

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, <i>Chair</i> 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	
Elizabeth Bonney, MD, MPH 7/1/17 – 6/30/22 Immunobiology, Molecular Biology, Obstetrics & Gynecology	Professor, Director of Research Division Department of Obstetrics, Gynecology, and Reproductive Sciences University of Vermont College of Medicine	
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	
William T. Dauer, MD* 7/1/18 – 6/30/23 Pediatrics, Pediatric Development & Behavior, Rare Diseases & Genetics, Neurobiology	Elinor Levine Professor of Neurology Professor of Cell and Developmental Biology Director, Movement Disorders Group & Udall Center of Excellence for Parkinson's Disease Research University of Michigan Medical School	
Frances E. Jensen, MD 7/1/15 – 6/30/20 Neuroscience	Professor and Chair, Neurology Department Perelman School of Medicine, University of Pennsylvania	
Deborah L. Johnson, PhD 7/1/17 – 6/30/22 Molecular & Cellular Biology	Professor, Department of Molecular and Cellular Biology Baylor College of Medicine	

Kojo A. Mensa-Wilmot, PhD 7/1/16 – 6/30/21 Cellular Biology Professor and Head, Department of Cellular Biology University of Georgia

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 2018 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: <u>http://annualreport.nichd.nih.gov</u>

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 populationbased 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 large 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. <u>http://clinicalcenter.nih.gov/translationalresearch-resources/U01/</u>

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 2018. I also invite you to reach out to me with your ideas and proposals for collaborative initiatives we may undertake together, at <u>stratakc@mail.nih.gov</u>.

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; see below) 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 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 and the 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., <u>PubMedCentral</u>, <u>Clinicaltrials.gov</u>).
- 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.
- Working 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 on the first Friday of December and in June of each year. The BSC reviews site visit reports, evaluations, and all other activities of the



Constantine A. Stratakis, MD, D(med)Sci, Scientific Director Sara K. King, Chief of Staff Jessica Rigby, Scientific Program Analyst Amaressa Bostwick, Administrative Support Specialist Jennifer Polney, Administrative Support Specialist OSD. Each NICHD investigator is subject to a review at least once every four years, according to NIH policy (NIH Sourcebook Process for Reviewing NIH Intramural Science, <u>https://oir.nih.gov/sourcebook/processes-reviewing-nih-intramural-science</u>). To ensure the most effective use of public dollars toward high-quality, high-impact research, the Board is made up of accomplished senior extramural researchers. Membership of the BSC is listed at (<u>https://annualreport.nichd.nih.gov/bsc.html</u>).

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

Office of Education

The Goals and objectives of the Office of Education can be summarized as follows. 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 new training initiatives. Our professional development workshops and activities typically include public speaking workshops, job interviewing, writing and editorial services for professional school and job applications, grantsmanship workshops, academic and non-academic career presentations, one-on-one counseling, teaching opportunities, and lab management programs. Additional areas of involvement include programming for career exploration, networking among fellows and alumni, grantsmanship, and the enhancement of fellows' competitiveness for research awards and future career opportunities, as well as support of new tenure-track investigators.

Notable accomplishments of the past year

The Office of Education organizes numerous workshops, programs, and individualized opportunities for a population averaging 250 trainees, including postdoctoral, visiting, and research fellows; clinical fellows and medical students; graduate students; and postbaccalaureate fellows and summer trainees.

Our **TmT (Three-minute Talks) competition**, now in its fifth year, is held in conjunction with NIDCR, NIAMS, NHGRI, and NEI. **Jakob Gutzmann** in the laboratory of <u>Dax Hoffman</u> received the third-place award.

We continued our **Graduate Student Talks** initiative, established in 2014, which provides the Institute's graduate students with experience in presenting their thesis research to a non-specialist scientific audience.

An **online Annual Progress Review** for fellows, developed by the Office and launched in 2016, tracks scientific and career development and progress. As part of investigators' assessments of mentoring, the reports by fellows are analyzed and provided for the Board of Scientific Counsellors site visits.

The **database of NICHD alumni** from 2008 to the present continues to be updated.

We compiled a valuable **list of organizations that accept grant applications** from NIH intramural fellows, through both NIH and non-NIH funding mechanisms.







Yvette R. Pittman, PhD, Director, Office of Education Erin Walsh, PhD, Program Manager Carol Carnahan, BS, Program Coordinator



In September 2018, the Division of Intramural Research gave its **11th Mentor of the Year awards** to **LiQi Li**, <u>Section on Cellular and Developmental Biology</u>, in the investigator category, and to **Marina Venero Galanternik**, <u>Section on Vertebrate Organogenesis</u>, as fellow.

For NICHD, **26 Fellows Award for Research Excellence (FARE) awards** were made for the 2019 competition.

In addition to the **Fellows Intramural Grants Supplement (FIGS)** continuing to recognize and stimulate grant applications among fellows, we launched the second cycle for our new competitive internal funding opportunity for NICHD postdoctoral and clinical fellows, our **Intramural Research Fellowships (IRFs)**. Its ultimate goal is to promote fellows' grant writing and to enhance their awareness of various components for an NIH grant application.

The Institute has 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 US and French scientists to obtain postdoctoral training with French and US mentors, respectively.

The **Fellows Recruitment Incentive Award (FRIA)** continues to support investigators who recruit postdocs from populations traditionally under-represented in science.

The alumni of our **NICHD Developing Talent Scholars program**, in its eighth year, now number 12 individuals; two of our new alumni are starting professional school at Cornell University and Pennsylvania

State University, and five new postbaccalaureate fellows joined the program in 2018. 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 13th year.

The Office of Education is fully committed to and actively involved in graduate and professional school advising and career counseling for all of our fellows. NICHD, along with four other institutes, launched a new career development program this year, specifically directed at those fellows interested in careers outside academia. The **Planning and Career Exploration (PACE) program** provides fellows with key resources to explore various scientific careers and helps them set achievable goals and build their professional networks.

The **14**th **annual meeting of fellows**, held for about 120 people to address scientific developments and careers, took place at the Smithsonian Museum of the American Indian and featured keynote speaker Dr. Yvette Seger, Director of Science Policy at the Federation of American Societies for Experimental Biology (FASEB), who shared her perspective on science policy careers. Each spring, this retreat includes presentations by fellows and a poster presentation by each attendee. The program is developed and run by a fellows' steering committee.

<u>The NICHD Connection</u> monthly newsletter continues its focus on mentoring, careers, and academic programs for young scientists, publishing its 100th issue in September 2018 and reaching all members of the intramural division and our alumni.

Yvette Pittman became Director of the Office, and Erin Walsh was added to its staff as Program Manager.

Contact

For further information, contact <u>*pittmanyv@mail.nih.gov*</u>, Dr. Erin Walsh (<u>erin.walsh@nih.gov</u>), or Carol Carnahan (<u>carnahac@mail.nih.gov</u>).

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 eleven 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's or women's health. 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 <u>fdporter@helix.nih.gov</u> or visit <u>https://</u> www.nichd.nih.gov/about/org/dir/osd/mt/cd.



Forbes D. Porter, MD, PhD, Clinical Director Meg Keil, PhD, Associate Director, Nursing and Protocol Navigation Marvellen Rechen, BS, RN, Special Assistant to the Clinical Director Donna Peterson, BS, RN, Protocol and Institutional Review Board (IRB) Coordinator Karim Calis, PharmD, MPH, Director of Clinical Research and Compliance Alan DeChernev, MD, Senior **Investigator** Simona Bianconi, MD, Staff Clinician An Dang Do, MD, PhD, Staff Clinician Denna Zeltser, MD, Staff Clinician Sheila Brady, CRNP, Nurse Practitioner Marianne Knue, CRNP, Nurse Practitioner John Perreault, CRNP, Nurse Practitioner Kisha Jenkins, BS, RN, Clinical Nurse Margarita Raygada, PhD, Genetic Counselor Charalampos Lyssikatos, MD, Protocol Coordinator Craig Abbott, PhD, Statistician Fathy Majadly, BS, Patient Specimen Coordinator Loc Trinh, Research Chemical Engineer Denise Phillips, Administrative and Credentialing Support Lisa Ast, Administrative Support Jennifer Hernandez, Administrative Support Monique Shelton, Administrative Support

Clinical Trials at NICHD

Numerous clinical protocols are run by the NICHD, Division of Intramural Research (for a complete listing, please visit <u>https://www.clinicaltrials.gov/ct/search;?term=nichd</u>). 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.

Bone and Matrix Biology in Development and Disease

- » Natural History Studies on children and adults with osteogenesis imperfecta, both dominant and recessive forms. Secondary features are a focus, including scoliosis, cardio-pulmonary and metabolic function, audiology and basilar invagination, as well as identification of causative genetic mutations. Patients may be referred to DR. JOAN MARINI at <u>oidoc@helix.nih.gov</u>.
- » 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 <u>oidoc@helix.nih.gov</u>.

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 <u>karel@mail.nih.gov</u>.
- » 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 <u>stratakc@mail.nih.gov</u> 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. MARI SUZUKI at mari.suzuki@nih.gov or MR. CHARALAMPOS LYSSIKATOS at <u>charalampos.lyssikatos@nih.gov</u> or 301-496-6633.
- » Research investigating the long-term effects of Cushing disease in childhood. Patients may be referred to DR. MEG KEIL at <u>keilm@mail.nih.gov</u> or 301-435-3391.
- » Studies into how genetics play a role in the development of obesity. Patients may be referred to DR. JACK YANOVSKI at <u>vanovskj@mail.nih.gov</u> or 301-435-8201.
- Study on the safety and efficacy of pegvisomant in children and adolescents with growth hormone excess, who have persistent disease after surgical and/or radiation treatment or are not eligible for those.
 Patients may be referred to DR. CONSTANTINE STRATAKIS at <u>stratakc@mail.nih.gov</u> or to DR. CHRISTINA TATSI at 301-451-7170.

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 <u>amir@helix.nih.gov</u>.

- » 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 <u>romeror@mail.nih.gov</u>.
- » 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 <u>romeror@mail.nih.gov</u>.
- » 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 <u>romeror@mail.nih.gov</u>.

Pediatric Endocrinology, Metabolism, and Genetics

- » Studies on pediatric disorders that are associated with the predisposition to develop obesity and diabetes including Bardet-Biedl Syndrome, Alström Syndrome, Prader-Willi Syndrome, leptin receptor deficiency, PCSK1 deficiency, and Pro-opiomelanocortin (POMC) deficiency. Patients may be referred to DR. JACK YANOVSKI at <u>yanovskj@mail.nih.gov</u> 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 <u>dmerke@nih.gov</u> 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 <u>delaneya@mail.nih.gov</u>.
- » 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 <u>fdporter@mail.nih.gov</u> 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 <u>an.dangdo@nih.gov</u> 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 **MR. JOHN PERREAULT** at 301-827-9235 or to **MS. KISHA JENKINS** at 301-594-2005.

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 <u>baronj@cc1.nichd.nih.gov</u> or DR. YOUN HEE JEE at <u>jeeyh@mail.nih.gov</u>.

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 <u>zimmerbj@mail.nih.gov</u> or MS. HANG WATERS at <u>watershn@mail.nih.gov</u>.

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 <u>decherna@mail.nih.gov</u> or 301-594-5494.
- » Research on reproductive function in sickle cell disease. Patients can contact **DR. ALAN DECHERNEY** at <u>decherna@mail.nih.gov</u> 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 highdimensional 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: <u>http://www.med.wayne.edu/prb</u>. 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.

NICHD-NIDDK-NIDCR Inter-Institute Endocrine Training Program

The Inter-Institute Endocrinology Training Program (IETP) is a three-year training program that 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.

Developing an independent career as a physician scientist is the primary focus during the second and third years of training; emphasis is placed on how to develop research questions and hypothesisdriven research protocols. To this end, the second and third year are spent primarily in the laboratory or conducting clinical research under the mentorship of a senior investigator in one of the several endocrinology branches of the NIH. During this research period, fellows continue to gain active clinical experience 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 other services within the Clinical Center, where patients are not selected with regard to endocrine problems. Thus, fellows gain experience with several common endocrine problems 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
- Karel Pacak, MD, PhD, DSc, Head, Section on Medical Neuroendocrinology, NICHD
- Constantine Stratakis, MD, D(med)Sci, Scientific Director and Head, Section on Endocrinology and Genetics, NICHD
- Andrew Demidowich, MD, *Staff Clinician*
- Katherine T. Arague, MD, *Clinical Fellow*
- Iris Hartley, MD, *Clinical Fellow* Crystal Kamilaris, MD, *Clinical*
- Fellow
- Marissa Lightbourne, MD, *Clinical Fellow*
- HongXiu Luo, MD, Clinical Fellow
- Maziar Rahmani, MD, PhD, *Clinical Fellow*
- Skand Shekar, MD, Clinical Fellow

The IETP provides a comprehensive training experience that involves not only the NIH clinical branches working in endocrinology but also 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

- 1. Sater ZA, Jha S, McGlotten R, Hartley I, El Lakis M, Araque KA, Nieman LK. Diverticular perforation: a fatal complication to forestall in Cushing syndrome. *J Clin Endocrinol Metab* 2018 103(8):2811-2814.
- 2. Araque KA, Kadayakkara DK, Gigauri N, Sheehan D, Majumdar S, Buller G, Flannery CA. Reducing severe hypoglycaemia in hospitalised patients with diabetes: early outcomes of standardised reporting and management. *BMJ Open Qual* 2018 7(2):e000120.
- 3. Kamilaris CDC, Stratakis CA. An update on adrenal endocrinology: significant discoveries in the last 10 years and where the field is heading in the next decade. *Hormones (Athens)* 2018 17(4):479-490.
- 4. Kamilaris CDC, Faucz FR, Voutetakis A, Stratakis CA. Carney complex. *Exp Clin Endocrinol Diabetes* 2018 Epub ahead of print.
- 5. Motazedi B, Rahmani M, Welch JM, Motazedi A. First report of concomitant pheochromocytoma and duodenal neuroendocrine tumour in a sporadic multiple endocrine neoplasia type 1. *BMJ Case Rep* 2018 bcr-2017-222947.

Collaborators

- Kenneth Berman, MD, Director, Endocrine Training Program, Washington Hospital Center, Washington, DC
- Rebecca Brown, MD, Diabetes, Endocrinology, and Obesity Branch, NIDDK, Bethesda, MD
- Alan H. DeCherney, MD, Reproductive Endocrinology and Infertility Training Program, NICHD, Bethesda, MD
- Rachel Gafni, MD, Craniofacial and Skeletal Diseases Branch, NIDCR, Bethesda, MD
- Phillip Gorden, MD, Diabetes, Endocrinology, and Obesity Branch, NIDDK, Bethesda, MD
- Nicholas Patronas, MD, Diagnostic Radiology, NIH Clinical Center, Bethesda, MD
- James C. Reynolds, MD, Nuclear Medicine Department, NIH Clinical Center, Bethesda, MD
- William F. Simonds, MD, Metabolic Diseases Branch, NIDDK, Bethesda, MD
- Joseph Verbalis, MD, Director, Endocrine Training Program, Georgetown University Medical Center, Washington, DC
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Contact

For more information, email <u>Ranganath.Muniyappa@nih.gov</u> or go to <u>https://www.niddk.nih.gov/research-</u> funding/at-niddk/training-employment/medical-student-md/inter-institute-endocrinology-fellowship-program.

Pediatric Endocrinology Inter-Institute Training Program

The Fellowship in Pediatric Endocrinology is a three-year, ACGMEaccredited 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 URL <u>http://pe.nichd.nih.gov</u> provides more detailed information about the program.

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 followup 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, among others, the treatment of adrenal and pituitary tumors, congenital adrenal hyperplasia, McCune-Albright syndrome, disorders of sexual development, obesity, Cushing's syndrome.

Second and third years. During the second and third years, mandatory clinical responsibilities are limited to one half-day continuity clinic per



Miranda Broadney, MD, MPH, Acting Program Director Constantine A. Stratakis, MD, D(med)Sci, Scientific Director, NICHD

Jeffrey Baron, MD, Head, Section on Growth and Development, NICHD

- Andrew Bremer, MD, PhD, Program Director, Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK
- Rebecca Brown, MD, Senior Clinical Fellow, Clinical Endocrinology Branch, NIDDK
- Stephanie Chung, MD, Assistant Clinical Investigator, NIDDK
- Andrew Dauber, MD, Division Chief, Children's National Health System
- Angela Delaney Freedman, MD, Office of the Clinical Director, NICHD
- Rachel Gafni, MD, Craniofacial and Skeletal Diseases Branch, NIDCR
- Margaret Keil, MS, CRNP, Director, Pediatric Endocrine Clinical Services, NICHD

(continued)

week and inpatient pediatric endocrine consultation on an on-call basis for three months per year. Fellows learn how to develop a research protocol, conduct a study, evaluate the results, and create a presentation or 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 or COMLEX 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 Youn Hee Jee, MD, Senior Fellow, NICHD

- Deborah Merke, MD, Chief of Pediatric Services Clinical Center, NIH
- James Mills, MD, Senior Investigator, Division of Epidemiology, Statistics, and Prevention Research, NICHD
- Kristina Rother, MD, Head, Section on Pediatric Diabetes and Metabolism, NIDDK
- Priya Vaidyanathan, MD, Site Director, Endocrinology, Children's National Health System, Washington, DC
- Fetima Worthington, *Program Coordinator, NICHD*
- Jack Yanovski, MD, PhD, Head, Section on Growth and Obesity, NICHD

August 31st, and interviews are conducted from September through November. Applicants must register with both NRMP and ERAS (<u>http://www.nrmp.org</u>, <u>https://www.aamc.org</u>).

Contact

Miranda Broadney, MD, MPH Acting Program Director <u>Miranda.Broadney@nih.gov</u> tel: 301-594-1176 Ms. Fetima Benjamin Program Coordinator <u>fetima.benjamin@nih.gov</u> tel: 301-451-1466

Pediatric Endocrinology Training Program NICHD, NIH Building 10, Room 2-5142 10 Center Drive Bethesda, MD 20892-23330

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 academic, government, and military institutions, and in order to establish and maintain high standards of training for students and residents in obstetrics and gynecology and to provide evidencebased, 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 Military Medical Center and the Shady Grove Fertility Center. Research is strongly emphasized. Trainees in the program must complete a thesis project and may choose among any research laboratory in the Institute's intramural research program. To complete



- Alan H. DeCherney, MD, Deputy Director, Reproductive Endocrinology and Infertility Fellowship Program
- Micah J. Hill, DO, Program Director of the Combined NICHD/Federal Fellowship in Reproductive Endocrinology
- Lauren A. Bishop, MD, *Clinical Fellow*
- Olivia J. Carpinello, MD, *Clinical Fellow*
- Anthony M. DeAngelis, MD, PhD, *Clinical Fellow*
- Nicole Doyle, MD, PhD, *Clinical Fellow*

Michael B. Evans, DO, *Clinical Fellow* Anne E. Martini, DO, *Clinical Fellow* Toral Parikh, MD, *Clinical Fellow* Justin Pilgrim, DO, *Clinical Fellow* Jessica R. Zolton, DO, *Clinical Fellow*



Reproductive Endocrinology and Infertility Fellows

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 the Shady Grove Fertility Center. Experience in 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 synergism 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 per year 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. Fellows also attend weekly research conferences sponsored by the NICHD and present updates on thesis work at the weekly "Research in Progress Conference." Core training objectives of the Accreditation Council for Graduate Medical Education (ACGME) 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

- 1. Zolton JR, Parikh TP, Hickstein DD, Holland SM, Hill MJ, DeCherney AH, Wolff EF. Oocyte cryopreservation for women with GATA2 deficiency. *J Assist Reprod Genet* 2018 35:1201-1207.
- 2. Hill MJ, Healy MW, Richter KS, Parikh TP, Devine K, DeCherney AH, Levy M, Widra EA, et al. Defining thresholds for abnormal premature progesterone levels during ovarian stimulation for assisted reproduction technologies. *Fertil Steril* 2018 110:671–679.e2.
- 3. Healy MW, Yamasaki M, Patounakis G, Richter KS, Devine K, DeCherney AH, Hill M. The slow growing embryo and premature progesterone elevation: compounding factors for embryo-endometrial asynchrony. *Hum Reprod* 2017 32:362-367.
- 4. Hill MJ, Royster GD, Taneja M, Healy MW, Zarek SM, Christy AY, DeCherney AH, Widra E, Devine K. Does elevated progesterone on day of oocyte maturation play a role in the racial disparities in IVF outcomes? *Reprod Biomed Online* 2017 34:154-161.
- 5. Heitmann RJ, Hill MJ, Csokmay JM, Pilgrim J, DeCherney AH, Deering S. Embryo transfer simulation improves pregnancy rates and decreases time to proficiency in the Reproductive Endocrinology and Infertility fellow embryo transfers. *Fertil Steril* 2017 107:1166-1172.

Collaborators

- William H. Catherino, MD, PhD, Uniformed Services University of the Health Sciences, Bethesda, MD
- John M. Csokmay, III, MD, Walter Reed National Military Medical Center, Bethesda, MD
- Saioa Torrealday, MD, Walter Reed National Military Medical Center, Bethesda, MD
- Eric A. Widra, MD, Shady Grove Fertility Center, Washington, DC

Contact

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Phone: 301-402-2141 Fax: 301-451-2857

Program Director: Micah J. Hill, DO; <u>hillmicah@mail.nih.gov</u> Program Coordinator: Marilyn Minor; <u>minormar@mail.nih.gov</u>

Online Application: https://students-residents.aamc.org/training-residency-fellowship/applying-fellowships-eras/

MANAGEMENT

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.

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.



Francie Kitzmiller, Chief, Administrative Management Branch Becky Preston, Deputy Branch Chief Valerie Leftwood, Deputy Branch Chief

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.

Contact

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AMB Staff

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Vincent Black, *Property Technician* Moona Braxton, *Property Technician*

Steve Norris, Space Analyst Antoinette Chavez, Administrative Technician Shamera Wallace, Administrative Technician John Burton, Purchasing Agent Jax Chang, Purchasing Agent Towanda Daniels, Purchasing Agent Roshonna Davis, *Purchasing Agent* William Davis, Purchasing Agent Jodi Ferrell, Purchasing Agent Rebecca Greenridge, Purchasing Agent Sherry Jones, Purchasing Agent Brittany May, *Purchasing Agent* David Shen, Purchasing Agent Hanumanth Vishnuvajjala, Purchasing Agent James Law, Procurement Technician Jennifer Smith, Procurement Technician Tamika Morgan, Facilities Assistant

Research Animal Management Branch

The Research Animal Management Branch (RAMB) supports animal use research in the NICHD Division of Intramural Research.

The RAMB operates and manages the Building 6B Shared Animal Facility (SAF), Suite 6C127 of the Ambulatory Care Research Facility (ACRF) Animal Facility, and the NICHD aquatics facilities. The DIR Animal Program and NICHD Animal Care Use Committee (ACUC) have oversight over these facilities with regard to animal use as well as over NICHD 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 NIH Institutes 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 includes a room for cavefish.

The NIH Animal Center (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 m² (1,299 ft²) 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



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- Ruth A. Woodward, DVM, Deputy Animal Program Director; Facility Veterinarian, NIHAC Shared Animal Facility
- Tannia S. Clark, DVM, Facility Veterinarian, 6B Shared Animal Facility/6 Shared ZF Facility
- Daniel T. Abebe, MS, *Research Technician*
- Julie A. Jacobs, Facility Manager, NIHAC Shared Animal Facility
- Christopher M. Rishell, BS, Facility Manager, 6B Shared Animal Facility
- Lauren Pandolfo, MS, Aquatics Facility Manager
- Klara Post, MS, Animal Care and Use Committee Coordinator

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.

Contact

For more information, email <u>schechj@mail.nih.gov</u> or visit <u>http://www.animalcare.nichd.nih.gov</u>.

CORE FACILITIES

NICHD Biomedical Mass Spectrometry Core Facility

The NICHD Biomedical Mass Spectrometry Core Facility was created 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 posttranslational 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 room 8S-261 of Building 10 on the NIH campus and 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 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 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.

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 Facility's staff are available for consultation on both project design and data interpretation. Staff members meet with the PI and other scientists involved in each study to discuss experimental goals and data requirements. The Facility has capabilities 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-



Peter S. Backlund, PhD, Staff Scientist, Acting Director Alfred L. Yergey, PhD, Scientist Emeritus

translational modifications, including phosphorylation, glutamylation, AMPylation, and disulfide bond formation; (4) quantification of specific post-translational modifications; and (5) *de novo* sequencing of peptides. 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 positiveor 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 (ultra-performance liquid chromatography) system with either an ESI (electrospray ionization) or APCI (atmospheric pressure chemical ionization) 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 was to investigate betacyclodextrin-cholesterol complexes in the presence of various 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. We also determined the reduced mobility (Ko) and CCS values for a group of analyte ions that had been previously characterized in other drift tube IM-MS instruments. In addition, we determined CCS values for both positively and negatively charged ions of cyclodextrins and maltodextrose [Reference 1]. The instrument was used to detect structural differences between two commercial preparations of hydroxypropyl-modified beta-cyclodextrins [Reference 2]. One of these preparations is currently being used in clinical trials to treat Neimann-Pick disease type C1 patients.

We are currently using IM-MS 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. The muscle PC species profiles under the phytol and control diet were similar, except that some additional species were detected in the phytoldiet 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.

The formation of PC complexes was also demonstrated using a mixture of two purified PCs, and we observed clusters of doubly charged positive ions ranging from trimers to octamers. The CCS values of the hepta- and octameric clusters show a significant departure from the trend line exhibited for trimers through hexamers, indicating a possible change in complex structure that may represent a transition to lipid self-organization.

ASSAY FOR QUANTITATION OF PHOSPHATIDYL-INOSITOL MONO-, DI- AND TRI-PHOSPHATES

We implemented an LC-MRM (multiple reaction monitoring) assay method to measure endogenous levels of phosphatidyl inositol phosphates in tissues and virus particles. The method is being used to quantitate levels of these compounds in brain tissues and influenza virus particles.

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

We developed an 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 monkeys, 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 elevations in *N*-acetyltryptamine at night to concentrations that exceed those of melatonin. We also used the technique to measure the 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 the tryptophan metabolite plays a significant physiological role as an endocrine or paracrine chrono-biotic through actions mediated by the melatonin receptor [Reference 3].

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. The assay also measures etiocholanolone, androsterone, and testosterone. More recently, we developed an MRM-based assay for quantification of glucocorticoids in serum, including cortisol, cortisone, 11-deoxycortisol, and corticosterone.

Publications

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CORE FACILITIES

The NICHD Zebrafish Core

The NICHD Zebrafish Core was established in May 2012 with the goal of providing its clients with consultation, access to equipment and reagents, and service in the area of zebrafish genetics. NICHD investigators as well as investigators from other NIH institutes and from outside the NIH are its clientele. The oversight committee for the Core comprises Harold Burgess, Ajay Chitnis, Brant Weinstein, and Katie Drerup. The Core's activities consist of (1) oversight and support of client-specific projects, (2) custom generation of genetic zebrafish models, (3) troubleshooting of new methodologies with promising application in zebrafish, (4) maintenance and improvement of equipment and infrastructure, and (5) service and educational outreach.

Oversight and support of client-specific projects

Over 2017–18, the Core engaged in research projects with seven labs.

Porter Lab (NICHD): Genetic Dissection and Creation of Human Disease Models of Sterol Metabolism. Niemann-Pick disease type C is a rare autosomal recessive disease caused by the accumulation of cholesterol and glycolipids in late endosomes/lysosomes. In 2018, we published a phenotypic characterization and proof-of-principle drugscreening approach for zebrafish carrying mutant alleles of *npc1*, the zebrafish ortholog to the human Niemann-Pick Type C disease gene *NPC1*, with one of these alleles having been generated by the Core in previous years. The Core had previously used CRISPR-Cas9 technology to create additional genetic mutant lines for the Porter lab in genes with roles in other steps of cholesterol metabolism, such as *dhcr7*, the zebrafish ortholog to the human Smith-Lemli-Opitz syndrome gene. Phenotypic characterization of these mutant lines 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 arises as a result of excess growth hormone (GH) secretion during childhood, before the growth plates close. Since 2012, the Core has supported this lab's investigation into the zebrafish ortholog of a human gene implicated as a driver of gigantism. The Core used CRISPR-Cas9 methods in previous years to generate



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

Yvonne Rosario, PhD, Postdoctoral Intramural Research Training Award Fellow



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

zebrafish carrying loss-of-function mutations in four zebrafish orthologs to human genes implicated by the Stratakis lab in human growth anomalies. Characterization of the resulting phenotypes is ongoing. (2) The Core also continues to support the Stratakis lab's investigation into the function of two zebrafish orthologs to human adrenal hyperplasia and Cushing disease–associated genes through the study of mutant lines that the Core had previously helped to generate or acquire. Phenotypic characterization found notable effects on juvenile growth in the case of one gene and on early embryogenesis in the case of the other. Phenotypic characterization of these lines will continue into 2018–19. Last year, we generated mutants for six new genes whose human orthologs are implicated in adrenal hyperplasia and/or Cushing disease, and we have begun phenotypic characterization of these mutations.

Kaler Lab (NICHD): Modeling Copper Deficiency-Associated Distal Motoneuropathy. Complete loss of the copper-binding ATPase ATP7A causes a severe human disease leading to childhood death. Two *ATP7A* missense mutations cause a milder syndrome, distal motoneuropathy, that is nevertheless debilitating in children and young adults. Since 2013, the Core has supported a project to clarify the structure-function relationship of ATP7A and motor-neuron defects from the perspective of these missense mutations. In early 2018, using CRISPR-Cas9 technology in combination with donor DNA, the Core successfully created a zebrafish cognate to one of these alleles. Initial phenotype analysis indicates an intermediate phenotype characterized by hypopigmentation, but no obvious diminishment of motor function within the first four months of life. The scope of the Directors Award includes creation of the second *Atp7a* allele, and preliminary results indicate a degree of success.

Marini lab (NICHD): see Precise Genome Editing below.

Blackshear lab (NIEHS): Assessing Functions of a Zinc-Finger Protein Gene Family in Zebrafish. The Blackshear lab is interested in dissecting the connections between a family of zinc-finger proteins and blood development, requesting that we create null mutations in seven zebrafish orthologs and assist with
preliminary phenotype characterization. Last year, we created null alleles for six out of the seven genes, and alleles for the seventh were obtained this year. Phenotypes for all alleles tested have proven to be weak or absent, and the Blackshear lab is now comparing the transcriptomes of mutants versus control siblings in case subtle molecular consequences of these gene disruptions can illuminate their function.

Meilleur lab (NINR): Testing the Ability of Small Molecules to Mitigate Myopathy in Zebrafish *ryr1b* Mutants. This year, Meilleur and colleagues began to test candidate drugs for their ability to potentially ameliorate muscle defects seen in zebrafish mutants that carry mutations in the *ryr1b* gene; mutations in its human counterpart are implicated in various myopathies. Last year, we acquired larvae carrying the *ryr1b* mutation from an outside source and raised them to adulthood. This year, we developed a behavioral assay using the Viewpoint Zebrabox system, which can identify *ryr1b* homozygous mutant larvae by virtue of their reduced movement over time. Three drugs were tested for their ability to ameliorate this movement deficit, establishing proof-of-principle confirmation that the system can be used to screen candidate drugs. We also developed strategies for pre-selecting equal numbers of wild-type and mutant siblings in advance of these assays, thereby increasing the efficiency of the screening pipeline.

Caldovic Lab (Children's National Medical Center): Finding Neuroprotective Drugs to Mitigate Hyperammonemia, a Consequence of Urea Cycle Defects and Liver Failure. Exposure of the brain to high ammonia levels causes neuro-cognitive deficits, intellectual disabilities, coma, and death. Since 2012, the Core has helped this lab to use zebrafish embryos to identify small molecules that are able to diminish the effects of hyperammonemia. In the initial few 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. A manuscript summarizing this work is currently being drafted. Over the last two years, the Core has supported a re-implementation of this screen, using a larger library of 10,000 compounds, bolstered by additional personnel from the Caldovic lab, and the Core's implementation of NICHD's massive embryo production systems as a source for embryos. Additional candidate compounds were thus identified, and ongoing secondary screens and dose-response studies on lead compounds will continue in 2018–19.

Basic gene knockouts

The Core continues to offer the creation of at least two novel CRISPR/Cas9 frame-shifting alleles per gene on a fee-for-service basis, and we have been able to create mutations in all genes requested. Demand was lower this year, likely because laboratories are still characterizing the mutant lines that we previously made for them.

Precise genome editing

In June 2018, the Core received supplemental Directors Award funding for a Postdoctoral position and consumables to compare various precise genome-editing methods and efficiencies for creating targeted point mutations that are cognates to known human disease-associated point mutations across several loci, using NextGen sequencing of microinjected embryos to quickly ascertain which methods work best for which loci. Between June and October 2018, we advertised the position and hired Yvonne Rosario and initiated her training. We also acquired reagents and designed detailed strategies to compare Cas9 vs. Cpf1 mutagenesis, and oligo- vs. plasmid-based templates vs. base editing. The planned point mutations include (1) two zebrafish *npc1* alleles for the Porter lab that are cognate to disease-causing alleles in humans and may potentially be uses to test drug therapies for Niemann-Pick disease; and (2) an amino-acid substitution

in the zebrafish *ifitm5* gene for the Marini lab that will be a cognate to the human the IFITM5S40L substitution known to cause type VI spectrum osteogenesis imperfecta that is normally associated with mutations in *PEDF*. The project aims to provide an inroad to understanding the potential link between IFTM5 and PEDF functions.

We continue to regularly meet and discuss progress with the laboratories of Raman Sood (NHGRI Zebrafish Core) and Shawn Burgess (NHGRI) to ensure that efforts in optimizing precise genome editing strategies are not redundant.

A 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. This year, we dedicated both MEPS to the TAB5 strain, which is better suited for precise genome-editing projects, and we have optimized collection to one day per week for each of two MEPS. The availability of these embryos has been useful to other NIH labs, and we have accordingly established a protocol for requesting TAB5 embryos.

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. 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. To establish a system to measure size over time without handling fish, we previously developed a first version of automated size-measurement software and, this year, we initiated a second round of software improvements. This year, we also designed and fabricated a system for rearing zebrafish individually during their first two months of larval and juvenile growth. Combining this hardware and software in the current research year is anticipated to finally allow direct comparisons of growth curves for individual wild-type and mutant zebrafish with a minimum of investigator effort.

Additional Funding

- NICHD Customers: \$12,375 in fee-for-use charges
- Non-NICHD Customers: \$10,800 in fee-for-use charges
- Director's Award: \$68,247 for year 1 of 2

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 (MGC) Facility supports NICHD investigators by providing next-generation deep sequencing and project data analysis.

Next-Generation sequencing and bioinformatics support

The MGC provides DNA and RNA sequencing services for genomic and genetic research to investigators within the NICHD. The MGC is currently operating with four sequencing machines. Most of our work is conducted on our high-capacity, production-scale machine: an Illumina HiSeg 2500. Two of the other sequencers, an Illumina MiSeg and an Ion Torrent Personal Genomics Machine, are smaller, faster machines, which can generate longer sequence reads of up to 400 base pairs. Our most-recent acquisition is a Pacific Biosciences (PacBio) Sequel, which can sequence long single molecules of more the 50,000 base pairs. To increase the throughput of the HiSeq 2500 even further, we have a cBot liquid handler for automated highprecision loading of sequencing chips. Our array of sequencers provides a suite of scales and capabilities. Our sequencing services include whole-genome, whole-exome, targeted exome, and genespecific DNA sequencing, as well as whole-transcriptome sequencing (RNA-Seq), microRNA sequencing, microbiome sequencing, bisulfite sequencing (DNA methylome), ChIP-Seq, and ribosomal profiling. The PacBio Sequel enables mutation phasing, structural variant analysis, transposon location identification, and other analyses that are not possible or practical with the other sequencers.

Recently, 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 into 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



Forbes D. Porter, MD, PhD, Director, Molecular Genomics Core Facility Steven L. Coon, PhD, Staff Scientist James R. Iben, PhD, Staff Scientist Tianwei Li, PhD, Staff Scientist Joseph Zoeller, BS, Postbaccalaureate Intramural Research Training Award Fellow sequencing for 97 projects (1,266 samples) across the full spectrum of sequencing types, generating 8,490 gigabases of sequence; the projects involved 32 NICHD Principal Investigators from 10 Affinity Groups. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support to 15 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 that promotes understanding of human health and development.

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- 3. Brown JL, Sun M, Kassis JA. Global changes of H3K27me3 domains and Polycomb group protein distribution in the absence of recruiters Spps or Pho. *Proc Natl Acad Sci USA* 2018 115(8):E1839-E1848.
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CORE FACILITIES

NICHD Microscopy and Imaging Core

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. The Facility is operated as a 'one-stop shop' where investigators can, with a minimum of efforts, go from their scientific question to the final data.

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 (<u>https://next.cirklo.org/nichd</u>). The MIC is available free of charge to all NICHD investigators and, resources allowing, to anyone within the Porter building.

Vincent Schram is the point person for light microscopy and image analysis. The EM branch of the Facility is staffed by Chip Dye, and Lynne Holtzclaw is in charge of sample preparation (histology). Chip Dye and Lynne Holtzclaw report directly to Vincent Schram, who serves as interim director under the management of Chris McBain (NICHD). Tamás Balla (NICHD) acts as scientific advisor for the Facility.

Vincent Schram has a bilateral agreement with Carolyn Smith, who manages the NINDS confocal Facility (LIF), also in the Porter building. Both Facilities freely exchange users, equipment, and support. Although this mode of operation was never codified officially, it is greatly 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.

Light microscopy

The Facility is well supported by the Office of the Scientific Director. The MIC is equipped with six modern confocal microscopes, each optimized for certain 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 highspeed confocal imaging or selective recording of membrane-bound



Vincent Schram, PhD, Staff Scientist Louis (Chip) Dye, BS, Research Assistant Lynne A. Holtzclaw, BS, Research Assistant events in live cells; (4) a Zeiss LSM 880 2-photon confocal for thick tissues and live animals. In addition, the Core recently received two new confocal systems: (5) a Zeiss 800 optimized for advanced tiling experiments; and (6) a Zeiss 880 Airy, which offers twice the spatial resolution of conventional microscopes without using special dyes or protocols.

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), and large specimens. High-end computer workstations with imaging software (Zeiss Zen, Nikon Element, Bitplane Imaris, SVI Hyugens, and ImageJ) are also available.

After an initial orientation, during which their project is researched by the staff and the best approach is decided upon, users receive hands-on training on the equipment and/or for software best suited to their goals, followed by continuous support when required. Once image acquisition is complete, the staff devise solutions and train users on how to extract usable data from their images. 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) an extensive yearly workshop covering light and electron microscopy, image analysis and sample processing; (3) MIC staff volunteer time to teach FAES classes; (4) the Facility organizes frequent on-campus demonstrations of new instruments and software by vendors in a dedicated space. The equipment demonstrations are open to the entire NIH community.

The MIC has a total of 291 registered users in 65 laboratories. At 11,933 instrument hours, overall usage has grown since the last fiscal year, a figure that now includes the animal perfusion station and the JEOL electron microscope. 60% of usage comes from NICHD investigators, most within the Porter building, 20% for training, internal projects, and pilot experiments, and 20% from other Institutes, predominantly NINDS. Usage of each confocal system was uneven, with the Zeiss 710 and 780 being most heavily used. However, it is worth noting that the Zeiss 800 has only been in service since January 2018 and the 880 Airy since April 2018.

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 for the light microscopy section, where end users do their own processing. In the past 12 months, Chip Dye processed a total of 67 samples: 53 from NICHD investigators, 2 for MIC internal test projects, and 12 from other Institutes. While this number represents a reduction from last year's figure, it should be stressed it includes technically challenging and labor-intensive projects for Tamás Balla and Michaela Serpe. The JEOL 1400 electron microscope continues to be available on the calendar for trained users. After the MIC, Joshua Zimmerberg (NICHD) is the major user of that instrument.

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 dedicates a significant amount of time to training users in various techniques, such as rodent perfusion, cryopreservation, cryosectioning, immunofluorescence, and tissue clearing (she trained 21 users during the past 12 months). She processed samples and acquired images for Drs. Balla, Buonanno, Fields, Heuser, Hoffman, Klein, Le Pichon, Loh, Marini, Ozato, Petros,

Porter, Sackett, Stojilkovic, and Stopfer (NICHD). She has also provided training and services to Drs. Gordon, Mankodi, Youle (NINDS), Penzo, Plenz, Usdin (NIMH), Chen (NIBIB), and Danner (CC).

A collaborative project with David Klein to characterize pineal cell types for which genes of interest had already been documented by RNA-seq was completed in July 2018. The manuscript is currently being prepared for publication. Lynne Holtzclaw has also joined a collaborative effort with the NINDS laboratory of Richard Youle to study the accumulation of ubiquitinated protein aggregates in brain and liver of a TAX1BP1 knock-out mouse.

Collaborators

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- Richard Youle, PhD, Neurogenetics Branch, NINDS, Bethesda, MD

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For more information, email <u>schramv@mail.nih.gov</u> or visit <u>http://mic.nichd.nih.gov</u>.

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 five key areas: core IT support, clinical informatics, custom software development for scientific and administrative support, biological visualization services, and bioinformatics.

During 2018, the team migrated its applications and database to new hardware, leading to an increase in storage capacity and computing resources.

Core IT Services

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

NETWORK AND DESKTOP SERVICES

During the past year, the Core supported reliable, secure, and efficient information technology solutions. These include acquisition, maintenance, and support for licensed software used by our DIR research community; e.g., EndNote/Reference Manager, Bookends, GraphPad Prism, PyMol, network services (e-mail, data backups, VPN, helix, PDAs, wireless configurations) and crossplatform desktop, server, and application hosting in the Rock Spring and Bldg. 35 Data Centers. 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. Hosting these licenses permits users to leverage their research with additional tools available on Helix and Biowulf. We also assist users in identifying, researching, and purchasing custom hardware configurations to match research instrument requirements.

DATA-RECOVERY SERVICES

CSSC 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 \$2,500 savings per instance to the DIR research budget.



Ryan Dale, PhD, Scientific Information Officer Marco Crosby, *Program Engineer* Michelle Duverneau, IT Specialist John Czapary, BS, *Laboratory* Technical Manager Patrica Pullen, MBA, Project Manager Sean Ivusic, MS, Database Support Lead Asma Idriss, PMP, MS, Program Manager Kesa Koresko, MS, Senior DB/BI Specialist Loc Vu, BS, *Lead Software Engineer* Matt Breymaier, BS, Senior Bio/ Application Software Engineer Nareg Bakirci, MS, Website Developer Jeremy Swan, BA, Senior Biovisualization Specialist Nicki Swan, BA, Graphic Designer Raquel Gray, MBA, Team Lead Tamara Prodanov, MD, **Documentation Specialist** Audrey Harrell, MS, Information Specialist

(continued)

Clinical Informatics

The CSSC continued to expand, support and develop the Clinical Trials Database (CTDB) application and its auxiliary tools. CTDB allows researchers to custom design, collect, and report clinical data related to natural history and interval-based studies. The total number of protocols and research projects supported by the CTDB team increased to approximately 570 for 15 NIH institutes, with an expansion of research questions to over 225,000. The CTDB clinical research team also provide data management support to Principal Investigators (PIs) of the NICHD's Division of Intramural Research (DIR). George Tran, Information Specialist Rolanda Bailey, BS, Documentation Specialist Vida Bayat Mokhtari, MD, Information Specialist Kami Emanuel, Information Specialist Joo Kang, BS, Information Specialist Joelle Khoriaty, MBA, Documentation Specialist Orlando Ortega, MS, Documentation Specialist

Our software development group completed one release that included features for electronic data upload and performance

improvements of a QA module. Our database development and reporting team continued to migrate protocol data from various NIH institutes, while supplying reporting for protocols within CTDB. New datamart and reporting functionality added to the CTDB datamart allows users to combine and retrieve additional information and data related to their research. While already providing encryption-atrest, the CTDB project interface to NIH's Biomedical Translational Research Information System (BTRIS) was upgraded to ensure that encryption-on-the-wire security policies were followed. We also supported the Clinical Trial Survey System (CTSS), an application for patient surveys, used for 165 active protocols.

Custom Software Development for Scientific and Administrative Support

The CSSC provides custom software development for the DIR scientific and administrative community.

We continued to enhance the Manuscript Tracking System (MTrac), a web-based application that automates the clearance and approval process for manuscripts in the DIR.

The DIRweb application supports several activities: the NICHD annual report, PI and Fellows retreats, Training tracking, Fellows progress report, and Administrative Management Branch (AMB) personnel and travel package tracking.

The team continued to release enhancements to the Fellows Annual Progress Report (APR); a unified means for tracking and mentoring intramural trainees along with easing the re-appointment process. Success with the Fellows APR has garnered interest from various other institutes, and plans to implement a scalable and cost-effective inter-institute service are under way. This solution provided the Office of Education with useful metrics regarding mentoring and training programs. An Exit Survey feature was also added for DIR Fellows: a short survey allowing DIR trainees a platform for providing feedback.

We developed a new Package Tracking module for the DIR AMB, granting AMB staff real-time accuracy metrics for personnel and travel package compilation.

The CSSC team continued working on Cost Tracker, an application that permits capturing, organizing, and

reporting of various expenses on a per-protocol basis. The work is done closely with the Office of the Clinical Director to improve protocol cost vs. effectiveness and a protocol-cost estimator module.

Biological visualization services

The CSSC team provided DIR laboratories with scientific communications and media services, including publication support, website support, audio/visual production, and print media.

Those services were provided to: The NICHD Annual Report, the DIR Annual Fellows and Scientific Retreats, the NIH Research Festival, the NICHD Exchange lecture series, and NICHD research labs and medical training programs, including the Pediatric Endocrinology Training Program and Inter-Institute Adult Endocrinology training program. Support services included recording audio and video of presenters, producing video abstracts to accompany publications, and offering professional photography. Moreover, the team produced several scientific figures in support of publications.

We supported the NICHD Office of Education by producing a monthly newsletter, <u>*The NICHD Connection*</u>, in collaboration with Intramural Fellows. We created print collateral for conferences including the NICHD Scientific Retreat, NICHD Fellows Retreat, and the MEN-1 symposium, as well as recruitment materials for NICHDs clinical training programs. We continued to facilitate the use of new technologies through demos, we helped coordinate several Virtual Reality demonstrations at the Intramural Research Festival, and we provided co-leadership of the Virtual and Augmented Reality Interest Group.

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. The CSSC continued to provide a platform for conducting scientific review by the Board of Scientific Counselors, administrative intranet support and business operations, in addition to public-facing laboratory websites through a Confluence wiki known as Science NICHD (*http://science.nichd.nih.gov*).

Bioinformatics

During the past year, the bioinformatics team assisted the new Molecular Genetics Laboratory with data collection and storage for high-throughput sequencing. The scientific informatics group also develops research tools to assist investigators with genomic data management and analysis. A high-performance computing (HPC) cluster was used to assist with genomics computational requirements. The Genomics workbench Galaxy was installed and configured on the HPC to make use of the parallel processing capabilities. The CSSC maintains and adds features to a web application, which allows researchers to search for and visualize RNA-seq data compiled by the Section on Neuroendocrinology. The CSSC also continues to provide and manage dozens of terabytes of storage to support genomic research.

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, NIMHD, and NIDDK.

Publications

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Collaborators

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- Peter Schmidt, MD, Behavioral Endocrinology Branch, NIMH, Bethesda, MD
- Steven Stanhope, PhD, University of Delaware, Newark, DE
- Constantine Stratakis, MD, D(med)Sci, Section on Endocrinology and Genetics, NICHD, Bethesda, MD
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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 Ajay Chitnis Katie Drerup Ben Feldman (Core) Brant Weinstein

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

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 Mihaela Serpe Yun-Bo Shi

Storz

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) Miranda Broadney (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 Pedro Rocha

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 novel *in vivo* microstructural and functional MRI methods designed to measure salient properties of the developing brain and assess and characterize their changes in diseases and disorders. These novel quantitative imaging biomarkers are also used in neuroscience application to characterize brain network connectivity and dynamics, as well as brain tissue architectural organization.

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, socialemotional, 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	Deborah Merke	Forbes Porter
Janice Chou	Anil Mukherjee	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

Every biochemical process in a eukaryotic cell relies on a molecular information flow that leads from receptors that inform the cell about its environment all the way to the molecular effectors that determine the appropriate cellular response. Proper information transmission requires a high degree of organization, where the molecular players are organized into different cellular compartments so that the specificity of the cellular response can be properly maintained. Breakdown of this organization is the ultimate cause of all human diseases, even if the affected molecular pathways differ according to the type of disease, such as cancer, diabetes or neurodegenerative diseases—just to name a few.

Research described in this report has focused on the question of how cells organize their internal membranes to provide a structural framework on which molecular signaling complexes assemble to ensure proper information processing. These cellular processes are often targeted by cellular pathogens, such as viruses, to force the cells to produce the pathogen instead of performing the cell's normal functions. Better understanding of such processes can not only provide new strategies to fight various human diseases but also intercept the life cycle of cellular pathogens, thus offering an alternative to antimicrobial drugs.

Phospholipid transport controlled by ORP5/8 proteins at plasma membrane-ER contact sites

The project addresses the role of non-vesicular lipid transport at contact sites formed between the plasma membrane (PM) and the endoplasmic reticulum (ER) in maintaining proper lipid composition and signaling at the PM. In the first series of studies, we characterized the roles of two lipid-transport proteins, oxysterol binding protein–like protein 5 and –8 (ORP5 and ORP8) in the transport of phosphatidylserine (PS) from the ER to the PM. The proteins use a phosphatidylinositol 4-phosphate (PI4P) gradient between the PM (high PI4P) and the ER (low PI4P) to support the transport of PS from the ER to the PM. The PI4P gradient between the PM and ER is maintained by the production of PI4P in the PM by the lipid kinase phosphatidylinositol 4-kinase alpha (PI4KA) and



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elimination of PI4P in the ER by the Sac1 lipid phosphatase enzyme. PI4P is a minor phospholipid, produced by four different PI4P-kinase enzymes, and has important functions in the cells, as it recruits and organizes protein complexes in endocytic membranes, such as in the Golgi and the late endosomes, but it is also a precursor of $PI(4,5)P_2$, one of the most important PM phosphoinositide lipids. The transport of PI4P from the PM to the ER by the ORP5/8 proteins represents a divergent pathway, i.e., in competition with the canonical route of PI4P conversion to $PI(4,5)P_2$.

In our studies, we found that PI4P transport by the ORP5/8 proteins from the PM to ER is, in fact, regulated by the $PI(4,5)P_2$ content of the PM. We showed that, under "normal" conditions, ORP5 is the major component of this PI4P transport process, but that its transport function is switched off when either PI4P or $PI(4,5)P_2$ levels are lowered in the PM. Such regulation is achieved through the N-terminal pleckstrin homology (PH) domain of ORP5, which provides contact with the PM and which requires both PI4P and $PI(4,5)P_2$ for proper PM interaction. Without this interaction, ORP5 is unable to transport lipids between the PM and the ER. The mechanism is a safeguard for the cells to maintain $PI(4,5)P_2$ levels in the PM. We also discovered that ORP8, which under normal conditions is a bystander, is recruited to the PM when $PI(4,5)P_2$ levels are elevated. Under these conditions, the transport of PI4P from the PM to the ER is increased, thereby reducing the availability of PI4P for $PI(4,5)P_2$ synthesis and thereby protecting cells from excess $PI(4,5)P_2$ in the PM. Through this rheostat mechanism, ORP5 and ORP8 play important roles not only in PS transport but also in maintaining a strict control over PM $PI(4,5)P_2$ levels.

The role of inositol lipids in peripheral nerve myelination

In the second project, we applied our knowledge on the role of PI4KA as a main controller of PM–ER lipid transfer to a whole animal model and studied the importance of these processes in peripheral nerve myelination in mice. Myelination is a process in which long neuronal processes are wrapped in a series of PM sheets, provided by Schwann cells, in the peripheral nerves. Several human diseases present with myelination defects, and a better understanding of the process can help us design strategies to cure or alleviate these pathologic conditions. As pointed out earlier, the PI4KA enzyme is crucial for maintaining the PI4P gradient between the PM and the ER. We genetically inactivated the PI4KA in mice, specifically in Schwann cells, and studied its effect on the myelination process. We found that mice with the PI4KA deletion in Schwann cells (referred to as mutants) show progressive loss of hind-leg function, noticeable from 30 days of age. The mice suffer from a severe myelination defect with substantially reduced myelin thickness and greatly reduced lipid content, most severely affecting phosphatidylserine (PS) and phosphatidylethanolamine (PE). Surprisingly, mouse Schwann cells kept in culture retain their PM PI(4,5)P₂ levels as well as their Akt (anti-apoptotic serine/threonine protein kinase, activator of mTORC1) and mTORC1 (controller of protein synthesis) responses even after prolonged PI4KA inhibition, in spite of the massive reduction in their PM PI4P levels.

The Akt and mTOR responses are a faithful reflection of the signaling initiated by $PI(3,4,5)P_3$, a lipid that is made by PI 3-kinases from $PI(4,5)P_2$. When $PI(4,5)P_2$ levels fall, it would be expected that $PI(3,4,5)P_3$ signaling would be impaired. So the intact mTOR and Akt responses are further confirmations that there is no impairment of this signaling angle, consistent with the unchanged $PI(4,5)P_2$ levels. This is important because one would expect that, if plasma membrane PI4P lipid levels fall (as is the case after PI4KA inhibition or inactivation), the lipid $PI(4,5)P_2$ would also decrease, given that it is made from PI4P. In contrast, PI4P depletion from the PM causes massive rearrangements of the actin cytoskeleton both in cultured cells and in the nerves of affected animals. Our studies highlight the central role of PI4KA in the myelination process and show that the role of the enzyme is more closely linked to actin dynamics and PS metabolism than to $PI(3,4,5)P_3$ -mediated signaling cascades.

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- NSERC Postdoctoral Fellowship supporting Dr. Joshua Pemberton
- Natural Sciences & Engineering Research Council of Canada (NSERC) Banting Postdoctoral Fellowship supporting Dr. Joshua Pemberton
- JSPS-NIH Fellowship supporting Dr. Takashi Baba

Publications

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Structural and Chemical Biology of Membrane Proteins

Molecular mechanism of posttranslational 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, which 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 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 is mutated in about one-third of all human cancers, is palmitoylated at the Golgi and subsequently targeted to the plasma membrane by vesicular transport. Palmitoylated Ras localizes to cholesterol-rich domains on the plasma membrane. However, it is subsequently depalmitoylated by the thioesterase APT1, dissociates from the plasma membrane, and redistributes on endomembranes, including the Golgi. Such dynamic recycling of Ras is critical for its function.

Protein *S*-acylation is catalyzed by a large group of enzymes known as DHHC–<u>p</u>almitoyl <u>a</u>cyl<u>t</u>ransferase (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 up to 24 putative DHHC–PATs encoded in their genome. Beyond the shared DHHC domain, DHHC–PATs vary considerably; some possess ankyrin repeats (structural protein motifs that mediate proteinprotein interactions), a few have six transmembrane helices instead of the usual 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



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FIGURE 1. Organization and properties of DHHC palmitoyltransferases (PATs)

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-PATs 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).

DHHC-PAT can palmitoylate many substrates, and, conversely, a given substrate can be palmitoylated by many DHHC-PATs. Such 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.

Besides its broad importance in cell biology, palmitoylation has been linked to several diseases, most notably neuro-psychiatric 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 coenzyme A (CoA) that serves as the acyl donor.

In a major breakthrough in this field, we solved the high-resolution crystal structures of two members of the DHHC family, human DHHC20 and zebrafish DHHC15 (Figure 3a). They are the first structures of any member of this family to be characterized and reveal a tepee-like transmembrane domain organization, which splays apart towards the cytoplasmic side and harbors the active site at the membrane-aqueous interfacial region (Figure 3b), thus readily explaining why membrane-proximal cysteines are palmitoylated. The structures also revealed that the tightly bound Zn²⁺ ions do not actively take part in catalysis but serve as structural Zn²⁺ ions. The C-terminus of DHHC–PATs is the least conserved overall but has two important conserved sequence motifs, and the structures show how these form critical points of contact with the DHHC–CRD (cysteine-rich domain) domain. Unexpectedly, the C-terminus has an amphipathic helix followed by a



FIGURE 2. Mitoferrin and mitochondrial iron transport

a. Iron is imported through the plasma membrane by the Transferrin/Transferrin Receptor (*blue*) cycle and is transported out of endosomes by divalent metal ion transporter (DMT) (*grey*). The iron is delivered to Mitoferrin (*yellow cylinders*) by unknown means. Mitoferrin delivers iron to unknown partners in mitochondria, which become available for heme and Fe-S cluster biosynthesis.

b. Schematic depiction of the simplest transporter cycle and the different putative states of Mitoferrin involved.

c. Assay of iron transport activity by TMfrn1, a form of Mitoferrin 1. Representative PGSK (fluorescent reporter of iron) quenching curves upon addition of iron to TMfrn1 proteoliposome. Red traces are increasing concentrations of iron. Black dotted traces show protein-free liposomes.

hydrophobic loop, which insert hydrophobic residues into the membrane and straddle the transmembrane domain, thus forming a supporting structure.

We also solved the structure of human DHHC20 irreversibly modified by a covalent inhibitor, 2-bromopalmitate. The structure mimics the auto-acylated intermediate state in the enzymatic pathway and thus reveals how the acyl group of fatty acyl–CoA binds in a cavity formed in the bilayer by the transmembrane domain (Figure 3c). Residues lining the cavity contact the acyl chain, and mutation of these residues affects enzymatic activity. By mutating two residues at the tapering end of the cavity, we also showed that we can change the acyl chain length selectivity of the mutant enzymes (Figure 3d). Thus, the cavity functions as a molecular ruler in determining the acyl chain length selectivity of human DHHC20. This is important because, although palmitate is the most prevalent fatty acid used by DHHC palmitoyltransferases, they can use fatty acyl–CoAs of other chain lengths, and this property varies between different members of the DHHC family.

Molecular mechanism of iron transport into mitochondria

The importance of iron in biology cannot 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 (Figures 2a,b). Mitoferrin-1 (Mfrn1) is expressed mainly in erythroid cells, while mitoferrin-2 is expressed ubiquitously. Knockout of Mfrn1 is embryonically lethal,

FIGURE 3. Structure of DHHC palmitoyltransferases

a. The structure of human DHHC20 and a catalytically inactive mutant of zebrafish DHHC15 shown in ribbon trace. The transmembrane domain (TM) is shown in green, the DHHC– containing cysteine-rich domain in blue and the C-terminal domain in orange. The grey spheres indicate Zn²⁺ ions. These are both Golgi-resident enzymes and thus the top side faces the Golgi lumen and the active site the cytoplasm.

b. Active site of human DHHC20 showing the catalytic triad containing the active-site cysteine.

c. Structure of human DHHC20 irreversibly modified with 2-bromopalmitate, which results in the active-site cysteine linking to the alphacarbon of palmitic acid. The acyl group of palmitic acid is shown in stick rendition. Also shown are two residues towards the top of the tapering cavity to where the palmitate binds.



d. The acyl chain length selectivity of wild-type (WT) human DHHC20. Mutation of tyrosine181 to alanine (Y181A) expands the cavity and shifts the acyl selectivity to the longer side. On the other hand, mutation of serine29 to phenylalanine (S29F) contracts the cavity and thus shifts the acyl selectivity to the shorter side.

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 the 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. Such 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 (Figure 2c). 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.

Molecular mechanism of Porcupine, an integral membrane enzyme of the MBOAT family, which catalyzes Wnt lipidation

In metazoans, Wnt proteins regulate a large number of processes, including cellular growth, differentiation, and tissue homeostasis through the highly conserved Wht signaling pathway. Porcupine is an endoplasmic reticulum (ER)-resident integral membrane enzyme that catalyzes post-translational modification of Wnts with palmitoleic acid, an unsaturated lipid. This unique form of lipidation with palmitoleic acid is a vital step in the biogenesis and secretion of Wnt, and Porcupine inhibitors are currently in clinical trials for cancer treatment. However, Porcupine-mediated Wnt lipidation has not been reconstituted in vitro with purified enzyme. We recently reported the first successful purification of human Porcupine and confirmed, through in vitro reconstitution with the purified enzyme, that Porcupine is necessary and sufficient for Wnt acylation. By systematically examining a series of substrate variants, we showed that Porcupine intimately recognizes the local structure of Wnt around the site of acylation. Our *in vitro* assay enabled us to examine the activity of Porcupine with a range of fatty acyl-CoAs with varying length and unsaturation. The selectivity of human Porcupine across a spectrum of fatty acyl–CoAs suggested that the kink in the unsaturated acyl chain is a key determinant in Porcupine-mediated catalysis. We also showed that two putative Porcupine inhibitors, which were discovered with cell-based assays, indeed target human Porcupine. Together, these results provide several, high-resolution biochemical insights into the mechanism of Porcupine-mediated Wnt acylation and pave the way for further detailed biochemical and structural studies.

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PEDIATRIC ENDOCRINOLOGY, METABOLISM, AND GENETICS

Regulation of Childhood Growth

Children grow taller because their bones grow longer. Bone elongation occurs at the growth plate, a thin layer of cartilage found near the ends of juvenile bones. In growth plates, new cartilage is produced through chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis, and then newly formed cartilage is 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. 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. If the genetic defect affects tissues other than the growth plate cartilage, the child may present with a more complex syndrome that includes other clinical abnormalities.

Many 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 and molecular studies of growth plate biology suggest that there are hundreds of genes that control linear growth. Therefore, there are likely many genetic causes of linear growth disorders that remain to be discovered.

We investigate the cellular and molecular mechanisms governing childhood growth and development. We focus particularly on growth at the growth plate. 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.

Novel genetic causes of childhood growth disorders

To discover new genetic causes of childhood growth disorders, we invite families with monogenic growth disorders to the NIH Clinical



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FIGURE 1. Heterozygous Weaver mice showed postnatal overgrowth.

CRISPR/Cas9 genome editing was used to generate a mouse model carrying a Weaverassociated Ezh2 variant (V626M).

A. Crosses of heterozygotes generated mice that were wild-type, heterozygous, or homozygous for the Weaver variant, which was confirmed by PCR amplification of a genomic region containing the variant followed by Sanger sequencing.

B. Body weight was measured in heterozygous Weaver mice and wild-type littermates weekly up until 8 weeks of age (homozygotes were perinatally lethal). Two-way ANOVA showed that body weights differed significantly between sexes and genotypes. Pairwise comparison showed that, in both males and females, body weights were significantly increased in heterozygous Weaver mice.

C-H. At 8 weeks of age, heterozygote Weaver mice showed increased weights of heart, kidney, and spleen, but no differences in weights of liver and lung, or in tibia length, compared with wild-type mice.

Center, where we evaluate the clinical, biochemical, and radiological features of the condition. We then obtain DNA samples from informative family members and use powerful genetic approaches, including SNP (single-nucleotide polymorphism) arrays, to detect deletions, duplications, mosaicism, and uniparental disomy, combined with exome sequencing to detect single-nucleotide variants and small insertions/deletions in coding regions and splice sites. When sequence variants that are likely to cause the disorder are identified, we study in the laboratory the variants and the genes in which they occur to confirm that the variant is pathogenic, to elucidate the pathogenesis of the disorder, and to explore the role of the gene in normal growth.

These analysis have previously led to our studies of heterozygous mutations in *ACAN*, causing autosomal dominant short stature with advanced bone age and premature osteoarthritis [Reference 3; also Nilsson O et al., *J Clin Endocrinol Metab* 2014;99:E1510; and Gkourogianni A et al., 2017 *J Clin Endocrinol Metab* 2017;102:460] and to our study of biallelic mutations in BRF1 in children with growth failure, central nervous system abnormalities, and facial anomalies [Reference 4].

We also studied a subject with tall stature, advanced bone age, and mild dysmorphic features [Reference 1]. Exome sequencing revealed a *de novo* missense mutation in *EZH2*. *EZH2* encodes a histone methyltransferase that methylates histone 3 at lysine 27 (H3K27). The finding was consistent with previous reports that heterozygous missense mutations in *EZH2* cause Weaver syndrome, which is characterized by tall stature,



FIGURE 2. Homozygous and heterozygous Weaver mice showed decreased H3K27 methylation *in vivo*.

Immunostaining of E14.5 embryos was used to examine difference in histone methyltransferase activity in vivo. Formalin-fixed wild-type, heterozygotes, and homozygotes were placed on the same slide and stained with hematoxylin and eosin for histology (A), antibody against total H3 (*B*), antibody against H3K27me2 (*C*), or antibody against H3K27me3 (D). The brown color indicates immunostaining, green represents counterstain. We found that both H3K27me2 and me3 levels were greatest in wild-type embryos, reduced in heterozygous embryos, and further reduced in homozygous embryos. No difference was observed for total H3 between different genotypes. Scale bar: 3mm. advanced bone age, characteristic facies, and variable intellectual disability. The molecular pathogenesis of the disorder is poorly understood. We previously showed that *EZH2* plays a critical role in the regulation of chondrocyte proliferation and hypertrophy in the growth plate, which are the central determinants of skeletal growth [Lui JC, et al., Nat Commun 2016;7:13685]. To determine whether the *EZH2* mutations found in Weaver syndrome cause a gain of function or a loss of function, *EZH2* with the mutation found in our patient was expressed in mouse growth plate chondrocytes [Reference 1]. The mutant protein showed lower histone methyltransferase activity than the wild-type protein. The *EZH2* mutation found in our subject was then introduced into mice using CRISPR/Cas9. Heterozygotes showed mild overgrowth, recapitulating the Weaver overgrowth phenotype (Figure 1). Both homozygous and heterozygous embryos showed decreased H3K27 methylation (Figure 2). The findings demonstrate that *EZH2* mutations found in Weaver syndrome cause a partial loss of function.

Molecular and cellular mechanisms by which specific genes and pathways regulate childhood growth

For normal bone growth to occur, cells in the growth plate must differentiate from proliferative zone (PZ) chondrocytes to hypertrophic zone (HZ) chondrocytes. The important role of microRNAs (miRNAs) in growth plate chondrocyte differentiation was previously revealed by cartilage-specific ablation of Dicer, an enzyme essential for the biogenesis of many miRNAs. We sought to identify specific miRNAs that regulate differentiation of PZ chondrocytes to HZ chondrocytes [Reference 2]. First, we microdissected individual growth plate zones and performed miRNA profiling, using a solution hybridization method, as well as miRNA-seq (miRNA sequencing) to identify miRNAs that are preferentially expressed in PZ compared with HZ. We found that some of these preferentially expressed miRNAs (mir-374-5p, mir-379-5p, and mir-503-5p) serve to promote proliferation and inhibit hypertrophic differentiation. We also found evidence that the observed differential expression of mir-374-5p, mir-379-5p, and mir-503-5p between PZ and HZ is induced by the normal concentration gradient across the growth

plate of parathyroid hormone–related protein (PTHrP). Taken together, our findings suggest that the PTHrP concentration gradient across the growth plate induces differential expression of mir-374-5p, mir-379-5p, and mir-503-5p between PZ and HZ. In PZ, the higher expression levels of these miRNAs promote proliferation and inhibit hypertrophic differentiation. In HZ, downregulation of these miRNAs inhibits proliferation and promotes hypertrophic differentiation.

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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. At a basic level, this work is directed toward making invisible structures and processes visible. 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 so well suited to many NICHD mission-critical applications; it is non-invasive, non-



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ionizing, requires (in most cases) no exogenous contrast agents or dyes, and is generally deemed safe and effective for use with fetuses and children in both clinical and research settings.

A technical objective of our lab 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 correlation studies, and for populating imaging databases with high-quality normative data.

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 clinically 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 have been used by radiologists and neuroscientists as noninvasive quantitative histological 'stains'. Remarkably, the MRI images are 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 cancer patients' response to therapy. Our measures of diffusion anisotropy (e.g., fractional anisotropy or FA) are used universally to follow changes in normally and abnormally developing white matter, including dysmyelination and demyelination and many other applications of brain white matter visualization. Our group also pioneered the use of fiber directionencoded 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 deepbrain gray-matter areas, we also proposed and developed DTI "Streamline" Tractography. Collectively, these advances helped inspire several large federally-funded research initiatives, such as the NIH Human Connectome Project (HCP)

More recently, we invented and developed a family of advanced *in vivo* diffusion MRI 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 human 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 typical MRI voxel. MAP-MRI also subsumes DTI, as well as providing a bevy of new *in vivo* quantitative 'stains' to measure features such as average axon diameter (AAD) and axon-diameter distribution (ADD) within and along large white-matter pathways, 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 is a determinant of axon or nerve conduction velocity and therefore the rate at which information flows along white matter pathways, helping to determine the delays or



FIGURE 1. New stains of white matter pathway morphology derived from differential geometric features

Our new method produces geometric features of white matter pathways, such as the way they splay, bend, and twist as they traverse the brain. First row: Results for multi-shell Human Connectome Project (HCP) data. Second row: DTI and NNSD results for HCP data, where various measures of orientational distortion are calculated.

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 maximizing information flow and minimizing metabolic demands. We also developed novel multiple pulsed-field gradient (mPFG) methods and demonstrated their feasibility for use *in vivo* on conventional clinical MRI scanners as a further means to extract quantitative features to measure and map in the central nervous system (CNS). The methods can also provide an independent measurement of the AAD and other features of cell size and shape.

Although gray matter appears featureless in DTI brain 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 architecture that are currently invisible in conventional MRI. One of our long-term goals 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 are using to study water mobility and exchange in gray matter and white matter. We believe these be promising in identifying inflammation and redistribution of tissue water in brain parenchyma, as well.
In general, we are continuing to develop translationally oriented methods to follow normal and abnormal development and aid in the diagnosis of various diseases and disorders of the brain, noninvasively and *in vivo*.

Quantitative fetal and pediatric MRI

MRI is considered safer than X-ray-based methods, such as computed tomography (CT), for scanning fetuses, infants and children. However, clinical MRI data still lack the guantitative 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. Clinical MRI also often 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, with which one obtains maps of meaningful intrinsic physical quantities or chemical variables that can be measured in physical units and compared among different tissue regions, in individual subjects, and in 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 continue to advance precision imaging studies, whereby MRI phenotypic data can be meaningfully combined with a subject's genotype.

Our group is known for carrying out key 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. One example is the NIH Study of Normal Brain Development, jointly sponsored by a consortium of four NIH Institutes (NICHD, NIMH, NINDS, and NIDA). This multicenter effort, initiated in 1998, was intended to advance our understanding of normal brain development in typical healthy children and adolescents. Remarkably, the Brain Development Cooperative Group (*http://www.brain-child.org/brain_group.html*), created by this forward-looking mechanism, is still active, publishing numerous papers each year, primarily by mining these rich data, many of which we processed, vetted, and uploaded. Our role in this interdisciplinary project was as the DTI Data-Processing Center (DPC). We have uploaded all admissible DTI data collected from this project into a database accessible to interested investigators, which are made publicly available through the National Database for Autism Research (NDAR; *http://ndar.nih.gov*). In collaboration with Carlo Pierpaoli, we continue to support and help update and disseminate this processing and analysis software, called "TORTOISE," which can be downloaded from *http://www.tortoisedti.org*.

Our involvement in traumatic brain injury (TBI) research, particularly in detecting mild TBI (mTBI), has continued to expand owing to its high relevance to the NICHD mission. TBI is an acute problem in the pediatric population, but it also affects and afflicts men and women in the military. DTI provides essential information for the diagnosis of TBI and has potential as an important tool for the assessment of structural damage in the brain. For clinical applications, however, reliable imaging protocols are needed. Part of our work has been to develop a robust DTI data-processing pipeline in order to improve the accuracy



FIGURE 2. Development of novel net displacement imaging method capable of measuring and mapping very slow flows

The method is designed to one day measure very slow flows of CSF and other body fluids, which currently cannot be measured with conventional MRI methods. Average displacement images of the different flow regimes observed in our novel pack-bed MRI phantom obtained under different experimental conditions. Note the signature Poiseuille flow profile of the bulk water images and the overall higher displacement of water transported through the bead pack compared with bulk water.

and reproducibility of DTI findings, in collaboration with scientists at the Center for Neuroscience and Regenerative Medicine (CNRM) 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) polymerbased diffusion MRI phantom that we developed, patented, and are disseminating to numerous clinical sites worldwide.

To permit analysis of novel MRI data as well as to develop new clinical and biological applications of quantitative MRI, we need to continue to create and disseminate 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 use 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 based on methods such the Kullback-Leibler divergence. Recently, our group developed 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



FIGURE 3. Demonstration of MRI-based method for measuring increased water exchange associated with neuronal excitation

The figure shows changes in neuronal activity and accompanying water cycling rate measured in organotypic brain cultures. Neuronal activity is measured by monitoring the change in fluorescence of an optical indicator/dye, whereas MRI is used to monitor changes in water exchange across neuronal membranes.

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 the physical underpinnings of these interactions, which underlie a myriad of biological behaviors.

To address this dearth in our understanding of ion-water-biopolymer interactions, we have developed a comprehensive multi-scale experimental framework to study them by combining macroscopic techniques (e.g., osmotic swelling pressure measurements, mechanical measurements) with high-resolution scattering methods, such as NMR and neutron and light scattering experiments. Swelling pressure measurements

provide information on the overall thermodynamic response, while high-resolution scattering methods 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.

We have been able to apply our understanding of water-polymer interactions to produce novel diffusion MRI phantoms that we use to calibrate MRI scanners, specifically to assure the 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 enabling quantitative diffusion MRI studies to be performed at many different sites. The polymer consisting of the phantom 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. Colleagues at NIST in Boulder, Colorado, have incorporated our PVP polymer into NIST's own diffusion MRI standard. The technology is also being promulgated commercially e.g., by <u>http://hpd-online.com/diffusion-phantom.php</u>.

Measuring and mapping functional properties of extracellular matrix

Our first goal is to understand and quantify the interactions among the major macromolecular components of extracellular matrix (ECM), which give rise to its unique functional properties. ECM is present in every tissue and performs a key role in determining normal and abnormal tissue and organ function. Specifically, we are studying interactions among the primary ECM components, namely collagen, proteoglycans (PG), water, and ions, which govern ECM's macroscopic mechanical and transport properties, using cartilage as a model system. The biomechanical behavior of cartilage and other ECMs reflects molecular composition and microstructure, which change 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 to 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 ECM. Previously, we designed and built a tissue micro-osmometer to perform highprecision 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 selfassemble 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 Ca²⁺ environments and in allowing aggrecan to serve as a calcium ion reservoir in cartilage and bone.

We recently began developing a new biomimetic model of cartilage ECM, consisting of polyacrylic acid (PAA) microgel particles dispersed and embedded within a polyvinyl alcohol (PVA) gel matrix. In this novel system, PAA mimics the behavior of proteoglycan (i.e., HA and aggrecan) microgel 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 and lubricating ability of cartilage. Similar studies cannot be obtained from measurements made on biological tissues because their composition, structure, 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 functional properties, such as tissue load-bearing ability. One major obstacle is that water molecules bound to immobile species (e.g., collagen) are largely invisible to conventional MRI approach. However, magnetization transfer (MT) MRI (as well as other methods) make it possible to detect the bound protons indirectly by transferring their magnetization to the free water surrounding them. It also makes it possible to obtain an estimate of the collagen content in tissue. In a pilot study with collaborators Uzi Eliav and Ed Mertz, 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 are qualitatively consistent with those obtained by histological techniques, such as high-definition infrared (HDIRI) spectroscopic imaging. This work was originally aided by a DIR Director's Award that we received with our collaborators Sergey Leikin and Edward Mertz.

Patents

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- "Development of Bench and Pre-Clinical MRI Methods to Assess Glymphatic Clearance in the Living Brain." 308811-4.01-60855, (CNRM-89-9237)
- "In vivo Brain Network Latency Mapping." NIH BRAIN Initiative grant 1-R24-MH-109068-01

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Biophysics of Large Membrane Channels

Healthy cell functioning and development require both a precise compartmentalization and effective communication between cells and cell organelles. Such processes are critically dependent on the systems regulating metabolism. Specialized membrane channel proteins constitute one such key system that regulates transport of metabolites across the plethora of cell membranes. Deviant alterations in these complex dynamic structures often cause the shift from health to disease. Keeping this in mind, we study "large" betabarrel channels of eukaryotes and bacteria, which are responsible for metabolite fluxes. The channels are fundamentally different from the conventional ion-selective channels both in their structure and in mechanisms of their selectivity and regulation.

We seek to understand the physical principles of channel 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, which allows us to study them at the single-molecule level. The combination of experimental work with physical theory gives us 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 channel-forming proteins and peptides. They include the 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*), Cytolysin A, (cytolytic pore-forming toxin expressed in virulent strains of Escherichia coli and Salmonella enterica), 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



Sergey M. Bezrukov, PhD, DSci, Head, Section on Molecular Transport Tatiana K. Rostovtseva, PhD, Associate Scientist Philip A. Gurnev, PhD, Staff Scientist Maria Queralt-Martin, PhD, Visiting Fellow Amandine Rovini, PhD, Visiting Fellow pentadecapeptide from *Bacillus brevis*) as a molecular sensor of membrane mechanical properties. With the goal of studying channel-forming proteins under controlled conditions, we first isolate them from the host organisms, purify, and then reconstitute them into planar lipid bilayers, the model membrane system with precisely defined physical properties. This allows us to explore the channels' interactions with the lipid membrane as modified by volatile anesthetics, by cytosolic proteins, such as tubulin and alphasynuclein, and by newly synthesized drugs that act as blockers of the translocation pores of bacterial toxins. Understanding the molecular mechanisms of channel functioning is vital for developing new approaches to treatment of various diseases, for which aberrations in ion channel transport are implicated in their pathogenesis.

Sequence diversity of tubulin isotypes in regulation of mitochondrial respiration

The microtubule protein tubulin is a heterodimer consisting of alpha/beta subunits, each subunit featuring several isotypes in vertebrates. For example, the human proteome contains as many as seven alpha-tubulin and eight beta-tubulin isotypes, which vary mostly in the length and primary sequence of the disordered anionic C-terminal tails (CTTs). The biological reason for such sequence diversity remains a topic of vigorous inquiry. We demonstrated that this diversity may be a key feature of tubulin's role in regulating the permeability of the mitochondrial outer-membrane voltage-dependent anion channel (VDAC). Previously, we showed that, in addition to their important functions involving interactions with motor proteins and other microtubule-associated proteins, tubulin CTTs are essential for tubulin's interaction with the VDAC. As the major transport channel and most abundant protein in the mitochondrial outer membrane, the channel is responsible for most of the metabolite flux in and out of mitochondria. The VDAC was proven to be involved in a wide variety of mitochondria-associated pathologies, ranging from various forms of cancer to neurodegeneration. This year, using recombinant yeast alpha/beta-tubulin constructs with alpha-CTTs, beta-CTTs, or both from various human tubulin isotypes, we probed their interactions with VDACs reconstituted into planar lipid bilayers. A comparative study of the blockage kinetics revealed that both alpha-CTTs and beta-CTTs block the VDAC pore, but the efficiency of blockage spans two orders of magnitude, depending on the CTT isotype. Beta-tubulin constructs, notably beta3, blocked the VDAC most effectively. We guantitatively described these experimental results, using a physical model that accounts only for the number and distribution of charges in the CTT, and not for the interactions between specific residues on the CTT and VDAC pore. Based on the results, we speculate that the effectiveness of VDAC regulation by tubulin depends on the predominant tubulin isotype in a cell. Consequently, the fluxes of ATP/ADP through the channel could vary significantly depending on the isotype. Our results suggest an intriguing link between VDAC regulation and the diversity of tubulin isotypes present in vertebrates.

Stochastic gating as a novel mechanism for channel selectivity

In contrast to the highly ion-selective channels studied in neurophysiology, which have narrow selectivity filters of the size of a partially dehydrated ion, metabolite channels are significantly wider. Indeed, they have to accommodate metabolite molecules that are typically much larger than simple mono- or divalent ions. It is clear that the large pore size of metabolite channels jeopardizes one of the main membrane functions, namely, to serve as a barrier for solutes other than the particular metabolites these channels have evolved to pass. For example, the VDAC, recognized to be the major pathway for ATP and ADP exchange between mitochondria and cytosol, is also highly permeable to molecules smaller than these metabolites. The VDAC



FIGURE 1. Cartoon of a model of VDAC pore blockage by the alpha- or beta-CTT of tubulin

The orientation of membrane-bound tubulin dimer was established by us earlier with alphatubulin as the membrane-bound subunit. In such alignment, the contribution of the length and especially the first amino acids of the CTT to the blocking kinetics should be different for alpha-CTTs (*a*) and beta-CTTs (*b*). There is some degree of rotational dynamics for the membrane-bound alpha-tubulin shown in (*b*) with the arrow and shadow image, which could also affect the kinetics of pore blockage by the beta-CTT [Reference 1]. is also the most abundant integral protein of the outer mitochondrial membrane. Interestingly however, several studies demonstrated that, under physiological conditions, these channels are predominantly closed, which allows the membrane to sustain its barrier function. This year, by applying our recent theory of the stochastic gating effect on channel-facilitated transport, we proposed that stochastic gating may provide a mechanism for metabolite channel selectivity in favor of slowly moving large solutes. Specifically, we showed that fast gating of a predominantly closed channel leads to an increase, by orders of magnitude, in the channel selectivity for large, slowly diffusing molecules versus small ions. We hypothesized that this, to our knowledge, newly described mechanism of the selectivity attributable to stochastic gating provides an explanation for the puzzling observation that the VDAC in intact mitochondria is mostly closed. Our conjecture is that the large number of predominantly closed VDAC channels are necessary to keep ATP/ADP transport at a sufficiently high level while effectively suppressing small-ion leakage through the mitochondrial outer membrane. It is also worth mentioning that the stochastic gating effect we propose might not be restricted to the VDAC case. We hypothesized that it can be used to minimize the shunting effect of other wide channels with respect to small solutes, thus shedding light on the functioning of various predominantly plugged beta-barrel channels (e.g., the OprF of Pseudomonas aeruginosa) and on other biological processes controlled by passage through fluctuating bottlenecks.

Lipid nanodomains in ion channel functioning

The function of lipid rafts in mammalian cell membranes remains an issue of controversy, though the association of many membrane proteins with the relatively detergent-resistant membrane fraction of plasma membranes has been well established. However, to the best of our knowledge, the ability of nano-sized membrane inhomogeneities to modify channel behavior has not yet been established. This year, using raft-forming model membrane systems containing cholesterol, we showed that lipid lateral phase separation at the nanoscale level directly affects the dissociation kinetics of the gramicidin dimer, a model ion channel. Gramicidin inserts into membranes and dimerizes to form cation-selective ion channels. The conducting



FIGURE 2. Flux through a stochastically gated channel

Depending on the molecular intra-channel diffusivity (indicated by the numbers near the curves), the flux through a channel can differ significantly from its conventional estimate. The flux ratio is the ratio of the flux at an arbitrary gating rate to its conventional counterpart [Reference 2].

lifetime of the channels is exquisitely sensitive not only to lipid composition, but also to compounds that alter membrane mechanics. For a lipid raft model, we studied mixtures of dioleoylphosphatidylcholine, porcine brain sphingomyelin, and cholesterol. We found that the gramicidin channel detects the presence of separate nanoscale domains in the 1/1/1 mixture well above the microscopic miscibility transition, as reported by fluorescence microscopy. Of particular interest for neurophysiology, we noted that the proportion of cholesterol in nerve cell membranes is relatively high, about 40%, and that cholesterol depletion in live mammalian cells inhibits both raft nanodomain formation and activation of the PI(3)K/Akt pathway. Our findings unequivocally demonstrate that nanodomains in cholesterol-rich membranes do affect ion channel function. While the structure of gramicidin indeed differs from that of mammalian ion channels, many of the fundamental principles of ion conduction through channels were first demonstrated with gramicidin, and its sensitivity to the surrounding lipid environment is a property shared by a significant number of ion channels involved in synaptic signaling. Thus, we suggest that perturbations of lipid phase mixing produced by application of membrane-active agents could affect nervous system function through the interactions at the nanoscale that were uncovered in our study.

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FIGURE 3. Cartoon illustrating influence of lipid rafts on ion channels

Lipid lateral phase separation at the nanoscale level directly affects the dissociation kinetics of the gramicidin dimer, a model ion channel. Modulation of channel protein function by the saturated chains and high cholesterol environment in lipid rafts occurs via the changes in the local mechanical properties of the membrane [Reference 3].

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Protein Sorting in the Endomembrane System

Our lab investigates the molecular mechanisms by which transmembrane proteins (referred to as "cargo") are sorted to different compartments of the endomembrane system in eukaryotic cells. In polarized cells, such as epithelial cells and neurons, the system comprises an array of membrane-enclosed organelles including the endoplasmic reticulum (ER), the Golgi apparatus, the trans-Golgi network (TGN), endosomes, lysosomes, lysosomerelated organelles (LROs) (e.g., melanosomes), and various domains of the plasma membrane. Transport of cargo between these compartments is mediated by carrier vesicles or tubules that bud from a donor compartment, translocate through the cytoplasm, and eventually fuse with an acceptor compartment. Work in our laboratory focuses on the molecular machineries that mediate these processes, including: (1) sorting signals and adaptor proteins that select cargo proteins for packaging into the transport carriers; (2) microtubule motors and organelle adaptors 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 basic research to the elucidation of disease mechanisms, including congenital disorders of protein traffic, such as the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS) and hereditary spastic paraplegias (HSPs), and to the exploitation of intracellular transport by pathogens such as HIV-1.

AP-4 mediates export of ATG9A from the TGN to promote autophagosome formation.

This past year, we discovered a role for the heterotetrameric AP-4 (adaptor protein 4) complex in the signal-mediated export of the autophagy protein ATG9A from the TGN, which contributes to the elucidation of the pathogenesis of a group of HSPs caused by mutations in AP-4 subunit genes. The HSPs are a clinically and genetically heterogeneous group of disorders characterized by progressive lower limb spasticity. Mutations in any of the four



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FIGURE 1. AP-4 epsilon KO causes retention of ATG9A at the TGN of mouse embryonic fibroblasts.

subunits of AP-4 cause an autosomal recessive form of complicated HSP referred to as "AP-4 deficiency syndrome." In addition to lower limb spasticity, the syndrome features intellectual disability, microcephaly, seizures, a thin corpus callosum, and upper limb spasticity. To elucidate the pathogenetic mechanism, we characterized a knockout (KO) mouse for the *AP4E1* gene encoding the epsilon subunit of AP-4. We found that AP-4 epsilon KO mice exhibit a range of neurological abnormalities, including hindlimb

clasping, reduced motor coordination, and weak grip strength. In addition, the KO mice display a thin corpus callosum and axonal swellings in various areas of the brain and spinal cord. Biochemical and cellular analyses identified ATG9A, the only multispanning membrane component of the core autophagy machinery, as a specific AP-4 cargo. Moreover, we found that AP-4 promotes signal-mediated export of ATG9A from the TGN to the peripheral cytoplasm, contributing to lipidation of the autophagy protein LC3B and maturation of pre-autophagosomal structures. Immunohistochemical analyses of various AP-4–deficient cell types, including non-neuronal cell lines, patient skin fibroblasts, and mouse neurons, showed retention of ATG9A at the TGN and its depletion from the peripheral cytoplasm (Figure 1). ATG9A mislocalization was associated with an increased tendency to accumulate mutant huntingtin (HTT) aggregates in the axons of AP-4 epsilon KO neurons. The findings indicated that the AP-4 epsilon KO mouse is a suitable animal model for human AP-4 deficiency syndrome and that defective mobilization of ATG9A from the TGN and impaired autophagic degradation of protein aggregates might contribute to neuroaxonal dystrophy in this disorder.

Segregation in the Golgi complex precedes export of endolysosomal proteins in distinct transport carriers, independently of AP complexes.

The studies described above demonstrated that ATG9A requires AP-4 for its signal-dependent export from the TGN. It has been proposed that other biosynthetic cargos destined for the endolysosomal system



FIGURE 2. TfR/LAMP are segregated from CD-MPR/Sortilin in the Golgi stack before their budding by AP-independent and – dependent mechanisms, respectively.

exit the TGN by interaction of sorting signals with adaptor proteins. However, there was no direct evidence for such a mechanism. Using advanced imaging methodologies, we found that this is indeed the case for the cation-dependent mannose 6-phoshate receptor (CD-MPR) and sortilin, which require interaction with the GGA adaptor proteins for export from the TGN into transport carriers bound for the endosomal system. In contrast, we found that the transferrin receptor (TfR) and the lysosomal protein LAMP1 exit the TGN independently of sorting signals and adaptor proteins. Moreover, they were transported into a different type of carrier directed towards the plasma membrane rather than to endosomes and lysosomes. These proteins subsequently undergo



FIGURE 3. Hypothetical model for the regulation of lysosome positioning by Ragulator and BORC

AP-2-dependent endocytic delivery to their corresponding compartments. Strikingly, we observed that these different TGN export mechanisms are preceded by early segregation of the corresponding proteins within distinct domains of the Golgi stack by virtue of the luminal and transmembrane domains of the proteins (Figure 2). The findings revealed a diversity of sorting mechanisms in the Golgi complex, including early segregation in the Golgi stack prior to export into distinct populations of transport carriers.

Function of the BORC complex in the regulation of lysosome movement

Another important accomplishment of the lab was the discovery of the BORC complex and its role in lysosome positioning. The multiple functions of lysosomes are critically dependent on their ability to move bidirectionally along microtubules between the center and the periphery of the cell. Centrifugal and centripetal movement of lysosomes are 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 towards the cell periphery (Figure 3). We showed that 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 establish BORC as a master regulator of lysosome positioning through coupling to different kinesins and microtubule tracks.

Interaction of BORC with Ragulator controls lysosome positioning in response to amino acid availability.

This past year, we discovered an additional role for BORC in the response of lysosomes to amino-acid deprivation. Depletion of amino acids from the medium turns off a signaling pathway involving the Ragulator complex (a pentameric protein complex that is required for mTORC1 activation) and the Rag GTPases, causing inactivation of the mammalian target of rapamycin complex 1 (mTORC1, a nutrient/energy/redox sensor that controls protein synthesis) kinase from the lysosomal membrane. Reduced 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 were poorly understood. We found that Ragulator directly interacts with BORC, inhibiting its ability to drive lysosomes towards the cell periphery. Amino-acid depletion strengthens this interaction, explaining the redistribution of lysosomes to the juxtanuclear area. The findings demonstrated that amino-acid availability controls lysosome positioning through Ragulator-dependent modulation of BORC (Figure 3).

Hijacking of the retromer complex by Legionella pneumophila

We collaborated with Aitor Hierro's group to characterize the mechanism by which the intracellular pathogen *Legionella pneumophila* hijacks retromer, a complex composed of VPS26, VPS29, and VPS35 subunits and



FIGURE 4. Structure of RidL in complex with retromer.

involved in protein recycling from endosomes. The hijacking involves a *L. pneumophila* effector named RidL, which interacts with the VPS29 subunit of retromer. We solved the crystal structure of RidL in complex with a VPS29-VPS35 retromer subcomplex (Figure 4). We found that a hairpin loop protruding from RidL inserts into a conserved pocket on VPS29 that is also used by cellular ligands, such as TBC1D5 and VARP, for VPS29 binding. Consistent with the idea of molecular mimicry in protein interactions, RidL outcompeted TBC1D5 for binding to VPS29. Furthermore, we found that the interaction of RidL with retromer does not interfere with retromer dimerization but is essential for association of RidL with retromer-coated vacuolar and tubular endosomes. The work thus provided structural and mechanistic evidence for how RidL is targeted to endosomal membranes.

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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 with 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 sociodemographic 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, France, Israel, Italy, Japan, Kenya, Peru, the Republic of South Korea, and the United Kingdom, as well as the United States; in all places, intra-cultural as well as crosscultural 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



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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

Command of language is a fundamental life skill, a cornerstone of cognitive and socio-emotional development and a necessary ingredient for successful functioning in society. We used 15-year prospective longitudinal data from the Avon Longitudinal Study of Parents and Children to evaluate two types of stability of core language skill in 5,036 typically developing and 1,056 atypically developing (preterm, dyslexic, autistic, and hearing-impaired) children in a multi-age, multi-domain, multi-measure, multi-reporter framework. A single core language skill was extracted from multiple measures at several ages, and the skill proved stable from infancy to adolescence in all groups, even accounting for child nonverbal intelligence and sociability and maternal age and education. Language skill is a highly conserved and robust individual-differences characteristic. Lagging language skills, a risk factor in child development, would profitably be addressed early in life.

The overall purpose of this within-family longitudinal study was to examine in close detail a variety of socio-emotional parenting cognitions and practices of mothers with their first- and second-born toddlers alongside socio-emotional characteristics of the siblings themselves. Mothers participated with their 20-month-old first-borns and again, an average of three years later, with their 20-month-old second-borns (55 families, 165 participants). We assessed and compared continuity and stability in maternal cognitions and practices between the two times, and similarities, differences, and correspondences in siblings' behaviors. Maternal socio-emotional parenting cognitions were continuous in mean level and stable in individual differences across siblings; maternal socio-emotional practices were continuous in mean level but unstable in individual differences. First-borns were more sociable and emotionally available to mothers than second-borns; first- and second-borns' socioemotional behaviors were largely unrelated. This study contributes to understanding socio-emotional domains of parenting and child development, birth order effects, and the shared and non-shared contexts of siblings' environments within the family.

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 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 care-giving cognitions engender care-giving 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 three-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 more supportive parenting two years later when their children were 4 years of age; 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.

Child development and parenting in multicultural perspective

Promoting children's pro-social behavior is a goal for parents, healthcare professionals, and nations. We are interested in the question of whether positive parenting promotes later child pro-social behavior, or whether children who are more pro-social elicit more positive parenting later, or both. To date, relations between parenting and pro-social behavior have been studied only in a narrow band of countries, mostly with mothers and not fathers, and child gender was infrequently explored as a moderator of parenting/ pro-social relations. Our cross-national study used 1,178 families (mothers, fathers, and children) from nine countries to explore developmental transactions between parental acceptance-rejection and girls' and boys' pro-social behavior from age and education, higher parental acceptance predicted increased child pro-social behavior from age 9 to 10 and from age 10 to 12. Higher age-9 child pro-social behavior also predicted increased parental acceptance from age 9 to 10. Such transactional paths were invariant across nine countries, mothers and fathers, and girls and boys. Parental acceptance increases child pro-social behaviors later, but child pro-social behaviors are not effective at increasing parental acceptance in the transition to adolescence. The study identifies widely applicable socialization processes across countries, mothers and fathers, and girls and boys.

Adults speak to infants differently than how they speak to adults. Compared with Adult-Directed Speech (ADS), Infant-Directed Speech (IDS) has a higher pitch (f0), a larger pitch range (f0 range), a slower tempo (longer phoneme duration), and is more rhythmic. Inoue *et al.* [*Neurosci Res* 2011;70:62] implemented a machine algorithm that, by using a mel-frequency cepstral coefficient and a hidden Markov model, discriminated IDS from ADS in Japanese. We applied the original algorithm to two other languages that are very different from Japanese—Italian and German—and then tested the algorithm on Italian and German databases of IDS and ADS. Our results showed that: first, in accord with the extant literature, IDS is realized in a similar way across languages; second, the algorithm performed well in both languages and close to that reported for Japanese.

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Activity-Dependent Regulation of Neurons by the Neuregulin-ErbB Signaling Pathway

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 an activitydependent fashion (i.e., experience) in the developing rodent 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, which are necessary for the generation and modulation of gamma oscillations. Using genetically targeted mouse mutant models (i.e., knockout [KO] mice), 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. ErbB4 receptors are also expressed prominently in dopaminergic neurons. In the recent studies described below, we generated mouse KO models that delete ErbB4 selectively in dopamine (DA)-producing neurons and showed that the mice exhibit a pronounced imbalance of DA levels and altered cognitive behaviors. More recently, our group also began to shed light on how the structure of different unprocessed Neuregulin ligands (i.e. proNRGs) determines their trafficking to distinct neuronal subcellular



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compartments, their post-translational processing in response to neuronal activity, and their functions in the developing and maturing nervous system.

In order to study the regulation of Neuregulin-ErbB4 signaling and its functional significance during neural development, our laboratory uses a combination of technical approaches, including: electrophysiological recordings of dissociated neurons or in acute brain slices prepared from normal and genetically altered mice; multi-electrode field recordings from brains of freely moving rats; reverse-microdialysis neurochemistry; confocal fluorescence microscopy in fixed and live tissue; fluorescence *in situ* hybridization using RNAscope and BaseScope; protein biochemistry including 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 that are altered in many psychiatric disorders.

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

Brain-expressed Neuregulins (NRGs), ligands for the ErbB family of receptor tyrosine kinases (ErbB2-ErbB4), originate from three different genes (NRG1-NRG3) and their diversity is greatly increased by the use of distinct promoters (NRG1: type-I, -II and -III) and alternative splicing, which generates numerous isoforms. While NRG complexity has been known for decades, the functional significance of this evolutionarily conserved diversity remained poorly understood. Recently, we made a series of discoveries that have begun to uncover how NRGs can be assigned to different classes of ligands based on their single (NRG1 type-I and -II, NRG2) or dual (NRG1 type-III, NRG3) trans-membrane (TM) topologies. First, we demonstrated that the single vs. dual TM topologies impart different subcellular trafficking properties to the unprocessed



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.



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 proforms 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.

Neuregulin proforms (proNRGs). Single TM proNRGs are targeted to the cell body plasma membrane and accumulate at contact sites between the endoplasmic reticulum and overlying plasma membrane (Figure 1A), in a specialized site known as subsurface cisternae (SSCs). Unexpectedly, we found no evidence of single TM NRGs in axons; instead, proNRGs accumulate at SSCs as large somato-dendritic puncta that are most prominent in CNS GABAergic interneurons or at C-boutons on spinal cord/medulla cholinergic neurons. In response to neural activity, specifically requiring activation of N-methyl-d-aspartate glutamate receptors (NMDARs), we observe that single TM proNRGs disperse from SSCs and become susceptible to proteolytic processing by extracellular alpha secretases. Importantly, proNRGs processing results in the release of the biologically active NRG1 (type-I or -II) or NRG2 ectodomains into the extracellular space (Figure 1B). We went on to demonstrate that the NRG2 ectodomain binds to ErbB4 on GABAergic interneurons, promotes signaling and association with NMDARs, and results in the internalization of NMDARs. Taken together, our work has uncovered a bidirectional signaling pathway between NRG/ErbB4 and NMDARs that can function as a homeostatic mechanism to regulate interneuron excitability [Reference 1].

Subcellular distribution of dual transmembrane Neuregulins in central neurons

In contrast to single TM NRGs, the dual TM NRG1-CRD (type-III) is targeted to axons where it signals in juxtacrine mode. Unexpectedly, we recently found that NRG3 is also a dual TM protein and, like NRG1-CRD (cysteine-rich domain), is targeted to axons (Figure 1C). Unlike single TM NRGs, dual TM NRGs are processed constitutively by beta-secretase (BACE), and cleavage occurs independently of NMDAR activity. Interestingly, processing is a prerequisite for axonal accumulation of dual TM NRGs; treatments that interfere with



FIGURE 3. Oligodendrocytes and neurons express distinct ErbB4 JM (juxtamembrane) transcripts.

The sensitivity and specificity of singlepair 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* 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 back-signaling (*G*,*H*), whereas GABAergic neurons express the cleavage-resistant JMb ErbB4 receptor (*I*,*J*).

processing cause intracellular accumulation of their pro-forms [Reference 1]. Taken together, our recent findings concerning the relationships between transmembrane topology, modes of proteolytic processing, subcellular distribution, and signaling modality suggest that single and dual TM NRGs regulate neuronal functions in fundamentally different ways (Figure 2). The work was supported by a Directors Investigator Award [Reference 1].

A novel ultrasensitive *in situ* hybridization approach to detect short sequences and splice variants with cellular resolution

Detection of short isoform-specific sequences requires RNA isolation for PCR analysis—an approach that does not register the regional and cell-type-specific distribution of isoforms. The ability to distinguish the differential expression of RNA variants in tissue is critical because alterations in mRNA splicing and editing, as well as the coding of single nucleotide polymorphisms, have been associated with numerous cancers and neurological and psychiatric disorders. Recently, we reported a novel highly specific and sensitive single-probe colorimetric/fluorescent *in situ* hybridization (ISH) approach, called BaseScope, that targets short exon/exon RNA splice junctions using single-pair oligonucleotide probes (50 bp). We used this approach to investigate, with single-cell resolution, the expression of four *ErbB4*–encoding transcripts that differ by alternative splicing of exons encoding two juxtamembrane (JMa/JMb) and two cytoplasmic (CYT–1/CYT-2) domains. First, by comparing ErbB4 hybridization on sections from wild-type and *ErbB4* knockout mice (missing exon 2), we demonstrated that single-pair probes have the specificity and sensitivity to visualize and quantify the differential expression of ErbB4 isoforms (Figures 3A–F). Next, we demonstrated that expression of ErbB4 isoforms (Figures 3G–J), and that



FIGURE 4. NRG2 null mice exhibit working memory deficits and reduced dopamine levels, which 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.

the differential expression of ErbB4 isoforms is evolutionarily conserved to humans. BaseScope could serve as an invaluable diagnostic tool to detect alternative spliced isoforms, and potentially single base polymorphisms, associated with disease [Reference 2].

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

Using RNAscope and novel antibodies generated in our lab, we found that NRG2 expression in the adult rodent brain does not overlap with that of NRG1 and is more extensive than originally reported. In agreement with earlier reports, we observed that NRG2 transcripts are expressed in granule cells of the cerebellum and dentate gyrus. In addition, we found that NRG2 transcripts and protein are expressed in the medial prefrontal cortex (mPFC) and striatum (Figure 4A). We generated NRG2 knockout mice (KO) to investigate the function of this single TM NRG in the developing brain. We found that NRG2 KO mice have higher extracellular dopamine levels in the dorsal striatum, but lower levels in the mPFC—a pattern that is similar to that reported for the imbalance of dopamine in schizophrenia patients. 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 dependent on hippocampal and mPFC function. Acute administration of the antipsychotic clozapine rapidly raised extracellular dopamine levels in the mPFC and improved alternation T-maze performance (Figure 4B). Similar to mice treated chronically with NMDAR antagonists, we demonstrate that NMDAR synaptic currents in NRG2 KOs are augmented at hippocampal glutamatergic synapses and are more sensitive to ifenprodil, indicating an increased



FIGURE 3. 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), compared with baseline walking by rats injected with saline (black). C,D. Effects of D4R drugs on ketamineinduced 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.

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].

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 NRG1 acutely increases extracellular DA levels and regulates long-term potentiation (LTP) and gamma oscillations, and ErbB4 is expressed in GABAergic (Pv⁺) and mesocortical DAergic (TH⁺) 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. Unexpectedly, we found that, in contrast to GABAergic neurons, ErbB4 is expressed DA neuron axons and that NRG regulates extracellular DA levels by modulating dopamine transporter (DAT) function. In contrast to mice harboring mutations in GABAergic neurons, which show sensory-motor gating deficits and increases in motor activity, ErbB4 TH KO mice exhibit deficits in cognitive-related tasks (in the T-, Y- and Barnes-mazes). Therefore, direct effects of NRG/ ErbB4 signaling in GABAergic vs. DAergic neurons differentially affect cortical circuits and DA homeostasis and behaviors relevant to schizophrenia [Reference 4]. Using mice that lack ErbB4 in all tissues, we are presently extending our research to the functional role of the receptor in modulating the levels of neurotransmitters and associated behaviors. Our preliminary results indicate that mice lacking ErbB4 exhibit an imbalance in extracellular dopamine levels in several brain regions, as well as behavioral deficits that resemble those observed in mice that harbor targeted mutations in either GABAergic or dopaminergic neurons.

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

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 phenocopies schizophrenia by eliciting psychoto-mimetic symptoms and affecting cognitive functions in healthy individuals. In collaboration with Judith Walters's lab, we used multielectrode 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 the dopamine D4 receptor (D4R) and ErbB4 regulates 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, which 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 (Figure 5). Interestingly, in the MD, the D4R agonist increased the power of ketamine-induced gamma oscillations [Reference 5]. Experiments are in progress to evaluate the effect of mutating D4R, in conjunction with pharmacological targeting of D4R receptors, on behaviors that pertain to deficits reported in patients with schizophrenia. We are using the 5-Choice Serial Reaction Time Task (5CSRTT) to quantify the effect of the dopamine receptors on measures of attention and impulsivity.

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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 and the pathways that sustain changes in behavioral state. In addition, we are developing a suite of genetic tools and behavioral assays to probe the nexus between neuronal function and behavior at singlecell resolution. The neuronal connections that allow the brain to integrate sensory and internal-state information are established through genetic interactions during development. We aim to identify genes and neurons that are required for 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.

Molecular identification of neurons that mediate prepulse inhibition

Startle responses are rapid reflexes that are triggered by sudden sensory stimuli and which help animals defend against or escape from potentially threatening stimuli. In both fish and mammals,



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FIGURE 1. Activity imaging during prepulse inhibition

Activity map for neurons that show elevated activity in response to a weak auditory stimulus (Prepulse active, *green*), and on trials where the prepulse inhibits responses to a subsequent startle stimulus (PPI effective, *red*), superimposed on a standard zebrafish reference brain (*grey*). Neurons that show activity correlated with behavioral prepulse inhibition are clustered in rhombomere 4, adjacent to the command neuron for startle responses, the Mauthner cell (indicated).

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 optogenetic inhibition eliminates PPI. The neurons 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. To identify the precise subset of *Gsx1* neurons that mediate PPI, we then used volumetric calcium imaging to simultaneously visualize the activity in tens of thousands of *Gsx1* neurons during PPI (Figure 1). The method enabled us to locate a specific brain region that contains *Gsx1* neurons whose activity correlates strongly with behavioral PPI. To determine where such neurons



FIGURE 2. Cre enhancer trap lines for neural circuit analysis in zebrafish

a. Whole brain projections of the expression pattern for new Cre transgenic lines

b. Cre lines with expression in specific rhombomeres enable the function of neurons in different hindbrain territories to be parsed.

are causally related to PPI, we optogenetically activated them before probing fish with a startle stimulus. Controlled firing of *Gsx1* neurons was able to reproduce the effect of a prepulse. We next visualized the morphology and projections of these neurons in order to reveal how they connect to the 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. We found a specific subset of *Gsx1* neurons that use the neurotransmitter glutamate and that project to an area of the lateral dendrite of the Mauthner cell, the command neuron for startle responses in fish, that receives auditory input. By directly visualizing neurotransmitter release from the auditory nerve, we discovered that acoustic information to the Mauthner cell is selectively depressed during PPI, while transmission to other brain regions is spared, which indicates that a key mechanism for PPI is presynaptic inhibition [Reference 1]. Given that PPI is abnormal in neuro-psychiatric 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.

Tools for decoding neuronal circuits

During our work to identify the neuronal basis for prepulse inhibition, we realized that existing transgenic tools did not allow manipulation of small groups of neurons with a high degree of specificity. We developed a system to leverage the thousands of Gal4 lines already available in zebrafish, by generating a new library of Cre enhancer trap lines with restricted patterns of expression within the brain (Figure 2a). The lines can be used 'intersectionally' to restrict the pattern of expression with Gal4 domains, allowing small clusters of

FIGURE 3. Unbiased analysis of brain structure and composition in mutant models

a–e. Computationally derived neuroanatomical regions based on cluster analysis of more than 200 transgene expression patterns. Images are single slices through a reference brain in horizontal (*a*), sagittal (*b*),



and coronal (*c*–*e*) planes. Planes for *c*–*e* are indicated in *b*.

f. Deformational field analysis of *atoh7* morphant larvae. Heat map reveals regions that are smaller in mutants, statistically significant after correction for comparison of more than 5.3 M voxels. A large cluster is detected within the neuropil region of the optic tectum (indicated), corresponding to the loss of projections from retinal ganglion cells.

neurons to be targeted. During our studies on prepulse inhibition, we used a subset of the lines to ablate *Gsx1* neurons within specific hindbrain domains to determine which specific neurons are required for prepulse inhibition (Figure 2b).

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 registration techniques enable data from several individual zebrafish 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 [Reference 2].

We postulated that the very high precision of alignment may enable statistically robust whole-brain analysis of neuronal composition and morphology in zebrafish models of neurological disorders. For this, we first developed a new computational method to automatically segment the larval zebrafish brain into 180 neuroanatomical regions, based on gene expression patterns (Figure 3a). We then generated new software, *Cobraz*, that uses those regions to identify changes in brain size, neurotransmitter distribution, or local morphology in confocal images of genetic mutant zebrafish larvae. We validated our algorithm by comparing embryos injected with the *atoh7* morpholino to wild-type siblings. Loss of *atoh7* is known to eliminate retinofugal projections. Indeed, we found that the optic tectum, the major retino-recipient area, was significantly smaller in morphants (Figure 3b) [Reference 3]. In collaboration with the Dawid laboratory, we used the software to assess brain development in zebrafish *kctd15* mutants, which show a growth reduction but

no overt change in brain structure. Mutant larvae showed a significant loss of the torus lateralis, a region known to produce growth hormone–releasing hormone [Reference 4]. The technique can be applied to almost any zebrafish neuro-developmental model, thus enabling robust and quantitative detection of subtle changes in brain structure or composition.

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 goal in neuroscience is to identify the neural systems that maintain shortterm behavioral states and to determine how they interact with central mechanisms for behavioral choice. 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 a light-search behavior. For the first two minutes in the dark, larval swim patterns are similar to a behavior characterized as arearestricted search across species, with high fractal dimension and repeated turning behavior. 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 5].

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Global Regulation of Gene Expression by ppGpp

Our continuing research goal is to understand the emerging powerful regulatory functions of compounds that are closely related to the four common precursor ribonucleotides (GTP, ATP, UTP, and CTP) and that are polymerized into RNA. RNA is a polymer of ribose (R) linked by a phosphate bonded at its two (3' and 5') sites [...pR(pR)pR...] with the bases G, A, U, or C bases attached to the ribose. Unlike the precursors, this family of analogs are all blocked from polymerization because their 3' and 5' ribose sites are already bonded to one or more phosphate. We focus on one subset of this family of analogs that have both ribose sites blocked by 3' and 5' polyphosphates (ppGpp and pppGpp [here termed (p)ppGpp]). For the other family subset, one ribose has its 3' and 5' sites attached to single phosphate to make a cyclic bond (cAMP, cGMP). Other examples are mononucleotides whose two phosphates similarly block each other's 3' and 5' sites (c-di-AMP, c-di-GMP, c-di-AG). Both (p)ppGpp and cAMP were discovered about a half century ago, and both fields are currently very active with discoveries of new analogs and new regulatory functions. Cyclic regulators are biologically ubiquitous; the ppGpp-like regulators are found in bacteria and plant chloroplasts but not in animals, making them a particularly appealing target for new antibiotics.

We focus on bacterial (p)ppGpp for several reasons: the regulatory effects in the model E. coli are global, owing to direct RNA polymerase binding, and can provide targets for antibiotic development that will not affect humans. The (p)ppGpp nucleotides sense a variety of nutritional and physical sources of stress that impair growth. They then accumulate to warn cells of stress and respond with an astonishing array of regulatory functions that allow survival. Many mechanisms are known that protect pathogens from host defenses to thereby aggravate pathogenicity. Evidence for the fundamental role of (p)ppGpp in pathogenicity is pervasive, both with respect to antibiotic resistance and the reduction of host carrier states. The current crisis occasioned by the lack of new antibiotics coupled with the emergence of "superbugs" has led to widespread interest in (p)ppGpp. Our lab is too small to undertake pharmaceutical development. Instead, we focus on ppGpp-like compounds in the hope that a fundamental understanding will continue to underlie practical advances in this field.



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ppGpp affects carbohydrate-dependent growth transitions, but in a different manner than cAMP.

When cells grow exponentially on a mixture of glucose and lactose, they first exhaust the glucose, then growth lags transiently because adjustments of gene expression are needed before growth can resume on lactose. The growth pause between serial use of two sugars is termed a "diauxic" lag. Early research on cAMP revealed that it represses the utilization of lactose until glucose is exhausted and subsequently discovered that AMP interacts with lac-operon promoter DNA to turn off lactose usage in preference to glucose. The relative abundance of (p)ppGpp is known to affect this process. Levels of ppGpp are determined by the balance of the activities of two synthetases and one hydrolase. We compared diauxic lags in wild-type cells with mutants of one or another ppGpp synthetase under many conditions, including controls that bypassed cAMP effects, and directly measured (p)ppGpp levels. Our efforts suggest a surprising explanation. Acetate is measurably formed and excreted during growth on glucose, only to be reabsorbed later and, in the presence of (p)ppGpp, converted to acetylphosphate (Ac-P). This pathway is the reverse of the normal pathway for making Ac-P from acetyl-CoA. Ac-P is a high-energy donor for post-translational acetylation of a large number of proteins that alter activities of glycolysis and the Krebs cycle. Enhanced protein acetylation was confirmed during the lag by Western plots with anti-acetyl-lysine monoclonal antibody. We also found that basal ppGpp levels correlate directly with basal Ac-P levels. While details of the mechanism underlying this correlation remain to be uncovered, this reveals a new level of regulatory complexity, i.e., global transcriptional regulation by ppGpp appears to intersect with regulation by a known post-translational regulator, Ac-P [Reference 5].

Phage T4 transcription during infection is reduced without *dksA* but not without ppGpp.

When in our lab, former postdoctoral fellow Tamara James observed that the plaque sizes of wild-type E. coli infected with phage T4 were larger than those of infected *E. coli* deleted for the RNA polymerase-binding transcription factor *dksA* or for ppGpp (ppGpp^o). This is of interest given that ppGpp and DksA both often act directly on RNA polymerase (RNAP). The 20-minute time course of the T4 infection cycle consists of early, middle, and late transcripts. Early promoters use unmodified RNA polymerase, and early transcripts do not terminate but instead read through middle promoters. Middle transcripts are made with a modified RNAP, which recruits two early phage phiX proteins, MotA and AsiA. Late promoters use a RNAP additionally modified by three middle proteins. Biological measures during infection included a new semi-automated statistical method devised to document plaque size populations along with classical estimates of burst sizes and latent periods. The NICHD Sequencing Core performed RNA-seq analysis complemented by our RTqPCR and primer extension analyses. The effect of $\Delta dksA$ is to double burst size without changing the timing of phage release and to increase early and some middle transcripts by readthrough. The increased middle gene transcripts appear to partially compensate for RNAP modification defects for mutant phage with a partly active MotA protein. In vitro assays of early phage phiX promoter transcripts with RNA polymerase revealed that DksA did not inhibit activity, which suggests that the effects of deletions *in vivo* are indirect. Surprisingly, ppGpp° had only modest effects on all parameters measured, despite statistical documentation of enlarged plaques. Direct ppGpp assays might address the possibility that infection quickly abolishes the ppGpp accumulation response. The work has already attracted attention because it may provide a way to enhance the yield of phage phiX surface vectors loaded with therapeutic payloads with cell-specific targets. This is the first study of effects of DksA and ppGpp on lytic bacterial phage development [Reference 1].

ppGpp alters RNAP structure to enhance DksA inhibition of *rrnB* P1 promoter initiation.

A key element for triggering the global stringent regulatory response is inhibition of ribosomal RNA synthesis at ribosomal promoters. Two distinct ppGpp binding sites on RNAP sites are known from our work and from that of others. Site 1 is located on a boundary of the large β ' subunit and the smallest ω subunit; Δ omega mutants are viable with modest effects on the stringent response. Site 2 is 60Å away from site 1 and formed only in the presence of DksA binding; Δ DksA adversely alters *rrnB* P1 inhibition and the stringent response. In collaboration with the Murakami laboratory, we soaked preformed RNAP holoenzyme crystals with DksA alone or with the added presence of ppGpp, which allowed structural documentation of conformational changes due to each addition. Bound DksA has access to both the template strand at the active site and a downstream DNA site. Addition of ppGpp modifies this complex in a manner that explains its ability to inhibit *rrnB* P1 initiation by collapsing open complexes before they can initiate RNA synthesis and forming the first diester bond. Our laboratory showed that cellular ppGpp is a more potent inhibitor than pppGpp and that site 1 binds to both ppGpp and pppGpp. Similar studies might reveal whether different affinities of the two isomers for site 2 might account for this behavior. These observations provide definitive structural evidence for allosteric effects of ppGpp and DksA on transcription regulation [Reference 2].

Opposing effects of (p)ppApp and (p)ppGpp on RNA polymerase structure and function

As mentioned in earlier annual reports, while a postdoctoral our lab, Katarzyna Potrykus discovered a substrate-specificity difference between a eukaryotic (p)ppGpp "Mesh" hydrolase and the *E.coli* and *Streptococcal equisimilis* hydrolases. The bacterial hydrolases cleave (p)ppGpp but not (p)ppApp, while the Mesh enzyme cleaves both. The most recent step in our continuing collaboration the Potrykus lab compared (p)ppApp isomers and (p)ppGpp isomers with respect to effects on the standard *rrnB* P1 promoter activity and, in collaboration with the Murakami laboratory, also identified new RNA polymerase binding sites. The adenine analogs were demonstrated to activate the standard *rrnB* P1 promoter transcripts. Regulatory features of the adenine analogs on transcription were assessed and found to be more modest than those of (p)pGpp and, surprisingly, were often the opposite of those known for (p)ppGpp. Three examples are: (1) (p)ppApp activates initiation of transcription, whereas (p)ppGpp inhibits; (2) pppApp is a more potent activator than ppApp, whereas ppGpp is more potent inhibitor than pppGpp; and (3) pppApp activation of transcription stabilizes open complexes, whereas ppGpp destabilizes open complexes. Evidently, a 'yin-yang' regulatory relationship exists between pppApp and ppGpp.

Order-of-addition experiments reveal that the analog added first dominates the regulatory outcome over the regulatory effect of the one added later and *vice versa*. The findings suggest different binding sites for the G and A analogs as well as a high degree of plasticity of RNAP conformational changes, seemingly each kinetically stable enough that, once formed, are able to persist, despite the added presence of the second analog. Soaking pppApp into RNA holoenzyme crystals reveals that pppApp binds to an entirely new site located near the catalytic polymerization site near the switch region. The new site is distinct from the DksAppGpp binding site 2 and distinct from ppGpp binding site 1. Moreover, the placement of the new pppApp binding site is consistent with its ability to stabilize open complexes. RNA polymerase gel shifts, detected by electromobility shift assay (EMSA) and occasioned by additions of pppApp and ppGpp, and order of addition experiments are also consistent with regulatory effects. Many years ago, (p)ppApp was reported to accumulate in extracts of *Bacillus subtilis*, a soil bacterium. The comparison of (p)ppApp and (p)ppGpp analogs suggests that, during evolution, RNA polymerase evolved to harbor a (p)ppApp–specific binding site, which could imply a widespread biological occurrence of (p)ppApp. Future experiments will be aimed at searching for cellular (p)ppApp [Reference 3].

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Membrane Rearrangements in Viral Entry and Developmental Fusion

Diverse biological processes, in which enveloped viruses infect cells and cells from all kingdoms of life secrete, internalize, traffic and sort integral proteins, sculpt their membranes, and bring together parent genomes in sexual reproduction, share a common stage: fusion of two membranes into one. Biological membrane remodeling is tightly controlled by protein machinery but is also dependent on the lipid composition of the membranes. Whereas each kind of protein has its own, individual personality, membrane lipid bilayers have rather general properties, manifested by their resistance to disruption and bending, and by their charge. Our long-term goal is to understand how proteins fuse membrane lipid bilayers. We expect that better understanding of important fusion reactions will bring about new ways of controlling them and will lead to new strategies for guelling diseases involving cell invasion by enveloped viruses and defects in intracellular trafficking or intercellular fusion. Our general strategy is to combine in-depth analysis of the best characterized fusion reactions with comparative analysis of diverse, less explored fusion reactions, which can reveal new kinds of fusion proteins and clarify the generality of emerging mechanistic insights. In our recent studies, we explored mechanisms of the myoblast fusion stage of development, of the regeneration of skeletal muscles, and of the osteoclast precursor fusion stage during the formation of multinucleated osteoclasts.

Distinct functions of myomaker and myomerger during myoblast fusion

Multinucleated skeletal muscle fibers form through the fusion of myoblasts during development and regeneration. Previous studies identified myomaker (Tmem8c) and myomerger as musclespecific membrane proteins essential for fusion [Millay DP, *et al. Nature* 2013;499:301; Quinn ME, *et al. Nat Commun* 2017;8:15665]. In our recent study (collaboration with Douglas Millay) we explored the localization of endogenous myomaker in muscle cells [Reference 1]. We found myomaker at the plasma membrane and also in the Golgi and post-Golgi vesicles. Trafficking of myomaker is regulated by palmitoylation of C-terminal cysteine residues, which allows Golgi localization. To examine whether myomaker functions at the myoblast fusion stage or only prepares the cells for fusion,



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FIGURE 1. Schematic of the division of fusogenic labor between myomaker and myomerger

Myomaker is indispensable for hemifusion, and myomerger is not required for this step. The fusion reaction would stall at this stage without the presence of myomerger, which at the second stage of the reaction drives opening of a fusion pore [Reference 2]. A subsequent expansion of the pore also depends on dynamin, cell metabolism and PIP2 [Leikina E, *et al. J Cell Biol* 2013;200:109]. Other factors that may be important for fusion (for instance annexin A5 [Leikina E, *et al. J Cell Biol* 2013;200:109]) are omitted for clarity.

we applied a synchronized fusion approach developed in earlier studies of our lab [Leikina E, *et al. J Cell Biol* 2013;200:109]. We reversibly blocked fusion of myoblasts without blocking pre-fusion stages using lysophosphatidylcholine (LPC), an inhibitor of membrane merger. We accumulated ready-to-fuse cells in the presence of LPC and then removed LPC to allow fusion. Myomaker antibody applied at the time of LPC removal blocked lipid mixing and content mixing, indicating that myomaker at the plasma membrane at the time and place of fusion is directly involved in the early stages of myoblast fusion.

In our most recent report [Reference 2], we furthered the understanding of myoblast fusion by demonstrating that the fusion reaction proceeds through a novel step-wise, bi-factorial mechanism whereby different proteins function at independent points in the pathway. We demonstrated that the myoblast fusion reaction proceeds through a novel step-wise, bi-factorial mechanism whereby different proteins function at independent points in the pathway. We demonstrated that the myoblast fusion reaction proceeds through a novel step-wise, bi-factorial mechanism whereby different proteins function at independent points in the pathway [Reference 2]. While myomaker function is essential for fusion initiation and hemifusion, myomerger drives subsequent fusion pore formation (Figure 1). As reported in our earlier work, fusion pore formation and expansion also depend on dynamin, cell metabolism, and phosphatidylinositol 4,5-bisphosphate [Leikina E, *et al. J Cell Biol* 2013;200:109]. In better characterized fusion systems (viral and intracellular membrane fusion), reactions can be stalled at the hemifusion stage, but both hemifusion and pore formation are driven by the same protein complex. Thus, we revealed a membrane fusion mechanism that is divergent from well established models of fusion such as SNARE-mediated intracellular fusion, hemagglutinin (HA)-mediated viral fusion, and developmental fusion mediated by the cell fusogen Eff-1. Myomaker does not require myomerger to mediate hemifusion, as evidenced by the finding that the treatments promoting hemifusion-to-fusion transition of myomerger^{-/-} myoblasts result in levels of complete fusion comparable to wild-type (WT) levels, showing that the lack of myomerger

does not impact myomaker-dependent hemifusion. Similarly, myomerger does require myomaker to drive the hemifusion-to-fusion transition, as evidenced by the finding that myomerger drives completion of heterologous fusion reaction mediated by the viral fusion protein HA, a system completely devoid of myomaker. In summary, we distinguished the functions served by single fusogens in other systems and assigned to two independently acting muscle-specific proteins that function to drive distinct fusion stages. Our results suggest that the division of the reaction could be a regulatory checkpoint for myoblast fusion. A deeper understanding of the myoblast fusion mechanism could help develop new therapeutic strategies for genetic and acquired muscle diseases.

Phosphatidylserine at the surface of osteoclast precursors regulates their fusion.

In our recent study [Reference 4], we focused on the cell-cell fusion stage of osteoclast formation. Multinucleated osteoclasts resorb bones to balance the bone-forming activity of osteoblasts in the continuous bone-remodeling process in both healthy animals and in pathological states. Osteoclasts are formed from precursor cells (OCPs) of monocyte/macrophage lineage in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kappa-B ligand (RANKL). Many groups have characterized the osteoclastogenesis using in vitro models based on human monocytes (HMs), murine bone marrow cells (BMC), and macrophage-like murine monocytic RAW 264.7 cells ("RAW cells"). Several proteins, including: a regulator of immune properties of dendritic cells, the dendritic cell-specific transmembrane protein DC-STAMP; the osteoclast-stimulatory transmembrane protein (OC-STAMP); purinergic receptors; S100 proteins; protein tyrosine phosphatase PEST; an adaptor protein Tks5; an intermediate-conductance calcium-activated potassium channel; and CD47, were shown to be involved in osteoclastogenesis, and it has been suggested that they are also involved in OCP fusion. Recent studies also demonstrated that the formation of multinucleated osteoclasts depends on clathrin-mediated endocytosis. The specific stages of osteoclastogenesis that are dependent on the proteins listed above (fusion vs. pre- or postfusion stages) remain to be clarified. Generation of multinucleated osteoclasts also involves syncytin-1 (Syn-1), the envelope protein of a human endogenous retrovirus, HERVW1. Syn-1 is highly expressed in placental trophoblasts and mediates their fusion in human placentogenesis. Fusogenic activity of Syn-1 is triggered by its interactions with ASCT1/2 receptors. Suppression of Syn-1 activity inhibits both formation of multinucleated human osteoclasts and expression of a biochemical marker of osteoclast maturation, tartrate-resistant acidic phosphatase (TRAP). Given that TRAP expression develops independently of cell-cell fusion, these findings suggest that Syn-1 either functions in both the fusion stage and the pre-fusion stages.

We uncoupled the cell-fusion step from both pre-fusion stages of osteoclastogenic differentiation and the post-fusion expansion of the nascent fusion connections [Reference 4]. As for myoblast fusion [Leikina E, *et al. J Cell Biol* 2013;200:109] and in our earlier work on osteoclast fusion [Verma SK, Leikina E, Melikov K, Chernomordik LV. *Biochem J* 2014;464:293], we accumulated ready-to-fuse cells in the presence of LPC and then removed it to study synchronized cell fusion. We found that osteoclast fusion requires the DC-STAMP-dependent non-apoptotic exposure of phosphatidylserine at the surface of fusion-committed cells. Fusion also depended on annexins, extracellular phosphatidylserine binding proteins (annexin A5 in human osteoclasts), which, along with annexin-binding protein S100A4, regulated fusogenic activity of syncytin 1. Thus, in contrast to fusion processes mediated by a single protein, such as epithelial cell fusion in *C. elegans*, cell fusion step in osteoclastogenesis is controlled by the phosphatidylserine-coordinated activity of several proteins.

In addition to identification of protein and lipid players in osteoclast fusion, in our recent studies we suggested new ways of unbiased presentation of cell fusion at given conditions that combine the empirical cumulative distribution function for the sizes of multinucleated cells with the total number of cell-cell fusion events, which generate these cells [References 3, 4].

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Building the Zebrafish Lateral Line System

Cells divide, move, adhere, and interact with their neighbors and their environment to determine the formation of multicellular organ systems with unique fates, morphologies, function, and behavior. Our goal is to understand how such interactions determine the self-organization of cell communities in the nervous system of the zebrafish embryo. The lateral line is a mechano-sensory system that helps sense the pattern of water flow over the fish and amphibian body and consists of sensory organs called neuromasts that are distributed in a stereotypic pattern over the body surface. Each neuromast has sensory hair cells at its center, surrounded by support cells, which serve as progenitors for production of more hair cells during growth and regeneration of neuromasts. The development of this superficial sensory system in zebrafish is spearheaded by the posterior lateral line primordia, groups of about 150 cells formed on either side of a day-old embryo near the ear. Cells in the primordia migrate collectively under the skin to the tip of the tail, as they divide and reorganize to form nascent neuromasts that are deposited sequentially from its trailing end. Their journey is easily observed in live transgenic embryos with fluorescent primordium cells. Furthermore, a range of genetic and cellular manipulations can be used to investigate gene function and morphogenesis in the system. Understanding the self-organization of this relatively simple and accessible system in zebrafish will help elucidate the broader principles that determine cell fate specification, morphogenesis, and collective cell migration in the developing vertebrate nervous system.

Self-organization of the zebrafish lateral line primordium

Formation of the posterior lateral line system in zebrafish is pioneered by the posterior Lateral Line (pLL) primordium, a group of about 150 cells that forms near the ear. While leading cells in the pLL primordium have a relatively mesenchymal morphology, trailing cells are more epithelial; they have distinct apical/basal polarity and reorganize to sequentially form nascent neuromasts or protoneuromasts. The pLL primordium begins migration toward the tip of the tail at about 22 hours post fertilization (hpf). Proliferation adds to the growth of the primordium; nevertheless, as the primordium migrates, the length of the column of cells undergoing



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The Whts (Wingless/Integrated) and Fgfs (fibroblast growth factors) are evolutionarily conserved secreted proteins that allow cells to communicate with their neighbors via distinct signaling pathways to influence various aspects of their neighbor's behavior, fate, shape and capacity to proliferate. Wht and Fgf signaling systems coordinate morphogenesis and migration of the primordium. Thus, Wnt signaling dominates at the leading end and is thought to determine the relatively mesenchymal morphology of leading cells, while Fgf signaling dominates in the trailing end. There, Fgf determines reorganization of groups of trailing cells to form rosettes, as the cells constrict at their apical ends. Furthermore, Fgf signaling determines the specification of a central cell in each rosette as a sensory hair cell progenitor and helps determine collective migration of the pLL primordium cells. Wnt signaling promotes its own activity and at the same time drives expression of fgf3 and fgf10. However, leading cells do not respond to these Fgf ligands because Wnt signaling simultaneously promotes expression of intracellular inhibitors of the Fgf receptor. Instead, the Fgfs activate Fgf receptors and initiate Fgf signaling at the trailing end of the primordium, where Wnt signaling is weakest. There, Fgf signaling determines expression of the diffusible Wnt antagonist Dkk1b, which counteracts Wht signaling to help establish stable Fgf-responsive centers. Once established, the trailing Fgf signaling system coordinates morphogenesis of nascent neuromasts by simultaneously promoting the reorganization of cells into epithelial rosettes and by initiating expression of factors that help specify a sensory hair cell progenitor at the center of each forming neuromast. Over time, the leading domain with active Wnt signaling shrinks closer to the leading edge, and additional Fgf signaling centers form sequentially in its wake, each associated with formation of additional protoneuromasts.

The interactions between the leading Wnt system and the trailing Fgf system provide a useful framework for understanding the self-organization of neuromast formation and deposition by the migrating pLL primordium; however, many questions remain unanswered. The Wnt and Fgf signaling systems act simply as a means of communication between cells, and it remains unclear what molecular mechanisms the systems regulate to specifically determine morphogenesis of epithelial rosettes and the collective migration of primordium cells. Furthermore, the mechanics of collective migration in the primordium remains poorly understood, specifically, how the pull of leading cells, which migrate in response to chemokine cues in their path, determines the Fgf-dependent collective migration of trailing cells in the primordium. The summary above suggests that morphogenesis of epithelial rosettes during the assembly of nascent neuromasts is entirely dependent on Fgf signaling. However, it has been observed that, in the absence of collective migration mediated by chemokines in the leading cells, the trailing cells in the primordium come together to form one or two large rosettes. These and other observations related to the changes in the number and size of epithelial rosettes in the presence and absence of collective migration suggest that primordium cells have an inherent potential to form epithelial rosettes and that the formation of rosettes can be influenced by a variety of signaling systems and/or by migratory behavior of leading cells. Our attention has now shifted to answering some of the questions outlined here.

Cxcl12a induces *snail1b* expression to initiate collective migration and sequential Fgf-dependent neuromast formation in the zebrafish pLL primordium.

Our discovery that *snail1b* is expressed in leading cells led us to speculate that its expression might be determined by Wnt signaling and that, as a factor known for its role in promoting epithelial-mesenchymal transition (EMT), it might determine mesenchymal morphology of leading cells. However, our analysis revealed that Wnt signaling does not determine *snail1b* expression in the leading zone. Instead, its expression in the leading zone is promoted by chemokine signals first encountered by leading cells of the primordium, while Fgf signaling prevents *snail1b* expression in trailing cells. The effect of knocking down *snail1b* function was also not what we expected. It did not compromise the ability of leading cells to adopt a mesenchymal morphology. Instead, sequential morphogenesis of epithelial rosettes associated with formation of protoneuromasts in the trailing domain was delayed in snail1b morphants. Our analysis revealed, however, that the delay in protoneuromast formation is not related to a specific role of Snail1b in morphogenesis of epithelial rosettes. Instead, it is indirectly related to the role of Snail1b in helping initiate effective collective migration of the primordium, a role consistent with its previously described role in promoting cell movement. Interestingly, we found that other manipulations that prevent effective primordium migration also cause a similar delay in sequential formation of Fgf signaling centers and associated protoneuromasts and a shrinking of the leading Wht system. Taken together, these observations reveal an unexpected role for collective migration of the primordium in kick-starting sequential formation of additional protoneuromasts. We also showed that problems in initiating collective migration in the primordium may, at least in part, be related to aberrant expansion of the cell-adhesion molecule Epcam into the leading zone and/or the loss of *cdh2* expression from the leading zone of the primordium following knock-down of snail1b function.

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

Many basic aspects of the biology of the pLL primordium, 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 pLL primordium, 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 pLL primordium from early in migration until termination (approximately 24 hours). From this dataset, we constructed digital models of the pLL primordium. We could query the 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 pLL primordium 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 pLL primordium. The fate of deposited cells as central sensory hair cell progenitors, within neuromasts, 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 pLL primordium 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 Fgf 10. 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 of 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 caused by a deficiency in the G6P transporter (G6PT or SLC37A4), and GSD-Irs, also known as severe congenital neutropenia syndrome type 4, is caused 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 on 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 the functionality of neutrophils. GSD-Ia and GSD-lb patients manifest a common metabolic phenotype of impaired glucose homeostasis not shared by GSD-Irs, whereas 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 the enhanced apoptosis that 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 diseases more precisely and develop new treatment approaches, including gene therapy. We have generated several efficacious G6Pase-α–expressing recombinant adeno-associated virus (rAAV) vectors and provided a proof-of-principle gene therapy in murine GSDla that is safe, efficacious, and appropriate for entering clinical trials. The rAAV vector expressing a codon-optimized G6Pase-α developed in this laboratory (US patent number: 9,644,216; European patent number: EP3074510) was selected in 2018 Phase I/II clinical trial for human GSD-Ia (ClinicalTrials.gov Identifier: NCT03517085).

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



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Intramural Research Training Award Fellow (IHCA, 52%) and β -catenin–mutated HCA (bHCA, 28%). We previously showed that non–tumor–bearing (NT), rAAV–treated GSD-la 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 nuclear factor kappaB (NF-kappaB), 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 NF-kappaB were reduced, as were expression of mesenchymal markers, STAT3 targets, NF-kappaB 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/NF-kappaB-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 HCA/HCC. We previously developed a G6Pase- α -expressing rAAV vector, rAAV-G6PC, and showed that rAAV-G6PC-treated $G6pc^{-2}$ 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 rAAVco-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-q activity maintained glucose homeostasis, developed no 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-a 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



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 PPARy, another lipogenic factor. Moreover, aberrant PPARy overexpression aggravates hepatic steatosis. The net outcome is downregulation of hepatic SIRT1 signaling. Impaired signaling by SIRT1 increases acetylation of ATG, reduces ATG12-ATG5 conjugation, and downregulates FoxO signaling, which induces autophagy genes. Accordingly, hepatic G6Pase-α deficiencymediated 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.

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 less than 6% 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.

Hepatic G6Pase- deficiency leads to metabolic reprogramming in GSD-Ia.

We had shown that hepatic G6Pase- α deficiency-mediated steatosis leads to defective autophagy that is frequently associated with carcinogenesis, and we recently showed that hepatic G6Pase- α deficiency also leads to enhancement of hepatic glycolysis and hexose monophosphate shunt (HMS), which can contribute to hepatocarcinogenesis. The enhanced hepatic glycolysis is reflected in increased lactate accumulation, increased expression of many glycolytic enzymes, and elevated expression of *c-Myc*, which stimulates glycolysis. The enhanced HMS is reflected in elevated G6P dehydrogenase activity, elevated production of NADPH, and reduced glutathione. We showed that that restoration of hepatic G6Pase- α expression normalizes both glycolysis and HMS in GSD-Ia. Moreover, the HCA/HCC lesions in L-*G6pc^{-/-}* mice exhibit elevated levels of hexokinase 2 and the M2 isoform of pyruvate kinase, which play an important role in aerobic glycolysis and cancer cell proliferation. Taken together, hepatic G6Pase- α deficiency causes metabolic reprogramming, leading to enhanced glycolysis and elevated HMS, which, along with impaired autophagy, can contribute to HCA/HCC development in GSD-Ia.

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Chromatin Remodeling and Gene Activation

Our main objective is to understand how genes are activated for transcription in the context of chromatin structure. Chromatin is generally repressive in nature, but its structure is manipulated by cells in a regulated way to determine which genes are transcriptionally active and which remain repressed in a given cell type. Chromatin is not just a packaging system for DNA in eukaryotic cells but also participates in gene regulation, which involves either attenuation of the inherently repressive properties of nucleosomes to facilitate gene expression, or enhancement of those properties to ensure complete repression, events that are choreographed by DNA sequence–specific transcription factors (activators and repressors) and chromatin-remodeling complexes. The complexes include histone- or DNA–modifying enzymes that implement the "epigenetic code", and ATP–dependent remodeling machines that move or displace nucleosomes.

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. The octamer is 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 beadson-a-string structure folds spontaneously to form a fiber about 30 nm wide, assisted by the linker histone (H1), which binds to the nucleosome core and to the linker DNA. Thus, collectively, the histones determine 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. Nucleosomes 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, histonemodifying complexes, and histone chaperones. The remodeling machines (e.g., the SWI/SNF, RSC, CHD, and ISWI complexes) use ATP to move nucleosomes along or off DNA or to substitute histone H2A in a nucleosome with the H2A.Z variant (e.g., the SWR complex). The histone-modifying complexes contain enzymes



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LauraAnn Schmidberger, BS, Postbaccalaureate Fellow 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 (e.g., Asf1 and the CAF-1 complex) mediate the histone transfer reactions that occur during transcription and DNA replication. These enzymes, together with DNA-methylating and demethylating enzymes, are central to epigenetics.

Aberrant gene regulation is the basis of many disease states, and 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.

We are exploiting and developing high-throughput technologies to obtain genome-wide maps of nucleosomes, chromatin-remodeling complexes and RNA polymerase II, etc. We have made major progress in our genomics studies, primarily using budding yeast (*Saccharomyces cerevisiae*) as a model organism. Budding yeast possesses homologs of most chromatin-modifying enzymes found in mice and humans. It provides a simple, experimentally tractable model for understanding the functions and mechanisms of action of these enzymes. Such studies are facilitating the design and execution of the more complex and difficult experiments that are necessary to understand the roles of chromatin-remodeling enzymes in higher organisms.

The current objectives of our yeast studies are to: (1) determine the roles of various chromatin remodeling complexes (RSC, SWI/SNF, ISW1, ISW2, and CHD1) in chromatin organization and gene expression, which are important because genes encoding homologous subunits of some of these enzymes are often mutated in various cancers; and (2) understand the nature and role of stable non-histone "barrier" complexes bound at active promoters. Through collaborations, we are now extending our studies on chromatin remodeling from yeast to mouse.

Roles of the SWI/SNF and RSC chromatin-remodeling complexes in gene activation by the Gcn4 transcription factor

We continued our yeast studies in collaboration with Alan Hinnebusch's lab. In our first collaborative project [Reference 1], we investigated transcriptional activation by the well studied yeast transcription factor Gcn4 *in vivo*. Gcn4 production is under translational control and is induced by amino acid starvation. Gcn4 then binds to and activates transcription from specific promoters for genes encoding proteins required for amino acid biosynthesis. We expected that specific Gcn4 binding would be limited to promoter sites. Surprisingly, our ChIP-seq data for Gcn4 showed that the majority of sequence-specific DNA binding sites are not in gene promoters, but inside coding regions. Moreover, Gcn4-bound sites within coding regions are not associated with the nucleosome-depleted regions (NDRs) typically observed at promoter sites. Nevertheless, we found that many of the internal Gcn4 sites activate cryptic internal promoters and, in some cases, neighboring promoters, in a manner reminiscent of the transcription enhancers typically found in higher eukaryotes (Figure 1).



FIGURE 1. Two modes of gene activation by the yeast Gcn4 transcription activator

1. Conventional promoter binding by Gcn4 results in transcription by Pol II.

2. Internal bidirectional cryptic promoter activation by Gcn4. Adapted from Reference 1.

In a second collaborative project with the Hinnebusch lab and with Chhabi Govind's lab, we explored the roles of the related RSC and SWI/SNF remodelers in gene activation [Reference 2]. We had previously shown that the RSC nucleosome remodeling complex functions throughout the yeast genome to set the positions of the nucleosomes flanking promoters (the +1 and -1 nucleosomes), thereby determining the widths of the NDRs. The related SWI/SNF complex participates in nucleosome remodeling/eviction and promoter activation at a subset of yeast genes, including those activated by Gcn4, but does not act globally to establish NDRs. By analyzing the large cohort of Gcn4–induced genes in mutants lacking the catalytic subunits of SWI/SNF or RSC, we uncovered cooperation between the two remodelers during transcriptional activation, which together widen the NDR. SWI/SNF functions on a par with RSC at the most highly transcribed, constitutively expressed genes, suggesting general cooperation by these remodelers for maximal transcription (Figure 2). SWI/SNF and RSC occupancies are greatest at the most highly expressed genes, consistent with cooperative functions in nucleosome remodeling and transcriptional activation. At less active promoters, RSC is more important for chromatin remodeling than SWI/SNF.

Major determinants of nucleosome positioning

The compact structure of the nucleosome limits DNA accessibility and inhibits the binding of most sequence-specific proteins. Nucleosomes are not randomly located on the DNA but positioned with respect to the DNA sequence, suggesting models in which critical binding sites are either exposed in the linker, resulting in activation, or buried inside a nucleosome, resulting in repression. The mechanisms determining nucleosome positioning are therefore of paramount importance for understanding gene regulation and other events that occur in chromatin, such as transcription, replication, and repair. In a review [Reference 3], we assessed our current understanding of the major determinants of nucleosome positioning: DNA sequence, non-histone DNA-binding proteins, chromatin-remodeling enzymes, and transcription. We outline the major challenges for the future: elucidating the precise mechanisms of chromatin opening and promoter activation, identifying the complexes that occupy promoters, and understanding the multiscale problem of chromatin fiber organization.



FIGURE 2. Model for cooperation between chromatin remodelers SWI/SNF and RSC in promoter nucleosome disassembly and re-positioning at highly expressed yeast genes

A. At weakly expressed genes, relatively low levels of RSC (green oval) suffice to maintain wild-type NDR formation, primarily by keeping nucleosomes (*sectored cylinders*) from crossing NDR boundaries. Cylinders with faint shades indicate nucleosome positions in cells depleted of functional RSC.

B. Upon gene activation by Gcn4 or at highly expressed constitutive genes, SWI/SNF (red oval) and RSC are actively recruited and cooperate in evicting and repositioning the –1 nucleosome and the +1 nucleosome to both widen the NDR and keep nucleosomes from assembling there. Cylinders with faint shades indicate nucleosome positions in cells lacking SWI/SNF or depleted of RSC. Pol II may also contribute to eviction of +1 nucleosomes. Adapted from Reference 2.

Conventional and pioneer modes of glucocorticoid receptor interaction with enhancer chromatin *in vivo*

The mechanism by which glucocorticoid hormone controls gene expression is central to an understanding of its role in human metabolism and disease. When the hormone enters a cell, it binds to the glucocorticoid receptor (GR), inducing its translocation from the cytosol to the nucleus, where it binds to glucocorticoid response elements (GREs) to activate or repress target genes. Importantly, GR binds to distinct GREs in different types of cell, resulting in different patterns of gene expression. This observation raises the question as to what distinguishes those sites that are bound from those that are not. Although the answer is unclear, chromatin structure clearly plays a major role. We collaborated with Gordon Hager's lab to study the interaction of the glucocorticoid receptor with enhancer chromatin in mouse adenocarcinoma cells [Reference 4]. We found that enhancers containing GREs occur in one of three major chromatin architectures: they can be nucleosome-depleted, marked by the histone variant H2A.Z in the flanking nucleosomes and associated with the Brg1 SWI/SNF chromatin remodeler; they can be covered by a nucleosome containing H2A.Z and associated with Brg1; or they can be nucleosomal and lack both H2A.Z and Brg1. Hormone-induced GR binding results in nucleosome shifts and increased levels of Brg1 at all three types of GR enhancer. We propose that nucleosome-depleted GR enhancers are created and maintained by hormone-independent transcription factors that recruit Brg1, allowing GR to bind to non-nucleosomal DNA (a "conventional" mode of transcription factor binding). In contrast, at nucleosomal GR enhancers, GR binds like a pioneer factor (i.e., a transcription factor that binds to protein-free and nucleosomal cognate sites with similar affinities) and then recruits Brg1 to remodel the local chromatin structure, promoting downstream events in gene regulation (Figure 3).

FIGURE 3. Conventional and pioneer modes of glucocorticoid receptor (GR) binding to responsive enhancers

A. GR does not bind to unresponsive enhancers. They are nucleosomal, inaccessible to DNase I, do not incorporate the histone variant H2A.Z, and lack the Brg1 subunit of the mouse SWI/SNF complex.

B. GR binds to pre-programmed nucleosome-depleted enhancers. The nucleosome-depleted region (NDR) may be created by other transcription factors bound to their cognate sites near the Glucocorticoid Response Element (GRE), in concert with the Brg1 complex, a chromatin remodeler capable of shifting nucleosomes. The NDR is flanked by nucleosomes incorporating H2A.Z and is accessible to DNase I. In response to hormone, GR binds to the GRE in the NDR, and the flanking nucleosomes are shifted aside (black arrows), presumably by Brg1.

C. GR binds to pre-programmed DNase I– accessible nucleosomal enhancers. The GRE is covered by a nucleosome incorporating



H2A.Z and associated with Brg1, both contributing to DNase I accessibility. In response to hormone, GR binds to the GRE, recruits more Brg1, and the nucleosome is shifted aside.

D. GR binds to DNase I–insensitive nucleosomal enhancers. The GRE is covered by a nucleosome that lacks H2A.Z and Brg1. In response to hormone, GR binds to the GRE, recruits Brg1, and the nucleosome is shifted. Adapted from Reference 3.

SPT6 interacts with NSD2 and facilitates interferon-induced transcription.

Transcription through chromatin by RNA polymerase II is facilitated by various factors, including the SPT6 histone chaperone. We collaborated with Keiko Ozato's lab to study the role of SPT6 in interferon-induced transcription in mouse cells [Reference 5]. We found that SPT6 recruitment to interferon-induced genes depends on an interaction with the histone H3-K36 methyltransferase NSD2, which boosts transcription of these genes.

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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 the 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.

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 aminoacid 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*D*DDD in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide).



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FIGURE 1. Two RNase H2 mutants with differential Ribonucleotide Excision Repair activity reveal a threshold of rNMPs for embryonic development.

RNase H2 has two distinct functions: initiation of the Ribonucleotide Excision Repair (RER) pathway by cleaving ribonucleotides (rNMPs) incorporated during DNA replication and processing the RNA portion of an R-loop formed during transcription. An RNase H2 mutant that lacked RER activity but supported R-loop removal revealed that rNMPs in DNA initiate p53dependent DNA damage response and cause early embryonic arrest in the mouse. However, an RNase H2 AGS-related mutant with residual RER activity develops to birth. Estimations of the number of rNMPs in DNA in these two mutants define a ribonucleotide threshold



above which p53 induces apoptosis. Below the threshold, rNMPs in DNA trigger an innate immune response. Cells containing both defective enzymes retain rNMPs above the threshold, indicating competition for RER substrates between active and inactive enzymes and suggesting that patients with compound heterozygous mutations in *RNASEH2* genes may not reflect the properties of recombinantly expressed proteins.

Several types of RNA/DNA hybrid structures are formed, and they 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. Such 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 distinct 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 such mutation, *RNASEH2A–G37S* (G37S), has significant loss of RNase H2 activity. Using the 3D structure of the human enzyme that 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 and in 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 blocking either an innate or adaptive immune response. In contrast, the G37Smutant perinatal lethality and the fact that RNase H2 KO mice die during early 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 to 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 into 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 cause *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 that 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 yeast, we demonstrated that yeast producing the RNase H2 ^{RED} enzyme acted *in vivo* by leaving embedded ribonucleotides (rNMPs) in DNA but was potent in removing 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 results in embryonic death. *Rnaseh2a*^{G375/RED} embryos also arrest at approximately the same stage as *Rnaseh2a*^{RED/RED} embryos because of better association of RNase H2^{RED} than RNase H2^{G375} with 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.

Detection of a threshold of ribonucleotide tolerance in DNA for embryonic development

Embryonic development in the absence of RNase H2 exhibits defects as early as E6.5 to 7.5, the period of gastrulation in which cell numbers double every 4–5 h. Previous studies have suggested that the high retention of rNMPs incorporated during DNA replication lead to p53-dependent DNA damage. We provided evidence for the prior speculation that rNMPs are indeed the cause of embryonic lethality. We used mice with a separation of function in the RNase H2 enzyme (RNase H2^{RED}) that retained RNA/DNA hydrolysis but was unable to remove rNMPs in DNA. Embryonic development was arrested at E10, the same day as seen for embryos with complete loss of both of RNase H2's functions. When there is complete loss of both functions, the abundance of rNMPs in DNA is about 65% of that seen in mouse embryo fibroblast cells. A mouse (RNase H2^{G37S}) with partial loss of both RNase H2 activities develops to birth and retains about 30% of the number of rNMPs in DNA compared with the cells with complete loss of RNase H2. A compound heterozygous mouse in which both RNase H2^{RED} and RNaseh2^{G375} are present is also early embryonic lethal, retaining about 40% as many rNMPs in as in the deletion cells. Embryos with complete loss of RNase H2, RNase H2^{RED}, and RNase H2^{RED/G37S} all exhibit a p53-dependent DNA-damage response. In contrast, mice with RNase H2^{G37S} develop to birth with little or no p53-dependent DNA damage. The weights of the embryos in which there is p53 DNA damage are only one to a few mg, whereas the RNase H^{G375} mouse is about 1000 mg at birth, an enormous difference, which indicates more than normal levels of rNMPs do not necessarily cause embryonic lethality. We conclude that a threshold of tolerance of rNMPs in DNA for embryonic development past E10 is exceeded in all mouse strains tested except RNase H^{G375}. Human patients with the *RNASEH2A* G37S mutation have Aicardi-Goutières syndrome. Although the patients with RNASEH2A G37S mutations are homozygous, similar to our RNase H2^{RED/G37S} mice, some AGS patients are compound heterozygous, with each allele having a different mutation in the same RNase H2 gene. In vitro studies of mutant forms of RNase H2 mutations reflect the properties of the RNase H2 mutant but may be unreliable for assessing the contribution of each of the two forms of RNase H2 when both are present *in vivo*. The strong effect on the stage of lethality of RNase H2^{RED/G37S} embryos indicates a competition between the rNMP active RNase H2^{G37S} and the inactive RNase H2^{RED} for some step in removal of rNMPs. The protein proliferating cell nuclear antigen (PCNA) is a critical component in removing rNMPs in DNA. We suggest that the competition between RNase H2RED and RNase H2G37S occurs when RNase H2 interacts with PCNA to repair rNMPs in DNA rather than binding to rNMPs in DNA.

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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 adapted CRISPR-based degron strategies to the study of these proteins; namely, we tagged the proteins with AID (auxininduced degrons; a degron is a short portion of a protein important for protein degradation rates), 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



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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*).

in each of these contexts has been complicated by their multi-faceted nature: for example, RNAi (RNA interference)–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 decline.

To better address the role of individual nucleoporins, we adapted 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 remaining nucleoporins, as well as collaborating extensively with groups who have developed approaches to address other issues of nucleoporin function.

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, for technical reasons these possibilities remain largely unexplored because the abundance and stability of nucleoporins makes



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 TIR1expressing 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).

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 the interpretation of resultant phenotypes. The rapid degradation of nucleoporins using AID-tagged cell lines has helped circumvent some 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 distinct NPC structural requirements. Third, we are examining gene expression by RNA-seq (RNA sequencing) 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 the nucleus, while GDP-bound Ran (Ran-GDP) is the predominant form in the 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. During 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 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, in vertebrate systems, is regulated in a particularly dramatic fashion during anaphase. The regulation has not been correlated with post-translational modifications of RCC1, and the underlying molecular mechanism has not been reported.

RanBP1 is a highly conserved Ran–GTP–binding protein, which acts as co-activator of RanGAP1 (Ran GTPase–Activating Protein 1) 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 determine whether RanBP1 plays an analogous role in mammalian cells during mitosis.

FIGURE 3. The SUMO pathway

SUMO proteins are post-translationally processed (step 1). Processed SUMO polypeptides possess a C-terminal diglycine motif, which is activated to form an ATPdependent 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 (<u>Ubiquitin-like protein proteases</u>) in yeast and SENPs (<u>Sentrin-specific protease</u>) 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 with the nuclear pore complex.

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 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 posttranslational 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,

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 were stained with DNA dye Hoechst 33258 (blue), and antibodies against Prospero (EE cells) and Escargot (EB and ISC cells). We observed a rapid and progressive increase in the fraction of Escargot-positive cells in the IRBIT^{-/-} flies over time. In conjunction with additional experiments, the 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.

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*). Therefore, 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 highly expressed 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 tissuearchitecture 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 of full-length IRBIT, and further experiments suggested that altered dNTP pools likely 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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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 that of 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. However, as we age, 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.

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 can 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. The mutations, which result from DNA replication errors as well as environmental insults, fall into two categories: cancer-driver mutations that initiate carcinogenesis, and genome-destabilizing mutations that promote aneuploidy



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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. The 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.

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, we inoculated immune-compromised mice subcutaneously with ESCs harboring conditional *Gmnn* alleles alone or together with a tamoxifen-dependent Cre recombinase gene. We then injected the mice 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.

DHS (trans-4,4-dihydroxystilbene) suppresses DNA replication and tumor growth by inhibiting ribonucleotide reductase regulatory subunit M2 (RRM2).

Given that inhibition of DNA replication can lead to replication fork stalling, resulting in DNA damage and apoptotic death, inhibitors of DNA replication are commonly used in cancer chemotherapy. Ribonucleotide reductase (RNR) is the rate-limiting enzyme in the biosynthesis of deoxyribonucleoside triphosphates (dNTPs), which are essential for DNA replication and DNA-damage repair. Gemcitabine, a nucleotide analog that inhibits RNR, has been used to treat various cancers. However, patients often develop resistance to this drug during treatment. Thus, the development of new drugs that inhibit RNR is needed. We identified a synthetic analog of resveratrol (3,5,4'-trihydroxy-trans-stilbene), termed DHS (trans-4,4'-dihydroxystilbene), that acts as a potent inhibitor of DNA replication. Molecular docking analysis identified the RRM2 of RNR as a direct target of DHS. At the molecular level, DHS induced cyclin F-mediated down-regulation of RRM2 by the proteasome. Thus, treatment of cells with DHS reduced RNR activity and consequently the synthesis of dNTPs, with concomitant inhibition of DNA replication, arrest of cells at S-phase, DNA damage, and finally apoptosis. In mouse models of tumor xenografts, DHS was efficacious against pancreatic, ovarian, and colorectal cancer cells. Moreover, DHS overcame both gemcitabine resistance in pancreatic cancer and cisplatin resistance in ovarian cancer. Thus, DHS is a novel anti-cancer agent that targets RRM2 with

therapeutic potential either alone or in combination with other agents to arrest cancer development.

CDK1 inhibition facilitates formation of syncytiotrophoblasts and expression of human chorionic gonadotropin.

Human placental syncytiotrophoblast (STB) cells play essential roles in embryo implantation and nutrient exchange between the mother and the fetus. STBs are polyploid cells, which are formed by fusion of diploid cytotrophoblast (CTB) cells. Abnormality in STB formation can result in pregnancy-related disorders. While several genes have been associated with CTB fusion, the initial events that trigger cell fusion are not well understood. Our primary objective was to enhance our understanding of the molecular mechanism of placental cell fusion.

We used FACS (fluorescence-activated cell sorting) and microscopic analysis to optimize Forskolin-induced fusion of BeWo cells (surrogate of CTBs) and we subsequently analyzed changes in the expression of various cell-cycle regulator genes through Western blotting and qPCR. We performed immunohistochemistry on first-trimester placental tissue sections to validate the results in the context of placental tissue. We studied the effect of the cyclin-dependent kinase 1 (CDK1) inhibitor RO3306 on BeWo cell fusion using microscopy and FACS, and we monitored the expression of human chorionic gonadotropin (hCG) with Western blotting and qPCR (quantitative polymerase chain reaction).

The data showed that the placental cell fusion was associated with down-regulation of CDK1 and its associated cyclin B, and significant reductions in DNA replication. Moreover, inhibition of CDK1 by an exogenous inhibitor induced placental cell fusion and expression of hCG. We thus showed that placental cell fusion can be induced by inhibiting CDK1. The study has a high therapeutic significance to manage pregnancy related abnormalities.

A family of PIKFYVE inhibitors with therapeutic potential against autophagy-dependent cancer cells disrupt several events in lysosome homeostasis.

High-throughput screening identified five chemical analogs (termed the WX8-family) that disrupted three events in lysosome homeostasis: (1) lysosome fission via tubulation without preventing homotypic lysosome fusion; (2) trafficking of molecules into lysosomes without altering lysosomal acidity; and (3) heterotypic fusion between lysosomes and autophagosomes. Remarkably, these compounds did not prevent homotypic fusion between lysosomes, despite the fact that homotypic fusion required some of the same machinery essential for heterotypic fusion. The effects varied 400-fold among WX8-family members, were time- and concentration-dependent, reversible, and resulted primarily from their ability to bind specifically to the PIKFYVE phosphoinositidekinase. The ability of the WX8 family to prevent lysosomes from participating in autophagy suggested that they have therapeutic potential in treating autophagy-dependent diseases. In fact, the most potent family member (WX8) was 100-times more lethal to 'autophagy-addicted' melanoma A375 cells than the lysosomal inhibitors hydroxychloroquine and chloroquine. In contrast, cells that were insensitive to hydroxychloroquine and chloroquine were also insensitive to WX8. Therefore, the WX8 family of PIKFYVE inhibitors provides a basis for developing drugs that could selectively kill autophagy-dependent cancer cells, as well as increasing the effectiveness of established anti-cancer therapies through combinatorial treatments.

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Mechanism and Regulation of Eukaryotic Protein Synthesis

We study the mechanism and regulation of protein synthesis, focusing on GTPases, protein kinases, translation factors and mRNA features 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 elF2alpha. We are characterizing elF2gamma mutations that are associated with 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 polyamineregulated gene-specific translational control mechanisms.

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

In collaboration with several other researchers including Lina Basel-Vanagaite, Guntram Borck, Vera Kalscheuer, Daniela Gasperikova, and Clesson Turner, we found that the human MEHMO syndrome, an X-linked intellectual disability (XLID) syndrome, is caused by mutations in the *EIF2S3* gene, which encodes the translation factor eIF2gamma. MEHMO syndrome is named based on the constellation of patient phenotypes: intellectual (<u>m</u>ental) disability, <u>e</u>pilepsy, <u>hypogonadism and hypogenitalism, <u>m</u>icrocephaly, and <u>obesity</u>. Genetic and biochemical studies of a yeast model of the first-characterized *EIF2S3* mutation linked to MEHMO syndrome revealed that the mutation disrupts eIF2 complex integrity and translation start-codon selection. Our studies on yeast models of</u>



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additional MEHMO syndrome mutations in elF2gamma likewise revealed impaired elF2 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 elF2alpha phosphorylation, is induced in patient cells. The findings directly link intellectual disability with impaired translation initiation and provide a mechanistic basis for MEHMO syndrome resulting from partial loss of elF2 function [Reference 1]. Our studies linking altered protein synthesis with intellectual disability are consistent with the critical role of protein synthesis in learning and memory in model systems. Based on our studies, we propose that more severe *ElF2S3* mutations cause the full MEHMO phenotype, while less deleterious mutations cause a milder form of the syndrome with only a subset of symptoms. Ongoing studies of additional MEHMO syndrome mutations in elF2gamma reveal protein synthesis defects associated with altered binding of the initiator methionyl-tRNA to elF2.

Molecular analysis of the hypusine-containing protein eIF5A and polyamine control of protein synthesis

In a series of molecular-genetic and biochemical studies, we found that the translation factor eIF5A, the sole protein containing the unusual amino acid hypusine [*N*^e-(4-amino-2-hydroxybutyl)]ysine], promotes translation elongation, an activity that depends on the hypusine modification. Using *in vivo* reporter assays, we showed that eIF5A in yeast, like its bacterial homolog EF-P, is critical for the synthesis of proteins containing runs of three or more consecutive proline residues. Consistent with our *in vivo* findings, we showed that eIF5A was necessary 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.

In collaboration with Rachel Green, we reported that eIF5A functions globally to promote both translation elongation and termination. Moreover, utilizing our *in vitro* reconstituted assay system, we showed that the structural rigidity of the amino acid proline contributes to its heightened requirement for eIF5A, and that eIF5A could functionally substitute for polyamines to stimulate general protein synthesis [Reference 2]. 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 3].

The eIF5A occupies the E site of the ribosome, with the hypusine residue projecting toward the acceptor stem of the P-site tRNA. Our studies suggest a function for eIF5A and its hypusine residue in repositioning the peptidyl-tRNA [Reference 3]. 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 P-site tRNA to promote peptide bond formation and that the body of eIF5A functions like polyamines to enhance general protein synthesis.

Over the last year, we linked eIF5A to the regulation of polyamine metabolism in mammalian cells [Reference 4]. The enzyme ornithine decarboxylase (ODC) catalyzes the first step in polyamine synthesis. ODC is regulated by a protein called antizyme, which, in turn, is regulated by another protein called antizyme inhibitor (AZIN1). The synthesis of AZIN1 is inhibited by polyamines, and this regulation is dependent on a conserved element in the 5' leader of the AZIN1 mRNA, which we refer to as a uCC (for upstream <u>C</u>onserved

Coding region.) Whereas translation initiation is typically restricted to AUG codons, and scanning eukaryotic ribosomes inefficiently recognize near-cognate start codons, we found that high polyamine levels enhance translation initiation from the near-cognate start site of the uCC. Surprisingly, the regulation is dependent on the sequence of encoded polypeptide. Ribosome profiling revealed polyamine-dependent pausing of elongating ribosomes on a conserved Pro-Pro-Trp (PPW) motif in the uCC. Mutation of the PPW motif impaired initiation at the near-cognate AUU start codon of the uCC and abolished polyamine control, leading to constitutive high-level expression of AZIN1. In contrast, substituting an alternate elongation pause sequence restored uCC translation. We proposed that most scanning ribosomes bypass the near-cognate start codon of the uCC without initiating and then translate AZIN1. However, a ribosome will occasionally initiate translation at the uCC start codon. Under conditions of high polyamine levels, the elongating ribosomes pause on the PPW motif. The paused ribosome serves as a roadblock to the scanning ribosomes that bypass the near-cognate start codon. The resultant queuing of scanning ribosomes behind the paused elongating ribosome positions a ribosome near the start site of the uCC, providing greater opportunity for initiation at the weak start site. Consistent with the notion that ribosome queuing is important for uCC translation, impairing ribosome loading reduced uCC translation and derepressed AZIN1 synthesis. We believe that the mechanism whereby a paused elongating ribosome promotes initiation at an upstream weak start site via ribosome queuing may underlie the control of translation of other mRNAs, especially those whose translation is derepressed by conditions that impair ribosome loading.

In further studies on the AZIN1-regulatory mechanism, we identified eIF5A as a sensor and effector for polyamine control of uCC translation. Using our reconstituted *in vitro* translation assay system, we found that synthesis of a PPW peptide, like translation of polyproline sequences, requires eIF5A. Moreover, the ability of eIF5A to stimulate PPW synthesis was inhibited by polyamines. Thus, we propose that polyamine inhibition of eIF5A serves as the trigger to cause the ribosome pause that governs uCC translation. Taken together, our studies showed that eIF5A functions generally in protein synthesis and that modulation of eIF5A function by polyamines can be exploited to regulate specific mRNA translation [Reference 4].

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

We also study 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 the rationale for its evolutionary conservation are not well understood, and, until now, 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, we found 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 bypasses canonical translation initiation and instead requires novel eEF2-directed pseudo-translocation reactions prior to peptide bond formation, was sensitive to the loss of diphthamide. 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 the notion that diphthamide facilitates proper translocation, our collaborators in Venki Ramakrishnan's lab used electron cryomicroscopy to reveal 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 appeared 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]. In ongoing studies, we are further exploring the role of diphthamide in promoting the accuracy and efficiency of translation elongation.

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, and, paradoxically, enhances the translation of a select group of mRNAs containing upstream open reading frames like the GCN4 mRNA in yeast and the ATF4, CHOP, and GADD34 mRNAs in mammalian cells.

We previously used structural, molecular, and biochemical studies to define how the elF2alpha kinases recognize their substrate. In collaboration with Frank Sicheri, we obtained the X-ray structure of elF2alpha 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 elF2alpha substrate recognition. We also studied how the protein phosphatase PP1 is targeted to dephosphorylate elF2alpha. We showed that a novel N-terminal extension on yeast elF2gamma binds to yeast PP1 (GLC7) and targets GLC7 to dephosphorylate elF2alpha. Moreover, we reconstituted the function of the human PP1 targeting subunit GADD34 in yeast cells and mapped a novel elF2alpha-binding motif to the C-terminus of GADD34. Interestingly, we showed that a conserved elF2alpha dephosphorylation. Taken together, our data demonstrate that GADD34 and its viral orthologs direct specific dephosphorylation of elF2alpha by interacting with both PP1 and elF2alpha through independent binding motifs.

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.

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Regulation and Functional Relevance of Retrograde Axonal Transport in Axons

Cytoplasmic dynein is the single motor responsible for microtubule minus-end (cell body-directed) axonal transport. The importance of this retrograde motor to neural health is apparent: mutations in the dynein-dynactin complex are causal in subtypes of neurological disease. Additionally, the abnormal localization of dynein-dependent cargos is associated with disease states. Though dynein function appears essential for neural health, the mechanisms that govern precise cargo movement by this motor and how that impacts neural circuit structure and function are almost completely unknown. One dynein cargo of critical importance to axonal physiology is the mitochondrion. Axons depend on the proper localization of mitochondria for sufficient ATP synthesis and calcium buffering, as well as for lesser known functions such as production or metabolites, synthesis of signaling molecules, and iron homeostasis. Abnormalities in mitochondrial localization are correlated with neurological disease; however, whether there is a causal relationship between organelle movement and disease has been difficult to determine. Our work on mitochondrial transport and function in relation to neural circuit activity aims at an understanding of the regulation of retrograde axonal transport and how it impacts the nervous system.

Using novel tools that we developed, namely, forward and reverse genetics, advanced imaging of intracellular phenomena *in vivo*, and analyses of neural circuit function, we interrogate the molecular regulation of cargo-specific retrograde transport in axons and determine the role that movement of each of these cargos plays in the formation and maintenance of functional neural circuits.

Regulation and functional significance of retrograde axonal transport

MITOCHONDRIAL RETROGRADE TRANSPORT MECHANISMS AND FUNCTION

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



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A. 5-dpf (days post-fertilization) *TgBAC(neurod:egfp)*ⁿ¹¹ transgenic zebrafish larva expressing GFP in all neurons. Mitochondrially localized TagRFP mosaically expressed.

A`. Posterior lateral line (pLL) ganglion with one neuron expressing TagRFP in mitochondria (circled). Arrowheads point to mitochondria in the single labeled axon.

A``. Mitochondrial transport in a single pLL axon. Arrowhead: retrograde. Arrow: antereograde.



A```. Arrowheads point to mitochondria in the axon terminal of this system.

B. Mitochondrial accumulation in actr10-mutant axon terminals (arrows).

C. Retrograde mitochondrial transport is disrupted in this mutant strain.

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 Actr10, a protein known to associate with the dynein motor. 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 (Figure 1). 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 working to address the normal rate and function of mitochondrial turnover in axons. For this, we made a stable transgenic zebrafish line expressing the photo-convertible protein mEos in the inner mitochondria had evacuated the axon terminal and new (unconverted) had taken their place (Figure 2). This is in contrast to the predictions based on shorter-term imaging done on the order of minutes in cultured neurons. The findings demonstrate the power of an entirely *in vivo* system for observing cellular



FIGURE 2. Mitochondria turn over in axon terminals within hours.

A. Photoconversion of mEos in the inner mitochondrial membrane space labels axon terminal mitochondria (*magenta* in A'). Twenty-four hours after conversion, no converted (old) mitochondria remain.

B. Quantification of old (*red*) to new (*green*) mitochondria before (pre), just after (post), and 24hr after (24hr post) conversion.

C. Time-lapse imaging revealed that mitochondrial turnover occurs about 3hrs after conversion in 4–5 dpf (days post-fertilization) larvae.

phenomena on a time-scale relevant to the organism. Finally, in collaboration with the Kindt lab, we are investigating 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*.

MICROTUBULES, DYNEIN STABILITY, AND NUDC

Our forward-genetic screen yielded several other mutant lines, including one with a loss-of-function mutation in the dynein-associated protein NudC. In mammalian systems, loss of NudC results in failed mitosis and abnormal neuronal migration. The function of NudC in these phenomena is still under investigation. During cell division, loss of NudC results in abnormal microtubule orientation and failure of kinetochore complex formation. Work in migrating neurons indicated that loss of NudC impacts the stability of the dynein motor protein complex. Whether NudC has similar or disparate functions during different stages of development in neurons is thus still highly debated. The NudC mutant identified in our screen will allow us to address the role of NudC in mature axons *in vivo*.

NudC mutants do not phenocopy dynein or dynactin loss-of-function mutants, with only axon branchpoint swellings in distal axons and no signs of either axonal or retinal degeneration. In collaboration with the Petralia lab, we used transmission electron microscopy to reveal that loss of NudC results in multilamellar body formation in the swellings (Figure 3). *In vivo* analyses of axonal transport of autophagosome-and endosome-related cargos revealed that most show disruption of their axonal transport; however, the disruptions are not consistent, leading us to ask whether the structure or arrangement of the microtubule cytoskeleton is effected in this line. Currently, we are using pharmacology, live imaging of microtubule dynamics, and expansion microscopy to gain insight into the structure, stability, and dynamics of the microtubule cytoskeleton in this line to understand the function of NudC in a mature axon. The culmination of this work will allow us to determine whether NudC serves a conserved function in developing neurons, which, when disrupted, leads to varying phenotypes based on developmental state.



FIGURE 3. *nudc* mutants show multilamellar body formation in axon terminal branchpoint swellings.

A. Wild-type axon terminal of an afferent axon terminal in the posterior lateral line system of a zebrafish larva at 4 dpf (days post-fertilization).

B. nudc axon terminal branchpoints show swellings by this time-point (arrow).

Bottom. Axon branchpoint swellings in *nudc* mutants show multilamellar body formation.

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 disease. Despite the importance of the process, little is known about how various cargoes attach to and are then 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 the immuno-labeling approaches and *in vivo* imaging techniques that we previously developed to determine whether the strains have deficits in the retrograde transport of specific cargoes. We can use the mutant strains to first identify the proteins involved in the retrograde transport of particular cargoes and then 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 (PRLR) receptor. 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 prolactin (PRL), estradiol and its receptor (liganded or un-liganded), epidermal growth factor (EGF), and 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, as well as 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.

The luteinizing hormone receptor

The luteinizing hormone receptor (LHR) is expressed primarily in the gonads, where it mediates LH signaling, which regulates ovarian and testicular function. The human *LHR* gene is transcriptionally controlled by diverse networks, in which coordination and interactions between regulatory effectors are essential for silencing/ activation of *LHR* expression. The proximal Sp1 promoter site for transcription factor Sp1 recruits histone (H) deacetylases (HDAC) and the Sin3A corepressor complex, which contribute to the silencing of *LHR* transcription. Site-specific acetylation/methylation induced by trichostatin A (TSA), an inhibitor of histone deacetylase, causes phosphatase release, which serves as an on switch for Sp1



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Activation of the *LHR* gene is achieved by the combined actions of chromatin changes, release of inhibitory factors (p107, HDAC1/2/mSin3A, and phosphatase PP1), phosphorylation of the transcription factor Sp1 at Ser641 by PI3K/PKC zeta, and recruitment of Positive Cofactor 4 (PC4). PC4 functions as a Sp1 coactivator and as a linker to bridge Sp1 to acetylated H3.3. These



modifications lead to an increase in chromatin accessibility, recruitment of Transcription Factor II B (TFIIB) and RNA polymerase II (Pol II), and transcriptional activation during *LHR* gene derepression induced by TSA [Reference 1].

phosphorylation, which in turn causes p107 repressor release from Sp1, recruitment of Transcription Factor II B (TFIIB) and RNA polymerase II (Pol II), and transcriptional activation. Positive Cofactor 4 (PC4) is recruited by Sp1, acts as its coactivator, and has an essential role in the formation/assembly of the preinitiation complex (PIC) and TFIIB and Pol II recruitment in TSA-mediated LH transcription.

Our recent studies demonstrated an association between PC4 and acetylated histone H3 in TSA-induced LHR de-repression in MCF7 cells. Tandem mass spectrometry (MS/MS) studies revealed an association of PC4 with the H3.3 variant acetylated at several Lys residues (K9, K14, K18, K23, K27). We further confirmed the presence of these modifications 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. Further, immunoprecipitation (IP) 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 A/B 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. In recent studies, we found that this resulted from a lowered accessibility of the chromatin at the promoter region of the LHR, as indicated by the relative increases in DNAse protection in cells with H3.3 knock-down. Recent pulldown studies demonstrated association of H3/H3.3 with GST-PC4 in MCF7-extracts, whereas we found no direct association with histone H4. Similarly, we observed direct association with recombinant H3 and H3.3 proteins in a GST-PC4 pull-down assay, indicating direct association with H3 and H3.3 but not with H4. Given that addition of H3-H4 or H3.3-H4 tetramer revealed H3 and H4 or H3.3 and H4 protein bands, respectively, we conclude that PC4 associates with the tetramer via H3 or H3.3 in vitro. This is in contrast to findings in Flag-PC4-transfected cells, in which PC4 associates with endogenous histone protein but not with the tetramer, as evidenced by the sole presence of H3 and absence of H4 in IP with Flag antibody. This could indicate dissociation of the tetramer resulting from the association with the PC4-Sp1 complex. The recruitment of PC4 to Sp1 and formation of PC4–Sp1 complex was previously shown to be essential for LHR transcription. PC4 expression is not affected by TSA, therefore PC4 levels do not relate to changes

in H3/H3.3–Ac expression. However, PC4–H3.3 interactions favor histone acetylation. Knock-down of endogenous PC4 in MC7 cells resulted in significant reduction of H3K–acetylated protein expression. TSA induced enrichment of acetylated H3K9 (H3K9–Ac) and H3.3K9–Ac at the promoter and consequently of LHR transcriptional activity.

Our studies have linked acetylation of H3 to PC4, given that its absence abolished H3 acetylation induced by TSA, despite of mayor increases in expression of both H3 and H3.3 protein induced by TSA. Acetylation of H3.3 leads to chromatin accessibility and gene transcription. Taken together, the findings indicate a critical role of PC4 association with acetylated H3.3 in TSA-induced Sp1-activated LHR transcription [Reference 1] (Figure 1).

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. We demonstrated the enzyme's participation in the nuclear export/transport of specific mRNAs, the structural integrity of the Chromatoid Body storage/processing of relevant mRNAs, and their transit/association to the actively translating polyribosomes, where it 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), where its expression is both cell- and stage-specific. Transgenic (Tg) mouse models generated in our laboratory 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) in germ cells (pachytene spermatocytes and round spermatids) and downstream in LCs, and its direct autocrine regulation by A/AR in LCs, and indirect paracrine regulation in germ cells. Functional *GRTH* binding sites for germ cell nuclear factor (GCNF, an orphan nuclear receptor-transcription factor that regulates GRTH transcription)) and the helicase's regulation by A/AR were identified in the distal region of the gene. The studies provided evidence for actions of A on GCNF cell-specific regulation of GRTH expression, which operates selectively in round spermatids. Also, GRTH exerts negative autocrine regulation of GCNF. Our in vivo/in vitro models, linking A actions to germ cells through GCNF as an A-regulated trans-factor that controls transcription/expression of GRTH, provide a connection between A action and two relevant germ cell genes essential for the progress of spermatogenesis and establish their regulatory relationship.

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 expressed 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. 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. The mouse model will permit us to discern the biological and biochemical impact of the phospho-species in GRTH function. In recent studies, we elucidated the GRTH phospho-site at a threonine structurally adjacent to the mutant site found in patients. Molecular modelling of the phospho-site, based on the crystal structure of DDX19, which shares 64% amino acid identity with the GRTH/DDX25 discovered in our lab (because we do not have the crystal structure of GRTH, we modeled the phospho-site based on the crystal structure of DDX19),

elucidated the relevant amino acids that form the GRTH/PKA (protein kinase A) interface. Studies based on the abolition of the phospho-form provide the basis for drug design and virtual and throughput screening to find a reversible chemical inhibitor that coould be used as a male contraceptive.

The prolactin receptor

The human prolactin receptor (PRLR) mediates the diverse cellular actions of prolactin (PRL) and has an important role in the etiology and progression of breast cancer, tumoral growth, and chemo-resistance. Our studies have elucidated the relevance of PRL, estradiol (E2) and its liganded or unliganded receptor, epidermal growth factor (EGF), epidermal growth factor receptors (ERBB1/EGFR), and ERBB2/HER2 (human epidermal growth factor receptor 2) in the up-regulation of the *PRLR* gene transcription/expression and their mechanistic commonalities for definition of PRL/PRLR–induced progression and metastasis of breast tumors, which could explain persistence and invasiveness in certain refractory states to adjuvant endocrine therapies. We demonstrated that the specific CDK7 kinase inhibitor THZ1, which inhibits E2–induced phosphorylation of estrogen receptor a (ERa) at S118, abrogated E2–induced *PRLR* transcription/expression and E2–induced cell migration. THZ1 singly or in combination with other approaches targeting PRLR expression, function/signaling, could abate *PRLR* transcription/expression and prevent deleterious effects of PRLR, fueled by tumor PRL, in breast cancer [Reference 2].

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NEUROSCIENCES

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 activitydependent 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, activitydependent 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



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Scholarship Program, Postbaccalaureate Research Fellow environmental experience may alter myelin formation in an activity-dependent manner, thereby improving function based on experience.

Learning is perhaps the most important function of childhood. Our research is delineating the molecular mechanisms that convert short-term memory into long-term memory. While we continue our long-standing research on synaptic plasticity, our laboratory is actively exploring new mechanisms of nervous system plasticity during learning that extend beyond the neuron doctrine, such as neurons firing antidromically (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 various 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 50fold. 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, cytokines, and other molecules 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. We showed that, through this axon-oligodendrocyte signaling mechanism, electrically active axons become preferentially myelinated by a factor of 8 to 1 over electrically inactive axons, thus regulating myelination of axons and neural circuit function according to functional experience. The findings are also relevant to such demyelinating disorders as multiple sclerosis and to remyelination after axon injury.

This research is expanding the biological significance of myelin. Myelin has been traditionally viewed in terms of conduction failure after damage, but we are exploring how myelin and changes in myelin affect spike time



FIGURE 1. Myelin plasticity to modulate impulse conduction velocity

The speed of neural impulse transmission is altered by astrocytes (*aqua*) at electrogenic nodes of Ranvier (*grey*), regulating detachment of the outer layers of the myelin sheath (*purple*) from the axon (*tan*) via thrombindependent cleavage of a cell adhesion molecule NF155. 3D reconstruction from electron microscopy.

arrival, 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.

MODIFICATION OF MYELIN STRUCTURE AND CONDUCTION VELOCITY BY ASTROCYTES

Many neurological and psychological dysfunctions can develop when optimal synchrony of spike time arrival and appropriate conduction latencies that are required to sustain neural oscillations are disturbed. Given that, to achieve temporal summation at synapses, optimal neural circuit function and synaptic plasticity require the proper impulse transmission speed through all axons to induce spike timingdependent plasticity, and to sustain oscillations at appropriate frequencies, mechanisms that determine and modify conduction time through axons could provide a non-synaptic mechanism of neural circuit plasticity. Conduction velocity in myelinated axons depends on the thickness of the myelin sheath and the morphology of electrogenic nodes of Ranvier along axons. Our research and that of others has shown that myelination of unmyelinated axons and the thickness of the myelin sheath can be increased in response to neural activity and environmental experience. Also, during growth of axons, the myelin sheath is thickened, but once formed, myelin structure was believed to be static. There was no known mechanism that could reduce the thickness of the mature myelin sheath, except in the context of pathology, but such a mechanism would be necessary to reduce conduction velocity to promote optimal spike time arrival from inputs that arrive at relay points in neural networks too soon.

Our research published this year shows that myelin thickness and nodal gap length are reversibly altered by astrocytes, the glial cells that contact nodes of Ranvier, and that this alters the speed of impulse transmission and neural network function. Myelin is attached to the axon by intercellular junctions adjacent to the node of Ranvier. We found that one of these cell adhesion molecules (neurofascin 155) has a binding site for the proteolytic enzyme thrombin, which is secreted by neurons and enters the brain from the vascular system. We found that thrombin-dependent cleavage of neurofascin 155 severs the tether between the axon and myelin, allowing it to detach and render the myelin sheath thinner. The process is inhibited by vesicular release of thrombin protease inhibitors from perinodal astrocytes. Previously, it was unknown how the myelin sheath could be thinned, and the functions of perinodal astrocytes were not well understood. The findings provide a new form of nervous system plasticity in which myelin structure and conduction velocity are adjusted by astrocytes. The thrombin-dependent cleavage of neurofascin 155 may also have relevance to myelin disruption and repair.

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 owing to disrupted neurotransmission from axons to oligodendrocytes may be an underlying cause of Gulf War Illness. Our research in progress is showing that proliferation and development of oligodendrocytes is affected in an animal model of Gulf War illness, and such perturbations could have long-lasting consequences in neural network functions involved in many of the symptoms associated with Gulf War Illness, including difficulties with working memory, mental focus, chronic pain, and others.

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 longterm 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, cDNA microarrays, and RNAseq 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 appropriate patterns of impulses, leading to different forms of synaptic plasticity, and is identifying new molecular mechanisms regulating synaptic strength.

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 optogenetics and by delivering electrical stimulation through platinum electrodes in specially designed cell culture dishes. After stimulation, we measured mRNA and protein expression by gene microarrays, quantitative RT-PCR (reverse transcriptase–polymerase chain reaction), RNAseq, 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). Moreover, regulation of gene expression in neurons by specific temporal patterns of impulse activity is not a property of special genes; the neuronal transcriptome in general is highly regulated by the pattern of membrane depolarization, with hundreds of genes differentially regulated by the temporal code of neuronal firing. Our experiments are thus revealing the intracellular 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 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, and our work is revealing fundamental differences in chromatin structure between neurons and glia (astrocytes and oligodendrocytes).

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Translational Biophotonics in Developmental Disorders and Diseases

Brain imaging and spectroscopy of developmental disorders

Functional near-infrared spectroscopy (fNIRS) is a non-invasive and wearable imaging technique that assesses brain function and is suitable for studies of children and toddlers, especially those with neuro-developmental disorders. Such measurements are based on local changes in the cerebral hemodynamic response associated with brain activity. NIR light (700–900 nm) can penetrate deep enough through tissue to probe the cortical region. The NIR absorption spectrum of tissue is sensitive to changes in the concentration of major tissue chromophores, such as hemoglobin. Therefore, measurements of temporal variation in backscattered light can capture functionally evoked changes in the cortex to assess brain function. We are currently pursuing two general tracks of research involving fNIRS in the brain: the developmental trajectories of cognitive abilities and the evaluation of fNIRS using cognitive tasks that are used in fMRI.

In our previous studies, we used fNIRS to examine prefrontal cortical activation as it relates to developmental level in toddlers [Reference 1]. We have continued this work through a series of projects and collaborations. First, as part of a previous collaboration with Audrey Thurm, we designed a study in which 24-month-old toddlers listened to speech sounds or watched gesture production while we recorded fNIRS in the prefrontal cortex (PFC). Over the past year, we analyzed the data from this project, and manuscripts are currently under review or being published. As a continuation of this work, we began another project that will examine the developmental trajectory of the mirror neuron network (MNN) in infants. The MNN is associated with the development of sophisticated social behaviors that emerge in typical infants. By modeling MNN development, we hope to uncover a sensitive measure of deviations in social communication development before clinical behavioral deficits can be detected. MNN activation has been indicated through mu rhythm suppression using EEG. In our pilot study, we are recruiting healthy adults (n=40)to determine whether MNN activation can be elicited, using a motor observation and a simultaneous execution paradigm and EEG/fNIRS systems. We will then examine the synchronicity of these signals as they relate to social communication and cognitive functioning.



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FIGURE 1. Differences in activation between highperformance (HP) and normal-performance (NP) groups

Both auditory and visual NP groups showed higher activation in the right dorsolateral PFC (DLPFC) during visual (*a*) and left DLPFC and medial PFC (MPFC) during auditory (*b*) tasks than did their HP cohorts.

Upon completion of the adult pilot, we intend to recruit typically developing infants (*n*=60) and infants at risk for developmental delays (*n*=60) from 9–12 months of age to collect fNIRS/EEG signals during the motor observation and the execution paradigm. At-risk infants will be brought in again at 24 months of age to evaluate any deviations in their social communicative development. We will examine their developmental status at 24 months in relation to their initial neural data to determine whether MNN activation can predict developmental outcomes. We aim to begin recruiting infants for this phase of the project in January 2019.

In a collaboration with Andrea Gropman, we are also examining brain function in children and adults with urea cycle disorders (UCD). UCDs are a set of rare genetic disorders caused by the loss of enzymatic activities (such as ornithine transcarbamylase deficiency [OTCD]) that convert ammonia to urea through the transfer of nitrogen. UCD often results in life-threatening hyperammonemia, resulting in a broad range of neurological impairments in working memory and executive function. Using functional magnetic resonance imaging (fMRI), Gropman's studies found that patients with OTCD show impairments in frontal-lobe processing through their performance on a working memory task, compared with a control group. We are replicating this work using fNIRS. We recorded hemodynamic activity from the prefrontal cortex of 26 children and adults (control and UCD) while they performed N-back tasks (which test processing speed) and Stroop tasks (which evaluate executive functions). Data collection for this project is ongoing, with a goal of examining 40 subjects.

We also used fNIRS to examine working memory in typically developing adults. Our results reveal that individual differences in learning style and performance can affect the lateralization of prefrontal cortex activation during the execution of a working memory task [Reference 2] (Figure 1). In another study, we implemented a moral judgment (MJ) task that is based on a series of questions in which subjects examine personal (emotionally salient) versus impersonal (less emotional and more logical) dilemmas. In two recently published studies [References 3 and 4], we investigated hemodynamic patterns for each dilemma and their correlation with psychopathic traits, as measured by the Psychopathic Personality Inventory-Revised Content Scale (PPI-R-CS). We analyzed fNIRS data using a non-linear classification method called a cubic support vector machine (SVM). We found that brain activity differs significantly during personal and impersonal dilemmas (mean accuracy of 85%). Consistent with fMRI studies, we found that left dorsolateral



FIGURE 2. Changes in mean HbO during personal and impersonal MJ tasks.

Changes in mean HbO which have been approximately mapped on different brain regions during (*a*) personal and (*b*) impersonal moral judgment (MJ) tasks. The captured brain activity during impersonal scenarios was significantly higher than during personal dilemmas. The average hemodynamic change in the left DLPFC for impersonal dilemmas was especially large. HbO: oxyhemoglobin.

PFC is highly activated when subjects make non-utilitarian decisions (i.e., benefiting the majority group) for impersonal moral dilemmas. Our results using Canonical Correlation Analysis (CCA) showed a significant correlation between PFC activation and psychopathic traits, as measured by the PPI-R-CS. Specifically, cold-heartedness and carefree non-planfulness were highly correlated with PFC activation during personal moral dilemmas. Machiavellian egocentricity, rebellious nonconformity, cold-heartedness, and carefree nonplanfulness are the core traits that exhibit similar dynamics with PFC activation during impersonal (more logical) moral dilemmas. Activation in ventromedial prefrontal cortex (vmPFC) and left lateral PFC were positively correlated with PPI-R-CS traits during personal dilemmas; on the other hand, the right vmPFC and right lateral PFC show positive correlation during impersonal dilemmas (Figure 2).

Additionally, we conducted a study to examine neural activation during a *go/no-go* behavior inhibition task that activates prefrontal cortical areas. The go/no-go task was administered to 44 typically developing adults while fNIRS and heart rate were recorded. We found that fNIRS detected differences between baseline and the go/no-go task and could be a suitable alternative to fMRI in the evaluation of behavior inhibition. We are further analyzing the data to determine whether fNIRS measurements are related to individuals' level of task performance or to more general measures of day-to-day behavior inhibition abilities. Analysis is ongoing, with the goal of submitting a manuscript by the end of 2018.

Tissue characterization and function

We are investigating photonic techniques to elucidate biomarkers for the diagnosis of disease or the assessment of treatment outcome across a variety of conditions. Our first study is in the assessment of facial plethora in Cushing's syndrome (CS), as it is one of the earliest described clinical features of the disease. In collaboration with Constantine Stratakis, we quantified changes of facial plethora in CS as an early assessment of cure. We performed non-invasive multi-spectral NIR imaging on the right cheek of patients before and 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 therapy. Results indicate that a reduction in facial plethora after surgery, as evidenced by decrease in blood



FIGURE 3. Fluorescence lifetime of imaging agent correlates with HER2 expression in cancer.

Results for different parameters for six tumor cell lines expressing various levels of HER2. Comparison between the tumor and the contralateral site. Fluorescence lifetime (*a*) and fluorescence intensity (*b*) measured *in vivo* at the tumor and contralateral sites, 1 h after injection of HER2-specific Affibody probe. ELISA results in (*c*) and (*d*).

volume fraction, is correlated with the cure of CS. The first set of results were published in 2015 [Afshari A *et al., J Clin Endocrinol Metab* 2015;100:3928]. We also showed that water content fraction could be used as a new biomarker of early cure in patients with CS. We are pursuing the technique to assess Kaposi Sarcoma (KS) in three ongoing NCI clinical trials. Given the capability of our technology, the goal is to further evaluate diffuse multispectral imaging in a relatively large sample as a potential supplement to existing response assessment in KS. Previously, we showed that successful treatment is indicated by a reduction in the blood volume in lesions, making this method a quantitative marker of tumor response to therapy. As a next step in our 'bench-to-bedside' goal, we developed a new hand-held multispectral camera to be used as a point-of-care system. The device uses a high-resolution complementary metal-oxide semiconductor (CMOS) camera with on-chip filters. High-resolution images are acquired simultaneously at eight different near-infrared wavelengths (700–980 nm). We also developed a user-friendly graphical interface for data processing.

We are also investigating the assessment of tumor development in patients with the goal of facilitating treatment strategies and identifying targets for early intervention. In our recently published study [Reference 5], we designed time-resolved fluorescence lifetime imaging to distinguish bound human epidermal growth factor 2 (HER2)–specific affibody probes to HER2 receptors in live animals. Our results show that changes in fluorescence lifetime of the bound contrast agent can be used to rapidly assess the high- to mid-level expression of HER2–expressing tumors *in vivo* (Figure 3). This patient-friendly approach allows the use of only one measurement for diagnosis and determining the efficacy of treatment intervention.

As a continuation of our work in preterm pregnancy complications, in collaboration with Jessica Ramella-Roman, we used the Preterm Imaging system based on colposcopy to characterize uterine cervix structure in a longitudinal study of low-risk and high-risk (i.e., prior preterm birth [PTB] or a sonographic short cervix) patients [Reference 6]. Polarization imaging is an effective tool to measure optical anisotropy in birefringent materials, such as the cervix's extracellular matrix, and to predict cervical ripening. For this reason, it has potential to predict pre-term birth. Through our collaboration with Roberto Romero's Branch and Ramella-Roman, we will test the system in a control population and those with PTB prevalence.

Placenta oxygenation from basics to point of care

Monitoring placenta oxygenation is critical to ensure a healthy pregnancy outcome. Abnormalities in placental oxygenation have been associated with preeclampsia, intrauterine growth restriction, fetal hypoxia, and cerebral palsy. Therefore, it is crucial to have a quantitative understanding of placental oxygenation. In designing a placental oximeter, the following criteria should be prioritized: it must be lightweight, relatively small, and battery operated and have wireless capability. Most importantly the device must be relatively inexpensive, so it can be used in low-resource settings, where the most high-risk cases are to be expected. fNIRS is a convenient technique for dynamic *in vivo* monitoring of anterior placental oxygenation. In parallel to the *in vivo* studies, we investigate the placenta at the cellular level to study the effect of oxygen level on cell metabolism. We intend to first find the baseline placental oxygenation for normal pregnancies to and standardize the oxygenation data across pregnancies, and second to study placental cell metabolism *in vitro* at physiologically relevant variations of oxygen level, using Dynamic Full Field Optical Coherence Tomography (DFFOCT).

For the *in vivo* study, we created a fast, non-invasive, and wearable device to allow continuous measurement of the oxygenation of the anterior placenta in a subject-friendly environment. The light-weight compact system can be positioned at various abdominal locations for localized measurement of oxygenation. The NIRS device uses light in near infrared region (750 and 850 nm) and consists of several source-detector pairs to simultaneously probe maternal and placental tissue. We investigated the efficiency of the device in separating oxygenation of the maternal and placental tissue while accounting for variations in melanin concentration and fat content. We developed a multi-layer model based on Monte Carlo simulation that includes the optical properties of skin, fat, uterus, and placental tissue. We further measured the optical properties of the placenta using dual wavelength LED sources with a photodiode array unit, which we built in-house, to calculate the attenuation coefficient (as a function of the scattering and absorption coefficients) based on the diffuse reflection curve from placental tissues. Using the above methods, we developed a system that includes parameters such as skin color and fat thickness in the calculation of oxygenation index.

In collaboration with the Sonia Hassan, Shad Deering, and Ramon Diaz-Arrastia, we are testing our device in pilot studies. In our first pilot study, we are measuring the oxygenation of the placenta during the last trimester in normal pregnancies to establish the baseline placental oxygenation. Meanwhile, we are continuing to refine our data analysis software by incorporating anatomical data from subjects. By taking advantage of electronic miniaturization of spectroscopy, along with Artificial Intelligence for the classification of data between typical and atypical pregnancies, we expect to provide earlier detection of pregnancy complications, which can improve maternal and fetal health in the future.

For the *in vitro* study, we are piloting an experiment to confirm DFFOCT's ability to capture metabolically coupled movements in *in vitro* samples. We are comparing the images of cells cultured at physiological hypoxia and hyperoxia with DFFOCT to detect the difference in the dynamics and using protein expression to verify

altered cellular metabolism. To accurately maintain the oxygen level, we pass pre-equilibrated media over the sample while imaging. We will statistically analyze the recorded data to validate the findings. Through this study, we expect to establish an understanding of placental metabolism under varying oxygenation states.

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 in order 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 eukaryotic intitiation factor 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 the 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, and poly(A)-binding protein (PABP) in mRNA activation, 48S PIC assembly, and scanning in vivo; (3) uncover the mechanisms of translational repression by the repressors Scd6, Pat1, and Dhh1; (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. During amino acid



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limitation, transcription of these genes is coordinately induced by the activator Gcn4 as the result of 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 (HAT) complexes in eviction of promoter nucleosomes at certain yeast genes, but it is 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, as well as the HAT complexes SAGA, NuA4, NuA3, and Rtt109/Asf1, in these processes are under study. We also recently discovered that Gcn4 can activate transcription from binding sites within the coding sequences (CDS) of its target genes, inducing internal subgenic sense and antisense (AS) transcripts in addition to the conventional full-length transcripts that initiate 5' of the CDS; and we are probing both the mechanism and possible regulatory functions of these internal AS transcripts.

eIF1A residues implicated in cancer stabilize translation preinitiation complexes and favor suboptimal initiation sites in yeast.

Our previous cryo-EM analysis of partial yeast PICs revealed distinct conformations relevant to different stages of initiation. A py48S-open complex exhibits upward movement of the 40S head from the body that widens both the mRNA binding cleft and the P site and eliminates interactions of the 40S subunit with Met-tRNAi and mRNA that are evident in the py48S-closed complex. The py48S-open conformation seems well suited for scanning of successive triplets for complementarity to Met-tRNAi, with TC anchored in the unstable P-out conformation; whereas py48S-closed exhibits the more stable P-in conformation required for start-codon selection. During the transition from py48S-open to py48S-closed, the unstructured N-terminal tail (NTT) of factor eIF1A assumes a defined structure and deploys five basic residues to interact extensively with the tRNAi anticodon or mRNA nucleotides surrounding the AUG codon or rRNA, thus suggesting that the eIF1A NTT directly stabilizes the P-in state. Interestingly, EIF1AX mutations altering the human eIF1A NTT are recurring mutations associated with uveal melanoma (UM). We found that substituting all five basic residues, and seven UM-associated substitutions, in yeast eIF1A suppresses initiation at nearcognate UUG codons and AUGs in poor sequence context. Ribosome profiling of the UM-associated NTT substitution R13P reveals heightened discrimination against unfavorable AUG context throughout the translatome. Both the R13P and K16D substitutions were shown to destabilize the closed complex at UUG codons in reconstituted PICs. We thus conclude that electrostatic interactions between eIF1A NTT basic residues and nucleotides in tRNAi, mRNA, or rRNA in the decoding center stabilize the closed conformation of the PIC and promote utilization of suboptimal start codons in vivo. We predict that UM-associated EIF1AX mutations alter the expression of human oncogenes or tumor suppressor genes by increasing discrimination against poor initiation codons.

eIF1 Loop 2 interactions with Met-tRNAi control the accuracy of start codon selection by the scanning preinitiation complex.

As described above, AUG recognition evokes rearrangement from an open PIC conformation with the TC in a P-out state to a closed conformation, with the TC more tightly bound in the P-in conformation. Factor eIF1 binds to the 40S subunit and exerts a dual role of enhancing TC binding to the open PIC conformation



FIGURE 1. Gcn4 binding in coding regions can activate internal and canonical 5' promoters in yeast.

Similar to other yeast transcriptional activators, Gcn4 typically activates transcription by binding upstream of the promoter region of its target genes (top). However, ChIP-seq analysis of Gcn4binding sites throughout the genome revealed that most of its occupied binding motifs reside inside coding sequences (CDSs) (bottom). Unlike Gcn4 binding upstream of promoters, in which the recognition motif resides within an extended nucleosome-depleted region (NDR) (top), Gcn4 binding within CDSs occurs at motifs generally positioned within the short DNA linkers separating nucleosomes. Gcn4 binding within CDS frequently activates cryptic internal promoters to induce bidirectional subgenic sense and antisense transcripts, and can also activate the canonical 5' positioned promoter located upstream of the CDS (bottom).

while antagonizing the P-in state, necessitating eIF1 dissociation for start codon selection to proceed. Our previous cryo–EM structures of partial yeast PICs revealed juxtaposition of eIF1 Loop 2 with the Met–tRNAi D loop in the P-in state and predict a distortion of Loop 2 from its conformation in the open complex to avoid a clash with Met–tRNAi. We showed that Ala substitutions in Loop 2 increase initiation at both near-cognate UUG codons and AUG codons in poor context *in vivo*. Consistently, the D71A–M74A double substitution stabilizes TC binding to 48S PICs reconstituted with mRNA harboring a UUG start codon, without affecting eIF1 affinity for 40S subunits. Similar but relatively stronger decreases in discrimination against poor start codons were conferred by arginine substitutions in Loop 2; and none of the Loop 2 substitutions perturbed the rate of TC loading on scanning 40S subunits *in vivo*. The findings indicate that electrostatic and steric clashing between the eIF1 Loop 2 and tRNAi D loop impede Met–tRNAi accommodation specifically in the P-in state of the closed complex without influencing the P-out mode of TC binding to the open complex; and Arg substitutions convert the Loop 2–tRNAi clash to an electrostatic attraction that stabilizes P-in and enhances selection of poor start codons *in vivo*. Thus, in contrast to the eIF1 ANTT that specifically stabilizes the closed/P-in state of the PIC and enables recognition of poor start codons, eIF1 Loop 2 destabilizes the P-in state and helps ensure relatively greater initiation frequencies for optimal start codons *in vivo*.

Tma64 (eIF2D), Tma20 (MCT-1), and Tma22 (DENR) recycle posttermination 40S subunits *in vivo*.

The recycling of ribosomal subunits after translation termination at stop codons is critical for efficient gene expression, as it liberates free ribosomal subunits, deacylated tRNA, and mRNA for use in further rounds of translation. Rli1/ABCE1 catalyzes the first stage of recycling, splitting the 80S ribosome into a free 60S subunit and a tRNA/mRNA-bound 40S subunit. The next step of recycling, dissociation of tRNA and mRNA from the 40S, has been reconstituted *in vitro* with the single protein ligatin/eIF2D, or the two interacting

proteins MCT-1 (also known as MCTS1) and DENR that are homologous to the N- and C-termini, respectively, of eIF2D. Earlier work suggested that the canonical initiation factors eIF1, eIF1A, eIF3, and eIF3j can also recycle 40S post-termination complexes, but it was unknown which, if either, of these redundant recycling mechanisms occurs in living cells. eIF2D was also shown to substitute for eIF2 in tRNAi recruitment for certain mRNAs *in vitro*, but evidence that eIF2D functions during initiation *in vivo* was lacking. To address these questions, we performed ribosome profiling on yeast mutants lacking eIF2D (Tma64) together with MCT-1 (Tma20) or DENR (Tma22). Both double mutants revealed that 80S ribosomes gueued immediately upstream of stop codons, consistent with a genome-wide block in 40S recycling at stop codons. We also found decisive evidence that the unrecycled 40S complexes in the mutants could reinitiate translation at AUG codons located downstream in the 3' UTR, as indicated by 80S peaks at such AUG codons in the profiling data, and by detecting expression of epitope-tagged 3' UTR translation products that was diminished by eliminating the presumptive AUG start codons. In vitro translation experiments using reporter mRNAs containing upstream ORFs (uORFs) further established that reinitiation at coding sequences downstream of the uORFs increased in cell extracts devoid of these proteins. In some cases, 40S ribosomes appeared to rejoin with 60S subunits and undergo an alternative 80S reinitiation process in 3' UTRs, previously observed in cells depleted of Rli1, that involves unrecycled 80S post-termination complexes. The results support a crucial role for eIF2D (Tma64), MCT-1 (Tma20), and DENR (Tma22) in the recycling of 40S ribosomal subunits at stop codons and thereby diminishing translation reinitiation following termination at both uORFs in 5' UTRs and at the stop codons of the main coding sequences of most yeast mRNAs.

SWI/SNF and RSC cooperate to reposition and evict promoter nucleosomes at highly expressed genes in yeast.

A key unsolved aspect of transcriptional activation by Gcn4 (as well as by other transcription factors) is how it mediates the eviction of the "-1" and "+1" nucleosomes that occlude promoter DNA and block access by general transcription factors (GTFs) and Pol II. We are addressing the mechanism of nucleosome eviction and the consequences of defects in this process on transcription for the hundreds of co-regulated genes in the Gcn4 transcriptome as well as for all other constitutively expressed genes. Previously, we showed, by H3 chromatin immunoprecipitation (ChIP) coupled to deep-sequencing (H3 ChIP-Seq) of wild-type and mutant yeast strains lacking Snf2, Gcn5, and Ydj1, that these cofactors collaborate in evicting H3 from the –1 and +1 nucleosomes and intervening nucleosome-depleted region (NDR) at a large fraction of Gcn4 target genes. Moreover, we found that the cofactors cooperate similarly at the majority of all other promoters. Having found that nucleosome eviction in the induced Gcn4 transcriptome is only partially impaired in cells lacking Snf2, we surmised that SWI/SNF cooperates with one or more other remodeling factors in evicting promoter nucleosomes. Considering that RSC and SWI/SNF belong to the same family of remodeling complexes, we studied whether SWI/SNF and RSC cooperate in nucleosome eviction at genes induced by Gcn4, and also at genes expressed constitutively at high levels where we had previously found that SWI/SNF cooperates with Gcn5 and Ydj1. We also explored whether SWI/SNF resembles RSC in determining the positions of -1 and +1 nucleosomes and hence, NDR width, at highly expressed genes.

Our findings reveal a previously undetected widening of NDRs, in addition to eviction of the –1 and +1 nucleosomes, on induction of Gcn4 target genes in wild-type cells, and demonstrate that SWI/SNF and RSC have distinct and equally critical roles in achieving wide, nucleosome-depleted NDRs for robust transcription at these induced genes. We also uncovered cooperation between SWI/SNF and RSC in nucleosome positioning and eviction at the most highly transcribed subset of constitutively expressed genes, suggesting

their general cooperation in achieving high transcription rates. The occupancies by both remodelers were found to be greatest at highly expressed or induced genes, supporting direct functions for both remodelers at this group of genes. Our results reveal an extensive division of labor between SWI/SNF and RSC in promoter nucleosome eviction and displacement at the most highly transcribed genes in yeast.

Gcn4 binding within coding regions can activate both internal and canonical 5 promoters in yeast.

We are also interested in determining the role of promoter nucleosome eviction in controlling binding of Gcn4 itself upstream from the promoters of its target genes, and set out first to define all the binding sites for Gcn4 throughout the genome in wild-type cells. ChIP-seq analysis of Gcn4 binding revealed 546 genomic sites occupied by Gcn4 in starved cells, representing only about 30% of all genomic sequences with significant matches to the consensus Gcn4 binding motif. Analysis of nucleosome occupancy data from MNase-seq analysis revealed that distance of a motif from the nearest nucleosome dyad and its match to the consensus sequence are the major determinants of Gcn4 binding *in vivo*. Surprisingly, only about 40% of the bound sites are in promoters, and analysis of genome-wide mRNA expression data and ChIPseq analysis of RNA Pol II in starvation conditions indicates that only about 60% of such promoter-located Gcn4 occupancy peaks activate transcription, revealing extensive negative control over Gcn4 function. Remarkably, most of the remaining around 300 Gcn4-bound motifs reside within coding sequences (CDS), with about 75 representing the only bound motif in the vicinity of a known Gcn4-induced gene. RNA-seq analysis revealed that many such unconventional Gcn4 occupancy peaks map between divergent antisense and sub-genic sense transcripts induced from within CDS under starvation conditions, and are also located adjacent to starvation-induced TBP (TATA-box binding protein) occupancy peaks detected by ChIP-seq analysis. The findings are consistent with Gcn4 activation of cryptic, bidirectional internal promoters at these genes. Mutational analysis confirmed that Gcn4-bound motifs within CDS can activate both sub-genic and full-length transcripts from the same or adjacent genes, demonstrating that functional Gcn4 binding is not confined to promoters. Our results show that internal promoters can be regulated by a well defined activator that also functions at conventional 5' positioned promoters.

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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.



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FIGURE 1. DPP6-KO mice show impaired spatial working memory in the T-maze and contextual fear conditioning task.

A. Schematic showing the experimental design in forced run and choice run of the T-maze task.

B. Percentage of correct choices for mice during the learning phase.

C. Percentage of correct choices comparing Day 1 with Day 8.

D. Percentage of correct choices for average scores over 8 days (n = 10 each group; p < 0.001), compared with Student's *t*-test.

E-F. Contextual fear conditioning task, at 24 hr after training mice, shows a significant reduction in time exhibiting freezing behavior when placed back in the test chamber (n = 10 for each group; p < 0.001).

Role of voltage-gated ion channels in synaptic development and disease ISOMERASE REGULATION OF POTASSIUM CHANNEL TRAFFICKING AND FUNCTION

Kv4.2 channels are key determinants of dendritic excitability and integration, spike timing-dependent plasticity, and long-term potentiation. Downregulation of Kv4.2 channel expression occurs following hippocampal seizures and in epilepsy, suggesting that A-type currents may be targets for novel therapeutics. 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, coexpression in HEK293T cells, 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, Travis Tabor 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 those of wild-type (WT) Kv4.2. The isomerizing activity may also regulate Kv4.2 binding to its auxiliary subunits. These data suggest that the isomerase plays a role in regulating Kv4.2 function.

To further study the physiological function of the isomerase and the Kv4.2 channel, we used Crispr-Cas9 techniques to generate a knockin (KI) mouse in which the isomerase binding site is specifically abolished.

The mice are viable, appear normal, and showed normal initial learning and memory in Morris Water Maze but better 'reversal' learning in Morris Water Maze than did WT mice. In the operant reversal lever press, the KI mice displayed improved reversal learning. The data strongly support the idea that activity-dependent regulation of Kv4.2 plays an important role in cognitive flexibility. Cognitive flexibility is the ability to appropriately adjust one's behavior to a changing environment and is impaired in various neurodevelopmental disorders such as the autism spectrum disorder (ASD).

In light of these findings, Cole Malloy used whole-cell patch clamp electrophysiology in acute hippocampal slices to investigate how isomerization of Kv4.2 impacts neuronal function. He used current-clamp recordings to detect alterations in action potential firing properties in the KI mice. In order to gain further insights into the molecular cascade impacting Kv4.2 function, pharmacological manipulation of isomerase and kinase activity are under way to address the dependence of phosphorylation and/or conformation change induced by the isomerase. Given the behavioral results showing altered cognitive flexibility, we plan experiments investigating synaptic function and plasticity in the KI mice.

CA²⁺ REGULATION OF POTASSIUM CHANNEL FUNCTION

In addition to pore-forming Kv4 subunits, native hippocampal A-type currents require non-conducting modulatory auxiliary subunits known as K-channel–interacting proteins (KChIPs) and dipeptidyl peptidase–like proteins (DPLPs). Both KChIPs and DPLPs work in concert to enhance Kv4 function. Interestingly, in recent unpublished work, we identified a mechanism by which Kv4.2 current density is regulated by Ca²⁺ via R-type voltage-gated Ca²⁺ channels (Cav2.3). Ca²⁺ regulation of Kv4.2 channels occurs despite an apparent lack of the structural determinants of the canonical Ca²⁺-activated K⁺ channels. We hypothesized that KChIP auxiliary proteins imbue Kv4 channels with Ca²⁺ sensitivity, as they contain four calcium-binding EF–hand domains (helix-loop-helix structural domains), two of which bind Ca²⁺.

To assess this possibility, Jon Murphy expressed KChIP2c, a short KChIP isoform, with Kv4.2 in HEK293T cells and performed whole-cell patch-clamp recordings in either nominal or 10 micromolar Ca²⁺. Under low Ca²⁺ conditions, coexpression of KChIP2c enhances Kv4.2 function in several ways: KChIP2c increases K⁺ current density, shifts the voltage dependence of inactivation to more hyperpolarized potentials, and accelerates recovery from inactivation. In the presence of 10 micromolar Ca²⁺, he measured a 1.5 fold increase in peak Kv4.2 current density while the other parameters of Kv4 function remained unchanged. Intriguingly, whereas the boosting effect of Ca²⁺ is conserved among the Kv4 family, including Kv4.1-4.3, it was only observed for short splice isoforms of KChIP2, KChIP2b/c, and the predominant KChIP3 isoform, KChIP3a. KChIP mRNAs contain multiple start sites, which generate considerable N-terminal variation and functional diversity in shaping A-type currents. While the KChIP core is cytoplasmic by nature, the variable N-termini regulate subcellular localization by encoding variable membrane-interaction motifs. Comparisons of the variable N-terminal domains of KChIP2 isoforms suggested that a conserved polybasic domain limits the Ca²⁺ sensitivity of longer KChIP2 isoforms. Ongoing studies are aimed at determining whether this previously unidentified polybasic domain regulates the plasma membrane trafficking and Ca²⁺ sensitivity of Kv4–KChIP2 complexes.

Proteomic and subcellular localization studies suggest that Cav2.3–containing voltage-gated calcium channels could be a potential calcium source for a modulatory effect on Kv4.2–mediated A-type K currents (IA) in CA1 hippocampal neurons. Jakob Gutzmann compared WT with Cav2.3 knock-out (KO) neurons and

observed a significant reduction in somatic IA. Cav2.3 KO neurons showed an elevated frequency of action potential firing after somatic injection of positive current. Further investigation revealed that individual action potentials showed profound changes in waveform: longer time-to-peak, and more significantly, a broader full-width at half-maximum. Additionally, the fast after-hyperpolarization following an action potential was shifted towards positive potentials in cells from the KO animals. Ultimately, he could track these changes pharmacologically to a functional lack of large-conductance potassium (BK) channel activity. This is the first time that Cav2.3 has been linked to BK channel activity, which so far has been thought to be regulated by other calcium channels. To support this novel finding, Lin Lin also performed coimmunoprecipitation (co-IP) experiments from acutely isolated adult mouse hippocampal tissue, and could successfully co-IP BK and Cav2.3, which hints at a physical interaction between the two channels, in addition to the functional interaction described above. A consequence of the altered action potential waveform in Cav2.3 KO neurons is an increase in short-term plasticity between CA1 and the informationally downstreamlying subiculum pyramidal neurons.

DPP6 PLAYS A ROLE IN BRAIN DEVELOPMENT, FUNCTION, AND BEHAVIOR.

DPP6 is well known as an auxiliary subunit of Kv4.2, which has been associated with numerous developmental and intellectual disorders and neuropsychiatric pathologies, especially ASD. We previously reported a novel role for DPP6 in regulating dendritic filopodia formation and stability, affecting synaptic development and function. Recently we reported that DPP6 knockout mice are impaired in hippocampus-dependent learning and memory. Results from the Morris water maze, T-maze, Object's spatial location, Novel object recognition, and Fear conditioning tasks showed that DPP6-KO mice exhibit slower learning and reduced memory performance [Reference 3].

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Translational Research in Inherited Neurodegenerative and Motor Neuron Diseases

The laboratory is committed to dissecting the mechanisms and pathophysiologies of inherited neurodegenerative and motor neuron diseases and using this knowledge to improve health through rational remedies, including gene therapy. Patients and families affected by these disorders provide the impetus for scientific inquiry. In addition to molecular genetics, the laboratory employs model organisms (yeast, mouse, zebrafish) and cellular and biochemical approaches, and conducts clinical protocols. Choroid plexus-targeted adenoassociated virus (AAV) gene therapy for alpha-mannosidosis, a prototypical lysosomal storage disease, and the study of motor neuron degeneration mediated by the p97/valosin-containing protein (p97/VCP) represent our current main directions. Copper histidinate (CuHis), a new molecular entity originally developed at the NIH Clinical Center for the Section's long-term clinical work on Menkes disease, received fast-track designation from the Food & Drug Administration (FDA) in 2018. New drug approval will pave the way for a trial of subcutaneous CuHis in combination with cerebrospinal spinal fluid (CSF)-directed ATP7A gene therapy for this illness.

Choroid plexus-targeted viral gene therapy for alpha-mannosidosis, a prototypical lysosomal storage disease

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 recombinant lysosomal enzymes into the cerebrospinal fluid [CSF] during a spinal tap) 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. We devised an alternative strategy 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, which are post-mitotic neuroectoderm-derived cells, this approach can provide a permanent source of enzyme production for secretion into the CSF and penetration into cerebral and cerebellar structures. We are using mouse and cat models (the latter in collaboration with



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Kaler Laboratory July 2018

John Wolfe) to evaluate choroid plexus transduction by several recombinant AAV (rAAV) vector serotypes and to determine the post-treatment lysosomal alpha-mannosidase (LAMAN) concentration and distribution in brain. Analyses of these two animal models are complementary and open the door to IND (investigational new drug)– generating studies for a future first-in-human clinical trial.

In a natural history study of alphamannosidosis, we evaluate human subjects with this condition 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 are invaluable as benchmarks for assessing response to investigational treatments.

ATP7A-related disorders, including motor neuron degeneration

ATP7A is an evolutionarily highly conserved copper-transporting P-type ATPase associated with X-linked recessive Menkes disease and occipital horn syndrome, a milder version of the phenotype. Recently, two unique ATP7A missense mutations, T994I and P1386S, were shown to cause adult-onset isolated distal motor neuropathy without clinical or biochemical features of other ATP7A disorders. The mutant alleles cause subtle defects in ATP7A intracellular trafficking, resulting in preferential plasma membrane localization compared with wild-type ATP7A. We identified an abnormal interaction between the vesicular trafficking protein p97/VCP and ATP7A-T994I. Mutations in p97/VCP cause at least three other motor neuron diseases: amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth disease, and inclusion body myopathy with early-onset Paget disease and fronto-temporal dementia (IBMPFD). We identified a putative UBX domain (found in ubiquitin-regulatory proteins) in the luminal loop of ATP7A immediately adjacent to the T994I mutation, at which p97/VCP binds. TIRF (total internal reflection fluorescence microscopy) imaging, cell fractionation, and immunoprecipitation analyses determined the intracellular localization of the interaction at or near the cell plasma membrane. The findings have implications for similar phenomena involving other proteins, and we are pursuing further studies to better understand the pathophysiological mechanism(s) implicated in motor neuron degeneration associated with p97/VCP.

Mutations in the acetyl CoA transporter SLC33A1 cause a complex autosomal recessive phenotype known as Huppke-Brendel syndrome. Low serum copper and ceruloplasmin and cerebellar atrophy similar to Menkes disease in affected patients, as well as hepatic copper overload similar to Wilson disease, imply possible effects on ATP7A and ATP7B, the copper-transporting ATPases implicated in those respective disorders. SLC33A1 normally mediates transport of acetyl-CoA, required for lysine acetylation, which is a reversible post-translational modification of some transmembrane proteins. To explore the possible connection between these diverse gene products, we used CRISPR/Cas9 to knock out SCL33A1 in Hek293T cells and studied ATP7A trafficking in response to copper stimulation. For these experiments, we over-expressed fluorescently tagged ATP7A or ATP7B constructs in Hek293T cells. In contrast to normal Hek293T cells, both copper ATPases failed to traffic from the *trans*-Golgi network to the plasma membrane in SLC33A1 knockout cells. We also studied fibroblasts available from four affected patients; all showed partial defects in endogenous ATP7A trafficking in response to copper. We then employed tandem mass spectroscopy to document acetylation of numerous lysine residues in ATP7A and ATP7B, which we now are systematically mutating to identify the critical site(s) implicated. This previously unappreciated post-translational modification appears to mediate normal Cu–ATPase trafficking and in part explains the Huppke-Brendel phenotype.

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

In collaboration with Elizabeth Higgs and Mosoka Fallah, the Section participated in the Trans–NIH response to the Ebola epidemic in West Africa, including a vaccine clinical trial (PREVAIL-1), and remains involved in a natural history study of Ebola survivors (PREVAIL-3), specifically children and adolescent survivors, as well as a Birth Cohort substudy that evaluates pregnancy outcomes in female Ebola survivors of child-bearing age. In contrast to AAV gene therapy, in which the brain's immuno-privileged status is advantageous, the Ebola filovirus poses neurocognitive and other health risks in survivors of the acute infection owing to immunological niches or sanctuary sites within the CNS.

Clinical research protocols

- 1. Principal Investigator (PI), 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

- U.S. Patent Application No. 62/620,811 filed January 23, 2018: Methods and Compositions for Treating Lysosomal Storage Diseases
- U.S. Patent Application No. 15/769,294 filed April 18, 2018: Codon-Optimized Reduced-Size ATP7A cDNA and Uses for Treatment of Copper Transport Disorders

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
- Cooperative Research and Development Agreement (CRADA) with Cyprium Therapeutics, Inc. New York, NY

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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. Over the years, our lab has identified Pho, Pho-like, Spps, and Combgap as DNA proteins that bind to PREs. Our recent genome-wide studies show that different PREs require different DNA-binding proteins. In addition, our work illustrates the combinatorial nature and redundancy of PcG recruitment in Drosophila.

A second major project in the lab is to determine how the PREs of the *engrailed/invected* (*en/inv*) gene complex 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 *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. In addition, we showed that a 79-kb transgene (*HA-en79*), which contains the *en* gene and flanking regulatory DNA, is able to rescue a deletion for the



Judith A. Kassis, PhD, Head, Section on Gene Expression J. Lesley Brown, PhD, Staff Scientist Yuzhong Cheng, PhD, Senior Research Technician Sandip De, PhD, Research Fellow Adrienne Perkins, PhD, Postdoctoral fellow Ming-an Sun, PhD, Postdoctoral Fellow Elizabeth Fishman, BS, Postbaccalaureate Fellow Natalie Gehred, BS, Postbaccalaureate Fellow Eliana Ramirez, Summer student entire *inv-en* locus. This laid the groundwork for current studies to explore the effects of the chromosomal neighborhood on gene expression.

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. 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. Different PREs have different architectures (Figure 1). Our laboratory identified four PRE-DNA binding proteins: Pho, Phol, Spps, and, most recently, Combgap. The Combgap protein has 10 zinc fingers and recognizes the sequence GTGTGT [Reference 2].

PRE activity can be studied in transgenes, where a single PRE can recruit PcG protein complexes and silence the expression of a reporter gene. In transgenes, mutation of Spps or Pho or Combgap binding sites within the PRE obliterates its ability to recruit PcG proteins, and the reporter gene is expressed. Thus, transcriptional silencing by a single PRE in a transgene requires the combinatorial activity of many DNA-binding proteins. We were interested in determining what happens when one of the PRE DNA-binding proteins (the 'recruiters') from the genome is removed and examined the effect on PcG recruitment genome-wide. We studied PcG binding genome-wide in mutants that lack the recruiters Spps or Pho [Reference 3]. We found that PcG recruitment to some PREs was completely disrupted, whereas recruitment



FIGURE 1. The order, number, and spatial arrangement of consensusfactor 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. Model of the *inv-en* PcG domain in the transcriptionally silenced state

The *inv* and *en* genes are covered with H3K27me3 and are transcriptionally silent. PcG proteins are associated with this domain. There are strong, constitutive PREs, as well as 'weak', tissue-specific PREs. 'Weak' PREs often overlap enhancers and are active in some tissues but inactive in other tissues. Actively transcribed genes remain segregated from the PcG domain and determine the limits of the PcG domain (reprinted from Reference 4).

of PcG proteins was hardly diminished at most PREs. Most PcG domains, which are covered by the chromatin mark H3K27me3, contain several PREs. We believe that the structure of the H3K27me3 domains stabilizes genomic PREs to the loss of one recruiter. However, there are different kinds of PREs, and some are uniquely sensitive to the loss of one recruiter. Our study highlights the complexity and diversity of PcG recruitment mechanisms.

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 disc, *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. *en* and *inv* are co-regulated and express proteins with largely redundant functions. Unlike *en, inv* is dispensable for viability in the laboratory.

The *en* and *inv* genes exist in a 113kb domain that is covered by the H3K27me3 chromatin mark (Figure 2). Within the *en/inv* domain there are four major PREs, strong peaks of PcG protein binding. One popular model posits that DNA-binding proteins bound to the PREs recruit PcG protein complexes and that PRC2 tri-methylates histone H3 throughout the domain until PRC2 comes to either an insulator or an actively transcribed gene. There are two PREs upstream of the *en* transcription unit, PRE1 and PRE2 (Figure 1). 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*

FIGURE 3. ChIP-seq profile showing H3K27me3 and H3K36me3 binding to the *e*(*Pc*), *inv*, *en*, and *tou* genes in *Drosophila*

H3K27me3, a mark deposited by PcG protein complex PRC2, is bound from the 3' end of the *tou* gene to the 3' end of the *E*(*Pc*) gene. Arrows indicate



the direction and extent of the transcription units for the genes shown. H3K36me3 is a mark of actively transcribed genes, and is bound to *E*(*Pc*) and *tou*. Samples from *Drosophila* 3rd instar larvae, brains and discs. In these tissues, at least 80% of the cells are not expressing *inv* or *en*. Data from Reference 5.

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 [Reference 4].

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 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 *inv-en* 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 those peaks may 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]. Our work shows that there are two types of PREs in *Drosophila*: strong, constitutive PREs, and tissue-specific PREs that tend to overlap with enhancers (Figure 2). We suggest than mammalian genes may lack strong, constitutive PREs. Thus, the identification of PREs is more challenging in mammals.

The *inv-en* gene complex is flanked by two ubiquitously expressed genes (Figures 2 & 3). The H3K27me3 mark stops at these two genes. We believe that it is the transcription of these two ubiquitously expressed genes that blocks the spreading of the H3K27me3 mark and stabilizes the repression of *inv* and *en* by PcG proteins. In order to test this assumption, we made a large transgene, marked by HA-tagged Engrailed protein. A 79-kb *HA-en* transgene was able to correctly express En and completely rescue *inv-en* double mutants. We inserted this transgene into other places in the *Drosophila* genome [Reference 5]. Our data showed that, while the information to form the H3K27me3 domain is contained within the 79-kb *HA-en* transgene, the structure of the H3K27me3 domains differed from at the endogenous locus. Specifically, the H3K27me3 mark spread beyond the transgene into flanking DNA. Further, enhancers within the 79-kb *HA-en* transgene could interact with some flanking genes and drive their expression in subsets of the En pattern. Finally, removal of the PREs from the transgene lead to loss of PcG silencing in the abdominal segments of



FIGURE 4. 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. Figure reprinted from Cheng Y *et al.*, *Dev Biol* 2014;395:131.

the flies. The data provide evidence that the endogenous *inv-en* domain imparts a stability to the locus and facilitates both transcriptional activation and silencing of these two developmentally important genes.

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 disc 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) (Figure 4). 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 4); 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 inven 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 and 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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Genomics of Development in Drosophila melanogaster

Our goal is to understand how linear information encoded in genomic DNA controls 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 humans 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 found 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



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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 pharateadult 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 343 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 15 known Polycomb-group genes (Pc, ph-d, ph-p, Psc, Su(z)2, Sce, E(z), Su(z)12, esc, Sfmbt, Asx, calypso, Pcl, Scm, and crm). The remaining mutations identify 45 additional genes required for silencing. For 31 of the silencing genes, we identified the corresponding transcription units using a combination of meiotic-recombination mapping and wholegenome 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



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).

chromatin-associated factors (*gpp*, *Tip60*, *CG9293*, *CG43736*, and *ptip*), insulator proteins, and two aminoacyl-tRNA synthetases (*ThrRS* and *AsnRS*).

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 several 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 malefertile 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. We

are using these translocations to test whether the disruption of male fertility is attributable to transcriptional defects (as first proposed almost fifty years ago) or to defects in chromatin remodeling and in condensation for packaging into the sperm head.

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Assembly and Function of Drosophila Chromatic Circuits

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 to (1) identify key neuronal types and map their synaptic connections, (2) examine functional requirements of identified neurons for color vision and spectral preference behaviors, and (3) determine the synaptic mechanisms that transform visual signals at different processing stages.

Using molecular genetics, histology, and serial-EM (electron microscope) 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 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 such 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,



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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. 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 Li3 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. By combining two orthogonal expression systems and two different peroxidases, HRP (horse radish peroxidase) and APX (ascorbate peroxidase), we expressed HAtagged mitochondrion-targeted APX in Tm5c and membrane-tethered HRP in their LT11 postsynaptic neurons, 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 had previously found that the amacrine Dm8 neurons, which receive UV–sensing R7 photoreceptor inputs, are both required and sufficient for animals' innate spectral preference for UV light. Using behavioral assays, we further found that inactivating Tm5c, one of Dm8's synaptic targets, abolishes 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 the receptors in Tm5c, which prevents Tm5c from receiving Dm8 inputs, abolished UV preference, indicating that the receptors are functionally required in Tm5c for UV preference behaviors. Thus, the R7s→Dm8→Tm5c connections constitute a hard-



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.

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 respective 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 several 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 the 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

Correct brain wiring 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 developmental origins and exhibit defects in dendritic morphology, such as changes in branching numbers and patterns. Dendritic

defects could cause neuronal connectivity defects, which likely underlie 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 in which 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 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 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; and (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 (calcium-dependent cell-adhesion proteins) 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 in layer-specific targeting of their dendritic arbors and their planar projection directions. Interestingly, the total dendritic length was unaffected, suggesting 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 reduce dendritic termination frequency but not branching frequency. Using a modified GRASP method that we had 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 or Rheb have a small dendritic tree. On the other hand, mutant Dm8 neurons lacking the negative regulators of the insulin signaling pathway, Pten or TSC1, have an abnormally expanded dendritic tree compared with wild-type neurons. 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 responsible for the structural integrity of tissues and organs as well as for maintaining an optimal environment for cellular function. ECM pathology 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. Collagens are triplehelical proteins forming the structural scaffolds of the ECM. Their procollagen precursors are assembled and folded from three proalpha chains in the endoplasmic reticulum (ER), trafficked through the Golgi apparatus, secreted, and then converted into mature collagen by enzymatic cleavage of propeptides. The most common collagen is type I, which is a heterotrimer of two pro-alpha1(I) chains and one pro-alpha2(I) chain. It is by far the most abundant protein in all vertebrates. Type I collagen fibers form the organic scaffold of bone, tendons, ligaments, and the matrix of skin and many other tissues. We focus on translational studies of developmental disorders of the ECM such as osteogenesis imperfecta (OI), Ehlers-Danlos syndrome (EDS), and chondrodysplasias, as well as related ECM pathologies in fibrosis, cancer, and osteoporosis. Our goal is to understand molecular mechanisms and treatment targets in ECM disorders, particularly those involving abnormal metabolism of type I collagen, and to bring this knowledge to clinical research and practice.

Procollagen folding and its role in bone disorders

Osteoblasts and fibroblasts produce and secrete the massive amounts of type I procollagen needed to build the skeleton. Given that type I procollagen is one of the most difficult proteins to fold, its massive production presents a unique challenge for protein quality control and trafficking. We discovered that the conformation of natively folded human procollagen is less 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 fibers. Unincorporated molecules denature within several hours of secretion and become susceptible to rapid proteolytic



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The most common hereditary cause of elevated 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 the 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 the pathophysiology mechanism because procollagen folding and the consequences of its misfolding for the cell remain poorly understood.

Cell biology of procollagen misfolding

Our current research thus focuses 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 entirely unexpected features of procollagen quality control and trafficking. We observed sorting of normally folded and misfolded procollagen molecules at ER exit sites (ERES) marked by coat protein complex II (COPII). Normally folded procollagen was loaded into giant (up to 500 nm) Golgi-bound transport vesicles. Contrary to widely held beliefs, the vesicles do not have a COPII coat nor do they not contain HSP47, a collagen-specific ER chaperone that preferentially binds to natively folded procollagen to assist in its folding and loading into ERES. Apparently HSP47 is removed from procollagen upon its entry into ERES. Misfolded procollagen is retained at ERES, resulting in a COPII-dependent modification of ERES membrane by ubiquitin and autophagic machinery. The resulting autophagic ERES are subsequently directly engulfed by lysosomes and degraded. The findings delineate a novel, COPII-dependent, non-conventional micro-autophagy-like pathway for recycling ERES-loaded cargo [Reference 1].

Importantly, our findings may have wide implications beyond procollagen and ECM biology. For instance, COPII coat involvement in regulating autophagic degradation and 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 pathologies in patients with COPII mutations as deficient autophagic degradation of difficult-to-fold proteins, another line of investigation in our and collaborating laboratories.

To validate physiological significance and further build on these findings, we are expanding our tools by exploiting emerging gene-editing technologies. We created an osteoblast cell line in which endogenous pro-alpha2(I) chain has a fluorescent tag and Flp-recombinase (site-directed recombination technology) target sites for manipulating the tag, e.g., changing the fluorescence color or completely replacing it. We demonstrated that the cells produce and mineralize bone-like ECM, enabling us to perform live-cell imaging of endogenous rather than transiently transfected procollagen. Presently, we are introducing additional Flp-recombinase target sites into the same gene to produce cell-culture models with a variety of different OI mutations. The same strategy can then be used to generate mouse models and to study other proteins.

In another approach, we are investigating the pathophysiology of cell-stress response to procollagen misfolding caused by a Gly610 to Cys substitution in the triple-helical region of pro-alpha2(I). We helped develop a mouse model of OI with this mutation (G610C mouse), which mimics the pathology found in a large group of patients from an Old Order Amish community in Pennsylvania. Our study of this model revealed misfolding and accumulation of mutant procollagen in the ER of fibroblasts and osteoblasts, resulting in cell stress and malfunction. We are investigating the mechanism and role of the cell stress in OI by altering how the cells to adapt to it. Building on our success in understanding rerouting of misfolded procollagen from ERES to autophagic degradation, we examined how reduced autophagy, and therefore increased accumulation of misfolded procollagen in the ER, affected the severity of OI in G610C mice. Reduced expression of Atg5, a protein we found to be involved in enhancing ERES micro-autophagy, resulted in about 40% perinatal lethality of the animals, apparently owing to malfunction of lung fibroblasts. Given that lung pathology is the most common cause of death in OI patients, we are examining the underlying molecular mechanisms and potential targets for therapeutic intervention. We also observed that cell-specific knock-out of the autophagy-related gene *Atg5* in mature osteoblasts reduced bone synthesis and raised bone fragility, explaining the pathogenicity of misfolded procollagen accumulation in osteoblast ER *in vivo*.

Development of novel OI treatments

Our observations suggested that pathology associated with procollagen misfolding may be at least partially reversed by improving cell adaptation to misfolded procollagen accumulation in the ER, thereby improving lung fibroblast and osteoblast function. Although this would not eliminate potentially detrimental effects of secreted mutant collagen in OI, pharmacological treatment of cell 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 cell malfunction caused by procollagen misfolding in cases that do not involve pathogenic mutations.

In one approach to pursuing this strategy, we are targeting procollagen autophagy, given that enhancing the natural ability of the cell to remove and degrade the misfolded molecules via autophagy is the simplest way to prevent their pathogenic accumulation molecules in the ER. Our preliminary study of autophagy enhancement by a low-protein diet (LPD) in G610C mice revealed improved osteoblast differentiation and function, resulting in better bone quality. However, prolonged LPD stunted animal growth. We are thus developing intermittent LPD and fasting approaches that might provide the same benefits of autophagy enhancement without the detrimental side effects of long-term nutrient deprivation. At the same time, we

are validating the general approach of autophagy enhancement by direct genetic modulation of autophagy efficiency. We were able to completely rescue the perinatal lethality in animals with altered endogenous Atg5 by introducing an *Atg5* transgene that normalized the Atg5 protein level. The experiments suggested that the lethality was caused by quantitative Atg5 deficiency rather than other effects of the altered gene sequence and that increasing *Atg5* expression can stimulate autophagy. We are completing the analysis of animals with transgenic *Atg5* overexpression. So far, our observations are providing encouraging evidence of improvements in bone geometry in these animals. However, additional measurements are still required for fully understanding the *Atg5* overexpression effects.

In another approach, we are collaborating with several extramural laboratories to test the effects of chemical chaperones, which are drugs known to reduce protein misfolding or the accumulation of misfolded proteins in the ER. In collaboration with Antonella Forlino, we found that 4-phenylbutyrate (4PBA) reduced OI severity in a zebrafish model. 4PBA is an FDA-approved scavenger of ammonia, but it also has chaperone and histone deacetylase inhibitor activities. It might also enhance autophagic degradation of misfolded procollagen, although the mechanism of this effect is presently unclear. In collaboration with Satoru Otsuru, we are testing effects of 4PBA on bone pathology in G610C mice. The study is still in its early stages, but preliminary data already indicate improved bone formation at least in 7-weak-old female animals.

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, with closely related skeletal dysplasias, and with more complex disorders that were 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 ERmembrane ion channel TRICB, Golgi-membrane metalloprotease S2P, the transmembrane anterior posterior transformation protein 1 (TAPT1), and collagen prolyl-4-hydroxylase 1 (P4H1). 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 posttranslational 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 the 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. As expected, we found that patient cells with mutant P4H1 secreted abnormal procollagen that had significantly reduced thermal stability owing to underhydroxylation of proline residues by P4H1 [Reference 2]. Surprisingly, however, we found no abnormalities in the procollagen folding or secretion rates, no evidence of misfolded procollagen accumulation in the cell, and no evidence of altered ER chaperone composition.

We also continued translational studies of OI caused by missense mutations in type I collagen that are not substitutions of obligatory Gly residues, specifically focusing on substitutions of Y-position arginine (Y-Arg) residues in the Gly-X-Y triplets within the triple helix. In an earlier collaboration with Joan Marini, we found that Y-Arg substitutions with cysteine (Cys) caused procollagen misfolding, but it remained unclear whether the primary cause of misfolding was the loss of Y-Arg or aberrant disulfide bond formation by Cys. In a more recent collaboration with Peter Byers, we were able to examine cells from two different OI patients, one of whom had a Y-Arg substitution with Cys and the other a substitution of the same Y-Arg with leucine (Leu). The latter study revealed that the loss of Y-Arg, which enhances the collagen triple helix stability and promotes triple helix folding through binding of HSP47, disrupted procollagen folding and caused its accumulation in the ER to almost the same level as Gly substitutions [Reference 3].

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's lab to investigate bone tumors caused by defects in protein kinase A (PKA), a key enzyme in the cAMP signaling pathway. Initially, we investigated synthesis of type I collagen homotrimers. However, over the last 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 4]. Improved understanding of bone tumors caused by PKA deficiencies may not only clarify the role of cAMP signaling but may 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 gene, 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 various 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 Emilios Dimitriadis and Ferenc Horkay to map cartilage elasticity by force microscopy at length scales appropriate for examining the material properties of the ECM and to combine it with our multimodal imaging technology [Reference 5].

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 are collaborating with Peter Basser on using the technology to calibrate and test newer methods for non-invasive *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

Our work is dedicated to the better understanding of common molecular and cellular mechanisms of neurodegeneration, with the ultimate goal of developing treatments for neurodegenerative diseases and even preventing them. The lab currently focuses on investigating an evolutionarily conserved neuronal stress response pathway under control of DLK (dual leucine zipper kinase), which plays an important role in several neuropathologies. As a cellular stress response pathway in neurons, its function is to promote recovery from injury; however, at the same time, it can drive several types of pathologies, including peripheral neuropathies and neurodegeneration.

The hypothesis driving our work is that common mechanisms are responsible for neurodegeneration during development, childhood, and aging. Most of what is currently understood about neurodegenerative disease stems from the identification of genetic linkages that are causative or predisposing, and from efforts to uncover the mechanisms underlying these linkages. However, the linkages only account for a relatively small proportion of all cases. The vast majority of cases have no established genetic etiology and therefore no clear pathway to target. An understanding of any common mechanisms involved in neurodegeneration would provide major breakthroughs for designing treatments. We showed that Dual Leucine Zipper Kinase (DLK; MAP3K12) acts as a crucial downstream node in neurodegeneration and neuropathy, two pathologies with very different causes and outcomes [References 1 & 2]. The lab is currently investigating how such diverse diseases converge upon this single pathway and how this pathway mediates divergent fates.

The DLK-dependent injury response promotes neurodegeneration in the mammalian CNS.

The existence of common mechanisms of neurodegeneration has long been hypothesized. In previous work, I focused on DLK, which is a MAP3 kinase (mitogen activated protein triple kinase) previously shown to initiate a retrograde stress signaling cascade from the axon to the cell body, and which has since become a promising drug target



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Josette J. Wlaschin, MSc, Postbaccalaureate Fellow for the treatment of several diseases. As a kinase that is enriched in neurons, DLK is an attractive drug target and was identified in several screens for genes that drive neurodegeneration. Moreover, DLK is upstream of JNK (c-Jun N-terminal kinase) signaling, which itself has long been proposed as a therapeutic target for neurodegeneration, but whose specific targeting has not proved feasible. Importantly, the work uncovered a powerful role for DLK signaling in several animal models of neurodegeneration and showed that human disease tissue bears markers of DLK/JNK signaling activation [Reference 2]. The most exciting implication of this study is that DLK is an important driver of neurodegeneration with diverse etiologies, suggesting it is part of a long sought common mechanism of neurodegeneration and is thus an attractive therapeutic target.

Intriguingly, and at first glance perhaps counter-intuitively, DLK signaling can result in many different outcomes, including neuronal death and long-term survival, depending on context. Several studies have shown that DLK can promote neuron death in the CNS (central nervous system), for example after injury to the optic nerve, and during normal development. However, DLK is also described as an important pathway for axon regeneration after neuron injury. Therefore, it is thought of as a regulator and coordinator of neuronal stress signaling, able to promote recovery or death. My lab is now focusing, in parallel, on two key questions: understanding how DLK performs these dual roles; and determining how distinct diseases converge upon this common pathway.

DLK is required for microgliosis and pain after traumatic injury to sensory neurons.

I have had a long-term interest in neurodegeneration and the involvement of DLK. After working at Genentech and prior to starting at NICHD, in the course of two years spent working semi-independently at the NINDS, and in collaboration with the Chesler and Hoon labs, my work in the field of somato-sensation and pain resulted in several co-authorships [e.g., References 3 & 4]. The work led to the idea of examining a potential role for DLK in pain. It was clear that peripheral nerve injury activates many molecules downstream of DLK. However, the possibility of links between injury, DLK, and neuropathic pain had not been examined. Notably, work in my lab established that DLK signaling plays a causative role in chronic pain, raising the tantalizing possibility that inhibition of DLK would also be an effective treatment for pain [Reference 1].

Partial sciatic nerve axotomy results in the development of mechanical hypersensitivity (allodynia), which can be measured by a reflexive paw withdrawal response. We demonstrated that DLK deletion blocks the development of this mechanical allodynia by preventing the full complement of transcriptional changes that normally occur following injury. Strikingly, we discovered a novel role for DLK in regulating a microglial reaction in the vicinity of injured neurons. DLK controls a distress call from injured neurons to microglia via transcriptional upregulation of the neuronal cytokine Csf1, resulting in a characteristic spinal cord microgliosis at the central terminals of the DRG neurons. The microgliosis is blocked in the DLK conditional knockout (DLK cKO). Our data corroborate recent work from others showing that neuronal expression of Csf1 after injury is required for the spinal cord microgliosis and necessary for the development of the mechanical allodynia (Guan et al., *Nat Neurosci* 2016;19:94).

Nerve injury initiates a cascade of events that evolve over time as pain becomes chronic. We therefore examined a time course of acute transcriptional changes in the dorsal root ganglion (DRG) for key genes, which we found to correlate with the onset of microgliosis. Knowing the time course of gene expression

changes aided design of a study in which we examined the protective effects of a pharmacological DLK inhibitor after nerve injury. Importantly, inhibitor treatment between 18 hours and 8 days post-injury was sufficient to suppress DLK-dependent transcriptional changes and spinal cord microgliosis and to prevent mechanical pain. The results expose DLK as a critical regulator of events leading from nerve injury to the development of neuropathic pain and suggest that targeting this pathway might be of therapeutic value. They also highlight non-cell autonomous aspects of the neuronal injury response, for example an injured neuron-to-microglia signal that has interesting implications in the context of neurodegeneration, and in which neuroinflammation is thought to be a key player.

Additional Funding

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The Biological Impact 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 the methods of highthroughput 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 the highly informative techniques of yeast genetics. As an example, we generated an expression technique that tags each integration with a highly specific serial number. With the 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 the datasets has greatly improved our understanding of integration and the mechanisms that select insertion sites.

Single nucleotide-specific targeting of the Tf1 retrotransposon promoted by the DNAbinding protein Sap1 of Schizosaccharomyces 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 such dense maps of integration is the inability to measure repeated insertions at specific nucleotide



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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 the 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 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 1]. At permissive temperature, Tf1 transposition was 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 1]. An example of this close association can be seen in a segment of chromosome 1 (Figure 1). Another important observation was a strong correlation between levels of integration in intragenic sequences and the amount of Sap1 bound. If Sap1 were directly responsible for positioning Tf1 integration, we would expect integration to take

place at specific nucleotide positions relative to the nucleotides bound by Sap1. Using the ChIP-Seq data, we were able to identify a Sap1-binding motif, which closely resembled previously published motifs. We used the FIMO program of the MEME Suite to perform genomic searches, which identified 5,013 locations that matched this motif. The alignment of all these motifs revealed that 82% of all integration events cluster within 1 kb of this motif. Importantly, 43% of all integrations occurred within 50 bp of the motif and they had two dominant positions: 9 bp upstream and 19 bp downstream of the motif. The clustering of inserts at the Sap1 motif would be expected to occur if Sap1 covers its binding site on the DNA and directs integration to either side of the protein. Thus far, we have been unable to detect a direct interaction between Sap1 and Tf1 integrase (IN) with pull-down assays. However, our two-hybrid assays detected a strong Sap1–IN interaction. The two-hybrid result together with the strong alignments of integration with Sap1 motif sequence and the reduction in integration in the *sap1-1* mutant argue that Sap1 plays an important role in Tf1 integration.

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 depends 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 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 2]. 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. We tested two candidates, Rhp18 and the NineTeen complex, in two-hybrid assays and found that they interact with Tf1 IN. Surprisingly, several pathways we identified were previously found 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.

Retrotransposon Tf1 induces genetic adaptation to environmental stress.

Ever since Barbara McClintock discovered transposable ("Controlling") elements in maize, it has been accepted that they are activated by changes in environmental conditions. Although increased mobility has long been thought to benefit the host, the precise impact and importance of this activity has not been directly studied. *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 finding, 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 the hypothesis by studying the fitness of cells with



FIGURE 2. Model for adaptation through stressinduced mobilization of transposable elements

Repeated exposure to stress results in cycles of increased transposition. Competition between cells containing new insertions results in the selection of sets of insertions that improve survival. 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 cell-regulatory TOR pathway. A critical feature of Tf1 activity and that of the closely related Tf2 is that their promoters participate in the core stress response, showing significant activation when cells are exposed to these stresses. Significantly, we found that the activation of Tf1 transcription results in increased integration frequencies. As a result, each of these features—increased mobility, targeting of promoters, and the stimulation of adjacent genes—promotes adaptation. Having observed these properties, we propose that Tf1 is a highly evolved mutagenic system that benefits the host by driving adaptation to environmental insults. In a model for adaptation through stressinduced mobilization of transposable elements, we propose that repeated exposure to stress results in cycles of increased transposition.

Competition between cells containing new insertions results in the selection of sets of insertions that improve survival (Figure 2). An intriguing additional possibility is that, through continued exposure to an unfamiliar stress, several insertions could accumulate in individual cells that together could form the foundation of a new gene-regulatory network (GRN). Such networks would be specific depending on the nature of the existing insult. The assembly of GRNs resulting from integration activity of TEs is a compelling model for how regulatory sequences of TEs have undergone wide-spread domestication in controlling GRNs. Supporting this model is our study of polymorphic Tf1 and Tf2 LTRs present in 57 wild isolates of *S. pombe.* The enrichment of LTRs in the promoters of heat-shock and sporulation genes provided evidence that TEs do promote adaptation in natural conditions. Together, our results indicate that integration activity provides substantial benefit when cells are subjected to stress.

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



FIGURE 3. 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.

resulted in the activation of proto-oncogenes and progression to T cell leukemia. Such adverse outcomes led to the use of lentivirus vectors for more 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 at 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 that 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 3]. 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 many spliced mRNAs and with transcription units that contain high numbers of introns (Figures 2A and 2B) [Reference 3]. The correlations were independent of transcription levels, size of transcription units, and length of the introns. Analysis of previously published HIV-1 integration site data showed that integration density in transcription units in mouse embryonic fibroblasts also correlated strongly with intron number and that the correlation was absent from cells lacking LEDGF (Figures 2C and 2D). The data suggest that LEDGF/p75 not only tethers HIV-1 integrase to the 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

Our long-term goal is to obtain a comprehensive understanding of how metabolic signaling pathways influence oocyte growth, development, and quality. 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, understanding how oocyte development and growth impact meiotic progression 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 the role of metabolism in oocyte development and maintenance.

The GATOR complex: integrating developmental and metabolic signals in oogenesis

The Target of Rapamycin Complex 1 (TORC1) regulates cell growth and metabolism in response to multiple inputs, including amino-acid availability and intracellular energy status. In the presence of sufficient nutrients and appropriate growth signals, the Ragulator and the Rag GTPases (a complex that regulates lysosomal signaling and trafficking) target TORC1 to lysosomal membranes where TORC1 associates with its activator, the small GTPase Rheb. Once activated, TORC1 is competent to phosphorylate its downstream targets. The Gap Activity Towards Rags (GATOR) complex is an upstream regulator of TORC1 activity.

The GATOR complex consists of two sub-complexes. The GATOR1 complex inhibits TORC1 activity in response to amino-acid starvation.



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FIGURE 1. GATOR1 prevents p53 hyperactivation in *Drosophila* early ovarian cysts.

Ovaries from (A) p53R–GFP, (B) nprl2¹; p53R–GFP, (C) p53R-GFP;nprl3¹/Df, (D) p53R-GFP; iml¹/ *Df*, and (*E*) *p53R–GFP*; *mei-P22^{p22}*, *nprl3*¹ were stained for GFP (green) and 1B1 (red). Germarial regions are defined by 1B1 staining. In wild-type ovaries the p53-GFP reporter is briefly activated in region 2 (indicated by arrow). Note the low level of GFP staining. In contrast, in GATOR1 mutants, p53R–GFP is robustly activated, with strong GFP signal often persisting into germarial region 3 and beyond. Additionally, in GATOR1mutant germaria, p53R-GFP is frequently activated in germline stem cells (GSC) and daughter cystoblasts (CB). In mei-P22p22, nprl31 double mutant germaria, the hyperactivation of p53R–GFP is rescued in region 2a ovarian cysts. However, p53–GFP activation in GSC and CB is retained in the double mutants (asterisk), indicating that in these cells the activation of p53



is not contingent on the presence of meiotic DSBs. Scale bars, 10μ m. (*F*) Percentage of germaria that sustain p53R–GFP signal in region 3. (*G*) Percentage of germaria with high p53R–GFP signal in region 2. (*H*) Percentage of germaria with p53R–GFP expression in GSC and CB. Unpaired T-student test was used to calculate the statistical significance. Error bars represent SD from at least three independent experiments. **P<0.001, ****P<0.0001.

GATOR1 is a trimeric protein complex consisting of the proteins Nprl2, Nprl3, and Iml1. 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. Notably, *Nprl2* and *Iml1* are tumor suppressor genes, while mutations in *Iml1*, known as *DEPDC5* in mammals, are a 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 Wdr24, 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* oocyte development and physiology.

The GATOR complex regulates an essential response to meiotic doublestranded breaks.

The TORC1 inhibitor GATOR1 controls meiotic entry and early meiotic events in yeast. However, how metabolic pathways influence meiotic progression in metazoans remains poorly understood. In the last



FIGURE 2. *seh1* RNAi depletion and codepletion 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. year, we determined that the TORC1 regulators GATOR1 and GATOR2 mediate a response to meiotic DNA double-stranded breaks (DSBs) during *Drosophila* oogenesis. The initiation of homologous recombination through the programmed generation of DNA DSBs is a universal feature of meiosis. DSBs represent a dangerous form of DNA damage that can result in dramatic and permanent changes to the germline genome. To minimize this destructive potential, the generation and repair of meiotic DSBs is tightly controlled in space and time. We showed that meiotic DSBs promote the GATOR1dependent downregulation of TORC1 activity. These data suggested that low TORC1 activity may be important for the efficient repair of meiotic DSB. In line with this hypothesis, we determined that GATOR1-mutant ovaries, which have high levels of TORC1 activity, exhibit many phenotypes consistent with the misregulation of meiotic DSBs, including an increase in the steady-state number of meiotic DSBs, the retention of meiotic DSBs into later stages of oogenesis, and the hyper-activation of p53, a transcription factor that mediates a highly conserved response to genotoxic stress (Figure 1). Importantly,

RNAi depletions of *Tsc1* phenocopied the GATOR1 ovarian defects. TSC1 is a component of the potent TORC1 inhibitor Tuberous Sclerosis Complex (TSC), confirming that the misregulation of meiotic DSBs observed in GATOR1–mutant oocytes is attributable to high TORC1 activity rather than to a TORC1–independent function of the GATOR1 complex. Further genetic analysis demonstrated that GATOR1 impacts the repair, rather than the generation, of meiotic DSBs. These data are particularly intriguing in light of similar meiotic defects observed in *npr3* mutants in *Saccharomyces cerevisiae*. Our results raise the possibility that GATOR1–mediated down regulation of TORC1 activity may be a common feature of the early meiotic cycle in many eukaryotes.

GATOR1 prevents the derepression of retrotransposons in response to meiotic double-stranded breaks.

Genotoxic stress has been implicated in the deregulation of retrotransposon expression in several organisms, including *Drosophila*. In line with these studies, we find that, in GATOR1 mutants, the double-stranded breaks that initiate meiotic recombination trigger the deregulation of retrotransposon expression. Similarly, it was previously shown that *p53*-mutant females de-repress retrotransposon expression during oogenesis, but as observed in GATOR1 mutants, primarily in the presence of meiotic DSBs. Through epistasis analysis we determined that *p53* and GATOR1 act through independent pathways to repress retrotransposon expression in the female germline. Surprisingly, we found that depletions of the potent TORC1 inhibitor TSC in the female germline resulted in little to no increase in retrotransposon expression. The data raise the interesting possibility that GATOR1 regulates retrotransposon expression independently of TORC1 activity. Notably, GATOR1 components, but not TSC components, were recently identified in a high-

throughput screen for genes that suppress LINE1 (Long Interspersed Element-1) expression in mammalian tissue culture cells. Taken together, our data indicate that the GATOR1 complex opposes retrotransposon expression during meiosis in a pathway that functions in parallel to p53 in the female germline of *Drosophila*.

A genetic screen for new regulators of the GATOR complex

We previously demonstrated that mutations in the GATOR2 components mio and seh1 cause constitutive activation of the GATOR1 complex in the female germline, resulting in permanent inhibition of TORC1 activity and a block in oocyte growth and development. Germline RNAi depletions of any of the GATOR1 components in the *mio* or *seh1* mutant backgrounds relieve the permanent TORC1 inhibition and rescue the mio and seh1 ovarian phenotypes (Figure 2). We utilized this epistatic relationship to conduct a highthroughput RNAi-based screen to identify upstream regulators and downstream effectors of the GATOR1 complex during obgenesis. To identify additional genes that, when co-depleted with seh1, rescue the seh1^{RNAi} ovarian phenotypes, we used RNAi lines from the Transgenic RNAi Project (TRiP) that have been optimized for germline expression. We screened 3,472 TRiP collection lines, representing 3,103 target genes, to identify genes that, when co-depleted with seh1, rescue the seh1^{RNAi} ovarian growth phenotype. From the screen, we identified 67 genes, 2.0% of those screened, in which co-expression in the female germline fully suppressed the seh1 ovarian growth deficit. Five genes were identified by two TRIP lines targeting independent sequences. Several lines of evidence indicate that the screen was successful. Importantly, we identified many genes that negatively regulate growth and metabolism, including negative regulators of TORC1/PI3K/ insulin signaling, such as pten, Ampk, gigas, mapk (rl), ras and p22A^{B55} (twins), and pp2A-C (mts), as well as many negative regulators of Hippo, a second signaling pathway that promotes cell proliferation and growth. Gene categories highly enriched in gene-ontology (GO, a comprehensive resource for knowledge regarding the functions of genes and gene products) biological processes include those for: (1) the mTor signaling pathway; (2) autophagy; and (3) the MAPK signaling pathway. Taken together, the findings strongly suggest that the screen provided a sensitized background that successfully identified several pathways regulating germline growth and metabolism. Importantly, our screen identified many genes and pathways not previously associated with growth control during oogenesis. The identified pathways include genes involved in glycolysis, fatty acid metabolism, DNA repair, and stress granule formation, as well as genes of unknown function. The genes will be a rich source for future studies on the metabolic regulation of growth control and meiotic progression during oogenesis.

The GATOR2 component Wdr24 regulates TORC1 activity and lysosome function.

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 in metazoans of the GATOR2 complex remains poorly understood. 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



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 autolysosomelike structures under nutrient-replete conditions.

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 such 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.

Spectral unmixing for simultaneous analysis of multiple fluorophores within cells

The ability to unambiguously distinguish more than a few different labels in a single fluorescence image has been severely hampered



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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. by the excitation cross-talk and emission bleed-through of fluorophores with highly overlapping spectra. To overcome the problem, we developed a cell-labeling, imageacquisition, and image-analysis approach to study the spatial distribution of six different organelles within eukaryotic cells. The work allowed us to present a systemslevel analysis of the organelle interactome using a multispectral image-acquisition method that overcomes the challenge of spectral overlap in the fluorescent protein palette. We employed confocal and lattice light sheet (LLS) instrumentation and an imaging informatics pipeline of five steps to achieve mapping of organelle numbers, volumes, speeds, positions, and dynamic inter-organelle contacts in live fibroblast cells. Cells were transfected with six fluorescent fusion protein markers of organelles or labeled with compartment-specific fluorescent chemical dyes to highlight six subcellular compartments: peroxisomes, lysosomes, ER, mitochondria, Golgi, and lipid droplets (LD). We acquired live-cell, time-lapse images and then applied linear unmixing algorithms to every pixel in the image, deconvolving spectrally-overlapping fluorophores. Our data allowed us to describe the frequency and

locality of two-, three-, four-, and five-way interactions among six different membrane-bound organelles (ER, Golgi, lysosome, peroxisome, mitochondria, and LD) and to show how these relationships change over time. We further demonstrated that each organelle has a characteristic distribution and dispersion pattern in three-dimensional space and that there is a reproducible pattern of contacts among the six organelles, impacted by microtubule and cell nutrient status. In this way, we provided a full systems-level description of the spatial organization of eukaryotic organelles under various physiological conditions. The live-cell confocal and LLS spectral-imaging approaches we developed in this study are applicable to any cell system expressing multiple fluorescent probes, whether in normal conditions or when cells are exposed to disturbances such as drugs, pathogens, or stress. The methodology thus offers a powerful new descriptive tool and source for hypotheses testing in the fields of cellular organization and dynamics.

ER exit sites regulate procollagen quality control through noncanonical autophagy.

Type I collagen is the main component of bone matrix and other connective tissues. Rerouting of its procollagen precursor to a degradative pathway is crucial for osteoblast survival in pathologies involving

excessive intracellular buildup of procollagen that is improperly folded and/or trafficked. What cellular mechanisms underlie this rerouting had been unclear. We studied these mechanisms in collaboration with the lab of Sergey Leikin, employing live-cell imaging and correlative light and electron microscopy (CLEM) to examine procollagen trafficking both in wild-type mouse osteoblasts and osteoblasts expressing a bone pathology-causing mutant procollagen. We found that, although most procollagen molecules successfully trafficked through the secretory pathway in these cells, a subpopulation did not. The latter molecules appeared in numerous dispersed puncta colocalizing with subunits of the COPII coatomer, autophagy markers, and ubiquitin machinery, with more puncta seen in mutant procollagen-expressing cells. Blocking endoplasmic reticulum exit site (ERES) formation suppressed the number of these puncta, suggesting they formed after procollagen entry into ERESs. The punctate structures containing procollagen, COPII, and autophagic markers did not move toward the Golgi but instead were relatively immobile. They appeared to be quickly engulfed by nearby lysosomes through a bafilomycin-insensitive pathway. CLEM and fluorescence recovery after photobleaching experiments suggested that engulfment occurred through a noncanonical form of autophagy resembling microautophagy of ERESs. The findings point to a non-canonical mode of procollagen quality control at ERESs involving lysosomal engulfment of ERESs that have been modified with autophagic machinery.

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 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 thought 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 discovery 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.

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Neurosecretory Proteins in Neuroprotection and Neurodevelopment

Mechanism of sorting, transport, and regulated secretion of neuroproteins

The intracellular sorting of pro-neuropeptides and neurotrophins to the regulated secretory pathway (RSP) is essential for processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of proopiomelanocortin (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,



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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 sympathochromaffin 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 (also known as Neurotrophic factor- α 1, NF- α 1) knock-out (KO) mouse to study the function of CPE/NF- α 1 *in vivo*. The 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- α 1 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/NF- α 1 in human disease. Using the *Cpe*-KO mice as a model in which to study its nervous system deficiencies, we showed defects in learning and memory by the Morris water maze and object-preference tests, and depressive-like behavior by the forced swim test. 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 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 or glutamate. Moreover, a non-enzymatically active form of CPE/NF- α 1 (E342Q) applied extracellularly had the same neuroprotective effect, indicating that it acts independently of enzymatic activity. Hence, 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 ¹²⁵I-CPE/NF-α1 binds to HT22 cells, an immortalized hippocampal neuronal cell line, in a saturable manner, and that the binding is specifically displaced by non-iodinated CPE/NF-α1, but not by bovine serum albumin, suggesting the existence of a receptor. Use of K235a, a Trk (tropomyosin receptor kinase) family inhibitor, and PD16285, a fibroblast growth factor receptor (FGFR1-3) inhibitor, did not prevent the neuroprotective action of CPE/NF- α 1 in hippocampal neurons treated with H₂O₂, suggesting that the CPE/NF-a1 likely uses a different class of receptors than those of the Trk family or FGFRs. We screened a human G protein-coupled receptor (GPCR) library for binding activity to CPE and identified a promising receptor candidate.

The mechanism of action of CPE/NF-α1 in neuroprotection involves the activation of the ERK1/2 (extracellular-signal-regulated kinase) signaling pathway and the Akt signaling pathway (an intracellular signal transduction pathway) during stress, which then leads to enhanced expression of a pro-survival mitochondrial protein, Bcl2, inhibition of caspase 3 activation, and promotion of neuronal survival [Reference 1]. Furthermore, this CPE/NF- α 1–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-apoptotic protein Bcl2, in the hippocampus. In situ hybridization studies indicated especially elevated CPE/NF-a1 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- α 1 and Bcl2 expression and a decline in Bax, a pro-apoptotic protein, after treatment with the synthetic glucocorticoid dexamethasone. The 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 upregulated 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]. Furthermore, CRS also elevated the expression of FGF2. We also demonstrated that primary hippocampal neurons treated with CPE/NF- α 1 raised FGF2 expression. Thus, another pathway for CPE/NF-α1 may be through FGF2, which is known to have neuroprotective effect.

The relevance of CPE/NF-α1 in neuroprotection in humans was underscored 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. Immuno-cytochemical 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 protein in the ER. Moreover, many cells appear unhealthy, indicating that they might be undergoing ER stress, unlike the cells expressing WT CPE, which show staining in the cell body and neurites. CPE-QQ was not secreted and even prevented WT CPE from being secreted by aggregating with it. Overexpression of CPE-QQ in rat primary hippocampal neurons resulted in elevated levels of the ER stress marker CHOP, reduced levels of pro-survival protein Bcl-2, and increased neuronal cell death. Thus, 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 of age, the animals exhibited memory deficits and depressive-like behavior compared with WT mice, but that their spatial learning ability was unimpaired. The CPE-QQ mice were neither obese nor diabetic, likely owing to some CPE activity in these mice, given that the endogenous WT gene was not deleted. However, they had 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 the microtubule-associated protein tau at ser³⁹⁵, a hallmark of AD. The studies identified a human mutation in the CPE gene resulting in expression of a CPE-QQ protein, which caused neurodegeneration and impairment of memory function in a mouse model, similar to AD, as well as depressive-like behavior.

Stress also induces depression. Huda Akil's group (University of Michigan) reported that FGF2 is an antidepressant. We found that prolonged (6h/day for 21 days) restraint stress reduced CPE/NF α -1 and FGF2 in the hippocampus of mice and induced depressive-like behavior. However, after short-term restraint stress, (1h/day for 7 days) mice did not show depressive-like behavior despite elevated corticosterone levels indicative of stress. Moreover, hippocampal CPE/NF α -1, FGF2, and doublecortin, a marker for neurogenesis, were elevated in these mice, suggesting that the anti-depressive effects of CPE/NF- α 1 are mediated at least in part, through increased neurogenesis. Indeed, we found that exogenously applied CPE/NF- α 1 could upregulate *FGF2* mRNA and protein expression in cultured hippocampal neurons, indicating that CPE/NF- α 1 regulates FGF2 expression. CPE/NF- α 1-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, we propose that CPE/NF- α 1 prevents stress-induced depressivelike behavior 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]. Rosiglitazone has previously been shown to be effective in treating diabetic patients with bi-polar disorders.

Role of CPE/NF-1 and CPE-deltaN in embryonic brain development

Embryonic mouse brains express three forms of CPE/NF- α 1 mRNA: 2.1kb, 1.9kb, 1.73kb in size, encoding a 53kD wild-type CPE/NF- α 1, and 2 isoforms: 47kD and 40kD N-terminal truncated forms (CPE/NF- α 1-DNs), respectively. The three mRNAs are expressed as early as E8.5 and increase significantly in two waves at E10.5 and postnatal day1 [Reference 5]. Interestingly, CPE/NF- α 1-DNs are not expressed in adult mouse brain. *In situ* hybridization studies indicate that CPE/NF- α 1 is expressed primarily in the forebrain in mouse

embryos, suggesting a role of CPE/NF- α 1 in neurodevelopment. We examined the effect of CPE/NF- α 1 on E13.5 neocortex-derived neurospheres, which contain stem cells and neuroprogenitors. Application of recombinant CPE/NF- α 1 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 downregulated the wnt pathway in the neurospheres, leading to reduced levels of β-catenin, a protein known to enhance proliferation, suggesting that CPE/NF- α 1's inhibitory effect on proliferation is brought about by negatively regulating the wnt pathway. We also 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 after CPE/NF- α 1 treatment, without alteration in the percentage of neuronal and oligodendrocyte populations. We also observed this phenomenon when the cultured embryonic stem cells were treated with a non-enzymatic form of CPE, indicating that the effect was independent of enzymatic activity. Interestingly, dissociated cells from neurospheres derived from Cpe/Nf- α 1-KO mouse embryos showed fewer astrocytes but more neurons, which was reversed with CPE/NF- α 1 application. In vivo, Cpe/Nf- α 1–KO mouse cortex (at P1, the time of astrocytogenesis) showed about half the astrocyte numbers compared with WT animals, confirming the ex vivo data. Our results suggest a novel role for CPE/NF- α 1 as an extracellular signal to inhibit proliferation and induce differentiation of neural stem cells into astrocytes, thus playing an important role in neurodevelopment [Reference 6].

Neurite outgrowth is key to the formation of synapses and the neural network during development. We found that CPE/NF- α 1 prevented Wnt-3a inhibition of nerve growth factor (NGF)–stimulated neurite outgrowth in PC12 cells and cortical neurons. Moreover, CPE/NF- α 1 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- α 1, could play an important role in regulating these positive and negative cues, which are critical for neurodevelopment.

We also studied the function of 40kD CPE/NF-α1-DN during development. Overexpression of 40kD CPE/ NF-α1-DN in HT-22 cells, a hippocampal cell line, resulted in an increase in expression of IGFBP2, DAP1, and Ephrin 1A mRNAs and proteins; we demonstrated that the IGFBP2 is involved in proliferation in HT22 and mouse cortical neurons [Reference 5]. Further studies aimed at determining the role of CPE/NF-α1-DN *in vivo* are in progress.

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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 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 elF4G; and the 40S subunit- and RNA-binding protein elF4B. 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, events that 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 elF2, which converts elF2 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, elF5B, 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

Our work on the DEAD-box RNA helicase eIF4A was published last year [Reference 1], as was work on the mechanistic consequences of mutations in eIF1A that are associated with uveal melanoma [Reference 2]. We completed two additional manuscripts in fiscal year (FY) 2018. One describes the cryo-electron microscopy structure of a yeast 48S pre-initiation complex in which eIF1 has departed following start codon recognition and has been replaced by the N-terminal domain of eIF5. Biochemical analyses done in our lab of variants of eIF5 made based on this new structure strongly support its mechanistic relevance as a key intermediate in the start codon recognition pathway. The work was done in close collaboration with the labs of Venki Ramakrishnan and Adesh Saini [Reference 3]. In studies on the yeast DEAD-box RNA helicase Ded1, we provided evidence that Ded1 acts as part of a Ded1-eIF4G-eIF4A-eIF4E tetrameric complex to unwind structures in the 5' UTRs of mRNAs that inhibit PIC attachment and scanning. We also showed that different mRNAs engage with this Ded1 complex in distinct ways, leading to multiple, mRNA-dependent mechanisms for Ded1 function [Reference 4].

We continued our genome-wide analysis of the effects of growth temperature on start codon usage. The work showed that translation of some, but not all, upstream open reading frames (uORFs) is modulated by growth temperature, but in different ways that depend on the specific mRNA. Most regulated uORFs starting with AUG behave similarly, with their translation repressed at low temperature and activated at higher temperature, whereas uORFs starting with near-cognate codons do not show coherent behavior but can be activated, repressed, or remain unchanged at lowered or elevated temperatures. We spent a considerable amount of effort this year looking into specific cases of altered uORF translation that might have physiological consequences, work that should set the stage for future studies, once the initial manuscript is published.

We also made progress on our studies of genome-wide translation initiation using our fully reconstituted yeast-based *in vitro* system. Using poly(dT)–purified mRNA from yeast, we can examine the initiation efficiency on each mRNA by isolating 48S complexes assembled *in vitro*, using sucrose gradient ultracentrifugation, followed by RNase footprinting, library construction, and deep sequencing. The power of the *in vitro* system lies in our ability to manipulate components in ways that are impossible *in vivo* and to isolate only the direct effects on translation initiation. For example, we can omit components or use lethal variants of factors and determine the effect on initiation for every message simultaneously.

We also initiated a mutational analysis to determine the mechanistic roles of each residue in the N-terminal tails (NTTs) of eIF1 and eIF5. The factors have coordinated but antagonistic functions in the initiation process, and their unstructured NTTs have been shown to be important; however, their exact roles remain largely undefined. We are systematically mutating each residue in the NTTs of these factors and determining the phenotypic effects *in vivo* in yeast. Variants with strong phenotypes will then be analyzed in the *in vitro* reconstituted system to define the effects of the changes on key steps in the initiation process.

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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 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 in our laboratory. Another recently initiated area of investigation focuses on hematopoietic stem cells (HSCs), which give rise to all bloodcell 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 DNAbinding 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.

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 tyrosinebased activation motifs or ITAMs, are contained within four distinct subunits of the multimeric TCR complex (CD3-zeta, CD3-gamma,



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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



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).

be influenced by potential compensatory mechanisms that operate during T cell maturation, and T cells in these mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. Experiments with the mice confirmed that the knockin zeta locus functions as predicted.

Over the past few years, 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). 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). We are currently using this model system to evaluate the role of ITAM multiplicity and ITAM-mediated signal amplification in T cell development, immune tolerance, and mature T cell function by inducing the zeta-6Y to zeta-6F 'switch' in peripheral T cells after they have completed their maturation in the thymus. We predict that these 'lateswitch' mice may show a T cell phenotype that is different from that observed in germline 6F/6F mice, given that 'tuning' can adjust for the alteration in TCR signaling in the latter but not the former mouse model. The experiments will be especially informative, as they more accurately mimic the effects of TCR signal attenuation by pharmacologic methods used for treatment of human diseases and therefore should provide valuable insights of translational relevance.

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 *Themis1-¹⁻* 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 Ca²⁺ 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 PLCgamma2 and found that Themis2 is required for normal tyrosine phosphorylation of Lyn and PLCgamma2; 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-^{r-}* 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 are 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 the F-box protein Fbxl12 in thymocyte development

A major aspect of the T cell maturation process is the precise regulation of cell proliferation. Rather than being a shared property among all or most developing thymocytes, proliferation is strictly limited to two stages during early development. The initial proliferative phase is driven by thymus-expressed cytokines, the second coincides with 'beta-selection' (i.e., is initiated in cells that have productively rearranged the TCRbeta chain and express a signaling complex called the pre-TCR). The proliferative burst that accompanies beta-selection is estimated to result in a 100–200 fold expansion and is essential for further differentiation and for maximizing TCR diversity. Previous work showed that beta-selection-associated proliferation requires concurrent signals by the pre-TCR and Notch, but how these signals induce cell-cycle progression and why they need to be coordinated has remained unclear. Initiation of proliferation in beta-selected thymocytes requires the ubiquitin-mediated degradation of the cyclin-dependent kinase inhibitor Cdkn1b, which acts to prevent cell-cycle progression. In a recent study, we examined the molecular control of betaselection-associated proliferation. We confirmed prior findings that Cdkn1b degradation is induced by an SCF E3 ubiquitin ligase that contains the ligand recognition subunit Fbxl1. Deletion of Fbxl1 partially blocked beta-selection-associated proliferation, and the defect was rescued by co-deletion of Cdkn1b. We identified a new F-box protein, Fbxl12, that is highly expressed in thymocytes. We found that Fbxl12 also functions as an SCF E3 ligase subunit that, like Fbxl1, directs Cdkn1b degradation. The phenotype of Fbxl12-deficient mice generated in our lab was strikingly similar to Fbxl1-deficient mice, and deletion of both Fbxl1 and

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.



Fbxl12 resulted in a severe block in beta-selection–associated proliferation, indicating that Fbxl1 and Fbxl12 act in concert to regulate thymocyte proliferation. Interestingly, we found that Fbxl1 expression is induced by Notch signaling, whereas Fbxl12 expression is induced by pre-TCR signaling. Both Fbxl1 and Fbxl12 are required for thymocyte proliferation; thus, their selective regulation by Notch and the pre-TCR, respectively, provides an explanation for why concurrent Notch and pre-TCR signaling are necessary for cell-cycle progression and proliferation at the beta-selection checkpoint.

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 LIMzinc 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 Lmo2tg 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 conditionally deleted Ldb1 in *Lmo2-tg* mice. We found that Ldb1 is required for *Lmo2*-tg-induced thymocyte self-renewal and T-ALL induction, indicating that *Lmo2* overexpression promotes T-ALL by functioning as a subunit of Ldb1 complexes. 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 our 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.

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- Dr. Amy Palin is applying for an NIH K99 award.
- NICHD Director's Award
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The Arms Race between KRAB–ZFPs and Transposable Elements and its Impact on Mammals

At the NICHD, our central mission is to ensure that every human is born healthy. Despite much progress in understanding the many ways the mother interacts with the fetus during development, we still know little about the molecular changes that promoted the emergence of placental mammals over 100 million years ago from our egg-laying relatives, nor those mechanisms that continue drive phenotypic differences amongst mammals. One attractive hypothesis is that retroviruses and their endogenization into the genomes of our ancestors played an important role in eutherian evolution, by providing protein coding genes such as syncytins (derived from retroviral env genes that cause cell fusions in placental trophoblasts) and novel gene-regulatory nodes that altered expression networks to allow for implantation and the emergence and continued evolution of the placenta. Our primary interest is to explore the impact of these endogenous retroviruses (ERVs), which account for about 10% of our genomic DNA, on embryonic development and on the evolution of new traits in mammals. This has led us to examine the rapidly evolving Kruppel-associated box zinc-finger protein (KZFP) family, the single largest family of transcription factors (TFs) in most, if not all, mammalian genomes. Our hypothesis is that KZFP gene expansion and diversification was driven primarily by the constant onslaught of ERVs and other transposable elements (TEs) to the genomes of our ancestors, as a means to transcriptionally repress them. The hypothesis is supported by recent evidence demonstrating that the majority of KZFPs bind to TEs and that TEs and nearby genes are activated in KZFP knockout mice. We will continue to explore the impacts of the TE/KZFP "arms race" on the evolution of mammals. We will also begin a new phase exploring whether KZFPs play broader roles in genome regulation, beyond gene silencing, and how these functions impact mammalian development.

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 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



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FIGURE 1. ZFP568 directly represses the *lgf2-P0* transcript.

A. Scatter plots of gene expression in *Zfp568* wildtype (WT) and knockout (KO) embryonic stem cells (ESCs) and trophoblast stem cells (TSCs), as determined by RNA-seq.

B. ZFP568 and H3K9me3 ChIPseq signals at the *lgf2* locus in *Zfp568* WT and KO ESCs and TSCs. DMR0 is a differentially methylated region overlapping exon 1 of the *lgf2-P0* transcript. *lgf2as* is the *lgf2* antisense transcript.

by 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 therefore begun a systematic interrogation of KRAB-ZFP function as a potential adaptive repression system against ERVs.

We focused on ZFP809 as a likely ERV-suppressing KRAB-ZFP, 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 hypothesized that ZFP809 might function *in vivo* to repress other ERVs that utilized the PBSpro. Using ChIP-seq of epitope-tagged ZFP809 in embryonic stems cells (ESCs) and embryonic carcinoma (EC) cells, we determined that ZFP809 binds to several sub-classes of ERV elements via the PBSpro. We generated *Zfp809* knockout mice to determine whether ZFP809 was required for silencing the ERV element VL30pro. 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



FIGURE 2. Structure of ZFP568 zinc fingers bound to the *lgf2-P0* target sequence

ZFP568 uses zinc fingers 3–11 to achieve high-affinity binding to the *Igf2-P0* target sequence. An A-T-rich stretch in the target sequence alters the minor grove distance, leading to non-canonical interactions between zinc fingers and DNA, highlighting the flexibility of zinc fingers in DNAbinding modes to accommodate altered DNA structures.

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. These studies 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 and other retrotransposons targets. The hypothesis is strongly supported by the distinct ERV reactivation phenotypes we observed in mouse ESC lines lacking one of five of the largest KRAB-ZFP gene clusters. Furthermore, our preliminary evidence suggests that KRAB-ZFP cluster KO mice are viable, but have elevated rates of somatic retrotransposition of specific retrotransposon families, providing the first direct genetic link between KRAB-ZFP gene diversification and retrotransposon mobility.

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 or 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 *lgf2* gene called *lgf2-P0* (Figure 1). Insulin-like growth factor 2 (lgf2) 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 *lgf2-P0* promoter, cause inappropriate *lgf2-P0* activation. We also showed that this lethality could be rescued by deletion of *lgf2*. The 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. In an exciting follow-up to these studies, we determined that ZFP568 allele

variants have lost the ability to bind to and repress Igf2-P0, which may have been driven by the loss of the *Igf2-p0* transcript in human placenta. We solve the crystal structure of mouse ZFP568 zinc fingers bound to the Igf2-P0 binding site, which reveals several non-canonical ZF-DNA contacts, highlighting the ability of individual ZFs to change confirmation depending upon ZF context and DNA structure (Figure 2). The structures also explain how mutations in human ZNF568 alleles disrupt *Igf2-P0* interactions, which contain either deleted ZFs or mutations of key ZF-DNA contact residues. Taken together, our studies provide important insights into the evolutionary and structural dynamics of ZF-DNA interactions, which play a key role in regulating mammalian development and evolution.

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- Director's Award, The role of retrotransposons and zinc finger proteins in neural development and disease, Co-PI Levin, on-going
- Human Placenta Project, Exploring KRAB-ZFP gene function in placental development

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Deciphering the Virulence Mechanisms of Microbial Pathogens

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. According to the CDC (Centers for Disease Control), the number of Legionnaires' disease cases in the U.S. has risen more than four-fold over the past 15 years, making *L. pneumophila* an emerging pathogen of increasing relevance. 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 the 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 the NICHD's mission.

Upon inhalation of contaminated water droplets, *L. pneumophila* enters the lung and is phagocytosed (taken up) by specialized immune cells known as alveolar macrophages (Figure 1). 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. The 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 the Dot/Icm type IV secretion system (T4SS) (Figure 1). *L. pneumophila* mutants with a non-functional T4SS 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 emerging 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 macrophage, *L. pneumophila* delivers a large number of effector proteins (*red*) through the Dot/ lcm 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 bacterium. 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 found that the crystal structure of RavN has only residual resemblance to conventional eukaryotic U-box ubiquitin ligases (Figure 2), yet its mode of interaction with E2 enzymes has been preserved throughout evolution, indicating that RavN is a ubiquitin ligase relic. In support of this conclusion, we demonstrated that substitution of residues within the predicted E2 binding interface rendered RavN inactive. The study confirmed that, despite having undergone extensive evolutionary changes, *L. pneumophila* effectors have retained features that are critical for their biological function, including the ability to hijack host factors that are part of the ubiquitylation machinery.

Discovery of inhibitors of the Legionella type IV secretion system

Most classical antibiotics kill bacteria or inhibit their growth by disrupting key aspects of their physiology. Coupled with over-prescription of antibiotics and patient non-compliance, this has led to the rapid emergence of multidrug-resistant strains that have become insensitive to the microbicidal or microbiostatic activity of existing compounds. In addition, recent insight into the complexity and sensitivity of the human microbiome and its importance for human health has raised concerns about the excessive use of antibiotics



FIGURE 2. RavN is a ubiquitin ligase relic from *L. pneumophila*.

Upper panel. Superposition of RavN and two related E3s in complex with their respective E2s (*gray*). A close-up view highlights binding-site residues.

Lower panel. Strep–HRP immunoblot to detect poly-ubiquitylation (indicated by the dark smearing pattern) catalyzed by purified RavN or RavN mutants in which binding site residues have been substituted with serine.

and their collateral effect on commensal microflora. Thus, there is an urgent need for the development of 'smarter' therapeutics that discriminate between pathogens and commensals by selectively targeting virulence components of microorganisms.

Given their essential role in virulence, bacterial secretion systems represent a compelling target for the development of novel therapeutic agents. In collaboration with Anton Simeonov, we designed a high-throughput fluorescence resonance energy transfer (FRET)-based beta-lactamase (B-Lac) reporter assay and screened a library of over 18,000 compounds for candidates that interfere with the ability of *L. pneumophila* to deliver a B-Lac-reporter protein into mouse macrophages (Figure 3). Upon vetting 501 candidate compounds in a variety of *in vitro* and cell-based secondary screens, we identified six lead compounds that fulfilled all criteria of genuine T4SS inhibitors. The compounds efficiently interfered with biological processes that depend on a functional T4SS, such as intracellular bacterial proliferation, but had no detectable effect on *L. pneumophila* growth in culture medium, conditions under which a T4SS is dispensable. Together, the results suggest that, by directly targeting functional aspects of the T4SS, the six lead compounds render *L. pneumophila* incapable of using this translocation system to deliver cargo into recipient cells.

A novel platform for the identification of kinase substrates

Eukaryotic cells receive a continuous stream of signals from the intracellular and extracellular environment that are converting into cascades of phosphorylation events that are catalyzed by protein kinases. Kinases covalently transfer the gamma phosphate group of adenosine triphosphate (ATP) onto side chains of substrate proteins, preferentially serine, threonine, or tyrosine residues, thereby altering the activity, localization, or stability of their substrates. Almost all cellular processes are controlled, at least in part, by protein phosphorylation, explaining why mammalian cells encode hundreds of kinases that target thousands of kinase substrates. Not surprisingly, pathogens like *L. pneumophila* encode molecular mimics of host



FIGURE 3. Screen for inhibitors of the L. pneumophila T4SS

Overview of the high throughput screen for compounds that interfere with reporter protein translocation by the T4SS and that, upon addition to macrophage monolayers (*blue nuclei*), block growth of Lp (*green*).

protein kinases in order to manipulate signaling pathways during infection.

Discovering substrates of bacterial or eukaryotic protein kinases has remained a major challenge because of the transient nature of kinase-substrate interactions and the complexity of the phosphoproteome, even at steady state. To bypass these obstacles, we developed a novel screening platform that combined a previously described thiophosphate labeling technique [Allen JJ *et al.*, *Nat Methods* 2007;4:511] with a high-density human protein microarray containing more than 9,000 human proteins (Figure 4). As phosphate donor for the kinase reaction, we used adenosine 50-O-(3-thiotriphosphate) (ATPgS). When conjugated onto amino acid residues of substrate proteins on the array, the thiophosphate moiety can be alkylated with p-nitrobenzyl mesylate (PNBM) and detected by a thiophosphate ester-specific antibody followed by a fluorescently labeled secondary antibody. Protein spots on the array that are labeled upon incubation with the wild-type form of a kinase but not its catalytically inactive variant represent putative kinase substrate proteins.

The *Legionella* effector kinase LegK7 hijacks the host Hippo pathway to promote infection.

Using Profile Hidden Markov Model-based protein structure prediction to identify cryptic catalytic domains within *L. pneumophila* effectors, we discovered that the protein LegK7 contains a central domain (residues 183–462) that has folding homology to eukaryotic protein kinases. Using the above-mentioned ATPgS labeling technique, we experimentally confirmed that LegK7 indeed exhibits kinase activity *in vitro*. Upon probing the protein microarray platform (Figure 4) with LegK7, we identified MOB1 as a direct substrate of LegK7 (Figure 5). MOB1 is a key scaffold protein within the Hippo kinase signaling pathway, which controls cell-cycle progression, cell proliferation, differentiation, and apoptosis in eukaryotes. In a variety of *in vitro* and cell-based assays, we subsequently showed that LegK7 hijacks the Hippo pathway by molecular mimicking of the host Hippo kinase (MST1 in mammals), which is the key regulator of pathway activation. LegK7, like Hippo/MST1, phosphorylates the scaffolding protein MOB1 on two residues, which

FIGURE 4. A novel platform for the identification of kinase substrates

A human protein microarray containing more than 9,000 human proteins (spotted in duplicate) is probed with a kinase (orange color). Substrates proteins (grey color) of the kinase that are phosphorylated on the microarrays are identified using a fluorescently labeled antibody.



triggers a signaling cascade resulting in the degradation of the transcriptional regulators TAZ and YAP1. Transcriptome analysis by RNAseq revealed that LegK7-mediated targeting of TAZ and YAP1 alters the transcriptional profile of mammalian macrophages, a key cellular target of *L. pneumophila* infection. Specifically, genes targeted by the transcription factor PPARg, which is regulated by TAZ, displayed altered expression, and continuous interference with PPARg activity rendered macrophages less permissive to *L. pneumophila* intracellular growth (Figure 5). Thus, a conserved *L. pneumophila* effector kinase exploits the Hippo pathway to promote bacterial growth and infection.



FIGURE 5. An important role of LegK7 during *L. pneumophila* infection

The conserved Hippo pathway controls cell proliferation and development in eukaryotes. LegK7 is an effector kinase in the pathogen *L. pneumophila* that functionally mimics host Hippo kinase by phosphorylating MOB1. Activated MOB1 triggers degradation of downstream transcriptional regulators, thus altering host gene expression to support bacterial growth.

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RNA Metabolism in Cell Biology, Growth, and Development

We are interested in tRNAs and certain mRNAs as well as some of their key interacting proteins and how the pathways involved in their biogenesis, maturation, and metabolism intersect with processes critical to cell proliferation, growth, and development during health and disease. One focus is the synthesis of tRNAs by RNA polymerase III (RNAPIII), as well as the early phases of their post-transcriptional processing and 'handling' by the eukaryote-ubiquitous RNA-binding protein known as La. The La protein was first discovered because, in some individuals, it becomes a target of autoantibodies and is part of an autoimmune process that leads to (and is diagnostic of) Sjögren's syndrome, systemic lupus erythematosus (SLE), and neonatal lupus. The autoimmunity to La occurs by an as yet undetermined mechanism, and the La protein is sometimes referred to as the La autoantigen. Beyond this, and critical to its normal essential function in vertebrates and other eukaryotes, La contains RNA-binding motifs as well as subcellular trafficking elements. La associates with noncoding (nc) RNA as well as with mRNAs, presumably 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. The major products of RNAP III are the tRNAs, although it also synthesizes 5S rRNA and certain other ncRNAs. We also investigate the biochemistry, genetics, and function of specific post-transcriptional modifications that impact tRNA function during translation of mRNAs into protein by the ribosome. We also study La-related protein-4 (LARP4), which is predominantly cytoplasmic, interacts with the 3' poly(A) tails of mRNAs, and contributes to their stability/metabolism and translational control [Reference 1].

In summary, we strive to understand the structure-function relationship, genetics, cell- and molecular biology of the La protein, LARP4, the tRNA modification enzymes tRNA isopentenyltransferase TRIT1, 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 and mouse tissue culture cells, and gene-altered mice.



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Data from our lab suggest that the levels of cytoplasmic tRNA may uniquely regulate the translation of LARP4 mRNA (and other mRNAs), which in turn promotes stabilization of mRNAs encoding ribosomal proteins [Reference 1]. Tumor suppressors and oncogenes mediate deregulation of tRNA production by RNAP III, collectively contributing to the increased translational capacity required for proliferation during growth and development and of cancer cells. However, tRNA levels/abundance alone do not account for their differential nor their regulated activity. tRNAs are the most heavily and the most diversely modified molecules in cells, and some of the modifications can control or regulate their codon-specific activity. As alluded to above, we study some of the key modifications that affect tRNA translational activity during mRNA decoding.

The tRNA-modification enzyme Trm1 synthesizes dimethyl-guanosine-26 (m2,2G26), which resides at the top of the anticodon stem of several tRNAs, and TRIT1 adds an isopentenyl group onto Adenine-37 in the anticodon loop of certain tRNA (i6A37) molecules. We showed that both modifications can activate their tRNAs for translation in codon-specific assays. We also examine effects of TRIT1 on tRNA activity during mammalian development, using *trit1*-gene–altered mice as a model system in which to understand how a deficiency in the enzyme leads to childhood mitochondrial dysfunction and metabolic disease. We complement our studies by investigating how differences in the copy numbers of tRNA genes can affect how the genetic code is deciphered via use of secondary information in the genetic code. In humans they number more than 500, and are distributed on all the chromosomes, many residing in clusters, the loci of which can vary, as do the loci of individual tRNA gene loci.

A model emerges from the integration of our studies whereby differential expression of tRNAs in a tissueand 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 of 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 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 sources contributing to disease penetrance, especially to multifactorial and multigene disorders.

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

The RNAP III enzyme consists of 17 subunits, several with homology to subunits of RNAPs I and II. In addition, the transcription factor TFIIIC, composed of six subunits, binds to A- and B-box promoters (promoter elements of tRNA genes) and recruits TFIIIB to direct RNAP III to the correct start site. TFIIIB-RNAP III complexes appear highly stable and demonstrate great productivity in supporting the many cycles of initiation, termination, and re-initiation necessary to produce the more than ten-fold molar excess of tRNAs relative to ribosomes that is required to drive translation during growth and development. In contrast to all other multisubunit RNA polymerases, termination and reinitiation by RNAP III (also known as Pol III) are functionally if not physically linked. Our laboratory has developed methods for *in vivo* and biochemical studies to examine the unique mechanisms used by RNAP III. Hereditary mutations in RNAP III cause hypomyelinating leukodysplasia, as well as defects in innate immunity. In addition to its essentiality for cell proliferation, RNAP III is also linked to aging.



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

Red-white colony differentiation by tRNA– mediated suppression

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. Our results reveal the RNAP III terminator to be 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 *maf1*Δ cells. By contrast, the efficiency of *N2,N2*-dimethyl G26 [m(2)2G26]] modification on certain tRNAs was reduced in response to *maf1* deletion and associated with anti-suppression, which we 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 *maf1*Δ cells leads to m(2)2G26 hypo-modification resulting from limiting Trm1, thus reducing the activity of suppressor tRNASerUCA (UCA is the anticodon for serine) 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 antisuppression. 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 [Reference 6].

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 *Schizosaccharomyces 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 the mutants' increased elongation rate. Importantly, similar mutations in spontaneous cancer suggest this as an unforeseen mechanism of RNAP III activation in disease.

The role of La-related protein-4 (LARP4) in poly(A)-mediated mRNA stabilization

Ubiquitous in eukaryotes, 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 (internal ribosome entry site) motifs. La-related protein-4 (LARP4) emerged later in evolution, and we found it to be an mRNA-associated cytoplasmic factor associated with poly(A)-binding protein C1 (PABPC1). LARP4 uses two regions to bind to PABPC1. We showed that the N-terminal domain of LARP4, comprising amino acids 1–286 and containing two RNA-binding motifs known as an 'La module,' exhibits preferential binding to poly(A). The La module is flanked on each side by a different motif that independently interacts with PABP. LARP4 is controlled at the level of mRNA stability: one level of control is by an A+U-rich element (ARE) in its 3' UTR via interactions with the protein tristetraproline (TTP), which is regulated in mammalian cells through TTP by tumor necrosis factor alpha (TNFa); a second level of control was found for LARP4 mRNA coding sequence in an unusual group of synonymous codons with poor match to cellular tRNA levels [Reference 1]. The LARP4 protein controls the metabolism/homeostasis and translation of heterologous mRNAs by affecting their poly(A) tail length [Reference 1].

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

More than 20 years ago, we began developing, refining, and advancing a tRNA-mediated suppression (TMS) system in fission yeast (*Schizosaccharomyces pombe*), which provides a red-white phenotypic real-time assay that can be used to investigate various aspects of tRNA biogenesis, maturation, and metabolism of tRNAs *in vivo*. In fission yeast, the human La protein can replace the tRNA processing/maturation function of Sla1p, the fission yeast equivalent of the 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 this phosphorylation event promotes pre-tRNA processing. We use this system to study transcription by RNAP III, post-transcriptional processing, and tRNA modifications by conserved enzymes that produce tRNA isopentenyl-adenosine-37 and dimethyl-guanosine-26.

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, information that is derived from mRNAs' biased use of synonymous codons. This can produce a layer of information beyond 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 to alterations in the stability of the mRNA. Other types of secondary information can also be encoded in 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 in 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 important 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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Extracellular Vesicles in Pathogenesis of Human Tissue

Our general goal is to understand the mechanisms of pathogenesis and transmission of human pathogens, in particular of the human immunodeficiency virus (HIV). Over the last several years, it has become clear that infected cells release not only viral particles but also extracellular vesicles (EVs) of viral size that play a role in viral infection. Moreover, the release of EVs is a part of normal cell physiology, and EVs play an important role in cellcell communication. During the past year, our efforts focused on establishing the role of EVs in health and disease, in particular in viral infection. It is important to study the role of EVs in the context of human tissues, in which the critical events of many human diseases occur. Our Section was a pioneer in establishing new experimental tissue system in which pathogenesis can be studied in *in vivo*-like systems under controlled laboratory conditions. Also, such systems are now used as a platform in preclinical testing of antivirals.

This year, we continued to develop such systems, in particular, a system of human placenta ex vivo to study its physiology in health and disease. Similarly, we developed a system of ex vivo atherosclerotic plaques to study atherogenesis under controlled laboratory conditions. In such systems, tissue cyto-architecture as well as native cell-cell communications are largely preserved. One of the main means of such communication are cytokines that are release by various cells. We investigated the role of EVs as carriers of cytokines and their role in viral infection in different systems of *ex* vivo tissues and in several body fluids. In particular, we found that, although cytokines are generally considered to function as classical soluble molecules mediating cell-cell communications in multicellular organisms, bioactive cytokines are also released in association with EVs, in particular encapsulated in EVs. The finding is important for our understanding of the mechanisms of cell-cell communication in tissues and may lead to a reconsideration of the analysis of cytokines in immuno-activated tissues, given that, by standard protocols of cytokine measurements, cytokines are not detectable within EVs.

We continued to investigate the role of viral infections on EVs. In work published in 2017, we showed that, in addition to infectious viral particles, HIV–infected cells release EVs that carry viral molecules, in particular membrane molecules. We hypothesized that



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Extracellular vesicles in hCMV infection

Human cytomegalovirus (hCMV) is an important pathogen and is implicated in immune stimulation in the course of HIV infection, even after productive HIV infection is fully suppressed. We investigated whether infected cells release EVs that carry hCMV proteins and may therefore play a role in viral pathogenesis, including host immune response. We studied EVs isolated from the cell-free supernatant of human lung fibroblasts (MRC-5 cells) infected with a recombinant hCMV labeled with an enhanced green fluorescent protein [EGFP] or of primary dermal fibroblast cells infected with the AD169 strain of hCMV, using an iodixanol step-gradient (a radiocontrast, non-osmotic density gradient medium). We determined the purity of this fraction by measuring hCMV DNA by qPCR and the size and distribution of EVs using Nanosight and transmission electron microscopy (TEM). Also, to identify contaminating hCMV virions, we stained all lipidcontaining particles in EGFP-hCMV viral preparations with the fluorescent dye Dil, which stains everything that has a lipid membrane. In our preparation, this was either viral particles (already labeled with EGFP) or vesicles. Thus, viruses become labeled with both dyes whereas vesicles with only one. This analysis of individual particles allowed us to distinguish between hCMV virions and EVs. Using specific fluorescent antibodies, we also analyzed the expression of gB and gH, two abundant envelope glycoproteins of hCMV, on EVs. We found that 15% of EVs were positive for gB and 5% were positive for gH, a proportion consistent with the relative representation of these glycoproteins in the envelope of the mature cell-free virus. A smaller fraction (4%) was positive for both viral proteins. We confirmed this conclusion with a flow technique, originally developed in our laboratory, that allows us to analyze individual EVs based on their capture by 15nm magnetic nanoparticles (MNPs) coupled to monoclonal antibodies. Specifically, we captured Dil-stained EVs, collected from infected or control uninfected MRC-5 cells, and purified them on an iodixanol density gradient, with MNPs coupled to specific anti-gB antibodies and labeled with the fluorescent probe Zenon AF488. To avoid aggregation, we used MNPs in large excess over the number of virions or EVs. We isolated the EV–MNP complexes on magnetic columns and eluted, and visualized them with a flow cytometer. Most of the GFP-negative events were double-positive for Dil and anti-gB AF488 antibodies, thus representing captured gB-positive EVs. Thresholding on AF488 fluorescence revealed a similar number of EVs. In control experiments using uninfected MRC-5 cells, we found no EVs carrying gB. Thus, similar to EVs released by HIV-infected cells, EVs released by hCMV-infected cells carry viral surface proteins. Such EVs may contribute to various physiological effects in which viruses have been implicated, given that these EVs and hCMV should target the same cells, i.e., cells expressing hCMV receptors.

Extracellular vesicles as cytokine carriers

We undertook a first comprehensive study of cytokine association with EVs in an attempt to answer the following questions: (1) how general the phenomenon of cytokine association with EVs is; (2) whether only particular cytokines are associated with EVs; (3) whether a cytokine released in association with EVs in one system can be released as a free (soluble) molecule in another; (4) whether the association of cytokines with EVs is a regulated process that can be modulated; (5) whether cytokines are encapsulated in EVs rather than merely attached to them; and (6) whether EV–encapsulated cytokines can be delivered to sensitive cells and trigger a physiological response.

To answer these questions, we systematically analyzed the association between 33 cytokines and EVs in eight *in vitro*, *ex vivo*, and *in vivo* biological systems (cultured T cells, cultured monocytes, explants of tonsillar, cervical, placental villous, and amnion tissues, amniotic fluid, and blood plasma of healthy volunteers). We demonstrated that the association of a given cytokine with EVs is not necessarily linked to the property of the cytokine but rather to the regulated property of a system. In the eight different systems, we found that a given cytokine can be released predominantly in a soluble form in one system, while in another it can be predominantly EV-associated. For example, the chemo-attracting cytokine MIG (monokine induced by gamma interferon) in the plasma of healthy donors and in amniotic fluid is present almost exclusively in a soluble form, while both monocytes and T cells release this cytokine almost exclusively in association with EVs. This is true also for several other cytokines. In general, placental villous explants secreted cytokines preferentially in soluble forms, including eight cytokines that were found over 90% in the free form. In contrast, T cells and monocytes released some of the same cytokines predominantly associated with EVs. The difference between placental villous explants and immune cells may be related to the high level of expression of cytokines by these tissue explants, while T cell and monocyte suspensions that express much lower levels of cytokines may need to concentrate them in EVs rather than dissolving them in solution.

Among EV-associated cytokines, IL-2, IL-4, IL-10, IL-12, IL-15, IL-16, IL-18, IL-21, IL-22, IL-33, Eotaxin, IP-10, ITAC, M-CSF, MIG, MIP-3, TGF-beta, and TNF-alpha were preferentially encapsulated in EVs across all systems. In some instances, all EV-associated cytokine was inside EVs (for example, IL-10 in cervix, T cell and monocyte cultures, and IFN-gamma in placental villous and amnion cultures, as well as in amniotic fluid). Thus, different biological systems differentially distribute the released cytokines between free and EV-associated forms. The pattern of the cytokine released is not a fixed property of the system but rather can be modulated: both tonsillar explants and cultured monocytes altered the relative fractions of free and EV-associated forms of cytokines upon activation. Moreover, the two stimuli used here to activate monocytes dramatically changed the pattern of cytokine association with EVs. Thus, the pattern of cytokine packaging in EVs strongly depends on the nature of the activator.

The nature of cytokine association with EVs also depends on the system and its activation state. For example, placenta release EV in which IL-22 is predominantly encapsulated while IL-13 is predominantly exposed on the outer EV surface for EVs released by the same tissue. The entire distribution of cytokines between inner and outer EV compartments is not constant but changes upon activation of the systems by different stimuli. Even though a significant fraction of cytokines are encapsulated in EVs and are thus not detected by standard target cell-free cytokine assays, these cytokines may play important physiological roles. Therefore, the interpretations of the roles of these cytokines in health and disease based on these standard assays should be now reconsidered. The system of EV–encapsulated cytokines we described may represent an important system of cell-cell communication in health and disease and may serve as a new therapeutic target.

Development of new ex vivo tissue systems to study pathogenesis

To study normal and pathologic cell interactions in tissues, as well as various tissue infections, it is important to develop adequate models that allow one to study these processes under controlled laboratory conditions. Earlier, we developed a model of lymphoid tissue *ex vivo* that is used to study HIV pathogenesis. We took advantage of this unique system of tissue culture to test dual-targeted compounds that inhibit HIV and TB infection. Also, we developed two new systems to study important aspect of human pathology. (1) To study the mechanisms of placenta function and the role of EVs in pregnancy, we developed an *ex vivo* system that retains placental cyto-architecture and its main metabolic aspects, in particular the release of EVs and soluble factors. (2) To study mechanisms of atherosclerosis, we developed an *ex vivo* system of atherosclerotic plaques.

(1) We used the *ex vivo* system of human placenta to investigate the pattern of secretion of cytokines, growth factors, and EVs by placental villous and amnion tissues. We cultured placental villous and amnion explants for two weeks at the air/liquid interface and analyzed their morphology and the released cytokines and EVs. Placental explants of both placental villous tissue and amnion were viable for at least 14 days. Both types of explants continue to secrete cytokines and growth factors over 14 days of culture, providing further evidence of tissue viability and functioning. We found that syncytiotrophoblast-specific EVs can be captured from placental villous culture supernatants by MNPs coupled to antibodies against specific placental antigens. These EVs carried the membrane proteins CD51, CD63, CD105, CD200, CD274, and syncytin-1. EVs produced by amnion and captured with anti-CD90 MNPs expressed CD29, CD44, CD105, CD140b, CD324, and CD326, which are involved in cell-cell and cell-matrix interactions, as well as cell adhesion, and migration. We investigated the expression of cytokines that were associated with EVs generated by placenta amnion and villous parts. The complex differential distribution of cytokines between EVs of different origin and phenotype suggests a fine regulation of their biogenesis and different biological functions. In general, a system of *ex vivo* placental villous and amnion tissues can be used as an adequate model to study placental metabolic activity in normal and complicated pregnancies.

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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, in the seach for causes of recessive OI, we identified several key genes. 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 we 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. We are also investigating melorheostosis, a very rare bone dysostosis, which is characterized by bone overgrowth in a radiographic pattern of "dripping candle wax." We recently identified mosaic mutations in the oncogene MAP2K1 as the cause of about half of cases of this benign condition. The causative mutations occur at a hot spot in the MAP2K1 negative regulatory domain and inhibit bone-morphometric protein 2 (BMP2)-induced bone differentiation. We are now developing animal models for studies of melorheostosis pathophysiology and treatment.

Mechanism of rare forms of osteogenesis imperfecta

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,



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Postbaccalaureate Intramural Research Training Award Fellow and bone histology with broad unmineralized osteoid and a fish-scale pattern. 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* (the gene encoding interferon-induced transmembrane protein 5, which is mutated in osteogenesis imperfecta type V) in one proband allele, causing a p.S40L substitution in the intracellular domain of BRIL, the encoded protein. The *IFITM5* transcript and BRIL 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. 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 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 in other OI types. However, the proportion of bone with low mineralization was higher in Type VIII bone than type VII, consistent with patchy osteoid only occurring in type VIII.

The third member of the complex, cyclophilin B (CyPB), encoded by *PPIB*, is an ER-resident peptidylprolyl *cis-trans* isomerase (PPIase). CyPB is the major PPIase catalyzing collagen folding. We characterized the first patient with deficiency in *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. 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 1 (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. However, CyPB deficiency results in increased hydroxylation at telopeptide crosslinking sites in tendon, with moderate increase in glycosylation.

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 inositol triphosphate receptor (IP₃R)-mediated calcium release from the ER to the cytoplasm. We found that TRIC-B was undetectable in fibroblasts and osteoblasts of three independent probands. Together with our collaborators Yoshi Yamada and Joshua Zimmerberg, we showed that absence of TRIC-B results in reduced calcium flux from the ER and abnormal store-operated calcium entry, but that ER steady-state calcium is normal. As expected, the disturbed calcium flux causes ER stress along the PERK (protein kinase RNA-like endoplasmic reticulum kinase, an ER-resident transmembrane protein kinase that initiates both pro-apoptotic and pro-survival signaling pathways) pathway of the unfolded protein response (UPR) and elevated BiP, an ER chaperone protein and regulator of ER stress. Disruption of 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%. Procollagen chain assembly is also delayed, likely through sequestration of protein disulfide isomerase (PDI) by calreticulin. 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.

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 lysyl hydroxylase in proband osteoblasts. Reduced collagen crosslinks presumably undermine bone strength. The mutations in *MBTPS2* demonstrate that RIP plays a fundamental role in bone development.

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 (procollagen proteinase C, also known BMP-1 or bone-morphometric protein). 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. The patients have elevated bone density DEXA Z-scores and, in bone histology, patchy unmineralized osteoid. The processing of the C-propeptide from collagen secreted by proband cells is delayed. Using bone mineralization density distribution (BMDD), we investigated mineralization 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 processing intermediate 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. PINP and TRAP, serum markers of bone remodeling, 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 our lab, is a knock-in mouse that contains a Gly349Cys substitution in the alpha1(I) collagen 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 (see below).

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. Alternatively, a single low dose of bisphosphonate concurrent with Scl-AB treatment facilitated the anabolic action of Scl-AB by increasing availability of trabecular surfaces for new bone formation.

Brtl mice also provided important information about the cytoskeletal organization in OI osteoblasts and their potential role in the phenotypic variability. Abnormal cytoskeletal organization was demonstrated 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 cofilin-1. The alterations affected 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 longitudinal length, weight, head circumference, and body mass index (BMI) data on 100 children with type III and IV OI, with known mutations in type I collagen, to generate sexand type-specific growth curves for OI. We examined effects of gender, OI type, and pathogenic variant, using multilevel modeling, and we constructed OI-specific centile curves, using a generalized additive model for location, scale, and shape (GAMLSS). The data show that gender and OI type, but not the collagen chain in which the causative mutation is located, have significant effects on height in OI. Boys are taller than girls, and type IV OI boys and girls are taller than type III. In both genders, length curves for types III and IV OI overlap and the type IV 95th centile curve overlaps the lower US Centers for Disease Control and Prevention (CDC) curves for the general population. A pubertal growth spurt is generally absent or blunted in types III/IV OI. The body-mass-index 50th and 95th centile curves are distinctly shifted above respective US CDC curves in both genders. 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 doses of the bisphosphonate pamidronate.

Melorheostosis: genetic and clinical delineation

Melorheostosis is a very rare sporadic bone dysostosis that is characterized by metabolically active bone in the appendicular skeleton, which leads to asymmetric bone overgrowth, seen radiographically as "dripping candle wax," functional impairment, and pain. Skin overlying the bone lesion often has a hyper-pigmented, vascular lesion. The etiology of melorheostosis had been uncertain; attempts to find germline mutations were unsuccessful, and we hypothesized somatic mutations. Our collaborative team (with investigators

Tim Bhattacharyya, Richard Siegel, and Nadja Fratzl-Zelman) was the first to look directly at bone samples. Fifteen patients with melorheostosis consented to paired biopsies of both affected and contralateral unaffected bone. DNA extracted from each bone tissue was subjected to 100x whole exome sequencing (WES), and each patient's affected samples were compared with their own unaffected samples.

Using WES, we identified somatic mosaic *MAP2K1* (which encodes mitogen-activated protein kinase kinase 1 and is also known as MEK1) mutations in the affected, but not in unaffected, bone of eight unrelated patients and in the skin overlying lesions, but not in blood. There was no evidence of an underlying germline mutation. In affected bone, the mutant allele frequency ranged from 3–34%. Given that melorheostosis is a progressive but not a metastatic condition, it was striking to identify causative mutations in an oncogene. The activating mutations (Q56P, K57E, and K57N) cluster tightly in the *MAP2K1* negative-regulatory domain and would be expected to raise MAP2K1 activity. Identical mutations have been found in several malignancies of other tissues (lung, blood, melanoma, colon), while only three instances of conversion of melorheostosis to osteosarcoma have been reported. Affected bone displays a mosaic pattern of increased p-ERK1/2 in osteoblast immunohistochemistry. Osteoblasts cultured from affected bone constitute two populations with distinct p-ERK1/2 levels by flow cytometry, enhanced ERK1/2 activation, and elevated cell proliferation. However, the *MAP2K1* mutations inhibit BMP2-mediated osteoblast mineralization and differentiation *in vitro*, underlying the markedly increased osteoid detected in affected bone histology. Our data show that the *MAP2K1* oncogene is important in human bone formation and implicate MAP2K1 inhibition as a potential treatment avenue for melorheostosis.

We also reported distinguishing clinical characteristics of melorheostosis patients with *MAP2K1* mutations (MAP2K1–positive melorheostosis). Eight patients with mutations in *MAP2K1* in affected bone were compared with the seven MAP2K1–negative patients to identify distinguishing characteristics. Patients with MAP2K1–positive melorheostosis had a distinct phenotype with the classic "dripping candle-wax" appearance on radiographs, characteristic vascular lesions on skin overlying affected bone, and higher prevalence of extraosseous mineralization and joint involvement. Melorheostotic bone from both MAP2K1–positive and MAP2K1–negative patients showed two zones of distinct morphology: an outer segment of parallel layers of primary lamellar bone and a deeper zone of intensely remodeled highly porous osteonal-like bone. Affected bone from MAP2K1–positive patients showed excessive osteoid, increased numbers of osteoblasts and osteoclasts, and increased vascularity on histology, compared with paired unaffected bone, which was not seen in affected bone in most MAP2K1–negative patients. The identification of a distinct phenotype of patients with MAP2K1–positive melorheostosis demonstrates clinical and genetic heterogeneity among patients with the disease. Further studies are needed to better understand the underlying pathophysiology and associated skin findings.

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Hippocampal Interneurons and Their Role in the Control of Network Excitability

Neocortical projection neurons instruct inhibitory interneuron circuit development in a lineage dependent manner.

Neocortical circuits, for all their complexity, demonstrate a stereotypical organization across regions, with many repeated motifs, which suggests there may be general principles that guide the self-assembly of these circuits during development. A particularly interesting problem for the developing cortex is how to integrate inhibitory interneurons (INs), which are produced by progenitors outside the telencephalon, into circuits among locally generated excitatory projection neurons (PNs). Upon reaching the nascent cortex, INs must coordinate with their new host environment to establish proper positioning, wiring, and total numbers. There is accumulating evidence that this process involves subtype-specific interactions between INs and neighboring PNs. Investigation of the process is daunting, given the large diversity among INs; however, it is possible that the interactions are organized within primary classes of PNs and INs. Recent evidence suggests that the subtype identity of both PNs and INs is important for the process. We knocked out the transcription factor Satb2 in PNs to induce those of the intratelencephalic (IT) type to adopt a pyramidal tract (PT) identity. Loss of IT PNs disrupted the lamination and molecular expression profile of INs derived selectively from caudal ganglionic eminence (CGE), and in a subtype-specific manner. Reprogrammed PNs demonstrated altered connectivity with local CGE INs, with a striking reduction in synaptic targeting of all subtypes. In deep layers of control mice, where IT and PT PNs are intermingled, IT PNs targeted neighboring CGE INs, while PT PNs did not, confirming this lineage-dependent motif. Our data show that both PN class and IN embryonic lineage are important general variables during the construction of cortical circuits.

Subunits of the glutamate receptor AMPAR differentially regulate cellular and synaptic maturation of CGE-derived hippocampal interneurons.

Hippocampal GABAergic local circuit INs target distinct domains of their postsynaptic targets to gate incoming excitatory input, control firing of PNs, and pace synchronized activity among neurons in both



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neonatal and adult brain. To date more than 20 subgroups of GABAergic INs have been identified in the rodent CA1 hippocampus. Fate mapping strategies revealed that distinct interneuron subtypes are derived from either the medial ganglionic eminence (MGE) precursor pools (e.g. parvalbumin [PV]–, somatostatin [SOM]–, and neuronal nitric oxide synthase [nNOS]–containing interneurons) or from CGE precursor pools (e.g., cholecystokinin [CCK]–, vasoactive intestinal polypeptide [VIP]–, reelin [RE]– and calretinin [CR]–containing interneurons). On exiting proliferation, interneurons migrate tangentially towards the hippocampus using both genetic [Pleasure et al., *Neuron* 2000;28:727] and environmental cues. Cell numbers are then consolidated through programmed apoptotic cell death that, in part, relies on activity-dependent regulation through a calcineurin-dependent mechanism. INs regulate all aspects of hippocampal circuit activity, and their dysregulation may contribute to numerous neural-circuit disorders. An understanding of how interneurons are integrated into the developing hippocampal circuit is crucial for establishing future therapeutic strategies. We showed that elimination of AMPAR–mediated excitatory input onto CGE–derived interneurons during development alters emerging anatomical, synaptic, and circuit properties, resulting in deficits in social interaction and hippocampal-dependent learning.

In the hippocampal CA1 microcircuit, CGE-derived INs are recruited by activation of glutamatergic synapses comprising GluA2-containing calcium-impermeable AMPARs. However, the role played by AMPARs in cell migration, survival, and maturation of the developing circuit is unknown. We generated two knockout mouse lines in which we selectively eliminated Gria2 (GluA2 KO), Gria1, Gria2, and Gria3 (GluA1–3 KO), genes that encode subtypes of ionotropic glutamate receptors, in 5HT3AR-Cre-expressing CGE-derived interneurons (5HT3AR is an ionotropic serotonin receptor on a subtype of GABAergic interneurons). In both neonatal (P5–9) and juvenile (P17–21) hippocampus spontaneous excitatory postsynaptic currents (sEPSC), the frequency onto CGE interneurons was significantly reduced in the GluA2 KO, which was coupled with a reduction in dendritic glutamatergic synapse density. Elimination of GluA1-3 almost completely eliminated sEPSCs without further reducing synapse density, but increased the complexity of dendritic branching. In GluA1–3 KOs, the number of interneurons invading the hippocampus was increased in the early postnatal period but converged with wild-type (WT) numbers by P21 due to an increased apoptosis. However, GluA1–3 KOs showed an increase in CCK-containing and a reduction in VIP-containing interneurons. Elimination of GluA1–3–containing synapses significantly reduced recruitment of both feedforward and feedback inhibitory input onto pyramidal neurons and altered the contribution of MGE- versus CGE-derived interneuronmediated inhibition. As a consequence of these combined anatomical, synaptic, and circuit alterations, GluA2 KO and GluA1-KO mice exhibited impaired learning and deficits in social behavior. Thus, AMPAR subunits differentially contribute to numerous aspects of the development and maturation of CGE-derived interneurons and hippocampal circuitry that are essential for normal animal behavior.

Molecular dissection of Neuroligin2 and Slitrk3 reveals an essential framework for GABAergic synapse development.

Synapse development is a multi-step process orchestrated by numerous molecules acting in a highly spatially and temporally controlled manner. Among these molecules, synaptic cell adhesion molecules (CAMs) are a class of membrane proteins essential for the establishment and maturation of synaptic connections. Recent studies identified a growing number of synaptic CAMs, including Neuroligins (NLs) and leucine-rich repeat transmembrane proteins (LRRTMs) that bind to presynaptic neurexins, Slit– and Trk–like family proteins (Slitrks) that bind to presynaptic protein tyrosine phosphatases (PTPs), immunoglobulin superfamily proteins (IgSFs) that mediate *trans*-synaptic homophilic or heterophilic adhesion interactions, cadherin family proteins, and transmembrane tyrosine kinase receptors. Although these molecules are involved in various stages of synapse development, how diverse synaptic CAMs work in concert to control synapse formation remains largely unclear.

In the brain, many types of interneurons make functionally diverse inhibitory synapses onto principal neurons. Although numerous molecules have been identified that function in inhibitory synapse development, it remains unknown whether there is a unifying mechanism for the development of diverse inhibitory synapses. We reported a general molecular mechanism underlying hippocampal inhibitory synapse development. In developing neurons, the establishment of GABAergic transmission depends on Neuroligin 2 (NL2), a synaptic CAM. During maturation, inhibitory synapse development requires both NL2 and Slitrk3 (ST3), another CAM. Importantly, NL2 and ST3 interact with nanomolar affinity through their extracellular domains to synergistically promote synapse development. Selective perturbation of the NL2–ST3 interaction impairs inhibitory synapse development, with consequent disruptions in hippocampal network activity and increased seizure susceptibility. Our findings reveal how unique postsynaptic CAMs work in concert to control synaptogenesis and establish a general framework for GABAergic synapse development.

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Pathophysiology, Genetics, and Treatment of Congenital Adrenal Hyperplasia

In its most severe classic form, congenital adrenal hyperplasia (CAH) is a life-threatening, rare orphan disease that is part of the neonatal screen performed in all 50 U.S. states. In its mildest non-classic form, CAH is considered one of the most common autosomal recessive diseases and may be a common cause of female infertility. Our intramural NIH research program strives to elucidate the pathophysiology and genetics of CAH, thus facilitating the development of new approaches to the diagnosis, evaluation, and treatment of the disease. We are conducting the largest ever Natural History Study of CAH, with over 450 patients enrolled. We were the first to identify adrenaline deficiency as a new hormonal imbalance in CAH and the first to report in CAH smaller-than-normal amygdala, the emotion regulator of the brain, providing insight into hormonal effects on the brain. We found that approximately 10 percent of patients with CAH due to 21-hydroxylase deficiency have a contiguous gene deletion syndrome resulting in CAH with a connective tissue dysplasia, Ehlers-Danlos syndrome, which represents a novel phenotype named CAH-X. Central to our work is the study of new treatments, including a long-term trial testing sex hormone blockade in children, and novel ways of replacing cortisol, aimed at mimicking the normal circadian rhythm of cortisol secretion. The NIH Clinical Center is the ideal venue in which to carry out these studies and is one of the few places in the world that facilitates the conduct of long-term studies of rare diseases.

Epidemiology of nonclassic congenital adrenal hyperplasia

The epidemiology of the classic or severe life-threatening form of CAH due to 21-hydroxylase deficiency is well established (1:10,000 to 1:20,000 live births) owing to the neonatal screening of millions of newborns in over 40 countries worldwide. Neonatal screening does not, however, accurately detect nonclassic CAH; data on the prevalence of the milder form of the disorder are therefore lacking. In a 1985 HLA-B linkage study of 210 families (43 families with nonclassic CAH), nonclassic CAH was estimated to be the most common autosomal recessive condition, with carrier rates especially high in Ashkenazi Jews. Surprisingly, similar studies had not been repeated. This past year, using state-of-the-art genetic analysis,



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FIGURE 1. Schematic of CYP21A1P/CYP21A2 and TNXA/ TNXB chimeric genes

Formation of chimeric genes occurs as a result of misalignment of homologous genes during meiosis. Active genes are in solid colors; pseudogenes are in grey and are framed with the color of the corresponding functional gene. Representative chimeric genes are shown. In total, there are nine known *CYP21A1P/CYP21A2* chimeras (CH-1 to CH-9), and we identified three different types of TNXA/TNXB chimeras (CAH-X CH-1 to CAH-X CH-3) with different junction sites. Approximately 10 percent of patients with CAH due to 21-hydroxylase deficiency carry at least one TNXA/TNXB chimera, resulting in hypermobility-type Ehlers-Danlos syndrome or CAH-X syndrome.

we found that nonclassic CAH is less common in US Ashkenazi Jews than previously suggested and that nonclassic CAH is commonly found in the general US Caucasian population. In collaboration with the Johns Hopkins University School of Medicine's Epidemiology-Genetics Program, we genotyped 200 unrelated healthy subjects of Ashkenazi Jewish descent and 200 healthy Caucasians who did not self-identify as a specific ethnicity. Nonclassic CAH carriership was found in 15% of Ashkenazi Jews and 9.5% of Caucasians, and one subject in each cohort had a genotype consistent with being affected with nonclassic CAH [Reference 1].

As nonclassic CAH may result in infertility that is easily treated with glucocorticoid therapy and nonclassic CAH women not receiving glucocorticoid therapy might have higher miscarriage rates than those receiving treatment, our data have important implications for pre-conception and infertility counseling. Screening for this common and mostly undiagnosed condition in the setting of female infertility, regardless of ethnicity, would offer the opportunity for treatment.

Genotype-phenotype studies of CAH-X

CAH is most commonly caused by 21-hydroxylase deficiency. The gene encoding 21-hydroxylase, *CYP21A2*, and a highly homologous pseudogene, *CYP21A1P*, map to the short arm of chromosome 6 within the human leukocyte antigen histocompatibility complex. Deleterious sequence in the *CYP21A1P* pseudogene can be transferred to the *CYP21A2* functional gene by homologous recombination, and such events produce common

mutations that account for approximately 95% of all *CYP21A2* disease–causing mutations. Of these common mutations, approximately 30% are large deletions. The *TNXB* gene encoding tenascin-X, an extracellular matrix protein that is highly expressed in connective tissue, and a highly homologous pseudogene, *TNXA*, flank *CYP21A2* and *CYP21A1P*, respectively. Autosomal recessive tenascin X deficiency was described as a cause of Ehlers Danlos syndrome in 2001. We hypothesized that deletions of *CYP21A2* might commonly extend into the *TNXB* gene, and we have been studying this phenomena in our Natural History Study.

The first evaluation of the potential clinical implications of *TNXB* heterozygosity in CAH patients was performed in our Natural History Study of CAH (www.ClinicalTrials.gov Identifier No. NCT00250159) at the NIH Clinical Center. In 2013, we prospectively studied 193 consecutive unrelated patients with CAH with clinical evaluations for manifestations of Ehlers Danlos syndrome and genetic evaluations for *TNXB* mutations. Heterozygosity for a *TNXB* deletion was present in 7% of CAH patients; these CAH patients were more likely than age-and sex-matched CAH patients with normal *TNXB* to have joint hypermobility, chronic joint pain, multiple joint dislocations, and a structural cardiac valve abnormality detected by echocardiography. Six of 13 probands had a cardiac abnormality, including the rare quadricuspid aortic valve, a left ventricular diverticulum, and an elongated anterior mitral valve leaflet. As a result of this study, the term CAH-X was coined to describe the subset of CAH patients who display an Ehlers Danlos syndrome phenotype resulting from to the monoallelic presence of a *CYP21A2* deletion extending into the *TNXB* gene.

The study of CAH-X has provided insight into the recombination events that occur in the class III region of the major histocompatibility complex (MHC) locus. This region of the genome is predisposed to genetic recombination and misalignment during meiosis. The majority of deletions that occur generate chimeric *CYP21A1P/CYP21A2* genes. Chimeric recombination between *TNXB* and *TNXA* also occurs (Figure 1). The recombination event deletes *CYP21A2* and therefore represents a CAH disease–causing allele. We described three unique types of *TNXA/TNXB* chimera (CH): CAH-X CH-1 renders the gene nonfunctional, resulting in reduced dermal and serum TNX expression; CAH-X CH-2 alters protein structure; and CAH-X CH-3 is predicted to reduce protein folding energy. Our laboratory continues to investigate how *TNXB* contributes to the phenotype of CAH patients.

To date, we have described 24 patients (19 families) with monoallelic CAH-X and three patients with biallelic CAH-X. Approximately 10 percent of patients with CAH owing to 21-hydroxylase deficiency are now estimated to be affected by CAH-X. Overall, CAH-X patients have generalized joint hypermobility, subluxations, and chronic arthralgia and about 25% have cardiac structural abnormalities. Patients with biallelic CAH-X show severe skin hyperextensibility with delayed wound healing and significant joint hypermobility. Other connective tissue disease manifestations in CAH-X patients include chronic tendonitis and/or bursitis, rectal prolapse, severe gastroesophageal reflux, and cardiac abnormalities.

The study of CAH-X syndrome provides insight into the complex clinical and genetic characteristics associated with CAH and promises to improve patient outcome through the development of focused medical management aimed at preventing long-term consequences.

Novel genetic causes of adrenal insufficiency

The most common cause of primary adrenal insufficiency in adults is autoimmunity, but a genetic etiology must be considered, especially in children. We studied four males from two unrelated families presenting



FIGURE 2. Classic and alternative steroidogenesis pathways leading to adrenal androgen production

In 21-hydroxylase deficiency, elevations of 17-hydroxyprogesterone and androstenedione can activate alternative steroidogenic pathways shown in yellow boxes. (El-Maouche D, Arlt W, Merke DP. *Lancet* 2017;390:2194).

with adrenal insufficiency in childhood, and we identified a genetic cause of their disease [Reference 2]. All had a nonclassic rare form of CAH resulting from a deficiency in the P450 cholesterol side-chain cleavage enzyme (P450scc), which is encoded by *CYP11A1*. All patients carried a *CYP11A1* p.E314K variant. This previously reported variant was predicted to be benign by some models, highlighting the importance of carrying out functional studies. We showed that the p.E314K variant affects P450scc stability and its halflife and clinically impairs both adrenal and gonadal function. Our patients had normal male genitalia, and older males had normal pubertal progression with eventual evidence of peripubertal gonadal failure. Our study highlights the importance of performing genetic studies in all children diagnosed with primary adrenal insufficiency and suggests that mild defects in *CYP11A1* may be common. Identifying the underlying cause of adrenal insufficiency is essential to providing appropriate clinical care.

Management of adrenal insufficiency

Patients with adrenal insufficiency are at risk for life-threatening salt-wasting adrenal crises. Management of illness episodes aims to prevent adrenal crises. This year, we evaluated rates of illnesses and associated factors in a large cohort of patients with adrenal insufficiency due to congenital adrenal hyperplasia followed prospectively at the NIH Clinical Center and receiving repeated glucocorticoid stress-dosing education. Also this year, longitudinal analysis of 156 CAH patients over 23 years was performed [Reference 3]. We evaluated a total of 2298 visits. During childhood there were more illness episodes and stress dosing than during adulthood; however, more emergency room visits and hospitalizations occurred during adulthood. The most robust predictors of stress dosing were young age, low hydrocortisone dose, and high fludrocortisone dose during childhood, and, during adulthood, female sex. Gastrointestinal and upper respiratory tract infections were the two most common precipitating events for adrenal crises and hospitalizations across all ages. Adrenal crisis with hypoglycemia occurred in 11 pediatric patients (ages 1.1–11.3 years). Undetectable epinephrine was associated with emergency room visits during childhood and illness episodes during adulthood. Based on this study, we revised our management of infectious illnesses for patients with adrenal

insufficiency to include more frequent glucocorticoid dosing and recommendations regarding intake of simple and complex carbohydrates. Our new age-appropriate guidelines aim to reduce adrenal crises and prevent hypoglycemia, particularly in children.

New and improved biomarkers of CAH

The diagnosis and management of CAH has been limited by inadequate biomarkers. Several pitfalls have been identified in the use of 17-hydroxyprogesterone, the most commonly used biomarker, for both diagnosis and management. In newborn screening, both false positives and false negatives are common. The development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) panels of adrenal steroids has expanded the repertoire of potential new and improved steroid biomarkers. We found that steroids synthesized with the participation of 11β-hydroxylase (11-oxygenated C19 steroids) are abundant in patients with CAH resulting from 21-hydroxylase deficiency (Figure 2). With our collaborators, we examined the relationship between the serum steroid metabolome of children and adults with classic 21-hydroxylase deficiency and the presence of long-term disease complications. We found that elevations of these 11-oxygenated C19 steroids are associated enlarged adrenal glands and testicular tumors [Reference 4]. These newly described steroids may be useful in the diagnosis and management of CAH.

Novel treatment approaches: circadian cortisol replacement

Humans have biological clocks with characteristic patterns of hormone secretion. Cortisol has a circadian rhythm, with levels low at sleep onset, rising between 0200hr and 0400hr, peaking in the early morning, and then declining throughout the day. Existing glucocorticoid replacement is non-physiologic, and the lack of diurnal rhythm may contribute to the many adverse outcomes observed in patients with adrenal insufficiency. In CAH, physiologic cortisol replacement might improve control of adrenal androgens at lower glucocorticoid doses, thus improving patient outcome. A promising treatment approach we are studying is physiologic cortisol replacement in patients with CAH.

In 2016, we successfully replaced cortisol in a physiologic manner through the use of a pump usually used to deliver insulin. A programmed 24-hour infusion of hydrocortisone was delivered subcutaneously for six months to eight patients with adrenal insufficiency due to 21-hydroxylase deficiency and with multiple co-morbidities. Following six months of pump therapy, patients experienced significant improvement in disease control at similar or lower daily doses of glucocorticoid, and significant improvement in quality-of-life and fatigue compared with oral conventional therapy. This year, we completed a long-term follow-up. Improvements achieved in androgen control, lean body mass, and health-related quality-of-life after six months of pump therapy were maintained at eighteen months [Reference 5].

Our group was the first to study circadian cortisol replacement in CAH patients with the use of a modifiedrelease formulation of hydrocortisone, Chronocort® (CRADA #02800). We successfully completed a phase 2, open-label trial of 16 adults with classic CAH. Compared with various forms of conventional therapy prior to entry, six months of twice daily modified-release hydrocortisone yielded improved disease control throughout the day, using lower hydrocortisone dose equivalent. Successful completion of this phase 2 study (NCT 01735617), carried out at the NIH Clinical Center, resulted in a multi-center international phase 3, parallel arm, randomized, open-label study to determine whether this new modified-release preparation of hydrocortisone improves short-term clinical outcome. We are carrying out long-term follow-up to evaluate outcomes. The studies provide insight into the role that circadian rhythm plays in the development of the comorbidities associated with adrenal insufficiency. Physiologic cortisol replacement represents a novel treatment approach that promises to improve treatment outcome for patients with CAH, as well as other forms of adrenal insufficiency.

Novel treatment approaches: sex steroid blockade in children

As an alternative approach to the treatment of CAH, the effects of elevated androgen and estrogen could be prevented through the use of sex steroid blockade. Short-term (2-year) administration of an antiandrogen, aromatase inhibitor and reduced hydrocortisone was shown to normalize linear growth rate and bone maturation. A prospective long-term randomized parallel study to adult height of an antiandrogen (flutamide) and an aromatase inhibitor (letrozole), and reduced hydrocortisone dose vs. conventional treatment is near completion. The main outcome is adult height, and we will compare data compared between the treatment groups. The goal of this novel treatment approach is to normalize the growth and development of children with CAH and, ultimately, to determine whether this treatment regimen is effective in improving the growth of children with CAH. The Clinical Center is the ideal place to carry out such a longterm study of a rare disease.

Since the inception of our study of peripheral blockade of sex hormones using an antiandrogen and aromatase inhibitor, new and improved drugs that block sex steroids have been developed. In collaboration with extramural investigators, we are studying Abiraterone, an irreversible inhibitor of 17α-hydroxylase, a key enzyme required for testosterone synthesis, in a multicenter Phase 1/2 study in pre-pubescent children (NCT 02574910).

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Childhood Neurodegenerative Lysosomal Storage Disorders

The Section on Developmental Genetics conducts both basic laboratory research and clinical investigations into a group of the most common childhood neurodegenerative lysosomal storage disorders (LSDs), called <u>n</u>euronal <u>c</u>eroid <u>lipofuscinoses</u> (NCLs), also commonly known as Batten disease. The diseases affect mostly children and there is no effective treatment for any of the NCLs. Mutations in at least 13 different genes (called the *CLNs*) underlie various types of NCLs. Among these genes, *CLN1, CLN2, CLN5, CLN10*, and *CLN13* encode soluble lysosomal enzymes; *CLN4* and *CLN14* encode peripherally associated cytoplasmic proteins; *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*.

Despite intense studies, the normal physiological functions of each of the *CLN* genes are poorly understood. Consequently, the development of mechanism-based therapeutic strategies remains challenging. Studies within the past decade have drastically changed our notion that the lysosomes are merely a terminal degradative organelle. Some of the emerging new roles of the lysosome include its central role in nutrient-dependent signal transduction regulating metabolism, cellular proliferation, or quiescence. Thus, endolysosomal dysfunction contributes to pathogenesis of virtually all LSDs.

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 13 NCL types share some common clinical and 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-



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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, the proteins must also be depalmitoylated for recycling or degradation in lysosome. PPT1 catalyzes the cleavage of thioester linkage *S*-palmitoylated proteins, important because *S*-palmitoylated proteins are refractory to degradation by lysosomal hydrolases, and PPT1 deficiency leads to lysosomal accumulation of the 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 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 1]. 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 2].

We also developed a non-invasive methods, using MRI and MRS (magnetic resonance spectroscopy), to evaluate the progression of neuro-degeneration in *Ppt1^{-/-}* mice. The methods permit repeated evaluations of potential therapeutic agents in treated animals. Application of such methods in our clinical trial with INCL

also allowed us to evaluate the progressive decline in brain volume and neuro-degeneration [Reference 3]. In collaboration with the NEI, we are also conducting studies to determine whether electro-retinography can be used to assess the progressive retinal deterioration in *Cln1-⁻⁻* as well as in the *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 thioesterasemimetic 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.

Lysosomal Ppt1 insufficiency may contribute to the pathogenesis of JNCL (CLN3-disease).

Even though mutations in at least 13 different genes cause various forms of NCLs, at the cellular level, all NCLs characteristically accumulate autofluorescent material (ceroid) in lysosomes and, clinically, patients develop seizures, visual failure, and experience a progressive decline in cognitive and motor functions. Such findings suggest shared pathogenic mechanism(s) among these diseases. It has been reported that *CLN3* mutations suppress the exit of CI-M6PR from the trans-Golgi network (TGN). Notably, CI-M6PR transports soluble proteins like PPT1 from the TGN to the lysosome, although how this defect contributes to JNCL pathogenesis remains unclear. We showed that the lysosomes in the brain of *Cln3^{-/-}* mice, which mimic JNCL, and those in cultured cells from JNCL patients contain significantly reduced levels of the Ppt1 protein and Ppt1 enzyme activity. Moreover, in *Cln3^{-/-}* mice, lysosomal accumulation of *S*-palmitoylated proteins (constituents of ceroid) and a progressive increase in intracellular autofluorescence occur in an age-dependent manner. Furthermore, in *Cln3^{-/-}* mice the V0a1 subunit of v-ATPase is mislocalized to the plasma membrane instead of its normal location on the lysosomal membrane, which elevates lysosomal pH, as previously reported in *Cln1^{-/-}* mice, a reliable animal model of INCL [Reference 2]. We propose that lysosomal Ppt1 insufficiency is a pathogenic link between INCL and JNCL.

Altered lysosomal membrane localization of Rab7 dysregulates autophagy in a mouse model of INCL.

In eukaryotic cells, vesicular transport enables intracellular proteins to reach their destinations. In this process, a large superfamily of Ras-like GTPases (called Rabs) play pivotal roles in vesicle formation, cargo selection, sorting, transport, and vesicular fusion, which are all critical for endocytic and autophagic degradation. Rab7, one of the proteins belonging to the Rab superfamily of GTPases, directly or indirectly performs several important functions in the vesicular trafficking and membrane fusion events that occur between early endosome and late endosome/lysosome. Moreover, Rab7 (also known as RAB7A) facilitates the membrane fusion of endosome to lysosome and autophagosome to lysosome. The processes allow

lysosomal acid hydrolases to encounter the cargo from both extracellular and intracellular sources that are delivered to the lysosome for degradation. Disruption of autophagosome-lysosome fusion is one of the suggested mechanisms for the accumulation of undegraded cargo in the lysosome leading to the LSDs.

As stated in the introduction, INCL is a devastating neurodegenerative LSD caused by mutations in the CLN1 gene encoding palmitoyl-protein thioesterase-1 (PPT1), which catalyzes depalmitoylation of Spalmitoylated proteins (constituents of ceroid) required for their degradation by lysosomal hydrolases. Thus, it is suggested that PPT1 deficiency causing lysosomal accumulation of ceroid leads to INCL. However, the molecular mechanism(s) of INCL pathogenesis remains poorly understood. Defective autophagy, due in part to impaired autophagosome-lysosome fusion, has been reported to underlie several LSDs, including some NCLs, but has not been reported in INCL. Even though impaired autophagosome-lysosome fusion is one of the suggested mechanism of dysfunctional autophagy, the precise mechanism of the defect remains unclear. A small GTPase, Rab7, plays critical roles in mediating autophagosome-lysosome fusion. We found that, in *Cln1^{-/-}* mice, which mimic INCL, and in cultured cells from INCL patients, autophagy is dysregulated. We also demonstrate that Rab7 undergoes S-palmitoylation, a reversible post-translational modification of proteins by fatty acids (predominantly palmitate), which promotes protein stability and membrane anchorage. Moreover, dynamic S-palmitoylation (palmitoylation-depalmitoylation) facilitates the protein trafficking and steady-state membrane localization required for the function of many proteins. We found that dynamic S-palmitoylation of Rab7 is essential for its trafficking to the lysosomal membrane. Notably, Rab7 localization on lysosomal membrane is appreciably reduced in Ppt1-deficient Cln1-/- mice. Further, the interaction of Rab7 with its activator, RILP (Rab-Interacting Lysosomal Protein), which is required for its GTPase activity, was suppressed in *Cln1^{-/-}* mice and in cultured INCL cells. The defect most likely impairs autophagosome-lysosome fusion, leading to intracellular accumulation of undegraded cargo (ceroid), thus contributing to INCL pathogenesis. Our findings uncover a previously unrecognized role of Cln1/Ppt1 in regulating Rab7 GTPase activity and suggest that suppression of autophagosome-lysosome fusion may contributes to INCL pathogenesis.

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 to the degradation of macromolecules of intra- and extracellular origin that are delivered to the lysosome. Moreover, it has been reported that dysregulation of lysosomal acidification contributes to pathogenesis in virtually all LSDs, including several NCLs. Furthermore, defective regulation of lysosomal pH has also been reported in common neuro-degenerative diseases such as Alzheimer's and Parkinson's disease. However, despite intense 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
FIGURE 2. Lysosomal acidification defect in a mouse model of infantile neuronal ceroid lipofuscinosis

Lysosomal acidification is accomplished by vacuolar ATPase (v-ATPase) localized to the lysosomal membrane. Consisting of a lysosomal membrane-localized V0 sector and cytosolic V1 sector, v-ATPase is a multi-subunit protein. We discovered that the V0a1 subunit of the v-ATPase requires S-palmitoylation (a reversible, posttranslational modification of proteins by the



16-carbon saturated fatty acid palmitate) for trafficking from the trans-Golgi network (TGN) to the lysosomal membrane. The schematic representation shows the endosomal sorting and trafficking of V0a1 in wild-type (WT) and *Cln1-¹⁻* mice, which mimic INCL. In *Cln1-¹⁻* mice, as opposed to WT littermates, the V0a1 subunit of v-ATPase is misrouted to the plasma membrane instead of its normal location on the lysosomal membrane. The defect dysregulates lysosomal acidification. Given that lysosomal acid hydrolases require an acidic pH in the lysosomal lumen, elevated lysosomal pH contributes to diminished degradative function of these enzymes, thereby contributing to INCL pathogenesis.

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 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 dynamic *S*-palmitoylation, which is required for its sorting and trafficking to the lysosomal membrane [Reference 2]. 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.

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 endoplasmic reticulum (ER) as a pre-propeptide with a molecular mass of about 50 kDa. The cleavage of the leader peptide in the ER generates the 48 kDa precursor of mature CD (pro-CD). In the Golgi complex, attachment of mannose 6-phosphate to pro-CD facilitates the protein's binding to endosomal/lysosomal sorting receptors. The receptor-ligand complexes then exit the *trans*-Golgi network in clathrin-coated intermediates and fuse with the endosomal system. The low pH of the late endosomal lumen facilitates dissociation of the receptor-ligand complexes and allows the ligand (i.e., pro-CD) to be delivered to lysosome. The pro-CD then undergoes further proteolytic cleavage by cathepsin B (CB) and cathepsin L (CL), 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 the 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.

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Gene Regulation in Innate Immunity

Macrophages and related cells such as microglia recognize incoming pathogens and produce cytokines, notably interferons (IFNs), the interleukins IL-1 and IL-6, and tumor necrosis factor alpha (TNFalpha). 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 NFkB. 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 at high levels in macrophages, microglia, and dendritic cells (DCs) 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.

The role of IRF8 in brain inflammation: Alzheimer's disease and Aicardi-Goutières syndrome

IRF8 is a transcription factor of the conserved interferon regulatory factor family. It is expressed in cells important for host anti-pathogen protection and is involved in inflammatory responses. Recent evidence shows that, through its expression in microglia, IRF8 plays a significant role in inflammation in the brain. SNP-based genome-



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FIGURE 1. Microglia in *IRF8* KO brain

Immunohistochemical analysis of microglia in wild-type and IRF8 KO (knockout) brain (onemonth-old). Microglia were stained with the microgliaspecific marker Iba1.

wide association studies revealed that inflammatory genes expressed in microglia are risk factors for Alzheimer's disease (AD), which causes memory loss followed by breakdown of broader cognitive function. We have begun to study the role of IRF8 in AD using the mouse model of AD. Histological studies found that microglia scattered over the entire brain have abnormal morphology in IRF8 knockout (KO) mice (Figure 1). IRF8 KO microglia were all devoid of extensive dendrites projecting next to neurons and appeared similar to those in AD models. RNA-seq analysis of wild-type (WT) and IRF8 KO microglia showed that a large array of AD-associated genes were activated in IRF8 KO microglia (Figure 2). Some of the genes (red dots) were found to be AD risk factors, including *ApoE* (encoding apolipoprotein E), which represented the highest risk factor.

Aicardi-Goutières syndrome (AGS) patients present with varying forms of encephalopathy with prominent neuroinflammation. Symptoms include vision/motor deficits, and cognitive deficiency and vascular damage in the brain associated with lupus (SLE)-like autoimmune conditions. Classically, AGS has been linked to mutations in enzymes that degrade endogenous nucleic acids (DNA and RNA), produced by normal biological processes, including replication, transcription, and DNA repair [Crow Y], Manel N. Nat Rev Immunol 2015;15:429]. Mutations in any of the three RNase H2 subunits account for those in more than 50% of AGS patients. Mutations in other nucleic acid-metabolizing factors linked to AGS are in TREX1 (encoding three-prime repair exonuclease 1), ADAR (encoding double-stranded RNA-specific adenosine deaminase), and *SAMHD1* (encoding deoxynucleoside triphosphate triphosphohydrolase). More recently, constitutively activating mutations in the signaling pathways that sense excess nucleic acids and activate innate immune responses have been found to also cause AGS-like diseases. The Crouch lab showed that defects in RNase H2 lead to accumulation of DNA and activate the cGAS-STING pathway, which detects the presence of cytosolic DNA; loss of Sting eliminates activation of interferon-stimulated genes (ISGs). Accumulation of RNA triggers the RIG-I/MDA5 pathway, which plays a major role in sensing RNA-virus infection by detecting cytoplasmic viral double-stranded RNA. Both pathways signal Tbk1 (a serine/threonine kinase that plays an essential role in regulating inflammatory responses to foreign agents), leading to activation of type I interferons and their downstream pathways. Thus, activation of ISGs, coined the "interferon signature," is a defining feature of AGS. ISG proteins are found in spinal fluids, presumably produced in microglia



FIGURE 2. Alzheimer's diseaseassociated genes in *IRF8* KO microglia

Microglia were sorted from wildtype and *IRF8* KO brain (3-month-old), and RNA seq (an RNA sequencing method) analyses were performed. Genes upregulated in IRF8 KO microglia were enriched with genes upregulated in microglia of the Alzheimer's disease mouse model.

(although not proven), and in peripheral monocytes/macrophages. Recent developments in the field have broadened the description of AGS with regard to its onset, phenotypic severity, and variability. Therefore, in collaboration with Robert Crouch and Yoh-suke Mukouyama, we are studying how IRF8 affects microglial inflammatory responses in mice carrying the mutation in the RNAseH2A, which causes AGS in humans.

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, long stretches of regulatory DNAs densely occupied by transcription factors and chromatin regulators. The super-enhancers 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 of acetyl-histones to the BET family proteins. The inhibitors, affecting mostly BRD4, antagonize cancer growth, particularly leukemia and lymphoma. Furthermore, BET inhibitors have been shown to inhibit inflammatory responses related to cardiovascular and autoimmune diseases. Such 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, the 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 owing 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 (KO) mice, focusing on hematopoiesis and macrophage responses. First, we tested mice in which *Brd4* is deleted in early hematopoiesis using the Vav-Cre technique. We showed that *Brd4* KO mice die during fetal development as a result of 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 macrophage and neutrophils); the resultant *Brd4* KO mice failed to start IL-4-dependent peritoneal macrophage expansion. The results strongly point to a central role of BRD4 in immune cell expansion, which is required for maintaining immunity.

We investigated genome-wide distribution of BRD4 in macrophages in a resting condition and after lipopolysaccharide (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 coding regions. BRD4 binding over genic regions markedly increased after LPS stimulation, indicating that BRD4 moves rapidly over the genome, presumably to accommodate a rapid alteration of histone acetylation.

Because it has been proposed that BRD4 is central to the generation of super-enhancers, we were interested to know whether *Brd4* KO macrophages possessed super-enhancers. We found that 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.

NSD2 guides interferon-induced H3.3 deposition and regulates transcription by recruiting the chromatin reader ZMYND.

IFNs 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, leading 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

Pheochromocytomas (PHEOs) and paragangliomas (PGLs) are rare and clinically important chromaffin cell tumors that typically arise, respectively, from the adrenal gland and from extra-adrenal paraganglia. The clinical features and consequences of PHEO/ PGL result from the release of catecholamines (norepinephrine and epinephrine). An undetected PHEO/PGL poses a hazard to patients undergoing surgery, childbirth, or general anesthesia, because of the potential for excess catecholamine secretion, which can result in significant, often catastrophic outcomes. Diagnosing and localizing a PHEO/PGL can be challenging. Plasma and urinary catecholamines as well as their metabolites and radio-iodinated metaiodobenzylguanidine (MIBG) scanning can yield false-positive/ negative results in patients harboring the tumor, and computed tomography (CT) and magnetic resonance imaging (MRI) lack sufficient specificity. The molecular mechanisms by which genotypic changes predispose to the development of PHEO/PGL remain unknown, even in patients with identified mutations. Moreover, in patients with hereditary predispositions, PHEOs/PGLs differ in terms of their growth, malignant potential, catecholamine phenotype, responses to standard screening tests, various imaging modalities, and therefore, subsequently, to different therapeutic options. Our studies focuses on developmental, molecular, genetic, epigenetic, proteomic, metabolomic, and other types of studies to investigate the bases for predisposition to develop PHEOs/PGLs and the expression of different neurochemical phenotypes and malignant potentials, including therapeutic responses.

Clinical and genetic aspects of pheochromocytoma and paraganglioma

Germline or somatic mutations of numerous genes have been implicated in the pathogenesis of pheochromocytomas/ paragangliomas (PPGLs), including the isocitrate dehydrogenase 1 (*IDH1*) gene and alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) gene. Although concurrent *IDH1* and *ATRX* mutations are frequently seen in gliomas, they have never been reported together in PPGLs. The aim of this study was to characterize one paraganglioma with concurrent *IDH1* and *ATRX* mutations identified by whole exome sequencing. We used leukocyte and tumor DNA for



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whole exome sequencing and Sanger sequencing. We measured the 2-hydroxyglurarate level and the global DNA methylation status in the tumor and analyzed *ATRX*'s cDNA transcripts. Tyrosine hydroxylase (TH), HIF1 α and ATRX staining, as well as telomere-specific FISH were also performed. In this particular case, we confirmed the presence of a somatic *IDH1* (c.394C \rightarrow T, p.R132C) mutation and a concurrent somatic *ATRX* splicing mutation (c.4318-2A \rightarrow G). Dramatic accumulation of 2-hydroxyglutarate was detected in the paraganglioma without the global DNA hypermethylation, and pseudohypoxia was also activated. Importantly, immunohistochemistry revealed negative TH staining in the tumor, and the first exon region of the *TH* gene was hypermethylated, resulting in normal plasma metanephrines. The

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splicing *ATRX* mutation resulted in two transcripts, causing frameshifts. Immunohistochemistry revealed scarce ATRX staining in the tumor. Alternative lengthening of telomeres (ALT) was detected by FISH. The case represented the first concurrence of *IDH1* and *ATRX* mutations in PPGLs. Although relatively rare, a somatic R132C mutation of *IDH1* might play a role in a small subset of sporadic PPGLs.

Our knowledge of the susceptibility genes for PPGLs has widened; however, data on its impact on surgical decision-making has not been described. The aim of this study was to determine the effect of routine preoperative genetic testing on the operative intervention in patients with PPGLs. We subjected 108 patients diagnosed with PPGLs, who underwent 118 operations, to preoperative genetic testing for 9 known PPGL susceptibility genes and performed a retrospective analysis of a prospective database to evaluate clinical factors associated with the surgical approach selected and the outcome of the surgical intervention. In 51 patients (47%), a germline mutation was detected and one-third had no family history of PPGL. In 77 operations (65%), it was the first operative intervention for the disease site (60 laparoscopic, 17 open), and 41 (35%) were reoperative interventions (36 open, 5 laparoscopic). For initial operations, variables associated with whether an open or laparoscopic approach was used were tumor size and the presence of germline mutation. Sixty-eight adrenal operations were performed (54 total, 14 cortical-sparing). Variables significantly associated with a cortical-sparing adrenalectomy being performed were the presence of germline mutation and tumor size. We concluded, that preoperative knowledge of the germline mutation status affects the surgical approach and extent of adrenalectomy.

We also provided the first direct molecular-genetic evidence of an association between a somatic ironregulatory protein 1 (*IRP1*) loss-of-function mutation and pheochromocytoma and secondary polycythemia. In patients diagnosed with PPGL and polycythemia with negative genetic testing for mutations in *HIF2A* (gene encoding hypoxia-inducible factor alpha), *PHD1/2* (gene encoding hypoxia-inducible factor prolyl hydroxylase 1), and *VHL* (Von Hippel–Lindau disease tumor suppressor gene), *IRP1* should be considered a candidate gene.

Imaging of pheochromocytomas and paragangliomas

We evaluated and compared the diagnostic performance of ⁶⁸Ga-DOTA(0)-Tyr(3)-octreotate (⁶⁸Ga-DOTATATE) with ¹⁸F-fluoro-2-deoxy-D-glucose (¹⁸F-FDG) positron emission tomography-computed tomography (PET/CT) and with anatomic imaging using computed tomography and/or magnetic resonance (CT/MR) imaging in the detection of mutated succinate dehydrogenase (SDHx)-related PPGLs in pediatric patients. Nine pediatric



patients (5:4, girls:boys; 14.6 \pm 2.0 years) with an SDHx-related mutation (SDHB:SDHA:SDHD, n = 7:1:1) were included in this retrospective study. At the time of initial diagnosis, 7 of 9 patients had metastatic disease. They all underwent CT/MR imaging along with PET/CT using 68 Ga-DOTATATE (n = 9), 18 F-FDG (n = 8), and positron emission tomography-magnetic resonance imaging (PET/MR) using ¹⁸F-FDG (n = 1). We compared the per-lesion, per-region, and per-patient detection rates and calculated them for each of the imaging modalities. A composite of all functional and anatomic imaging studies served as the imaging comparator. Eight out of nine patients were positive for PPGLs on the imaging studies, which demonstrated 107 lesions in 22 anatomic regions on the imaging comparator. The per-lesion detection rates for ⁶⁸Ga-DOTATATE PET/ CT, ¹⁸F-FDG PET/CT (¹⁸F-FDG PET/CT refers to both ¹⁸F-FDG PET/CT and ¹⁸F-FDG PET/MR), and CT/MR imaging were 93.5% (95% CI: 87.0–97.3%), 79.4% (95% CI: 70.5–86.6%), and 73.8% (95% CI: 64.5–81.9%), respectively. The per-lesion detection rate for ⁶⁸Ga-DOTATATE PET/CT was significantly higher than that for ¹⁸F-FDG PET/ CT or for CT/MR imaging. In all anatomic regions except the abdomen, the per-lesion detection rates for ⁶⁸Ga-DOTATATE PET/CT was found to be equal or superior to ¹⁸F-FDG PET/CT, and CT/MR imaging. The per-region detection rate was 100% (95% CI: 84.6–100%) for ⁶⁸Ga-DOTATATE PET/CT and 90.9% (95% CI: 70.8–98.9%) for both ¹⁸F-FDG PET/CT and CT/MR imaging. The per-patient detection rates for ⁶⁸Ga-DOTATATE PET/CT, ¹⁸FDG PET/CT, and CT/MR imaging were all 100% (95% CI: 63.1–100%). We thus demonstrated the superiority of ⁶⁸Ga-DOTATATE PET/CT in localization of SDHx-related PPGLs in pediatric population over ¹⁸F-FDG PET/CT and

CT/MR imaging with the exception of abdominal (excluding adrenal and liver) lesions, which suggests that it might be considered as a first-line imaging modality in pediatric patients with SDHx–related PPGLs.

MYC-associated factor X (MAX) has been recently described as a new susceptibility pheochromocytoma (PHEO) gene with a total of about 40 reported cases. At present, no study has specifically described the functional imaging phenotype of MAX-related pheochromocytoma. We reported our experience with contrast-enhanced computed tomography (CT) and ¹⁸F-fluorodihydroxyphenylalanine (¹⁸F-FDOPA) positron emission tomography (PET)/CT in six consecutive patients (four at the initial diagnosis and two at the followup evaluation) with rare, but clinically important, MAX-related pheochromocytomas. In five patients, we compared ¹⁸F-FDOPA with other radiopharmaceutical agents. The patients had five different mutations in the MAX gene that caused disruption of Max/Myc interaction and/or abolished interaction with DNA, as determined by in silico analyses. All but one patient developed bilateral pheochromocytomas during their lifetime. In all cases, ¹⁸F-FDOPA PET/CT accurately visualized pheochromocytomas, which were often multiple within the same gland or bilaterally, and detected more adrenal and extra-adrenal lesions than did CT (perlesion sensitivity, 90.9% vs 52.4% for CT/magnetic resonance imaging). The two pheochromocytomas missed on ¹⁸F-FDOPA PET/CT were less than 1 cm in size, corresponding to nodular adrenomedullary hyperplasia. ⁶⁸Ga-DOTA,Tyr3-octreotate PET/CT detected fewer lesions than did ¹⁸F-FDOPA PET/CT in one of three patients, and ¹⁸F-FDG PET/CT was only faintly positive in two of four patients, with underestimation of extra-adrenal lesions in one patient. We concluded that MAX-related pheochromocytomas exhibit a marked ¹⁸F-FDOPA uptake, a finding that illustrates the common well differentiated chromaffin pattern of pheochromocytomas associated with activation of kinase signaling pathways. ¹⁸F-FDOPA PET/CT should be considered as the firstline functional imaging modality for diagnostic or follow-up evaluations for these patients.

In recent years, inherited and acquired mutations in genes encoding the tricarboxylic acid (TCA) cycle enzymes have been reported in diverse cancers. PPGLs often exhibit dysregulation of glucose metabolism, which is also driven by mutations in genes encoding the TCA cycle enzymes or by activation of hypoxia signaling. PPGLs associated with SDH deficiency are characterized by high ¹⁸F-FDG avidity, an association that is currently only partially understood. We therefore hypothesized that accumulation of succinate resulting from the TCA-cycle defect could be the major connecting hub between SDH-mutated tumors and the ¹⁸F-FDG uptake profile. To test whether succinate modifies the ¹⁸F-FDG metabolic profile of tumors, we performed in vitro and in vivo (small-animal PET/CT imaging and autoradiography) experiments in the presence of succinate, fumarate, and phosphate-buffered saline (PBS) in various cell models. As a control, we also evaluated the impact of succinate on ¹⁸F-fluorocholine uptake and retention. We performed glucose transporter 1 (GLUT1) immunohistochemistry to assess whether ¹⁸F-FDG uptake correlates with GLUT1 staining. Intra-tumoral injection of succinate significantly increased ¹⁸F-FDG uptake at 24 h on small-animal PET/CT imaging and autoradiography. No effect of succinate was observed on cancer cells in vitro, but interestingly we found that succinate elevated ¹⁸F-FDG uptake by human umbilical vein endothelial cells in a concentration-dependent manner. No significant effect was observed after intra-tumoral injection of fumarate or PBS. Succinate, fumarate, and PBS have no effect on cell viability, regardless of cell lineage. Intramuscular injection of succinate also significantly increases ¹⁸F-FDG uptake by muscle when compared with either PBS or fumarate, highlighting the effect of succinate on connective tissues. We observed no difference between PBS and succinate on ¹⁸F-fluorocholine uptake in the tumor or muscle or in hind limb blood flow. GLUT1 expression quantification did not significantly differ between the study groups. We showed that succinate stimulates ¹⁸F-FDG uptake by endothelial cells, a finding that partially explains

the ¹⁸F-FDG metabotype observed in tumors with SDH deficiency. Although this study is an ¹⁸F-FDG-based approach, it provides an impetus to better characterize the determinants of ¹⁸F-FDG uptake in various tumors and their surrounding microenvironment, with special emphasis on the role of tumor-specific oncometabolites.

Therapeutic aspects of pheochromocytoma and paraganglioma

Patients harboring germline mutations in SDHB present with PPGLs that are more likely to be malignant and clinically aggressive. We retrospectively evaluated the combination chemotherapy of cyclophosphamide, vincristine, and dacarbazine (CVD) in patients with SDHB-associated metastatic PPGL. Twelve metastatic PPGL patients harboring SDHB mutations/polymorphisms with undetectable SDHB immunostaining were treated with CVD. CVD therapy consisted of 750 mg/m² cyclophosphamide with 1.4 mg/m² vincristine on day 1 and 600 mg/m² dacarbazine on days 1 and 2, every 21–28 days. We determined treatment outcome by RECIST (response evaluation criteria in solid tumors) criteria as by response duration and progression-free and overall survivals. A median of 20.5 cycles (range 4–41) was administered. All patients experienced tumor reduction (12–100% by RECIST). Complete response was seen in two patients, while partial response was observed in 8. The median number of cycles to response was 5.5. Median duration of response was 478 days, with progression-free and overall survivals of 930 and 1190 days, respectively. Serial [18F]-FDG PET and CT imaging demonstrated continued incremental reduction in maximal standardized uptake values (SUV_{max}) values in 26/30 lesions. During treatment administration, the median SUV decreased from over 25 to less than 6, indicating the efficacy of chemotherapy over a prolonged period of time. Prolonged therapy results in continued incremental tumor reduction and is consistent with persistent drug sensitivity. CVD chemotherapy is recommended as part of the initial management in patients with metastatic SDHB-related PPGL.

Metastatic PPGLs are frequently associated with *SDHB* mutations. The CVD regimen is recommended as standard chemotherapy for advanced mPPGL. There is limited evidence to support the role of metronomic schemes (MS) of chemotherapy in metastatic PHEO/PGL treatment. We report two patients with *SDHB*-related metastatic PGL who received a regimen consisting of MS temozolomide (TMZ) and high-dose lanreotide after progression on both CVD chemotherapy and high-dose lanreotide. Molecular profiling of the tumor tissue from both patients revealed hypermethylation of the O-6-methylguanine-DNA-methyltransferase (*MGMT*) promoter (MGMT is a DNA repair enzyme). In one patient, progression-free survival was 13 months and the second patient remained under treatment after 27 months of stabilization of the metabolic response of his disease. Treatment was well tolerated, and adverse effects were virtually absent. A modification in the scheme of TMZ from standard schemes to MS is safe and feasible and can be considered in patients with progressive metastatic PPGL refractory to dacarbazine in standard doses.

Cluster I PPGLs tend to develop malignant transformation, tumor recurrence, and multiplicity. Transcriptomic profiling suggests that cluster I PCPGs and other related tumors exhibit distinctive changes in the TCA cycle (the central driver of cellular respiration), the hypoxia signaling pathway, the mitochondrial electron transport chain, and methylation status, suggesting that the therapeutic regimen might be optimized by targeting these signature molecular pathways. We thus investigated the molecular signatures in clinical specimens from cluster I PPGLs in comparison with cluster II PPGLs that are related to kinase signaling and often present as benign tumors. We found that cluster I PPGLs develop a dependency to mitochondrial complex I, evidenced by the upregulation of complex I components and enhanced NADH dehydrogenation. Alteration in mitochondrial function resulted in strengthened NAD⁺ metabolism, here considered a key mechanism of chemoresistance, in particular of succinate dehydrogenase subunit B (*SDHB*)-mutated cluster I PPGLs via the PARP1/BER DNA repair pathway. Combining a PARP inhibitor with temozolomide, a conventional chemotherapeutic agent, not only improved cytotoxicity but also reduced metastatic lesions, with prolonged overall survival of mice with *SDHB* knockdown PPGLs allograft. Our findings provided novel insights into an effective strategy for targeting.

Proteasome inhibitors have been used frequently 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 FDA-approved proteasome inhibitor bortezomib alone and in combination with another proteasome inhibitor, salinosporamid A, in pheochromocytoma cells. Given that PPGLs are neuroendocrine tumors for which no definite cure is yet available, drugs with a wide spectrum of mechanisms of action are being tested to identify suitable candidates for PPGL treatment. We showed that bortezomib induces pheochromocytoma cell death via the apoptotic pathway both *in vitro* and *in vivo*. The combination of bortezomib with salinosporamid A exhibits additive effect on these cells and inhibits proliferation, cell migration and invasion, and angiogenesis more potently than bortezomib alone. Altogether, we suggest the 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.

Metabolic aspects of PHEO/PGL

Metabolic aberrations have been described in neoplasms with pathogenic variants in the Krebs cycle genes encoding SDH, fumarate hydratase (FH), and isocitrate dehydrogenase (IDH). In turn, accumulation of the oncometabolites succinate, fumarate, and 2-hydroxyglutarate can be employed to identify tumors with those pathogenic variants. Additionally, such metabolic readouts may aid in genetic variant interpretation and improve diagnostics. Using liquid chromatography-mass spectrometry, 395 PPGLs from 391 patients were screened for metabolites to indicate Krebs cycle aberrations. We used multigene panel sequencing to detect driver pathogenic variants in cases with indicative metabolite profiles but undetermined genetic drivers. Aberrant Krebs cycle metabolomes identified rare cases of PPGLs with germline pathogenic variants in FH and somatic pathogenic variants in IDH and SDH, including the first case of a somatic IDH2 pathogenic variants in PPGL. Metabolomics also reliably identified PPGLs with SDH loss-of-function (LoF) pathogenic variants. Therefore, we utilized tumor metabolite profiles to further classify variants of unknown significance in SDH, thereby enabling missense variants associated with SDH LoF to be distinguished from benign variants. We proposed incorporation of metabolome data into the diagnostics algorithm in PPGLs to guide genetic testing and variant interpretation and to help identify rare cases with pathogenic variants in FH and IDH.

Animal model of pheochromocytoma and cell culture studies

Ongoing studies are focusing on the development of SDHB– and HIF2A–related PHEO/PGL. We also started working on animal experiments using innate immunity approaches to treat subcutaneous and on metastatic PHEO in mice.

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NEUROSCIENCES

Mechanisms Regulating GABAergic Cell 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 such as single-cell sequencing to dissect gene expression and connectivity patterns, the classification of interneurons into specific subtypes is ever-evolving. Interneurons and GABAergic projection neurons are born in the ventral forebrain during embryogenesis and undergo a prolonged migratory period to populate nearly every brain region. However, our general understanding of the developmental mechanisms that generate this GABAergic cell diversity remains poorly understood. The goal of our lab is to dissect the genetic and molecular programs that underlie initial fate decisions during embryogenesis and to explore how the environment and genetic cascades interact to give rise to such a stunning diversity of GABAergic cell subtypes. We take a multifaceted approach, 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. We believe these pursuits will act as a springboard for future research and provide new insight into both normal development and various neurodevelopmental diseases.

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



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FIGURE 1. MGE-derived GABAergic cells populate many different brain regions.

The mage 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.

parvalbumin-expressing (SST⁺ and PV⁺) subtypes, and some nNOS (neuronal nitric oxide synthetase)– expressing neurogliaform and ivy cells in the hippocampus. The MGE is a transient, dynamic structure that arises around E10 and bulges into the lateral ventricle over the next several days before dissipating towards the end of embryogenesis. Given that 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 to 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, whereupon 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 interneurons, 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 that 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



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 to 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 (SST⁺) interneurons, whereas basal divisions primarily give rise to parvalbumin (PV⁺) cells.

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 wildtype hosts either homotopically (cortex-to-cortex) or heterotopically (cortex-to-hippocampus or cortex-tostriatum). The technique allows us to determine whether transplanted interneurons adopt properties of 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. We are currently following up on these studies using single-cell RNA sequencing to characterize, in an unbiased manner, how a cell's transcriptome is altered when grafted into a new brain environment.



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. Ctx: cortex; Hip: hippocampus.

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 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 the 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), which 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 fatedetermining genes expressed in specific interneuron populations. Our hope is that the 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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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. We showed that the molecular function of the H19ICR is to organize the region into alternative 3D structures. 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 *lgf2* promoter from these enhancer interactions. Upon paternal inheritance, the H19ICR is methylated and cannot bind to CTCF. Therefore, alternative loop structures form, which allow *lgf2* 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. Especially, we strive to understand the role of development in disease progression. In addition, to the *lgf2/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 [Reference 1].

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 depends on the 2.4 kb *H19* <u>Imprinting Control Region</u> (*H19ICR*), which lies between the two genes, just upstream of the *H19* promoter (Figure 2). On the maternal chromosome, binding of the CTCF protein, a transcriptional regulator, to the *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 cytosine residues within the ICR DNA sequences 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



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.

acquired until relatively late in development, after the embryo implants into the uterus. In contrast, at the endogenous locus, ICR methylation occurs during spermatogenesis. The findings thus imply that DNA methylation is not the primary imprinting mark that distinguishes maternally from paternally inherited ICRs.

The *Nctc1* gene lies downstream of H19 and encodes a long non-coding RNA that is transcribed across the muscle enhancer element (ME in Figure 2), which is shared by *lgf2* 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 paternal *lgf2* promoters. We showed that all three co-regulated promoters (*lgf2*, *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 such 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 [Reference 1].

Molecular mechanisms for tissue-specific promoter activation by distal enhancers [Reference 1]

Normal mammalian development is absolutely dependent on establishing the appropriate patterns of expression of thousands of developmentally regulated genes. Most often, development-specific expression depends on promoter activation by distal enhancer elements. The *Igf2/H19* locus is a highly useful model system for investigating mechanisms of enhancer activation. First, the biological significance of the model





is clear, given that expression of these genes is so strictly regulated. Even two-fold changes in RNA levels are associated with developmental disorders and with cancer. Second, we already know much about the enhancers in this region and have established powerful genetic tools to investigate their function. *Igf2* and *H19* are co-expressed throughout embryonic development and depend on a series of tissue-specific enhancers that lie between 8 and more than 150 kb downstream of the *H19* promoter (or between 88 and more than 130 kb downstream of the *Igf2* promoters). The endodermal and muscle enhancers have been precisely defined, and we generated mouse strains carrying deletions that completely abrogate enhancer function. We also generated insulator insertion mutations that specifically block muscle enhancer activity. We used these strains to generate primary myoblast cell lines so that we can combine genetic, molecular, biochemical, and genomic analyses to understand the molecular bases for enhancer functions.

A LONG NON-CODING RNA IS AN ESSENTIAL ELEMENT OF THE MUSCLE ENHANCER [REFERENCE 2].

Transient transfection analyses define a 300-bp element that is both necessary and sufficient for maximal enhancer activity. However, stable transfection and mouse mutations indicate that this core element is not sufficient for enhancer function in a chromosomal context. Instead, the *Nctc1* promoter element is also essential (*Nctc1* encodes a spliced, polyadenylated long non-coding RNA); *Nctc1* RNA itself is not required (at least in *trans*). Instead mutational analysis demonstrates that it is *Nctc1* transcription through the core enhancer that is necessary for enhancer function. Curiously, the *Nctc1* promoter has chromatin features typical of both a classic enhancer and a classic peptide-encoding promoter. Several recent genomic studies also suggested a role for non-coding RNAs in gene regulation and enhancer function. We will use our model system to characterize the role of *Nctc1* transcription in establishing enhancer orientation, enhancer promoter specificity, and enhancer tissue specificity.

THE MUSCLE ENHANCER (ME) DIRECTS RNA POLYMERASE (RNAP) II NOT ONLY TO ITS COGNATE PROMOTERS (I.E., TO THE H19 AND *IGF*2 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 enhancerdeletion (DME) cell lines (Figure 3). As expected, RNAP binding to the *H19* and *lgf2* 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.



FIGURE 4. Chromatin patterns at the *lgf2/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.

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 *lgf2* and *H19* genes is both enhancer-dependent and insulator-sensitive; that is, a functional insulator located between an enhancer and its regulated gene prevents RNAP binding and likewise prevents RNA transcription. Across the intergenic regions, RNAP binding and RNA transcription are similarly enhancer-dependent (see above). However, intergenic RNAP binding and transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as a physical block for RNAP progression, but rather they specifically interfere with certain RNAP states or activities.



FIGURE 5. Muscle celldifferentiation defects in *lgf2/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.

THE MUSCLE ENHANCER REGULATES RNAP BINDING AND RNA TRANSCRIPTION, BUT DOES NOT ESTABLISH CHROMATIN STRUCTURES.

Both RNA transcription and RNAP binding across the *lgf2/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 the *lgf2/H19* domain.

Function of the *H19* and *Igf2* genes in muscle cell growth and differentiation [References 2 & 3]

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 6. The long noncoding H19 RNA is required for normal myotube fusion and hypertrophy.

Loss-of-imprinting defects at the Igf2/H19 locus result in extra expression of lgf2 and defects in myotube differentiation: Compare W.T (wild-type) with ΔICR/ DICR and Δ ICR/lgf2+ cells. Mutation of the paternal lgf2 gene can restore normal Igf2 expression levels and thus restore normal differentiation (see Δ ICR/Igf2cells). However, these cells still do not make the H19 long non-coding RNA, do not fuse efficiently, and do not respond to Wnt7a signaling.





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/lgf2* act through MAP kinase to regulate differentiation of myoblast cells.

To distinguish the roles of *lgf2* 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 *lgf2* expression via a second mutation in the paternal *lgf2* gene. Analyses of cell lines from such mice demonstrate that extra *lgf2* 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 (BWS) 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 demonstrate that balletic *lgf2* and loss of *H19* play independent and distinct roles in generating the BWS phenotype.

Biallelic expression of *lgf2* results in increased levels of circulating IGF2 peptide, which superactivates insulin and insulin-like receptor kinases in cardiomyocytes, resulting in hyper-activation of AKT/mTOR signaling

pathways, which in turn causes cardiomyocyte hypertrophy and hyperplasia. Such effects result in a cardiac hypertrophy that is non-pathologic and transient, that is, the hearts function normally and, as long as *H19* levels are normal, the heart size normalizes after birth, once *Igf2* expression is repressed. Thus, there are no significant health effects associated with loss of imprinting of Igf2 only.

Loss of expression of *H19* is pathologic. Hearts show progressive heart disease as manifested by hypertrophy, increased fibrosis, expression of cardiac failure markers, and reduced and abnormal heart function, as measured by echocardiography. In hearts, *H19* expression is restricted to endothelial cells. *In vivo* analyses of whole hearts and *in vitro* analyses of isolated endothelial cells show that reduced *H19* results in increased endothelial-to-mesenchymal transition (EMT). EMT is a process that is an essential feature of normal cardiac development; for example, formation of cardiac valves requires EMT. However, elevated frequency of EMT is associated with heart disease. Our data support the idea that *H19* regulates the cell fate of endothelial cells, and future experiments aim to identify the molecular mechanisms.

Role of calsequestrin2 in regulating cardiac function [Reference 5)]

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 Ca²⁺ ions in excitation-contraction (EC) coupling and the presumed critical roles of CASQ2 in regulating Ca²⁺ 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 Ca²⁺ storage in the SR of the cardiomyocyte. Rather, a compensatory increase in SR volume and surface area in mutant mice appears to maintain normal Ca²⁺ storage capacity. Second, CASQ2 is not required for the rapid, triggered release of Ca²⁺ from the SR during cardiomyocyte contraction. Rather, the RyR receptor, an intracellular calcium ion channel, opens appropriately, resulting in normal, rapid flow of Ca²⁺ 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 Ca²⁺ 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.

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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-betacyclodextrin (VTS-270) for NPC1; a multicenter, multinational, clinical efficacy trial of intrathecal VTS-270 was recently completed. We also recently initiated a natural history study of CLN3 disease (juvenile Batten disease) and, in collaboration with NCATS, a multicenter natural history study of creatine transporter deficiency.

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



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FIGURE 1.

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

malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol.

Our laboratory initially cloned the human 3β-hydroxysterol 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*^{delta3-5}/delta3-5</sup>) exhibit variable craniofacial anomalies, are growth-retarded, 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/1793M}* and *Dhcr7^{T93M/delta3-5}* mice are viable and demonstrate SLOS with a gradient of biochemical severity (*Dhcr7^{delta3-5}/delta³⁻⁵* > *Dhcr7^{T93M/delta3-5}* > *Dhcr7^{93M/T93M}*). We used *Dhcr7^{T93M/delta3-5}* 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^{delta3-5/delta3-5*) expressing a *CYP27* (sterol 27-hydroxylase) transgene. *CYP27Tg* mice display increased *CYP27* expression and elevated 27-hydroxycholesterol levels but normal cholesterol levels. While *Dhcr7^{delta3-5/delta3-5* mice are growth-retarded, exhibit a low incidence of cleft palate (9%), and die during the first day of life, *Dhcr7^{delta3-5/delta3-5*:*CYP27Tg*}}}



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.

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 also 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*^{delta3-5}:*CYP27Tg* than in *Dhcr7*^{delta3-5}/del^{ta3-5} 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 further evaluate this finding.



FIGURE 2. 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 3 β -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 phase 1/2a 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 the histone deacetylase (HDAC) inhibitor vorinostat in adult NPC1 patients. The collaboration also includes Paul Helquist and Olaf Wiest 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 in order 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.

Creatine transport deficiency and CLN3 Disease

Recently, we initiated natural history protocols to study children with creatine transport deficiency (CTD) and CLN3 disease. CTD is an X-linked disorder arising from mutation of *SLC6A8* (which encodes Solute Carrier Family 6 Member 8, a protein called sodium- and chloride-dependent creatine transporter 1). Individuals with CTD manifest significant developmental delay and have frequent seizures. The work on CTD is a multicenter trial being conducted in collaboration with NCATS and Lumos Pharma. Our goal is to obtain detailed natural history data, establish a biorepository, find biomarkers, and identify potential clinical outcome measures in preparation for a therapeutic trial.

CLN3 disease (juvenile Batten disease) is an autosomal recessive, progressive neurodegeneration arising from mutation of *CLN3*, the gene encoding the lysosomal/endosomal protein battenin. The function of the battenin is not known, but its absence leads to a lysosomal storage disorder. Children with CLN3 disease typically first lose vision, followed by progressive cognitive and motor impairment. Similar to the other disorders that we study, our goal is to conduct a natural history study in order to facilitate studies designed to understand the pathology underlying these disorders as well as development of therapeutic interventions.

Additional Funding

- 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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Genome Three-Dimensional Organization as a Determinant of Cell-Fate Decisions

Our lab is interested in understanding cell-lineage differentiation, gene regulation, and how non-coding DNA elements and the 3D architecture of chromosomes contribute to these processes during development and disease. We are also interested in early mammalian development as a system in which decipher how cells make lineage decisions and how gene-regulatory networks are established.

Eukaryotic cells need to deal with the biophysical constrains of packaging two meters of DNA inside a tiny nucleus (2–10 microns) and retain the ability to access both its coding and non-coding elements to precisely orchestrate gene expression programs. Research over the past decade has begun to elucidate the mechanisms through which DNA condensation and organization in the nucleus are achieved. The results of such research suggest that the processes are tightly controlled and are themselves critical components of gene regulation. Our long-term goal is understand how such processes occur *in vivo* and how their regulation dictates cell identity and cell-fate decisions in mammals.

To do so, our research program combines the robustness of mouse genome editing and genetics with cutting-edge sequencing-based genomic techniques such as ATAC-seq, ChIP-seq, and Hi-C, as well as live-imaging approaches. We believe that the early mouse embryo is an ideal model system in which to determine how nuclear architecture is regulated in the context of an organism and how it impacts cell behavior and identity.

Fertilization is the ultimate reprograming experiment where two highly differentiated cells (oocyte and sperm) fuse to form a zygote with totipotent potential. This involves a massive rearrangement of epigenetic modifications, both at the level of the DNA and of the histones, and the activity of several transcriptional regulators. Our studies aim to understand how 3D chromatin structures are established during this period, and how this impacts future developmental decisions.

Following fertilization and within a few cell divisions, the first cell lineages are established, and different gene-expression programs are put into action. In mammals, the result is the formation of the



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Sarah K. Frail, BSc, Postbaccalaureate Intramural Research Training Award Fellow blastocyst, a structure that contains three different cell types, each with a defined differentiation potential. The trophectoderm is responsible for forming the placenta, the primitive endoderm leads to the yolk sac, and the epiblast gives rise to all remaining embryonic tissues. We will build on decades of lineage-fate experiments and precisely characterized signaling pathways known to regulate early mouse development to understand the contribution of nuclear organization to gene regulation during these early cell fate decisions.

We are also interested in understanding not only how DNA organization impacts cell behavior, and ultimately animal development and health, but also the mechanisms through which DNA folding itself is established and regulated, and which proteins are involved in these processes. To broadly address these questions, we will employ a number of high-throughput technologies that we have established in the lab, in combination with genome-wide CRISPR screens. Ultimately, candidates identified this way will be fully characterized *in vivo* to stringently determine their impact on gene regulation during mammalian development.

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FIGURE 1.

Representative image of the lab's research

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Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. The 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. Previously, we reported that intraamniotic inflammation, which affects at least one of every three preterm neonates, is characterized by the activation of amnioticfluid neutrophils, cells that represent the first line of defense against infection. Using DNA fingerprinting, we determined that amnioticfluid 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 further



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major 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. This year, the sensitivity and specificity of FINE in fetuses with normal hearts and congenital heart disease in the second and third trimesters was reported for the first time.

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 used noninvasive methods to interrogate neuro-connectivity. In previous work, we reported a study showing that fetuses subsequently born preterm have a disorder of neuro-connectivity not seen in 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.

Vaginal progesterone for preventing preterm birth and adverse perinatal outcomes in singleton gestations [Reference 1]

We conducted a meta-analysis of individual patient data to evaluate the efficacy of vaginal progesterone for preventing preterm birth and adverse perinatal outcomes in singleton gestations with a sonographically detected short cervix in the midtrimester, including the data of the OPPTIMUM study. Trials comparing vaginal progesterone vs. placebo/no treatment in women with a singleton gestation and a midtrimester sonographic cervical length of 25 mm were included in this study. The primary outcome was preterm birth

Study	Relative risk (fixed) (95% CI)	Vaginal progesterone n/N	Placebo n/N	Weight (%)	Relative risk (95% Cl)
Fonseca 2007		19/114	31/112	28.5	0.60 (0.36-1.00)
O'Brien 2007		1/12	4/19	2.8	0.40 (0.05-3.13)
Hassan 2011		21/235	36/223	33.6	0.55 (0.33-0.92)
Cetingoz 2011 ←		→ _{0/4}	1/4	1.4	0.33 (0.02-6.37)
Norman 2016		29/133	35/118	33.7	0.74 (0.48-1.12)
Combined		70/498	107/476	100.0	0.62 (0.47-0.81)
0.05 0.1 0.2 Favors vagi	0.1 0.2 0.3 0.5 1 2 3 5 Test for heterogeneity: $l^2 = 0\%$ Favors vaginal progesterone Favors placebo Test for overall effect: Z = 3.44, P = 0.0006				

FIGURE 1.

The effect of vaginal progesterone on preterm birth at less than 33 weeks of gestation

FIGURE 2. Schematic comparison of the fetal immune system between term and preterm labor

A. At baseline in term pregnancy, fetal DCs (dendritic cells) express low levels of co-stimulatory molecules, few maternal cells circulate in the fetal blood stream (maternal microchimerism, MMc), inflammatory cytokines in the fetal plasma are low, and the majority of fetal T cells are naive.

B. In the context of preterm labor, the MMc is elevated, and there is a possible presence of bacteria owing



to PPROM, which could both serve as sources of antigens for DCs. Fetal DCs express higher levels of co-stimulatory molecules and present these antigens to naive T cells that subsequently will differentiate into effector cells with a Th1 phenotype and producing IFN- γ . Release of inflammatory cytokines such as TNF- α by activated fetal T cells results in uterine contraction.

at less than (<) 33 weeks of gestation. Secondary outcomes included adverse perinatal outcomes and neuro-developmental and health outcomes at two years of age. Data were available from 974 women (498 allocated to vaginal progesterone, 476 allocated to placebo) with a cervical length of 25 mm participating in five high-quality trials.

Vaginal progesterone was associated with a significant reduction in the risk of preterm birth at <33 weeks of gestation (Figure 1). Moreover, vaginal progesterone significantly reduced the risk of preterm birth at <36, <35, <34, <32, <30, or <28 weeks of gestation; spontaneous preterm birth at <33 and <34 weeks of gestation; respiratory distress syndrome; composite neonatal morbidity and mortality; birthweight of <1500 and <2500 g; and admission to the neonatal intensive care unit. Maternal adverse events, congenital anomalies, and adverse neuro-developmental and health outcomes at two years of age did not differ between groups.

In summary, vaginal progesterone reduces the risk of preterm birth and improves perinatal outcomes in singleton gestations with a midtrimester sonographic short cervix, without any demonstrable harmful effects on childhood neuro-development.

Vaginal progesterone is as effective as cervical cerclage to prevent preterm birth in women with a singleton gestation [Reference 2].

An indirect-comparison meta-analysis of individual patient data compared the efficacy of vaginal progesterone and cerclage in preventing preterm birth and adverse perinatal outcomes in women with a singleton gestation, previous spontaneous preterm birth, and a midtrimester sonographic short cervix. Both vaginal progesterone and cerclage, compared with placebo or no cerclage, significantly reduced the risk of preterm birth <35 and <32 weeks of gestation and composite perinatal morbidity/mortality. Adjusted indirect comparison meta-analyses did not show statistically significant differences between vaginal progesterone and cerclage in the reduction of preterm birth or adverse perinatal outcomes. We conclude

that vaginal progesterone and cerclage are equally effective for preventing preterm birth and improving perinatal outcomes in women with a singleton gestation, previous spontaneous preterm birth, and a midtrimester sonographic short cervix.

Alloreactive fetal T cells promote uterine contractility in preterm labor [Reference 3].

Pregnancy is considered as a successful form of immune tolerance in which the mother accepts the semiallogeneic fetus. Given that a breakdown of such tolerance is considered a mechanism for disease, several studies have focused on the mechanisms whereby the maternal immune system rejects the fetus. However, the fetal immune system has been somewhat disregarded. We hypothesized that the fetal immune system must also tolerate the mother and that a breakdown of such tolerance may likewise lead to pregnancy complications. Therefore, we investigated whether maternal antigen-specific activated fetal T cells contribute to the pathogenesis of spontaneous preterm labor.

We first demonstrated that antigen-presenting cells are activated in the umbilical cord blood of preterm neonates, which was accompanied by an increased proportion of pro-inflammatory memory CD4⁺ T cells together with a corresponding decline in naive CD4⁺ T cells, thus establishing that preterm neonates possess a distinct population of activated pro-inflammatory memory CD4⁺ T cells. Maternal cells can be present in the fetal circulation during pregnancy, where they promote the generation of fetal regulatory T cells in order to suppress fetal immune responses. However, the number of maternal cells present in the fetal circulation can increase in women with pregnancy complications. We therefore determined whether changes in these maternal cells in the cord blood are associated with preterm labor. We found that maternal cells were increased in the circulation of preterm neonates. CD4⁺ and CD8⁺ T cells from preterm neonates underwent more proliferation when presented with maternal antigens than those from term neonates; moreover, the response was specific to the mother, given that proliferation was not observed in response to antigens from third-party donors. Furthermore, T cells from preterm neonates expressed increased pro-inflammatory signals in response to stimulation with maternal antigens. Using an established model of myometrial contractility, we showed that unstimulated CD4⁺ and CD8⁺ T cells from preterm neonates caused increased contractility, suggesting that T cells from preterm neonates contribute to the onset of labor (Figure 2).

Using a mouse model of *in utero* adoptive transfer, we also established that the presence of activated T cells in mouse fetuses in mid-pregnancy can result in pregnancy loss. Collectively, the findings demonstrate that fetal anti-maternal rejection is a novel mechanism for spontaneous preterm labor and are the first demonstration that the fetal immune system can reject the mother, leading to pregnancy termination.

A new customized fetal growth standard for African American women [Reference 4].

Current nomograms for the assessment of fetal growth in African American women were derived either from neonatal (rather than fetal) biometry data or were not customized for maternal ethnicity, weight, height, parity, and fetal sex. The goal of this study was to: (1) develop a new customized fetal growth standard for African American mothers; and (2) compare such a standard with three existing standards for the classification of fetuses as small (SGA) or large (LGA) for gestational age. A retrospective cohort study included 4183 women (4001 African American and 182 Caucasian) from the Detroit metropolitan area who



FIGURE 3. Effect of covariates on fetal growth in African American women

Unless otherwise stated in the legend of each panel, continuous lines represent the estimated fetal weight median and the 10th/90th centiles for a female (F) fetus born at term to a nulliparous African American mother, having a height of 163 cm, weighing 64 kg at first visit. Interrupted lines show how the chart would change for a male fetus (A), for 10 additional kg of maternal weight (*B*), for a mother in her third pregnancy (parity=2) (*C*), and for a combination of factors (10 additional kg in maternal weight [Wt.], 10 additional centimeters in height [Ht.], parity of 2, and a male [M] fetus) (D).

underwent ultrasound examinations between 14 and 40 weeks of gestation (the median number of scans per pregnancy was 5, interquartile range 3–7) and for whom relevant covariate data were available. We used longitudinal quantile regression to build models defining the "normal" estimated fetal weight (EFW) centiles for gestational age in African American women, adjusted for maternal height, weight, parity and fetal sex, and excluding pathologic factors with a significant effect on fetal weight (Figure 3). We compared the resulting PRB/NICHD growth standard with three other existing standards: the customized gestation-related optimal weight (GROW) standard; the NICHD African American standard; and the multinational World Health Organization (WHO) standard, which is used to screen fetuses for SGA (<10th centile) or LGA (>90th centile) based on the last available ultrasound examination for each pregnancy.

The screen-positive rate for SGA was 7.2% for the NICHD African American standard, 12.3% for the GROW standard, 13% for the WHO standard customized by fetal sex, and 14.4% for the PRB/NICHD customized standard. For all standards, the screen-positive rate for SGA was at least two-fold higher among fetuses delivered preterm than at term. The screen-positive rate for LGA was 8.7% for the GROW standard, 9.2% for the PRB/NICHD customized standard, 10.8% for the WHO standard customized by fetal sex, and 12.3% for the NICHD African American standard. Finally, the highest overall agreement among standards was between the GROW and PRB/NICHD customized standards.

We developed a novel customized PRB/NICHD fetal growth standard from fetal data in an African American population without assuming proportionality of the effects of covariates, and without assuming that these effects are equal on all centiles of weight; we also provide an easy-to-use centile calculator. The standard classified more fetuses as being at risk for SGA than do existing standards, especially among fetuses



FIGURE 4. Application of the FINE method to a fetus with tetralogy of Fallot at 23 weeks of gestation

The six echocardiography views are abnormal and demonstrate the typical features of this cardiac defect.

delivered preterm, but it classified about the same number of LGA. The comparison among the four growth standards also revealed that the most important factor determining agreement among standards is whether they account for the same factors known to affect fetal growth.

Fetal intelligent navigation echocardiography (FINE) detects 98% of congenital heart disease [Reference 5].

FINE is a novel sonographic method that we invented and that automatically generates and displays nine standard fetal echocardiography views in normal hearts by applying "intelligent navigation" technology to spatiotemporal image correlation (STIC) volume datasets of the fetal heart. We set out to determine the sensitivity and specificity of FINE in the prenatal detection of congenital heart disease (CHD). We conducted a case-control study of 50 fetuses with a broad spectrum of CHD (cases) (Figure 4) and 100 fetuses with normal hearts (controls) in the second and third trimesters. Using 4-dimensional ultrasound with STIC technology, we acquired volume data sets. After all identifying information was removed, the data sets were randomly distributed to a different investigator for analysis using the FINE method. The diagnostic performance of FINE for the prenatal detection of CHD was: sensitivity of 98% (49 of 50), specificity of 93% (93 of 100), positive likelihood ratio of 14, and negative likelihood ratio of 0.02. Among cases with confirmed CHD, the diagnosis with use of FINE completely matched the final diagnosis in 74% (37 of 50); minor discrepancies were seen in 12% (6 of 50), and major discrepancies were seen in 14% (7 of 50). This is the first time that the sensitivity and specificity of FINE in fetuses with normal hearts and CHD in the second and third trimesters have been reported. Because FINE identifies a broad spectrum of CHD with 98% sensitivity, the method could be used prenatally to screen for and diagnose CHD.

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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 involved in 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 IRFs 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 irondependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration—all the likely result of functional iron deficiency. Biochemically and with expression arrays, we studied, in IRP2^{-/-} mice, the mechanisms that lead to anemia and neurodegeneration with motor neuron loss. 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 resulting from 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 ironsulfur-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 ironsulfur recipient proteins, we developed an algorithm that facilitates



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discovery of previously unrecognized mammalian iron-sulfur proteins. Our work suggests that there are hundreds of previously unrecognized mammalian ironsulfur 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 the regulation of intracellular iron metabolism in mammals

In previous years, our laboratory identified and characterized the *cis* and *trans* elements mediating irondependent 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 IRP1 and IRP2, two essential iron-sensing proteins. 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 ironsulfur 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, which 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. Unexpectedly, we discovered that ferroportin is an abundant protein on mature red cells, where, as our work showed, it is needed to export free iron released from heme by oxidation. Using erythroid ferroportin knockout animals, we showed that absence of ferroportin results in accumulation of intracellular iron, increased oxidative stress, and reduced viability of cells in circulation. Upon realizing that ferroportin is key to reducing free iron levels in red cells, we analyzed the Q248H mutation of ferroportin, which confers gain of function and reduces iron abundance in red cells.

The Q248H mutation underwent positive selection in malarious regions of Africa, and we hypothesized that it conferred resistance to malaria by diminishing iron available to support growth of the malaria parasite in red cells. Upon infecting mice that lacked erythroid ferroportin with several malaria strains, we demonstrated that the mice experienced increased morbidity and mortality, likely because iron concentrations in red cells were high and supported parasite growth well. We noted that more than 8% of African Americans carry this allele, which has the potential to cause tissue iron overload in liver and kidney, perhaps accounting for some of the morbidities to which African Americans are unusually predisposed.

We recently discovered that loss of IRP1 causes polycythemia and pulmonary hypertension through derepression of hypoxia-inducible factor 2-alpha (HIF2 α) translation in renal interstitial through the IRE–IRP system. We confirmed that overexpression of HIF2 α drives production of erythropoietin and polycythemia in a mouse model of Chuvash polycythemia, and we discovered that we could reverse disease by activating Irp1 to repress HIF2 α translation using TEMPOL, which converts Irp1 from the aconitase to the IRE–binding form. Phlebotomy has not been a very helpful therapy to the thousands of patients with Chuvash polycythemia is Russia, and we propose that oral Tempol supplementation could constitute a good therapeutic intervention.

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 wildtype animals to supply animals with functional macrophages; the bone marrow transplants were successful. We then discovered that the bone marrow transplant was not necessary; we demonstrated that exogenously expanded wild-type macrophages can repopulate the reticuloendothelial system of *Hmox1-'-* mice and restore normal erythrophagocytosis, reverse renal iron overload, and reverse anemia. 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. We also seek to understand the role of iron-sulfur cluster assembly in the regulation of mitochondrial iron homeostasis and in 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 the 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 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. 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. In one patient, we found that a splicing abnormality of glutaredoxin 5 was associated with sideroblastic anemia. In the affected tissues, mitochondrial iron overload is a feature common to all three diseases.

In collaboration with Brian Robinson, Victor Gordeuk, Marston Linehan, and John Tisdale, 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 the 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 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 the delivery of iron-sulfur clusters to respiratory chain complexes I–II, 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.

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.

Additional Funding

• Bench-to-Bedside Award: Analysis of whether the Ferroportin Q248H mutation prevalent in Africans and African Americans predisposes to unrecognized pathological tissue iron overload and disease

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Molecular Mechanisms of Synapse Development and Homeostasis

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, whose purpose is to mediate rapid and efficient transmission of signals across the synaptic cleft. Synaptic 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 post-synaptic 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, superresolution 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



Mihaela Serpe, PhD, Head, Section on Cellular Communication Peter Nguyen, Biological Laboratory Technician Tae Hee Han, PhD, Research Fellow Saumitra Dey Choudhury, PhD, Visiting Fellow Lindsey Friend, PhD, Visiting Fellow Tho Huu Nguyen, PhD, Visiting Fellow Rosario Vicidomini, PhD, Visiting Fellow Qi Wang, PhD, Visiting Fellow 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.

Neto, an essential protein that recruits neurotransmitter receptors and organizes post-synaptic densities at the *Drosophila* NMJ

Many neurological disorders are linked to defects in synaptogenesis. The initial clustering functions of receptors in synaptogenesis are poorly understood. Prior to motor neuron arrival at its target muscle, 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, which 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 post-synaptic density (PSD) scaffolds, and importantly, their channel properties. iGluRs assembled from different subunits have strikingly different biophysical properties; their association with different auxiliary subunits increases this diversity even further. In flies, as in humans, synapse strength and plasticity are determined by the interplay between different iGluRs 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.

We previously reported that the *Drosophila* Neto, a transmembrane protein, plays a key role in the initial clustering of the iGluRs at nascent synapses. First, in the absence of Neto, iGluRs are not recruited at synaptic locations and remain scattered as small, nascent clusters away from the presynaptic arbor. In fact, Neto is limiting for iGluRs clustering: Reducing the Neto levels, by RNAi-mediated knock down or in a strong neto hypomorph (neto¹⁰⁹), drastically diminishes the synaptic iGluRs levels. The net levels of muscle iGluRs remain normal, indicating a redistribution of receptors from junctional to extrajunctional locations. Second, masking the extracellular structural motifs of Neto called CUB domains does not prevent Neto from engaging iGluRs and forming functional receptors, but effectively blocks Neto's ability to mediate iGluRs clustering. Post-synaptic differentiation is not even initiated at these synapses, indicating that clustering of iGluRs and not synapse activity triggers synapse development. Thus, self-aggregation and/or interactions with other extracellular cues enable the Neto-dependent clustering of iGluRs. Third, our recent results indicate that the intracellular domain of Neto- β directly engages iGluRs as well as other intracellular proteins to selectively regulate the distribution of iGluR subtypes, the recruitment of post-synaptic proteins, and the organization of post-synaptic structures. Fourth, our studies on functional receptors reconstituted in heterologous systems revealed that Neto modulates the function of iGluRs but not their assembly or surface delivery. Given that iGluR gating properties control the distribution and trafficking of these receptors in vivo, Neto could influence the synaptic recruitment of iGluRs by simultaneously controlling several steps in receptor trafficking and clustering and/or receptor function.

To further characterize the functional domains of Neto, we generated truncated Neto variants and tested their cellular distribution and ability to rescue Neto function during development. Neto is expressed in and apically targeted in several epithelia, permitting us to distinguish between surface delivery defects and synaptic recruitment. We found that the extracellular part of Neto is required for apical targeting as well as for clustering of Neto/iGluR complexes at the NMJ. The intracellular domains of Neto could be completely removed without preventing targeting and clustering at the NMJ. We also determined that Neto binds to iGluRs via its LDLa (low-density lipoprotein class A) motif and transmembrane domain (Tm). Our studies indicate that: (1) that the extracellular and Tm part of Neto are both required and sufficient to (a) interact with iGluRs, (b) target Neto to post-synaptic sites, and (c) stabilize the synaptic iGluRs/Neto clusters; and that (2) the intracellular domains regulate the synaptic targeting of Neto/iGluR complexes.

Our current efforts focus on identifying proteins that interact with Neto and provide iGluR-clustering activities at the developing NMJ. To this end, we initiated several complementary screens: (1) pull-down and mass-spectroscopy comparisons of proteins interacting with the intracellular domains of the fly Neto proteins (α and β); and (2) a synthetic lethality screen (below).

Tenectin, an integrin ligand critical for structural and functional integrity of the fly NMJ

To search for novel extracellular matrix (ECM) proteins important for NMJ development, we took advantage of *neto*¹⁰⁹, a strong *neto* hypomorph mutant that we isolated and characterized in our laboratory. The mutant has drastically diminished levels of synaptic iGluRs, but normal net levels of muscle receptors, indicating a defect in the trafficking and/or stabilization of receptors at junctional locations. Given that 50% of *neto*¹⁰⁹ hypomorphs die during development, further reduction of synaptogenic proteins in hemizygous animals should increase lethality. Using this rationale, we set up a synthetic lethality screen to identify proteins that interact genetically with Neto and that control the development of NMJ. In a pilot, proof-of-concept screen of candidate interactors, we confirmed known NMJ modulators, such as Glass bottom boat (Gbb), a BMP ligand with critical roles during NMJ development, and Mind the gap (Mtg), a neuronal secreted protein previously implicated in the assembly of functional post-synaptic domain. In a search for ECM candidates, we identified a set of overlapping deficiencies that dramatically increased the lethality of *neto*¹⁰⁹ hemizygotes. Among the common loci disrupted by these deficiencies was *tenectin*, a gene encoding a large, secreted protein conserved in many insects but with no obvious mammalian homolog.

Previous studies indicated that Tenectin is a large secreted mucin that confers structural integrity to tubular structures. Also, reduction of Tenectin via RNAi produced flightless adults with locomotor defects. We found that Tenectin is secreted from motor neurons and striated muscles and accumulates in the synaptic cleft. Using genetics, biochemistry, electrophysiology, histology, and electron microscopy, we found that Tenectin recruits the α PS2/ β PS integrin (a transmembrane protein that flexibly links the inside to the outside surface of cells) at synaptic terminals and forms pre- and post-synaptic biologically active *cis* complexes with distinct functions: The presynaptic Tenectin/integrin complexes control neurotransmitter release, while the post-synaptic complexes ensure the architectural integrity of synaptic boutons. Interestingly, removal of Tenectin disrupted integrin recruitment selectively at synaptic locations, without affecting integrin anchoring at muscle attachment sites. Also, excess Tenectin in the striated muscle depleted the synaptic pool of integrin, presumably by sequestering integrin at ectopic locations. We exploited the remarkable features of this selective integrin ligand to reveal an unprecedented role for integrin in connecting the ECM of the

synaptic cleft with the cytoskeletal protein spectrin, in particular to the spectrin-based membrane skeleton. These Tenectin/integrin/spectrin complexes are crucial for the integrity and function of synaptic structures. Our identification and genetic manipulation of a highly selective ligand, such as Tenectin, opens the door for similar strategies to deplete integrins locally and parse out compartment-specific functions for integrins, and thus reveal hidden aspects of the integrin/ECM biology and their functions at synapses.

Local BMP/BMPR complexes regulate synaptic plasticity.

Synaptic activity and synapse development are intimately linked, but our understanding of the coupling mechanisms is limited. In particular, how synapse activity status is monitored and communicated across the synaptic cleft remains poorly understood. Our studies uncovered a role for bone morphogenetic proteins (BMPs) in sensing the activity of post-synaptic receptors and relaying this information across the synaptic cleft.

At the *Drosophila* NMJ, BMP signaling is critical for NMJ growth and neurotransmitter release. BMP signaling fulfills these functions via a canonical signaling pathway triggered primarily by muscle-secreted Glassbottom boat (Gbb), a BMP7 homolog. Gbb binding to the presynaptic BMP type-II receptor (BMPRII) Wishful thinking (Wit) and to the BMPRIs Thickveins (Tkv) and Saxophone (Sax) induces formation of BMP signaling complexes that are retrogradely transported from the synaptic terminals to the motor-neuron soma, which resides in the ventral ganglia. The BMP/BMPR signaling complexes phosphorylate the pathway effector Mad, which associates with a co-Smad and translocates into the motor-neuron nuclei. The nuclear pMad/co-Smad complexes activate transcriptional programs with distinct roles in the structural and functional development of the NMJ.

Interestingly, pMad also accumulates at synaptic locations. We recently demonstrated that synaptic pMad localizes presynaptically at the active zone and constitutes a sensor for synapse activity because it correlates with the post-synaptic iGluRs activity. Furthermore, synaptic pMad marks a novel BMP signaling modality that is genetically distinct 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, the type-A iGluRs. 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 the post-synaptic levels of 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 post-synaptic densities, we proposed that presynaptic pMad marks sites where active postsynaptic type-A iGluRs induce the assembly of trans-synaptic complexes with presynaptic BMP/BMPRs. In this model, the BMP signaling modalities are coordinated by shared, limited components, in particular the BMPRs, which are tightly regulated at transcriptional, translational, and post-translational levels. The canonical BMP signaling pathway requires endocytosis of the BMP/BMPR complexes and their retrograde transport to the motor neuron soma, whereas the novel pathway requires that BMP/BMPRs 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. Thus, BMP signaling may monitor synapse activity and coordinate it with synapse growth and maturation.

Our current efforts focus on elucidating the composition, regulation, and function of the synaptic BMP/BMPR

complexes, using genetics and cell-biology approaches. We had already established genetically that the accumulation of synaptic pMad requires the BMPRII Wit and the BMPRIS Tkv and Sax. In addition, we found that Tkv tagged with the red fluorescent tracer mCherry is distributed to presynaptic aggregates that appear to co-localize with the synaptic pMad at active zones. In recent studies, we screened the entire collection of *Mad* mutants for variant(s) that uncouple the accumulation of synaptic vs. nuclear pMad. We found a mutation that appears to selectively impair the accumulation of synaptic but not nuclear pMad. Importantly, this *Mad* mutant also has reduced GluRIIA synaptic levels, as indicated by immunohistochemistry and electrophysiological assays. Modeling the interaction between this *Mad* variant and the BMP/BMPR complexes should provide insights into the structural elements required for the deployment and function of this new BMP signaling modality.

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Thyroid Hormone Regulation of Vertebrate Postembryonic Development

This laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during postembryonic development. The main models are the metamorphoses of *Xenopus laevis* and *X. tropicalis*, two closely related species, which offer unique but complementary advantages. The control of the developmental process by TH offers a paradigm to study gene function in postembryonic organ development. During metamorphosis, various 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 the 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, through specific larval epithelial cell death, it is transformed into an organ with a many-folded adult epithelium surrounded by elaborate connective tissue and muscles and by 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 their 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 to knock down or knock out endogenous genes for functional analyses.

Investigation of the function of endogenous TR genes using knockout animals

By using the TALEN technology, we had previously generated *X. tropicalis* animals lacking any functional TRα and observed that TRα knockout animals are surprisingly able to complete metamorphosis at a similar age as the wild-typing siblings [Reference 1]. However, careful analyses during development revealed many roles of TRα during *Xenopus* development, including



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FIGURE 1. Schematics showing the effects of TRa (thyroid hormone a) knockout on *Xenopus tropicalis* development

TRa knockout has little effect on embryogenesis, and 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 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 TR α (due to the lack of thyroid hormone) functions to control metamorphic timing, whereas, when thyroid hormone becomes available during metamorphosis.

preventing precocious initiation of metamorphosis when TH is absent, regulating the rate of metamorphic progression, and coordinating temporal regulation of metamorphosis in different tissues/organs [Reference 1]. We have now also generated TRβ heterozygous knockout animals and begun to analyze the role of TRβ in *Xenopus* development. In addition, global RNA sequencing (RNA-seq) and chromatin-immunoprecipitation (ChIP) sequencing (ChIP-seq) are being carried out on wild-type and TR–knockout animal tissues to determine the molecular pathways regulated by distinct TRs in different tissues during metamorphosis.

Genome-wide identification of thyroid hormone receptor targets in the remodeling intestine during *X. tropicalis* metamorphosis

We have been studying how TH regulates adult stem cell development by using TH–regulated intestinal metamorphosis as a model system. A key step is to identify and functionally characterize direct TH target genes during this process. Prior to the release of a well annotated *Xenopus* genome, we generated a set of genomic microarray chips covering about 8,000 bp flanking each predicted transcription start site in *X. tropicalis* genome. We used the chips for chromatin immunoprecipitation assays (ChIP-on-chip) on the intestine of premetamorphic tadpoles treated with or without TH for genome-wide identification of TR binding sites. We have now completed and published this study [Reference 2]. Our work led to the identification of 278 candidate direct TR target genes. Further, we provided evidence that the genes are regulated by TH and likely involved in the TH–induced formation of adult intestinal stem cells during metamorphosis [Reference 2].

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.

The histone methyltransferase DOT1L is a coactivator for the thyroid hormone receptor during *Xenopus* development.

Histone modifications are associated with transcriptional regulation by diverse transcription factors. Genome-wide correlation studies revealed that histone activation marks and repression marks are associated with, respectively, activated and repressed gene expression. One of the histone activation marks is histone H3 K79 methylation, which is carried out by only a single methyltransferase, Dot1L. Interestingly, the ChIP-on-chip analysis mentioned above revealed that Dot1L is regulated by TH at the transcriptional level, and our previous studies showed that H3K79 methylation levels are induced at TH target genes during natural and TH-induced metamorphosis. The findings suggest that TH induces Dot1L expression and that Dot1L, in turn, functions as a TR coactivator to promote vertebrate development. In co-transfection studies or in the reconstituted frog oocyte *in vivo* transcription system, we showed that overexpression of Dot1L enhances gene activation by TR in the presence of TH. Making use of the ability to carry out transgenesis in *X. laevis* and gene knockdown in *X. tropicalis*, we further demonstrated that endogenous Dot1L is critical for TH-induced activation of endogenous TR target genes, while transgenic Dot1L enhances endogenous TR function in premetamorphic tadpoles in the presence of TH. Our studies thus provide, for the first time, complementary gain- and loss-of-functional *in vivo* evidence for a cofactor, Dot1L, in gene activation by TR during vertebrate development [Reference 3].

EVI and MDS/EVI are required for adult intestinal stem cell formation during postembryonic vertebrate development.

As indicated above, we have been using intestinal metamorphosis as a model to study the development of adult organ-specific stem cells in vertebrates. During metamorphosis, the larval epithelial cells in the tadpole intestine undergo apoptosis in response to the rising concentration of TH. Concurrently, a small fraction of the epithelial cells, by a yet unknown mechanism, undergo TH–dependent dedifferentiation to become adult stem cells [Reference 4]. We had previously carried out a microarray analysis to identify genes that are regulated by TH in the intestinal epithelium. Among the genes discovered are the genes ectopic viral integration site 1 (EVI) and its variant, myelodysplastic syndrome 1 (MDS/EVI). They both encode zinc-finger proteins, which have been recognized as important oncogenes in various types of cancer. In contrast to the established role of EVI and MDS/EVI in cancer development, their potential function during vertebrate postembryonic development, especially in organ-specific adult stem cells, is unclear. We showed that high levels of EVI and MDS/EVI are expressed in the intestine at the climax of metamorphosis and induced by TH. By using the TALEN gene-editing technology, we knocked out both EVI and MDS/EVI and showed that EVI and MDS/EVI are not essential for embryogenesis and premetamorphosis in X. tropicalis [Reference 5]. On the other hand, knocking out EVI and MDS/EVI causes severe retardation in the growth and development of the tadpoles during metamorphosis and leads to tadpole lethality at the climax of metamorphosis. Furthermore, the homozygous knockout animals exhibit reduced adult intestinal epithelial stem cell proliferation at the end of metamorphosis (for the few that survive through metamorphosis) or during THinduced metamorphosis. The findings reveal a novel role of EVI and/or MDS/EVI in regulating the formation and/or proliferation of adult intestinal stem cells during post-embryonic development in vertebrates [Reference 5].

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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 make possible the electrical and chemical potential differences that are used to transmit signals and perform work. However, the membrane must be broken frequently 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 boundaryestablishing 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 with 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.

The question as to how molecular scale features influence big biological processes must be answered in the language of physical laws. Physics is the language of mechanism at the molecular scale. The challenge is linking these physics to "big" processes that happen in life. The technique our Unit applies is to use detailed physics-driven molecular simulation to "build up" models that can be applied at the much larger level of the cell, which requires retaining important information and eliminating irrelevant details. The software our lab develops is based around the models that we are building. Thus, 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.

Another key component of our research is to seek the best possible validation of our models. Few techniques are able to yield molecular



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information about lipids. Recent breakthroughs that break the diffraction-limit barrier are typically only applicable to static structures much larger than a molecular dye. In contrast, lipids are small and dynamic. We briefly discuss below our breakthroughs for comparing neutron scattering with our molecular simulations of complex lipid bilayers.

The projects use the NIH Biowulf computing cluster to run simulations and models. We use molecular dynamics software (such as NAMD and CHARMM) to conduct molecular simulations. In-house software development for eventual public distribution is a key element of the lab's work.

Modeling the response of protein structure to lateral stress

The properties of a biological membrane are determined by all components present. The outer bacterial membrane is highly enriched in "beta barrel" proteins, so-called because they are roughly cylindrical and consist of beta sheets, one of the two main forms of secondary structure found in proteins. In a computational study, we determined the response of the beta barrel proteins to lateral stress, reported as an elastic coefficient. In addition, we determined the molecular mechanism for how the protein responds to stress, in terms of the molecular features (sheet-to-sheet hydrogen bonding) that are stressed. We reported a method for determining the elastic response of membrane-embedded biomolecules to stress [Reference 1]. The results are key to determining the response of a cell or bacterium to external forces.

Methodology to compare molecular simulations of bilayers to smallangle neutron scattering (SANS) experiments

Neutron scattering can be applied to determine the molecular details of a complex structure like those of the cell. Neutrons effectively "bounce off" nuclei, creating an interference pattern at a detector, a process that is inherently quantum-mechanical. Unlike crystallography, which takes advantage of repeated elements to generate precise positional information, the information from a scattering experiment is "smeared out," representing the dynamic nature of the lipids' movement. We developed a technique to predict this "smeared out" information from molecular simulations, permitting the validation of the structure of complex membranes predicted by computational application of physical laws.

Comparison with scattering data generated by our colleagues at Oak Ridge National Laboratory indicates that our modeled structure of a complex ordered phase of lipid bilayers is an excellent match, which validates our observation that saturated lipids, when supported by cholesterol, cluster at the nanometer scale. The structure supports the hypothesis that specific lipid-lipid interactions are sufficient to drive changes in protein function in the cell.

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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 are addressing 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 inositoltrisphosphate receptors in the oscillatory calcium release of 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 voltagegated channels expressed in neuroendocrine cells, the cell typespecific 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.

Ligand-gated receptor channels: regulation and function

Former Visiting Fellow Claudio Coddou started two projects on the regulation and function of P2X receptor channels (P2XRs). The work was recently completed and published. The first project focused on the kinetics of P2X2R desensitization. The channels exhibited a slow desensitization during the initial ATP application and a progressive, calcium-dependent increase in rates of desensitization during repetitive stimulation. He observed the pattern in whole-cell recordings from cells expressing recombinant and native P2X2R, and it was termed use-dependent desensitization (UDD).



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FIGURE 1. Cell type-specific expression of neurotransmitters and their receptors in pituitary gland

Ligand-gated receptor channel agonists (GABA, ACh, GLU, and ATP) are synthesized in hypothalamus and transported by the hypophysial portal blood flow to anterior pituitary cells and/ or synthesized and released by anterior pituitary cells. Evidence confirming functional expression of their receptors in different pituitary cell subtypes was obtained after their identification. Secretory anterior pituitary cells other than lactotrophs, as well as folliculostellate cells, also synthetize glutamate, but



functional iGluRs (ionotropic glutamate receptors) have not yet been electrophysiologically detected in any type of pituitary cells. Gray area indicates membrane potential values from -100 mV to +50 mV (Y-axis) and duration of recordings (melanotrophs: 30 s; corticotrophs: 80 s; gonadotrophs and somatotrophs: 40 s; thyrotrophs: 75 s; and lactotrophs: 95 s [X-axis]).

However, no desensitization was observed in perforated-patched cells and in two-electrode voltage clamped oocytes. Addition of ATP, but not of ATPS or GTP, also abolished UDD, whereas intracellular injection of apyrase facilitated receptor desensitization. Experiments with injection of alkaline phosphatase or addition of staurosporine and ATP in the intracellular solution suggested a role for a phosphorylation-dephosphorylation in receptor desensitization. Mutation of residues that are potential phosphorylation sites identified a critical role of the S363 residue in the intracellular ATP action. The findings indicate that intracellular calcium and ATP have opposing effects on P2X2R gating: calcium allosterically facilitates receptor desensitization, and ATP covalently prevents the action of calcium. Single-cell measurements further revealed that intracellular calcium remains elevated after washout in P2X2R-expressing cells, and the blockade of mitochondrial sodium/calcium exchanger lowers calcium concentrations during washout periods to basal levels, suggesting a role of mitochondria in this process. Therefore, the metabolic state of the cell can influence P2X2R gating [Reference 1].

Coddou's second project focused on the role of cyclin-dependent kinase 5 (Cdk5) in P2X2R gating. A putative Cdk5 phosphorylation site is present in the full-size variant P2X2aR, which is absent from the splice variant P2X2bR. We found an interaction between P2X2aR and Cdk5/p35 by co-immunofluorescence when expressed in HEK293 culture cells. We also found that threonine phosphorylation was significantly higher in HEK293 cells co-expressing P2X2a and p35 than in cells expressing only P2X2aR. 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 UDD of P2X2aR, but not of P2X2bR, in cells co-expressing these receptors and p35. In *Xenopus* oocytes, P2X2aR showed a slower UDD than in HEK293 cells, and Cdk5 activation prevented this effect. A similar effect was found in P2X2a/3R heteromeric channels.

The P2X2a-T372A receptor mutant was resistant to UDD. By co-localization using immunofluorescence in primary culture of nociceptive neurons, we observed similar distribution between P2X2aR and Cdk5/ p35 in endogenous cells. Moreover, co-immunoprecipitation experiments showed an interaction between Cdk5 and P2X2aR in mouse trigeminal ganglia. Endogenous P2X2aR-mediated currents in PC12 cells and P2X2/3R-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 P2X2aR is a novel target for Cdk5-mediated phosphorylation, which might play important physiological roles in, for example, pain signaling [Reference 2].

In general, the functions of anterior pituitary cells are controlled by two major groups of hypothalamic and intrapituitary ligands: one exclusively acts on G protein-coupled receptors and the other activates both G protein-coupled receptors and ligand-gated receptor channels. The second group of ligands operate as neurotransmitters in neuronal cells, and their receptors are termed as neurotransmitter receptors. Most information about pituitary neurotransmitter receptors was obtained from secretory studies, RT-PCR (reverse transcriptase-polymerase chain reaction) analyses of mRNA expression, and immunohistochemical and biochemical analyses, which were all performed using a mixed population of pituitary cells. However, recent electrophysiological and imaging experiments characterized GABA-, acetylcholine-, and ATP- activated receptors and channels in single pituitary cell types, expanding this picture and revealing surprising differences in the receptors' and channels' expression between subtypes of secretory cells and between native and immortalized pituitary cells. Recently, we summarized the current knowledge on the electrophysiological and pharmacological properties of these receptors and their roles in calcium signaling and calcium-controlled hormone secretion [Reference 3]. Figure 1 summarizes the current knowledge and hypotheses regarding the expression and role of these channels in secretory pituitary cells.

Voltage-gated channels: regulation and function

In collaboration with Patrick Fletcher and Arthur Sherman, we examined common and diverse elements of ion channels and receptors underlying electrical activity in six major secretory pituitary cells: corticotrophs, melanotrophs, gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs. The cell types are all 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 compared 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. Using a generic mathematical model, we supported these ideas, showing that the model reproduces many of the observed features of pituitary electrical signaling. Mapping our observations to the developmental lineage suggests possible modes of regulation that may give rise to mature pituitary cell types [Reference 4].

Role of phosphatidylinositol 4-kinase in cellular functions

Tamás Balla's group recently reported that sciatic nerves of mice lacking phosphatidylinositol 4-kinase alpha (PI4KA) in Schwann cells show substantially reduced myelin thickness, with grave consequences for

nerve conductivity and motor functions. However, prolonged inhibition of PI4KA in immortalized mouse Schwann cells failed to lower plasma-membrane phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) levels or PI 3-kinase (PI3K) activation, in spite of large reductions in plasma-membrane PI4P levels. Instead, it caused rearrangements of the actin cytoskeleton, which was also observed in sciatic nerves of knockout animals. These and other studies define a role for PI4KA in myelin formation primarily affecting metabolism of key phospholipids and the actin cytoskeleton. Our group contributed to this work with sciatic nerve histochemistry and immunohistochemistry [Reference 5].

Our ongoing collaborative studies with Tamás Balla's group focus on the role of PI4K in pituitary cell functions. The preliminary data, obtained with the phosphatidylinositol kinase inhibitor wortmannin (Wm), at a concentration that inhibits both PI3Ks and PI4Ks (10 µM), indicated that high basal growth hormone release was not affected, and neither was basal and GnRH-stimulated follicle-stimulating hormone (FSH) secretion. In contrast, high basal prolactin (PRL) release was inhibited in a time-dependent manner by 10 µM Wm. Basal luteinizing hormone (LH) release was very low and not affected by Wm, but the sustained GnRH-stimulated secretion was abolished without affecting the initial peak response. Thyrotropin-releasing hormone (TRH)-induced early and sustained PRL release was both inhibited by Wm. Also, concentrationdependent effects of Wm on basal PRL release in static cultures showed enhancement at 100 nM and inhibition at 10 µM, consistent with opposing contribution of Wm–sensitive PI kinases to control of actionpotential secretion coupling in lactotrophs. These results clearly show that Wm affects regulated but not constitutive exocytosis in pituitary gonadotrophs and lactotrophs, two cell types critical for control of reproductive functions. Based on the inhibitory potency curves, the involvement, in lactotrophs, of type III PI4Ks and/or PI3Ks of low Wm sensitivity was implicated in inhibition of PRL and LH secretion, while PI3Ks of high Vm sensitivity (presumably the class-I of these enzymes) in facilitation of action-potential secretioncoupling. Therefore, analysis of the roles of phosphoinositides in secretory functions of gonadotrophs and lactotrophs is warranted. It is important to stress that all our preliminary experiments were performed with pituitaries from females, and that studies have to be extended to males. The first goal of ongoing investigations is to pharmacologically identify the PI kinases involved in LH and PRL secretion. The subsequent goal of ongoing investigations is to explore which step(s) of the complex sequences of the signaling and/or secretion pathways is affected and by which phosphoinositide. We are also progressing in development of appropriate conditional knockout mice models for *in vitro* and *in vivo* studies.

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Olfactory Coding and Decoding by Neuron Ensembles

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 coincidentally from multiple presynaptic neurons 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 in both brain slices and 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 tested 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.



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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 alternative proposal is that the basic tastes are represented by populations of cells, with each cell sensitive to several 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 monosynaptically connected second-order gustatory neurons, before, during, and after tastant delivery.

Surprisingly, we found no evidence consistent with a basic taste model of gustation. Instead, we found that the moth's gustatory system represents individual tastant chemicals as spatio-temporal patterns of activity distributed across the population of gustatory receptor neurons. We further 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. Furthermore, 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 many antennal lobe tracts. In several species, one of these tracts, the mediolateral antennal lobe tract (mIALT), 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 the 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 to another brain area, the mushroom body. Recent studies showed 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 of GABA and ACh in the retina. These unexpected results suggest new roles for the vPN population in olfactory information processing.

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Regulatory Small RNAs and Small Proteins

The group currently has two main interests: (1) identification and characterization of small noncoding RNAs and (2) identification and characterization of small proteins of less than 50 amino acids. Both small RNAs and small proteins have been overlooked because they are not detected in biochemical assays and the corresponding genes are missed by genome annotation and are poor targets for genetic approaches. However, both classes of small molecules are now being found to have important regulatory roles in organisms ranging from bacteria to humans.

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 coimmunoprecipitation with the RNA-binding protein Hfq. To further extend our identification of small RNAs in a range of bacteria species, we recently examined small RNA expression using deep sequencing.

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. We have now also started to explore the role of ProQ, a second RNA chaperone in *E. coli* and other bacteria [Reference 1].

It is 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, as well as during the interaction between bacteria and bacteriophage [Reference 2]. For example, we showed that the Spot 42 RNA, whose levels are highest when glucose is present, plays a broad role in catabolite repression by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse non-preferred



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FIGURE 1. Model for impact of convergently transcribed small protein and sRNA genes on intracellular Mg²⁺

In response to limiting Mg²⁺, the PhoQP twocomponent system induces the transcription of the mRNA encoding the 31-amino acid MgtS protein and MgrR sRNA. These small gene products were first shown to regulate the MgtA Mg²⁺ importer and the *eptB* mRNA, respectively. Our recent studies show that they both also modulate the PitA phosphate symporter to increase intracellular Mg²⁺, pointing to a detrimental role of PitA under limiting Mg²⁺ conditions. carbon sources. Similarly, we discovered that the Sigma(E)-dependent small RNA MicL, transcribed from a promoter located within the 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 coppersensitivity phenotype previously ascribed to inactivation of the *cutC* gene is actually derived from the loss of MicL and elevated Lpp levels. Our observation raises the possibility that other phenotypes currently attributed to protein defects can rather be attributed to deficiencies in unappreciated regulatory RNAs. Most recently, we characterized a set of small RNAs expressed from a locus we named sdsN. 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 nitroreductase NfsA, which catalyzes the reduction of the nitrogroup (NO₂) in nitroaromatic compounds, and of 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 because 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 *E. coli mntP* gene encoding a manganese (Mn²⁺) exporter, directly binds Mn²⁺, resulting in a conformation that liberates the ribosome-binding site. Remarkably, we were able to recapitulate the effect of Mn²⁺–dependent activation of translation *in vitro*. We also found that the *yybP-ykoY* motif responds directly to Mn²⁺ in *Bacillus subtilis*. The identification of the *yybP-ykoY* motif as a Mn²⁺ sensor suggests that the genes preceded by this motif, and which 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 regulatory RNAs that bind to other proteins such as ProQ and 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 a number of short RNAs encode small proteins [Reference 3]. 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 such 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 *E. coli* genome. We tested expression of these predicted as well as previously annotated small proteins by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and assaying for synthesis using immunoblot assays. This approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids are synthesized. We have now initiated a complementary approach, based on genome-wide ribosome profiling of ribosomes arrested in start codons, to identify additional small proteins.

More than half the newly discovered proteins were predicted to consist of a single transmembrane alphahelix and, by biochemical fractionation, were found to be located in the inner membrane. 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.

To elucidate the functions of the small proteins, we are now 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. The combined approaches are beginning to yield insights into how the small proteins act 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 Mn²⁺ through MntR. The lack of MntS leads to reduced activity of Mn²⁺-dependent enzymes under Mn²⁺-poor conditions, whereas overproduction of MntS leads to very high intracellular Mn²⁺ and bacteriostasis under Mn²⁺-rich conditions. These and other phenotypes led us to propose a model whereby, during transitions between low- and high-Mn²⁺environments, *E. coli* uses the Mn²⁺ exporter MntP to compensate for overactivity of the Mn²⁺ importer MntH and MntS to compensate for MntP overactivity.

We also showed that the 31-amino acid inner membrane protein MgtS (formerly denoted YneM), whose synthesis is induced by very low magnesium (Mg²⁺) by the PhoPQ two-component system in *E. coli*, acts to increase Mg²⁺ levels and maintain cell integrity upon Mg²⁺ depletion (Figure 1). Upon development of a functional tagged derivative of MgtS, we showed that MgtS interacts with MgtA to increase the levels of this P-type ATPase Mg²⁺ transporter under Mg²⁺-limiting conditions [Reference 4]. MgtS stabilization of MgtA

provides an additional layer of regulation of this tightly controlled transporter. Surprisingly, we found that overexpression of the MgtS protein also leads to induction of the low-phosphate regulon controlled by the PhoRB two-component system. Studies to understand this activation showed that MgtS, although consisiting of only 31 amino acids, forms a complex with a second protein, PitA, a cation-phosphate symporter [Reference 5]. Given that the additive effects of deleting *mgtA* and *mgtS* on intracellular Mg²⁺ concentrations seen previously are lost in the *pitA* mutant, we suggest that MgtS binds to and prevents Mg²⁺ leakage through PitA under Mg²⁺-limiting conditions. Consistent with a previously unappreciated detrimental role of PitA in low Mg²⁺, we also observed MgrR sRNA repression of PitA synthesis. Thus, in response to Mg²⁺ limitation, PhoQP induces the expression of two convergent small genes whose products act to modulate PitA at different levels to increase intracellular Mg²⁺.

We discovered the 49–amino acid inner membrane protein AcrZ (formerly named YbhT), whose synthesis is increased in response to 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 that this interaction occurs through the inner membrane protein AcrB. Mutants lacking AcrZ are sensitive to many, 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. Detailed structural and mutational studies are now giving insight into how AcrZ changes AcrB activity.

This work, together with our ongoing studies on other small proteins and related findings by others in eukaryotic cells, support 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 signaling pathways involved 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 these 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 exist, 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 in 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 thereof 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 tumor formation pathway in cAMP-responsive, steroidogenic, or related tissues. Ongoing work with collaborating NCI laboratories aims to clarify the role of PDE in the 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 those very similar to iMAD to primary bimorphic adrenocortical disease (PBAD) and McCune-Albright syndrome caused by somatic mutations in the GNAS gene (encoding the G protein-stimulatory subunit alpha [Gsa]). Although a few of the patients with MMAD/AIMAH have germline PDE11A, PDE8B, or somatic GNAS mutations, others have mutations in the genes encoding germline fumarate hydratase (FH), menin (MEN1), or adenomatous polyposis coli (APC), pointing to the range of possible pathways that may be involved. Particularly interesting among these are FH mutations that are associated with mitochondrial oxidation defects linked to adrenomedullary tumors, which led us to investigate a disorder known as the Carney Triad. The Carney Triad is the only known disease that, among its clinical manifestations, has 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 found to harbor 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 and is now widely known as 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 (*ARMC5*) was identified that, when mutated, causes more than a third of the known PMAH cases. The

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FIGURE 1. The Stratakis Lab

The Section staff with Dr. Constantine Stratakis in August 2018 at their NIH, CRC laboratory

function of the gene is unknown, and we thus 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.

Although PPNAD appears to be less heterogeneous and is mostly caused by *PRKAR1A* mutations, 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 as well as 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 the Carney complex that is not associated with *PRKAR1A* mutations. Our laboratory is now investigating the 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 the 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 bone and in the adrenal and are 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 so as to target 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 the 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, which 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, that 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, is caused by Xq26.3 genomic duplication, and is characterized by early-onset gigantism resulting from excess GPR101 function and consequent elevation of GH. Another recent discovery was the identification of SGPL1 (sphingosine-1-phosphate lyase 1) deficiency in patients with primary adrenocortical insufficiency.

Carney complex (CNC) genetics

We have collected families with CNC and related syndromes from several collaborating institutions worldwide.

Through genetic linkage analysis, we identified loci harboring genes for CNC on chromosomes 2 (2p16) and 17 (17q22–24) and are currently searching for other possible loci for this genetically heterogeneous condition. With the application of state-of-the-art molecular cytogenetic techniques, we are investigating the participation of the currently identified genomic loci in expression of the disease, and we constructed a comprehensive genetic and physical map of the 2p16 chromosomal region for the cloning of 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 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 other diseases

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). Such mutations 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.

In 2018, we were successful in obtaining funded through a Uniformed Services University of the Health Sciences Award on the "Genetics of human susceptibility to infections and/or complications of Zika virus: variants of the cyclic AMP-dependent PKA pathway." The resulting publication [Reference 4] described an association between Zika virus disease burden and certain variants of genes involved in the cAMP signaling pathway.

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 demonstrate protein kinase A subunit involvement in additional phenotypes. An example of the ongoing work using PKA-subunit animal models is described in one of the references of this report [Reference 2]. Ongoing work with several animal crosses is investigating various aspects of PKA subunit functions and the possible involvement of cAMP-pathway perturbations in various pathophysiologic and/or disease-related states.

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.

Genes encoding phosphodiesterase (PDE) 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 (PDE11A), 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 that control 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 to treat 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 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, which 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 the 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, which contains 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 to work on identifying new genetic defects in other forms of adrenal tumors and/or hyperplasias. The most noteworthy discovery of the past year was the identification of a somatic PRKACB defect in a cortisol-producing tumor of a patient with Cushing's syndrome (Figure 2). The S54L *PRKACB* defect is the first shown to be linked to adrenocortical Cushing's syndrome in a patient who had non-genetic



FIGURE 2. A *PRKACB* mutation in cortisol-producing adenomas

The image shows the S54L PRKACB mutation that was identified by our collaborators at the Cochin Hospital in Paris, France, as part of our joint grant for the investigation of protein kinase A (PKA) subunit defects in patients with adrenocortical tumors [see also Reference 1]. The functional investigations of this (and other, yet unpublished) PRKACB defect(s) were completed in the Stratakis Lab.

Cushing's syndrome. Our laboratory continues work on PRKACB (and other PKA subunit) defects both at the clinical genetic and molecular levels.

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*, which encodes an orphan G protein–coupled receptor (GPCR) and is 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, is caused by Xq26.3 genomic duplication, and is 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 in Belgium, but all the molecular work for gene identification was carried out here at the NIH. We found that the gene is expressed in areas of the brain that regulate growth, and we are actively investigating small-molecule compounds that may bind to GPR101 (unpublished).

In addition, we studied patients with pediatric Cushing disease (CD) resulting from 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 that predispose to pediatric CD. Last year, we reported *CABLES1* (encoding a cyclindependent 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 [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 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 tyrosine kinase–encoding *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. One such example is the recent identification of SGPL1 defects in patients with primary adrenal insufficiency.

Additional Funding

- INSERM, Paris, France (Co-Principal Investigator): "Clinical and molecular genetics of Carney complex," 06/2003-present
- Several small grants supporting staff members from France, Brazil, Greece, Spain, and elsewhere
- Bench-to-Bedside 2017 Award: "Therapeutic targets in African Americans with primary aldosteronism"
- Gifts on Cushing's syndrome research (various private donations)
- Pfizer #W1215907 2017-2018 US ASPIRE ENDOCRINE study titled "Characterization of GPR101-mediated growth regulation and receptor deorphanization"
- Uniformed Services University of the Health Sciences 2018 Award "Genetics of human susceptibility to infections and/or complications of Zika virus: variants of the cyclic AMP-dependent PKA pathway"

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Genetic and Environmental Determinants of Primate Biobehavioral Development

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, handreared 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 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 cerebrospinal fluid [CSF] 5-HIAA [5-hydroxy-3-indole acetic acid, a serotonin metabolite]). 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* (serotonin transporter polymorphic region) gene. During the past two years, we expanded the range of



Stephen J. Suomi, PhD, Head, Section on Comparative Behavioral Genetics Annika Paukner, PhD, Senior Visiting Fellow outcomes for which G x E interactions involving the *5-HTTLPR* polymorphism and early rearing condition differences appear, including in 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, we observed a similar pattern, i.e., the less efficient (from a transcriptional point of view) allele was associated with a negative outcome among PR-reared monkeys but with a neutral or, in some cases, even with 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 monkey 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 DNA 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. Comparing the two groups, the analyses revealed that (1) more than 4,400 genes were differentially methylated in both the PFC and lymphocytes; (2) however, approximately 25% of the affected genes were identical in both PFC and lymphocytes, although there was considerable tissue specificity; 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. The differences continued after weaning, albeit with some attenuation, but increased again by two 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 DNA 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 monkey 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) monkey 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 monkey 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 monkey 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 monkey 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 hypothalamic-pituitary axis (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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Organ and Tissue Formation during Development

The major focus of the Section is to understand how the elaborate networks of blood and lymphatic vessels arise during vertebrate development. 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.

In addition to our work on vessel development, we are also pursuing studies to understand the role of epigenetics during early development, in particular how DNA methylation and other epigenetic mechanisms help to coordinate cell, tissue, and organ specification and differentiation.

Specification and patterning of developing blood vessels

We are working to elucidate the cellular and molecular mechanisms responsible for the specification, patterning, and differentiation of blood vessels during development. Blood vessels are ubiquitous and vital components of vertebrate animals, innervating and supplying every tissue and organ with oxygen and nutrients. Many of the recent insights into mechanisms of blood vessel formation have come from studies in model organisms including the zebrafish.



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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.

In zebrafish every blood vessel can be observed in living animals with high resolution, and simple, rapid screening can be accomplished for even subtle vascular-specific mutants (Figure 1). We are carrying out a several related projects using the fish, including:

(1) *New tools for experimental analysis of vascular development.* This includes generating novel transgenic lines for visualizing different endothelial cell and perivascular cell types and for driving gene expression or performing molecular profiling of mRNAs and microRNAs in these same cell populations.

(2) *Genetic analysis of vascular development*. We have identified many novel mutants affecting vascular development in our transgene-assisted forward genetic screens, and we are currently characterizing the phenotypes and molecular basis for a number of these mutants.

(3) *Analysis of vascular specification, patterning, and morphogenesis.* We are studying the development of several vascular beds including the vasculature of the pectoral fin, the fish equivalent of the mammalian forelimb.

Specification and patterning of the lymphatic system

The lymphatic system is a completely separate vascular system from the blood circulatory system and comprises an elaborate blind-ended tree of vessels that extensively innervate most of the body, emptying lymph fluid into the venous blood vascular system via several evolutionarily conserved drainage points. The lymphatic system is essential for immune responses, fluid homeostasis, and fat absorption, and is involved in many pathological processes, including tumor metastasis and lymphedema. However, 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. Our groundbreaking studies demonstrated that zebrafish possess 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



FIGURE 2. Novel lymphatic vascular reporter

Lateral view confocal image of the trunk of a 12 dpf (days post-fertilization) Tg(kdrl:cherry); Tg(mrc1a:egfp) double-transgenic zebrafish with red fluorescent blood vessels and green fluorescent lymphatics. See Reference 1 for more information. purpose of imaging and studying lymphatic development. We are currently pursuing further study of the formation of the lymphatic system through several ongoing projects:

(1) We generated new transgenic lines that permit direct, specific visualization of the developing lymphatic vasculature and are using sophisticated imaging of these transgenic animals to characterize lymphatic development (Figure 2).

(2) We carried out forward-genetic ENU (*N*ethyl-*N*-nitrosourea) mutagenesis screens using our lymphatic reporter transgenic lines to identify new lymphatic-specific mutants with defects in novel genes that play important roles in lymphatic development.

(3) We are characterizing and studying novel micro RNAs expressed in the lymphatic endothelium and how these small regulatory RNAs influence lymphatic gene expression and lymphatic development.

The results of our studies, combining the genetic and experimental tools available in the zebrafish with the ability to perform high-resolution microscopic imaging of developing vascular structures in living animals, will continue to lead to important new insights into the origins and growth of the lymphatic system and molecular mechanisms that are critical in lymphatic development and lymphatic pathologies.

Regulation of vascular integrity

We are using the zebrafish to understand the cellular and molecular mechanisms responsible for proper vessel morphogenesis and for the generation and maintenance of vascular integrity. Disruption of vascular integrity is associated with hemorrhagic stroke, a severe and debilitating form of stroke associated with high morbidity and mortality. Meningeal vascular dysfunction is also associated with a neurocognitive deficits and neurodegenerative disease. Many of the recent insights into molecular mechanisms regulating vascular integrity have come from studies in model organisms such as the zebrafish. We are pursuing several related projects.

(1) *Genes regulating vascular integrity*. We used forward-genetic screens to identify new zebrafish mutants that disrupt cranial vascular integrity in the zebrafish (Figure 3), using exome sequencing and SNP (single nucleotide polymorphism) analysis with a newly developed SNP database to perform higher-throughput cloning of mutants. We already characterized the role of *GDF6* (growth differentiation factor 6, also



FIGURE 3. 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 hemorrhageprone larva deficient in *rap1b* (*B*). known as *BMP13*) in vascular integrity, demonstrating that the gene promotes maintenance of vascular integrity by suppressing excess VEGF (vascular endothelial growth factor) signaling. We are currently characterizing the molecular nature of the defects in several additional mutants.

(2) Acquisition and function of supporting vascular smooth muscle cells. The vascular smooth cells (VSMC) that surround the endothelial tube play a critical role in regulating vascular tone and vascular integrity. We examined the early origins of these cells, how the interaction of these cells with endothelial tubes helps maintain the vascular basement membrane and restricts vessel diameter, and the molecular mechanisms underlying the arterial (versus venous) specific recruitment of VSMC.

(3) Vasculature and vascular-associated cells in the meninges. The meninges are an external enveloping connective tissue that encases the brain, producing cerebrospinal fluid, acting as a cushion against trauma, nourishing the brain via nutrient circulation, and removing waste. Despite its importance, the cell types present in the meninges and the function and embryonic origins of this tissue are still not well understood. We recently discovered and characterized fluorescent granular perithelial cells (FGPs) in the zebrafish, a novel endothelium-derived perivascular cell population closely associated with meningeal blood vessels that is likely to have a critical role in meningeal function (Figure 4). We are currently carrying out several different studies to understand the function of these and other novel meningeal vascular-associated cell populations.

Epigenetics of development

We are using the genetically and experimentally accessible zebrafish and Mexican tetra (*Astyanax mexicanus*) models to uncover the molecular basis for organ- and tissue-specific epigenetic regulation during development in the following interrelated projects:

(1) *Role of the DNA methylase Dnmt3bb.1 in hematopoiesis and eye development*. DNA methyltransferases (DNMTs) are responsible for placing methyl marks on DNA. Promoter DNA methylation is typically associated with gene repression, while gene body methylation is generally associated with activation of a gene locus. We showed that Dnmt3bb.1 mediates hematopoietic stem and progenitor cell (HSPC) maintenance by methylating the gene body region of the *cmyb* locus, leading to the sustained expression of this essential transcription factor for maintaining hematopoietic specification. More recently, we showed that the same DNA methyltransferase mediates global repression of eye development genes, and that excess



FIGURE 4. Novel perivascular cells on the zebrafish brain

Confocal micrograph of "Fluorescent Granular Perithelial Cells" (FGPs, in green) adhering to the outside of meningeal blood vessels (in purple) on the brain of a Tg(mrc1a:egfp); Tg(kdrl:cherry) doubletransgenic adult zebrafish. We recently showed that FGPs are unique endotheliumderived perivascular cells with unusual scavenging properties that are likely critical for brain homeostasis. See Reference 4 for additional details.



FIGURE 5. Mexican tetra cave and surface fish

The Mexican tetra *Astyanax mexicanus* is a freshwater fish native to parts of southern Texas and eastern and central Mexico, which exists in both surface-dwelling ("surface morphs," top right) and very closely related cave-dwelling ("cave morphs," bottom left) populations. Cave morphs have a series of uniquely evolved adaptations including loss of eyes and pigment, dramatically altered metabolism, altered vascular function, and altered sleep regulation and behavior. Results from our laboratory suggest that altered DNA methylation and resulting coordinated changes in expression of large sets of genes have helped to drive at least some of this rapid evolutionary change. See Reference 5 for details. *DNMT3B* expression promotes eye loss in blind eyeless *Astyanax* cavefish (Figure 5).

(2) *Epigenetic regulation of metabolism using* Astyanax *cavefish*. The fish have extreme and unusual metabolic adaptations that allow them to survive chronic and long-term food deprivation. We are using molecular profiling and other methods to examine changes in the regulation of metabolic genes and the role of epigenetics in these changes.

(3) Forward genetic screen for epigenetic regulatory factors. Genetic screens carried out in Drosophila and *C. elegans* have been highly successful in identifying genes regulating cell-type specific epigenetic gene regulation in invertebrates, but the molecular mechanisms involved in organ- and tissue-specific epigenetic regulation in vertebrates are still relatively unknown. We developed a novel zebrafish transgenic reporter line that allows us to monitor dynamic changes in epigenetic regulation in intact animals during development. Using this transgenic line, we are performing the first large-scale F3 genetic screen in a vertebrate to identify recessive mutants in regulators of epigenetic gene silencing or activation (Figure 6).



FIGURE 6. An epigenetic silencing mutant in the zebrafish

Lateral views of the head and anterior trunk of a wild type (*top*) and tissue-specific epigenetic silencing mutant (*bottom*) zebrafish. The mutant causes loss of epigenetic silencing specifically in the liver (*red arrows*), as visualized with a novel transgenic reporter line developed in our lab that permits dynamic, tissue-specific visualization of epigenetic silencing in living animals.

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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.

Prokaryotic mutagenesis

As part of an international scientific collaboration with Andrew Robinson, Antoine van Oijen, Myron Goodman, and Michael Cox, we investigated the sub-cellular localization of the TLS polymerase pol IV in Escherichia coli. DNA polymerase pol IV is one of three TLS polymerases found in *E. coli*. All three polymerases are produced at elevated levels in bacteria as part of the SOS response to DNA damage. Historically, they have been thought to serve as a lastresort DNA damage-tolerance mechanism, re-starting replication forks that have stalled at damage sites on the DNA. TLS polymerases are highly error prone: inducing their activities leads to increased rates of mutation (error rates of up to 1 in every 100 nucleotides incorporated into DNA). TLS is an important source of mutations that fuel bacterial evolution. For several species of bacteria, deleting genes for TLS polymerases dramatically reduces rates of antibiotic resistance development in laboratory measurements, and in some cases even reduces infectivity. Many of the drugs used to treat



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bacterial infections cause an increase in mutation rates as a result of TLS. It remains unclear, however, whether TLS polymerases contribute to resistance by providing damage tolerance, increasing cell survival, and thus the chances that a resistant mutant will be found, or by facilitating adaptive mutation, i.e., selectively increasing mutation rates to speed the evolution of drug resistance.

It is thought that pol IV is the most abundant TLS polymerase in *E. coli*. From Western blots, it has been estimated that levels of pol IV increase from approximately 250 molecules per cell in the absence of DNA damage to 2,500 molecules per cell upon activation of the SOS damage response. On a variety of different lesion-containing DNA substrates pol IV promotes TLS, although its tendency for misincorporation varies with lesion type. The polymerase bypasses adducts to the N² position of guanines and a variety of alkylation lesions in a mostly error-free fashion. When overexpressed, pol IV induces -1 frameshift mutations in cells treated with alkylating agents. In addition to these lesion bypass activities, pol IV participates in transcription and double-strand break repair and contributes significantly to cell fitness in late stationary phase cultures in the absence of any exogenous DNA damage. It is also been reported that pol IV is required for the formation of adaptive point mutations in the *lac* operon and was found to be a major determinant in the development of ciprofloxacin resistance in a laboratory culture model.

Visualization of pol IV within live bacterial cells would make it possible to better understand how pol IV activity is regulated in response to DNA damage and test proposed models for its TLS activity at replisomes. To do so, we reported a single-molecule time-lapse approach to investigate pol IV dynamics and kinetics in live *E. coli* cells under normal growth conditions and following treatment with the antibiotic ciprofloxacin, the DNA-damaging agent MMS, or ultraviolet (UV) light. Twenty minutes after treating cells with the DNAdamaging antibiotic ciprofloxacin, we observed a striking increase in pol IV fluorescence, indicative of SOSdependent up-regulation. During the same period, we observed an increase in the formation of punctate foci, consistent with individual molecules of pol IV binding to DNA. The canonical view in the field is that pol IV primarily acts at replisomes that have stalled on the damaged DNA template. In contrast with this view, we observed that only a small proportion (about 10%) of pol IV foci colocalize with replisome markers. Initially, the proportion of replisomes that contain pol IV tracked with the increasing concentration of pol IV. In the period 90–180 min after ciprofloxacin addition, however, colocalization dropped dramatically, despite pol IV concentrations remaining relatively constant. Our data strongly suggest that pol IV is only licensed to carry out TLS at stalled replication forks during the early stages of SOS, whereas it continues to act on substrates outside of replication forks throughout the SOS response. In an SOS-constitutive mutant that expressed high levels of pol IV, we observed few foci in the absence of damage, indicating that access of pol IV to DNA is dependent on the presence of damage, as opposed to concentration-driven competition for binding sites.

As part of a scientific collaboration with Martin Gonzalez, we investigated how the mutagenesis-promoting activity of the *E. coli* polV ortholog polV_{ICE391} is normally kept to a minimum. polV_{ICE391} demonstrates a higher frequency of spontaneous mutagenesis and transversion mutations than the other cloned polV orthologs that have been previously characterized. However, the high mutation frequency promoted by polV_{ICE391} is only evident with the sub-cloned operon and is not seen when it is expressed from the native 88kb Integrating Conjugative Element ICE391. ICE391 is a mobile genetic element capable of transfer between different species of bacteria. Therefore, it seemed plausible that ICE391 carries factor(s) necessary to minimize aberrant polV_{ICE391} –mediated mutagenesis. In particular, we identified SetR_{ICE391} as a regulator of

the polV_{ICE391}-encoded *rumAB* operon, given that *E.coli* expressing SetR_{ICE391} demonstrated lower levels of polV_{ICE391}-mediated spontaneous mutagenesis than cells lacking SetR_{ICE391}. Electrophoretic mobility shift assays revealed that SetR_{ICE391} acts as a transcriptional repressor of polV_{ICE391} by binding to a site overlapping the -35 region of the *rumAB* operon promoter. SetR_{ICE391} regulation was shown to be specific for the *rumAB* operon, and *in vitro* studies with highly purified SetR_{ICE391} revealed that, under alkaline conditions, as well as in the presence of activated RecA, SetR_{ICE391} undergoes a self-mediated cleavage reaction that inactivates its repressor functions. Conversely, a non-cleavable SetR_{ICE391} mutant capable of maintaining repressor activity, even in the presence of activated RecA, exhibited low levels of polV_{ICE391}-dependent mutagenesis. Our studies therefore provided compelling evidence that SetR_{ICE391} acts as a transcriptional repressor of the ICE391-encoded mutagenic response *in vivo*.

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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, an alarming rise in body weight that 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, suggesting that 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 endophenotypes 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 lead to more targeted, etiology-based prevention and treatment strategies for pediatric obesity.

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 unaffected or heterozygous children and appear to eat more at



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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 such sequence alterations may affect body weight. We therefore developed transgenic 'knockin' mice expressing the human wild-type and human doublemutant *MC3R*. 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 compared with *MC3R*^{hWT/hWT}. *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 in *MC3R^{hDM/hDM}* human subjects (Figure 2). *MC3R*^{hDM/hDM} bone- and adipose tissue-derived mesenchymal stem cells (MSCs) differentiate into adipocytes that

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accumulate more triglyceride than wild-type *MC3R*^{hWT/hWT} MSCs. *MC3R*^{hDM/hDM} thus impacts nutrient partitioning to generate increased adipose tissue that appears metabolically healthy. The 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 the regulation of 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 levels gain even more weight when followed longitudinally. We also documented that hyperinsulinemia is positively related to energy intake in non-diabetic, obese children, leading to treatment studies to reduce





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 than 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 (*a*) and lower fat-free mass (*b*), but surprisingly greater adiponectin concentrations (*c*) than mice with the normal human *MC3R* (*MC3R^{hWT/hWT}*). Humans with the double-mutant receptor also showed greater adiponectin (*d*).

hyperinsulinemia. 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. These associations suggest mechanisms whereby insulin resistance may contribute to excessive weight gain in children and have informed some of our treatment approaches to pediatric obesity (described below).

Our evaluations concerning 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. Thus, children reporting bingeeating behaviors, such as loss of control over eating, gained an average additional 2.4 kg of weight per year compared with children who did not engage in binge eating. 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 lower subsequent satiety, may play a role in the greater weight gain found in binge-eating children. Among cohorts of lean and obese youth, we demonstrated that youth with loss of control (LOC) eating had higher serum leptin and were at significantly greater risk for worsening of components of the metabolic syndrome than those without LOC episodes, even after adjusting for adjosity and other relevant covariates. Our data also suggest that anxiety symptoms may interact with LOC eating to become an important co-factor for excessive weight gain among children [Reference 1]. These data also suggest that interventions targeting disordered eating behaviors may potentially be useful in preventing excessive fat gain in children prone to obesity and have led to trials of preventative strategies related to binge eating. Because binge eating appears to be a heritable trait, we also initiated studies to investigate potential genetic factors linked to LOC over-eating.



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/m²), baseline triglycerides, and time in study (P = 0.02).

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. We examine body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, and genetic factors believed to regulate metabolic rate and body composition. 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 homeostasis was markedly improved in overweight or obese children who engaged in moderate activity for just three minutes every half hour, compared with remaining sedentary [Reference 2]. 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 U.S. population.

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

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in children and adults is urgently needed, yet current pharmacologic approaches are extremely limited for both children and adults. In several clinical protocols, we have 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 controls. We



FIGURE 4. Effect of short, moderate intensity walking breaks on children's glucose tolerance

Children with overweight or obesity who walked for three minutes every 30 minutes (*blue*) had lower insulin and C-peptide concentrations during an oral glucose tolerance test than when they sat uninterrupted for three hours (*black*).

concluded that orlistat added to a behavioral program significantly increased weight loss over a 6-month interval; however, it had little impact on obesity-related co-morbid conditions in obese adolescents. 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, in those randomized to metformin BMI, BMI-Z score, and body fat mass declined to a significantly greater extent. Serum glucose and HOMA-IR (homeostatic model assessment of insulin resistance) also declined significantly more in metformin-treated than in placebo-treated children. We recently published studies on the pharmacokinetics of metformin and on 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. The *SLC22A1* variant carriers had significantly smaller reductions in percentage of total trunk fat after metformin therapy. The median percentage change in trunk fat was –1.20% (–2.40% to 7.30%) for the *SLC22A1* subjects and –2.20% (–9.00% to 0.900%) for those without the variant.

A third study examined prevention of weight gain using interpersonal therapy (IPT) versus a control health education program (HE) in adolescents reporting loss of control eating behaviors [Reference 1]. At 3-year follow-up, baseline social-adjustment problems and trait anxiety moderated outcome. Among girls with high self-reported baseline social-adjustment problems or anxiety, IPT was 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 3].

Among girls with greater (moderate) baseline depressive symptoms (N = 78), those in cognitive behavioral therapy (CBT) developed lower 2-hr insulin than those in HE. Additional metabolic benefits of CBT were seen for this subgroup between post-treatment analyses and those conducted until the one-year follow-up.

We also participated in a multi-site randomized, placebo-controlled trial of beloranib, a methionyl aminopeptidase 2 inhibitor, to treat the hyperphagia of patients with the Prader-Willi syndrome [Reference 4] (Figure 5). The medication was effective in reducing body weight: compared with placebo, weight change was significantly greater with 1.8 mg (mean difference –8.2%, 95% CI: –10.8 to –5.6) and 2.4 mg beloranib (–9.5%, 95% CI: –12.1 to –6.8). However, the trial had to be stopped because of serious venous thrombotic events in beloranib-treated participants. An ongoing study based on lab data demonstrating attentional biases to high-palatability foods in children with obesity is investigating whether adolescents' attentional biases can be retrained. We also initiated a translational trial studying the effects of modulating 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; the initial data are encouraging. In the coming year, we will also begin another study of specific pharmacotherapy for patients with Prader-Willi syndrome. These latest trials are examples of a precision medicine approach [Reference 5] to treat obesity. Finally, a novel intervention, which has completed enrolment and is currently being analyzed, is a randomized-controlled pilot trial of colchicine to ameliorate the inflammation of obesity and thus improve its complications.

Additional Funding

 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 (random control trial) testing a new methionine aminopeptidase inhibitor in patients with Prader-Willi syndrome and obesity. 2014–2016



FIGURE 5. Effects of placebo and beloranib on body weight in patients with hyperphagia due to the Prader-Willi syndrome

Patients with Prader-Willi syndrome who were treated with the experimental medication beloranib had significantly more weight loss than those treated with placebo, who gained weight during the study.
- NIH Clinical Center/ORWH "Bench-to-Bedside" Award: Attention Bias Retraining in Adolescents with Loss of Control Eating. 2016–2017
- NIH Clinical Center/ORD "Bench-to-Bedside" Award: Melanocortin agonist to bypass leptin resistance of Bardet-Biedl syndrome. 2017–2018
- 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
- 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
- Soleno Therapeutics, Inc. Grant support to fund an RCT testing a diazoxide choline sustained release tablets in patients with Prader-Willi syndrome and hyperphagia. 2018–2020

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The Biophysics 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 for 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) for 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, one 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 highenergy 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 on the influenza virus. At the same time, we are (1) actively investigating the interaction of proteins and lipids in regulating the membrane-remodeling stages of the malaria parasite, to better understand how to produce improved anti-malarial compounds, and (2) trying to determine the relationship between human subject activity and the leakage of cytosolic components of skeletal muscle into the blood stream for individuals with fragile-membrane muscular dystrophies.

Membrane fusion, fission, and enzyme phosphorylation in the pathophysiology of influenza and insulin resistance

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. But 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 hemagglutinin (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.' 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 declined when the liposome cholesterol level matched physiological concentrations. In a separate study, we showed that the acylation of the cytosolic tail of HA changes membrane curvature [Reference 1].

LIPID-DEPENDENCE OF TARGET MEMBRANE STABILITY DURING INFLUENZA VIRAL FUSION

Although influenza kills about a half million people every year, even after excluding pandemics, there is only one set of antiviral drugs: the neuraminidase inhibitors. By using a new approach utilizing giant unilamellar vesicles and infectious X-31 influenza virus, and testing for the newly identified pore intermediate of membrane fusion, we observed about 30–87% poration, depending upon lipid composition. Testing the



FIGURE 1. Sequence of events leading to release of malaria parasite from red blood cells

Diagram showing the sequence of events involved in rupture of the vacuole and cell membrane. Using chemical inhibitors, we showed that it is possible to block each event in the sequence.

BAPTA-AM: 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid acetoxymethyl ester, a cell-permeable Ca²⁺ chelator; C2: compound 2, or 4-[7-[(dimethylamino)methyl]-2-(4-fluorphenyl)imidazo[1,2-α]pyridine-3-yl]pyrimidin-2-amine); CWHM-117: compound 8p; E-64: thiol protease inhibitor; SERA-6 KO: knock out of the *SERA 6* gene.

hypothesis that spontaneous curvature (SC) of the lipid monolayer controls membrane poration, our Poisson model and Boltzmann energetic considerations suggest a transition from a leaky to a non-leaky fusion pathway depending on the SC of the target membrane [Reference 4]. When the target membrane SC is below approximately –0.20 nm⁻¹, fusion between influenza virus and target membrane is predominantly non-leaky, whereas above that, fusion is predominantly leaky, suggesting that influenza HA-catalyzed topological conversion of target membranes during fusion is associated with a loss of membrane integrity.

SUBCUTANEOUS ADIPOSE TISSUE IMAGING OF HUMAN OBESITY REVEALS TWO TYPES OF ADIPOCYTE MEMBRANES: INSULIN-RESPONSIVE AND NON-RESPONSIVE.

In adipose tissue, resistance to insulin's ability to increase glucose uptake can be induced by many factors, including obesity. Impairment of insulin action may occur at various spatial loci at the cellular or subcellular level. To begin to understand the spatial response to insulin in human subcutaneous adipose tissue (hSAT), we continued to develop our quantitative imaging method for activation of a major signaling node in the gluco-regulatory insulin signaling pathway. After treatment with insulin or control media, we immuno-stained biopsied tissues for Akt phosphorylation at Thr308/9 (pAkt) and then imaged by confocal fluorescence microscopy automated to collect a large grid of high-resolution fields. In hSAT from 40 obese men and women, substantial heterogeneity of pAkt densities in adipocyte membranes were quantified in each image mosaic, using a spatial unit of at least twice the size of the point-spread function. Statistical analysis of the distribution of pAkt spatial units was best fit as the weighted sum of two separate distributions, corresponding to either a low or high pAkt density. A "high-pAkt fraction" metric was calculated from the fraction of high-pAkt distributed units over the total units. Importantly, upon insulin stimulation, tissues from the same biopsy showed either a minimal or a substantial change in the high-pAkt fraction. Further supporting a two-state response to insulin stimulation, subjects with similar insulin sensitivity indices are also segregated into either of two clusters identified by the amount of membrane-localized pAkt.

Membranes during egress of *Plasmodium falciparum*, the causative agent of malaria

We focus on our continued research on the physiology of the deadly malaria parasite *Plasmodium falciparum*. Despite some progress in the combating malaria, *Plasmodium spp*. cause over 200 million annually reported malaria cases with more than 400,000 deaths, mostly in children under the age of 5. With no vaccine, and drug resistance rising, we are focusing on the unique membrane biology of the parasites to find new targets for therapy. 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, by providing, e.g., the first recordings of *P. falciparum* egress and invasion of erythrocytes, and by describing new phenomena such as shape transformation of infected cells, which signals the egress initiation and membrane transformation upon egress. We developed several non-interventional methods that permit 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.

Last year, we reported 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 2]. In contrast, by controlling maturation of the subtilisin-like serine protease SUB1 in exoneme secretory vesicles, PMX 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. In another study, we showed a new potential route for drug delivery. Despite its membrane impermeability, heparin, a natural glycosaminoglycan, inhibited malaria parasite egress, trapping merozoites within infected erythrocytes. Heparin does not bind to the erythrocyte surface, but rather enters the infected red blood cell (iRBC) at the last minute of the parasite cycle through parasite-induced pores that we discovered in iRBC. This short encounter is 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 for how neutralizing antibodies can block egress, we intend to exploit membrane perforation as a new physiological strategy to target therapeutics intracellularly.

EXP2 IS A NUTRIENT-PERMEABLE CHANNEL IN THE VACUOLAR MEMBRANE OF *PLASMODIUM* AND IS ESSENTIAL FOR PROTEIN EXPORT VIA PTEX.

The blood stage of the parasite is responsible for the symptoms of malaria. Understanding how the parasite establishes a red blood cell infection and acquires nutrients is critical to devise new ways to combat a parasite that repeatedly developed resistance to frontline treatments. Blood-stage malaria parasites reside within a parasitophorous vacuolar membrane (PVM); PVMs form when parasites invade their host cell. Establishment of infection requires the parasite to export effector proteins into the red blood cell cytosol, as well as to import nutrients past the PVM. Protein export is achieved by a protein complex, the *Plasmodium* translocon of exported proteins (PTEX). Its putative membrane-spanning pore complex consists of the protein EXP2, which shares sequence homology with nutrient-permeable pores of other apicomplexans, suggesting a potential dual role of the protein in nutrient uptake and protein export. Using regulated gene expression, we showed that EXP2 is essential for protein export [Reference 3]. Further, EXP2 expression correlates with the occurrence of a previously characterized nutrient-permeable PVM channel of unknown

molecular identity in cell-attached patch-clamp experiments. To show that EXP2 indeed constitutes the nutrient-permeable PVM channel, charged amino acid residues of EXP2 were truncated, which diminished the response of the nutrient-permeable channel to applied voltages, thus identifying EXP2 as the channel-forming protein. The results put EXP2 in the center of focus for understanding nutrient import and protein export past the PVM in blood-stage malaria, and therefore how to disrupt it. The realization represents an important step in understanding the interaction of the malaria parasite with its host cell.

ROUNDING PRECEDES RUPTURE AND BREAKDOWN OF VACUOLAR MEMBRANES MINUTES BEFORE MALARIA PARASITE EGRESS FROM ERYTHROCYTES.

Because *Plasmodium falciparum* replicates inside of a parasitophorous vacuole (PV) within a human erythrocyte, parasite egress requires the rupture of two limiting membranes. Parasite Ca²⁺, kinases, and proteases contribute to efficient egress; however, their coordination in space and time is not known. We linked the kinetics of parasite egress to specific steps with specific compartment markers, using live-cell microscopy of parasites expressing PV-targeted fluorescent proteins, and specific egress inhibitors [Reference 5]. Several minutes before egress, under control of parasite [Ca²⁺], the parasitophorous vacuole began rounding. Then after about 1.5 minutes, under control of PfPKG (*Plasmodium falciparum* cGMP–dependent protein kinase) and the transcriptional regulator SUB1, there was abrupt rupture of the PV membrane and release of vacuolar contents. Over the next approximately six minutes, simultaneously with erythrocyte membrane distortion, the vacuolar membrane progressively deteriorated, which lasted until the final minute of the egress program, when newly-formed parasites mobilized and erythrocyte membranes permeabilized and then ruptured—a dramatic finale to the parasite cycle of replication. The new stage discovered in this project has features that suggest the possibility of a new target for antimalarial drug development.

Developing methods to test activity and dietary regimen modification to treat fragile membrane muscular dystrophy

There are currently no routinely available, easily accessible, objective outcome measurements for either disease progression or treatment efficacy in the muscular dystrophies. A major theme under development for the past few years 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. Last year, we introduced proof-of-principle studies in dietary changes and mass spectrometry measurements of membrane lipids to advance our ability to study the effects of specific lipids on membrane stability and to test lipids as therapies in models of muscular dystrophy. A new assay for phytanic acid lipids was developed. With feeding, we succeeded in increasing the muscle content of this specific lipid in mice.

TOWARDS A SURROGATE BIOMARKER FOR EFFICACY IN MUSCULAR DYSTROPHIES.

A major barrier to clinical trials for muscular dystrophy is the lack of a good measure of therapeutic efficacy. We are developing a time-series analysis procedure that uses event-driven, kinetic properties of high-frequency blood samples to reveal significant changes associated with minimal subject activity. Our approach showed that creatine kinase (CK), previously considered minimally informative because of large population variability, can be used as a biomarker when each subject is their own control. We further

demonstrated that the amino transferases ALT and AST follow kinetics similar to that of CK and are significantly correlated. We predict that additional biomarkers exist and that their identification will facilitate the development of discriminants useful in the assessment of disease progression and/or treatment efficacy. We used the SOMAscan assay, a featured platform in the Center for Human Immunology (CHI), NIH, for the purpose of conducting novel proteomic biomarker discovery. For proof-of-principle and validation of the SOMAscan assay, we examined a subset of available samples representing blood collected before and after physical activity from the first visits of 10 subjects. Using an aptamer-based platform, we identified analytes in blood that significantly increased after the subjects arose from night-time sleep. As hypothesized, muscle-specific proteins (myoglobin and CK) were identified, confirming our earlier analyses based on clinical chemistry evaluations. In addition, we identified significant increases in signaling and chemokine proteins after arising. The results support our hypothesis that activity-correlated analyses is a viable procedure for identifying candidate biomarkers related to both muscle-specific membrane-damaging events and systemic physiological changes. We are in the process of extending our analyses; we believe that this newly developed procedure can aid in identifying disease progression and treatment efficacy.

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Colophon

About the Cover Image

By Laura Pillay and Daniel Castranova of the <u>Weinstein Lab</u>. The image features transgenic zebrafish larvae showing blood vessels in green, blood cells in red, and angiography in magenta.

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