2019

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*

Elizabeth Bonney, MD, MPH, *Acting Chair* 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
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
Hugo J. Bellen, DVM, PhD* 7/1/19 – 6/30/24 Genetics, Neuroscience, Model Organisms	Investigator, Howard Hughes Medical Institute Professor, Departments of Molecular and Human Genetics and Neuroscience, Baylor 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
Nancy Carrasco, MD* 7/1/19 – 6/30/24 Molecular Medicine, Pharmacology, & Physiology	Professor and Chair, Department of Molecular Physiology and Biophysics Vanderbilt School of Medicine
William T. Dauer, MD* 7/1/18 – 6/30/23 Neurodevelopment & Behavior, Rare Diseases & Genetics, Neurobiology	Director, Peter O'Donnell Jr. Brain Institute Professor of Neurology & Neurotherapeutics, and Neuroscience Lois C.A. and Darwin E. Smith Distinguished Chair in Neurological Mobility Research UT Southwestern Medical Center
P. Ellen Grant, MD* 7/1/19 – 6/30/24 Newborn Medicine, Radiology	Director, Fetal-Neonatal Neuroimaging and Developmental Science Center Professor of Radiology and Pediatrics Harvard Medical School

Frances E. Jensen, MD, FACP 7/1/15 – 6/30/20 Neuroscience	Professor and Chair, Department of Neurology Co-Director Penn Medicine Translational Neuroscience Center Perelman School of Medicine, University of Pennsylvania
Deborah L. Johnson, PhD 7/1/17 – 6/30/22 Molecular & Cellular Biology	President and Chief Executive Officer of Keystone Symposia Professor, Department of Molecular and Cellular Biology Baylor College of Medicine
Ursula Kaiser, MD* 7/1/19 – 6/30/24 Endocrinology, Diabetes & Hypertension	Chief, Division of Endocrinology, Diabetes, and Hypertension Co-Director, Brigham Research Institute Brigham and Women's Hospital Professor of Medicine, Harvard Medical School
Kojo A. Mensa-Wilmot, PhD 7/1/16 – 6/30/21 Cellular Biology	Professor and Head, Department of Cellular Biology University of Georgia
Errol Norwitz, MD, PhD, MBA* 7/1/19 – 6/30/24 Obstetrics & Gynecology	Louis E. Phaneuf Professor of Obstetrics and Gynecology Tufts University School of Medicine Chief Scientific Officer Chair, Department of Obstetrics and Gynecology Tufts Medical Center
Eric Vilain, MD, PhD 7/1/15 – 6/30/20 Molecular & Human Genetics	James A. Clark Distinguished Professor of Molecular Genetics 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 School of Public Health Boston University

Message *from the* Scientific Director

Our 2019 annual report for the Division of Intramural Research (DIR) of 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: *annualreport.nichd.nih.gov*

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

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

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

We use a range of model systems in the areas of developmental biology, molecular and cellular biology, neurosciences, structural biology, imaging, 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 bioinformatics, imaging, 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.

We are delighted to announce the recruitment of Dr. Veronica Gomez-Lobo this year as founding Director of the Pediatric and Adolescent Gynecology Program. Dr. Gomez-Lobo and her team will provide clinical and research support to women's health, gynecological diseases, adolescent medicine, and the transition to adulthood, all central to the mission of NICHD.

If you have not yet done so, I encourage you all to read NICHD's 2020 Strategic Plan, available at *www.nichd.nih.gov/about/org/strategicplan*. This document will help guide the institute's research agenda for the next five years. We are now working with our director, Dr. Diana Bianchi, on the plan's implementation and we anticipate that the priorities identified in the plan will inform the DIR's future recruitments.

I invite you to read through the selection of our *Clinical Research Protocols* listed in this report 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. You can learn more about the U01 opportunities as *clinicalcenter.nih.gov/translational-research-resources/U01/*

The DIR researchers whose names appear in this publication are committed to training the next generation of scientists and physician scientists; they include tenure-track investigators who have recently joined us and accomplished investigators who continue to forge new scientific paths. Link to their reports on the web to learn about their work in 2019. I also invite you to reach out to me with your ideas and proposals for collaborative initiatives we may undertake together, at *dassom@mail.nih.gov*.

Our drive and purpose, on behalf of the American public and the international community, is to strive to uncover fundamental answers to our existence, whether the basic science that underpins life or the complexities of human health and disease. This is our privilege and responsibility to our chosen professions.

Sincerely yours,

Mary C. Dasso, PhD
Acting Scientific Director, NICHD, NIH
February 2020

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, and, as a high priority, ensuring that we provide state-of-the-art training in basic, translational, and clinical research for the next generation of scientific and clinical leaders.
- 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., *PubMedCentral*, *Clinicaltrials.gov*).
- Serving in an advisory role to the Institute Director and other senior leadership of the Institute and other institutes, offices, and centers across NIH.
- Actively participating in the governance of the NIH Intramural Research Program (IRP).
- Contributing to the development and support of trans-NIH initiatives.
- 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

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*

Olga Cherkasova, *Administrative Support Specialist*

**In February 2020, Dr. Constantine Stratakis stepped down as Scientific Director, NICHD. Dr. Mary Dasso was appointed Acting Scientific Director on February 6, 2020.*

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 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, *https://oir.nih.gov/sourcebook/processes-reviewing-nih-intramural-science*). 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 *https://annualreport.nichd.nih.gov/bsc.html*. Annually, the Scientific Director reports on the activities of the OSD, the reviews of our investigators and their accomplishments, and 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 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 sixth year, is held in conjunction with NCATS, NIDCR, NIAMS, NHGRI, and NEI. **Dr. Mengying Li** in the Epidemiology Branch in the Division of Intramural Population Health Research received the second-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.

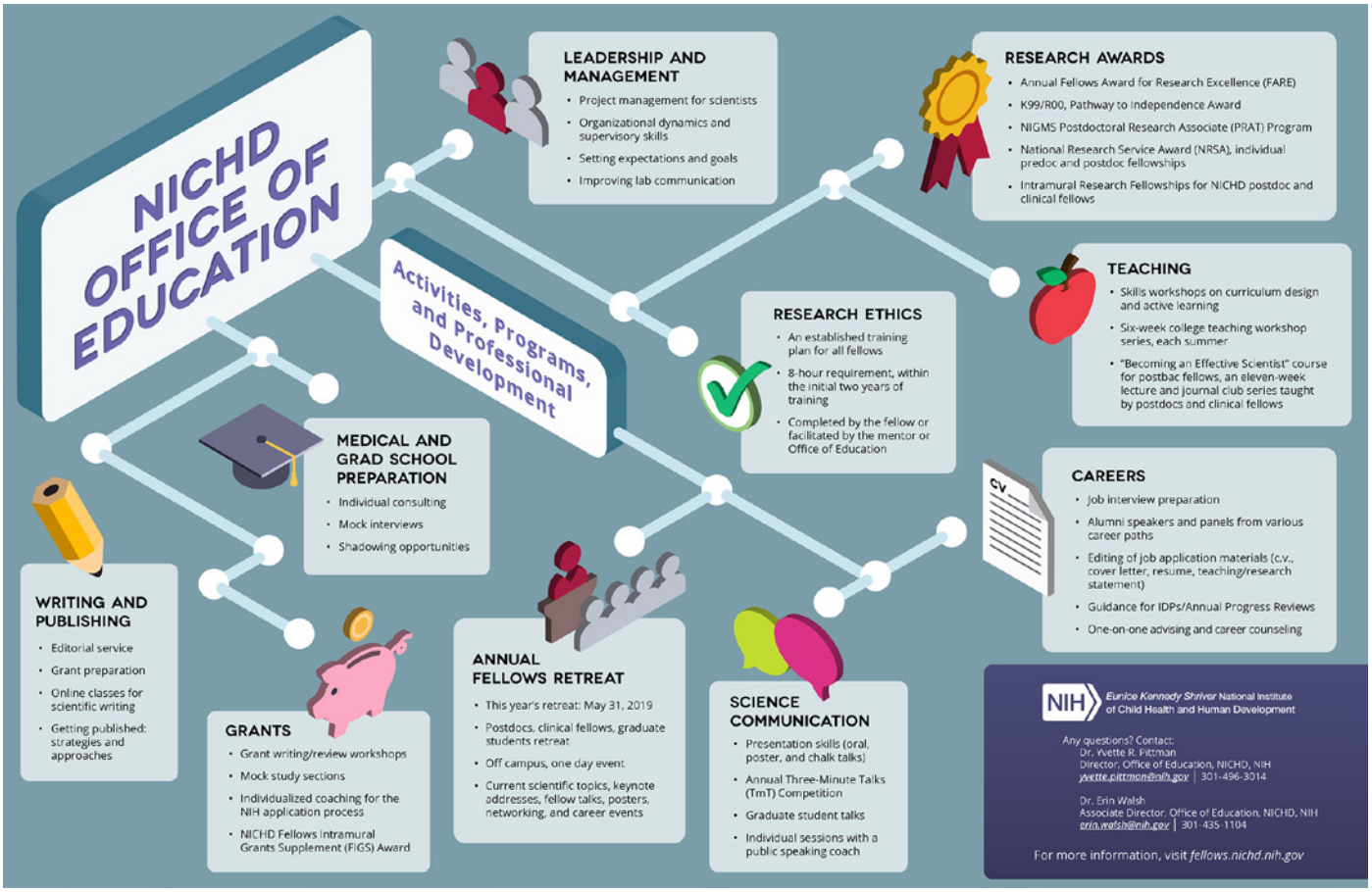
An **online Annual Progress Review** for fellows, developed by the Office and launched in 2016, tracks scientific and career development and progress. As part of the assessments of investigators' mentoring, the reports by fellows are analyzed and provided to the site visits of the Board of Scientific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

In September 2019, the Division of Intramural Research gave its **12th Mentor of the Year awards** to **Alan Hinnebusch**, *Section on Nutrient Control of Gene Expression*, in the investigator category, and to

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

Erin Walsh, PhD, *Associate Director*

Carol Carnahan, BS, *Program Coordinator*



María Queralt-Martín, *Section on Molecular Transport*, as fellow.

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. It can be found on the *NICHD fellows' wiki site*. For NICHD, 24 Fellows Award for Research Excellence (FARE) awards were made for the 2020 competition.

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

The Institute continues its **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, and, since 2016, up to three second- and third-year medical students from Santa Casa de São Paulo School of Medical Sciences (Brazil) train with NICHD investigators for the **Future Researchers Program**.

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

The alumni group for our **NICHD Developing Talent Scholars program**, in its ninth year, now numbers 16 individuals, and two new postbaccalaureate fellows joined the program in 2019. The Scholars program focuses on developing talent and supporting trainees' academic and career progression.

Postdoctoral fellows were also given the opportunity to organize and teach our **annual course for postbaccalaureate trainees**, which entered its 14th 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 career development program in 2018, specifically directed at those fellows interested in careers outside academia, and 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 **15th Annual Fellows Meeting** was held for about 120 people to address scientific developments and careers, took place at the William F. Bolger Center in Potomac, Maryland, and featured keynote speaker Dr. Elaine Ostrander, NIH Distinguished Investigator. Each spring, this retreat includes presentations by fellows, career panel sessions, and a poster presentation by each attendee. The program is developed and run by a fellows' steering committee.

The NICHD Connection **monthly newsletter** continues its focus on mentoring, careers, and academic programs for young scientists, publishing its 115th issue in December 2019 and reaching all members of the intramural division and our alumni.

Dr. Erin Walsh became Associate Director of the Office.

Contact

For further information, contact *yvette.pittman@nih.gov*, Dr. Erin Walsh (*erin.walsh@nih.gov*), or Carol Carnahan (*carnahac@mail.nih.gov*).

Office *of the* Clinical Director, NICHD

The NICHD intramural clinical research program currently includes 71 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. Over the past year, we initiated a new program focused on adolescent gynecology. The protocols are conducted by 30 NICHD Principal Investigators and 166 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.

Over the past year we transitioned from an NICHD–based Institutional Review Board (IRB) to a central NIH Clinical Center IRB. The chair of the NICHD IRB, Dr. Karim Calis, continues as one of the chairs for the consolidated IRB. The NICHD Office of the Clinical Director (OCD) continues to support the NICHD Data and Safety Monitoring Committee (DSMC), which is chaired by Dr. Frank Pucino.

Contact

For more information, email *fdporter@helix.nih.gov* or visit *https://www.nichd.nih.gov/about/org/dir/osd/mt/cd*.

Members of the new Pediatric and Adolescent Gynecology (PAG) team and Children's National Colleagues
Lauren Damle (attending Children's/MedStar), Shashwati Patel (Postgraduate year 6 [PGY 6] PAG, NICHD), Jill Rekart (PAG nurse, Children's National), Tazim Dowlut-McElroy (PAG Staff Clinician, NICHD), Veronica Gomez-Lobo (Director of PAG, DIR), Allison Mayhew (PGY 5 PAG, NICHD), Jacqueline Yano Maher (Staff Clinician PAG, NICHD)

Forbes D. Porter, MD, PhD, *Clinical Director*
Meg Keil, PhD, *Associate Director, Nursing and Protocol Navigation*
Maryellen Rechen, BS, RN, *Special Assistant to the Clinical Director*
Donna Peterson, BS, RN, *Protocol and Institutional Review Board (IRB) Coordinator*
Karim Calis, PharmD, MPH, *Director of Clinical Research and Compliance*
Alan DeCherney, 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*
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 *https://www.clinicaltrials.gov/ct/search;?term=nichd*). 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 *oidoc@helix.nih.gov*.
- Screening and diagnosis on patients with suspected connective tissue disorders. Patients and their families receive comprehensive evaluations, counseling, and risk assessment. Patients may be referred to **DR. JOAN MARINI** at *oidoc@helix.nih.gov*.

Developmental Endocrinology, Metabolism, Genetics, & Endocrine Oncology

- Patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma (PHEO) and paraganglioma (PGL). Patients may be referred to **DR. KAREL PACAK** at *karel@mail.nih.gov* or **MARIANNE KNUE** at *marianne.knue@nih.gov* or 301-827-3355.
- Research on endocrine, genetic, and other pediatric disorders that are associated with the predisposition to endocrine and other tumors, abnormal development in fetal or later life and may affect the pituitary, the adrenal and other related organs. Patients may be referred to **DR. CONSTANTINE STRATAKIS** at *stratakc@mail.nih.gov* or to **DR. ELENA BELYAVSKAYA** at 301-496-0862.
- Research investigating the causes, complications, and treatment of Primary Aldosteronism. Patients may be referred to **DR. FADY HANNAH-SHMOUNI** at *fady.hannah-shmouni@nih.gov* or **DR. CRYSTAL KAMILARIS** at *crystal.kamilaris@nih.gov* or 301-496-6633.
- Research investigating the long-term effects of Cushing disease in childhood. Patients may be referred to **DR. MEG KEIL** at *keilm@mail.nih.gov* or 301-435-3391.
- 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 *stratakc@mail.nih.gov* or to **DR. CHRISTINA TATSI** at 301-451-7170.
- Studies into how genetics play a role in the development of obesity. Patients may be referred to **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-435-8201.
- 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 *yanovskj@mail.nih.gov* or 301-496-4168.
- Pharmacotherapy of excessive hunger and obesity in patients with Prader-Willi syndrome, Bardet-Biedl syndrome, and other rare disorders with known genetic causes. Patients may be referred to **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-496-6726.

» Evaluation of patients with endocrine disorders that are associated with excess androgen, including different forms of congenital adrenal hyperplasia. Patients may be referred to **DR. DEBORAH MERKE** at *dmerke@nih.gov* or **MS. ELIZABETH JOYAL** at *ejoyal@nih.gov*.

» Studies on patients with genetic disorders related to altered cholesterol metabolism. This includes patients with Smith-Lemli-Opitz syndrome (SLOS) and Niemann-Pick Disease, type C (NPC). Patients may be referred to **DR. FORBES PORTER** at *fdporter@mail.nih.gov* or **MS. NICOLE FARHAT** at 301-594-1765.

» Study of individuals with CLN3, or Juvenile Neuronal Ceroid-Lipofuscinosis (Juvenile Batten Disease) and their family members. Interested participants may be referred to **DR. AN NGOC DANG DO** at *an.dangdo@nih.gov* or **MS. KISHA JENKINS** at 301-594-2005.

» Studies of patients with genetic disorders related to an abnormal function of the creatine transporter gene causing creatine transport deficiency (CTD). Patients may be referred to **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 *baronj@cc1.nichd.nih.gov* or **DR. YOUN HEE JEE** at *jeeyh@mail.nih.gov*.

» Studies on metabolic effects of food additives (high intensity sweeteners) with special focus on pregnancy, and prenatal and infantile development. Interested participants may be referred to **DR. KRISTINA ROTHER** at 301-435-4639 or *kristina.rother@nih.gov*.

Maternal–Fetal Medicine & Translational Imaging

» 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, go-nogo tests will be administered to quantify spatial and temporal brain activity. Subjects may be referred to **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.

» Mirror neuron network dysfunction as an early biomarker of neurodevelopmental disorder. In this study, functional near-infrared spectroscopy (fNIRS) combined with electroencephalography (EEG) to measure brain activity in the mirror neuron network (MNN). 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. The first part of the study involves adult subjects to determine whether MNN activation can be elicited, using a motor observation and a simultaneous execution paradigm using EEG/fNIRS systems. The synchronicity of these signals using more advanced machine learning methods to examine how the features from both signals relate to each other and help characterize brain function in the mirror neuron network. In the next step, typically developing infants and infants at risk for developmental delays from 9–12 months of age are recruited. 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. For more information on the study, please contact **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.

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

Pediatric & Adolescent Gynecology

Pediatric and Adolescent Gynecology (PAG) is a relatively new subspecialty within the field of Obstetrics and Gynecology and encompasses gynecologic care from the fetal period into adulthood. Many of the conditional managed by pediatric gynecology are rare diseases and have been poorly studied. Our vision is to create a comprehensive and innovative pediatric and adolescent gynecology program within NICHD which encompasses community clinical and research resources. This program integrates regional clinical care with a post-graduate training program to populate primarily human clinical studies of gynecologic disorders that begin before puberty, to understand both childhood and adult gynecologic disease and fertility.

Proposed future studies include:

» Gonadal Tissue Freezing for Fertility Preservation in Girls at Risk for Ovarian Dysfunction and Primary Ovarian Insufficiency. This study is designed to evaluate the feasibility of experimental ovarian tissue cryopreservation for fertility preservation in children with increased risk of loss of ovarian function due to Turner syndrome or galactosemia and post-menarcheal adolescents with recent development of idiopathic primary ovarian insufficiency. For additional information, contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

» Androgen Receptor, Implications for Health and Wellbeing: Natural History Study of Patients with Androgen Insensitivity. Research in individuals with the androgen receptor gene and consequently receptor abnormalities will allow better health care for these individuals and may also begin to elucidate possible androgen receptor mediated mechanisms for differences in physiology and health in other populations. For additional information, contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

» Idiopathic Premature Ovarian Insufficiency. Little is known about the clinical phenotype of adolescents with this condition. In collaboration with Harvard Medical School/Boston Children's Hospital, this project combines careful clinical phenotyping to increase our understanding of POI, an understudied disorder, with an evaluation of current estrogen treatment strategies, as well as novel research regarding fertility preservation options. Our results will provide new information that will be of high impact for clinical care. For additional information, contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

Physical Biology & 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, Miyoshi Myopathy (MM), Becker Muscular Dystrophy (BMD), Miyoshi Muscular Dystrophy–3 (MMD3). Patients may be referred to **DR. JOSHUA ZIMMERBERG** at *zimmerbj@mail.nih.gov* or **MS. HANG WATERS** at *watershn@mail.nih.gov*.

Reproductive Endocrinology & Infertility

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

» Research on reproductive function in sickle cell disease. Patients can contact **DR. ALAN DECHERNEY** at *decherna@mail.nih.gov* or 301-594-5494.

Combined Maternal–Fetal Medicine/Medical Genetics 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 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, labor and delivery, intensive care unit, 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 science 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 multidisciplinary 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, and advanced imaging techniques such as Doppler velocimetry, as well as ultrasound-guided invasive procedures such as amniocentesis. Opportunities for laboratory-based research and training are available at the PRB in the fields of parturition, reproductive immunology, placental pathology and biology, biomarker discovery, and systems biology in reproduction. Alternative opportunities are available at the C.S. Mott Center of Wayne State University.

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

Detailed information about the training program is available at: *http://mfmfellowship.org*. The website also contains information about the faculty and their publications and awards. The Director of the Fellowship Program is Dr. Lami Yeo. 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.

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

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Mohammad Al-Jundi, MD, *Clinical Fellow*

Iris Hartley, MD, *Clinical Fellow*

Rasha Haykal, MD, *Clinical Fellow*

Crystal Kamilaris, MD, *Clinical Fellow*

Maziar Rahmani, MD, PhD, *Clinical Fellow*

Skand Shekar, MD, *Clinical Fellow*

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 hypothesis-driven research protocols. To this end, the second and third years 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 by participating 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 the 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.

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. Kamilaris CDC, Stratakis CA. Multiple endocrine neoplasia type 1 (MEN1): an update and the significance of early genetic and clinical diagnosis. *Front Endocrinol (Lausanne)* 2019;10:339.
2. Hartley IR, Costa Beber Nunes J, Lodish M, Stratakis CA. Cushing disease in a patient with nonbullous congenital ichthyosiform erythroderma: lessons in avoiding glucocorticoids in ichthyosis. *J Pediatr Endocrinol Metab* 2019;32(8):911-914.
3. Hartley I, Zhadina M, Collins MT, Boyce AM. Fibrous dysplasia of bone and McCune-Albright syndrome: a bench to bedside review. *Calcif Tissue Int* 2019;104(5):517-529.
4. Kamilaris CDC, Faucz FR, Voutetakis A, Stratakis CA. Carney complex. *Exp Clin Endocrinol Diabetes* 2019;127(2-03):156-164.
5. Kushchayeva Y, Lightbourne M, Lodish M, Stratakis CA. Genetic tumor syndromes with endocrine involvement: a compendium and an update. *Pediatr Endocrinol Rev* 2019;16(3):311-334.

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Pediatric Endocrinology Inter-Institute Training Program

The Fellowship in Pediatric Endocrinology is a three-year, ACGME–accredited program. Applicants must have completed a residency in Pediatrics or Medicine/Pediatrics and be eligible 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 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 *https://www.nichd.nih.gov/about/org/dir/osd/tp/peitp* 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 four months at the NIH Clinical Research Center, four months at Children's National Health Systems (CNHS), two months at Walter Reed National Military Medical Center, one month on consult service, and one month elective (e.g., at The Johns Hopkins University Hospital, Baltimore, MD). Continuity clinics are held once a week and alternate between the NIH outpatient pediatric endocrine clinic and the diabetes and general endocrine outpatient clinics at CNHS. In addition, multi-disciplinary clinics in long-term follow-up for bone disorders, neuroendocrine tumors, disorders of sexual development, obesity, and other conditions 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, and lipodystrophy.

Second and third years. During the second and third years, mandatory clinical responsibilities are limited to one half-day continuity clinic per week and inpatient pediatric endocrine consultation on an on-call basis for three months per year. Fellows learn how to develop a research protocol, conduct a clinical study or basic research, evaluate the results,

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Fetima Worthington, *Program Coordinator, NICHD*

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

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Margaret Keil, MS, CRNP, *Director, Pediatric Endocrine Clinical Services, NICHD*

James Mills, MD, *Senior Investigator, Division of Epidemiology, Statistics, and Prevention Research, NICHD*

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and generate presentations and manuscripts 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 the Scholarship Oversight Committee. Many of our fellows 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 August 31st, and interviews are conducted from September through November. Applicants must register with both NRMP and ERAS (*http://www.nrmp.org*; *https://www.aamc.org*).

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Rachel Gafni, MD, *Craniofacial and Skeletal Diseases Branch, NIDCR*

Ellen Leschek, MD, *Program Director, Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK*

Deborah Merke, MD, *Adjunct Investigator, NICHD & Chief, Section of Congenital Disorders, CC*

NICHD Pediatric and Adolescent Gynecology Training Program

The Pediatric and Adolescent Gynecology (PAG) Fellowship training program is a two-year program, not accredited by the ACGME (Accreditation Council for Graduate Medical Education), comprising faculty from the National Institute of Child Health and Human Development (NICHD), Children's National Health Systems (CNHS), and MedStar Washington Hospital Center (MWHC). The mission of the fellowship program is to ensure that the graduate possesses the knowledge, skills, and professional attributes essential for the function as a consultant to pediatricians, family practitioners, obstetricians, and gynecologists for girls from birth up to age 18–21 years with pediatric gynecologic concerns, as well as for women born with congenital anomalies. Qualified candidates must be U.S. Citizens or Green Card holders and have completed an accredited residency in Obstetrics and Gynecology in the United States and be Board-eligible in this specialty.

Veronica Gomez-Lobo, MD, *Program Director*

Tazim Dowlut-McElroy, MD, *Associate Program Director*

Jacqueline Maher, MD, *Staff Clinician*

Allison Mayhew, MD, *Clinical Fellow*

Shashwati Pradhan, MD, *Clinical Fellow*

Program structure

The first-year fellow will attend most PAG clinics as well as Heavy Menses and Special Gynecologic Endocrine clinics at MedStar and CNHS. The second-year fellow will attend Vulvar Dermatology clinics. Fellows alternate attendance at Turner syndrome clinics, PROUD (positive reevaluation of urogenital differences clinics, and DSD (disorders of sex development) clinics at CNHS and consult services at NICHD. These outpatient services do not depend on fellows for clinical care, which will allow flexibility to maximize the educational experience. Surgeries will be performed by the fellow on call with a resident, and the fellow will function as the surgical instructor during such cases. Complex surgeries will be performed by both fellows as co-surgeons. Rotations, including one month in Child Abuse and 2–4 weeks of pediatric surgery at CNHS, will be scheduled during the second year of fellowship; electives in Adolescent Medicine (eating disorders), as well as to travel to other sites for further complex anomaly training will be available.

For successful completion of this training program, the fellow will design, implement, complete, describe, and report at least one research study. A second multi-center project is to be designed and submitted, or collaboration with a project submitted to the Fellows Research Network at North American Society for Pediatric and

Adolescent Gynecology (NASPAG) will be required. During the fellowship, fellows will have thirty percent of their time protected for clinical research. The research will take place throughout the two years of training on 1.5 weekdays, except during outside rotations.

Structured training includes a series of introductory seminars geared to the first-year fellows, which takes place from July to September of the first year. The introductory seminars provide a historical perspective and basic understanding of the practice of Pediatric and Adolescent Gynecology and Reproductive Endocrinology, as well as of statistics. Fellows are required to attend a weekly colorectal conference, a monthly DSD clinic conference, a monthly Turner's clinic conference, PAG Journal Club/research meeting, and lectures. In addition, fellows are encouraged to attend the monthly Reproductive Endocrine Journal club and DSD-TRN (Translational Research Network) case conference.

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 North American Society for Pediatric and Adolescent Gynecology, the American Society for Reproductive Medicine, the Society for Gynecologic Investigation, and the Pediatric Endocrine Society. Moreover, fellows are encouraged to attend specialty meetings in their chosen interest areas, such as the Oncofertility Consortium and Differences in Sex Development–Androgen Insensitivity annual meetings.

Application information

Applications are submitted using the common application form on NASPAG. Applications are accepted in July before the anticipated start date, and interviews are typically held in August or September before the start date. Selection of candidates is processed through the National Resident Matching Program (NRMP). One to two positions are available in alternating years.

Please visit the URL below for detailed program information.
https://www.cc.nih.gov/training/gme/programs/pediatric_adolescent_gynecology.html

Publications

1. Moravek MB, Appiah LC, Anazodo A, Burns KC, Gomez-Lobo V, Hoefgen HR, Jaworek Frias O, Laronda MM, Levine J, Meacham LR, Pavone ME, Quinn GP, Rowell EE, Strine AC, Woodruff TK, Nahata L. Development of a pediatric fertility preservation program: a report from the Pediatric Initiative Network of the Oncofertility Consortium. *J Adolesc Health* 2019 64(5):563-573.
2. Grimstad FW, Fowler KG, New EP, Ferrando CA, Pollard RR, Chapman G, Gomez-Lobo V, Gray M. Uterine pathology in transmasculine persons on testosterone: a retrospective multicenter case series. *Am J Obstet Gynecol* 2019 220(3):257.
3. Nahata L, Chen D, Moravek MD, Quinn GP, Sutter ME, Taylor J, Tishelman AC, Gomez-Lobo V. Understudied and under-reported: fertility issues in transgender youth—a narrative review. *J Pediatr* 2019 205:265-271.
4. Dowlut-McElroy T, Higgins J, Williams KB, Strickland JL. Treatment of prepubertal labial adhesions: a randomized controlled trial. *J Pediatr Adolesc Gynecol* 2019 32(3):259-263.
5. Light A, Wang LF, Zeymo A, Gomez-Lobo V. Family planning and contraception use in transgender men. *Contraception* 2018 98(4):266-269.

6. Pecker LH, Maher JY, Law JY, Beach MC, Lanzkron S, Christianson MS. Risks associated with fertility preservation for women with sickle cell anemia. *Fertil Steril* 2018 110(4):720-731.

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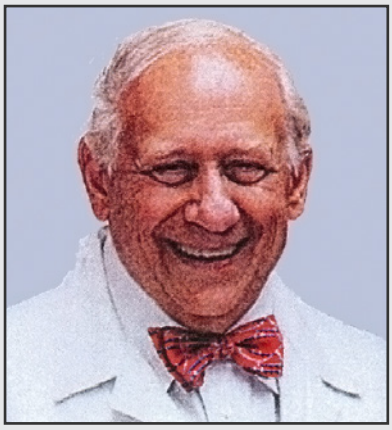
Reproductive Endocrinology and Infertility Training Program

The Intramural NICHD Reproductive Endocrinology and Infertility Training Program sponsors a three-year clinical fellowship in Reproductive Endocrinology and Infertility, which is accredited by the Accreditation Council for Graduate Medical Education (ACGME). The objective of this graduate medical education program is to train clinicians to serve as researchers and future leaders in the field of reproductive endocrinology with a view toward advancing basic, translational, and clinical science in reproduction. The program was started in 1978 and has since trained over 60 physicians in reproductive endocrinology. Upon completion of the rigorous scientific, clinical, and surgical curriculum, fellows may apply to the American Board of Obstetrics and Gynecology for certification in the subspecialty of Reproductive Endocrinology. The Fellowship in Reproductive Endocrinology and Infertility is served by faculty from four institutions: the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), the Uniformed Services University of the Health Sciences (USUHS), Walter Reed National Military Medical Center (WRNMMC), and the Shady Grove Fertility Center. The program accepts civilian and military graduates of U.S. residencies in Obstetrics and Gynecology and has the mission to train reproductive endocrinology and infertility fellows who will serve as faculty in 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 evidence-based, cutting-edge treatments to couples with infertility. Program graduates have become assistant, associate, and tenured professors and departmental chairs.

Fellows in the program rotate on clinical services of NICHD–supported intramural graduate medical programs in Medical and Pediatric Endocrinology as well as in Medical Genetics. The clinical training is robust. Thus, within the unique environment of the NIH, fellows participate in evaluation and management of rare and challenging endocrine conditions on the NIH Reproductive Endocrine Teaching Service. Clinical and surgical rotations take place at the Walter Reed National 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, Combined NICHD/Federal Fellowship in Reproductive Endocrinology*

Jasmine M. Aly, MD, *Clinical Fellow*

Lauren A. Bishop, MD, *Clinical Fellow*

Olivia J. Carpinello, MD, *Clinical Fellow*

Joshua C. Combs, MD, *Clinical Fellow*

Anthony M. DeAngelis, MD, PhD, *Clinical Fellow*

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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.

Nicole Doyle, MD, PhD, *Clinical Fellow*
Michael B. Evans, DO, *Clinical Fellow*
Steven A. Gay, MD, *Clinical Fellow*
Anne E. Martini, DO, *Clinical Fellow*
Jessica A. Lentscher, MD, *Clinical Fellow*
Karrie Walker, DO, *Clinical Fellow*
Jessica R. Zolton, DO, *Clinical Fellow*
Carter Monique O. Owen, MD, *Collaborator*

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 rotations 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 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 ACGME 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 weekly NIH teaching-rounds conferences, 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 ACGME are covered in special NIH grand rounds and by courses at the NIH or the WRNMMC 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 those of 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.

Publications

1. Carpinello O, DeCherney A. Theory of relativity of progress. *Fertil Steril* 2019 111:895-896.
2. Connell MT, Richter KS, Devine K, Hill MJ, DeCherney AH, Doyle JO, Tucker MJ, Levy MJ. Larger oocyte cohorts maximize fresh IVF cycle birth rates and availability of surplus high-quality blastocysts for cryopreservation. *Reprod Biomed Online* 2019 38:711-723.
3. 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.
4. Hill MJ, Healy MW, Richter KS, Parikh TP, Devine K, DeCherney AH, Levy M, Widra EA, Patounakis G. Defining thresholds for abnormal premature progesterone levels during ovarian stimulation for assisted reproduction technologies. *Fertil Steril* 2018 110:671–679.e2.
5. Martini AE, Zolton JR, DeCherney AH. Isolated absent thelarche in a patient with neurofibromatosis type 1 and acromegaly. *Obstet Gynecol* 2018 131:96-99.

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

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 resource to NICHD's Scientific Director in the management and overall planning for the DIR.

The senior leadership within the AMB works directly with the Scientific Director and Deputy Scientific Director in 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 at 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. These include 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 and branches that they support and the many other entities at the NIH, such as the Office of the Scientific Director, NICHD; the Office of the Director, NICHD; the Office of Intramural Research, Office of the Director; the Fogarty International Center; Human Resources offices; the Office of Research Services including the Division of International Services; the Office of Research Facilities; and the Technology Transfer Branch.

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Research Animal Management Branch

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

In addition to providing animal research support, research, and veterinary consultation services to NICHD investigators, the RAMB represents the interests of the NICHD DIR on all aspects of animal research conducted within the Intramural Research Program (IRP).

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 Division of Intramural Research (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, in the Building 14C Rodent Facility, 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 the NIH Institutes NICHD, NHGRI, NHLBI, and NCI.

The Building 6B SAF houses up to 12,000 cages of rodents and provides support to the animal research activities of three Institutes (NICHD, NEI, and NIAMS). In addition to traditional rodent research support, the program supports NICHD behavioral research studies being conducted in the dedicated 6B behavioral suite. The Building 6B SAF is a restricted-access, disease-free rodent facility and has one room dedicated to the NICHD Aquatics program, which houses cavefish for use on NICHD research studies.

The NICHD ACRF Animal Facility located in Building 10, suite 6C127, supports the animal research activities of the NICHD. The facility has four animal rooms and two procedure rooms available for NICHD researcher use. The RAMB provides care and housing for rodent and aquatic species housed within this restricted-access, conventional animal holding facility.

The Building 6 Shared Zebrafish Facility (SZF) supports NICHD and NHGRI with 15,000 two-liter tanks; the total capacity of the SZF is

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

Ruth A. Woodward, DVM, *Animal Program Director & Building 6B Facility Veterinarian*

Christopher M. Rishell, BS, *Senior Facilities Manager*

Lauren Pandolfo, MS, *Aquatics Program Manager*

Julie A. Jacobs, *Facilities Contract Manager*

Daniel T. Abebe, MS, *Research Behavioral Technician*

Maria Publico, BS, *Animal Care and Use Committee Coordinator*

** As of October 2019, Dr. Schech is now at Baylor School of Medicine in Houston, TX.*

approximately 330,000 zebrafish. The Building 6 SZF program provides care and research support to animals maintained on NICHD and NHGRI research projects.

The RAMB operates a satellite frog facility housing *Xenopus* species within the Building 49 vivarium. This satellite facility provides primary animal care and research support to over 450 tanks of *Xenopus* utilized by NICHD DIR researchers.

The RAMB Aquatics Program also provides aquatics animal care and support to several institutes and centers (ICs) within their satellite facilities across the NIH. NICHD is considered a leader in the field of aquatics research animal care and support at the NIH.

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 2017, 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 many animal-user investigators have been active contributors to the NIH Animal Research Advisory Committee's (ARAC) efforts to adopt the new "Guide to Care and Use of Laboratory Animals," which is a primary-source set of guidelines used by the AAALACi and the NIH Office of Animal Welfare.

Additional Funding

- In addition to direct funding by the Intramural Research Programs of NICHD, the RAMB is also funded by facility users from other NIH Institutes and Centers.

Publications

1. Hernandez-Ramon EE, Si N, Lachir YY, Cline JM, Wood CE, Asaki E, Edelman DC, Petersen D, Sanchez V, Woodward RA, Wang Y, Poirier MC. Transcriptional regulation of Tamoxifen in human and non-human primate endometrium and cultured breast and endometrial cells. *Curr Top Toxicol* 2019 15:1-15.

Contact

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Bioinformatics and Scientific Programming Core Facility

The goal of the Bioinformatics and Scientific Programming Core (BSPC) is to provide expert bioinformatics support to NICHD researchers, assisting at all stages from experimental design through multiple iterations of analysis to final manuscript preparation. In addition, we develop software tools that can be applied to a wide range of bioinformatics, genomics, and general data analysis both at NICHD and in the larger international scientific community. We coordinate training for staff and trainees in basic programming and genomic analyses to help build bioinformatics support directly within labs.

Ryan Dale, MS, PhD, *Scientific Information Officer*

Caroline Esnault, PhD, *Bioinformatics Scientist*

Gennady Margolin, PhD, *Bioinformatics Scientist*

Apratim Mitra, PhD, *Bioinformatics Scientist*

Hongen (Henry) Zhang, PhD, *Staff Scientist*

Eric Chang, BS, *Postbaccalaureate Fellow*

Sydney Hertafeld, BS, *Postbaccalaureate Fellow*

Nicholas Johnson, BS, *Postbaccalaureate Fellow*

Structure

NICHD's Bioinformatics and Scientific Programming Core (BSPC) uses a "hub and spoke" model, consisting of a central core of staff (currently in Building 10) coordinating with embedded bioinformaticians (currently in Buildings 6 and 49) working directly in laboratories. This allows us to build centralized infrastructure that can be re-used across many research programs while at the same time maintaining focused and custom local support in labs. Joint meetings and discussion allow everyone, central and embedded, to share lessons learned and identify new tools and methods.

Projects overview

In 2019, the BSPC worked on 49 projects, collaborating with 36 PIs, fellows, staff scientists, and staff clinicians across 23 laboratories. The projects included assays such as bulk RNA-seq, single-cell RNA-seq, ChIP-seq, whole exome sequencing, whole genome sequencing, DNA methylation, CUT&RUN, bulk ATAC-seq, and single-cell ATAC seq. Some projects involved custom algorithm development and tool development, and many projects required integration with published studies. Roughly a third of the projects involved RNA-seq differential expression analysis; roughly half of the projects from this year have been completed and the rest remain ongoing.

Projects often begin with an in-depth discussion with researchers to understand the background and goals of the project. It is important for us to understand the underlying biology and details of the experimental design (when applicable) for each project, so that we can make the most informed analysis decisions. We then provide a

prioritized plan for the first round of analysis and schedule the work. There are often several iterations of analysis, as a project progresses. Each iteration may add more sophisticated analyses, new data generated by the lab, or integrate results with published data. As expected for a no-cost shared resource, the time it takes for one iteration on one project is highly dependent on the existing workload across all other projects that we are handling in the Institute.

After each iteration, we meet to discuss the results in detail. The meeting includes a walk-through of the results, the computational background, discussion of how to use and interpret the tables, figures, and other output, and recommendations for next steps. Depending on the researchers' interests, this can also include a discussion of the code and help with running it or adapting it to other projects in the lab. The next iteration of analysis is then planned, prioritized, and scheduled.

Projects: computation and code

Most projects are multi-week or multi-month projects that continue after many iterations and often require authoring substantial amounts of custom R and Python code. We work closely with NICHD's Molecular Genomics Core, where much of the raw high-throughput sequencing data for NICHD are generated. We can access these data directly, avoiding the need to coordinate data transfer and/or storage space with researchers. Analysis performed by the BSPC makes extensive use of NIH's Biowulf high-performance computing cluster, and there is no direct cost to researchers for work done by the BSPC.

To ensure long-term computational reproducibility, we build a complete software environment for each project, which allows us to track all versions of software and dependencies, and any one project's environment can be updated without affecting any others. All source code is kept under version control so that the entire history of the project can be tracked. We also build reproducible workflows for each project that keep track of which results have been updated, and wherever possible, provide output as standalone, interactive HTML files, so that researchers can easily explore their results.

Additional software development and computational resources

The BSPC continues to develop and maintain publicly available open-source tools. One example is lcdb-wf, a system of workflows and pipelines to process high-throughput sequencing data, run extensive quality control, and perform differential ChIP-seq or RNA-seq analyses and which runs on NIH's Biowulf high-performance computing cluster. We also continue to contribute to the Bioconda project, a system used by bioinformaticians worldwide to easily install biology-related software tools.

The BSPC maintains an RStudio Connect Server instance, which allows us to publish interactive applications that researchers can use to interactively explore and plot their data. We also maintain a GitLab instance in NICHD's data center, which provides source code version control, issue tracking, and documentation for projects we work on in such a way that they can be shared with collaborators. These repositories currently store tens of thousands of lines of Python and R code and documentation written by the BSPC and used in various projects.

Contact

For more information, email *ryan.dale@nih.gov* or visit *https://www.nichd.nih.gov/about/org/dir/other-facilities/cores/bioinformatics*.

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, and biological visualization services.

Core IT Services

The CSSC continued to expand its services to the Division of Intramural Research (DIR) community in the following core IT areas:

NETWORK AND DESKTOP SERVICES

During the past year, the CSSC continued supporting reliable, secure, and efficient information technology solutions. This includes acquisition, maintenance, and support for licensed software used by the DIR community e.g., GraphPad Prism, Amira, DNASTAR Lasergene, MathWorks MATLAB, SnapGene, and FlowJo, as well as network services (email, data backups, VPN, helix, PDAs, wireless configurations) and cross-platform desktop, server and application hosting in the Rock Spring and Bldg. 35 Data Centers. We also assist users in identifying, researching, and purchasing custom hardware configurations to match research instrument requirements.

DATA-RECOVERY SERVICES

The CSSC implemented core data recovery tools for all media: hard drive, SSD, and flash etc., including RAID 0 and 5 recovery tools. Since 2005, the Core has recovered over five terabytes of research data from failed drives and media, saving many thousands of dollars in recovery costs.

Clinical informatics

The CSSC continued to support and develop applications related to clinical and translational medicine, including the Clinical Trials Database (CTDB) project. Such informatics tools allow researchers to design, collect, and report clinical observations related to natural history and interval-based studies. The total number of protocols and research projects supported by the CTDB team increased to 605 for 15 NIH institutes, with an expansion of research questions to over 240,000. Since its inception, data from CTDB supported 1,500 NICHD publications.

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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*
Biplav Khadka, BS, *Software Developer*
Nareg Bakirci, MS, *Website Developer*
Nicki Swan, BA, *Graphic Designer*
Raquel Gray, MBA, *Team Lead*
Rana Alneaimy, MD, *Documentation Specialist*
Rolanda Bailey, BS, *Documentation Specialist*

(continued)

Tamara Prodanov, MD, *Documentation Specialist*
Kami Emanuel, *Information Specialist*
Audrey Harrell, MS, *Information Specialist*
Vida Bayat Mokhtari, MD, *Information Specialist*
Alesia Robinson, BS, *Information Specialist*
George Tran, *Information Specialist*

Our software development group completed one release that included features for QA module, Biorepository, Form Data download and various interface improvements. Our database development and reporting team continued to migrate protocol data from various NIH institutes, while supplying reporting for protocols within CTDB. New data-marts (pft, exercise data, and six minutes walk) and reporting functionalities were added to the CTDB data-mart, allowing users to combine and retrieve additional information. We also supported the Clinical Trial Survey System (CTSS), an application for patient surveys, used for 68 active protocols; 101 CTSS websites were archived in 2019.

Custom software development for scientific and administrative support

The CSSC provides custom software development for the DIR's 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.

DIRweb includes lab training web services for the NIH Enterprise Directory and Division of Occupational Health and Safety Training.

The team continued to release enhancements to the Fellows Annual Progress Report, a unified means for tracking and mentoring intramural trainees along with easing the re-appointment process. This solution provided the Office of Education with useful metrics regarding mentoring and training programs. The Exit Survey feature, a short survey allowing DIR trainees a platform for providing feedback, has also been updated for DIR Fellows.

We continued to develop the new Package Tracking module for the DIR AMB, providing AMB staff real-time accuracy metrics for personnel and travel package compilation. A feedback system was developed to support real-time customer satisfaction collection for the AMB, Office of the Clinical Director, and laboratory administrative support staff. The system also offers more detailed feedback submissions periodically along with comprehensive response metrics.

The CSSC team continued to work 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.

The services were provided to: The NICHD DIR Annual Report, the DIR Annual Fellows and Scientific Retreats, the DIR Annual Fellows and Scientific Retreats, the Anita B. Roberts Lecture Series, the annual Mortimer B. Lipsett Lecture, the NICHD Exchange lecture series, NICHD research labs, and medical training programs, including the Pediatric Endocrinology Training Program and the Inter-Institute Adult Endocrinology Training Program. Services included recording audio and video of presenters.

We supported the NICHD Office of Education by producing a monthly newsletter, *The NICHD Connection*, in collaboration with Intramural Fellows. We created print collateral for conferences, including the NICHD Scientific Retreat and NICHD Fellows Retreat, and updated recruitment materials for NICHD's clinical training programs. We also continued maintaining websites for the *NICHD DIR Annual Report* and *Annual Fellows Retreat*.

The CSSC continued to provide a platform for conducting scientific review by the Board of Scientific Counselors, administrative intranet support, and business operations. In 2019, we supported the migration of the public facing laboratory websites from Confluence wiki to Drupal. The plan for Science@NICHD (*http://science.nichd.nih.gov*) is to keep hosting NICHD DIR internal sites.

Collaborators

- Richard Childs, MD, *Clinical Director, NHLBI, Bethesda, MD*
- Maryland Pao, MD, *Clinical Director, NIMH, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- Steven Stanhope, PhD, *University of Delaware, Newark, DE*
- Constantine Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

Contact

For more information, email *ryan.dale@nih.gov* or visit *https://www.nichd.nih.gov/about/org/dir/osd/cf/ucss*.

NICHD Microscopy and Imaging Core

The mission of the NICHD Microscopy and Imaging Core (MIC) is to provide service in four different areas: (1) sample preparation for light and electron microscopy studies; (2) wide-field and confocal light microscopy; (3) transmission electron microscopy (TEM); and (4) image analysis and data extraction. The Facility is operated as a 'one-stop shop,' where investigators can, with minimum effort, go from their scientific question to the final data.

Vincent Schram, PhD, *Staff Scientist*

Lynne A. Holtzclaw, BS, *Research Assistant*

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

Sara Felsen, BS, MS, *Postbaccalaureate Fellow*

Mode of operation

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

Vincent Schram is the point person for light microscopy and data 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 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.

The MIC has an open-door policy with the NINDS Light Imaging Facility (LIF, Building 35), where the two cores freely exchange users, equipment, and support. Although not officially sanctioned, this mode of operation provides extended support hours, wider expertise, and access to more equipment than each Institute could afford on its own.

The MIC serves over 300 registered users in 68 laboratories. NICHD uses 80% of the Facility resources, NINDS 15%, and other Institutes (NIBIB, NIA, and NIMH) the remaining 5%.

Light microscopy

The MIC is equipped with six modern confocal microscopes, each optimized for certain applications:

1. Zeiss LSM 710 for high-resolution confocal imaging of fixed specimen and live cells;

2. Zeiss LSM 780 for challenging specimens that require both high resolution and high sensitivity;
3. Nikon Spinning Disk/Total Internal Reflection Fluorescence (TIRF) hybrid microscope for high-speed confocal imaging and selective recording of membrane-bound events in live cells;
4. Zeiss LSM 880 2-photon confocal for thick tissues and live animals;
5. Zeiss 800 optimized for confocal imaging of large specimens (tiling);
6. Zeiss 880 Airy, which offers near superresolution without the need for 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.

After an initial orientation, during which the staff research the project and decide upon the best approach, users receive hands-on training on the equipment and/or for the software best suited to their goals, followed by continuous support, when required. Once image acquisition is complete, the staff devise solutions and train users in 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 (NIH's Foundation for Advanced Education in the Sciences) classes; and (4) the Facility organizes frequent on-campus demonstrations of new imaging equipment, technology, and software by vendors in a dedicated space. The equipment demonstrations are open to the entire NIH community.

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 196 samples; 6 of them were immuno–EM studies. John Heuser, part of Joshua Zimmerberg's unit (NICHD), continued to spend a significant amount of time on the JEOL electron microscope.

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. Drs. Dever, Dufau, Fields, Hoffman, Klein, Le Pichon, Loh, Marini, McBain, Pfeifer, Sackett, Stojilkovic, and Stopfer (NICHD), Mankodi, Roche, Youle, and Ward (NINDS), and Chen (NIBIB) received support and training from Mrs. Holtzclaw.

In a collaborative endeavor with David Klein (NICHD), we characterizing pineal cell types for which genes of interest had been documented by RNA-seq [Reference 1]. A collaborative effort with the NINDS laboratory of Katherine Roche (NINDS) also resulted in a publication [Reference 2]. The continuing collaboration with the laboratory of Richard Youle (NINDS) to study the accumulation of ubiquitinated protein aggregates in brain and liver of a TAX1BP1 knock-out mouse continued during the year and is expected to be completed by year's end.

Image analysis

Extracting usable information from the collected images is an essential part of the Facility's services. Vincent Schram provides training and support for image analysis on two high-end computers running advanced image analysis packages: Imaris from Bitplane (3D rendering, segmentation and analysis), Huygens from Scientific Volume Imaging for deconvolution of regular confocal images, and ImageJ, Zeiss Zen and Nikon Elements for regular image processing. The latter now includes Denoise.AI, a unique denoising module for point scanner images based on machine learning.

Collaborators

- Tamás Balla, PhD, *Section on Molecular Signal Transduction, NICHD, Bethesda, MD*
- David C. Klein, PhD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Katherine W. Roche, PhD, *Receptor Biology Section, NINDS, Bethesda, MD*
- Carolyn L. Smith, PhD, *Light Imaging Facility, NINDS, Bethesda, MD*
- Richard J. Youle, PhD, *Biochemistry Section, NINDS, Bethesda, MD*

Contact

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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.

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

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 HiSeq 2500. Two of the other sequencers, an Illumina MiSeq 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 than 100,000 base pairs. To increase the throughput of the HiSeq 2500 even further, we have a cBot liquid handler for automated high-precision 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 gene-specific DNA sequencing, as well as whole-transcriptome sequencing (RNA-Seq), microRNA sequencing, microbiome sequencing, bisulfite sequencing (DNA methylome), ChIP-Seq, and ribosomal profiling. The PacBio Sequel permits 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

sequencing for 166 projects (2,798 samples) across the full spectrum of sequencing types, generating 12,395 gigabases of sequence; the projects involved 35 NICHD Principal Investigators from 11 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.

Publications

1. Mays JC, Kelly MC, Coon SL, Holtzclaw L, Rath MF, Kelley MW, Klein DC. Single-cell RNA sequencing of the mammalian pineal gland identifies two pinealocyte subtypes and cell type-specific daily patterns of gene expression. *PLoS One* 2018 13(10):e0205883.
2. Coon SL, Fu C, Hartley SW, Holtzclaw L, Mays JC, Kelly MC, Kelley MW, Mullikin JC, Rath MF, Savastano LE, Klein DC. Single cell sequencing of the pineal gland: the next chapter. *Front Endocrinol (Lausanne)* 2019 10:590.
3. Fletcher PA, Smiljanic K, Previde RM, Iben J, Li T, Rokic MB, Sherman A, Coon SL, Stojilkovic SS. Cell type- and sex-dependent transcriptome profiles of rat anterior pituitary cells. *Front Endocrinol (Lausanne)* 2019 10:623.
4. Wester JC, Mahadevan V, Rhodes CT, Calvigioni D, Vankatesh S, Maric D, Hunt S, Yuan X, Zhang Y, Petros TJ, McBain CJ. Neocortical projection neurons instruct inhibitory interneuron circuit development in a lineage dependent manner. *Neuron* 2019 102(5):960-975.
5. Tabor KM, Marquart GD, Hurt C, Smith TS, Geoca AK, Bhandiwad AA, Subedi A, Sinclair JL, Rose HM, Polys NF, Burgess HA. Brain-wide cellular resolution imaging of Cre transgenic zebrafish lines for functional circuit-mapping. *eLife* 2019 8:e42687.

Collaborators

- Jeffrey Baron, MD, *Section on Growth and Development, NICHD, Bethesda, MD*
- Juan Bonifacino, PhD, *Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD*
- Andres Buonanno, PhD, *Section on Molecular Neurobiology, NICHD, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Michael Cashel, MD, PhD, *Section on Molecular Regulation, NICHD, Bethesda, MD*
- David J. Clark, PhD, *Section on Chromatin & Gene Expression, NICHD, Bethesda, MD*
- Robert J. Crouch, PhD, *Section on the Formation of RNA, NICHD, Bethesda, MD*
- Mary Dasso, PhD, *Section on Cell Cycle Regulation, NICHD, Bethesda, MD*
- Angela Delaney Freedman, MD, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Melvin Depamphilis, PhD, *Section on Eukaryotic DNA Replication, NICHD, Bethesda, MD*
- Thomas Dever, PhD, *Section on Protein Biosynthesis, NICHD, Bethesda, MD*
- Katie Drerup, PhD, *Unit on Neuronal Cell Biology, NICHD, Bethesda, MD*
- Benjamin Feldman, PhD, *Zebrafish Core, NICHD, Bethesda, MD*
- David Klein, PhD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Claire E. Le Pichon, PhD, *Unit on the Development of Neurodegeneration, NICHD, Bethesda, MD*
- Henry L. Levin, PhD, *Section on Eukaryotic Transposable Elements, NICHD, Bethesda, MD*

- Mary Lilly, PhD, *Section on Gamete Development, NICHD, Bethesda, MD*
- Paul Love, MD, PhD, *Section on Cellular and Developmental Biology, NICHD, Bethesda, MD*
- Todd Macfarlan, PhD, *Unit on Mammalian Epigenome Reprogramming, NICHD, Bethesda, MD*
- Matthias Machner, PhD, *Section on Microbial Pathogenesis, NICHD, Bethesda, MD*
- Richard Maraia, MD, *Section on Molecular and Cellular Biology, NICHD, Bethesda, MD*
- Joan C. Marini, MD, PhD, *Section on Heritable Disorders of Bone & Extracellular Matrix, NICHD, Bethesda, MD*
- Chris McBain, PhD, *Section on Cellular and Synaptic Physiology, NICHD, Bethesda, MD*
- Keiko Ozato, PhD, *Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD*
- Timothy J. Petros, PhD, *Unit on Cellular and Molecular Neurodevelopment, NICHD, Bethesda, MD*
- Karl Pfeifer, PhD, *Section on Epigenetics, NICHD, Bethesda, MD*
- Pedro Rocha, PhD, *Unit on Genome Structure and Regulation, NICHD, Bethesda, MD*
- Dan Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*
- Mihaela Serpe, PhD, *Section on Cellular Communication, NICHD, Bethesda, MD*
- Yun-Bo Shi, PhD, *Section on Molecular Morphogenesis, NICHD, Bethesda, MD*
- Stanko S. Stojilkovic, PhD, *Section on Cellular Signaling, NICHD, Bethesda, MD*
- Gisela Storz, PhD, *Section on Environmental Gene Regulation, NICHD, Bethesda, MD*
- Constantine Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*
- Brant Weinstein, PhD, *Section on Vertebrate Organogenesis, NICHD, Bethesda, MD*

Contact

For more information, email *fdporter@helix.nih.gov* or visit *http://mgl.nichd.nih.gov*.

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.

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

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

Yvonne Rosario, PhD, *Postdoctoral Intramural Research Training Award Fellow*

Emily N. Katz, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Oversight and support of client–specific projects

Over 2018–2019, the Core engaged in research projects with fourteen labs and two cores.

Porter Lab, NICHD: *Genetic dissection and creation of human disease models of sterol metabolism*

In previous years, the Core used CRISPR-Cas9 technology to create genetic mutant zebrafish lines for the Porter lab in four genes: *npc1*, *npc2, cln3,* and *ebp,* which have roles in various steps of sterol metabolism and lipid storage. In the previous year, we characterized and published some phenotypes of *npc1* mutants and, this year, we investigated other *npc1* phenotypes as well as phenotypes of *cln3* and *ebp* mutants. This year, the Core also used CRISPR-Cas9 technology to regenerate mutant alleles of *dhcr7* and *cln3* in the TAB5 strain of zebrafish, with the goal of reducing the phenotypic variability seen in the more outbred EK strain of zebrafish that was originally used.

Stratakis Lab, NICHD: *Function of zebrafish orthologs to human genes implicated in disorders of the pituitary-adrenal axis*

In previous years, the Core used CRISPR-Cas9 technology to generate zebrafish carrying loss-of-function mutations in four zebrafish orthologs to human genes implicated by the Stratakis lab in human growth anomalies and eight zebrafish orthologs to human adrenal hyperplasia and Cushing's disease–associated genes. Over the last

year, we made considerable progress in the characterization of one of these genes, with new findings of temperature-sensitive maternal effects on viability and central nervous system anatomy.

Blackshear Lab, NIEHS: *Functions of a zinc-finger protein gene family in zebrafish*
The Core has been engaged over the past several years to use CRISPR-Cas9 technology to generate zebrafish carrying loss-of-function mutations in seven zebrafish orthologs and assist with preliminary phenotype characterization. We observed no abnormal phenotypes for thirteen out of thirteen mutant alleles for these seven genes. This year, the Core sent tissue samples to the Blackshear lab to compare the transcriptomes of mutants with those of control siblings to determine whether subtle molecular consequences of these gene disruptions can illuminate their function and whether unexpected functional splice forms and/or genetic compensation might be responsible for the lack of abnormal phenotypes. Eleven of the thirteen alleles were cryopreserved, and cryopreservation of the remaining three alleles is scheduled in the upcoming months.

Larson Lab, NCI: *Transgenic zebrafish carrying mutant and WT Runx1-mKate protein fusions*
This year, the Larson lab engaged the Core to generate transgenic zebrafish carrying mutant and wild-type Runx1-mKate protein fusions. Optimal microinjection conditions for three constructs were determined, and a screen for germ-line transmitters of each is ongoing.

Lawal Lab, NINR: *Function of* ryr1b *mutants*
Tokunbor Lawal spent the first part of the year continuing previous efforts to optimize an assay to test candidate drugs for their potential to ameliorate muscle defects seen in zebrafish mutants that carry mutations in *ryr1b*, a gene whose human counterpart is implicated in various myopathies. Owing to an insufficient magnitude of the assay read-out as designed, we are now troubleshooting an alternative assay. We are also launching a new project to test the functionality of *ryr1b* alleles using a common genetic platform.

Caldovic Lab, Children's National Medical Center: *Neuroprotective drugs to mitigate hyperammonemia*
Exposure of the brain to high ammonia, a consequence of urea cycle defects and liver failure, causes neurocognitive deficits, intellectual disabilities, coma, and death. Since 2012, the Core has helped this lab use zebrafish embryos to identify small molecules that can diminish the effects of hyperammonemia. In the first 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 past three years, the Core has supported a reimplementation of this screen, using a larger library of 10,000 compounds, bolstered by additional personnel from the Caldovic lab. The screen was completed during the last year, and the team is writing up the data and conducting validation assays.

Swartz Lab (NINDS)
This year the Swartz lab engaged the Core to provide consultation and access to zebrafish embryos, equipment, and reagents, enabling them to determine that zebrafish *tmem266* is expressed in the cerebellum. Follow-on studies are currently being planned.

Olivier Lab (NHBLI)
The Core began providing embryos to the Olivier lab this year, enabling them to conduct experiments on infection in their own laboratory and on their own animal protocol.

Kemper (NHLBI) and Afzalii (NIDDK) Labs

These labs are interested in the human immune system and the role of zebrafish *rca2.1*, a CD-46 ortholog, in development and health. Our initial goal was to generate *rca2.1* null zebrafish using CRISPR/Cas9. Based on concerns that novel translational start sites and/or decay of otherwise nonfunctional RNAs can elicit genetic compensation, we sought to disrupt all *rca2.1* transcription. To identify transcriptional start sites (TSSs) and determine which predicted alternate transcripts are expressed during early development, we identified public databases of zebrafish RNA sequences and locations of their 5' termini and, with help from the NICHD Computer Support Services Core (Dale), integrated them into a searchable private track on the UCSC Genome Browser. We used the technique track to select gRNA (guide RNA) combinations aimed at cooperatively deleting the TSS target and downstream spans of *rca2.1*. Fragment analysis of coinjected embryos indicated the presence of diagnostic peaks arising from the desired 20 Kb deletion in close to 20% of F_0 embryos. Germline transmission from seven out of eight F_0 adults was subsequently achieved. Thus, using several gRNAs, we efficiently recovered large genomic *rca2.1* deletion alleles. Unlike classic CRISPR/Cas9 insertion-deletion (in-del) alleles, interpretation of phenotypes observed should be uncomplicated by concerns of extant or novel alternative transcripts or genetic compensation linked to nonsense-mediated decay.

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 were able to create mutations in all genes requested. Demand was lower this year, limited to generation of mutant alleles of *dhcr7* and *cln3*, as most laboratories are still characterizing mutant lines that we had previously made for them. We also explored making larger deletions and were successful in our first trial, for which we made a 20 KB deletion in *rca2.1*. This should model a general strategy for creating knockout alleles that avoid the risk of false-negative results (i.e., reduction of phenotype strength), which are sometimes associated with standard CRISPR/Cas9 in-del alleles.

Independent research by the NICHD Zebrafish Core: precise genome editing

OPTIMIZING STRATEGIES FOR CRISPR/CAS9–BASED HOMOLOGY-DIRECTED REPAIR (HDR)

In the previous year, the Core used CRISPR-Cas9 technology in combination with donor DNA to generate a zebrafish line with an Atp7a amino acid (AA) substitution that is cognate to an ATP7A (AA) substitution of interest to the Kaler lab (NICHD), and which causes distal motoneuropathy. The phenotype is characterized by hypopigmentation, with no obvious reduction in motor function. We devised a novel screening strategy for this project that included synonymous changes alongside the targeted nonsynonymous change. The synonymous changes introduced RFLPs (restriction fragment length polymorphisms) amenable to our molecular screening strategy. Over the past year, in collaboration with the NICHD Bioinformatics and Scientific Programming Core (Dale), we developed software for generalizing this approach to any locus of interest.

This year, in collaboration with the NICHD Molecular Genomics Core (Coon), the Core continued to conduct Director's Award–funded research to compare precise genome editing methods and efficiencies. Our initial comparison examines the efficiency of five distinct methods to generate seven distinct alleles of interest to labs in the NICHD and elsewhere at the NIH, namely: two *npc1* alleles (Porter lab, NICHD), one *rhoaa* allele (Weinstein lab, NICHD), one *ifitm5* allele (Marini lab, NICHD), two *ryr1b* alleles (Meilleur/Lawal lab, NINR), and one *cacna1c* allele (Golden lab, NIDDK). We prescreened candidate gRNAs and performed microinjections with four biological replicates to compare at least one method for each allele and at least two alleles for each

method. Lysates from these injections and a variety of control samples were then PCR–amplified and the amplicons purified to enable bar-code labeling and Next-Gen sequencing by the Molecular Genomics Core, which is currently under way.

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.

Additional Funding

- Director's Award for year 2 of 2

Publications

1. Tseng WC, Loeb HE, Pei W, Tsai-Morris CH, Xu L, Cluzeau CV, Wassif CA, Feldman B, Burgess SM, Pavan WJ, Porter FD. Modeling Niemann-Pick disease type C1 in zebrafish: a robust platform for screening of candidate therapeutic compounds. *Dis Model Mech* 2018 11(9):034165.

Collaborators

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- Ljubica Caldovic, PhD, *Children's National Medical Center, Washington, DC*
- Steven Coon, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Ryan Dale, MS, PhD, *Bioinformatics and Scientific Programming Core; Computer Support Services Core, NICHD, Bethesda, MD*
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Affinity Groups

Scientists and physicians in the NICHD Division of Intramural Research (DIR) are organized into 12 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, *Head*
Ajay Chitnis
Katie Drerup
Ben Feldman (Core)
Brant Weinstein

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.

Joan Marini, *Head*
Sergey Leikin

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.

Alan Hinnebusch, *Head*
Mary Dasso
Tom Dever
Maria Dufau
Henry Levin
Jon Lorsch (NIGMS)
Mihaela Serpe
Yun-Bo Shi

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.

Matthias Machner, *Head*
Anirban Banerjee
Juan Bonifacino
Andres Buonanno
Mary Lilly
Gisela Storz

Developmental Endocrinology, Metabolism, Genetics, and Endocrine Oncology

The mission of the **Developmental Endocrinology, Metabolism, Genetics and Endocrine Oncology (DEMGEO) Affinity Group** is to advance our understanding of endocrine, genetic, and metabolic disorders and endocrine tumors 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.

Karel Pacak, *Head*
Jeff Baron
Janice Chou
Fady Hannah-Shmouni (Training Program)
Deborah Merke (CC)
Anil Mukherjee
Forbes Porter
Kristina Rother (Training Program)
Stanko Stojilkovic
Constantine Stratakis
Jack Yanovski

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, *Head*
Jim Kennison
Paul Love
Todd Macfarlan
Keiko Ozato
Karl Pfeifer
Pedro Rocha

Genomics and Basic Mechanisms of Growth and Development

The mission of the **Genomics and Basic Mechanisms of Growth and Development (GBMGD) 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 understanding of both health and disease. The members of the GBMGD group have a strong history of producing knowledge-changing advances in a number of fundamental processes that are 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 GBMGD is a unique group 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 GBMGD

members, as well as others, has revealed that although these processes are fundamental and ubiquitous, defects in them are often manifested as specific health disorders with distinctive phenotypes affecting human development and with surprising tissue-specificity, or in cancer. Indeed, a substantial number of the most common clinically relevant genetic disorders affecting children are due to polymorphisms/mutations in factors required for the maintenance of basal cellular functions. Inherent to the GBMGD mission is to increase understanding of how natural genetic diversity in the human population contributes to these fundamental processes in ways that affect health and disease, and to apply such knowledge so that specific strategies toward improving health can be developed.

The vision of the GBMGD group is to elucidate new knowledge about fundamental processes that will promote 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, GBMGD group members will continue to generate new knowledge relevant to the fundamental processes essential to growth, development and health.

The GBMGD 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 or discipline, they extend beyond classical developmental biology and the gene pattern-specific expression aspects of animal development. By focusing on basal cellular functions, the GBMGD approach provides unique and complimentary perspectives on developmental biology and growth in health and disease states. Given the NIH's tolerance to high-risk endeavors not usually fostered elsewhere, the GBMGD group, and the many collaborations among its members, fosters a greater depth and breadth of fundamental discovery than would exist in its absence.

Rich Maraia, *Head*	David Clark	Mel DePamphilis
Mike Cashel	Bob Crouch	Roger Woodgate

Maternal-Fetal Medicine and Translational Imaging

The mission of the **Maternal-Fetal Medicine and Translational Imaging Affinity Group** is to understand the biology of normal pregnancy and its complications, as well as normal/abnormal neurodevelopment during childhood by the application of innovative, state-of-the art cellular, molecular, and imaging methods to improve the diagnosis, treatment, and prevention of conditions responsible for maternal, perinatal, and infant morbidity and mortality.

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.

Nervous System Development and Plasticity (Fields): Our research is concerned with understanding the molecular and cellular mechanisms by which neural activity and experience regulates development and plasticity of the nervous system, with three main areas of emphasis: (1) myelination and neuron-glia interactions, (2) cellular mechanisms of learning, and (3) gene regulation by neuronal firing.

Translational 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.

Biomedical Optics (Tromberg) develops models, methods, and devices for understanding and controlling light interactions with biological tissues. These methods are used to perform real-time quantitative measurements of clinically-relevant information, including: tissue blood flow, oxygen extraction, metabolic rate of oxygen consumption, and body/tissue composition (lean mass, hydration, and fat mass). Advanced capabilities include continuous dynamic monitoring of intrinsic physiological signals that can be used in feedback optimization for guiding therapies and clinical decision making. Our technology development effort includes portable, bedside, non-contact and wearable sensor platforms, as well as the design and integration of probes into instruments for minimally invasive surgical feedback and guidance.

Roberto Romero, *Head*	R. Douglas Fields	Leonid Margolis
Peter Basser	Amir Gandjbakhche	Bruce Tromberg (NIBIB)

Molecular Medicine

The **Molecular Medicine Group** brings together basic research programs that share the ultimate goal of developing treatments for human diseases through a better understanding of their pathophysiology.

Main areas of research focus include the maintenance of iron homeostasis and mechanisms underlying neurodegeneration. At the intersection of our work, we hope to shed light on a group of rare diseases called neurodegeneration with brain iron accumulation or NBIA, which may also have broader implications for other types of related diseases.

Claire Le Pichon, *Head*
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.

Mark Stopfer, *Head*
Tamás Balla
Dax Hoffman
Y. Peng Loh
Chris McBain
Tim Petros

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.

Leonid Chernomordik, *Head*
Sergey Bezrukov
Alexander Sodt
Joshua Zimmerberg

Reproductive Endocrinology and Infertility and Pediatric and Adolescent Gynecology

The **Reproductive Endocrinology & Infertility and Pediatric & Adolescent 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, *Head* (Training Program)
Veronica Gomez-Lobo (Training Program)

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.

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

Yeun Ju Kim, PhD, *Staff Scientist*

Alejandro Alvarez-Prats, PhD, *Postdoctoral Fellow*

Takashi Baba, PhD, *Postdoctoral Fellow*

Gergo Gulyas, MD, PhD, *Postdoctoral Fellow*

Joshua Pemberton, PhD, *Postdoctoral Fellow*

Nivedita Sengupta, PhD, *Postdoctoral Fellow*

Marek Korzeniowski, PhD, *Special volunteer*

Ljubisa Vitkovic, PhD, *Special Volunteer*

Elizabeth Schott, BS, *Postbacccalureate and Pre-Intramural Training Award Fellow*

Phosphatidylinositol 4,5-bisphosphate controls autophagosome-lysosome fusion.

Inositol phospholipids constitute a class of phospholipids that are present in tiny amounts but that have very important regulatory functions, as they organize protein signaling complexes on specific membrane compartments. They are produced by phosphoinositide kinases that can phosphorylate specifically one of three positions of the inositol ring of phosphatidylinositol (PI). We studied the role of phosphatidylinositol 4-kinase alpha (PI4K2A) in autophagosome-lysosome fusion. Autophagy is an important cellular process that helps clear damaged organelles and also allows cells to recycle useful nutrients from degraded organelles during starvation. In earlier studies we found that PI4K2A interacted with the GABARAP protein, one of a family of proteins that are important for autophagy. Our observation was confirmed in a subsequent study by the

Albanesi group, which also showed that PI4K2A was important for the acidification of autophagic vesicles. Therefore, we generated HEK293 cells (human embryonic kidney tissue culture cells) with inactivated PI4K2A using CRISPR/Cas9 gene editing and studied their properties, including those important for autophagy. We found increased number of LC3 (a widely used marker of autophagy)–positive vesicles that failed to acidify in the PI4K2A knockout (KO) cells. We determined the distribution of phosphatidylinositol 4-phosphate (PI4P) along the endosomal network by introducing a novel bioluminescence resonance energy transfer (BRET)–based assay, which permitted the quantification of levels of PI4P within various Rab–positive endosomes (phosphatidylinositol 4,5-bisphosphate controls autophagosome-lysosome fusion by activating cycling of the GTPase Rab7). The analysis showed that the levels of PI4P are highest in Rab7–positive endosomes and are kept very low in Rab4, Rab5, and Rab11 compartments. PI4K2A was responsible for 80% of the PI4P produced in the Rab7 compartment, with the rest produced by PI4K2B. Conversion of PI4P to PI(4,5)P2 caused the inactivation of Rab7 and release of PLEKHM1, an important adapter protein linking autophagic vesicles with lysosomes, from the Rab7 compartment. We also found that PI4P in the Rab7 compartment was converted to PI(4,5)P2 by endogenous PIP5Kgamma in wild-type, but not in PI4K2A KO cells and that PIP5Kgamma knock-down in parental HEK293 reproduced the effects of PI4K2A inactivation by inhibiting autophagic vesicle acidification. These data suggested a sequence of events by which PI(4,5)P2 generation from the PI4P produced by PI4K2A in Rab7–positive endosomes caused Rab7 inactivation, presumably by activating one or more Rab7 GTPase–activating (GAP) protein(s). This chain of events contributes to the cycling of some Rab7 effectors, including PLEKHM1, which is necessary for the fusion of lysosomes with autophagosomes. The importance of these studies is that they reveal a hitherto unknown role of PI4P to PI(4,5)P2 conversion in the control of Rab7 activation state, thus serving as a regulator of trafficking decisions in the endocytic pathway.

Identification of chemical inhibitors of phosphatidylinositol 4-kinase type II alpha

In light of the pivotal role of PI4P in virus replication and the role of type II PI4Ks in endosomal functions, we wanted to identify small-molecule inhibitors for the PI4K2A enzyme. To date, pharmaceutical companies have focused exclusively on inhibitors for type III PI4Ks, such as PI4KA and PI4KB, but no efforts have been devoted by the industry to identifying inhibitors of type II PI4Ks. Part of the reason is that type II PI4Ks represent a different class of lipid kinases, which are resistant to wortmannin and to all known PI3K inhibitors, whereas the type III PI4Ks are sensitive to some PI3K inhibitors, thus providing chemical scaffolds that companies could modify to enhance their selectivity and potency to type III PI4Ks. We developed a PI4K activity assay for a small format suitable for high-throughput screening (HTS) using a bacterially expressed human PI4K2A enzyme produced by Evžen Boura's group. A high throughput screening was then performed by our collaborators Marc Ferrer and Juan Marugan with about 400,000 compounds from small molecule diversity collections. Sytravon (library of novel small molecules), NPC (NCGC Pharmaceutical Collection of approved and investigational drugs) and MLPCN (NIH's Molecular Libraries Probe Production Centers Network of thousands of small molecules) collections were screened at top two doses (76 & 15 μM final concentration). Based on the screening results, we identified over 580 compounds with over 50% inhibitory activity. The compounds were re-tested at seven doses in 1:3 serial dilution to confirm their activity. Further, they were counter-screened with ADP-Glo™ reagents (a luminescent ADP detection assay) in the absence of the enzyme to exclude artificially luminescent compounds, and with PI4KB, another structurally unrelated PI4K. The list was further narrowed by eliminating compounds that were known inhibitors of protein kinases or were deemed structurally unsuitable for further development, yielding 14 inhibitors shortlisted for further studies. We then tested the 14 inhibitors in a cellular assay using the BRET method to monitor PI4P levels in Rab7 endosomes that require PI4K2A. Two inhibitors

were then found to have an inhibitory effect on PI4K2A in the cell with an IC_{50} of about 30 μM. The two lead compounds (code named NC03 and NC02) were then modified by NCATS (NC03) and by Radim Nencka, our medicinal chemist collaborator (NC02) to increase the potency and cellular availability. Unfortunately, these significant efforts did not yield any improvement over the lead compounds. Since then, a new screen was performed, and testing is under way to improve the newly identified lead compounds.

Reversible oxidation of the PtdIns(4)P phosphatase Sac1 by H_2O_2

In a collaborative project with the group of Sue Goo Rhee and Dongmin Kang, the effects of reactive oxygen radicals were examined on the Sac1 phosphatase enzyme. Sac1 dephosphorylates PI4P in the endoplasmic reticulum (ER) and plays crucial roles in the control of non-vesicular lipid transport driven by PI4P gradients between various organelles and the ER. We found that hydrogen peroxide (H_2O_2) has a profound effect on the cellular level of PI4P, which was most prominent in the Golgi and Rab7–positive endosomes, but also manifested in a PI4P increase in the plasma membrane (PM). Upon H_2O_2 exposure, Sac1 undergoes reversible inactivation in mammalian cells owing to the oxidation of its catalytic Cys389 residue, which then forms an intramolecular disulfide with Cys392. The Korean group also showed that this oxidation process also takes place during stimulation of cells by EGF and that Duox (dual oxidase) enzymes are responsible for the endogenous H_2O_2 production under these conditions. The findings revealed an important regulation of the Sac1 phosphatase by reversible oxidation, thereby controlling both the signaling function of PI4P and its effectiveness in driving lipid transport at membrane contact sites.

Additional Funding

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Structural and Chemical Biology of Membrane Proteins

Molecular mechanism of posttranslational protein lipidation by DHHC palmitoyltransferases

Posttranslational modifications greatly expand the structural, chemical, and functional diversity of the proteome. Of these, protein lipidation, which collectively refers to covalent modification of proteins by lipids, constitutes a centrally important class of posttranslational modification. Protein palmitoylation, or more generally, protein *S*-acylation is a specific form of protein lipidation whereby long-chain, typically C16, fatty acids become covalently attached to internal cysteines through a thioester linkage. Palmitoylation is one of the most pervasive and physiologically important posttranslational modifications, and the targets of palmitoylation span a very wide range of proteins, ranging from ion channels to cell-surface receptors, neuronal scaffolding proteins, and small GTPases. The repertoire of palmitoylated proteins has expanded rapidly in recent years, with thousands of proteins now known to be part of the cellular "palmitoylome." The physicochemical 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. This makes *S*-acylation one of the few dynamic posttranslational modifications and unique among different forms of protein lipidation. The physiological effects of *S*-acylation are diverse and have critical cellular importance; for example, 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–palmitoyl acyltransferase (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

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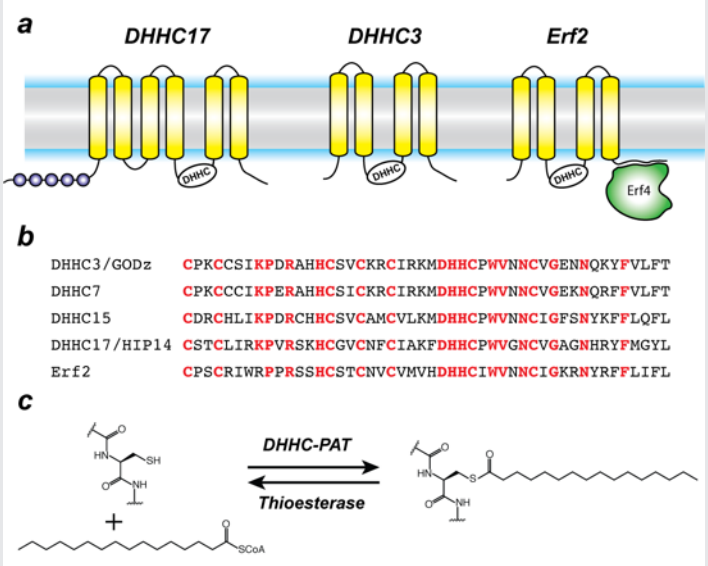


FIGURE 1. Organization and properties of DHHC palmitoyltransferases (PATs)

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).

compartments. Humans have 23 DHHC-PATs encoded in their genome. Beyond the shared DHHC domain, DHHC-PATs vary considerably; some possess ankyrin repeats (structural protein motifs that mediate protein-protein 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 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 DHHC-PAT 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 neuropsychiatric disorders such as Huntington's disease and various forms of cancer. Recently, it was shown that DHHC20 palmitoylates epidermal growth factor (EGFR) and is thus a potential therapeutic target for a wide range of cancers. More recently, DHHC3 has been proposed as a target for cancer treatment owing to its activity as the palmitoyltransferase for programmed-death ligand 1(PD-L1). However, despite their importance across a broad spectrum of biological pathways and their biomedical importance, very little was known about the molecular mechanism of DHHC palmitoyltransferases when we started working on this family. Nothing was known about their structural organization or how they interact with substrates and the fatty acyl coenzyme A (CoA), which serves as the acyl donor.

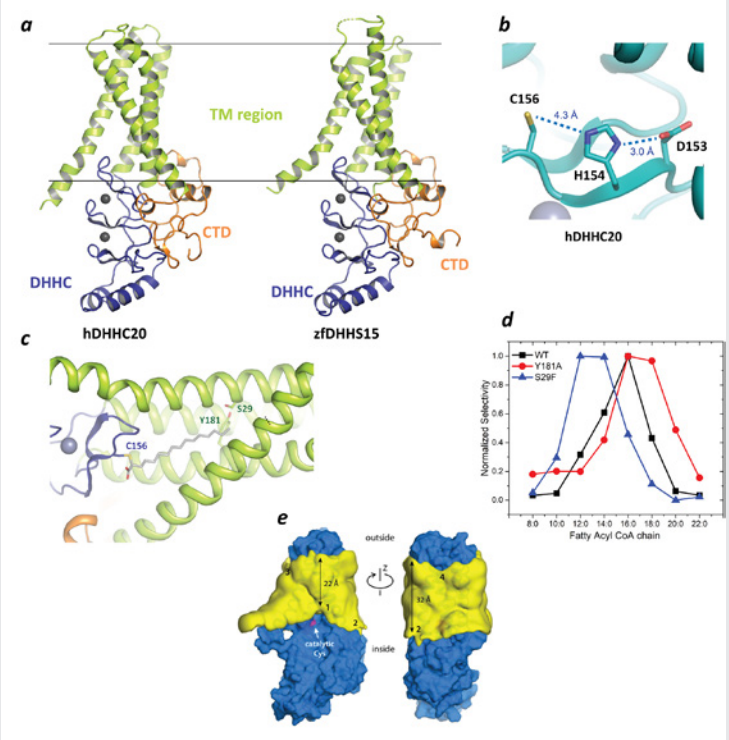
In a major breakthrough in this field, we solved the high-resolution crystal structures of two members of the DHHC family, human DHHC20 and zebrafish DHHC15 (Figure 2a). They are the first structures of any member of this family to be characterized and reveal a tepee-like transmembrane domain organization, which splays apart towards the cytoplasmic side and harbors the active site at the membrane-aqueous interfacial region

FIGURE 2. Structure, function and membrane deformation 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 Zn^{2+} 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 alpha-carbon 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.

e. 3D density maps for the POPC alkyl-chain core (*yellow surface*) near the protein surface (*blue*), calculated from the molecular dynamics (MD) trajectories. The catalytic cysteine (Cys156) is highlighted (*magenta*). The four regions where the membrane is deformed are indicated. The approximate minimum and maximum widths of the alkyl-chain core of the bilayer are also indicated, showing that the maximum deformation is around the active site.

(Figure 2b), thus readily explaining why membrane-proximal cysteines are palmitoylated. 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 2c). 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 2d). 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.

In the past year, we focused on several aspects of DHHC function, particularly the mechanism of recognition of acyl CoA in the membrane. Starting from our high-resolution structure and using molecular dynamics

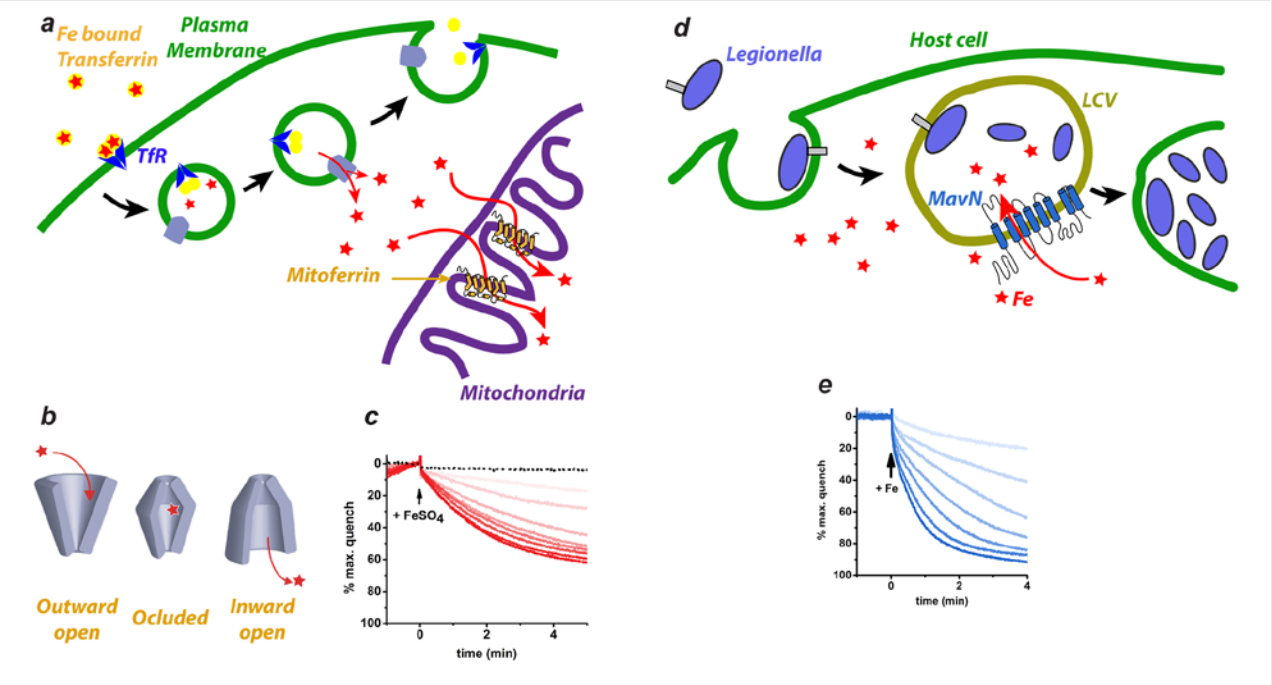


FIGURE 3. Iron transport by Mitoferrin and MavN

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 Mitoferrin1 homolog. 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.

d. Schematic showing *Legionella* entering a host cell and sequestering itself in a *Legionella*-containing vacuole (LCV). MavN is inserted into the membrane of the LCV and hijacks iron from the host cell.

e. Assay showing iron transport by protealiposome reconstituted MavN, using the identical assay as in *c*.

simulations and in collaboration with José Faraldo-Gómez, we showed that the protein surface of human DHHC20 severely distorts the membrane, which causes the active site to be exposed to the aqueous side of the membrane. Otherwise, the active site would have been buried in the hydrophobic part of the membrane and would have impaired catalysis, given that this kind of catalysis involves charge separation. We also determined a high-resolution structure of human DHHC20 in complex with an intact palmitoyl CoA. The structure represents the initial encounter complex when hDHHC20 binds to palmitoyl CoA and reveals how the polar CoA part of the fatty acyl CoA is recognized by hDHHC20. Mutation of residues involved in binding to CoA impair catalysis, thus lending credence to our structural model.

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

In metazoans, Wnt proteins regulate many processes, including cellular growth, differentiation, and tissue homeostasis, through the highly conserved Wnt signaling pathway. Porcupine is an endoplasmic reticulum (ER)–resident integral membrane enzyme that catalyzes posttranslational 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 of 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, the 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.

Molecular mechanism of iron transport across cellular membranes

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 3a and 3b). Mitoferrin-1 (Mfrn1) is expressed mainly in erythroid cells, while mitoferrin-2 is expressed ubiquitously. Knockout of Mfrn1 is embryonically lethal, reflecting the importance of mitoferrins in vertebrate physiology.

Mfrn1 and Mfrn2 were discovered more than 10 years ago. However, the proposed iron transport activity had not been demonstrated using an *in vitro* functional reconstitution assay. Also no report about their interaction with iron or other related metal ions existed, most likely because heterologous overexpression and purification of mitoferrins were not reported in the literature. We carried out heterologous purification and *in vitro* functional reconstitution and mutational dissection of a vertebrate Mfrn1 (Figure 3c). 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 provided 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.

In the past year, we used our *in vitro* proteoliposome-reconstituted iron transport assay, the first such assay to be reported in the literature, to dissect the iron transport activity of MavN, another proposed iron transporter, in the bacterial pathogen *Legionella pneumophila*. *Legionella* is an intracellular pathogen that enters the host cell and

sequesters itself in the Legionella-Containing Vacuole (LCV), where it survives and proliferates. This is achieved by secreting hundreds of so-called "effector proteins," which are secreted through the Type IV secretion system (T4SS) into the host cell. Of the hundreds of effector proteins, there are seven core effector proteins and MavN is one of them. MavN is inserted into the membrane of the LCV. Using genetic and cell-based studies, the laboratory of Ralph Isberg proposed that MavN is an iron transporter. We showed by heterologous purification and protealiposome reconstitution that MavN is indeed an iron transporter. Mutations in MavN that impaired iron transport *in vitro* also impaired *Legionella* growth in a cell-based assay, pointing to the importance of MavN for *Legionella*.

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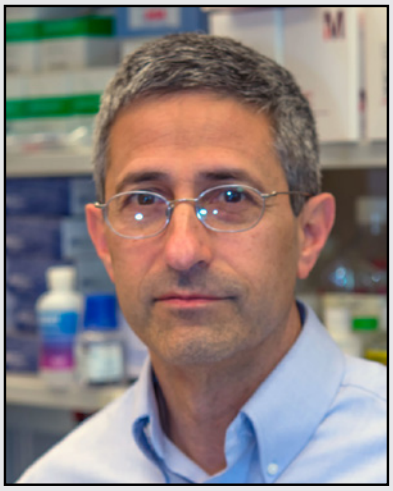
Regulation of Childhood Growth

Children grow taller because their bones grow longer. The bone elongation occurs at the growth plate, a thin layer of cartilage found near the ends of juvenile bones. In the growth plates, new cartilage is produced through chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis, and then the 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 further clinical abnormalities.

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



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Novel genetic causes of childhood growth disorders

For many children who are brought to medical attention for linear growth disorders, clinical, laboratory, and genetic evaluation fails 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.

To discover new genetic causes of childhood growth disorders, we invite families with monogenic growth disorders to the NIH Clinical Center, where we evaluate the clinical, biochemical, and radiological

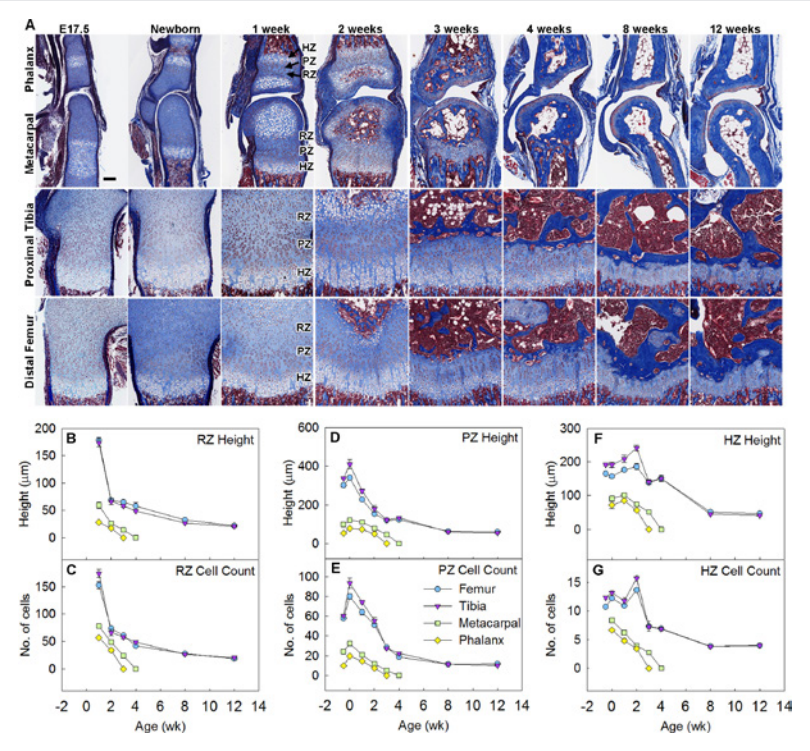

FIGURE 1. Growth plate senescence is more advanced in shorter bones than in longer bones.

Masson Trichrome–stained histological sections of proximal tibias, distal femurs, distal metacarpals, and proximal forelimb phalanges from C57BL/6 mice at various ages. Cartilage matrix stains light blue, bone matrix dark blue. Epiphyseal fusion (disappearance of growth plate) occurs at approximately 3 weeks in phalanges and 4 weeks in metacarpals but has not yet occurred at 12 weeks in tibias or femurs. Scale bar, 100 μm. (*B*–*G*) Quantitative histological measurements of RZ height (*panel B*) and cell count (*panel C*), PZ height (*panel D*) and cell count per column (*panel E*), HZ height (*panel F*) and cell count per column (*panel G*), in each of the 4 growth plates at various ages. HZ: hypertrophic zone; PZ: proliferative zone; RZ: resting zone.

features of the condition. We then obtain DNA samples from informative family members and use powerful genetic approaches, including SNP 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.

Recently, such analyses showed that mutations in a gene called *QRICH1* impairs growth at the growth plates, causing short stature [Reference 1]. We studied a child with short stature, irregular growth plates of the proximal phalanges, developmental delay, and mildly dysmorphic facial features. Exome sequencing identified a *de novo*, heterozygous, nonsense mutation in *QRICH1*. Our *in vitro* studies confirmed that the mutation impaired expression of the QRICH1 protein. Knockdown of *Qrich1* in primary mouse epiphyseal chondrocytes mediated by siRNA caused downregulation of gene expression associated with hypertrophic differentiation, a step that is critical for bone elongation. We then identified an unrelated individual with another heterozygous *de novo* nonsense mutation in *QRICH1*, who had a similar phenotype. A recently published study identified *QRICH1* mutations in three patients with developmental delay, one of whom had short stature. Our findings indicate that *QRICH1* mutations cause not only developmental delay but also a chondrodysplasia characterized by diminished linear growth and abnormal growth plate morphology resulting from impaired growth plate chondrocyte hypertrophic differentiation [Reference 1].

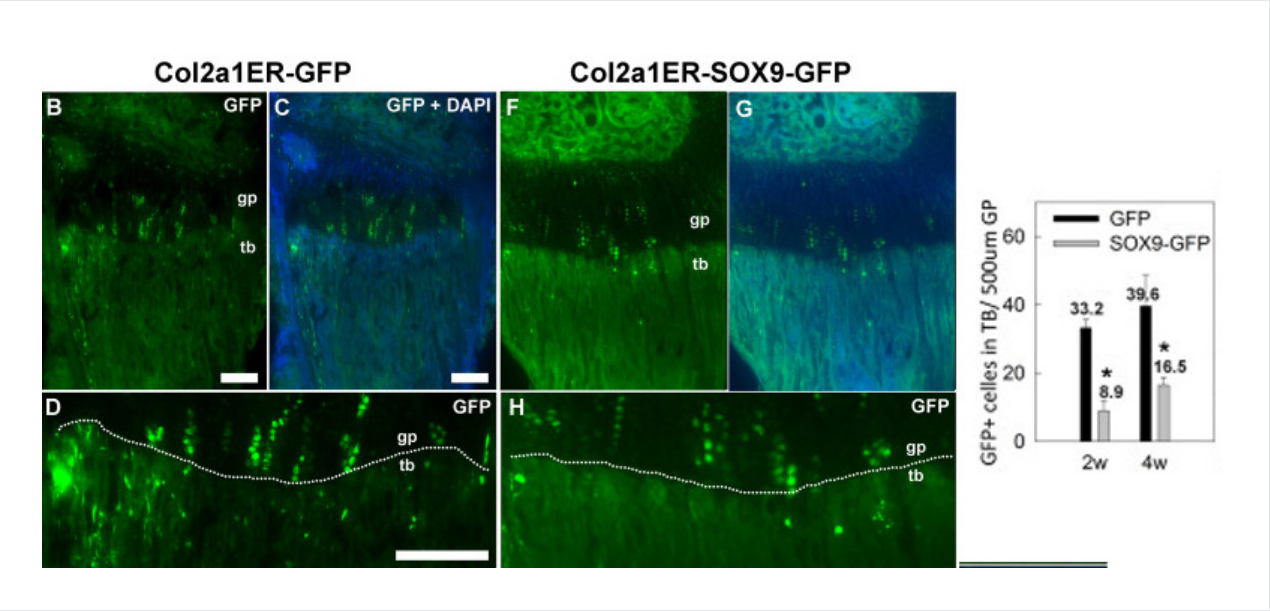

FIGURE 2. Transgenic *Sox9* expression suppressed transition of chondrocytes into trabecular bone.

Lineage-tracing experiments were performed by crossing mice with tamoxifen-inducible cre expression driven by a collagen 2 promoter (Col2a1ER-cre) with either the transgenic *Sox9*-GFP mice or transgenic mice expressing only GFP. Fluorescent microscopy (*B–G* lower magnification, *D–H* higher magnification) showed that GFP–positive chondrocyte columns were present in the growth plate (gp, area above the white dotted line) in both Col2a1ER-GFP and Col2a1ER-Sox9-GFP mice. However, in mice with *Sox9* misexpression, there were significantly fewer GFP–positive cells in the metaphyseal trabecular bone (tb, area below the white dotted line) at both 2 and 4 weeks of age (graph). *, P<0.05, ANOVA.

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

Our group also studies the fundamental mechanisms governing skeletal growth. Recently, we focused on why bones at different anatomical locations vary dramatically in size [Reference 2]. For example, human femurs are 20-fold longer than the phalanges in the fingers and toes. The mechanisms responsible for such size differences are poorly understood. Bone elongation occurs at the growth plates and advances rapidly in early life but then progressively slows as a consequence of a developmental program termed growth plate senescence. This developmental program includes declines in cell proliferation and hypertrophy, depletion of cells in all growth plate zones, and extensive underlying changes in the expression of growth-regulating genes. We found evidence that these functional, structural, and molecular senescent changes occur earlier in the growth plates of smaller bones (metacarpals, phalanges) than in the growth plates of larger bones (femurs, tibias), and that such differential aging contributes to the disparities in bone length (Figure 1). We also found evidence that the molecular mechanisms that underlie the differential aging between different bones involve modulation of critical paracrine regulatory pathways, including Igf, Bmp, and Wnt signaling. Taken together, the findings reveal that the striking disparities in lengths of different bones, which characterize normal mammalian skeletal proportions, are achieved in part by modulating the progression of growth-plate senescence [Reference 2].

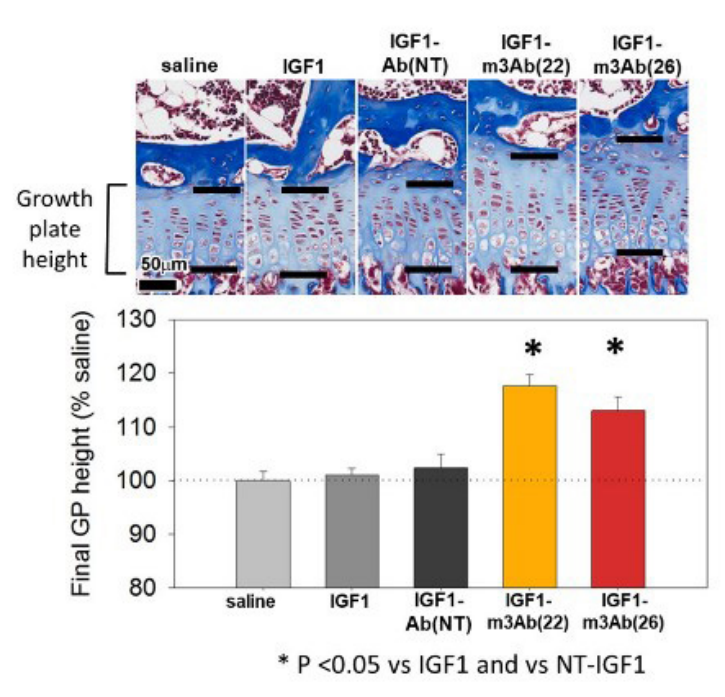

FIGURE 3. Effect of IGF-1 on growth plate height in lit (GH–deficient) mice

Treatment with fusion proteins that combine IGF-1 with cartilage-targeting fragments [IGF1-m3Ab(22), IGF1-m3Ab(26)] increased growth plate height, whereas neither IGF-1 itself nor a nontargeted fusion protein [IGF1-Ab(NT)] had an effect. *p < 0.05.

We also explored the transdifferentiation of growth plate chondrocytes into osteoblasts [Reference 3]. In the postnatal growth plate, as hypertrophic chondrocytes approach the chondro-osseous junction, they may undergo apoptosis or directly transdifferentiate into osteoblasts. The molecular mechanisms governing the switch in cell lineage are poorly understood. We found that, in hypertrophic chondrocytes, the physiological downregulation of *Sox9* (a transcription factor critical for chondrogenesis) is associated with upregulation of osteoblast-associated genes (such as *Mmp13*, *Col1a1*, *Ibsp*), before the chondrocytes enter the metaphyseal bone. In transgenic mice that continued to express *Sox9* in all cells derived from the chondrocytic lineage, upregulation of these osteoblast-associated genes in the hypertrophic zone failed to occur. Furthermore, lineage tracing experiments showed that, in transgenic mice expressing *Sox9*, the number of chondrocytes transdifferentiating into osteoblasts was markedly reduced (Figure 2). Collectively, our findings suggest that *Sox9* downregulation in hypertrophic chondrocytes promotes expression of osteoblast-associated genes in hypertrophic chondrocytes and promotes the subsequent transdifferentiation of these cells into osteoblasts [Reference 3].

New treatment approaches for growth plate disorders

Currently, treatment approaches for linear growth disorders are limited. Recombinant human growth hormone (GH) is used for both GH deficiency and certain causes of short stature not attributable to GH deficiency. However, the efficacy of GH treatment is often suboptimal in severe, non–GH deficient conditions such as skeletal dysplasias. Consequently, the conditions for which treatment is most needed, such as achondroplasia, are often the conditions for which GH is least effective. Because GH has limited efficacy for severe disease and significant known and potential adverse effects, better treatments for growth disorders are needed.

In addition to GH, there are also many paracrine factors that positively regulate growth-plate chondrogenesis and might therefore be used therapeutically. The development of these paracrine factors into effective treatment has been hampered by their mechanism of action; the growth factors are produced locally and act

locally in the growth plate and thus do not lend themselves to systemic therapeutic approaches. We envision that these locally acting molecules could be targeted to the growth plate by linking them to cartilage-binding proteins, such as antibody fragments. When administered systemically, such hybrid molecules would be preferentially taken up by growth plate cartilage and thus might greatly augment the therapeutic effect on the target organ while diminishing adverse effects due to action on other tissues. Similarly, growth-promoting endocrine factors, such as GH and IGF-I, might be targeted to the growth plate to enhance the therapeutic effects on chondrogenesis and reduce adverse effects on nontarget tissues.

For this purpose, we developed cartilage-targeting single-chain human antibody fragments and then created fusion proteins of such antibody fragments, combined with insulin-like growth factor 1 (IGF-1), an endocrine/paracrine factor that positively regulates chondrogenesis [Reference 4]. The fusion proteins retained both cartilage-binding and IGF-1 biological activity and were able to stimulate bone growth in an organ culture system. Using a growth hormone–deficient mouse model, we found evidence that subcutaneous injections of the fusion proteins had greater on-target efficacy at the growth plate (Figure 3) and less off-target effect than IGF-1 alone. Our findings provide proof-of-principle that targeting therapeutics to growth plate cartilage has the potential to improve treatment for childhood growth disorders [Reference 4].

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2. Lui JC, Jee YH, Garrison P, Iben JR, Yue S, Ad M, Nguyen Q, Kikani B, Wakabayashi Y, Baron J. Differential aging of growth plate cartilage underlies differences in bone length and thus helps determine skeletal proportions. *PLoS Biol* 2018 23:16(7):e2005263.
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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 behavior 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 also 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 understanding and 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, which is intended to generate measurements and maps of intrinsic physical quantities, including diffusivities, relaxivities, or exchange rates, rather than the qualitative stains and images conventionally used in radiology. At a basic level, the 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, as well as insights gleaned from our tissue-sciences research to discover and develop novel imaging biomarkers that can detect changes in tissue composition, microstructure, or microdynamics more sensitively and specifically. The ultimate translational goal is to be able 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 applications critical to NICHD's mission; it

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(continued)

is noninvasive, nonionizing, 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 has been to transform clinical MRI scanners into scientific instruments capable of producing reproducible, highly accurate, and precise imaging data with which to measure and map useful imaging quantities for various clinical applications, including single scans, longitudinal and multi-site studies, personalized medicine, and genotype/phenotype correlation studies, as well as for populating imaging databases with high-quality normative data. We also develop our various MRI tools and methodologies to advance the field of neuroscience, providing means to explore brain structure and function.

Michal Komlosh, PhD, *Collaborating Scientist funded via the Henry Jackson Foundation and the Center for Neuroscience and Regenerative Medicine*

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In vivo MRI histology

The most mature *in vivo* MRI 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 non-invasive 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 direction–encoded color (DEC) maps to display the orientation of the main association, projection, and commissural white matter pathways in the brain. To assess anatomical connectivity among various cortical and deep-brain gray-matter areas, we also proposed and developed DTI "Streamline" Tractography. 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 and map. We also developed a family of diffusion MRI methods to 'drill down into the voxel' and measure features such as average axon diameter (AAD) and axon-diameter distribution (ADD) within 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

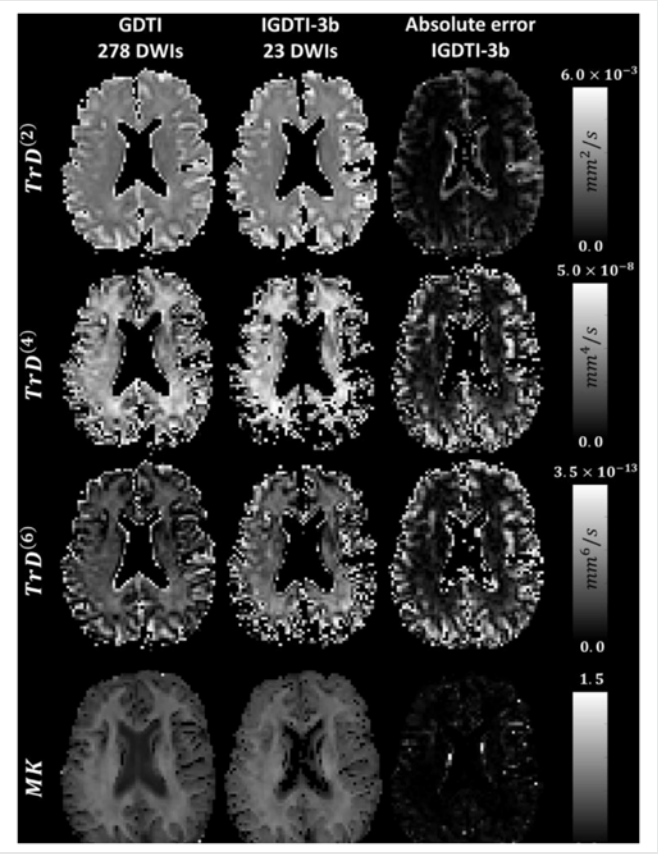

FIGURE 1. New isotropically weighted diffusion MRI method shows novel anatomical features in living human brain.

Comparison of orientationally averaged Trace of diffusion tensors, $TrD^{(n)}$, for tensors of orders 2, 4, and 6, and mean t-kurtosis, $\bar{W}$ measured *in vivo* with Generalized Diffusion Tensor Imaging (GDTI) and Isotropic Generalized Diffusion Tensor Imaging (IGDTI) with 3 b-values. Figure shows the ability of IGDTI to quantify rotation-invariant higher-order tensor (HOT)–derived diffusion parameters in brain tissue within a clinically feasible scan duration.

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 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. We pioneered several promising two-dimensional MRI relaxometry, exchange, and diffusometry-based methods to study water mobility and exchange in gray and white matter. We believe these will help identify changes in normal and abnormal development, as well as inflammation and trauma.

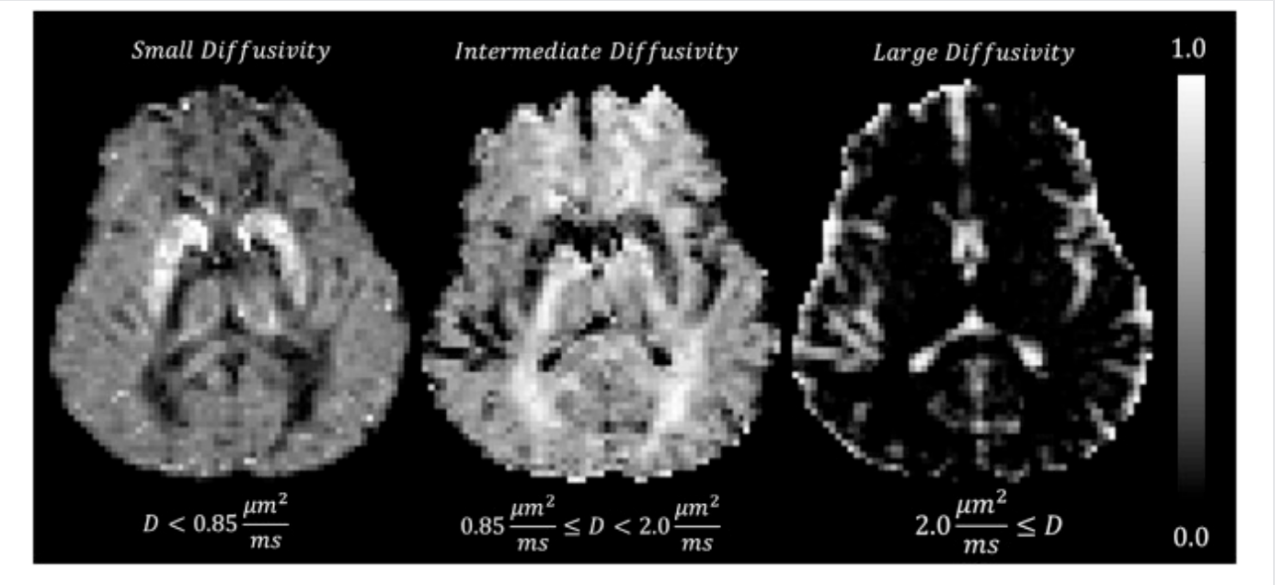

FIGURE 2. Spectra of mean water diffusivities within living human brain

Low, intermediate, and large diffusivity signal components in normalized mean diffusion distributions (MDDs) measured in live human brain. Subcortical gray matter, in particular the putamen, the globus pallidus, the caudate nucleus, and, to a lesser extent the thalamus, contain mostly low diffusivity components ≤ 0.85 μm^2/ms. Brain regions containing primary white matter (WM) pathways are dominated by intermediate diffusivity components in the range of 0.85−2.0 μm^2/ms. Peaks in grey matter (GM) contain significant signal fractions of small and intermediate mean diffusivities. As expected, the largest diffusivity values are observed mainly in regions of the CSF (cerebrospinal fluid). This approach allows us to cleanly cluster different tissue types and assess the spectrum of mean water diffusivities within them.

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 still lacks the quantitative character of CT. 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. It is also prone to more acquisition and imaging artifacts. 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 benefited greatly from the introduction of "quantitative" clinical MRI techniques, with which one obtains maps of meaningful intrinsic physical quantities or chemical variables, which can be measured in physical units and compared among different tissue regions, in individual subjects, and within 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 obtained from a healthy population. Quantitative MRI methods

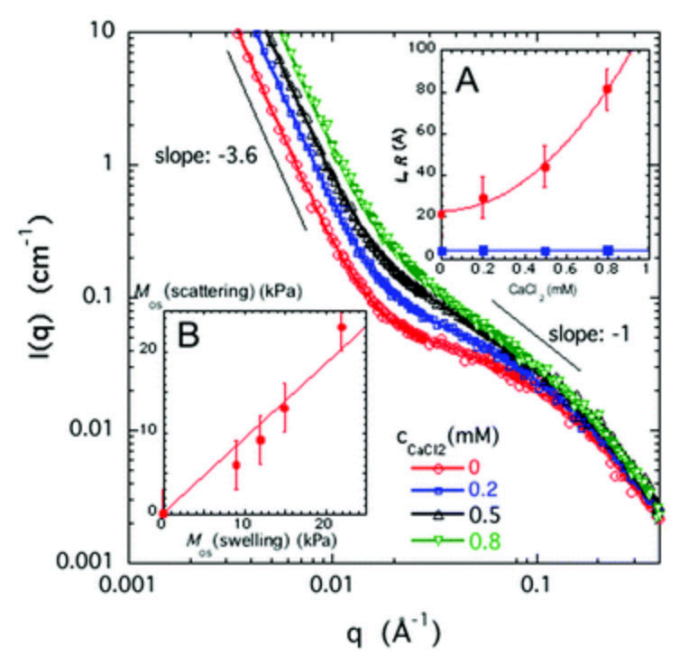

FIGURE 3. Remarkable correspondence between load bearing ability of polyelectrolyte gels measure by Small Angle Neutron Scattering and Osmotic Stress techniquesn

Small-angle neutron scattering profiles of sodium polyacrylate (NaPA) gels (φ = 0.04) swollen in 40 mM NaCl containing increasing amounts of $CaCl_2$ as indicated in the figure. Inset (*A*): Variation of the correlation length L (*red dots*) and the cross-sectional radius, R (*blue squares*) as a function of the $CaCl_2$ concentration. SANS provides a powerful high-resolution method to probe molecular structure over a large range of length scales. Inset (*B*): Osmotic modulus, M_{os}(scattering) vs. osmotic modulus M_{os}(swelling) for NaPA gels. Notice the excellent agreement between the swelling and scattering measurements.

should continue to advance "precision imaging" studies, in which MRI phenotypic and genotypic data can be meaningfully melded and used for improved diagnosis and prognosis assessments.

To date, we have developed algorithms that generate a continuous, smooth approximation to 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 the tensor-valued random variables that we use in designing optimal DTI experiments and interpreting their results. In tandem, we developed nonparametric 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 nonparametric 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 the Kullback-Leibler divergence. However, much work remains to be done in order to address and remedy MRI artifacts so as to permit one to draw statistically significant inferences from clinical DTI data, obtained in longitudinal and multi-center studies, and particularly in single-subject studies.

We carry 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 the NICHD, NIMH, NINDS, and NIDA. This multi-center consortium, initiated in 1998, was intended to advance our understanding of normal brain development in typical healthy children and adolescents. The *Brain Development Cooperative Group*, created by this funding mechanism, is still active, publishing numerous papers

each year, primarily by mining these rich high-quality data, many of which we processed, vetted, and uploaded. We served as the DTI Data-Processing Center (DPC) in this interdisciplinary project. The processed DTI data collected from this project were uploaded into a database accessible to interested investigators, and made publicly available through the *National Database for Autism Research (NDAR)*. In collaboration with Carlo Pierpaoli, we continue to support and help update and disseminate the processing and analysis software that grew out of this effort, 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 and in partnership with various Department of Defense (DoD) affiliates. TBI is an acute problem in the pediatric population, but it also affects and afflicts young men and women in the military. Diffusion MRI provides essential information to aid in the assessment of TBI. Because of the subtlety of changes seen in TBI, quantitative imaging protocols are essential, as are the development of advanced imaging methods. To this end, we developed a DTI data–processing pipeline in order to improve the accuracy and reproducibility of MAP–MRI findings, and, in collaboration with scientists at the DoD Center for Neuroscience and Regenerative Medicine (CNRM), performed the first normative MAP–MRI studies, as well as applied this new and powerful method to individuals with mild and moderate TBI. To enable this application, we built on our existing state-of-the-art TORTOISE pipeline to include and incorporate MAP–MRI data. We are continuing to develop and use novel MRI phantoms, such as our polyvinyl pyrrolidone (PVP) polymer and Glass Capillary Array (GCA) MRI phantoms that we developed and are disseminating to improve the performance of advanced diffusion MRI methods.

In collaborative research fostered by our dynamic Affinity Group in the area of fetal MRI, an in collaboration with Roberto Romero, we are exploring ways to "freeze" the motion of fetal brains as the fetus moves throughout the scan. We are also developing approaches to characterize microstructure and function, initially in the fetal brain, but eventually within the entire placenta.

Biopolymer physics: water–ion–biopolymer interactions

Water-ion-biopolymer interactions play a key role in the physical-chemical interactions among tissue components. Our primary objective is to understand the forces that govern these interactions, ranging from the movement of water and ions across channels, to the self-assembly of proteins into nanofibers in neuro-degenerative processes, such as Creutzfeldt-Jakob disease, Alzheimer's disease, and chronic traumatic encephalopathy (CTE). Little is understood about the physical underpinnings of these interactions, which underlie a myriad of biological processes and behaviors.

To determine the effect of ions on the structure and dynamics of biopolymers, we developed a multiscale experimental framework by combining macroscopic techniques (osmotic swelling pressure measurements, mechanical measurements) with scattering methods (e.g., SANS and SAXS), which probe the structure and interactions over a broad range of length and time scales. Macroscopic swelling pressure measurements provide information on the overall thermodynamic response of the system, while SANS and SAXS allow us to investigate biopolymers at the molecular and supramolecular length scales and to quantify the effect of changes in the environment (e.g., ion concentration, ion valance, pH, temperature) on the structure and interactions among biopolymers, water, and ions. Studies made on well-defined model systems that mimic certain essential features of tissue provide important insights that cannot be obtained from experimental studies made on biological systems. Mathematical models based on well-established polymer physics concepts makes it

possible to design and analyze fundamental experiments, which quantify and explain tissue behavior. We are now combining molecular dynamics simulations with cutting-edge experimental studies to gain insight into the underlying molecular mechanism that governs key aspects of tissue structure-function relationships.

Better understanding of the structure and interactions among tissue components is also necessary to design and develop tissue models and novel tissue phantoms that mimic tissue behavior. Biomimetic phantoms with well-controlled physical properties (osmotic, mechanical, relaxation, etc.) are critically important in quantitative MRI to validate measurements and advanced MRI applications from bench to bedside (*in vivo* MRI histology). We produced novel diffusion MRI phantoms, which 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 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 has ideal properties for this demanding application. 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 *http://hpd-online.com/diffusion-phantom.php*. We developed a variety of NMR and MRI phantoms that possess various salient features of cell or tissue systems, providing 'ground truth' to test the validity of our models and experimental designs.

Measuring and mapping functional properties of extracellular matrix

Extracellular matrix (ECM) is present in every tissue and performs a key role in determining normal and abnormal tissue and organ function. We study interactions among the main ECM components using cartilage as a model system. In cartilage ECM, the most abundant macromolecule is collagen (type II), which is organized into fiber bundles that form a meshwork. The major proteoglycan (PG) is the bottlebrush-shaped aggrecan. Intracellularly synthesized aggrecan molecules are secreted into the ECM, where they aggregate to form a secondary bottlebrush with hyaluronic acid (HA), which is stabilized by a link protein. The biomechanical behavior of cartilage and other ECMs reflects their molecular composition and microstructure, which change during development, disease, degeneration, and aging. To determine 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). Understanding the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential to predicting cartilage load-bearing properties, which are mainly governed by osmotic and electrostatic forces. To quantify the effect of hydration on cartilage properties, we previously developed a tissue micro-osmometer to perform precise measurements in a rapid manner. The instrument is capable of determining very small changes in the amount of water absorbed by small tissue samples (less than 1 microgram) as a function of the equilibrium water activity (vapor pressure). We make osmotic pressure measurements to determine how the individual components of cartilage ECM (e.g., aggrecan, HA, and collagen) contribute to the total load-bearing capacity of the tissue. We demonstrated that aggrecan–HA aggregates self-assemble into microgels, contributing to improved dimensional stability and the tissue's lubricating ability. We also found that aggrecan is highly insensitive to changes in the ionic environment, particularly to the concentration of calcium ions, which is critically important to maintaining the tissue's mechanical integrity and to allowing aggrecan to serve as a calcium ion reservoir in cartilage and bone.

We developed 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 composite system,

PAA mimics the behavior of proteoglycan (i.e., HA–aggrecan complexes), while PVA mimics the behavior 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, etc.) on the macroscopic mechanical/swelling properties, and ultimately the origin of the load-bearing and lubricating ability of cartilage.

We are also using osmotic pressure measurements to determine the contributions of individual components of ECM to the total tissue swelling pressure. In collaboration with Emilios Dimitriadis, we developed a method for mapping the local elastic and osmotic properties of cartilage, using AFM together with the tissue micro-osmometer. Many of the impediments that previously hindered the use of AFM to probe inhomogeneous samples, particularly biological tissues, were addressed by this approach that utilizes the precise scanning capabilities of a commercial AFM to generate large volumes of compliance data, from which the relevant tissue elastic properties can be extracted. In conjunction with results obtained from high-resolution scattering measurements and biochemical analysis, the technique allows us to map the spatial variations in the osmotic modulus within tissue specimens and thus the compressive resistance of the tissue to external load.

We are attempting to translate this critical tissue-science understanding of the structure-function relationships of ECM components 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 diseases of cartilage and other tissue and organs, as well as to provide a means for following normal and abnormal development of the ECM, which entails making 'invisible' components of ECM, (e.g., collagen and PGs) 'visible' and then using our understanding of biopolymer interactions to predict functional properties of the tissue, such as its load-bearing ability. One major obstacle is that water molecules bound to immobile species (e.g., collagen) are largely invisible to conventional MRI approaches. However, magnetization exchange (MEX) 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 enables us to estimate the collagen content in tissue. In a pilot study with collaborators Uzi Eliav and Ed Mertz, we applied the new MEX MRI method to determine the concentration and distribution of the main macromolecular constituents in bovine femoral-head cartilage samples. The results obtained by the MEX MRI method are qualitatively consistent with those obtained by histological techniques, such as high-definition infrared (HDIRI) spectroscopic imaging. The work was originally supported by a DIR Director's Award that we received with our collaborators Sergey Leikin and Edward Mertz, and are continuing to pursue collaboratively. Our novel approach has the potential to map tissue structure and functional properties *in vivo* and noninvasively, so while it is "high-risk" it has the potential for a "high-reward."

Patents

1. Benjamini D, Basser PJ. Multi-dimensional spectroscopic NMR and MRI using marginal distributions. *USPTO Patent No. WO2018031942-A1* 2018;N/A.
2. Horkay F, Pierpaoli C, Basser PJ. Phantom for diffusion MRI imaging. *USPTO Patent No. 10,078,124* 2018;N/A.

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- "Characterizing brain microstructure in patients with mTBI using Mean Apparent Propagator (MAP) MRI." HJF Award Number: 308049-8.01-60855, (CNRM-89-3895), which is under the joint auspices of the NIH, DoD, CNRM, and USUHS.
- "Development of Bench and Pre-Clinical MRI Methods to Assess Glymphatic Clearance in the Living Brain." 308811-4.01-60855, (CNRM-89-9237), which is under the joint auspices of the NIH, DoD, CNRM, and USUHS.
- "In vivo Brain Network Latency Mapping." NIH BRAIN Initiative grant 1-R24-MH-109068-01
- “Connectome 2.0: Developing the next generation human MRI scanner for bridging studies of the micro-, meso- and macro-connectome”, NIH BRAIN Initiative-funded 1U01EB026996-01
- "MRI methods aimed at the detection of pathologies involving myelin, collagen and amyloid plaques" Bi-National Science Foundation (BSF) grant; 2013-2018, #2013253
- “Neuroradiology/Neuropathology Correlation/Integration Core”, 309698-4.01-65310, (CNRM-89-9921)
- “Localization of Epileptogenic Foci using Diffusion Weighted MRI”, Bench-to-Bedside (BtB) Award, NIH Director’s Challenge Innovation Grant, NIH IRP

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Biophysics of Large Membrane Channels

We study mitochondrial and bacterial membrane proteins that form "large" beta-barrel channels responsible for metabolite fluxes between cells and cellular compartments. Healthy cell functioning and development require effective communication between cells and cell organelles, which is facilitated by beta-barrel membrane channels. We are interested in the physical mechanisms that regulate such channels under normal and pathological conditions. Among many wet-lab approaches, such as fluorescence correlation spectroscopy, bilayer overtone analysis, and confocal microscopy, our hallmark method is to reconstitute channel-forming proteins into planar lipid membranes, which allows us to study them at the single-molecule level. Empirical findings obtained in these experiments are rationalized within a framework of a physical theory of channel-facilitated transport, which brings an understanding necessary to design new strategies to effectively correct the deviant interactions associated with disease.

In contrast to the highly ion-selective channels studied in neurophysiology, which have narrow selectivity filters to match the size of partially dehydrated ions, metabolite, or "nutrient," channels are significantly wider. Indeed, they have to accommodate metabolite molecules that are typically much larger than simple mono- or divalent ions. Because of their size and their primarily beta-barrel scaffolds, the mechanisms of the selectivity and gating of such channels are quite different from those of the ion-selective channels formed from alpha-helical subunits. To grasp the general principles of beta-barrel channel functioning and regulation, we work with a variety of proteins and channel-forming peptides.

The channel-forming proteins and peptides we work with include 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 *C. perfringens* (Ib) binary toxins, Epsilon toxin (from *C. perfringens*), OmpF (general bacterial porin from *Escherichia coli*), LamB (sugar-specific bacterial porin from *E. coli*), OprF (porin from *Pseudomonas aeruginosa*), MspA (major outer-membrane porin from *Mycobacterium smegmatis*), Alamethicin (amphiphilic peptide toxin from *Trichoderma viride*), Syringomycin E (lipopeptide toxin from *Pseudomonas syringae*), and the bacterial peptide TisB involved in

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persister cell formation. We also use gramicidin A (linear pentadecapeptide from *B. brevis*) as a molecular sensor of membrane mechanical properties. With the goal of studying the channel-forming proteins under controlled conditions, we first isolate them from the host organisms, purify them, and then reconstitute them into planar lipid bilayers, the model system with precisely defined physical properties. This allows us to explore channel interactions with the lipid membrane as modified by volatile anesthetics, cytosolic proteins, such as tubulin and alpha-synuclein, and newly synthesized drugs that act as blockers of the translocation pores of bacterial toxins. Our motivation is that learning the molecular mechanisms of channel functioning is vital for developing new approaches for the treatment of various diseases, for which regulation of transport through ion channels plays the key role.

Membrane association of alpha-synuclein domains studied using VDAC nanopore reveals an unexpected binding pattern.

The exceptional diversity of interactions between peripheral membrane proteins and bilayer lipid membranes makes the membrane surface a rich scene for cellular functions and their regulation. However, this diversity presents significant experimental barriers to the studies of interaction mechanisms among the components. One of the main challenges is that the energies of interaction between individual protein residues and lipid molecules are small; thus, statistical effects play a significant role. A variety of techniques to characterize binding of peripheral membrane proteins to liposome or planar lipid bilayer platforms have been developed, but it is increasingly clear that, except in the simplest of systems, no isolated technique yields a clear picture of the membrane binding process. In part, this is because these assays measure the average binding parameters of an ensemble of molecules, highlighting the need to characterize membrane-bound proteins at the single-molecule level.

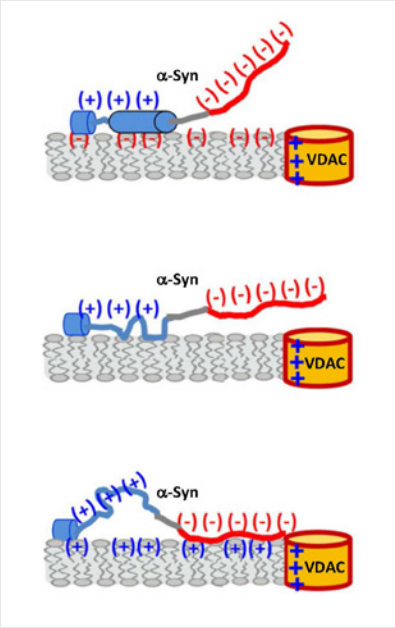


FIGURE 1. Membrane-catalyzed alpha-synuclein interaction with VDAC

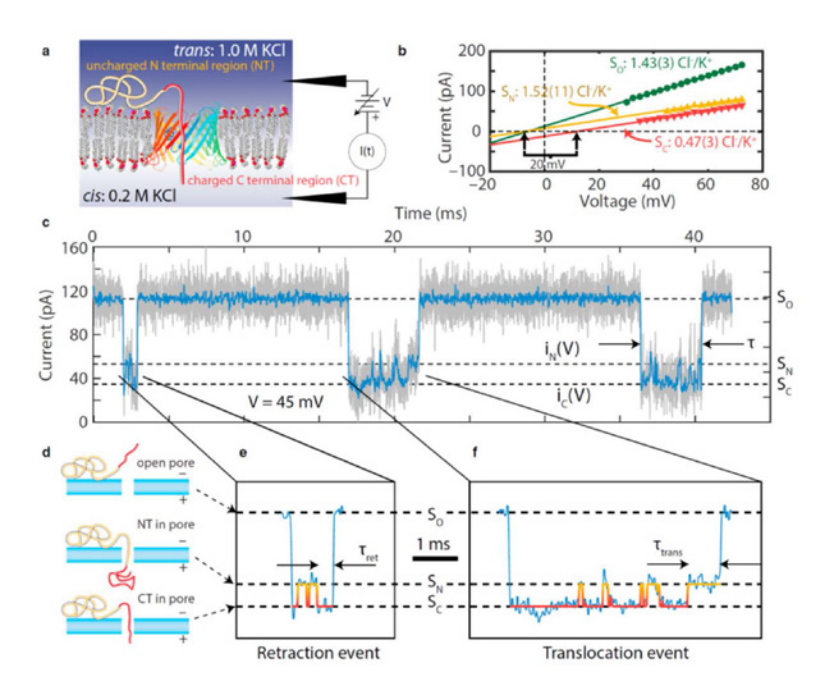
A tentative model qualitatively accounting for the electrostatic and hydrophobic interactions and conformational variability of α-syn on the membrane surface. The availability of the anionic C-terminal domain of α-syn for VDAC pore blockage depends on the membrane-lipid charge and salt concentration. Interaction of each α-syn domain with the membrane affects the molecule's overall membrane affinity, as well as the rate of capture of the C-terminal domain by the VDAC nanopore. The membrane-binding region is shown in blue (net 3 positive charges), nonpolar NAC (non-amyloid-β component) domain in grey, and the anionic C-terminal domain in red (net 15 negative charges). Anionic lipids enhance α-syn binding and promote the formation of two helical membrane-binding domains in the N-terminus, but they electrostatically repel the anionic C-terminal domain from the membrane surface, reducing its availability for the capture by the nanopore. For zwitterionic, and perhaps cationic lipids, a more random configuration of the N-terminus can be expected, resulting in multiple α-syn conformations on the membrane surface.

FIGURE 2. Watching translocation of single proteins

Salt concentration gradient–enhanced observation of α-syn dynamics in a VDAC nanopore.

a. Experimental setup (not to scale). The "diblock-copolymer"–like structure of α-syn consists of two "selectivity tags" of differing charge density (light yellow and dark red) that modulate the electrical properties of the VDAC nanopore differently under a salt concentration gradient.

b. Current-voltage curves of the open pore high-conducting (S_O, dark green circles) and the two low-conducting substates when α-syn is inside the pore (S_N, yellow triangles and S_C, red inverted triangles); selectivities were calculated from the reversal potentials (vertical arrows).



c. Sample current record; as-recorded data are shown in light gray and software-filtered data in dark blue.

d. Identification of the substates by noting that the capture of the C-terminus in the nanopore corresponds to the lower-conducting substate.

e. and *f.* Details of a retraction (*e*) and a translocation (*f*) event.

This year, we used the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane as a single-molecule probe for alpha-synuclein (α-syn), a protein of considerable clinical interest owing to its well-established involvement in the pathology of Parkinson's disease. It is well-established that α-syn binding from solution to the surface of membranes composed of negatively charged and/or non-lamellar lipids can be characterized by equilibrium dissociation constants in the order of tens of micromolar. However, we previously found that VDAC, reconstituted into planar bilayers of a plant-derived lipid, responds to α-syn at nanomolar solution concentrations. Now, using lipid mixtures that mimic the composition of mitochondrial outer membranes, we showed that functionally important binding does indeed take place in the nanomolar range. We demonstrated that the voltage-dependent rate at which a membrane-embedded VDAC nanopore captures α-syn is a strong function of membrane composition. Comparison of the nanopore results with those obtained by the bilayer overtone analysis of membrane binding demonstrated a pronounced correlation between the two datasets. The stronger the binding, the larger the on-rate, but with some notable exceptions. This leads to a tentative model of α-syn–membrane interactions, which assigns different lipid-dependent roles to the N- and C-terminal domains of α-syn, accounting for both electrostatic and hydrophobic effects. As a result, the rate of α-syn capture by the nanopore is not simply proportional to the α-syn concentration on the membrane surface but sensitive to the specific interactions of each domain with the membrane and nanopore.

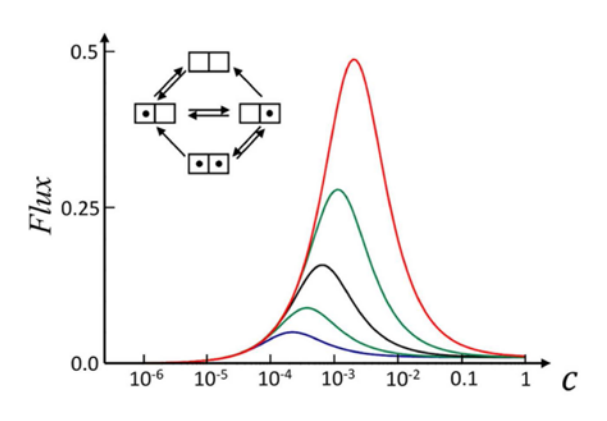


FIGURE 3. Crossover in flux concentration dependence

A simple two-site model of channel-facilitated membrane transport allows for a description of not only flux saturation with the solute concentration (*C*) but also more subtle effects, such as superlinearity and crossover behavior. In particular, solute attraction to the channel walls in combination with strong short-range solute-solute attraction may result in a sharp peak in the concentration dependence of the flux. Different curves correspond to different strengths of solute-solute interactions, increasing from bottom to top. This rich behavior may play a role in both sensory and regulation mechanisms in cellular metabolic circuits.

Real-time nanopore-based recognition of protein translocation success

A growing number of new technologies are supported by a single- or multi-nanopore architecture for capture, sensing, and delivery of polymeric biomolecules. Nanopore-based single-molecule DNA sequencing is the premier example. The method relies on the uniform linear charge density of DNA, so that each DNA strand is overwhelmingly likely to pass through the nanopore and across the separating membrane. By contrast, translocation is not assured for disordered peptides, folded proteins, or block copolymers with heterogeneous charge densities, and additional strategies to monitor the progress of the polymer molecule through a nanopore are required. This year, we studied the translocation of a heterogeneously charged polypeptide using model-free detection of the effect of the polymer charge on the electrical environment inside the nanopore to monitor the translocation process of single polypeptides in real time. This was accomplished using "selectivity tags," regions of different but uniform charge density at the ends of a polypeptide, that produce different selectivity of the nanopore to cations and anions, and hence ionic current levels, in a voltage-biased nanopore under a salt concentration gradient. We employed the natural, disordered "diblock copolymer"–like 140 amino-acid polypeptide α-syn, which comprises two such tags, a highly negatively charged C-terminal region (CT; 43 amino acids, total charge –15e) and a largely neutral N-terminal region (NT; 97 amino acids, total charge +3e). By using these features, we demonstrated a single-molecule method for direct, model-free, real-time monitoring of the translocation of a disordered, heterogeneously charged polypeptide through a nanopore. The two selectivity tags at the ends of the polypeptide enabled us to discriminate between α-syn translocation and retraction. Our results demonstrated exquisite sensitivity of polypeptide translocation to the applied transmembrane potential and proved the principle that nanopore selectivity reports on biopolymer substructure. We anticipate that the selectivity tag technique will be broadly applicable to nanopore-based protein detection, analysis, and separation technologies, and to the elucidation of protein translocation processes in normal cellular function and in disease.

Mapping intra-channel diffusive dynamics of interacting molecules onto a two-site model: crossover in flux concentration dependence

Transport of various solutes through membrane channels is an extremely complex phenomenon. One of the reasons for this complexity is interactions of solute molecules between themselves and with the channel. This year, we addressed this problem by analyzing how such interactions affect the flux dependence on the solute

concentration. The study focused on narrow membrane channels, for which it was assumed that the molecules cannot bypass each other because of their hard-core repulsion. In addition, other short- and long-range solute-solute interactions were also considered. Such interactions make it impossible to develop an analytical theory for the flux in the framework of the continuum diffusion model of solute dynamics in the channel, developed in our lab during recent years. To overcome this difficulty, we coarse-grained the diffusion model by mapping it onto a two-site one where the rate constants describing the solute dynamics were expressed in terms of the parameters of the initial diffusion model. This allowed us, first, to find an analytical solution for the flux as a function of the solute concentration and, second, to characterize the solute-solute interactions by two dimensionless parameters. Such a characterization proved to be very informative, as it resulted in a clear classification of the effects of the solute-solute interactions on the concentration dependence of the flux. Unexpectedly, it turned out that this dependence can be nonmonotonic, exhibiting a sharp maximum as a function of system parameters. In other words, we found that the effect is quite nontrivial: the flux can reach a well-pronounced maximum at a certain "optimal" concentration, the value of which is defined by the interaction parameters. We hypothesize that this phenomenon may be used by nature as a sensory mechanism of a regulatory circuit, wherein an optimal solute concentration is reported upon by maximizing the transmembrane flux of the molecules.

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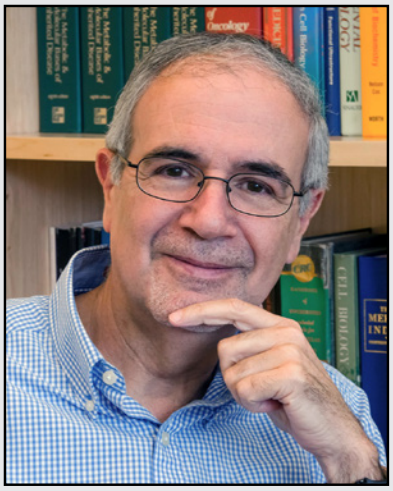
Protein Sorting in the Endomembrane System

Our laboratory investigates the molecular mechanisms by which transmembrane proteins (referred to as "cargo") are sorted to different compartments of the endomembrane system in eukaryotic cells. The system comprises an array of membrane-enclosed organelles including the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi network (TGN), endosomes, lysosomes, and lysosome-related organelles (LROs) (e.g., melanosomes), as well as different domains of the plasma membrane in polarized cells such as epithelial cells and neurons (Figure 1). Transport of cargo between the compartments is mediated by vesicular/tubular carriers 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 different intracellular transport pathways, including endocytosis, recycling to the plasma membrane, retrograde transport from endosomes to the TGN, biogenesis of lysosomes and LROs, and polarized sorting in epithelial cells and neurons. Knowledge gained from this basic research is applied to the elucidation of disease mechanisms, including congenital disorders of protein trafficking, such as the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS), hereditary spastic paraplegias (HSPs), and ponto-cerebellar hypoplasias.



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Lysosome positioning influences mTORC1 and mTORC2 signaling.

This past year, we examined the role of lysosome positioning on mTOR signaling in response to growth factors. Binding of growth factors to cognate receptors at the cell surface initiates intracellular signaling cascades that eventually reach organelles such as lysosomes. A key component of these signaling cascades is the serine/threonine kinase mTOR, which exists as a subunit of two complexes named mTORC1 and mTORC2 (Figure 2). Whereas mTORC1 associates with lysosomes, the intracellular distribution of mTORC2 is less well understood. We

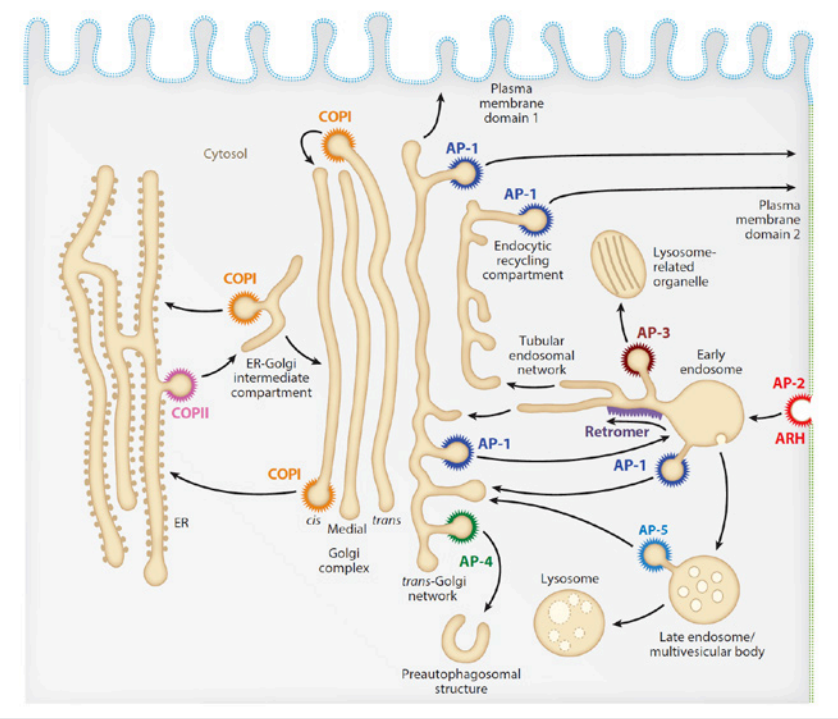

FIGURE 1. Schematic representation of the endomembrane system of eukaryotic cells showing the localization of coats involved in protein sorting

found that perinuclear clustering of lysosomes induced by uncoupling lysosomes from kinesin motors delayed the reactivation of mTORC1 caused by addition of serum (a source of growth factors). The finding indicated that increasing the distance of lysosome-associated mTORC1 from the source of growth-factor signaling at the plasma membrane delays the relay of signals through the cytoplasm. In addition, we made the surprising finding that mTORC2 reactivation after serum replenishment was also delayed by perinuclear clustering of lysosomes. The experiments demonstrated the existence of pools of both mTORC1 and mTORC2 that are sensitive to lysosome positioning, a finding that may explain how changes in lysosome positioning in cancer cells promote their proliferation.

Reversible association with motor proteins (RAMP): a streptavidin-based method to manipulate organelle positioning

We also developed a novel method, named "reversible association with motor proteins" (RAMP), to manipulate organelle positioning within the cytoplasm (Figure 3). RAMP involves coexpressing (1) an organellar protein fused to the streptavidin-binding peptide (SBP), and (2) motor domains from plus-end–directed or minus-end–directed kinesin motors fused to streptavidin. The SBP–streptavidin interaction drives accumulation of organelles at the plus or minus end of microtubules, respectively. Importantly, addition of biotin dissociates the motor from the organelle, allowing restoration of normal patterns of organelle transport and distribution. We demonstrated that the method can be used to manipulate the distribution of various intracellular organelles, including lysosomes, mitochondria, peroxisomes, and the endoplasmic reticulum. The method should be useful for examining how the positioning of these organelles affects their functions, and for analyzing the movement of organelle cohorts upon release from the kinesin motor.

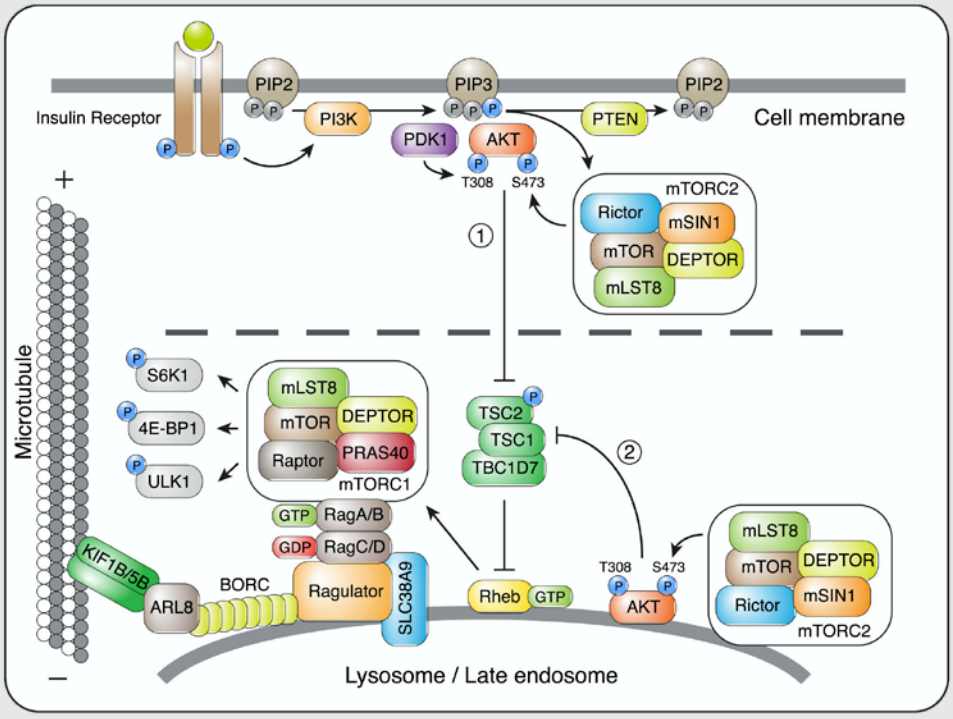

FIGURE 2. Lysosome positioning influences mTORC2 and AKT signaling.

A novel neurodevelopmental disorder caused by mutations in the VPS51 subunit of the GARP and EARP complexes

The Golgi-associated retrograde protein (GARP) and endosome-associated recycling protein (EARP) complexes are related heterotetrameric complexes that associate with the TGN and recycling endosomes, respectively. GARP and EARP function to coordinate the SNARE–dependent fusion of endosome-derived transport carriers with their corresponding compartments, enabling retrograde transport to the TGN and recycling to the plasma membrane. GARP is composed of VPS51, VPS52, VPS53, and VPS54 subunits, whereas EARP is composed of VPS50, VPS51, VPS52, and VPS53 subunits. Although the complexes are known to play key roles in intracellular protein trafficking, their importance in human physiology remains poorly understood. In collaboration with David Everman, we recently identified compound heterozygous mutations in the gene encoding the shared GARP/EARP subunit VPS51 in a 6-year-old patient with severe global developmental delay, microcephaly, hypotonia, epilepsy, ponto-cerebellar abnormalities, liver dysfunction, lower extremity edema, and dysmorphic features. Biochemical and cellular analyses showed that the mutation in one allele causes a frameshift that produces a longer but highly unstable protein, whereas the mutation in the other allele produces a protein with a single amino-acid substitution that is stable but assembles less efficiently with the other GARP/EARP subunits. The mutations consequently result in reduced levels of GARP and EARP complexes in the patient's cells. In addition, the patient's cells exhibit lysosomal abnormalities, consistent with the requirement of GARP for the sorting of acid hydrolases to lysosomes. The findings thus identified a novel genetic locus for a neurodevelopmental disorder and highlighted the critical importance of GARP/EARP function in cellular and organismal physiology.

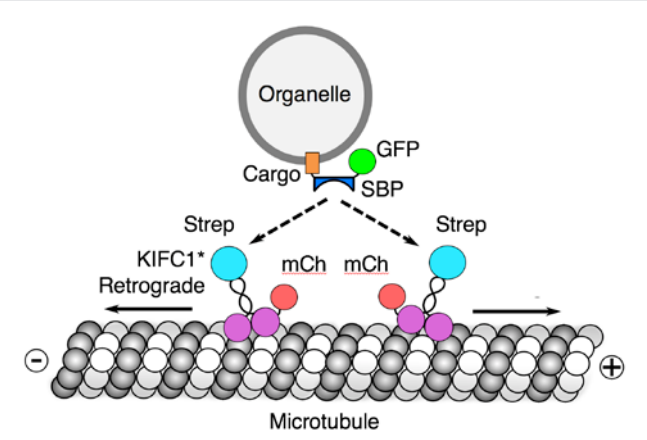

FIGURE 3. Reversible association with motor proteins (RAMP)

ARFRP1 functions upstream of both ARL1 and ARL5 to coordinate the recruitment of distinct classes of tethering factors to the TGN.

Despite the importance of GARP in cellular and organismal physiology, until recently it was unclear how it was recruited to the TGN and how its function was coordinated with that of a different class of tethering factors, long coiled-coil tethers of the golgin family. The golgins mediate long-distance capture of endosome-derived transport carriers, whereas GARP promotes SNARE–dependent fusion of the carriers with the TGN. This past year, we discovered that the ARF–like (ARL) GTPase ARFRP1 is an upstream activator of two other ARL GTPases, ARL1 and ARL5, which in turn recruit golgins and GARP, respectively, to the TGN (Figure 4). In addition, we found that this GTPase cascade is essential for the delivery of retrograde cargos to the TGN. From these findings, we concluded that ARFRP1 is a master regulator of retrograde-carrier tethering to the TGN. The mechanism involving the recruitment of distinct classes of tethering factors by a bifurcated GTPase cascade may be paradigmatic of other vesicular fusion events that take place within the cell.

The autophagy protein ATG9A promotes HIV–1 infectivity.

Nef is an accessory protein encoded by the primate immunodeficiency viruses HIV-1, HIV-2, and SIV and contributes to viral replication, assembly, budding, infectivity, and immune evasion, through engagement of various host-cell pathways. To gain a better understanding of the role of host-cell proteins in the functions of Nef, we carried out tandem affinity purification–mass spectrometry analysis and identified over 70 HIV-1 Nef–interacting proteins, including the autophagy-related 9A (ATG9A) protein. ATG9A is a transmembrane component of the machinery for autophagy, a catabolic process in which cytoplasmic components are degraded in lysosomal compartments. Pulldown experiments demonstrated that ATG9A interacts with Nef from not only HIV-1 and but also from SIV. However, expression of HIV-1 Nef had no effect on the levels and localization of ATG9A, or on autophagy, in the host cells. To investigate a possible role for ATG9A in virus replication, we used CRISPR/Cas9 to knock out (KO) ATG9A in HeLa cervical carcinoma and Jurkat T cells, and analyzed virus release and infectivity. We observed that ATG9A KO had no effect on the release of wild-type (WT) or Nef–defective HIV-1 in these cells. However, the infectivity of WT virus produced from ATG9A–KO HeLa and Jurkat cells was reduced 4-fold and 8-fold, respectively, compared with virus produced from WT cells. The reduction in infectivity was independent of the interaction of Nef with ATG9A and was not attributable to reduced incorporation of the viral envelope (Env) glycoprotein into the virus. The loss of HIV-1 infectivity was rescued by pseudotyping HIV-1 virions with the vesicular stomatitis virus G glycoprotein. From these studies, we concluded that ATG9A promotes HIV-1 infectivity in an Env–dependent but Nef–independent manner. ATG9A could promote infectivity by participating in either the removal of a factor that inhibits infectivity or in the incorporation of a factor that enhances infectivity of the viral particles. ATG9A is thus a novel host-cell factor implicated in HIV-1 infectivity.

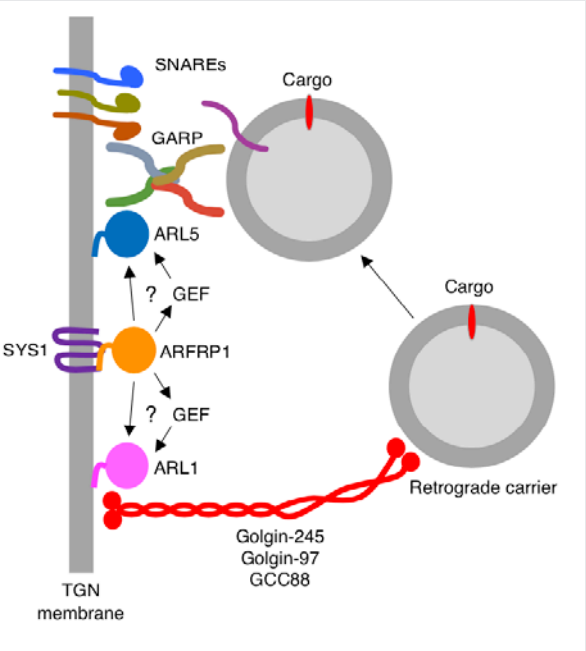

FIGURE 4. Model for the function of ARFRP1 in the coordinated recruitment of golgins and GARP to the TGN

Negative regulation of autophagy by UBA6–BIRC6–mediated ubiquitination of LC3

Although the process of autophagy has been extensively studied, the mechanisms that regulate it remain insufficiently understood. To identify novel autophagy regulators, we performed a whole-genome CRISPR/Cas9 knockout screen in H4 human neuroglioma cells expressing endogenous LC3B tagged with a tandem of GFP and mCherry. Using this methodology, we identified the ubiquitin-activating enzyme UBA6 and the hybrid ubiquitin-conjugating enzyme/ubiquitin ligase BIRC6 as autophagy regulators. We found that these enzymes cooperate to monoubiquitinate LC3B (a component of the autophagy machinery that participates in substrate selection and autophagosome formation), targeting it for proteasomal degradation (Figure 5). Knockout of UBA6 or BIRC6 increased autophagic flux under conditions of nutrient deprivation or protein synthesis inhibition. Moreover, depletion of UBA6 or BIRC6 reduced the formation of aggresome-like induced structures in H4 cells and α-synuclein aggregates in rat hippocampal neurons. The findings demonstrate that UBA6 and BIRC6 negatively regulate autophagy by limiting the availability of LC3B. Inhibition of UBA6/BIRC6 could be used to enhance autophagic clearance of protein aggregates in neurodegenerative disorders.

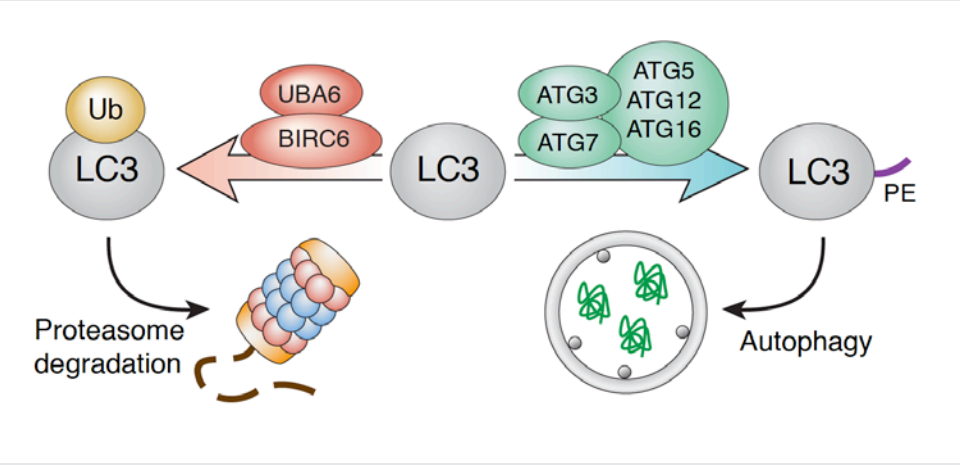

FIGURE 5. Negative regulation of autophagy by UBA6-BIRC6–mediated ubiquitination of LC3

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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 psychiatric disorders and may account for their cognitive and behavioral symptoms. Our lab is interested in how Neuregulin and its receptor ErbB4, which are both genetically linked to psychiatric disorders, function in an activity-dependent fashion (i.e., experience) in the developing brain to regulate synaptic plasticity, neuronal network activity, and behaviors that model features of psychiatric disorders in rodents. We identified a functional interaction between Neuregulin/ErbB4, glutamatergic (NMDA receptors), and dopamine signaling in GABAergic interneurons that is critical for understanding how Neuregulin can regulate E–I balance and synchronous activity in neuronal networks, both processes being important for cognitive functions altered in psychiatric disorders.

Our earlier studies demonstrated that, in the hippocampus and neocortex, expression of ErbB4, the major Neuregulin neuronal receptor, is restricted to GABAergic interneurons, particularly the parvalbumin-positive (Pv+) fast spiking interneurons, which are critically important for modulating gamma oscillation induction and strength (i.e., power). Using genetically targeted mouse models, we discovered that Neuregulin–ErbB4 signaling regulates synaptic plasticity, neuronal network activity, and behaviors associated with psychiatric disorders. More recently our group has been investigating other aspects of Neuregulin expression throughout the brain, its processing in response to neuronal activity, and its function in distinct neuronal populations of the developing and maturing nervous system. Distinct Neuregulin isoforms originate either from four different genes (Neuregulins 1–4) or, in the case of the Neuregulin-1 gene, the use of alternate transcriptional promoters. These mRNAs encode proteins that have either one or two transmembrane (TM) domains. We recently discovered that, contrary to the prior notion that all Neuregulins are targeted to axons to signal in juxtracrine or paracrine

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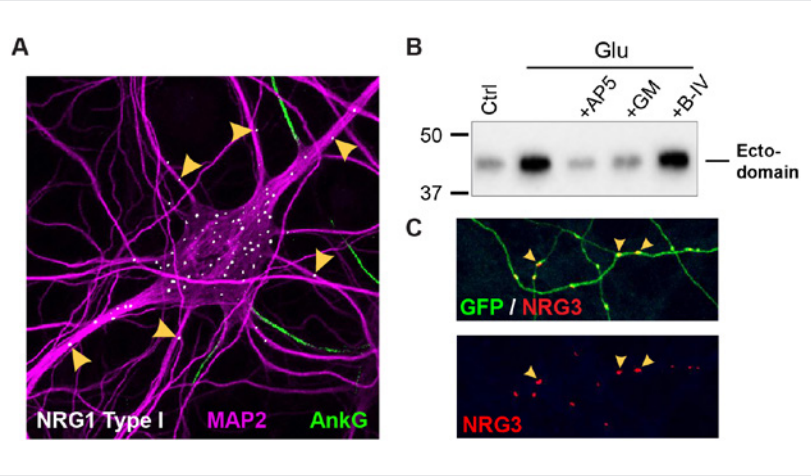

FIGURE 1. Differential trafficking of NRG isoforms in transfected hippocampal neurons

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

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

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

fashion, single–TM Neuregulins are targeted to the soma of neurons while dual–TM Neuregulins are trafficked into axons. Interestingly, we uncovered a novel bidirectional signaling pathway in which single–TM Neuregulins are processed and released from neuronal soma in response to excitatory transmission selectively through NMDARs; following release, Neuregulins signal in autocrine fashion by activating ErbB4 receptors on GABAergic interneurons and promote internalization of NMDARs.

Research in our laboratory uses a combination of experimental approaches, including electrophysiological recordings in acute brain slices prepared from normal and genetically altered mice, multi-electrode field recordings from brains of freely moving rats, reverse-microdialysis neurochemistry, confocal fluorescence microscopy in fixed and live tissue, proteomics analyses, and behavioral testing. The ultimate goal of this multi-disciplinary approach is to generate holistic models to investigate the developmental impact of genes that modulate E–I balance and neuronal network activity, and that consequently affect behaviors and cognitive functions altered in psychiatric and neurological disorders.

Subcellular distribution and functions of single- vs. dual-transmembrane (TM) Neuregulins in central neurons

Numerous Neuregulins (NRGs) are generated through the use of four different genes (*NRG1–NRG4*) and promoters (NRG1: types -I, -II and -II)), but the functional significance of this evolutionarily conserved diversity remains poorly understood. As summarized below, we recently discovered that NRGs can be categorized into two groups based on their distinct transmembrane (TM) topologies, which impart different subcellular trafficking properties. Using RNAScope and newly generated monoclonal antibodies, we found that NRG2 mRNA and protein are prominently expressed in the developing and adult ErbB4–positive GABAergic interneurons, suggesting that NRG2 can engage in autocrine ErbB4 signaling. Interestingly, we found no evidence of NRG2 protein in axons. Instead, we found that unprocessed pro-NRG2 accumulates at endoplasmic reticulum–plasma membrane (ER–PM) junctions in neuronal soma and proximal dendrites of GABAergic interneurons. Our more recent studies on the other single–TM NRGs (NRG1 types I and II)

demonstrate a similar subcellular distribution (Figure 1). Moreover, we found that the ectodomains of all single–TM NRGs are cleaved by alpha-secretases in an activity-dependent manner and shed from the cell surface to signal in paracrine/autocrine fashion. We identified a novel bidirectional signaling pathway in cortical GABAergic interneurons, in which NMDA receptor activation promotes pro-NRG2 shedding and binding to cell-surface ErbB4 receptors, and, in turn, activation of ErbB4 promotes its association with NMDARs and the internalization of these complexes. Consequently, in this fashion, bidirectional signaling between the NRG/ErbB4 and NMDAR pathways function as a homeostatic mechanism to regulate GABAergic interneuron excitatory drive and intrinsic excitatory properties [Vullhorst D, et al., *Nat Commun* 2015;6:7222]. By contrast to single–TM NRGs, we found that the following dual–TM domain NRGs are targeted to axons, where they signal in juxtacrine mode: CRD (cysteine-rich domain)–NRG1 (type III) and NRG3 (our recent studies uncovered it also is a dual–TM NRG). The findings reveal a previously unknown functional relationship between transmembrane topology, subcellular targeting, and processing (Figure 2), and suggest that single– and dual–TM NRGs regulate neuronal functions in fundamentally different ways [Reference 1]. This work was supported by a Directors Investigator Award.

NMDA receptors regulate NRG2 binding to ER–PM junctions and ectodomain release by ADAM10.

We investigated the temporal and downstream targets of NMDAR activity that mediate pro-NRG2 processing in hippocampal neurons. Using pharmacological and genetic approaches, we found that activation of NMDARs causes the dissociation of single-TM pro-NRGs from SSCs (sub-surface cisternae)/ER–PM junctions and subsequent processing by the sheddase ADAM10 but not by ADAM17, as initially reported in studies using

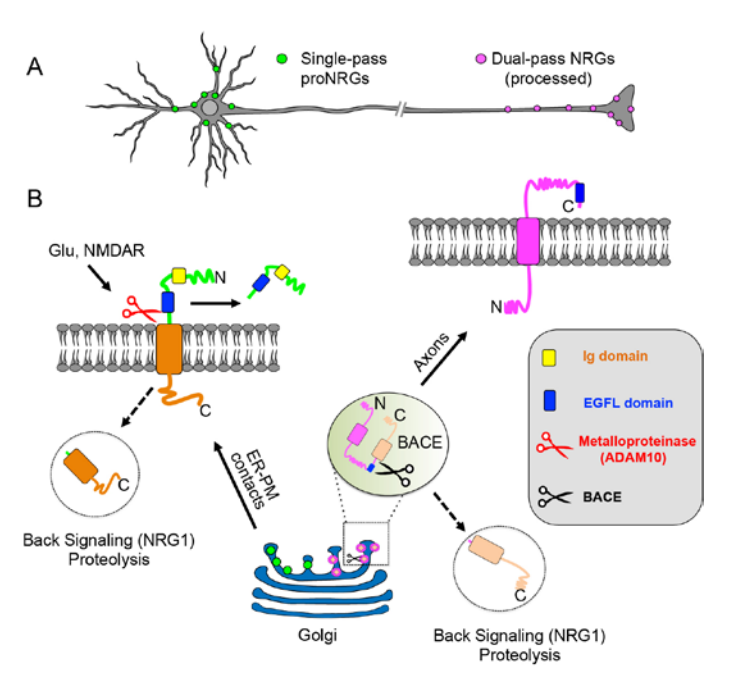

FIGURE 2. Transmembrane (TM) topology of unprocessed pro–NRGs determines their neuronal subcellular targeting and processing in central neurons.

A. A schematic presentation, based on our findings [References 1 & 2], that depicts single TM NRGs accumulating at somatic subsurface cistern and at proximal dendrites to mediate NRG\ErbB4 signaling, whereas dual TM NRGs accumulate on axons and their terminals to drive long-range signaling.

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

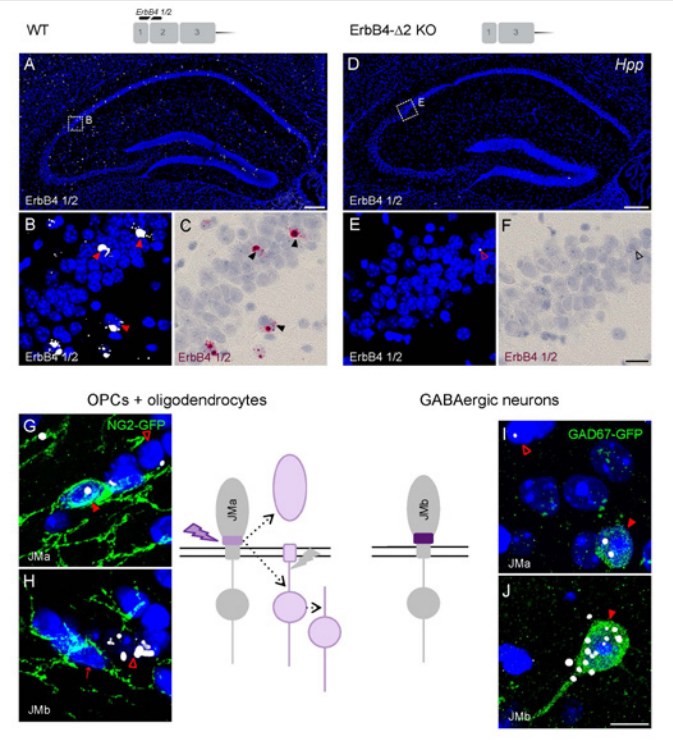

FIGURE 3. Oligodendrocytes and neurons express distinct ErbB4 JM (juxtamembrane) transcripts.

The sensitivity and specificity of single-pair probe *in situ* hybridization was demonstrated by the presence of signals in sparse GABAergic hippocampal neurons of wild-type (WT) mice hybridized with a probe corresponding to exon 2 of the *ErbB4* gene (*A–C*), and by the absence of signal in sections prepared from *ErbB4* knockout mice that lack exon 2 (*D–F*). Oligodendrocyte precursor 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*).

nonneuronal cell lines. Concomitantly, we observed the dephosphorylation in a conserved Ser/Thr–rich region in the intracellular domain of pro-NRG2. Most of the biologically active NRG1 (type-I or -II) or NRG2 ectodomains are shed into the extracellular space within minutes of NMDAR activation, which can then promote signaling via ErbB4 receptors [Reference 2].

A novel ultrasensitive *in situ* hybridization (ISH) 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 loses 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 coding single nucleotide polymorphisms, have been associated with numerous cancers and neurological and psychiatric disorders. We reported a novel, highly specific, and sensitive single-probe colorimetric/fluorescent 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. Next, we demonstrated that expression of ErbB4 isoforms differs between neurons and oligodendrocytes (Figure 3). BaseScope could serve as an invaluable diagnostic tool to detect alternative spliced isoforms, as well as single-base polymorphisms, associated with disease [Reference 3].

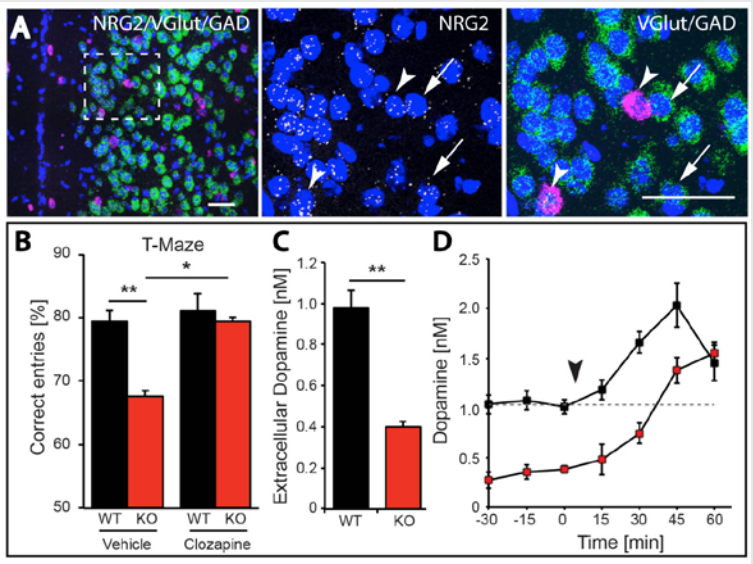

FIGURE 4. NRG2 null mice exhibit working memory deficits and reduced dopamine levels, which are restored by clozapine.

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 drug 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.

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

We found that NRG2 expression in the adult rodent brain does not overlap with NRG1 and is more extensive than originally reported, including expression in the striatum and medial prefrontal cortex (mPFC). We therefore generated NRG2 knockout mice (KO) to study its function. NRG2 KOs have higher extracellular dopamine levels in the dorsal striatum but lower levels in the mPFC, a pattern with similarities to the dopamine dysbalance in schizophrenia. Like ErbB4 KO mice, NRG2 KO mice performed abnormally in a battery of behavioral tasks relevant to psychiatric disorders. They 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 alternation reward test, a task that is dependent on hippocampal and mPFC function. Acute administration of clozapine rapidly increased extracellular dopamine levels in the mPFC and improved alternation T-maze performance (Figure 4). Similar to mice treated chronically with *N*-methyl-D-aspartate receptor (NMDAR) antagonists, we demonstrated that NMDAR synaptic currents in NRG2 KOs are augmented at hippocampal glutamatergic synapses and are more sensitive to ifenprodil, indicating an increased contribution of GluN2B–containing NMDARs. Our findings reveal a novel role for NRG2 in the modulation of behaviors with relevance to psychiatric disorders [Reference 4].

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. Because NRG1 acutely increases extracellular DA levels and regulates LTP (long-term potentiation) 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 of

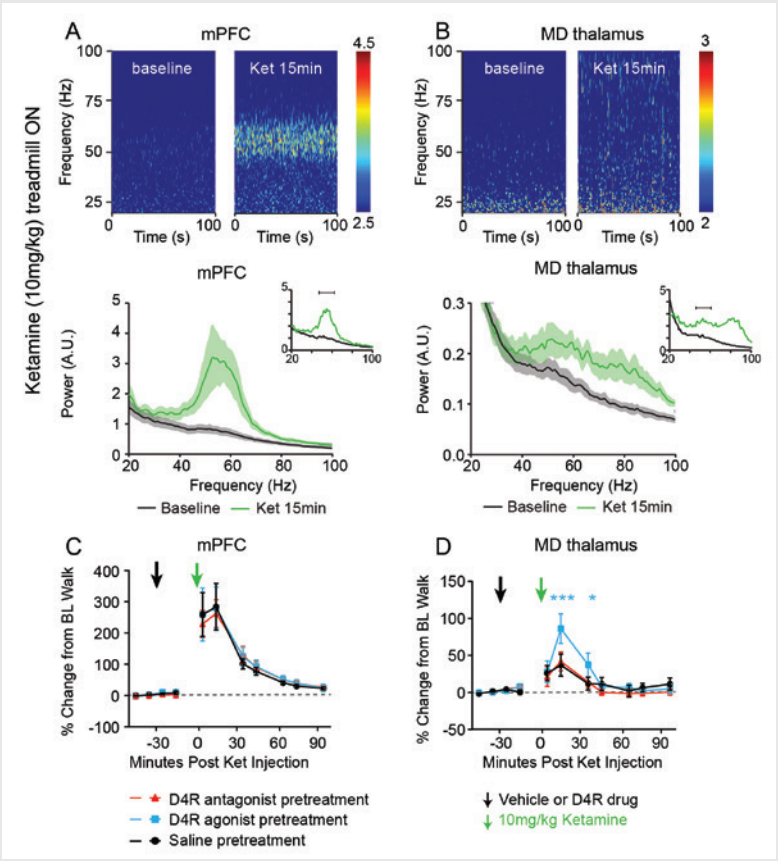

FIGURE 5. Ketamine-induced gamma oscillations in the medial prefrontal cortex (mPFC) and medial (MD) thalamus of walking rats, and regulation by D4R agonist.

Multielectrode 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 ketamine-induced gamma power. Administration of saline or a D4R antagonist (*red*) had no effect on ketamine-induced gamma power in either the (*C*) mPFC or the (*D*) MD thalamus, whereas the D4R agonist A-412997 (*cyan*) selectively increased gamma power in the MD thalamus.

mice harboring targeted mutations of ErbB4 in either PV+ or TH+ neurons. Unexpectedly, we found that, in contrast to GABAergic neurons, ErbB4 is expressed in DA neuron axons, and that NRG regulates extracellular DA levels by modulating DA transporter (DAT) function. In contrast to mice harboring mutations in GABAergic neurons, which show sensorimotor 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, NRG/ErbB4 signaling in GABAergic vs. DAergic neurons differentially and directly affects cortical circuits and DA homeostasis, as well as behaviors relevant to schizophrenia [Reference 5].

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 (attention deficit/hyperactivity disorder). 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 multi-electrode recordings from the medial prefrontal cortex (mPFC) and mediodorsal thalamus (MD) of rats acutely treated with ketamine, which serves as a rodent model with "face validity" for schizophrenia, to study the drug's effects on spiking and gamma local field potentials in the mPFC

and MD of freely moving rats. We found that ketamine raises gamma local field potentials and frequencies in both brain areas, but does not increase thalamocortical synchronization. Based on our prior *in vitro* studies, showing that a "cross-talk" between 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. 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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- Center for Compulsive Behaviors Fellowship Award

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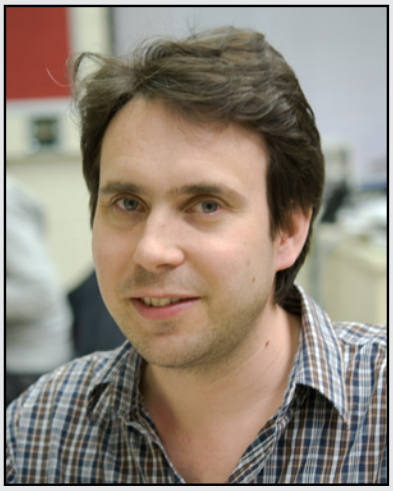
Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

Our goal is to understand how, under diverse environmental contexts, the nervous system selects appropriate behavioral responses to sensory information in a way that best satisfies internal motivational objectives. We use the larval zebrafish as a model because 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, zebrafish 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 measured, 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 neuronal mechanisms by which sensory context regulates behavioral decisions and the pathways that sustain changes in behavioral state. Neuronal connections that allow the brain to integrate sensory and internal-state information are established through genetic interactions during development, and are frequently disrupted by gene mutations associated with neurodevelopmental disorders. We are therefore able to use discoveries about sensorimotor integration pathways to understand how human disease genes disrupt brain development. Finally, to support these objectives, we develop new genetic tools and behavioral assays to probe the nexus between neuronal function and behavior at single-cell resolution.

Neuronal pathways for auditory sensory processing

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, 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



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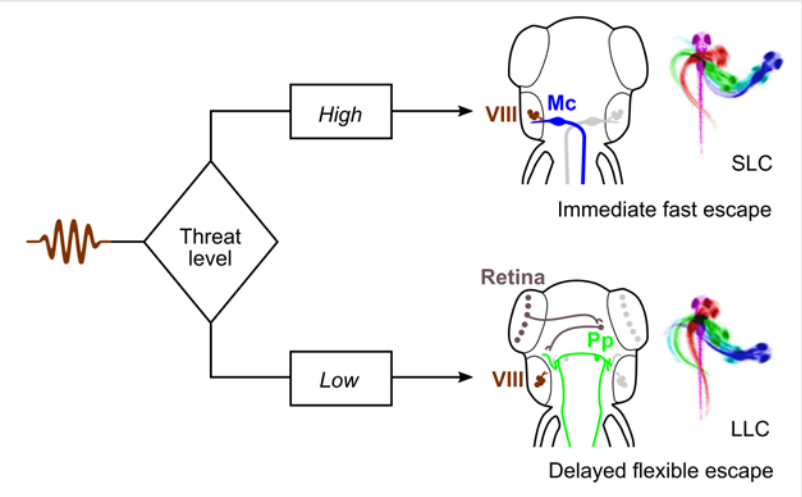

FIGURE 1. Behavioral selection in the auditory escape pathway

Threat levels select between rapid Mauthner cell (Mc)–driven escape responses and delayed flexible escape behavior initiated by a cluster of neurons in the prepontine hindbrain (Pp).

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. We aim to resolve the core neuronal pathway that mediates PPI to then understand how gene mutations linked to neurodevelopmental disorders disrupt sensory processing.

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 preexposure 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. 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. 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 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 such 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 the B3 recombinase in zebrafish to achieve stochastic labeling of isolated neurons in cells that co-express the Gal4 transcription factor and the 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, which 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, indicating that a key mechanism for PPI is presynaptic inhibition [Reference 1]. Given that PPI is abnormal in neuropsychiatric disorders with developmental origins, including schizophrenia and autism, our work will help identify and probe fundamental defects in circuitry that are abnormal in these conditions.

As in humans, zebrafish have more than one pathway for escape behavior. At larval stages, auditory stimuli drive either an extremely rapid Mauthner cell–mediated response, or a slightly delayed non-Mauthner behavior. Delayed responses are of special interest as they integrate additional information, including visual cues, into decisions on escape trajectory, but almost nothing is known about the neuronal pathway that mediates such behavior. We performed a circuit-breaking screen to identify neurons that initiate delayed escape and discovered a population of cells adjacent to the cerebellum in the prepontine hindbrain that initiate delayed escapes. Auditory cues that are insufficiently threatening to drive an immediate escape behavior activate the prepontine delayed escape population, allowing larvae to perform a flexible and graded response (Figure 1). The identification of such neurons provides a new paradigm for dissecting the circuit that mediates very rapid behavioral selection in an ethologically critical pathway [Reference 2].

Tools for decoding neuronal circuits

During our work to identify the neuronal basis for prepulse inhibition, we realized that existing transgenic tools did not permit 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. The lines can be used 'intersectionally' to restrict the pattern of expression with Gal4 domains, allowing small clusters of neurons to be targeted. Thus, 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. A unique feature of brain imaging in zebrafish is the ability to visualize the total architecture of the brain while simultaneously recording the position of every constituent labeled neuron. Brain registration techniques enable data from several individual zebrafish to be co-aligned to very high precision. Thus after generating our new toolkit of Cre lines, we performed whole-brain imaging for each line, then registered the image of each line to the same reference brain. We then developed a new online brain atlas for larval zebrafish (*http://vis.arc.vt.edu/projects/zbb/*) that enables researchers to quickly visualize the larval brain and locate transgenic lines to aid experiments (Figure 2). The Zebrafish Brain Browser leverages the power of Web3D to provide 3D visualization of the zebrafish brain within any web browser [Reference 3].

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. 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 retinorecipient area, was significantly smaller in morphants [Reference 4]. The technique can be applied to almost any zebrafish neurodevelopmental model, thus enabling robust and quantitative detection of subtle changes in brain structure or composition.

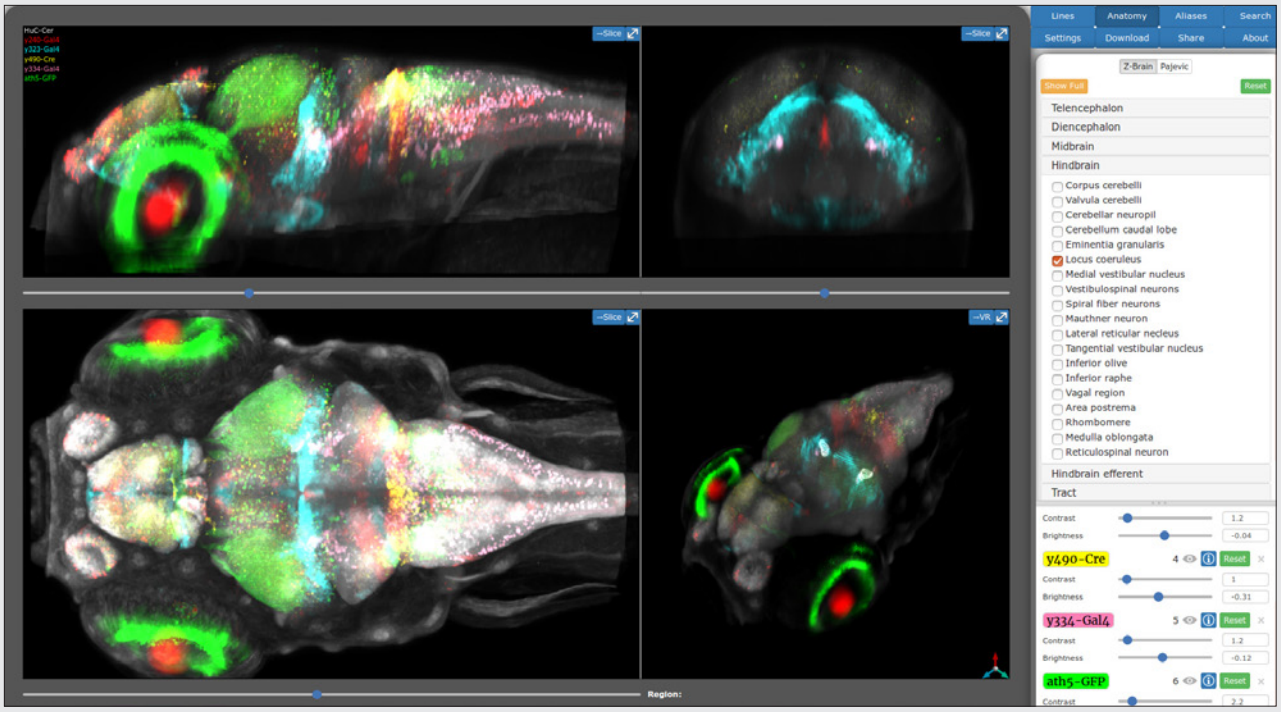

FIGURE 2. Zebrafish Brain Browser Web atlas

The new online Zebrafish Brain Browser visualizes transgene expression in three dimensions: by enabling users to toggle between projections through selected brain regions, or to view single slices through the brain in each cardinal axis. The atlas comprises almost 300 expression patterns, facilitating selection of transgenic lines to experimentally manipulate selected neurons within the larval brain.

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Global Regulation of Bacterial Gene Expression by ppGpp

Our continuing research goals are to understand the emerging fundamental and widespread regulatory functions in bacteria of a special class of purine ribonucleotide analogs of GTP and ATP. The only function of these analogs is regulation because their ribose 3' and 5' hydroxyl groups are blocked, which prevents their participation in all metabolic reactions except their hydrolysis. There are now three structural categories of these 3'–5' blocked nucleotide analogs: mono-cyclics, di-cyclics, and polyphosphorylated ribonucleotides. We focus on the last category, present in nearly all bacteria and plant chloroplasts but not in eukaryotes. Fifty years ago, we discovered (p)ppGpp, the *Escherichia coli* analogs of GTP and GDP. Two years ago, with collaborators, we added (p)ppApp as new ATP and ADP analog members of this subset. All bind to *E. coli* RNA polymerase (RNAP), and the regulatory effects of (p)ppApp *in vitro* suggest that it is an antagonist to (p)ppGpp. Our overarching objective is to understand sources and functions of both analogs, abbreviated collectively as ppApp or ppGpp.

Current research efforts of many laboratories led to a consensus that (p)ppGpp functions as the first responder to virtually all sources of physiological stress in bacteria, ranging from nutritional and physical to metabolic stress. Cellular defenses through (p)ppGpp serve to ensure bacterial adaptation and survival, including survival against host efforts to kill pathogens. We focus on *E. coli* as a model enteric organism, for which much is still to be learned regarding regulation at the transcriptional level, which arises from ppGpp binding at two sites on RNA polymerase. Our past work and that of several others has defined structural and regulatory features of both sites, which, in the presence of high levels of ppGpp, lead to activation as well as inhibition of nearly one third of the genome. Currently, our first goal is to examine the effects of very modest changes in (p)ppGpp basal levels on cellular physiology. This subtle regulation arises from responses to otherwise imperceptible perturbations in amino-acid, carbon, lipid, or energy metabolism that change the balance between ppGpp synthesis and hydrolysis. Our second goal is to understand roles for ppApp, a very newly discovered analog. The evidence that pathogenicity is enhanced by (p)ppGpp is now pervasive from the behavior of ppGpp0 cells that completely lack ppGpp. The profligate misuse of antibiotics coupled with very few new drugs makes fundamental studies of ppGpp acutely relevant if we are not to enter an era in which all antibiotics are ineffective.

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Global regulation of bacterial gene expression by ppGpp

We have made progress towards our first goal of understanding physiological effects of low basal ppGpp levels. As classically defined, growth rates are said to be "balanced" when they are determined by the efficacy of use of different nutrients present in excess rather than by limited abundance. This caveat allows cells to fine-tune gene expression, which contrasts starkly with starvation, when adjustments to prevent death are very different from the fine-tuning during growth. Our commentary last year reviewed markedly different effects of starvation [Reference 1]. Classically, a large range of balanced growth rates are correlated with changes in the cellular content of protein, RNA, and DNA; the slower the growth, the lower the macromolecular content. We established early on that (p)ppGpp determines rates of balanced exponential bacterial growth [Mechold U, et al. *Nucleic Acids Res* 2013;41:6175]. Macromolecular content of ppGpp0 strains does not vary but remains at high levels even during slow growth. Hydrolase mutants that elevate ppGpp basal levels four- or eight-fold without stress, as well as RNAP suppressor mutants whose gene expression mimic effects of ppGpp in a ppGpp0 strain, were used to establish that ppGpp not only determines growth rates but is also both necessary and sufficient to account for growth-rate changes of RNA and protein content.

Recently we submitted a manuscript describing current experiments that allow a similar conclusion to be reached regarding the effects of ppGpp on the variation in initiation rates of chromosomal DNA replication. Bacteria divide in as quickly as every 30 minutes, but it takes an hour for cells to duplicate their circular chromosome. This disparity is resolved by multiple bidirectional initiation forks from a single origin (*ori*) region, which occur before DNA replication is completed at a single terminator (*ter*) sequence. Measurements of *ori/ter* DNA ratios by PCR provide accurate estimates of even small differences of initiation frequencies. It is also known that ppGpp mildly inhibits DNA elongation through primase. Our genomic sequencing results reinforce *ori/ter* ratio differences for slow-growing ppGpp0 strains and show that elongation inhibition sites are random without affecting *ori/ter*. The *ori/ter* ratios of ppGpp–containing cells drop about three-fold when comparing fast and slow growth, while the *ori/ter* ratios of ppGpp^0 cells remain constantly high, even when their rate of balanced growth is slow. Parallel measurements of *ori/ter* ratios with suppressor mutants of ppGpp hydrolase and of RNAP again led to the conclusion that ppGpp is necessary and sufficient for initiation of DNA replication.

While this work was under way, a report appeared [Kraemer JA, et al. *MBio* 2019;10:pii e01330] proposing that ppGpp inhibition of ribosomal RNA operon (*rrn*) transcription from seven operons, comprising more than half of genomic transcripts, resulted in transmission of topological changes to the *ori* region that inhibit initiation. We tested this hypothesis with a strain deleted for all seven chromosomal *rrn* operons. The strain remains viable because two high-copy plasmids carry either a single *rrnB* operon or several essential tRNA genes with the *rrn* deletions. Thus, topological changes resulting from ppGpp inhibition of chromosomal *rrn* transcription are not possible. Inhibition of *ori/ter* ratios at slow growth rates persists in this strain as well as in its ppGpp0 derivative, a prediction not sustained by the hypothesis. A direct inhibitory mechanism is suggested by PCR showing three-fold more gyrase transcripts in wild-type but not in ppGpp0 cells. A manuscript describing this work manuscript has been submitted.

Our second goal is to characterize ppApp. In collaboration with the lab of Katarzyna Potrykus, we have now established the biological and structural relevance of ppApp binding to RNA polymerase. Bioinformatics identified a gene in *Methylobacterium extorquens* encoding SAH*Mex*, a small ortholog of the MESH-1 eukaryotic ppGpp hydrolase discovered by Katarzyna Potrykus in our lab, which also degrades ppApp. The ppGpp/ppApp hydrolase activities of the small SAH*Mex* protein were confirmed *in vitro.* This led us to seek the source of

ppApp in this organism. It is a large RSH_{Mex} enzyme commonly associated with ppGpp synthesis in all Gram-positive bacteria. The pure enzyme synthesizes ppApp and ppGpp *in vitro*, providing the first rigorous biochemical proof that ppApp is the catalytic product of this enzyme in bacteria. Induced ectopic expression of RSH*Mex* in *E. coli* led to appreciable (p)ppApp formation, providing evidence of its regulatory activity. Surprisingly, *E. coli* controls without ectopic RSH_{Mex} protein also revealed traces of (p)ppApp, constituting the first observation of (p)ppApp in *E. coli.* [Reference 2]. Searches are under way in *E. coli* for physiological conditions provoking (p)ppApp to determine whether cellular (p)ppApp might function to antagonize (p) ppGpp regulation, as suggested *in vitro*. Interestingly, not all RSH enzymes synthesize or hydrolyze (p)ppApp. Synthesis and hydrolase activities of a streptococcal RSH_{Seq} are limited to ppGpp but not to ppApp. A future goal will be to exploit the RSH*Mex* and RSH_{Seq} proteins to achieve preferential accumulation of either ppApp or pppApp in order to assign specific functions for each, as we did for ppGpp and pppGpp [Mechold U, et al. *Nucleic Acids Res* 2013;41:6175]. It is noteworthy that the RNAP binding sites for (p)ppApp are found in both Gram-positive and -negative bacteria, whereas direct (p)ppGpp–regulatory RNAP interactions are absent from Gram-positives, suggesting that another level of diversity exists for roughly half the bacterial kingdom.

Advances this year build on past work. Work described for the first goal, resulting in a very recent submission, has been under way for more than two years and as noted, was influenced by Katarzyna Potrykus's studies as a postdoctoral fellow in this lab [Mechold U, et al. *Nucleic Acids Res* 2013;41:6175] and well as by our recent peer-reviewed commentary on starvation for sources of carbon, nitrogen, and phosphate [Reference 1]. Collaborative work on the second goal also originated from another postdoctoral discovery by Katarzyna Potrykus, namely that MESH-1 hydrolase cleaves both ppGpp and ppApp. Our collaborative work on this topic was published earlier [Reference 5]. Similarly, our characterization of adaptive growth on different sugars in last year's report [Reference 6] included development of an innovative high-throughput ppGpp isotope assay method, which was published as a video this year [Reference 4]. Finally, Llorenc Fernández-Coll's experience with RNAP secondary channel-binding proteins led to a peer-reviewed commentary this year on oxidation of zinc finger cysteines that inactivate DksA, a RNAP–binding protein co-factor for ppGpp regulation [Reference 3].

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Membrane Rearrangements in Developmental Fusion and Cancer Progression

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 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 a better understanding of important fusion reactions to bring about new ways of controlling them and to lead to new strategies for quelling 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, a strategy that can reveal new kinds of fusion proteins and clarify the generality of emerging mechanistic insights. In our recent studies, we focused on the role of the fusion of cancer cells in cancer progression.

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Interactions with muscle cells boost fusion, stemness, and drug resistance of prostate cancer cells.

Mechanisms by which interactions between cancer cells and non-malignant cells within the tumor microenvironment influence cancer progression and metastasis are still not understood. In the early 1900's, Otto Aichel suggested that the leukocyte-like characteristics of metastatic cancer cells , which facilitate their migration through the blood, are acquired by their fusion with white blood cells [Aichel O. *Vorträge und Aufsätze Über Entwicklungsmechanik der Organismen. Leipzig, Germany: Wilhelm Engelmann* 1911;92-111]. The hypothesis that cell fusion contributes to initiation and progression of cancer and, specifically, aneuploidy, drug resistance, and metastatic potential characteristic of malignant cells has been further developed in several recent studies [reviewed in Reference 2 and by Noubissi FK and Ogle BM *Int J Mol Sci* 2016;17:pii e1587].

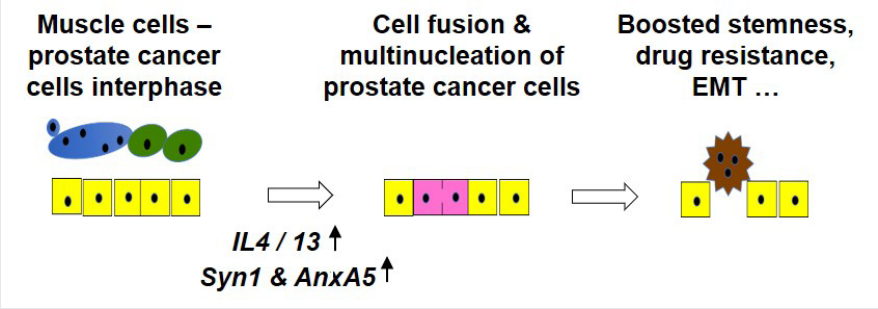

FIGURE 1. Suggested pathway by which muscle cells in the microenvironment of prostate cancer cells promote its progression

We propose that interactions of the cancer cells (*yellow shapes*) with smooth and skeletal muscle cells (*green and blue shapes*) promote the epithelial-mesenchymal transition (EMT), yielding more invasive mesenchymal cells, and expand the subpopulations of cancer stem-like cells. Such changes in the properties of cancer cells depended on: (1) the muscle cell–induced increases in the concentrations of interleukins 4 and 13 (IL-4/IL-13); (2) the cytokine-induced upregulation of the expression of Syncytin 1 and Annexin A5; and (3) Annexin A5– and Syncytin 1–dependent cancer-cell fusion, which generates multi-nucleated cancer cells (*pink shapes*), developing the features characteristic of the cancer stem-like cells, including elevated expression of the stem-cell marker CD133, anchorage-independent growth, and drug resistance.

In our recent study [Reference 3], we focused on the role of cell fusion in prostate cancer. The prostate gland is surrounded by the smooth muscle of the prostate stroma and the striated muscle fibers of the rhabdosphincter. While both smooth and skeletal muscle cells are known to produce and secrete many signaling molecules, the effects, if any, of the muscle cells on the development and progression of primary prostate tumors are yet unexplored. In the study, we modeled interactions between cancer cells and muscle cells in *in vitro* co-cultures and found that primary smooth or skeletal muscle cells increase clonogenic potential and drug resistance of the primary prostate cancer cells and PC3 cells. These interactions also expanded the fraction of the cancer cells expressing the stem-cell marker CD133. We dissected the pathway of these muscle cell–induced changes in the properties of the cancer cells into several distinct steps. First, the interactions of the cancer cells with muscle cells upregulate expression and secretion of interleukins IL-4 and IL-13. The cytokines share receptors and have been linked in earlier studies to both prostate cancer progression and fusion of muscle cells [reviewed in Reference 3]. Second, the cytokines upregulate expression of the fusogens syncytin 1 and annexin A5 [Reference 3]. Third, upregulation of these proteins promotes efficient fusion of the cancer cells with 10–20% of cell nuclei located in fusion-generated multinucleated cells, facilitating the analysis of the underlying mechanisms [Reference 3].

Fusion between cancer cells and fusion between cancer cells and muscle cells is associated with upregulation of syncytin 1 and annexin A5 and is inhibited by blocking their expression. The case for the direct involvement in cancer cell fusion is especially strong for syncytin 1, as blocking fusogenic refolding of syncytin 1 with a peptide inhibitor abolishes fusion [Reference 3]. Suppressing either the activity of Il-4/IL-4 cytokines or cell fusion blocks the increases in the stemness and drug resistance of the cancer cells co-cultured with muscle cells. On the other hand, the effects of the muscle cells can be reconstituted by either treating cancer cells with recombinant IL-4 and IL-13 or by fusing the cancer cells with heterologously expressed syncytin 1. Our work is the first to show that interactions with primary smooth and skeletal muscle cells promote changes in the properties of prostate cancer cells that are consistent with their malignant progression. Furthermore, our study identifies three required and sufficient steps in the underlying pathway and thus provides definitive mechanistic insights into discovered phenomenon. Our finding that, in the human prostate tissue microarrays,

prostate cancer cells have higher levels of syncytin 1 and annexin A5 expression than non-malignant tissues suggests the importance of syncytin 1– and annexin A5–dependent cancer cell fusion in the cancer progression *in vivo*. This novel pathway of microenvironment-driven progression of prostate cancer identified in our *in vitro* experiments and *in vivo* analysis (Figure 1) represents a novel paradigm of cancer progression and may present new therapeutic targets. Furthermore, the work, as well as our earlier studies on cell fusion stages in muscle and bone remodeling [References 1 and 4], has emphasized the dependence of multistep fusion pathway on the activity of many proteins, including annexins and syncytin.

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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; it 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 the production of more hair cells during growth and for the regeneration of neuromasts. The development of this superficial sensory system in zebrafish is spearheaded by the posterior lateral line primordia (pLLp), 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, which 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.

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Self-organization of the zebrafish lateral line primordium

Formation of the posterior lateral line system in zebrafish is pioneered by the pLLp. While leading cells in the pLLp 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 pLLp 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 collective migration progressively shrinks, as cells stop migrating and are deposited from the trailing end. Thus, cells that were incorporated into protoneuromasts are deposited as neuromasts, while cells that

were not, are deposited between neuromasts as interneuromast cells. Eventually, the primordium ends its migration a day later, after depositing 5–6 neuromasts and by resolving into 2–3 terminal neuromasts.

Wnts (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. Wnt 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 pLLp 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 Wnt 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 pLLp; 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. We built agent-based models using the NetLogo programming environment to visualize how both signaling and mechanical interactions could contribute to periodic formation of neuromasts in the migrating primordium.

A sheath of motile cells supports collective migration of the zebrafish posterior lateral line primordium under the skin.

The zebrafish pLLp migrates in a channel formed by the underlying horizontal myoseptum and somites, and the overlying skin. While cells in the leading part of the pLLp are flat and have a more mesenchymal

morphology, cells in the trailing part progressively reorganize to form epithelial rosettes, called protoneuromasts. The epithelial cells extend basal cryptic lamellipodia in the direction of migration in response to both chemokine and Fgf signals. We showed that, in addition to these cryptic lamellipodia, the core tall epithelial cells are in fact surrounded by a population of flat motile cells, which extend actin-rich migratory processes apposed to the overlying skin. These thin cells wrap around the protoneuromasts, forming a continuous sheath of cells around the apical and lateral surface of the pLLp. The processes extended by these cells are highly polarized in the direction of migration, and such directionality, like that of the basal lamellipodia, is dependent on Fgf signaling. Consistent with interactions of sheath cells with the overlying skin contributing to migration, removal of the skin stalls migration. However, this is accompanied by some surprising changes; there is a profound change in the morphology of the sheath cells, with directional superficial lamellipodia being replaced by undirected blebs or ruffles. Furthermore, removal of the skin not only affects underlying lamellipodia, it simultaneously alters the morphology and behavior of the deeper basal cryptic lamellipodia, even though these cells do not directly contact the skin. Directional actin-rich protrusions on both the apical and basal surface and migration are completely and simultaneously restored upon regrowth of the skin over the pLLp. We suggest that this system utilizes a circumferential sheath of motile cells to allow the internal epithelial cells to migrate collectively in the confined space of the horizontal myoseptum and that elastic confinement provided by the overlying skin is essential for effective collective migratory behavior of primordium cells.

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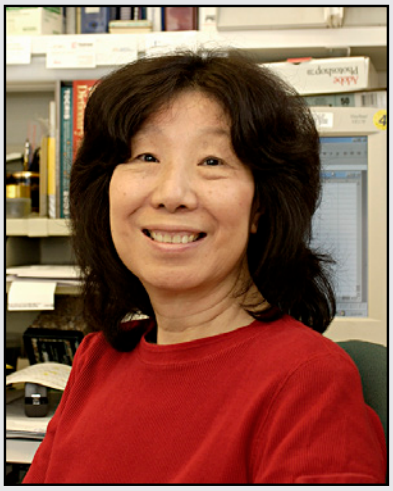
Molecular Genetics of Heritable Human Disorders

We conduct research to delineate the pathophysiology and develop novel therapies for the three subtypes of type I glycogen storage disease (GSD-I), GSD-Ia, GSD-Ib, and GSD-Irs (GSD-I–related syndrome). GSD-Ia is caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC), GSD-Ib by a deficiency in the G6P transporter (G6PT or SLC37A4), and GSD-Irs, also known as severe congenital neutropenia syndrome type 4, by a deficiency in G6Pase-β. G6Pase-α and G6Pase-β are endoplasmic reticulum (ER)–bound G6P hydrolases, with active sites lying inside the lumen, which depend upon G6PT to translocate G6P from the cytoplasm into the ER lumen. The G6PT/G6Pase-α complex maintains interprandial glucose homeostasis, while the G6PT/G6Pase-β complex maintains energy homeostasis and functionality of neutrophils. GSD-Ia and GSD-Ib patients manifest a common metabolic phenotype of impaired glucose homeostasis not shared by GSD-Irs. GSD-Ib and GSD-Irs patients manifest a common myeloid phenotype of neutropenia and myeloid dysfunction not shared by GSD-Ia. Inactivation of G6PT or G6Pase-β in neutrophils leads to enhanced apoptosis, which underlies neutropenia in GSD-Ib and GSD-Irs. A deficiency in either G6PT or G6Pase-β in neutrophils prevents recycling of glucose from the ER to the cytoplasm, leading to the impaired energy homeostasis that underlies neutrophil dysfunction in GSD-Ib and GSD-Irs. There is no cure for GSD-Ia, GSD-Ib, or GSD-Irs. Animal models of the three disorders are available and are being exploited to both delineate the disease more precisely and develop new treatment approaches, including gene therapy. We generated efficacious G6Pase-α–expressing and G6PT–expressing recombinant adeno-associated virus (rAAV) vectors and provided a proof-of-principle gene therapy in murine GSD-Ia and GSD-Ib that is safe, efficacious, and appropriate for entering clinical trials. In 2018, a human G6Pase-α–expressing rAAV vector developed in this laboratory (US patent number: 9,644,216; European patent number: EP3074510) was selected for the phase I/II clinical trial for human GSD-Ia (*ClinicalTrials.gov Identifier: NCT03517085*).



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Sirtuin signaling controls mitochondrial function in GSD-Ia.

Mitochondrial dysfunction has been implicated in GSD-Ia, but the underlying mechanism and its contribution to hepatocellular adenoma

and carcinoma (HCA/HCC) development remain unclear. We showed that hepatic G6Pase-α deficiency leads to downregulation of SIRT1 signaling, which underlies defective hepatic autophagy in GSD-Ia. SIRT1 is an NAD–dependent deacetylase that can deacetylate and PGC-1α, a master regulator of mitochondrial integrity, biogenesis, and function. We hypothesized that downregulation of hepatic SIRT1 signaling in G6Pase-α–deficient livers impairs PGC-1α activity, leading to mitochondrial dysfunction. Using liver-specific *G6pc*-knockout (L-*G6pc*$^{-/-}$) mice, we showed that the G6Pase–deficient livers display defective PGC-1α signaling, reduced numbers of functional mitochondria, and impaired oxidative phosphorylation. Overexpression of hepatic SIRT1 restores PGC-1α activity, normalizes the expression of electron transport chain components, and increases mitochondrial complex IV activity. We previously showed that restoration of hepatic G6Pase-α expression normalizes SIRT1 signaling. More recently, we showed that restoration of hepatic G6Pase-α expression also restores PGC-1α activity and mitochondrial function. We also demonstrated that HCA/HCC lesions found in G6Pase-α–deficient livers display marked mitochondrial and oxidative DNA damage. Taken together, our study shows that downregulation of hepatic SIRT1/PGC-1α signaling underlies mitochondrial dysfunction and that oxidative DNA damage incurred by damaged mitochondria may contribute to HCA/HCC development in GSD-Ia [Reference 1].

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

We had shown that hepatic G6Pase-α deficiency–mediated steatosis leads to the defective autophagy that is frequently associated with carcinogenesis. We showed that hepatic G6Pase-α deficiency also leads to enhancement of hepatic glycolysis and the hexose monophosphate shunt (HMS), which can contribute to hepatocarcinogenesis. The enhanced hepatic glycolysis is reflected in augmented lactate accumulation, increased expression of many glycolytic enzymes, and elevated expression of c-Myc, which stimulates glycolysis. The increased HMS is reflected in increased G6P dehydrogenase activity, elevated production of NADPH, and reduced glutathione. We showed 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 and mitochondrial dysfunction, can contribute to HCA/HCC development in GSD-Ia [Reference 3].

Gene therapy prevents hepatic tumor development in murine GSD-Ia at the tumor-developing stage.

The hallmarks of GSD-Ia are impaired glucose homeostasis and long-term risk of HCA/HCC. Currently, there is no therapy to address HCA/HCC in GSD-Ia. We previously developed an rAAV vector–mediated gene therapy for GSD-Ia and showed that rAAV-G6PC–treated *G6pc*$^{-/-}$ mice expressing 3% or more of normal hepatic G6Pase-α activity maintain glucose homeostasis and do not develop HCA/HCC. However, it remains unclear whether *G6PC* gene transfer at the tumor-developing stage of GSD-Ia can prevent tumor initiation or abrogate the pre-existing tumors. Using L-*G6pc*$^{-/-}$ mice that develop HCA/HCC, we showed that treating the mice at the tumor-developing stage with rAAV-G6PC restores hepatic G6Pase-α expression, normalizes glucose homeostasis, and prevents *de novo* HCA/HCC development. The rAAV-G6PC treatment also normalizes defective hepatic autophagy and corrects metabolic abnormalities. However, gene therapy cannot restore G6Pase-α expression in the HCA/HCC lesions and fails to abrogate any pre-existing tumors.

The major regulators of hepatic glucose metabolism are the glucocorticoids that promote gluconeogenesis. We examined the expression of 11 β-hydroxysteroid dehydrogenase type-1 (11βHSD1), which mediates local glucocorticoid activation by converting inert cortisone (in human) and dehydrocorticosterone (in rodents) into active cortisol and corticosterone, respectively. We showed that the expression of 11βHSD1 is downregulated in HCA/HCC lesions, leading to impairment in glucocorticoid signaling, which is critical for gluconeogenesis activation. This suggests that downregulation of local glucocorticoid action in the HCA/HCC lesions may suppress gene therapy–mediated G6Pase-α restoration. Collectively, our data show that rAAV–mediated gene therapy can prevent *de novo* HCA/HCC development in L-*G6pc*$^{-/-}$ mice at the tumor developing stage, but that it cannot reduce any pre-existing tumor burden [Reference 4].

An evolutionary approach to optimizing G6Pase-α activity for gene therapy of GSD-Ia

GSD-Ia is characterized by impaired glucose homeostasis with the hallmark of hypoglycemia following a short fast. Previously, we developed rAAV vectors expressing either the wild-type (WT) (rAAV-hG6PC-WT) or codon-optimized (co) (rAAV-co-hG6PC) human (h) G6Pase-α. We showed that *G6pc*$^{-/-}$ mice treated with either rAAV-hG6PC-WT or rAAV-co-hG6PC maintain glucose homeostasis and do not develop HCA/HCC if they restore 3% or more of normal hepatic G6Pase-α activity. The codon-optimized vector, which has a higher potency, is currently being used in a phase I/II clinical trial for human GSD-Ia (NCT 03517085). While routinely used in clinical therapies, codon-optimized vectors may not always be optimal. Codon-optimization can impact RNA secondary structure, change RNA/DNA protein binding sites, affect protein conformation and function, and alter posttranscriptional modifications that may reduce potency or efficacy. We therefore sought to develop alternative approaches that could improve the expression yet minimize the impact of sequence changes resulting from broad codon optimization. The human, dog, mouse, and rat G6Pase-α share 87–91% sequence identity. Intriguingly, *in vitro* expression assays have routinely shown that the canine G6Pase-α isozyme is significantly more active than hG6Pase-α. We therefore expanded our analysis to compare *G6PC* genes across the evolutionary tree, seeking potential codon changes that could enhance enzymatic activity of hG6Pase-α. We identified a Ser-298 to Cys-298 substitution naturally found in canine, mouse, rat, and several primate G6Pase-α isozymes, which, when incorporated hG6Pase-α sequence, markedly enhanced enzymatic activity. Using *G6pc*$^{-/-}$ mice, we showed that the efficacy of the rAAV-hG6PC-S298C vector was threefold higher than that of the rAAV-hG6PC-WT vector. The rAAV-hG6PC-S298C vector with increased efficacy, that minimizes the potential problems associated with codon-optimization, offers a valuable vector for clinical translation in human GSD-Ia [Reference 5].

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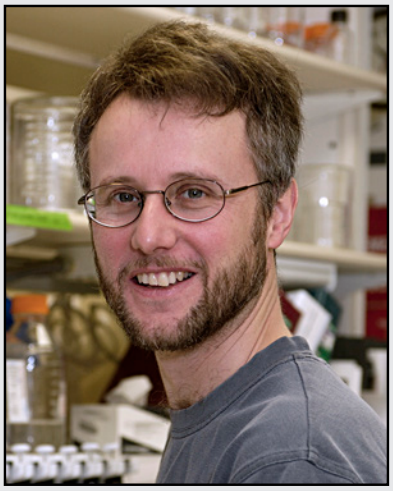
Chromatin Remodeling and Gene Activation

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

The structural subunit of chromatin is the nucleosome core, which contains 147 bp of DNA wrapped 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 beads-on-a-string structure folds spontaneously to form a 30 nm–wide fiber, assisted by the linker histone (H1), which binds to the nucleosome core and to the linker DNA. Collectively, the histones thus 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 in transcription. For transcription to occur, the promoter must be cleared of nucleosomes to allow transcription complex formation, and then Pol II must negotiate the nucleosomes on the gene. Nucleosomes are compact structures capable of blocking transcription at every step. To circumvent and regulate such chromatin blocks, eukaryotic cells possess dedicated enzymes, including ATP–dependent chromatin remodeling machines, histone-modifying complexes, and histone chaperones. The remodeling machines use ATP to move nucleosomes along or off DNA (e.g., the SWI/SNF, RSC, CHD, and ISWI complexes), or to exchange histone variants between nucleosomes (e.g., the SWR complex). The histone-modifying complexes contain enzymes that modify the histones posttranslationally to alter their DNA–binding properties and to mark them for recognition by other complexes, which have activating or repressive roles (the "histone code" hypothesis). Histone-modifying enzymes include histone acetylases (HATs), deacetylases (HDACs), methylases, and kinases. Histone chaperones mediate histone transfer reactions that occur during transcription and DNA replication (e.g., FACT, Asf1, and the CAF-1



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complex). These enzymes, together with the DNA–methylating and –demethylating enzymes present in higher eukaryotes, are central to epigenetics.

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

Interplay between the RSC, ISW1, CHD1, and ISW2 chromatin-remodeling complexes

Our aim is to dissect chromatin remodeling mechanisms *in vivo* and to understand their contributions to gene regulation. Our current efforts focus on elucidating the contributions of the various ATP–dependent remodeling complexes to chromatin organization *in vivo*. During this Fiscal Year, we made significant progress towards understanding the roles of the RSC (remodeling the structure of chromatin) ATP–dependent remodeling complex in gene regulation. RSC is an essential SWI/SNF–like remodeler that is similar to the mammalian PBAF chromatin-remodeling complex and plays an important role in determining the size of the nucleosome-depleted region (NDR) typically found at potentially active gene promoters. In both mammals and yeast, promoter NDRs are flanked by arrays of regularly spaced nucleosomes, which are phased relative to the transcription start site.

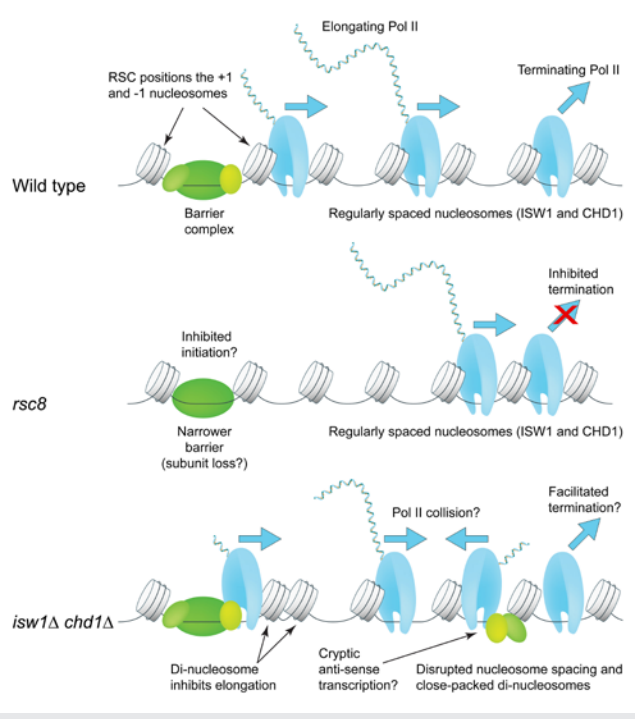

FIGURE 1. Roles of the RSC, ISW1, and CHD1 ATP–dependent chromatin remodelers in RNA polymerase II initiation, elongation and termination

RSC widens the nucleosome-depleted region at the promoter by repositioning the flanking +1 and –1 nucleosomes farther apart to accommodate a complete barrier complex. CHD1 (short spacing) and ISW1 (longer spacing) compete to set the nucleosome spacing on a gene using the +1 nucleosome as a reference. In cells lacking RSC (*rsc8*), nucleosomes shift toward the promoter, but the spacing is the same as in wild-type cells; Pol II lingers 3' of the transcript-termination site. In the *isw1 chd1* double mutant, nucleosome spacing is disrupted, with the +2 nucleosome in particular being pushed against the +1 or +3 nucleosome. High cryptic initiation in these cells may result in collisions between transcribing cryptic anti-sense Pol II and promoter-initiated Pol II (elongation defect). From Reference 1.

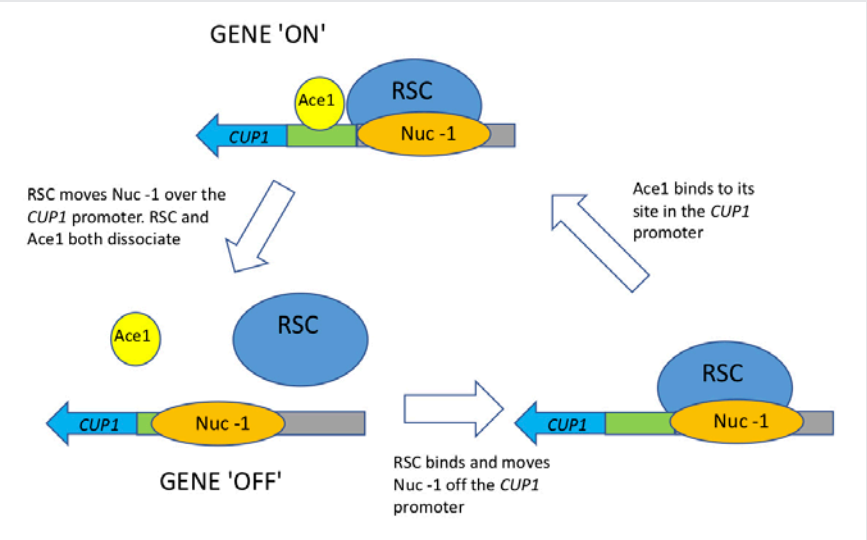

FIGURE 2. Linked cycles of RSC chromatin remodeling and Ace1 transcription factor binding in yeast

Single-molecule tracking and smFISH reveal the link between chromatin remodeling by the RSC complex and Ace1 transcription factor binding. Cycles of Ace1 binding at the *CUP1* promoter lead to bursts of *CUP1* transcription, which depend on short cycles of nucleosome mobilization by RSC (the –1 nucleosome on the promoter). RSC ensures a homogeneous response of the cell population to copper-mediated oxidative stress. From Reference 2.

We examined the interplay between RSC and the ISW1, CHD1, and ISW2 ATP–dependent nucleosome-spacing enzymes in chromatin organization and transcription, using isogenic yeast strains lacking all combinations of these enzymes [Reference 1]. The contributions of these remodelers to chromatin organization are largely combinatorial, distinct, and non-redundant, supporting a model in which the first (+1) nucleosome on the gene is positioned by RSC and then used as a reference nucleosome by the spacing enzymes to create phased nucleosomal arrays. We observed that defective chromatin organization correlates with altered Pol II distribution: RSC–depleted cells exhibit lower levels of elongating Pol II and higher levels of terminating Pol II, consistent with defects in both termination and initiation, suggesting that RSC facilitates both. Cells lacking both ISW1 and CHD1 show the opposite Pol II distribution, suggesting opposite elongation and termination defects. Such cells have extremely disrupted chromatin, with high levels of closely packed dinucleosomes (i.e., two nucleosomes with no intervening linker DNA), primarily involving the second (+2) nucleosome on the gene (i.e., dinucleosomes composed of either the +1 and +2 nucleosomes or the +2 and +3 nucleosomes). We propose that ISW1 and CHD1 facilitate Pol II elongation by separating nucleosomes that are too close together (Figure 1).

Role of the RSC ATP–dependent chromatin remodeling complex in transcription factor dynamics

In a collaboration with the Karpova lab, we investigated the role of RSC in transcription factor dynamics [Reference 2]. Generally, the binding of sequence-specific transcription factors to cognate sites is highly dynamic. However, how such binding is linked to chromatin remodeling and transcription is unclear. The *CUP1* gene encodes a metallothionein responsible for protecting cells against the toxic effects of copper ions; it is induced when excess copper ions bind to the Ace1p transcription factor, which then binds to upstream activating sequences (UAS elements) in the *CUP1* promoter to activate transcription. Using single-molecule tracking, we showed that RSC reduces the time it takes for the Ace1p transcription factor to locate and bind to the *CUP1* promoter. We quantified smFISH (single molecule–fluorescence *in situ* hybridization) mRNA data using a gene-bursting model and demonstrated that RSC regulates *CUP1* transcription bursts by modulating Ace1p

transcription factor occupancy, rather than by affecting initiation and elongation rates. The single-molecule tracking data show that RSC binds to the activated *CUP1* promoter transiently. We also observed that RSC does not affect nucleosome occupancy at *CUP1*. We propose that transient binding of Ace1p and rapid bursts of *CUP1* transcription may depend on short, repetitive cycles of RSC–mediated nucleosome mobilization (Figure 2). This type of regulation would reduce transcriptional noise and ensure a homogeneous response of the cell population to copper stress.

Role of tRNA genes in chromosome organization in yeast

We also collaborated with the Kamakaka lab to investigate the role of tRNA genes in chromosomal organization in the nucleus [Reference 3]. The genome is packaged and organized in an ordered, nonrandom manner within the nucleus, involving contacts between specific chromatin segments and nuclear substructures. tRNA genes are binding sites for transcription factors as well as for architectural proteins and are thought to play an important role in such organization. We tested the hypothesis by removing all tRNA genes from chromosome III, either by deletion or by transfer to another chromosome. Surprisingly, loss of all tRNA genes does not grossly affect chromatin architecture or chromosome tethering and mobility. However, loss of tRNA genes does affect local chromatin structure by altering both nucleosome positioning and the binding of proteins involved in maintaining chromosome architecture (cohesins and condensins), as expected. The absence of tRNA genes also alters centromere clustering and reduces the frequency of long-range heterochromatin clustering, with concomitant effects on gene silencing.

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

Damaged DNA is a leading cause 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 . 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. Aicardi-Goutières syndrome (AGS), a severe neurological disorder with symptoms appearing at or soon after birth, can be caused by defective human RNase H2. We are examining mouse models of AGS to gain insight into the human disorder. 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, in eukaryotes RNase H2 is a complex of three distinct polypeptides but a single polypeptide in prokaryotes. The catalytic subunit of the hetero-trimeric RNase H2 of eukaryotes is similar in its primary amino-acid sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave both RNA/DNA hybrids and a single ribonucleotide [References 1 and 2] or the transition from the ribonucleotide in the case of RNA–primed DNA synthesis (e.g., rrrr*r*DDD in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide).

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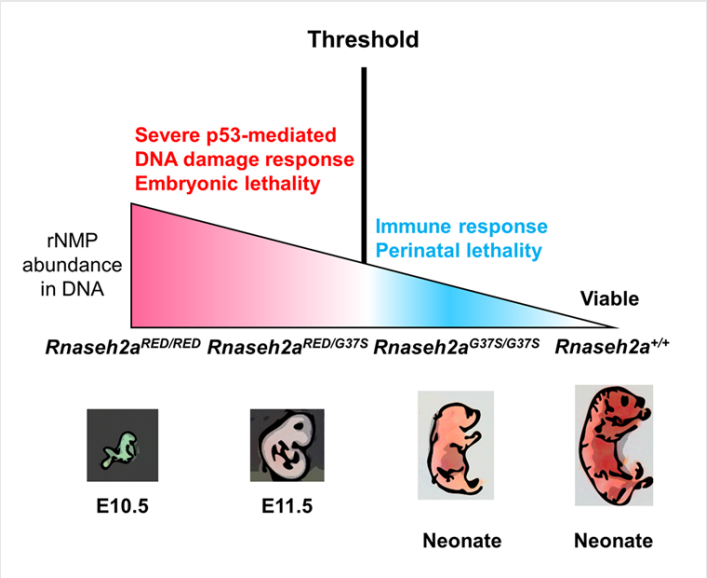
FIGURE 1. Two RNase H2 mutants with differential Ribonucleotide Excision Repair activity reveal a threshold of rNMPs for embryonic development.



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 the p53–dependent 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 1]. 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, as well as 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 1]. 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 1]. 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 G37S–mutant 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, which produces the small molecule cGAMP. cGAMP 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. Further studies are under way, which we expect will lead us to the cause of lethality.

To distinguish between 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 embryonic 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. *Rnaseh2α*$^{G37S/RED}$ embryos also arrest at approximately the same stage as *Rnaseh2α*$^{RED/RED}$ embryos because of better association of RNase $H2^{RED}$ than RNase $H2^{G37S}$ with substrate containing 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 ribonucleoside monophosphates (rNMPs) incorporated during DNA replication leads 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 RNase H2 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 $RNaseh2^{G37S}$ 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^{G37S} mouse weighs about 1000 mg at birth, an enormous difference, indicating that 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 in RNase H^{G37S} mice.

Human patients with the *RNASEH2A* G37S mutation have Aicardi-Goutières syndrome. Although the patients with such 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 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 PCNA (proliferating cell nuclear antigen) is critical for 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.

Enzyme or substrate: which is it?

CHANGES IN THE ENZYME

Our studies on RNase $H2^{RED}$ have permitted us and others to assign specific substrates to each of the two activities are related to various phenotypes seen when RNase H2 is absent [References 1 and 2]. In collaboration with the Yasukawa group [Reference 3], we examined human RNases H2 with alterations seen in AGS patients. We observed differences in protein stability, mono- and divalent salt concentrations, protein shape, and enzymatic activities of these aberrant enzymes. Activities of the altered enzymes ranged from a few per cent to more than 50%.

ABASIC SUBSTRATES

Previously, together with the Storici and Tell groups, we examined the abilities of eukaryotic RNases H2 to cleave substrates containing ribose abasic sites. Both prokaryotic and eukaryotic RNases H2 can recognize

and cleave at rNMPs in duplex DNA, but we found that eukaryotic RNases H2 were unable to incise when the base was absent on the ribose-phosphate moiety, an abasic site. We recently showed [Reference 4] that the bacterial *Escherichia coli* RNase HII does cleave at these abasic site–containing duplex DNA substrates. However, RNase HII from the archaeon *Pyrococcus abyssi* failed to hydrolyze the abasic site–containing substrate, even though we found that it binds quite well to the substrate. Eukaryotic RNases H2 are heterotrimeric enzymes with the A-subunit carrying the catalytic portion of the enzyme. Prokaryotic RNases HII such as those found in *E. coli* and *P. abyssi* enzymes are monomeric proteins with similar primary amino acid sequences to their eukaryotic counterparts. However, there are two regions in which the prokaryotic proteins differ from each other. We modeled the tertiary structures of the RNases HII and found that the additional/different sequences are likely to cause the differential ability of these enzymes to incise at the abasic ribose phosphate site.

RNASE H2C, A PROTEIN WITHOUT THE HOMOTRIMERIC PARTNERS RNASE H2A AND RNASE H2B, IS A SUSCEPTIBILITY FACTOR FOR METASTATIC BREAST CANCER.

In collaboration with Sarah Deasy in Kent Hunter's group, we examined cell lines that cause metastatic breast cancer. Deasy *et al.* had found that high levels of expression of mouse RNase H2c, the protein that has a major function to connect the A and B subunits to form the heterotrimeric RNase H2, is a novel metastasis susceptibility factor. Somewhat surprisingly, we found that the role of Rnaseh2c in metastatic disease is independent of RNase H2 enzymatic activity and exhibited few if any properties of AGS cells. However, RNA–sequencing analysis revealed engagement of the T cell–mediated adaptive immune response. The results suggest an important novel, non-enzymatic role for RNASEH2C during breast cancer progression [Reference 5].

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- NICHD IRP Director's Award

Publications

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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), which separates the nuclear and cytoplasmic compartments of the cell. The sequestration of chromosomes within the nucleus has profound consequences for almost all aspects of gene expression and cell function. Communication between the nucleus and cytoplasm occurs through conduits called nuclear pore complexes (NPCs), which are embedded in the NE and consist of about 30 proteins called nucleoporins (Figure 1). Beyond nucleocytoplasmic trafficking, nucleoporins are important for chromosome organization, transcriptional control, RNA processing, cell signaling, and cell-cycle control. Both nucleoporins and soluble components of the nuclear trafficking machinery also perform transport-independent functions in mitotic chromosome segregation. The involvement of nucleoporins in such diverse events offers the intriguing possibility that they might coordinate these processes with nuclear trafficking and with each other. Moreover, nucleoporin dysfunction has important clinical implications; nucleoporin genes are frequently misregulated in cancers, and nucleoporin mutations cause congenital defects, pediatric nephrotic syndromes, and premature ovarian insufficiency. Nucleoporins are critical viral targets, and their disruption contributes to neurodegenerative conditions, including amyotrophic lateral sclerosis, frontotemporal dementia, and Huntington's disease.

Our research studies 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. Our goal is to define their biochemical roles and how their dysregulation causes human disease. We have taken a multifaceted approach toward this question, using both CRISPR–based strategies in mammalian cells and *Drosophila* developmental genetics.

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Selective degradation of nucleocytoplasmic transport proteins and their interphase function

Understanding the activities of individual nucleoporins has been complicated by their multifaceted nature, abundance, and unusual stability. To better address this question, we employed strategies for

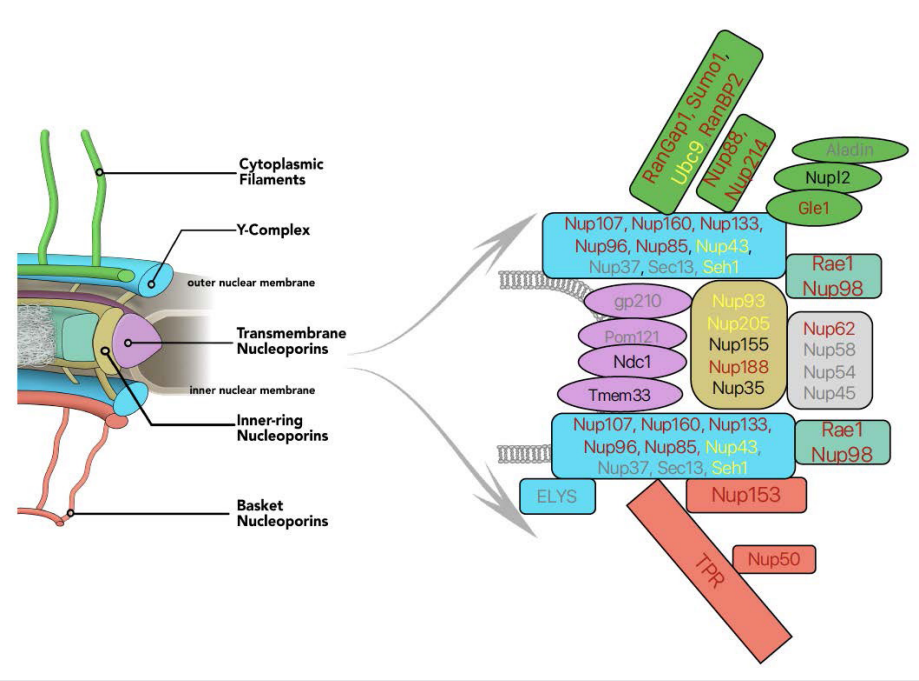


FIGURE 1. AID tagging of nucleoporins

Left side shows model of nuclear pore structure with cytoplasmic fibers (*green*) oriented upward and nucleoplasmic basket (*red*) oriented downward. Other domains of the NPC as indicated. Right side represents the polypeptides associated with NPC sub-complexes, with fill colors corresponding to model domains. Text shows progress in obtaining AID–tagged cell lines for individual nucleoporins: *red*: successful tagging; *black*: in progress; *yellow*: tagging achieved but cell line quality issues; *grey*: tagging unsuccessfully attempted.

selective and rapid degradation of individual proteins within human tissue-culture cells (Figure 2). Specifically, we used CRISPR/Cas9 to construct cell lines in which sequences encoding auxin-induced degron (AID) domains are inserted into both alleles of targeted genes within cells that also 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. We frequently also add a fluorescent tag to the targeted proteins, allowing their degradation to be monitored visually as well as biochemically. We have been successful in developing cell lines that allow conditional depletions of nucleoporins associated with different regions of the NPC (Figure 1).

Our current studies regarding the roles of nucleoporins during interphase address three issues. First, we are examining the role of individual nucleoporins in NPC assembly and stability. Our results indicate that different regions of the NPC can persist independently after depletion of individual nucleoporins, suggesting that the NPC is a surprisingly modular structure. Second, we are examining the role of individual nucleoporins in different nuclear trafficking pathways, an assessment that includes evaluation of 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 with and without auxin, to observe the impact of acute or prolonged nucleoporin depletion. Our results suggest that particular nucleoporins have distinct and nonredundant roles in gene expression. Defining which individual nucleoporins contribute to each of these processes will allow us to better design future experiments examining the role of those selected proteins in the regulation of cellular events.

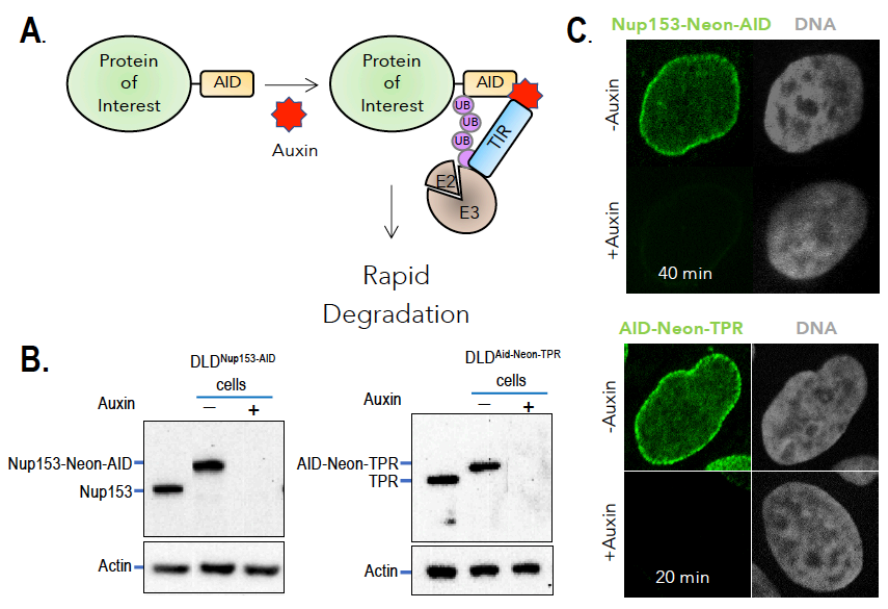


FIGURE 2. Auxin-induced degradation of AID–tagged nucleoporins

A. TIR1–expressing cells ubiquitinate and degrade proteins tagged with auxin-induced degron (AID) domains upon auxin addition. Nup153 and TPR were homozygously fused with the AID tag and a fluorescent marker (*neon Green*) in TIR1–expressing cells. Rapid and uniform degradation is observed after auxin addition by Western blotting (*B*) or destruction of the fluorescent tag (*C*).

Roles of nucleoporins in gene expression and RNA trafficking

One example of how our studies have addressed important questions in the nucleoporin field is our findings regarding the NPC nuclear basket. The vertebrate NPC nuclear basket consists of three basket nucleoporins (BSK-NUPs) called Nup153, Tpr, and Nup50. Beyond trafficking, BSK-NUPs have been implicated in chromatin remodeling, control of gene expression and protein modification, and mRNA processing. To understand the role of BSK-NUPs in these processes, we analyzed AID–tagged cell lines for each of these nucleoporins. We found that Nup153 and Tpr each remain associated with NPCs in the absence of the other, but that Nup50 localization depends on Nup153. No other nucleoporins showed significant changes in localization after depletion of any BSK-NUP. Loss of each nucleoporin caused unique transport and transcriptomic changes, validating our central hypothesis that individual nucleoporins play distinct and specific roles. Notably, not only were transcriptomic changes distinct among BSK-NUPs, but their profiles also differed from changes after depletion of RanGAP1 and the loss of Ran–mediated nuclear trafficking.

We further examined the role of Tpr in gene expression, because it showed the most rapid and significant gene expression changes. GANP is a scaffolding subunit of the TRanscription and EXport 2 (TREX-2) complex, which bridges the transcription and export machineries in yeast through association with the Mediator complex and the NPC. We observed that the GANP protein associated with the NPC in a Tpr–dependent manner, suggesting a possible link between Tpr–dependent transcriptomic changes and altered RNA export. We analyzed cell lines in which GANP or other key RNA export factors were AID–tagged and observed changes in RNA abundance closely related to changes after Tpr depletion (Figure 3), suggesting an integral role of Tpr in TREX-2–dependent RNA export. Both up- and downregulated transcripts showed retention within nuclei in the absence of either Tpr or GANP, further illustrating a critical role of Tpr in TREX-2–mediated mRNA export. Our ongoing experiments are investigating the mechanisms through which Tpr facilitates TREX-2–dependent mRNA export.

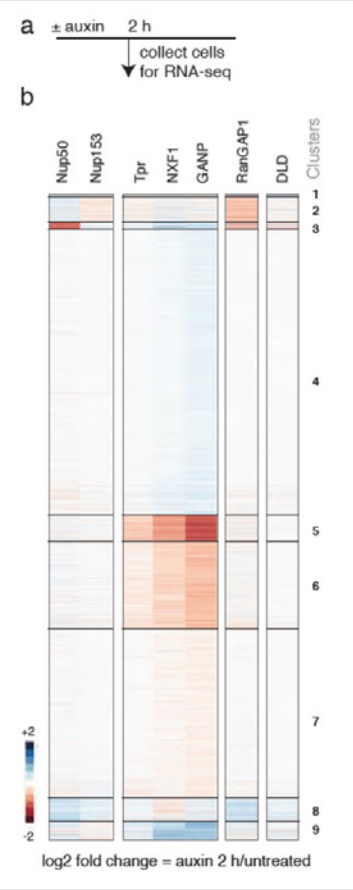


FIGURE 3. Loss of Tpr or TREX-2 (DGANP) causes similar rapid changes in mRNA abundance.

Heatmaps of unsupervised k-means clustering of differentially expressed genes 2 h after auxin treatment of cells expressing corresponding AID–tagged proteins. DLD: parental DLD1 cells treated with auxin. DNXF1 shows a broader arrest of RNA trafficking, and DRanGAP1 shows arrest of Ran–dependent transport. Note the distinct patterns after depletion of other BSK–NUPs, Nup153, and Nup50.

Mitotic roles of the nuclear trafficking machinery

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. The Ran GTPase pathway and nucleoporins promote chromosome segregation through their important roles in spindle assembly and cell-cycle progression. To segregate chromosomes accurately, Ran–GTP distribution must be tightly regulated, both spatially and temporally. Ran's cytoplasmic GTPase–activating protein, RanGAP1, and its chromatin-bound nucleotide exchange factor, RCC1, establish an interphase distribution, with GTP–bound Ran (Ran-GTP) in the nucleus and GDP–bound Ran (Ran-GDP) in the cytosol. Such asymmetry drives nucleocytoplasmic trafficking through controlled cargo binding and release by karyopherins, a family of Ran-GTP–binding transport receptors. After mitotic NE breakdown (NEB), RCC1 generates Ran-GTP near chromosomes, while Ran distal to chromosomes is GDP–bound, directing spindle assembly through spatially regulated release of spindle assembly factors (SAFs) from karyopherins. Notably, the error-prone nature of Ran–dependent meiotic spindle assembly may contribute to human pregnancy losses and genetic disorders, including Down's syndrome. RanBP1 is a Ran-GTP–binding protein that forms a stable heterotrimeric complex with Ran and RCC1 *in vitro* (RRR complex), inhibiting RCC1's nucleotide exchange activity.

Xenopus egg extracts (XEEs) are a well-established *ex vivo* model system for mitotic studies. XEEs possess large amounts of RCC1, which is stockpiled in eggs to facilitate early development. We previously reported that RRR complex formation in XEEs determines RCC1's partitioning between its chromatin-bound and soluble forms and inhibits the exchange activity of soluble RCC1. We found that dissolution of the RRR complex at anaphase onset creates a large pool of free RCC1, which becomes available for chromatin binding. We further investigated how RRR–complex dynamics influences kinetochore and centromere function within XEE. Kinetochores are proteinaceous structures that act as attachment sites for spindle microtubules. Kinetochores assemble during each mitosis on centromeres, specialized chromatin domains that are present at a single locus on each of the sister chromatids. Centromeres are also sites of chromatid cohesion, which is maintained until anaphase onset, when centromeres must be rapidly and synchronously re-modeled to release sister chromatid cohesion and

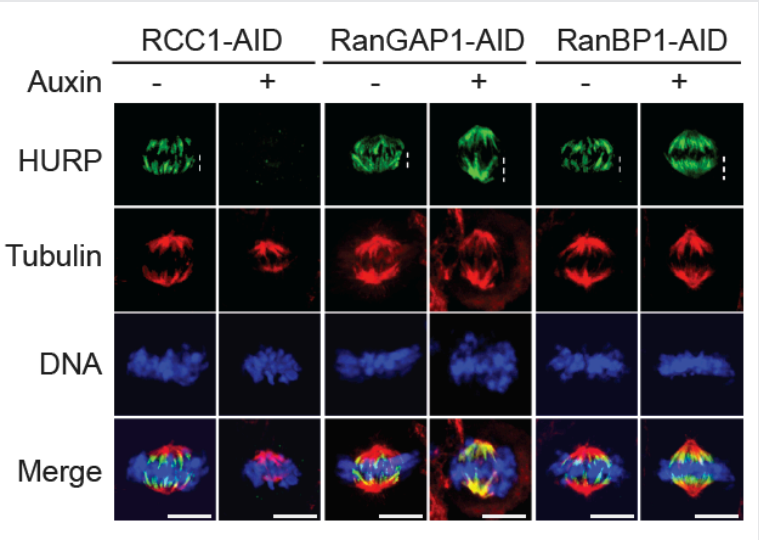


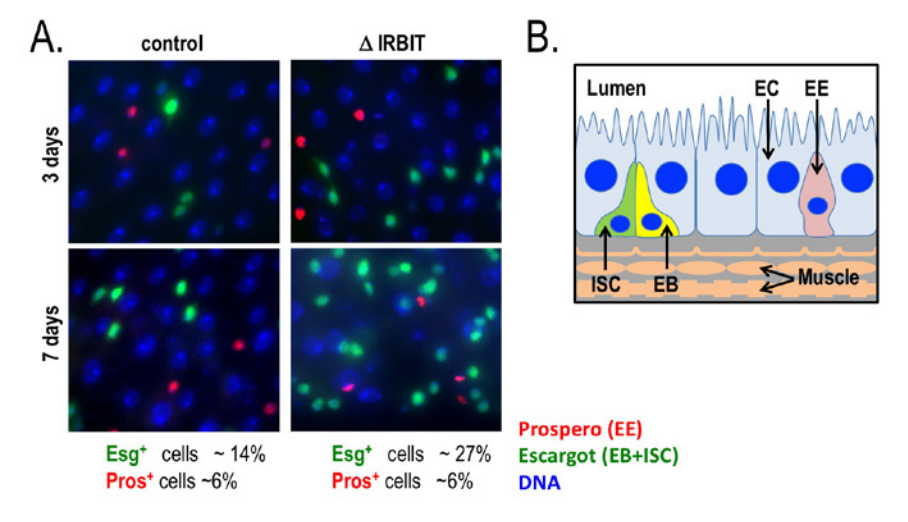
FIGURE 4. Regulation of mitotic Ran–GTP gradients by RRR complex

Immuno-staining with HURP and Tubulin antibodies of cells in HCT116$^{RCC1\text{-}\mu AID\text{-}3xFLAG}$, HCT116$^{RanGAP1\text{-}3mAID}$, and HCT116$^{RanBP1\text{-}\mu AID\text{-}HA}$ cells treated with or without 1 mM Auxin for 5, 2, and 3 h, respectively. Cells also express TIR1. White dashed lines represent the length of HURP signal (top row). Scale bars = 10 μm.

thus allow chromosome segregation into daughter cells and cytokinesis. We studied replicated chromosomes in XEE that possess physiologically paired sister chromatids and found that mimicking the anaphase wave of free RCC1 by adding exogenous RCC1 causes the specific release of chromatid cohesion. Strikingly, RCC1 mutants that lacked activity as a Ran exchange factor were as effective wild-type RCC1 in this assay, whereas active but chromatin binding–deficient RCC1 mutants or Ran-GTP itself were not active. Together, these data indicate that RCC1 can regulate anaphase kinetochore composition in a Ran-GTP–independent fashion; this is the first report of a biological function of RCC1 that is independent of its activity as a Ran exchange factor.

We recently investigated the relevance of the RRR complex to mitosis in somatic cells. Most RCC1 localizes on or near chromosomes throughout mitosis in non-embryonic systems, which have relatively low levels of soluble RCC1, raising questions about whether RRR complex formation significantly controls RCC1 dynamics after early development. Nevertheless, RanBP1 depletion by RNAi disrupts mitotic progression in mammalian tissue-culture cells. Notably, the dynamics for chromosome-bound RCC1 are not uniform as tissue culture cells progress through mitosis. To better understand mitotic RCC1 dynamics, we investigated whether RanBP1 or a related protein called RanBP3 might be important for controlling mitotic Ran-GTP gradients within somatic cells. To address this question, we systematically varied RanBP1 and RanBP3 levels in HCT116 or DLD1 cell lines through overexpression or fusion with AID tags. Consistent with earlier reports, we found that RanBP1 was dispensable for interphase import or export of a model substrate, while RanBP3 appears to facilitate nuclear export via the Crm1 karyopherin. Within mitosis, we observed that altering RanBP1 concentrations substantially altered RCC1 dynamics on metaphase chromosomes, while altering RanBP3 did not. Moreover, we found dramatic re-localization of the SAF Hepatoma Up-Regulated Protein (HURP, a component of the spindle-assembly pathway) during metaphase in direct correspondence with changes in RCC1 dynamics (Figure 4), showing that Ran-GTP levels and SAF activity near chromosomes correlate with altered RCC1 behavior. Analogous to findings in XEE, these data indicate an important mitotic role in human somatic cells for RanBP1 in controlling RCC1 dynamics and determining the accurate the spatial distribution and magnitude of the Ran-GTP gradients, thus ensuring correct execution of Ran–dependent mitotic events.

FIGURE 5. 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.

The role of the IRBIT protein in tissue homeostasis

We previously 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 genome stability that was potentially disrupted in the absence of IRBIT, and 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 5B): 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 5A). The midguts of one-day-old wild-type and IRBIT$^{-/-}$ flies were essentially indistinguishable at the tissue-architecture level. However, we observed a rapid loss of tissue homeostasis in the IRBIT$^{-/-}$ flies, with a progressive increase

in relative numbers of undifferentiated enteroblast progenitor cells and tissue dysplasia. IRBIT$^{-/-}$ flies also show fewer cell-cell contacts when stained for junctional proteins in the posterior midgut epithelium and altered gene expression patterns, reminiscent of changes associate with inflammation and aging. The phenotypes are fully rescued through expression of full-length IRBIT, and further experiments suggested that altered dNTP pools likely contribute to the IRBIT$^{-/-}$ phenotypes. Further analysis showed that the IRBIT–RNR pathway is essential to ensure correct differentiation of intestinal stem cell (ISC) progeny, and that it is a key downstream target of the GATAe transcription factor. Moreover, the GATAe–IRBIT–RNR pathway becomes dysfunctional as flies age, contributing to a characteristic accumulation of undifferentiated ISC progeny that can be reversed by specifically inhibiting RNR within progeny cells. Collectively, our findings showed that RNR suppression by IRBIT is an important mechanism that directs differentiation of ISC progeny to maintain intestinal tissue homeostasis.

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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 rereplicate 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.

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CDK1 inhibition facilitates formation of syncytiotrophoblasts and expression of human chorionic gonadotropin [Reference 4].

Human placental syncytiotrophoblast (STB) cells play essential roles in embryo implantation and nutrient exchange between the mother and the fetus. STBs are polyploid, formed by fusion of diploid cytotrophoblast (CTB) cells. Abnormality in STBs 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. The primary objective of this study was to enhance our understanding of the molecular mechanism of placental cell fusion.

FACS (fluorescence-activated cell sorting) and microscopic analysis were used to optimize Forskolin-induced fusion of BeWo cells (surrogate of CTBs) and subsequently, changes in the expression of different cell-cycle regulator genes were analyzed through Western blotting and qPCR (quantitative polymerase chain reaction). Immunohistochemistry was performed 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 by microscopy and FACS, and by monitoring the expression of human chorionic gonadotropin (hCG) by Western blotting and qPCR.

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

DHS (4,4′-dihydroxy-*trans*-stilbene) suppresses DNA replication and tumor growth by inhibiting RRM2 (ribonucleotide reductase regulatory subunit M2) [Reference 3].

DNA replication machinery is responsible for accurate and efficient duplication of the chromosome. 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 (4,4'-dihydroxy-*trans*-stilbene), that acts as a potent inhibitor of DNA replication. Molecular docking analysis identified the RRM2 (ribonucleotide reductase regulatory subunit M2) 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 decreased 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 anticancer agent that targets RRM2 with therapeutic potential either alone or in combination with other agents to arrest cancer development.

A family of PIKFYVE inhibitors with therapeutic potential against autophagy-dependent cancer cells disrupt multiple events in lysosome homeostasis [Reference 2].

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, the compounds did not prevent homotypic fusion between lysosomes, despite the fact that homotypic fusion required some of the same machinery essential for heterotypic fusion. These 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 phosphoinositide kinase. The ability of the WX8 family to prevent lysosomes from participating in macroautophagy/autophagy suggested that they have therapeutic potential in treating autophagy-dependent diseases. In fact, the most potent WX8 family member 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 the WX8 family. Therefore, the WX8 family of PIKFYVE inhibitors provides a basis for developing drugs that could selectively kill autophagy-dependent cancer cells, as well as for increasing the effectiveness of established anticancer therapies through combinatorial treatments.

The Cdk2–c–Myc–miR–571 axis regulates DNA replication and genomic stability by targeting geminin [Reference 1].

DNA rereplication leads to genomic instability and has been implicated in the pathology of a variety of human cancers. Eukaryotic DNA replication is tightly controlled to ensure that it occurs only once during each cell cycle. Geminin is a critical component of this control: it prevents DNA rereplication from occurring during S, G_2, and early M phases by preventing MCM helicases (essential for genomic DNA replication) from forming prereplication complexes. Geminin is targeted for degradation by the anaphase-promoting complex (APC/C) from anaphase through G_1 phase. However, accumulating evidence indicates that Geminin is downregulated in late S-phase owing to an unknown mechanism. We used a high-throughput screen to identify miRNAs that can induce excess DNA replication, and we found that the microRNA miR-571 could reduce the protein level of Geminin in late S-phase independently of the APC/C. Furthermore, miR-571 regulated efficient DNA replication and S-phase cell-cycle progression. Strikingly, the transcription factor c-Myc suppressed miR-571 expression by binding directly to the miR-571 promoter. At the beginning of S-phase, the cell cycle regulator Cdk2 (cyclin-dependent kinase 2) phosphorylated c-Myc at Serine 62, promoting its association with the miR-571 promoter region. Collectively, we identified miR-571 as the first miRNA that prevents aberrant DNA replication and the Cdk2–c-Myc–miR-571 axis as a new pathway for regulating DNA replication, the cell cycle, and genomic stability in cancer cells. The significance of these finding is that they identify a novel regulatory mechanism critical for maintaining genome integrity by regulating DNA replication and cell-cycle progression.

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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 eIF2alpha. We are characterizing eIF2gamma mutations that are associated with the MEHMO syndrome, a novel X-linked intellectual disability syndrome, and we are investigating the function of the translation factor eIF5A, with a focus on its ability to stimulate the peptidyl transferase activity of the ribosome and facilitate the reactivity of poor substrates such as proline. We are also examining the role of the hypusine modification on eIF5A and the role this factor plays in polyamine-regulated gene-specific translational control mechanisms, and we are characterizing metabolite control of translation via upstream Conserved Coding (uCC) regions in select mRNAs.

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

Protein synthesis plays a critical role in learning and memory in model systems, and our studies have linked a human X-linked intellectual disability (XLID) syndrome to altered function of eIF2. In previous studies, with collaborators in Israel, Germany, Slovakia, and at the US Walter Reed National Military Medical Center, we showed that the MEHMO syndrome (named based on the constellation of patient phenotypes: mental [intellectual] disability, epilepsy, hypogonadism and hypogenitalism, microcephaly, and obesity) is caused by mutations in the *EIF2S3* gene, which encodes the gamma subunit of eIF2. Our prior studies, using genetic and biochemical techniques in yeast models of human MEHMO syndrome mutations revealed that the

mutations disrupt eIF2 complex integrity and translation start-codon selection. Over the past year, we generated yeast models of two additional EIF2S3 mutations linked to MEHMO syndrome. One of the mutations impaired methionyl-tRNA binding to eIF2 [Reference 1], and both mutations impaired eIF2 function, altered translational control of specific mRNAs, and reduced the stringency of translation start-site selection. Our collaborators in London linked a novel EIF2S3 mutation with hypopituitarism and glucose dysregulation, potentially expanding the clinical symptoms of MEHMO syndrome [Reference 2]. More recently, we studied induced pluripotent stem (iPS) cells derived from a patient with MEHMO syndrome. We observed a general reduction in protein synthesis, constitutive induction of the integrated stress response, a translational regulatory response typically associated with eIF2alpha phosphorylation, and heightened expression of the transcriptional activators ATF4 and CHOP and the protein phosphatase regulatory subunit GADD34 under stress conditions in the cells. Moreover, upon differentiation into neurons, the mutant cells exhibited reduced dendritic arborization. Based on our studies, we propose that the mutations in eIF2gamma impair the efficiency and fidelity of protein synthesis, and that this altered control of protein synthesis underlies the MEHMO syndrome. 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 *EIF2S3* 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 are examining additional MEHMO syndrome mutations in eIF2gamma, using both yeast and mammalian cell systems.

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^{ϵ}-(4-amino-2-hydroxybutyl)lysine], promotes translation elongation and that this activity depends on the hypusine modification. Using *in vivo* reporter assays, we showed that eIF5A in yeast, like its bacterial homolog EF-P, is especially critical for the synthesis of proteins containing runs of three or more consecutive proline residues. Consistent with these *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. 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 3]. 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. With the hypusine residue projecting toward the acceptor stem of the P-site tRNA, eIF5A occupies the E site of the ribosome. Our studies support a model in which eIF5A and its hypusine residue function to reposition the acceptor arm of the P site to enhance reactivity towards either an aminoacyl-tRNA, for peptide bond formation, or a release factor, for translation termination.

Over the past year, we further investigated the hypusine modification on eIF5A. The modification is formed in two steps: first, an *n*-butylamine moiety from spermidine is transferred to a specific Lys side chain on eIF5A, whereupon hydroxylation on the added moiety completes formation of hypusine. Whereas deoxyhypusine synthase, which catalyzes the first step in hypusine formation, is essential in yeast, the *LIA1* gene encoding the hydroxylase is non-essential. We identified mutations in eIF5A that cause synthetic growth defects in cells lacking the hydroxylase. Our results are consistent with the notion that the hydroxyl modification helps bind and

position eIF5A and its hypusine residue to effectively promote the reactivity of the peptidyl-tRNA on the ribosome.

Recently, 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 such regulation is dependent on an element in the leader of the AZIN1 mRNA. The element resembles an upstream open reading frame (uORF); however, we refer to it as an upstream Conserved Coding (uCC) region, because it lacks an AUG start codon and initiates at a near cognate codon instead. 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. Remarkably, such regulation is dependent on the sequence of encoded polypeptide, including a highly conserved Pro-Pro-Trp (PPW) motif. Ribosome profiling revealed polyamine-dependent pausing of elongating ribosomes on the PPW motif in the uCC, and 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. We proposed that scanning ribosomes typically bypass the near-cognate start codon of the uCC without initiating and then translate AZIN1. However, occasionally a ribosome will initiate translation at the uCC start codon. Under conditions of high polyamines, these elongating ribosomes pause on the PPW motif. The paused ribosome serves as a roadblock to subsequent scanning ribosomes that bypass the near-cognate start codon. The resultant queue 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 this queuing model, we found that impairing ribosome loading, and thus queue formation, reduced uCC translation and derepressed AZIN1 synthesis.

In further studies on the AZIN1 regulatory mechanism, we identified eIF5A as a sensor and effector for polyamine control of uCC translation. Using reconstituted *in vitro* translation assays, 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 and could be rescued by increasing eIF5A levels. We propose that polyamines interfere with eIF5A binding on the ribosome and that 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]. We are now exploring the possibility that polyamine regulation of eIF5A underlies translational control of mRNAs encoding other enzymes and regulators of polyamine biosynthesis.

Translational control by metabolite–sensing nascent peptides

In recent studies, we searched for additional mRNAs containing potential uCCs. Reporter assays in mammalian cells and *in vitro* revealed that a uORF–like element in the mRNA encoding plant GDP-L-galactose phosphorylase (GGP), a control enzyme in the vitamin C biosynthetic pathway, is a uCC. We propose that interaction of vitamin C with the GGP uCC nascent peptide in the ribosome exit tunnel causes the ribosome to pause and that queuing of subsequent scanning ribosomes results in increased initiation on the uCC and prevents ribosome access to the GGP ORF. We believe that the mechanism of a paused elongating ribosome promoting 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.

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

We are also studying the translation elongation factor eEF2. Like its bacterial ortholog EF-G, eEF2 promotes translocation of tRNAs and mRNA from the A site to the P site on the ribosome, following peptide bond formation. In all eukaryotes and archaea, a conserved histidine residue at the tip of eEF2 is posttranslationally modified to diphthamide through the action of seven nonessential proteins. The function of diphthamide and the rationale for its evolutionary conservation are not well understood, and to date the only clear 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 screened for mutations that sensitize eEF2 to loss of the diphthamide modification and we are currently characterizing the mutants. We also examined peptide synthesis in a reconstituted yeast *in vitro* translation system using unmodified eEF2 or eEF2 containing the diphthamide modification. Using the canonical initiation pathway to direct the synthesis of the peptide Met-Phe-Lys revealed no distinction between unmodified eEF2 and eEF2 with the diphthamide modification. In contrast, synthesis of the same peptide directed by the novel cricket paralysis virus (CrPV) internal ribosome entry site (IRES), which bypasses canonical translation initiation and instead requires novel eEF2–directed pseudotranslocation reactions prior to peptide bond formation, was sensitive to the loss of diphthamide. We propose that the precise phasing of pseudotranslocation, in which a tRNA–mimicking RNA element from the virus is translocated through the ribosome, is dependent on the diphthamide modification on eEF2.

Further insights into the function of diphthamide were obtained by our collaborators in Venki Ramakrishnan's lab. Using electron cryomicroscopy, they revealed that eEF2 interacts with the CrPV–IRES on the ribosome and stabilizes the IRES in a conformation reminiscent of a hybrid tRNA state. Interestingly, diphthamide 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. Our studies provide the evidence that diphthamide plays a role in protein synthesis, and we propose that diphthamide has at least two functions: first, to disrupt the decoding interactions of rRNA with the codon-anticodon duplex in the A site; and second, to help chaperone the codon-anticodon interaction 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.

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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 the cause of 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.

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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 systems have revealed several mediators of mitochondrial movement. Anterograde

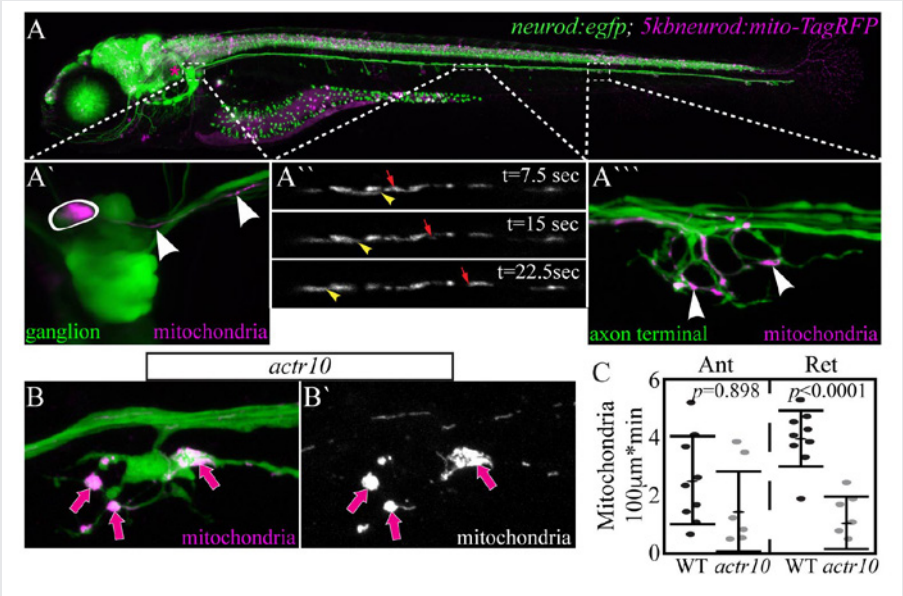

FIGURE 1. Mitochondria accumulate in *actr10*–mutant axon terminals as a result of failed retrograde transport.

A. 5–dpf (days postfertilization) *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: anterograde.

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.

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 results in 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 photoconvertible protein mEos in the inner mitochondrial membrane space. Somewhat surprisingly, just three hours after photoconversion, the old (converted) mitochondria had evacuated from the axon terminal and new (unconverted) had taken their place (Figure 2). This is in contrast to the predictions based on shorter-term imaging conducted within the order of minutes in cultured neurons. The findings demonstrate the power of an entirely *in vivo* system for observing cellular phenomena on a time-scale relevant to the organism.

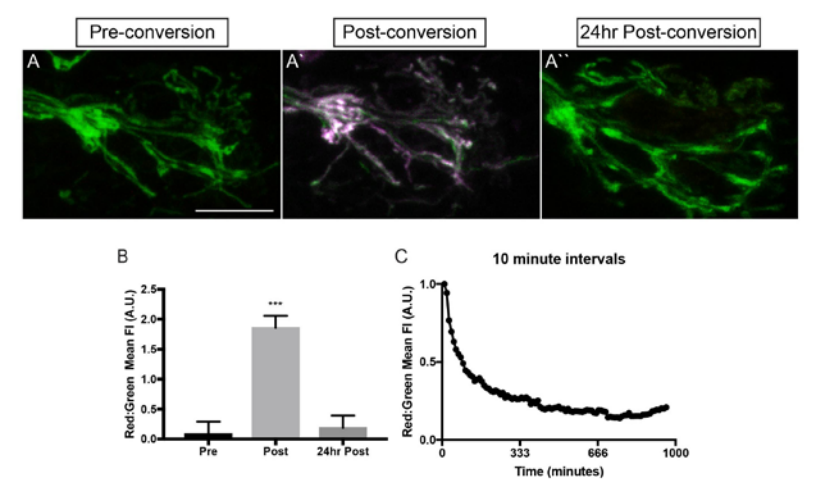

FIGURE 2. Mitochondria turn over in axon terminals within hours.

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 postfertilization) larvae.

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 such phenomena is still under investigation. During cell division, loss of NudC results in abnormal microtubule orientation and failure of kinetochore complex formation. Studies on 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 in order to understand the function of NudC in a mature axon. The work will ultimately permit us to determine whether NudC serves a conserved function in developing neurons, which, when disrupted, leads to varying phenotypes based on developmental state.

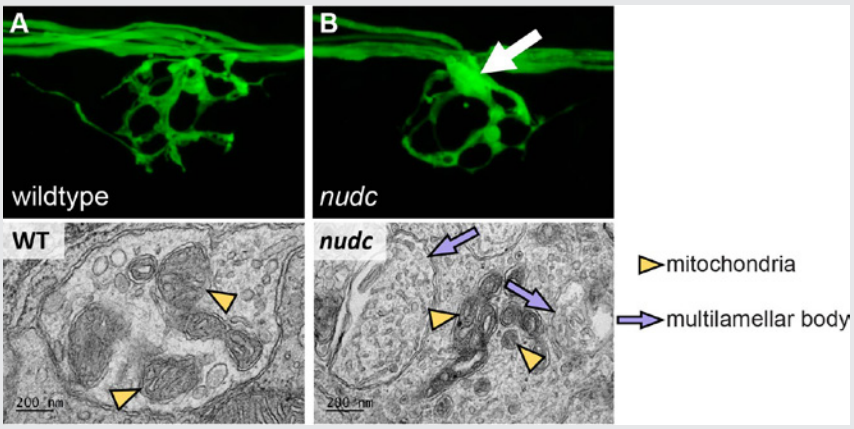

FIGURE 3. In axon terminal branchpoint swellings *nudc* mutants show multilamellar body formation.

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

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 the 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, including the structure and regulation of the genes encoding the luteinizing hormone receptor and prolactin receptor. We also investigate the regulatory mechanism(s) involved in the progression of spermatogenesis and the control of Leydig cell function. Some of this laboratory work focuses on aspects of LHR gene transcription, which is regulated by diverse networks essential for silencing/activation of LHR expression. We also investigate novel gonadotropin-regulated genes relevant to the progression of testicular gametogenesis, Leydig cell function, and other endocrine processes. Major emphasis is placed on studying the function and regulation of the gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), an essential posttranscriptional 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 non-hormonal male contraceptive.

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The luteinizing hormone receptor

The luteinizing hormone receptor (LHR) is expressed primarily in the gonads, where it mediates signals that regulate ovarian and testicular function. The *LHR* gene is transcriptionally regulated by diverse networks in which coordination and interactions between regulatory effectors are essential for silencing/activation of *LHR* expression. The proximal Sp1 site of the promoter recruits histone (H) deacetylases and the Sin3A corepressor complex, which contribute to the silencing of *LHR* transcription. Site-specific acetylation/methylation, induced by the histone deactylase inhibitor trichostatin A (TSA), causes phosphatase release, which serves as a switch for Sp1 phosphorylation, recruitment of the transcription factor II B (TFIIB) and of RNA polymerase II (Pol II), and transcriptional activation. For TSA–mediated *LHR* transcription, positive coactivator 4 (PC4), recruited by Sp1, acts as its coactivator and has an essential role in the formation/assembly of the preinitiation complex (PIC) and in TFIIB and Pol II recruitment. Further studies demonstrated association between PC4 and acetylated H3 in TSA–induced *LHR* derepression in MCF7 cells. The recruitment of PC4 to Sp1 and formation of the PC4–Sp1 complex was thus shown to be essential for *LHR* transcription.

More recently, tandem mass spectrometry (MS/MS) analysis revealed that PC4 associates with the histone variant H3.3, acetylated at several Lys residues. Using an H3.3–specific antibody, immunoprecipitation demonstrated interaction of PC4 with H3.3 induced by TSA, and the presence of the complex PC4–H3.3 at the LHR promoter was demonstrated by reChiP (a sequential chromatin immunoprecipitation protocol). Depletion of endogenous PC4 or H3.3A/B by siRNA (small interfering RNA) caused marked reduction in TSA–induced formation of the complex, its recruitment to the *LHR* promoter, and transcriptional activation of the *LHR* gene, which resulted from a reduction in accessibility of the chromatin at the promoter region of the *LHR* gene. Pull-down studies demonstrated association of H3/H3.3 with GST–PC4 in MCF7 extracts, while we found no direct association with H4. From these and other findings we concluded that PC4 associates with the tetramer via H3 or H3.3. PC4–H3.3 interaction favors acetylation of H3.3, which leads to chromatin accessibility and gene transcription. The findings indicate a critical role of PC4 association with acetylated H3.3 in TSA–induced, Sp1–activated LHR transcription [Reference 1].

Gonadotropin-regulated testicular RNA helicase

Gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), discovered in our laboratory, is a testis-specific member of the DEAD-box family of RNA helicases and is essential for the completion of spermatogenesis. It is present in Leydig cells (LC), meiotic (pachytene spermatocytes), and haploid germ cells (round and elongated spermatids) [Reference 4]. Males lacking GRTH are sterile owing to azoospermia, which results from failure of round spermatids to elongate. We demonstrated its participation in the nuclear export/transport of specific mRNAs, the structural integrity of the chromatoid body (CB), and in storage/processing of relevant mRNAs and their transit/association to the actively translating polyribosomes, where GRTH may regulate translational initiation of genes. GRTH is the only family member of the DEAD-box family regulated by hormones. GRTH transcription is stimulated in LCs by LH/cAMP through direct actions of androgen (A)/the A receptor (AR) (autocrine), and, in paracrine fashion, in germ cells through the AR in Sertoli cells. The upstream region of the *GRTH* gene directs its expression in germ cells and downstream in the LC. Through these regions, A/AR exerts its direct (endogenous) regulation of the *GRTH* gene in LC, and indirectly in germ cells. Functional binding sites for germ cell nuclear factor (GCNF) present in round spermatids (RS) and spermatocytes (SP) and its regulation by A/AR were identified in the distal region of the *GRTH* gene, operative selectively in RS. Current knowledge indicates actions of A on GCNF cell-specific regulation of GRTH expression in germ cells (RS). Also, GRTH exerts negative autocrine regulation of GCNF, linking A actions to germ cells through GCNF as an A–regulated transfactor that controls transcription/expression of GRTH.

These findings provide a connection of androgen action to two relevant germ-cell genes (*GRTH* and *GCNF*), which are essential for the progress of spermatogenesis, and established their regulatory interrelationship. Our early studies revealed that missense mutation of R to H at amino acid 242 of GRTH, found in 5.8% of patients with complete loss of sperm, causes loss of the 61 kDa phospho-species (pGRTH), with preservation of the 52 kDa nonphospho form. The finding provided an avenue through which to elucidate the function of pGRTH in spermatogenesis. We generated humanized mutant *GRTH* knock-in (KI) mice. The mice are sterile, with reduced testicular size and lack of sperm (arrest at step 8 of round spermatids [RS]), and demonstrate complete loss of the pGRTH species but with preservation of the nuclear 52 kDa form. The mouse model permits the study of the biological/biochemical functions of the cytoplasmic pGRTH. In KI mice, the nuclear export transport and functions of GRTH are preserved (i.e., mRNA export, miRNA regulation), while the cytoplasmic functions, including shuttling of messages, storage in the CB, and translational events all requiring pGRTH, are absent. We observed marked reduction of the CB size in RS and lack of pGRTH in the CBs. Germ cell apoptosis was present

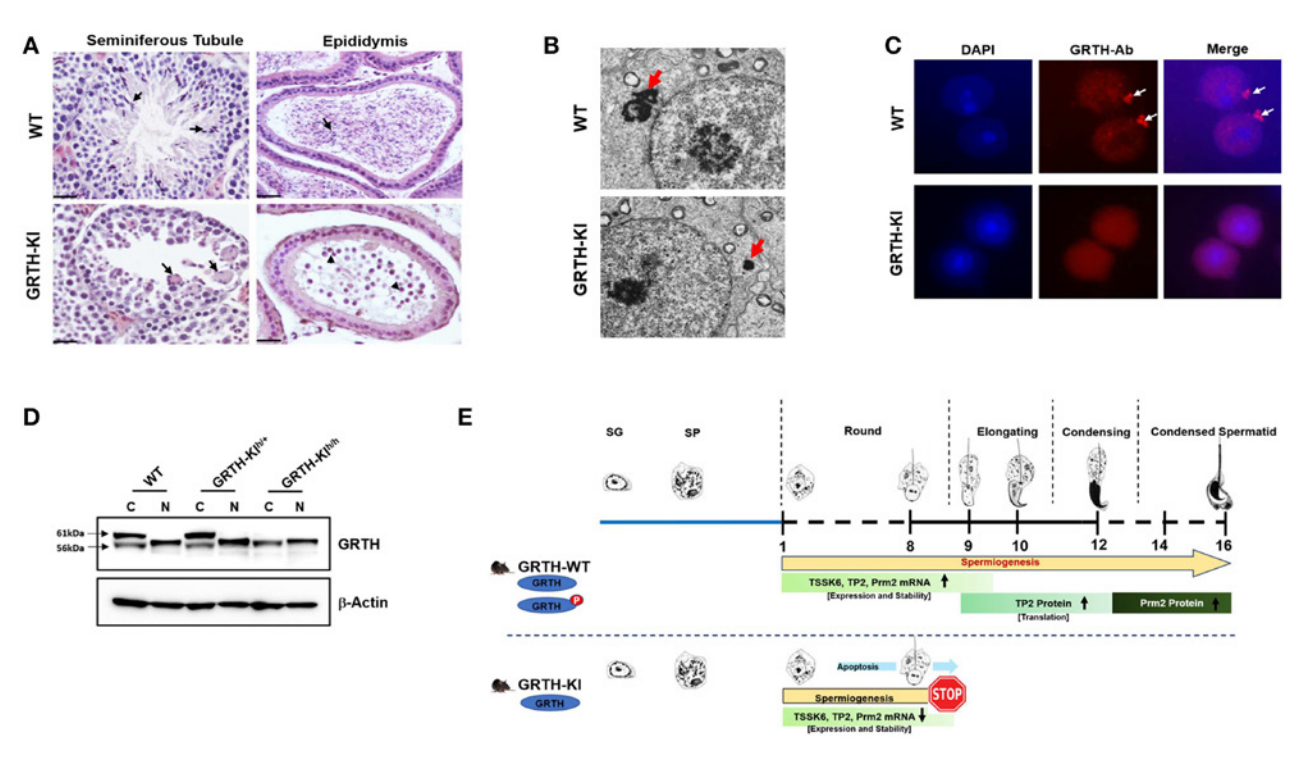

FIGURE 1. Essential role of phospho-GRTH (DDX25) in round spermatid during spermiogenesis

A. H&E staining of WT and GRTH–KI mice testis with the human R242H mutation that abolishes phosphorylation of GRTH at T239, showing the seminiferous tubules and epididymis. Lack of pGRTH abolishes the progress of spermatogenesis at step 8 of round spermatids (RS). In WT mice, seminiferous tubules (*arrows*) contain elongated spermatids, while GRTH–KI mice show the presence of degenerating multinucleated giant cells and the absence of elongated spermatids. The epididymis of WT mice is filled with mature sperm. In contrast, KI mice lack sperm and contain degenerating germ cells.

B. EM sections of testis show marked reduction in the size of the chromatoid body (CB) and of round spermatids (red arrows) in KI compared with WT (CB: non-membranous filamentous cytoplasmic body, which resides in the cytoplasm adjacent to the nucleus of RS and serves as a repository of long-lived mRNAs associated as mRNPs, awaiting translation during spermatogenesis).

C. Immunofluorescence staining of GRTH protein (*red*) in the nucleus, cytoplasm, and CB of round spermatids of WT mice. GRTH is absent from the CB of KI. Nuclear staining DAPI (*blue*).

D. Western Blot showing 56 KDa non-pGRTH expression in WT, heterozygous ($^{+/-}$) and homozygous ($^{-/-}$) KI mice, while 61 KDa pGRTH was absent from KI$^{-/-}$ mice compared with WT and KI$^{+/-}$ mice.

E. Diagram showing progression of mice spermatogenesis from spermatogonia to RS and the various subsequent steps that give rise to sperm in WT, while in KI$^{-/-}$ mice, owing to the lack of pGRTH, there is a blockade at step 8 of RS. In WT mice, during the process of spermiogenesis, we observed stable expression of Tssk6, TP2, and protamine 2 mRNAs in round spermatids; pGRTH plays a role in the stability of these germ cell–specific mRNAs until they are ready for translation at later steps, during elongation of spermatids. During spermatid elongation, transition proteins are replaced by protamines 1/2, and the chromatin is further condensed. The stability of relevant mRNAs is hampered in KI mice owing to the lack of phospho-GRTH, with a consequent reduction in levels of or abolition of relevant mRNAs expression and degradation [Reference 4].

in pachytene spermatocytes (PS) and RS. In contrast to knockout (KO) mice, KI mice showed no changes in miRNA biosynthesis, which excludes participation of pGRTH as transcriptional regulator of the microprocessor complex (the RNase III Drosha and the RNA–binding protein DCGR) that affects primiRNAs formation but indicates the participation of non-phospho GRTH in these processes. In KI mice, there is loss of chromatin remodeling and of related proteins including TP2, PRM2, and TSSK6. Significant decreases of their mRNA and half-lives indicate that their association with pGRTH in the cytoplasm protects these mRNAs from degradation. Also, our work showed that pGRTH stimulates TP2 translation in a 3' UTR–dependent manner [Reference 2].

In recent studies, we elucidated the GRTH phospho-site at a threonine (Tr239) structurally adjacent to the mutant site found in patients (R242H). Molecular modelling of the phospho-site, based on the RecA domain 1 of the DDX9 crystal structure, elucidated the relevant amino acids that formed the GRTH/PKA interface, solvent accessibility, and H-bonding. In addition to the core residues T239 and R242, these include amino acids E165, K240, and D237 [Reference 3]. Disruption of these relevant amino acids (single or double mutations) caused reduction in or abolition of the p-GRTH at Tr239, which is the cytoplasmic form of GRTH, essential for the progress of spermatogenesis beyond step 8 of round spermatids, with consequent lack of sperm formation. It is worth noting that the deleterious effects on GRTH phosphorylation caused by the mutations did not result from changes of protein kinase A alpha (PKAα) catalytic binding affinity but rather from consequential structural changes that can affect PKA catalytic efficiency. Studies based on the abolition of the phospho-form provide the basis for drug design and virtual and throughput screening for discovery of a reversible chemical inhibitor for use as male contraceptive [Reference 4].

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Nervous System Development and Plasticity

Healthy brain and cognitive development in children is central to the mission of NICHD. The prolonged postnatal period of brain development in humans, extending through adolescence and into early adult life, allows environmental experiences to influence brain structure and function. 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. In addition to synaptic plasticity, we are interested in novel mechanisms of activity-dependent nervous system plasticity that are particularly relevant to the period of childhood, including the involvement of glia (non-neuronal brain cells). 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 glia sense neural impulse activity and how activity-dependent regulation of glia contributes to development, plasticity, and the cellular mechanisms of learning. A major emphasis of our current research is to understand how myelin (white matter in the brain) is regulated by functional activity. By changing conduction velocity, activity-dependent myelination may be a non-synaptic form of plasticity, regulating nervous system function by optimizing the speed and synchrony of information transmission through neural networks. Our studies identified several cellular and molecular mechanisms for activity-dependent myelination, and the findings have important implications for normal brain development, learning, cognition, and psychiatric disorders. Our research shows that myelination of axons by glia (oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system [PNS]) is regulated by impulse activity, and we identified several molecular mechanisms that control proliferation and differentiation of myelinating glia and myelination. Most recently we determined that myelin thickness can be adjusted through a treadmilling process that adds and removes layers of myelin from the sheath to adjust conduction velocity and

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improve functional performance by optimizing spike time arrival at synapses. The findings provide evidence for a new form of nervous system plasticity and learning that would be particularly important in child development, but which also operates in adulthood, thereby improving function based on experience.

Learning is perhaps the most important function of childhood. Our research is determining the molecular mechanisms that convert short-term memory into long-term memory. 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 research shows 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 [AP-LTD]).

All 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 neurons and glia.

Regulation of myelination by neural impulse activity

Myelin, the multilayered membrane of insulation wrapped around nerve fibers (axons) by glial cells, is essential for proper neural impulse transmission and nervous system function. 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.

The biological significance of myelin is expanding as a result of our research findings. Myelin has been traditionally viewed in terms of conduction failure after damage (for example in multiple sclerosis), but we are exploring how myelin and changes in myelin affect spike time arrival, the frequency, phase, and amplitude coupling of oscillations in the brain, as well as the propagation of brain waves. Many neurological and psychological dysfunctions can develop when optimal neural synchrony of spike-time arrival and the appropriate conduction latencies required to sustain neural oscillations are disturbed. Abnormalities in brain waves and synchrony are associated with many psychiatric and developmental conditions, including, among others, schizophrenia, epilepsy, dyslexia, and autism.

Our research shows that neurotransmitters that are released along axons firing action potentials activate receptors on myelinating glia, 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 triggers 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

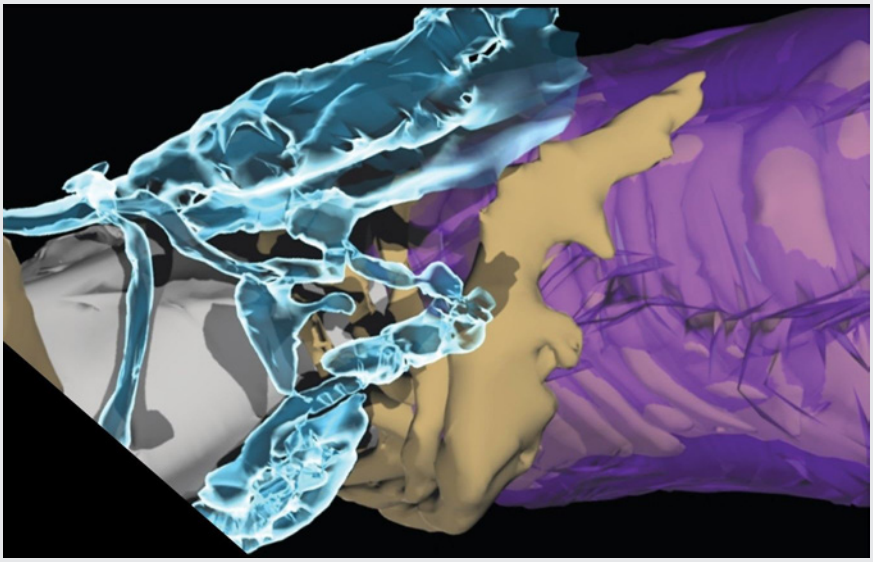

FIGURE 1. Myelin plasticity to modulate impulse conduction velocity

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 thrombin-dependent cleavage of the cell-adhesion molecule NF155. 3D reconstruction from electron microscopy.

electrically inactive axons, thus regulating myelination of axons and neural circuit function according to functional experience. This would be particularly important, for example, in the adolescent brain, where environmental experience during sensitive periods can have long-lasting effects on neural circuit development and behavior. The findings are also relevant to such demyelinating disorders as multiple sclerosis and to remyelination after axon injury.

MODIFICATION OF MYELIN STRUCTURE AND CONDUCTION VELOCITY BY ASTROCYTES

Given that optimal neural circuit function and synaptic plasticity require the proper impulse transmission speed through all axons to induce spike timing–dependent 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 the 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, the myelin sheath is thickened during the growth of axons, but once formed, myelin structure was believed to be static. Prior to our research 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 achieve optimal spike-time arrival from inputs that arrive at relay points in neural networks too soon.

Our research 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 nodes 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 the latter to detach and rendering 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. Our findings uncover 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 discovery that glutamatergic transmission between axons and oligodendrocytes triggers myelination led us to propose that impairments in myelination caused by disrupted neurotransmission from axons to oligodendrocytes may be an underlying cause of Gulf War Illness. Our research, published this year [Reference 3], shows that proliferation and development of oligodendrocytes is affected in an animal model of Gulf War illness and in cell cultures exposed to agents like sarin nerve gas (acetylcholinesterase inhibitors). Perturbations by these agents of axon-glial interactions that take place through acetylcholine signaling could have long-lasting consequences in neural network functions underlying many of the symptoms associated with Gulf War Illness, including difficulties with working memory, mental focus, chronic pain, and others. Organophosphate pesticides operate in a similar manner, and exposures to pesticide contamination, especially in childhood, would impair normal development of oligodendrocytes and myelin formation, contributing to cognitive and psychological dysfunctions.

Regulation of gene expression by action potential firing patterns

All information in the nervous system is encoded in the temporal pattern of neural impulse firing. Long-lasting changes in the nervous system require regulated gene expression, but how neuronal firing patterns control gene transcription is a fundamental question central to the processes of experience-dependent plasticity during development and learning. Our experiments are revealing the intracellular signaling and gene-regulatory networks that respond selectively to appropriate temporal patterns of action-potential firing to generate adaptive responses.

Long-lasting changes in the nervous system require regulation of gene expression, and environmental experience can drive changes in nervous system plasticity. 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. Live-cell calcium imaging shows that temporal aspects of intracellular calcium signaling are particularly important in regulating gene expression according to neural-impulse firing patterns in normal and pathological conditions. After stimulation, we measured mRNA and protein expression by gene microarrays, quantitative RT-PCR (reverse transcriptase–polymerase chain reaction), RNA-seq (RNA sequencing), 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, our research shows that regulation of gene expression in neurons by specific temporal patterns of impulse activity is not a property of special genes; in general, the neuronal transcriptome is highly regulated by the pattern of membrane depolarization, with hundreds of genes differentially regulated by the temporal code of neuronal firing.

We are also pioneering new methods of transcriptional analysis in neurons. The standard approach to analyzing gene expression is by measuring the abundance of tens of thousands specific gene transcripts in cells by microarray or RNA-seq, as described above, but this approach fails to capture the unique feature of transcriptional regulation in neurons. In contrast to other cells responding to external signals that may drive cells to a steady-state equilibrium, transcriptional networks in neurons are continually being modulated dynamically by temporally varying action potential firing frequencies and burst patterns, together with synchrony and phase relationships among populations of interconnected neurons. Such activity may not alter the abundance of a gene transcript significantly; nevertheless, the coordinated activity within transcriptional networks is being modulated dynamically to modify function.

To address this problem, we applied a covariance approach, using a Pearson correlation analysis to determine how pairs of genes in mouse dorsal root ganglion (DRG) neurons are coordinately expressed in response to stimulation producing the same number of action potentials in different temporal patterns. Our analysis of 4,728 distinct gene pairs related to calcium signaling, 435,711 pairs of transcription factors, 820 pairs of voltage-gated ion channels, and 86,862 calcium signaling genes paired with transcription factors, indicates that genes become coordinately activated by distinct action potential firing patterns. Thus, in addition to regulating the expression level of numerous genes, the temporal pattern of action potential firing profoundly modulates how genes are networked in functional pathways.

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.

DIFFERENCES IN CHROMATIN STRUCTURE BETWEEN NEURONS AND GLIA

In collaboration with David Clark, our research is revealing fundamental differences in chromatin structure between neurons and glia. Chromatin can be visualized by electron microscopy as regularly spaced 'beads-on-a-string,' in which the beads represent nucleosome cores and the string is the intervening linker DNA. Using MNase digestion and RNA-seq, we compared the chromatin structure of purified mouse DRG neurons, cortical oligodendrocyte precursor cells (OPCs), and cortical astrocytes. We found that DRG neurons have shorter average nucleosome spacing (approximately 165 base pairs) than either glial cells (OPCs, with approximately 182 base pair spacing) or astrocytes (with approximately 183 base pairs). The significance of these basic differences in chromatin structure between DRG neurons and these glial cells is unknown and is currently being investigated. Interestingly, the atypical nucleosome spacing of neuronal chromatin does not extend to promoter-proximal regions.

Synaptic plasticity

It is widely appreciated that there are two types of memory, short-term and long-term, and that sleep plays a critical role in memory consolidation. Gene expression is necessary to convert short-term into long-term memory, and our research concerns how signals reach the nucleus to initiate this process and 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, RNA-seq, calcium imaging, and two-photon *in vivo* imaging to investigate the signaling pathways, genes, and proteins involved in LTP and LTD in primary cell culture and hippocampal

brain slice. 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.

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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 noninvasive and wearable imaging technique that assesses brain function and is suitable for studies of children and toddlers, especially those with neurodevelopmental 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 functional magnetic resonance imaging (fMRI).

In one line of research we use fNIRS to examine prefrontal cortical activation as it relates to developmental level in toddlers. Specifically, we examined brain activity in prefrontal regions in 24-month-old toddlers while they listened to speech sounds or watched gesture production, while we simultaneously recorded fNIRS in the prefrontal cortex (PFC). The stimuli allowed us to contrast brain activation across different types of communication and communicative intent. Our most recent publication demonstrated differential activation to gesture compared with speech stimuli, as well as differential activation to meaningful versus non-meaningful stimuli. Importantly, the differences in mean activation in the left PFC in response to meaningful gesture (when controlling for meaningless gesture) at age 2 predicted verbal ability at age 3 (Figure 1A). Differences in mean activation in response to meaningful speech compared with meaningful gesture at age 2 also predicted verbal ability at age 3 (Figure 1B). The findings may reflect potential biomarkers for aspects of language development.

In another study, we are using fNIRS combined with electroencephalography (EEG) to measure brain activity in the mirror-neuron network (MNN). The MNN is associated with the development of sophisticated social behaviors that emerge in typical infants.

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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. Through a clinical protocol "Mirror neuron network dysfunction as an early biomarker of neurodevelopmental disorder" (18-CH-N001), we are now finalizing our adult pilot study (n= 52) to determine whether MNN activation can be elicited, using a motor observation and a simultaneous execution paradigm and EEG/fNIRS systems. Preliminary results show a strong negative correlation between mu rhythm suppression in EEG electrodes and fNIRS channels for action execution (Figure 2A). In addition, as hypothesized, the greatest correlations are found between EEG electrodes placed on central clusters (C3) and fNIRS channels placed on the left somato-sensorimotor regions, namely the left postcentral gyrus, left superior frontal gyrus, and left precentral gyrus (Figure 2B). We will further examine the synchronicity of these signals using more advanced machine-learning methods to examine how the features from both signals relate to each other and to help characterize brain function in the MNN. We will start recruiting typically developing infants (n=60) and infants at risk for developmental delays (n=60) from 9–12 months of age for the second phase of this project by January 2020. 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.

In a collaboration with Andrea Gropman, we are also examining brain function in patients with urea-cycle disorders (UCD). UCDs are a set of rare genetic disorders caused by the loss of those enzymatic activities

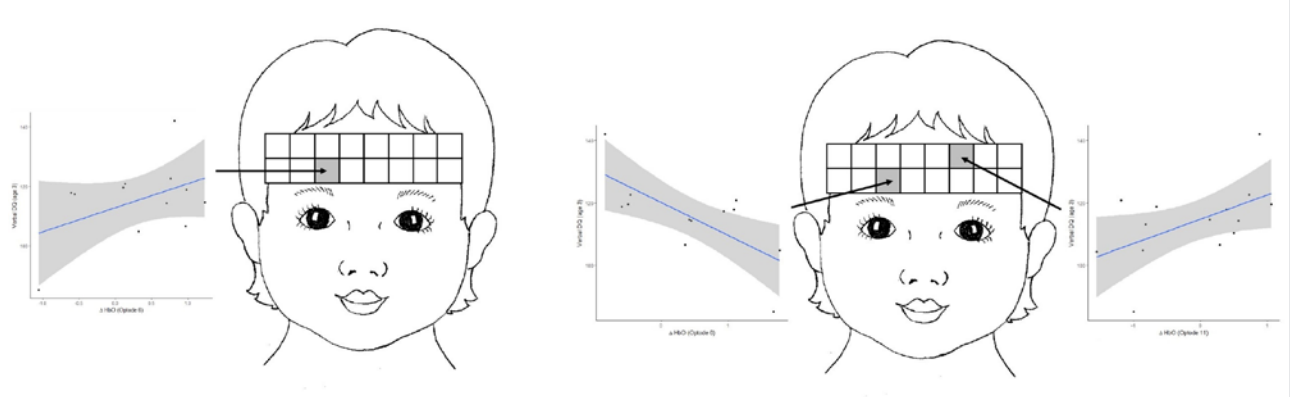

FIGURE 1. Developmental quotient (DQ) and age

A. Difference in HbO z-scores between meaningful gesture and non-meaningful gesture conditions predicts verbal developmental quotient at age three.

B. The difference in HbO z-scores between meaningful speech and meaningful gesture conditions predicts verbal DQ at age three.

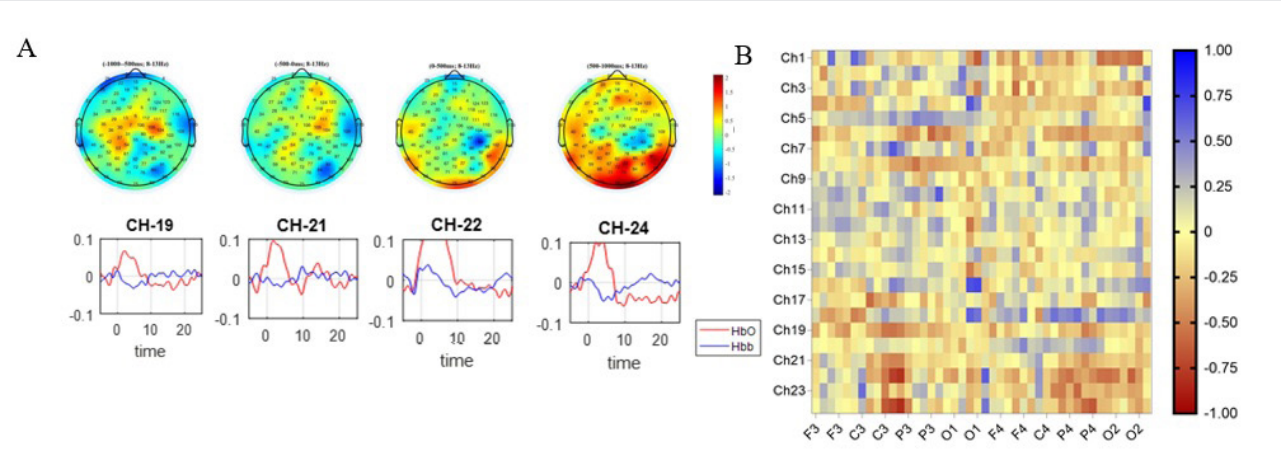

FIGURE 2. Mirror neuron network assessed with fNIRS and EEG

A, top. Topoplot of action execution between –1000 ms and +1000 ms of start action.

A, bottom. Hemodynamic response function in channels recorded from channels placed over left postcentral gyrus, left superior frontal gyrus, and left precentral gyrus (channels 19, 21, 22, 24).

B. Heat map showing the correlations between the 2 signals. The greatest negative correlation is found between fNIRS channels placed over the left postcentral gyrus, left superior frontal gyrus, and left precentral gyrus and EEG electrodes placed on C3 and P3 clusters.

(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. We used fNIRS to measure brain activity in the PFC while patients performed a working memory task, namely a Stroop Interference task. Our results show that activation based on changes in HbO signal in left PFC is higher in controls than in UCD subjects. In addition, we used the concept of Hilbert Transform to calculate the instantaneous amplitude of total hemoglobin (HbO+HbR) in very low frequency band (VLF, <0.03 Hz) related to cerebral autoregulation. We applied this approach on a twin study of siblings with and without UCD. Our results, to be published soon, showed that, in the VLF region, the UCD sibling exhibited a lower degree of oscillation in instantaneous hemodynamic amplitude than did the control sibling during performance of working memory task.

We also used fNIRS to examine working memory in typically developing adults. To this end, we used an N-back working memory task, which provides a measure of mental workload. We tested 23 typically developing adults while fNIRS was recorded in the PFC. We compared the two task levels and the beginning and end of the 3-back task. Our findings indicated that there was a significant difference between the two task levels as a result of the task complexity. We also found a difference between the beginning and end of the 3-back task. We interpreted these findings as an improvement of subject's performance resulting from the learning process of the brain. Thus, more correct answers were recorded, and less hemodynamic activation was seen, at the end of the 3-back task [Reference 1].

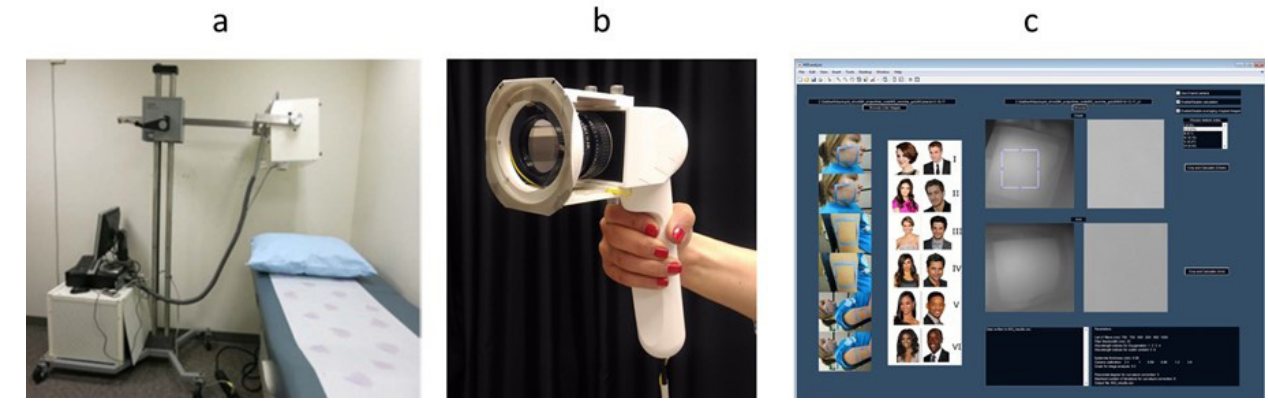

FIGURE 3. Multispectral Imager from portable to point of care

Left. Portable multispectral imaging device.

Middle. New hand-held multispectral imager.

Right. Graphical user interface for data analysis.

We conducted a study to examine neural activation during a 'go/no-go' behavior inhibition task that activates PFC 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. The analysis is ongoing, with the goal of submitting a manuscript by the end of 2019. We also drafted a manuscript examining heart-rate dynamics in relation to prefrontal activation, which will be submitted as part of a special issue in February 2020.

We used machine learning as a tool to identify features from fNIRS signal during a mental arithmetic task. We identified a set of features from the HbO signal to determine functionally connected channels during the task, along with latency differences among different brain regions. The features extracted to characterize functional connectivity during MA included the mean, variance, start, and slope of oxygenation activity. Three clusters corresponding to channels placed across the dorsolateral PFC (DLPFC), the temporal cortex, the posterior superior frontal cortex, and the ventrolateral PFC (VLPFC) were formed across different experimental runs for each participant, depicting functionally connected regions. The results indicated earlier activation of medial PFC than in the DLPFC, which might be related to the recruitment of retrieval-based techniques before the recruitment of additional cognitive skills such as a combination of decomposition, retrieval-based techniques, and updating processes. Our study shows that the technique can show temporal differences in activation across regions of the cortex and has the potential to distinguish between typical and impaired brain connectivity while performing a cognitive task [Reference 2].

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. We are assessing facial plethora in Cushing's syndrome (CS), as it was 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 noninvasive multispectral NIR imaging on the right cheek of patients before and after surgery. Patients were defined as cured by postoperative 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 volume fraction, is correlated with the cure of CS. The first set of results were published in 2015. In our follow-up paper [Reference 4], we also showed that water content fraction could be used as a new biomarker of early cure in patients with CS. We recorded data for 29 new patients, and follow-up imaging was done for 26 patients. We developed and tested the new hand-held system that has improved performance over the existing portable system. We plan to use this system as a point-of-care imaging device. In brief, the new imager uses a high-resolution CMOS camera with on-chip filters. Images are acquired simultaneously at eight different near-infrared wavelengths (700–980 nm). Our graphical user interface (Figure 3c) now supports both portable and the hand-held multispectral imagers.

Annually, about 15 million preterm infants are born in the world. Of these, about 1 million would die before the age of five because of complications resulting from their premature birth. Given that the high incidence of preterm birth (PTB) is partially the result of the lack of effective diagnostic modalities, methodologies are needed to determine the risk of PTB. We proposed a noninvasive tool based on polarized light imaging aimed at measuring the organization of collagen in the cervix. Cervical collagen has been shown to remodel with the approach of parturition. We used a full-field Mueller matrix polarimetric colposcope to assess and compare cervical collagen content and structure in nonpregnant and pregnant women *in vivo*. Local collagen directional azimuth was used and a total of eight cervices were imaged. In continued collaboration with Jessica Ramella-Roman on preterm pregnancy complications, 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 PTB or a sonographic short cervix) patients. 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 preterm 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 [Reference 5].

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. A placental oximeter should be light-weight, relatively small, 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. We fabricated a wearable oximeter using fNIRS technique for dynamic *in vivo* monitoring of anterior placental oxygenation. We intend to find the baseline placental oxygenation for normal pregnancies to standardize the oxygenation data across pregnancies and correlate pregnancy outcome with the placental oxygenation. In parallel, we are using a novel technique called Dynamic Full-Field Optical Coherence Tomography (DFFOCT) to study placental cell metabolism *in vitro* at physiologically relevant variations of oxygen levels.

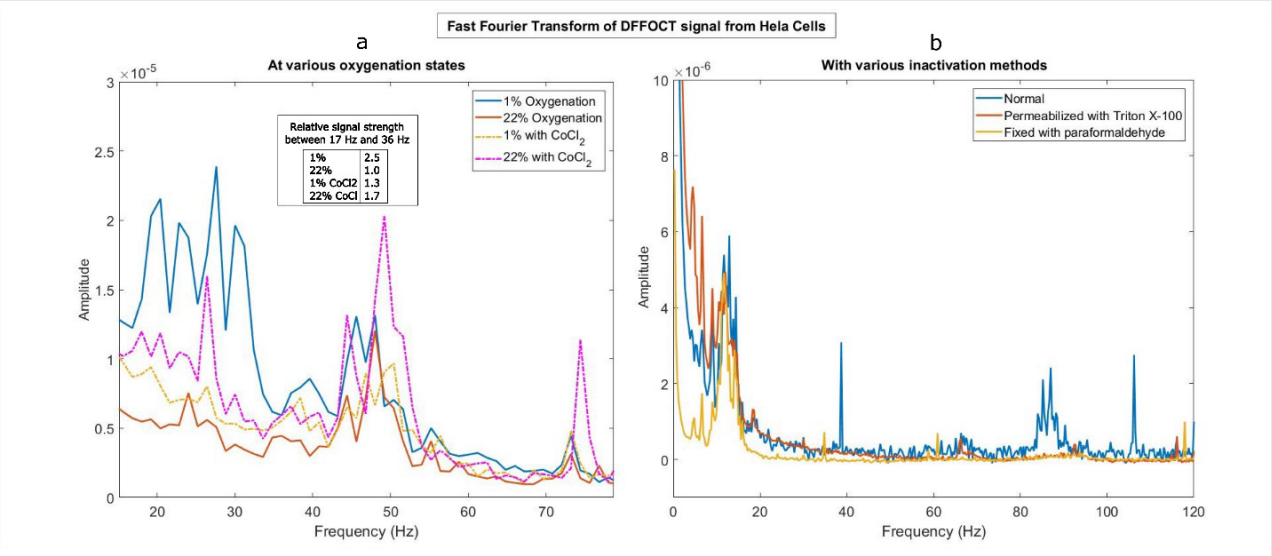

FIGURE 4. Dynamic full-field optical coherence tomography for cell dynamics

a. FFT spectra of HeLa cells grown under the following conditions: oxygenation of 22% (*red*), 1% (*blue*), 22% with $CoCl_2$ (*pink, dotted*) and 1% with $CoCl_2$ (*yellow, dotted*). Inset shows the normalized strength of the spectra in the range 17Hz to 36 Hz as integration (normalized with respect to 22% oxygenation).

b. FFT spectra of Hela Cells under three conditions: normal (*blue*), permeabilized with TritonX 100 (*red*), and fixed with 4% paraformaldehyde for 20 minutes (*yellow*).

Our oximeter device for *in vivo* studies is fast, non-invasive, and wearable, so that it allows continuous measurement of the oxygenation of the anterior placenta in a subject-friendly environment. The light-weight compact system is flexible and can thus be positioned at various abdominal locations for localized measurement of oxygenation. The NIRS device uses light in near-infrared region (760 and 840 nm) and consists of six 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 method to negate the effect of maternal tissues on the measurements using our multi-layer tissue model based on Monte Carlo simulation. The model includes the optical properties of skin, fat, uterus, and placental tissue. We further measured the optical properties of the placenta *ex vivo* using dual-wavelength LED sources with a higher resolution photodiode array unit 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 Shad Deering and with Roberto Romero's Maternal-Fetal Medicine, Imaging, and Behavioral Development Affinity Group, 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 and *ex vivo* placental tissue measurements. 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.

We established DFFOCT as a useful tool to detect the changes in intracellular activity as a function of ambient oxygenation. The *in vitro* experiments were performed on HeLa cells. DFFOCT signal from the cells grown on porous membrane was isolated using segmentation algorithms and analyzed using Fourier transform to understand the dynamic activity occurring within the cells. Signals from cells grown under hypoxia and physoxia (physiological oxygenation) show unique differences in the Fourier spectra under these conditions. Control experiments with cobalt chloride were used to mimic hypoxia. We also ran Western blots to confirm the effects of hypoxia on expression of the Hif-1α protein in the cells. Further, to identify the cellular activities that are responsible for DFFOCT signal, we inactivated cells in a variety of ways, such as para-formaldehyde fixation and treatment with Triton x100. The experiments show that loss of DFFOCT signal correlates with the treatment (Figure 4). Currently, we are investigating the cellular mechanisms affected by oxygenation by designing precise control experiments. This will help us use DFFOCT as a tool to assess activity of placental cells under altered oxygenation conditions.

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 the GTP–bound eukaryotic initiation 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, eIF5, eIF3, 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 the helicase Dhh1; (4) elucidate the *in vivo* functions of yeast orthologs of eIF2D and the MCT-1/DENR complex in 40S ribosome recycling at stop codons and reinitiation in 3' untranslated regions *in vivo*; and (5) elucidate the roles of yeast orthologs of eIF2A and eIF2D in eIF2–independent initiation of translation in stress conditions.

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 limitation, transcription of these genes is coordinately induced by the

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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 [Rawal Y, et al. *Genes Dev* 2018;32:695].

eIF1 interactions with Met–tRNAi and eIF2β 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 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 reductions 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 eIF1A N-terminal tail (NTT), which 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* [Thakur A, Hinnebusch AG. *Proc Natl Acad Sci USA* 2018;115:E4159].

The β-subunit of eIF2 interacts with eIF1, eIF1A, and the anticodon stem of tRNAi exclusively in the open complex and thus should exclusively stabilize the scanning conformation of the PIC. Supporting this assumption, eIF2β and eIF1 substitutions designed to weaken their mutual interaction increase UUG initiation *in vivo* and stabilize TC binding at UUG codons in reconstituted PICs *in vitro*. Moreover, compound substitutions at the interface additionally derepress *GCN4* translation, signifying a reduced rate of TC loading to the PIC, which was also reconstituted *in vitro*. Such genetic and biochemical phenotypes indicate destabilization of the open complex and a shift to the closed/P_{IN} state. Remarkably, an eIF1 substitution designed to strengthen, not

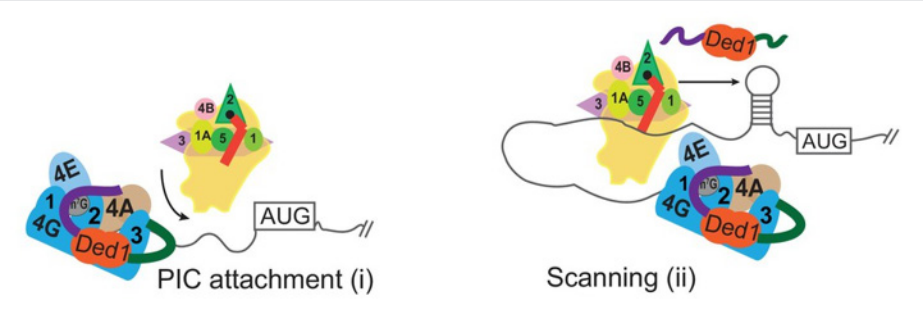

FIGURE 1. Different modes of Ded1 function in stimulating PIC attachment to mRNA or subsequent scanning for the start codon

Model depicting how different mRNAs might differ in the extent to which attachment to the mRNA or subsequent scanning of the leader sequence are the rate-limiting steps in 48S PIC assembly. Depending on which step is rate-limiting, the requirements for Ded1, either acting alone or within the eIF4G·eIF4E·eIF4A·Ded1 complex, could be different on different mRNAs.

weaken, the eIF2β:eIF1 interface had the opposite genetic and biochemical phenotypes. The position of eIF2β in the open complex is also predicted to clash with Met-tRNA$_i$ in the closed/P$_{IN}$ state, and substitutions designed to diminish this clash increased UUG initiation *in vivo* and stabilized Met-tRNA$_i$ binding at UUG codons *in vitro*, but they did not confer a Gcd$^-$ phenotype and had little effect on TC loading *in vitro*, thus showing that eIF2β's clash with Met-tRNA$_i$ disfavors transition to the closed complex without affecting TC binding to the open complex. In summary, eIF2β resembles eIF1 in both (1) stabilizing the open/P$_{OUT}$ conformation by its direct binding to eIF1 and Met-tRNA$_i$, and (2) impeding transition to P$_{IN}$ at non-AUG codons through a clash with tRNA$_i$.

Reconstitution of RNA helicase Ded1 function in eIF4F/eIF4A–dependent acceleration of 48S PIC assembly on structured native mRNAs

RNA helicases eIF4A and Ded1 are believed to resolve mRNA structures that impede ribosome attachment or scanning to the start codon, but whether they perform distinct functions *in vivo* has not been unequivocally established. Previously, we compared the effects of mutations in Ded1 or eIF4A on genome-wide translational efficiencies (TEs) by ribosome profiling. Despite similar reductions in bulk translation, inactivation of Ded1 substantially reduced the relative TEs of more than 600 mRNAs, whereas inactivation of eIF4A affected less than 40 mRNAs in a similar manner. Ded1–dependent mRNAs show greater than average 5' UTR lengths and propensity for secondary structures, implicating Ded1 in scanning though structured 5' UTRs. We measured the kinetics of 48S PIC assembly in the yeast reconstituted system for the native mRNAs that we identified as being Ded1 hyper- or hypodependent *in vivo* by ribosome profiling. Whereas eIF4A was essential for 48S PIC assembly on all mRNAs tested, Ded1–hypodependent mRNAs could be recruited rapidly without Ded1, and addition of Ded1 only moderately accelerated their recruitment. Ded1–hyperdependent mRNAs, by contrast, were recruited poorly in the absence of Ded1, and Ded1 greatly accelerated their recruitment. Eliminating stem-loop (SL) structures in the 5' UTRs enhanced Ded1–independent recruitment, and diminished Ded1 acceleration of 48S assembly, on several hyperdependent mRNAs. Inserting SLs into a synthetic unstructured 5' UTR conferred a strong Ded1 requirement for rapid recruitment. Previous biochemical findings indicated that Ded1 unwinding activity is stimulated by its interactions with eIF4A and eIF4G, subunits of the cap–binding complex eIF4F. Consistent with this, we found that eliminating domains in Ded1 that mediate its association with eIF4A or eIF4G increased the Ded1 concentration required for maximal rate acceleration

($K_{1/2}^{Ded1}$) and, for some mRNAs, also reduced the maximal rate achieved with saturating Ded1 (k_{max}). Remarkably, the requirements for particular Ded1 interactions with the various components of eIF4F differed substantially for different mRNAs. Thus, Ded1 accelerates 48S assembly by resolving 5' UTR structures in a manner stimulated by its interactions with eIF4G and eIF4A, supporting the model that Ded1 operates primarily in the context of the quaternary complex eIF4E/eIF4G/eIF4A/Ded1.

Functional interplay between RNA helicases Ded1 and Dbp1 in stimulating translation of structured mRNAs *in vivo*

To illuminate the *in vivo* function of the Ded1 paralog Dbp1, we conducted ribosome profiling of *dbp1Δ* and *dbp1Δded1* double mutants. The results indicated that Dbp1 functionally cooperates with Ded1 throughout the translatome in stimulating translation of mRNAs with long, structure-prone 5' UTRs, as the TE reductions in the double mutant generally exceeded those in the *ded1* single mutant. For many such mRNAs, Dbp1 largely masks the involvement of Ded1. Importantly, Dbp1 mimics Ded1 in accelerating 48S PIC assembly in the reconstituted system on Ded1–hyperdependent mRNAs containing structured 5' UTRs. Using the recently developed method of TCP-seq, for genome-wide profiling of 40S subunits to quantify PIC occupancies in 5' UTRs, we found that 40S subunits tend to accumulate in the 5' UTRs of mRNAs in the helicase mutants, particularly for mRNAs judged to be Ded1/Dbp1–hyperdependent by conventional 80S profiling, providing direct evidence that Ded1/Dbp1 stimulate scanning through structured 5' UTRs *in vivo* to enhance translation. We also uncovered cooperation between these helicases in promoting 43S PIC attachment to a subset of helicase-dependent mRNAs, which exhibit reduced 40S occupancies in 5' UTRs in the helicase double mutant.

Conserved mRNA–granule component Scd6 utilizes helicase Dhh1 to repress translation initiation and activates Dcp2–mediated mRNA decay *in vivo.*

Scd6 protein family members are evolutionarily conserved components of mRNA granules. Scd6, and two other proteins with RGG domains, Sbp1 and Npl3, were implicated as translational repressors that act by binding to the RNA2 or RNA3 domains of eIF4G. Scd6 can interact with Dcp2, a catalytic subunit of decapping enzyme, and also with the decapping activator and general translational repressor Dhh1. To provide evidence that Scd6, Sbp1, or Npl3 can function as repressors of particular mRNAs in cells, we tethered each protein, as a fusion to MS2 viral coat protein, to a GFP reporter mRNA containing MS2 coat-protein binding sites in the 3' UTR region, in wild-type (WT) cells or mutants lacking *DCP2* or *DHH1*. We found that tethering Scd6, but not Sbp1 or Npl3, repressed reporter mRNA abundance in a manner requiring Dcp2, and that it also suppressed reporter mRNA translation via Dhh1. Ribosome profiling and RNA-Seq analysis of *scd6Δ* and *dhh1Δ* mutants, and of double mutants also lacking *DCP2*, indicated that Scd6 cooperates with Dhh1 in both degradation and translational repression of a group of native mRNAs, and that both processes require *DCP2*, as derepression of both mRNA levels and TEs on deletion of *SCD6* or *DHH1* was suppressed by *dcp2Δ*. These last findings lead to the surprising conclusion that translational repression conferred by Scd6 and Dhh1 depend on the decapping enzyme, and possibly generates a pool of uncapped but relatively stable mRNAs of low TEs owing to their inability to bind eIF4F.

Chromatin remodeler Ino80C acts independently of histone variant H2A.Z to evict promoter nucleosomes and stimulate transcription of highly expressed genes.

Previously, we used histone H3 ChIP-Seq analysis to show that that the ATP–dependent chromatin remodeling complexes SWI/SNF and RSC cooperate in achieving wide, nucleosome-depleted regions in the promoters of

genes transcriptionally activated by Gcn4, by evicting and repositioning the –1 and +1 promoter nucleosomes, and that defects in this process in mutants lacking these chromatin remodelers (CRs) are associated with reduced transcription. Similar findings were made for the small subset of about 200 most highly transcribed, constitutively expressed genes, suggesting a general cooperation by these CRs in achieving high-level transcription in yeast. The CR Ino80C has been implicated in nucleosome editing by catalyzing replacement of the histone variant H2A.Z (encoded by *HTZ1*) with canonical H2A. The removal of an H2A.Z:H2B dimer by Ino80C could render the partially disassembled nucleosome more susceptible to eviction; however, an important role for Ino80C in promoter nucleosome eviction had not been reported. By analyzing an *ino80Δ* mutant lacking the catalytic subunit of Ino80C, we found that this CR functions on *par* with SWI/SNF in eviction of promoter nucleosomes and transcriptional activation of Gcn4 target genes, and that it plays an even greater role than SWI/SNF at a group of several hundred Ino80C–hyperdependent genes. At Gcn4 target genes, defects in nucleosome eviction are accompanied by reduced promoter occupancies of TATA–binding protein, and hence PIC assembly, and also with reduced transcription. ChIP-seq analysis of Ino80 itself shows that Ino80C is enriched at both Gcn4 target genes and Ino80C–hyperdependent genes.

If Ino80C enhances nucleosome eviction strictly in the course of editing H2A.Z-H2B dimers, then deleting *HTZ1* should mimic the effect of deleting *INO80* on promoter nucleosome eviction. Moreover, depleting Ino80 should have no effect on nucleosome occupancies in cells lacking *HTZ1.* At odds with these predictions, the *htz1Δ* mutation was found to have much smaller effects than *ino80Δ* on eviction of promoter nucleosomes. Moreover, depleting Ino80 from the nucleus by "anchor-away" impaired histone eviction in the absence of *HTZ1*. Thus, Ino80C can function similarly to SWI/SNF family members SWI/SNF and RSC in promoting chromatin access independently of nucleosome editing.

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 we 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 ChIP-seq analysis of RNA polymerase 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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Role of voltage-gated ion channels in synaptic development and disease

REGULATION OF POTASSIUM CHANNEL TRAFFICKING AND FUNCTION

Kv4.2 channels, the major contributors to somatodendritic A-type

potassium 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, co-expression 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, former Postbaccalaureate Fellow Travis Tabor generated bungarotoxin binding site–tagged Kv4.2 at the second extracellular loop for visualizing Kv4.2 in live neurons while in our lab. 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.

In an effort to study Kv4.2 trafficking in the context of ion channel homeostasis during perturbations to neuron intrinsic excitability, we discovered quantitative and qualitative differences in microtubule-based transport in axons versus dendrites. Adriano Bellotti characterized the differences by recording time series of over 500 neurites, and we validated an unexpected result using mathematical models of cargo transport. We developed a deterministic model that corroborates differences in cargo frequency and a stochastic model that validates differences in puncta speed, superdiffusivity, and frequency in axons vs dendrites. In a secondary project, we are studying deterministic models of nonlinear cargo transport with feedback control and with branching. We are now embarking on such simulations with realistic pyramidal cell morphologies.

Travis Tabor developed a novel tool for labeling only the cell-surface fraction of Kv4.2 channels. The tool will be

useful for studying surface expression levels and surface trafficking of Kv4.2 channels.

CA^{2+} 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 Ca^{2+} via R-type voltage-gated Ca^{2+} channels (Cav2.3). Ca^{2+} regulation of Kv4.2 channels occurs despite an apparent lack of the structural determinants of the canonical Ca^{2+}-activated K^+ channels. We hypothesized that KChIP auxiliary proteins imbue Kv4 channels with Ca^{2+} sensitivity, as they contain four calcium-binding EF–hand domains (helix-loop-helix structural domains), two of which bind Ca^{2+}.

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 Ca^{2+}. Under low Ca^{2+} 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 Ca^{2+}, 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 Ca^{2+} 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 many 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 Ca^{2+} sensitivity of longer KChIP2 isoforms. Ongoing studies are aimed at determining whether this previously unidentified polybasic domain regulates the plasma membrane trafficking and Ca^{2+} sensitivity of Kv4–KChIP2 complexes.

Proteomic and subcellular localization studies suggest that Cav2.3–containing voltage-gated calcium channels are a potential calcium source for a modulatory effect on Kv4.2–mediated A-type K currents (IA) in CA1 hippocampal neurons. While in our lab, former Visiting Fellow 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 co-immunoprecipitation (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 downstream-lying subiculum pyramidal neurons.

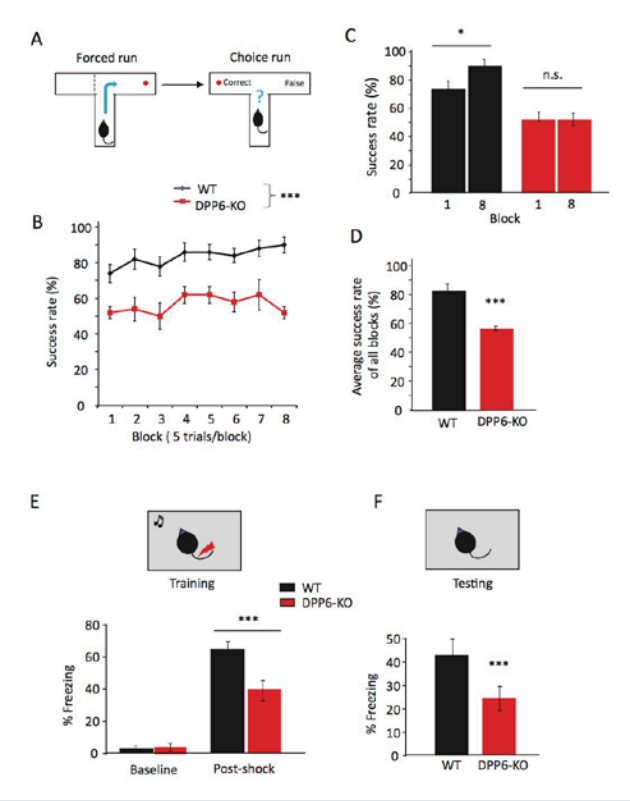

FIGURE 1. DPP6-KO mice show impaired spatial working memory in the T-maze and contextual fear conditioning task.

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; $p < 0.001$).

We found that signaling through metabotropic glutamate receptors supports activity-dependent translation of the voltage-gated calcium channel Cav2.3. The regulation is dependent on the Fragile X mental retardation protein (FMRP) and is therefore lost in FMRP KO mice. The KO mice exhibit exaggerated levels of Cav2.3, which likely contributes to the dendritic hyperexcitability found by other groups.

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

Well known as an auxiliary subunit of Kv4.2, DPP6 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. More recently, we observed that DPP6 KO mice have significantly smaller brains than control mice. To determine which regions are affected, we performed *in vivo* MRI to scan the live mouse brain. Our results showed that DPP6–KO mice have significantly reduced volume specifically in the hippocampus and cerebellum. Our findings indicate that DPP6 loss drives microcephaly and learning and memory impairment in DPP6–KO mice.

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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 adeno-associated 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.

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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 postmitotic neuroectoderm-derived cells, the approach can provide a permanent source of enzyme production for secretion into the CSF and penetration into cerebral and cerebellar structures. We are using a mouse model to evaluate choroid plexus transduction by several recombinant AAV (rAAV) vector serotypes and

to determine the posttreatment lysosomal alpha-mannosidase (LAMAN) activity and distribution in the brain globally. These preclinical studies open the door to IND (investigational new drug)–generating studies for a future first-in-human clinical trial.

In a natural history study of alpha-mannosidosis, 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 potentially useful benchmarks for assessing response to investigational treatments.

ATP7A–related disorders, including motor neuron degeneration

ATP7A is an evolutionarily conserved copper-transporting P-type ATPase associated with X-linked recessive Menkes disease and occipital horn syndrome, a milder version of this phenotype. Based on clinical trials conducted by the Section using subcutaneous injections of a lyophilized form of copper histidinate (CuHis), this new molecular entity was granted Fast Track status by the FDA and is anticipated to be the first federally approved treatment for this difficult neurometabolic disorder. Recent companion preclinical studies in a mouse model of Menkes' disease illustrate the synergistic effect of AAV serotype 9 gene therapy with subcutaneous CuHis. The Section's therapeutic advances in Menkes' disease highlight the importance of future newborn screening for this disorder, for which targeted next-generation sequencing appears to be a reliable and cost-effective approach.

Recently, two unique ATP7A missense mutations, T994I and P1386S, were shown to cause adult-onset isolated distal motor neuropathy without the 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's disease and frontotemporal 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's 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 culture cells and studied ATP7A trafficking in response to copper stimulation. For these experiments, we overexpressed 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 knock-out 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 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 immunoprivileged 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

- U01-CH-079066-04. Choroid plexus-directed gene therapy for alpha-mannosidosis
- U01-HL121842-01A1. Phase II study of AAV9-GAA gene transfer in Pompe disease
- Cooperative Research and Development Agreement (CRADA) with Cyprium Therapeutics, Inc. New York, NY

Publications

1. Sharma P, Reichert M, Lu Y, Markello TC, Adams DR, Steinbach PJ, Fuqua BK, Parisi X, Kaler SG, Vulpe CD, Anderson GJ, Gahl WA, Malicdan MCV. Biallelic HEPHL1 variants impair ferroxidase activity and cause an abnormal hair phenotype. *PLoS Genet* 2019 15(5):e1008143.

2. Yi L, Kaler SG. Interaction between the AAA ATPase p97/VCP and a concealed UBX domain in the copper transporter ATP7A is associated with motor neuron degeneration. *J Biol Chem* 2018 293:7606-7617.
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4. Hu Frisk JM, Kjellén L, Kaler SG, Pejler G, Öhrvik H. Copper regulates maturation and expression of an MITF:tryptase axis in mast cells. *J Immunol* 2017 199:4132-4141.
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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 distinct 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 entire *inv-en* locus. Our current studies explore the effects of the chromosomal neighborhood on gene expression.

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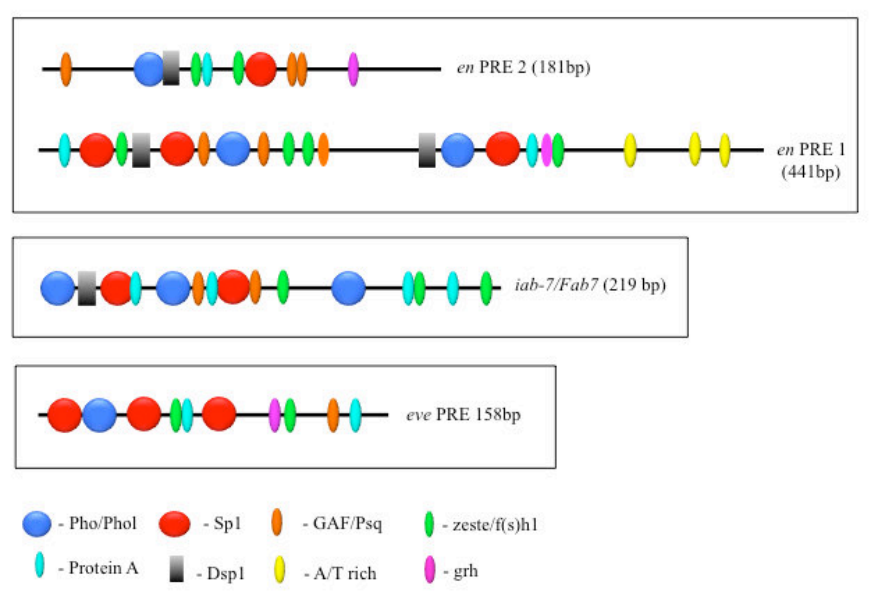

FIGURE 1. The order, number, and spatial arrangement of consensus-factor binding sites varies in different PREs

en PRE1 and 2 are from the *engrailed* gene; *iab-7/Fab-7* PRE is from the *Abd-B* gene; *eve* PRE is from the *even-skipped* gene. The symbols represent consensus binding sites for the proteins indicated below. Figure reprinted from Brown JL, Kassis JA. *Genetics* 2013;195:433.

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.

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 the Spps, 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 to determine what happens when one of the PRE DNA–binding proteins (the 'recruiters') from the genome is removed, and we examined the effect on PcG recruitment genome-wide. We studied PcG binding genome-wide in mutants that lack the recruiters Spps or Pho [Reference 2]. We found that PcG recruitment to some PREs was completely disrupted, whereas recruitment 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.

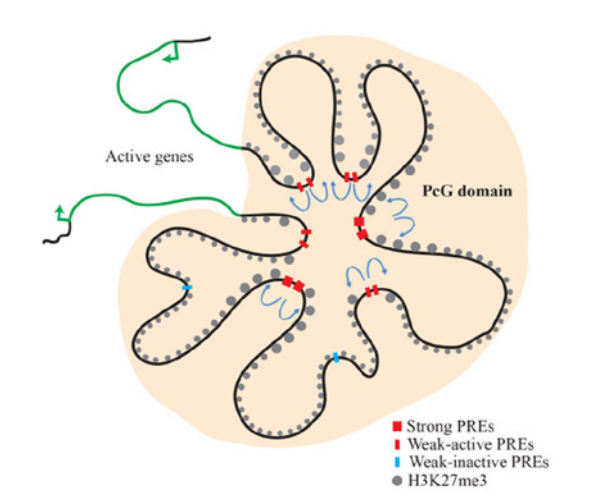

FIGURE 2. Model of the *inv-en* PcG domain in the transcriptionally silenced state

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 others. Actively transcribed genes remain segregated from the PcG domain and determine the limits of the PcG domain (reprinted from Reference 3).

In addition to identifying hundreds of presumptive PREs within Polycomb (H3K27me3) domains, there are thousands of presumptive PREs outside the H3K27me3 domains in larvae [Reference 2]. What is the function of these PREs? Our data suggest that many are tissue-specific PREs, given that H3K27me3 is detected in these locations in some cell types. However, at other locations, no H3K27me3 is present in any cell type. Our on-going studies are addressing the interesting question as to whether these are PREs and to the nature of their function.

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 coregulated and express proteins with largely redundant functions. Unlike *en*, *inv* is dispensable for *Drosophila* 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*

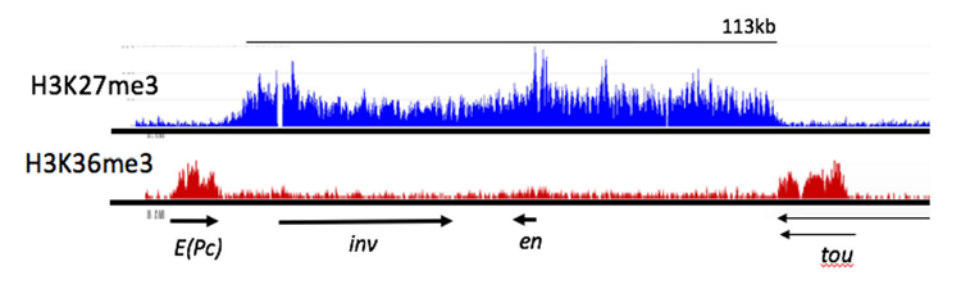

FIGURE 3. ChIP-seq profile showing H3K27me3 and H3K36me3 binding to the *e(Pc)*, *inv*, *en*, and *tou* genes in *Drosophila*

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 4.

PREs yielded viable flies with no misexpression of *en* or *inv*. Importantly, the H3K27me3 *en/inv* domain is not disrupted in either of these mutants [Reference 3].

In *Drosophila*, PREs are easily recognizable in chromatin immunoprecipitation 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 3]. Surprisingly, the flies are viable, and there is no misexpression 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 3]. 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).

The *inv-en* gene complex is flanked by *tou* and *E(Pc)*, two ubiquitously expressed genes (Figures 2 & 3). The H3K27me3 mark stops at the two genes. We believe that it is the transcription of these two genes that blocks the spreading of the H3K27me3 mark and stabilizes the repression of *inv* and *en* by PcG proteins. 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 the transgene into other places in the *Drosophila* genome [Reference 4]. 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 differs from that 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 led to loss of PcG silencing in the abdominal segments of the flies. The data provide evidence that the endogenous *inv-en* domain imparts stability to the locus and facilitates both transcriptional activation and silencing of these two developmentally important genes.

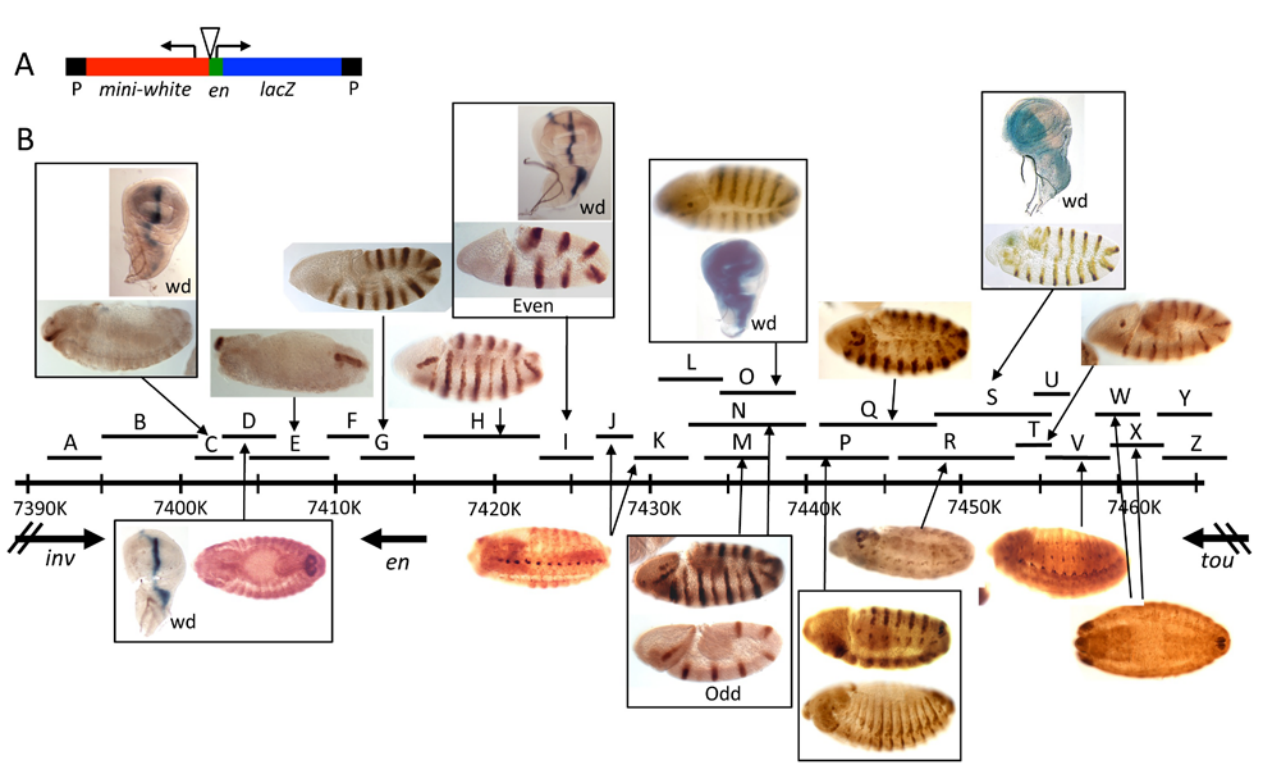

FIGURE 4. Enhancers of the *invected* and *engrailed* genes

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.

Enhancer–promoter communication

Enhancers are often located tens or even hundreds of kb away from their promoter, sometimes even closer to the 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 *en* and the closely-linked co-regulated gene *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 *inv-en* regulation. First, *inv* and *en* share regulatory DNA, most of which flanks 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 several 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.

Publications

1. Kassis JA, Kennison JA, Tamkun JW. Polycomb and Trithorax group genes in Drosophila. *Genetics* 2017 206:1699-1725.
2. 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 E1839-E1848.
3. De S, Mitra A, Cheng A, Pfeifer Y, Kassis JA. Formation of a Polycomb-domain in the absence of strong Polycomb Response Elements. *PLoS Genetics* 2016 12:e1006200.
4. De S, Cheng Y, Sun M, Gehred ND, Kassis JA. Structure and function of an ectopic Polycomb chromatin domain. *Sci Adv* 2019 5:eaau9739.
5. Kuroda MI, Kang H, De S, Kassis JA. Dynamic competition of Polycomb and Trithorax in transcriptional programming. *Ann Rev Biochem* 2019; Epub ahead of print.

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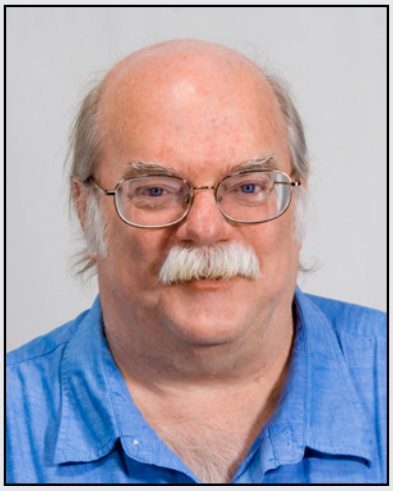
Genomics of Development in *Drosophila*

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.



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** Dr. Kennison has retired as of January 1, 2020.*

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 activate transcription of the *Scr* promoter that is 35 kb distant but

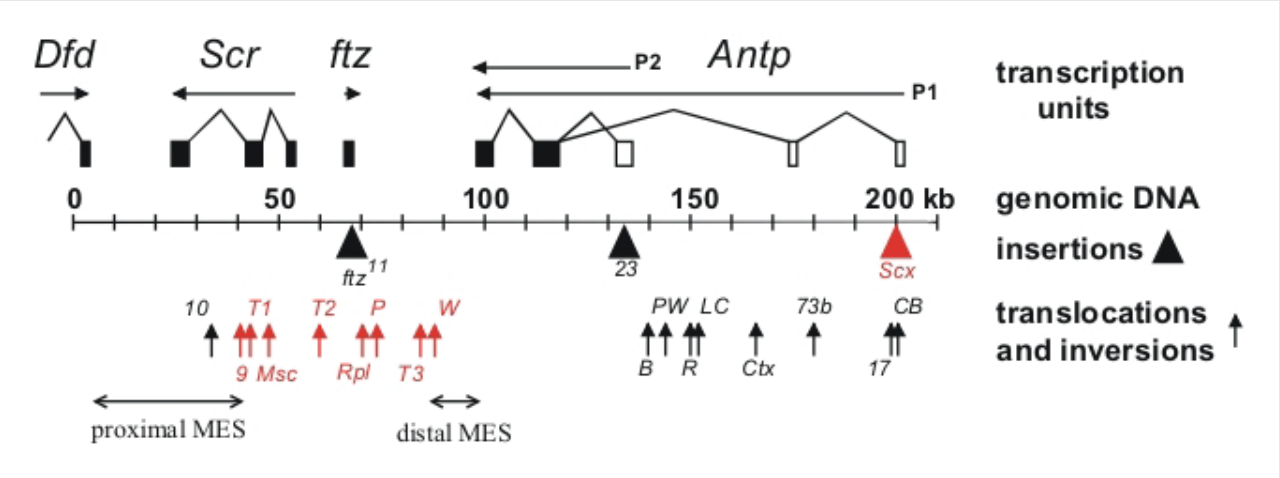


FIGURE 1. Chromosomal aberrations in the distal half of the *Antennapedia* complex

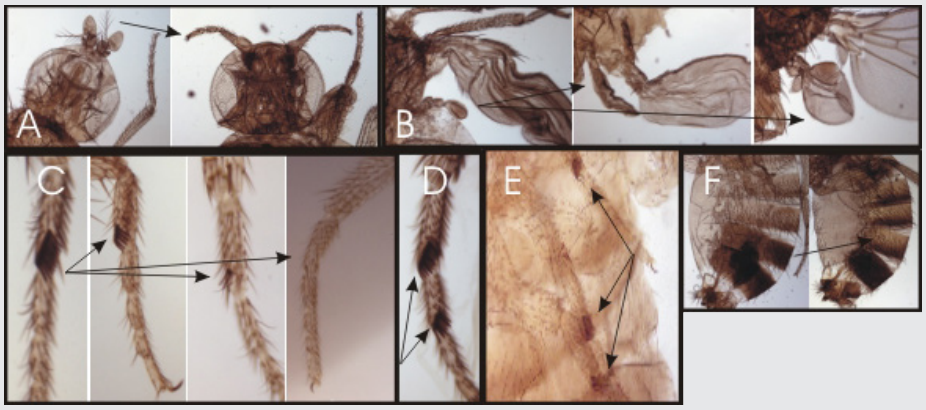
The transcription units are shown above the genomic DNA, while chromosomal aberrations are shown below (solid triangles indicate insertions of transposable elements and upward arrows indicate breakpoints of translocations and inversions). Chromosomal aberrations (*red*) interfere with silencing in the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

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 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.

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



A. Wild-type on the left and the transformation of aristae to distal leg on the right.

B. Wild-type haltere on the left and transformations of anterior and posterior haltere to anterior and posterior wing in the middle and right, respectively.

C. First legs from a wild-type male on the left and three different mutants with reduced sex combs on the right.

D. Mutant male with sex combs on both the first and second tarsal segments.

E. Mutant male with sex combs on all three pairs of legs.

F. Abdominal segments from a wild-type male on the left; mutant male with transformation of the fifth abdominal segment to a more anterior identity on the right.

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 whole-genome sequencing. The new silencing genes encode DNA–binding proteins (*Spps*, *ftz-f1*, *grh*, *Dsp1*, *cut*, *Nf-YB*, *CG17829*, and *ocm*), chromatin-remodeling factors (*Mi-2*, *Iswi*, and *CG16908*), chromatin-modifying and chromatin-associated factors (*gpp*, *Tip60*, *CG9293*, *CG43736*, and *ptip*), insulator proteins, and two aminoacyl-tRNA synthetases (*ThrRS* and *AsnRS*).

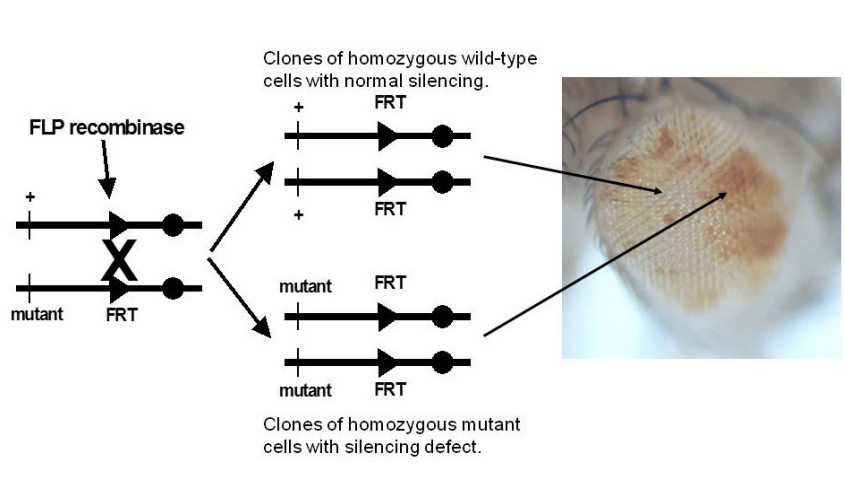


FIGURE 3. Genetic screen for new mutations that disrupt pairing-sensitive silencing

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

Structure and function of the *Drosophila* genome

The *Drosophila melanogaster* genome has been intensely studied for over 100 years. Recently, sequencing of the majority of the genomic DNA revealed much about the structure and organization of the genome. Despite those molecular advances, much remains to be discovered about the functions encoded within the genome. In addition to transcriptional regulation exerted through *cis*-regulatory elements by *trans*-acting factors, gene regulation can also be influenced by the gene's 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 hypertranscription 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 male-fertile X-autosome translocations are not random; the nonrandom pattern led investigators 50 years ago to propose a model in which the X chromosome is precociously inactivated during spermatogenesis and in which 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 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 or in condensation for packaging into the sperm head.

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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 triple-helical proteins forming the structural scaffolds of the ECM. Their procollagen precursors are assembled and folded from three pro-alpha 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 thus identify treatment targets in ECM disorders, particularly those involving abnormal metabolism of type I collagen, and to bring this knowledge to clinical research and practice.

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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. 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 degradation. Up to 10–15% of procollagen is misfolded even under normal conditions, necessitating

activation of cell stress–response pathways responsible for degradation of misfolded molecules and forcing the cell to always function in a high-stress mode. Our findings indicate that one of the key factors in bone pathology is osteoblast malfunction resulting from excessive cell stress, which is often caused by increased procollagen misfolding, inability of the cell to handle the normal load of misfolded procollagen, or both.

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 focuses on the cell biology of procollagen misfolding. In one approach, we are using live-cell imaging to investigate the synthesis, folding, trafficking, and degradation of fluorescently tagged procollagen in osteoblasts. Such imaging of osteoblasts transiently transfected with fluorescent procollagen chains revealed new pathways of sorting and trafficking of normally folded and misfolded procollagen molecules in the cell. As expected, normally folded procollagen is loaded into Golgi-bound transport vesicles at ER exit sites (ERES) marked by the coat protein complex II (COPII). Contrary to widely held beliefs, however, these vesicles do not have a COPII coat nor do they contain HSP47, a collagen-specific ER chaperone that preferentially binds to natively folded procollagen to assist in its folding and loading into ERES. Transport vesicle formation involves fusion of ER-Golgi Intermediate Compartment (ERGIC) membranes with ERES, which appears to facilitate HSP47 removal from procollagen. Misfolded procollagen is retained at ERES, resulting in a COPII–dependent modification of ERES membranes by ubiquitin and autophagic machinery. The resulting autophagic ERES are then directly engulfed by lysosomes and degraded.

This novel ERES microautophagy pathway for ERES–loaded cargo may have wide implications, given that COPII coat involvement in regulating autophagic degradation and cargo rerouting from the secretory to degradative pathway at ERES is likely to be a general rather than collagen-specific phenomenon. 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 the 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 the 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 the 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 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 microautophagy, 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*.

New approaches to analysis and treatment of bone pathology

Our observations suggested that the pathology associated with procollagen misfolding may be 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 the detrimental effects of secreted mutant collagen, pharmacological treatment of cell malfunction is the most realistic short-term strategy for targeting the causes rather than the effect of bone pathology in OI. It is also likely to be a good long-term strategy for treatment of cell malfunction caused by procollagen misfolding in cases that do not involve pathogenic mutations.

To pursue this strategy, we are examining the effects of enhancing the natural ability of cells to remove and degrade misfolded molecules via autophagy, which is the simplest way to prevent their pathogenic accumulation 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, but prolonged LPD stunted animal growth. We are thus testing intermittent LPD and fasting approaches that might provide the same benefits of autophagy enhancement without long-term nutrient deficiency. At the same time, we are validating the general approach of autophagy enhancement by direct genetic modulation of autophagy efficiency. Our study on the effects of altered *Atg5* expression on bone pathology in G610C mice confirmed that decreased autophagy worsens, and increased autophagy alleviates OI symptoms. However, we also found that osteoblasts degrade misfolded procollagen primarily by ERES microautophagy, which is only moderately enhanced rather than regulated by Atg5, necessitating a search for other therapeutic targets.

We are therefore testing drugs known to reduce the accumulation of misfolded proteins in the ER by enhancing their secretion and autophagy (e.g., 4-phenylbutyrate or 4PBA) and drugs known to reduce the impact of this accumulation on protein synthesis (e.g., ISRIB). We found that 4PBA reduces bone pathology in a zebrafish model of OI and in G610C mice, but that 4PBA is very rapidly metabolized and therefore difficult to deliver to the targeted tissues in the proper therapeutic dosage. Our studies of ISRIB and other drugs are still in exploratory stages.

A key issue in monitoring treatment efficiency in animal models as well as in general diagnostic analysis of bone pathology is the lack of reliable methods for characterizing the function of bone cells. Traditional histopathology relies on subjective analysis of bone cell morphology in tissue sections, which is not a reliable indicator of cell function. Over the last year, we developed a new approach for visualizing and quantifying mRNA expression in individual cells in bone sections. The approach enables objective and reliable cell identification as well as *in situ* characterization of cell differentiation and function. Based on the interest of bone histomorphometry experts in coming to our laboratory to learn this approach, we hope that it will soon be adapted not only in research but also in clinical practice.

Translational studies on patients with novel or unusual forms of skeletal dysplasia

Over the last several years, we assisted several clinical research groups in characterizing collagen metabolism pathology in cells from patients with newly discovered skeletal dysplasias caused by mutations in cartilage-associated protein (CRTAP), prolyl-3-hydrohylase (P3H1), cyclophilin B (CYPB), the collagen-binding molecular chaperone FKBP65, the signaling protein WNT1, the ER–membrane ion channel TRICB, Golgi-membrane metalloprotease S2P, the transmembrane anterior-posterior transformation protein 1 (TAPT1), and collagen prolyl-4-hydroxylase 1 (P4H1). Our studies 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 roles remain unclear. More surprisingly, we found no detectable changes in the procollagen folding rate in cultured fibroblasts from patients with FKBP65 mutations. Our data suggested 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's disease), 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. 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 collagen triple helix. We have found that Y-Arg substitutions cause procollagen misfolding and accumulation in the ER almost to the same extent as Gly substitutions, because Y-Arg enhances collagen triple-helix stability and promotes triple helix folding through binding of HSP47.

Presently, we are examining the molecular mechanism of OI caused by mutations in the ER–membrane stress receptor CREB3L1/OASIS. Preliminary analysis of RNA-seq and qPCR data combined with our new mRNA–based histopathology assay suggests common regulation CREB3L1 and some COPII proteins involved in secretory and/or autophagic procollagen export from the ER. However, this study is still in an early phase, and much work remains to be done.

Extracellular matrix pathology in tumors and fibrosis

Another aspect of our collagen metabolism pathology studies has been characterization of this pathology in fibromas and tumors, e.g., abnormal collagen composition of uterine fibroids and the potential role of type I collagen homotrimers in cancer. 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 investigated bone pathology and tumors caused by defects in cAMP signaling, e.g., associated with mutation in protein kinase A (PKA), which is a key enzyme in the cAMP signaling pathway. Initially, we studied synthesis of type I collagen homotrimers. However, over the last 3–5 years, our focus has shifted to abnormal differentiation of osteoblastic cells and deposition of bone. 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 diminishing mineralization away from the central core. We assisted clinical researchers in characterizing abnormal osteoblast maturation, the role of an abnormal inflammatory response, and effects of antiinflammatory drug treatments in these animals. 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.

Multimodal microspectroscopic imaging and mapping of tissues

Given that tissue analysis plays crucial role in understanding and treating collagen metabolism disorders, we are developing methods to characterize not only cell function in tissue sections but also ECM composition and structure. Label-free microspectroscopic infrared and Raman imaging of tissues and cell cultures provides important information about the chemical composition, organization, and biological reactions inaccessible by traditional histology. By resolving the problem of light-path variations with passive thermomechanical stabilization, we developed high-definition (HD) infrared imaging and Raman microspectroscopic methods, achieving spectral reproducibility of 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 illustrated by our studies of ECM structure and composition in a mouse model of diastrophic dysplasia (DTD). DTD is an autosomal recessive dysplasia that affects cartilage and bone development and is caused by mutations in the S*LC26A2* sulfate transporter gene, deficient sulfate uptake by chondrocytes, and resulting undersulfation of glycosaminoglycans in cartilage matrix. For instance, we found that chondroitin undersulfation leads to disorientation of collagen fibers, disrupting a thin protective layer at the articular surface and causing subsequent cartilage degradation. We also investigated the relationship between chondroitin undersulfation and the rate of its synthesis across the growing epiphyseal cartilage and built a mathematical model for the sulfation pathway, predicting treatment targets for sulfation-related chondrodysplasias and genes that might contribute to the juvenile idiopathic arthritis recently associated with single-nucleotide polymorphisms in the gene encoding the SLC26A2 transporter. We are further extending the technology by combining imaging of bone and cartilage ECM composition and structure with biomechanical measurements at the same length scales and *in vivo* ECM studies at large scales by solid-state magnetic resonance imaging (MRI) being developed by our collaborators.

As a test of the technology and important translational application, we are presently working on combining our advances in mRNA–based and microspectroscopy-based histopathological analysis for understanding normal growth plate homeostasis and growth plate pathology in OI. General growth deficiency and disproportional development of proximal and distal limb bones (rhizomelia) are common, clinically important, and yet poorly understood features of this disease. Spatially resolved imaging of mRNA at a single-cell level is enabling us to identify the progression of growth-plate chondrocytes through differentiation steps and expression of different ECM components at these steps. Spatially resolved microspectroscopic analysis of ECM organization at the same distance scales in the same tissue sections is enabling us to relate the cell differentiation and function to ECM composition, structure, and function.

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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 account for only 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, also known as 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.

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

The existence of common mechanisms of neurodegeneration has long been hypothesized. In my previous work at Genentech, I focused on DLK, a MAP3 kinase (mitogen activated protein triple kinase) that had been known, upon injury, to initiate a retrograde stress-signaling cascade from the axon to the cell body. Using several different animal

models of neurodegeneration, the work uncovered an important role for DLK signaling in promoting neuronal death as well as in controlling synapse density. We also showed that human disease tissue bears markers of activation of the DLK/JNK N-terminal kinase signaling pathway [Reference 2]. The most exciting implication of this study is that DLK is part of a long-sought common mechanism of neurodegeneration, which has led to its becoming a promising drug target for the treatment of several diseases.

Intriguingly, and at first glance perhaps counter-intuitively, DLK signaling can result in many different outcomes, including neuronal death, long-term survival, and even regeneration, 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 as well as 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. The 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.

Perhaps rather unsurprisingly, peripheral nerve injury activates many transcripts downstream of DLK. However, the possibility of links between injury, DLK, and neuropathic pain had not previously been examined. Notably, work in the lab established that DLK signaling plays a causative role in neuropathic pain, raising the tantalizing possibility that DLK inhibition could 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 the 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 the 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 Z, et al. *Nat Neurosci* 2016;19:94-101).

Nerve injury initiates a cascade of events that evolve over time as pain becomes chronic. Our 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 may be of therapeutic value. They also highlight non-cell-autonomous aspects of the neuronal injury response, for example as a signal from injured neurons to microglia that has important implications for neurodevelopmental as well as neurodegenerative diseases and that we intend to pursue in future work.

Our most recent publication is collaborative work with the lab of Nick Ryba, in which we performed single-nucleus RNA sequencing of sensory neurons with or without nerve injury, and examined neuron fate on an individual cell basis [Reference 3]. Central findings from the work include that, firstly, nerve injury induces a profound transcriptional switch from sensory neurons of different subtypes to a completely novel,

injured neuronal identity. Secondly, sensory neurons have the potential to recover, back to their native transcriptional state. Ongoing work will examine the specific contributions of injured as well as of intact neurons to the development of neuropathic pain.

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The Biological Impact of Transposable Elements

Mobile genetic elements create genetic diversity which, through natural selection, promotes adaptation and innovation. A wealth of examples document how the action of transposable elements (TEs) leads to important modifications in morphology, metabolism, and ultimately, fitness. Recent work reveals that large gene networks co-regulated by key transcription factors are built on regulatory sequences derived from TEs. Evolution of the networks depends on the dispersal of regulatory elements to genes that, when coexpressed, provide the host with novel functions such as innate immunity or the pregnancy of mammals. The adaptation and innovation produced by mobile elements comes at the cost of gene disruption resulting from indiscriminate insertion and homologous recombination. In each host species a tenuous balance is struck between the mutagenic activity of TEs and the benefits provided by the genetic diversity they produce. This genetic conflict is affected by many factors, including host mechanisms that silence TEs, and the activity of mobile elements, which can be triggered by environmental stress. When the balance between genome defense and TE activity is perturbed, transposition in gonads leads to infertility and, in the case of humans, *de novo* inserts generate disease alleles. The HIV-1 virus is an example of an infectious mobile element that can overwhelm defense mechanisms. Its efficient integration into the genome of immune cells has resulted in over 35 million deaths.

Long terminal repeat (LTR) retrotransposons of yeast provide a unique opportunity to study in real time the biology and impact of TEs in highly characterized model systems. The Ty1, Ty3, and Ty5 elements of *Saccharomyces cerevisiae* each possess unique mechanisms that limit the disruption of important sequences by directing integration to "safe havens" such as heterochromatin and sequences upstream of RNA polymerase III (pol III) promoters. Our studies on the LTR retrotransposon Tf1 of *Schizosaccharomyces pombe* revealed integration behavior that contrasts sharply with the Ty elements. Our large datasets of *de novo* integration show that Tf1 integrates principally into the promoters of RNA pol II–transcribed genes. Promoters are clearly not "safe havens," raising questions about the biological significance of this integration behavior. Several experiments from our lab showed that Tf1 integration alters promoter activity and is well suited to promote adaptation to environmental stress.

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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 *S. pombe* is studied as a model for oncogenic retroviruses because it integrates into the promoters of stress-response genes. Although integrases (INs) encoded by retroviruses and LTR retrotransposons are responsible for catalyzing the insertion of cDNA into the host genome, distinct host factors are required for the efficiency and specificity of integration. Our finding that the *Saccharomyces* protein Sap1 (switch-activating protein 1) is located at positions of integration but does not interact with IN 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.

Transposable element insertions in fission yeast drive adaptation to environmental stress.

Cells are regularly challenged by environmental stress to which rapid and robust responses are critical for survival. To cope with adverse conditions, cells activate transient programs of transcription that alter expression of hundreds to thousands of genes. Pre-wired transcription responses have evolved owing to frequent exposure to a common set of external stresses. However, it is not clear how cells cope when confronted with environmental shock, defined here as novel stresses or conditions for which existing responses are inadequate to support survival. Although genetic modifications that improve survival can clearly be achieved through single-nucleotide mutations, these are mostly neutral or detrimental, and would rarely allow survival to abrupt changes in environment. Alternatively, TEs are activated by stress and, because they carry regulatory sequence, TEs can readily alter gene expression. Such a possibility is in line with McClintock's original proposal that TEs provide a means to overcome the threat of environmental stress by reorganizing the genome [McClintock B. *Science* 1984;226:792]. However, the tenet has not been directly tested. Tf1 provides a unique opportunity to study the role of transposition in cells' survival in response to changes in environmental conditions. A stress-response enhancer embedded in Tf1 causes integration to induce the expression of adjacent promoters. The prominent clustering of integration in promoters and the influence of the Tf1 enhancer on adjacent genes suggest the intriguing possibility that Tf1 may be wired to provide efficient adaptation to environmental stress.

To test directly whether transposition plays a significant role in adaptation we passaged populations of cells in medium containing various concentrations of $CoCl_2$, which generates reactive oxygen species, causes DNA

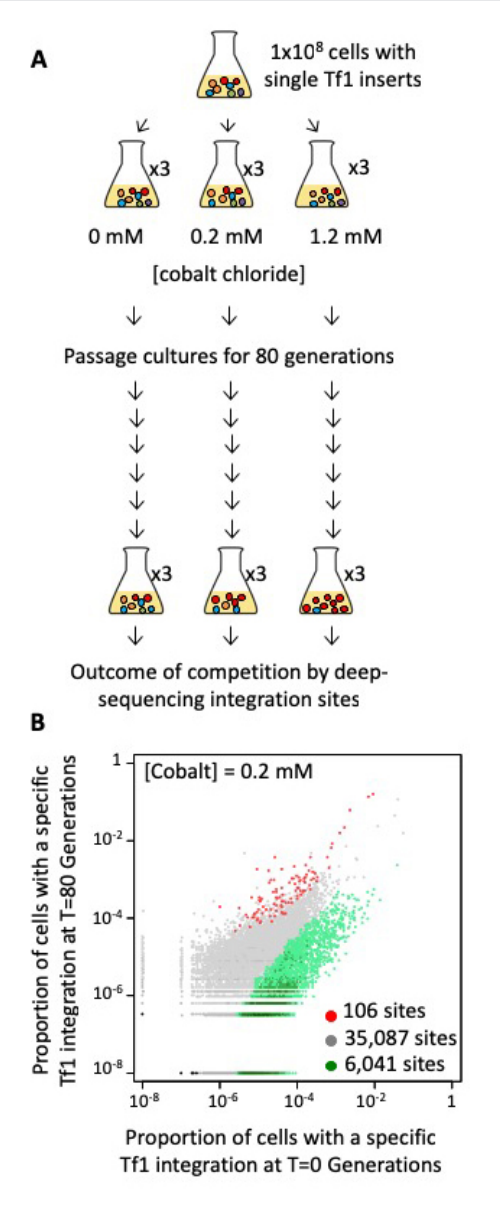

FIGURE 1. A reproducible set of Tf1 integration tags showed improved growth in $CoCl_2$

A. Cells with Tf1 integration tags were passaged for 80 generations in $CoCl_2$. Changes in clonal populations were monitored by sequencing the tags at T = 0 and T = 80 generations.

B. Proportions of cells in the cultures containing a Tf1 integration tag at each of the insertion sites at the beginning (T = 0; x-axis) versus the end (T = 80; y-axis) of the passages in the presence of 0.2 mM $CoCl_2$.

damage, induces apoptosis, and mimics hypoxia. We used cultures in which each cell contained one of 41,000 pre-established insertions of Tf1-*neo* that we created by overexpression of Tf1 followed by selection for integration events (Figure 1A). Each cell contained a specific insertion that served as a tag, which we used to measure clonal expansion during competitive growth. With high-throughput sequencing of the Tf1-*neo* tags we monitored clonal expansion in cultures grown for 80 generations in 0.0 mM, 0.2 mM, or 1.2 mM $CoCl_2$. Three independent passaging experiments were conducted for each $CoCl_2$ concentration. We assessed whether the Tf1 insertions themselves provided a prominent path to improved growth by identifying clones that expanded in each of the three independent passaging experiments. In cultures grown without $CoCl_2$, the bulk of the insertions maintained constant proportions in the cultures. However, large changes occurred in the cultures that contained $CoCl_2$. With 0.2 mM $CoCl_2$, cells with 106 integration positions reproducibly expanded two-fold or more (Figure 1B). The positions accounted for 3.1% of the initial culture and expanded markedly to become 58%, 52%, and 31% (average of 47%) of the final cultures for passaging experiments 1, 2, and 3, respectively. Such substantial percentages of reproducible tags indicate that the Tf1 insertions contributed significantly to improved growth. Importantly, a significant proportion of the competition-enriched positions were adjacent to genes that participate in TOR pathways, indicating that TOR provides resistance to $CoCl_2$. In *S. pombe*, the TORC1 and TORC2 kinase pathways activate genes involved in cell growth and stress response, respectively. Deletion of genes involved in both TORC1 and TORC2 pathways, together with studies of strains with recreated insertions, demonstrated that Tf1 integration functioned as the major path to adaptation. To test whether TEs mediate adaptation in natural settings, we analyzed the genomes of 57 wild isolates of *S. pombe*. We found that polymorphic LTR insertions clustered significantly adjacent to genes that contribute to sporulation frequency, heat shock, and osmotic stress. Our analysis thus indicates that Tf elements function in the wild to provide resistance to environmental stress.

Dense transposon integration reveals essential cleavage and polyadenylation factors promote heterochromatin formation.

In eukaryotes the assembly of DNA into highly condensed heterochromatin is critical for a broad range of functions related to genome integrity. The methylation of histone H3 on lysine 9 (H3K9me) is central to the formation of heterochromatin by creating binding sites for a range of chromatin proteins important for silencing transposable elements, chromosome segregation, and epigenetic inheritance. Used extensively for this purpose, *S. pombe* is an excellent model in which to study the molecular mechanisms that generate and regulate heterochromatin. Centromeres, subtelomeres, and the mating-type region are packaged into constitutive heterochromatin, while meiosis genes are silenced by facultative heterochromatin until cells are starved of nitrogen. Importantly, Clr4, the H3K9–specific histone methyltransferase, is recruited to heterochromatin regions by several mechanisms. Constitutive heterochromatin results from RNAi factors that include the Ago1–containing RNA–induced transcriptional silencing complex (RITS). Facultative heterochromatin at meiosis genes is independent of RNAi and relies on the RNA elimination (i.e., degradation) factors Red1 and Mmi1 and on the nuclear exosome. However, gaps exist in our understanding of how RNA elimination generates heterochromatin. A new approach for identifying gene function is the high-throughput sequencing of integration profiles, also known as Tn-seq, which identifies genes important for growth under selective conditions. Genes necessary to sustain growth under a specific condition do not tolerate insertions in that condition. Tn-seq has been applied to identify pathogenic genes in bacteria. However, we were the first to develop the method for a eukaryote; we developed a method for identifying essential genes in yeast, and others have subsequently applied the strategy with single-cell eukaryotes.

With the goal of identifying novel factors important for heterochromatin, we produced dense profiles of integrations using the Hermes transposable element and a silencing reporter (*ura4*) positioned in the outer repeats of centromere 1. Inserts that disrupted genes important for heterochromatin activated *ura4*, and thus the cells were unable to grow when passaged in 5-fluoroorotic acid (FOA) (Figure 2A). Genes with established roles in heterochromatin assembly had significantly fewer insertions in cells with the centromere reporter *otr1R::ura4* than in cells lacking the reporter (Figure 2B). The list of candidates consisted of a total of 199 genes and, importantly, 65 are known to be essential for viability. These essential genes were candidates because they tolerated many insertions in their 3' sequences that reduced heterochromatin but not viability. The high number of essential genes is significant in that most proteins found to be important for heterochromatin are identified in screens of deletion strains that cannot include essential genes. The 199 candidates showed highly significant enrichments for functions in silencing at centromere outer repeats and included all four factors that produce siRNA. Other RNA processing factors were identified that were not previously linked to heterochromatin structure. Strikingly, four of the RNA processing candidates form an interaction module of the canonical mRNA polyadenylation factor and the cleavage factor CPF, as predicted from highly homologous proteins in *S. cerevisiae*. To determine whether polyadenylation and cleavage contribute to heterochromatin structure at the centromere repeats, we focused on the function of Iss1, a subunit of CPF. We generated a C-terminal truncation of Iss1 (Iss1-ΔC) by removing 38 amino acids that, based on the Hermes insertions, were not important for viability. Iss1-ΔC showed no growth restriction on nonselective medium but exhibited a heterochromatin defect, as demonstrated by growth in the absence of uracil and reduced levels of H3K9 dimethylation (H3K9me2) at *otr1R::ura4*. The results demonstrated that the Hermes screen correctly identified Iss1 as important for heterochromatin structure at the *otr1R::ura4* reporter. Interestingly, we found that Iss1 contributes to the heterochromatin of centromere repeats in cells that lack the *otr1R::ura4* reporter but, in this case, the contribution to H3K9me2 was only observed when the RNAi

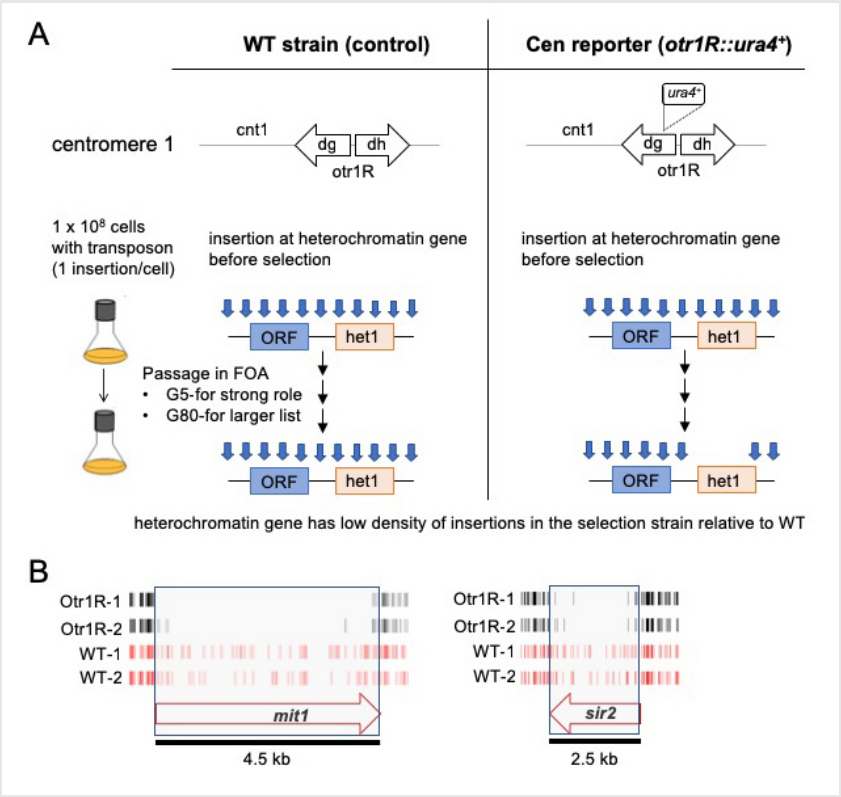

FIGURE 2. Dense maps of transposable element integration identifies genes important for heterochromatin at centromere repeats.

A. Single insertions of the transposable element Hermes were generated in cells with WT cen1 and cen1 *otr1R::ura4*. Cultures were passaged in 5-fluoroorotic acid (FOA) for 5 or 80 generations. Cells with insertions in heterochromatin genes (het1) express *ura4* and cannot grow in FOA. After growth on FOA fewer insertions were detected in het genes in cells with cen1 *otr1R::ura4*.

B. Genes involved in forming centromere heterochromatin such as *mit1* and *sir2* had fewer inserts in cells with the cen1 *otr1R::ura4* (*black*, dupl. libraries) than cells with WT cen1 (*red*, dupl. libraries).

pathway was disabled by deletion of *ago1.* This role at the outer centromere repeats is therefore independent or redundant with RNAi.

We expanded our study of the Iss1-ΔC mutation to evaluate changes in expression and transcription termination genome-wide. RNA-seq data revealed that Iss1-ΔC did not significantly impact canonical transcription termination, but 73 genes were found to have higher expression. Importantly, the genes overlapped significantly with genes upregulated in cells lacking Rrp6, the 3'-5' exonuclease subunit of the nuclear exosome. As a key subunit of the nuclear exosome, Rrp6 plays an important role in RNA surveillance in the degradation of meiotic transcripts expressed during vegetative growth and the resulting formation of heterochromatin at these genes. The elimination of meiotic mRNAs depends on the RNA–binding protein Mmi1 to bind to the determinant of selective removal (DSR) sequence in order to recruit the exosome. Our co-IP experiments revealed that Iss1 interacted with Rrp6, Mmi1, and the polyA polymerase Pla1, indicating that Iss1 is associated with this network of elimination factors. Significantly, the interaction with Mmi1 was disrupted by the Iss1-ΔC mutation, a mutation that greatly reduced H3K9me2 at meiotic genes. We tested whether Iss1 plays a direct role in the heterochromatin of meiotic genes by performing ChIP-seq of Iss1-FLAG. While a subset of Iss1–bound genes was highly-expressed and was associated with the canonical function of Iss1 in mRNA termination, most Iss1–bound peaks showed a strong correlation with genes regulated by RNA elimination and heterochromatin. Importantly, the iss1-ΔC mutation caused significant increases in RNA levels of these genes. Taken together, our studies of RNA levels, Iss1 association with chromatin, and H3K9me2 indicate that Iss1 plays a direct role in the formation of heterochromatin at meiotic genes. Our application of

Hermes profiles to identify genes important for heterochromatin formation demonstrates the significance of the approach, especially given that we were able to identify large numbers of essential genes, a result that is not obtainable with other screens.

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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.

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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 many 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 subcomplexes (Figure 1). The GATOR1 complex inhibits TORC1 activity in response to amino acid

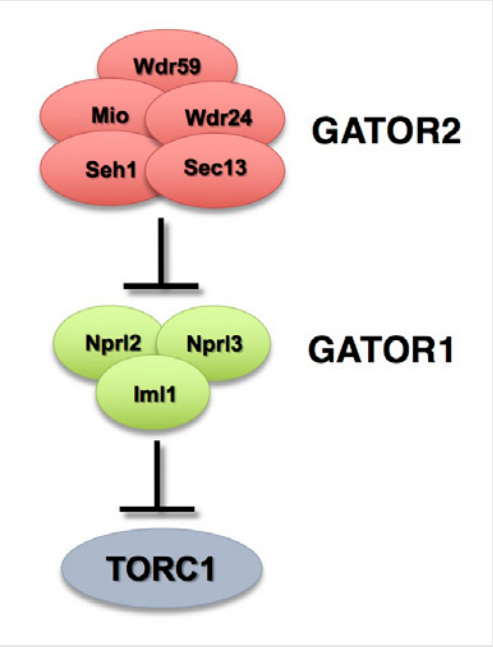

FIGURE 1. The GATOR complex regulates TORC1 activity.

The GATOR2 complex opposes the activity of the TORC1 inhibitor GATOR1.

starvation. 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 double-stranded 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. During the last year, we expanded our examination of how 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, which can result in dramatic and permanent changes to the germline genome. To minimize their destructive potential, the generation and repair of meiotic DSBs is tightly controlled in space and time. We showed that meiotic DSBs promote the GATOR1–dependent down-regulation of TORC1 activity. Consistent with this observation, we found that mutants in genes such as the *Rad51* homolog *spnA*, which retain meiotic DSBs into late stages of oogenesis, exhibit a profound reduction in TORC1 activity in the female germline (Figure 1), data that suggest 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

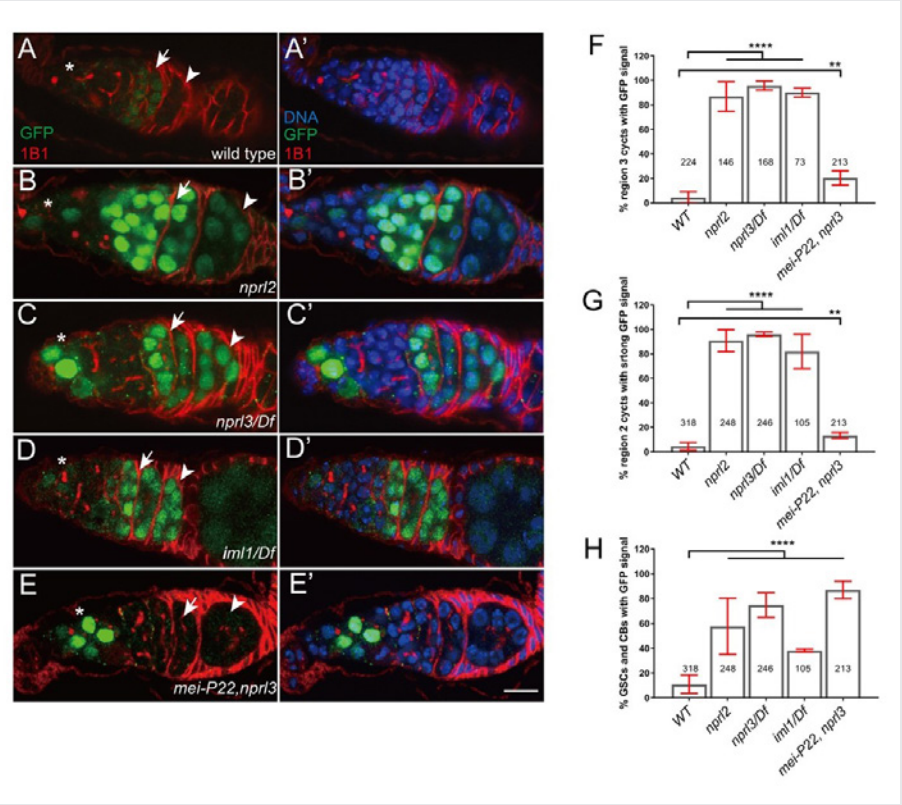

FIGURE 2. GATOR1 prevents p53 hyperactivation in *Drosophila* early ovarian cysts.

Ovaries from (*A*) *p53R–GFP*, (*B*) *nprl2*^{1}; *p53R–GFP*, (*C*) *p53R–GFP;nprl3*^{1}*/Df*, (*D*) *p53R–GFP; iml*^{1}*/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 the strong GFP signal often persisting into germarial region 3 and beyond. Additionally, in GATOR1–mutant germaria, p53R–GFP is frequently activated in germline stem cells (GSC) and daughter cystoblasts (CB). In *mei-P22*^{p22}*, nprl3*^{1} 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 double-stranded breaks (DSBs). Scale bars, 10μm. (*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 < 0.01, ****P < 0.0001.

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 hyperactivation of p53, a transcription factor that mediates a highly conserved response to genotoxic stress (Figure 2). 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 revealed that many of the phenotypes associated with high TORC1 activity observed in GATOR1–mutant ovaries are the result of the hyperactivation of the downstream TORC1 target S6K. We also demonstrated that GATOR1 impacts the repair, rather than the generation, of meiotic DSBs. Our data are particularly intriguing in light of similar meiotic defects observed in *npr3* mutants in *Saccharomyces cerevisiae*. These results raise the possibility that GATOR1–mediated downregulation of TORC1 activity may be a common feature of the early meiotic cycle in many eukaryotes.

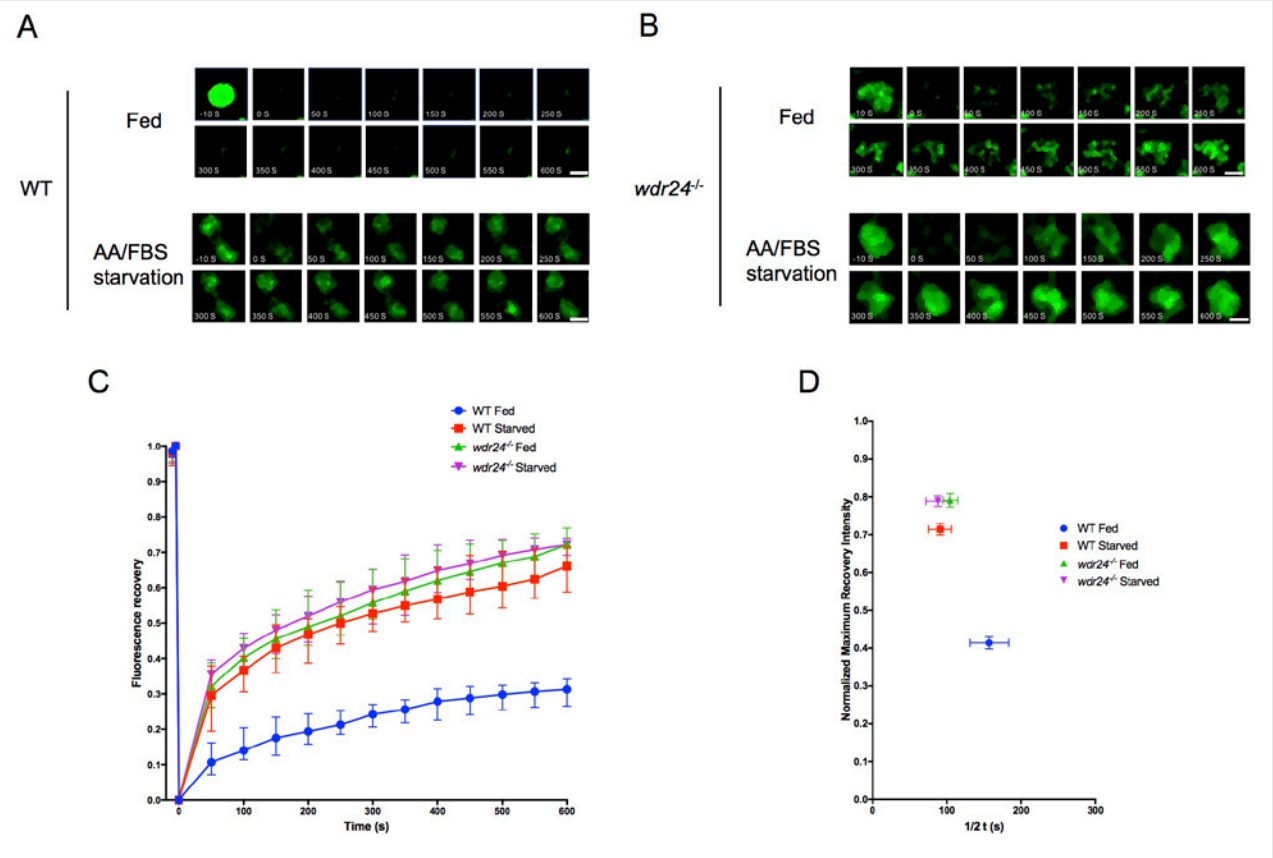

FIGURE 3. The GATOR2 complex regulates the dynamic recruitment of TSC to lysosomes.

A. The time-point pictures in 50s intervals from after lysosomal FRAP of the Halo-TSC2 signal in WT HeLa cell. Note that fluorescent recovery was faster on lysosomes in starvation conditions than in complete medium. The 0s image represents photobleaching. Time point images were taken for 600s at intervals of 5s after photobleaching. Scale bar: 0.5 μm.

B. In WDR24-KO cell, there is no difference in the recovery of the Halo-TSC2 on lysosome in both nutrient conditions. Scale bar: 0.5 μm.

C. Fluorescence recovery versus time curves from the FRAP experiments in *A* and *B*. A total of 30 lysosomes in different cells under each treatment were analyzed. Error bar represents standard error.

D. Plot showing the relationship between the normalized maximum fluorescence recovery intensity versus half time (1/2 t) from the curves in *C*. Error bar represents standard error.

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 TORC1 inhibitor TSC in the female germline resulted in little or 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.*

The GATOR2 complex regulates the Rag GTPase–dependent recruitment of the TSC to lysosomes.

Tuberous sclerosis is a rare multiorgan genetic disorder affecting 1 in 6,000 newborns per year. Mutations in TSC components result in the hyperactivation of the metabolic regulator TORC1, causing the growth of benign tumors in many parts of the body. Although the importance of the TSC in cell metabolism is well-established, a detailed understanding of its regulation has remained elusive. We used *Drosophila melanogaster* and tissue culture cells to demonstrate that the GATOR2 complex is a novel regulator of TSC. The GATOR complex acts as an upstream regulator of TORC1. Its subcomplex GATOR1 inhibits the activity of TORC1 by preventing the recruitment of the complex to lysosomes where it encounters its activator Rheb. Specifically, GATOR1 regulates TORC1 localization by serving as a GTPase–activating protein for RagA/B; in their GTP–bound status, RagA/B recruit TORC1 to lysosomes.

Using fluorescence recovery after photobleaching (FRAP), we determined that knocking out WDR24, one of the subunits of the GATOR2 complex, resulted in the rapid recruitment of the TSC subunits TSC2 and TSC1 to lysosomes in both Hela cells and in the *Drosophila* ovary. Furthermore, we demonstrated that the GATOR2 complex regulates TSC2 dynamics by regulating the guanine nucleotide–binding status of the RagA or RagC small GTPases. Specifically, GDP–bound RagA and GTP–bound RagC promote the dynamic recruitment of TSC2 to lysosome. Moreover, by using a photoconvertible protein–tagged TSC2, we determined that the rapid association of TSC2 to lysosomes in *wdr24*$^{-/-}$ cells is accompanied by its rapid dissociation. Taking together, we provided both *in vitro* and *in vivo* evidence to support the model that the GATOR complex regulates the dynamic cycling of the TSC between lysosomes and the cytoplasm.

Publications

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Neurosecretory Proteins in Neuroprotection and Neurodevelopment

Mechanism of sorting, transport, and regulated secretion of neuroproteins

The intracellular sorting of proneuropeptides 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 proproteins 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 proproteins are sorted into the dense-core granules of the RSP for processing by prohormone convertases and carboxypeptidase E (CPE) and then secreted. We showed that the sorting of prohormones to the RSP occurs by a receptor-mediated mechanism. Site-directed mutagenesis studies identified a 3-D consensus sorting motif consisting of two acidic residues found in POMC, proinsulin, and BDNF. We identified the transmembrane form of CPE as an RSP sorting receptor that is specific for the sorting signal of these proproteins.

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

Transport of vesicles containing hormone or BDNF to the plasma membrane for activity-dependent secretion is critical for endocrine function and synaptic plasticity. We showed that the cytoplasmic tail of a transmembrane form of CPE in hormone- or BDNF–containing dense-core secretory vesicles plays an important role in their transport to the vesicles' release site. Overexpression of the CPE tail inhibited the movement of BDNF– and POMC/CPE–containing vesicles to the processes in hippocampal neurons and pituitary cells, respectively. The transmembrane CPE tails on the POMC/ACTH and

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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 thus emerge as novel β-adrenergic inotropic and lusitropic modulators. Together, they can play a key role in the myocardium's orchestration of its complex response to sympatho-chromaffin stimulation. Additionally, pGlu serpinin is a powerful cardio-protectant after ischemia. The mechanism involves 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-alpha1 in neuroprotection and anti-depression during stress

To study the function of CPE/NF-alpha1 *in vivo,* we generated a CPE (CPE is also known as Neurotrophic factor-alpha1, NF-alpha1) knock-out (KO) mouse. 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-alpha1 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-alpha1 in human disease. Using the *Cpe*-KO mice as a model, 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. 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 CA3 neurons in the hippocampus. Hippocampal neurons in the 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 glucocorticoid-induced epileptic-like neuronal firing of the granule cells in the dentate gyrus, releasing large amounts of glutamate during the weaning stress paradigm, which includes emotional stress from maternal separation, and physical stress from ear-tagging and tail clipping for genotyping. Hence, CPE/NF-alpha1 is important for the survival of CA3 neurons during stress. We then showed that a mutant mouse expressing an enzymatically inactive form of CPE/NF-alpha1 (E342Q), had a normal hippocampus and learning and memory after the weaning stress paradigm, indicating that the neuroprotective action is independent of enzymatic activity. CPE/NF-alpha1 (E342Q), 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 treatment. Likewise, the enzymatically inactive form of CPE/NF-alpha1 (E342Q) applied extracellularly had the same neuroprotective effect. We thus demonstrated that CPE/NF-alpha1 acts extracellularly as a signaling molecule to mediate neuroprotection. To this end, we showed that ^{125}I-CPE/NF-alpha1 binds to the cell surface of HT22 cells, an immortalized hippocampal neuronal cell line, in a saturable manner, and that the binding is specifically displaced by non-iodinated CPE/NF-alpha1, 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-alpha1 in hippocampal neurons treated with H_2O_2, suggesting that the CPE/NF-alpha1 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-alpha1 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 prosurvival mitochondrial protein, Bcl2, inhibition of caspase 3 activation, and promotion of neuronal survival [Reference 1]. Furthermore, this CPE/NF-alpha1–mediated neuroprotection pathway is activated by rosiglitazone, a PPARg ligand (a peroxisome proliferator-activated receptor, a transcription factor), 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 1hour per day for seven days, mice showed significantly elevated levels of CPE/NF-alpha1 mRNA and protein, as well as of the anti-apoptotic protein Bcl2, in the hippocampus. *In situ* hybridization studies indicated especially elevated CPE/NF-alphalpha1 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-alpha1 and Bcl2 expression and a decline in Bax, a proapoptotic 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-alpha1 and Bcl2 expression are coordinately up-regulated to mediate neuroprotection of hippocampal neurons. The importance of CPE as a neuroprotective agent was demonstrated by the absence of an increase in Bcl2 in the hippocampus of *Cpe*-KO mice after CRS, leading to the degeneration of the CA3 neurons. Furthermore, CRS also elevated the expression of the signaling protein FGF2. We demonstrated that primary hippocampal neurons treated with CPE/NF-alpha1 raised FGF2 expression. Thus, another pathway for CPE/NF-alpha1 may be through FGF2, which is known to have neuroprotective effect.

The relevance of CPE/NF-alpha1 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 2]. Our search in the GenBank 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 reduced 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 endoplasmic reticulum (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 the prosurvival 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 ser^{395}, 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, as well as depressive-like behavior in a mouse model, linking this gene for the first time to neurodegenerative disease and depression [Reference 2].

Stress also induces depression. Huda Akil's group (University of Michigan) reported that FGF2 is an anti-depressant. We found that prolonged (6 hours per day for 21 days) restraint stress reduced CPE/NF-alpha1 and FGF2 in the hippocampus of mice and induced depressive-like behavior. However, after short-term restraint stress, (1 hour per day for 7 days) mice did not show depressive-like behavior despite elevated corticosterone levels indicative of stress. Moreover, hippocampal CPE/NF-alpha1, FGF2, and doublecortin, a marker for neurogenesis, were elevated in these mice, suggesting that the anti-depressive effects of CPE/NF-alpha1 are mediated, at least in part, through increased neurogenesis. Indeed, we found that exogenously applied CPE/NF-alpha1 could up-regulate *FGF2* mRNA and protein expression in cultured hippocampal neurons, indicating that CPE/NF-alpha1 regulates FGF2 expression. CPE/NF-alpha1-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-alpha1 prevents stress-induced depressive-like behavior by up-regulating hippocampal FGF2 expression, which leads to enhanced neurogenesis and anti-depressive activity [Reference 3]. Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway [Reference 3]. Rosiglitazone has previously been shown to be effective in treating diabetic patients with bi-polar disorders.

Role of CPE/NF-alpha1 and CPE-deltaN in embryonic brain development

Embryonic mouse brains express three forms of CPE/NF-alpha1 mRNA(2.1kb, 1.9kb, and 1.73kb in size) encoding a 53kD wild-type CPE/NF-alpha1, and two terminal-truncated isoforms of CPE/NF-alpha1-DN (47kD and 40kD). The three mRNAs are expressed as early as E8.5 and increase significantly in two waves at E10.5

and postnatal day 1 [Reference 4]. Interestingly, CPE/NF-alpha1-DNs are not expressed in adult mouse brain. *In situ* hybridization studies indicate that CPE/NF-alpha1 is expressed primarily in the forebrain in mouse embryos, suggesting a role of CPE/NF-alpha1 in neurodevelopment. We examined the effect of CPE/NF-alpha1 on E13.5 neocortex-derived neurospheres, which contain stem cells and neuroprogenitors. Application of recombinant CPE/NF-alpha1 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-alpha1 down-regulated the wnt pathway in the neurospheres, leading to reduced levels of beta-catenin, a protein known to enhance proliferation, suggesting that CPE/NF-alpha1'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-alpha1 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/NFalpha1*–KO mouse embryos showed fewer astrocytes but more neurons, which was reversed with CPE/NF-alpha1 application. *In vivo*, *Cpe/NF-alpha1*–KO mouse cortex (at P1, the time of astrocytogenesis) showed about half the astrocyte numbers of those of WT animals, confirming the *ex vivo* data. Our results suggest a novel role for CPE/NF-alpha1 as an extracellular signal to inhibit proliferation and induce differentiation of neural stem cells into astrocytes, thus playing an important role in neurodevelopment [Reference 5].

Neurite outgrowth is key to the formation of synapses and the neural network during development. We found that CPE/NF-alpha1 prevented Wnt-3a inhibition of nerve growth factor (NGF)–stimulated neurite outgrowth in PC12 cells, a neuroendocrine cell line, and in cortical neurons. Moreover, CPE/NF-alpha1 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-alpha1, could play an important role in regulating these positive and negative cues, which are critical for neurodevelopment. 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.

We also studied the function of 40kD CPE/NF-alpha1-DN and showed that it is translocated from the cytoplasm into the nucleus of rat embryonic neurons. Overexpression of 40kD CPE/NFalpha1-DN in HT-22 cells, a hippocampal cell line, resulted in an increase in expression of IGF binding protein2 (IGFBP2), Death Associated Protein (DAP1), and Ephrin 1A mRNAs and proteins that are involved in neuronal proliferation, programmed cell death, and neuronal migration, respectively. We demonstrated that IGFBP2 is involved in proliferation in a CPE/NF-alpha1-DN–dependent manner in HT22 and mouse cortical neurons [Reference 4]. Thus 40kD CPE/NF-alpha1-DN functions to regulate expression of genes important in neurodevelopment. Further studies aimed at determining the role of CPE/NF-alpha1-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.

Jon Lorsch, PhD, *Chief, Laboratory on the Mechanism and Regulation of Protein Synthesis*

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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 preinitiation complex (PIC) is then loaded onto the 5' end of an mRNA with the aid of eIF3 and the eIF4 group of factors: the RNA helicase eIF4A; the 5' 7-methylguanosine cap-binding protein eIF4E; the scaffolding protein eIF4G; and the 40S subunit– and RNA–binding protein eIF4B. Both eIF4A and eIF4E bind to eIF4G and form the eIF4F complex. Once loaded onto the mRNA, the 43S PIC is thought to scan the mRNA in search of an AUG start codon. The process is ATP–dependent and likely requires several RNA helicases, including the DEAD–box protein Ded1p. Recognition of the start site begins with base pairing between the anticodon of tRNAi and the AUG codon. Base pairing then triggers downstream events that commit the PIC to continuing initiation from that point on the mRNA, 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 eIF2, which converts eIF2 to its GDP–bound state. In addition, the initiator tRNA moves from a position that is not fully engaged in the ribosomal P site [termed $P_{(OUT)}$] to one that is [$P_{(IN)}$], and the PIC as a whole converts from an open conformation that is conducive to scanning to a closed one that is not. At this stage, eIF2•GDP dissociates from the PIC, and eIF1A and a second GTPase factor, eIF5B, coordinate joining of the large ribosomal subunit to form the 80S initiation complex. In a process that appears to result in conformational reorganization of the complex, eIF5B hydrolyzes GTP and then dissociates along with eIF1A.

The molecular mechanics of eukaryotic translation initiation

We completed and submitted for publication a manuscript describing studies

on the effect of temperature on translation of upstream open reading frames in *S. cerevisiae*. The paper describes our transcriptome-wide studies on the effects of temperature on translation in yeast. Our work showed that translation of a subset of upstream open reading frames (uORFs) is regulated by changes in temperature. In some cases, the alterations in translational efficiency of the uORFs affects translation of the downstream main ORF, leading to changes in gene expression. The paper has now been accepted for publication [Reference 2].

We also advanced our work aimed at leveraging our *in vitro* reconstituted yeast translation initiation system to measure the effects of initiation factors and other components on the rates of initiation in every yeast mRNA simultaneously. In this approach, using deep-sequencing methodology analogous to *in vivo* ribosome profiling, we use poly(dT)–purified total yeast mRNA and monitor 48S translation preinitiation complex (PIC) assembly on every mRNA at the same time. We use internal standard mRNAs to measure in absolute terms assembly rates instead of rates relative to the average of all mRNAs in the experiment. We are now optimizing the protocol and have already used it to study the effects of eIF4B and the DEAD box helicase Ded1 on mRNA recruitment to the PIC.

We made significant progress in our studies on the functions of the N-terminal tails (NTTs) of eIFs 1 and 5. We conducted a targeted alanine-scanning genetic analysis to determine which residues, when substituted with alanine, produce significant phenotypes such as lethality, slow growth, or an increase or decrease in the stringency of start codon recognition. We found several positions in both factors at which a change to alanine is lethal, and several others that increase or decrease the fidelity of start codon recognition. We also assessed the effects of viable mutations in both factors' NTTs on the autoregulation of eIF1 translation, which depends on changes in recognition of the poor-context AUG start codon of eIF1 mRNA. We are now expressing and purifying variant proteins that produce the strongest phenotypes for in-depth mechanistic studies in the reconstituted translation initiation system *in vitro*.

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Publications

1. Sen ND, Gupta N, K Archer S, Preiss T, Lorsch JR, Hinnebusch AG. Functional interplay between DEAD-box RNA helicases Ded1 and Dbp1 in preinitiation complex attachment and scanning on structured mRNAs in vivo. *Nucleic Acids Res* 2019 47:8785-8806.
2. Kulkarni SD, Zhou F, Sen ND, Zhang H, Hinnebusch AG, Lorsch JR. Temperature-dependent regulation of upstream open reading frame translation in S. cerevisiae. *BMC Biol* 2019 17(1):101.

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Genes and Signals Regulating Mammalian Hematopoiesis

Our research focuses on the development of the mammalian hematopoietic system. A long-term area of interest for our laboratory is the study of signal transduction molecules and pathways that regulate T lymphocyte maturation in the thymus. Currently, we are analyzing transgenic and conditional nonsignaling mutants of T cell antigen receptor (TCR) signaling subunits, made in our lab, to evaluate the importance of individual TCR signaling chains and motifs at specific stages of T cell development and in mature T cells. We are also using gene profiling to identify proteins that are important for fine-tuning the T cell–signaling response in developing and mature T lymphocytes. In conjunction with checkpoint inhibitors for immunotherapy in humans, these molecules may also be targets. We are also investigating the function of new T cell–specific proteins that we identified by subtraction library screening. Our studies have revealed a critical role for one of these proteins, called Themis, in T cell development by enhancing the TCR–signaling response in thymocytes. We found that another newly identified protein, Fbxl12, is important for regulating proliferation during T cell development. Another area of investigation focuses on hematopoietic stem cells (HSCs) and early stages of T cell, B cell, and erythrocyte development. We initiated characterization of a protein (Ldb1) that is important for the generation and maintenance/self-renewal of HSCs. The studies revealed a critical function for Ldb1 as a key subunit of mutltimeric DNA–binding complexes in controlling the self-renewal/differentiation cell-fate decision in HSCs. Current work seeks to extend our knowledge of the role of Ldb1 complexes in regulating gene transcription and to explore the function of such complexes in other hematopoietic lineages. For example, we have begun to investigate the importance of Ldb1 complexes for regulating self-renewal in immature thymocytes and in the induction of T cell acute lymphoblastic leukemia (T-ALL). Our results suggest that Ldb1 complexes represent potential therapeutic targets for the treatment of an aggressive form of childhood leukemia called early T progenitor T cell acute lymphoblastic leukemia (ETP-T-ALL).

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T cell antigen receptor signaling in thymocyte development

Much of our research has focused on the role of TCR signal transduction in thymocyte development. Signal transduction

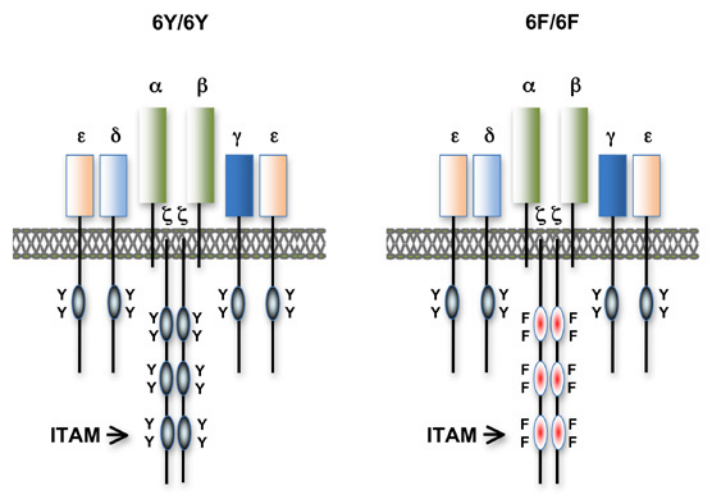


FIGURE 1. T cell antigen receptors expressed in 6Y/6Y and 6F/6F knock-in mice

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).

sequences, termed immuno-receptor tyrosine-based activation motifs or ITAMs, are contained within four distinct subunits of the multimeric TCR complex (CD3-zeta, CD3-gamma, CD3-delta, and CD3-epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement; their function is to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell–activation cascade. Though conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be partly regulated by distinct ITAMs through the recruitment of different effector molecules. 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 autoreactive cells, which may cause autoimmune disease, are deleted in the thymus (negative selection).

We also found that a complete complement of TCR–ITAMs is not required for most mature T cell effector functions. However, recent work demonstrated a requirement for ITAM multiplicity for the generation of T follicular helper cells, which are required for optimal B cell antibody responses. One possible explanation for the relatively mild phenotype observed in the TCR ITAM–reduced mice is that ITAM–mediated signal amplification is not required for most mature T cell activation responses; another is that, in ITAM–mutant mice, T cells exhibit normal functional responsiveness because of compensatory mechanisms (such as regulated expression of other signaling molecules) imposed during development. To resolve this question, we recently generated a TCR–zeta chain conditional knockin mouse in which T cell development and selection can occur without attenuation of TCR signaling (i.e., in the presence of a wild-type 3-ITAM “6Y” zeta chain), but in which mature, post-selection T cells may be induced to express TCRs containing signaling-defective (0-ITAM "6F") zeta chains in lieu of wild-type zeta chains (Figure 1). Thus, mature T cell signaling should not be influenced by potential compensatory mechanisms that operate during T cell maturation, and T cells in these mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. Preliminary

experiments confirmed that the knockin zeta locus functions as predicted. We are currently evaluating the effect of late 'switching' from 6Y zeta to 6F zeta in mature T cells generated with wild-type 6Y zeta containing TCRs. These experimental conditions will more closely mimic those in which TCR signaling is attenuated pharmacologically to treat human diseases such as autoimmunity. In addition, these experiments should provide information relevant to the design of chimeric antigen receptor T cells (CAR T cells), which currently are configured to express a full-length zeta (3 ITAM) signaling module.

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 center on identifying the mechanism by which CD5 inhibits TCR signaling and on 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 identified Themis, now known as Themis1, a novel T cell–specific adapter protein (Figure 2). To investigate the function of Themis1 in T cell signaling and development, we generated Themis1 knockdown cell lines, Themis1 knockout mice (conventional and conditional), and Themis1 transgenic mice. Analysis of the effects of modulating Themis1 expression revealed a critical role for the protein in late T cell development. We obtained the following results. (1) The Themis1 paralog Themis2, which is expressed in B cells, can substitute for Themis1 in T cell development; we found that the ability of the B cell–specific family Themis member Themis2 was equivalent to that of Themis1 to restore normal T cell development in *Themis1*$^{-/-}$ mice, thus demonstrating functional redundancy of Themis1 and Themis2. (2) We generated retroviruses encoding domain-deletion mutants of Themis1, infected *Themis1*$^{-/-}$ bone marrow progenitors, and made bone marrow chimeras to determine which regions

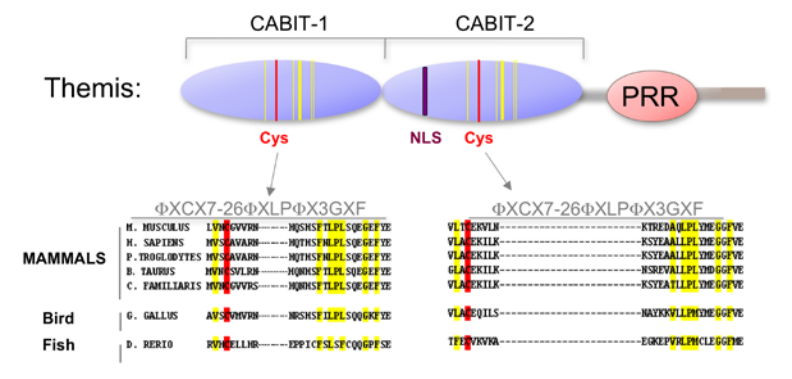


FIGURE 2. Themis is highly conserved in vertebrates.

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).

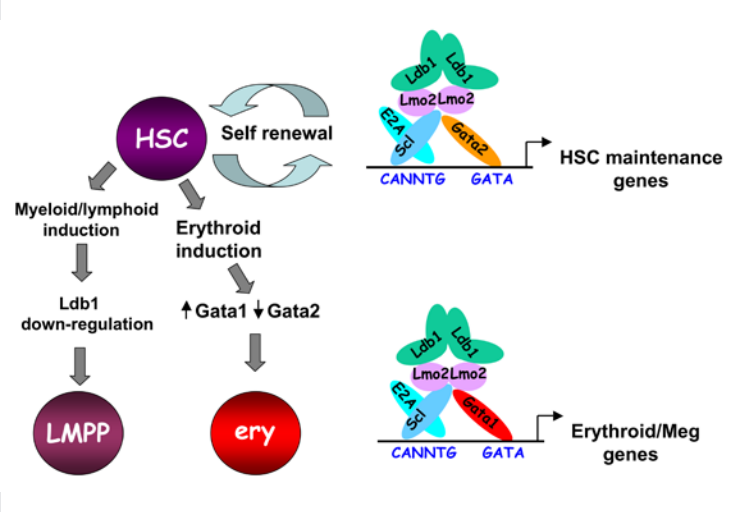
of Themis1 are important for *in vivo* function; we found that the Themis1 proline-rich sequence (PRS), which mediates binding to the signaling protein Grb2, was required for *in vivo* function, as assessed by rescue of the developmental block in *Themis1*$^{-/-}$ thymocytes, but that the CABIT (cysteine-containing, all beta in Themis)–domain cysteines are not essential. (3) We generated *Themis2*$^{-/-}$ mice and began a collaboration with Richard Cornall to characterize the mice; our results identified an important role for Themis2 in facilitating B cell activation by low-avidity, but not high-avidity, B cell receptor (BCR)–antigen interactions; Themis2 was required to elicit normal Ca^{2+} 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*$^{-/-}$ mice, indicates that Themis1 and Themis2 perform similar functions in T and B cells.

In the past few years, we have 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 SHP-1, a key hematopoietic protein tyrosine phosphatase. 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 are mature T cells, but the reason for this sensitivity was unknown. The function

FIGURE 3. Model of Ldb1 function in the hematopoietic lineage

Ldb1 forms a multimeric DNA–binding complex in hematopoietic cells with the adapter Lmo2 and the transcription factors Scl and Gata1 or Gata2. In hematopoietic stem cells (HSCs), in which Gata2 is highly expressed, Ldb1-Lmo2-Scl-Gata2 complexes positively regulate expression of HSC maintenance genes. Differentiation of HSCs to the myeloid or lymphoid lineage (LMPP) is triggered by downregulation of Ldb1, whereas commitment to the erythroid lineage (ery) is triggered by induction of Gata1 and downregulation of Gata2, resulting in the formation of an Ldb1-Lmo2-Scl-Gata1 complex, which positively regulates expression of erythroid-specific genes.



of Themis1, together with its high expression in thymocytes, provides an explanation for the sensitivity of thymocytes to TCR signaling. By showing that deletion of the gene encoding SHP-1 rescues T cell development in *Themis1*$^{-/-}$ mice, we confirmed that the primary role of Themis1 is to inhibit SHP-1. 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 the receptor 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 beta selection–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 codeletion 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 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 LIM–zinc finger protein–interaction motif and a dimerization domain. In hematopoietic cells, Ldb1 functions by interacting with and/or recruiting specific partners (including the LIM–only protein Lmo2 and the transcription factors SCL/Tal1 and Gata1 or Gata2) to form multimolecular 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 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 at 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 cell lineages downstream of the HSC identified an essential function in the erythroid lineage but not in myeloid cells or lymphoid cells. Interestingly, ChIP-seq analysis of Ldb1 DNA–binding complexes demonstrated that, in HSCs, Ldb1 complexes contain the transcription factor Gata2, whereas, in erythroid progenitors, Ldb1 complexes contain Gata1 (which is highly expressed in the erythroid lineage). The results indicate that multimeric Ldb1 transcription complexes have distinct functions in the hematopoietic system depending on their subunit composition, with Gata2–containing complexes regulating expression of HSC–maintenance genes and Gata1 complexes regulating expression of erythroid-specific genes (Figure 3). Current studies aim to determine how Ldb1 complexes regulate gene expression and the role of Ldb1 dimerization in mediating long-range promoter-enhancer interactions in hematopoietic cells. In addition, we are investigating a potential role for Ldb1 in regulating self-renewal of T cell progenitors in the thymus.

Acute lymphoblastic leukemias are the most common type of cancer in children. T cell acute lymphoblastic leukemia (T-ALL) results from oncogenic transformation of immature T cell progenitors (thymocytes). Mouse models of T-ALL have been generated, and one of the most informative is the Lmo2–transgenic (*Lmo2*-tg) mouse, which expresses high levels of the nuclear adapter Lmo2 in thymocytes. The model closely mimics a prevalent type of human T-ALL, which is associated with chromosomal mutations that result in increased expression of LMO2. We recently reported that overexpression of Lmo2 in mouse thymocytes induces T-ALL

at two distinct stages of development (an early 'ETP' stage and a later 'DN3' stage). Notably, human T-ALLs can also occur at two similar stages of thymocyte maturation. The most immature forms of T-ALL in *Lmo2*-tg mice and in humans express high levels of the transcription factor Hhex and are designated early T progenitor (ETP) T-ALL, whereas later-stage tumors are low in Hhex but express high levels of more mature markers of T cell development, including Notch1, Dtx1, Ptcra, and Hes1. Lmo2 functions as a subunit of the multimeric Ldb1–nucleated DNA–binding complexes described above. We found that normal ETP thymocyte progenitor cells express the same Ldb1 complex subunits that are present in HSCs and that ETPs exhibit HSC characteristics, including the 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 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.

Additional Funding

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The Arms Race between Transposable Elements and KRAB-ZFPs 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 from our egg-laying relatives over 100 million years ago, nor about 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 such 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 coelacanths, birds, and tetrapods, constituting 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 by closely related species

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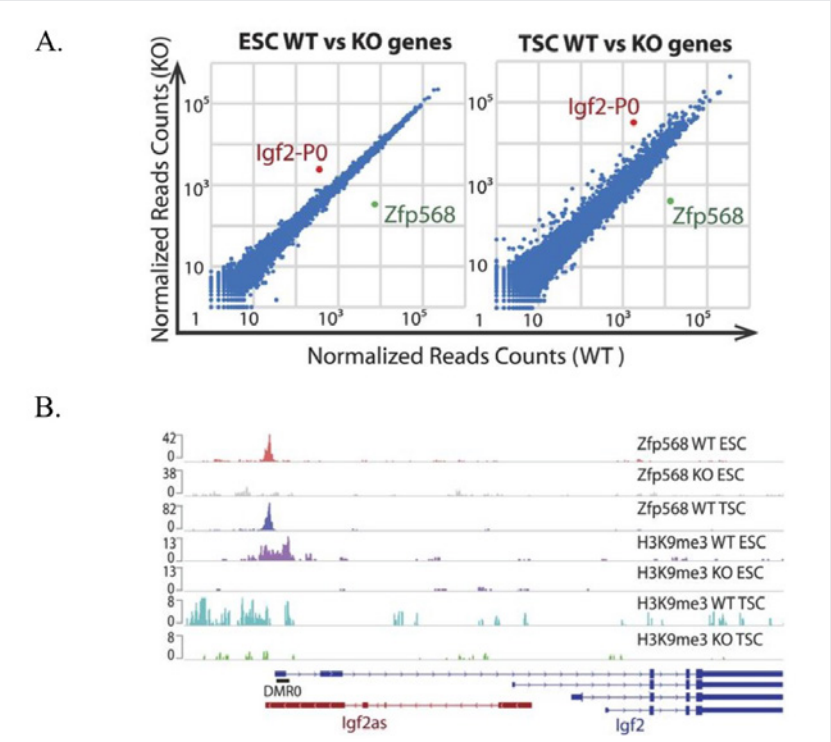

FIGURE 1. ZFP568 directly represses the *Igf2-P0* transcript.

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

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

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 combination of

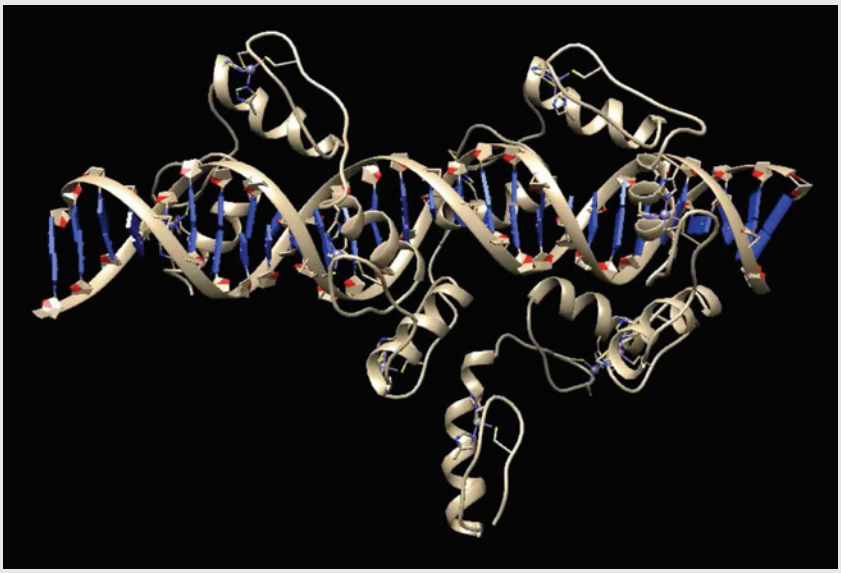

FIGURE 2. Structure of ZFP568 zinc fingers bound to the *Igf2-P0* target sequence

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 DNA–binding modes to accommodate altered DNA structures.

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 of 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 ERVs 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 knockout (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 *Igf2* gene called *Igf2-P0* (Figure 1). Insulin-like growth factor 2 (Igf2) is the major fetal growth hormone in mammals. We demonstrated that loss of *Zfp568*, which causes gastrulation failure, or mutation of the ZFP568 binding site at the *Igf2-P0* promoter, cause inappropriate *Igf2-P0* activation. We also showed that this lethality could be rescued by deletion of *Igf2*. 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 is highly conserved and under purifying selection in eutheria with the exception of human. Human ZNF568 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 solved 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.

Additional Funding

- 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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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 NICHD's mission.

Within freshwater environments, *L. pneumophila* exists as an intracellular parasite of single cell organisms know as amoeba. 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 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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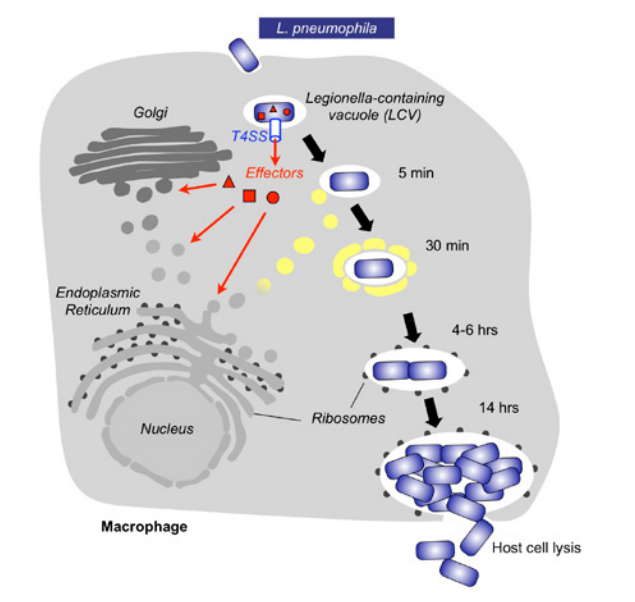


FIGURE 1. Intracellular replication cycle of *Legionella pneumophila*

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

A 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 such as *L. pneumophila* encode molecular mimics of host protein kinases in order to manipulate signaling pathways during infection.

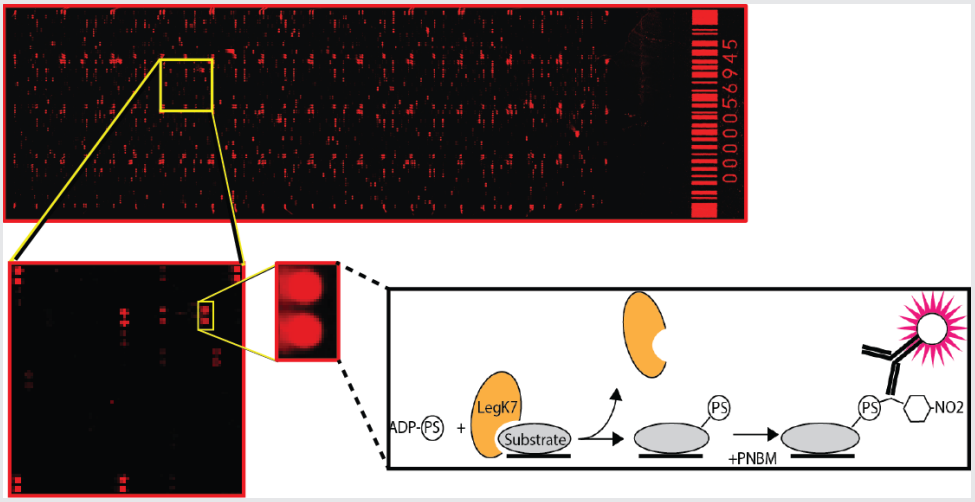
Discovering substrates of bacterial or eukaryotic protein kinases has remained a major challenge because of the transient nature of kinase-substrate interactions and the complexity of the phospho-proteome, even at steady state. To bypass these obstacles, we developed a novel screening platform that combines 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 2). As phosphate donor for the kinase reaction, we used adenosine 5'-O-(3-thio)triphosphate (ATPgammaS). 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 and, subsequently, 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

FIGURE 2. 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*). Substrate proteins (*grey color*) of the kinase that are phosphorylated on the microarrays are identified using a fluorescently labeled antibody.



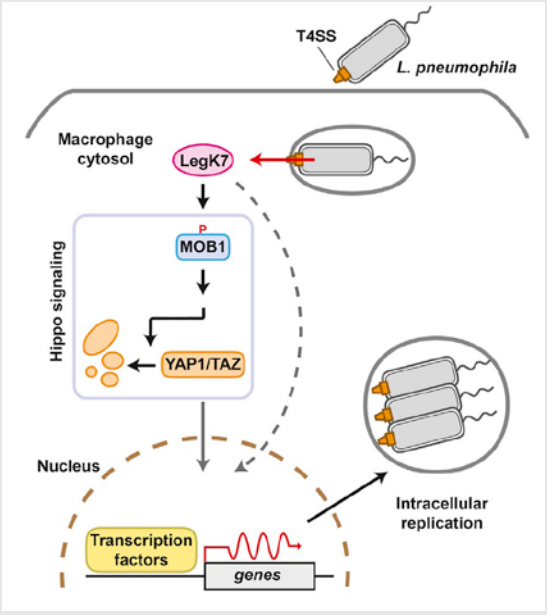


FIGURE 3. An important role of LegK7 during *L. pneumophila* infection

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.

(residues 183–462) that has folding homology to eukaryotic protein kinases. Using the above-mentioned ATPgammaS labeling technique, we experimentally confirmed that LegK7 indeed exhibits kinase activity *in vitro*. Upon probing the protein microarray platform with LegK7, we identified MOB1 as a direct substrate of LegK7 (Figure 3). MOB1 is a key scaffold protein within the Hippo kinase signaling pathway, which, in eukaryotes, controls cell-cycle progression, cell proliferation and differentiation, and apoptosis. 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 triggers a signaling cascade, resulting in the degradation of the transcriptional regulators TAZ and YAP1. Transcriptome analysis by RNA-seq 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

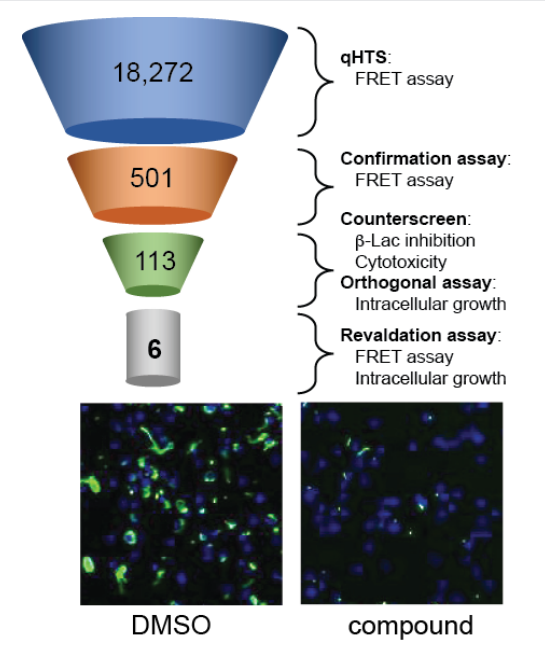


FIGURE 4. Screen for inhibitors of the *L. pneumophila* T4SS

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*).

permissive to *L. pneumophila* intracellular growth (Figure 3). Thus, a conserved *L. pneumophila* effector kinase exploits the Hippo pathway to promote bacterial growth and infection.

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 overprescription of antibiotics and patient noncompliance, 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 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 4). 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.

Publications

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RNA Metabolism in Cell Biology, Growth, and Development

We are interested in tRNAs and mRNAs as well as some of their 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 their posttranscriptional processing and 'handling' by the eukaryotic RNA–binding protein La. The La protein was first discovered because it becomes a target of autoantibodies in patients who suffer from, and is integral to, an autoimmune process that leads to (and is diagnostic of) Sjögren's syndrome, systemic lupus erythematosus (SLE), and neonatal lupus. Autoimmunity to La occurs by a complex, as yet incompletely understood mechanism, and the protein is sometimes referred to as the La autoantigen. Critical to its normal essential function, the conserved La protein contains multiple RNA–binding motifs and subcellular trafficking elements, and associates with noncoding (nc) RNAs, mostly in the nucleus, as well as with mRNAs in the cytoplasm. In the nucleus, La binds to the 3' oligo(U) motif common to all RNAP III transcripts as discrete small RNPs (ribonucleoproteins) and functions by protecting its most abundant ligands, the nascent precursor tRNAs, from 3' exonucleolytic digestion and by serving as a chaperone to prevent their misfolding. Although the major products of RNAP III are the tRNAs, it also synthesizes 5S rRNA and some other essential noncoding RNAs (ncRNAs) involved in fundamental processes necessary for translating the genetic information in mRNA during protein synthesis. Our investigations also include specific posttranscriptional modifications of tRNAs that impact their metabolism and function during translation by cytoplasmic and mitochondrial ribosomes.
We also study La–related protein-4 (LARP4), which, in contrast to La protein, is predominantly cytoplasmic at steady state, and interacts mostly with mRNAs rather than ncRNAs. However, similar to La, LARP4 interacts with the 3' end regions of its RNA ligands, in this case the mRNA poly(A) tails (PATs), and contributes to their stability/metabolism and translation. Genome-wide mRNA-Seq and analysis of PATs indicate that LARP4 interacts with a large number of mRNAs and promotes their stability. Such analyses of mice whose LARP4 gene is disrupted reveal shorter PATs, whereas over-expression of LARP4 leads to PAT lengthening. Studies on the mechanisms by which mRNA PAT metabolism and mRNA stability are linked suggest that LARP4 affects nascent PAT metabolism. Mechanistic studies are under way.

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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 and 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.

Data from our lab suggest that levels of cytoplasmic tRNAs may regulate translation-mediated decay of LARP4 mRNA and LARP4 levels, which in turn promotes stabilization of mRNAs encoding ribosomal proteins. 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 or 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 translation 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 ($m^{2,2}G26$), which resides at the top of the anticodon stem of several tRNAs, whereas TRIT1 adds an isopentenyl group onto adenine-37 in the anticodon loop of certain tRNA (i^6A37) molecules. We showed that each of these modifications can activate their tRNAs for translation in codon-specific assays. We also examine effects of TRIT1 on tRNA activity during mammalian development, using *TRIT*-gene–altered mice as a model system to understand how a deficiency in this 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, active tRNA genes number more than 300, and are distributed on all the chromosomes, many residing in clusters, the loci of which can vary, as do the loci of individual tRNA genes. A system we use to examine codon-specific effects of loss of the i^6A37 modification on a specific tRNA is the fission yeast *S. pombe*, in which human TRIT1 can complement the phenotype caused by deletion of the homologous enzyme Tit1. We also use the *S. pombe* system to study La and pol III, in which the human La protein can complement the phenotype caused by deletion of the fission-yeast La-homolog Sla1.

One theme of our and others' work is that differential expression of tRNAs occurs in a tissue- and temporal-specific manner and, 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 tRNA gene copy numbers 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 multisubuit enzyme complex consists of 17 subunits, several with homology to subunits of RNAPs I and II. The transcription factor TFIIIC, composed of six subunits, binds to A- and B-box promoters

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

Red-white colony differentiation by tRNA–mediated suppression

(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 reinitiation necessary to produce the more than tenfold 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 being essential for cell proliferation, RNAP III is also linked to aging.

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 nontemplate 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 in the 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 nontemplate 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 nontemplate strand provides distinct sequence-specific destabilizing information through interactions with the C37 subunit.

Control of the differential abundance or activity of tRNAs can be an important determinant of gene regulation. RNAP III synthesizes all tRNAs in eukaryotes, and its derepression is associated with cancer. Maf1 is a conserved general repressor of RNAP III under the control of TOR (target of rapamycin), which acts to integrate transcriptional output and protein-synthetic demand toward metabolic economy. We used tRNA-HydroSeq to document that little change occurred in the relative levels of different tRNAs in *maf1Δ* cells. By contrast, the efficiency of *N*2,*N*2-dimethyl G26 [m(2)2G26)] modification on certain tRNAs was reduced in response to *maf1* deletion and associated with antisuppression, which we validated by other methods. Overexpression of Trm1, which produces m(2)2G26, reversed *maf1* antisuppression. The model that emerges is that competition by elevated tRNA levels in *maf1Δ* cells leads to m(2)2G26 hypomodification resulting from limiting Trm1, thus reducing the activity of suppressor tRNASerUCA (UCA is the anticodon for serine) and accounting for antisuppression. Consistent with this, RNAP III mutations associated with hypomyelinating 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, a response that 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 of RNAP III. 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: first, 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 second, 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), the latter of which is regulated in mammals by tumor necrosis factor alpha (TNFa); a second level of control was found for the 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, posttranscriptional 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 increase 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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Immune Activation and Viral Pathogenesis

Our 2019 activity focused on two interrelated projects regarding pathologic immune activation in tissues. Viruses, in particular HIV-1 and cytomegalovirus (CMV) are involved in the immune activation, leading to various diseases, in particular cardiovascular diseases.

IMMUNE ACTIVATION IN TISSUES INFECTED WITH HIV-1 AND TREATED WITH ANTIVIRALS

Immune activation is now considered to be a driving force of various human pathologies, including HIV-1 disease. Currently, antiretroviral therapy (ART) has proven to be efficient in suppressing HIV-1 replication. However, lengthy suppression of HIV-1 replication by ART is associated with an increased risk of complications, including neurological and cardiovascular diseases, which appear to be related to the residual immune activation in patients undergoing ART. Cytokines may play an important role in the residual immune activation. Earlier, we found that cytokines, which are generally considered to be classical soluble immune-regulating molecules, can be associated with extracellular vesicles (EVs). In this form, they can be delivered to (target) cells and elicit cellular responses. We found that the spectra of EV–associated cytokines in HIV-1–infected tissues is different from that of soluble cytokines. We focused on both soluble and EV–associated cytokines that become up-regulated in HIV infection in human tissues *ex vivo*. After HIV-1 is fully suppressed by antiviral drugs, cytokines remain upregulated, with EV–associated cytokines more likely to be elevated than soluble ones. Similarly, cytokines are upregulated in myocardial infarction, and EV–associated cytokines form a distinct group regulated differently from soluble cytokines. The findings indicate a physiological role of the EV–associated cytokines distinct from that of soluble molecules.

While ART efficiently suppresses viral replication in infected individuals, various diseases develop years earlier than in the control population. Moreover, some infected individuals with total suppression of HIV replication fail to fully restore their immune system (immune non-responder [INR]); in particular, they have an inability to reconstitute the CD4+ T cell pool after antiretroviral therapy. General residual immune activation seems to be involved in these pathologies. Understanding the mechanisms of these phenomena requires the development of *ex vivo* models in which these mechanisms can be

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studied under controlled laboratory conditions. We developed a model of human tissues infected *ex vivo* with HIV-1 and investigated immune activation after HIV-1 was suppressed by antivirals. Also, we identified mitochondrial defects in the INRs that may contribute to their failure to reconstitute CD4+ T cells.

IMMUNE ACTIVATION IN CARDIOVASCULAR DISEASE: THE ROLE OF CMV (CYTOMEGALOVIRUS)

Various human diseases have immune activation as a common denominator; myocardial infarction is one such diseases. In blood, immune activation is associated with activation of various cells, in particular, platelets that release vesicles upon activation. We studied this phenomenon and found that these EVs lead to aggregation of monocytes with platelets or with vesicles released by platelets. Aggregation of monocytes with platelets and platelet-derived EVs may represent an early marker of disease progression. It remains to be determined what triggers such persistent immune activation. Earlier, we found that the development of unstable angina is associated with the presence of CMV RNA in blood. We further studied the role of this virus in immune activation and the development of atherosclerosis. We found that productive CMV infection negatively correlates with endothelial function in myocardial infarction. The results further implicate CMV in cardiovascular disease.

HIV–1 pathogenesis and immune activation in tissue treated with antiretroviral therapy

To investigate the role that cytokines play in residual immune activation, it is necessary to develop an *ex vivo* laboratory-controlled system reflecting what happens *in vivo*. As an experimental model, we used *ex vivo* human lymphoid tissues, where critical events in HIV-1 infection occur *in vivo*. We evaluated concentrations of 33 cytokines released by donor-matched human lymphoid tissues *ex vivo* productively infected with HIV-1 over 16 days of infection and treated or not with the antivirals. We evaluated concentrations of soluble and EV–associated cytokines separately.

SOLUBLE AND EV–ASSOCIATED CYTOKINES IN HIV INFECTION OF HUMAN TISSUES *EX VIVO*.

Specifically, we found that two strains of HIV-1 (R5 and X4) efficiently replicated in tissues and triggered an upregulation of numerous soluble cytokines as early as day three post infection. Some of the cytokines are typical of the acute TH1 (T helper cell 1) response, which protects against intracellular pathogens. Evaluation of EV–associated cytokines demonstrated that some of the same cytokines upregulated in soluble form were also upregulated in EV–associated form. The beta-chemokines (MIP-1a, MIP-1b, and RANTES), in particular, were consistently upregulated throughout infection with both virus strains in soluble and EV–associated forms. However, several cytokines were uniquely upregulated in the EV form, particularly upon X4 infection. Also, in early HIV-1 infection, there was a significant increase in the percentage of the soluble chemokines RANTES and TNF-alpha compared with EV–associated cytokines. Additionally, RANTES significantly increased in the percentage of surface-associated EVs compared with internal EVs.

PERSISTENT IMMUNE ACTIVATION IN *EX VIVO* HIV–INFECTED TISSUES UNDER ART

We investigated whether the HIV-1–triggered immune activation is reduced when viral replication is suppressed. In particular, we demonstrated that ritonavir and AZT-3TC treatment of tissues efficiently suppressed viral replication (over 99% suppression for both treatments). Despite control of viral replication, cytokines remained upregulated after 13 days of ART treatment, and EV–associated cytokines were less likely to decrease than soluble ones. Also, X4 elicited stronger immune responses, as measured by increased soluble

and EV–associated cytokines, particularly pro-inflammatory cytokines and the beta-chemokines compared with the R5 strain.

We identified mitochondrial defects in the INRs that may contribute to their failure to reconstitute CD4+ T cells. Thus, the phenomenon of residual immune activation after successful ART can be reproduced in a laboratory experimental system with human lymphoid tissue *ex vivo*, opening a way to study this phenomenon under controlled laboratory conditions. We showed that cells with the phenotype and transcriptional profile of Tregs (regulatory T cells) were enriched among cycling cells in health and in HIV infection. However, there were diminished frequencies and numbers of Tregs among cycling CD4+ T cells in INRs, and cycling CD4+ T cells from INR subjects displayed transcriptional profiles associated with the impaired development and maintenance of functional Tregs. Flow-cytometric assessment of TGF-beta activity confirmed the dysfunction of Tregs in INR subjects. Transcriptional profiling and flow cytometry revealed diminished mitochondrial fitness in Tregs among INRs, and cycling Tregs from INRs had low expression of the mitochondrial biogenesis regulators peroxisome proliferator activated receptor coactivator 1- (PGC1) and transcription factor A for mitochondria. *In vitro* exposure to the cytokine IL-15 (interleukin-15) allowed cells to complete division, restored the expression of PGC1, and regenerated mitochondrial fitness in the cycling Tregs of INRs. Our data suggest that rescuing mitochondrial function could correct the immune dysfunction characteristic of Tregs in INRs and enhance immune restoration in these subjects.

In conclusion, our analysis showed that HIV-1–infected lymphoid tissues *ex vivo* upregulated production of many cytokines, both free and EV–associated, and that the majority of the cytokines remained upregulated despite suppression of viral replication by ART. Also, in spite of ART, many patients do not reconstitute their immune system, in part owing to mitochondrial dysfunction. The mechanisms of both these phenomena can now be investigated under controlled laboratory conditions, which should result in the development of new therapeutic strategies.

Cytomegalovirus and EVs in atherosclerosis: immune activation

Acute cardiovascular syndrome (ACS) is associated with a general activation of the immune system, which includes activation of many cells, in particular monocytes and platelets, leading to destabilization and rupture of coronary atherosclerotic plaques and to acute myocardial infarction (AMI). Recently, it was found that immune activation is associated with the release of EVs by activated cells; the EVs mediate cell-cell communication and play an important role in immune activation. One of the manifestations of the activation of monocytes and platelets is the formation of monocyte-platelet complexes (MPCs).

We analyzed MPCs *in vivo* and *in vitro* and investigated the abilities of various monocyte subclasses to form MPCs, the characteristics of the cells and EVs involved in MPC formation, and MPC changes in AMI. We identified MPCs by co-staining for the platelet antigen CD41a and for the monocyte antigens CD14 and CD16. Platelet activation was evaluated by expression of phosphatidylserine (PS). Monocytes of some classes disproportionately formed MPCs: although classical monocytes (CD14++CD16–) constituted the majority, MPCs were preferentially formed by intermediate monocytes (CD14++CD16+). CD41a–positive events in MPCs exposed more PS than in the circulating monocytes. AMI was associated with a 50% increase in circulating monocytes and with a threefold increase in MPCs, in particular in those formed by classical monocytes. In AMI patients, MPCs formed by intermediate monocytes contained more CD41a–positive events than MPCs with other monocyte subsets, whereas in controls, MPCs formed by classical monocytes carried more platelets

than other MPCs. The sizes of some of the CD41a+ events in MPCs were smaller than those of regular platelets and may represent platelet-derived EVs. Some of the aggregates seem to consist of monocytes and platelet-derived EVs. Binding of EVs to monocytes was confirmed in *in vitro* experiments when monocytes were co-incubated with platelet-generated EVs. There was association between complications of AMI and the increase in MPCs and their composition. Aggregation of EVs and platelets with monocytes in AMI patients is another manifestation of immune activation associated with atherosclerosis that plays an important role in this pathology and can be used as an AMI correlate.

However, it is not yet understood what causes the immune activation, given that, in general, the destabilization of atherosclerotic plaques and development of recurrent cardiovascular events are strongly associated with activation of the immune system. Perturbation of the T cell repertoire, with higher expression of effector memory and activation markers, was found not only in blood of patients with cardiovascular diseases but also within their atherosclerotic plaques. Herpes viruses are one of the main candidates causing persistent immunoactivation within plaques owing to their ubiquity and ability to cycle between dormancy and replication. Specifically, it has been shown that accumulation of intermediate and late-differentiated T effector memory cells in blood is associated with CMV. In our previous work, we showed that productive CMV infection is more common in patients with acute coronary syndrome than in patients with chronic coronary artery disease or in healthy volunteers. Moreover, we also found a positive correlation between the cytomegaloviral DNA load and T lymphocyte differentiation within the atherosclerotic plaques of patients with cardiovascular diseases.

We analyzed the presence of CMV DNA in plasma and endothelial function in 33 patients with ST-elevation myocardial infarction (STEMI) and 33 volunteers without cardiovascular diseases, using real-time polymerase chain reaction and a noninvasive test of flow-mediated dilation (FMD). We found that the presence of CMV DNA in plasma of STEMI patients was significantly higher than in volunteers without cardiovascular diseases. Also, we found a significant prevalence in the number of copies of CMV DNA in older hypertensive patients, indicating that one of the possible mechanisms of the development of hypertension in patients with CMV infection could be the development of endothelial dysfunction triggered by CMV; endothelial function plays an important role in patients with atherosclerosis. We addressed this issue by using FMD test given that CMV was shown earlier, and confirmed here, to be an independent risk factor of STEMI. Using this test, we demonstrated for the first time the negative correlation between productive CMV infection and endothelial function in patients with STEMI. In a multivariate analysis, we confirmed that the identified correlation persisted, regardless of sex, hypertension, CRP (C-reactive protein) level, or patient age, factors that were significantly associated with CMV production and endothelial function. In summary, we found that CMV infection is strongly associated with endothelial dysfunction in STEMI patients.

Although we found an association between CMV in plasma and endothelial dysfunction, it is possible that other pathogens, not studied in the present work, may also be associated with this pathological state. In general, our results support a hypothesis that acute coronary events are synchronized with CMV replication, and that this viral reactivation contributes to endothelial dysfunction. Therefore, it is conceivable that preventing the reactivation of CMV would enhance endothelial function and affect the outcome of coronary artery disease.

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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 collectively 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. We recently identified several key genes in the search for causes of recessive OI. Discoveries of defects in collagen modification have generated a new paradigm for OI as a collagen-related disorder of matrix. We established that structural defects in collagen cause dominant OI, while deficiency of proteins that interact with collagen for folding, posttranslational 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 predominantly 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

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serum PEDF, elevated alkaline phosphatase (ALPL) as children, 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 type V OI) 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. 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 in type VII, consistent with patchy osteoid occurring only in type VIII.

The third member of the complex, cyclophilin B (CyPB), encoded by *PPIB*, is an ER–resident peptidyl-prolyl *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. In our recent collaboration with Mitsuo Yamauchi and colleagues [Reference 1], we explored the role of CyPB in posttranslational modifications of collagen in skin. As in bone, hydroxylation of collagen crosslinking sites was almost absent in mutant mice, and the key cross-linking residue alpha1(I)K87 was underglycosylated. Absence of CyPB led to the occurrence of two novel types of collagen crosslinks that are not present in normal skin. Atomic force microscopy showed that this was associated with a lower nanoindentation modulus in KO than in normal skin. The studies underscore the tissue-dependent effects of CyPB, which have common effects on cross-linking and mechanical properties.

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. 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 intramembrane 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 procollagen C-proteinase (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* (the gene encoding the pro-alpha1 chain of type 1 collagen) 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 small, and their femora exhibit extreme brittleness on mechanical testing, as well as reduced fracture load. BMDD measurement on femora 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.

Mutations in the COL1A1 C-propeptide

The C-propeptide of type I collagen (COL1A1 C-propeptide) is processed after collagen is secreted from the cell and before it is incorporated into matrix. Interestingly, mutations in the C-propeptide account for about 6% of OI patients. Our investigation of the biochemical consequences of C-propeptide mutations in comparison to collagen helical mutations revealed both intra- an extracellular differences [Reference 2]. Immunofluorescence microscopy indicated that procollagen with C-propeptide defects was mislocalized to the ER lumen, in contrast to the ER membrane localization of normal procollagen and helical mutations. Second, although the mutations were not located in the processing site itself, pericellular cleavage of the C-propeptide was defective in both pericellular processing assays and cleavage assays with purified C-proteinase. These consequences are expected to contribute to abnormal osteoblast differentiation and matrix function, respectively.

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 OI mechanism and treatment.

We also collaborated with Kenneth Kozloff's group to investigate a potential anabolic therapy, sclerostin antibody (Scl-AB), which stimulates osteoblasts via the canonical Wnt pathway. Scl-AB stimulated bone formation in young Brtl mice and increased bone mass and load-to-fracture. Treatment with Scl-AB caused no detrimental change in Brtl bone material properties. Nanoindentation studies indicated unchanged mineralization, unlike the hypermineralization induced by bisphosphonate treatment. 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. Furthermore, because a lifelong deficiency of sclerostin leads to patterns of excessive cranial bone growth and nerve compression, we undertook dimensional and volumetric measurements of the skulls of Brtl mice treated with Scl-AB. Treated mice showed calvarial thickening but minimal effects on cranial morphology and anatomic landmarks. Narrowing of vascular but not neural foramina was seen.

Brtl mice provided important information about the cytoskeletal organization in OI osteoblasts and their potential role in phenotypic variability. We observed abnormal cytoskeletal organization involving vimentin, stathmin, and cofilin-1 in lethal pups. The alterations affected osteoblast proliferation, collagen deposition, integrin, and TGF-beta signaling. The data open the possibility that cytoskeletal elements may present novel OI treatment targets. Another potential novel treatment may be 4-PBA, a chemical chaperone. When this drug is used to treat OI cells, it enhances autophagy, as opposed to apoptosis, of the cells and stimulates protein secretion. Interestingly, the enhanced protein secretion reflects a broad range of cellular proteins rather than simply the retained mutant collagen and relieves the ER stress along the PERK pathway.

Two basic insights have emerged from Brtl studies. The first concerns hypermineralization of OI bone, which was previously thought to be a passive process. Altered levels for osteocyte transcripts involved in bone mineralization, such as Dmp1 and Sost, demonstrated, however, the presence of an actively directed component. We used acoustic transmission microscopy to characterize the properties of Brtl cortical bone. The periodically oriented collagen organization in periosteal cortex of Brtl bone was strongly reduced compared to that of normal bone. Young’s modulus and ER sound velocity were significantly increased in Brtl cortex. The data demonstrate that the mutant collagen of Brtl mice affects the mechanical behavior of bone predominantly in the endosteal region by altering collagen orientation.

Second, the osteoclast is important to the OI phenotype, with elevated numbers of osteoclasts. Co-culture experiments with Brtl and wild-type (WT) mesenchymal stem cells (MSCs) and osteoclast precursors yielded elevated osteoclast numbers from WT or Brtl precursors grown with Brtl MSCs, but not with WT MSCs. The results indicate that an osteoblast product is necessary to elevate osteoclast numbers.

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.

Previously, OI–specific growth curves were 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 types III and IV OI, with known mutations in type I collagen, to generate sex- and type-specific growth curves for OI [Reference 3]. 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. Our preliminary analysis indicates that OI children obtain comparable benefits from lower and higher doses of 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 hyperpigmented, vascular lesion. Given that attempts to identify germline mutations causing melorheostosis were unsuccessful, 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 had paired biopsies of both affected and contralateral unaffected bone, and DNA from each patient's affected and unaffected samples were compared.

Using whole exome sequencing (WES), we identified somatic mosaic *MAP2K1* mutations in the affected, but not in unaffected, bone of eight unrelated patients and in the skin overlying lesions, but not in blood [Reference 4]. 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 rather than 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 increase MAP2K1 activity. Identical mutations have been found in malignancies of other tissues, but only three instances of conversion of melorheostosis to osteosarcoma have been reported. Increased MAPK activity leads to increased phosphorylation and activation of ERK1/2, accounting for the mosaic pattern of increased p-ERK1/2 in osteoblast immunohistochemistry of affected bone. 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.

The bone lesions of MAP2K1–positive melorheostosis were investigated using conventional histology, quantitative backscattered imaging, μCT and nanoindentation [Reference 5]. The lesions have two zones, one with intensively remodeled osteonal structure and increased osteoid, which is covered by a zone containing compact multi-layered lamellae. The remodeling zone has lower than normal bone mineralization and a high porosity, reflecting increased tissue vascularity. The lamellar portion is even less mineralized than the remodeling zone, indicating a younger tissue age. Nanoindentation was not increased in the lamellar zone, indicating that the surgical hardness of this bone reflects its lamellar structure and not its material properties. We propose that the genetically induced deterioration of bone microarchitecture in the remodeling zone triggers a periosteal reaction and leads to overall cortical outgrowth.

We also reported distinguishing clinical characteristics of melorheostosis patients with MAP2K1–positive melorheostosis. These patients have 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 inner remodeling zone and an outer zone of primary lamellar bone. The identification of a distinct phenotype of patients with MAP2K1–positive melorheostosis demonstrates clinical and genetic heterogeneity among patients with the disease.

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Hippocampal Interneurons and Their Role in the Control of Network Excitability

Cortical and hippocampal GABAergic inhibitory interneurons (INs) are "tailor-made" to control cellular and network excitability by providing synaptic and extrasynaptic input to their downstream targets via $GABA_A$ and $GABA_B$ receptors. The axons of this diverse cell population make local, short-range projections (although some subpopulations project their axons over considerable distances) and release the inhibitory neurotransmitter γ-aminobutyric acid (GABA) onto a diverse array of targets. Distinct cohorts of INs regulate sub- and suprathreshold intrinsic conductances, regulate Na^+- and Ca^{2+}-dependent action potential generation, modulate synaptic transmission and plasticity, and pace both local- and long-range large-scale synchronous oscillatory activity. An increasing appreciation of the roles played by INs in several neural circuit disorders, such as epilepsy, stroke, Alzheimer's disease, and schizophrenia, has seen this important cell type take center stage in cortical circuit research. With almost 30 years of interest in this cell type, the main objectives of my lab have been to understand (1) the developmental trajectories taken by specific cohorts of INs as they populate the nascent hippocampus and cortex; (2) how ionic and synaptic mechanisms regulate the activity of both local circuit GABAergic INs and principal neurons (PN) at the level of small, well-defined networks; and (3) how perturbations in their function alter the cortical network in several neural circuit disorders. To this end, we use a variety of electrophysiological, imaging, optogenetic, immunohistochemical, biochemical, molecular, and genetic approaches with both wild-type and transgenic animals.

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

For all their complexity, neocortical circuits demonstrate a stereotypical organization across regions, with many repeated motifs, which suggests there are general principles that guide the self-assembly of these circuits during development. A particularly interesting problem for the developing cortex is how to integrate INs, which are produced by progenitors outside the telencephalon, into circuits among locally generated excitatory 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 IN and neighboring projection neurons. 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 projection neurons and INs. Recent evidence suggests that the subtype identity of both projection neurons and IN is important for the process.

Jason Wester and Vivek Mahadevan knocked out the transcription factor Satb2 in projection neurons to induce those of the intratelencephalic (IT) type to adopt a pyramidal tract (PT) identity. Loss of IT projection neurons disrupted the lamination and molecular expression profile of INs derived selectively from the 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 projection neurons are intermingled, IT projection neurons targeted neighboring CGE INs, while PT projection neurons did not, confirming the lineage-dependent motif. Single-cell RNA sequencing revealed that major CGE IN subtypes were conserved, but with differential transcription of synaptic proteins and signaling molecules. Our data show that both projection neuron class and IN embryonic lineage are important general variables during the construction of cortical circuits.

AMPARs differentially regulate cellular and synaptic maturation of CGE-derived hippocampal interneurons.

In this study, led by former Postdoctoral Fellow Gulcan Akgul, we generated two knockout (KO) 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. In both the neonatal (postnatal day [P] 5–9) and juvenile (P17–21) hippocampus the frequency of spontaneous excitatory postsynaptic currents (sEPSC) was significantly reduced in *GluA2* KO, which was coupled with a reduction in dendritic glutamatergic synapse density. Elimination of *GluA1-3* 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 owing to increased apoptosis. However, *GluA1-3* KOs showed an increase in interneurons containing the neurotransmitter CCK and a reduction in those containing the neurotransmitter VIP. 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 (medial ganglionic eminence)– versus CGE–derived interneuron-mediated 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.

Shisa7, a $GABA_A$ receptor auxiliary subunit controlling benzodiazepine actions

GABA type A receptors ($GABA_A$Rs) mediate fast inhibitory transmission in the mammalian brain and are ligand-gated pentameric anion channels assembled from various combinations of 19 subunits. The abundance and kinetics of $GABA_A$Rs at synapses fundamentally control inhibitory synapse strength and neural circuit information processing. Whether native $GABA_A$Rs contain additional auxiliary subunits that control both trafficking and kinetics of the receptor remains unknown. $GABA_A$Rs are also the primary targets for several drugs, notably benzodiazepines (BDZs), barbiturates, anesthetics, and ethanol. The function and

pharmacology of $GABA_A$Rs are of great physiological and clinical importance and have long been thought to be determined by the channel pore–forming subunits. In a collaboration with the lab of Wei Lu, we discovered that Shisa7, a single-passing transmembrane protein, localizes at GABAergic inhibitory synapses and interacts with $GABA_A$Rs. Shisa7 controls receptor abundance at synapses and accelerates the channel deactivation kinetics. Shisa7 also potently enhances the action of diazepam, a classic benzodiazepine, on $GABA_A$Rs. Genetic deletion of Shisa7 selectively impairs GABAergic transmission and diminishes the effects of diazepam in mice. Our data indicate that Shisa7 regulates $GABA_A$R trafficking, function, and pharmacology and reveal a previously unknown molecular interaction that modulates benzodiazepine action in the brain.

Circuit malformation and rearrangement in a mouse model of classic type I lissencephaly

Classical lissencephaly is a rare neurodevelopmental disease caused by haploinsufficiency of the *LIS1* gene and is characterized in humans by brain malformation, developmental delays, and hyperexcitability. The deficits are recapitulated in a mouse model of lissencephaly. As Lis1 protein is required for cellular migration, we used this model to investigate the impact of disrupted neuronal migration on both PN and IN microcircuit formation.

PNs of the developing mouse hippocampus arise in proliferative waves of cell birth and migration between E12–17. Later, PNs appear as a densely packed monolayer of neurons, in a principal cell layer (PCL). Emerging evidence suggests the existence of many excitatory cell subtypes within the PCL. James (Jim) D'amour is investigating two populations of PNs readily identified in the superficial-deep axis of the hippocampus: calbindin (CB)-positive and calbindin-negative PNs. CB immunostaining labels superficial PNs of the PCL, while the deeper half of the PCL is largely devoid of CB expression. In normal animals, CB expression confers a complex dendritic morphology, sensitivity to endocannabinoids, greater sag current conductance, and preferential targeting of IN subtypes. These data suggest that CB–expressing PCs form a previously underappreciated layer within the PCL, akin to the layers within the neocortex, but compressed into a single band within the evolutionarily older hippocampus. Jim next studied this CB subpopulation of cells in the mouse model of lissencephaly. Mice heterozygous for the *Lis1* gene display fractured PCLs into deep and superficial bands. He found that calbindin-expressing PNs are preferentially located in the deeper band (opposite of their normal superficial positioning) and is currently probing the interplay between cellular positioning, genetic identity, and cellular birthdate in the formation of brain circuits. Preliminary data suggest that ectopically positioned CB–PNs retain their intrinsic properties, including morphology and ion channel expression. However, connectivity to extrinsic afferents and local IN subpopulations appear to be perturbed, altering the role played by these PNs in generating coherent oscillations.

In a second related study, Tyler Ekins characterized the distributions of INs in the *Lis1*$^{+/-}$ CA1 hippocampus. Although the total density of IN classes (PV-, SOM-, RN-, CCK-) reach the hippocampus in normal quantities, PV–containing INs migrate towards more superficial regions and are found in ectopic positions deep in stratum radiatum in the *Lis1*$^{+/-}$ mouse. He next eliminated one copy of LIS1 from specific cell types: Emx-Cre (Lis1 removed from PNs), Nkx2.1-Cre (Lis1 removed from MGE–derived INs). Interestingly, Emx-Cre and Nkx-Cre lines resulted in a pattern similar to the global *Lis1*$^{+/-}$ line: no change in overall cell density, but shift PV INs towards superficial regions of the CA1, indicating both cell-autonomous and non-autonomous roles for Lis1 in IN migration. Cluster analysis of PV–IN electrophysiological properties identified two subpopulations: fast-Spiking (FS) with higher rheobase and firing frequency and lower input resistance, and fast-spiking-like (FSL) cells with lower rheobase and firing frequency and higher input resistance. FS cells consist of the standard

subtypes (basket, bistratified, and axo-axonic cells), but FSL cells had atypical PV cell morphologies and, in WT, represent a minor population. In contrast, although *Lis1*$^{+/-}$ PV cells also sorted into clusters of FS and FSL cells, FSL cells were the dominant population, particularly among cells positioned in the stratum radiatum. He next recorded the output of PV INs and found that basket cells provide stronger inhibition to morphologically defined "simple" PNs (see above), which tend to occupy the deeper half of the PCL than "complex" superficially located PNs. This pattern held true in the LIS1 KO, even among PNs in ectopic positions. Synaptically coupled PV and PN pairs demonstrate that unitary inhibitory connections from WT basket cells are strongest on simple PNs than complex PNs. Further paired recordings will elucidate the connectivity patterns and dynamics of FS/FSL cells in the LIS hippocampus and help us gain a better understanding of how cellular migration drives synaptogenesis and circuit formation.

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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 nonclassic form, CAH is 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 owing to 21-hydroxylase deficiency have a contiguous gene deletion syndrome resulting in connective tissue dysplasia, the 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.

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Adrenal crisis prevention

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 attributable to to congenital adrenal hyperplasia, who were followed prospectively at the NIH Clinical Center and received repeated glucocorticoid stress-dosing education. Longitudinal analysis of over 2,200 visits from 156 CAH patients over 23 years was performed [Reference 1]. 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. Life-threatening 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.

This longitudinal assessment of illnesses, glucocorticoid stress-dosing practices and illness sequelae in patients with adrenal insufficiency from CAH resulted in recommendations to revise age-appropriate glucocorticoid stress-dosing guidelines to include more frequent glucocorticoid dosing and frequent intake of simple and complex carbohydrates. Our new age-appropriate guidelines aim to reduce adrenal crises and prevent hypoglycemia, particularly in children. These suggestions were incorporated into the Endocrine Society Clinical Practice Guideline for Congenital Adrenal Hyperplasia.

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. The 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 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.

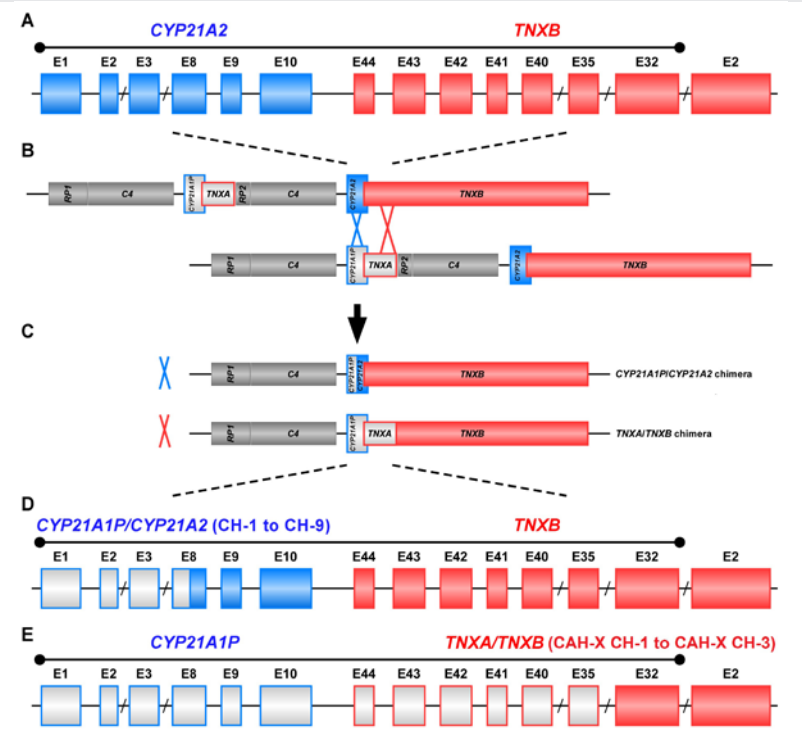

FIGURE 1. Schematic of *CYP21A1P/CYP21A2* and *TNXA/TNXB* chimeric genes

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 owing to 21-hydroxylase deficiency carry at least one *TNXA/TNXB* chimera, resulting in hypermobility-type Ehlers-Danlos syndrome or CAH-X syndrome.

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. Genetic testing for CAH-X is complex and complicated by pseudogene interference and the large 70kb size of the *TNXB* gene. This year, we developed a PCR–based, high-throughput, cost-effective assay that accurately identifies CAH-X [Reference 2]. The assay had 100% sensitivity and 99.2% specificity.

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.

The influence of complement component 4 (C4) copy number on phenotype in patients with CAH

We evaluated *C4A* and *C4B* copy number in relation to *CYP21A2* genotype, psychiatric and autoimmune comorbidity, and serum C4 concentrations in 145 patients with CAH and 108 carrier relatives [Reference 3]. We found *CYP21A2* mutation-specific *C4* structural variations. *C4B* copy number determined serum C4 concentration; high *C4B* copy number was associated with the most common nonclassic CAH mutation

(p.V281L), while low *C4B* copy number was associated with 30kb deletions. Only 30% of subjects had the expected two copies each of the two *C4* genes. High *C4A* copy number (3 or more) was associated with increased risk of having an externalizing psychiatric condition. No association was found between *C4* copy number and autoimmune disease, possibly because of the young age of our patients or the influence of their glucocorticoid treatment. We showed, for the first time, an association at the genomic level between C4 copy number and susceptibility to psychopathology in CAH.

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 with adrenal insufficiency in childhood, and we identified a genetic cause of their disease [Reference 4]. 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 half-life and clinically impairs both adrenal and gonadal function. Our patients had normal male genitalia, and older males had normal pubertal progression but 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. Children with isolated glucocorticoid deficiency should be genetically evaluated for steroidogenic enzyme deficiencies.

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 11beta-hydroxylase (11-oxygenated C19 steroids) are abundant in patients with CAH resulting from 21-hydroxylase deficiency (Figure 2). With our collaborators Richard Auchus and Adina Turcu, 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 with enlarged adrenal glands and testicular tumors. 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.

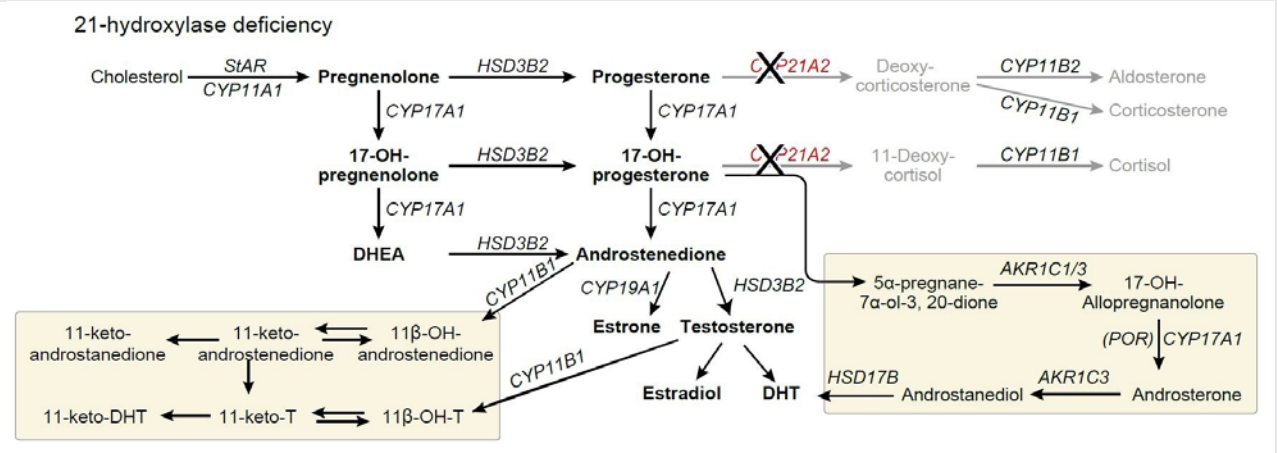

FIGURE 2. Classic and alternative steroidogenesis pathways leading to adrenal androgen production

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.

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 owing to 21-hydroxylase deficiency and with multiple comorbidities. 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. The 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 modified-release 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 a lower hydrocortisone dose equivalent. Successful completion of this phase 2 study (NCT 01735617), carried out at the NIH Clinical Center, resulted in a multicenter 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 and 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 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 long-term 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 the group of Perrin White, we are studying Abiraterone, an irreversible inhibitor of 17a-hydroxylase, a key enzyme required for testosterone synthesis, in a multicenter Phase 1/2 study in prepubescent children (*NCT02574910*).

Additional Funding

- Cooperative Research and Development Agreement (CRADA) #02800 for Age-Appropriate Hydrocortisone Formulations for the Treatment of Adrenal Insufficiency including Congenital Adrenal Hyperplasia
- NIH U Grant: Abiraterone Acetate in Children with Classic 21-Hydroxylase Deficiency
- Cooperative Research and Development Agreement (CRADA) #03222 for Clinical Development of a Corticotropin Releasing Factor-1 Receptor Antagonist for the Treatment of Congenital Adrenal Hyperplasia

Publications

1. El-Maouche D, Hargreaves CJ, Sinaii N, Mallappa A, Veeraraghavan P, Merke DP. Longitudinal assessment of illnesses, stress dosing and illness sequelae in patients with congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2018 103:2336-2345.
2. Lao Q, Brookner B, Merke DP. High throughput screening for CYP21A1P-TNXA/TNXB chimeric genes responsible for Ehlers Danlos syndrome in patients with congenital adrenal hyperplasia. *J Mol Diagn* 2019 21:924-931.
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4. Kolli V, Kim H, Torky A, Lao Q, Tatsi C, Mallappa A, Merke DP. Characterization of the CYP11A1 non-synonymous variant p.E314K in children presenting with adrenal insufficiency. *J Clin Endocrinol Metab* 2019 104:269-276.
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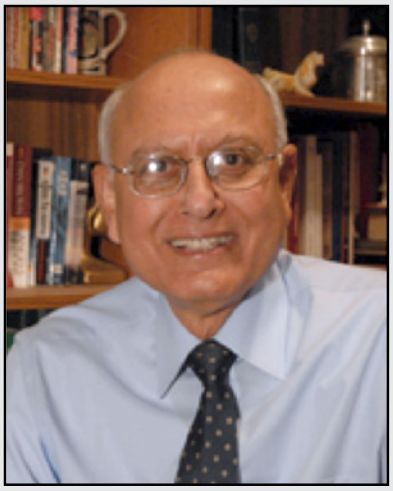
Childhood Neurodegenerative Lysosomal Storage Disorders

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 neuronal ceroid lipofuscinoses (NCLs), also known as Batten disease. The diseases mostly affect children and there is no effective treatment for any of the NCLs. Mutations in at least 13 different genes (called the *CLNs*) underlie various forms 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 various subcellular localizations are encoded by *CLN3*, *CLN6*, *CLN7*, *CLN8*, and *CLN12*.

Despite intense studies, the 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 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 that regulates 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, which is caused by mutations in the *CLN1* gene, a gene that encodes the lysosomal depalmitoylating enzyme palmitoyl-protein thioesterase-1 (PPT1). Numerous proteins in the body, especially in the brain, undergo posttranslational modification called *S*-palmitoylation (also called *S*-acylation), whereby a 16-carbon, saturated fatty acid (generally palmitic acid) is attached



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to specific cysteine residues in polypeptides via thioester linkage. While *S*-palmitoylation plays important roles in membrane anchorage of soluble proteins, protein-protein interaction, protein trafficking, and protein stability, these lipid-modified proteins must also be depalmitoylated for recycling or degradation and clearance by lysosomal hydrolases. PPT1 catalyzes the cleavage of thioester linkage of *S*-palmitoylated proteins, which is vitally important because *S*-palmitoylated proteins are refractory to degradation by lysosomal hydrolases. Thus, PPT1 deficiency leads to lysosomal accumulation of the S-palmitoylated proteins (constituents of ceroid), which has been proposed to be the mechanism of INCL pathogenesis. However, the precise molecular mechanism underlying INCL pathogenesis has remained elusive for more than two decades after the discovery that *CLN1* mutations cause INCL. Children afflicted with INCL are normal at birth but, by 11 to 18 months of age, they exhibit signs of psychomotor retardation. By two years of age, they are completely blind owing to retinal degeneration and, by age four, they manifest no brain activity and remain in a vegetative state for several 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 our laboratory investigations to develop novel therapeutic strategies for Batten disease. The results of our earlier investigations on INCL led to a bench-to-bedside clinical trial. 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 and thus 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 dysregulates lysosomal acidification, causing elevated pH and thus adversely affecting lysosomal degradative function.

We also developed a noninvasive method, using MRI and MRS (magnetic resonance spectroscopy), to evaluate the progression of neurodegeneration in $Cln1^{-/-}$ mice. The methods permit repeated evaluation 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 neurodegeneration. In collaboration with Wadih Zein, we are also conducting studies to determine whether electroretinography 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 *Cln1*$^{-/-}$ mice and that the pathology is ameliorated by treatment with resveratrol, which has antioxidant 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. These and related studies provide insight into the complex mechanisms of heritable disorders of neurodegeneration such as INCL (CLN1 disease) and identify several potential therapeutic targets. Our results suggest that thioesterase-mimetic small molecules such as NtBuHA are potential therapeutics 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.

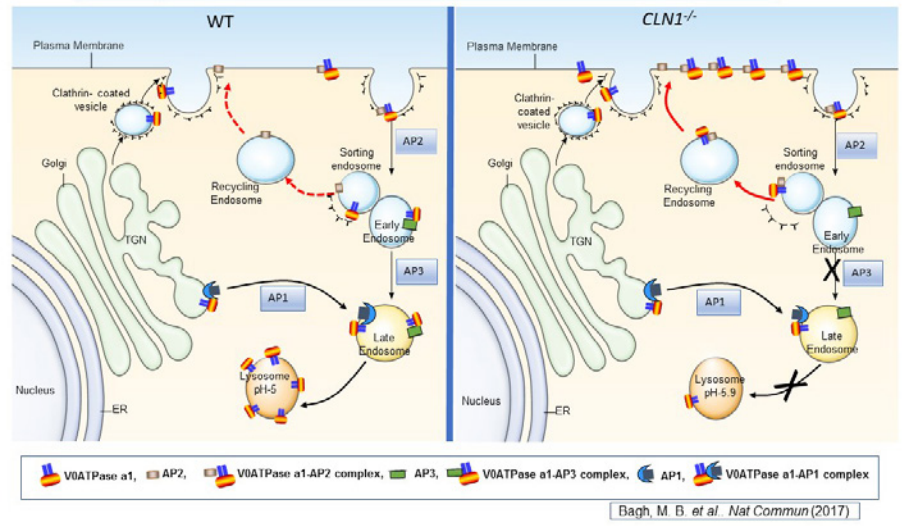
Palmitoyl-protein thioesterase-1 deficiency suppresses Rab7–RILP interaction dysregulating autophagy in *Cln1*$^{-/-}$ mice.

Despite the discovery more than two decades ago that PPT1 deficiency underlies INCL, the precise molecular mechanisms of pathogenesis have remained elusive. We found that autophagy is dysregulated in *Cln1*$^{-/-}$ mice, which mimic INCL, and in postmortem brain tissues from an INCL patient as well as in cultured INCL fibroblasts. Moreover, Rab7, a small GTPase, critical for autophagosome-lysosome fusion, requires *S*-palmitoylation for trafficking to the late endosomal/lysosomal membrane and is dysregulated in *Cln1*$^{-/-}$ mice. Notably, the defect inhibited the Rab7–RILP (Rab–interacting lysosomal protein) interaction, which is essential for Rab7 GTPase activity and required for autophagosome-lysosome fusion. The defect impaired degradative functions of the autolysosome (a hybrid structure arising from the fusion of autophagosome with lysosome), causing lysosomal accumulation of undegraded cargo, leading to INCL pathogenesis. Importantly, treatment of INCL fibroblasts with NtBuHA, a brain-penetrant, PPT1–mimetic small molecule, ameliorated the defective Rab7–RILP interaction. Our findings reveal a previously unrecognized role of *CLN1*/PPT1 in autophagy and suggest that thioesterase-mimetic small molecules may ameliorate the dysregulated autophagy, with therapeutic implications for INCL.

Ppt1 deficiency causes microglia proliferation and generates reactive astrocytes, mediating neuropathology in a mouse model of INCL.

In the central nervous system, the astrocytes and microglia are components of the innate immune system; however, under certain circumstances their proliferation and activation can lead to neurotoxicity. Although the role of the innate immune responses in neurodegenerative diseases have been reported to be initiated early in the disease process, the infiltration of the immune cells from the blood to the brain does not appear to play a role in the neuropathology. Recently, it was reported that the proliferation and activation of microglia leads to the conversion of astrocytes to the neurotoxic A1 phenotype, causing pathogenesis in models of common neurodegenerative diseases. In common neurodegenerative diseases such as Alzheimer's and Parkinson's, causal gene mutations associated with these diseases have been reported. However, establishing the cause and effect relationship linking mutations in a specific gene with neurodegeneration has remained challenging.

FIGURE 1. Lysosomal acidification defect in a mouse model of infantile neuronal ceroid lipofuscinosis



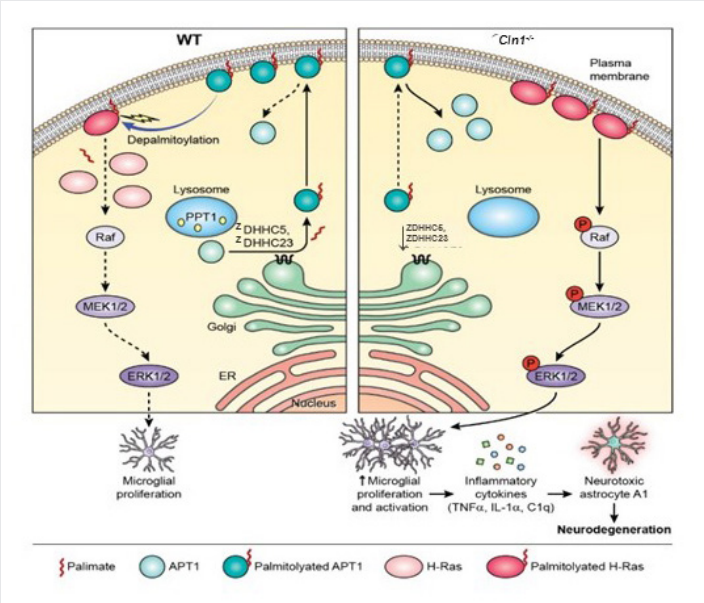
Lysosomal acidification is accomplished by vacuolar ATPase (v-ATPase) localized to the lysosomal membrane. Consisting of a lysosomal membrane–localized V0 sector and cytosolic V1 sector, v-ATPase is a multi-subunit protein. We discovered that the V0a1 subunit of the v-ATPase requires *S*-palmitoylation (a reversible, post-translational 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*$^{-/-}$ mice, which mimic INCL. In *Cln1*$^{-/-}$ 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.

We sought to determine whether the conversion of astrocytes to the neurotoxic A1 phenotype underlie neuropathology in a rare monogenic neurodegenerative disease such as INCL. The availability of *Cln1*$^{-/-}$ mice, a well-recognized reliable animal model of INCL, provided an opportunity to address this question. We found that in *Cln1*$^{-/-}$ mice, Ppt1 deficiency suppressed ZDHHC5 (zinc finger DHHC-type palmitoyltransferase 5) and ZDHHC23 (zinc finger DHHC-type palmitoyltransferase 23), which catalyze *S*-palmitoylation of the cytosolic thioesterase Apt1, which is essential for the enzyme's membrane localization. Given that Apt1 depalmitoylates membrane-localized H-Ras, reduced Apt1 levels in *Cln1*$^{-/-}$ mice significantly elevated H-Ras levels on the plasma membrane, thus activating its signaling pathway, which stimulated microglia proliferation. Increased cytokine produced by microglia and elevated complement C1q levels stimulated the generation of neurotoxic A1 astrocytes. Importantly, treating *Cln1*$^{-/-}$ mice with NtBuHA substantially ameliorated these abnormalities. Our results reveal a previously unrecognized pathway to neurodegeneration in INCL and demonstrate that NtBuHA exerts its neuro-protective effects by suppressing the generation of neurotoxic A1 astrocytes in the brain.

Persistent mTORC1–signaling contributes to neuropathology in INCL mice: amelioration by recombinant PPT1 or Akt1 inhibitor.

Although classically, the lysosome has been considered the terminal organelle for degradation and cellular clearance, emerging evidence indicates that it also relays several nutrient cues to the master growth regulator, the mechanistic target of rapamycin complex 1 (mTORC1) kinase. Previously, we reported that in *Cln1*$^{-/-}$ mice, the reliable animal model of INCL, lysosomal acidification is dysregulated owing to mislocalization of a critical subunit (V0a1) of v-ATPase, which regulates lysosomal acidification. The v-ATPase along with Lamptor1 and SLC38A9 are essential components of the lysosomal nutrient-sensing machinery, which regulates mTORC1

FIGURE 2. Dysregulated neuro-immune interaction as a mechanism of neurodegeneration in INCL



Schematic model explaining how Ppt1 deficiency in *Cln1*$^{-/-}$ mice may cause increased H-Ras signaling, which stimulates activated microglia proliferation and its conversion to A1 phenotype, leading to neurodegeneration.

Left panel: In WT mice, dynamic *S*-palmitoylation of APT1 mediated by ZDHHC5 and ZDHHC23 and APT1 depalmitoylation, respectively, are responsible for steady state membrane localization of both APT1 and H-Ras.

Right panel: In *Cln1*$^{-/-}$ mice, the lower level of ZDHHC5 and ZDHHC23 results in a reduced level of *S*-palmitoylated APT1, which increases the levels of plasma membrane–anchored, *S*-palmitoylated H-Ras, thus transducing proliferation signals to microglia to produce proinflammatory cytokines. The cytokines stimulate the conversion of astrocytes to the neurotoxic A1-phenotype. Elevated numbers of A1-astrocytes release yet to be identified neurotoxins, causing progressive neuronal loss in the *Cln1*$^{-/-}$ mouse brain.

signaling. We found that Lamptor1 and SLC38A9, which are components of the lysosomal nutrient-sensing apparatus, require *S*-palmitoylation for their localization on the lysosomal membrane and are misrouted in PPT1–deficient *Cln1*$^{-/-}$ mice. However, despite the disruption of the lysosomal nutrient-sensing machinery, mTORC1 signaling is persistently high in *Cln1*$^{-/-}$ mouse brain. Notably, in *Cln1*$^{-/-}$ mice sustained high levels of p-Akt (a serine/threonine-specific protein kinase), p-TSC2 (phospho-tubersclerosis 2), and pPRAS40 (proline-rich Akt substrate of 40 kDa), which promoted the recruitment of Rheb to the lysosomal surface, mediating mTORC1-activation. Remarkably, while mTORC1 signaling in the brain was detectable only on postnatal day 1 of wild-type (WT) mice and was virtually undetectable through adulthood, it was persistently detectable in the brain of *Cln1*$^{-/-}$ prenatal mice through adulthood. The dysregulated autophagy contributing to neuropathology. Importantly, Akt1 inhibitor or recombinant PPT1 substantially reduced mTORC1 signaling. Our results reveal a previously unrecognized role of PPT1 in regulating mTORC1 signaling and suggest that inhibitors of Akt1 may have therapeutic implications for INCL.

Misrouting of the v–ATPase subunit Voa1 dysregulates lysosomal acidification in *Cln1*$^{-/-}$ mice.

The lysosome, the primary organelle for intracellular digestion in eukaryotic organisms, 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 neurodegenerative diseases such as Alzheimer's

and Parkinson's disease. However, despite intense studies, the mechanisms underlying the lysosomal acidification defect remain 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 zinc finger proteins containing Asp-His-His-Cys (DHHC) in the active site. Thus, the enzymes are commonly known as ZDHHC-PATs or ZDHHCs. In contrast, there are four thioesterases that have been so far characterized. Two are cytosolic (acyl-protein thioesterase-1 [Apt1] and Apt2) and two (palmitoyl-protein thioesterase-1 [PPT1] and PPT2) are localized to the lysosome. Dynamic palmitoylation (palmitoylation-depalmitoylation), requiring coordinated action of both the ZDHHC-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 and clearance of *S*-palmitoylated proteins by lysosomal hydrolases.

Despite its fundamental importance, the mechanisms underlying the defect remain unclear. We found that in *Cln1*$^{-/-}$ mice, which mimic INCL, reduced v-ATPase activity correlates with elevated lysosomal pH. Moreover, v-ATPase subunit a1 of the V0 sector (V0a1) requires palmitoylation for interacting with adaptor protein-2 (AP-2) and AP-3, respectively, for trafficking to the lysosomal membrane. Notably, treatment of *Cln1*$^{-/-}$ mice with NtBuHA ameliorated this defect. Our findings reveal an unanticipated role of Cln1/Ppt1 in regulating lysosomal targeting of V0a1 and suggest that varying factors adversely affecting v-ATPase function dysregulate lysosomal acidification in other LSDs and common neurodegenerative diseases.

Cln3–mutations, which underlie juvenile NCL, cause significantly reduced levels of Ppt1 protein and Ppt1 enzyme activity in the lysosome.

Given that intracellular accumulation of ceroid is a characteristic of all NCLs, a common pathogenic link for INCL and JNCL has been suggested. It has been reported that *CLN3* mutations suppress the exit of cation-independent mannose 6-phosphate receptor (CI-M6PR) from the *trans*-Golgi network (TGN). CI-M6PR transports soluble proteins such as PPT1 from the TGN to the lysosome, so we hypothesized that *CLN3* mutations may cause lysosomal PPT1 insufficiency, contributing to JNCL pathogenesis. We found that the lysosomes in *Cln3*–mutant mice, which mimic JNCL, and those in cultured cells from JNCL patients, contain significantly reduced levels of Ppt1 protein and Ppt1 enzyme activity and progressively accumulate autofluorescent ceroid. Furthermore, in JNCL fibroblasts the V0a1 subunit of v-ATPase is mislocalized to the plasma membrane instead of its normal location on lysosomal membrane. The defect dysregulates lysosomal acidification, as we previously reported in *Cln1*$^{-/-}$ mice, which mimic INCL. Our findings uncover a previously unrecognized role of CLN3 in lysosomal homeostasis and suggest that *CLN3* mutations causing lysosomal Ppt1 insufficiency may at least partly contribute to JNCL pathogenesis.

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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 (TNF-alpha). While IFNs impart antiviral and antimicrobial protection to the host, the latter cytokines are associated with inflammatory responses. IFNs are produced upon activation of the IRF (interferon regulatory factor) family of transcription factors, while inflammatory cytokines are produced by activation of the transcription factor NFκB. Our goal is to study the molecular pathways that direct the development and function of macrophages and other myeloid cells. To this end, we focus on the role of IRF8 in innate immunity. IRF8, a member of the IRF family, is expressed 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 antipathogen 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 (single nucleotide

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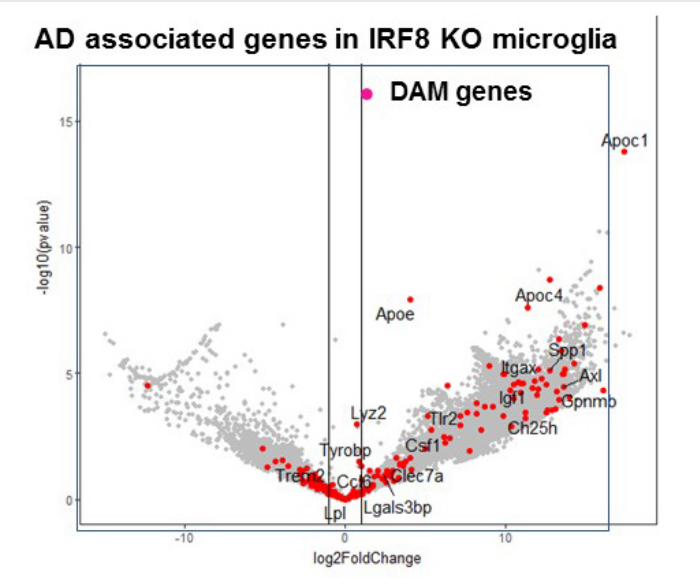

FIGURE 1. Alzheimer's disease–associated genes in *IRF8* KO microglia

Microglia were sorted from wild-type 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.

polymorphism)–based genome-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 in 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 various forms of encephalopathy with prominent neuroinflammation. Symptoms include vision/motor deficits, 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 YJ, 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,

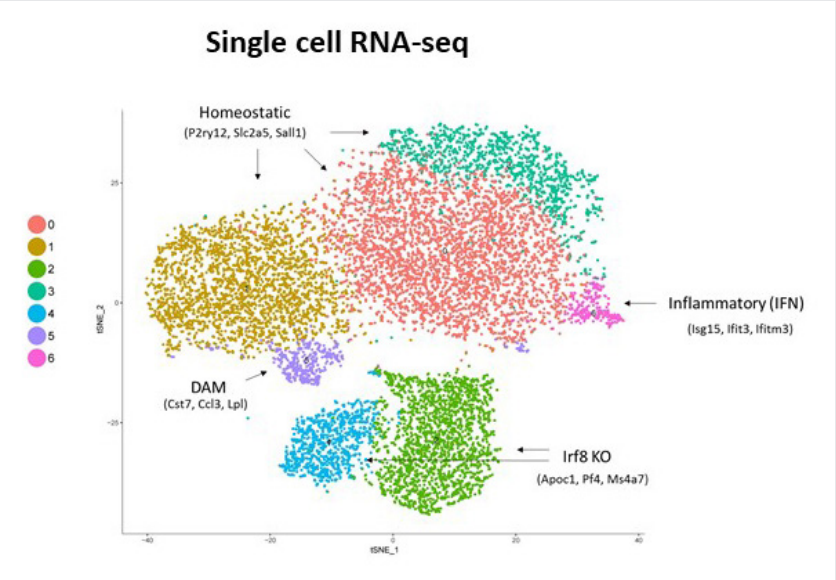

FIGURE 2. Single-cell RNA-seq was performed with the 10X genomics system.

Seven clusters were identified among WT, IRF8 KO, 5xFAD, and the cross of the latter two. IRF8KO microglia show a unique cluster.

DAM: disease-associated microglia genes

activation of ISGs, coined the "interferon signature," is a defining feature of AGS. ISG proteins are found in spinal fluids, presumably produced in microglia (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.

To further investigate the role of IRF8 in AD, we crossed IRF8KO mice with 5xFAD mice, a model of AD. We found that amyloidβ accumulation was reduced in 5xFAD microglia when crossed with IRF8KO. Consistent with these data, we found that the lysosomal activation marker CD68 was less in the absence of IRF8 (Figure 1). With the help of Steven Coon, we performed single cell RNA-seq for microglia from these mice. Our results indicated that IRF8KO microglia form a cell population with transcriptome profiles distinct from those of WT or 5xFAD. Our analysis indicated that IRF8KO and AD mice share transcriptome profiles more closely than WT (Figure 2).

Context-dependent role for BRD4

RD4 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 plays a critical role in forming superenhancers. Superenhancers are long stretches of regulatory DNAs densely occupied by transcription factors and chromatin regulators. They direct strong transcription of select genes and thus help define cellular and lineage identity. In the past several years, research on BRD4 has seen a dramatic upturn owing to the development of small-molecule inhibitors that inhibit binding of acetyl-histones to the BET family proteins. These inhibitors, affecting mostly the BET protein BRD4, antagonize cancer growth,

particularly leukemia and lymphoma. Furthermore, BET inhibitors have been shown to inhibit inflammatory responses related to cardiovascular and autoimmune diseases. 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 mice, focusing on hematopoiesis and macrophage responses. First, we tested mice in which *Brd4* is deleted in early hematopoiesis by using the Vav-Cre technique. We showed that *Brd4* KO mice die during fetal development owing to severe defects in the expansion of hematopoietic stem cells (HSC) and in the development of hematopoietic progenitor cells. As a consequence, *Brd4* KO embryos fail to develop immune cells of all lineages, including lymphocytes and myeloid cells, which are important for innate and adaptive immunity. We also found that BRD4 is essential for the proliferation of macrophages, based on LysM-Cre–dependent deletion of *Brd4* (LysM-Cre selectively targets macrophages and neutrophils); the resultant *Brd4* KO mice failed to start IL-4–dependent peritoneal macrophage expansion. The results strongly point to a central role of BRD4 in immune cell expansion, required for maintaining immunity.

We investigated the genome-wide distribution of BRD4 in macrophages in a resting condition and after LPS stimulation. LPS is a pathogen component that rapidly induces inflammatory genes and interferon-stimulated genes important for protection against pathogens. We found that BRD4 broadly occupies genic and intergenic regions. Within the genic region, BRD4 binding peaked at the transcription start site (TSS), although binding was detected over the 5' promoter and within the coding regions. BRD4 binding over the genic regions markedly increased after LPS stimulation, indicating that BRD4 moves rapidly over the genome, presumably to accommodate a rapid alteration of histone acetylation. Furthermore, BRD4 displayed dense clustering over distant regulatory regions that represented superenhancers. BRD4 clusters coincided with the H3K27 chromatin mark, which denotes superenhancers as well as RNA polymerase II clustering. BRD4–containing superenhancers localized to genes important for basic macrophage phenotypes and innate immune responses. We also showed that superenhancers rapidly redistributed in response to LPS in WT macrophages. Interestingly, superenhancers were found even in Brd4 KO macrophages. These superenhancers contained large clusters of pol II and H3K27ac marks, but without BRD4, and their distribution patterns were anomalous. The results led us to conclude that BRD4 plays an important role in shaping superenhancers necessary for inflammatory responses in macrophages.

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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 to different therapeutic options. We focus on developmental, molecular, genetic, epigenetic, proteomic, metabolomic, immunologic, 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.

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Clinical and genetic aspects of pheochromocytoma and paraganglioma

Pheochromocytoma and PHEO/PGL can be divided into at least four molecular subgroups. Whether such categorizations are independent factors for prognosis or metastatic disease is unknown. We performed a systematic review and individual patient meta-analysis aimed at estimating whether driver mutation status can predict metastatic disease and survival. Driver mutations were used to categorize patients according to three different molecular systems: two subgroups (*SDHB* mutated or wild-type [WT]), three subgroups (pseudohypoxia, kinase signaling or Wnt/unknown) and four subgroups (tricarboxylic acid cycle, *VHL/EPAS1*, kinase signaling or

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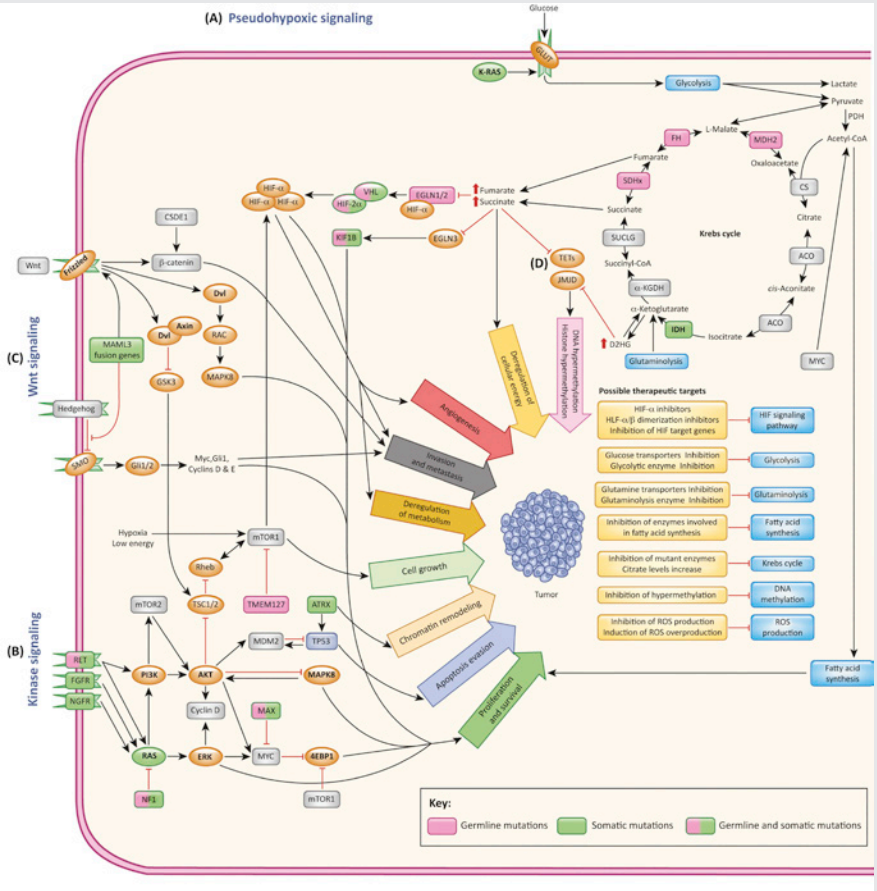
Wnt/unknown). We analyzed 21 studies and 703 patients. Multivariate models for association with metastasis showed correlation with the *SDHB* mutation, as well as with norepinephrine and dopamine but not with PHEO/PGL location. Other molecular systems were not associated with metastasis. In multivariate models for association with survival, age, and metastases neither paraganglioma nor *SDHB* mutation remained significant. Other molecular subgroups did not correlate with survival. We concluded that molecular categorization according to *SDHB* provided independent information on the risk of metastasis. Driver-mutation status did not correlate independently with survival. We believe that these data may ultimately be used to guide current and future risk stratification of PHEO/PGL.

Adrenocortical carcinoma (ACC) and PHEO/PGL are defined by clinico-pathological criteria and can be further subdivided based on various molecular features. Whether differences between these molecular subgroups are significant enough to rechallenge their current clinico-pathological classification is currently unknown. It is also not fully understood to which other cancers ACC and PHEO/PGL show similarity. To address these questions, we included recent RNA-seq data from the TCGA (Cancer Genome Atlas) and TARGET (Therapeutically Applicable Research to Generate Effective Treatments) datasets. Two bioinformatics pipelines were used for unsupervised clustering and principal components analysis and results validated using a consensus clustering model and interpreted according to previous pan-cancer experiments. We studied two datasets consisting of 3319 tumors from 35 disease categories. Consistent with the current classification, ACCs clustered as a homogenous group in a pan-cancer context. It also clustered close to neural crest–derived tumors, including gliomas, neuroblastomas, pancreatic neuroendocrine tumors, and PHEOs/PGLs. By contrast, some PPGLs were mixed with pancreatic neuroendocrine tumors or neuroblastomas. Thus, our unbiased gene-expression analysis of PHEO/PGL did not overlap with their current clinico-pathological classification. The results emphasize the importance of the shared embryological origin of these tumors, all either related or close to neural crest tumors, and open the way for investigation of a complementary categorization based on gene-expression features.

Somatic mutations in hypoxia-inducible factor 2α gene (*HIF2A*) are associated with the polycythemia-paraganglioma syndrome. Specifically, for the first time, our group described the classic presentation of female patients with recurrent paragangliomas (PGLs), polycythemia (at birth or in early childhood), and duodenal somatostatinomas. Studies demonstrated that somatic *HIF2A* mutations occur as postzygotic events, while some are associated with somatic mosaicism affecting hematopoietic and other tissue precursors. The phenomenon could explain the development of the early onset of polycythemia in the absence of erythropoietin-secreting tumors. Somatic *HIF2A* mutations (p.A530V, p.P531S, and p.D539N) were identified in DNA extracted from PGLs of three patients. No somatic mosaicism was detected through deep sequencing of blood genomic DNA. Compared with the classic syndrome, both polycythemia and PGL in all three patients developed at an advanced age, with polycythemia at age 30, 30, and 17 years and PGLs at age 34, 30, and 55 years, respectively. Somatostatinomas were not detected, and two patients had ophthalmic findings. The biochemical phenotype in all three patients was noradrenergic, with ^{18}F-FDOPA PET/CT as the most sensitive imaging modality. All patients demonstrated multiplicity, i.e., lesions in multiple sites, and none developed metastatic disease. The findings suggest that newer techniques need to be developed to detect somatic mosaicism in patients with this syndrome. Absence of *HIF2A* mosaicism in patients with somatic HIF2A



Genomic landscape of pheochromocytoma and paraganglioma

From: Jochmanova I, Pacak K. *Trends Cancer* 2018;4(1):6-9.

mutations supports association with late onset of the disease, milder clinical phenotype, and an improved prognosis compared with patients who have *HIF2A* mosaicism.

We also extensively reviewed cardiomyopathies in patients with catecholamine-secreting PHEO/PGL. We also emphasized the necessity of using adrenoceptor blockade in all patients with these tumors. Furthermore, a pharmacologic catecholamine blockade is achieved with α-adrenoceptor and β-adrenoceptor blockers, calcium-channel blockers, or both, with or without the catecholamine synthesis inhibitor metyrosine (Demser). Studies on other medications for catecholamine-induced sinus tachycardia are limited. For the first time, we introduced the use of Ivabradine in the treatment of catecholamine-induced tachyarrhythmia and heart failure. Finally, for the first time we outlined recommended postoperative care of patients with surgically removed PHEO/PGL.

Cancer cells without mitochondrial DNA (mtDNA) do not form tumors unless they reconstitute oxidative phosphorylation (OXPHOS) by mitochondria acquired from host stroma. To understand why functional respiration is crucial for tumorigenesis, we used time-resolved analysis of tumor formation by mtDNA–depleted cells and genetic manipulations of OXPHOS. We showed that pyrimidine biosynthesis dependent on respiration-linked dihydroorotate dehydrogenase (DHODH) is required to overcome cell-cycle arrest, while mitochondrial ATP generation is dispensable for tumorigenesis. Latent DHODH in mtDNA–deficient cells is fully activated with restoration of complex III/IV activity and coenzyme Q redox cycling after mitochondrial transfer,

or by introduction of an alternative oxidase. Furthermore, deletion of DHODH interferes with tumor formation in cells with fully functional OXPHOS, while disruption of mitochondrial ATP synthase has little effect. Our results show that DHODH–driven pyrimidine biosynthesis is an essential pathway linking respiration to tumorigenesis, pointing to inhibitors of DHODH as potential anticancer agents.

Imaging of pheochromocytomas and paragangliomas

Phenotyping disease on the basis of nuclear imaging findings heavily depends on genetic background, metabolites, cell membrane specific targets and signaling pathways. PHEOs/PGLs that are related to succinate dehydrogenase subunits mutations (*SDHx* mutations) are less differentiated than other subgroups and may therefore fail to concentrate ^{18}F-FDOPA, a precursor of catecholamines biosynthesis. However, the ^{18}F-FDOPA–negative phenotype has been reported mostly in *SDHx*-PHEO/PGL of sympathetic origin, suggesting that both genotype status and location (from sympathetic vs. parasympathetic paraganglia; adrenal vs. extra-adrenal) could influence ^{18}F-FDOPA uptake. The aim of this study was to test whether *SDHx* drives ^{18}F-FDOPA uptake in the presence of normal epinephrine/norepinephrine concentrations. A cohort of 86 head and neck PHEO/PGL patients (including three metastatic) with normal metanephrines underwent ^{18}F-FDOPA PET/CT. The relationships between ^{18}F-FDOPA uptake and tumor genotype were evaluated. We found that in non-metastatic head and neck PGLs (50 non-*SDHx*/33 *SDHx*), no significant difference was observed between these two groups for SUVmax and total lesion uptake. Metastatic head and neck PGLs also had highly elevated uptake values. Our results suggest that neither *SDH* deficiency nor metastatic behavior influence on ^{18}F-FDOPA uptake in head and neck PGL, probably owing to their high differentiation status, even at metastatic stage. The potential prognosticator value of ^{18}F-FDOPA uptake would need to be further explored in the setting of metastatic PHEO/PGL of sympathetic origin.

An additional multicenter retrospective study included patients from the period 2003 to 2017 with an appropriate CT examination and a histological diagnosis of adrenal adenoma, pheochromocytoma, adrenocortical carcinoma, or metastasis. In total, 346 patients were suitable for the CT image analysis, which included evaluation of the largest diameter, the shape of the lesion, the presence of central necrosis and its margins, and the presence of an enhancing peripheral rim ("ring sign"). PHEOs had a significantly more spherical shape, whereas an elliptical shape significantly reduced the probability of PHEO, as did another shape. A "ring sign" was also more frequent in PHEOs than in other adrenal tumors. A sharp necrosis also increased the probability of PHEO more than unsharp necrosis. The probability calculation model created on the basis of the results confirms a high sensitivity and specificity.

We also summarized recent data related to targeted radionuclide therapy (TRT), which we feel should preferably be performed at specialized centers with an experienced interdisciplinary team. In future, radiotherapy should include the introduction of dosimetry and biomarkers for therapeutic responses for more individualized treatment plans, α-emitting isotopes, as well as the combination of targeted radiotherapies with other systemic therapies.

Immune and metabolic aspects of pheochromocytoma and paraganglioma

Therapeutic options for metastatic PHEO/PGL are limited. We therefore tested an immuno-therapeutic approach based on intratumoral injections of the antibiotic complex mannan-BAM with toll-like receptor ligands into subcutaneous PHEO in a mouse model. The therapy elicited a strong innate immunity-mediated

antitumor response and resulted in a significantly lower PHEO volume compared with the phosphate buffered saline (PBS)–treated group and in a significant improvement in mouse survival. The cytotoxic effect of neutrophils, as innate immune cells predominantly infiltrating treated tumors, was verified *in vitro*. Moreover, the combination of mannan-BAM and toll-like receptor ligands with agonistic anti–CD40 was associated with increased mouse survival. Subsequent tumor rechallenge also supported adaptive immunity activation, reflected primarily by long-term tumor-specific memory. We verified these results further in metastatic PHEO, where the intratumoral injections of mannan-BAM, toll-like receptor ligands, and anti-CD40 into subcutaneous tumors resulted in significantly less intense bioluminescence signals of liver metastatic lesions induced by tail vein injection compared with the PBS–treated group. Subsequent experiments focusing on the depletion of T cell subpopulations confirmed the crucial role of $CD8^+$ T cells in the inhibition of bioluminescence signal intensity of liver metastatic lesions. The results call for a new therapeutic approach in patients with metastatic PHEO/PGL by using immunotherapy that initially activates innate immunity, followed by an adaptive immune response.

Therapeutic aspects of pheochromocytoma and paraganglioma

Neuroendocrine tumors (NETs) express somatostatin receptors, which can be targeted with radiolabeled peptides. In a variety of solid tumors, radio-guided surgery (RGS) has been used to guide surgical resection. ^{68}Ga-DOTA analogs have been shown to be more accurate than other radioisotopes for detecting NETs. A pilot study previously demonstrated the feasibility and safety of ^{68}Ga-DOTATATE RGS for patients with NETs. We evaluated which intra-operative techniques and thresholds define positive lesions that warrant resection during ^{68}Ga-DOTATATE RGS. This prospective cohort study, conducted between October, 2013, and February, 2018, included 44 patients with NETs who underwent ^{68}Ga-DOTATATE RGS. Forty-four patients (22 women and 22 men) had 133 lesions detected on preoperative imaging scans, with a diagnosis of a pancreatic NET (19 of 44), gastrointestinal NET (22 of 44), and PHEO/PGL (3 of 44). The target-to-background ratio (TBR) was obtained by normalizing to the omentum (106 of 133) or other solid organs (27 of 133). The omentum had a significantly lower mean count than other solid organs for background count activity three hours after injection. The lesions containing NETs had a significantly higher TBR than those that did not contain NETs. On a receiver operating characteristic curve analysis, a TBR of 2.5 had a sensitivity of 90% and a specificity of 25%, and a TBR of 16 had a sensitivity of 54% and a specificity of 81%. We concluded that a TBR of 2.5 or greater is a highly sensitive threshold for indicating a lesion to be consistent with a NET on histologic findings and thus warranting surgical resection.

In another study, we investigated the anti-tumor potential of novel molecular-targeted approaches in murine pheochromocytoma cell lines (MPC/MTT), immortalized mouse chromaffin $Sdhb^{-/-}$ cells, 3D-pheochromocytoma tumor models (MPC/MTT spheroids), and human pheochromocytoma primary cultures. We identified the specific PI3Kα inhibitor BYL719 and the mTORC1 inhibitor everolimus as the most effective combination in all models. Single treatment with clinically relevant doses of BYL719 and everolimus significantly decreased MPC/MTT and $Sdhb^{-/-}$ cell viability. A targeted combination of both inhibitors synergistically reduced MPC and $Sdhb^{-/-}$ cell viability and showed an additive effect on MTT cells. In MPC/MTT spheroids, treatment with clinically relevant doses of BYL719 alone or in combination with everolimus was highly effective, leading to a significant shrinkage or even a complete collapse of the spheroids. We confirmed the synergism of clinically relevant doses of BYL719 plus everolimus in human pheochromocytoma primary cultures of individual patient tumors, with BYL719 attenuating everolimus-induced AKT activation. We thus established a method to assess molecular-targeted therapies in human PHEO cultures and identified a highly

effective combination therapy. Our data pave the way to customized combination therapy to target individual patient PHEOs/PGLs.

We also continue to work closely with NCI on treating metastatic PHEO/PGL with ^{177}Lu-DOTATATE (Lutathera). Pacak is lead coinvestigator on Frank Lin's NCI protocol treating these patients.

We wrote a review as a guide for practicing clinicians summarizing current management of PHEO/PGL according to tumor size, location, age of first diagnosis, presence of metastases, and especially underlying mutations in the era of precision medicine.

Animal model of pheochromocytoma and cell culture studies

Our aim was to develop transgenic mice with a gain-of-function *Epas1*^{A529V} mutation (corresponding to the human *EPAS1*^{A530V} mutation; *EPAS* encodes HIF2α) to recapitulate some clinical findings and to discover some new developmental associations with *EPAS* mutations. We demonstrated elevated levels of erythropoietin and polycythemia, a reduced urinary metanephrine-to-normetanephrine ratio, and elevated expression of somatostatin in the ampullary region of duodenum. Further, inhibition of HIF2α with its specific inhibitor PT2385 significantly reduced erythropoietin levels in the mutant mice. However, polycythemia persisted after PT2385 treatment, suggesting an alternative erythropoietin-independent mechanism of polycythemia. The findings demonstrated the vital roles of *EPAS1* mutations in the *EPAS1*–related syndrome development and the great potential of the *Epas1*^{A529V} animal model for further pathogenesis and therapeutics studies.

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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, connectivities, neurochemical markers, and electrophysiological properties. 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 such 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* strategies 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 that these pursuits will act as a springboard for future research and provide new insights into both normal development and various neurodevelopmental diseases.

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Mechanisms regulating initial fate decisions within the medial ganglionic eminence

The medial ganglionic eminence (MGE) gives rise to the majority of forebrain interneurons, most notably the somatostatin- and parvalbumin-expressing (SST^+ and PV^+) subtypes, and some nNOS

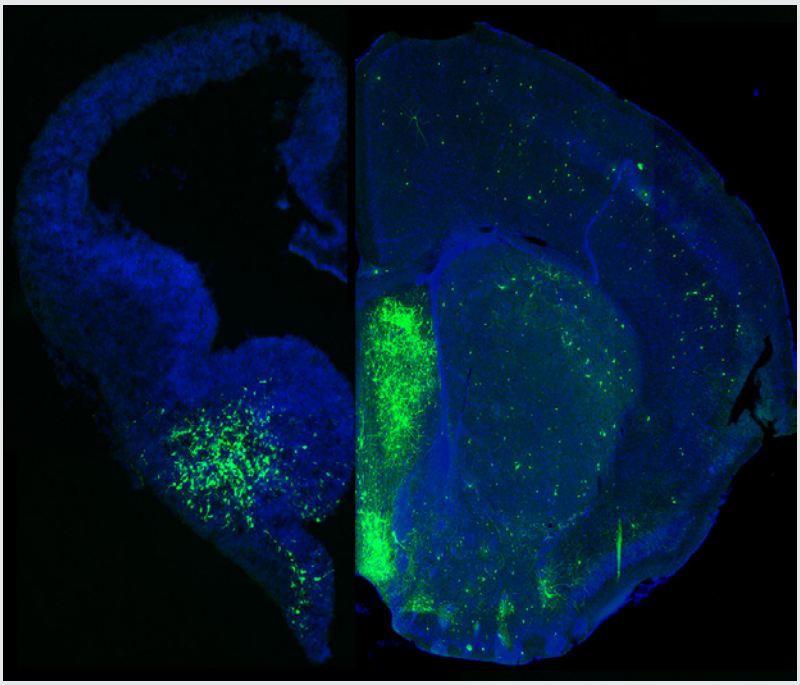

FIGURE 1. MGE–derived GABAergic cells populate many different brain regions.

The image depicts a section of an embryonic brain (*left*) that has been electroplated to label cells derived from the medial ganglionic eminence (MGE), merged with an section of an adult brain (*right*), displaying the incredible spatial and morphological diversity of 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.

(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 to become either PV^+ or SST^+ interneurons. SST^+ interneurons are preferentially born early in embryogenesis from the dorsoposterior MGE, whereas PV^+ interneurons are born throughout embryogenesis with a bias of originating from the ventroanterior 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.

Characterization of the epigenetic landscape during embryonic neurogenesis

In multicellular organisms, cells are genetically homogenous but structurally and functionally heterogenous as a result of differential gene expression that is often mitotically heritable. The mechanisms regulating such expression are 'epigenetic,' as they do not involve altering the DNA sequence itself; they include DNA methylation (DNAme), histone modifications, and higher-order chromatin structure. In particular, DNA and histone modifications often follow specific rules termed the “epigenetic code”, similar to the genetic code. Collectively, DNAme and histone modification have been reported to regulate transcription and chromatin structure in many stem cell and developmentally critical processes. Previous scRNA-seq (single-cell RNA

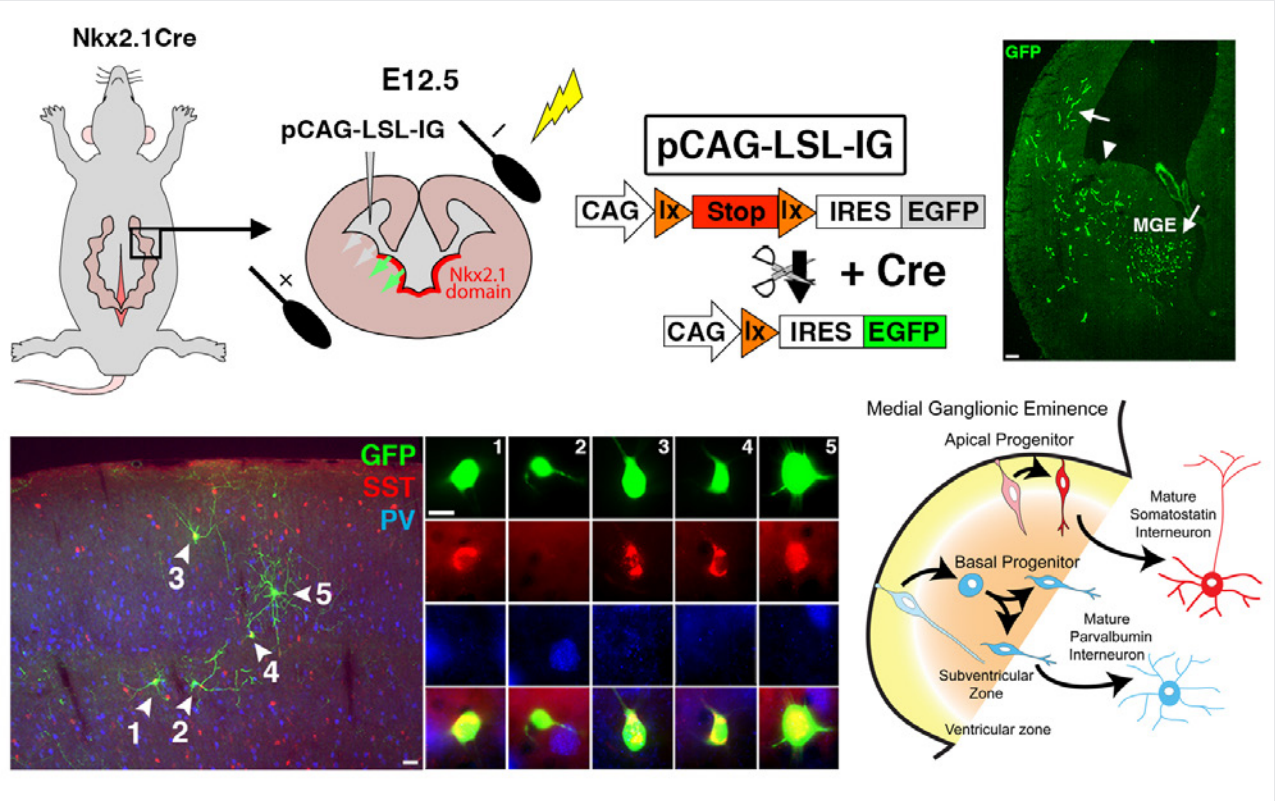

FIGURE 2. Manipulation of gene expression in the MGE by *in utero* electroporation (IUE)

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.

sequencing) experiments on the ganglionic eminences (GEs) identified surprisingly few region-specific genes in cycling progenitors (immature cells that are still cycling and have not exited the cell cycle) despite the fact that these regions produce distinct GABAergic cell populations. Because there are dynamic changes in the chromatin landscape during development, a prevailing hypothesis is that epigenomic signatures may be a better predictor of cell fate during development, revealing both potential distal enhancers and/or genetic loci that may be 'poised' but not yet expressed. However, direct support for this hypothesis is lacking. This idea is particularly relevant, given that epigenetic changes are observed in many neurological and psychiatric diseases and that most single-nucleotide variants (SNVs) identified in diseases-specific GWAS (genome-wide association studies) map to non-coding regions, implying that epigenetic regulation of gene expression may underlie some disease etiologies. We are currently utilizing cutting-edge techniques to define the chromatin state of different embryonic neurogenic regions to better understand epigenetic changes in distinct cell types during development, with the hope applying this knowledge to various neurodevelopmental diseases.

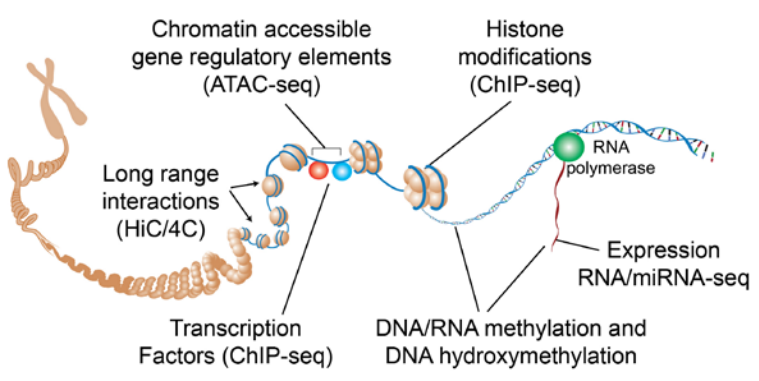

FIGURE 3. Schematic of epigenetic regulation mechanisms and applicable assays

While gene expression ultimately arises from RNA transcription, there are numerous epigenetic mechanisms that can regulate a cell's transcriptome, including DNA methylation, histone modifications, chromatin accessibility, and long-range chromatin interactions. There are numerous techniques available to assess all these distinct genomic DNA modifications. Image from Duke University Center for Genomic and Computational Biology.

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 postmitotic 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 hardwired into definitive subgroups, and that the environment more passively sculpts the maturation of these cells. To test these competing hypotheses, we are harvesting early postnatal interneuron precursors (P0–P2) in specific brain regions and transplanting them into wild-type hosts either homotopically (cortex-to-cortex) or heterotopically (cortex-to-hippocampus or cortex-to-striatum). The technique allows us to determine whether transplanted interneurons adopt properties of 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 scRNA-seq to characterize, in an unbiased manner, how a cell's transcriptome is altered when grafted into a new brain environment.

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

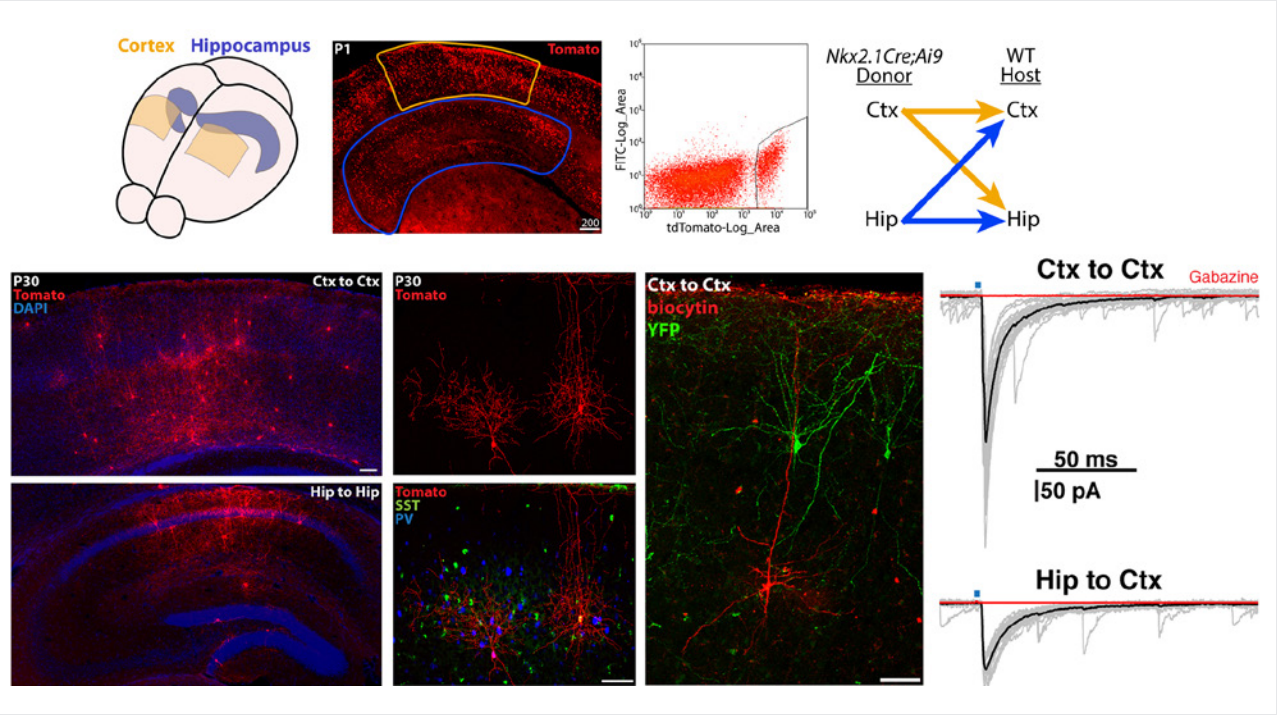

FIGURE 4. Transplantation of MGE–derived interneuron precursors into postnatal brains

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.

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 between specific interneuron cell types. Then, the methylated genomic DNA will be analyzed, allowing us to look back in time to identify candidate fate-determining genes expressed in specific interneuron populations. Our hope is that 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 noncoding 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 *Igf2* promoter from these enhancer interactions. Upon paternal inheritance, the *H19ICR* is methylated and cannot bind to CTCF. Therefore, alternative loop structures form, which allow *Igf2* promoters to interact with the shared enhancers while preventing *H19* promoter/enhancer interactions. In humans, epigenetic mutations that disrupt *H19ICR* function result in loss of monoallelic expression. Mutations in the paternal *H19ICR* lead to loss of *Igf2* expression and biallelic (2X) *H19* expression and are associated with the Russell-Silver syndrome. Mutations in the maternal *H19ICR* lead to loss of *H19* but biallelic (2x) *Igf2* expression and are associated with the Beckwith-Wiedemann syndrome and several pediatric cancers. Our lab generated mouse models that phenocopy the human diseases, and our goal is to characterize the molecular defects associated with misimpression of *Igf2/H19* and to understand how these molecular defects lead to disease and cancer. In particular, we strive to understand the role of development in disease progression. In addition, to the *Igf2/H19* locus, we are completing analyses of mice carrying conditional mutations at the *Casq2* locus to understand the role of development in cardiac disease progression.

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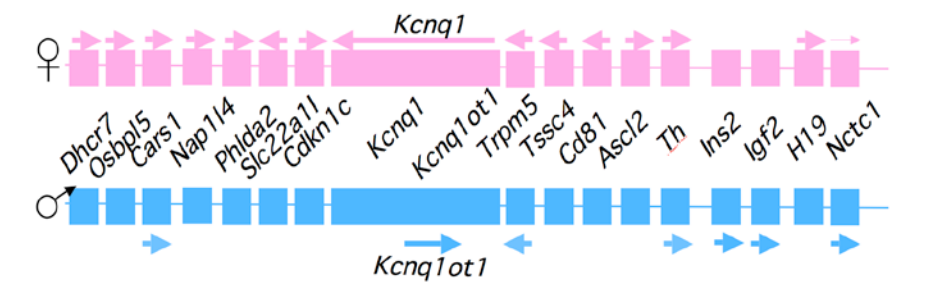

FIGURE 1. An imprinted domain on mouse distal chromosome 7

Maternal (*pink*) and paternal (*blue*) chromosomes are indicated. Horizontal arrows denote RNA transcription.

Alternative long-range interactions between distal regulatory elements establish allele-specific expression at the *Igf2/H19* locus

Our studies on the mechanisms of genomic imprinting focus on the *H19* and *Igf2* genes. Paternally expressed *Igf2* lies about 80 kb upstream of the maternal-specific *H19* gene. Using cell-culture systems as well as transgene and knockout experiments *in vivo*, we identified the enhancer elements responsible for activation of the two genes. The elements are shared and are all located downstream of the *H19* gene (Figure 2).

As mentioned above, imprinting at the *Igf2/H19* locus depends on the 2.4 kb *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. Taken together, 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 ICRs is not acquired until relatively late in development, after the embryo implants in 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 noncoding RNA that is transcribed across the muscle enhancer element (ME in Figure 2), which is shared by *Igf2* and *H19*. *Nctc1* expression depends on this enhancer element. Concordantly, the shared enhancer interacts with the *Nctc1* promoter, just as it interacts with the maternal *H19* and paternal *Igf2* promoters. We showed that all three co-regulated promoters

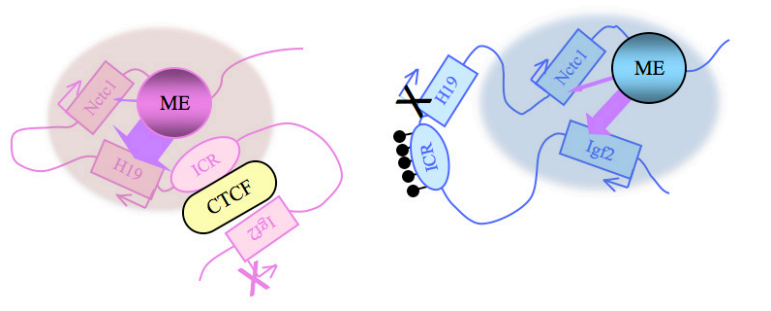

FIGURE 2. Distinct maternal and paternal chromosomal conformations at the distal 7 locus

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.

(*Igf2*, *H19*, and *Nctc1*) also physically interact with each other in a manner that depends on their interactions with the shared enhancer. Thus, enhancer interactions with one promoter do not preclude interactions with another promoter. Moreover, we demonstrated that 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.

Molecular mechanisms for tissue-specific promoter activation by distal enhancers

Normal mammalian development is absolutely dependent on establishing the appropriate patterns of expression of thousands of developmentally regulated genes. Most often, development-specific expression depends on promoter activation by distal enhancer elements. The *Igf2/H19* locus is a highly useful model system for investigating mechanisms of enhancer activation. First, the biological significance of the model is clear, given that expression of these genes is so strictly regulated. Even twofold changes in RNA levels are associated with cancer and developmental disorders. 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 NONCODING RNA IS AN ESSENTIAL ELEMENT OF THE MUSCLE ENHANCER.

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 noncoding RNA); *Nctc1* RNA itself is not required (at least in *trans*). Instead mutational analysis demonstrates that it is *Nctc1* transcription through the core

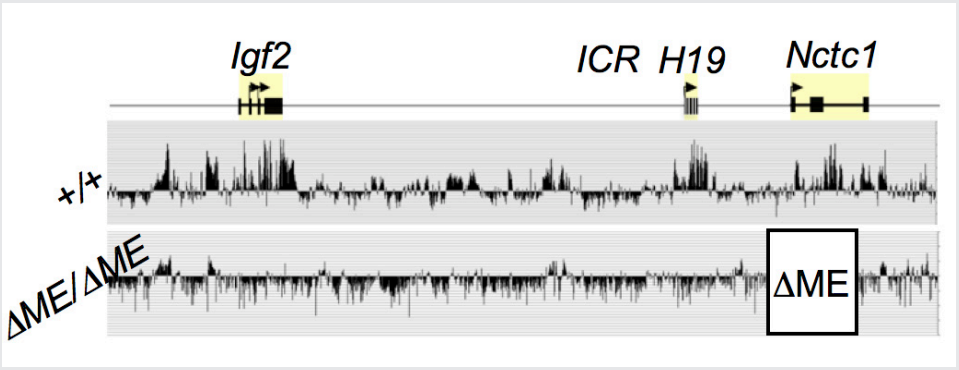

FIGURE 3. The shared muscle enhancer (ME) directs RNAP binding and RNA transcription across the entire 150 kb locus.

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 noncoding RNAs in gene regulation and enhancer function. We will use our model system to characterize the role of *Nctc1* transcription in establishing enhancer orientation, enhancer promoter specificity, and enhancer tissue specificity.

THE MUSCLE ENHANCER (ME) DIRECTS RNA POLYMERASE (RNAP) II NOT ONLY TO ITS COGNATE PROMOTERS (I.E., TO THE *H19* AND *IGF2* PROMOTERS) BUT ALSO ACROSS THE ENTIRE INTERGENIC REGION.

We used ChIP-on-chip to analyze RNAP localization on chromatin prepared from wild-type and from enhancer-deletion (DME) cell lines (Figure 3). As expected, RNAP binding to the *H19* and *Igf2* promoters is entirely enhancer-dependent. Curiously, we also noted enhancer-dependent RNAP localization across the entire locus, including the large intergenic domain between the two genes. Furthermore, RNAP binding is associated with RNA transcription. Thus, the enhancer regulates accessibility and RNAP binding not solely at specific localized sites but across the entire domain. The results support a facilitated tracking model for enhancer activity.

RNAP BINDING AT 'REAL' GENES AND ACROSS THE INTERGENIC REGIONS IS QUALITATIVELY DIFFERENT.

We used naturally occurring single-nucleotide polymorphisms (SNPs) to investigate allelic differences in

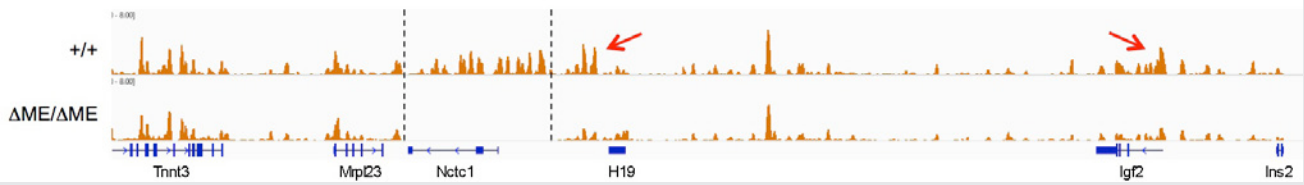

FIGURE 4. Chromatin patterns at the *Igf2/H19* locus are independent of enhancer activity.

Chromatin was isolated from wild-type and enhancer-deletion muscle cells, using antibodies to H3K4me1, and analyzed by DNA sequencing.

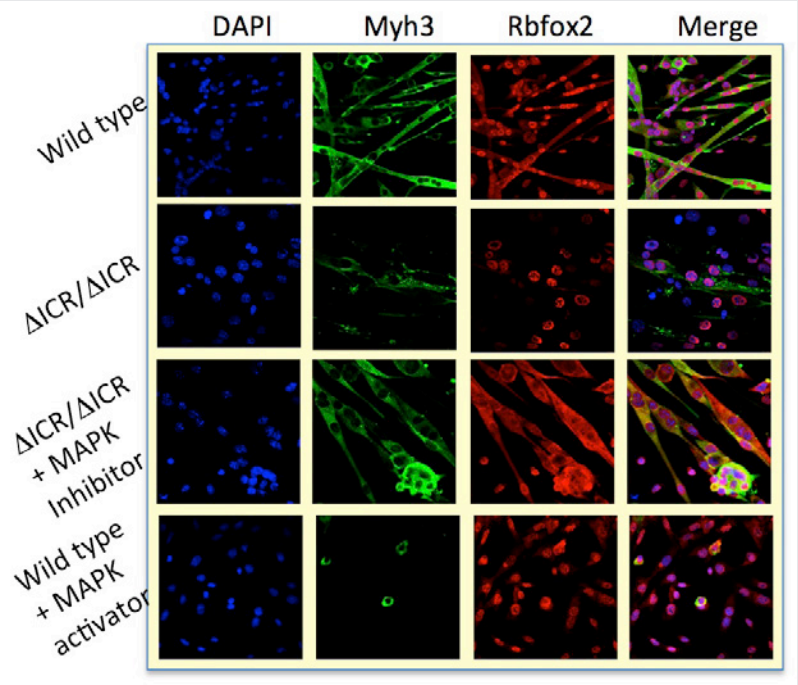

FIGURE 5. Muscle cell–differentiation defects in *Igf2/H19* loss-of-imprinting mice

Differentiation defects in loss-of-imprinting (ΔICR) myoblasts can be rescued by blocking MAP kinase 3 activity. Conversely, artificial activation of the MAPK activity in wild-type cells mimics the genetic defect.

binding of RNAP and activation of gene expression in wild-type cells and in cells carrying enhancer deletions or insulator insertion mutations. RNAP binding across the *Igf2* and *H19* genes is both enhancer-dependent and insulator-sensitive; that is, a functional insulator located between an enhancer and its regulated gene prevents RNAP binding and likewise prevents RNA transcription. Across the intergenic regions, RNAP binding and RNA transcription are similarly enhancer-dependent (see above). However, intergenic RNAP binding and transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as a physical block for RNAP progression, but rather they specifically interfere with certain RNAP states or activities.

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

Both RNA transcription and RNAP binding across the *Igf2/H19* domain are entirely dependent upon the muscle enhancer. For example, levels of *H19* RNA are reduced more than 10,000-fold in muscle cells in which the enhancer has been deleted. To test the dependence of chromatin structure on enhancer activity, we performed ChiP-Seq on wild-type and on enhancer-deletion cell lines using antibodies to the histones H3K4me1, H3K43me3, and H3K36me3. Surprisingly, we saw no changes in the patterns of chromatin modification (Figure 4). Thus, a functional enhancer and active RNA transcription are not important for establishing chromatin structures at the *Igf2/H19* domain.

Function of the *H19* and *Igf2* genes in muscle-cell growth and differentiation [Reference 1]

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

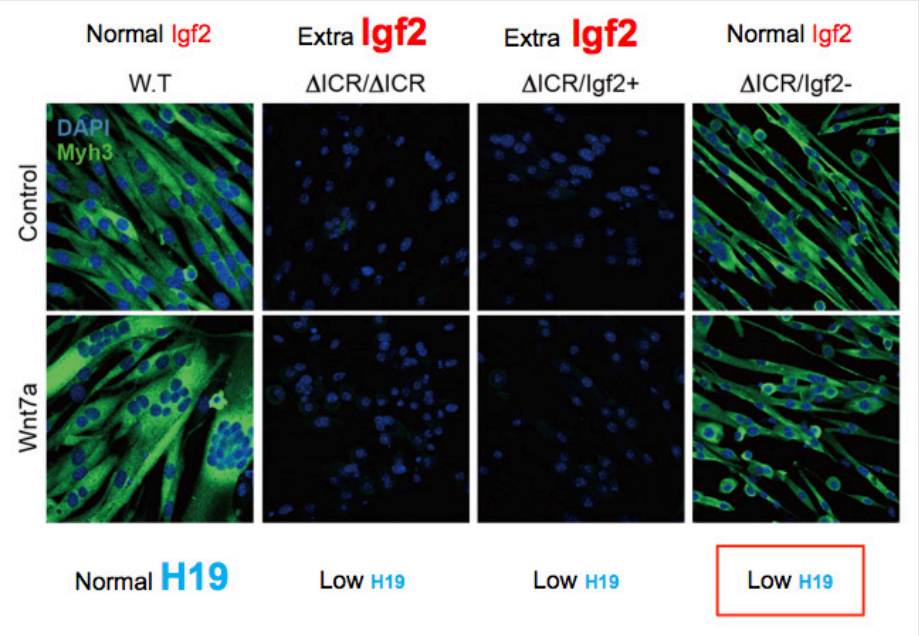

FIGURE 6. The long noncoding H19 RNA is required for normal myotube fusion and hypertrophy.

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

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* 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).

To understand the molecular basis for the differentiation phenotype, we performed RNA sequencing and identified several hundred genes whose expression levels are altered by the *ICR* deletion. GO (gene ontology) pathway analysis demonstrates that these differentially expressed genes were highly enriched in the MAP kinase signaling pathway. Of special note, expression of the *Mapk3* gene is elevated in mutant cell lines.

To determine the significance of the changes in *Mapk3*, we used drug inhibitors to block MAP kinase activity. In mutant cell lines, we can restore normal differentiation by blocking activation of the MAP kinase target MEK1. Similarly, treatments that activate MAP kinase in wild-type cells can mimic the ICR–deletion phenotype. The results suggest that *H19*/*Igf2* act through MAP kinase to regulate differentiation of myoblast cells.

To distinguish the roles of *Igf2* overexpression and *H19* underexpression, we analyzed additional mouse strains that restore *H19* via a bacterial artificial chromosome transgene or that restore normal levels of *Igf2* expression via a second mutation in the paternal *Igf2* gene. Analyses of cell lines from such mice demonstrate that extra *Igf2* is the direct cause of failure to differentiate in loss-of-imprinting mutations, but that *H19* is essential for normal fusion and for muscle hypertrophy in response to Wnt pathways (Figure 6). Molecular and genetic analyses indicate that H19 normally functions to bind to p53 and reduce its bioavailability. Thus, loss of H19 results in increased p53 function and therefore to enhanced activation of the mTOR/AKT signaling pathways.

Function of *H19* and *Igf2* genes in cardiac development

Beckwith-Wiedeman syndrome (BWS) is a developmental disorder characterized by generalized overgrowth of the fetus and a high risk for several neonatal cancers. Many BWS patients also display cardiac problems. BWS can be explained by one of two different genetic lesions: loss of function of the *CDKN1C* gene or maternal loss of imprinting at the H19/Igf2 locus. Maternal loss of imprinting has the effect of doubling Igf2 expression while concomitantly reducing H19 RNA levels. Curiously, children born via artificial reproductive technology (ART) show increased incidence of BWS, which can be explained by increasingly frequent loss of H19/Igf2 imprinting in these children. Moreover the children show high frequency of cardiac dysfunction. Altogether, these results suggest that abnormal expression of the H19/Igf2 locus can lead to cardiac problems.

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 biallelic *Igf2* and loss of *H19* play independent and distinct roles in generating the BWS phenotype.

Biallelic expression of *Igf2* 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 reduction in *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 3]

Mutations in the *CASQ2* gene, which encodes cardiac calsequestrin (CASQ2), are associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden death. The survival of individuals homozygous for loss-of-function mutations in *CASQ2* was surprising, given the central role of Ca^{2+} ions in excitation-contraction (EC) coupling and the presumed critical roles of CASQ2 in regulating Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytoplasm. To address this paradox, we generated a mouse model for loss of *Casq2* gene activity. Comprehensive analysis of cardiac function and structure yielded several important insights into CASQ2 function. First, CASQ2 is not essential to provide sufficient Ca^{2+} storage in the SR of the cardiomyocyte. Rather, a compensatory increase in SR volume and surface area in mutant mice appears to maintain normal Ca^{2+} storage capacity. Second, CASQ2 is not required for the rapid, triggered release of Ca^{2+} from the SR during cardiomyocyte contraction. Rather, the RyR receptor, an intracellular calcium-ion channel, opens appropriately, resulting in normal, rapid flow of Ca^{2+} into the cytoplasm, thus allowing normal contraction of the cardiomyocyte. Third, CASQ2 is required for normal function of the RyR during cardiomyocyte relaxation. In the absence of CASQ2, significant Ca^{2+} leaks occur through the RyR and

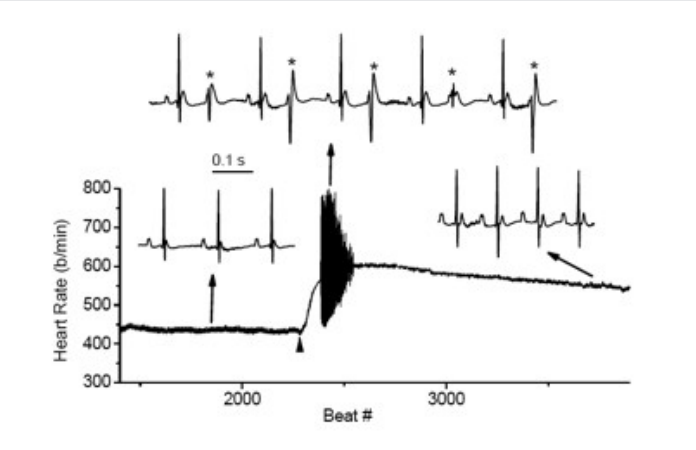

FIGURE 7. Cardiac arrhythmias in calsequestrin-2–deficient mice phenocopy the human disease.

Premature ventricular complexes (*) are induced by stress in *Casq2*–deficient but not in wild-type mice.

lead to premature contractions and cardiac arrhythmias (Figure 7). Fourth, CASQ2 function is required to maintain normal levels of the SR proteins junctin and triadin. We do not yet understand what role, if any, the compensatory changes in these two SR proteins play in modulating the loss of *Casq2* phenotype.

To address these issues and to model cardiac disorders associated with late-onset (not congenital) loss of CASQ2 activity, we established and are analyzing two new mouse models in which changes in *Casq2* gene structure are induced by tissue-specific transgenes activated by tamoxifen treatment. In the first model, an invested/null allele is restored to normal function by the addition of the drug. In the past year, we demonstrated the effectiveness of this model and noted that full CASQ2 protein levels are restored within one week of treatment. In the second model, a functional gene is ablated by the addition of the drug. The *Casq2* gene and mRNAs are deleted from cardiac cells within four days of hormone treatment. Phenotypic analyses shows that restoration of CASQ2 in adult animals is sufficient to fully restore cardiac function. Moreover, restoration solely in pacemaking cells is also enough to rescue function, suggesting an important role for reduced heart rate in the CPVT phenotype as well as a new target for therapeutic interventions.

Publications

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2. Gebert C, Correia L, Li Z, Petrie HT, Love PE, Pfeifer K. Chromosome choice for initiation of V-(D)-J recombination is not governed by genomic imprinting. *Immunol Cell Biol* 2017 95:473-477.
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Cholesterol Homeostasis and Lysosomal Disorders

We study the molecular, biochemical, and cellular processes that underlie genetic disorders resulting from impaired cholesterol homeostasis and lysosomal dysfunction. 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. More recently, we focused on Batten disease, which is attributable to mutation of *CLN3*, the gene that encodes the transmembrane protein battenin. Our research group uses basic, translational, and clinical research approaches with the ultimate goal of developing and testing therapeutic interventions for rare genetic disorders. Our basic research uses neuronal, zebrafish, and 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 SLOS, CLN3, and NPC1 are ongoing. Our emphasis on both basic and clinical research allows us to integrate laboratory and clinical data in order to improve our understanding of the pathological mechanisms underlying SLOS, CLN3, and NPC, with the goal of improving clinical care of these patients. Therapeutic trials have been conducted for SLOS and NPC1. In collaboration with NCATS (the National Center for Advancing Translational Sciences), our research group has been involved in a multicenter trial of creatine transporter deficiency.

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(continued)

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

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spectrum, infants often die as result of multiple major malformations, while mild SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol.

Our laboratory initially cloned the human 3beta-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*$^{delta3–5/delta3–5}$) 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 missense allele (*Dhcr7*^{T93M}). The T93M mutation is the second most common mutation found in SLOS patients. *Dhcr7*$^{T93M/T93M}$ and *Dhcr7*$^{T93M/delta3–5}$ mice are viable and demonstrate SLOS with a gradient of biochemical severity (*Dhcr7*$^{delta3–5/delta3–5}$ greater than *Dhcr7*$^{T93M/delta3–5}$ greater than *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.

FIGURE 1.

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

As part of our clinical studies on SLOS, we identified a novel oxysterol, 27-hydroxy-7-dehydrocholesterol (27-7DHC), derived from 7-DHC in SLOS patients. We therefore investigated whether 27-7DHC contributes to the pathology of SLOS and found a strong negative correlation between plasma 27-7DHC and cholesterol levels in these patients. In addition, previous work showed that low cholesterol levels impair hedgehog signaling (a signaling pathway required for proper cell differentiation). 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

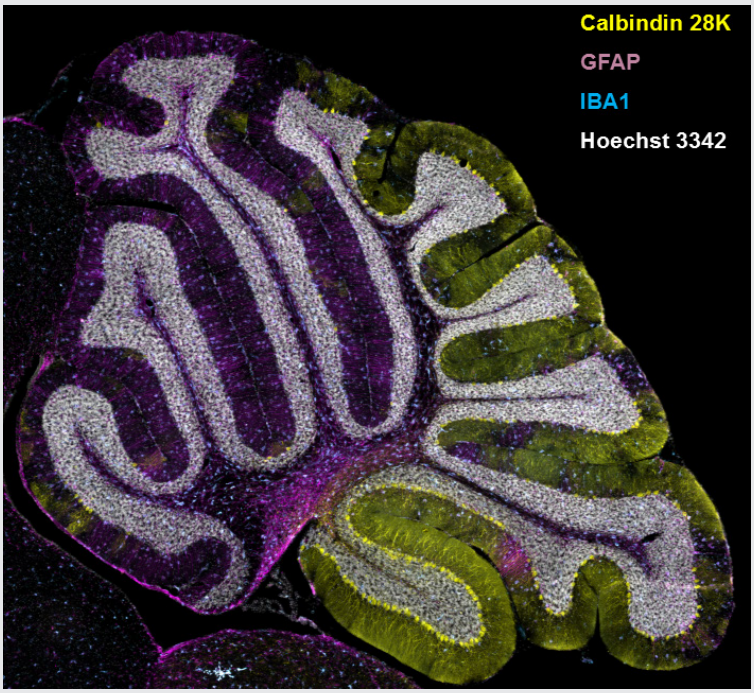

FIGURE 2. Gliosis in NPC1 mouse cerebellum

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.

display increased *CYP27* expression and elevated 27-hydroxycholesterol levels but normal cholesterol levels. While *Dhcr7*$^{delta3\text{–}5/delta3\text{–}5}$ mice are growth-retarded, exhibit a low incidence of cleft palate (9%), and die during the first day of life, *Dhcr7*$^{delta3\text{–}5/delta3\text{–}5}$*:CYP27Tg* embryos are stillborn and have multiple malformations, including growth retardation, micrognathia, cleft palate (77%), lingual and dental hypoplasia, ankyloglossia, umbilical hernia, cardiac defects, cloacae, curled tails, and limb defects; we 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*$^{delta3\text{–}5/delta3\text{–}5}$*:CYP27Tg* than in *Dhcr7*$^{delta3\text{–}5/delta3\text{–}5}$ embryos. The fact that 27-7DHC plays a role in SLOS may explain some of the phenotypic variability and may lead to development of a therapeutic intervention. The project is a good example of the benefits of integrating clinical and basic science to both understand the pathology of SLOS and develop potential therapeutic interventions. We are currently investigating the pathological role of other 7-DHC–derived oxysterols, such as DHCEO (3beta,5alpha-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.

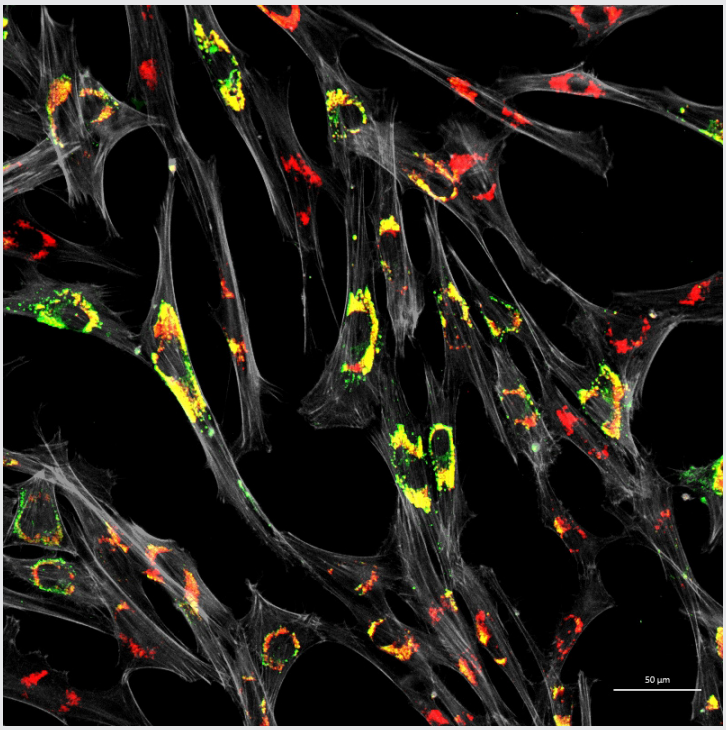

FIGURE 3. Accumulation of unesterified cholesterol in NPC1 patient fibroblasts

Human NPC1 fibroblasts were immuno-stained for Lamp1 (*green*) and stained with filipin (*red*); filipin stains unesterified cholesterol, which accumulates in the Lamp1–positive endolysosomal compartment. Cell structure was outlined by immuno-staining for actin (*gray*).

LATHOSTEROLOSIS AND DESMOSTEROLOSIS

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

Desmosterolosis is another inborn error of cholesterol synthesis that resembles SLOS. It results from a mutation in the 3beta-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 nonenzymatically 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 nonenzymatically produced oxysterols. We also tested the safety and efficacy of the histone deacetylase (HDAC) inhibitor vorinostat in adult NPC1 patients. In collaboration with the Therapeutics of Rare and Neglected Disease Program of NCATS, we completed a phase 1/2a therapeutic trial of lumbar intrathecal cyclodextrin (VTS-270, adrabetadex) therapy in NPC1. We have now transitioned to a multicenter, multinational phase 2b/3, which is being evaluated. We also initiated a study to evaluate the safety and efficacy of combined intrathecal and intravenous cyclodextrin.

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 CSF (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 (juvenile Batten 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

- Ara Parshegian Medical Research Fund, University of Notre Dame
- Together Strong NPC Foundation
- Dana's Angels Research Trust

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Three-Dimensional Organization of the Genome 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 to decipher how cells make lineage decisions and how gene-regulatory networks are established.

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Eukaryotic cells need to deal with the biophysical constraints of packaging two meters of DNA inside a tiny nucleus (2–10 microns) and still 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 reprogramming 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 many transcriptional regulators. Our studies aim to understand how 3D chromatin structures are established during this period and how this impacts future developmental decisions.

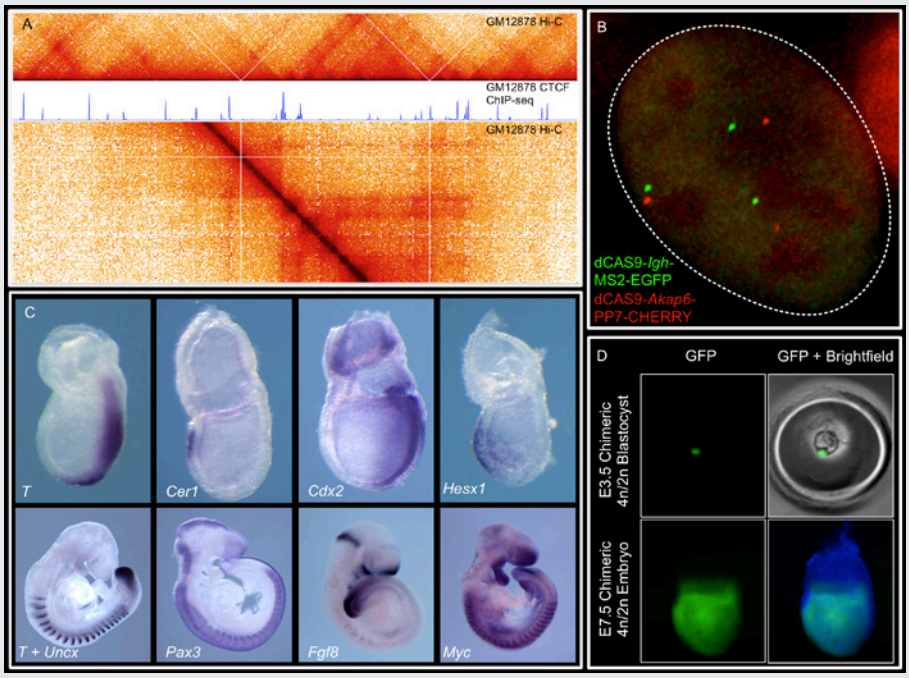
Following fertilization and within a few cell divisions, the first cell lineages are established and different gene-expression programs

FIGURE 1. Representative image of the lab's research

We combine imaging techniques in both fixed and living cells with sequencing-based genomic techniques that assess DNA–DNA interactions.

A. Hi-C and CTCF ChIP-seq of GM1278 cells, which allow characterization of chromatin structure and identification of binding sites of an important architectural protein.

B. dCAS9 MCP-EGFP and PCP-CHERRY live imaging of the *Igh* and *Akap6* loci. The mouse embryo is an unparalleled system in mammalian biology for understanding how tissue-specific gene expression is achieved.



C. Whole mount *in situ* hybridization for patterning markers in mid and late gastrulating embryos

D. Tetraploid aggregation with GFP ES (embryonic stem) cells allows generation of fully ES–cell derived embryos.

are put into action. In mammals, the result is the formation of the 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 several 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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Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies

Roberto Romero, MD, DMedSci,
Chief, Perinatology Research Branch

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. The cost of prematurity in the U.S. alone is estimated to be $26 billion per year. An important goal is to understand the mechanisms of disease responsible for spontaneous preterm birth and fetal injury and to develop methods for the prediction and prevention of preterm birth.

The Perinatology Research Branch (PRB) has proposed that preterm parturition is a syndrome caused by many 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 antifetal rejection (chronic inflammatory lesions of the placenta), cervical disease, and a decline in progesterone action. Previously, we reported that intra-amniotic inflammation, which affects at least one out of every three preterm neonates, is characterized by the activation of amniotic-fluid neutrophils, cells that represent the first line of defense against infection. Using DNA fingerprinting, we determined that amniotic-fluid neutrophils are of fetal origin in cases of preterm labor, maternal origin in cases of clinical chorioamnionitis at term, and mixed origin in patients who have inflammatory processes near term. Moreover, in a series of studies, we were able to demonstrate that neutrophils produce antimicrobial peptides and exhibit the formation of extracellular traps, whereby they immobilize and kill bacteria.

The Branch also studies other obstetrical syndromes that account for the high rate of infant mortality in the United States, including clinical chorioamnionitis, which is the most common infection-related diagnosis in delivery units around the world, as well as meconium aspiration syndrome and amniotic fluid embolism.

Congenital anomalies continue to be a leading cause of perinatal mortality in the U.S. Imaging, a powerful tool for scientific discovery, has changed the practice of obstetrics and maternal-fetal medicine. Imaging with ultrasound allows the definition of fetal anatomy, biometry, growth, and the study of physiologic parameters, such as cardiac function, fetal sleep, and breathing. We invented a new method for the examination of the fetal heart, called fetal intelligent navigation echocardiography (FINE). We recently reported a further

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. We also determined the sensitivity and specificity of FINE in fetuses with normal hearts and congenital heart disease in the second and third trimesters.

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 neuroconnectivity, as well as the potential relationship between insults that could alter fetal neurodevelopment. Given that preterm birth is a leading cause of neurodevelopmental disorders, we used noninvasive methods to interrogate neuroconnectivity. In previous work, we reported a study showing that fetuses subsequently born preterm have a disorder of neuroconnectivity not seen in fetuses of the same gestational age subsequently born at term. Neuroconnectivity was reduced in the left hemisphere, close to the prelanguage region, providing the first evidence that a disorder of functional connectivity is present in the fetus before birth.

Does the human placenta delivered at term have a microbiota?

Traditionally, the human placenta has been viewed as sterile, and colonization of the placenta by microorganisms has been associated with adverse pregnancy outcomes. Yet, recent reports employing DNA sequencing techniques have reported that the human placenta at term contains a unique microbiota. However, when studying low microbial biomass samples, sequence-based approaches can capture background contaminating DNA from DNA extraction kits, PCR reagents, and laboratory environments. The objective of this study was therefore to determine whether the human placenta delivered at term in patients without labor, undergoing Cesarean delivery, harbors a resident microbiota. This was a cross-sectional study of placentas from 29 women who had a Cesarean delivery without labor at term. The study included technical controls to account for potential background contaminating DNA. The bacterial profiles of placental tissues and background technical controls were characterized and compared using bacterial culture, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomic surveys. Twenty-eight of 29 placental tissues were negative for bacterial culture. The few bacteria cultured from the remaining sample were likely laboratory contaminants because corresponding 16S rRNA genes from the cultivars were not detected in the same placental sample. Quantitative real-time PCR did not indicate greater abundances of bacterial 16S rRNA genes in placental tissues than in background technical controls. Furthermore, 16S rRNA gene sequencing did not reveal consistent differences in the composition or structure of bacterial profiles between placental samples and background technical controls (Figure 1).

Most of the bacterial sequences obtained from metagenomic sequencing of placental tissues were from cyanobacteria, aquatic bacteria, or plant pathogens; the placenta is therefore unlikely to provide a suitable ecological niche for these bacteria and, as such, these data are not likely indicative of a placental microbiota. In summary, a resident microbiota could not be identified in human placentas delivered at term from women without labor, using several modes of microbiologic inquiry. A consistently significant difference in the abundance and/or presence of a microbiota between placental tissue and background technical controls

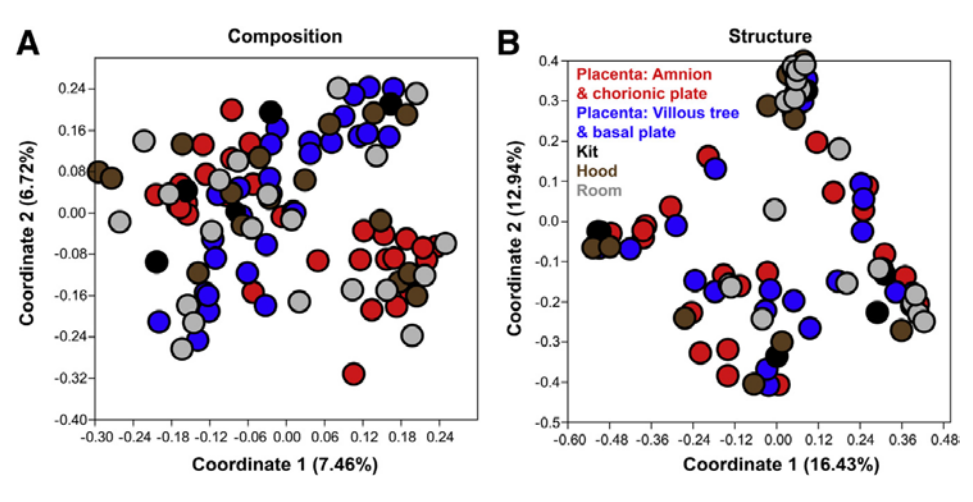


FIGURE 1. Principal coordinates analysis

The analyses illustrate similarity in 16S rRNA gene profiles among the amnion and chorionic plate, villous tree and basal plate, and technical control samples.

could not be found. The study demonstrates that incorporating technical controls for potential sources of background contaminating DNA into studies of low microbial biomass samples, such as the placenta, is necessary in order to derive reliable conclusions about host-associated microbiota.

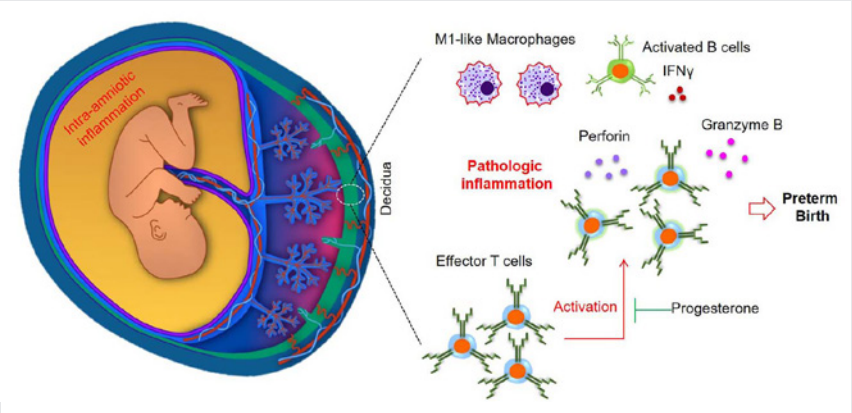
Antibiotics can eradicate intra-amniotic infection or inflammation.

In a case study, 50 women with singleton gestations between 20–34 weeks, preterm labor with intact membranes, and evidence of intra-amniotic infection or inflammation, by analysis of amniotic fluid obtained by abdominal amniocentesis, received an antibiotic treatment that consisted of ceftriaxone, clarithromycin, and metronidazole. Follow-up amniocentesis was performed in 19 of 29 patients who were undelivered for 7 or more days. Treatment success was defined as resolution of intra-amniotic infection/inflammation or delivery at 37 or more weeks. Resolution of intra-amniotic infection/inflammation was confirmed in 79% of patients (15/19) who had a follow-up amniocentesis. Treatment success occurred in 84% of patients (16/19) who had a follow-up amniocentesis and in 32% of patients (16/50) who received the antibiotic regimen. In conclusion, the administration of antibiotics to patients with preterm labor and intact membranes with proven intra-amniotic infection/inflammation is associated with eradication of infection and inflammation in a subset of patients.

Progesterone treatment prevents preterm labor and birth induced by effector and activated T cells.

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide, which is commonly preceded by spontaneous preterm labor. Many etiologies have been associated with preterm labor; yet, only pathological inflammation has been causally linked to preterm birth. Pathological inflammation can be acute or chronic in nature. Most research has focused on the mechanisms whereby the maternal innate immune system induces acute inflammation, leading to preterm labor and birth. However, the role of the maternal adaptive immune system, specifically T cells, in the pathogenesis of preterm labor and birth was poorly understood. In the above-mentioned study, we used human tissues and animal models to investigate a causal link between maternal T cell activation and preterm labor and birth.

FIGURE 2. Conceptual framework



Effector and activated T cells expressing granzyme B and perforin can induce pathologic inflammation by initiating local immune responses at the maternal-fetal interface (decidua) (i.e., activation of B cells and an M1-like macrophage polarization without an increased influx of neutrophils), which, in turns, leads to preterm labor and birth. Activation of T cells also induces inflammatory responses in the maternal circulation and the amniotic cavity, inducing fetal damage prior to preterm labor and birth. These effects can be abrogated by treatment with progesterone, a clinically approved strategy.

First, by performing exhaustive immunophenotyping of the decidual tissues, we found that effector and activated T cells are present at the human maternal-fetal interface. Importantly, the cells were enriched in women who underwent spontaneous preterm labor and birth. Next, using a murine model of *in vivo* T cell activation, we reported that the activation of maternal T cells induces preterm birth and adverse neonatal outcomes. The mechanisms whereby the activation of T cells induces preterm birth included stereotypical immune responses at the maternal-fetal interface as well as in the mother and the fetus, which were different from those initiated by microbial products (e.g., endotoxin) or progesterone withdrawal function (e.g., treatment with RU486). We also showed that activation of T cells induces proinflammatory responses in the myometrium and cervix. Lastly, we showed that pretreatment with progesterone, a clinically proven strategy, has systemic and local anti-inflammatory effects preventing preterm birth and adverse neonatal outcomes. Collectively, the findings reported in this study provide the first mechanistic demonstration of a role for maternal effector and activated T cells in the pathophysiology of preterm labor and birth (Figure 2).

Fetal biometry as a predictor of adverse perinatal outcomes

A basic need in prenatal care is an evaluation of adequacy of fetal growth. Although many fetal growth standards have been proposed, there is no consensus on the best standard to use in practice. We therefore compared the predictive ability of estimated fetal weight (EFW) percentiles, according to seven growth standards, to detect fetuses at risk for adverse perinatal outcomes in a retrospective cohort of 3,437 African-American women. The standards are as follows: the one-fits-all (Hadlock, INTERGROWTH-21st, WHO, FMF), ethnic-specific (NICHD), fetal sex and maternal characteristics–customized (GROW), and African-American–customized (PRB/NICHD) growth standards were applied to the last available scan prior to delivery. We found, among others, that fetuses with an EFW at or below the 10th percentile as well as those with an EFW at or above 90th percentile were at increased risk for adverse perinatal outcomes according to all, or some, of the seven standards, respectively. The relative risk carried by an EFW at or below the 10th percentile for the composite adverse perinatal outcome was higher for the most stringent standard (NICHD) compared with the least stringent standard (FMF) in small-for-gestational-age screening. The complementary analysis based on the ROC curve analysis suggests a slightly improved detection of adverse perinatal outcomes by more recent

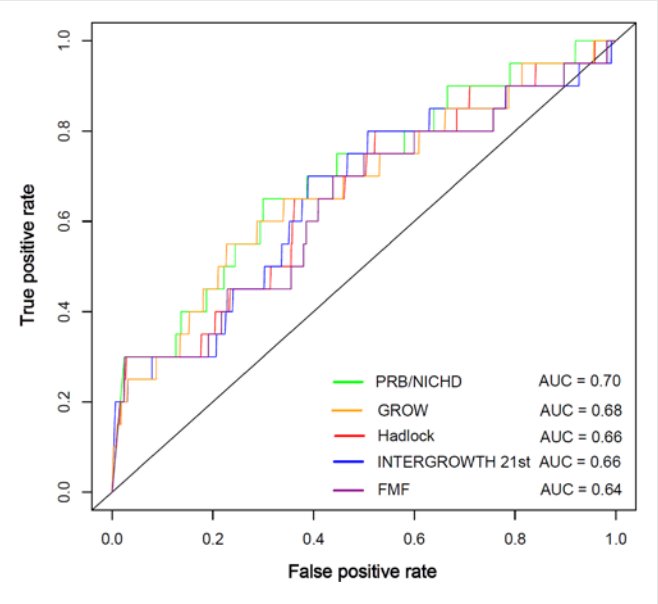


FIGURE 3. Receiver Operating Characteristic (ROC) curves for the prediction of perinatal death

The ROC curves are constructed from the percentile of estimated fetal weight according to each standard, and the area under the curves (AUC) is shown in the legend. GROW: Gestational-age Related Optimal Weight; FMF: Fetal Medicine Foundation; PRB: Perinatology Research Branch.

population-based (INTERGROWTH-21st) and customized (PRB/NICHD) standards compared with the Hadlock and FMF standards (Figure 3).

QUEST MRI to detect oxidative stress in the fetal brain

Intra-amniotic inflammation has been implicated as a major risk for fetal brain injury, which is only diagnosable in postmortem excised tissue. *In vivo* and *ex vivo* studies have shown that fetal brain injury can be induced by oxidative stress. Therefore, the early detection of fetal brain oxidative stress may be useful to diagnose fetal injury and to provide personalized antioxidant treatment. In the above-mentioned study, we reengineered the QUEnch-assiSTed (QUEST) MRI method to measure excessive free radical production in the fetal brain. First, we created 3D printed clip to gently secure individual fetuses *in situ* to stabilize spontaneous breathing movements. Second, we determined the best MRI sequence (R1 data) to obtain signals without artifacts. Third, we used surface coil reception to maximize the filling factor, which resulted in improvement of the signal-to-noise ratio over a whole-body coil. Lastly, the MRI data (R1 data) were normalized to reduce coil inhomogeneities and slice bias for a more precise R1 estimate. This reengineered method was then tested in an animal model in which an ultrasound-guided intra-amniotic injection of endotoxin induced preterm birth, a pregnancy complication that is prevented by treatment with the antioxidant rosiglitazone. We report that the QUEST MRI method is capable of detecting oxidative stress in the fetal brain (green-yellow signal), which is reduced by treatment with the antioxidant rosiglitazone. The study represents the first demonstration that oxidative stress can be determined in the fetal brain using QUEST MRI.

Oxygenation and perfusion to evaluate cerebral hemodynamics in growth-restricted fetuses

We estimated venous blood oxygenation (S_vO_2) of the fetal brain's superior sagittal sinus by susceptibility weighted imaging and measured the blood perfusion by power Doppler ultrasound and fractional moving blood volume from the frontal lobe in a midsagittal plane of the fetal brain. The experiments were performed

on 33 healthy fetuses and 10 abnormal fetuses with fetal growth restriction. We found that S_vO_2 values and the trend across gestations were similar in fetal growth–restricted and healthy fetuses, while in healthy fetuses fractional moving blood volume values were higher and the trend across gestations was the opposite. We also found that, in fetal growth–restricted fetuses, the S_vO_2 and fractional moving blood volume association was significantly positive. Such a dual-imaging modality approach could be beneficial for early detection of fetal brain sparing and brain oxygenation saturation changes in high-risk pregnancies.

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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 IREs near the 5' end of the ferritin H and L chains. IRP1 is an iron-sulfur protein that functions as an aconitase in iron-replete cells. IRP2 is homologous to IRP1 but undergoes iron-dependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration—all 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 iron-sulfur cluster biogenesis machinery. We developed a treatment for the rare disease ISCU (iron-sulfur cluster assembly enzyme) myopathy. By identifying a tripeptide motif common to many iron-sulfur recipient proteins, we developed an algorithm that facilitates discovery of previously unrecognized mammalian iron-sulfur proteins. Our work suggests

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that there are hundreds of previously unrecognized mammalian iron-sulfur proteins. Discovery of iron-sulfur cofactors will lead to breakthroughs in several research areas involving DNA repair, ribosomal biogenesis, mRNA translation, intermediary metabolism, and the 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 iron-dependent alterations in the abundance of ferritin and the transferrin receptor. IREs are RNA stem-loops found in the 5' end of ferritin mRNA and the 3' end of transferrin receptor mRNA. We cloned, expressed, and characterized 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 iron-sulfur cluster (see "Mammalian iron-sulfur cluster biogenesis" below). IRP2 also binds to IREs in iron-depleted cells but, unlike IRP1, in iron-replete cells it is selectively ubiquitinated and then degraded by the proteasome.

To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we initially observed no abnormalities in iron metabolism associated with loss of IRP1 function. *Irp2*$^{-/-}$ mice develop a progressive neurologic syndrome characterized by gait abnormalities and axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. *Irp2*$^{-/-}$ animals develop iron-insufficiency anemia and erythropoietic protoporphyria. In animals that lack IRP1, IRP2 compensates for loss of IRP1's regulatory activity in most cell types, but we discovered several cell types and accompanying phenotypes in which *Irp2* expression cannot be sufficiently increased to compensate. Animals that lack both IRP1 and IRP2 die as early embryos. The adult-onset neurodegeneration of adult *Irp2*$^{-/-}$ mice is exacerbated when one copy of *Irp1* is also deleted. *Irp2*$^{-/-}$ mice offer a unique example of spontaneous adult-onset, slowly progressive neurodegeneration; analyses of gene expression and iron status at various stages of disease are ongoing. Dietary supplementation with the stable nitroxide Tempol prevents neurodegeneration; the treatment appears to work by recruiting the IRE–binding activity of IRP1. We found that motor neurons were the most adversely affected neurons in *Irp2*$^{-/-}$ mice and that neuronal degeneration accounted for the gait abnormalities. In collaboration with Grace Yoon, we discovered two *IRP2*$^{-/-}$ patients who suffered from severe neurodegenerative disease in infancy, and were bed-ridden or died as adolescents.

We discovered a form of the iron exporter ferroportin lacking 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 (HIF2a) translation in renal interstitial through the IRE–IRP system. We confirmed that overexpression of HIF2a 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 HIF2a 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 are conducting experiments with HIF2alpha inhibitors, which reveal that the drugs reverse polycythemia and pulmonary hypertension in our $Irp1^{-/-}$ and Chuvash polycythemia models.

We also elucidated the pathophysiology of intravascular hemolysis and hyposplenism in animals that lack heme oxygenase 1 (HMOX1). Their tissue macrophages die because they cannot metabolize heme after phagocytosis of red cells. To mitigate or reverse disease, we performed bone marrow transplants from wild-type animals to supply animals with functional macrophages; the bone marrow transplants were successful. We then discovered that the bone marrow transplant was not necessary by demonstrating that exogenously expanded wild-type macrophages can repopulate the reticuloendothelial system of *Hmox1*$^{-/-}$ mice, restore normal erythrophagocytosis, and reverse renal iron overload and anemia. Five human *HMOX1*$^{-/-}$ patients have been identified, but we believe this represents an underdiagnosed rare human disease that is often misdiagnosed.

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, the cytosol, and the 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 the 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 antisense 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 antisense 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.

We identified a tripeptide motif, LYR, in apoproteins that are recipients of nascent iron-sulfur clusters. The cochaperone 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 transfer apparatus by binding to the C-terminus of HSC20, facilitating delivery of the three iron-sulfur clusters of SDHB. 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 had been previously appreciated. More recently, we discovered that, through recognition of LYR-like motifs in these recipient proteins, HSC20 is responsible for the delivery of iron-sulfur clusters to respiratory chain complexes I–II.

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 the functioning and sensing of numerous pathways important in cellular functions.

We discovered that the mitochondrial protein ABCB7 (ATP-binding cassette sub-family B member 7) forms a complex with dimeric ferrochelatase, which binds ABCB10 to the other half of the ferrochelatase dimer. Our preliminary results suggest that ABCB7 may represent a mitochondrial heme exporter.

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- 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 Assembly, Plasticity, and Homeostasis

The purpose of our research is to understand the mechanisms of synapse assembly, plasticity, and homeostasis. The chemical synapse is the fundamental communication unit connecting neurons in the nervous system to one another and to nonneuronal cells and 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 postsynaptic compartments and by neuronal activity itself. Our long-term goal is to elucidate the molecular mechanisms that regulate formation of functional synapses during development and which fine-tune them during plasticity and homeostasis. We focus on four key processes in synaptogenesis: (1) trafficking of components to the proper site; (2) organizing those components to build synaptic structures; (3) maturation of the synapse to optimize its activity; and (4) homeostatic mechanisms that restore synapse activity after various perturbations in the system. 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 relies entirely on kainate receptors, which impact synaptic transmission and neuronal excitability in the mammalian central nervous system but remain poorly understood. The *Drosophila* NMJ

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can thus be used to analyze and model defects in the structural and physiological plasticity of glutamatergic synapses, which are associated with a variety of human pathologies, from learning 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 postsynaptic 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 iGluR 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: (1) trafficking the components to the proper site; and (2) organizing those components to build synaptic structures. Recent advances, particularly from vertebrate iGluR biology, reveal that the solution to these problems is entirely dependent on the activity of a rich array of auxiliary subunits that associate with the receptors. These highly diverse transmembrane proteins associate with iGluRs at all stages of the receptor life-cycle and mediate the delivery of receptors to the cell surface, their distribution, synaptic recruitment, association with various postsynaptic density (PSD) scaffolds, and importantly, their channel properties. iGluRs assembled from different subunits have strikingly different biophysical properties; their association with different auxiliary subunits increases this diversity even further.

The *Drosophila* NMJ utilizes at least six kainate receptor (KAR) subunits, which form two distinct postsynaptic complexes (type-A and type-B) that co-exist within individual PSDs and enable NMJ functionality and plasticity and a presynaptic KAR (KaiRID)–containing complex that modulates basal neurotransmission. The postsynaptic KARs are heterotetrameric complexes composed of three shared subunits, GluRIIC, GluRIID, and GluRIIE, and either GluRIIA (type-A receptors) or GluRIIB (type-B). The shared subunits are essential for viability and for iGluR synaptic recruitment. Our previous studies identified *Drosophila* Neto as an obligatory subunit of the fly NMJ iGluR complexes. Neto belongs to a family of highly conserved auxiliary proteins that share an ancestral role in the formation and modulation of glutamatergic synapses. Vertebrate Neto1 and Neto2 and *Caenorhabditis elegans* Neto/SOL-2 were recently shown to modulate the properties of selective iGluRs, mostly KARs. *Neto1/Neto2* double knockout mice have defects in long-term potentiation and in learning and memory, but the underlying mechanisms are extremely difficult to study owing to the low abundance of these channels and the small currents they elicit. In contrast, we found that *Drosophila* utilizes Neto and KARs at the NMJ, a synapse essential for viability. Using live imaging, we showed that Neto clusters at nascent NMJs at the time when iGluRs begin to accumulate and cluster [Kim YJ, et al. *Genes Dev* 2012;26:974]. Similar to animals lacking essential, shared iGluR subunits, *neto*^{nul} mutants are completely paralyzed and die as embryos, with the iGluRs scattered as small aggregates, away from the neuronal arbor. Importantly, Neto does not cluster at synaptic locations in the absence of iGluRs. Our studies demonstrate that Neto engages the iGluRs on the muscle membrane and that they traffic together to synaptic sites where they form clusters. By controlling the clustering and trafficking of functional iGluR complexes, Neto directly controls synapse assembly, organization and maintenance of PSDs, and synapse functionality [Kim YJ, et al. *Genes Dev* 2012;26:974; Kim YJ, et al. *PLoS Genet* 2015;11:e1004988; Kim YJ, Serpe M. *Fly (Austin)* 2013;7:146; Ramos CI, et al. *PLoS Genet* 2015;11:e1005191].

Neto-mediated intracellular interactions sculpt the postsynaptic iGluR fields.

The Neto proteins are multidomain transmembrane proteins with two extracellular CUB (for complement C1r/C1s, UEGF, BMP-1) domains followed by an LDLa (low-density lipoprotein receptor domain class A) motif. CUB domains are BMP–binding, protein-interaction domains that could promote aggregation. *Drosophila neto* encodes two isoforms, Neto-α and Neto-β, with different cytoplasmic domains generated by alternative splicing. The cytoplasmic domains, both rich in putative phosphorylation motifs and docking sites, are highly divergent in Neto proteins across species, presumably reflecting cell- and/or tissue-specific roles. To characterize the functional domains of Neto, we generated truncated Neto variants and tested their cellular distribution and ability to rescue Neto function during development. 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. Muscle expression of a Neto variant with no intracellular domain (Neto-ΔCTD) can rescue the iGluRs recruitment in $neto^{null}$ mutants. Neto activities are restricted by an inhibitory prodomain which must be removed by Furin-mediated proteolysis (Kim YJ, et al. *PLoS Genet* 2015;11:e1004988). When the prodomain cleavage is blocked, Neto is properly targeted to the muscle membrane and engages the iGluR complexes *in vivo* but fails to enable the incorporation of iGluRs in stable synaptic clusters. The recruitment of PSD components is partly attributable to Neto-mediated intracellular interactions.

Type-A and type-B glutamate receptor complexes differ in their trafficking to the synapses, subsynaptic localization, and synaptic responses; at synapses with both type-A and -B receptors, the dose of GluRIIA vs. GluRIIB is a key determinant of quantal size (the response of the muscle to the spontaneous release of a single synaptic vesicle). Previous work from our lab and others established that the synaptic recruitment of GluRIIA requires several postsynaptic components. Such postsynaptic contribution complements Neto's ability to retain iGluRs at synaptic sites via extracellular interactions, the "clustering capacity." We found that mutants with high iGluR "clustering capacity" that lack the relevant postsynaptic interactions cannot stabilize synaptic type-A receptors and incorporate type-B instead. We propose that the type-B receptors are the "default receptors" at synapses with adequate clustering capacity, while the type-A receptors require an extensive network for their synaptic stabilization.

A first evidence in support of this model comes from our studies on Neto-β, the predominant Neto isoform at the larval NMJ (Ramos CI, et al. *PLoS Genet* 2015;11:e1005191). Our developmental studies indicate that Neto-β controls the synaptic recruitment of iGluRs and of other postsynaptic components, such as P21-activating kinase (PAK), an important PSD component previously implicated in the stabilization of type-A receptors at postsynaptic sites. The $neto\text{-}\beta^{null}$ synapses have reduced iGluRs synaptic clusters, in particular the type-A subtype; this reflects the drastic reduction of Neto net levels. However, a $neto\text{-}\beta^{short}$ allele, which truncates part of the cytoplasmic domain and produces a shorter Neto-β variant, shows increased accumulation of synaptic GluRIIB and much reduced GluRIIA compared with control synapses. Thus, short Neto-β, which cannot recruit type-A stabilizers, clusters type-B receptors, suggesting that Neto-β uses its cytoplasmic domain as an organizing platform to sculpt postsynaptic composition.

Interestingly, loss of Neto-α has no detectable effect on iGluR synaptic accumulation [Reference 3]. Instead, 3D-SIM (secondary-ion mass spectrometry) analysis captured the enlarged receptor fields, which appeared to fill the small $neto\text{-}\alpha^{null}$ boutons (Figure 1). Muscle overexpression of a *neto-α* transgene fully rescued the PSD size of $neto\text{-}\alpha^{null}$ synapses. Thus, Neto-α limits the size of the postsynaptic receptor fields but has no detectable role in the organization of presynaptic specializations.

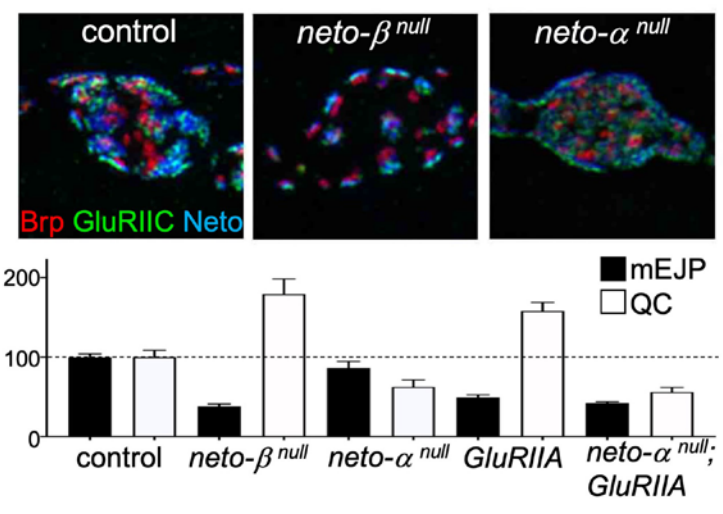

FIGURE 1. Neto isoforms have distinct roles in PSD organization (upper) and NMJ function (lower).

3D-SIM images of synaptic boutons stained for the active zone scaffold, Brp (*red*), GluRIIC (*green*) and Neto (*blue*). Individual synapses are much reduced in the absence of Neto-β and enlarged in the absence of Neto-α.

Reduced postsynaptic sensitivities (mEJP) trigger a compensatory increase in quantal content (QC), the number of vesicle released by the neuron in most genotypes, except for *neto-α^{null}*.

Neto-α controls basal neurotransmission and synapse homeostasis.

In flies as in vertebrates, neuronal activity induces input-specific changes in the synaptic strength; at the larval NMJ, the postsynaptic sensitivity is primarily modulated by synapse-specific recruitment of type-A (GluRIIA-containing) receptors. Robust homeostatic mechanisms keep synapses within an appropriate dynamic range, so that the evoked potentials measured in the muscle remain constant from embryo to third instar larvae; reduced postsynaptic sensitivities (i.e., reduced GluRIIA activity) trigger a compensatory increase in quantal content (QC), the number of vesicle released by the neuron, referred to as presynaptic homeostatic potentiation (PHP).

The drastic reduction of synaptic iGluRs (primarily GluRIIA) at *neto-β^{null}* NMJs causes reduced mini frequency and amplitudes (mEJP), but these NMJs have normal evoked potentials owing to increased QC [Ramos CI, et al. *PLoS Genet* 2015;11:e1005191] and (Figure 1). Neto-α accounts for less than 10% of the Neto synaptic pool. Nonetheless, *neto-α^{null}* NMJs have normal mini amplitudes, but reduced basal neurotransmission [Reference 3]. Interestingly, neuronal but not muscle expression of a *neto-α* transgene rescues the basal neurotransmission, indicating that Neto-α functions in the presynaptic compartment to modulate basal neurotransmission. We examined the homeostatic responses at *neto-α^{null}* NMJs using well-established paradigms, including chronic (developmental) and acute (pharmacological) induction of PHP. Loss of presynaptic Neto-α renders these NMJs unable to express PHP [Reference 3]. Specifically, (1) removal of GluRIIA during development leads to reduced quantal size (mEJP) and triggers PHP (increased QC), a PHP response that is not detectable in *neto-α^{null};GluRIIA* double mutants (Figure 1); also, (2) application of sub-blocking concentrations of philanthotoxin (PhTx), a polyamine toxin derived from wasp venom, to semi-intact larval preparations triggers a fast reduction in quantal size and an increase in QC, so that the basal neurotransmission recovers within minutes. PhTx reduces the quantal size at *neto-α^{null}* NMJs, but the basal neurotransmission never recovers.

Given that a presynaptic KAR, KaiRID, has been recently implicated in the control of basal neurotransmission and the expression of PHP [Kiragasi B, et al. *Cell Rep* 2017;19:2694], we examined whether Neto-α modulates KaiRID synaptic distribution and function. We found that Neto-α controls neurotransmitter release in a KAR–dependent manner. Furthermore, Neto-α is both required and sufficient for the PHP response, which

includes an expansion of the vesicle release machinery. Interestingly, neuronal expression of Neto-β cannot rescue *neto-α^{null}* PHP deficits because Neto-β cannot traffic to the synaptic terminals and instead remains restricted to the somato-dendritic compartment. In contrast, a Neto variant with no intracellular domains (Neto-ΔCTD) can reach the presynaptic terminal and rescue the basal neurotransmission defects of *neto-α^{null}*, but cannot restore the PHP. Our studies demonstrate that the intracellular part of Neto-α functions as a bona fide effector of PHP. The limiting Neto-α seems to be recruited at synapses by the presynaptic KaiRID. This finding challenges our current thinking that auxiliary subunits "assist" iGluRs, and it provides an exquisite example of an auxiliary protein that performs a key synaptic function with assistance from iGluRs.

Our current efforts focus on identifying proteins that interact with Neto both inside and outside the cell and that provide critical activities at the developing NMJ, including iGluR–clustering, iGluR-recruitment and stabilization at PSDs, and mediation of PHP. To this end, we initiated complementary screens: pull-down and mass-spectroscopy comparisons of proteins interacting with the intracellular domains of the fly Neto proteins (α and β); and a synthetic lethality screen (see below).

Tenectin, an integrin ligand critical for structural and functional integrity of the fly NMJ

To search for novel extracellular matrix proteins important for NMJ development, we took advantage of *neto^{109}*, a strong *neto* hypomorph mutant that we isolated and characterized in our lab. 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. In a search for ECM (extracellular matrix) 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 (tnc)*, a gene encoding a large, secreted protein conserved in many insects but with no obvious mammalian homolog.

Tnc is a large secreted mucin that forms a gel-like structure used as luminal scaffolds during the development of tubular epithelia. We found that Tnc is also secreted from motor neurons and muscles and accumulates in the synaptic cleft at larval NMJ. Reduction of Tnc via RNAi produced flightless adults with locomotor defects. To examine the role of *tnc* in synapse development, we generated *tnc* alleles and found that *tnc* is essential for normal NMJ morphology and function. Tnc–deprived NMJs have small, distorted boutons and reduced probability of vesicle release. Interestingly, Tnc recruits αPS2/βPS integrin at synaptic terminals, but not at the muscle attachment sites. Such selectivity was instrumental in probing for integrin functions during synapse development. We found that Tnc and αPS2/βPS integrin form *cis*-active complexes with distinct pre- and postsynaptic functions. In muscle, Tnc/integrin regulates the size and architecture of synaptic boutons, partly through recruitment of the spectrin-based membrane skeleton. In motor neurons, Tnc/integrin ensures the proper assembly and function of active zones by recruiting key players in neurotransmitter release, including the voltage-gated Ca^{2+} channel Cacophony (Cac), and the active-zone scaffold Bruchpilot (Brp). Neuronal knockdown of α-spectrin also diminished synaptic Cac and Brp, prompting us to examine whether Tnc recruits presynaptic spectrin.

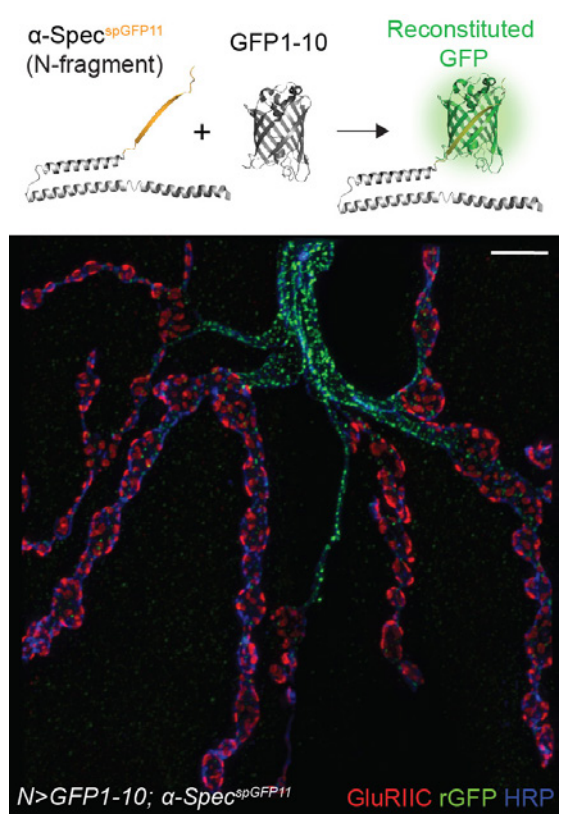

FIGURE 2. Reconstituted GFP (rGFP) marks the endogenous spectrin lattice in larval motor neurons.

3D-SIM image of a motor neuron terminal stained for rGFP, GluRIIC, and HPR (which labels the neuronal membrane).

A presynaptic spectrin network controls active zone assembly and neurotransmitter release.

To detect neuronal α-Spectrin, we employed a GFP reconstitution approach whereby one fragment of the split GFP, spGFP11, was introduced into the *α-Spectrin* locus via CRISPR/Cas9, and the second part, GFP1-10, was expressed intracellularly. In motor neurons, the GFP–marked lattice was bright along the axons, and moderate from the point where motor neuron arbors sink into the muscle cortex (Figure 2). Autocorrelation analyses indicated that the lattice spacing was about 200 nm, consistent with previous measurements. Within the synaptic terminal, the GFP–marked α-Spec fanned out and filled the synaptic boutons but coalesced into a bundle in the interbouton regions. In the absence of Tnc, the GFP–marked α-Spec remained bright along the axons but was drastically diminished at the synaptic terminals. Interestingly, we found that presynaptic α-Spectrin is both required and sufficient for the recruitment of Cac and Brp. We propose that Tnc/integrin complexes anchor the presynaptic spectrin network and ensure proper assembly and function of active zones. Together with our previous finding that postsynaptic Tnc/integrin recruits spectrin to modulate the structural integrity of synaptic boutons, our work identifies two spectrin-dependent integrin signaling pathways that coordinate synapse development and function.

Integrin activation generally promotes formation of focal adhesion complexes and cell migration. In the presence of Tnc, S2 cells (insect cells) accumulate integrin and spectrin at their membranes, but they neither migrate nor form focal adhesion points; instead, the cells round up. This is reminiscent of erythrocytes that maintain their shape and function through the membrane-associated skeleton. Several patients with acanthocytosis (spiky erythrocytes) caused by spectrin tetramerization deficits also present nervous system abnormalities, underscoring the relevance of spectrin network for normal neuronal development and function. Our studies reveal how dynamic changes in the extracellular matrix could be transduced via ligand-activated integrin and spectrin to coordinate changes in synapse structure and function.

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 postsynaptic receptors and relaying this information across the synaptic cleft.

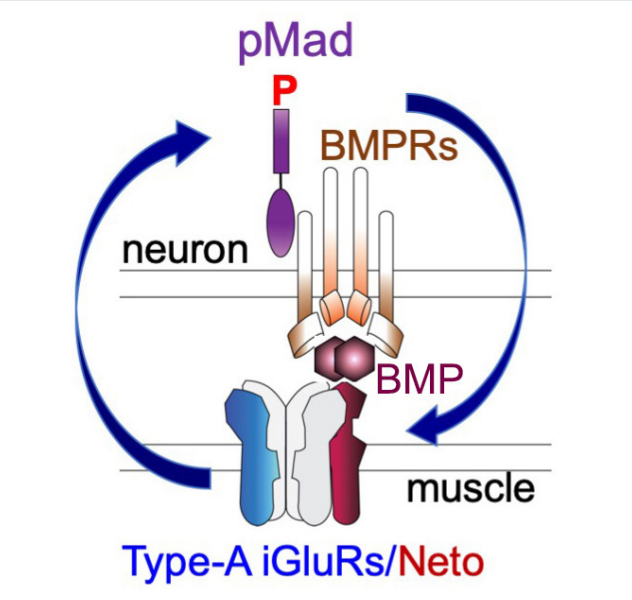

FIGURE 3. The positive feedback loop model

Neto, via its BMP–binding CUB domains, connects active postsynaptic type-A channels with presynaptic BMP/BMP Receptor complexes. Active type-A channels trigger accumulation of presynaptic BMP/BMPR complexes (marked by pMad), which in turn stabilize the type-A channels at postsynaptic sites.

At the *Drosophila* NMJ, BMP signaling is critical for NMJ growth, neurotransmitter release, and synapse plasticity and homeostasis. Our work uncovered a novel BMP signaling modality that operates in conjunction with the canonical BMP pathway to ensure these functions. The canonical BMP pathway, triggered by muscle-derived Gbb binding to presynaptic BMP type-II receptor (BMPRII), Wishful thinking (Wit), BMPRIs Thickveins (Tkv), and Saxophone (Sax), induces accumulation of phosphorylated Smad (pMad) in motor neuron nuclei and activates transcriptional programs with distinct roles in the structural and functional development of the NMJ. Gbb and Wit also signal noncanonically through the effector protein LIM kinase 1 (LIMK1) to regulate synapse stability. Interestingly, pMad also accumulates at synaptic locations but the biological relevance of this phenomenon remained a mystery for over a decade. We found that synaptic pMad constitutes a sensor for synapse activity [Sulkowski M, et al. *Development* 2014;141:436]. Furthermore, synaptic pMad marks a novel, noncanonical BMP pathway, genetically distinguishable from all other known BMP signaling cascades [Reference 4; Sulkowski M, et al. *PLoS Genet* 2016;12:e100581]. This novel pathway stabilizes postsynaptic type-A (GluRIIA–containing) glutamate receptors as a function of their activity.

Type-A receptors are the first to arrive at a nascent synapse; they form the "core" of receptor field and are surrounded by type-B receptors. Previous work in our lab and others established that the incorporation of type-A in stable synaptic complexes depends on GluRIIA activity and requires an extensive postsynaptic network. Our studies on the novel BMP signaling modality indicate that the synaptic stabilization of type-A receptors also requires transsynaptic complexes. The question arises as to how postsynaptic glutamate receptors modulate presynaptic pMad and are in turn stabilized by it. Given that synaptic pMad depends on active type-A receptors, we favor a model whereby Neto, via its BMP-binding CUB domains, connects active postsynaptic type-A receptors with presynaptic BMP/BMPR complexes (Figure 3). Such transsynaptic complexes could offer a versatile means for relaying synapse activity status to the presynaptic neuron via fast conformational modifications. At the same time, these transsynaptic complexes may facilitate interactions that stabilize the type-A receptors at the PSD, a positive feedback that could explain the Hebbian mode of GluRIIA

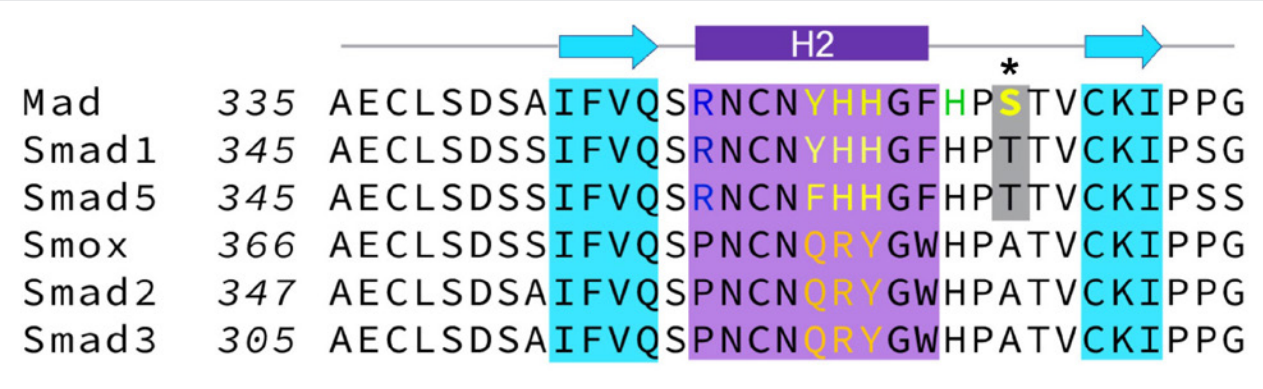

FIGURE 4. The H2 helix is a critical molecular determinant for the Smad–BMPRI interaction.

Alignment indicating class-specific residues in the H2 helix: Y^{352}HH in Smads of the BMP pathway, and QRY in the equivalent position in Smads of the Activin pathway.

incorporation at the PSD and maturation of iGluR fields at larval NMJ. As BMPRs are limiting and shared among different BMP signaling modalities, the neurons may use this novel BMP pathway to monitor synapse activity and then coordinate NMJ growth with synapse maturation and stabilization.

Selective disruption of synaptic BMP signaling by a Smad mutation adjacent to the conserved H2 helix

In search of Mad features that influence its association with the BMPRs, we collected most existing *Drosophila Mad* alleles and compared them for their ability to sustain the two Smad–dependent signaling modalities: canonical BMP signaling, marked by pMad accumulation in motor neuron nuclei, and Smad–dependent noncanonical signaling, marked by pMad accumulation at synaptic terminals. Within this comprehensive collection, we found that strong *Mad* alleles generally disrupt both synaptic and nuclear pMad accumulation, whereas moderate *Mad* alleles have a wider range of phenotypes and selectively impact different BMP signaling modalities. In particular, Mad^{8} showed drastically reduced synaptic pMad levels but only moderately diminished nuclear pMad signals. The postsynaptic composition and electrophysiological properties of Mad^{8} NMJs were likewise altered. Using biochemical assays and structural modeling, we examined how point mutations such as S359L (marked*, Figure 4), present in Mad^{8}, could influence the Mad–Tkv interface. Our study identified a new molecular determinant for this Mad–Tkv interaction, the highly conserved H2 helix [Reference 4]. Several genetic variants identified in human patients map to H2, underscoring the relevance of this motif for normal development and function.

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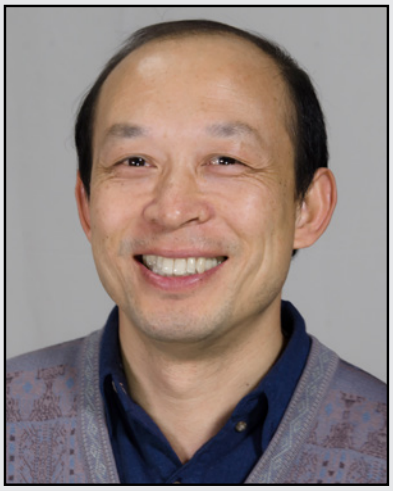
Thyroid Hormone Regulation of Vertebrate Postembryonic Development

This laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during postembryonic development, a period around birth in mammals when plasma levels of TH peak. The main model we use is the metamorphosis of *Xenopus laevis* and *X. tropicalis*, two highly related species that offer unique but complementary advantages. The control of this developmental process by TH offers a paradigm to study gene function in postembryonic organ development. During metamorphosis, different 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 and *de novo* development of the adult epithelial stem cells, followed by their proliferation and differentiation, the intestine is transformed into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both *in vivo*, using genetic approaches or hormone treatment of whole animals, and *in vitro* in organ cultures offer an excellent opportunity to (1) study the developmental function of TH receptors (TRs) and the underlying mechanisms *in vivo* and (2) identify and functionally characterize genes that are critical for organogenesis, particularly the formation of the adult intestinal epithelial stem cells, during postembryonic development in vertebrates. A major recent focus has been to make use of the TALEN and CRISPR/Cas9 technologies to knockdown or knockout the endogenous genes to permit functional analyses. In addition, we complement our frog studies by investigating the genes found to be important for frog intestinal stem cell development in developing mouse intestine by making conditional knockouts.



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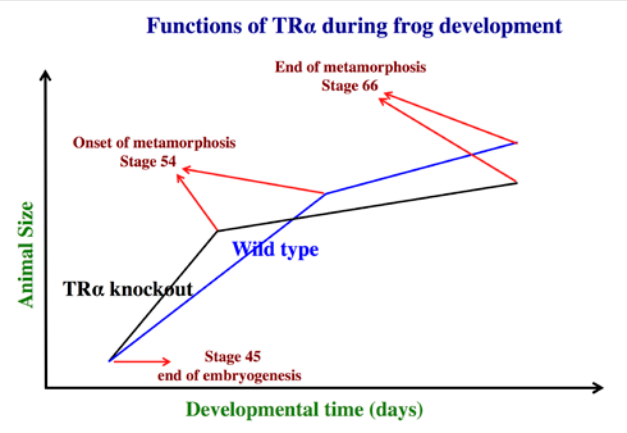
Unique role of TRβ in regulating notochord resorption during *Xenopus* metamorphosis

Tail resorption during anuran metamorphosis is perhaps the most dramatic tissue transformation that occurs during vertebrate development. Like all other process during metamorphosis, tail

FIGURE 1. Schematics showing the effects of TRα (thyroid hormone receptor α) knockout on *Xenopus tropicalis* development



TRα knockout has little effect on embryogenesis, and resulting tadpoles are normal by feeding stage (stage 45). However, 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 smaller at the end of metamorphosis than do the wild-type siblings. Thus, in premetamorphic tadpoles prior to stage 54, unliganded TRα (due to the lack of thyroid hormone) functions to control metamorphic timing, whereas, when thyroid hormone becomes available during metamorphosis, TRα helps increase the rate of metamorphosis.

resorption is controlled by TH. Earlier studies in the anuran species *X. laevis* and *X. tropicalis* had shown that the TR plays a necessary and essential role for metamorphosis. Of the two known TR genes in all vertebrates, *trα* is highly expressed during both premetamorphosis and metamorphosis, while *trβ* expression is low in premetamorphic tadpoles but highly upregulated as a direct target gene of TH during metamorphosis. In addition, the two TR genes have distinct temporal regulation patterns in different organs during development, suggesting that the two TRs have different functions during metamorphosis. Indeed, gene knockout studies by us and others showed that *trα* is not essential for metamorphosis but controls metamorphic timing and the rate of metamorphosis progression during early metamorphosis. Knockout of *trβ*, however, has no effect on metamorphic timing or early metamorphosis, but significantly delays late metamorphosis, particularly tail resorption. Homozygous *trβ* knockout tadpoles become tailed frogs well after their wild-type siblings complete metamorphosis. Most noticeably, in *trβ*-knockout tadpoles, an apparently normal notochord is present in the tail as late as three days after the initiation of tail shortening (stage 62), while in wild-type and *trα*-knockout tadpoles, the tail notochord disappears in about one day. We investigated how tail notochord resorption is regulated by TR [Reference 1]. We showed that *trβ* is selectively much more highly expressed in the notochord than is *trα*.

We also discovered differential regulation of several matrix metalloproteinases (MMPs), which are known to be upregulated by TH and thought to play a role in tissue resorption by degrading the extracellular matrix (ECM). In particular, MMP9-TH and MMP13 are extremely highly expressed in the notochord compared with the rest of the tail. *In situ* hybridization analyses show that these MMPs are expressed in the outer sheath cells and/or the connective tissue sheath surrounding the notochord. Our findings suggest that high levels of *trβ*

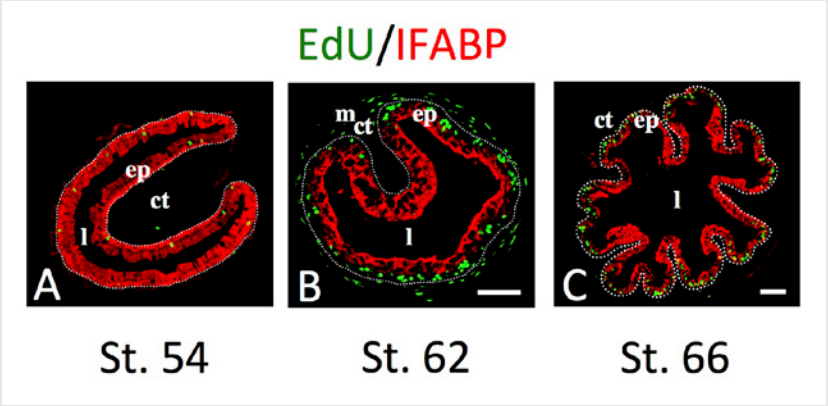


FIGURE 2. Intestinal metamorphosis involves the formation of clusters of proliferating, undifferentiated epithelial cells at the climax.

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), which 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.

expression in the notochord specifically upregulate the MMPs, which in turn degrades the ECM, leading to the collapse of the notochord and its subsequent resorption during metamorphosis.

Gene expression program underlying tail resorption during thyroid hormone–dependent metamorphosis of the ornamented pygmy frog *Microhyla fissipes*

Studies on amphibian metamorphosis have largely focused on the two highly related species *X. laevis* and *X. tropicalis*. Adult *X. laevis* and *X. tropicalis* animals remain aquatic, in contrast to most other anurans, which change from aquatic to terrestrial during metamorphosis, and thus more closely mimic the postembryonic development in mammals, making it important to study metamorphosis in a truly terrestrial frog species. In this regard, the anuran *Microhyla fissipes* offers several advantages as an alternative model for developmental and genetic studies. We made use of the advances in sequencing technologies to investigate the gene regulation profiles underlying the tail resorption program during metamorphosis in *M. fissipes* [Reference 2]. We first used single-molecule real-time (SMRT) sequencing to obtain 67,939 expressed transcripts in *M. fissipes.* We next identified 4,555 differentially expressed transcripts (DETs) during tail resorption by using Illumina sequencing on RNA samples from tails at different metamorphic stages. Bioinformatics analyses revealed that several KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and GO (Gene Ontology) terms associated with tail resorption were enriched. Our findings suggest that tail resorption during *M. fissipes* and *X. laevis* shares many of the same programs. Future investigations into the function and regulation of these genes and pathways should help to reveal the mechanisms governing amphibian tail resorption and adaptive evolution from aquatic to terrestrial life. Furthermore, analysis of the *M. fissipes* model, with particular regard to the changes in other organs associated with the transition from aquatic to terrestrial living, should help to yield important mechanistic insights into mammalian postembryonic developments.

TH activates the *Xenopus mbd3* gene via an intronic TRE during intestinal stem cell development.

To identify direct TH–response genes during the formation of adult intestinal stem cells, we previously carried out a ChIP (chromatin immunoprecipitation)-on-chip analysis with a polyclonal anti-TR antibody on the tadpole intestine and identified many putative TR target genes. Among them is the methyl-CpG binding domain protein 3 (*mbd3*) gene, which has been implicated in epigenetic regulation of cellular processes as a subunit of the Mi-2/NuRD (Nucleosome Remodeling Deacetylase) complex. We showed that *mbd3* is upregulated in the intestine by TH and that its expression peaks at stage 62, the climax of metamorphosis [Reference 3]. We further discovered a putative thyroid hormone response element (TRE) within the first intron of the *mbd3* gene that binds to TR/RXR *in vitro* and *in vivo* and mediates TH regulation of the *mbd3* promoter *in vivo*.

TRα mutations lead to epithelial defects in the adult intestine in a mouse model of resistance to thyroid hormone.

Intestinal maturation in mammals takes place around birth when TH levels are high, mimicking frog metamorphosis and implicating a role of TH in mammalian intestinal development and function. Interestingly, a several human patients carrying heterozygous mutations in the *TRα* gene (*RTHα*, or resistance to TH due to TRα mutation) have been discovered in recent years and found to suffer from constipation, implicating intestinal defects caused by *TRα* gene mutations. To determine how *TRα1* mutations affect the intestine, we analyzed a mutant mouse expressing a strong dominantly negative TRα1 mutant, (denoted TRα1PV; *Thra1*^{PV} mice) [Reference 4]. The mutant mouse faithfully reproduces RTHα phenotypes observed in human patients. In adult *Thra1*$^{PV/+}$ mice, we observed constipation just like in patients with *TRα* mutations. Importantly, we discovered significant intestinal defects, including shorter villi and higher numbers of differentiated cells in the crypt, accompanied by reduced stem-cell proliferation in the intestine. Our findings suggest an evolutionary conservation of TH function in the intestine. Further analysis of this mouse model should help reveal the molecular and physiological defects in the intestine caused by *TRα* mutations and determine the underlying mechanisms.

LAT1 regulates osteoclastogenesis and bone homeostasis through the mTORC1 pathway.

To regulate cellular processes, TH has to be actively transported into cells, a process that is mediated by several different types of transporters. *LAT1*, one of our previously identified TH–response genes in the intestine, encodes the light chain of a heterodimeric system L type of TH transporter, which also transports several amino acids. Interestingly, *Lat1* is highly upregulated at the climax of metamorphosis in the tadpole intestine, coinciding with the formation and rapid proliferation of adult intestinal stem cells. We also found out that LAT1 was also highly expressed in the mouse intestine during the neonatal period when the mouse intestine matures into the adult form, a process that appears also involves TH–dependent formation and/proliferation of the adult intestinal stem cells. In a collaborative study, we generated a mouse line with the *Lat1* gene floxed, which allows conditional knockout of the *Lat1* upon expression of the Cre recombinase. We are still in the process investigating whether LAT1 affects adult intestinal stem-cell development in mouse, but we recently showed, through another collaboration, that LAT1 is an important amino acid transporter for the regulation of bone homeostasis through its function in osteoclasts [Reference 5]. *Lat1* expression was significantly reduced in osteoclasts in a mouse model of ovariectomy-induced osteoporosis. The osteoclast-specific deletion of *Lat1* in mice led to osteoclast activation and bone loss *in vivo*, and LAT1 deficiency elevated osteoclastogenesis *in vitro*. Loss of LAT1 impaired activation of the mechanistic target of rapamycin complex

1 (mTORC1) pathway in osteoclasts, whereas genetic activation of mTORC1 corrected the activation of osteoclastogenesis and bone loss resulting from LAT1 deficiency. The findings suggest that the LAT1–mTORC1 axis plays a pivotal role in bone resorption and bone homeostasis, thereby providing a novel molecular connection between amino-acid intake and skeletal integrity.

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- Keisuke Nakajima was supported by Hiroshima University, Japan

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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 boundary-establishing membrane fidelity and the necessary ability of the cell to change these boundaries.

The challenge in studying the membrane is its complexity. The membrane is a thin sheet of small molecules, i.e., lipids. There are hundreds of types of lipids in the cell. Each lipid changes the properties of the membrane in its vicinity, sometimes making the sheet stiffer, sometimes softer, and sometimes acting to bend the membrane into a ball or tube. Furthermore, the lipids are constantly jostling and tangling with both each other and 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 extensive 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. Our Unit uses 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 on 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 information about lipids. Recent breakthroughs that break the

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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 public distribution is a key element of the lab's work.

Simple differences in the protein–membrane attachment mechanism have functional consequences for surface mechanics.

We developed two methods for propagating coupled membrane and embedded particle dynamics with ensembles that yield the correct thermodynamics of membrane fluctuations [Reference 1]. Proteins and functional lipids associate with cellular membranes, and their attachments influence the membrane's physical and dynamical properties. Therefore, it is necessary to accurately model the coupled dynamics of the membrane and any associated material of interest. The two methods differ in the binding mechanism of the particle to the surface. The “on-surface” mechanism should be used for particles that slide along the membrane; this description leads to an effective reduction in the membrane surface tension. The “in-surface” mechanism treats the particles as tightly bound to the lipidic binding sites; the method avoids the double-counting of the lateral entropy of implicitly modeled lipids.

The difference between the two attachment mechanisms has significant consequences for crowded membranes in living systems. Local tension is a major factor in determining the ease with which protein machinery can bud off membrane material from the plasma membrane. Likewise, for *in vitro* experiments on membrane reshaping with ad hoc membrane attachment mechanisms, the presence or lack of additional tension applied is critical for interpreting mechanism.

Efficient computation of protein–lipid interactions in reaction–diffusion modeling

In this study, together with our collaborators in Margaret Johnson's lab, we devised a theoretical treatment of how to properly and efficiently account for proteins interacting with high concentrations of lipids in reaction-diffusion modeling [Reference 2]. Compared with most molecular processes, modeling cellular processes requires large time and length scales. Moreover, the molecules of the cell do not typically move like the objects we interact with in our daily lives. Instead, they move randomly by a diffusive mechanism. Reaction-diffusion modeling uses kinetic data for macromolecular reactions to account for both the slow diffusive motion of molecules as well as for the empirically determined kinetics of their binding and unbinding. In conjunction with our lab's continuum membrane modeling, we are developing a software approach to account for how macromolecular complexes reshape membranes. The study fills an important need for a method that can implicitly model the high concentrations of signaling lipids, such as phosphoinositides, while retaining the rigor and efficiency of reaction diffusion modeling. A critical piece of programming and mathematical analysis was provided by two summer interns in our lab, Ruchita Kothari and Gudrun Thorkelsdottir.

A new mechanism of cholesterol redistribution amongst leaflets of the plasma membrane

In collaboration with Michael Schick and David Allender, we determined the role that curvature stress plays in determining how cholesterol redistributes between the leaflets of the plasma membrane [Reference 3]. A lipid membrane bilayer is made up of two apposed sheets of lipid molecules, oriented so that they form a chemical barrier, which prevents polar material from passively crossing. The inner and outer leaflets of the plasma membrane are composed of very different lipids; the inner is enriched in phosphatidylethanolamine head groups, while the outer leaflet is enriched in sphingolipids. Our lab refined experimental findings on these two lipids, determining that, for example, sphingomyelin prefers significant positive curvature. Cholesterol couples very differently to these lipids' curvature preferences, i.e., it strongly influences the shape of the bilayer around it; thus, around disordered (very fluid) lipids it makes concave leaflets, while around ordered lipids (with the lipid acyl chain tails nearly straight, somewhat gelatinous) it tends to make the surface convex. Our study, which combined simulation and theory, determined that its asymmetric curvature preference prevents cholesterol from accumulating to a high degree in the outer leaflet, where it would otherwise be attracted to the leaflet's saturated lipid content.

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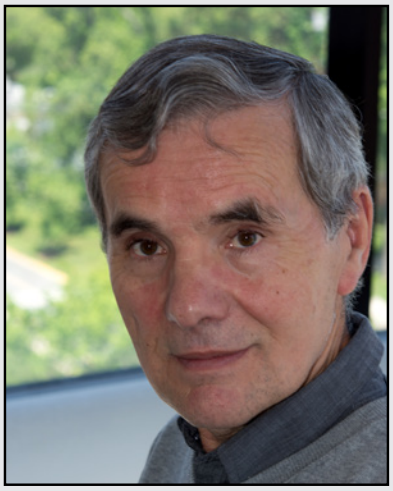
Signaling and Secretion in Neuroendocrine Cells

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 inositol-trisphosphate 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 voltage-gated channels expressed in neuroendocrine cells, the cell type–specific patterns of electrical activity and channels involved, the physiological relevance of such activity, and the crosstalk between G protein–coupled receptors and ion channels. More recently, we characterized ligand-gated receptor channels expressed in pituitary cells, including the ATP–gated P2X receptor channels. Our current work focuses on age-, sex-, and tissue structure–specific signaling, transcription, and secretion in the pituitary gland, the heterogeneity of secretory pituitary cells reflecting their embryonal and postnatal genesis, and cell type–specific exocytic pathways. We are also studying how the structural features of P2X receptors relate to the channels' functions and how plasma-membrane receptors and the intracellular signaling milieu affect channel activity.



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Transcriptome profiles of rat anterior pituitary cells

We continued our investigations on receptors and channels expressed in neuronal and endocrine cells and their roles in signaling, gene transcription, and hormone secretion. To gain a better understanding of the cell type-specific expression and role of these and other proteins in anterior pituitary cell functions and related disorders, we performed single-cell RNA sequencing on freshly dispersed cells from adult male and female rats. Our analysis, based on over 7,000 cells, confirmed the presence of folliculostellate cells (FSC) and hormone producing cells (HPC): corticotrophs, gonadotrophs, thyrotrophs,

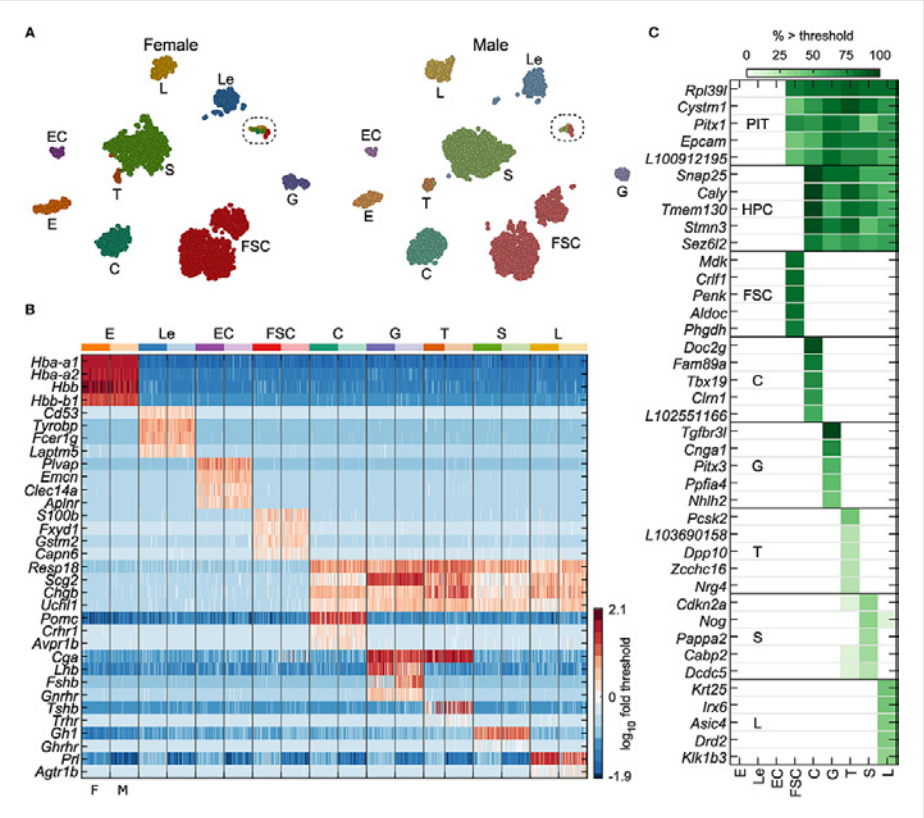

FIGURE 1. Identification of anterior pituitary cell types

A. tSNE map showing identified cell types for both female and male cells: erythrocytes (E), leukocytes (Le), endothelial cells (EC), folliculostellate cells (FSC), corticotrophs (C), gonadotrophs (G), thyrotrophs (T), somatotrophs (S), and lactotrophs (L). The results of marker-based classification, performed separately per sex, align very closely with the independent clustering done by tSNE.

B. Expression of genes used for cell-type classification in a random subsample of 50 cells per sex for each cell type, shown as log10 fold change relative to gene threshold. Columns alternate between female and male cells (F, M; bottom).

C. Selected genes identified as specific markers or cell type–dominant genes, excluding classification genes. PIT: pituitary cells, comprising FSCs and HPCs. The gene name prefix LOC was shortened to L. Cell types E, Le, EC, and FSC were defined by expression greater than threshold of at least two of the four marker genes indicated per group. Hormone-producing cells (HPCs) comprised C, G, T, S, and L, all of which expressed at least two of *Resp18*, *Scg2*, *Chgb*, and *Uchl1*. Specific HPC types expressed additional genes as follows: C, at least one of *Pomc*, *Crhr1*, or *Avpr1b*; G, at least two of *Cga*, *Lhb*, *Gnrhr*, or *Fshb*; T, at least two of *Cga*, *Tshb*, or *Trhr*; S, *Gh1* or *Ghrhr*; L, *Prl* or *Agtr1b*.

somatotrophs, and lactotrophs. Also recognized were endothelial and blood cells from the pituitary capillary network (Figure 1). We identified cell type–specific gene expression in HPCs and FSCs, examined cellular heterogeneity in these populations, and compared them with pituitary-nonspecific cell populations from portal blood capillaries.

Comparison of males and females, the latter in the diestrus phase of their estrous cycle, allowed us to identify sex-specific gene expression profiles in pituitary-specific cell types. The t-distributed stochastic neighbor embedding maps shown in Figure 2 demonstrating clear sexual dimorphism in some cell types but not in others. This is evident for lactotrophs and gonadotrophs, both of which appear as two distinct clusters, whereas, for somatotrophs, a subset of female cells appears distinct from a mixed main cluster. FSCs also formed two clusters, but both clusters contained a mix of cells from both sexes, indicating that sexual dimorphism was not the cause of subclustering. The t-distributed stochastic neighbor embedding map also suggests very little sexual dimorphism in corticotrophs, based on uniform mixing of male and female cells in that cluster.

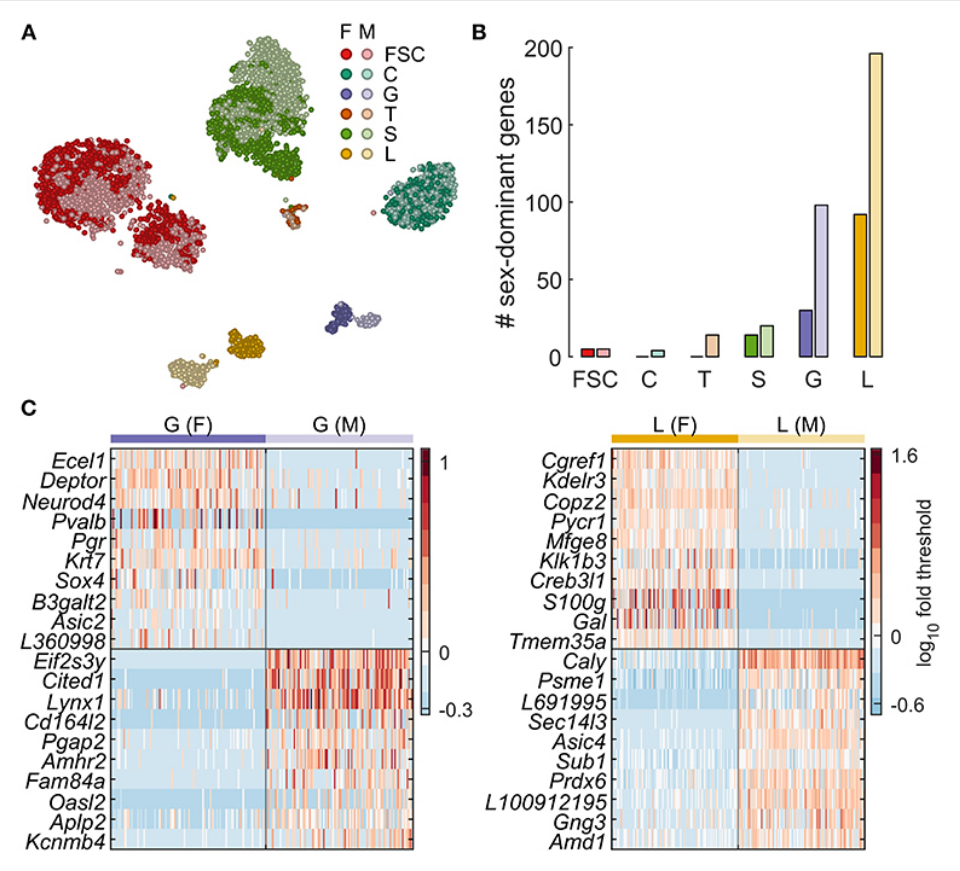

FIGURE 2. Sexually dimorphic gene expression underlies lactotroph and gonadotroph subclustering.

A. Sex-specific clustering of pituitary cells shown as a tSNE plot. For a given cell type, females are indicated with a darker color.

B. Number of genes identified as dominantly expressed per sex for each cell type.

C. Expression of genes with the highest difference in % cells expressing more than threshold between sexes for gonadotrophs and lactotrophs. A random subsample of 100 female (left of vertical line) and 100 male cells (right of vertical line) are shown. F, female; M, male. The gene name prefix LOC was shortened to L.

To study the genetic relationship between FSCs and HPCs, we focused on two groups of genes suggested by our differential expression analysis: development and neuroendocrine marker genes. Out of 64 development-differentiation genes expressed in pituitary cells we identified 13 FSC–specific (including *Prrx1*, *Prop1*, and *Sox9*), 11 specific for one or more hormone-producing cell types (including *Tbx19*, *Gata2*, and *Pou1f1*), and 9 genes common to FSCs and at least one HPC type (including *Pitx1*, *Pitx3*, and *Six1*). Many neuroendocrine marker genes were widely expressed in HPCs; 28 were expressed in at least 80% of one or more cell type of this group. Among this gene group, *Fuz*, *Gnas*, *Nptn*, and *Rtn1* were expressed in both cell types. *Snap25*, *Snap91*, *Stmn3*, *Syt4*, and *Syt7* were well expressed in all HPC types and were identified as specific markers for this population of pituitary cells. *Ndrg2* and *Tagln* were expressed dominantly and specifically in FSCs, respectively. In total, only 9 genes were FSC–specific, 47 were HPC–specific, and 14 were dominantly co-expressed by both FSC and HPC types. The enrichment in neuroendocrine genes related to exocytosis in HPCs is consistent with the physiological role of hormone secretion in these cells. Together, the findings suggest that HPCs and FSCs are either sister cells, i.e., cells of a common origin, or that a subset of FSCs are pituitary stem cells [Reference 1].

FSCs exhibit impressive transcriptome diversity, indicating their major roles in the production of endogenous ligands and detoxification enzymes, and organization of the extracellular matrix. Transcriptome profiles of HPCs also indicate contributions toward those functions. Among endogenous ligand genes, 15 genes were FSC–specific, including *Anxa1*, *Cxcl12*, *Igfbp2*, *Mdk*, and *Penk*; 18 genes were HPC–specific, including *C1qtnf4*

and *Fgf9* in all HPCs, *Inha*, and *Vegfa* in gonadotrophs, and *Bmp15* and *Nmu* in thyrotrophs; and 11 genes were common to FSCs and one or more HPC type, including *Anxa7, Ccl27, Copa*, and *Cntn1*. Among 79 detoxification enzyme genes expressed in pituitary cells, 20 were FSC–specific, six were common for FSCs and HPCs, and only seven genes were HPC–specific. Among 53 extracellular matrix genes expressed in pituitary cells, only five were FSC–specific, eight HPC-specific, and three common to pituitary cells. HPCs but not FSCs also express numerous genes encoding voltage-gated, ligand-gated, and other channels, clearly indicating their endocrine function. The findings point to complex interactions between HPCs and FSCs in the production of endogenous ligands and detoxification enzymes, organization of the extracellular matrix, and expression of cell-adhesion molecules [Reference 1].

Gating properties of purinergic receptor channels

Single-cell RNA sequencing also confirmed that all HPC types express ionotropic receptor channels activated by extracellular ATP, termed P2X4 channels, whereas gonadotrophs and folliculostellate cells express the P2X2 receptor gene. Both channels have unique properties. Our work on P2X2 receptors focused on the kinetics of receptor desensitization. The channels exhibit a slow desensitization during the initial ATP application and a progressive, calcium-dependent increase in rates of desensitization during repetitive stimulation. The pattern is observed in whole-cell recordings from cells expressing recombinant and native P2X2, but not in perforated-patched cells and in two-electrode voltage clamped oocytes. Addition of ATP, but not of ATPgS or GTP, to the pipette solution also abolishes progressive desensitization, whereas intracellular injection of apyrase facilitates receptor desensitization. Experiments with injection of alkaline phosphatase or addition of staurosporine and ATP to the intracellular solution suggest 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 of P2X2R in the intracellular ATP action. The findings indicate that the metabolic state of the cell can influence P2X2R gating [Reference 2].

We also used electrophysiology and recombinant channels to study in detail the antagonistic effect of 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD) on ATP–induced currents mediated by the rat P2X4 receptor and compared its specificity with another rat P2X receptors. We found that 5-BDBD is a potent P2X4 receptor antagonist, with an IC_{50} in submicromolar concentration range. In contrast, 5-BDBD did not affect the ATP–induced P2X2a, P2X2bR, and P2X7 receptor current amplitude or the pattern of receptor desensitization when applied in a high micromolar concentration range. However, it partially inhibited the P2X1 and P2X3 receptor–gated currents. Moreover, we studied the effects of 5-BDBD in long-term potentiation experiments performed in rat hippocampal slices, with the finding that this antagonist can partially reduce long-term potentiation, a response that is believed to be mediated in part by endogenous P2X4 receptors. These results indicate that 5-BDBD could be used to study the endogenous effects of the P2X4 receptor in the central nervous system and that this antagonist can distinguish between this and other P2X receptors, when they are co-expressed in the same tissue [Reference 3].

Expression of small integrin–binding ligand, *N*–linked glycophosphoproteins (SIBLINGs) in gonadotrophs

The cell–extracellular matrix tridimensional network is critical for the proper functioning of all tissues, including anterior pituitary gland. In addition to proteoglycans and fibrous proteins, the extracellular matrix contains other proteins, including SIBLINGs, which are soluble, secreted proteins that can act as modulators of cell adhesion as well as autocrine and paracrine ligands for extracellular matrix receptors. Our single-

cell RNA-seq analysis revealed that the pituitary gland expresses two SIBLING genes, *Dmp1* (dentin matrix protein-1) and *Spp1* (secreted phosphoprotein-1) encoding DMP1 and osteopontin proteins, respectively. Both genes were expressed exclusively in pituitary gonadotrophs, indicating that these cells have specific roles in the function of the cell–extracellular matrix tridimensional network of anterior pituitary gland. The qRT-PCR and immunocytochemical analyses confirmed that *Spp1*/osteopontin and *Dmp*1/DMP are expressed in a sex- and age-specific manner. The *Spp1*/osteopontin expression is higher in male gonadotrophs, whereas *Dmp1*/DMP1 is predominantly expressed in female gonadotrophs. Like *Lhb*, *Fshb*, and *Gnrhr*, the marker genes for gonadotrophs, *Dmp1* expression is stimulated by gonadotropin-releasing hormone (GnRH). In contrast, the expression of *Spp1* is not regulated by GnRH *in vivo* or *in vitro*. However, *Spp1* expression increases progressively after pituitary cell dispersion in both female and male cultures. We speculate that gonadotrophs signal to other pituitary cell types about changes in the structure of pituitary cell-matrix network by osteopontin and DMP1, a function consistent with the role of these secretory proteins in postnatal tissue remodeling, extracellular matrix reorganization after injury, and tumorigenesis. Our ongoing work focuses on secretion of these two proteins by gonadotrophs under different experimental paradigms, on characterization of their receptors, integrins, and CD44, within secretory and non-secretory anterior pituitary cells and their signaling pathways, and on their function in pituitary gland [Reference 4].

Continuous GnRH treatment blocks *Fshb* but not *Lhb* expression.

The pulsatile pattern of GnRH secretion is critical for proper gonadotropin synthesis and release, and continuous GnRH infusion leads to a marked reduction in blood gonadotropin levels. However, the mechanism of downregulation of gonadotropin release by continuous GnRH treatment has not been clarified. Our ongoing experiments focus on the expression profile of gonadotropin subunit genes *Cga*, *Lhb*, and *Fshb* and of *Gnrhr* in rat pituitary *in vitro* and *in vivo* to clarify their expression profiles in the absence or continuous presence of GnRH. Figure 1 shows that the genes are expressed in pituitary gonadotrophs but not in other pituitary cell types. Culturing mixed populations of anterior pituitary cells in GnRH–free conditions downregulated *Fshb*, *Cga*, and *Gnrhr* expression, whereas continuous treatment with GnRH receptor agonists upregulated *Cga* expression progressively but *Gnrhr* and *Fshb* expression transiently, accompanied with a prolonged blockade of *Fshb* but not of *Gnrhr* expression. In contrast, *Lhb* expression was relatively insensitive to loss of endogenous GnRH and continuous treatment with GnRH agonists, probably reflecting the status of *Egr1* and *Nr5a1* expression. Similar patterns of responses were observed *in vivo* after administration of a GnRH agonist. However, continuous treatment with GnRH stimulated luteinizing hormone (LH) secretion *in vitro* and *in vivo*, leading to decrease in LH cell content despite high basal *Lhb* expression. The data suggest that blockade of *Fshb* expression and depletion of the LH secretory pool are two major factors accounting for weakening of the gonadotroph secretory function during continuous GnRH treatment [Reference 5].

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Olfactory Coding and Decoding by Neuron Ensembles

Response heterogeneity and adaptation in olfactory receptor neurons

The olfactory system, consisting of relatively few layers of neurons with structures and mechanisms that appear repeatedly in widely divergent species, provides unique advantages for the analysis of information processing by neurons. Olfaction begins when odorants bind to olfactory receptor neurons, triggering them to fire patterns of action potentials. Recently, using new electrophysiological recording tools, we found that the spiking responses of olfactory receptor neurons are surprisingly diverse and include powerful and variable history dependencies. Single, lengthy odor pulses elicit patterns of excitation and inhibition that cluster into four basic types. Different response types undergo different forms of adaptation during lengthy or repeated stimuli. A computational analysis showed that this diversity of odor-elicited spiking patterns helps the olfactory system efficiently encode odor identity, concentration, novelty, and timing, particularly in turbulent, naturalistic environments.

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 postsynaptic neuron. Cyclic integration windows could allow a neuron to respond preferentially to spikes arriving coincidentally from several 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

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effectively in a preferred window of the oscillation cycle. With a computational model, we established that the nonuniform 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.

A population of projection neurons that inhibit the lateral horn but excite 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 (mlALT), contains projection neurons expressing GABA, a neurotransmitter that usually elicits inhibition; in the *Drosophila* brain, the great majority of ventral projection neurons (vPNs) are GABAergic and project through the tract to a brain area called the lateral horn. Most projection neurons, which are excitatory (ePNs), project through the medial ALT (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 postsynaptic 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 coexpress 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 at the dendritic presynaptic terminals in the antennal lobe. Indeed, several examples of mammalian neurons that can release multiple fast excitatory or inhibitory neurotransmitters have been reported, such as spatially segregated release of GABA and ACh in the retina. These unexpected results suggest new roles for the vPN population in olfactory information processing.

Spatiotemporal coding of individual chemicals by the gustatory system

Four of the five major sensory systems (vision, olfaction, somatosensation, and audition) are thought to be encoded by spatiotemporal patterns of neural activity. The only exception is gustation. Gustatory coding by the nervous system is thought to be relatively simple: every chemical ('tastant') is associated with one of a small number of basic tastes, and the presence of a basic taste, rather than the specific tastant, is represented by the brain. In mammals as well as insects, five basic tastes are usually recognized: sweet, salty, sour, bitter, and

umami. The neural mechanism for representing basic tastes is unclear. The most widely accepted postulate is that, in both mammals and insects, gustatory information is carried through labelled lines of cells sensitive to a single basic taste, that is, in separate channels from the periphery to sites deep in the brain. 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 spatiotemporal 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, spatiotemporal activity, which is a fast and accurate code. The results provide a dramatically new view of taste 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 have included computational searches for conservation of intergenic regions and direct detection after size selection or co-immunoprecipitation with the RNA binding protein Hfq. We recently examined small RNA expression using deep sequencing to further extend our identification of small RNAs in a range of bacteria species.

A major focus for the group has been to elucidate the functions of the small RNAs that 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 now have also begun to explore the role of ProQ, a second RNA chaperone in *E. coli*.

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 1]. For example, we showed that the Spot 42 RNA, whose levels are highest when glucose is present, plays a broad role in catabolite repression by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse nonpreferred carbon sources. Similarly, we discovered that a Sigma(E)-dependent small RNA, MicL, transcribed

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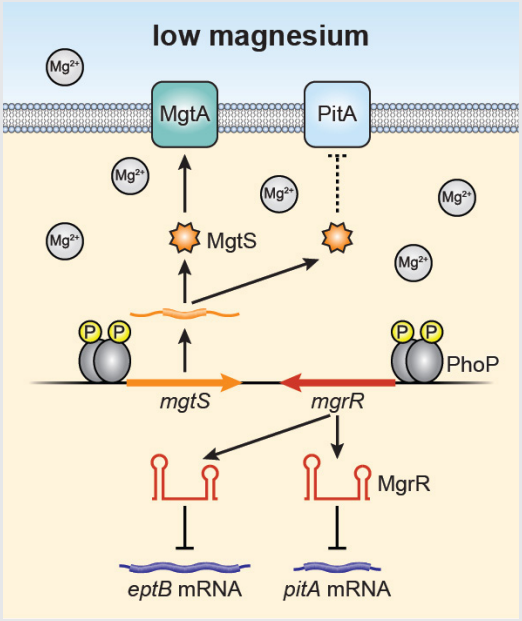

FIGURE 1. Model for impact of convergently transcribed small protein and sRNA genes on intracellular Mg^{2+}

In response to limiting Mg^{2+}, the PhoQP two-component 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 Mg^{2+} importer and the *eptB* mRNA, respectively. Our recent studies show that they both also modulate the PitA phosphate symporter to increase intracellular Mg^{2+}, pointing to a detrimental role of PitA under limiting Mg^{2+} conditions.

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 copper sensitivity phenotype previously ascribed to inactivation of the *cutC* gene is actually derived from the loss of MicL and from elevated Lpp levels. This observation raises the possibility that other phenotypes currently attributed to protein defects can be attributed to deficiencies in unappreciated regulatory RNAs. Studies to determine the factors that direct the cleavage of MicL, and likely other small RNAs from the 3' untranslated regions (UTRs) of mRNAs, showed that 3' stem-loops are critical for the highly specific processing [Reference 2]. Most recently, while characterizing the response to limited magnesium, we found that the adjacently encoded MgrR small RNA and MgtS small protein both down regulate the pitA–encoded cation-phosphate symporter to increase intracellular magnesium levels [Reference 3].

In addition to small RNAs that act via limited base-pairing, we are interested in regulatory RNAs that act by other mechanisms. For example, early work showed that the 6S RNA binds to and modulates RNA polymerase by mimicking the structure of an open promoter. In a more recent study, we discovered that a broadly conserved RNA structure motif, the yybP–ykoY motif, found in the 5'-UTR of the *mntP* gene encoding a manganese exporter, directly binds manganese, resulting in a conformation that liberates the ribosome-binding site. Remarkably, we were able to recapitulate the effect of manganese-dependent activation of translation *in vitro*. We also found that the yybP–ykoY motif responds directly to manganese ions in *Bacillus subtilis*. The identification of the yybP–ykoY motif as a manganese-ion sensor suggests that the genes preceded by this motif and encoding 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 [Reference 4] 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 several short RNAs do indeed encode small proteins. The correct annotation of the smallest proteins is one of the biggest challenges of genome annotation, and there is limited evidence that annotated short ORFs encode synthesized proteins. Although these proteins have

been largely missed, the few small proteins that have been studied in detail in bacterial and mammalian cells have been shown to have important functions in regulation, signaling, and cellular defenses [Reference 5]. We thus established a project to identify and characterize proteins of less than 50 amino acids.

We first used sequence conservation and ribosome-binding-site models to predict genes encoding small proteins of 16–50 amino acids in the intergenic regions of the model *E. coli* genome. We tested expression of these predicted as well as previously annotated small proteins by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and assaying for synthesis using immunoblot assays. The approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids are synthesized. We recently carried out a complementary approach based on genome-wide ribosome profiling of ribosomes arrested in start codons to identify many additional candidates; the synthesis of 38 of these small proteins was confirmed by chromosomal tagging [Reference 6].

Many of the initially discovered proteins were predicted to consist of a single transmembrane alpha-helix and were found, by biochemical fractionation, to be 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.

We now are using the tagged derivatives and information about synthesis and subcellular localization and employing many of the approaches the group has used to characterize the functions of small regulatory RNAs to elucidate the functions of the small proteins. The combined approaches are beginning to yield insights into how the small proteins are acting in *E. coli*. We found that synthesis of a 42–amino acid protein, now denoted MntS, is repressed by high levels of manganese through MntR. The lack of MntS leads to reduced activities of manganese-dependent enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese levels and bacteriostasis under manganese-rich conditions. These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP.

We also showed that the 31–amino acid inner-membrane protein MgtS (formerly denoted YneM), whose synthesis is induced by very low magnesium in a PhoPQ–dependent manner, acts to increase intracellular magnesium levels and maintain cell integrity upon magnesium depletion. Upon development of a functional tagged derivative of MgtS, we found that MgtS interacts with MgtA to increase the levels of this P-type ATPase magnesium transporter under magnesium-limiting conditions. Correspondingly, the effects of MgtS upon magnesium limitation are lost in an *mgtA* mutant, and MgtA overexpression can suppress the *mgtS* phenotype. MgtS stabilization of MgtA provides an additional layer of regulation of this tightly controlled magnesium transporter. Most recently, we found that MgtS also interacts with and modulates the activity of a second protein, the PitA cation-phosphate symporter, to further increase intracellular magnesium levels [Reference 3].

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. Copurification of AcrZ with AcrB, in the absence of both AcrA and TolC, two-hybrid assays, and suppressor mutations indicate this interaction occurs through the inner-membrane protein AcrB. Mutants lacking AcrZ are sensitive to many, but not all, 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 of other small proteins and related findings by others in eukaryotic cells, supports our hypothesis that many small proteins act as regulators of larger membrane proteins.

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Molecular Genetics of Endocrine Tumors and Related Disorders

The project "Molecular Genetics of Endocrine Tumors and Related Disorders" was started in the late 1990s. The goal has always been to identify molecular pathways involved in the first steps of tumor formation. Our approach was to study patients with rare endocrine conditions, mostly inherited, identify the causative genes, and then study the 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 (BAHs). 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 nonpigmented forms of BAH exist, and a new nomenclature was proposed, which we first suggested in 2008 and is since 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 hyperplasia (MMAD/

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(continued)

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 histomorphological 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 (D-seq). 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.

Mutations in *PRKAR1A* and protein kinase A activity in CNC 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 [Rossi ÁD, et al. *J Intern Med* 2019;285(2):215-222] 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 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 that yet another cAMP–specific PDE is involved in endocrine conditions. We also studied both *Pde11a* and *Pde8b* animal models.

Genetic investigations into other adrenocortical diseases and related tumors

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

This past 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

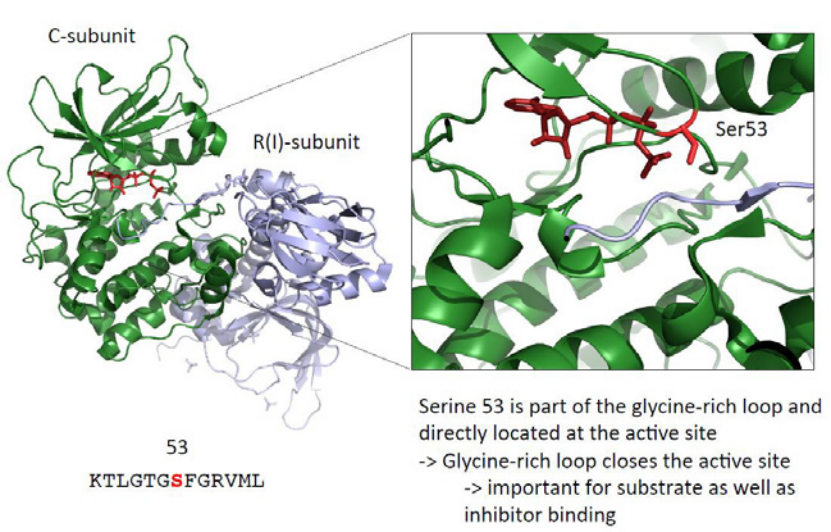

FIGURE 2. A *PRKACB* mutation in cortisol-producing adenomas

The image shows the S54L *PRKACB* mutation that was identified by our collaborators at the Hôpital Cochin 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.

first shown to be linked to adrenocortical Cushing's syndrome in a patient who had nongenetic 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), which results 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 cyclin-dependent kinase-binding protein) and *USP8* (encoding ubiquitin carboxyl-terminal hydrolase 8) mutations in patients with CD (*CABLES1*) and/or their tumors (*USP8*).

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

As part of a collaboration with other investigators at the NIH and elsewhere (including an international consortium organized by our laboratory), we are studying the genetics of the Carney Triad, a rare syndrome that predisposes to adrenal and other tumors, and of related conditions (associated with gastrointestinal stromal tumors [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 postoperative 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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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*), a small tropical freshwater fish, 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 accessible 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 on the role of epigenetics during early development, in particular how DNA methylation and other epigenetic mechanisms help 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. In zebrafish every blood vessel can be observed in living animals with high resolution, and simple, rapid screening can be accomplished for even subtle

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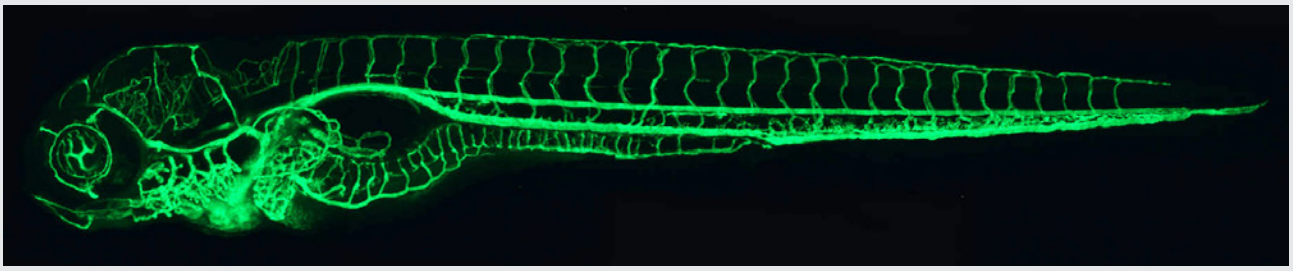

FIGURE 1. The zebrafish vascular system

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.

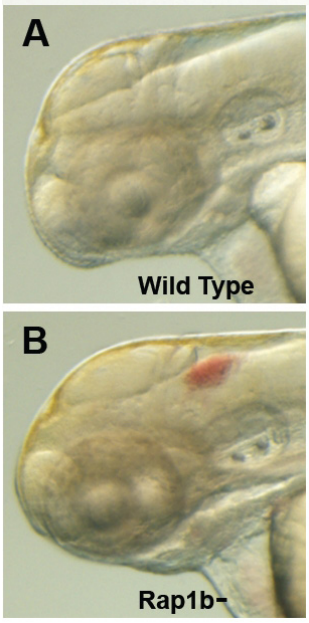

FIGURE 2. Intracranial hemorrhage (ICH) in the developing zebrafish

The clarity of zebrafish larvae also makes it straightforward to screen for animals with intracranial hemorrhage, as is evident in comparing lateral views of a 2-day-old wild-type larva (*A*) with a hemorrhage-prone larva deficient in *rap1b* (*B*).

vascular-specific mutants (Figure 1). We are carrying out several related projects using the fish, which are described below.

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.

GENETIC ANALYSIS OF VASCULAR DEVELOPMENT

We have identified many novel mutants affecting vascular development in our transgene-assisted forward genetic screens and are currently characterizing the phenotypes and molecular basis for several of these mutants.

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.

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 neurocognitive deficits and

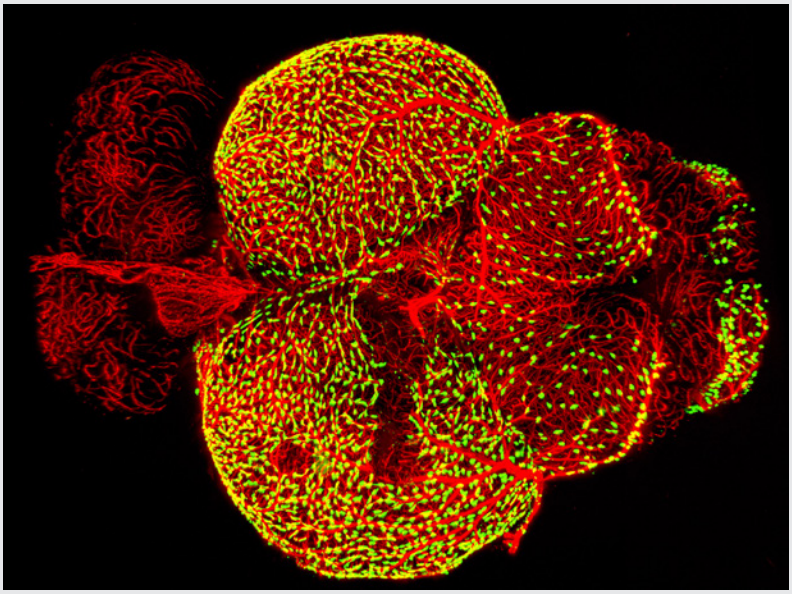

FIGURE 3. Novel perivascular cells on the zebrafish brain

Confocal micrograph of fluorescent granular perithelial cells (FGPs, *green*) adhering to the outside of meningeal blood vessels (*purple*) on the brain of a Tg(mrc1a:egfp); Tg(kdrl:cherry) double-transgenic adult zebrafish. We recently showed that FGPs are unique endothelium-derived perivascular cells with unusual scavenging properties that are likely to be critical for brain homeostasis. See Reference 5 for additional details.

neurodegenerative disease. Many of the recent insights into the molecular mechanisms regulating vascular integrity have come from studies in model organisms such as the zebrafish. We are pursuing several related projects.

GENES REGULATING VASCULAR INTEGRITY

We used forward-genetic screens to identify new zebrafish mutants that disrupt cranial vascular integrity in the zebrafish (Figure 2), using next-gen sequencing methods to accomplish higher-throughput cloning of mutants. We already characterized the role of *GDF6* (growth differentiation factor 6, also 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 defects in the regulatory protein RHOA (involved in cytoskeletal dynamics, transcription, cell cycle progression, and cell transformation) resulting in vascular integrity defects.

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 the cells, how their interaction 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.

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

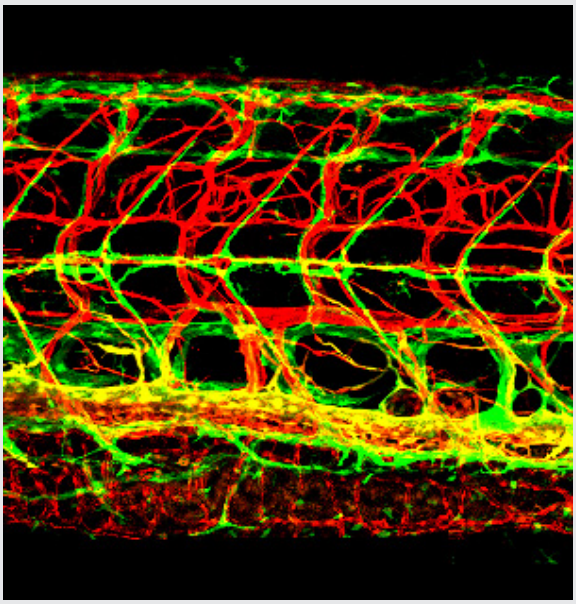

FIGURE 4. Novel lymphatic vascular reporter

Lateral view confocal image of the trunk of a 12 dpf (days postfertilization) *Tg(kdrl:cherry); Tg(mrc1a:egfp)* double-transgenic zebrafish with red fluorescent blood vessels and green fluorescent lymphatics. See Jung HM, et al. *Development* 2017;144:2070 for additional details.

meningeal blood vessels, which is likely to play a critical role in meningeal function (Figure 3). We are currently carrying out additional studies to understand the function of FGPs and other novel meningeal vascular-associated cell populations.

Specification and patterning of the lymphatic system

The lymphatic system is a vascular system completely separate 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 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 4).

(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 microRNAs expressed in the lymphatic endothelium and how these small regulatory RNAs influence lymphatic gene expression and lymphatic development.

(4) We are studying the formation of previously uncharacterized lymphatic vascular networks surrounding the zebrafish brain. Like similar brain lymphatic vessels recently discovered in the mammalian brain, the zebrafish

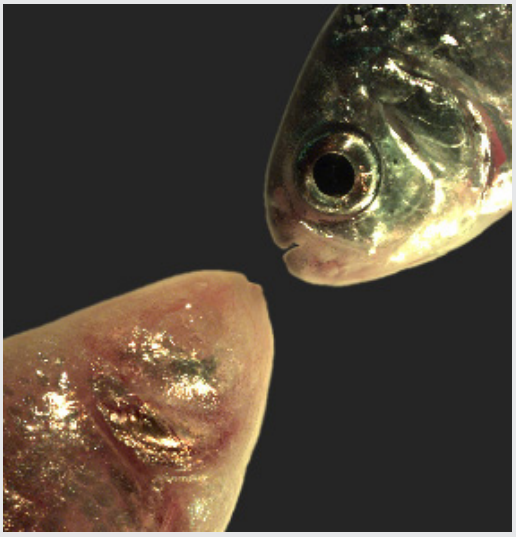

FIGURE 5. Mexican tetra cave and surface fish

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 drive at least some of this rapid evolutionary change. See Reference 3 for details.

vessels are likely to play critical roles in maintaining homeostasis and protecting the brain, and we are carrying out a detailed analysis of the development, form, and function of these critical vessels.

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.

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.

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 *DNMT3B* expression promotes eye loss in blind, eyeless *Astyanax* cavefish (Figure 5).

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.

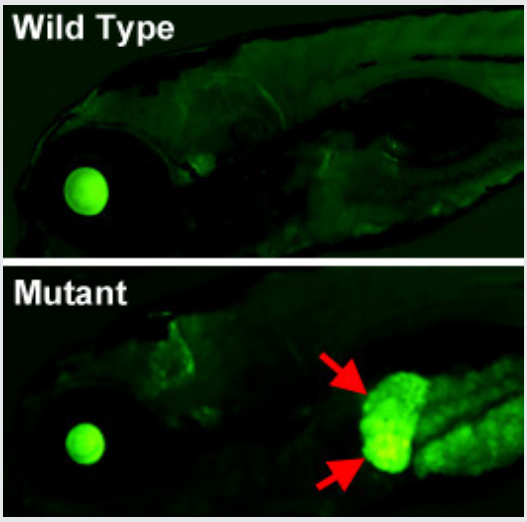

FIGURE 6. An epigenetic silencing mutant in the zebrafish

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.

FORWARD GENETIC SCREEN FOR EPIGENETIC REGULATORY FACTORS

Genetic screens carried out in *Drosophila* and the nematode *Caenorhabditi 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 the 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).

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- K99/R00 Award (to M. Venero Galanternik)
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Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells

Under optimal conditions, the fidelity of DNA replication is extremely high. Indeed, it is estimated that, on average, only one error occurs for every 10 billion bases replicated. However, given that living organisms are continually subjected to a variety of endogenous and exogenous DNA–damaging agents, optimal conditions rarely prevail *in vivo*. While all organisms have evolved elaborate repair pathways to deal with such damage, the pathways rarely operate with 100% efficiency. Thus, persisting DNA lesions are replicated, but with much lower fidelity than in undamaged DNA. Our aim is to understand the molecular mechanisms by which mutations are introduced into damaged DNA. The process, commonly referred to as translesion DNA synthesis (TLS), is facilitated by one or more members of the Y-family of DNA polymerases, which are conserved from bacteria to humans. Based on phylogenetic relationships, Y-family polymerases may be broadly classified into five subfamilies: DinB–like (polIV/pol kappa–like) proteins are ubiquitous and found in all domains of life; in contrast, the Rev1–like, Rad30A (pol eta)–like, and Rad30B (pol iota)–like polymerases are found only in eukaryotes; and the UmuC (polV)–like polymerases only in prokaryotes. We continue to investigate TLS in all three domains of life: bacteria, archaea, and eukaryotes.

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Prokaryotic mutagenesis

As part of an international scientific collaboration with Antoine van Oijen and Michael Cox, we investigated the subcellular localization of the *E.coli* RecA protein, which orchestrates the cellular response to DNA damage via its multiple roles in the bacterial SOS response. The lack of tools to provide unambiguous access to the various RecA states within the cell has previously prevented an understanding of the spatial and temporal changes in RecA structure/function that underlie control of the DNA damage response. To circumvent these problems, Antoine van Oijen's group developed a monomeric C-terminal fragment of the λ repressor as a novel fluorescent probe that specifically interacts with RecA filaments on single-stranded DNA (RecA*). Single-molecule imaging techniques in live cells revealed that RecA is largely sequestered in storage structures during normal metabolism. Upon DNA damage, the storage structures dissolve, and the cytosolic pool of RecA rapidly nucleates to form early SOS–signaling complexes, maturing into DNA–bound RecA bundles at later

time points. Both before and after SOS induction, RecA* largely appears at locations distal from replisomes. Interestingly, upon completion of DNA repair, RecA storage structures reform [Reference 1].

In a collaboration with Myron Goodman and Michael Cox, we investigated how *E.coli* DNA polymerase V (pol V) is regulated by a conformational change upon interacting with ATP and the RecA protein). Mutagenic translesion DNA polymerase V ($UmuD'_2C$) is induced as part of the DNA damage–induced SOS response in *Escherichia coli*, and is subjected to several levels of regulation. The UmuC subunit is sequestered on the cell membrane (spatial regulation) and enters the cytosol after forming a $UmuD'_2C$ complex, about 45 min after SOS induction (temporal regulation). However, DNA binding and synthesis cannot occur until pol V interacts with a RecA nucleoprotein filament (RecA*) and ATP to form the mutasome complex pol V Mut ($UmuD'_2C$-RecA-ATP). The location of RecA relative to UmuC determines whether pol V Mut is catalytically on or off (conformational regulation). In our collaborative study, we reported three interrelated experiments to address the biochemical basis of conformational regulation. We first investigated dynamic deactivation during DNA synthesis and static deactivation in the absence of DNA synthesis. Single-molecule (sm) TIRF-FRET microscopy was then used to explore several aspects of pol V Mut dynamics. Binding of ATP/ATPγS triggers a conformational switch that reorients RecA relative to UmuC to activate pol V Mut. The process is required for polymerase–DNA binding and synthesis. Both dynamic and static deactivation processes are governed by temperature and time, in which on to off switching is "rapid" at 37°C (about 1 to 1.5 h), "slow" at 30°C (about 3 to 4 h) and does not require ATP hydrolysis. We found that Pol V Mut retains RecA in both activated and deactivated states but binding to a primer-template (p/t) DNA occurred only when Pol V Mut is activated [Reference 2].

In another study related to polV, we generated a “so-called” steric gate mutant of pol V_{ICE391} found on the integrating conjugative element ICE391 (formerly known as IncJ R391), which incorporates high levels of ribonucleotides into the *E. coli* genome to investigate the molecular mechanisms of ribonucleotide excision repair (RER). The study was part of an international scientific collaboration with Andrew Robinson, Myron Goodman, and Iwona Fijalkowska. As noted above, pol V_{ICE391} ($RumA'_2B$) is a low-fidelity polymerase that promotes considerably higher levels of spontaneous “SOS–induced” mutagenesis than the related *E. coli* pol V ($UmuD'_2C$). The molecular basis for the enhanced mutagenesis was previously unknown. Using single-molecule fluorescence microscopy to visualize pol V enzymes, we discovered that the elevated levels of mutagenesis are likely attributable, in part, to prolonged binding of RumB to genomic DNA, leading to higher levels of DNA synthesis than with UmuC. We then generated a steric-gate pol V_{ICE391} variant (pol V_{ICE391}–Y13A) that readily misincorporated ribonucleotides into the *E. coli* genome and used the enzyme to investigate the molecular mechanisms of RER under conditions of elevated ribonucleotide-induced stress. To do so, we compared the extent of spontaneous mutagenesis promoted by *E. coli* pol V and pol V_{ICE391} with that of their respective steric gate variants. Levels of mutagenesis promoted by the steric gate variants that were lower than that of the wild-type enzyme were indicative of active RER, which removes misincorporated ribonucleotides but also misincorporated deoxyribonucleotides from the genome. Using such an approach, we confirmed that RNase HII plays a pivotal role in RER. In the absence of RNase HII, nucleotide excision repair (NER) proteins help remove misincorporated ribonucleotides. However, significant RER occurred in the absence of RNase HII and NER. Most of the RNase HII and NER–independent RER occurred on the lagging strand during genome duplication. We suggest that this is most likely attributable to efficient RNase HI–dependent RER, which recognizes the polyribonucleotide tracts generated by pol V_{ICE391}–Y13A. These activities are critical for the maintenance of genomic integrity when RNase HII is overwhelmed, or inactivated, as demonstrated by the

observation that delta *rnhB* or delta *rnhB* delta *uvrA* strains expressing pol V_{ICE391}–Y13A exhibited genome and plasmid instability in the absence of RNase HI [Reference 3].

Eukaryotic mutagenesis

We extended our RER studies to *Saccharomyces cerevisiae* in a collaboration with Anders Clausen. To do so, we utilized a steric-gate mutant of DNA polymerase η (pol η) that we had previously generated in our lab in 2015. Although best known for its ability to bypass UV-induced thymine-thymine (T-T) dimers and other bulky DNA lesions, pol η also has other cellular roles. In our study, we presented evidence that pol η competes genome-wide with DNA polymerases α and δ for the synthesis of the lagging strand, where it also shows a preference for T-T in the DNA template. Moreover, we found that the C-terminus of pol η, which contains a proliferating cell nuclear antigen (PCNA)–Interacting Protein motif is required for pol η to function in lagging-strand synthesis. We also presented evidence that a pol η–dependent signature is also found to be lagging-strand–specific in patients with skin cancer. Taken together, the findings provided insight into the physiological role of DNA synthesis by pol η and have implications for our understanding of how our genome is replicated to avoid mutagenesis, genome instability, and cancer [Reference 4].

In another international scientific collaboration with Justyna McIntyre, we investigated the regulation of human DNA polymerase iota (Polι), which was discovered by scientists in our lab twenty-one years ago. DNA polymerase iota (Polι) belongs to the Y-family of DNA polymerases that are involved in DNA–damage tolerance through their role in translesion DNA synthesis. Like all other Y-family polymerases, Polι interacts with PCNA, Rev1, ubiquitin and ubiquitinated PCNA and is also ubiquitinated itself. In our collaborative study, we reported that Polι also interacts with the p300 acetyltransferase and is acetylated. The primary acetylation site is K550, located in the Rev1–interacting region. However, K550 amino acid substitutions have no effect on Polι's ability to interact with Rev1. Interestingly, we found that acetylation of Polι significantly and specifically increased in response to S_N2 alkylating agents and, to a lower extent, to S_N1 alkylating and oxidative agents. Given that we did not observe acetylation of Polι's closest paralog, DNA polymerase eta (Polη), with which Polι shares many functional similarities, we believe that acetylation of Polι might exclusively regulate as yet to be determined and separate function(s) of Polι [Reference 5].

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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.

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(continued)

Genetic factors important for childhood body weight regulation

To identify gene variants affecting body composition, we have been examining polymorphisms in genes involved in the leptin signaling pathway. Genes include the leptin receptor (*LEPR*), genes that appear to alter leptin receptor signal transduction such as those that are part of the BBSome (an octameric protein complex component of the basal body involved in trafficking cargos to the primary cilium), 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 adults [Reference 1] and appears to have functional significance for MC3R signal transduction. Children and adults who were homozygous variant for both C17A and G241A polymorphisms have significantly greater fat

mass and higher plasma levels of insulin and leptin than unaffected or heterozygous children and appear to eat more at laboratory test meals (Figure 1). *In vitro* studies subsequently found that signal transduction and protein expression were significantly lower for the double mutant MC3R. Our ongoing studies attempt to understand the mechanisms by which these sequence alterations may affect body weight. We therefore developed transgenic 'knock-in' mice expressing the human wild-type and human double-mutant 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 $MC3R^{hDM/hDM}$ mice 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 inflammatory cell infiltration of adipose tissue 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 accumulate more triglyceride than $MC3R^{hWT/hWT}$ *MSCs*. $MC3R^{hDM/hDM}$ thus impacts nutrient partitioning to generate increased adipose tissue that appears metabolically healthy. The data thus 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 the mice. We are investigating a novel role for the MC3R in regulating hepatic autophagy, the role of MC3R in stem cell fate, and how variations in *Mc3r* may alter signaling of several downstream signaling pathways. Using tissue-specific knockout- and reactivation models, we are also studying the importance of hepatic and adipose tissue MC3R for whole body homeostasis.

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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

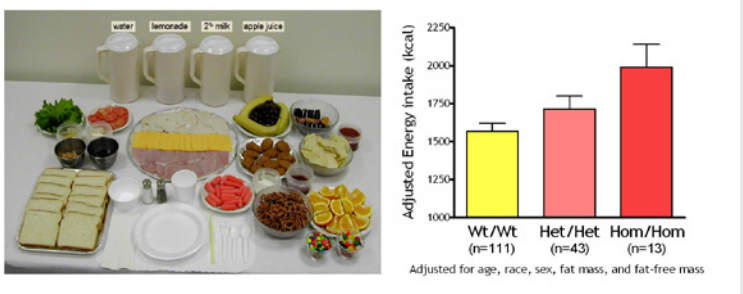


FIGURE 1. Energy intake studied by using free-access buffet meals of palatable foods

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).

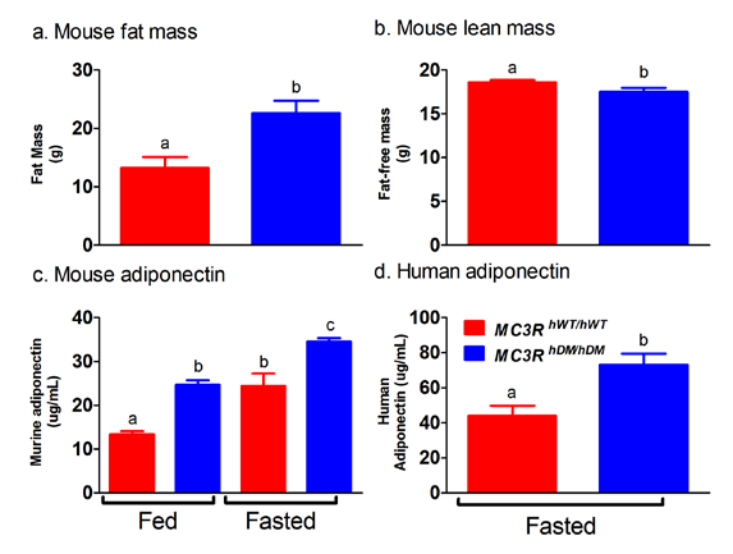


FIGURE 2. Studies of a human *MC3R* variant containing two naturally occurring polymorphisms

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*).

eating-related psychopathology, insulin sensitivity, changes in body composition, energy intake, and energy expenditure in children, and we studied the short- and long-term stability of the components of metabolic syndrome. We found that leptin is an important predictor of weight gain in children: those with high leptin gain even more weight when followed longitudinally. We also documented that hyperinsulinemia is positively related to energy intake in nondiabetic, obese children, leading to treatment studies to reduce 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. Such associations suggest mechanisms whereby insulin resistance may contribute to excessive weight gain in children and have informed some of our treatment approaches to pediatric obesity (described below).

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors also are associated with adiposity in children and abnormalities in metabolism. We found that binge-eating behaviors may predict future weight gain in children at risk for obesity. Thus children reporting binge-eating behaviors, such as loss of control over eating, gained, on average, 2.4 kg more weight per year than non-binge-eating children. Our data also suggest that children endorsing binge eating consume more energy during meals. Actual intake during buffet meals averaged 400 kcal more in children with binge eating, but, despite their greater intake, such children reported shorter-lived satiety than children without binge-eating episodes. The ability to consume large quantities of palatable foods, especially when coupled with decreased subsequent satiety, may play a role in the greater weight gain found in binge-eating children. Among cohorts of lean and obese youth, we demonstrated that youth with LOC (loss of control) eating have higher serum leptin and are at significantly greater risk for worsening of components of the metabolic syndrome than those without LOC episodes, even after adjusting for adiposity 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. The data further suggest that interventions targeting disordered eating behaviors may be useful in preventing excessive fat gain in children prone to obesity and have led to trials of preventative strategies

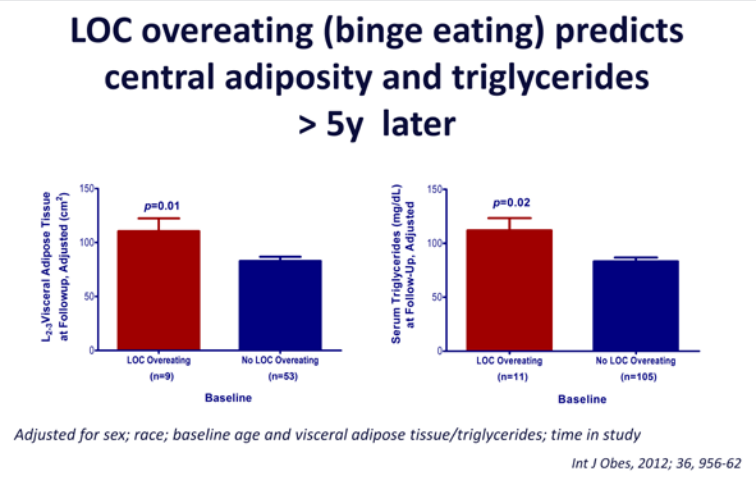


FIGURE 3. Loss of control (LOC) eating and metabolic complications in a longitudinal study

On average (±SE), children who engaged in binge eating at baseline had more visceral adipose tissue at L_{2-3} intervertebral space at follow-up than children who did not engage in binge eating at baseline, adjusting for sex, race, baseline age, baseline visceral adipose tissue at L_{2-3}, and time in study ($P = 0.01$). On average (±SE), children who engaged in binge eating at baseline had higher follow-up triglycerides than children who did not engage in binge eating at baseline, adjusting for sex, race, baseline age, body mass index (kg/m^2), baseline triglycerides, and time in study ($P = 0.02$).

related to binge eating. Because binge eating appears to be a heritable trait, we also initiated studies to investigate possible genetic factors linked to LOC over eating.

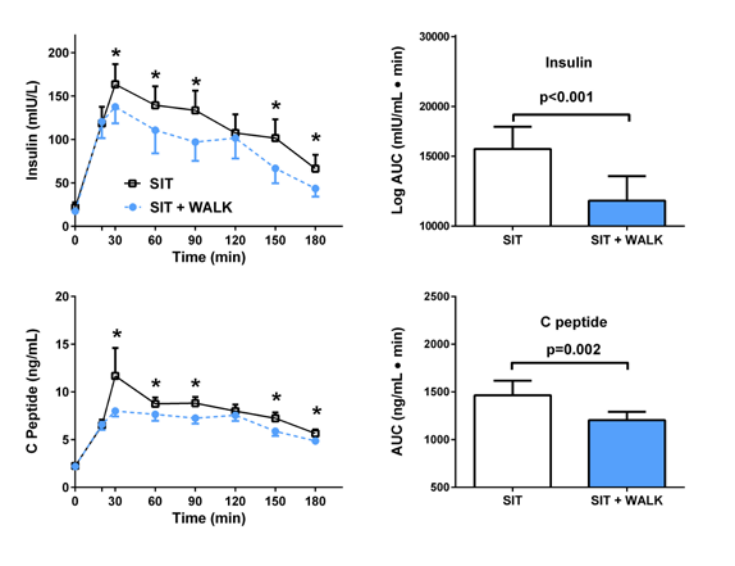
In order to determine the factors that are most important for development of the complications of obesity in youth, we study normal weight children and adolescents, children who are already obese, and the children of obese parents who are not obese. We examine body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, and genetic factors believed to regulate metabolic rate and body composition. Psychological and behavioral factors, such as propensity to engage in binge-eating behavior (Figure 3), and sleep [Reference 2] are also studied. Children are being followed longitudinally into adulthood. In two protocols, we study actual food consumption of children during meals, to elucidate differences in the calorie and macronutrient content of meals and the circulating hormones related to hunger and satiety in those who either endorse binge-eating behaviors or report no such behaviors. We found that eating in the absence of physiological hunger is a replicable trait that appears linked to obesity. We 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 children with overweight or obesity who engaged in moderate activity for just three minutes every half hour, versus remaining sedentary [Reference 3]. A new study extends these observations over a longer intervention period. We hypothesize that differences in these factors will predict the development of obesity in the populations studied and may be of great importance in developing rational approaches for the prevention and treatment of obesity in the diverse US population.

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

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in children and adults is urgently needed, yet current pharmacologic approaches are extremely limited for both children and adults. In several clinical protocols, we examined approaches for the prevention and treatment of excessive body weight. We completed a randomized controlled trial to examine the mechanism by which metformin may affect the body weight of younger children who have hyperinsulinemia and are therefore at

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 without interruption for three hours (*black*).



risk for later development of type 2 diabetes. Compared with placebo-treated children, those randomized to metformin significantly decreased BMI, BMI-Z score, and body fat mass. Serum glucose and HOMA-IR also decreased significantly more in metformin-treated than in placebo-treated children. We also published studies of the pharmacokinetics of metformin and how polymorphisms in enzymes affecting metformin clearance impact weight change. Mean population apparent clearance (CL/F) was 68.1 L/h, and mean apparent volume of distribution (V/F) was 28.8 L. Body weight was a covariate of CL/F and V/F. Estimated glomerular filtration rate was a significant covariate of CL/F. Carriers of an SLC22A1 variant (SLC22A1 is a protein required for metformin uptake by cells) had significantly smaller reductions in percentage of total trunk fat after metformin therapy. The median change in trunk fat was –2.20% (–9.00% to 0.900%) and –1.20% (–2.40% to 7.30%) for the SLC22A1 wild-type subjects and variant carriers, respectively.

A third study examined prevention of weight gain using interpersonal therapy (IPT) versus a control health education program (HE) in adolescents reporting loss of control eating behaviors. At three-year follow-up, baseline social-adjustment problems and trait anxiety significantly moderated outcome. Among girls with high self-reported baseline social-adjustment problems or anxiety, IPT, compared with HE, was associated with the steepest declines in BMIz. 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. 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. Among 78 girls with greater (moderate) baseline depressive symptoms, those in cognitive behavioral therapy (CBT) developed significantly lower 2-hr insulin than those in HE. Additional metabolic benefits of CBT were seen for this subgroup between post-treatment and 1-year follow-up in post-hoc analyses.

An ongoing investigation, based on prior lab data that found links between attentional biases to high-palatability foods in children with obesity, studies whether adolescents' attentional biases can be retrained. We also initiated a translational trial studying the effects of modulation of the leptin signaling pathway with the melanocortin agonist setmelanotide in patients with proximal signaling defects such as PCSK1 (an enzyme that processes prohormones) insufficiency and in patients with Bardet-Biedl syndrome. Initial data support the

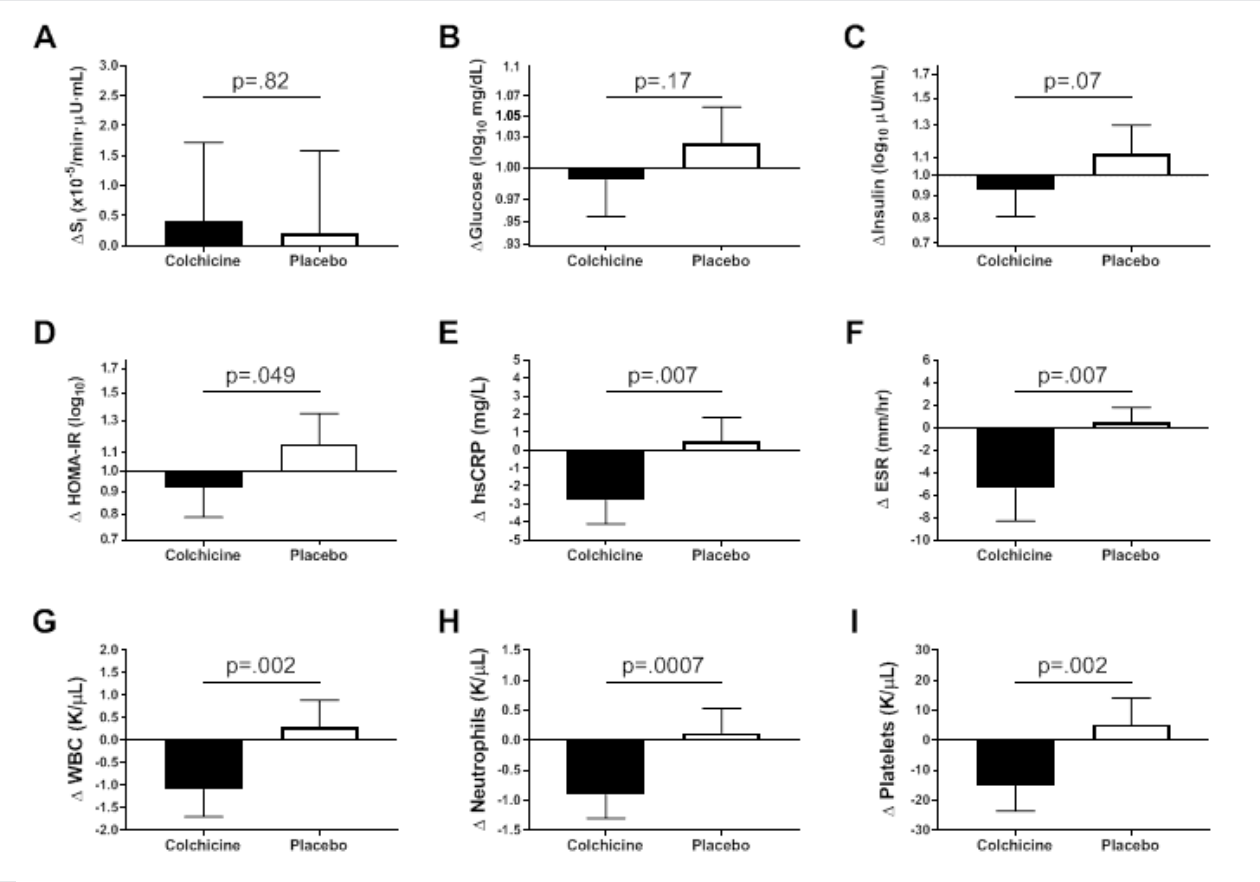


FIGURE 5. Effects of colchicine on inflammatory and metabolic measures

Metabolic and inflammatory changes after three months of study medication in participants randomized to colchicine (N=21) or placebo (N=19).

A. Insulin sensitivity (S_I).

B. Fasting glucose.

C. Fasting insulin.

D. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR).

E. High sensitivity C-reactive protein (hsCRP).

F. Erythrocyte sedimentation rate (ESR).

G. White blood cell count (WBC).

H. Neutrophil count.

I. Platelet count.

Data are presented as mean ± SEM.

hypothesis that increasing activity at melanocortin receptors reduces food intake in people with monogenic disorders affecting the proximal leptin pathway. Most recently, we initiated another study of specific pharmacotherapy for patients with Prader-Willi syndrome using diazoxide. These latest trials are examples of precision medicine approaches [Reference 4] to treat obesity.

We also recently completed a novel randomized controlled pilot trial of colchicine to ameliorate the inflammation of obesity and thus improve its complications [Reference 5]. Adults with obesity and metabolic syndrome, but who did not have diabetes, were randomized to colchicine 0.6 mg or placebo capsules twice daily for three months. Compared with placebo, colchicine significantly reduced C-reactive protein and erythrocyte sedimentation rate. The changes in homeostatic model assessment of insulin resistance, fasting insulin, and glucose effectiveness suggested significant metabolic improvements in the colchicine-treated versus placebo group. These results suggest a larger, adequately powered study should be conducted to determine whether colchicine improves insulin resistance and other measures of metabolic health in at-risk individuals.

Additional Funding

- NIH Clinical Center/ORD "Bench-to-Bedside" Award: Melanocortin agonist to bypass leptin resistance of Bardet-Biedl syndrome. 2017–2018
- Rhythm Pharmaceuticals, Inc.: Setmelanotide (RM-493; Rhythm Pharmaceuticals, Inc.) phase 2 open-label treatment trials in patients with rare genetic disorders of obesity. 2017–2020
- 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
- NICHD Division of Intramural Research Director's Investigator Award, 2018–2019

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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 are a simplification that ignores 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 high 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 with 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 the 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

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in great profusion. 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 high-energy region that the membrane seeks to minimize. Nevertheless, we observe that 'free edges' do indeed outnumber hemifusion diaphragms for certain lipid compositions of target membranes. Such edges only occur in close vicinity to activated HA molecules, indicating that edges are triggered to form by the same event that triggers full fusion, namely, the amphipathic helix of HA being ejected from HA and binding to the target bilayer. We can only presume for the moment that the HA fusion peptide somehow stabilizes the observed membrane edges, i.e., drastically lowers bilayer line-tension. This observation and the resultant hypothesis form the basis of our future work on the influenza virus.

While most medical research aimed at reducing the impact of the influenza virus is focused on vaccines, anti-viral therapeutic strategies should not lag behind. Influenza is estimated to result in between 9.3 million to 49.0 million illnesses, between 140,000 to 960,000 hospitalizations and between 12,000 to 79,000 deaths annually. One crucial stage of infection is the moment when the virus first assembles on the cellular membrane to bud out, starting a new life cycle of replication. Some of the unique lipids that abound in cellular functions are known as the phosphoinositides. These lipids, such as phosphoinositolphosphates PIP, PIP2, and PIP3, have an essential role in exocytosis that has not yet been elucidated. They are found in clusters, yet no one can define the mechanism of lipid clustering or the dependence of lipid clustering on membrane proteins. For these questions we turned to high-resolution light microscopy of HA, a known membrane protein involved in membrane fusion, and we discovered that HA clusters PIP2.

Also this year, we continued research on the physiology of the malaria parasite *Plasmodium falciparum*. Despite some progress in the combating malaria, there were 219 million cases of malaria in 2017, up from 217 million cases in 2016. The latest estimate of deaths from this disease is still very high, i.e. over 445,000 deaths per year in 2016 and 2017, mostly in children under the age of 5. With no vaccine, and drug resistance climbing, we are also focused on the unique membrane biology of the parasites that cause malaria, to find new targets for therapy. In our work, methods have been compiled allowing a comprehensive quantitative evaluation of parasite replication in erythrocytes. Last year, we showed that the *Plasmodium* translocon of exported proteins (PTEX) in the parasite vacuolar membrane critically transports proteins from the parasite to the erythrocytic cytosol and membrane to create protein infrastructure important for virulence. The components of PTEX are stored within the dense granule, which is secreted from the parasite during invasion. We showed that EXP2 (exported protein 2), one component of PTEX, also formed the nutrient channel of the parasite vacuole membrane. We described a protein, RON3, from another invasion organelle, the rhoptry, that is also secreted during invasion. We found that RON3 is required for the protein transport function of the PTEX and for glucose transport from the red blood cell cytoplasm to the parasite, a function thought to be mediated by PTEX component EXP2. Other parasite vacuole proteins include highly expressed single-pass transmembrane proteins such as EXP1 (exported protein 1). We found that EXP1 is required for the EXP2–based nutrient-permeable channel activity of the parasite vacuole.

Towards the goal of understanding the pathophysiology of mild blast induced TBI (bTBI), we also devoted research time to identifying the physical forces associated with the primary injury phase.

Mechanisms of poly-phosphoinositide clustering by the influenza viral hemagglutinin, HA

Although the lateral organization of proteins and lipids (clustering) in the cell plasma membrane (PM) is crucial to diverse fundamental cellular processes, there is considerable disagreement on the organizational mechanisms that govern such clustering, e.g., first, confinement by cytoskeleton-based fences, second, protein-specific partitioning into liquid-ordered lipid rafts, or third, tethering of groups of molecules to the underlying actin cytoskeleton, among others. One reason a mechanistic understanding of the organizing principles has remained elusive is that such nanoscale molecular assemblies are highly dynamic, requiring recordings of individual molecules at a higher temporal bandwidth than hitherto possible to gain a better understanding of the physicochemical principles that regulate membrane clustering. In addition to physiological processes, the pathophysiological basis of disease states is increasingly focused on clusters. HA localized to the PM of host cells clusters spontaneously and is crucial for fusion, viral budding, and infection; high HA density on resultant virions is needed for entry into and fusion with the next host cell. Yet, even this model system generates conflicting data on the mechanism of lipid clustering with HA, and there is not even qualitative agreement as to which lipids cocluster with HA. In contrast to other mechanisms of protein-lipid interactions, such as ordering of molecules into lipid rafts, lipid confinement by protein fences, tethering of lipid motion, or buffering by fixed binding sites, our findings describe and explain spatial PIP2 distributions and how they change in time via a distinctly dynamic mechanism: a potential gradient resulting from binding sites that are themselves both mobile and clustered.

The lipid phosphatidylinositol 4,5-bisphosphate (PIP2) forms nanoscopic clusters in cell plasma membranes; however, the processes determining PIP2 mobility and thus its spatial patterns are not fully understood. Using superresolution imaging of living cells, we found that PIP2 is tightly colocalized with and modulated by overexpression of the influenza viral protein hemagglutinin (HA). Within and near clusters, HA and PIP2 follow a similar spatial dependence, which can be described by an HA–dependent potential gradient; PIP2 molecules move as if they are attracted to the center of clusters by a radial force of 0.079 0.002 pN in HAb2 cells. The measured clustering and dynamics of PIP2 are inconsistent with the unmodified forms of the raft, tether, and fence models. Rather, we found that the spatial PIP2 distributions and how they change in time are explained by a novel, to our knowledge, dynamic mechanism: a radial gradient of PIP2 binding sites that are themselves mobile. The model may be useful for understanding other biological membrane domains whose distributions display gradients in density while maintaining their mobility.

Membranes during invasion of *Plasmodium falciparum*, the causative agent of malaria

We focus on our continued research on the physiology of the deadly malaria parasite *Plasmodium falciparum.* 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 into 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 noninterventional 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.

Intracellular malaria parasites grow in a vacuole delimited by the parasitophorous vacuolar membrane (PVM). The membrane fulfills critical roles for survival of the parasite in its intracellular niche such as in protein

export and nutrient acquisition. Using a conditional knockout, this year we demonstrated that the abundant integral PVM protein EXP1 is essential for parasite survival but that this is independent of its previously postulated function as a glutathione *S*-transferase. Patch-clamp experiments indicated that EXP1 is critical for the nutrient-permeable channel activity at the PVM. Loss of EXP1 abolished the correct localization of EXP2, a pore-forming protein required for the nutrient-permeable channel activity and protein export at the PVM. Unexpectedly however, loss of EXP1 affected only the nutrient-permeable channel activity of the PVM but not protein export. Parasites with low levels of EXP1 became hypersensitive to low nutrient conditions, indicating that EXP1 is indeed needed for nutrient uptake and experimentally confirming the long-standing hypothesis that the channel activity measured at the PVM is required for parasite nutrient acquisition. Hence, EXP1 is specifically required for the functional expression of EXP2, the nutrient-permeable channel. Both EXP1 and EXP2 are critical for the metabolite supply of malaria parasites.

The survival of *Plasmodium spp*. within the host red blood cell (RBC) depends on the function of a membrane protein complex, termed the *Plasmodium* translocon of exported proteins (PTEX), that exports certain parasite proteins, collectively referred to as the exportome, across the parasitophorous vacuolar membrane (PVM) that encases the parasite in the host RBC cytoplasm. The core of PTEX consists of three proteins: EXP2, PTEX150, and the HSP101 ATPase; of these three proteins, only EXP2 is a membrane protein. Studying the PTEX–dependent transport of members of the exportome, we discovered that exported proteins, such as ring-infected erythrocyte surface antigen (RESA), failed to be transported in parasites in which the parasite rhoptry protein RON3 was conditionally disrupted. RON3–deficient parasites also failed to develop beyond the ring stage, and glucose uptake was significantly reduced. These findings provide evidence that RON3 influences two translocation functions, namely, transport of the parasite exportome through PTEX and the transport of glucose from the RBC cytoplasm to the parasitophorous vacuolar (PV) space, where it can enter the parasite via the hexose transporter (HT) in the parasite plasma membrane.

Physical forces that drive CNS cell excitation during mild blast–induced traumatic brain injury

We developed a system that couples a pneumatic blast to a microfluidic channel to precisely and reproducibly deliver shear transients to dissociated human central nervous system (CNS) cells, on a time scale comparable to an explosive blast but with minimal pressure transients. Using fluorescent beads, we characterized the shear transients experienced by the cells, and demonstrated that the system is capable of accurately and reproducibly delivering uniform shear transients with minimal pressure across the cell-culture volume. The system is compatible with high-resolution, time-lapse optical microscopy. Using this system, we demonstrated that blast-like shear transients produced with minimal pressure transients and sub-millisecond rise times activate calcium responses in dissociated human CNS cultures. Cells respond with increased cytosolic free calcium to a threshold shear stress between 8-25 Pa; the propagation of this calcium response is a result of purinergic signaling. We propose that this system models, *in vitro*, the fundamental injury wave produced by shear forces consequent to blast shock waves passing through density inhomogeneity in human CNS.

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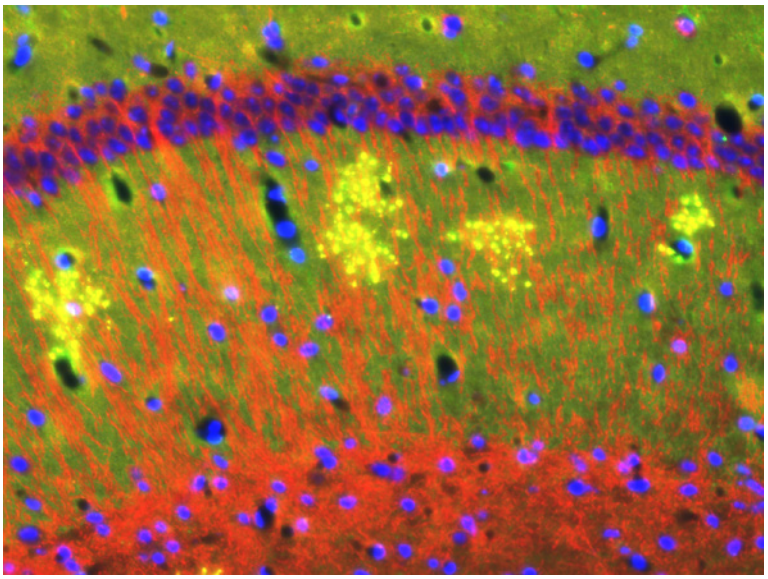

Image by Lin Lin, PhD, of the *Hoffman lab*.
In aged (12m) DPP6-KO mice, hippocampal area CA1 shows abnormal structures that are associated with postsynaptic apical dendrites (MAP2+) and presynaptic (synaptophysin+) sites. Cell bodies are in blue.

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