadic PPGLs. No difference was observed for LAT1/LAT2 mRNA levels. LAT1 protein was expressed in 15 out of 16 (93.75%) SDHx tumors, regardless of the ^{18}F-FDOPA positivity. LAT1 and CD98hc were co-expressed in 6/8 ^{18}F-FDOPA-negative PPGLs. In contrast, in one case with absence of LAT1/CD98hc, ^{18}F-FDOPA uptake was positive and attributed to LAT4 expression. We conclude that down-regulation of LAT1/CD98hc cannot explain the imaging phenotype of SDHx–related PPGLs. A reduced activity of LAT1 remains the primary hypothesis possibly due to a modification of intracellular amino acid content which may reduce ^{18}F-FDOPA uptake.

Therapeutic aspects of pheochromocytoma and paraganglioma

No definite cure is yet available for the neuroendocrine tumors PPGLs. Therefore, drugs with a wide spectrum of mechanisms of action are being tested to identify suitable candidates for PPGL treatment. Proteasome inhibitors have been frequently used in treating hematologic and solid tumors. They are administered individually or in combination with other regimens, to prevent severe side effects and resistance development. Because they have been shown to be efficient and are pharmaceutically available, we tested the first Food and Drug Administration–approved proteasome inhibitor bortezomib alone and in combination with another proteasome inhibitor, salinosporamid A, in PHEO cells. We showed that bortezomib induces PHEO cell death via the apoptotic pathway both *in vitro* and *in vivo*. The combination of bortezomib with salinosporamid A exhibits an additive effect on these cells and inhibits proliferation, cell migration and invasion, and angiogenesis more potently than bortezomib alone. We suggest these proteasome inhibitors, especially bortezomib, could be tested in PPGL patients, who might benefit from treatment with either the inhibitors alone or in combination with other treatment options.

Histone deacetylase inhibitors (HDACis) are a potent class of tumor-suppressive agents traditionally believed to exert their effects by loosening tightly wound chromatin, resulting in de-inhibition of various tumor suppressive genes. Recent literature however has shown altered intra-tumoral hypoxia signaling with HDACi administration not attributable to changes in chromatin structure. We sought to determine the precise mechanism of HDACi–mediated hypoxia signaling attenuation using vorinostat (SAHA), an FDA–approved class I/IIb/IV HDACi. Through an *in vitro* and *in vivo* approach utilizing cell lines for hepatocellular carcinoma (HCC), osteosarcoma (OS), and glioblastoma (GBM), we demonstrated that SAHA potently inhibits HIF-α

nuclear translocation via direct acetylation of its associated chaperone, heat shock protein 90 (Hsp90). In the presence of SAHA, we found elevated levels of acetyl-Hsp90, reduced interaction between acetyl-Hsp90 and HIF-α, reduced nuclear/cytoplasmic HIF-α expression, no HIF-α association with its nuclear karyopharyin Importin, and markedly reduced HIF-α transcriptional activity. These changes were associated with downregulation of downstream hypoxia molecules such as endothelin 1, erythropoietin, glucose transporter 1, and vascular endothelial growth factor. The findings were replicated in an *in vivo* Hep3B HRE-Luc expressing xenograft and were associated with significant decreases in xenograft tumor size. The study highlights a novel mechanism of action of an important class of chemotherapeutic.

We investigated whether anthracyclines were able to suppress the progression of metastatic PHEO. We explored their effects on experimental mouse PHEO tumor cells, using *in vitro* and *in vivo* models, and demonstrated that anthracyclines, particularly idarubicin (IDA), suppressed hypoxia signaling by preventing the binding of hypoxia-inducible factor 1 and 2 (HIF-1 and HIF-2) to the hypoxia response element (HRE) sites on DNA. This resulted in reduced transcriptional activation of HIF target genes, including erythropoietin (*EPO*), phosphoglycerate kinase 1 (*PGK1*), endothelin 1 (*EDN1*), glucose transporter 1 (*GLUT1*), lactate dehydrogenase A (*LDHA*), and vascular endothelial growth factor (*VEGFA*), which consequently inhibited the growth of metastatic PHEO. Additionally, IDA downregulated hypoxia signaling by interfering with the transcriptional activation of HIF1A and HIF2A. Furthermore, our animal model demonstrated the dose-dependent suppressive effect of IDA on metastatic PHEO growth *in vivo*. Our results indicate that anthracyclines are prospective candidates for inclusion in metastatic PPGL therapy, especially in patients with gene mutations involved in the hypoxia signaling pathway.

Animal model of pheochromocytoma and cell culture studies

Approximately 30–40% of PPGLs result from germline mutations in one of the susceptibility genes, including those encoding the succinate dehydrogenase subunits A-D (*SDHA–D*). Up to two thirds of patients affected by *SDHB* mutated PPGL develop metastatic disease with no successful cure at present. For the first time, we evaluated the effects of *SDHB* silencing in a three dimensional (3D) culture using spheroids of a mouse PHEO cell line, in which the SDHB subunit was either silenced or not (wild-type). We investigated the role of the micro-environment on spheroid growth and migration/invasion by co-culturing *SDHB*–silenced or wild-type spheroids with primary cancer-activated fibroblasts (CAFs). When spheroids were co-cultured with fibroblasts, *SDHB*–silenced cells showed a statistically significant increase in matrigel invasion, as demonstrated by the computation of the migratory areas. Moreover, cells detaching from the *SDHB*–silenced spheroids moved collectively, unlike the cells of wild-type spheroids, which moved individually. Additionally, *SDHB*–silenced spheroids developed long filamentous formations along which clusters of cells migrated far away from the spheroid, whereas these structures were not present in wild-type spheroids. We found that lactate, largely secreted by CAFs, plays a specific role in promoting migration only of *SDHB*–silenced cells. Thus, in this study, we demonstrated that *SDHB* silencing *per se* increases tumor cell migration/invasion and that this micro-environment, as represented by CAFs, plays a pivotal role in enhancing collective migration/invasion in PHEO *SDHB*–silenced tumor cells, suggesting their role in increasing the tumor metastasizing potential.

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Mechanisms Regulating Interneuron Development

The incredible diversity and heterogeneity of interneurons was observed over a century ago, with Ramon y Cajal hypothesizing in 'Recollections of My Life' that "The functional superiority of the human brain is intimately linked up with the prodigious abundance and unaccustomed wealth of the so-called neurons with short axons." Although interneurons constitute the minority (20%) of neurons in the brain, they are the primary source of inhibition and are critical components in the modulation and refinement of the flow of information throughout the nervous system. Abnormal development and function of interneurons has been linked to the pathobiology of numerous brain diseases such as epilepsy, schizophrenia, and autism. Interneurons are an extremely heterogeneous cell population with distinct morphologies, connectivity, neurochemical markers, and electrophysiological properties, and, with the advent of new technologies to dissect gene expression and connectivity patterns, the classification of interneurons into specific subtypes is ever-evolving. However, our general understanding of the developmental mechanisms that generate this diversity remains largely poor. The goal of our lab is to dissect the genetic programs that determine the initial interneuron fate decisions during embryogenesis and to explore how the environment and genetic cascades interact to give rise to such stunning diversity of interneuron subtypes. We take a multifaceted approach to this issue, utilizing both *in vitro* and *in vivo* approaches to identify candidate mechanisms that regulate interneuron fate decisions. We strive to develop cutting edge techniques that will overcome the many challenges faced when studying interneuron development. Our ultimate goal is to discover the genetic cascades and signaling mechanisms that direct interneuron differentiation and maturation, which should act as a springboard for future research.

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Mechanisms regulating initial fate decisions within the medial ganglionic eminence

The medial ganglionic eminence (MGE) gives rise to the majority of forebrain interneurons, most notably the somatostatin- and parvalbumin-expressing (SST^+ and PV^+) subtypes. The MGE is a transient, dynamic structure that arises around E10 and bulges into the lateral ventricle over the next several days before dissipating at

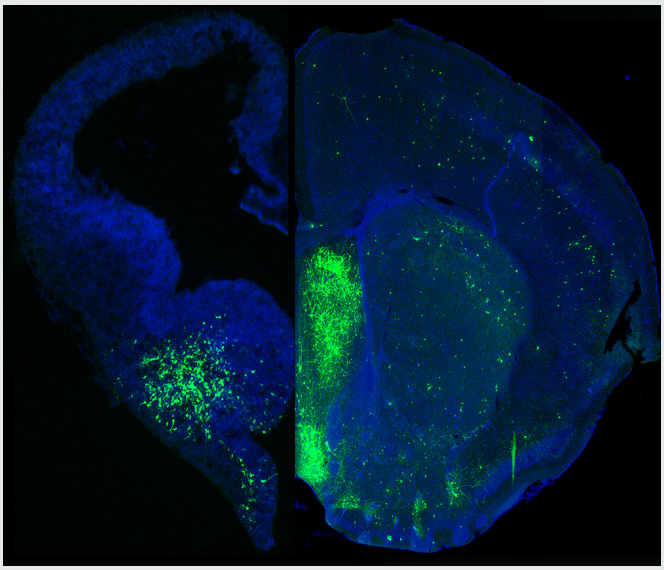

FIGURE 1. MGE–derived GABAergic cells populate many different brain regions.

Image depicts a section of an embryonic brain (left) that has been electroplated to label cells derived from the medial ganglionic eminence (MGE), merged with an section of an adult brain (right) displaying the incredible spatial and morphological diversity MGE–derived cells in the mature brain. Understanding how this heterogeneous population is generated from one embryonic brain structure is the focus of this laboratory.

the end of embryogenesis. As initial fate decisions are generated within the MGE, there has been much focus on identifying a logic for interneuron generation from this region. Previous experiments characterized both a spatial and temporal gradient within the MGE that regulates the initial fate decision of becoming either PV^+ or SST^+ interneurons. SST^+ interneurons are preferentially born early in embryogenesis from the dorso-posterior MGE whereas PV^+ interneurons are born throughout embryogenesis with a bias of originating from the ventro-anterior MGE. Our work discovered an additional mechanism regulating this fate decision: the mode of neurogenesis. Using *in utero* electroporations, we found that PV^+ interneurons are preferentially born from basal progenitors (also known as intermediate progenitors) whereas SST^+ interneurons arise more commonly from apical progenitors. We hope build on this observation to discover how these distinct spatial, temporal, and neurogenic gradients coordinate to regulate initial fate decisions of MGE progenitors.

How the environment sculpts interneuron diversity and maturation

Interneurons undergo an extensive tangential migration period before reaching their terminal brain region, upon which they interact with the local environment to differentiate and mature. The composition of interneuron subtypes varies significantly between different brain regions. Numerous experiments indicate that general interneuron classes, e.g., PV^+– or SST^+–expressing, are determined as cells become post-mitotic during embryogenesis, but when other features that define a mature interneuron subtype (neurochemical markers, cell type, and subcellular location of synaptic partners, electrophysiology properties, etc.) are established remains unknown. One hypothesis is that interneurons undergo an initial differentiation into 'cardinal' classes during embryogenesis, and maturation into 'definitive' subgroups requires active interaction with their mature environment. An alternate hypothesis is that immature interneurons are already genetically hard-wired into definitive subgroups, and that the environment more passively sculpts the maturation of these cells. To test these competing hypotheses, we are harvesting early postnatal interneuron precursors (P0-P2) in specific brain regions and transplanting them into wild-type hosts either homotopically (cortex-to-cortex) or heterotopically (cortex-to-hippocampus or cortex-to-striatum). The technique allows us to determine whether transplanted interneurons adopt properties of

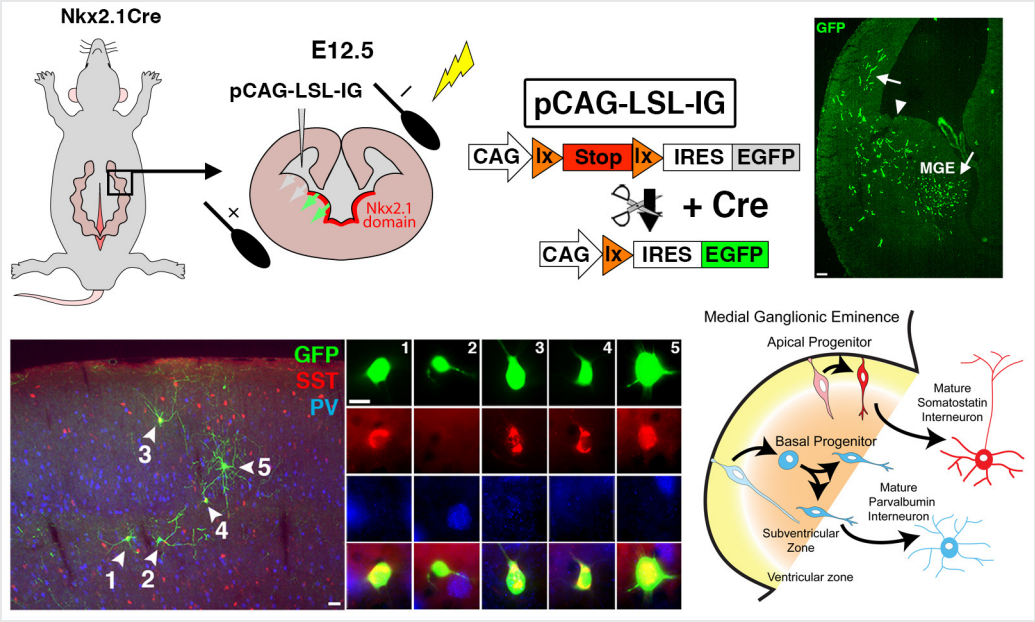

FIGURE 2. Manipulation of gene expression in the MGE by *in utero* electroporation (IUE)

Top. By using *Nkx2.1-Cre* mice, we can restrict expression of cre-dependent plasmids to the MGE. Note that the GFP$^+$ cells are restricted to the MGE and cells migrating away from the MGE at 2 days post-electroporation.

Bottom. Representative example from a P25 cortex of a mouse whose MGE was electroplated at E12.5. In this series of experiments, we used IUE to demonstrate that the mode of neurogenesis plays a role in interneuron fate determination: apical neurogenic divisions are biased to generate somatostatin interneurons whereas basal divisions primarily give rise to parvalbumin cells.

the host environment (indicating a strong role for the environment in regulating interneuron diversity) or retain subtype features more consistent with the donor region. Our initial experiments indicate that the environment largely determines the composition of interneuron subtypes in a brain region regardless of donor region. However, some interneuron subtypes appear to be more genetically predefined and resistant to environmental influences than others. In the future, we hope to combine this transplantation approach with other analytic techniques to more fully characterize how the environment sculpts interneuron diversity.

Novel approach to identify genetic cascades underlying interneuron fate decisions

The ability to longitudinally track gene expression within defined populations is essential for understanding how changes in expression mediate both development and plasticity. Previous screens that were designed to identify genes and transcription factors specific to SST– or PV–fated interneurons were largely unsuccessful because several issues significantly hinder these types of studies. First, these interneurons originate from the medial ganglionic eminence (MGE), which is a heterogeneous population of progenitors that give rise to both interneurons and a variety of GABAergic projection neurons, making it difficult to segregate interneuron progenitors from other cell types. Additionally, many markers that define mature interneuron subtypes are not expressed embryonically, and thus these class-defining markers are not helpful for studying MGE progenitors. In an ideal scenario, we would like to identify actively transcribed genes in MGE progenitors undergoing fate decisions while retaining the capacity to identify whether these cells become PV– or SST–expressing interneurons in the postnatal brain. To this end, we are developing a spatially and temporally inducible form of DNA adenine methylase identification (DamID) that will allow

us to label the transcriptome of MGE progenitors. Labeled cells can be harvested at maturity once we have the tools to distinguish specific interneuron cell types. Then the methylated genomic DNA will be analyzed, allowing us to look back in time to identify candidate fate-determining genes expressed in specific interneuron populations. Our hope is that this strategy could be widely applicable so that an investigator could characterize the temporal gene expression pattern of the cell type of interest.

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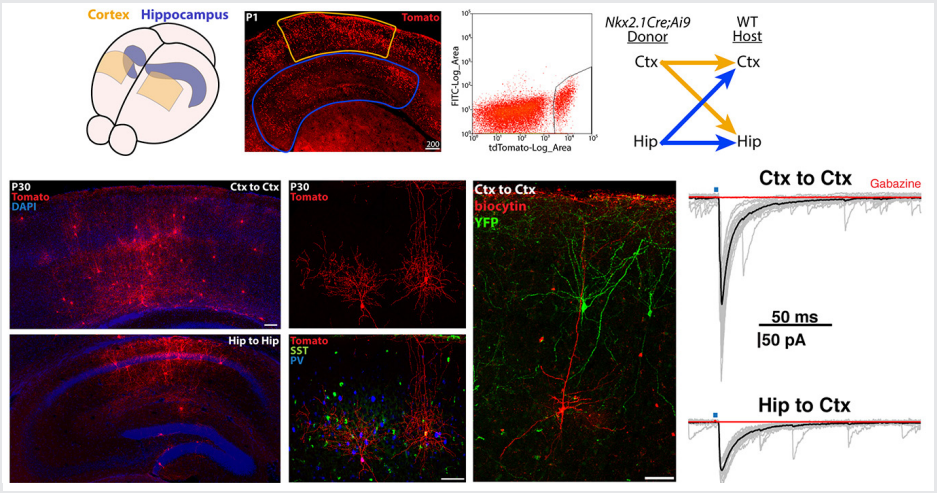

FIGURE 3. Transplantation of MGE–derived interneuron precursors into postnatal brains

Top. MGE–derived interneuron precursors are harvested from the cortex and hippocampus of P1 *Nkx2.1-Cre;Ai9* mice, FACS–purified, and transplanted either homotopically (Ctx-to-Ctx, Hip-to-Hip) or heterotopically (Ctx-to-Hip, Hip-to-Ctx) into P1 wild-type (WT) mice.

Bottom. 30 days post-transplantation, tomato$^+$ cells are dispersed throughout the host regions, displaying morphologies and neurochemical markers similar to endogenous interneurons. Grafted interneurons integrate into the host circuitry, as indicated by the postsynaptic responses in pyramidal cells upon stimulation of adjacent *Nkx2.1-Cre;Ai32*–derived, channel rhodopsin–expressing interneurons.

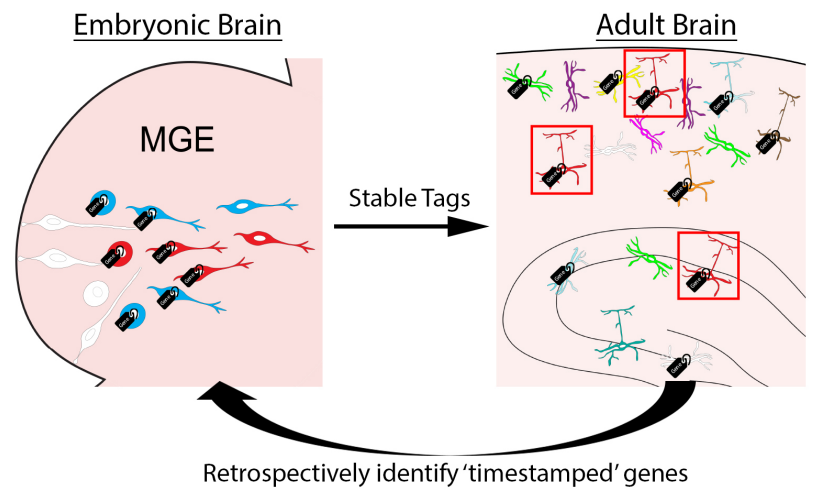

FIGURE 4. Timestamp of actively transcribed genes during development for future analysis

The goal of this approach is to label actively transcribed genes with stable methylation tags during embryogenesis as progenitors are undergoing initial fate decisions in the MGE. Then we can harvest specific interneuron subtypes in the adult brain using various transgenic mouse lines. Retrospective identification of actively transcribed gene during embryogenesis will provide us with candidate fate-determining genes for specific interneuron subtypes.

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, where monoallelic expression is regulated by a common *cis*-acting DNA regulatory element called the Imprinting Control Region (ICR). We study a cluster of imprinted genes 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. Imprinting of *H19* and of *Igf2* is regulated by the *H19ICR*, which is located just upstream of the *H19* promoter. Upon maternal inheritance, the *H19ICR* is not methylated, binds to the transcriptional repressor CTCF, and organizes the locus into loop structures that bring the *H19* promoter into contact with downstream enhancers but exclude the *Igf2* promoter from these enhancer interactions. Upon paternal inheritance, the *H19ICR* is methylated and cannot bind to CTCF. Therefore alternative loop structures form that allow *Igf2* promoters to interact with the shared enhancers while preventing *H19* promoter/enhancer interactions. In humans, epigenetic mutations that disrupt *H19ICR* function result in loss of monoallelic expression. Mutations on the paternal *H19ICR* lead to loss of *Igf2* expression and biallelic (2X) *H19* expression and are associated with the Russell-Silver syndrome. Mutations on the maternal *H19ICR* lead to loss of *H19* but biallelic (2x) *Igf2* expression and are associated with the Beckwith Wiedemann syndrome and several pediatric cancers. Our lab generated mouse models that phenocopy the human diseases, and our goal is to characterize the molecular defects associated with misimpression of *Igf2/H19* and to understand how these molecular defects lead to disease and cancer. In particular, we strive to understand the role of development in disease progression. In addition, to the *Igf2/H19* locus, we are completing analyses of mice carrying conditional mutations at the *Casq2* locus to understand the role of development in cardiac disease progression.

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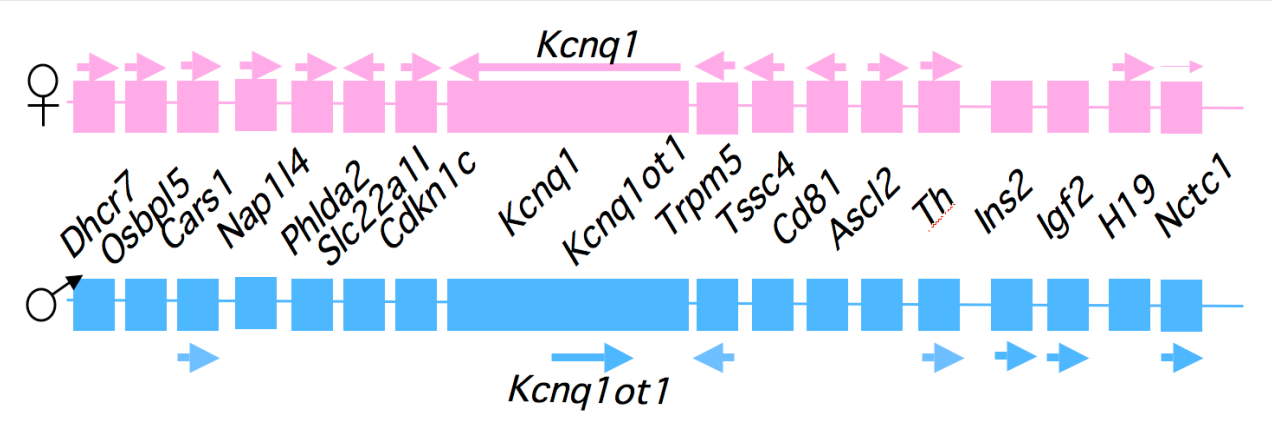


FIGURE 1. An imprinted domain on mouse distal chromosome 7

Maternal (*pink*) and paternal (*blue*) chromosomes are indicated. Horizontal arrows denote RNA transcription.

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. 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* (*H19ICR*), which lies between the two genes, just upstream of the *H19* promoter (Figure 2) (Reference 1). On the maternal chromosome, binding of the CTCF protein, a transcriptional regulator, to the *H19ICR* 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 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 *ICR*s is not acquired until relatively late in development, after the embryo implants into the uterus. In contrast, at

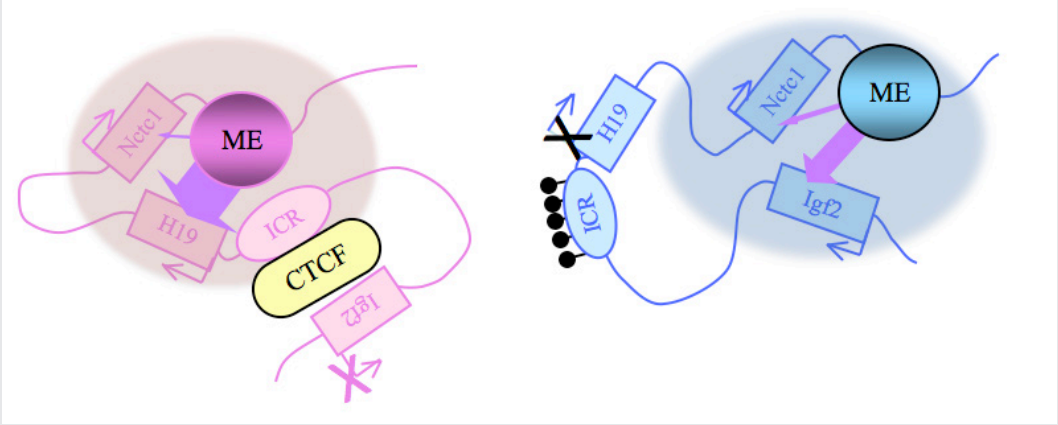


FIGURE 2. Distinct maternal and paternal chromosomal conformations at the distal 7 locus

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.

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 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; 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, 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

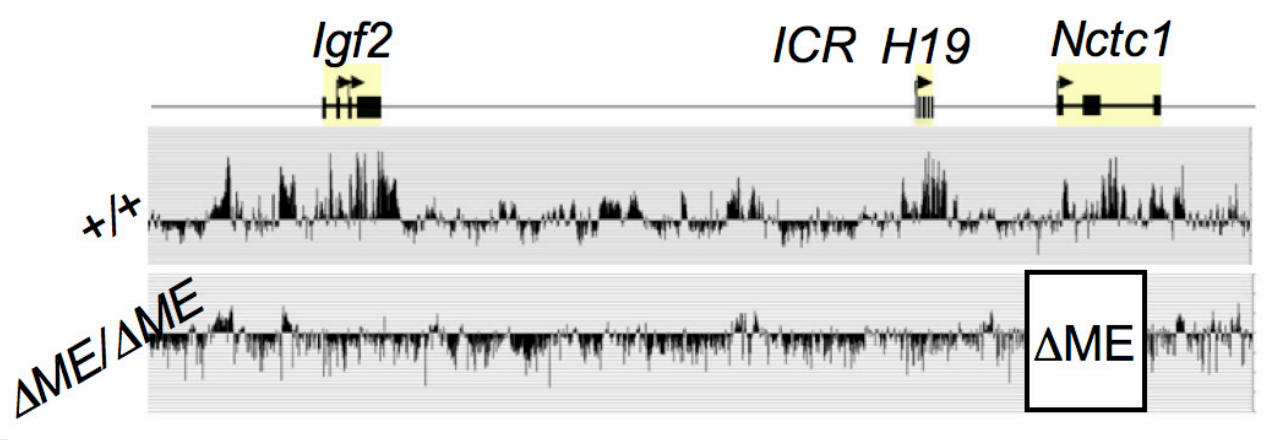


FIGURE 3. Distinct maternal and paternal chromosomal conformations at the distal 7 locus

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.

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

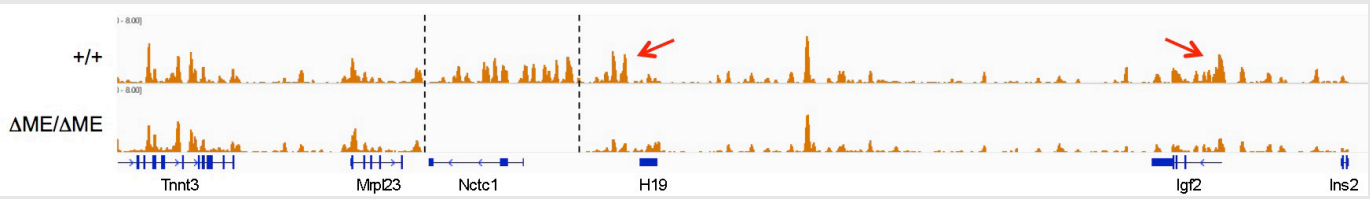


FIGURE 4. Chromatin patterns at the *Igf2/H19* locus are independent of enhancer activity.

Chromatin was isolated from wild-type and enhancer-deletion muscle cells, using antibodies to H3K4me1, and analyzed by DNA sequencing.

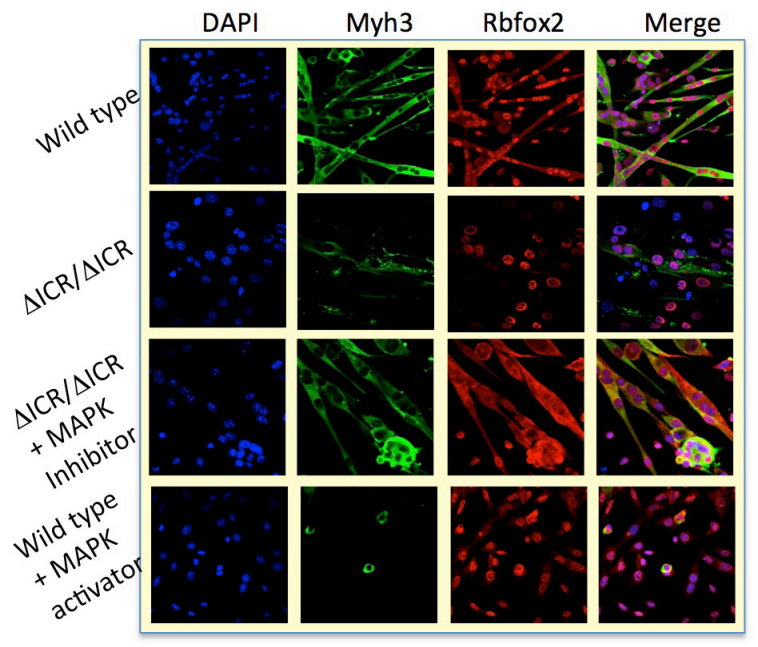


FIGURE 5. Muscle cell–differentiation defects in *Igf2/H19* loss-of-imprinting mice

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.

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 (RNAP) II NOT ONLY TO ITS COGNATE PROMOTERS (I.E., TO THE H19 AND *IGF2* PROMOTERS) BUT ALSO ACROSS THE ENTIRE INTERGENIC REGION.

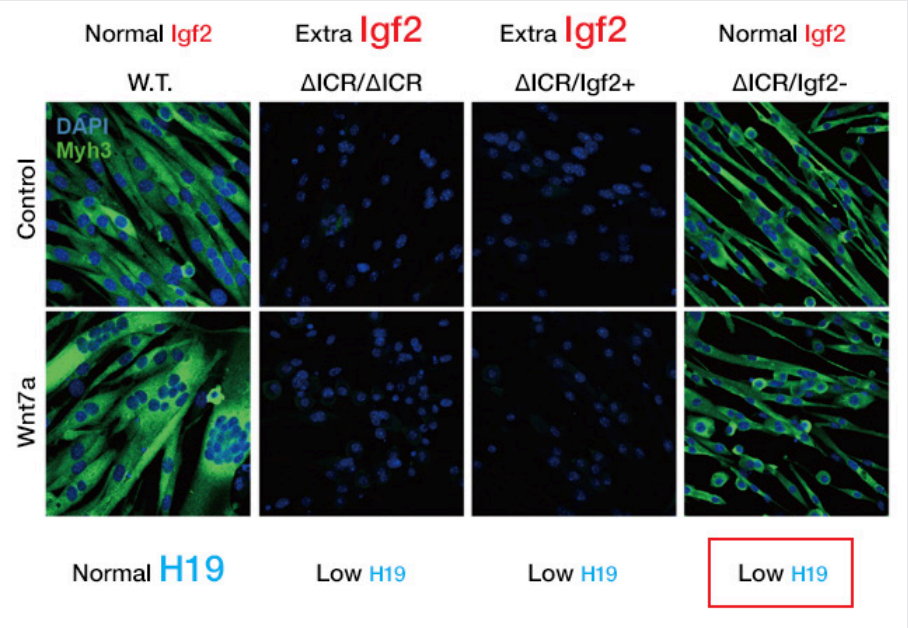
We used ChIP-on-chip to analyze RNAP localization on chromatin prepared from wild-type and from enhancer-deletion (DME) cell lines (Figure 3). As expected, RNAP binding to the *H19* and *Igf2* promoters is entirely enhancer-dependent. Curiously, we also noted enhancer-dependent RNAP localization across 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

FIGURE 6. The long non-coding 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 ΔICR/DICR and ΔICR/Igf2+ cells. Mutation of the paternal *Igf2* gene can restore normal Igf2 expression levels and thus restore normal differentiation (see ΔICR/Igf2– cells). However, these cells still do not make the H19 long non-coding RNA, do not fuse efficiently, and do not respond to Wnt7a signaling.



transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as 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.

Both RNA transcription and RNAP binding across the *Igf2*/*H19* domain are 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 (References 3, 4)

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, *H19ICR*–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 5).

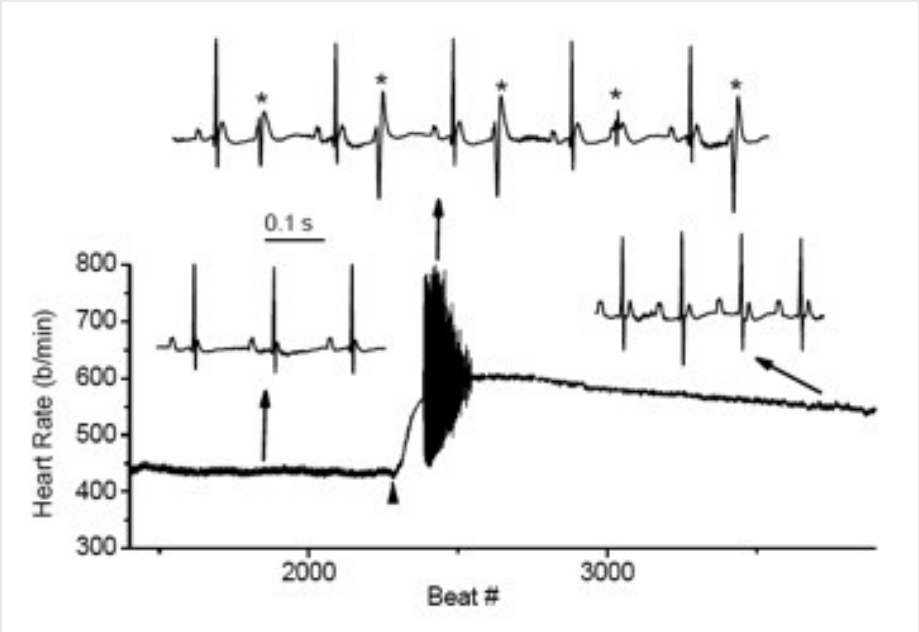


FIGURE 7. Cardiac arrhythmias in calsequestrin-2–deficient mice phenocopy the human disease.

Premature ventricular complexes (*) are induced by stress in *Casq2*–deficient but not in wild-type mice.

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 elevated 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 of *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 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). Molecular and genetic analyses indicate that H19 normally functions to bind to p53 and reduce its bioavailability. Thus, loss of H19 results in increased p53 function and therefore to enhanced activation of the mTOR/AKT signaling pathways.

Function of H19 and Igf2 genes in cardiac development

Cardiac dysfunction is a common phenotype in Beckwith Wiedemann syndrome patients. We observed that our BWS mouse model also results in cardiac dysfunction as measured by echocardiography and ECG analyses. Molecular and molecular genetic analyses indicate that these defects derive from two independent problems. In fetal animals, ballelic expression of *Igf2* leads to increased circulating IGF2 peptide, which, by

neonatal day 1, results in hyper-activation of AKT signaling pathways, and to cellular hyperplasia and hypertrophy. The hypertrophy is not clearly pathological but resembles the hypertrophy caused by exercise. However, loss of *H19* expression in the postnatal heart itself does lead to pathologic hypertrophy. At the molecular level, the problem is associated with reduced repression of p53 signaling pathways.

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 restoration of Casq2 in adult animals is sufficient to fully restore cardiac function. Moreover, restoration solely in pacemaking cells is also enough to rescue function, suggesting an important role for reduced heart rate in the CPVT phenotype as well as a new target for therapeutic interventions.

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. Park K-S, Mitra A, Rahat B, Kim B, Pfeifer K. Loss of imprinting mutations define both distinct and overlapping roles for misexpression of IGF2 and of H19 lncRNA. *Nucleic Acids Res* 2017 45(22):12766–12779.

5. Gebert C, Correia L, Li Z, Petrie HT, Love PE, Pfeifer K. Chromosome choice for initiation of V-(D)-J recombination is not governed by genomic imprinting. *Immunol Cell Biol* 2017 95:473-477.

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Cholesterol Homeostasis and Genetic Syndromes

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 and neurodegenerative disorders resulting from impaired intracellular cholesterol and lipid transport. 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. Niemann-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 recently completed a Phase I/IIa therapeutic trial of intrathecal 2-hydroxypropyl-beta-cyclodextrin (VTS-270) for NPC1, and a multicenter, multinational clinical efficacy trial of intrathecal VTS-270 is currently in progress. We also recently completed a Phase I/IIa trial of vorinostat (Zolinza) for the treatment of NPC1.

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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 various 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

FIGURE 1.

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

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 delta 7-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 several 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* $^{93M/T93M}$). 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, which results from inhibition of Wnt signaling owing to a toxic effect of 7-DHC

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 as a result of 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

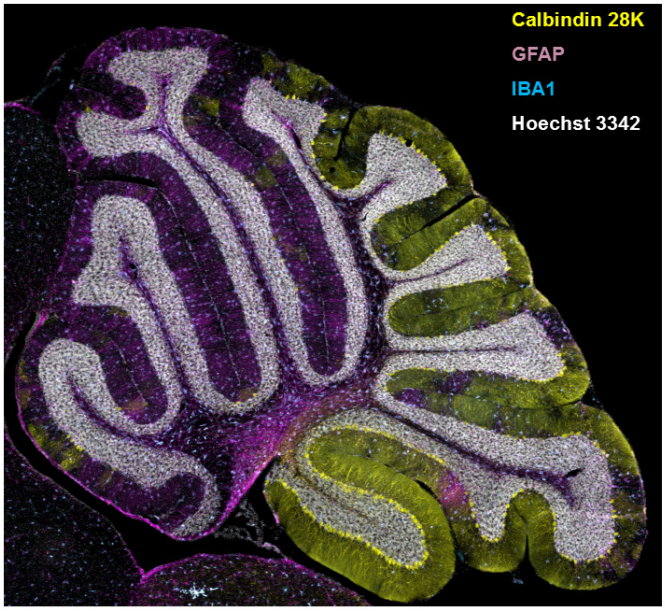

FIGURE 2. Gliosis in NPC1 mouse cerebellum

Immuno-staining of a sagittal section from the cerebellum of an NPC1–mutant mouse. Cerebellar Purkinje neurons are stained for calbindin 28K, and the expected loss of anterior Purkinje neurons is readily apparent. Expression of GFAP and IBA1 are used to detect astrogliosis and microgliosis, respectively. Nuclei are stained with Hoechst 3342.

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. We are currently investigating the pathological role of other 7-DHC–derived oxysterols such as DHCEO (3β,5α-dihydroxy-cholest-7-en-6-one).

Development of patient-derived induced pluripotent stem cells has given us insight into fundamental mechanisms that impair neuronal development 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 100 SLOS patients.

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.

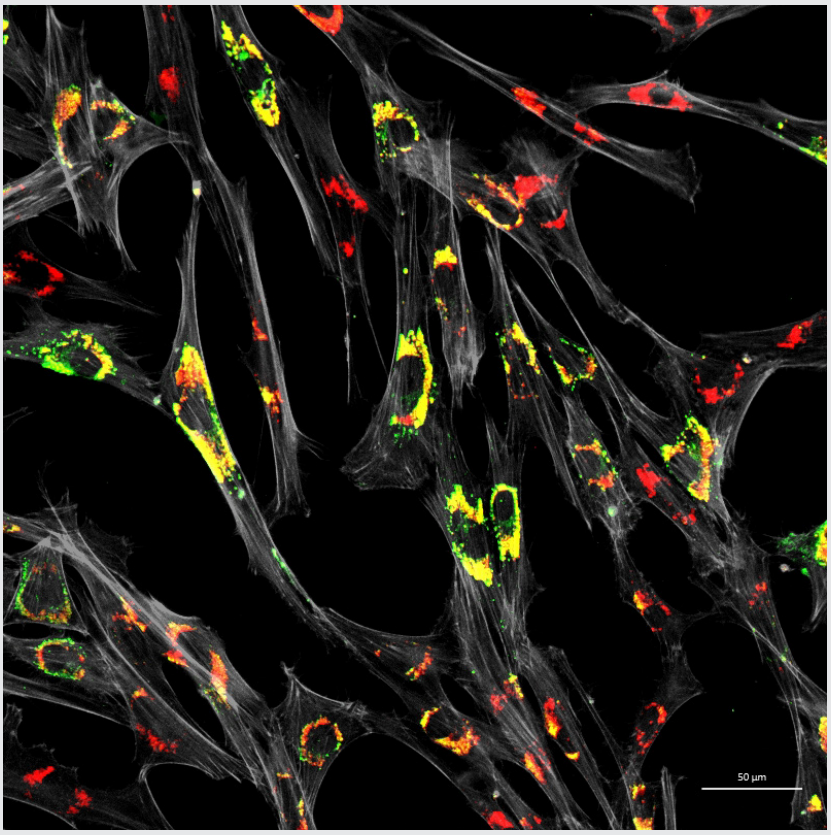

FIGURE 3. Accumulation of unesterified cholesterol in NPC1 patient fibroblasts

Human NPC1 fibroblasts were immuno-stained for Lamp1 (*green*) and stained with filipin (*red*). Filipin stains unesterified cholesterol, which accumulates in the Lamp1–positive endolysosomal compartment. Cell structure was outlined by immuno-staining for actin (*gray*).

LATHOSTEROLOSIS AND DESMOSTEROLOSIS

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 levels 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.

Desmosterolosis is another inborn error of cholesterol synthesis that resembles SLOS. It results from a mutation in the 3β-hydroxysterol delta 24-reductase gene (*DHCR24*). DHCR24 catalyzes the reduction of desmosterol to cholesterol. We disrupted the mouse *Dhcr24* gene with targeted homologous recombination in embryonic stem cells. Surprisingly, although most *Dhcr24* mutant mice die at birth, the pups are phenotypically normal.

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 100 NPC1 patients in 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, testing for oxysterols or bile acid derivatives has now become a standard method of diagnosis.

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 lower levels of the non-enzymatically produced oxysterols. In collaboration with the Therapeutics of Rare and Neglected Disease Program of NCATS, we recently completed a phase1/2a therapeutic trial of lumbar intrathecal cyclodextrin therapy in NPC1. We have now transitioned 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 a histone deacetylase (HDAC) inhibitor, vorinostat, in adult NPC1 patients. The collaboration also includes scientists from the University of 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
- Bench to Bedside award: Investigations of Juvenile Neuronal Ceroid Lipofuscinosis (CLN3)
- U01HD0990845: Intravenous delivery of 2-hydroxypropyl-beta-cyclodextrin for treatment of Niemann-Pick C disease

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Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies

Roberto Romero, MD, DMedSci,
Chief, Perinatology Research Branch

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. The cost of prematurity in the U.S. alone is estimated to be $26 billion per year. An important goal is to understand the mechanisms of disease responsible for spontaneous preterm birth and fetal injury and to develop methods for the prediction and prevention of preterm birth.

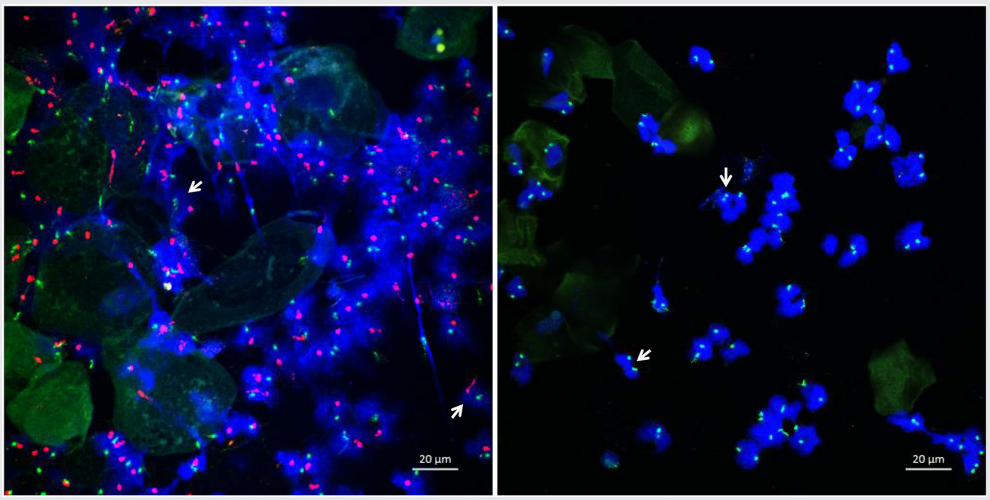
The Perinatology Research Branch (PRB) has proposed that preterm parturition is a syndrome caused by multiple pathologic processes, i.e., that preterm labor is one syndrome but has many causes. The emphasis of our Branch is to study intra-amniotic infection and inflammation, vascular disorders, maternal anti-fetal rejection (chronic inflammatory lesions of the placenta), cervical disease, and a decline in progesterone action. This year, we reported that intra-amniotic inflammation, which affects at least one of every three preterm neonates, is characterized by the activation of amniotic-fluid neutrophils, cells that represent the first line of defense against infection. Using DNA fingerprinting, we determined that amniotic-fluid neutrophils are of fetal origin in cases of preterm labor, maternal origin in cases of clinical chorioamnionitis at term, and mixed origin in patients who have inflammatory processes near term. Moreover, in a series of studies, we were able to demonstrate that neutrophils produce antimicrobial peptides and exhibit the formation of extracellular traps, whereby they immobilize and kill bacteria.

The Branch also studies other obstetrical syndromes that account for the high rate of infant mortality in the United States, including clinical chorioamnionitis, which is the most common infection-related diagnosis in delivery units around the world, as well as meconium aspiration syndrome and amniotic fluid embolism.

Congenital anomalies continue to be a leading cause of perinatal mortality in the U. S. Imaging, a powerful tool for scientific discovery, has changed the practice of obstetrics and maternal-fetal medicine. Imaging with ultrasound allows the definition of fetal anatomy, biometry, growth, and the study of physiologic parameters, such as cardiac function, fetal sleep, and breathing. We invented a new method for the examination of the fetal heart, called fetal intelligent navigation echocardiography (FINE). This year, we reported a major

FIGURE 1.

A) Left - fetal leukocytes;
B) Right - maternal leukocytes



breakthrough: Color Doppler FINE. Color-flow mapping is essential for adequate examination of the fetal heart in those suspected of having congenital anomalies. We demonstrated how Color Doppler FINE can be used to improve the diagnosis of congenital anomalies. The technology has been licensed and is now commercially available to sonographers worldwide.

Although ultrasound is the standard imaging modality in pregnancy, magnetic resonance imaging (MRI) has also been used to characterize fetal anatomy when ultrasound cannot provide definitive diagnostic answers. MRI provides unique information about fetal physiologic parameters (i.e., perfusion, oxygenation, and biochemistry) that are outside the domain of ultrasound. Moreover, MRI can be used to characterize the ontogeny of functional neuro-connectivity, as well as the potential relationship between insults that could alter fetal neuro-development. Given that preterm birth is a leading cause of neuro-developmental disorders, we have used noninvasive methods to interrogate neuro-connectivity. In previous work, we characterized the fetal neuroconnectome using fMRI. This year, we reported a study showing that fetuses subsequently born preterm have a disorder of neuro-connectivity compared with fetuses of the same gestational age subsequently born at term. Neuro-connectivity was reduced in the left hemisphere, close to the pre-language region, providing the first evidence that a disorder of functional connectivity is present in the fetus before birth.

A simple maternal blood test at 24–28 weeks can identify 80% of patients who will subsequently experience a fetal death (Reference 1).

In 2013, fetal death affected 23,595 pregnancies in the U. S., and an estimated 2.6 million third-trimester fetal deaths occurred worldwide. We previously reported that patients who experienced a fetal death had a lower concentration of placental growth factor (PIGF), a higher concentration of soluble endoglin (sENG), as well as a higher expression of soluble vascular endothelial growth factor Receptor-1 (sVERGFR-1) than those with a normal pregnancy after 20 weeks gestation. The objective of this study was to determine whether maternal plasma concentrations of angiogenic and anti-angiogenic factors measured at 24–28 weeks of gestation could predict subsequent fetal death.

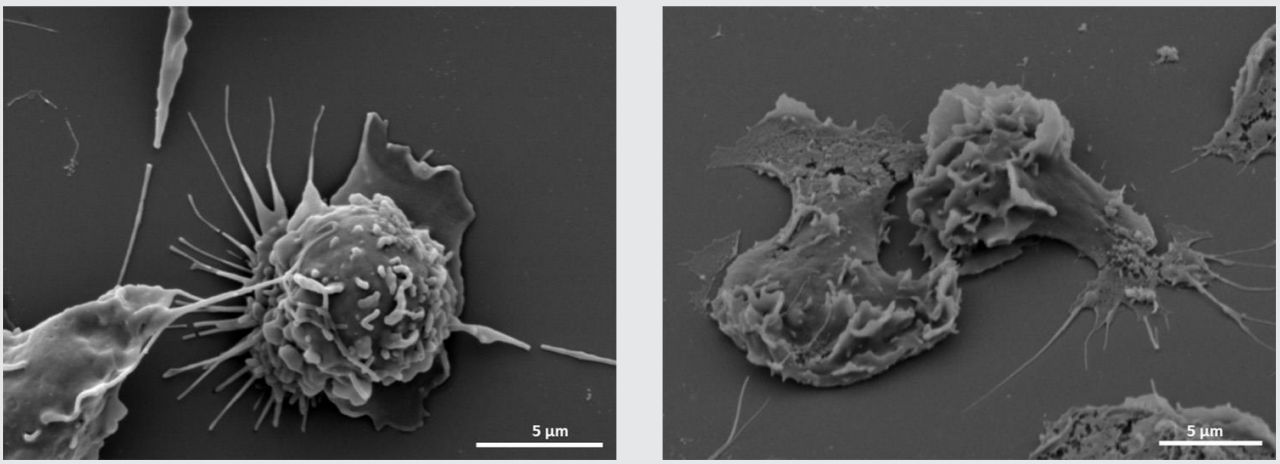


FIGURE 2.

Scanning electron microscopy. Left: maternal neutrophils in a resting stage with a round shape. Right: an amniotic fluid neutrophil forming a neutrophil extracellular trap with a flattened shape.

We conducted a case-cohort study that included 11 fetal deaths and 829 controls. The placentas of all fetal deaths were examined, and PlGF, sEng, and sVEGFR-1 concentrations were measured between 24–28 weeks of gestation. A positive test was defined as analyte concentrations (or ratios) of lower than 2.5th and 10th centiles [PlGF, PlGF/sVEGFR-1 (angiogenic index-1), and PlGF/sEng)] or greater than 90th and 97.5th centiles (sVEGFR-1 and sEng). The rate of placental lesions consistent with maternal vascular under-perfusion was 33.3% (1/3) among those who had a fetal death at less than 28 weeks and 87.5% (7/8) of those who had this complication at less than or at 28 weeks of gestation. A maternal plasma angiogenic index-1 value lower than the 2.5th centile (0.126) at 24–28 weeks of gestation carries a 29-fold increase in the risk of subsequent fetal death and identifies 55% of subsequent fetal deaths with a false-positive rate of 3.5%. Of note, 61% of women who have a false-positive angiogenic index-1 result will subsequently experience adverse pregnancy outcomes.

In women with intra-amniotic infection and/or inflammation, amniotic fluid neutrophils can be of either fetal or maternal origin (Reference 2).

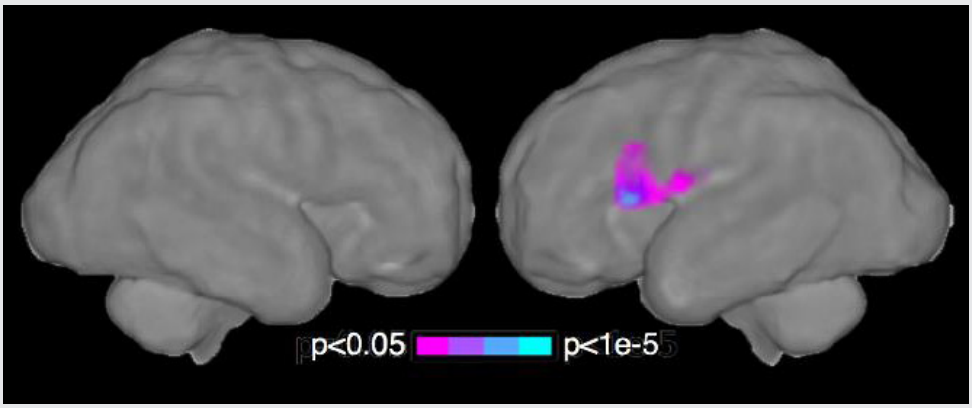
Neutrophils are the most abundant white blood cells in the amniotic cavity of women with intra-amniotic infection and/or inflammation. The current belief is that these neutrophils are of fetal origin. By using DNA fingerprinting, our study provided evidence that, in women with intra-amniotic infection and/or inflammation, amniotic fluid neutrophils can be of either fetal or maternal origin or a mixture of both fetal and maternal. The origin of amniotic fluid neutrophils was confirmed by detecting XY chromosomes (fetal, Figure 1A) or two XX chromosomes (maternal, Figure 1B) in samples collected from women who delivered a male neonate. The findings suggest that both the fetal and maternal innate immune system participate in the mechanisms of host defense against intra-amniotic infection.

Amniotic fluid neutrophils can form neutrophil extracellular traps, or NETs (Reference 3).

Amniotic fluid neutrophils were thought to participate in the mechanisms of defense against intra-amniotic

FIGURE 3.

We have discovered that weaker connections exist in the proto-language network of the preterm brain prior to the complications of early delivery, providing novel insight into the relationship between premature birth and subsequent neurological and behavioral health disorders.



infection. However, no functional evidence had validated this concept. The study provided *in vivo* and *ex vivo* evidence that amniotic fluid neutrophils can form neutrophil extracellular traps, or NETs, a mechanism whereby neutrophils immobilize and kill bacteria invading the amniotic cavity of women with intra-amniotic infection. The findings show that amniotic fluid neutrophils actively participate in the mechanisms of defense against intra-amniotic infection (Figure 2).

Altered functional connectivity in the preterm brain is identifiable before birth (Reference 4).

It has been suggested that neurological problems more frequent in those born preterm are expressed prior to birth, but given the technical limitations, this has been difficult to test in humans. We applied novel fetal resting-state functional MRI to measure brain function in 32 human fetuses *in utero* and found that systems-level neural functional connectivity was diminished in fetuses that would be subsequently born preterm. Neural connectivity was reduced in a left-hemisphere pre-language region, and the degree to which connectivity of this left language region extended to right-hemisphere homologs was positively associated with the time elapsed between fMRI assessment and delivery. The results provide the first evidence that altered functional connectivity in the preterm brain is identifiable before birth and suggest that neuro-developmental disorders associated with preterm birth may result from neurological insults that begin *in utero* (Figure 3).

Color and power Doppler combined with Fetal Intelligent Navigation Echocardiography (FINE) to evaluate the fetal heart (Reference 5)

Color Doppler flow mapping is a valuable and integral component of fetal cardiac examination, given that it allows identification of cardiac structures and vasculature, as well as the pattern and direction of blood flow throughout the heart. Indeed, some investigators have recommended that color Doppler should be routinely employed in fetal cardiac screening. In fetuses with congenital heart disease (CHD), color Doppler sonography is essential to identify and characterize abnormal cardiovascular anatomy, flow patterns/disturbances, and cardiac function. For the first time, we combined color and bidirectional power Doppler (S-flow) with Fetal Intelligent Navigation Echocardiography (FINE) to examine the fetal heart. FINE is a novel method invented by our group that interrogates fetal cardiac volume datasets and allows the automatic display of nine standard fetal echocardiography views required to diagnose most cardiac defects. We

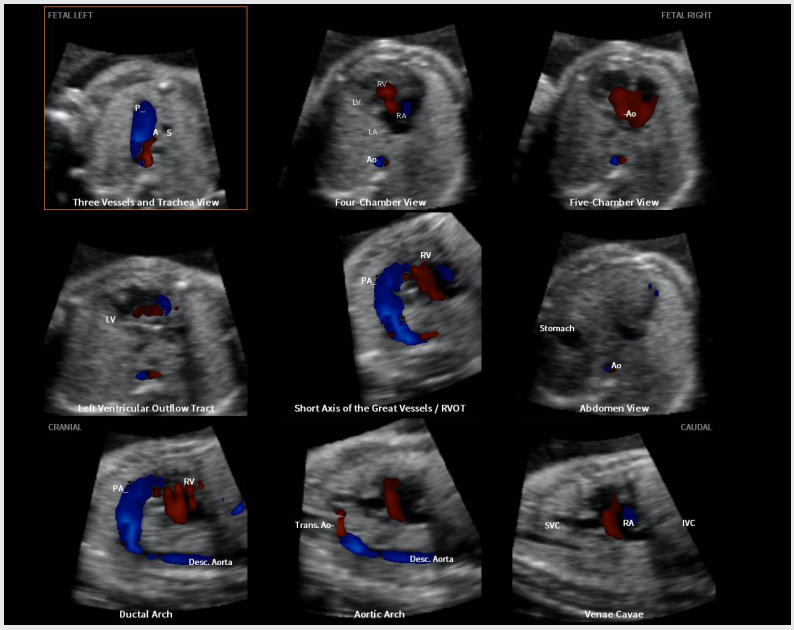


FIGURE 4.

This figure of a fetus with hypoplastic left heart and coarctation of the aorta diagnosed prenatally using color Doppler FINE was featured on the cover of Reference 5.

conducted a prospective cohort study in fetuses with normal hearts or CHD in the second and third trimesters. Sixty cardiac volumes (color Doppler, n=27; S-flow Doppler, n=33) were analyzed using FINE. The rate of successfully generating eight fetal echocardiography views with appropriate color and S-flow Doppler information was 89–100% and 91–100% of cases, respectively, using a combination of diagnostic planes and/or Virtual Intelligent Sonographer Assistance (VIS-Assistance®). However, the success rate for the ninth echocardiography view (i.e., superior and inferior vena cava) was 33% and 30% of cases for color and S-flow Doppler, respectively. In all four cases of congenital heart disease, color Doppler FINE demonstrated evidence of abnormal fetal cardiac anatomy and/or hemodynamic flow. Thus, for the first time, we showed that the FINE method, applied to cardiac volume datasets of normal fetal hearts acquired with color or bidirectional power Doppler information, can successfully generate eight to nine standard fetal echocardiography views (via color or power Doppler) in the second to third trimesters. In addition, for cases of CHD (Figure 4), color Doppler FINE successfully demonstrated abnormal anatomy and/or flow characteristics.

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Regulation of Mammalian Intracellular Iron Metabolism and Biogenesis of Iron–Sulfur Proteins

Our goal is to understand how mammals regulate intracellular and systemic iron metabolism to support processes that require iron and iron-sulfur clusters. 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 with motor neuron loss 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. 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 hypoxia-inducible factor (HIF) 2a through the IRE–IRP system. Our discovery introduces a new level of physiological regulation of erythropoiesis and provides a model for early pulmonary hypertension.

Our ongoing work on iron-sulfur cluster biogenesis has led to new insights into how mammalian iron-sulfur clusters are synthesized and transferred to appropriate recipient proteins. Several human diseases are now known to be caused by deficiencies in the iron-sulfur–cluster biogenesis machinery. We developed a treatment for the rare disease ISCU (iron-sulfur cluster assembly enzyme) myopathy. By identifying a tri-peptide motif common to many iron-sulfur recipient proteins, we developed an algorithm that facilitates discovery of previously unrecognized mammalian iron-sulfur

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proteins. Our work suggests that there are hundreds of previously unrecognized mammalian iron-sulfur proteins. Discovery of iron-sulfur cofactors will lead to breakthroughs in several research areas involving DNA repair, ribosomal biogenesis, mRNA translation, intermediary metabolism, and regulation of the growth and energy-sensing pathways that are critical for determining the fates of many cell types.

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, in iron-replete cells 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 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 the 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 the IRE at its 5' end that is important in permitting iron to cross the duodenal mucosa in iron-deficient animals and in preventing developing erythroid cells from retaining high amounts of iron in iron-deficient animals. Our findings explain why microcytic anemia is usually the first physiological manifestation of iron deficiency in humans. In addition, we recently discovered that loss of IRP1 causes polycythemia and pulmonary hypertension through derepression of hypoxia-inducible factor 2a (HIF2a) 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 (HMOX1). 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-

Members of the Section on Human Iron Metabolism

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 cultured macrophages from the *Hmox1*$^{-/-}$ mice, experiments that would pave the way to treating heme oxygenase 1–deficient human patients through bone marrow transplants or direct injection of corrected macrophages. Five human *HMOX1*$^{-/-}$ patients have been identified, but we believe this represents an under-diagnosed rare human disease that is often mis-diagnosed.

Mammalian iron–sulfur cluster biogenesis

Our goal in studying mammalian iron-sulfur biogenesis is 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, a protein that promotes the biosynthesis of heme and assembly and repair of iron-sulfur clusters by delivering enhancing early steps of iron-sulfur cluster biogenesis, 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 the iron-sulfur cluster assembly enzyme 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 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 that mutations of two iron-sulfur cluster assembly proteins, NFU1 and BOLA3, are required for correct lipoylation of many critical metabolic complexes, including pyruvate dehydrogenase. We identified a tripeptide motif, LYR, in apoproteins that are recipients of nascent iron-sulfur clusters. The co-chaperone HSC20 binds to HSPA9, its partner HSP70–type chaperone, and that chaperone complex binds to ISCU bearing a nascent iron-sulfur cluster and to iron-sulfur cluster–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 (SDHB), we discovered that several 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 the identification of unknown iron-sulfur proteins, which are likely to be much more common in mammalian cells than has been previously appreciated. More recently, we discovered that HSC20 is responsible for delivery of iron-sulfur clusters to respiratory chain complexes I-III, through recognition of LYR-like motifs in these recipient proteins.

Using expression arrays, we analyzed 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 discovered that anti-sense therapy would likely work as a treatment for ISCU myopathy patients, as we were 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.

Using informatics, over-expression of candidate proteins, and iron detection using ICP-MS (inductively coupled mass spectrometry), we identified many more iron-sulfur proteins that are involved in a wide range of metabolic pathways, ranging from intermediary metabolism, DNA repair and RNA synthesis, and possibly regulation of cellular growth control. Iron-sulfur proteins will prove to be integral to function and sensing of numerous pathways important in cellular functions.

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Control of Ectodermal Development in Vertebrate Embryos

The laboratory focuses on mechanisms regulating the differentiation of cranial neural crest cells, which give rise to the bone and cartilage of the vertebrate jaw, neuro-cranium, 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. We 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 enhancers 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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Dlx gene function in cranial neural crest development

In the previous year we focused on loss-of-function experiments with the Dlx family of homeodomain transcription factors. Following reports from numerous labs, including our own, indicating that

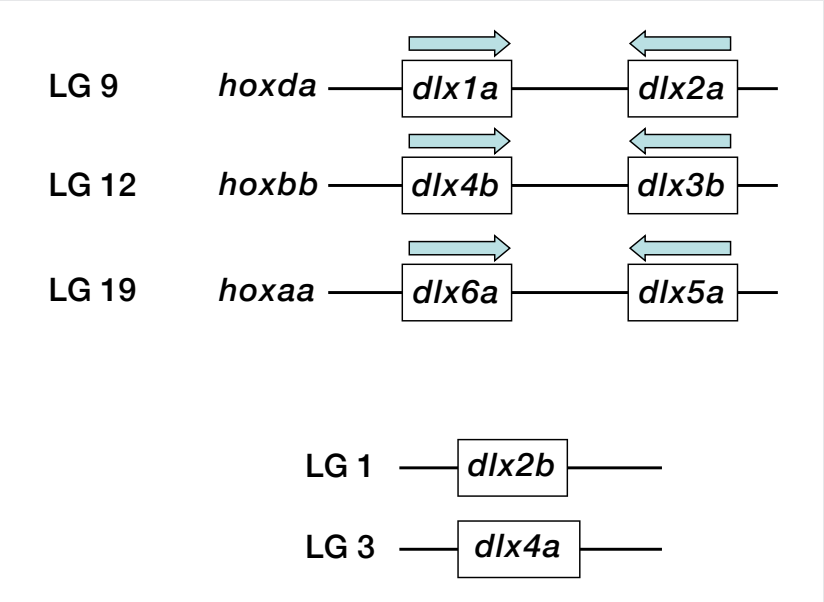

FIGURE 1. Genomic organization of *dlx* genes

Zebrafish *dlx* genes *dlx1a*, *dlx2a*, *dlx3b*, *dlx4b*, *dlx5a*, and *dlx6a* are organized into three pairs of convergently transcribed loci with about 10kb of intergenic 3' sequence. Each pair is linked to one hox gene cluster, mirroring the pattern found in other vertebrates such as human. The remaining two, *dlx2b* and *dlx4a*, are located at unlinked positions in the genome. Drawing is not to scale.

"knock down" analysis of gene function using antisense morpholinos frequently yielded results that were 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. So far, we identified INDEL (insertion and deletion) mutations that disrupt protein function of all six of the canonical *dlx* genes (i.e., *dlx1–6*), and we are in the process of targeting the two "extra" zebrafish *Dlx* loci (*dlx2b* and *dlx4a*). By in-crossing heterozygous carriers, we generated null embryos for all six individual *dlx* loci, as well as double-nulls for the linked pairs of genes (*dlx1a+dlx2a*, *dlx3b+dlx4b,* and *dlx5a+dlx6a*). In most cases, we observed little if any effect on early development. In particular, craniofacial cartilage formation was not affected. The exceptions were *dlx2a* and *dlx1a/dlx2a* double mutants, which showed abnormal jaw development in a portion of null embryos (i.e., incomplete penetrance). Most surprising is the observation that the *dlx5a/dlx6a* double-mutant embryos have apparently normal craniofacial development; equivalent mutations in the mouse result in massive defects in cranial morphology and limb formation. Our general conclusion is that, in zebrafish, *dlx* genes are much less critical for craniofacial or limb development (fins are also unaffected) than in the mouse. The finding has important evolutionary implications and is also relevant to the use of zebrafish as a general model system for functional analysis of genes found associated with disease in humans.

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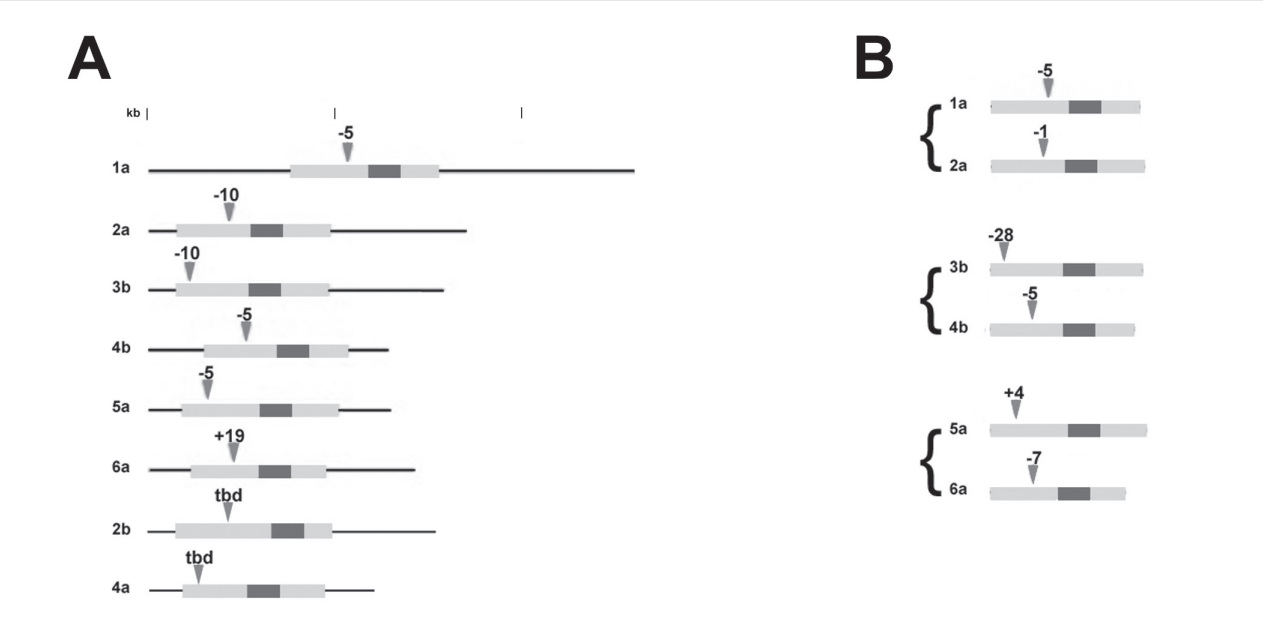

FIGURE 2. Summary of INDEL mutations in zebrafish *dlx* genes

A) The mRNAs of individual *dlx* genes are shown, with the open reading frames (ORFs) indicated in light gray and the homeodomains in dark gray. The location and size of deletions (-) or insertions (+) are indicated by the arrow heads. INDELs in *dlx2b* and *dlx4a* have not been fully characterized; tbd, to be determined.

B) Summary of the paired null mutations in linked loci (only ORFs are shown).

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Mechanisms of Synapse Assembly, Maturation, and Growth during Development

The purpose of our research is to understand the mechanisms of synapse development and homeostasis. The chemical synapse is the fundamental communication unit, connecting neurons in the nervous system to one another and to non-neuronal cells, and is designed to mediate rapid and efficient transmission of signals across the synaptic cleft. Such transmission forms the basis of the biological computations that underlie and enable our complex behavior. Crucial to this function is the ability of a synapse to change its properties, so that it can optimize its activity and adapt to the status of the cells engaged in communication and/or to the larger network comprising them. Consequently, 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 that 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 that include genetics, biochemistry, molecular biology, super-resolution imaging, and electrophysiology recordings in live animals and reconstituted systems.

Because of its many advantages, we choose to study these events in a powerful genetics system, *Drosophila melanogaster*, and to use the neuromuscular junction (NMJ) as a model for glutamatergic synapse development and function. The fact that individual NMJs 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. In addition, the richness of genetic manipulations that can be performed in *Drosophila* permits independent control of individual synaptic components in distinct cellular compartments. Furthermore, 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

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in the structural and physiological plasticity of glutamatergic synapses, which are associated with a variety of human pathologies from learning and 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.

Synapse assembly at the *Drosophila* neuromuscular junction (NMJ) requires the obligatory auxiliary subunit Neto.

At the fly and vertebrate NMJ, synaptogenesis follows the arrival of a motor neuron at its target muscle. Prior to neuron arrival, the ionotropic glutamate receptors (iGluRs) form small, nascent clusters on the muscle, which are distributed in the vicinity of future synaptic sites. Neuron arrival triggers formation of large synaptic iGluRs aggregates and promotes expression of more iGluRs to permit synapse maturation and growth. The iGluR clusters interact with the local cytoskeleton and other synaptic structures to maintain local density. This involves solving two fundamental problems common to all chemical synapses: first, trafficking the components to the proper site, and second, organizing those components to build synaptic structures. Recent advances, particularly from vertebrate iGluR biology, reveal that the solution to these problems is entirely dependent on the activity of a rich array of auxiliary subunits that associate with the receptors. These highly diverse transmembrane proteins associate with iGluRs at all stages of the receptor life-cycle and mediate the delivery of receptors to the cell surface, their distribution, synaptic recruitment, association with various postsynaptic density (PSD) scaffolds, and importantly, their channel properties. As they are assembled from different subunits, iGluRs have strikingly varied biophysical properties; their association with different auxiliary subunits increases this diversity even further.

We recently discovered that an obligatory auxiliary protein, Neto, is absolutely required for iGluRs to cluster and for NMJ functionality. To date, Neto is the only auxiliary protein characterized in *Drosophila*. Neto belongs to a family of highly conserved auxiliary proteins that regulate glutamatergic synapses. 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. We found 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 iGluR postsynaptic composition, and (3) modulates iGluR function but not their assembly or surface delivery. More specifically, we found that Neto activities are regulated by Furin-mediated limited proteolysis, which removes an inhibitory prodomain. When the prodomain cleavage is blocked, Neto engages iGluRs *in vivo*, but cannot mediate their stable incorporation/clustering at synaptic sites and fails to initiate postsynaptic differentiation.

To identify proteins that interact with Neto and provide iGluR–clustering activities at the developing NMJ, we initiated two complementary screens: a synthetic lethality screen, and mass-spectroscopy comparison of proteins present in the NMJ synaptosomal fractions isolated from control and *neto* mutant larval carcasses. In both cases, we took advantage of a strong *neto* hypomorph mutant that we isolated and characterized in our laboratory, *neto*^{109}. The mutant has drastically diminished levels of synaptic iGluRs, albeit normal net levels of muscle receptors. Intriguingly, the iGluRs are expressed on the muscle surface but have a diffuse, extra-junctional distribution, indicating that *neto*^{109} has a defect in the trafficking and/or stabilization

of receptors at junctional locations. Our preliminary analysis of the spectral counts in synaptosomal fractions from control and mutant larvae revealed several interesting candidates, present only in control fraction. We are in the process of validating the candidates and generating reagents to reveal their roles in synaptogenesis. In addition, 50% of *neto*^{109} hypomorphs die during development; further reduction of synaptogenic proteins in hemizygous animals should increase lethality. Using this rationale, we have started to screen the deficiencies available on chromosomes 2 and 3 and also test candidate genes previously reported to affect the NMJ function. We expect that the screen will reveal molecules that function in iGluR clustering, as well as proteins that control synaptic trafficking of Neto/iGluR complexes. In addition, the screen may identify other pre- and postsynaptic components important for synapse development.

Modulation of iGluRs function

Assembled from different subunits, iGluRs have strikingly varied biophysical properties; their association with different auxiliary subunits increases this diversity even further. Until recently, our investigations on iGluR function were limited by the inability to reconstitute functional *Drosophila* NMJ receptors in heterologous systems. In collaboration with Mark Mayer, we recently solved this problem by accomplishing the first functional reconstitution of NMJ iGluRs in *Xenopus* oocytes. Using this system, we found that Neto increases glutamate-activated currents by several orders of magnitude, but has a comparatively modest effect on the surface delivery of the iGluRs (up to a four-fold increase). Also, heterotetrameric iGluRs are absolutely 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, kainite, or NMDA.

In flies as in humans, synapse strength and plasticity are determined by the interplay between different iGluR subtypes. At the fly NMJ, the type-A and type-B iGluRs consist of four different subunits: either GluRIIA or GluRIIB, plus GluRIIC, GluRIID, and GluRIIE. Different, genetically distinct mechanisms control the synaptic recruitment of type-A and type-B receptors at the NMJ; however, we found that both receptor subtypes absolutely require Neto for their function. Given that the synaptic recruitment of iGluRs is also influenced by receptor activity, Neto may regulate iGluR clustering partly by modulating the receptor function.

Drosophila neto codes for two isoforms (α and β), which have different intracellular domains generated by alternative splicing. Muscle expression of either Neto-α or Neto-β could rescue the embryonic lethality and iGluR clustering defects of *neto* null mutants, although only Neto-β appears to efficiently recruit postsynaptic components. Both isoforms increase the glutamate-activated currents of *Drosophila* NMJ iGluRs in *Xenopus* oocytes, with Neto-β having a slightly stronger effect than Neto-α. Interestingly, a truncated Neto-ΔCTD variant, which lacks any intracellular part but retains the highly conserved extracellular and transmembrane domains, could also elicit NMJ iGluR–mediated currents in *Xenopus* oocytes. When overexpressed in the larval muscle, Neto-ΔCTD is required and sufficient for clustering of receptors *in vivo*.

To further characterize the receptor properties and determine how Neto modulates iGluR function, we reconstituted iGluRs in HEK293T-17 cells and examined single-channel currents evoked repeatedly by fast glutamate applications to (outside-out) patches containing only a few recombinant receptors. We had already found that Neto is absolutely required to elicit glutamate-gated currents. As in the *Xenopus* system, both type-A and type-B receptors are present on the surface of the HEK293T-17 cells, but without Neto there are no detectable currents. We are currently applying this new methodology to individual type-A and -B

receptors in complexes with Neto-α or Neto-β to determine subtype specific properties. These studies will allow us to directly measure mutant NMJ iGluR currents and tease apart the role of Neto in receptor function versus synaptic recruitment.

Local BMP/BMPR complexes regulate synaptic plasticity and homeostasis.

Synaptic activity and synapse development are intimately linked, but our understanding of the coupling mechanisms remains limited. Anterograde and retrograde signals together with trans-synaptic complexes enable intercellular communication. How synapse activity status is monitored and relayed across the synaptic cleft remains poorly understood. Our studies uncovered a role for Bone Morphogenetic Proteins (BMP) in sensing the activity of postsynaptic receptors across the synaptic cleft. At the *Drosophila* NMJ, BMP signaling is critical for NMJ growth and neurotransmitter release. It is generally thought that BMP signaling fulfills these functions via canonical and noncanonical pathways triggered primarily by muscle-secreted Glass-bottom boat (Gbb), a BMP7 homolog. Gbb signals by binding to the presynaptic BMP type-II receptor (BMPRII) Wishful thinking (Wit) and to the BMPRIs Thickveins (Tkv) and Saxophone (Sax). The canonical BMP pathway induces the accumulation of the pathway effector pMad (phosphorylated Smad) in motor neuron nuclei, which activates presynaptic transcriptional programs with distinct roles in the structural and functional development of the NMJ. Gbb and Wit signal non-canonically through the effector protein LIM kinase 1 (LIMK1) to regulate synapse stability. pMad also accumulates at synaptic locations but the biological relevance of this phenomenon remained a mystery for over a decade.

We recently found that presynaptic (neuronal) pMad correlates with postsynaptic (muscle) sensitivity and constitutes a sensor for synapse activity. Furthermore, synaptic pMad marks a novel, noncanonical BMP–signaling modality that is genetically distinguishable from all other known BMP–signaling cascades. This novel pathway does not require Gbb, but depends on presynaptic Wit and Sax and the activity of a particular subtype of postsynaptic glutamate receptors, type-A receptors. Unlike canonical BMP signaling, synaptic pMad plays no role in the regulation of NMJ growth. Instead, we found that selective disruption of presynaptic pMad accumulation reduces postsynaptic levels of the receptor GluRIIA, revealing a positive feedback loop that appears to function to stabilize active type-A receptors at synaptic sites. Thus, the novel BMP signaling modality appears to sculpt synapse composition and maturation as a function of synapse activity. Given that synaptic pMad accumulates at the active zone, near the presynaptic membrane, in close juxtaposition with the iGluRs containing postsynaptic densities, we proposed that presynaptic pMad marks sites where active postsynaptic type-A receptors induce the assembly of trans-synaptic complexes with presynaptic BMP and BMPRs (BMP receptors).

In recent work, we started to define the composition of these local BMP/BMPR complexes using genetics and cell biology approaches. Using mutants and RNAi lines to genetically manipulate the levels of receptors, we have already established that synaptic pMad accumulation requires the type-II BMPR Wit and type-I BMPRs Tkv and Sax. In addition, we found that endogenously tagged Tkv is distributed to presynaptic aggregates that appear to co-localize with the presynaptic pMad signals. Current studies in the laboratory focus on determining the composition and regulation of the pMad–containing complexes at synaptic terminals.

It is important to recognize that all the BMP signaling modalities are coordinated by shared, limited components, in particular the BMP receptors, which are tightly regulated at transcriptional, translational,

and post-translational levels. The canonical BMP signaling pathway requires endocytosis of the BMP/BMPR–signaling complexes and their retrograde transport to the motor neuron soma, whereas the noncanonical pathways rely on BMP/BMPR complexes to function at synaptic terminals. Given that the pathways share limited pools of BMPRs, the motor neurons must balance the partitioning of BMPRs among different BMP signaling modalities. Consequently, BMP signaling may monitor synapse activity and coordinate it with synapse growth and maturation.

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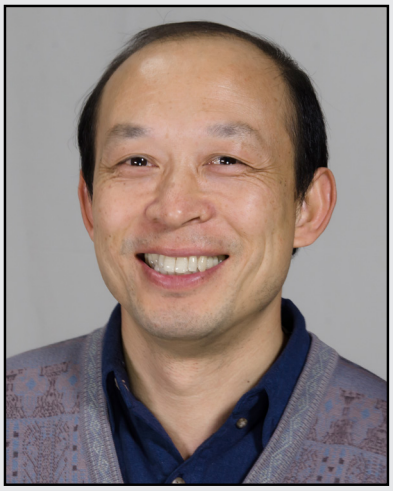
Thyroid Hormone Regulation of Vertebrate Postembryonic Development

This laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during post-embryonic development. The principal model is the metamorphosis of *Xenopus laevis* and *X. tropicalis*, two closely related species, which offer unique but complementary advantages. The control of this developmental process by TH provides a paradigm to study gene function in postembryonic organ development. During metamorphosis, distinct organs undergo vastly different changes. Some, like the tail, undergo complete resorption, while others, such as the limb, are developed *de novo*. The majority 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 involves 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 (1) study the developmental function of TH receptors (TRs) and the underlying mechanisms *in vivo* and (2) identify and functionally characterize genes that are critical for organogenesis, particularly for the formation of the adult organ-specific stem cells, during postembryonic development in vertebrates. A major recent focus has been to make use of the TALEN and CRISPR/Cas9 technologies (References 1,2) to knockdown or knockout the endogenous genes for functional analyses.



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Animals lacking TR complete metamorphosis around the same age as their wild-type siblings.

Using the TALEN technology, we generated *X. tropicalis* animals lacking any functional TRα and observed surprisingly that TRα knockout animals are able to complete metamorphosis at a similar age as their wild-typing siblings (Reference 1). Careful analyses during development, however, revealed that the TRα knockout animals initiated metamorphosis at a younger age and with a smaller

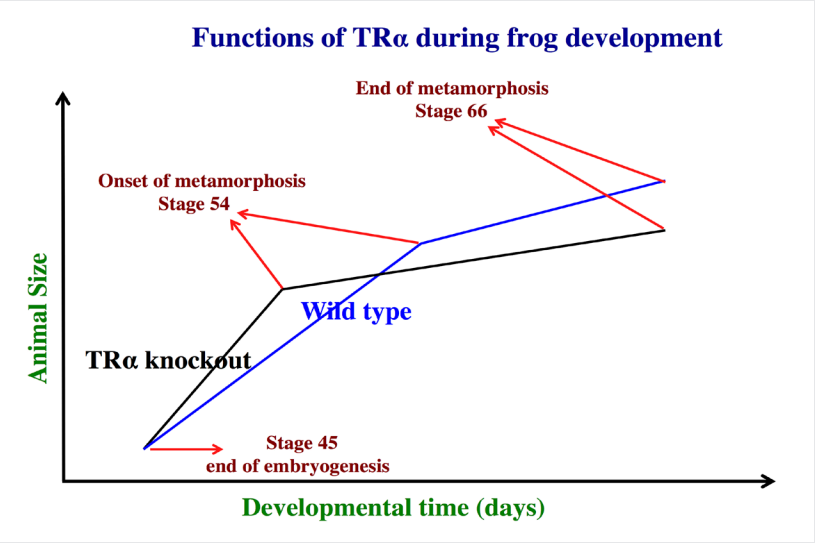

FIGURE 1. Schematics showing the effects of TRα (thyroid hormone α) knockout on *Xenopus tropicalis* development

TRα knockout has little effect on embryogenesis, and the resulting tadpoles are normal by feeding stage (stage 45). Once feeding begins, the animals grow at different rates, with the knockouts growing faster; they are thus larger than to wild-type siblings at the same age (in days) (comparing the vertical axis values of the lines for the knockout and wild-type animals at any given position along the horizontal axis between stages 45 and 54). The knockout animals also develop faster, reaching developmentally more advanced stages than wild-type siblings at the same age (in days). Thus, the knockout animals reach stage 54, the onset of metamorphosis, at a younger age (see the horizontal axis locations for the upper end of the lines). Interestingly, when the animals are compared at stage 54, the wild-type are larger than the knockout 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-types to catch up and surpass the knockouts in size. After the initiation of metamorphosis at stage 54, the knockout tadpoles metamorphose more slowly than the wild-type ones, enabling the latter to catch up in development, with both groups finishing metamorphosis at around the same age. The knockout animals initiate metamorphosis at a smaller size and also end up with a smaller size at the end of metamorphosis than do the wild-type siblings. Thus, in premetamorphic tadpoles prior to stage 54, unliganded TRα (due to the lack of thyroid hormone) functions to control metamorphic timing, whereas, when thyroid hormone becomes available during metamorphosis, TRα helps increase the rate of metamorphosis.

size but progressed more slowly through metamorphosis. The wild-type siblings initiated metamorphosis at an older age but progressed more quickly, eventually completing metamorphosis around the same time as the knockout animals. As the TRα knockout animals initiated metamorphosis at a smaller size, they were also smaller at the end of metamorphosis. The findings are consistent with our earlier studies with TRα knockdown animals and reveal a critical role of endogenous TRα both in mediating the metamorphic effect of TH during metamorphosis and in preventing precocious initiation of metamorphosis when TH is absent. They thus provide direct evidence to support the dual function model for TR in *Xenopus* development that we proposed over a decade ago based on gene expression and *in vitro* function studies.

Direct transcriptional regulation of the histidine ammonia-lyase 2 gene by the thyroid hormone receptor in developing adult intestinal stem cells

Our early studies in *Xenopus laevis* showed that intestinal remodeling involves complete degeneration of the larval epithelium and *de novo* formation of adult stem cells through de-differentiation of some larval epithelial cells. We further discovered that the histidine ammonia-lyase (HAL, also known as histidase or histidinase)-2 gene is strongly and specifically activated by TH in the proliferating adult stem cells of the intestine during metamorphosis, implicating a role of histidine catabolism in the development of adult intestinal stem cells. To determine the mechanism by which TH regulates the *HAL2* gene, we carried out a

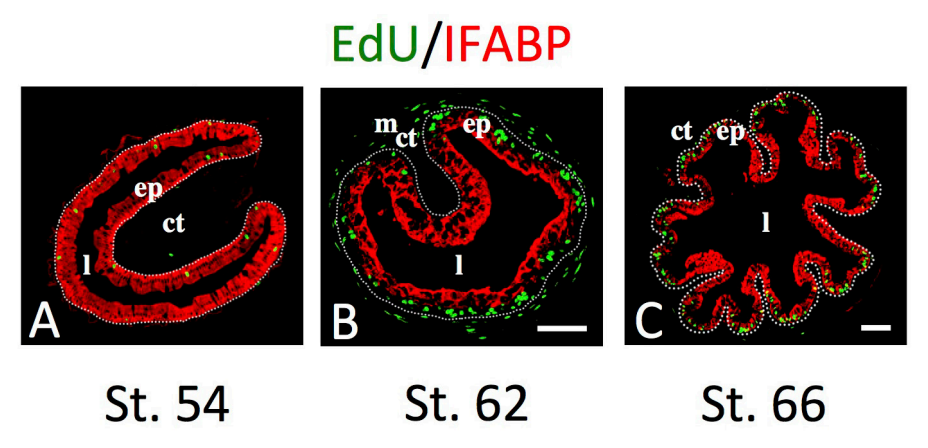

FIGURE 2. Intestinal metamorphosis involves the formation of clusters of proliferating, undifferentiated epithelial cells at the climax.

Tadpoles at premetamorphic stage 54 (A), climax (B, stage 62), and end of metamorphosis (C, stage 66) were injected with 5-ethynyl-2'-deoxyuridine (EdU) one hour before being sacrificed. Cross-sections of the intestine from the resulting tadpoles were double-stained by EdU labeling of newly synthesized DNA and by immunohistochemistry of IFABP (intestinal fatty acid binding protein), a marker for differentiated epithelial cells. The dotted lines depict the epithelium-mesenchyme boundary. Note that there are few EdU–labeled proliferating cells in the epithelium and that they express IFABP at premetamorphosis (A) and increase in the form of clustered cells (proliferating adult stem cells) that lack IFABP at the climax of metamorphosis (B). At the end of metamorphosis, EdU–labeled proliferating cells are localized mainly in the troughs of the epithelial folds, where IFABP expression is low (C). ep, epithelium; ct, connective tissue; m, muscles; l, lumen.

bioinformatics analysis and discovered a putative TH response element (TRE) in the *HAL2* gene (Reference 3). Importantly, we showed that this TRE is bound by TH receptor (TR) in the intestine during metamorphosis. The TRE is capable of binding to the heterodimer of TR and 9-*cis* retinoic acid receptor (RXR) *in vitro* and mediating transcriptional activation by liganded TR/RXR in frog oocytes. More importantly, the *HAL2* promoter containing the TRE can drive TH–dependent reporter gene expression to mimic endogenous *HAL2* expression in transgenic animals. Our results suggest that the TRE mediates the transcriptional activation of the *HAL2* gene by TH in the developing adult intestinal stem cells during metamorphosis.

A balance of Mad and Myc expression dictates larval cell apoptosis and adult stem cell development during *Xenopus* intestinal metamorphosis.

The Myc/Mad/Max network has long been known to be an important player in regulating cell proliferation, death and differentiation in diverse cell types. In general, Myc-Max heterodimers activate target gene expression to promote cell proliferation, although excess of c-Myc can also induce apoptosis. In contrast, Mad competes against Myc to form Mad-Max heterodimers that bind to the same target genes to repress their expression and promote differentiation. The role of the Myc/Mad/Max network during vertebrate development, particularly so-called post-embryonic development, a period around birth in mammals, is unclear. We discovered that Mad1 is induced by TH in the intestine during metamorphosis when larval epithelial cell death and adult epithelial stem cell development take place (Reference 4). More importantly, we demonstrated that Mad1 is expressed in larval cells undergoing apoptosis while c-Myc is expressed in proliferating adult stem cells during intestinal metamorphosis, suggesting that Mad1 plays a role in cell death during development. By using the TALEN–mediated gene-editing technology, we generated Mad1 knockout *Xenopus* animals, revealing that Mad1 is not essential for embryogenesis or metamorphosis. On the other hand, consistent with its spatio-temporal expression profile, Mad1 knockout leads to reduced

larval epithelial apoptosis but, surprisingly, also results in increased adult stem cell proliferation. The findings not only reveal a novel role of Mad1 in regulating developmental cell death but also suggest that a balance between Mad and Myc controls cell fate determination during adult organ development.

Requirement for thyroid hormone–induced activation of Notch signaling for adult intestinal stem cell development during metamorphosis

We showed earlier that TH–induced intestinal remodeling involves regulation of various genes including Notch receptor. To study the role of Notch signaling pathway, we used real-time RT-PCR and *in situ* hybridization or immunohistochemistry to analyze the expression of various components of this pathway, including the ligands DLL and Jag, the Notch receptor, and targets such as Hairy genes, in the metamorphosing intestine (Reference 5). We showed that they are up-regulated during both natural and TH–induced metamorphosis in a tissue-specific manner. Particularly, *Hairy1* is specifically expressed in the adult epithelial stem cells. Moreover, up-regulation of *Hairy1* and *Hairy2b* by TH was prevented by treating tadpoles with a γ-secretase inhibitor (GSI), which inhibits Notch signaling. More importantly, TH–induced up-regulation of LGR5, an adult intestinal stem cell marker, was suppressed by GSI treatment. Our results suggest that Notch signaling plays a role in stem cell development by regulating the expression of Hairy genes during intestinal remodeling. Furthermore, we demonstrated in organ cultures that prolonged exposure of tadpole intestine to TH plus GSI leads to hyperplasia of secretory cells and a reduction in absorptive cells. Our findings thus provide evidence for an evolutionarily conserved role of Notch signaling in intestinal cell fate determination but more importantly reveal, for the first time, an important role of the Notch pathway in the formation of adult intestinal stem cells during vertebrate development.

Methods development

SIMPLE AND EFFICIENT METHODS TO VISUALIZE AND QUANTIFY THE EFFICIENCY OF CHROMOSOMAL MUTATIONS FROM GENOME EDITING

Genome editing with designer nucleases such as TALEN and CRISPR/Cas enzymes has broad applications. Delivery of these designer nucleases into organisms induces various genetic mutations including deletions, insertions, and nucleotide substitutions. Characterizing those mutations is critical for evaluating the efficacy and specificity of targeted genome editing. While a number of methods have been developed to identify the mutations, none other than sequencing allow the identification of the most desired mutations, i.e., out-of-frame insertions/deletions that disrupt genes. During our studies on gene editing in *Xenopus* development, we developed a simple and efficient method to visualize and quantify the efficiency of genomic mutations induced by gene editing (Reference 2). Our approach is based on the expression of a two-color fusion protein in a vector that allows the insertion of the edited region in the genome between the two color moieties. We showed that our approach not only easily identifies developing animals with desired mutations but also efficiently quantifies the mutation rate *in vivo*. Furthermore, by using LacZα and GFP as the color moieties, our approach can even eliminate the need for a fluorescent microscope, allowing the analysis with simple bright field visualization. Such an approach will greatly simplify screening for effective gene-editing enzymes and identify the desired mutant cells and/or animals.

EFFICIENT, SIMPLE, NON-INVASIVE PROCEDURE FOR GENOTYPING AQUATIC AND NON-AQUATIC LABORATORY ANIMALS

Various animal models are indispensable in biomedical research. Increasing awareness and regulations have prompted the adaptation of more humane approaches in the use of laboratory animals. With the

development of easier and faster methodologies to generate genetically altered animals, convenient and humane methods to genotype these animals are important for research involving such animals. To facilitate genotyping of gene-edited *Xenopus* animals, we developed skin swabbing as a simple and noninvasive method for extracting genomic DNA from tadpoles and frogs for genotyping. The method is highly reliable and suitable for both immature and adult animals, not only for frogs but for also mice. Our approach thus allows a simpler and more humane approach for genotyping vertebrate animals.

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- Lataijah C. Crawford was an NICHD Developing Talent Scholar.

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Modeling the Biophysics of the Membrane

The integrity of lipid membranes is essential for life. They provide spatial separation of the chemical contents of the cell and thus the enable the electrical and chemical potential differences that are used to transmit signals and perform work. However, the membrane must frequently be broken to form, for example, new membrane structures in the cell. The simplest structure is a vesicle to transport cargo. Such vesicles are constantly cycled between organelles and the outer plasma membrane. Thus, there is a careful balance between boundary-establishing membrane fidelity and the necessary ability of the cell to change these boundaries.

The challenge in studying the membrane is its complexity. The membrane is a thin sheet of small molecules, i.e., lipids. There are hundreds of types of lipids in the cell. Each lipid changes the properties of the membrane in its vicinity, sometimes making the sheet stiffer, sometimes softer, and sometimes acting to bend the membrane into a ball or tube. Furthermore, the lipids are constantly jostling and tangling with both each other and proteins embedded in the membrane. To predict of how membranes are reshaped thus requires not only knowing how lipids affect the properties of the membrane surface, but also the location of specific lipids.

A broad objective of our research is to create a publicly available software package that can be used either as a stand-alone application for analyzing membrane-reshaping processes or as a library for cellular-scale modeling packages for which the role of the membrane may be unclear or unanticipated. The model will incorporate the physical mechanisms we are investigating in the narrower objectives of the lab. These include: the influence of lipid curvature stress on the conformation of a protein; whether through-bilayer coupling can amplify a signaling state (for example, of a G protein–coupled receptor); and whether protein motifs (e.g., a thick hydrophobic region of a protein) can enhance the local lipid composition around a protein. These projects will address how the lipid micro-environment is controlled by the cell to change coupling between protein signaling states.

The projects use the NIH Biowulf computing cluster to run simulations and models. Molecular dynamics software (such as

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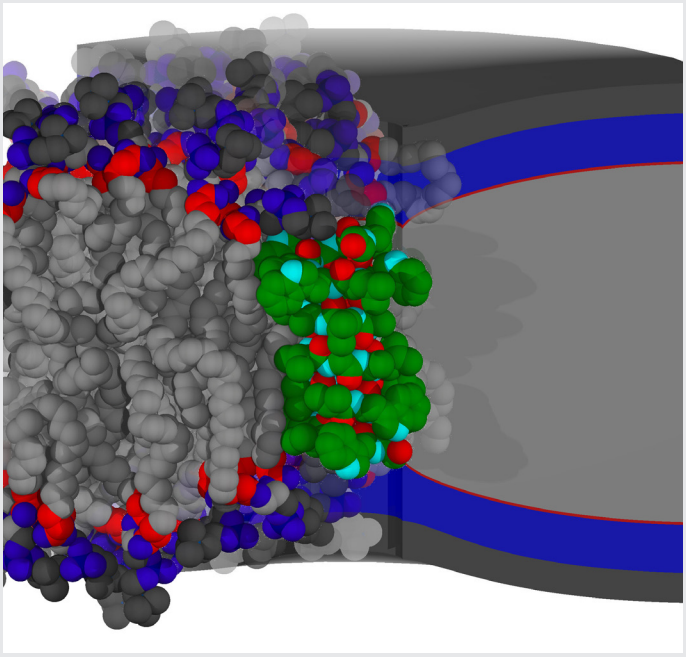

FIGURE 1. Continuum modeling of protein-membrane coupling

Shown in green is a short peptide channel deforming a thick bilayer in which it is embedded. At right is a continuum model of the bilayer color-coded by the layers of chemistry present. At left is a molecular simulation of the same peptide with equivalent coloring.

NAMD and CHARMM) are used to conduct molecular simulations. In-house software development for eventual public distribution is a key element of the lab's work.

Methodology to compare molecular simulations of bilayers to small-angle neutron scattering (SANS) experiments

We frame the question of "where are the lipids?" in terms of redistribution away from a null hypothesis in which lipids do not: (1) prefer to solvate a particular protein; (2) localize on structures of particular curvature (i.e., onto a vesicle); or (3) prefer to associate with lipids with specific chemistry. The broad lipid field literature has hypothesized extensions away from this null hypothesis based on fundamental biophysical experiments, namely that protein function varies based on local lipid competition; that lipid chemistry directly impacts the phase stability of vesicles and other curved phases; and that lipids can laterally "phase separate" based on preferred interactions between lipids. The challenge for research is that a lipid has a diameter less than a nanometer, while optical methods cannot characterize redistribution below approximately 200 nanometers.

This past year, we targeted small-angle neutron scattering (SANS) as a technique for determining the nano-scale co-localization of lipids. A SANS experiment averages all of the molecular correlations in a sample to a simple one dimensional intensity, essentially yielding an ensemble average of the Fourier transform of the internal structure. The effectiveness of the SANS experiment thus directly depends on the quality of the model used to explain the convoluted SANS intensity. We solved an important problem for modeling the SANS experiment: how to translate molecular simulations that employ periodic boundary conditions into the SANS intensity, while retaining lateral correlations. The method will be critical for interpreting the SANS intensity of sphingolipids; this includes a neutron spin echo experiment, which can show the timescale for bilayer fluctuations.

Modeling lipid redistribution governed by the peptide–lipid interface of gramicidin A

The question of how the lipid microenvironment changes protein function is a prime challenge in biophysics. A classic tool for this study is the small peptide gramicidin. Two gramicidin 'monomers,' which span a leaflet (half the bilayer), join to form a complete membrane-spanning channel. The channel is of a specific thickness that must be accommodated by the membrane. The accommodation deforms the membrane to match the channel. This simple two-state system thus responds exquisitely to a change in membrane properties, such as its thickness and stiffness. The most important lipids in this process are those directly next to the channel—they are perturbed the most, and thus the perturbation required is sensitive to their properties.

This project combined experiment, simulation, and theoretical analysis to describe how the lipid-peptide interface is differentially favored by lipids; in this case, it is favored by shorter lipids that match the peptide thickness. This was shown experimentally (by our collaborators in the Andersen lab) by comparing channel stability in two nearly equivalent bilayers: a bilayer with one lipid of medium height, and a mixture of short and tall lipids. Even though the average thickness of the two bilayers was equal, the channel stability was increased in the mixture. Theory and simulation not only co-validated the experimental finding, but provided molecular and material justification for the enhanced stability, also indicating how the channel would be stable vis-a-vis other changes in lipid chemistry, such as those leading to curvature preferences (Reference 3). In a third study (Reference 2), a connection was made between protein side-chain chemistry and the structure of the protein-lipid interface, namely, hydrogen bonding between peptide and lipid. Figure 1 is a cartoon showing the structure of the gramicidin channel, the molecular structure of the surrounding bilayer, and a depiction of the continuum model used to make predictions on a grander scale.

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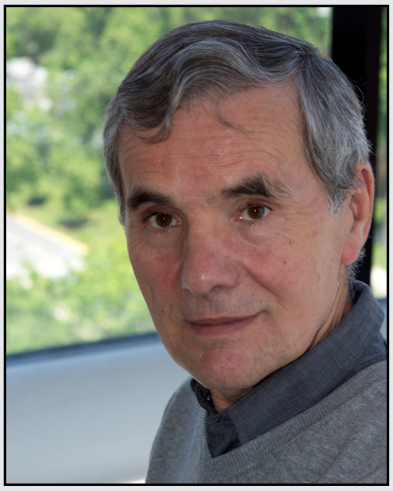
Signaling and Secretion in Neuroendocrine Cells

We investigate cellular signaling cascades, gene expression, and hormone secretion in hypothalamic and pituitary cells, with a special emphasis on the interactions between plasma-membrane electrical events and receptor-controlled pathways. Specifically, we address how these neuroendocrine cells use ion channels and G protein–coupled receptors as signaling platforms to efficiently process information. To this end, we characterize both native and recombinant receptors and channels that have been cloned from neuroendocrine cells. In the past, our work has focused on the role of inositol-trisphosphate receptors in the oscillatory calcium release by pituitary cells, the mechanism of periodic activation of these channels, and the complex mode of synchronization of calcium release from intracellular stores with electrical activity of cells. We also characterized voltage-gated channels expressed in neuroendocrine cells, the cell type–specific patterns of electrical activity and channels involved, the physiological relevance of such activity, and the crosstalk between G protein–coupled receptors and ion channels. More recently, we characterized ligand-gated receptor channels expressed in pituitary cells, including the ATP–gated P2X receptor channels. Our current work focuses on age-, sex-, and tissue structure–specific signaling, transcription, and secretion in the pituitary gland, the heterogeneity of secretory pituitary cells reflecting their embryonal and postnatal genesis, and cell type–specific exocytic pathways. We are also studying how the structural features of P2X receptors relate to the channels' functions and how plasma membrane receptors and the intracellular signaling milieu affect channel activity.



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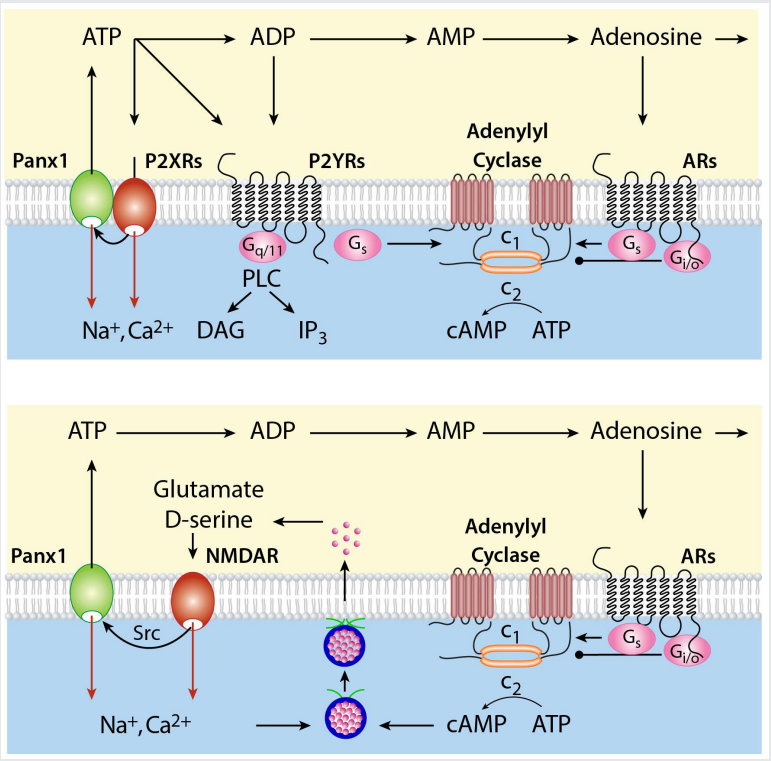
Characterization of purinergic P2X receptor channel functions

In collaboration with Ivan Milenkovic, we studied the role of P2X receptor channels in electrical activity of maturing auditory neurons. Using brain-slice recordings before hearing onset and *in vivo* recordings with iontophoretic drug applications after hearing onset, we showed that cell-specific purinergic modulation follows a precise tonotopic pattern in the ventral cochlear nucleus of developing gerbils. In high-frequency regions, ATP responsiveness diminished before hearing onset. In low-to-mid frequency regions, ATP

FIGURE 1. The crosstalk between Panx1 channels and ligand-gated receptor channels



Top panel: Interactions between Panx1 channels and P2X receptors (P2XRs). Activated P2XRs cross-activate Panx1 channels probably through the Src family kinases. This leads to potentiation of P2XR signaling. Two pathways have been proposed: enhancement of the current (*red arrows*) and continued activation of P2XRs and co-activation of P2YRs, and ARs (*black arrows*). Opening of the Panx1 channel facilitates ATP release, which acts as agonist at P2XRs and P2YRs before ectonucleotidases metabolize it into ADP, AMP, and adenosine. ADP also acts as natural ligand for some P2YRs, whereas adenosine is natural ligand for ARs. P2YRs signals through $G_{q/11}$ and G_s signaling pathway, whereas ARs facilitate adenylyl cyclase activity through G_s coupling and inhibit this enzyme through $G_{i/o}$ coupling. Both autocrine and paracrine modes of action were reported.

Bottom panel: Interactions between Panx1 channels and *N*-methyl-D-aspartate receptor (NMDAR) channels. Activated NMDAR cross-activates Panx1 through the Src family kinases, which in turn potentiate the NMDAR signaling, by enhancement of the current (*red arrows*) and/or through ATP released from Panx1 and ATP's dephosphorylation to adenosine, which binds to adenosine receptors to diminish neuronal excitability ($G_{i/o}$ coupling) or provides sustained NMDR activity, probably through release of glutamate and D-serine (G_s-coupling). ARs, adenosine receptors; P2XRs, ATP–gated purinergic P2X receptor channels; P2YRs, purinergic G protein–coupled P2Y receptors; PLC, phospholipase C; DAG diacylglycerol; IP_3, inositol (1,4,5) trisphosphate; NMDAR, *N*-methyl-D-aspartate receptor.

modulation persisted after hearing onset in a subset of low-frequency bushy cells (characteristic frequency lower than 10 kHz). Down-regulation of P2X2/3R currents along the tonotopic axis occurs simultaneously with an increase in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor currents, thus suggesting a high-to-low frequency maturation pattern. Facilitated action potential (AP) generation, measured as higher firing frequency, shorter excitatory postsynaptic potential–AP delay *in vivo*, and shorter spike latency in slice experiments, is consistent with increased synaptic efficacy caused by ATP. By combining recordings and pharmacology *in vivo*, in slices and in human embryonic kidney 293 cells, we showed that the long-lasting change in intrinsic neuronal excitability is mediated by the P2X2/3R (Reference 1).

In collaboration with Anmar Khadra, we also studied allosteric regulation of P2X4 channels by ivermectin (IVM). In general, treatment with IVM increases the efficacy of ATP to activate the P2X4 channel, slows both receptor desensitization during sustained ATP application and receptor deactivation after ATP washout, and makes the channel pore permeable to $NMDG^+$, a large organic cation. Previously, we developed a Markov model based on the presence of one IVM binding site, which described some effects of this compound on rat P2X4 channels. Recently, we presented two novel models, both with three IVM binding sites. The simpler one-layer model can reproduce many of the observed time series of evoked currents but does not

capture well the short time scales of activation, desensitization, and deactivation. A more complex two-layer model can reproduce the transient changes in desensitization observed upon IVM application, the significant increase in ATP–induced current amplitudes at low IVM concentrations, and the modest increase in the unitary conductance. In addition, the two-layer model suggests that this receptor can exist in a deeply inactivated state, not responsive to ATP, and that its desensitization rate can be altered by each of the three IVM binding sites. In summary, the study provides a detailed analysis of P2X4 receptor channel kinetics and elucidates the orthosteric and allosteric mechanisms regulating its channel gating (Reference 2).

The cyclin-dependent kinase 5 (Cdk5) plays key roles in brain development and function, including neuronal migration, membrane transport, axon guidance, and pain signaling. Earlier, we had reported that Cdk5 phosphorylates the transient receptor potential vanilloid 1, a key ion channel implicated in pain, increasing its function. In collaboration with Claudio Coddou, we thus focused on the potential role of a putative Cdk5 phosphorylation site in the full-size variant P2X2a receptor channel, which is absent from the splice variant P2X2b channel. We found an interaction between P2X2a and Cdk5/p35 by co-immunofluorescence and co-immunoprecipitation in HEK293 cells. We also found that threonine phosphorylation was significantly higher in HEK293 cells co-expressing P2X2a and p35 than in cells expressing only P2X2a channels. Moreover, P2X2a–derived peptides encompassing the Cdk5 consensus motif were phosphorylated by Cdk5/p35. Whole-cell patch-clamp recordings indicated a delay in development of use-dependent desensitization (UDD) of P2X2a but not of P2X2b receptor in HEK293 cells co-expressing these receptors and p35. In *Xenopus* oocytes, P2X2a receptors showed a slower UDD than in HEK293 cells and Cdk5 activation prevented this effect. A similar effect was found in P2X2a/3R heteromeric currents in HEK293 cells. The P2X2a-T372A receptor mutant was resistant to UDD. In endogenous cells, we observed similar distribution between P2X2a receptor and Cdk5/p35 by co-localization using immunofluorescence in primary culture of nociceptive neurons. Moreover, co-immunoprecipitation experiments showed an interaction between Cdk5 and P2X2a receptor in mouse trigeminal ganglia. Endogenous P2X2a receptor–mediated currents in PC12 cells and P2X2/3 channel–mediated increases of intracellular calcium in trigeminal neurons were Cdk5–dependent, given that inhibition with the cyclin-dependent kinase inhibitor roscovitine accelerated the desensitization kinetics of these responses. The results indicate that the P2X2a receptor is a novel target for Cdk5–mediated phosphorylation, which might play important physiological roles including pain signaling (Reference 3).

We also studied interactions of pannexin1 channels with purinergic and *N*-methyl-D-aspartate (NMDA) receptor channels. Pannexins are a three-member family of vertebrate plasma membrane–spanning molecules that have homology to innexins, the invertebrate gap junction–forming proteins. However, pannexins do not form gap junctions but operate as plasma membrane channels. It has been suggested that the best characterized member of this protein family, Pannexin1 (Panx1), is functionally associated with purinergic P2X and NMDA receptor channels. Activation of these receptor channels by their endogenous ligands leads to cross-activation of Panx1 channels, which in turn potentiates P2X and NMDA receptor–channel signaling. Two potentiation concepts have been suggested: enhancement of the current responses and/or sustained receptor-channel activation by ATP released through the Panx1 pore and adenosine generated by ectonucleotidase-dependent dephosphorylation of ATP. Figure 1 summarizes the current knowledge and our hypotheses about interactions of Panx1 channels with P2X and NMDA receptor channels.

Electrophysiological properties of secretory pituitary cells

Recently, we summarized our investigations on electrical properties and calcium signaling in pituitary

gonadotrophs, which are basophilic cells of the anterior pituitary gland specialized to secrete gonadotropins in response to elevation in intracellular calcium concentration. The cells fire APs spontaneously, coupled with voltage-gated calcium influx of insufficient amplitude to trigger gonadotropin release. The spontaneous excitability of gonadotrophs reflects the expression of voltage-gated sodium, calcium, potassium, non-selective cation-conducting, and chloride channels at their plasma membrane. The cells also express the hyperpolarization-activated and cyclic nucleotide–gated cation channels at the plasma membrane, as well as the GABA receptor γ-aminobutyric acid-A, nicotinic, and purinergic P2X receptor channels gated by GABA, acetylcholine, and ATP, respectively. Activation of the channels leads to initiation or amplification of the pacemaking activity, facilitation of calcium influx, and activation of the exocytic pathway. Gonadotrophs also express calcium-conducting channels at the endoplasmic reticulum membranes gated by inositol trisphosphate and intracellular calcium. These channels are activated potently by hypothalamic gonadotropin-releasing hormone (GnRH) and less potently by several paracrine calcium-mobilizing agonists, including pituitary adenylate cyclase–activating peptides, endothelins, acetylcholine, vasopressin, and oxytocin. Activation of the channels causes oscillatory calcium release and a rapid gonadotropin release, accompanied by a shift from tonic firing of single APs to periodic bursting type of electrical activity, which accounts for sustained calcium signaling and gonadotropin secretion (Reference 4).

Our collaborative work with Arthur Sherman focused on modeling the diversity of spontaneous and agonist-induced electrical activity in cultured corticotrophs, the anterior pituitary cell type critical in stress response. These cells fire APs spontaneously and in response to stimulation with corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), and such electrical activity is critical for calcium signaling and calcium-dependent adrenocorticotropic hormone secretion. The cells typically fire tall, sharp APs when spontaneously active, but a variety of other spontaneous patterns have also been reported, including various modes of bursting. Reports vary as to the fraction of corticotrophs that are electrically active, as well as their patterns of activity, and the sources of this variation are not well understood. The ionic mechanisms responsible for CRH– and AVP–triggered electrical activity in corticotrophs are also poorly characterized. We use electrophysiological measurements in single cultured corticotrophs and mathematical modeling to investigate possible sources of variability in patterns of spontaneous and agonist-induced electrical activity. In the model, variation in as few as two parameters can give rise to many of the types of patterns observed in electrophysiological recordings of corticotrophs. We compared the known mechanisms for CRH, AVP, and glucocorticoid action and found that various ionic mechanisms can contribute in different but complementary ways to generate the complex time courses of CRH and AVP responses. In summary, our modeling suggests that corticotrophs have several mechanisms at their disposal to achieve their primary function of pacemaking depolarization and increased electrical activity in response to CRH and AVP. The finding is consistent with critical roles of electrical activity in function of these cells (Reference 5).

In collaboration with the same group, we also examined common and diverse elements of ion channels and receptors underling electrical activity in six major secretory pituitary cells: corticotrophs, melanotrophs, gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs. All these cell types are electrically excitable, and voltage-gated calcium influx is the major trigger for their hormone secretion. Along with hormone intracellular content, G protein–coupled receptor and ion channel expression can also be considered as defining cell-type identity. While many aspects of the developmental and activity-dependent regulation of hormone and G protein–coupled receptor expression have been elucidated, much less is known about the regulation of the ion channels needed for excitation-secretion coupling in these cells. We compare the

spontaneous and receptor-controlled patterns of electrical signaling among endocrine pituitary cell types, including insights gained from mathematical modeling. We argue that a common set of ionic currents unites these cells, while differential expression of another subset of ionic currents could underlie cell type–specific patterns. We supported these ideas using a generic mathematical model, showing that it reproduces many of the observed features of pituitary electrical signaling. Mapping these observations to the developmental lineage suggests possible modes of regulation that may give rise to mature pituitary cell types.

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Olfactory Coding and Decoding by Neuron Ensembles

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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Oscillatory integration windows in neurons

Oscillatory synchronization of neurons occurs in many brain regions, including the olfactory systems of vertebrates and invertebrates, and is indispensable for precise olfactory coding. One mechanism by which oscillations have been proposed to influence coding is through the creation of cyclic integration windows—specific times within the oscillation cycle when synaptic input is most efficiently integrated by a post-synaptic neuron. Cyclic integration windows could allow a neuron to respond preferentially to spikes arriving from multiple presynaptic neurons coincidentally in a specific part of the cycle. Thus, coincidence detection mediated by integration windows could help read precise temporal codes for odors. Phase-specific effects of synaptic inputs have been described both in brain slices and in simulations. However, the existence of cyclic integration windows has not been demonstrated, and their functional requirements are unknown.

With paired local field potential (LFP) and intracellular recordings and controlled stimulus manipulations we directly test this idea in the locust olfactory system. We focused on the responses of Kenyon cells, which are high-order neurons in a brain area analogous to the vertebrate piriform cortex and which fire spikes when the animal is presented with an odor pulse. We found that inputs arriving in Kenyon cells sum most effectively in a preferred window of the oscillation cycle. With a computational model, we established that the non-uniform structure of noisy activity in the membrane potential helps mediate this process. Further experiments performed *in vivo* demonstrated that integration windows can form in the absence of inhibition and at a broad range of oscillation frequencies.

Our results establish that cyclic integration windows can be formed from very few ingredients: oscillatory input and noise in the membrane potential. Given the ubiquity of membrane noise, the mechanisms we describe likely apply to a wide variety of neurons that receive oscillatory inputs, with or without inhibition and across a range of frequencies. Our results reveal how a fundamental coincidence-detection mechanism in a neural circuit functions to decode temporally organized spiking.

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 (sub-esophageal) 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, we expressed the fluorescence-tagged acetylcholine receptor subunit (Da7) and active-zone marker (Brp) in the gustatory second-order neurons, respectively.

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 from 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.

Spatio-temporal 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 spatio-temporal 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 postulate is that, in both mammals and insects, gustatory information is carried through labelled lines, that is, in 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 highly 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 mono-synaptically 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 spatio-temporal patterns of activity distributed across the population of gustatory receptor neurons. Further, we found that the representations are transformed substantially, given that many 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, the information is carried not by labelled lines but rather by distributed, spatio-temporal activity, which is a fast and accurate code. The results provide a dramatically new view of taste processing.

A population of projection neurons that inhibits the lateral horn but excites the antennal lobe through chemical synapses in *Drosophila*

The insect antennal lobe is a useful model system in which to study neural computations. *Drosophila* has been a particularly beneficial model system because it offers numerous genetic tools for labeling and manipulating the activity of neurons. In the insect olfactory system, odor information is transferred from the antennal lobe to higher brain areas by projection neurons running through multiple antennal lobe tracts. In several species, one of these tracts, the mediolateral antennal lobe tract (mlALT), contains projection neurons expressing GABA, a neurotransmitter that usually elicits inhibition; in the *Drosophila* brain, the great majority of ventral projection neurons (vPNs) are GABAergic and project through this tract to a brain area called the lateral horn. Most projection neurons, which are excitatory (ePNs), project through the mALT to the lateral horn and another brain area, the mushroom body. Recent studies have shown that GABAergic vPNs play inhibitory roles at their axon terminals in the lateral horn. However, little is known about the properties and functions of vPNs at their dendritic branches in the antennal lobe.

We used genetic manipulations and optogenetic and patch clamp techniques to investigate the functional roles of vPNs in the antennal lobe. Surprisingly, our results show that specific activation of vPNs always elicits strong excitatory post-synaptic potentials in ePNs, even though most vPNs are GABAergic. Moreover, we found that the connections between vPNs and ePNs are mediated by direct chemical synapses rather than, as has been previously reported, by gap junctions. Neither pulses of GABA nor pharmacological or genetic blockade of GABAergic transmission gave results consistent with the involvement of GABA in vPN–ePN excitatory transmission. A possibility we cannot rule out is that GABAergic vPNs co-express an excitatory neurotransmitter and release it at specific compartments within cells; for example, GABA could be released at the axonal terminals in the lateral horn and an excitatory neurotransmitter released at the dendritic presynaptic terminals in the antennal lobe. Indeed, several examples of mammalian neurons that can release multiple fast excitatory or inhibitory neurotransmitters have been reported, such as spatially segregated release of GABA and ACh in the retina. These unexpected results suggest new roles for the vPN population in olfactory information processing.

Classification of odorants across layers in locust olfactory pathway

Olfactory processing takes place across multiple layers of neurons from the transduction of odorants in the periphery, to odor quality processing, learning, and decision making in higher olfactory structures. In insects, projection neurons in the antennal lobe send odor information to the Kenyon cells of the mushroom bodies and lateral horn neurons. To examine the odor information content in different structures of the insect brain (antennal lobe, mushroom bodies, and lateral horn), we designed a model of the olfactory network based on electrophysiological recordings made *in vivo* in the locust. We found that classification performance was better for all types of cells (projection neurons, lateral horn neurons, and Kenyon cells) when populations of cells were considered rather than individual cells. Classification success was even greater when the neurons constituting these populations were each tuned to different odor features. This finding therefore reflects an emergent network property. Odor classification improved with increasing stimulus duration: for similar odorants, Kenyon cells and lateral horn neuron ensembles reached optimal discrimination within the first 300–500 ms of the odor response. Performance improvement with time was much greater for a population of cells than for individual neurons. We conclude that, for projection neurons, lateral horn neurons, and Kenyon cells, ensemble responses are always much more informative than single-cell responses, despite the accumulation of noise along with odor information.

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Regulatory Small RNAs and Small Proteins

Currently, we have two main interests: identification and characterization of small noncoding RNAs and identification and characterization of 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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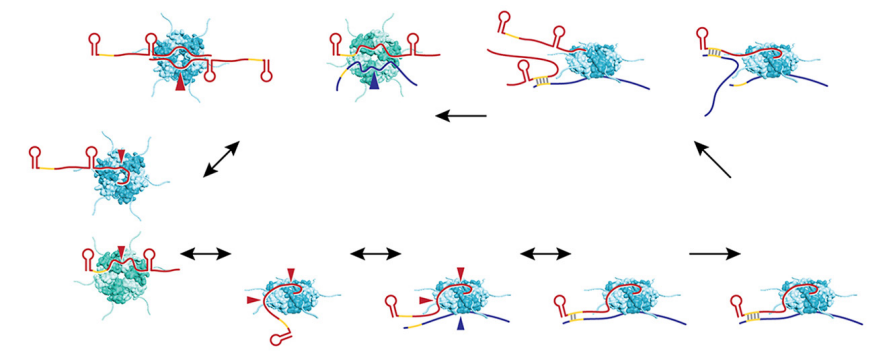
Identification and characterization of small regulatory RNAs

During the past 20 years, we have carried out several different systematic screens for small regulatory RNA genes in *Escherichia coli*. The screens 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.

A major focus for the group has been to elucidate the functions of the small RNAs we and others 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 acts as a chaperone to facilitate OxyS RNA base pairing with its target mRNAs (Reference 1). Recently, we also started to explore the role of ProQ, a second RNA chaperone in *E. coli* (Reference 2).

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* and other bacteria (Reference 3). 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 nonpreferred carbon sources. Similarly, we discovered that a Sigma(E)-dependent small RNA, MicL, transcribed from a promoter located within the

FIGURE 1. Model of Hfq–facilitated base pairing between sRNAs and mRNAs



E. coli Hfq (teal for the proximal face and rim views and green for the distal face view) employs four solvent-exposed surfaces to interact with RNA; sRNAs (red) have been found to contact the proximal and distal faces, rim, and C-terminus, and mRNAs (blue) have been shown to contact the distal face, rim, and C-terminus. Red and blue arrows denote sRNA and mRNA binding to Hfq, respectively. Hfq binding to mRNAs and sRNAs is thought to occur in random order. Initial binding is likely to involve only a subset of subunits, allowing for rapid displacement by other RNAs. Many RNAs bind to multiple surfaces, resulting in changes in RNA secondary structure and protection against RNase degradation, particularly for sRNAs. The free surface(s) of Hfq not already bound to RNA interacts with the cognate RNA partner, positioning the unbound seed region of the sRNA in close proximity to the unbound complementary region of the mRNA, facilitating base pairing. The lower affinity of duplex RNA for Hfq causes free RNAs to compete off sRNA–mRNA pairs, allowing Hfq to serve as a matchmaker for another pair of RNAs.

coding sequence of the *cutC* gene, represses synthesis of the lipoprotein Lpp, the most abundant protein in the cell, to oppose membrane stress. 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 raises the possibility that other phenotypes currently attributed to protein defects are attributable to deficiencies in unappreciated regulatory RNAs.

Most recently, we characterized a set of small RNAs expressed from a locus we denoted sdsN (Reference 4). Two longer sRNAs, SdsN137 and SdsN178, are transcribed from two Sigma(S)-dependent promoters but share the same terminator. Whole genome expression analysis after pulse overexpression of SdsN137 and assays of lacZ fusions revealed that SdsN137 directly represses the synthesis of the nitro-reductase NfsA, which catalyzes the reduction of the nitro-group (NO_2) in nitro-aromatic compounds, and the flavohemoglobin HmpA, which has aerobic nitric oxide (NO) dioxygenase activity. Consistent with this regulation, SdsN137 confers resistance to nitrofurans. Interestingly, SdsN178 is defective in regulating the above targets as a result of unusual binding to the Hfq protein, but cleavage leads to a shorter form, SdsN124, able to repress nfsA and hmpA.

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, which encodes a manganese exporter, directly binds to manganese, resulting in a conformation that liberates the ribosome-binding site. Remarkably, we were able to recapitulate the effect of manganese-dependent activation of translation *in vitro*. We also found that the yybP-ykoY motif responds directly to manganese ions in *Bacillus subtilis*. The identification of the yybP-ykoY motif as a manganese ion sensor suggests that the genes preceded by this motif that encode a diverse set of poorly characterized membrane proteins, have roles in metal homeostasis.

Further studies to characterize other Hfq–binding RNAs and their evolution as well as 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 actually encode small proteins. The correct annotation of the smallest proteins is one of the biggest challenges of genome annotation, and there is little evidence that annotated short open reading frames (ORFs) encode 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. 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 of 16–50 amino acids, in the intergenic regions of the model *Escherichia coli* genome. We tested expression of these predicted proteins 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.

More than half the newly discovered proteins were predicted to consist of a single transmembrane alpha-helix and were found to be in the inner membrane in biochemical fractionation. Interestingly, assays of topology-reporter fusions and strains with defects in membrane insertion proteins revealed that, despite their diminutive size, small membrane proteins display considerable diversity in topology and insertion pathways. Additionally, 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.

We now are using the tagged derivatives and information about synthesis and subcellular localization and 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. The combined approaches are beginning to give insights into how the small proteins are acting in *E. coli*.

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. The lack of MntS leads to reduced activities of manganese-dependent enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese and bacteriostasis under manganese-rich conditions. These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP (Reference 5).

We also discovered the 49–amino acid inner membrane protein AcrZ (formerly named YbhT), whose expression is induced by noxious compounds such as antibiotics and oxidizing agents, 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 this 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. The differential antibiotic sensitivity suggests that AcrZ enhances the ability of the AcrAB-TolC pump to export certain classes of substrates.

We showed that the 31–amino acid inner membrane protein MgtS (formerly denoted YneM), whose synthesis is induced by very low Mg^{2+} in a PhoPQ–dependent manner, acts to increase intracellular Mg^{2+} levels and maintain cell integrity upon Mg^{2+} depletion (Reference 6). Upon development of a functional tagged derivative of MgtS, we found that MgtS interacts with MgtA to increase the levels of this P-type ATPase Mg^{2+} transporter under Mg^{2+}-limiting conditions. Correspondingly, the effects of MgtS upon Mg^{2+} limitation are lost in an mgtA mutant, and MgtA overexpression can suppress the mgtS phenotype. MgtS stabilization of MgtA provides an additional layer of regulation of this tightly controlled Mg^{2+} transporter.

This work, together with our ongoing studies of other small proteins (Reference 7) and related findings by others in eukaryotic cells, supports our hypothesis that many small proteins act as regulators of larger membrane proteins.

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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 are 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 discovered 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

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(continued)

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 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 possible 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).

In 2013, 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

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SEGEN Laboratory Staff and Dr. Stratakis

Our staff and Dr. Constantine Stratakis in March 2016 at our laboratory at the NIH CRC (1-East-3216)

mutated, causes more than a third of the known PMAH cases. 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 studying them 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.

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 was our recent 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 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 are 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 pluripotent cells (TSPCs) in adult tissues such as the adult skeleton. 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 that consists 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 fruitful area of research, especially for our clinical fellows who matriculate at our laboratory during their two-year research time. The approach also leads to important new discoveries that may steer us into new directions.

One such discovery was our recent identification of the defect that explains the vast majority of cases of early pediatric overgrowth or gigantism. 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

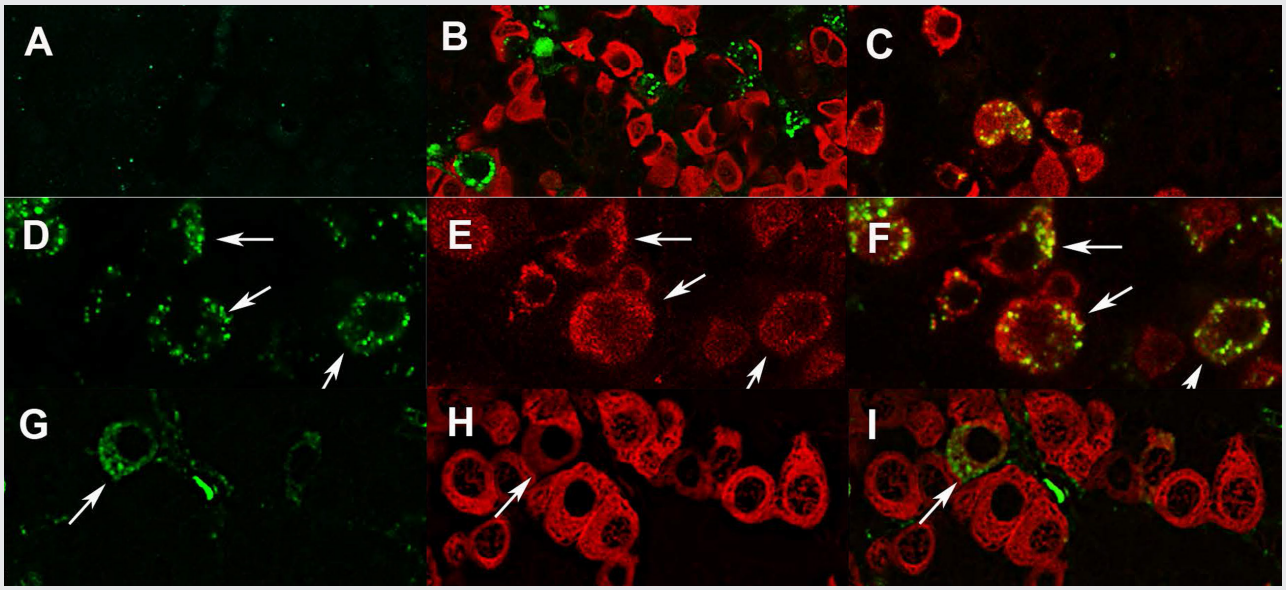

FIGURE 1. GPR101 expression in the pituitary and hypothalamus (Reference 1)

Labeling of GPR101 in the anterior pituitary gland of female rhesus monkey (A–F) and rat (G–I). (A) Preincubation of GPR101 antibody with blocking peptide, resulted in no staining. (B) Punctuate GPR101 (*green*) labeling could not be attributed to somatotrophs (*red*). (C) GPR101 (*green*) is expressed by LHβ–positive cells (*red*). (D–F) High magnification images showing membranous and cytoplasmic distribution of GPR101 in monkey gonadotrophs, excluding nucleus. (G–I) GPR101 (*green*) is expressed by subpopulation of rat somatotrophs (*red*), as indicated by arrows. Scale bars: A–D: 20 µm; E: 10 µm.

heterogeneous condition. With the application of state-of-the-art molecular cytogenetic techniques, we are investigating the participation of the 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-alpha) 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 the 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

Several years ago we 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. 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.

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 also studied both *Pde11a* and *Pde8b* animal models.

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.

This last year, in collaboration with a group in France, we investigated the genetic defects in GIP–dependent Cushing's syndrome that is caused by ectopic expression of glucose-dependent insulinotropic polypeptide receptor (GIPR) in cortisol-producing adrenal adenomas or in bilateral macronodular adrenal hyperplasias. We performed molecular analyses on adrenocortical adenomas and bilateral macronodular adrenal hyperplasias obtained from 14 patients with GIP–dependent adrenal Cushing's syndrome and one patient with GIP–dependent aldosteronism. *GIPR* expression in all adenoma and hyperplasia samples occurred through transcriptional activation of a single allele of the *GIPR* gene. While no abnormality was detected in proximal *GIPR* promoter methylation, we identified somatic duplications in chromosome region 19q13.32 containing the *GIPR* locus in the adrenocortical lesions derived from three patients. In two adenoma samples, the duplicated 19q13.32 region was rearranged with other chromosome regions, whereas a single tissue sample with hyperplasia had a 19q duplication only. Our French collaborators showed that juxtaposition with *cis*-acting regulatory sequences, such as glucocorticoid response elements in the newly identified genomic environment, drives abnormal expression of the translocated *GIPR* allele in adenoma cells.

We continue our work on identifying new genetic defects in other forms of adrenal tumors and/or hyperplasias.

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 identified novel genetic abnormalities.

We identified the gene *GPR101* encoding an orphan G protein–coupled receptor (GPCR), 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 headed by Albert Beckers, but all the molecular work for the gene identification was carried out here at the NIH. We found that the gene is expressed in areas of the brain that regulate growth (Figure 1) and we are actively investigating small-molecule compounds that may bind to GPR101 (unpublished).

In addition, we studied patients with pediatric Cushing disease (CD) due to corticotropin (ACTH)–secreting pituitary tumors, as part of our studies on Cushing's syndrome. Almost everything known today in the literature about pediatric CD, from its molecular investigations to its diagnosis and treatment, is derived from work that was done at the NIH. This laboratory is currently intensely involved in the identification of genetic defects predisposing to pediatric CD. This year, we reported *CABLES1* (encoding a cyclin-dependent kinase-binding protein) and *USP8* (encoding ubiquitin carboxyl-terminal hydrolase 8) mutations in patients with CD (*CABLES1*) and/or their tumors (*USP8*).

Genetic investigations into the Carney Triad, other endocrine neoplasias, and related syndromes and into 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 the Carney Triad, a rare syndrome

that predisposes to adrenal and other tumors, and of 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 in the *PDFGRA* gene. 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 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.

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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 distinctive bio-behavioral phenotypes in our rhesus monkey colony, determine how genetic and environmental factors interact to shape the developmental trajectories of each phenotype, and 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 repertories 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 (*Sapajus apella*).

As in previous years, we conducted detailed longitudinal studies on the behavioral and biological consequences of differential early social rearing, most notably by 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 or housed in individual cages containing an inanimate surrogate mother and given two hours of daily interaction with similarly reared peers (NR). At 7–8 months of age, MR and NR infants were all moved into one large pen, where they lived together until puberty. Thus, the

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differential social rearing occurred only for the first 7–8 months; thereafter MR and NR monkeys shared the same physical and social environment. We previously demonstrated that NR 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, and that they also exhibit deficits in serotonin metabolism (as indexed by chronically low values of CSF 5-HIAA). Additionally, they exhibit significantly lower levels of 5-HTT (serotonin transporter) binding throughout many brain regions than do MR subjects. Many of these differences between MR and NR monkeys persist throughout the juvenile years in the absence of experimental interventions. For example, we recently published data extending these rearing condition differences to measures of social dominance status, maternal competence, telomere length, and physical health during childhood, adolescence, and adulthood. However, our most recent studies indicate 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 has been to characterize interactions between differential early social rearing and polymorphisms in several candidate genes (G x E interactions), most notably the *5-HTTLPR* gene. During the past two years, we expanded the range of outcomes for which G x 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 aggression, play behavior, social buffering, behavioral and HPA (hypothalamic-pituitary-adrenal axis) 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. NR) on genome-wide patterns of mRNA expression in leukocytes and on methylation patterns in the 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 NR infants, whereas the reverse was the case for another 717 genes. In general, NR–reared infants showed enhanced expression of genes involved in inflammation, T lymphocyte activation, and cell proliferation and suppression of antiviral and antibacterial responses. 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. These new findings are currently being prepared for publication.

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 the first 6–7 weeks of life and thereafter maintained under identical conditions until adulthood. The analyses revealed that (1) more than 4,400 genes were differentially methylated in both the PFC and lymphocytes; (2) although there was considerable tissue specificity, approximately 25% of the affected genes were identical in both PFC and lymphocytes; and (3) in both the 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 NR monkeys at exactly the same time points as in the aforementioned longitudinal study of mRNA expression. We published the results of a long-term longitudinal study detailing genome-wide epigenetic changes in in MR and NR monkeys over their first two years of life. We found dramatic changes in methylation patterns of lymphocytes from infancy to 6 months in both males and females affecting wide swaths of the genome, but sex differences were largely reversed prior to weaning. These differences continued after weaning, albeit with some attenuation, but increased again by 2 years of age. Each sex of NR monkeys exhibited very different developmental trajectories over the same developmental period. In sum, genome-wide patterns of methylation in lymphocytes were highly dynamic throughout pre-pubertal development and varied dramatically as a function of both sex and early rearing history.

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 (Reference 5). 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 also 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.

Human mothers interact emotionally with their newborns through exaggerated facial expressions and mutual gaze, a capacity that has long been considered uniquely human. We previously began a research program on early face-to-face interactions in rhesus monkeys after we made the serendipitous discovery 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 finished our initial investigation

of 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 also 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.

Using eye-tracking technology, we also demonstrated 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 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 higher 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. Similar differences in social behavior persisted well into the second year of life.

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 more affiliative gesturing towards a human experimenter following oxytocin administration.

We completed a project begun last year involving 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 on 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 two additional studies completed this past year. First, hair cortisol levels measured shortly after birth, which presumably reflect 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.

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.

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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 they play an important role in immune responses. Our studies on the formation of blood and lymphatic vessels are of great clinical interest because of the roles both types of vessels 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 that are readily available for observation and experimental manipulation. Such features permit observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific defects.

Our 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 the roles of novel vascular-associated cells.

Tools for experimental analysis of vascular development in the zebrafish

The development of new tools to facilitate vascular studies in the zebrafish has been an important ongoing aim of this project. In previous work we (1) developed a widely used confocal micro-angiography method (Figure 1); (2) compiled an atlas of the anatomy of the developing zebrafish vasculature; (3) generated a variety of transgenic zebrafish lines expressing different fluorescent proteins within vascular or lymphatic endothelial cells, making it possible for us to visualize vessel formation in intact, living embryos; and (4) developed methodologies for long-term multiphoton confocal time-lapse imaging of vascular development in transgenic fish.

We are currently developing many new transgenic lines useful for *in vivo* vascular imaging as well as for *in vivo* blood or lymphatic

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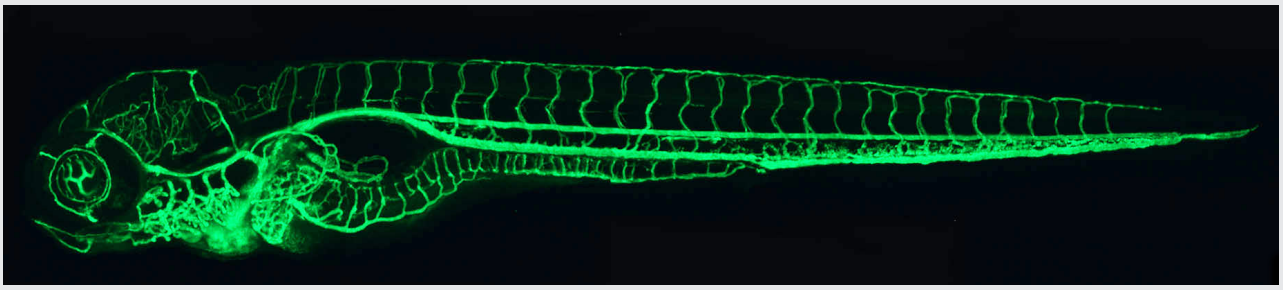

FIGURE 1. The zebrafish vascular system

Confocal micro-angiogram of the vascular system of a 4½-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.

endothelial-specific functional manipulation of signaling pathways involved in vascular specification, patterning, and morphogenesis. Notably, we generated "RiboTag" zebrafish, which facilitate high-throughput whole genome profiling of gene expression in blood and lymphatic vascular endothelium and in vascular smooth muscle cells, and we demonstrated that these transgenic lines can be used to perform *in vivo* profiling of vascular signaling pathways.

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 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 have 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 and used 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 or RNAseq. 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 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 critical for vascular development and vascular disease.

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

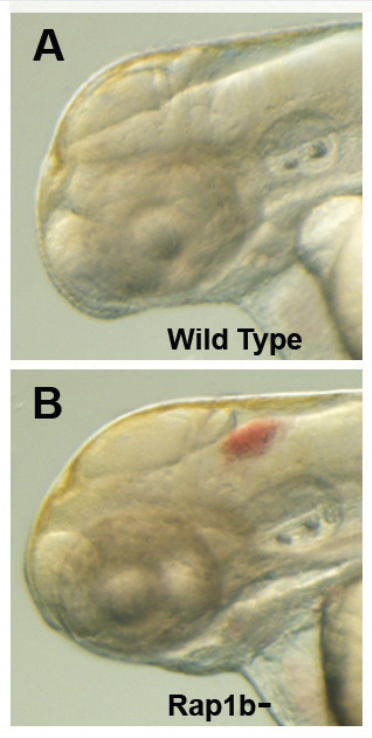

FIGURE 2. Intracranial hemorrhage (ICH) in the developing zebrafish

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).

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.

We used multiphoton time-lapse imaging to characterize patterns of vessel assembly throughout the developing zebrafish, and molecular and experimental analysis to understand how this pattern arises and what cues guide vascular specification, differentiation, and network assembly during development. Our discoveries included evidence that neuronal guidance factors play an important previously unknown role in vascular guidance and vascular patterning.

We also developed zebrafish models for ICH and uncovered a variety of different mutant zebrafish with defects in genes critical for vascular integrity (Figure 2).

Our current work includes projects aimed at (1) studying the specification, differentiation, and patterning of vascular smooth muscle in the zebrafish, making use of newly developed transgenic tools; (2) understanding the role of intracellular signaling substrates in regulating vascular endothelial signaling; (3) exploring the role of BMP–family ligands in modulating vessel growth and vascular integrity; and (4) analyzing additional pro- or anti-angiogenic factors.

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 sites.

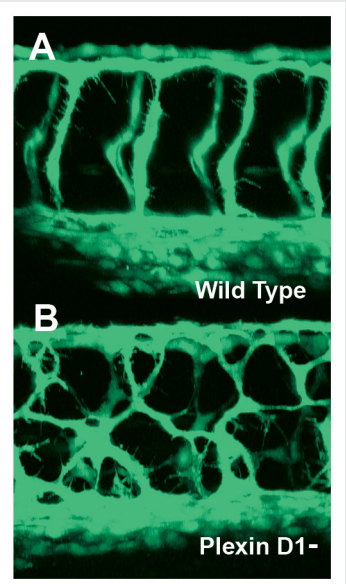

FIGURE 3. Mis-patterned trunk vessels in larvae lacking the vascular semaphorin receptor plexin D1

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 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 showed that the zebrafish possesses a lymphatic system that shares many of the morphological, molecular, and functional characteristics of lymphatic vessels found in other vertebrates, providing a powerful model for the purpose of 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 numerous 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. We have also been studying a novel lymphatic-related perivascular cell population that plays a supportive role in the brain. 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 DNA methyltransferase 3bb.1 (*dnmt3bb.1*) 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 *dnmt3bb.1* 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 continuing to study the role of *dnmt3bb.1* and DNA methylation in development and disease.

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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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Eukaryotic DNA repair and mutagenesis

The cDNA encoding human DNA polymerase iota (*POLI*) was cloned in 1999. At that time, it was believed that the *POLI* gene encoded a protein of 715 amino acids. Advances in DNA sequencing technologies lead to the realization that there is an upstream, in-frame initiation codon that would encode a DNA polymerase iota (pol iota) protein of 740 amino acids. The extra 25 amino–acid region is rich in acidic residues (11/25) and is reasonably conserved in eukaryotes ranging from fish to humans. As a consequence, the curated Reference Sequence (RefSeq) database identified pol iota as a 740 amino–acid protein. However, the existence of the 740 amino–acid pol iota has never been shown experimentally. Using highly specific antibodies to the 25 N-terminal amino acids of pol iota, we were unable to detect the longer 740 amino–acid (iota-long) isoform in western blots. However, trace amounts of the iota-long isoform were detected after enrichment by immunoprecipitation.

One might argue that the longer isoform has a distinct biological function, if it exhibits significant differences in its enzymatic properties from the shorter, well characterized 715 amino–acid pol iota. We therefore purified and characterized recombinant full-length (740 amino acid) pol iota-long and compared it with full-length (715 amino–acid) pol iota-short *in vitro*. The metal ion requirements for optimal catalytic activity differ slightly between iota-long and iota-short, but under optimal conditions, both isoforms exhibit indistinguishable enzymatic properties *in vitro*. We also reported that, like iota-short, the iota-long isoform can be mono-ubiquitinated and poly-ubiquitinated *in vivo*, as well as form damage-induced foci *in vivo*. We conclude that the predominant isoform of DNA pol iota in human cells is the shorter 715 amino–acid protein and that if, or when, expressed the longer 740 amino–acid isoform has identical properties to the considerably more abundant shorter isoform.

In 2003, we reported that mice derived from the 129 strain (129-derived strain) carry a naturally occurring nonsense mutation at codon 27 of the *Poli* gene that would produce a pol iota peptide of just 26 amino acids, rather than the full-length 717 amino–acid wild-type polymerase. In support of the genomic analysis, no pol iota protein was detected in testes extracts from 129X1/SvJ mice, where wild-type pol iota is normally highly expressed. The early truncation in pol iota occurs before any structural domains of the polymerase are synthesized and, as a consequence, we reasoned that 129-derived strains of mice should be considered as functionally defective in pol iota activity. However, it was recently reported that, during the maturation of the *Poli* mRNA in 129-derived strains, exon-2 is sometimes skipped and that an exon-2–less pol iota protein of 675 amino acids is synthesized that retains catalytic activity *in vitro* and *in vivo*. From a structural perspective, we found this idea untenable, given that the amino acids encoded by exon-2 include residues critical for the coordination of the metal ions required for catalysis, as well as the structural integrity of the DNA polymerase. To determine whether the exon-2–less pol iota isoform possesses catalytic activity *in vitro*, we purified a glutathione-tagged full-length exon-2–less (675 amino–acid) pol iota protein from baculovirus-infected insect cells and compared the activity of the isoform to full-length (717 amino–acid) GST–tagged wild-type mouse pol iota *in vitro*. Reactions were performed under a range of magnesium or manganese concentrations, as well as various template sequence contexts. Wild-type mouse pol iota exhibited robust characteristic properties previously associated with human pol iota's biochemical properties. However, we did not detect any polymerase activity associated with the exon-2–less pol iota enzyme under the same reaction conditions and concluded that exon-2–less pol iota is indeed rendered catalytically inactive *in vitro*.

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Physiology, Psychology, and Genetics of Obesity

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 cohorts 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 thus lead to more targeted, etiology-based prevention and treatment strategies for pediatric obesity.

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(continued)

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*). 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

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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. Using homozygous knock-in mouse models replacing murine *Mc3r* with wild-type human ($MC3R^{hWT/hWT}$) and double-mutant (C17A+G241A) human ($MC3R^{hDM/hDM}$) *MC3R*, we found that $MC3R^{hDM/hDM}$ have greater weight and fat mass (Figure 2), increased energy intake and feeding efficiency, but reduced length and fat-free mass than $MC3R^{hWT/hWT}$ (Reference 1). $MC3R^{hDM/hDM}$ mice do not have increased adipose tissue inflammatory-cell infiltration or greater expression of inflammatory markers despite their greater fat mass. Serum adiponectin is increased in $MC3R^{hDM/hDM}$ mice and $MC3R^{hDM/hDM}$ human subjects (Figure 2). $MC3R^{hDM/hDM}$ bone- and adipose tissue–derived mesenchymal stem cells (MSCs) differentiate into adipocytes that accumulate more triglyceride than do $MC3R^{hWT/hWT}$ *MSCs*. $MC3R^{hDM/hDM}$ thus impacts nutrient partitioning to generate increased adipose tissue that appears metabolically healthy. These data confirm the importance of MC3R signaling in human metabolism and suggest a previously unrecognized role for the MC3R in adipose tissue development. Ongoing studies continue to improve our understanding of the phenotype of these mice. We are investigating a novel role for the MC3R in regulating hepatic autophagy, the role of MC3R in stem cell fate, and how variations in *Mc3r* may alter signaling of several downstream signaling pathways.

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 we 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

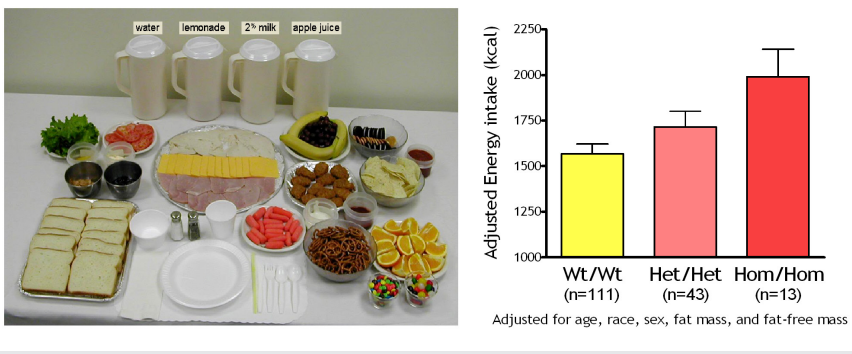

FIGURE 1. Energy intake studied by using free-access buffet meals of palatable foods

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).

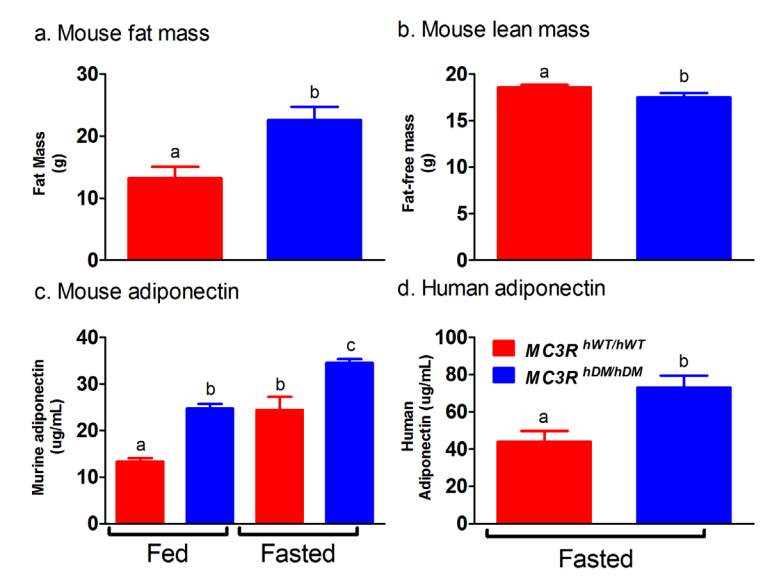

FIGURE 2. Studies of a human *MC3R* variant containing two naturally occurring polymorphisms

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).

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 they 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. 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 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 lowered 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 loss of control (LOC) over eating had significantly higher serum leptin levels 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 they have led to trials of preventative strategies related to binge eating. Because binge eating appears to be a heritable trait, we have also initiated studies to investigate possible genetic factors linked to loss of control over eating.

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 the development of complications of obesity in youth. Body composition, leptin concentration metabolic rate, insulin sensitivity

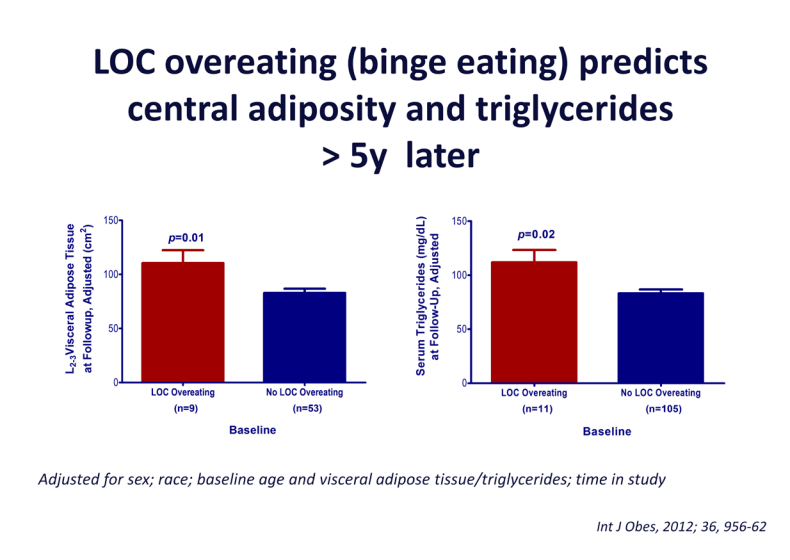

FIGURE 3. Loss of control (LOC) eating and metabolic complications in a longitudinal study

On average (±SE), children who engaged in binge eating at baseline had more visceral adipose tissue at L_{2-3} intervertebral space at follow-up than children who did 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 engaged in binge eating at baseline had higher follow-up triglycerides than children who did 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 ($P = 0.02$).

(Reference 2), glucose disposal, energy intake at buffet meals, and genetic factors believed to regulate metabolic rate and body composition are examined. We also study psychological and behavioral factors, such as propensity to engage in binge-eating behavior (Figure 3). 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 also investigated the role of sedentary behaviors, such as television watching, as a factor that alters metabolism. In a randomized, controlled, crossover trial (Figure 4), we found that glucose tolerance was markedly improved in children who engaged in moderate activity for just three minutes every half hour, versus remaining sedentary (Reference 3). We hypothesize that differences in these factors will predict the development of obesity in the populations studied, and 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, both for children and adults. In several clinical protocols, we examined approaches for the prevention and treatment of excessive body weight. We completed a randomized controlled trial 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. Adolescents treated with orlistat lost significantly more weight, BMI units, and fat mass than those that were not. We concluded that, added to a behavioral program, orlistat significantly improved weight loss over a 6-month interval. However, the drug had little impact on obesity-related co-morbid conditions in obese adolescents.

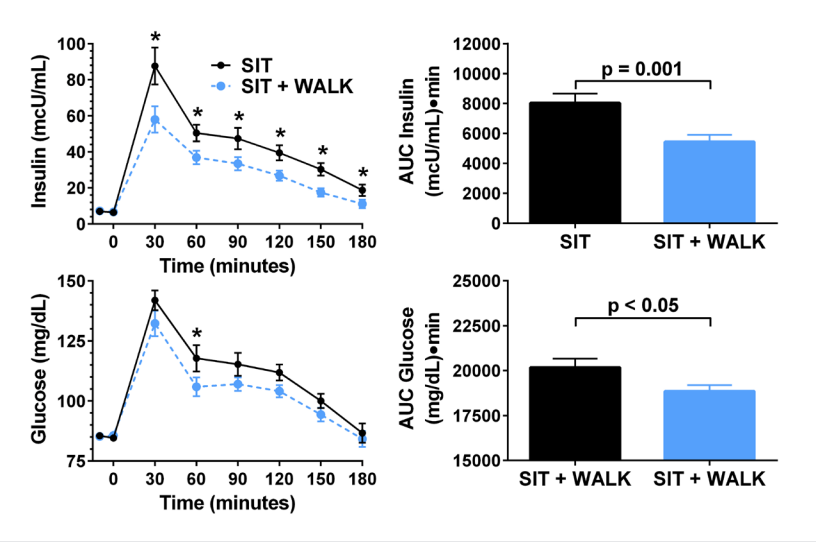

FIGURE 4. Effect of short, moderate intensity walking breaks on children's glucose tolerance

Children who walked for three minutes every 30 minutes (in blue) had lower glucose and insulin concentrations during an oral glucose tolerance test than when they sat uninterrupted for three hours (in black).

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. Compared with placebo-treated children, those randomized to metformin reduced BMI, BMI-Z score, and body fat mass to significantly greater extents. Serum glucose and HOMA-IR also decreased more in metformin-treated than in placebo-treated children. We recently published studies on the pharmacokinetics of metformin and how polymorphisms in enzymes affecting metformin clearance impact weight change. Mean population apparent clearance (CL/F) was 68.1 L/h, and mean apparent volume of distribution (V/F) was 28.8 L. Body weight was a covariate of CL/F and V/F. Estimated glomerular filtration rate was a significant covariate of CL/F. Carriers of a variant of the polyspecific organic cation transporter gene *SLC22A1* had significantly smaller reductions in percentage of total trunk fat after metformin therapy. The median percentage change in trunk fat was –2.20% (–9.00% to 0.900%) and –1.20% (–2.40% to 7.30%) for the normal *SLC22A1* subjects and variant carriers, respectively.

A third study examined prevention of weight gain using interpersonal therapy (IPT) versus a control health education program (HE) in adolescents reporting LOC eating behaviors (Reference 4). At three-year follow-up, baseline social-adjustment problems and trait anxiety significantly moderated outcome. Among girls with high self-reported baseline social-adjustment problems or anxiety, IPT was significantly associated with the steepest declines in BMIz compared with HE. For adiposity, girls with high or low anxiety in HE and girls with low anxiety in IPT experienced gains, while girls in IPT with high anxiety stabilized. Parent reports yielded complementary findings. The results have stimulated ongoing research to examine how anxiety may stimulate energy intake. This year, we also published preliminary data from a fourth study examining IPT approaches in younger children, finding good tolerability for such a program. A fifth study examined whether reducing depressive symptoms could ameliorate insulin resistance in adolescents at risk for Type 2 Diabetes (Reference 5). Among girls with greater (moderate) baseline depressive symptoms (N = 78), those in cognitive behavior therapy (CBT) developed lower 2-hr insulin than those in HE. Additional metabolic benefits of CBT were seen for this subgroup in *post hoc* analyses of post-treatment to one-year change.

We also participated in a multi-site randomized, placebo-controlled trial of beloranib, an inhibitor of methionyl aminopeptidase 2, to treat the hyperphagia of patients with the Prader Willi syndrome (Figure 5). The medication was effective in reducing body weight: compared with placebo, weight change was greater with 1.8 mg (mean difference –8.2%) and 2.4 mg beloranib (–9.5%). Unfortunately, the trial had to be halted due to an imbalance in venous thrombotic events in beloranib-treated participants (two fatal events of pulmonary embolism and two events of deep vein thrombosis) compared with placebo.

This year, we initiated a translational trial studying the effects of modulation of the leptin signaling pathway with the melanocortin agonist setmelanotide in patients with proximal signaling defects such as *PCSK1* insufficiency and in patients with Bardet Biedl syndrome. A novel intervention, which should complete enrollment in 2018, is a randomized controlled trial of colchicine to ameliorate the inflammation associated with obesity and thus reduce its complications.

Additional Funding

- NIH Clinical Center/ORD "Bench to Bedside" Award: Melanocortin agonist to bypass leptin resistance of Bardet-Biedl syndrome. 2017-2018
- NIH Clinical Center/ORWH "Bench to Bedside" Award: Attention Bias Retraining in Adolescents with Loss of Control Eating. 2016-2017
- Office of Disease Prevention, NIH: Grant supplement to support the clinical protocol "Effects of Interrupting Sedentary Behavior on Metabolic and Cognitive Outcomes in Children." 2016–2017
- 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. 2014–2016
- Rhythm Pharmaceuticals, Inc: Setmelanotide (RM-493; Rhythm Pharmaceuticals, Inc.) Phase 2 Open-Label Treatment Trials in Patients with Rare Genetic Disorders of Obesity. 2017-2019

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The Regulation or Disturbance of Protein/Lipid Interactions in Influenza, Malaria, and Muscular Dystrophy

Fusion and fission, the instances when organelles gain or lose their identities, are the essence of complex membrane dynamics in living cells and are key elements of synapses and other dynamic cellular trafficking networks. Without fusion and fission, enveloped viruses and parasites could not enter cells, replicate, or exit cells, nor would inflammatory cells respond and kill such invaders or deal with sick cells. Our earliest work concentrated on model membrane systems, the physical properties and theoretical pathways required for membrane fusion to occur, and the discovery that tension spreads headgroups for hemifusion, then pulls open fusion pores to allow coalescence of adherent bilayers. However, while able to focus on basic membrane biophysical properties and help develop a theoretical framework to understanding membrane interactions, model systems were a simplification that ignored the important roles of proteins. Including the role of proteins in these fundamental biophysical processes was both fruitful and informative, culminating in what we believed to be a canonical framework for understanding both fusion and fission. We introduced a simple paradigm: proteins act as catalysts (bilayer topoisomerases) lowering the huge energy barriers to membrane remodeling steps. A few amino acids of a specialized protein domain can reversibly enter the hydrophobic membrane matrix or cover the headgroups as inclusions or scaffolds, respectively, and thus transiently alter the thermodynamics of the system by specific protein-lipid interactions. By combining quantitative light microscopy with electrophysiology, and reconstitution of fusion and fission in lipid bilayer membranes, we constructed hypotheses with predicted fusion intermediates whose dimensions were deduced by continuum theory and fits to experiments. The predicted sizes were detectable by cryo-electron microscopy, so we labored to achieve the highest-resolution electron microscopy of hydrated membrane fusion events in order to understand how proteins catalyze the new configurations of lipids that ultimately mediate these processes. By successfully installing a new technology at NIH, the Volta Phase Plate, we were able to visualize the predicted hemifusion diaphragm mediated by the hemagglutinin (HA) of influenza virus (IFV), and the measurements of its dimensions fit the predictions of continuum theory. However, another result was unexpected: HA catalyzed the breakage of membranes, leading to free membrane edges—often in great profusion.

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To understand why this was unexpected, we must consider the physical forces that act on lipids in solution. Membranes avoid edges. The lipid bilayer is self-assembling because its free energy of cohesion (which derives in part from enthalpic attractive forces between hydrocarbon chains and in part from the entropic hydrophobic effect that minimizes interfacial area) automatically ensures stability of the lipid bilayer. Formally, the edge of an otherwise lamellar membrane has a large linear tension, i.e., should be a high-energy region that the membrane seeks to minimize. Nevertheless, we observe that 'free edges' do indeed outnumber hemifusion diaphragms for certain lipid compositions of target membranes. Such edges only occur in close vicinity to activated HA molecules, indicating that edges are triggered to form by the same event that triggers full fusion: namely, the amphipathic helix of HA being ejected from HA and binding to the target bilayer. We can only presume for the moment that the HA fusion peptide somehow stabilizes the observed membrane edges, i.e., drastically lowers bilayer line-tension. This observation and resultant hypothesis forms the basis of our future work.

Membrane fusion, fission, and the role of cholesterol in the assembly of influenza virus

There are two threads that run through all of the Section's work: the use of quantitative measurements of dynamic systems to test hypotheses deduced from biophysical reasoning. Our impact goes beyond our single experiment when we develop new technology that paves the road to future work. In the past, capacitance measurements opened up the field of the fusion pore to experimentation, as did simultaneous imaging with electro-physiology, and laser excitation of photo-activatable fluorophores opened up single-molecule imaging to living cells. More recently, the implementation of electrical and fluorescent measurements of cylindrical lipid nanotubes allowed us to test hypotheses regarding the interaction of dynamin and membrane curvature–scaffolding proteins with membranes. We found that the GTPase dynamin acts as a scaffold with many small amino acid insertions into the outer monolayer of a lipidic structure called the fission pore, to form a hemi-fission intermediate. The hemi-fusion intermediate was induced by influenza virus HA incorporating in virosomes and interacting with targeted lipid vesicles at low pH. Ultra-thin films were plunge-frozen and visualized by Volta phase plate cryo-electron tomography (VPP-cET). We identified two distinctly different hemifusion structures: a hemifusion diaphragm and a highly unexpected novel structure termed a 'lipidic junction' (Reference 1). The edges of liposomes' lipidic junctions were ruptured and stabilized by HA. The high frequency of lipidic junctions exclude their artefactual origin. Both rupture frequency and hemifusion diaphragm diameter decreased when the liposome cholesterol level matched physiological concentrations.

Another unexpected ultrastructural observation came when we deacylated HA, leading to our new hypothesis that HA acylation contributes to membrane curvature in influenza virus A assembly and HA–mediated membrane fusion. Indeed, de-acylation of the cytoplasmic tail of HA interrupted interactions between HA and matrix (M1) and affected viral envelope curvature, without influencing viral-like particles' (VLP) shape, lipid composition, or HA lateral spacing. Observed VLP size distribution data were supported by molecular dynamic simulations data based on the spontaneous curvature of palmitate. HA acylation also controls curvature-dependent fusion-pore widening, an important step in membrane fusion. In addition, HA acylation regulates the binding of M1 matrix protein on the budding virus during assembly. Thus, HA acylation contributes to the ability of HA to control curvature to bend membranes and control curvature-dependent fusion-pore widening, emphasizing the importance of protein-lipid interactions in membrane

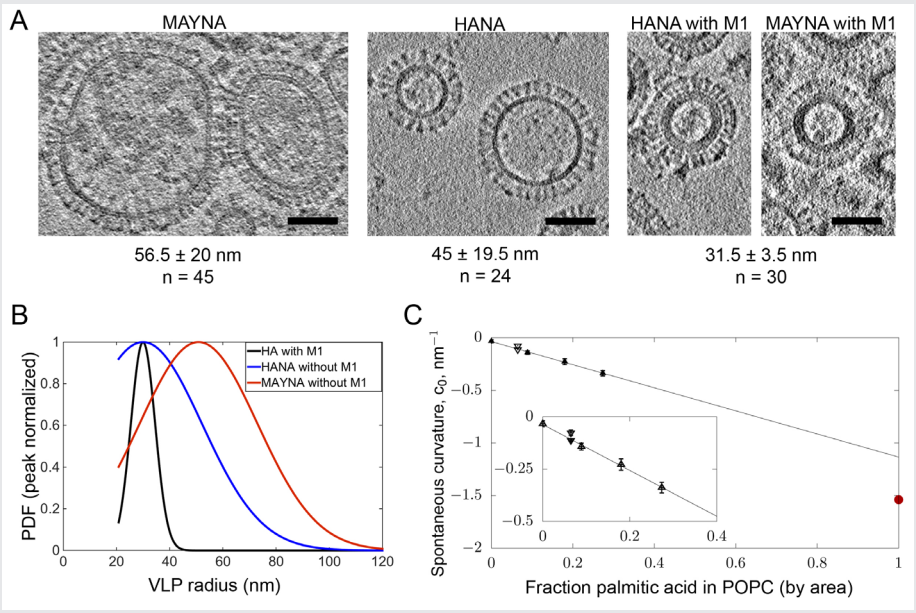

FIGURE 1. HA acylation affects curvature of the released particles.

(A) Representative slices of tomograms showing MAYNA (no M1), HANA (no M1), and HANAM1M2 spheroidal particles. Respective average radii, standard deviations, and numbers of measurements are shown below the images. (B) Peak normalized probability density functions (PDF) for the three species, HA with and without M1 and MAY without M1, calculated using the truncated Gaussian parameters identified from the global fit of the cumulative distributions of VLP radii. HA and MAY differ in their most probable size (peak of the PDF), and the distribution width is significantly greater in the absence of M1. (C) Spontaneous curvature of palmitic acid and palmitoylation on short alpha-helical peptide sequences computed from simulation. Extrapolation of the data to the 100% area fraction of palmitic acid yields an estimate of the spontaneous curvature of palmitic acid. Shown for reference (red point on the right curvature axis) is the same value estimated from the change in average size of the VLPs with HA compared to that with MAY. Abbreviations: MAYNA, mutated neuraminidase (NA) in which the mutant amino acids are M, A, and Y; HANA, viral particle made with wild-type HA and NA; M1 and M2, matrix proteins; MAY, mutant HA.

remodeling (Reference 2). For wild-type HA, the interaction of palmitate augments the natural electrostatics of M1/surface binding to negatively charged envelope. The layering and subsequent uncoating of influenza M1 at low pH provides enough tension to rupture membranes. In neutral media, adsorption of M1 protein to a lipid bilayer is strong, electrostatic, and reversible. Acidification alters M1 charged groups, driving a conformational change and partially desorbing M1 from the membrane because of increased repulsion between remaining M1. Lipid vesicles coated with M1 ruptured at low pH owing to this repulsive force. These studies indicate how protein motifs and their interactions with lipids can create the forces required for membrane transformations driven by fission, fusion, and lipidic edge formation.

To understand the origin of the negative charged lipids within the viral envelope, a fusion protein/lipid interactome project aimed to assess the intimate relationship of viral fusion protein with the biological membrane at a nm spatial scale. Cholesterol-sphingolipids distribution was linked to influenza virus budding. Secondary ion mass spectrometry (SIMS) was employed to image, with about 100 nm lateral resolution, the distributions of isotope-labeled cholesterol and labeled viral protein in the plasma membrane during virus budding (ongoing work). The data show that, in MDCK cells (mammalian cell-culture cells), influenza virus does not bud from cholesterol-enriched membrane domains. However, sphingolipid domains need not be enriched with cholesterol. SIMS was used to map the distributions of isotope-labeled cholesterol and sphingolipids in the plasma membranes of fibroblast cells; HA clusters were neither enriched with cholesterol nor co-localized with sphingolipid domains. HA clustering and localization in the plasma

membrane is not controlled by cohesive interactions between HA and liquid-ordered domains enriched with cholesterol and sphingolipids, or from specific binding interactions between hemagglutinin, cholesterol, and/or the majority of sphingolipid plasma membrane species.

Membranes during egress of the causative agent of malaria

Malaria continues to devastate children worldwide, with over 300,000 deaths from malaria in children under five in 2015 alone. With no vaccine available and drug resistance climbing, we are focusing on the unique membrane biology of the parasites that cause malaria, to find new targets for therapy. In our work, methods have been compiled allowing a comprehensive quantitative evaluation of parasite replication in erythrocytes. By developing, publishing, and promulgating new methods to study the biology of the malaria parasite, our work has impacted the field by transforming qualitative imaging to quantitative measures, providing the first recordings of *P. falciparum* egress and invasion of erythrocytes, describing new phenomena such as shape transformation of infected cells signaling the egress initiation and membrane transformation upon egress. We developed several non-interventional methods that allow fine staging of cell phenotype and quantification of the parasite replication cycle as it naturally progresses from parasite invasion of erythrocytes to parasite egress from the host cells. As a result, we were able, for the first time, to assess an intimate relationship between egress and invasion steps in parasites defective in an egress/invasion protease cascade. In addition, we corrected several mistaken conclusions about parasite biology and drug effects, by avoiding artifacts caused by the mechanical and osmotic fragility of live, infected erythrocytes (iRBC) and their exceptional sensitivity to illumination.

The methods we developed include: (1) quantitative assay for parasite intra-erythrocytic multiplication factor (IMF), which is the first assay to quantify the number of progeny per individual cell and thus permits precise assessment of the role of parasite and host on parasite multiplication; (2) quantitative assay of the length of parasite cycle, which permits assessment of the effects of any intervention into parasite physiology on cycle progression and host-cell biophysical characteristics, such as volume, hemoglobin content, cell hydration, ion homeostasis (the assay also provides cell phenotyping and timing of any physiological block to cycle progression); (3) quantitative parasite egress assay for short-duration treatments of iRBC allows testing of ionophores or other fast-acting drugs or conditions on parasite egress, extending the range of interventions into parasite physiology that now can be quantified; (4) assessment of photo-damage to iRBC by inhibition of parasite egress from erythrocyte upon controlled cell illumination (intensities and wavelength) to extend the parameters for microscopic work without damage; (5) a new method to release intact parasitophorous vacuoles (PV) from live iRBC for electrophysiological studies on the PV membrane using a patch clamp technique, a new method that exploits the K^+ permeability of infected erythrocytes, which swells the PV till it ejects from the iRBC, with vacuolar membranes intact (i.e., vacuoles preserve their vacuolar content) and easily patched by glass pipets; (6) quantitative parasite invasion assay, which allows naturally egressed parasites to invade neighboring normal erythrocytes (Reference 3), an invasion method that is independent of the parasite egress rate and is able to test pulse exposure to drugs (15–30 min), thus excluding the effect of drugs on parasite development.

Using these methods, new targets continue to be discovered in *Plasmodium falciparum,* the most clinically devastating species. The most promising to date is a pair of 'druggable' mediators of parasite egress and invasion, namely, aspartic proteases Plasmepsins IX and X. We determined that the aspartic protease PMIX (acting from within the 'rhoptry', apical secretory organelles of *Plasmodia*) is essential for erythrocyte

invasion (Reference 3). In contrast, PMX, by controlling maturation of the subtilisin-like serine protease SUB1 in exoneme secretory vesicles, is essential for both egress and invasion. A lead compound, C-117, is currently under intense evaluation by pharmaceutical companies, because it works in the high nanomolar range and is well tolerated orally by mice (Reference 3). In another study, we showed a new potential route for drug delivery thought to be impossible. Despite its membrane impermeability, the natural glycosaminoglycan heparin inhibited malaria parasite egress, trapping merozoites within infected erythrocytes (Reference 4). Heparin does not bind to the erythrocyte surface, rather it enters iRBC at the last minute of the parasite cycle through parasite-induced pores that we discovered in iRBC. This short encounter was sufficient to significantly inhibit parasite egress and dispersion. Heparin blocks egress by interacting with both the surface of merozoites and the inner aspect of erythrocyte membranes, preventing the rupture of infected erythrocytes but not of parasitophorous vacuoles, and independently interfering with merozoite disaggregation. Given that this action of heparin offers a plausible explanation of how neutralizing antibodies can block egress, we intend to exploit membrane perforation as a new physiological strategy to target therapeutics intracellularly (Reference 4). While we continue to search for the molecular identity of the pores before merozoite egress, we learned that a perforin-like protein, PPLP2, permeabilizes the iRBC membrane during gametocytes egress. Like merozoites, egress of malaria gametocytes from iRBC requires the concerted rupture of vacuolar and erythrocyte membranes. We showed that PPLP2 is essential for normal egress of gametocytes from iRBC but that it is dispensable for parasite asexual cycle. PPLP2$^-$ gametocytes were trapped within erythrocytes owing to failure to permeabilize the erythrocyte membrane despite a normal disruption of the vacuole membrane. As a consequence, transmission of PPLP2$^-$ parasites to the *Anopheles* vector was reduced. Thus, the hemolytic activity of PPLP2 is essential for gametocyte egress and transmission to mosquitoes.

One clinical issue for patients with malaria relates to its pathophysiology in hematological disorders. By using our IMF assay (above) to quantify the number of merozoites released from an individual schizont, and infecting blood, lab or clinical isolates, from patients with various thalassemias or sickle disease with *P. falciparum*, we determined that IMF strongly correlates with hemoglobin concentration of the host cells. We undertook mathematical modeling of malaria progression to learn that a lower IMF limits parasite density and anemia severity over the first two weeks of parasite replication. Thus, *P. falciparum* IMF is a parasite heritable virulence trait that correlates with erythrocyte indices and is reduced in thalassemia-trait erythrocytes.

By using the above photo-damage assay, we showed that low fluence red light is the safest to study parasite physiology with continuous or time lapse microscopy. Photo-sensitizing fluorescent dyes should be used with extreme caution in the study of cell physiology of malaria parasites. We are developing a comprehensive method to assess the malaria parasite replication cycle, using live cell microscopy that takes into consideration the photosensitivity of iRBC.

Muscle membrane modification to treat muscular dystrophy

A major theme under development is that membrane transformations and dynamics, including the newly identified membrane “edge”, are strongly influenced by lipid composition. To extend our research to physiology and pathophysiology, we explored the possibility that lipid composition can be altered with diet. We designed a dietary intervention trial in the dysferlin-deficient A/J mouse (a model of muscular dystrophy), which develops a mild myopathy after 6 months of age, to test whether a diet rich in alpha-linoleic acid alters lipid content by an iso-caloric substitution of flaxseed oil for soybean oil in a standard

defined diet. After five months, the test (flax) group had greater weight and muscle gain than the soy group, with no evidence for detrimental side effects. Lipidomics analysis of muscle tissue from the two diet groups demonstrated that levels of arachidonic acid (AA)–containing phospholipids were four-fold lower, and levels of alpha-linoleic acid–containing phospholipids were four-fold higher, in the flax-diet group. The results indicate that membrane lipid composition can be modified through changes in diet. While evidence exists for a beneficial role for dietary omega-3 fatty acids, less is known about other fatty acids or analogs with potential therapeutic benefit when incorporated into lipids. One such fatty acid is phytanic acid, which can be incorporated into membrane lipids. However, the ability of phytanic acid to modify biological membranes through a diet that would incorporate such stabilizing lipids into membranes was unknown. In addition, methodologies to quantify the incorporation of phytanic acid were not developed. We therefore initiated both a dietary study to evaluate phytanic acid incorporation into phospholipids and a new technique to measure phytanic acid species: ion mobility mass spectrometry. Dysferlin-deficient A/J mice were maintained on a defined diet supplemented with 2% phytol (a metabolite of which is phytanic acid) for three weeks. The muscle tissue was then analyzed using ion mobility mass spectrometry. Lipid species were differentially detected in the phytol diet muscle (m/z 790.7 and 862.7) and tentatively identified as phosphatidylcholine (PC) 20:0-16:0 and PC 20:0-22:6. To confirm the presence of phytanic acid, the ion mobility of these species was compared with standards, and determined to be a PC having one phytanic acid chain. We estimate that these species represent 10% of PC, validating a dietary strategy in altering muscle membranes. These 'proof-of-principle' studies in dietary changes and mass spectrometry measurements of membrane lipids advance our ability to study the effects of specific lipids on membrane stability and to test lipids as therapies in muscular dystrophy.

Technological advances in this arena were focused on imaging muscle and protein relations within muscle. (1) Continuing our long-standing contributions to super-resolution microscopy, we found that adaptive optics improves multiphoton super-resolution imaging. Thus, using a reference beam to determine optical aberrations and a deformable mirror to correct them, signal intensity was increased up to 40-fold with a lateral resolution down to 176 nm at depths up to 0.25 mm in embryos and other tissues (Reference 5). (2) Fluorescent proteins are in the static regime when excited; in this regime, estimating separations between Förster resonance energy transfer (FRET) pairs is problematic. We exploited the experimentally measured average FRET efficiency to estimate separations between fluorescent proteins.

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Publications

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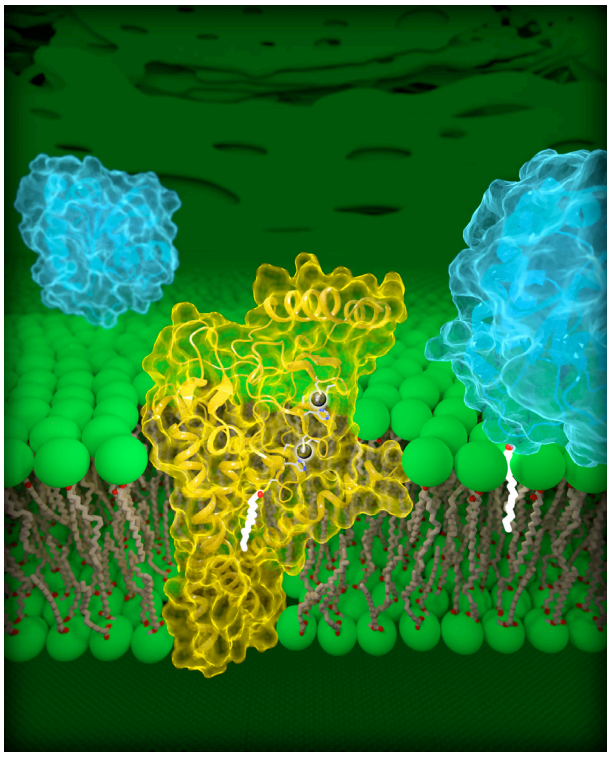
About the Cover Image



X-ray crystallography by Mitra Rana, a Visiting Fellow in the *Banerjee Lab*. 3D rendering by Jeremy Swan of the *Computer Support Services Core*.

Molecular view of DHHC palmitoyltransferases. Human DHHC20 palmitoyltransferase (yellow) shown localized in the Golgi body membrane (green stacks). The Zn^{2+} ions are shown as gray spheres and the acyl chain of the palmitoyl group in white sticks. A hypothetical substrate (blue) approaches the palmitoyltransferase from the left and, after palmitoylation, is localized to the Golgi body membrane through anchoring of the palmitoyl group, now transferred to the substrate.

The 3D render was created using *Blender*, a free, open source 3D creation program.

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