2021

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, *Chair* 7/1/17 – 6/30/22 Immunobiology, Molecular Biology, Obstetrics, and Gynecology	Professor and Head, Division of Reproductive Sciences, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Vermont College of Medicine
David R. Beier, MD, PhD* 7/1/21 – 6/30/26 Pediatrics, Genetics	Professor in Pediatrics and Director, Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute
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
Nancy Carrasco, MD* 7/1/19 – 6/30/24 Molecular Medicine, Pharmacology, and Physiology	Professor and Chair, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine
William T. Dauer, MD 7/1/18 – 6/30/23 Neurodevelopment and Behavior, Rare Diseases and Genetics, Neurobiology	Professor and Director, Peter O'Donnell Jr. Brain Institute, UT Southwestern Medical Center
P. Ellen Grant, MD* 7/1/19 – 6/30/24 Newborn Medicine, Radiology	Professor of Radiology and Pediatrics, Harvard Medical School Director of Fetal and Neonatal Neuroimaging Research, Endowed Chair in Neonatology, Boston Children's Hospital
Deborah L. Johnson, PhD 7/1/17 – 6/30/22 Molecular and Cellular Biology	President and Chief Executive Officer, Keystone Symposia Professor, Department of Molecular and Cellular Biology, Baylor College of Medicine

Ursula Kaiser, MD* 7/1/19 – 6/30/24 Endocrinology, Diabetes, and Hypertension	Chief, Division of Endocrinology, Diabetes, and Hypertension Co-Director, Brigham Research Institute, Brigham and Women's Hospital
Mary C. Mullins, PhD* 7/1/20 – 6/30/25 Developmental Biology	Professor and Vice Chair, Department of Cell and Developmental Biology, Perelman School of Medicine, University of Pennsylvania
Errol Norwitz, MD, PhD, MBA* 7/1/19 – 6/30/24 Obstetrics and Gynecology	President and CEO, Newton-Wellesley Hospital
Linda Overstreet-Wadiche, PhD* 7/1/20 – 6/30/25 Neurobiology	Professor, Department of Neurobiology, University of Alabama at Birmingham
Sandra L. Schmid, PhD* 7/1/21 – 6/30/26 Biophysics, Cell Biology, Molecular Biology, Model Organisms	Chief Scientific Officer, Chan-Zuckerberg Biohub
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 2021 annual report of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Division of Intramural Research (DIR) is now available to you at: *http://annualreport.nichd.nih.gov*

The DIR, like other institutions across the world, has continued to be impacted by the SARS-CoV-2 virus and COVID-19 pandemic. Labs had to put nearly all of their experiments on hold in March 2020 and, while our scientists have been gradually returning to the lab since summer 2020, as of the time of this writing, lab occupancy is still limited. Despite this limitation, our laboratories have remained scientifically engaged and productive.

We invite you to look through the report site, review our medical and scientific discoveries of the past year, see what work a colleague may currently be engaged in, and 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 and 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 working with a number of animal models, from fruit flies to rats and mice, are supported by a wide array of core services including bioinformatics, imaging, molecular genomics, and a large zebrafish core facility. Each investigator participates in at least one, and typically more, affinity groups. These affinity groups are team-based and future-oriented—to build on thematic interests while responding to rapidly shifting scientific priorities as new knowledge is uncovered.

I invite you to read through the selection of our Clinical Research Protocols listed in this flyer and to consider how we may collaborate, through the NIH U01 grant mechanism at the NIH Clinical Research Center. Such activities can lead to our next new success in therapeutics, the next miracle drug, if we combine our expertise and 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 U-01 opportunities at *https://ocr.od.nih.gov/new_u01/new_u01.html*.

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. Visit their sections of the report on the web to learn about their work in 2021. I also invite you to reach out to me with your ideas and proposals for collaborative initiatives we may undertake together, at *mcbainc@mail.nih.gov*.

Our drive and purpose, on behalf of the American public and the international community, is to strive to uncover the fundamental principles that underpin reproduction and development, as well as to apply these findings to improve human reproductive health and support healthy growth and maturation of children and adolescents.

Sincerely yours,
Chris J. McBain, PhD
Acting Scientific Director, NICHD, NIH
December 2021

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 biannually on the first Friday of December and in June of each year. The

Chris J. McBain, PhD, *Acting Scientific Director*

Sara K. King, *Chief of Staff*

Jessica Rigby, *Scientific Program Analyst*

Amaressa Abiodun, *Administrative Support Specialist*

Olga Cherkasova, *Administrative Support Specialist*

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.

Erin Walsh, PhD, *Program Director, Office of Education*

Triesta Fowler, MD, *Director of Communications and Outreach*

Veronica Harker, *Program Coordinator*

Katherine Lamb, *Program Specialist*

Notable accomplishments of the past year

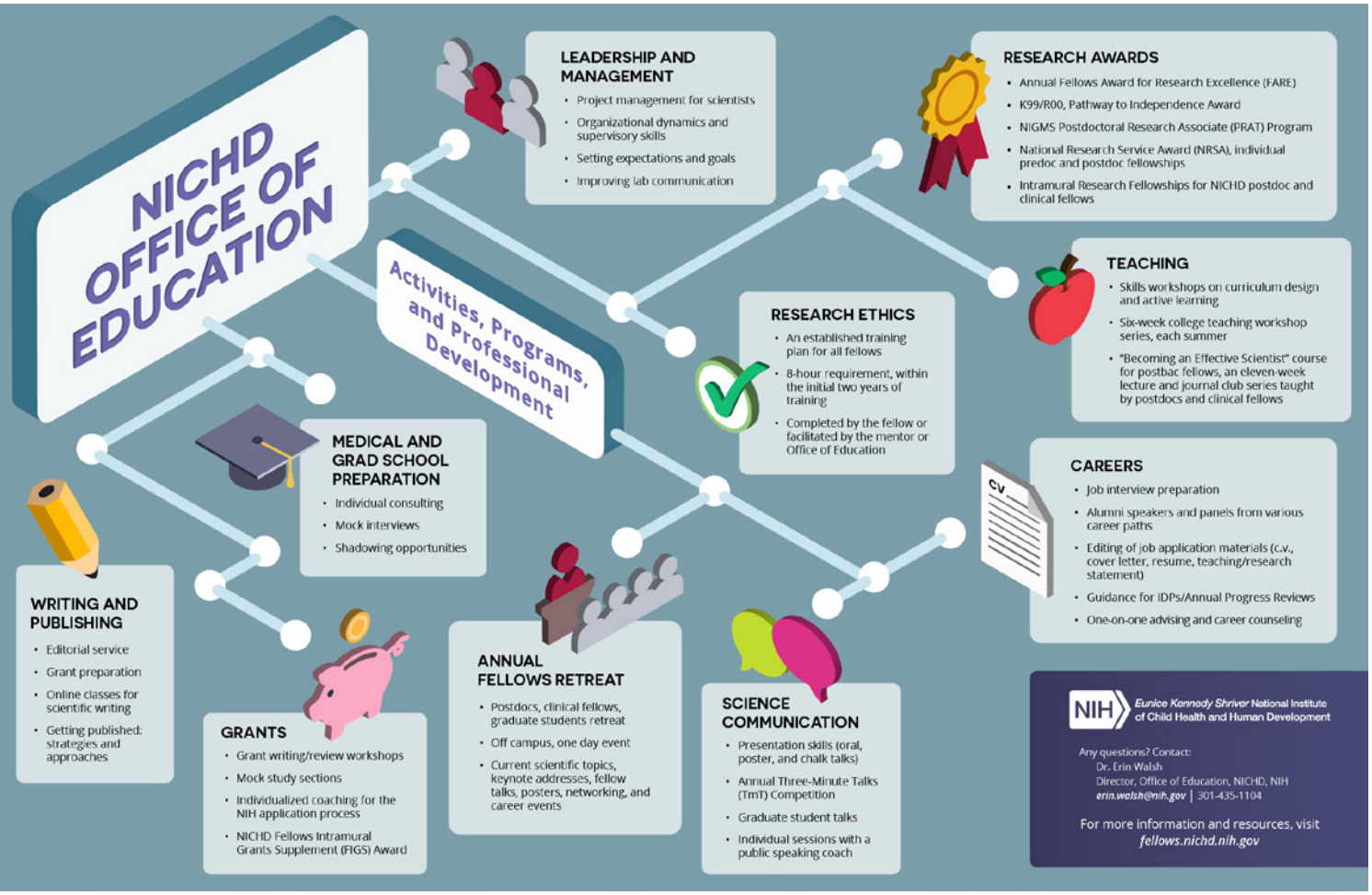
The Office of Education organizes numerous workshops, programs, and individualized opportunities for a population averaging 300 trainees, including: postdoctoral, visiting, and research fellows; clinical fellows and medical students; graduate students; postbaccalaureate fellows; and summer trainees.

We continued our **Graduate Student Talks** initiative, established in 2014, which provides the NICHD 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 2021, the Division of Intramural Research (DIR) gave its **14th Mentor of the Year Awards** to **Dr. Thomas Dever**, Section on Protein Biosynthesis, DIR, and **Dr. Tonja Nansel**, Social and Behavioral Sciences Branch, Division of Intramural Population Health Research (DIPHR), in the investigator category; and to **Dr. Cole Malloy**, Molecular Neurophysiology and Biophysics Section, DIR, as fellow.

Our **TmT (Three-minute Talks) competition** now in its seventh year, was held in conjunction with NCATS, NIDCR, NIAMS, NHGRI, NEI, NINR,



LEFT TO RIGHT:
Triesta Fowler, MD; Veronica Harker; Katherine Lamb

NIDCD, NIAMS, and NIDDK. **Dr. Susanna Mitro** in the Epidemiology Branch, DIPHR, received the second-place award.

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, 15 FARE awards (Fellows Award for Research Excellence) were made.

The Office of Education also worked collaboratively with both administrative and scientific groups within DIR to achieve overall training goals and develop policies on various topics, such as **tenure-track mentoring** and **Affinity Group Seminar Series** for invited speakers.

The **Fellows Intramural Grants Supplement (FIGS) program** continues to recognize and stimulate grant applications among fellows, and we launched the fifth cycle for our **Intramural Research Fellowships (IRFs)**, a 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**. We are also actively working on our partnership with **Howard University**, Washington, DC, for research, training, and mentoring opportunities.

The **Fellows Recruitment Incentive Award (FRIA)** continues to support investigators who recruit postdocs from populations traditionally under-represented in science, and these investigators also exhibit superior mentoring ability and demonstrate a commitment to the career development and academic progress of their fellows.

The alumni group for our **NICHD Developing Talent Scholars Program**, in its 11th year, now numbers 25 individuals, and two new postbaccalaureate fellows joined the program in 2021. The Developing Scholars Program focuses on providing trainees with a customized academic and professional development plan, which includes exposure to intramural activities and resources as well as supporting their career advancement.

We developed a new **Online Fellows Exit Survey**, which will be released soon for our trainee population. It aims to obtain a comprehensive view of trainees' experiences at NICHD, to stay connected with the trainees, and learn of their career moves, as well as to help us improve our training program.

Postdoctoral fellows will be given the opportunity to organize and teach our **annual seminar series** for postbaccalaureate trainees, which entered its 16th year in fall of 2021.

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, as well as to providing fellows with key resources to explore various scientific careers, help them set achievable goals, and build their professional networks.

The **16th Annual Fellows Meeting** was held in May 2021, using a virtual platform. Each spring, this retreat is held for about 120 trainees to address scientific developments and careers and 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 138th issue in November 2021 and reaching all members of the intramural division and our alumni.

The Office welcomed new staff members, and **Erin Walsh** became Director of the Office. **Katherine Lamb** and **Veronica Harker** joined the Office of Education as program coordinators.

Contact

For further information, contact Dr. Erin Walsh (*erin.walsh@nih.gov*).

Office *of the* Clinical Director, NICHD

The NICHD intramural clinical research program currently includes 71 protocols with six main areas of focus: (1) adult, pediatric, and reproductive endocrinology; (2) genetic diseases; (3) normal growth and development; (4) national/international public health; (5) women's health; and (6) adolescent gynecology. The protocols are conducted by 30 NICHD Principal Investigators and 210 associate investigators. The NICHD clinical protocol portfolio spans the spectrum from Natural History to therapeutic trials. Seventeen protocols involve an investigational drug or device. Approximately half the protocols include pediatric patients, and many focus on rare diseases. The NICHD Office of the Clinical Director provides support for investigational new drugs and continues to support the NICHD Data and Safety Monitoring Committee (DSMC), which is chaired by Dr. Frank Pucino.

Office of the Clinical Director: Professional, Administrative, and Biorepository Staff
Top: Karim Calis, Fathy Majadly, Laverne Mensah
Center: Maryellen Rechen, Glynnis Vance, Loc Trinh
Bottom: Denise Phillips, Vincent Black, Meg Keil

Forbes D. Porter, MD, PhD, *Clinical Director*
Karim Calis, PharmD, MPH, *Director of Clinical Research and Compliance*
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*
Denise Phillips, *Administrative and Credentialing Support*
An Dang Do, MD, PhD, *Staff Clinician*
Alan DeCherney, MD, *Senior Investigator*
Christina Tatsi, MD, *Staff Clinician*
Denna Zeltser, MD, *Staff Clinician*
Sheila Brady, CRNP, *Nurse Practitioner*
Josephine Ezemobi, CRNP, *Nurse Practitioner*
Marianne Knue, CRNP, *Nurse Practitioner*
John Perreault, CRNP, *Nurse Practitioner*
Kisha Jenkins, BS, RN, *Clinical Nurse*
Lola Saidkhodjaeva, RN, *Clinical Nurse*
Sara Talvacchio, RN, *Clinical Nurse*
Glynnis Vance, *Protocol Coordinator*
Craig Abbott, PhD, *Statistician*
Fathy Majadly, BS, *Patient Specimen Coordinator*
Vincent Black, *Patient Care Coordinator*
Craig Abbott, PhD, *Statistician*
Loc Trinh, *Research Chemical Engineer*
Lisa Ast, *Administrative Support*

New NICHD Clinical Investigators
Left to Right: Dr. Christina Tatsi, Assistant Clinical Investigator; Dr. An Dang Do, Assistant Clinical Investigator; Dr. Sarah Sheppard, Tenure-Track Investigator

Contact

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

Clinical Trials *at* NICHD

NICHD's Division of Intramural Research (DIR) runs numerous clinical protocols. (For a complete listing of NICHD clinical trials, visit *https://www.clinicaltrials.gov/ct/search;?term=nichd*.) The following lists names and contact information for DIR investigators who recruit patients for their studies. 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 of 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. For more information on the study, please contact **DR. JOAN MARINI** at *oidoc@helix.nih.gov*.

» Screening and diagnosis of patients with suspected connective tissue disorders, especially rare forms of osteogenesis imperfecta, other bone fragility disorders and melorheostosis. Patients and their families receive comprehensive evaluations, counseling, and risk assessment. For more information on the study, please contact **DR. JOAN MARINI** at *oidoc@helix.nih.gov*.

Developmental Endocrine Oncology and Genetics

» Patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma and paraganglioma. For more information on the study, please contact **DR. KAREL PACAK** at *karel@mail.nih.gov* or **MS. SARA TALVACCHIO** at *sara.talvacchio@nih.gov*.

» Research on endocrine, genetic, and other pediatric disorders associated with endocrine and other tumors that may affect the pituitary and other related organs. For more information on the study, please contact **DR. CHRISTINA TATSI** at *christina.tatsi3@nih.gov* or 301-451-7170, **DR. CONSTANTINE STRATAKIS** at *stratakc@mail.nih.gov*, or **DR. ELENA BELYAVSKAYA** at 301-496-0862.

» Research investigating the causes, complications, and treatment of primary aldosteronism. For more information on the study, please contact **DR. CRYSTAL KAMILARIS** at *crystal.kamilaris@nih.gov*.

» Research investigating the long-term effects of Cushing disease in childhood. For more information on the study, please contact **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. For more information on the study, please contact **DR. CHRISTINA TATSI** at 301-451-7170 or **DR. CONSTANTINE STRATAKIS** at *stratakc@mail.nih.gov*.

» Studies into how genetics play a role in the development of obesity. For more information on the study, please contact **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-451-3783.

» 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 deficiency. For more information on the study, please contact **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-451-3783.

» Pharmacotherapy of excessive hunger and obesity in patients with Prader-Willi syndrome, Bardet-Biedl syndrome, and other rare disorders with known genetic causes. For more information on the study, please contact **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-451-3783.

- Evaluation of patients with endocrine disorders associated with excess androgen, including different forms of congenital adrenal hyperplasia. For more information on the study, please contact **DR. DEBORAH MERKE** at *dmerke@nih.gov*, **MS. AMY MOON** at *amy.moon@nih.gov*, or **MS. LEE ANN KEENER** at *leeann.keener@nih.gov* or 240-858-9033.
- Studies of 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). For SLOS, please contact **DR. FORBES PORTER** at *fdporter@mail.nih.gov*, **DR. SAMAR RAHHAL** at *samar.rahhal@nih.gov*, or **MR. TRISTAN FURNARY** at *tristan.furnary@nih.gov*. For NPC, please contact **DR. FORBES PORTER** at *fdporter@mail.nih.gov*, **MS. NICOLE FARHAT** at 301-594-1765, or **MR. DEREK ALEXANDER** at 301-827-0387.
- Study of individuals with CLN3, or Juvenile Neuronal Ceroid-Lipofuscinosis/Juvenile Batten Disease, and their family members. For more information on the study, please contact **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. For more information on the study, please contact **MR. JOHN PERREAULT** at 301-827-9235 or **MR. DEREK ALEXANDER** at 301-827-0387.
- Studies using exome sequencing to identify novel genetic causes of idiopathic growth disorders in children and adults with either short stature or tall stature without a known cause. For more information on the study, please contact For more information on the study, please contact **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. For more information on the study, please contact **DR. KRISTINA ROTHER** at *kristina.rother@nih.gov* or 301-435-4639.

Maternal-Fetal Medicine, Imaging, and Behavioral Development

- *Studies to test and calibrate noninvasive optical imaging technology for functional brain imaging in healthy subjects.* 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. For more information on the study, please contact **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.
- *Studies of 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*.

» *A pilot study to evaluate a noninvasive multimodal biosensing device for screening and monitoring response to treatment of infectious respiratory diseases.* This observational pilot study will characterize the performance of a multimodal biosensor device (portable NIRS device, PPG and temperature sensor) in measuring human vital signs, which later will be explored as a point-of-care method for screening and treatment response monitoring of individuals with an infectious respiratory illness such as COVID-19 infection. The device will measure heart, respiratory, and tissue oxygenation parameters in healthy subjects at rest and during induced hypercapnia, breath holding, and paced breathing. For more information on the study, please contact **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.

» *A longitudinal study of biological markers for the prediction of the "great obstetrical syndromes."* This is a prospective cohort study of biomarkers in the great obstetrical syndromes to examine the natural history of normal pregnancy and the most common 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 and Adolescent Gynecology

» *Data collection study of pediatric and adolescent gynecology conditions.* This study is designed to perform deep phenotyping and data collection of children and adolescents presenting with gynecologic conditions including congenital anomalies. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

» *Gonadal tissue freezing for fertility preservation in girls at risk for ovarian dysfunction and primary ovarian insufficiency.* This study is designed to evaluate possible mechanisms of follicle loss/dysfunction in children with Turner syndrome and classic galactosemia, and in adolescents recently diagnosed with premature ovarian insufficiency. Ovarian tissue will be cryopreserved and stored for patient's own future use. For more information on the study, please 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 on androgen receptor genes and receptor abnormalities to improve care for those affected and elucidate possible androgen receptor-mediated explanations for differences in physiology and health in other populations. For more information on the study, please contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

Physical Biology and Medicine

» Studies of genetic disorders related to fragile sarcolemma muscular dystrophy, including Limb-Girdle Muscular Dystrophy type 2B-F, I, L, Myoshi Myopathy, Becker Muscular Dystrophy, and Myoshi Muscular

Dystrophy-3. For more information on the study, please contact **DR. JOSHUA ZIMMERBERG** at *zimmerbj@mail.nih.gov* or **MS. HANG WATERS** at *watershn@mail.nih.gov*.

Reproductive Endocrinology and Gynecology

» Studies, using endometrial biopsy, of reproductive disorders that affect the endometrium, such as recurrent implantation failure. For more information on the study, please contact **DR. ALAN DECHERNEY** at *decherna@mail.nih.gov* or 301-594-5494.

» Studies of reproductive function in sickle cell disease and individuals undergoing cytotoxic gonadal therapy, including fertility preservation (oocyte freezing). For more information on the study, please 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 Ph.D. 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, 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. Tinnakorn Chaiworapongsa. 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 *Eunice Kennedy Shriver* 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*

Karel Pacak, MD, PhD, DSc, *Head, Section on Medical Neuroendocrinology, NICHD*

Crystal Kamilaris, MD, *Staff Clinician*

Rasha Haykal, MD, *Clinical Fellow*

Sanjay Jumani, MD, *Clinical Fellow*

Maziar Rahmani, MD, PhD, *Clinical Fellow*

Skand Shekar, MD, *Clinical Fellow*

Sonal Vaid, MD, *Clinical Fellow*

Clinical and research training under the NICHD–NIDDK Inter–Institute Endocrine Training Program

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, diabetes, and metabolic diseases. Study design, outcome measures, statistical analysis, and ethical and regulatory issues are stressed.

The IETP provides a comprehensive training experience that involves not only the NIH clinical branches working in endocrinology but also 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, Hannah-Shmouni F. Molecular genetic and genomic alterations in Cushing's syndrome and primary aldosteronism. *Front Endocrinol* 2021;12:632543.
2. Gubbi S, Al-Jundi M, Del Rivero J, Jha A, Knue M, Zou J, Turkbey B, Carrasquillo JA, Lin E, Pacak K, Klubo-Gwiezdzinska J, Lin Fi. Case Report: Primary hypothyroidism associated with lutetium 177-DOTATATE therapy for metastatic paraganglioma. *Front Endocrinol* 2021;11:587065.
3. Shekhar S, Sinaii N, Irizarry-Caro JA, Gahl WA, Estrada-Veras JI, Dave R, Papadakis GZ, Tirosh A, Abel BS, Klubo-Gwiezdzinska J, Skarulis MC, Gochuico BR, O'Brien K, Hannah-Shmouni F. Prevalence of hypothyroidism in patients with Erdheim-Chester disease. *JAMA Netw Open* 2020;3(10):e2019169.
4. Al-Jundi M, Thakur S, Gubbi S, Klubo-Gwiezdzinska J. Novel targeted therapies for metastatic thyroid cancer—a comprehensive review. *Cancers (Basel)* 2020;12(8):2104.

Collaborators

- Kenneth Berman, MD, *Director, Endocrine Training Program, Washington Hospital Center, Washington, DC*
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Pediatric Endocrinology Inter-Institute Training Program

The Fellowship in Pediatric Endocrinology is a three-year, Accreditation Council for Graduate Medical Education (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 (exceptions can be made on an individual basis according to ACGME rules). The fellowship is now closed to new trainees, although it is possible for research-oriented fellows at other institutions who are selected to participate in the Pediatric Scientist Development Program to complete their training in the program. Training takes place predominately at the National Institutes of Health Clinical Center, which is one of the largest and most sophisticated research institutions in the United States. Additional clinical training takes place at Children's Hospital in Washington, DC, and at Walter Reed National Military Medical Center in Bethesda, MD. The fellowship is designed to provide clinical and research exposure that fosters the development of academic pediatric endocrinologists with experience in clinical, translational, and/or basic research.

The URL *https://www.nichd.nih.gov/about/org/dir/osd/tp/peitp* provides more detailed information about the program.

Pediatric Endocrine Fellows and Faculty

Kristina I. Rother, MD, MHSc, *Director, Pediatric Endocrine Fellowship Program, NICHD*

Christina Tatsi, MD, PhD, *Deputy Program Director, NICHD*

Marissa Lightbourne, MD, *Assistant Research Investigator/ Staff Clinician, NICHD*

Ashwini Mallapa, MD, MHSc, *Staff Clinician, NIH Clinical Center (CC)*

Deena Zeltser, MD, *Staff Clinician, NICHD*

Youn Hee Jee, MD, *Senior Fellow, NICHD*

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

Jack Yanovski, MD, PhD, *Head, Section on Growth and Obesity, NICHD*

Rachel Gafni, MD, *Senior Research Physician, Craniofacial and Skeletal Diseases Branch, NIDCR*

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

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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, 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.

Andrew Bremer, MD, PhD, *Program Director, Pediatric Growth and Nutrition Branch, NICHD*

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

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

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Alison M. Boyce, MD, *Lasker Tenure-Track Investigator, Section on Skeletal Disorders and Mineral Homeostasis, NIDCR*

Rebecca J. Brown, MD, MHSc, *Lasker Tenure-Track Investigator, Diabetes, Endocrinology, and Obesity Branch, NIDDK*

Stephanie Chung, MD, *Lasker Tenure-Track Investigator, Diabetes, Endocrinology, and Obesity Branch NIDDK*

Brittney A. Corbin, BA, *Pediatric Endocrine Fellowship Program Coordinator, NICHD*

Application information

As stated above, the program is now closed to new first-year applicants, but physician-scientists interested in completing their training at the NIH should seek admission to the Pediatric Scientist Development Program at *https://amspdc-psdp.org*.

Publications

1. Zenno A, Nadler EP. Surgical treatment of type 2 diabetes mellitus in youth. *Adv Exp Med Biol* 2021 1307:321–330.
2. Gladding A, Szymczuk V, Auble BA, Boyce AM. Burosumab treatment for fibrous dysplasia. *Bone* 2021 150:116004.

3. Meyers AG, Hudson J, Cravalho CKL, Matta ST, Villalobos-Perez A, Cogen F, Chung ST. Metformin treatment and gastrointestinal symptoms in youth: findings from a large tertiary care referral center. *Pediatr Diabetes* 2021 22:182–94.

Collaborators

- Andrew Dauber, MD, MMSc, *Director, Pediatric Endocrinology, Children's National Hospital, Washington, DC*
- Karen S. Vogt, MD, *Program Director, Pediatric Endocrinology, Walter Reed Medical Center, Bethesda, MD*

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NICHD Pediatric and Adolescent Gynecology Training Program

Training in the Pediatric and Adolescent Gynecology (PAG) subspecialty is achieved through non–ACGME approved fellowships, which are loosely coordinated through collaboration with the PAG Fellowship Director's Committee of the North American Society for Pediatric and Adolescent Gynecology (NASPAG). PAG is a two-year program with faculty from the National Institute of Child Health and Human Development (NICHD), Children's National Hospital (CNH), and the 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 to be able to function as a consultant for children and adolescents with 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.

PAG is uniquely qualified to meet the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development's mission to: "lead research and training to understand human development, improve reproductive health, enhance the lives of children and adolescents, and optimize abilities for all." PAG is a relatively new subspecialty within the field of Obstetrics and Gynecology and encompasses gynecologic care from the fetal period into adulthood. In 2018, the American Board of Obstetrics and Gynecology implemented a Focused Practice Designation in Pediatric and Adolescent Gynecology. Many of the conditions managed by pediatric gynecology are rare diseases and have been poorly studied. In addition, as experts in complex gynecology, many pediatric and adolescent gynecologists care for adult women with these and similar conditions. Thus, an emphasis on PAG offers unique opportunities that align with NICHD research priorities.

Program structure

Currently, the fellowship alternates between recruiting one or two fellows per year. A schedule is established by which fellows rotate through general pediatric and adolescent gynecology clinics (including a heavy-menses clinic), and surgeries, specialty clinics (reproductive endocrine, Turner syndrome, PROUD [positive re-evaluation of urogenital differences clinics, or disorders of sex development] and

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Swetha Naroji, MD, *Clinical Fellow*

Jamie Merkinson, MD, *Special Volunteer*

Rashmi Prasad, DO Candidate, *Special Volunteer*

Bohyon Yun, MD, *Special Volunteer*

Sarina Hanfling, BS, *Intramural Research Training Award Fellow*

Victoria Huynh, MD Candidate, *Medical Research Scholar Program*

Allison Kumnick, MD/MPH Candidate, *Medical Research Scholar Program*

Hong Lou, MD, *Laboratory Manager/Biologist*

Harveen Kaur, MPH, *Clinical Research Coordinator*

Sofia L. Getachew, MSPM, *Fellowship Program Coordinator*

vulvar dermatology clinics), colorectal surgery at CNH, and research and consult/clinics at the NIH Clinical Center. The outpatient services do not depend on fellows for clinical care, which allows flexibility to maximize the educational experience. Surgeries are performed by the fellow on call with a resident, and the fellow functions as the surgical instructor during such cases. Complex surgeries are performed by both fellows as co-surgeons. Rotations, including one month in Child Abuse, are scheduled during the second year of the fellowship. Electives in Adolescent Medicine (eating disorders), as well as travel to other sites for further complex anomaly training are available.

For successful completion of the training program, the fellow is required to design, implement, complete, describe, and report at least one research study. A second multi-center project must 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) is required. During the fellowship, fellows have thirty percent of their time protected for clinical research. The research takes 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 take place from July to September in the first year. The introductory seminars provide a historical perspective and basic understanding of the practice of Pediatric and Adolescent Gynecology and Reproductive Endocrinology (REI), as well as statistics. Fellows are required to attend a weekly colorectal conference, a monthly DSD (Differences of Sexual Development) clinic conference, a monthly Turner's clinic conference, combined REI/PAG Journal Club/case conferences, lectures, and quarterly research meetings. 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 with which the fellow is affiliated generally meet on a weekly basis to review research progress. Furthermore, fellows are encouraged to participate in the didactic training offered at national meetings, such as the NASPAG, 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. They 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. Kanakatti Shankar R, Dowlut-McElroy T, Dauber A, Gomez-Lobo V. Clinical utility of anti-Mullerian hormone in pediatrics. *J Clin Endocrinol Metab* 2021. *https://doi.org/10.1210/clinem/dgab687*

2. Dwiggins M, Brookner B, Fowler K, Veeraraghavan P, Gomez-Lobo V, Merke DP. Multidimensional aspects of female sexual function in congenital adrenal hyperplasia: a case-control study. *J Endocr Soc* 2020 4(11):1–10.
3. Practice Committee of the Oncofertility Consortium. Installing oncofertility programs for common cancers in optimum resource settings (Repro-Can-OPEN Study Part II): a committee opinion. *J Assist Reprod Genet* 2021 38(1):163–176.
4. Chattopadhyay S, Arnold JD, Malayil L, Hittle L, Mongodin EF, Marathe KS, Gomez-Lobo V, Sapkota AR. Potential role of the skin and gut microbiota in premenarchal vulvar lichen sclerosus: A pilot case-control study. *PLoS One* 2021 16(1):1–14.
5. Woodruff TK, Ataman-Millhouse L, Acharya KS, Almeida-Santos T, Anazodo A, Anderson RA, Appiah L, Bader J, Becktell K, Brannigan RE, Breech L, Bourlon MT, Bumbuliene Ž, Burns K, Campo-Engelstein L, Campos JR, Centola GM, Chehin MB, Chen D, De Vos M, Duncan FE, El-Damen A, Fair D, Famuyiwa Y, Fechner PY, Fontoura P, Frias O, Gerkowicz SA, Ginsberg J, Gracia CR, Goldman K, Gomez-Lobo V. A view from the past into our collective future: the oncofertility consortium vision statement. *J Assist Reprod Genet* 2021 38(1):3–15.

Collaborators

- Steven L. Coon, PhD, *Molecular Genetics Core, NICHD, Bethesda, MD*
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- Alan H. DeCherney, MD, *Reproductive Endocrinology and Infertility Fellowship Training Program, NICHD, Bethesda, MD*
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- Judith L. Fridovich-Keil, PhD, *Emory University, Atlanta, GA*
- Catherine Gordon, MD, *Boston Children's Hospital, Boston MA*
- Yasmin Jayasinghe, MBBS, FRANZCOG, PhD, *Royal Children's Hospital Melbourne, Parkville, Australia*
- David Lee, MD, PhD, *Oregon Health & Science University, Portland, OR*
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- Kyle Orwig, PhD, *University of Pittsburgh, Pittsburgh, PA*
- James Segars, MD, *The Johns Hopkins School of Medicine, Baltimore, MD*
- Ariella Shikanov, PhD, *University of Michigan, Ann Arbor, MI*
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- Mary Zelinsky, PhD, *Oregon Health & Science University, Portland, OR*

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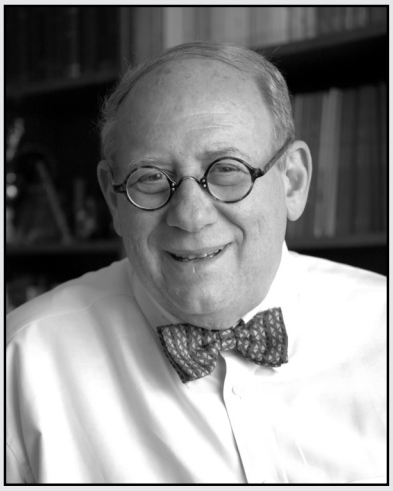
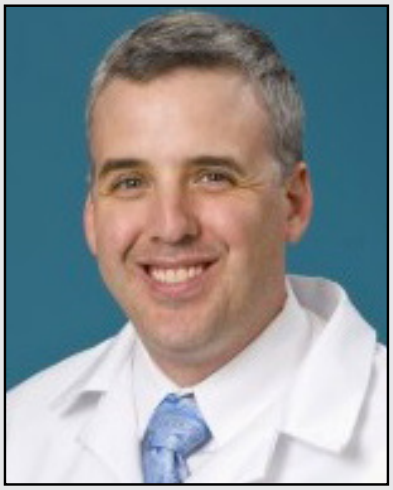
Reproductive Endocrinology and Infertility Training Program

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 the 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 90 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 to establish and maintain high standards of training for students and residents in obstetrics and gynecology, so that they can 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 WRNMMC and the Shady Grove Fertility Center. Research is strongly emphasized. Trainees in the program must complete a thesis project and may choose among any research laboratory in the Institute's intramural research program. To complete the research project, fellows are given 20 months of protected research time. The curriculum includes





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Micah J. Hill, DO, *Program Director, Combined NICHD/Federal Fellowship in Reproductive Endocrinology*

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Jonah Bardos, MD, MBE, *Clinical Fellow*

Marja Brolinson, MD, *Clinical Fellow*

(continued)

two university-based graduate courses, one in biostatistics, the other in reproduction. In the past year, faculty and fellows published 70 peer-reviewed articles. Over the past five years, each graduate 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.

Joshua C. Combs, MD, *Clinical Fellow*
Shelley Dolitsky, MD, *Clinical Fellow*
Charlene Echague, DO, *Clinical Fellow*
Steven A. Gay, MD, *Clinical Fellow*
Adina Schwartz, MD, *Clinical Fellow*
Karrie Walker, DO, *Clinical Fellow*
Grace Whiteley, DO, *Clinical Fellow*
Meghan Yamasaki, DO, *Clinical Fellow*

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 USUHS. The program is intended to achieve synergism between the four sponsoring institutions and to 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 Preoperative and Fellows' conferences. Fellows also attend weekly research conferences sponsored by the NICHD, and they 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. 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. Healy MW, Dolitsky SN, Villancio-Wolter M, Raghavan M, Tillman AR, Morgan NY, DeCherney AH, Park S, Wolff EF. Creating an artificial 3-dimensional ovarian follicle culture system using a microfluidic system. *Micromachines (Basel)* 2021 12:261.
2. Carpinello OJ, Jahandideh S, Yamasaki M, Hill MJ, DeCherney AH, Stentz N, Moon KS, Devine K. Does ovarian stimulation benefit ovulatory women undergoing therapeutic donor insemination? *Fertil Steril* 2021 115:638–645.
3. Hill MJ, Eubanks AE, Csokmay JM, Christy AY, Jahandideh S, DeCherney AH, Devine K, Levens ED, Connell MT. Is transferring a lower-quality embryo with a good-quality blastocyst detrimental to the likelihood of live birth? *Fertil Steril* 2020 114:338–345.
4. Aserlind A, Martini A, Dong J, Zolton J, Carpinello O, DeCherney A. Fertility preservation before hematopoietic stem cell transplantation: a case series of women with GATA binding protein 2 deficiency, dedicator of cytokinesis 8 deficiency, and sickle cell disease. *F S Rep* 2020 1:287–293.
5. Combs JC, Hill MJ, DeCherney AH. Polycystic ovarian syndrome genetics and epigenetics. *Clin Obstet Gynecol* 2020 64:20–25.

Collaborators

- William H. Catherino, MD, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Alicia Christy, MD, *Reproductive Health, Veterans Administration Central Office, Washington, DC*
- John M. Csokmay III, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Kate M. Devine, MD, *Shady Grove Fertility, Washington, DC*
- Torie Plowden, MD, MPH, *Womack Army Medical Center, North Carolina*
- Saioa Torrealday, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Eric A. Widra, MD, *Shady Grove Fertility, Washington, DC*

Contact

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Program Coordinator: Marilyn Minor; *minormar@mail.nih.gov*

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 and 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, *Administrative Officer, Chief, Administrative Management Branch*

Valerie Leftwood, *Administrative Officer - Deputy Branch Chief*

Becky Preston, *Administrative Officer - 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.

Contact

For more information, email *franciek@mail.nih.gov* or visit *https://www.nichd.nih.gov/about/org/dir/osd/mt/amb*.

AMB Staff

Izet Beckwith, *Administrative Officer*
Antoinette Chavez, *Administrative Officer*
Gina Elmore, *Administrative Officer – Budget*
Dena Flipping, *Administrative Officer*
Michelle Hudson, *Administrative Officer – Team Leader*
Joy Johnson, *Administrative Officer*
Bonnie Lancey, *Administrative Officer*
Keta Lawson-Davis, *Administrative Officer*
Wanda Logan, *Administrative Officer*
Lakeasha Mingo, *Administrative Officer – Team Leader*
Charlene Patrick, *Administrative Officer – Team Leader*
Mia Pulley, *Administrative Officer – Team Leader*
Natacha Rene, *Administrative Officer*
Nancy Richman, *Administrative Officer*
Sylvia Robinson, *Administrative Officer – Procurement Lead*
Beverley Todd, *Administrative Officer*
Marlene Taulton, *Administrative Officer*
Brittany Corum, *Administrative Technician*
Barbara Hardy, *Administrative Technician*
Ashley Mason, *Budget Analyst*
Michael Anderson, *Facilities Assistant*
Tamika Morgan, *Facilities Assistant*
Steven Norris, *Facilities Manager*
Giulia Mason, *Management Analyst*
Moona Braxton, *Procurement Technician*
Jennifer Smith, *Procurement Technician*
Anju Vergheese, *Program Analyst*
John Burton, *Purchasing Agent*
Jax Chang, *Purchasing Agent*
Towanda Daniels, *Purchasing Agent*
Roshonna Davis, *Purchasing Agent*
William Davis, *Purchasing Agent*
Sherry Jones, *Purchasing Agent*
James Law, *Purchasing Agent*
David Shen, *Purchasing Agent*
Hanumanth Vishnuvajjala, *Purchasing Agent*

Research Animal Management Branch

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

In addition to providing animal research support and veterinary consultation 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, the Building 6 Shared Zebrafish Facility (SZF), and the Building 49 Xenopus Facility. The DIR Animal Program and NICHD Animal Care Use Committee (ACUC) have oversight over these facilities with regard to animal use, as well as over NICHD animals in the Porter Neurosciences Research Center (PNRC) SAF, and in Central Animal Facilities (CAF). The RAMB Aquatics Program also provides aquatics animal care and husbandry 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.

The Building 6B SAF houses over 11,000 cages of specific pathogen-free (SPF) rodents and provides lead institute 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 with a dedicated technician. The NICHD ACRF Animal Facility supports the animal research activities of the NICHD with four animal rooms and two procedure rooms. The RAMB provides care and housing for rodent and aquatic species housed within this restricted-access, disease-free animal holding facility.

NICHD Aquatics are housed within the Building 6 SZF, the ACRF, and in Building 49. The Building 6 SZF supports NICHD and NHGRI with 15,000 two-liter tanks with a total capacity of over 330,000 zebrafish. The Xenopus satellite frog Facility in Building 49 provides primary animal care and research support to over 300 tanks of *Xenopus* used by NICHD DIR researchers.

Robin Kastenmayer, DVM, PhD, *Acting Animal Program Director and Senior Veterinarian*

Rachael Labitt, DVM, MS, *Rodent Veterinarian*

Terrance McMeans, *Rodent Facilities Manager*

Mike Wisnieski, BS, *Aquatic Facilities Manager*

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

Julie Jacobs, *Animal Program Manager*

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

Christopher Rishell, BS, *Senior Facilities Manager*

As part of the NIH, RAMB staff participate in the formulation of policies and procedures that impact the care and use of laboratory animals throughout the country. RAMB leads the effort for triennial re-certification by AAALAC (American Association for Accreditation of Laboratory Animal Care) International. RAMB staff and many animal-user investigators have been active contributors to the NIH Animal Research Advisory Committee (ARAC) and other trans-NIH committees.

Additional Funding

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

Contact

For more information, email *robin.kastenmayer@nih.gov* or visit *https://www.nichd.nih.gov/about/org/dir/osd/mt/ramb* or *https://ramb.nichd.nih.gov*.

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 several 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 also coordinate training for staff and trainees in basic programming and genomic analyses to help build bioinformatics support directly within labs.

Structure

The BSPC uses a "hub and spoke" model, consisting of a central core of staff in Building 6A coordinating with embedded bioinformaticians (currently in Buildings 6, 49, and 35) working directly in laboratories. This allows us to build a 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 2021, the BSPC worked on 97 projects, collaborating with principal investigators (PIs), fellows, staff scientists, and staff clinicians across 33 laboratories. Of these, 55 were new projects and 42 were carried over from the previous year. 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. In addition, new projects this year included analysis of TRIP (Thousands of Reporters in Parallel) data, CRISPRi methods development, and long-read assembly. Some projects involved custom algorithm development and tool development, and many projects required integration with published studies. New this year is the development of several new R Shiny web applications that our collaborators use to interactively explore and dig deeper into the analysis results we provide.

Projects often begin with an in-depth discussion with researchers to understand the background and goals of the project. It is important

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Caroline Esnault, PhD, *Staff Scientist*

Apratim Mitra, PhD, *Staff Scientist*

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

Gennady Margolin, PhD, *Bioinformatics Scientist*

Mira Sohn, PhD, *Bioinformatics Scientist*

Kiersten Campbell, BS, *Postbaccalaureate fellow*

Gus Fridell, BS, *Postbaccalaureate fellow*

Eva Jason, BS, *Postbaccalaureate Fellow*

Nicholas Johnson, BS, *Postbaccalaureate Fellow*

Arjun Mittal, BS, *Postbaccalaureate Fellow*

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 researcher's 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.

The BSPC's collaboration includes writing the manuscript, producing figures and tables, consulting on interpretation, writing detailed computational methods, reviewing code, and submitting code to public repositories along with the complete software environments required to make the analyses reproducible.

Projects: computation and code

Most projects are multi-week or multi-month projects, which 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.

We also maintain R Shiny applications into which we load analysis results. After our collaborators authenticate in the system, they are able to explore their results with interactive plots and tables, which allow them to dig deeper without requiring additional computational resources or bioinformatics skills. These applications are continuously updated based on feedback from our collaborators to ensure that they remain easy to use and helpful.

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 run on NIH's Biowulf 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.

Publications

1. Adams PP, Baniulyte G, Esnault C, Chegireddy K, Singh N, Monge M, Dale RK, Storz S, Wade JT. Regulatory roles of *Escherichia coli* 5′ UTR and ORF-internal RNAs detected by 3′ end mapping. *eLife* 2021; doi.org/10.7554/eLife.62438.
2. Rodriguez-Gil JL, Baxter LL, Watkins-Chow DE, Johnson NL, Davidson CD, Carlson SR, Incao AA, NISC Comparative Sequencing Program, Wallom KL, Farhat NY, Platt FM, Dale RK, Porter FD, Pavan WJ. Transcriptome of HPβCD-treated Niemann-Pick disease type C1 cells highlights GPNMB as a biomarker for therapeutics. *Human Mol Genet* 2021; doi.org/10.1093/hmg/ddab194.
3. Mahadevan V, Mitra AK, Zhang Y, Yuan X, Peltekian AA, Chittajallu VK, Esnault CM, Maric D, Rhodes CT, Pelkey KA, Dale RK, Petros T, McBain CJ. NMDARs drive the expression of neuropsychiatric disorder risk genes within GABAergic interneurons subtypes in the juvenile brain. *Front Mol Neurosci* 2021; doi.org/10.3389/fnmol.2021.712609.
4. Tseng WC, Johnson Escauriza AJ, Tsai-Morris CH, Feldman B, Dale RK, Wassif CA, Porter FD. The role of Niemann-Pick type C2 in zebrafish embryonic development. *Development* 2021 148(7):dev194258; doi.org/10.1242/dev.194258.
5. Gaikwad S, Ghobakhlou F, Young DJ, Visweswaraiah J, Zhang H, Hinnebusch AG. Reprogramming of translation in yeast cells impaired for ribosome recycling favors short, efficiently translated mRNAs. *eLife* 2021; doi.org/10.7554/eLife.64283.

Collaborators

- Joanna Klubo-Gwiezdzinska, MD, PhD, *Metabolic Diseases Branch, NIDDK*
- William J. Pavan, PhD, *Genomics, Development and Disease Section, NHGRI, Bethesda, MD*
- Shyamal Peddada, PhD, *Biostatistics and Bioinformatics Branch, DIPHR, NICHD, Bethesda, MD*
- Michael E. Ward, MD, PhD, *Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda, MD*

Contact

For more information, 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 of the Division of Intramural Research (DIR), NICHD, in the following key areas: core IT support, clinical informatics, custom software development for scientific and administrative support, and biological visualization services.

Ryan Dale, MS, PhD, *Scientific Information Officer, Head, Computer Support Services Core*

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*

(continued)

Core IT Services

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

NETWORK AND DESKTOP SERVICES

During the past year, the 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, PyMol, and FlowJo, as well as network services (e-mail, data backups, VPN, 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 their 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 clinical 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 for 15 NIH Institutes increased to 697, including six COVID studies. The Global

Rolanda Bailey, BS, *Documentation Specialist*
Tamara Prodanov, MD, *Documentation Specialist*
Kelly Colligan, BS, *Information Specialist*
Kami Emanuel, *Information Specialist*
Audrey Harrell, MS, *Information Specialist*
Vida Bayat Mokhtari, MD, *Information Specialist*
George Tran, *Information Specialist*

Question Library expanded to over 265,000 research questions. Our software development group completed two CTDB releases. Features included the addition of a new matrix question type, Patients Data Report, and the addition of audio function to questions. The CTDB releases also incorporated improvements to the functionality of the data-entry, samples, quality assurance, and e-binder modules. We supported the Clinical Trial Survey System (CTSS), an application for patient self-reporting, servicing 93 active protocols. The team completed one CTSS release and is working on finalizing a CTSS redesign. CTDB applications also support the NICHD Office of Clinical Director central biorepository, as well as several Institutes' research teams' CVs, trainings, and certificates documentation. In 2021, the CTDB project went through a third-party audit and was re-certified with the 21 CFR Part 11 compliance. Since its inception, data from CTDB supported over 1,500 NICHD publications.

The database development and reporting team continued integrations with other NIH Institutes. The team is in the process of building integrations with NHBLI and NIMH systems to incorporate data from these systems into the CTDB data-reporting environment. The team successfully upgraded the Cognos reporting environment to IBM Cognos 11.4. We continued supporting data marts, as new reporting requirements appear, and migrating data as needed. Additionally, the team worked closely with various principal investigators (PIs) across the Institutes to provide both management and research-related reports for clinical related studies, publishing over 664 reports in the past year. The team applies the latest patches to all production database environments to ensure continued uninterrupted services, and monitors the successful completion of backup and data-mart transformation services.

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. The 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 as well as for easing the re-appointment process. This solution provided the Office of Education with useful metrics regarding mentoring and training programs. We also updated the Exit Survey feature, a short survey allowing DIR Fellows a platform for providing feedback.

We continued developing new features and improvements for the Package Tracking module used by the DIR AMB, providing AMB staff real-time accuracy metrics for personnel and travel package compilation. The CSSC team continues to develop and support several feedback systems to support real-time customer satisfaction

collection. These include surveys for the AMB, Office of the Clinical Director, laboratory administrative support staff, and NICHDs Administrative Services Branch. The system also offers more detailed feedback submissions periodically, along with comprehensive response metrics.

The team is also finalizing a new feature: the Capital Equipment/Expenditure Request Tracking System. The system will enable users to efficiently submit requests through the review process while allowing administrative staff to track requests through the workflow process.

The CSSC team continued to improve Cost Tracker, an application that permits capturing, organizing, and reporting 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, and website support. Those services were provided to the NICHD DIR Annual Report, the DIR Annual Fellows, the DIR Annual Scientific Retreats, the Anita B. Roberts Lecture Series, and the NICHD research labs and medical training programs. We supported the NICHD Office of Education by producing a monthly newsletter, the monthly *SD Bulletin*, and *The NICHD Connection* in collaboration with intramural fellows. We continued maintaining websites for the NICHD DIR Annual Report.

The CSSC continued to provide a platform for conducting scientific review by the *Board of Scientific Counselors*, administrative intranet support, and business operations.

Additional Funding

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

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, Clinical Director, NICHD, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

Contact

For more information, 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) histology and sample preparation for light and electron microscopy; (2) wide-field and confocal light microscopy; (3) transmission electron microscopy (TEM); and (4) image analysis and data extraction. The Facility operates as a 'one-stop shop,' where investigators can, with a minimum of effort, go from their scientific question to the final data.

Although they were scaled back in June 2021, the MIC staff and its users continue to be affected by COVID–related NIH restrictions on physical occupancy.

Vincent Schram, PhD, *Acting Executive Director, Staff Scientist*

Tamás Balla, MD, PhD, *Scientific Advisor and Administrative Director*

Ling Yi, PhD, *Staff Scientist*

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

Lynne A Holtzclaw, BS, *Research Assistant*

Sara E Felsen, MS, *Intramural Research Training Award 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*. 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, NICHD. The MIC is under the joined management of Tamás Balla (scientific advisor and administrative director) and Vincent Schram (acting executive director).

Vincent Schram is the point person for light microscopy and data analysis. Following the retirement of Lynne Holtzclaw in August 2021, Ling Yi joined the MIC to head the sample-preparation/histology unit. Sara Felsen, our Intramural Research Training Award Fellow, left the MIC in August 2021. The EM branch of the Facility is staffed by Chip Dye. Ling Yi and Chip Dye report to Vincent Schram, who coordinates activities with Tamás Balla.

The MIC has an open-door policy with the NINDS Light Imaging Facility (LIF, Building 35). The two cores freely exchange users, share equipment, and trade 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's resources, NINDS 15%, and other Institutes (NIBIB, NIA, and NIMH) the remaining 5%.

Since its inception in 2004, the contribution of the MIC to the NICHD mission has been acknowledged in over 200 publications. For a complete list, visit *https://www.nichd.nih.gov/about/org/dir/other-facilities/cores/microscopyandimaging/publications*.

Light microscopy

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

1. Zeiss LSM 710 inverted for high-resolution confocal imaging;
2. Zeiss LSM 780 equipped with a spectral detector;
3. a new Nikon Spinning Disk/Total Internal Reflection Fluorescence (TIRF) hybrid microscope, received in March 2021, an advanced motorized rotating module that allows for unparalleled image quality;
4. Zeiss LSM 880 2-photon confocal for thick tissues and live animals;
5. Zeiss 800 optimized for advanced tiling experiments;
6. Zeiss 880 AiryScan with higher spatial resolution.

Additionally, the MIC operates two wide-field microscopes: a fully automated Zeiss Axioscan Z1 slide scanner that was delivered in February 2021, and an older wide-angle microscope. Both units are fully motorized and capable of fluorescence and transmission imaging on large tissue sections. The Axioscan Z1 can process up to 100 slides unattended.

After an initial orientation, during which the staff research the project and decide on 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 on how to extract usable data from their images.

Electron microscopy

The electron microscopy branch of the Facility processes specimens from start to finish: fixation, embedding, semi-thin and ultra-thin 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 branch, where end users do their own processing. In the past 12 months, Chip Dye processed a total of 110 samples for morphology studies.

Tissue preparation

Until her retirement, Lynne Holtzclaw was in charge of sample-processing training and services at the MIC. Six users were trained in person in rodent perfusion, cryopreservation, cryosectioning, and immunofluorescence. Five instances of perfusion and cryosectioning services were provided. NICHD users of the histology suite include Drs. Balla, Buonanno, Crouch, Fields, Hoffman, Le Pichon, Loh, McBain, Ozato, Pfeifer, Sackett, Stojilkovic, and Stopfer. Users from other Institutes include Drs. Mario Penzo (NIMH), Dietmar Plenz (NIMH), and Mark Cookson (NIA).

We pursued a collaborative effort with Robert Crouch to investigate developmental cerebellar protein expression disruptions. We concluded RNAScope experiments for a collaborative project with David Klein with the assistance of Sara Felsen. These experiments targeted important developmental genes in the rat pineal gland and corroborated RNASeq data from pineal glands.

Ying Yi took over the sample preparation branch in September 2021.

Image analysis

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

Image processing based on neural networks (Artificial Intelligence or AI) is a remarkably powerful tool for image restoration, segmentation, and resolution improvement. The MIC is actively looking at AI–powered solutions for image restoration and segmentation. The Nikon NIS-AI suite, an advanced software for noise removal and segmentation not possible with conventional methods, is available in the Core.

Collaborators

- Robert J. Crouch, PhD, *Section on Formation of RNA, NICHD, Bethesda, MD*
- David C. Klein, PhD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Carolyn L. Smith, PhD, *Light Imaging Facility, NINDS, Bethesda, MD*

Contact

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

Molecular Genomics Core Facility

With the goal of understanding the 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*
Rachel Lazris, 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 five sequencing machines. Most of our work is conducted on our two high-capacity, production-scale machines: an Illumina HiSeq 2500 and an Illumina NovaSeq 6000. To increase the throughput of the HiSeq 2500 even further, we have a cBot liquid handler for automated high-precision loading of sequencing chips. 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. The fifth sequencer, is a Pacific Biosciences (PacBio) Sequel, which can sequence long single molecules of more than 100,000 base pairs. This array of sequencers provides a suite of scales and capabilities. Our sequencing services include whole-genome, whole-exome, targeted exome, and gene-specific DNA sequencing, as well as whole-transcriptome sequencing (RNA-Seq), microRNA sequencing, microbiome sequencing, bisulfite sequencing (DNA methylome), ChIP-Seq, and ribosomal profiling. 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.

The MGC also operates a 10X Genomics Chromium Single Cell Controller. The Chromium converts a suspension of single cells or nuclei into cDNA libraries that are barcoded by cell or nucleus of origin. The cDNAs can then be converted into sequenceable libraries and run on our Illumina HiSeq 2500 or NovaSeq 6000 machines to generate thousands of 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 245 projects (2,902 samples) across the full spectrum of sequencing types, generating 20,177 gigabases of sequence; the projects involved 37 NICHD Principal Investigators from 13 Affinity Groups. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support to 18 NICHD investigators across eight 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

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2. Cherkasova V, Iben JR, Pridham KJ, Kessler AC, Maraia RJ. The leucine-NH4+ uptake regulator Any1 limits growth as part of a general amino acid control response to loss of La protein by fission yeast. *PLoS One* 2021 16(6):e0253494.
3. Coon SL, Li T, Iben JR, Mattijssen S, Maraia RJ. Single-molecule polyadenylated tail sequencing (SM-PAT-Seq) to measure polyA tail lengths transcriptome-wide. *Methods Enzymol* 2021 655:119–137.
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5. Patterson-West J, Tai CH, Son B, Hsieh ML, Iben JR, Hinton DM. Overexpression of the bacteriophage T4 motB gene alters H-NS dependent repression of specific host DNA. *Viruses* 2021 13(1):84.

Collaborators

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- Juan Bonifacino, PhD, *Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Michael Cashel, MD, PhD, *Section on Molecular Regulation, NICHD, Bethesda, MD*
- Ajay Chitnis, MBBS, PhD, *Section on Neural Developmental Dynamics, NICHD, Bethesda, MD*
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- Richard Maraia, MD, *Section on Molecular and Cellular Biology, NICHD, Bethesda, MD*
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- Deborah Merke, MD, *Section on Congenital Disorders, NICHD, Bethesda, MD*
- Keiko Ozato, PhD, *Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD*
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- Michael E. Ward, MD, PhD, *3-D Tissue Bioprinting Laboratory, NINDS, Bethesda, MD*
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- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

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The NICHD Zebrafish Core

The NICHD Zebrafish Core was established in 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, and Brant Weinstein. 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, Director of the NICHD Zebrafish Core*

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

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

Felicia Benoit, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Oversight and support of client-specific projects

Over 2020–2021, the Core engaged in research projects with nine labs and other customers and with two cores.

GENETIC DISSECTION AND CREATION OF HUMAN DISEASE MODELS OF STEROL METABOLISM *(PORTER LAB, NICHD)*

In previous years, the Core used CRISPR-Cas9 technology to create genetic mutant zebrafish lines for the Porter lab in five genes: *dhcr7*, *npc1*, *npc2*, *cln3*, and *ebp*, which play roles in various steps of cholesterol metabolism. A paper on *npc2* phenotypes was published in April 2021. This last year the Core also recovered germ-line carriers for an edited zebrafish *npc1* mutant line with a non-synonymous amino acid substitution that is cognate to a human disease-associated mutation of interest.

FUNCTIONAL ASSESSMENT OF TUBULIN ISOFORMS *(SACKETT LAB, EX-NICHD)*

This year, the Core initiated a two-pronged project to address two questions with the Sackett lab.

1. Are post-translational modifications of alpha-tubulin involving taurine conserved in zebrafish?
2. Do any zebrafish beta tubulin isoforms display tissue-specific expression and if so, what is the consequence of their genetic removal?

FUNCTION OF ZEBRAFISH ORTHOLOGS TO HUMAN GENES IMPLICATED IN DISORDERS OF THE PITUITARY–ADRENAL AXIS *(STRATAKIS LAB, NICHD)*

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. A manuscript summarizing the characterization of one of these genes, *gpr101*, was published in January 2021. This last year, the Core also generated and recovered a precisely edited zebrafish *satb1* mutant line with a non-synonymous amino acid substitution that is cognate to a human disease-associated mutation of interest.

FUNCTION OF ZEBRAFISH *RCA2.1 (KEMPER LAB, NHLBI)*

The Kemper lab is interested in zebrafish *rca2.1*'s function, because it is similar to human CD46 (a transmembrane glycoprotein that plays a role in regulating the complement system) and because an analogous gene that is expressed cannot be found in the mouse genome. In 2019–20, we generated and recovered a precise-edited deletion spanning the entire gene (about 20 KB) by simultaneously injecting gRNAs targeting the length of the gene along with Cas9 to assure complete disruption of gene function. Phenotype characterization this year indicated that mutants have a profound growth deficit accompanied by cardiac hypertrophy that is ultimately lethal. The Core also generated a standard small CRISPR-Cas9 deletion allele in *rca2.1* this year for comparison, which appears to cause the same phenotypes.

ROLE OF MINERALS IN BONE HEALTH *(LAVERNE BROWN, OFFICE OF DIETARY SUPPLEMENTS)*

The NICHD Zebrafish Core has been working with LaVerne Brown from the NIH Office of Dietary Supplements in planning and implementing preliminary steps for a nutritional study to explore how certain minerals influence bone health in the presence or absence of adequate vitamin D, a topic of relevance to human bone health. This year, the Core obtained six defined feeds from the Nutrition Obesity Research Center of the University of Alabama (Birmingham), and is planning the details of a study to begin in early 2022.

Independent research by the NICHD Zebrafish Core

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

Since 2018, the Core has explored the use of the CRISPR-Cas9 technology in combination with donor DNA to generate zebrafish lines with amino-acid substitutions cognate to human disease alleles of interest. The Core's first success with this approach was for an Atp7a amino-acid (AA) substitution that is cognate to an ATP7A (AA) substitution of interest to the Kaler lab (ex-NICHD), a substitution that causes distal motoneuropathy. The phenotype is characterized by hypopigmentation. The Core devised a novel screening strategy for this project that included synonymous changes alongside the targeted non-synonymous change. The synonymous changes introduced RFLPs (restriction fragment length polymorphisms) amenable to their standard molecular screening strategy. In collaboration with the NICHD Bioinformatics & Scientific Programming Core, software for generalizing this approach to any locus of interest was developed. During the last year, Feldman prepared and deposited on BioRXiv a manuscript summarizing these achievements.

Previously, in 2018–2019, the Core had also conducted Directors Award–funded research in collaboration with the NICHD Molecular Genomics Core to compare precise genome-editing methods and efficiencies using high-throughput sequencing. The Core compared five methods for generating seven alleles of interest to labs in NICHD and elsewhere at NIH, namely: two *npc1* alleles (Porter lab, NICHD), one *rhoaa* allele (Weinstein lab, NICHD), one *ifitm5* allele (Marini lab, NICHD), two *ryr1b* alleles (Lawal lab, NINR), and one *cacna1c* allele (Golden

lab, NIDDK). Over the last year the Core focused exclusively on recovering these alleles, developing preferred strategies along the way. Of particular promise, Feldman recently successfully extracted gametes (sperm or eggs) from F_0fish and subjected them to high-throughput sequencing as an efficient route to identifying F_0 germ-line transmitting founders. These efforts led to allele recovery and/or identification of germ-line transmitters for three of the seven initially targeted aa–substituted alleles: *cacna1c*, *ifitm5* and *npc1* (one of two), as well as a more recently added allele of *satb1*.

Feldman continues 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.

CRYO–PRESERVATION AND *IN VITRO* FERTILIZATION OF ZEBRAFISH SPERM

Over the last two years, Feldman has made efforts to improve quality-control measures to ensure the viability of cryo-preserved zebrafish lines and minimize variability in viability. This year, the project was led by Felicia Benoit from the lab of Alan DeCherney (NICHD), who had an interest in the subject. The project has been fraught with technical challenges, but the Core recently overcame these obstacles and identified a combination of conditions that assure high-yield recovery of cryo-preserved specimens.

Service

ACUC MEMBERSHIP

Feldman has served on the NICHD Animal Care and Use Committee (ACUC) since 2015 and continued in this capacity this year, meeting monthly to evaluate and decide upon animal-study proposals, renewals and amendments, and ad hoc issues relevant to animal welfare. This year, Feldman contributed toward renewal of the NICHD animal program's AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) Accreditation.

TAKE YOUR CHILD TO WORK DAY

Feldman worked with the Office of Communications to produce a video log for the 2021 Virtual Take Your Child to Work Day, showing how he breeds zebrafish and examines their embryo progeny.

SITE VISIT

2021 included Feldman’s quadrennial site visit, and Feldman accordingly prepared a packet and oral presentation for outside reviewers with excellent results.

FACULTY REPORTS

As a broader contribution to the scientific community, Feldman regularly pens recommendations for scientific articles of interest as a member of Faculty Opinions in the Developmental Biology/Pattern Formation Section. This year, he contributed four recommendations.

Additional Funding

- One-year salary support for Postbaccalaureate Fellow Felicia Benoit from the Office of Intramural Training & Education, NIH

Publications

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2. Trivellin G, Tirosh A, Hernández-Ramírez LC, Gupta T, Tsai-Morris C-H, Faucz F R, Burgess HA, Feldman B, Stratakis CA. The X-linked acrogigantism-associated gene gpr101 is a maternal regulator of early embryonic development and somatic growth in zebrafish. *Mol Cell Endocrinol* 2021 520:111091.

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- Andy Golden, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Claudia Kemper, PhD, *Laboratory for Complement and Inflammation Research, NHLBI, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- Daniel Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*
- Constantine Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*

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Affinity Groups

Scientists and physicians in the NICHD Division of Intramural Research (DIR) are organized into 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
Ben Feldman (Core)
Katherine Rogers
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 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*
Philip Adams
Anirban Banerjee
Juan Bonifacino
Andres Buonanno
Mary Lilly
Gisela Storz

Cell Regulation and Development

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

By combining expertise in the genetics of model organisms (including yeast, fruit fly, frog, zebrafish, and mouse), cell biology, biochemistry, molecular biology, electrophysiology, biophysics, genomics, and structural biology, 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 underlie past accomplishments and will facilitate future discoveries by the Group in areas including the mechanisms and regulation of protein synthesis and transcriptional activation of gene expression, the functions and regulation of hormone receptors, signaling events and their responsive genes, mechanisms governing accurate segregation of the genetic information during cell division, mechanisms of nucleocytoplasmic trafficking, RNA processing

and RNA export, 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, technologies for analyzing gene expression at the single cell level during development, transcriptional control of the specification and differentiation of cells during vertebrate embryogenesis, the mechanisms governing adult organ formation during postembryonic vertebrate development, and molecular mechanisms of synaptic circuit assembly and function.

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

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
Deborah Merke (CC)
Anil Mukherjee
Forbes Porter
Kristina Rother (Training Program)
Stanko Stojilkovic
Jack Yanovski
NIH Inter-Institute Endocrinology Training Program

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*
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
Bob Crouch
Mel DePamphilis
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*
Peter Basser
R. Douglas Fields
Amir Gandjbakhche
Leonid Margolis
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
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Reproductive Endocrinology & Infertility and Pediatric & 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)

RNA–Mediated Gene Regulation in the Lyme Disease Pathogen

The goal of this research is to identify and characterize gene regulation in the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease. We use genetics, RNA sequencing, and murine infection models to pinpoint genes for mechanistic study.

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The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease, an emerging infectious disease and the foremost vector-borne bacterial infection in the world. Given that *B. burgdorferi* inhabits tick and mammalian hosts, environments with very different temperatures, immune responses, and sources of metabolites, the bacterium must harbor robust gene-regulatory mechanisms in order to survive.

The major focus of the group has been to discover and characterize *B. burgdorferi* genes important for infectivity and stress response. We recognized the importance of mapping RNA boundaries (their 5' and 3' ends), which is critical for gene annotation, the discovery of novel transcripts, and mechanistic characterization of genes. Therefore, we performed 5'RNA-Seq on total RNA isolated from *B. burgdorferi* grown in culture. The method identified RNA 5' ends and distinguished transcription starts sites (TSSs) from 5' processed ends. These data were then compared with a previous genetic screen that identified transcriptionally active sequences on the *B. burgdorferi* genome during a mouse infection. This resulted in a dataset of putative mammalian-specific *B. burgdorferi* transcriptional events and identified numerous genes for future study.

One gene identified by our approach, *bb0562*, was an annotated hypothetical protein. Targeted deletion of *bb0562* revealed that the gene encodes a protein important for disseminated infection in mice by needle inoculation and tick-bite transmission. The gene *bb0562* was also found to be important for spirochete growth in low-serum media, leading to a growth defect that could be rescued with the addition of various long-chain fatty acids. We identified two canonical lipase motifs within BB0562 and demonstrated *in vitro* lipolytic activity with purified BB0562 protein. Collectively, the work established *bb0562* as a novel *B. burgdorferi* nutritional virulence determinant [Reference 1].

Ongoing work in our lab has been focused on the characterization of other genes, particularly regulatory RNAs. This is an unstudied area of

B. burgdorferi gene regulation. To expand upon our previous study, we augmented our RNA-Seq approach and are now combining three techniques to further refine RNA boundaries: 5'RNA-Seq, 3'RNA-Seq (which captures termination events and identifies RNA 3' ends), and total RNA-Seq (which sequences genes in their entirety). As proof of concept, we applied this approach to the model organism *Escherichia coli* [Reference 2]. In doing so, we identified numerous RNA fragments derived from 5' regions of mRNAs and internal to open reading frames (ORFs). We documented regulation for multiple transcripts and identified a function for a small RNA encoded internal to an essential cell division gene. The work revealed that regulatory transcripts are derived from a wide range of locations in bacterial genomes [Reference 3].

Further studies to identify *B. burgdorferi* RNA regulators and characterize their physiological roles are ongoing.

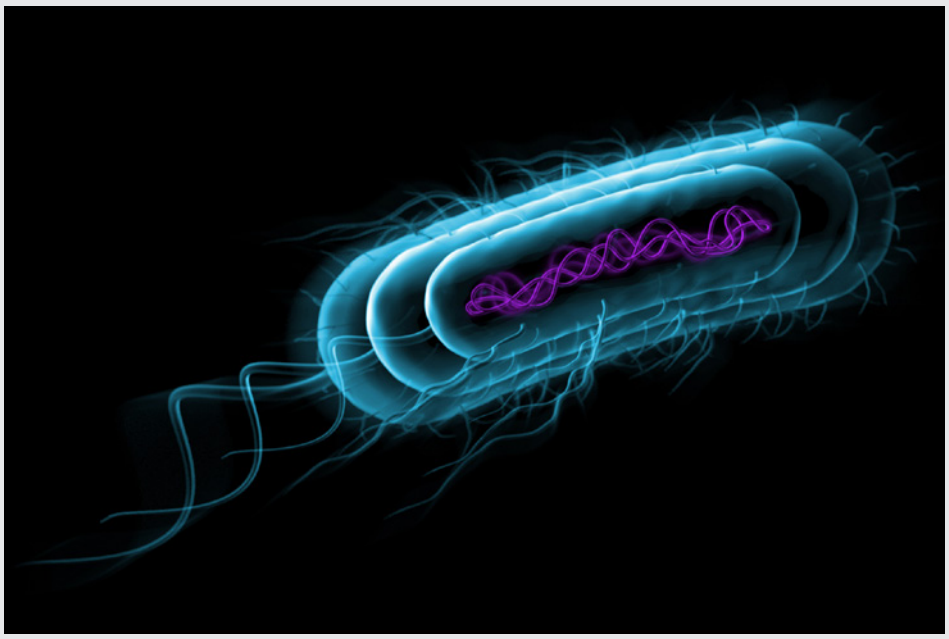

FIGURE 1. Spot the Regulators

Mapping of bacterial RNA ends led to the discovery of regulatory RNAs encoded within protein-coding sequences. Image credit: NIH Medical Arts (CCBY 4.0)

Additional Funding

- NICHD Early Career Award (2021) to Dr. Philip Adams, concluded

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Phosphoinositide Messengers in Cellular Signaling and Trafficking

Every biochemical process in a eukaryotic cell relies on a molecular information flow that leads from receptors, which 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.

Our research 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. Lipid composition of cellular membranes is a major determinant of their biophysical properties and is unique to the different cellular organelles. However, how cells achieve and maintain the proper lipid composition of their membranes is poorly understood. Cellular processes that affect the membrane lipid composition of organelles are often targeted by cellular pathogens, such as viruses, to force the cells to produce the pathogen instead of performing the cells' normal functions. Better understanding of these processes can provide new strategies not only to fight various human diseases but also to intercept the life cycle of cellular pathogens, thus offering an alternative to antimicrobial drugs.

The role of ORP3 proteins in cell regulation

Membrane contacts sites (MCSs) between various organelles are emerging as key structural elements where important communication between organelles takes place. MCSs are defined as membrane appositions between membranes of two organelles with a distance of no greater than 30 nm. MCSs have primarily featured in non-vesicular lipid transfer and Ca^{2+} signal propagation, but their importance is likely to reach beyond these two processes. While MCSs can be dynamic, they are stabilized by tethering proteins that also have functional roles. Several proteins have been identified that function in contact sites, most of which have been implicated in non-vesicular lipid

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transfer between the contributing membranes. An important class of molecules that function at MCSs are the ORP (oxysterol-binding protein–related protein) proteins, which are the mammalian orthologs of the yeast Osh proteins. The Osh/ORP proteins mediate the transport of specific lipids between cellular membranes, their lipid cargo preference being defined by their lipid transfer ORD domains. One of the salient features of Osh/ORP proteins is that they use a phosphatidylinositol-4-phosphate (PI4P) gradient as a driving force as they counter-transport PI4P in exchange for the specific lipids they move between membranes. Therefore, lipid transport by Osh/ORP proteins is linked to the activity of PI4 kinases.

In this research period, we investigated the role of ORP3, one of the less characterized members of this family, in cellular physiology. Specifically, we investigated the role of inositol lipids in the control of ORP3 function and, conversely, the possible role of ORP3 in the organization of endoplasmic reticulum (ER)–plasma membrane (PM) contacts with respect to PI4P status and store-operated Ca^{2+} entry (SOCE). SOCE is regulated by the ER–luminal Ca^{2+} concentration, which is sensed by the ER–localized STIM1/STIM2 proteins, which activate the plasma-membrane, Ca^{2+}–selective Orai channels at ER–PM contact sites. In previous studies, we and others showed that PI lipids play important roles in the control of SOCE. We found that PM association of ORP3 is triggered by protein kinase C (PKC) activation, especially when combined with cytoplasmic Ca^{2+} increases. Once activated by PKC, ORP3–PM association is determined by both the inositol lipids PI(4,5)P2 and PI4P. After activation, ORP3 efficiently extracts PI4P, and to a smaller extent phosphatidic acid, from the PM while it slightly increases PM cholesterol levels. Full activation of ORP3 results in reduced PM PI4P levels, which, in turn, inhibit Ca^{2+} entry via SOCE. We also identified the C-terminal region of ORP3 that follows the strictly defined lipid transfer ORD domain as critical for the proper localization and function of the protein. The importance of these studies is that they highlight the intimate connection between regulation of PI4P levels and Ca^{2+} entry at PM–ER contact sites and the critical role of ORP3 in this process. Notably, defects in SOCE have been found to cause severe human immunodeficiencies. Therefore, understanding its regulation is of major importance.

The role of PI 4-kinase type III beta (PI4KB) in peripheral nerve myelination

Myelination of peripheral nerves is a complex process requiring a coordinated series of molecular events executed by Schwann cells (SCs). Improper myelination and axonal sorting defects cause peripheral neuropathies such as the several forms of Charcot-Marie-Tooth (CMT) disease. Among the genes that are associated with CMT, many control vesicular trafficking. The fine architecture of myelin, exemplified by the delicate structure of the nodes of Ranvier, requires communication between the axon and the surrounding SCs and relies upon the proper delivery of molecular cues to their final destinations. The Golgi plays an important role in most of these processes. There is little information about the role of the Golgi in peripheral myelination by SCs. The minor phospholipid, phosphatidylinositol 4-phosphate (PI4P), is a key regulator of Golgi function. It plays a role in defining post-Golgi vesicle exit sites and recruits various adaptors for membrane coats. PI4P also controls delivery of ceramide, glycosyl ceramide, and cholesterol from the ER to the Golgi. One of the major regulators of Golgi function is the lipid kinase PI4KB. Therefore, to gain further insight into the role of Golgi in peripheral nerve myelination, we created and characterized a mouse model with genetic inactivation of PI4KB, specifically in SCs. We characterized the phenotype of these mice, focusing on the sciatic nerves. The mice display highly subtle functional defects, which do not show obvious progression with time. Yet, the conduction velocity of the sciatic nerves of mutant animals decreases dramatically, and major structural defects were revealed by histochemical and EM analyses. We found that the mutant mice developed a myelination defect characterized by thinner myelin only affecting the large-diameter axons, with gross alterations in the structure

of the nodes of Ranvier and a striking inability of non-myelinating SCs to wrap small diameter fibers in Remak bundles (bundles of a type of unmyelinated nerve fiber). Such changes were linked to Golgi functions affecting cholesterol transport, glycosylation, and to a hitherto unrecognized role of PI4KB in the SC microvilli at the nodes of Ranvier. The studies showed that PI4KB is an important component of the myelination process in peripheral nerves, supporting several aspects of Golgi function, including sterol and sphingolipid transport, glycosylation, and most likely the trafficking of proteins that are important for the process. The unexpected presence of the enzyme in the microvilli of SCs at the nodes of Ranvier together with the defective microvilli in the nodes of mutant mice revealed an important function of PI4KB within the peripheral nervous system, which requires further studies.

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- NICHD Intramural Research Fellowship for Gergo Gulyás

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Structural and Chemical Biology of Membrane Proteins

Molecular mechanism of post-translational protein lipidation by zDHHC protein *S*-acyltransferases

Post-translational 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 post-translational modification. Protein *S*-acylation, commonly known as protein palmitoylation, is a specific form of protein lipidation whereby long-chain fatty acids, typically C16, become covalently attached to cytosol-facing cysteines through a thioester linkage. Palmitoylation is one of the most pervasive and physiologically important post-translational 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 physico-chemical effect of palmitoylation is to alter the local hydrophobicity of the substrate protein. The thioester bond makes *S*-acylation unique in that it is a labile moiety and can be cleaved, in the cellular context, by thioesterase enzymes, which makes *S*-acylation one of the few dynamic post-translational 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. On the other hand, in recent work (see below), we showed that the Spike protein of SARS-CoV-2, the causative agent of COVID-19, is *S*-acylated, which is important in the viral life cycle.

Protein *S*-acylation is catalyzed by a large group of enzymes known as zDHHC palmitoyl acyltransferases (also referred to as DHHC

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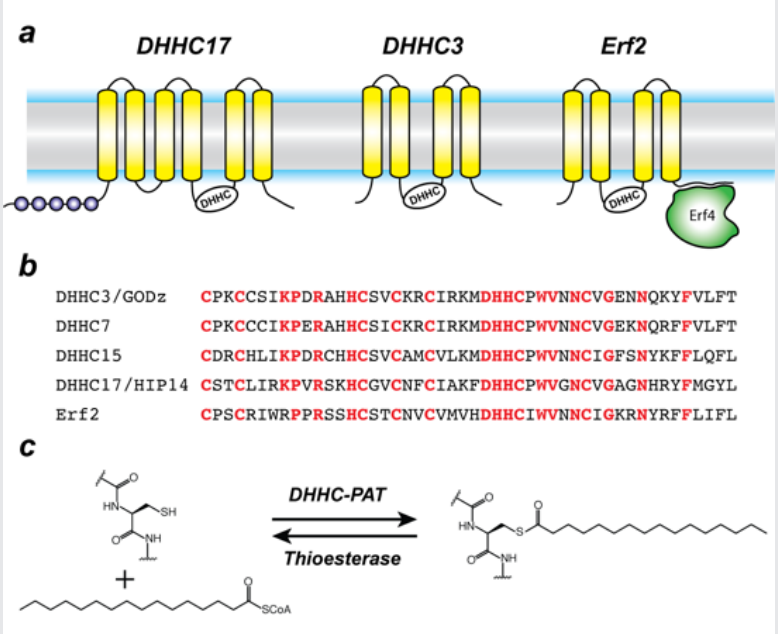


FIGURE 1. Organization and properties of DHHC palmitoyltransferases (PATs)

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.

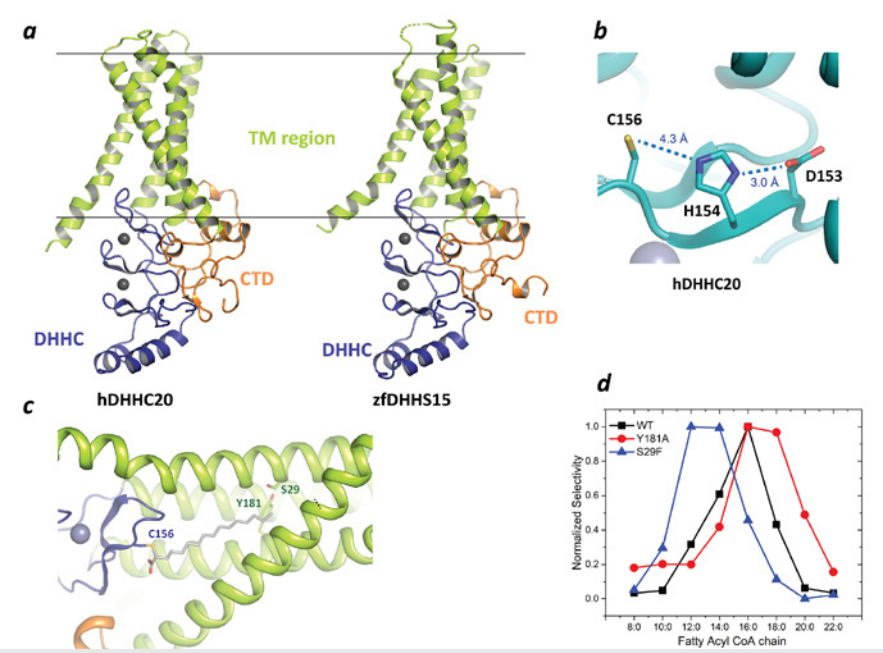
b. The DHHC-CRD region of a few representative DHHC-PATs are aligned. The conserved amino acids are shown in red.

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

enzymes or DHHC-PAT), so named because they contain a signature D-H-H-C motif (aspartate-histidine-histidine-cysteine) in a cysteine-rich domain (CRD) in an intracellular loop (Figure 1). These are low-abundance polytopic integral membrane proteins localized to a variety of cellular 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. To date, no consensus sequences have been reported 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 is 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 also 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 zDHHC20 palmitoylates epidermal growth factor (EGFR) and is thus a potential therapeutic target for a wide range of cancers. More recently, zDHHC3 has been proposed as a target for cancer treatment owing to its activity as the palmitoyltransferase for the programmed cell-death ligand 1 (PD-L1). However, when we started working on this family, very little was known about the molecular mechanism of zDHHC palmitoyltransferases, despite their importance across a broad spectrum of biological pathways and their biomedical importance. 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.

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.

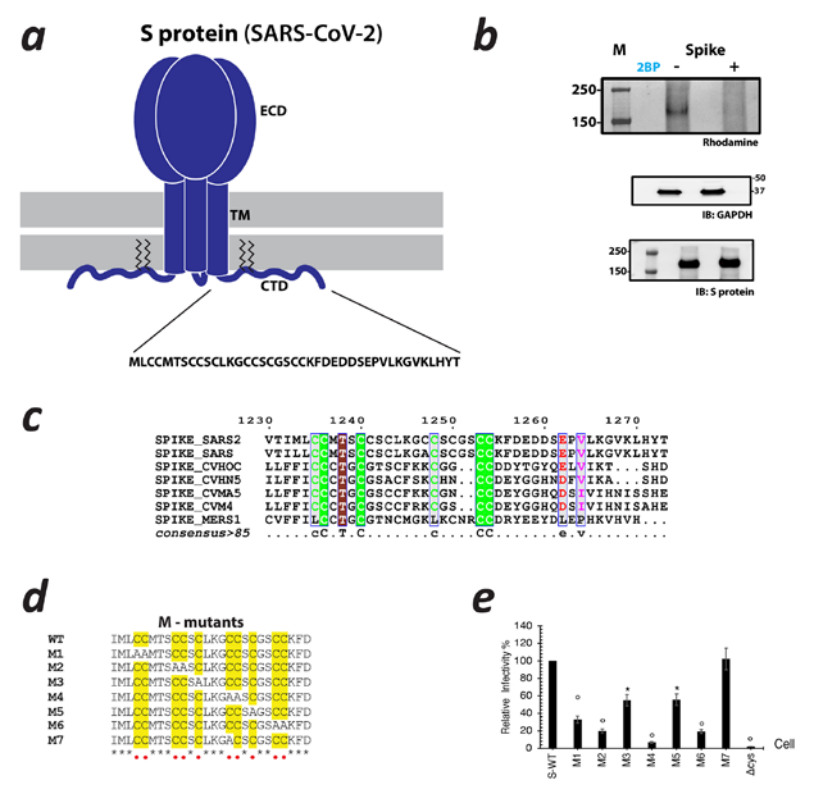
c. Structure of human DHHC20 irreversibly modified with 2-bromopalmitate, which results in the active-site cysteine linking to the alpha-carbon of palmitic acid. The acyl group of palmitic acid is shown in stick rendition. Also shown are two residues towards the top of the tapering cavity to where the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

In a major breakthrough in this field, we had earlier solved the high-resolution crystal structures of two members of the zDHHC family, human zDHHC20 (hDHHC20) and zebrafish zDHHC15 (Figure 2a), the first structures of any member of this family to be characterized. They 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 2b), thus readily explaining why membrane-proximal cysteines are palmitoylated. We also solved the structure of hDHHC20 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 their mutation 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 hDHHC20, important because, although palmitate is the most prevalent fatty acid used by DHHC palmitoyltransferases, they can use fatty acyl–CoAs of other chain lengths, a property that varies between different members of the DHHC family.

S-acylation was first discovered in viral proteins and in the wake of the COVID-19 pandemic, so we turned our attention to this. It had been shown that the Spike protein of other coronaviruses or its equivalent in other pathogenic viruses are *S*-acylated and, in a subset of these, *S*-acylation is crucial to the viral life cycle. However,

FIGURE 3. *S*-acylation of the SARS-CoV-2 Spike protein



a. Schematic showing the trimeric form of SARS-CoV-2 Spike protein with the ectodomain (ECD), Transmembrane (TM) region, and C-terminal Domain (CTD). The CTD is further enhanced to show the cystine clusters, which are potential sites of S-acylation.

b. S-acylation of the Spike protein determined by click chemistry. Gel images detecting rhodamine signal at the position of Spike protein in the presence and absence of 2-bromopalmitate (2BP), a global inhibitor of *S*-acylation. Western blots probed with antibodies against GAPDH and the Spike protein respectively serve as controls for loading and total Spike protein expression.

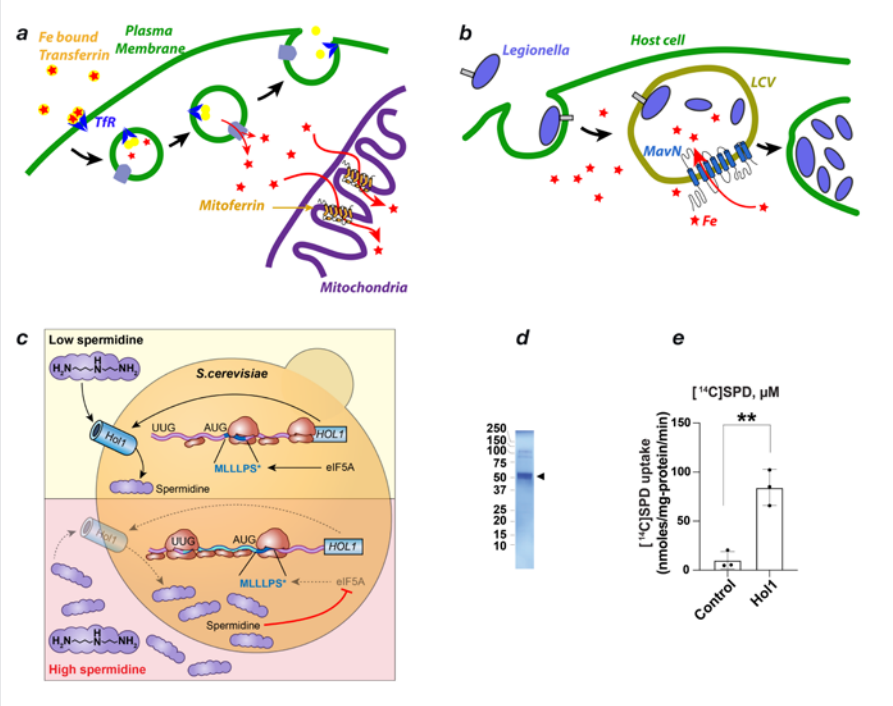
c. Sequence alignment of the CTDs of Spike protein from various coronaviruses.

d. Sequence alignment showing Spike protein mutants. Cysteine residues are highlighted in yellow. They are built on the backbone of wild-type (WT) Spike protein, M1 through M6 indicating either an individual cysteine or di-cysteine motif that is mutated to alanine. The M7 mutant represents a construct in which the additional cysteine in SARS-CoV-2 with respect to SARS-Co-V has been mutated out.

e. Infectivity of HIV-1 particles pseudotyped with WT Spike protein and the cysteine mutants.

it was not known whether the Spike protein of SARS-CoV-2, the causative agent of COVID-19, is *S*-acylated and, if so, how that affects the viral life cycle. Remarkably, even 40 years after the discovery of *S*-acylation, there were still no *in vitro* reconstitution of *S*-acylation of any viral protein with purified components. We started a project approved by the NIH COVID-19 committee to answer these questions. Our experiments demonstrated, using a click chemistry–based *in cellulo* assay, that the Spike protein of SARS-CoV-2 is *S*-acylated (Figure 3). We went on to identify specific cysteine clusters that are targets of *S*-acylation. Interestingly, when we investigated the effect of the cysteine clusters using pseudotyped virus, in collaboration with Eric Freed's lab, mutation of the same three clusters of cysteines (that are *S*-acylated) severely compromised viral infectivity. We developed a library of expression constructs of human zDHHC enzymes and used them to identify zDHHC enzymes that can *S*-acylate the SARS-CoV-2 Spike protein. We reconstituted *S*-acylation of the SARS-CoV-2 Spike protein *in vitro* using purified zDHHC enzymes and observed a striking heterogeneity in the *S*-acylation status of the different cysteines in our *in cellulo* experiments, which, remarkably, was recapitulated by the *in vitro* assay. Taken together, these results bolster our understanding of a poorly understood post-translational modification integral to the SARS-CoV-2 Spike protein. The study also opens up avenues for further mechanistic dissection and lays the groundwork for developing future strategies that could aid in the identification of targeted small-molecule modulators.

FIGURE 4. Transporters of iron and polyamine



a. Iron is imported through the plasma membrane by the transferrin/transferrin receptor *(blue)* cycle and is transported out of endosomes by the divalent metal ion transporter (DMT) *(grey)*. 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 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.

c. Assay showing iron transport by proteoliposome-reconstituted MavN. Hol1 as the major high-affinity polyamine transporter in *S. cerevisiae*. Polyamines autoregulate *HOL1* expression through feedback inhibition of *HOL1* mRNA translation mediated by polyamine inhibition of eIF5A–dependent translation termination on a conserved upstream open reading frame in the *HOL1* mRNA leader.

d. SDS-PAGE gel of detergent-purified *K. lactis* Hol1 *(arrowhead)*.

e. [^{14}C]Spermidine uptake by control or *K. lactis* Hol1 proteoliposomes. Error bars denote SD, **p<0.01 (Student's two-tailed t test, n=3).

Molecular mechanism of iron and polyamine 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 Fe-S clusters are biosynthesized and iron is inserted into heme there. Mitochondrial iron homeostasis plays a critical role in cellular iron homeostasis and in the overall physiology of the cell. In vertebrates, the only known major transporters of iron into mitochondria are mitoferrin-1 and mitoferrin-2, two homologous members of a large group of mitochondrial transporters known as the Mitochondrial Carrier family (Figure 4). 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 15 years ago. However, the proposed iron-transport activity had not been demonstrated using an *in vitro* functional reconstitution assay, and nothing was known about their interaction with iron or other related metal ions, most likely because heterologous overexpression and purification of mitoferrins were not reported in the literature. There were also no reports of a reconstituted iron

transport assay starting from purified protein. In earlier work, we carried out heterologous purification, *in vitro* functional reconstitution, and mutational dissection of a vertebrate Mfrn1 (Figure 4), the first demonstration that Mfrn1 can indeed transport iron. Our studies provided the first biochemical insights into Mfrn function. In earlier work, we also 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 highly conserved iron transporter, in the bacterial pathogen *Legionella pneumophila* (Figure 4b).

In the past year we shifted our interest to transporters that move polyamines across the membrane. Polyamines, small organic polycations, are essential for cell viability, and their physiological levels are homeostatically maintained by post-transcriptional regulation of key biosynthetic enzymes. In addition to *de novo* synthesis, cells can also take up polyamines; however, until recently, dedicated polyamine transporters were not known. Tom Dever's lab discovered that the *S. cerevisiae HOL1* mRNA is under translational control by polyamines. They also showed that Hol1 is required for yeast growth under limiting polyamine conditions. These experiments suggested that Hol1 is a high-affinity polyamine transporter (Figure 4c). We purified recombinant Hol1 and reconstituted it in protealiposomes to demonstrate that it indeed shows robust polyamine transport activity. An extensive set of experiments from the Dever lab bolstered the conclusions that Hol1 is a high-affinity polyamine transporter and under translational autoregulation by polyamines in yeast, highlighting the extensive control cells impose on polyamine levels. We are now investigating the underlying molecular mechanisms behind Hol1 function.

Structure and molecular mechanism of ATG9, the only transmembrane component of the core autophagy machinery

Autophagy is a critical process in both health and disease. Serving mainly to adapt organisms to a diverse range of conditions such as metabolic stress, pathogenic infection, and ageing, autophagy is conserved from yeast to humans. In addition, autophagy is involved in a large number of human diseases, including neuro-degenerative disorders, various forms of cancer, and inflammatory diseases. Although autophagy was discovered more than 50 years ago, some its fundamental aspects remain rather poorly understood and are only now coming to light. An outstanding example of this lack of knowledge is the structure and function of ATG9, the only essential transmembrane protein in the pathway. ATG9 localizes to small, 30–60 nm vesicles known as 'ATG9 vesicles,' which are critically important for the expansion of the autophagosome membrane. ATG9 vesicles traffic between the *trans*-Golgi network (TGN) and pre-autophagosomal structures in response to stimuli that initiate autophagy. Despite the clear and obvious importance of ATG9 in autophagy, its atomic structure and molecular function remain unknown. It has been speculated that ATG9 transports lipids that contribute to the growth of the double-membrane structure of the autophagosome. However, progress in this regard has been completely thwarted by the lack of an atomic structure that could serve as a starting point for a range of experiments, from cell-biological to biochemical, to elucidate its function.

In order to address this knowledge gap, we initiated a collaboration with the labs of Juan Bonifacino and Jiansen Jiang. This led to a 2.9 Å–resolution structure of the ubiquitous human ATG9A isoform by cryo-EM (Figure 5a). The structure revealed that ATG9A adopts a unique fold (Figure 5b, 5c), assembling into a domain-swapped homotrimer, with each wedge-shaped protomer comprising four transmembrane α-helices and two α-helices that are only partially embedded in the membrane. The transmembrane domain is capped by a cytosolic domain, which is mostly α-helical. Classification of the cryo-EM imaging revealed two predominant ATG9A conformers (states A and B). An important feature of the ATG9A structure is the presence of a branched

FIGURE 5. Structure and membrane interactions of human ATG9A

a. Cryo-EM density map of ATG9A in state A is shown in side and top views and colored according to the protomer *(cyan, magenta,* and *orange)*. Translucent surface shows disordered detergent molecules surrounding the transmembrane surface of the trimer.

b. Structure of an isolated protomer *(cyan)* and two alpha helices from another protomer *(magenta)*. Numbers correspond to alpha helices.

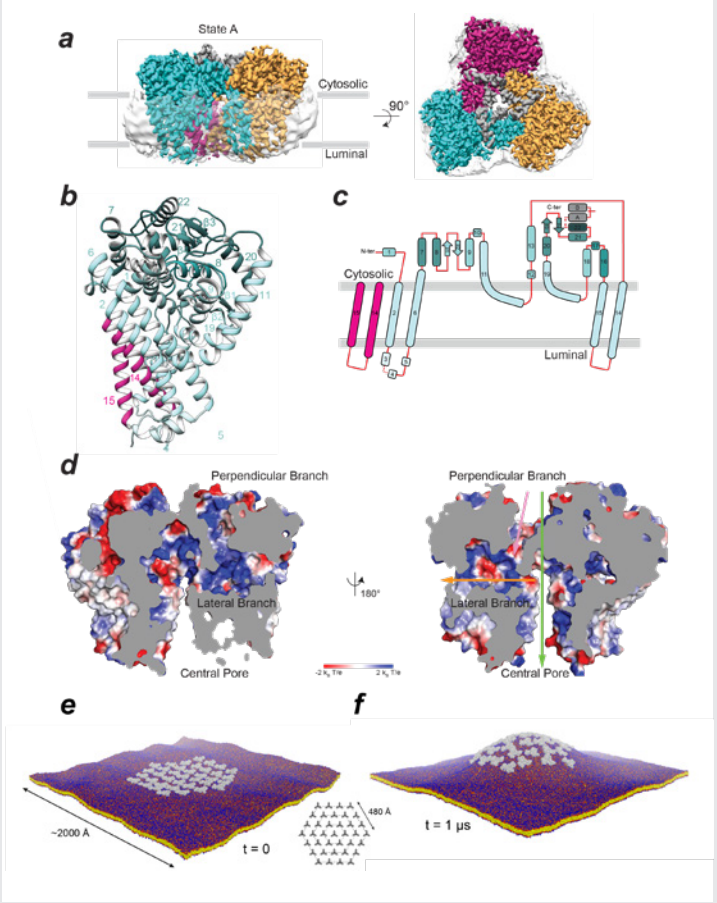
c. Topology of the ATG9A protomer showing numbered alpha helices as rounded rectangles and beta strands as filled arrows.

d. Branched network of internal pores in the ATG9A trimer shown by electrostatic potential surface of the central pore, lateral branch, and perpendicular branch (with 180° rotation). Green, yellow, and pink arrows are used to show the positions of the central pore, lateral branch, and perpendicular branch, respectively.

e. and *f.* Molecular dynamics simulation of a hypothetical ATG9A cluster in a POPC (2-oleoyl-1-pamlitoyl-sn-glyecro-3-phosphocholine) bilayer.

e. Configuration of the system at the beginning of the simulation; 36 ATG9A proteins *(gray)* are arranged around a central trimer in three concentric hexagons *(inset)*. Note the proteins are not in contact. The system amounts to about 11,220,000 particles.

f. At the end of the calculated trajectory of 1 ms.



network of pores through the protein, with outlets facing the cytosol, the outer leaflet lipid head groups, and the lumen (Figure 5d). Cell-based functional assays demonstrated the importance of pore-lining residues, suggesting an essential function of the pore. The dimensions of the pore are sufficient to accommodate phospholipids and likely permit lipid transport from or to a lipid chaperone such as ATG2. Alternatively ATG9 may act as a lipid scramblase, distributing the lipids that arrive through ATG2 among two leaflets of the bilayer. The branched pores may also function as solvent conduits to relieve osmotic pressures in growing and shrinking ATG9 vesicles. ATG9A directly interacts with ATG2A, interactions that are mediated by the ATG9A cytosolic domain, deletion of which abrogates both ATG2A co-immunoprecipitation and autophagosome maturation. Because ATG2A has demonstrated lipid-transport capability *in vitro*, the plausible functions of ATG9A are to: (1) facilitate movement of lipids across the bilayer of the growing phagophore, which could be achieved by using the branched pores we see in our structure for moving polar lipid headgroups or by distorting the bilayer, lowering the energetic barrier for trans-bilayer lipid movement; (2) act as a membrane-anchored 'lasso' to capture and accurately target ATG2A or other lipid chaperones. It is interesting to note that

two helices, which plug the central pore of the trimer, are discernible only in state A, indicating that flexibility of the putative 'lasso' could be partly dependent on the conformation of the transmembrane core.

The early stages of autophagy involve a reorganization of intracellular membranes to form the nascent autophagosome. ATG9 is essential for this process, and our structure provides a framework for interrogating its function in bilayer remodeling. Using molecular dynamics simulations in collaboration with the lab of José Faraldo-Gómez, and based on the cryo-EM data, we found that the architecture of the ATG9A trimer elicits a long-range positive membrane curvature, owing to the geometric features and amino-acid make-up of the protein surface exposed to the lipid. The simulations also show that co-localization of multiple ATG9A trimers greatly amplifies this effect and induces macroscopic changes in membrane morphology (Figure 5e, 5f). The finding corresponds with observations that ATG9 is present in small (30–60 nm) vesicles and thin tubules, and, at least in yeast, localizes to the edges of the growing phagophore. Interestingly, states A and B differ in the tilt-angle of the protomers relative to the membrane normal, implying a tolerance for a range of bilayer curvatures. This leads to the intriguing possibility that ATG9A–induced curvature may modulate the energetic scale and effect lipid diffusion out of the vesicles into another membrane.

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Regulation of Childhood Growth

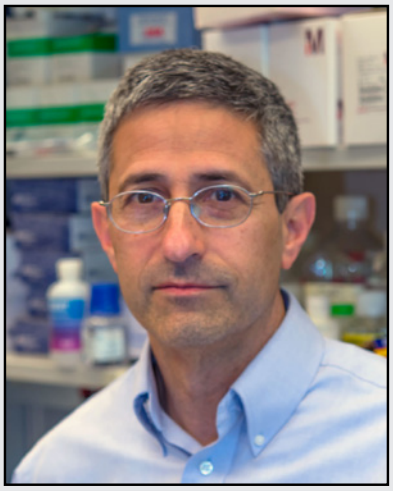
Children grow taller because their bones grow longer. Bone elongation occurs at the growth plate, a thin layer of cartilage found near the ends of juvenile bones (Figure 1). In the growth plates, new cartilage is produced through chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis, 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 other 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.

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 remaining 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



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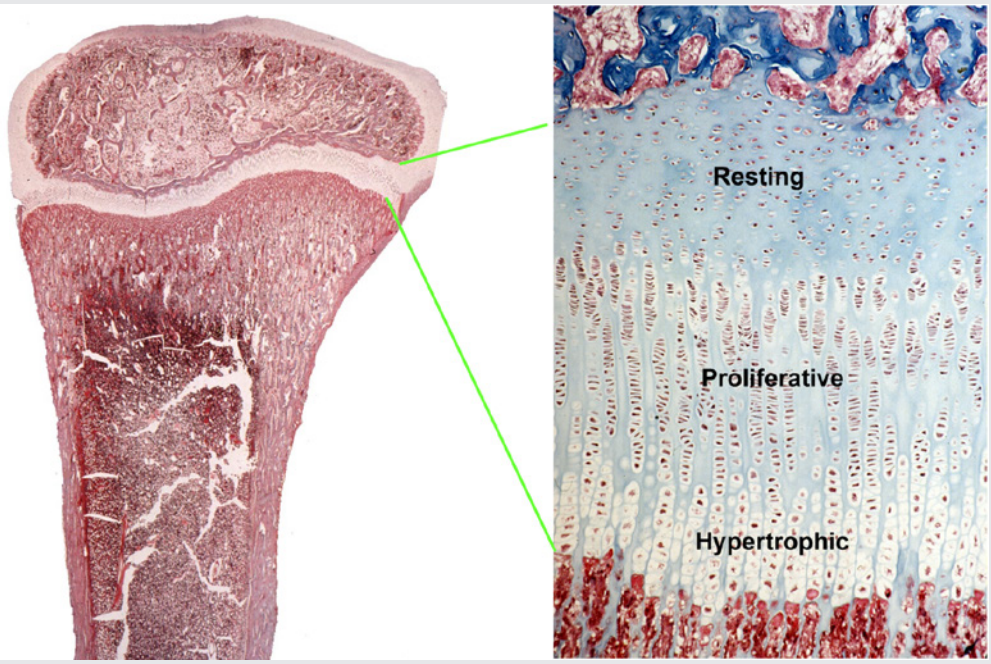

FIGURE 1.
Histological image of a growth plate, showing the three principal zones

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, the variants and the genes in which they occur are studied in the laboratory 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.

We used this approach to identify new causes of childhood growth disorders. For example, we previously found that variants in aggrecan (*ACAN*), a component of cartilage extracellular matrix, cause autosomal-dominant short stature with advanced skeletal maturation, and that such patients also tend to develop early-onset osteoarthritis. We also found evidence that heterozygous deletion of *CYP26A1* and *CYP26C1*, which encode enzymes that metabolize retinoic acid (RA), cause elevated RA concentrations, which accelerate bone and dental maturation in humans and cause developmental defects involving the eye and central nervous system. In additional studies, we found that variants in *QRICH1*, a gene of unknown function, cause a chondrodysplasia as a result of impaired growth-plate chondrocyte hypertrophic differentiation. Using this approach, we also discovered that variants in *DLG2* (encoding a membrane-associated guanylate kinase) cause delayed puberty and contribute to isolated hypogondotropic hypogonadism by a mechanism that involves decreased NMDA receptor phosphorylation and signaling, and consequently decreased GnRH expression [Reference 1].

We recently used similar approaches to study the etiology of congenital hypopituitarism. Children with this disorder present with pituitary hormone deficiencies and can have a small or absent anterior pituitary gland and an ectopic posterior pituitary. Occasionally the condition is familial, but more often it occurs sporadically. In most patients, the etiology remains unknown. We studied 13 children with sporadic congenital hypopituitarism. Children with non-endocrine, non-familial idiopathic short stature served as a control group.

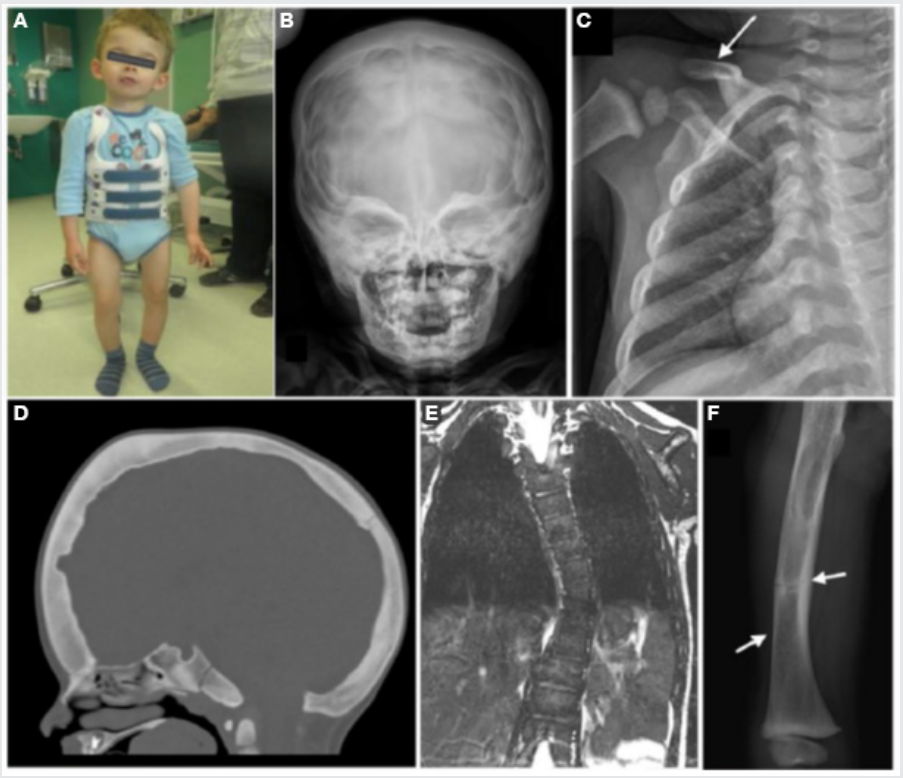

FIGURE 2. A child with a complex skeletal dysplasia caused by a neomorphic variant in SP7

Exome sequencing was performed in each proband and both unaffected parents. We used a burden-testing approach to compare the number of candidate variants in the two groups. First, we assessed the frequency of rare, predicted pathogenic variants in 42 genes previously reported to be associated with pituitary gland development. The average number of variants per individual was greater in probands with congenital hypopituitarism than those with non-familial short stature. Similarly, the number of probands with at least one variant in a pituitary-associated gene was greater in congenital hypopituitarism than in non-familial short stature. Second, we assessed the frequency of rare, predicted pathogenic variants in any protein-coding gene in the genome (to capture undiscovered causes) that were inherited in a fashion that could explain the sporadic occurrence of the probands' condition (congenital hypopituitarism or non-familial short stature) with a monogenic etiology (*de novo* mutation, autosomal recessive, or X-linked recessive). There were fewer monogenic candidates in probands with congenital hypopituitarism than those with non-familial short stature. Our findings provide evidence that the etiology of sporadic congenital hypopituitarism has a major genetic component but is infrequently monogenic with full penetrance, suggesting that the disorder often has a more complex etiology [Reference 2].

We also recently studied a child with a complex skeletal dysplasia, which included severe scoliosis, thickened calvarium, craniosynostosis, osteosclerosis of the clavicles and spine, and recurring fractures in the lower extremities (Figure 2). Some regions of the skeleton showed increased bone density and others showed decreased bone density. The number of osteoblasts was elevated but bone mineralization was impaired. We found that the disorder was caused by a *de novo* mutation in *SP7*, a gene also known as osterix. *SP7* encodes a transcription factor required for differentiation of osteoblasts, a cell type required for bone formation.

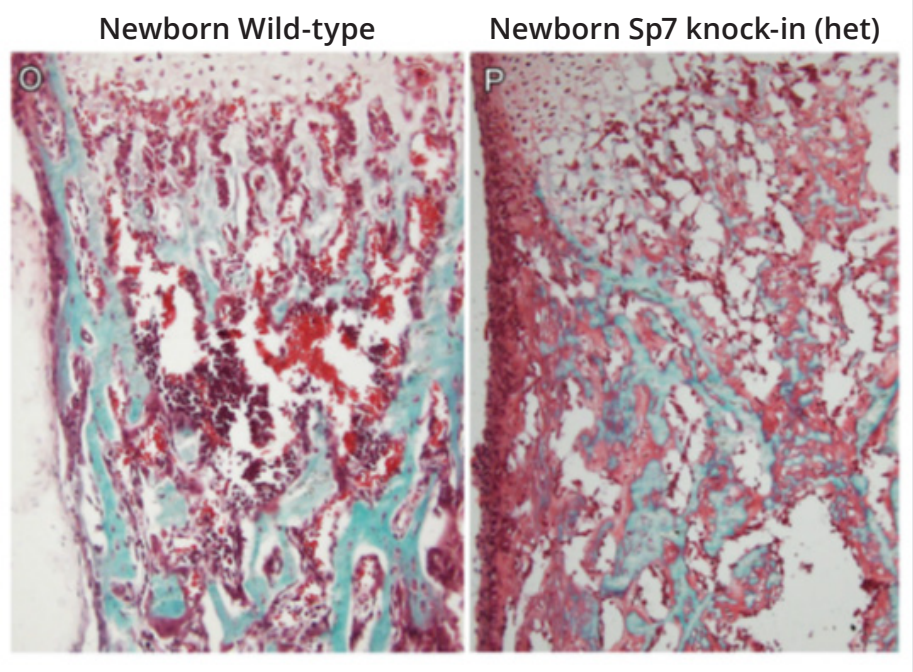

FIGURE 3. Bone histology from mice with an Sp7 mutation that is orthologous to the mutation found in the study subject

The histology reveals a complex skeletal phenotype.

Previously, recessive loss-of-function sequence variants in *SP7* were identified as a cause of osteogenesis imperfecta in which bone formation is markedly diminished, a phenotype strikingly different from that of the subject we studied. We generated mice with a variant orthologous to that of our subject. The mice showed a similar complex skeletal phenotype, confirming that the variant was pathogenic (Figure 3). The mutation shifted the DNA–binding specificity of SP7 from AT-rich motifs to a GC–consensus sequence (typical of other SP family members), resulting in an aberrant gene expression profile and abnormal osteoblast differentiation (Figure 4). Our study identifies a novel pathogenic mechanism in which a mutation in a transcription factor shifts DNA–binding specificity and provides the first *in vivo* evidence that the affinity of SP7 for AT–rich motifs, unique among SP proteins, is critical for normal osteoblast differentiation [Reference 3].

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

Our group also studies the fundamental mechanisms governing skeletal growth. Much of our work has focused on the growth plate, a thin layer of cartilage which is responsible for bone growth in children and therefore for height gain. Growth at the growth plate is controlled by many interacting regulatory systems, involving endocrine, paracrine, extracellular matrix-related, and intracellular pathways. Previously, our group studied growth-plate regulation by FGFs (fibroblast growth factors), BMPs (bone morphometric proteins), C-type natriuretic peptide, retinoids, WNTs (growth factors), PTHrP/IHH (signaling pathway that regulates chondrocyte differentiation), IGFs (insulin-like growth factors), estrogens, glucocorticoids, and microRNAs. More recently, we showed evidence that SOX9, a transcription factor, regulates the trans-differentiation of growth-plate chondrocytes into osteoblasts.

We also investigated the mechanisms that cause bone growth to occur rapidly in early life but then to progressively slow with age and eventually cease. We found evidence that the developmental program

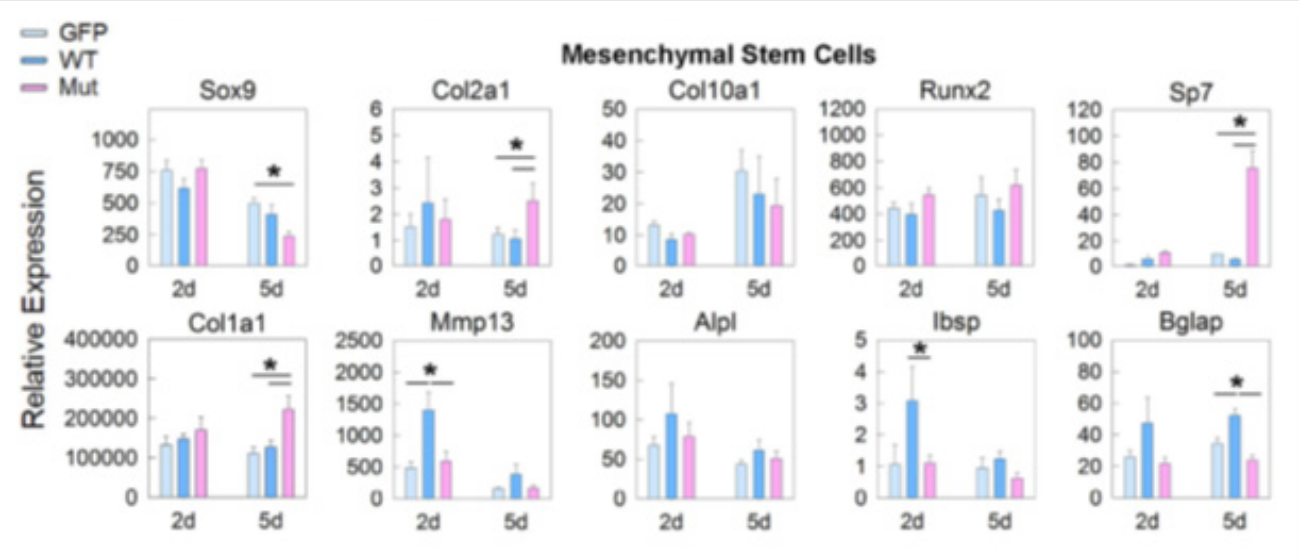

FIGURE 4. The pathogenic SP7 variant produces an aberrant gene expression profile when expressed in mesenchymal stem cells.

responsible for the decline in growth-plate function plays out more slowly in larger bones than in smaller bones and that such differential aging contributes to the disparities in bone length and therefore to establishing normal mammalian skeletal proportions [Reference 4].

We also studied the role of EZH2 in the growth plate. EZH2 encodes a histone methyltransferase that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27), which serves as an epigenetic signal for chromatin condensation and transcriptional repression. We found that loss of EZH2 and its paralog EZH1 in mice impairs bone growth, and we explored the cellular and molecular mechanisms involved. Although loss of EZH1/2 impairs growth, some heterozygous missense variants in this gene cause the Weaver overgrowth syndrome. We found that the variants responsible for the Weaver syndrome cause a partial loss of function with reduced histone methyltransferase activity. We created a mouse model that showed mild overgrowth, recapitulating the Weaver phenotype. Thus, our findings demonstrate that the Weaver syndrome is the result of EZH2 variants that cause a partial loss of function.

New treatment approaches for growth plate disorders

Recombinant human growth hormone (GH) is commonly used to treat short stature in children. However, GH treatment has limited efficacy, particularly in severe, non-GH–deficient conditions such as chondrodysplasias, and has off-target effects. Systemic insulin-like growth factor-1 (IGF-1) treatment has similar deficiencies. There are many endocrine and paracrine factors that promote chondrogenesis at the growth plate, which could potentially be used to treat such disorders. Targeting these growth factors specifically to the growth plate might augment the therapeutic skeletal effect while diminishing undesirable effects on non-target tissues. To develop growth plate–targeted therapy, we previously used yeast display to identify single-chain human antibody fragments that bind to cartilage with high affinity and specificity. As a first test of this approach, we created fusion proteins combining the cartilage-targeting antibody fragments with IGF-1, an endocrine/

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paracrine factor that positively regulates chondrogenesis. Such 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 GH–deficient mouse model, we found that subcutaneous injections of the fusion proteins increased growth-plate height without increasing proliferation in kidney cortical cells, demonstrating greater on-target efficacy at the growth plate and less off-target effect on the kidney than IGF-1 alone. Our findings provide proof of principle that targeting therapeutics to growth-plate cartilage can potentially improve treatment for childhood growth disorders [Reference 5].

We are currently applying this approach to target other chondrogenic endocrine and paracrine factors to the growth plate. We are exploring the utility of the approach both to stimulate growth-plate chondrogenesis non-specifically and also to reverse specific genetic defects in growth-plate function by modulating the abnormal molecular pathway responsible for the growth failure.

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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. Specifically, we are interested in how tissue microstructure, hierarchical organization, composition, and material properties all work together to affect biological function or dysfunction. We investigate biological and physical model systems at various length and time scales, performing biophysical measurements and developing novel physical/mathematical models (including molecular dynamics [MD] and continuum models) to explain their functional properties and behavior. Experimentally, we often use water to probe tissue structure and function from nanometers to centimeters and from microseconds to lifetimes. Our armamentarium includes atomic force microscopy (AFM), small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), static light scattering (SLS), dynamic light scattering (DLS), osmometry, and multi-dimensional nuclear magnetic resonance (NMR) relaxometry and diffusometry. A goal is to develop understanding and tools that can be translated from bench-based quantitative methodologies to the bedside to aid in diagnosis and therapy.

These 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, exchange rates, etc., rather than the qualitative 'weighted' MR images conventionally used in radiology. At a basic level, our work is directed toward making critical 'invisible' biological structures and processes 'visible.' Our quantitative imaging group uses their knowledge of physics, engineering, applied mathematics, imaging and computer sciences, as well as key insights gleaned from our tissue-sciences research, to discover and develop novel quantitative imaging biomarkers that can detect changes in tissue composition, microstructure, and/or microdynamics with high sensitivity and specificity. The ultimate translational goal is to assess normal and abnormal developmental trajectories, diagnose childhood diseases and disorders, and characterize degeneration and trauma (such as mild traumatic brain injury). Primarily, we use MRI as our imaging modality of choice because it is so well suited to many applications critical to the NICHD mission; it is non-invasive, non-ionizing, usually requires no exogenous contrast agents or dyes, and is generally

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(continued)

deemed safe and effective for use with mothers, fetuses, and children in both clinical and research settings.

One of our technical translational goals has been to transform clinical MRI scanners into scientific instruments capable of producing reproducible, accurate, and precise imaging data with which to measure and map useful imaging biomarkers 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. From a more basic perspective, another goal has been to apply our various MRI tools and methodologies to advance neuroscience, providing new methods to explore brain structure/function relationships, such as the human connectome.

Jackson Foundation and the Center for Neuroscience and Regenerative Medicine

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Rea Ravin, PhD, *Contract Scientist*

In vivo MRI histology

The most mature *in vivo* MRI histological technology that we invented, developed, and clinically translated is Diffusion Tensor MRI (DTI), by which we measure and map *D*, a diffusion tensor of water within an imaging volume. Information derived from this quantity includes white-matter fiber-tract orientation, the orientationally averaged mean apparent diffusion constant (mADC), and other intrinsic scalar (invariant) quantities. Such imaging parameters have been used by radiologists and neuroscientists as non-invasive quantitative histological 'stains,' MRI images that are obtained by probing endogenous tissue water *in vivo* without requiring any exogenous contrast agents or dyes. The mADC is the most widely used diffusion imaging parameter in neuroradiology to identify ischemic areas in the brain during acute stroke and to follow cancer patients' responses to therapy. The measures of diffusion anisotropy we first proposed (e.g., the fractional anisotropy or FA) are also widely used to follow changes in normally and abnormally developing white matter and in many other clinical and neuroscience applications, such as 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, which is used brain track white-matter fibers to help establish 'anatomical connectivity' and by neuroradiologists and neurosurgeons to plan surgical procedures so that they can spare 'eloquent' areas of the brain, advances that helped inspire several large federally-funded research initiatives, including the NIH Human Connectome Project (HCP) and, more recently, the NIH Brain Initiative.

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 (also known as 'microstructure imaging'), which otherwise could only be assessed using laborious *ex vivo* histological or pathological 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, an approach that can detect 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' or biomarkers to measure and map. We also developed CHARMED MRI, which measures the average axon diameter (AAD),

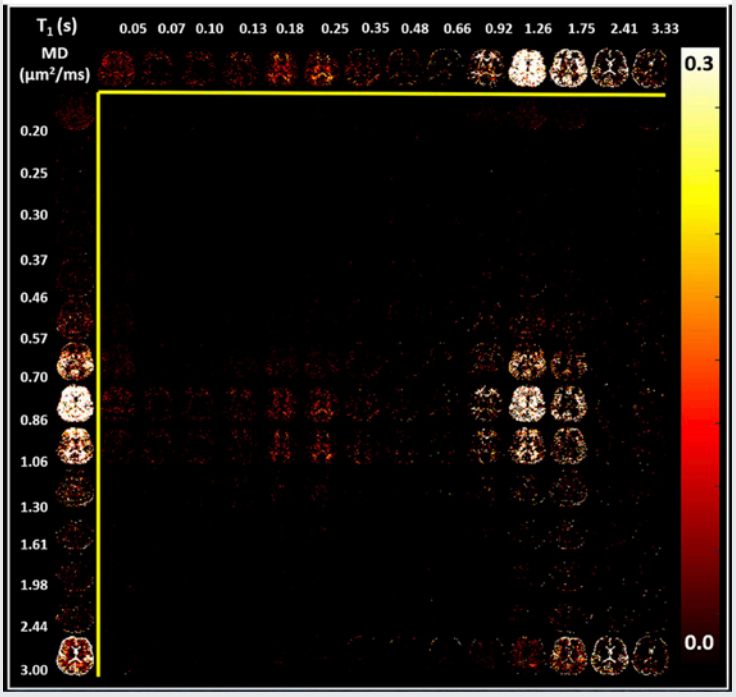


FIGURE 1. Maps of an estimated T_1–MD (mean diffusivity) 2-D spectrum obtained in human brain *in vivo* in a clinically feasible 20-minute MRI scan

Top row. The corresponding 1-D marginal distribution of T_1 (nuclear spin relaxation time) values.

Left column. The corresponding 1-D marginal distribution of MD values. The high-dimensional relaxometry-diffusometry imaging approach allows us to identify and characterize the state of different and distinct water pools within each voxel, rather than report only their voxel-averaged values, which has traditionally been done. One can see various water compartments, such as high T_1 and high MD, corresponding to cerebral spinal fluid; intermediate T_1 and MD, corresponding to gray and white matter parenchymal tissue; and short T_1 and intermediate MD, corresponding to white matter. This new method offers great promise for probing different tissue components occupying the voxel on a whole-brain basis to try to detect normal and abnormal development, degeneration, or trauma.

and AxCaliber MRI, which measures the axon-diameter distribution (ADD) along white-matter pathways, and we reported the first *in vivo* measurement of ADDs within the rodent corpus callosum. The ADD is functionally important, given that axon diameter is a critical determinant of conduction velocity and therefore the rate at which information travels along axon bundles, and helps determine the latencies or time delays between and among different brain areas. This led us to propose a novel MRI–based method to measure the 'latency connectome,' including a latency matrix that reports conduction times between different brain areas, and to develop 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 developed novel multiple pulsed-field gradient (mPFG) methods and demonstrated their feasibility *in vivo* on conventional clinical MRI scanners as a further means to extract quantitative features in the central nervous system (CNS), such as 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 non-invasive, *in vivo* methods to measure unique features of cortical gray-matter microstructure and architecture that are visible in electron microscopy (EM) applications but currently invisible in conventional MRI. One example is diffusion tensor distribution (DTD) MRI, in which we use our normal tensor-variate distribution to characterize heterogeneities in complex tissue structure. One of our long-term goals is to 'parcellate' or segment the cerebral cortex *in vivo* into its approximately 500 distinct cyto-architechtonic areas using non-invasive imaging methods. To this end, we are developing advanced MRI sequences to probe correlations among microscopic displacements of water molecules in the cortex as well as sophisticated

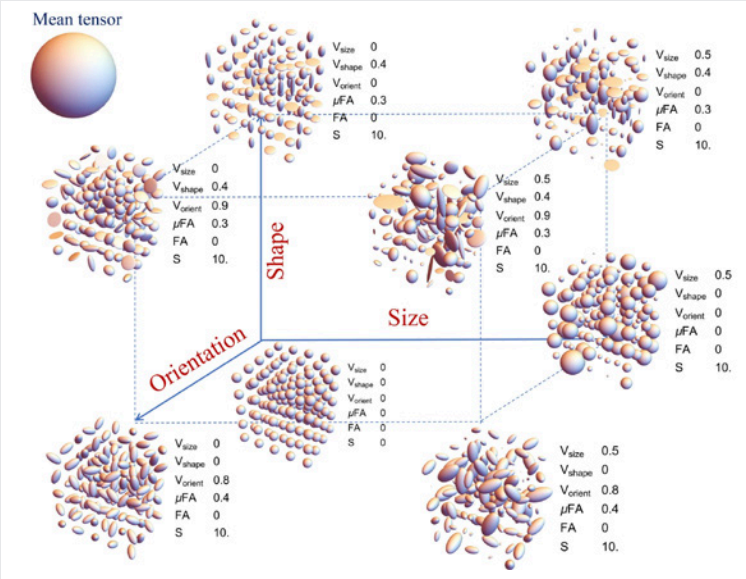


FIGURE 2. Diffusion Tensor Distribution (DTD) MRI resolves microscopic heterogeneity in brain tissue.

A key issue we addressed is that diffusion tensor MRI (DTI) can produce the same tensor estimates in different voxels that may have vastly different underlying tissue microstructures. In order to resolve this problem, we developed various new parametric quantitative imaging 'stains' to depict the shape, size, and orientation heterogeneity of the underlying distribution of diffusion tensors within a voxel reflecting different microstructural motifs. These are shown here for a macroscopically isotropic mean diffusion tensor (shown as a sphere in the upper left corner). The quantities are derived from a new imaging method we developed to measure or estimate a DTD in each voxel. Values of the various stains are shown as points in the 3D grid. Size axis refers to heterogeneity in the trace or mean diffusivity of the diffusion tensors; the shape axis refers to heterogeneity in the shapes of the diffusion tensors (i.e., oblate, prolate, spherical); and the orientation axis refers to the heterogeneity vs. coherence of the orientations of the diffusion tensors. V_{size}, stain quantifying the variation in the size (i.e., trace) of the diffusion tensors; V_{shape}, stain quantifying the variation in the shape of diffusion tensors; V_{orient}, stain quantifying the orientation dispersion of diffusion tensors. The micro Fractional Anisotropy, micro FA, and macro FA obtained from DTI are also shown, as well as S, the overall entropy of the DTD, as a measure of tissue complexity.

mathematical models to infer distinguishing microstructural and morphological features of cortical gray matter. One promising avenue is to use multi-dimensional MRI relaxometry and diffusometry-based methods to study water mobility and exchange in gray and white matter, enabling us to probe individual water pools or compartments that are too small to observe using conventional MRI. We continue to work to translate these and other methods to the clinic to help identify changes in normal and abnormal development, as well as in inflammation and trauma.

Quantitative MRI biomarker development for pediatric and fetal imaging applications

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. The scope of conventional MRI clinical applications is limited to revealing either gross morphological features or focal abnormalities. Clinical MRI also often lacks the biological specificity 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 measures and maps meaningful intrinsic physical quantities or chemical variables that possess physical units and can be compared among different tissue regions. 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 against normative values obtained from a healthy population. Quantitative MRI methods should continue to advance 'precision imaging' studies, in which MRI phenotypic and genotypic data can be meaningfully incorporated and used for improved diagnosis and prognosis assessments.

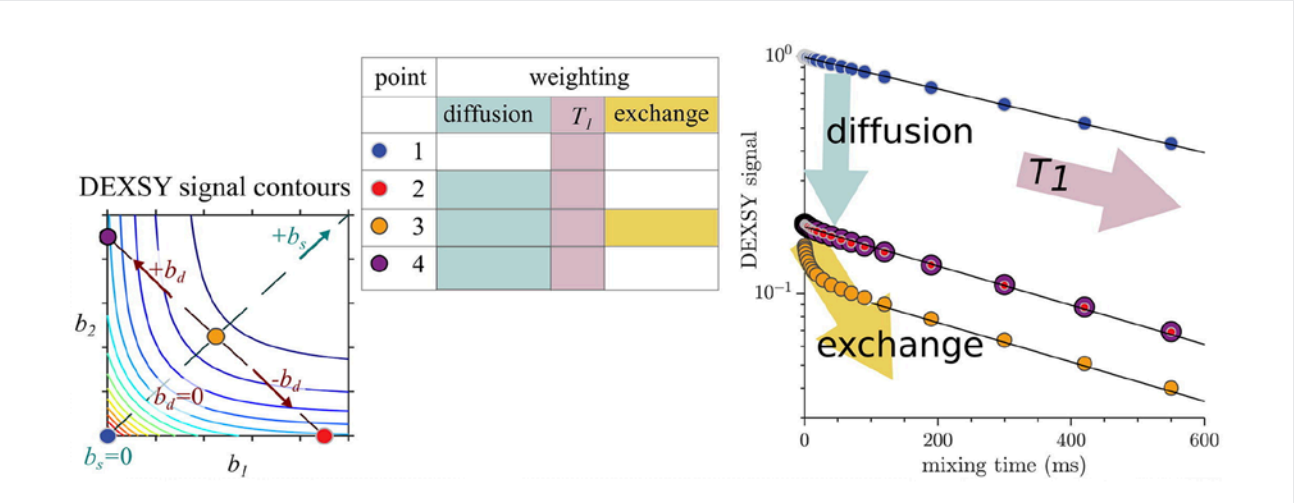


FIGURE 3. Deconflating effects of restriction and exchange in diffusion MR data

We developed a novel method for acquiring diffusion exchange spectroscopy (DEXSY) MR data, and a novel use as a potential *in vivo* functional imaging (fMRI) tool, an innovation that required (a) vastly speeding the MR DEXSY signal acquisition and (b) being able to disambiguate the effects of two key processes previously conflated in the analysis of DEXSY data: restriction or confinement of water in pools or compartments, and exchange between different water compartments.

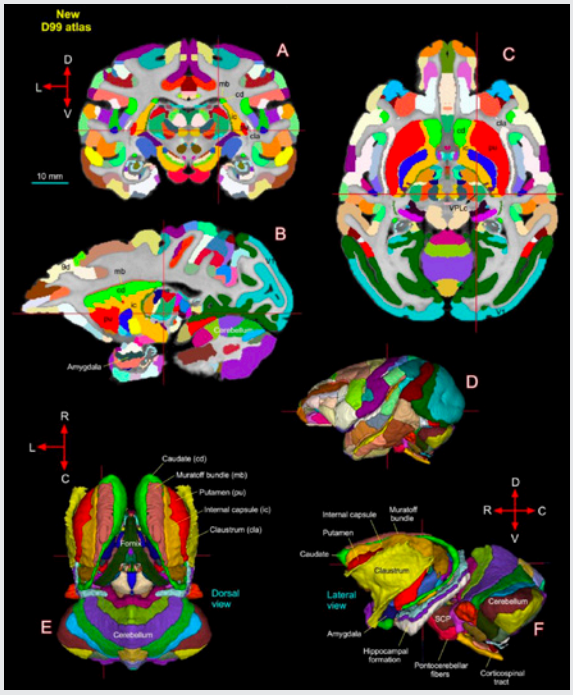
To advance our quantitative imaging activities, we developed numerical and statistical methods, including algorithms that generate a continuous, smooth approximation to the discrete, noisy, measured DTI field data so as to reduce noise and which allowed us to implement Streamline Tractography. We proposed a novel Gaussian distribution for the tensor-valued random variables that we use to design optimal DTI experiments and interpret their results. In tandem, we developed non-parametric empirical (e.g., Bootstrap) methods to determine the statistical distribution of DTI–derived quantities in order to study, e.g., the inherent variability and reliability of computed white-matter trajectories, enabling us to apply powerful hypothesis tests to assess the statistical significance of findings in a wide range of important biological and clinical applications that had been tested using *ad hoc* statistical methods. We are also developing novel methods to register different brain volumes and to generate group-average DTI data or atlases from various subject populations based on the Kullback-Leibler divergence.

Previously, we carried out clinical studies that utilize novel quantitative MRI acquisition and analysis methods and whose aim is to improve accuracy and reproducibility of diagnosis and to detect and follow normal and abnormal development. One early example is the NIH Study of Normal Brain Development, jointly sponsored by the NICHD, NIMH, NINDS, and NIDA, which was initiated in 1998 and intended to advance our understanding of normal brain development in typical healthy children and adolescents. The *Brain Development Cooperative Group* is still actively publishing papers, primarily by mining the rich high-quality MRI data, many of which our lab processed serving as the DTI Data-Processing Center (DPC). The processed DTI data collected from the project were uploaded into a database and made publicly available through the *National Database for Autism Research* (NDAR). Our former colleague Carlo Pierpaoli, who spearheaded

FIGURE 4. New macaque brain atlas uses Mean Apparent Propagator (MAP) MRI to improve delineation of different brain areas and structures.

A–C. New D99 digital macaque atlas (version 2.0) with combined cortical and subcortical segmentation overlaid on the coronal, sagittal, and horizontal D99 *ex vivo* MRI template, respectively. The cross-hairs in *A–C* show the same location of thalamic subregion VPLc (ventral posterior lateral caudal nucleus). The corresponding anteroposterior location of VPLc is also shown with the D99 rendered brain volume in *D*.

E–F. The spatial location of segmented subcortical regions shown on the dorsal and lateral views in 3D. The selected subcortical regions in *E–F* are also indicated with cortical areas in A–C. 9d, dorsal prefrontal area; SCP, superior cerebellar peduncle; V1, primary visual cortex. D, dorsal; V, ventral; R, rostral; C, caudal; L, lateral. Scale bar: 10 mm, applies to *A–C* only.

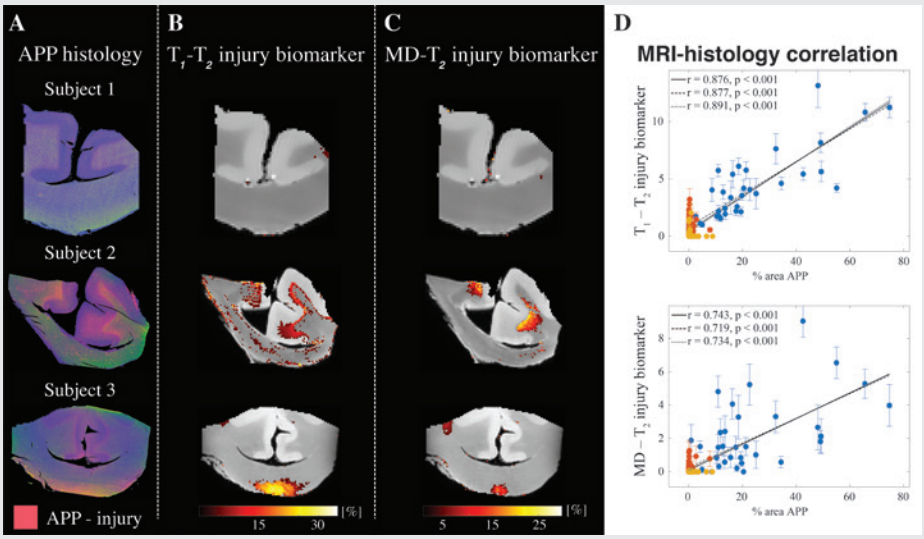


this work, continues to support, update, and disseminate the processing and analysis software called "TORTOISE," which grew out of this effort and which can be downloaded from http://www.tortoisedti.org.

Traumatic Brain Injury (TBI) represents a significant public health challenge for our pediatric population, but also for our young men and women who serve in the military. Our involvement in TBI research, particularly in detecting mild TBI (mTBI), has continued to expand through partnerships with various Department of Defense (DoD) entities. Diffusion MRI (dMRI) provides essential information to aid in the assessment of TBI, but conventional dMRI methods have lacked sufficient specificity. To improve the accuracy and reproducibility of MAP–MRI findings, we developed a data–processing pipeline, and, in collaboration with scientists at the DoD Center for Neuroscience and Regenerative Medicine (CNRM), performed the first normative MAP–MRI studies, and applied this new and powerful method to detect tissue damage in brains of individuals who have suffered TBI, extending our NICHD TORTOISE pipeline to be able to analyze MAP–MRI data. We are now employing multi-dimensional MRI relaxometry-diffusometry methods to study the etiology of various types of TBI, in collaboration with the USUHS Neuropathology Research Division and under the auspices of the CNRM, and to improve the correlation and integration of neuropathology and neuroradiological imaging data to speed the deployment of new MRI methods to assess various forms of TBI. We have also partnered with CNRM to study ways to measure very slow flows that occur during glymphatic transport, a mechanism the brain uses to wash away harmful macromolecules, just as the lymphatic system uses in other organs. With our partners at the University of Arizona, this research is providing experimental data to enable us migrate these imaging approaches to the clinical, to be able to assess normal and pathological glymphatic transport *in vivo*.

We are also collaborating with Sara Inati, who studies focal epilepsy, a devastating disorder that is difficult to detect using conventional neuroradiological methods. We are developing and testing various new MRI–

FIGURE 5. Potential MRI biomarker for TBI tested in a neuropathology brain specimen



The figure suggests that MRI can now be used to detect sequelae of Traumatic Axonal Injury in neuropathological brain tissue specimens. Histological images juxtaposed with multidimensional MRI–derived injury biomarker maps of three representative TBI (traumatic axonal injury) cases (subjects 1, 2, and 3 are control and two TBI cases, respectively).

A. Deconvolved histological amyloid precursor protein (APP) images (co-registered with MR images) are shown on the left panel, *red* = APP.

B. T_1–T_2 and *C* Mean Diffusivity (MD)–T_2 multidimensional injury maps show absence of significant injury, substantial injury along the white-gray matter interface, and substantial injury at the base of the corpus callosum, for subjects 1 to 3, respectively. All multidimensional injury maps were thresholded at 10% of the maximal intensity and overlaid on grayscale proton-density images.

D. APP density (% area) from 132 tissue regions in 11 different cases, consisting of 4 APP–positive regions from each TBI case (total of 32, *blue dots*), 4 to 6 normal-appearing white matter (WM) regions from all cases (total of 56, *red dots*), and 4 cortical grey matter (GM) regions from all cases (total of 44, *yellow dots*), and the corresponding MR parameter correlations. Individual data points represent the mean ROI value from each post-mortem tissue sample. Scatterplots of the mean (with 95% confidence interval error bars) % area APP and T_1–T_2 (*top*) or MD-T_2 (*bottom*).

based methods that we believe may reveal pathological microstructural features and changes in architectural organization of the brain in this disorder, for example, in cortical dysplasia, to improve localization and assessment of cortical lesions.

We have been partnering with our NICHD colleagues Roberto Romero and Mark Haacke to develop novel fetal MR imaging applications. Currently, it is challenging to measure quantitative imaging biomarkers *in utero*, particularly diffusion MRI–based biomarkers, owing to large-scale fetal and maternal motion during the scanning session, and to the difficulty in acquiring image volumes with sufficient coverage, quality, and spatial resolution in a clinically feasible amount of time. Our lab has been developing novel approaches to address these critical issues.

Biopolymer physics: water–ion–biopolymer interactions

Remarkably, despite their crucial role, little is known about the physical underpinnings of water-ion-biopolymer interactions, particularly in the physiological ionic strength regime. To determine the effect of ions on the structure and dynamics of key biopolymers, we developed a multi-scale experimental framework by combining macroscopic techniques (osmotic swelling-pressure measurements and mechanical measurements) with high-resolution scattering methods (e.g., SANS and SAXS) that 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 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 carried out on well defined model systems that mimic essential features of tissues provide important insights that cannot be obtained from experimental studies made on biological systems themselves. Mathematical models based on well established polymer-physics concepts and, more recently, molecular dynamics (MD) simulation approaches make it possible to design experiments, to help us quantify and explain aspects of tissue behavior and thus the underlying molecular and macroscopic mechanisms that govern key aspects of a tissue's normal functional properties.

We produced numerous novel biomimetic MRI phantoms, including diffusion MRI phantoms, which we use to calibrate scanners, specifically to assure the quality and fidelity of the imaging data in single-subject, longitudinal, and multi-site studies. For instance, our U.S. Patent for a 'Phantom for diffusion MRI imaging' is now enabling quantitative diffusion MRI studies to be performed at many different sites. Our colleagues at NIST Boulder, Colorado, have incorporated our polyvinylpyrrolidone (PVP) polymer into their own diffusion MRI NIST standard (*https://www.nist.gov/programs-projects/quantitative-mri*). We also developed a variety of NMR and MRI phantoms, such as 3-D printed polymer phantoms, that possess various salient features of cell or tissue systems, providing data with which to test the validity of our models and experimental designs, for instance in the testing of methods like our 'AxCaliber MRI,' which measures axon diameter distributions (ADD) in white matter pathways *in vivo*.

Measuring and mapping functional properties of extracellular matrix (ECM)

We study interactions among the main extracellular matrix (ECM) components, often using cartilage as a model system because it is aneural and avascular. In cartilage ECM, collagen (type II) is organized into fiber bundles that form a network that entraps the major proteoglycan (PG), a bottlebrush-shaped aggrecan. 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, and AFM. Understanding the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential to predicting its load-bearing ability, which is mainly governed by osmotic and electrostatic forces. To quantify the effect of hydration on cartilage properties, we previously developed a novel tissue micro-osmometer to perform precise and rapid measurements on small tissue samples (less than 1 microgram) as a function of the equilibrium water activity (vapor pressure). We also make osmotic pressure measurements to determine how the individual components of cartilage ECM (e.g., aggrecan and collagen) contribute to the total load-bearing capacity of the tissue. We also demonstrated that aggrecan-hyaluronic aggregates self-assemble into microgels, contributing to improved dimensional stability of the tissue and its lubricating ability. We also found that aggrecan is highly insensitive to changes in the ionic environment, particularly to calcium ions, which is critical for maintaining the tissue's mechanical integrity and allowing aggrecan to serve as a calcium ion reservoir in cartilage and bone.

To model cartilage ECM, we invented and developed a new biomimetic composite material consisting of polyacrylic acid (PAA) microgel particles dispersed and embedded within a polyvinyl alcohol (PVA) gel matrix.

PAA mimics the proteoglycan (i.e., hyaluronic-aggrecan complexes), while PVA mimics the fibrous collagen network entrapping them. Remarkably, the PVA/PAA biomimetic model system reproduces not only the shape of the cartilage swelling pressure curves, but also the numerical stiffness values reported for healthy and osteoarthritic human cartilage samples. Studies on these model composite hydrogels should continue to yield invaluable insights into the effects of various macromolecular factors (matrix stiffness, swelling pressure, fixed-charge density, etc.) on the tissue's macroscopic mechanical/swelling properties, and ultimately its remarkable load-bearing and lubricating abilities, and their loss in various diseases and disorders, including osteoarthritis.

We are now attempting to translate our understanding of the structure-function relationships of ECM components to develop and design novel non-invasive MRI 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 to follow normal and abnormal ECM development, which entails making 'invisible' components of ECM, (e.g., collagen and PGs) 'visible' to predict functional properties of the composite tissue, such as its load-bearing ability. One major obstacle is that protons bound to immobile species (e.g., collagen) are largely invisible with conventional MRI methods. However, magnetization exchange (MEX) MRI (as well as other related methods) make it possible to detect the bound protons indirectly by transferring their magnetization to the abundant free water protons surrounding them. It also enables us to estimate collagen content in tissue quantitatively. In pilot studies with Uzi Eliav (deceased) 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 were qualitatively consistent with those obtained by histological techniques, such as high-definition infrared (HDIRI) spectroscopic imaging. Our novel approach has the potential to map tissue structure and functional properties *in vivo* and non-invasively. We are now developing molecular dynamics (MD)–based models of cartilage and cartilage ECM analogs in order to interpret our experimental findings, develop and test novel hypotheses, and predict the behavior of our model system under different experimental conditions.

We also recently began using several novel one-sided NMR methodologies to study water relaxation, diffusion, and exchange behaviors in ECM as a means to characterize its critical functional properties. Velencia Witherspoon has been using these approaches to study the organization and structure of fascia. Our specialized NMR scanner/profilers are ideally suited to these tasks, as they can probe layered media, such as cartilage and fascia, using ultra-thin slices, almost as thin as a confocal microscope provides.

Patents

1. Benjamini D, Basser PJ. Determination of a Joint Probability Distribution of Radius and Length of Anisotropic Pores from Double Pulsed Field Gradient MRI Data. Patent number: 10871539. Filed: November 24, 2015; Date of Patent: December 22, 2020
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Additional Funding

- "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
- "Neuroradiology/Neuropathology Correlation/Integration Core." 309698-4.01-65310, (CNRM-89-9921)

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Biophysics of Large Membrane Channels

The Section studies mechanisms of channel-facilitated transport across cell and organelle membranes by combining experiments on channel reconstitution into planar lipid membranes with physical theory, allowing for both qualitative and quantitative understanding of the transport processes. We focus mostly on large, metabolite-transporting channels such as the VDAC (voltage-dependent anion channel) from the outer membrane of mitochondria, OmpF (general bacterial porin) from *Escherichia coli*, LamB (sugar-specific bacterial porin) from *Escherichia coli*, OprF (porin from *Pseudomonas aeruginosa*), MspA (major outer-membrane porin from *Mycobacterium smegmatis*), alpha-Hemolysin (toxin from *Staphylococcus aureus*), and translocation pores of *Bacillus anthracis* (PA63), *Clostridium botulinum* (C2IIa), and *Clostridium perfringens* (Ib) binary toxins, Epsilon toxin (from *Clostridium perfringens*), Alamethicin (amphiphilic peptide toxin from *Trichoderma viride*), Syringomycin E (lipopeptide toxin from *Pseudomonas syringae*), and the bacterial peptide TisB involved in persister cell formation. We also use Gramicidin A (linear pentadecapeptide from *Bacillus brevis*) as a molecular sensor of membrane mechanical properties.

To investigate these mostly beta-barrel channels under precisely controlled conditions, we first isolate the channel-forming proteins from their host organisms, or use recombinant proteins, and then reconstitute them into planar lipid membranes. Healthy cell functioning and development require effective communication between cells and cell organelles, which is facilitated by membrane channels. Our main goal is to elucidate the physical principles of the regulation of such channels under normal and pathological conditions. Among many wet-lab approaches, such as fluorescence correlation spectroscopy, bilayer overtone analysis, and confocal microscopy, single-channel electrophysiology is our hallmark technique that allows us to study transport processes at the single-molecule level. In our latest projects, we combine biophysical methods with those of cell biology. Empirical findings obtained in experiments are rationalized within the 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.

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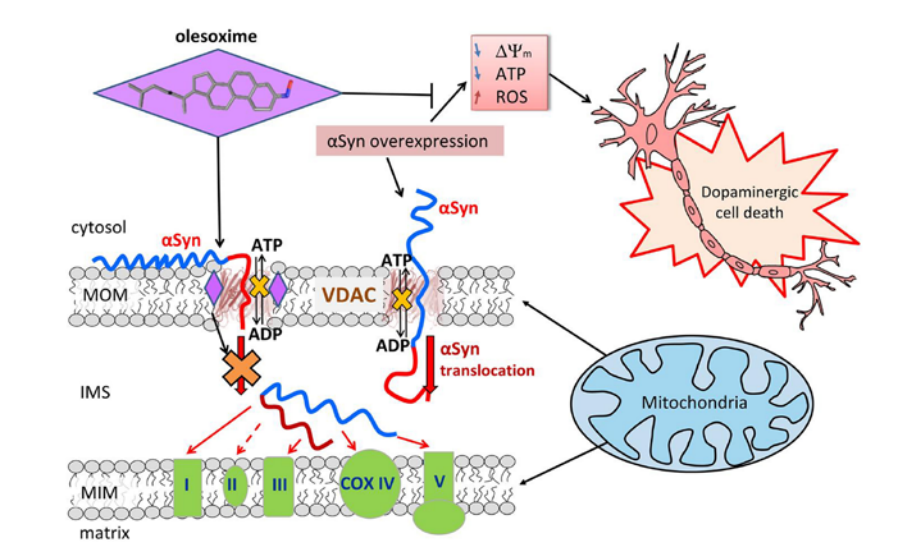
FIGURE 1. Proposed model of the olesoxime neuroprotective effect



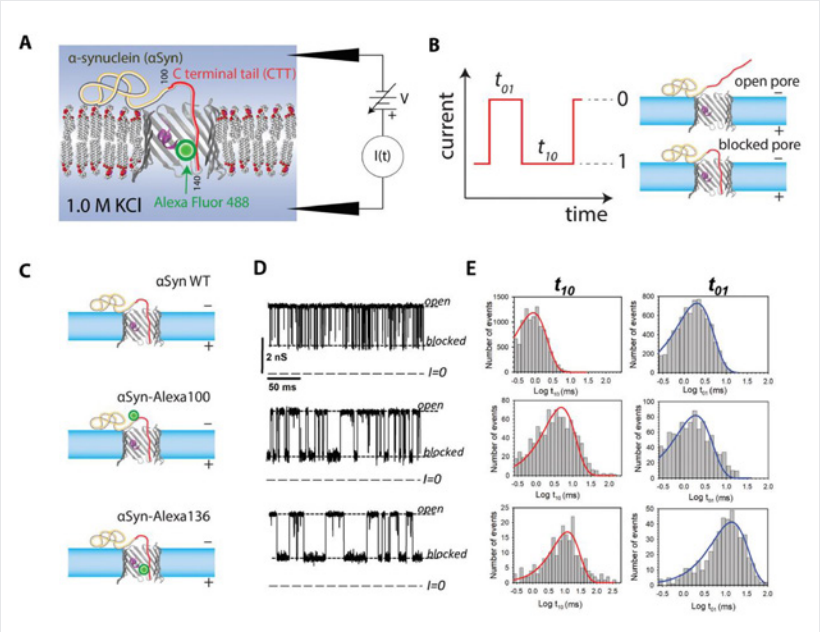
When alpha-synuclein (αSyn) is captured by the VDAC pore, it disrupts ATP/ADP fluxes through the VDAC. Under normal conditions, endogenous αSyn regulates the fluxes by reversibly and dynamically blocking the VDAC. Stress resulting from αSyn overexpression during Parkinson's disease induces αSyn translocation across the mitochondrial outer membrane (MOM) via the VDAC and targets electron transport chain complexes (ETC) in the inner membrane (MIM), causing their impairment, mitochondrial dysfunction, and eventually neuronal cell death. Olesoxime partitions into the MOM and hinders αSyn translocation through the VDAC by interacting with the pore-lipid interface. The model suggests a tentative mechanism of olesoxime protection of mitochondria integrity and promotion of neuronal cell survival.

Molecular mechanism of neuro-protective drug action through targeting alpha-synuclein interaction with the mitochondrial VDAC

The second most common neuro-degenerative disorder in the U.S. is Parkinson's disease (PD), a neurological condition that causes severe movement problems. It is well known that the disease is associated with abnormal accumulation of a protein called alpha-synuclein, which is naturally produced in healthy neurons. Previous studies have shown that, when alpha-synuclein builds up in cells, it somehow moves through the mitochondrial outer membrane and targets respiratory complexes at the inner membrane, causing mitochondrial dysfunction. However, the gateway that alpha-synuclein uses to enter mitochondria has long been a mystery. A hint to this puzzle appeared several years ago when our lab, using *in vitro* experiments with reconstituted mitochondrial VDACs, found that alpha-synuclein can translocate through this channel. This year, working in collaboration with Parkinson's disease researchers in the lab of Mark Cookson, we showed that the VDAC is indeed a pathway for alpha-synuclein translocation into the mitochondria in cells. In this work, we studied effects of a cholesterol-like synthetic compound called olesoxime, which was previously shown to defend mitochondria from the alpha-synuclein–induced toxicity [Reference 1]. Olesoxime generated considerable interest for its ability to protect neurons and mice in a range of neuro-degenerative conditions, including PD. It was also established that olesoxime binds to VDACs in cells.

Following our interest in alpha-synuclein interaction with the VDAC, we undertook to understand whether the molecular mechanism of olesoxime neuro-protection involves this interaction. Using neuronally differentiated human cells overexpressing wild-type alpha-synuclein as a cell model of PD, we found that olesoxime inhibits alpha-synuclein translocation into mitochondria. By applying a set of complementary electrophysiological and biophysical approaches, we provided mechanistic insights into the interplay between alpha-synuclein, the VDAC, and olesoxime. Consistent with past studies, we found that overexpressed alpha-synuclein induces cell

FIGURE 2. Probing alpha-synuclein modifications with a VDAC nanopore



A. Experimental setup (not to scale): the acidic C-terminal tail (CTT) of membrane-bound αSyn is drawn into the nanopore by an externally applied transmembrane voltage.

B. Definitions of open (0) and blocked (1) states; durations in each state are denoted t01 and t10, respectively.

C. Schematic of three αSyn constructs: the wild-type (WT), and αSyn labeled with Alexa Fluor 488 at residues 100 and 136, αSyn-Alexa100 and αSyn-Alexa136, respectively.

D. Experimentally observed stochastic fluctuations of single VDAC–pore conductance between open and blocked states for three αSyn constructs at −30 mV applied voltage; dashed lines indicate VDAC–open and αSyn–blocked states and zero current.

E. Corresponding to the traces in *D*, distributions of state t01 and t10 durations show quantitative differences in the kinetics of the αSyn–nanopore interaction introduced by Alexa Fluor 488 functionalization.

death and that olesoxime treatment dramatically reduced its rate by keeping mitochondria healthy. Working at the single-molecule level, from the data obtained in channel-reconstitution experiments, we deduced that olesoxime interacts with the VDAC beta-barrel at the lipid-protein interface in such a way that it hinders alpha-synuclein translocation through the VDAC pore and affects VDAC voltage gating. The findings suggest that the alpha-synuclein interaction with the VDAC is a new target for the development of drugs against alpha-synuclein toxicity. In particular, the use of molecules interacting with the alpha-synuclein–VDAC complex could be a promising and effective pharmacological treatment for a wide range of neuro-degenerative conditions, such as spinal muscular atrophy, amyotrophic lateral sclerosis, Parkinson's and Huntington's diseases, and chemotherapy side effects, aimed to decrease mitochondrial deficiencies in affected neurons.

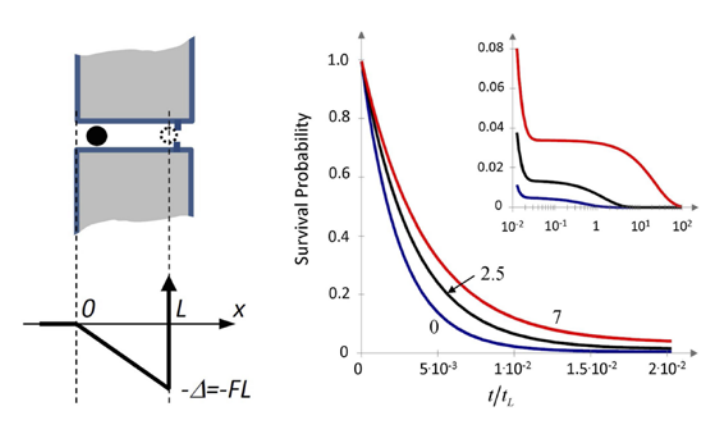
Effect of a post-translational modification mimic on protein translocation through a nanopore

Post-translational modifications (PTMs) of proteins are recognized as crucial components of cell-signaling pathways by modulating folding, altering stability, changing interactions with ligands, and therefore serving many regulatory functions. PTMs occur as covalent changes in the protein's amino acid side chains or the length and composition of their termini. Inspired by the importance of PTMs in cell signaling, this year we studied the functional consequences of PTMs for alpha-synuclein interactions with mitochondrial VDACs. We mimicked PTMs of the 140–amino-acid cytosolic protein alpha-synuclein by attaching divalent Alexa Fluor 488 to the beginning and end of its C-terminal tail. Each of these modifications increases the total negative charge of the tail by two elementary charges and introduces extra bulkiness at the modification location, significantly

FIGURE 3. Kinetics of blocker escape from a membrane channel

Left. Cartoon of a blocker in a cylindrical channel and the potential well of depth FL created for the blocker by the biasing force F>0 in the channel of length L available for the center of the blocking molecule.

Right. Survival probability of the blocker molecule in the channel S(t) at different values of the biasing force and hence the dimensionless well depth increasing from 0 to 2.5 to 7 thermal energy units (lower, middle, and upper curves, respectively) as functions of the dimensionless time; increasing bias results in a slowdown of the decay kinetics of S(t); the inset shows the long-time "tails" of the curves in a finer scale for S(t) and the logarithmic scale for the dimensionless time.



changing the dynamics of the alpha-synuclein interaction with the VDAC pore. Using single-channel reconstitution into planar lipid membranes, we found that such PTM–like modifications change interactions drastically in both the efficiency of VDAC inhibition by alpha-synuclein and its translocation through the VDAC pore. We analyzed time-resolved single-molecule events of alpha-synuclein capture by the VDAC pore within a framework of a one-dimensional diffusion model, using an interaction "quasi-potential" that incorporates mostly electrostatic and entropic components of alpha-synuclein interaction with the pore. The analysis proved to be an effective means of quantitatively describing the PTM–like modification effects on the kinetics of the capture, release, and translocation and yielded the positions of the modifications with an excellent precision of about three residues. Notably, the technique is general and can readily be applied to other biological channels and nanopores, suggesting that it could be extended to quantify populations of proteins that have undergone PTMs. Thus, in view of the recently established role of disordered charged termini of cytosolic proteins in the control of VDAC–facilitated transport, our findings establish a new mechanism of PTM–induced regulation of protein function.

Blocker escape kinetics from a membrane channel analyzed by mapping blocker diffusive dynamics onto a two-site model

Understanding the blockage of ion channels in biological membranes by natural and synthetic compounds is important from both theoretical and practical points of view. On the one hand, the phenomenon of blockage gives a rich example of the behavior of a solute molecule in strong confinement involving multiple interactions with the protein residues lining the channel walls and, in the case of a charged molecule, with the transmembrane electric field. On the other hand, its practical value is clear from the fact that about 13% of all present drugs of the world pharmacopeia act as ion-channel modifiers, with many of them working as channel blockers. This year, we developed an analytical theory of the blocker escape kinetics from the channel, assuming that a charged blocking molecule cannot pass through a constriction region (bottleneck). If the molecule spends a sufficiently long time in the channel, individual blockades in ionic current can be resolved in single-channel experiments. We focused on the effect of the external voltage bias on the blocker survival probability and characteristic times in the channel. The bias creates a potential well for the charged blocker, with the minimum located near the channel bottleneck. When the bias is strong, escape from the channel is a slow process, which allows for time-resolved

observation of individual blocking events. We performed our analysis in the framework of a two-site model of the blocker dynamics in the channel. Importantly, the rate constants, fully determining this model, were derived from a more realistic continuum diffusion model. This was done by mapping the latter onto its two-site counterpart which, while being much simpler, captures the main features of the blocker escape kinetics.

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- Colgate University award to William Rosencrans

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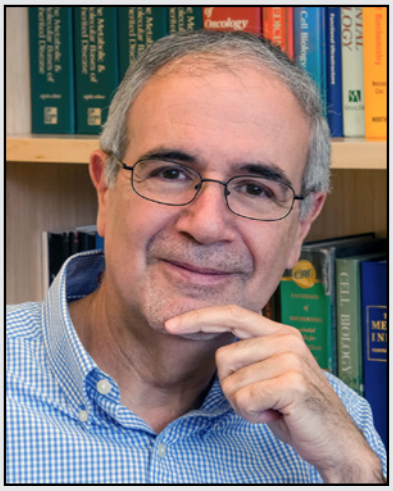
Protein Sorting in the Endomembrane System

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, lysosome-related organelles (LROs, e.g., melanosomes), and various domains of the plasma membrane in polarized cells, such as epithelial cells and neurons (Figure 1). Transport of cargo between these 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 (MT) motors and organelle adaptors that drive movement of the transport carriers and other organelles through the cytoplasm, and (3) tethering factors that promote fusion of the transport carriers to acceptor compartments. We study the machineries in the context of various intracellular transport pathways, including endocytosis, recycling to the plasma membrane, retrograde transport from endosomes to the TGN, biogenesis of lysosomes and LROs, autophagy, and polarized sorting in epithelial cells and neurons. We apply knowledge gained from this basic research to the elucidation of disease mechanisms, including congenital disorders of protein traffic, such as the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS), hereditary spastic paraplegias (HSPs), and other neurodevelopmental disorders.



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ARL8 relieves SKIP autoinhibition to enable coupling of lysosomes to kinesin–1.

Long-range movement of organelles relies on coupling to microtubule motors, a process that is often mediated by adaptor proteins. In many cases, the coupling involves organelle- or adaptor-induced activation of the microtubule motors by conformational reversal of an auto-inhibited state. This past year, we discovered that a similar regulatory mechanism operates for an adaptor protein named SKIP (also known as PLEKHM2). SKIP binds to the small GTPase ARL8 on the lysosomal

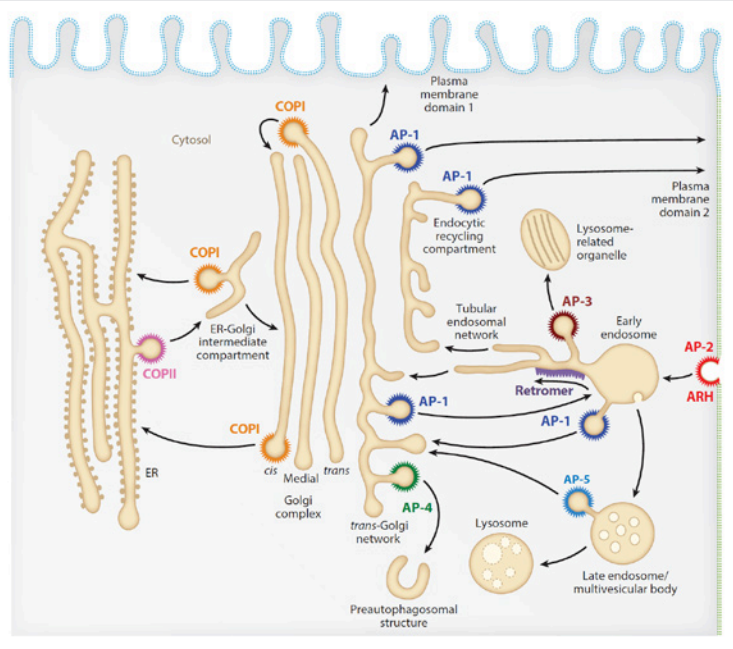

FIGURE 1. Schematic representation of the endomembrane system of eukaryotic cells showing the localization of coats involved in protein sorting

membrane to couple lysosomes to the anterograde microtubule motor kinesin-1. Structure-function analyses of SKIP revealed that the *C*-terminal region, comprising three pleckstrin homology (PH) domains, interacts with the *N*-terminal region, comprising ARL8– and kinesin-1–binding sites. The interaction inhibits coupling of lysosomes to kinesin-1 and, consequently, lysosome movement toward the cell periphery. We also found that ARL8 not only recruits SKIP to the lysosomal membrane, but also relieves SKIP auto-inhibition, promoting kinesin-1–driven, anterograde lysosome transport. The findings demonstrate that SKIP is not merely a passive connector of lysosome-bound ARL8 to kinesin-1 but is itself subject to both intra- and inter-molecular interactions that regulate its function.

SNX19 restricts endolysosome motility through contacts with the endoplasmic reticulum.

In addition to coupling to microtubule motors, interactions with other organelles also regulate the movement of endolysosomes within the cytoplasm. This past year, we found that the sorting nexin protein SNX19 tethers endolysosomes to the ER, reducing their motility and contributing to their concentration in the perinuclear area of the cell. Tethering depends on two *N*-terminal transmembrane domains that anchor SNX19 to the ER, and a Phox homology domain (PX) domain that binds to phosphatidylinositol 3-phosphate on the endolysosomal membrane. Two other domains named PXA and PXC negatively regulate the interaction of SNX19 with endolysosomes. The positioning and movement of endolysosomes within the cell are thus the result of a balance between movement driven by microtubule motors and immobilization by tethering to the ER.

Structure of human ATG9A, the only transmembrane protein of the core autophagy machinery

We also continued our studies on autophagy. A major achievement was the resolution of the atomic structure of the transmembrane autophagy protein ATG9A. In collaboration with the groups of Anirban Banerjee, Jiansen

Jiang, and José Faraldo-Gomez, we succeeded in obtaining a 2.9-Ångstrom resolution cryo–EM structure of human ATG9A. The structure revealed a novel fold with a homotrimeric domain-swapped architecture, several membrane spans, and a network of branched cavities, consistent with ATG9A being a transmembrane lipid transporter. In addition, structure-guided molecular simulations predicted that ATG9A causes membrane bending, explaining the localization of this protein to small vesicles and highly curved edges of growing autophagosomes.

ATG9A transport to the cell periphery by RUSC2–mediated coupling to kinesin–1

ATG9A cycles between the TGN in the perinuclear area and pre-autophagosomal structures in the peripheral area of the cell. In previous work, we showed that export of ATG9A from the TGN into transport vesicles is mediated by the adaptor protein 4 (AP-4) complex. We recently found that the AP-4 accessory protein RUSC2 couples ATG9A–containing vesicles to the plus-end-directed microtubule motor kinesin-1 via an interaction between a disordered region of RUSC2 and the kinesin-1 light chain (KLC). The interaction is counteracted by the microtubule-associated WD40–repeat domain 47 protein (WDR47). The findings uncovered a mechanism for the peripheral distribution of ATG9A–containing vesicles, involving the function of RUSC2 as a kinesin-1 adaptor and WDR47 as a negative regulator of this function.

ATG9A enables lipid mobilization from lipid droplets.

Further work on ATG9A showed that depletion of the protein not only inhibits autophagy but also increases the size and/or number of lipid droplets in human cell lines and in *C. elegans*. Moreover, ATG9A depletion blocks transfer of fatty acids from lipid droplets to mitochondria and, consequently, utilization of fatty acids in mitochondrial respiration. ATG9A localizes to vesicular-tubular clusters (VTCs) that are tightly associated with an ER subdomain enriched in another transmembrane protein, TMEM41B, and are also in close proximity to phagophores, lipid droplets, and mitochondria. The findings indicate that ATG9A plays a critical role in lipid mobilization from lipid droplets to autophagosomes and mitochondria, highlighting the importance of ATG9A in both autophagic and non-autophagic processes.

Regulation of LC3B levels by ubiquitination and proteasomal degradation

In addition to working on ATG9A structure and trafficking, we investigated the mechanisms of autophagy regulation. To this end, we conducted a genome-wide CRISPR-Cas9 knockout screen using cells expressing endogenous LC3B (a microtubule-associated protein that is central to the autophagy pathway) tagged with GFP–mCherry as a reporter, an approach that allowed us to identify the ubiquitin-activating enzyme UBA6 and the hybrid ubiquitin-conjugating enzyme/ubiquitin ligase BIRC6 as novel autophagy regulators. We found that the enzymes cooperate to mediate mono-ubiquitination and proteasomal degradation of LC3B, thus limiting the pool of LC3B available for autophagy. Depletion of UBA6 or BIRC6 raised the level of cytosolic LC3B, enhancing the degradation of autophagy adaptors and the clearance of intracellular proteins aggregates. The finding could be the basis for the development of pharmacological inhibitors of UBA6 or BIRC6 for the treatment of protein-aggregation disorders.

The Golgi–associated retrograde protein (GARP) complex is critical for maintenance of the Golgi glycosylation machinery.

The Golgi apparatus is a central hub for intracellular protein trafficking and glycosylation. This past year and in collaboration with the group of Vladimir Lupashin, we found that the Golgi-associated retrograde protein

(GARP) complex is critical for the maintenance of the Golgi glycosylation machinery in the Golgi apparatus. We observed that depletion of GARP subunits impairs the modification of *N*- and *O*-glycans and reduces the stability of glycoproteins and Golgi enzymes. Moreover, GARP–knockout (KO) cells exhibit reduced retention of glycosylation enzymes in the Golgi apparatus and their mis-sorting to the endolysosomal system. The findings led us to propose that the endosomal system is part of the trafficking itinerary of Golgi enzymes and that the GARP complex is essential for recycling and stabilization of the Golgi glycosylation machinery.

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Neuregulin–ErbB Signaling in Neuronal Development and Psychiatric Disorders

Failure of cortical microcircuits to properly regulate excitatory-inhibitory (E-I) balance is a key feature in the etiology of several developmental psychiatric disorders and neurological diseases, such as schizophrenia, autism, ADHD, and epilepsy. E-I balance is important to synchronize the firing pattern of local neuron ensembles, and its dysregulation can degrade cognitive functions and, in extreme cases, result in epileptiform activity. Alterations in neuronal network activity, in particular oscillations in the gamma-frequency range (30–80 Hz), are associated with behaviors and cognitive deficits in psychiatric disorders. Our Section has been investigating if and how Neuregulins (NRG 1–3) and their major neuronal receptor ErbB4, which are genetically linked to psychiatric disorders, function in an activity-dependent fashion (i.e., experience) in the developing brain to regulate synaptic and neuronal network properties. To this end, we utilized genetically modified NRG and ErbB4 mouse models, in combination with electrophysiological and molecular/cellular approaches, to identify novel interactions between the NRG/ErbB4, glutamatergic, dopaminergic, and GABAergic signaling pathways that affect E-I balance, synaptic plasticity, neuronal network activity, and behaviors repeatedly associated with cognitive deficits in psychiatric disorders.

Earlier studies focused on the functions of ErbB4 signaling in GABAergic interneurons in the hippocampus and neocortex, especially in fast-spiking parvalbumin-positive (Pv^+) interneurons that modulate gamma oscillations, and in mesencephalic dopaminergic neurons. Those studies demonstrated that ErbB4 activity in GABAergic interneurons regulate gamma oscillation amplitude (power) and numerous behaviors with relevance to schizophrenia, whereas ErbB4 in mesencephalic neurons modulates extracellular dopamine levels and cognitive performance in mice. Recently, we investigated how different NRG ligands are proteolytically processed and trafficked in neurons to understand how they mediate their biological functions during brain development. NRGs are synthesized as unprocessed pro-proteins (proNRGs) that either have a single- or a dual-transmembrane (TM) domain structure. We discovered that, contrary to the dogma that all proNRGs are targeted to axons to signal in juxtracrine/paracrine fashion, single–TM proNRGs are targeted to endoplasmic reticulum–plasma membrane (ER–PM) junctions on neuronal soma, whereas dual–TM proNRGs are initially processed in the Golgi and then trafficked to axons. The differential processing and

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trafficking of NRGs affect their function. Single–TM NRGs are initially cleaved from somatic ER–PM junctions in response to calcium influx through NMDA receptors (NMDARs) and, in turn, the released NRGs activate ErbB4 receptors on GABAergic interneurons in autocrine fashion to promote NMDAR internalization; this novel bidirectional autocrine signaling mode can homeostatically regulate excitation onto GABAergic interneurons. By contrast, dual–TM NRGs are initially cleaved by the aspartic acid protease BACE-1 in the *trans*-Golgi network, sorted into axons by transcytosis, and then selectively retained at presynaptic glutamatergic terminals via juxtacrine transsynaptic interactions with ErbB4 receptors on dendrites of GABAergic interneurons, in this fashion regulating excitatory glutamatergic inputs that drive GABAergic interneurons.

Our laboratory uses a combination of experimental approaches, which include molecular/cell biology, electrophysiology, optogenetics, fiber photometry together with genetically encoded sensors, confocal fluorescence microscopy, and behavioral analyses in genetically targeted mice, with the ultimate goal of generating holistic models to investigate the developmental impact of genes that modulate the neuronal networks that underlie behaviors and cognitive functions affected in psychiatric and neurological disorders.

Subcellular distribution, processing and function of single–TM NRGs in central neurons

We recently found that single–TM NRGs, such as NRG1 type II and NRG2, traffic as unprocessed pro-forms to the neuronal cell surface, where they accumulate at ER–PM junctions on neuronal soma and proximal dendrites (Figure 1). Shedding of the signaling-competent ectodomain of single–TM NRGs is triggered by calcium entry through excitatory glutamatergic NMDA receptors and mediated by membrane-bound ADAM (a disintegrin and metalloproteinase)–type metalloproteinases (Vullhorst *et al*., *J Neurosci* 2017;37:5232). NMDA receptor activity promotes dissociation of unprocessed proNRGs from ER–PM contact sites through dephosphorylation of multiple conserved Ser/Thr residues located in their intracellular region and ectodomain shedding by ADAM10, but not by ADAM 17 as originally proposed (Figure 2). Together, the two processes promote rapid regulated release of biologically active single–TM NRGs within minutes of NMDA receptor activation to promote ErbB4 signaling [Reference 1]. In turn, the processed NRG is released and binds to ErbB4 receptors expressed at the excitatory postsynaptic densities of GABAergic interneurons, where it selectively down-regulates NMDA, but not AMPA,

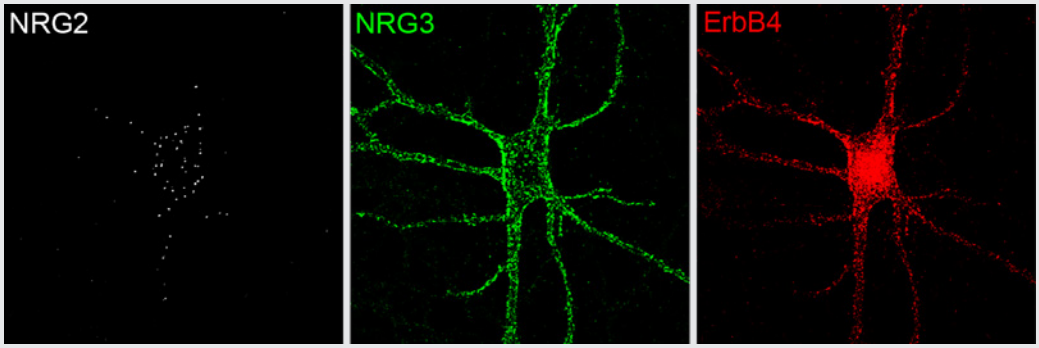


FIGURE 1. Distinct subcellular distribution of NRG2 and NRG3 in a cultured hippocampal ErbB4⁺ GABAergic interneuron

Surface puncta for pro-NRG2 (*white*) and processed NRG3 (*green*) were visualized by live labeling, followed by post-fixation labeling of ErbB4 (*red*). Note the restricted distribution of pro-NRG2 on the soma and proximal dendrites. In stark contrast, NRG3 and ErbB4 signals show extensive overlap, indicative of their transsynaptic interactions at excitatory synapses onto inhibitory interneurons.

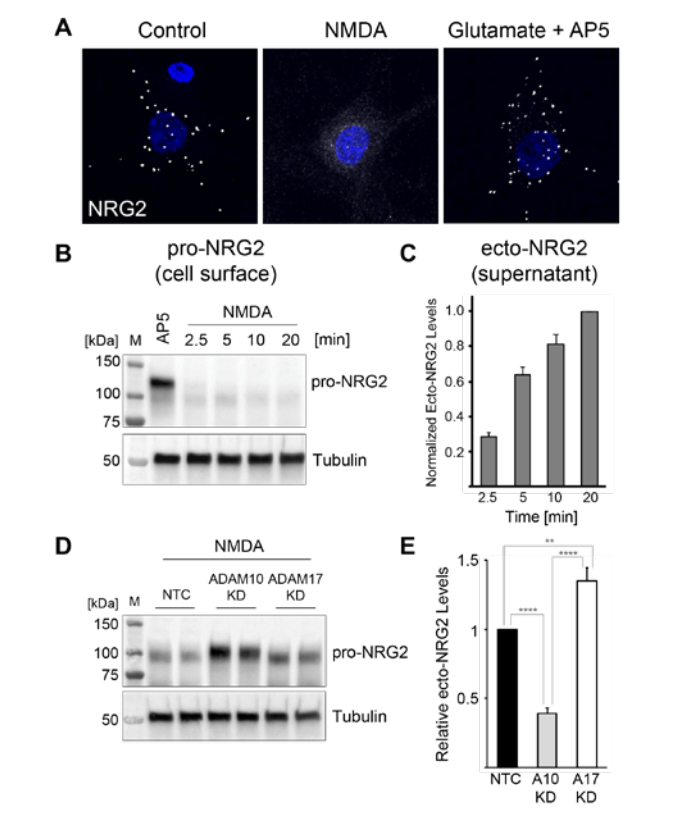


FIGURE 2. Activity-dependent pro-NRG2 processing by ADAM10, not ADAM17

A. Downregulation of NRG2 puncta on cultured hippocampal neurons in response to acute NMDA receptor stimulation.

B. Rapid downregulation of pro-NRG2 protein in whole-cell lysates of cultured neurons treated with 50 µm NMDA for the indicated times.

C. Accumulation of the NRG2 ectodomain in hippocampal culture supernatants following NMDA receptor stimulation, measured by ELISA.

D,E. shRNA–mediated knockdown of ADAM10, but not of the closely related ADAM17, blocks NMDAR–dependent processing of pro-NRG2 (*D*) and release of the NRG2 ectodomain (*E*).

receptors. We hypothesized (Vullhorst *et al*., *Nat Comm* 2015;6:7222) that this bidirectional autocrine NRG/ErbB4 signaling mode could serve as a homeostatic mechanism to regulate calcium entry in GABAergic interneurons.

Polarized axonal expression of dual–TM NRGs in central neurons by transsynaptic retention

In stark contrast to single–TM NRGs, dual–TM NRG1 type III and NRG3 are targeted to axons and accumulate at glutamatergic presynaptic terminals onto GABAergic interneurons, where they signal in juxtacrine mode via postsynaptic ErbB4 receptors expressed at postsynaptic densities on GABAergic interneurons (Figure 1). Our published (Vullhorst *et al*., *Neurosci* 2017;37:5232) and ongoing (Ahmad *et al*., submitted) studies demonstrate that cleavage of proNRG3 in the *trans*-Golgi network is required for its release from this organelle (Figure 3). Once cleaved near the second TM domain by BACE-1, the membrane-bound fragment containing the first cytoplasmic sequence, the TM and EGF–like domain (necessary for ErbB binding), is sorted and trafficked to axons by transcytosis. Through a novel mechanism that we denote 'transsynaptic retention,' the polarized accumulation of NRG3 on axonal presynaptic terminals is maintained through its transsynaptic interaction with postsynaptic ErbB4 receptors expressed on dendrites of GABAergic interneurons (Figure 3).

NRG2 and ErbB4 knockout mice exhibit dopamine dysregulation and severe behavioral phenotypes with relevance to psychiatric disorders.

We found that NRG2 expression is more extensive than originally reported, extending to striatal and medial prefrontal cortical (mPFC) neurons, and its receptor ErbB4 is expressed on presynaptic process from

FIGURE 3. Stable presynaptic accumulation of mature (processed) NRG3 depends on transsynaptic retention by its cognate receptor ErbB4.

A. Immunofluorescence cytochemistry showing co-localization of NRG3, ErbB4, and the vesicular glutamate transporter 1 (vGluT1) on a cultured GABAergic interneuron dendrite.

B,C. Reduced presynaptic NRG3 signals in shRNA–treated interneurons to knock down ErbB4 expression.

D. Model summarizing the results of our NRG3 trafficking studies. Unprocessed pro-NRG3 is cleaved by the protease BACE1 in the *trans*-Golgi network (TGN). Processed NRG3 then traffics to the somatodendritic cell surface by transcytosis, a process that involves re-endocytosis into Rab5^{+} early endosomes, sorting, and anterograde axonal transport in Rab4^{+} vesicles. Presynaptic NRG3 is stabilized by binding of postsynaptic ErbB4 receptors.

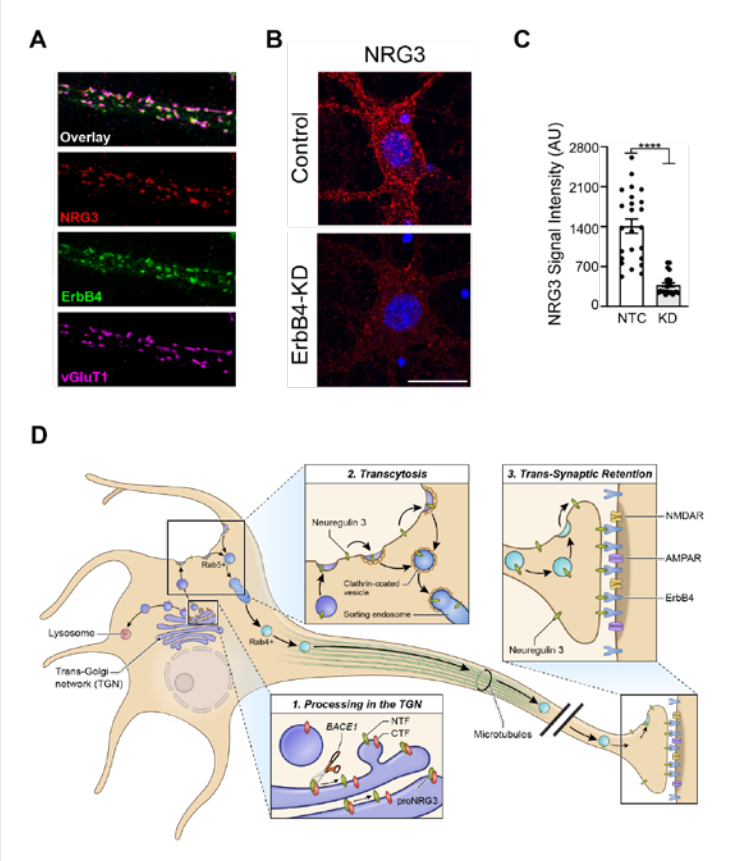


FIGURE 4. Overlapping behavioral and neurochemical phenotypes in NRG2 and ErbB4 KO mice

Lack of either NRG2 or ErbB4 in genetically engineered mice elicits similar phenotypic alterations with relevance to psychiatric disorders, demonstrating that NRG2 is an important and non-redundant ErbB4 receptor ligand in the postnatal brain.

	NRG2$^{-/-}$	ErbB4$^{-/-}$
Open field (Hyperactivity)	increased	increased
Elevated Plus Maze (Anxiety)	reduced	reduced
Prepulse Inhibition (Sensorimotor gating)	reduced	reduced
T-Maze (Working Memory)	reduced	reduced
Amphetamine Sensitivity	increased	increased

mesencephalic dopamine (DA) neurons that innervate these structures. Therefore, to investigate the function of NRG2–ErbB4 signaling, we generated NRG2 and ErbB4 knockout (KO) mice. We found that NRG2 and ErbB4 KOs have higher extracellular DA levels in the dorsal striatum but lower levels in the mPFC and hippocampus, a pattern with similarities to DA dysbalance in schizophrenia. NRG2 and ErbB4 KO mice performed abnormally in a battery of behavioral tasks relevant to psychiatric disorders (Figure 4). They exhibit hyperactivity in a novelty-induced open field, deficits in pre-pulse inhibition, hypersensitivity to amphetamine, antisocial behaviors, reduced anxiety-like behavior in the elevated plus maze, and deficits in the T-maze alteration reward test, a task dependent on hippocampal and mPFC function. Acute administration of clozapine, a potent atypical antipsychotic, to NRG2 KO mice rapidly reduced extracellular DA levels in the mPFC and improved alternation T-maze performance (Figure 5). The work emphasizes the importance of the NRG2–ErbB4 signaling pathway on the nigrostriatal, mesocortical, and mesolimbic DA systems [References 2 and 3].

Analysis of ErbB4 function in mice harboring targeted mutations in either GABAergic or DAergic neurons

Dysfunctional NRG-ErbB4 signaling in the hippocampus, pre-frontal cortex (PFC), and striatum may contribute to alterations in DA function associated with several schizophrenia symptoms. Because NRG1 acutely increases extracellular DA levels and regulates long-term potentiation (LTP) and gamma oscillations, and ErbB4 is expressed in GABAergic (Pv^+) and mesocortical DAergic (TH^+) neurons, we used genetic, biochemical, and behavioral approaches to measure DA function in the hippocampus, PFC, and striatum in mice harboring targeted mutations of ErbB4 in either PV^+ or TH^+ neurons. Unexpectedly, we have 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 sensory-motor gating deficits and increases in motor activity, ErbB4 TH KO mice exhibit cognitive-

FIGURE 5. Working memory deficits and reduced dopamine levels in KO mice are temporarily restored by clozapine administration.

A. Expression of NRG2 in the prefrontal 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 wild-type (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.

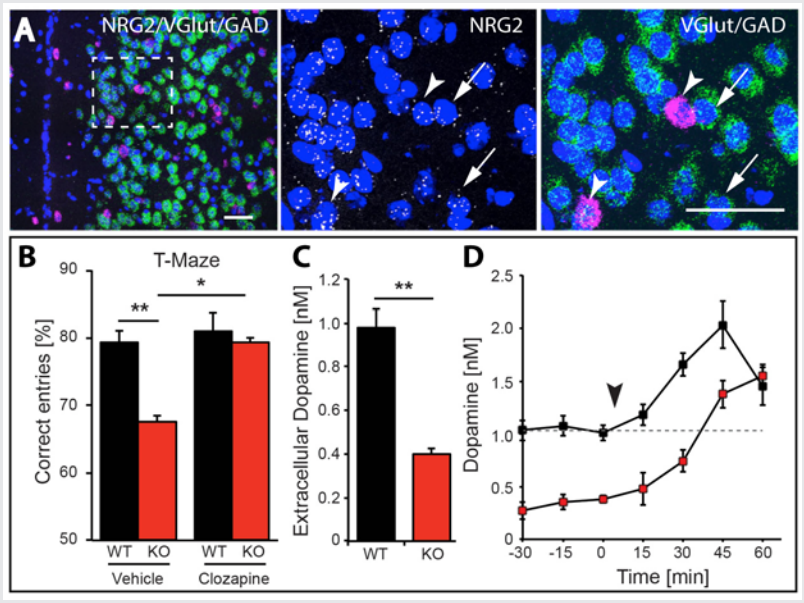
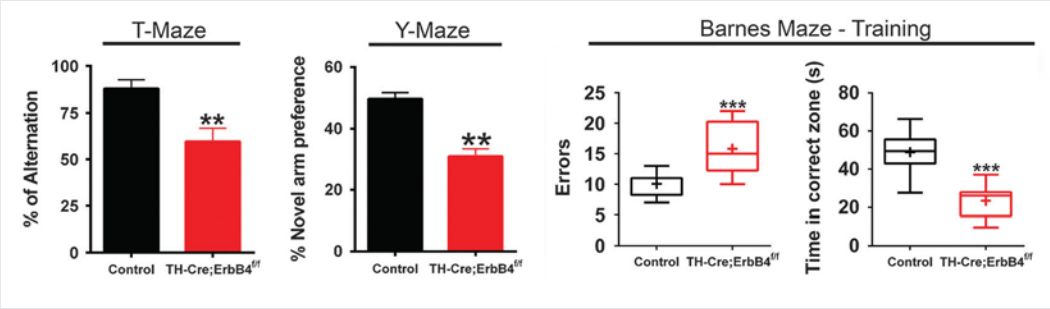


FIGURE 6. Working memory deficits in mice lacking ErbB4 in DAergic neurons



Mice were assessed in three different working memory tasks (T-, Y-, and Barnes maze) and found to perform significantly less well than their control littermates. By contrast, mice lacking ErbB4 in PV⁺ GABAergic interneurons perform normally in these tasks (not shown).

related deficits in the T-, Y- and Barnes- mazes (Figure 6). Therefore, direct effects of NRG/ErbB4 signaling in GABAergic vs. DAergic neurons differentially affect cortical circuits, DA homeostasis, and behaviors relevant to schizophrenia [Reference 4].

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 with neurological and psychiatric disorders. We reported on 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. 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 (Figure 7). First, by comparing ErbB4 hybridization on sections from wild-type and ErbB4–knockout mice (lacking 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. BaseScope could serve as an invaluable diagnostic tool to detect alternative spliced isoforms, and potentially single-base polymorphisms, associated with disease [References 5 and 6].

Transduction of mesencephalic DAergic neurons via AAV–Cre microinjection can lead to off-target toxicity.

Stereotaxic microinjection of adeno-associated virus (AAV)–expressing Cre recombinase (AAV–Cre) into specific brain regions has become a popular approach to ablate genes by *loxP/Cre* recombination in a regionally and temporally specific fashion. As part of our investigations into the functional role of the ErbB4 Cyt-1 isoform in DAergic neurons, we microinjected AAV–Cre into the ventral tegmental area (VTA) of mice harboring a conditional (floxed) ErbB4 Cyt-1 allele (ErbB4 Cyt-1$^{fl/fl}$ mice), using AAV–Cre titers commonly reported in the literature (about 10^{13} genome copies [GC]/mL). To our surprise, we found that this concentration is toxic to mesencephalic DA neurons. Furthermore, we found that the toxicity is independent of the ErbB4 deletion, as

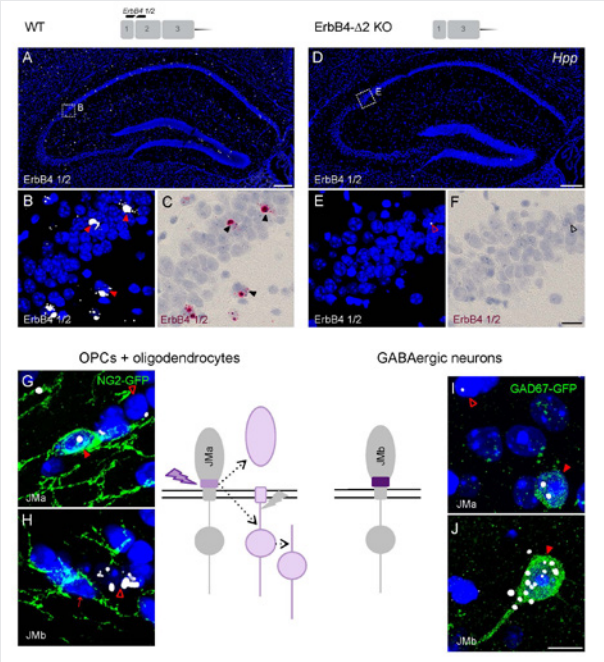


FIGURE 7. BaseScope analysis demonstrates that oligodendrocytes and neurons express distinct ErbB4 juxtamembrane (JM) transcripts.

The sensitivity and specificity of single-pair probe *in situ* hybridization was demonstrated by the presence of signals in sparse GABAergic hippocampal neurons of 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* KO 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*).

AAV–Cre microinjection in both ErbB4 Cyt-1$^{fl/fl}$ and WT C57Bl/6J mice resulted in similar losses of DA transporter (DAT) and tyrosine hydroxylase (TH) immunoreactivity, proteins critical for DA function, and elicited behavioral changes known to be associated with loss of DA neurotransmission. Furthermore, such phenotypes were observed with different AAV serotypes and proteins expressed (Cre/GFP mixture or Cre-GFP fusion protein). Interestingly, we observed that the AAV1 serotype affects DAT and TH expression, whereas AAV9 is toxic to DAergic neurons when used at about 10^{13} GC/mL. Importantly, we found that diluting the viral titer 1000-fold to 10^{10} GC/mL effectively prevents the undesired effects while still efficiently recombining floxed targets (Erben *et al.*, submitted). The work highlights the critical need for thorough and appropriate controls, such as including WT mice in experimental design, to account for potential off-target effects when working with AAV–Cre recombinase.

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- Center for Compulsive Behaviors Fellowship Award

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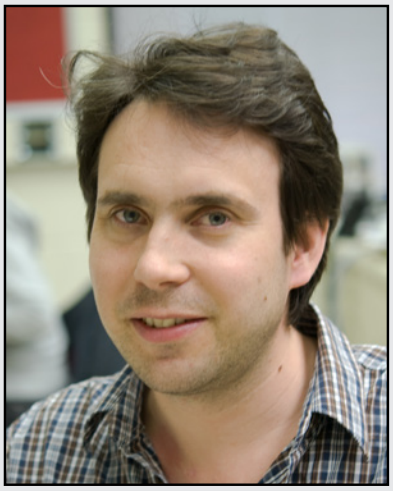
Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

The Section on Behavioral Neurogenetics studies 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 thus 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 neuro-developmental disorders. We can therefore use discoveries about sensorimotor integration pathways to understand how human disease genes disrupt brain development. 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.



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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

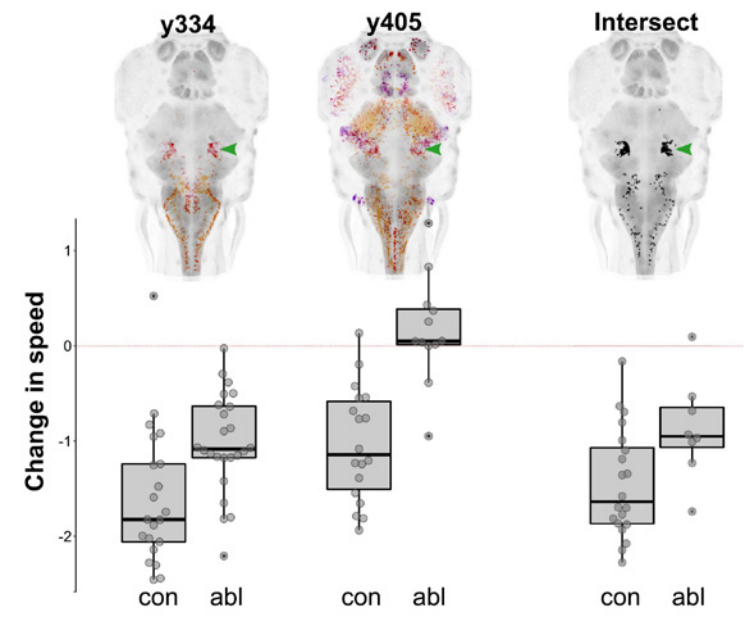


FIGURE 1. Prepontine tegmental neurons mediate tonic immobility in zebrafish.

Depth-coded projections of Gal4 expression in two lines (y334 and y405) that label neurons whose ablation interferes with the reduction in spontaneous swimming in response to an intense auditory stimulus (*bar graphs*). The computational intersectional expression of y334 and y405 is shown, revealing a common cluster of neurons in the prepontine tegmentum (*green arrowhead*). Laser ablation of the prepontine cluster diminishes immobility in response to an intensity stimulus.

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,' a form of startle modulation, termed prepulse inhibition, that is diminished in several neurological conditions. Previously, we showed that in zebrafish, as in mammals, several distinct cellular mechanisms mediate prepulse inhibition depending on the time interval between the prepulse and the startle stimulus, with NMDA–receptor signaling playing a key role for intervals greater than 100 ms. Our work on resolving the core neuronal pathway that mediates prepulse inhibition provides a basis to probe how gene mutations linked to neuro-developmental disorders disrupt sensory processing. NMDA–receptor mutations have been linked to both autism-spectrum disorders and schizophrenia. Working collaboratively with the Sirotkin laboratory, we demonstrated that mutations in the NMDA–receptor subunit grin1 disrupt prepulse inhibition [Reference 1].

While studying defensive responses to auditory stimuli, we noted that cues of about an order of magnitude larger than those needed to provoke startle responses drove an unexpected freezing behavior, especially when repeatedly presented over a short period of time. In many species, overwhelming stimuli elicit a behavior known as tonic immobility, an ultimate response to inescapable threat. In humans, the experience of tonic immobility correlates with development of the post-traumatic stress disorder, yet very little is known about its underlying neural basis. Leveraging our library of Gal4 (a tool to modulate gene activity)–transgenic lines, we performed a screen to discover neurons that are required for such behavior in zebrafish. We isolated a cluster of neurons in the prepontine tegmentum that are necessary for sustained immobility after an intense auditory stimulus (Figure 1). By manually isolating and performing RNA-Seq on these neurons, we found that they express several stress-associated neuropeptides, including markers that make them likely homologs of part of the mammalian parabrachial complex, an area recently implicated in responses to noxious stimuli. Our screen also demonstrated a central role for cerebellar signaling in tonic immobility, and we found a direct projection from Purkinje neurons to the prepontine neurons, similar to recent work showing that a subset

Purkinje neurons in mammals also directly project to the parabrachial nucleus. The study has identified for the first time a cellular pathway that mediates tonic immobility and suggests that the parabrachial has a deep evolutionary history in mediating defensive behavior [Reference 2].

Neural mechanisms for behavioral-state control

Over the course of the day, motivational goals change in response to both internal and external cues. At any given moment, an individual's behavioral state strongly influences decisions on how to interact with the environment. A major goal in neuroscience is to identify the neural systems that maintain short-term behavioral states and to determine how they interact with central mechanisms for behavioral choice. In zebrafish, loss of illumination triggers a short-term behavioral state in which larvae show heightened locomotor activity. We previously demonstrated that light-sensitive neurons in the hypothalamus trigger this state of hyperactivity and that the response to loss of illumination is a part of a light-search behavior.

Unexpectedly, we noted that during the first two minutes in the dark, zebrafish repeatedly turn in a single direction, resulting in a circular swimming pattern, and that individual fish have a tendency to circle in a left or rightward direction. Left/right motor identity is stable across several days, and correlates also with left/right startle preference, when larvae are tested in the absence of overriding visual cues. Motor asymmetry is common across the animal kingdom and is manifested in humans as hand-preference. Although human handedness is known to be disrupted in several neuro-developmental disorders and has been the subject of much research, little is known about how it is generated during development, or maintained within the brain. We therefore used the opportunity to leverage our observation that zebrafish have an intrinsic motor asymmetry to investigate developmental and neural mechanisms.

To achieve this, we performed a circuit-breaking screen before testing for changes in motor bias. Two lines that labeled neurons required for motor bias both included a small group of around 60 cells in the posterior tuberculum, a forebrain region ventral to the thalamus. Unilateral laser ablation of posterior tubercular neurons imposed a motor bias on treated larvae, such that larvae with an intact left posterior tuberculum tended to circle rightward, and vice versa. We also found that an output pathway running through the habenula was required for such neurons to drive motor asymmetry. The experiments define the specific neuronal basis for motor asymmetry in zebrafish.

While conducting these studies, a spontaneous mutation occurred in our wild-type stock that strongly suppressed motor asymmetry. Using a high-throughput sequencing approach, we mapped the underlying genetic lesion to a small deletion of the first two exons in the gene *epb41l5*, and then used an independent mutation to confirm that fish with a heterozygous mutation in this gene fail to develop normal motor asymmetry. Given that *epb41l5* interacts with members of the *notch* signaling pathway, we also tested left/right bias in heterozygous mutants with *mib* (an ubiquitin ligase that is essential for notch signaling) mutations, and again discovered loss of motor asymmetry. The findings indicate that normal acquisition of left/right motor identity is disrupted by gene mutations that quantitatively perturb Notch signaling levels during embryonic development [Reference 3].

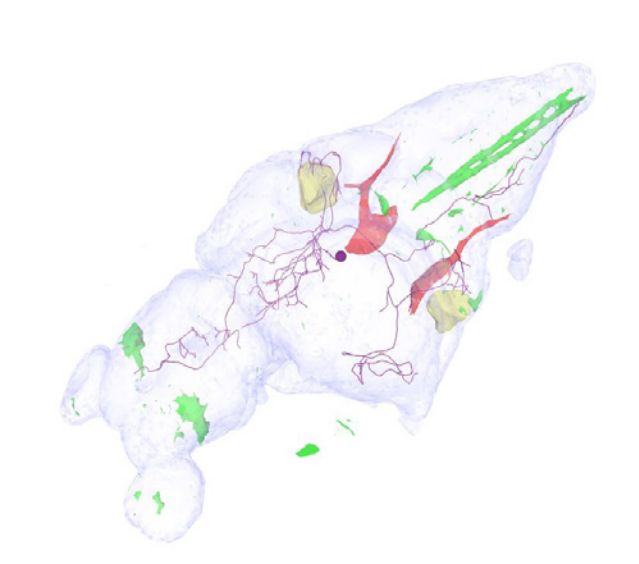
Tools for decoding neuronal circuits

To enable circuit-breaking screens, we generated several hundred new Gal4 and Cre lines that can provide intersectional targeting of small groups of neurons with a high degree of specificity. A unique feature of brain imaging in zebrafish is the ability to visualize the total architecture of the brain while simultaneously recording

FIGURE 2. Three-dimensional visualization tools facilitate analysis of transgene expression patterns and neuronal morphology in the context of neuroanatomical labels.

Example of a serotonergic neuron from the raphe nucleus (*purple*) that projects broadly throughout the brain, including into the eminentia granularis (*yellow*) and closely adjacent to the Mauthner escape neuron (*green*).

View an interactive version of this visualization tool at https://annualreport.nichd.nih.gov/burgess.html.



the position and morphology of every constituent labeled neuron. Thus, to make these tools accessible to the broader research community, we performed whole-brain imaging for each line, then registered the image of each line to the same reference brain. In collaboration with Nicholas Polys, we then developed an *online brain atlas* that enables researchers to quickly visualize the larval brain and locate transgenic lines to aid experiments. Such powerful visualization tools facilitate integrated analysis of reconstructed neuronal morphology in the context of the three-dimensional anatomy of the brain (Figure 2). Collaborating with several other groups, we noted that, as in mice, Cre lines have varying levels of susceptibility to germline recombination, necessitating care in using this tool for transgenic experiments [Reference 4].

In order to build a brain atlas, we optimized a protocol that permits highly precise brain registration. We showed that such high precision of alignment permits statistically robust whole-brain analysis of neuronal composition and morphology in zebrafish models of neurological disorders. The technique can be applied to almost any zebrafish neuro-developmental model, thus enabling robust and quantitative detection of subtle changes in brain structure or composition. In collaboration with the Stratakis lab, we applied brain morphometry to a zebrafish model of X-linked acrogigantism, revealing a developmental enlargement of the hypothalamus [Reference 5].

We then used the method to test brain structure and composition in zebrafish that carry mutations in genes that are homologous to human genes known to be disrupted in autism-spectrum disorder. By combining analysis of changes in brain structure and startle behavior in these mutants, we can define subsets of autism genes that have similar effects and are therefore likely to act in the same developmental pathways.

Additional Funding

- Simons Foundation Autism Research Initiative (2019) #653377 to Dr. Burgess “Generation of zebrafish mutants in homologs of ASD candidate genes”. Concluded.

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Cell Fusion Stages of Myogenesis and Osteoclastogenesis: Mechanisms and Physiological Role

Diverse biological processes, in which enveloped viruses infect cells and cells from all kingdoms of life secrete, internalize, traffic and sort integral proteins, sculpt their membranes and bring together parent genomes in sexual reproduction, share a common stage: fusion of two membranes into one. Biological membrane remodeling is tightly controlled by protein machinery but is also dependent on the lipid composition of the membranes. Whereas each kind of protein has its own individual personality, membrane lipid bilayers have rather general properties, manifested by their resistance to disruption and bending and by their charge. Our long-term goal is to understand how proteins fuse membrane lipid bilayers. We expect that a better understanding of important fusion reactions will bring about new ways of controlling them and lead to new strategies for quelling diseases involving cell invasion by enveloped viruses and defects in intracellular trafficking or in 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 that can reveal new kinds of fusion proteins and clarify the generality of emerging mechanistic insights. In our recent studies, we explored the mechanisms of myoblast fusion in the formation of multinucleated myofibers and the link between efficiency of formation of multinucleated osteoclasts and several IL-1–mediated diseases.

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Myomerger promotes fusion pore by elastic coupling between proximal membrane leaflets and hemifusion diaphragm.

Myoblast fusion, a key process in the development and regeneration of skeletal muscle, is one of the best characterized examples of cell-cell fusion [Brukman, Uygur, Podbilewicz, Chernomordik *J Cell Biol* 2019;218:1436]. Two muscle-specific proteins were discovered to be essential for myoblast fusion: Myomaker [Millay *et al*. *Nature* 2013;499:301] and Myomerger/Myomixer/Minion [Bi P *et al*., *Science* 2017;356:323; Quinn *et al*. *Nat Commun* 2017;8:15665; Zhang *et al*. *Nat Commun* 2017;8:15664]. In our earlier work, we found that Myomaker is required for hemifusion, whereas the subsequent transition from hemifusion to complete fusion depends on the extracellularly localized region (ectodomain) of Myomerger [Reference 1]. The role of Myomerger in myoblast fusion represents a paradigm applicable to all protein-

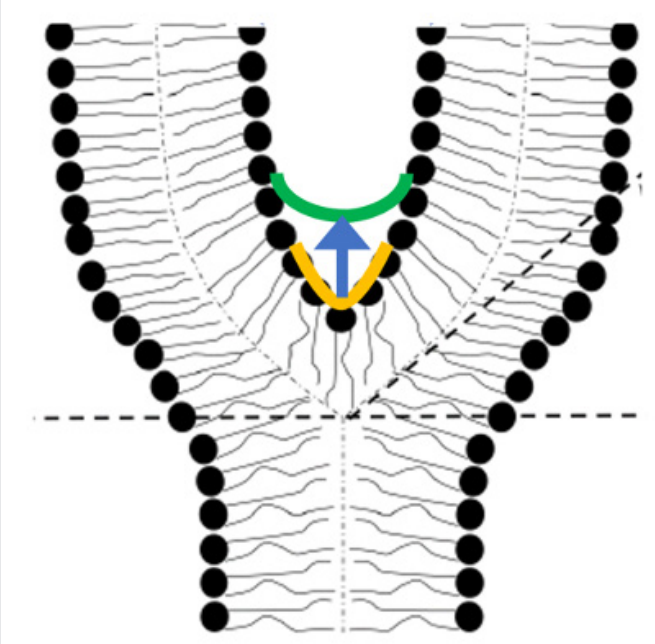

FIGURE 1. The hypothetical mechanism for the function of Myomerger in myoblast fusion

We suggest that a Myomerger-induced increase in the spontaneous curvature of the proximal monolayers of the fusing membrane bilayers changes the junction angle and the hemifusion diaphragm radius. This generates additional elastic stresses in the distal monolayers, resulting in an extra lateral tension in the diaphragm that accelerates the fusion-pore formation.

mediated fusion events, the essence of which is the promotion of fusion-pore formation in the hemifusion diaphragm (HD), which consists of the distal (inner) monolayers of the fusing membranes by proteins/protein regions that do not directly interact with these membrane monolayers. Considering that Myomerger is not involved in hemifusion but drives the transition from hemifusion to fusion, this protein presents an important model for analysis of the physical mechanisms underlying an, apparently, indirect effect of Myomerger and other proximal leaflet-associated factors on the fusion pore formation.

In our recent study [Reference 2], collaboration with the lab of Michael Kozlov, to explain how Myomerger promotes hemi-to-full fusion transition in myoblast fusion, we hypothesized that Myomerger shifts the spontaneous curvature of the proximal membrane monolayers towards positive values (Figure 1). The shift generates additional tension in the distal monolayers composing the HD and, hence, promotes the fusion-pore formation. We theoretically analyzed the effects of the positive spontaneous curvature of proximal membrane monolayers on the elastic stresses in HD and uncovered an HD rim–mediated elastic crosstalk between the proximal membrane monolayers and the HD. The crosstalk elicits growing HD tension and fusion pore-formation following increased proximal monolayer spontaneous curvature.

We supported the suggested mechanism by experiments in which we found that a synthetic polypeptide sMyomerger26-84, with an amino acid sequence corresponding to Myomerger protein lacking the N-terminal transmembrane domain of the protein (amino acids 1–25), generates positive spontaneous curvature of the lipid monolayer. Furthermore, the fusion defect in Myomerger-deficient myoblasts can be partially rescued not only by application of sMyomerger26-84 but also by application of lysophosphatidylcholine (LPC), a lipid of positive spontaneous curvature. A sufficiently strong shift of the spontaneous curvature in the proximal monolayers to positive values by LPC or by sMyomerger26-84 inhibited hemifusion and, consequently, fusion. However, the concentrations of sMyomerger26-84 and LPC that promoted hemifusion-to-fusion transition were

considerably lower than those required for the hemifusion inhibition. We suggest that levels of Myomerger expression characteristic for fusion-committed myoblasts are in a range that allows Myomerger to promote rather than inhibit myoblast fusion. Our estimate of the surface density of sMyomerger26-84 that rescues fusion of Myomerger-deficient cells is comparable to the surface densities reported for viral fusogens. The physical mechanism, by which, according to our analysis, Myomerger drives fusion pore opening, can also underlie the effects of other proximal leaflet-associated factors on the fusion pore formation in diverse fusion processes.

Novel Majeed syndrome–causing LPIN2 mutations link bone inflammation to inflammatory M2 macrophages and accelerated osteoclastogenesis.

In our recent work, as a part of multi-laboratory collaboration led by Raphaela Goldbach-Mansky, we identified novel heterozygous mutations in lipin 2 (LPIN2), a magnesium-dependent phosphatide phosphatase enzyme, in a patient with Majeed syndrome, and we explored the mechanisms that link these mutations to the development of sterile osteomyelitis [Reference 3]. Mutations in LPIN) differentially affect monocytes, monocyte-derived M1-like macrophages, and M2-macrophages, as well as osteoclastogenesis in various IL-1 (interleukin-1)–mediated diseases. Our data indicate that the mutations promote accelerated fusion of M2–like macrophage osteoclast precursors and the formation of multinucleated osteoclasts. The promotion of osteoclast fusion is associated with higher levels of nuclear factor–activated T cells c1 (NFATc1), a master transcription regulator of osteoclast differentiation that controls several osteoclast-specific genes, such as TRAP and cathepsin, and is required for sufficient osteoclast differentiation. LPIN2 deficiency also enhances the mitogen-activated protein kinase (MAPK) signaling pathway, including c-Jun N-terminal kinase (JNK), another important regulator of osteoclast differentiation activated by the pro-inflammatory cytokine IL-1. The proinflammatory effects of the LPIN2 mutation and fusion of osteoclasts were inhibited by IL-1 blockade with the human IL-1 receptor antagonist Anakinra. Our findings suggest a novel role of LPIN2 in macrophage polarization and the formation of bone-resorbing multinucleated osteoclasts, and they provide a model for the pathogenesis of sterile osteomyelitis, which distinguishes the Majeed syndrome from other IL-1–mediated autoinflammatory diseases.

Additional Funding

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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, which are distributed in a stereotypic pattern over the body surface. Each neuromast has sensory hair cells at its center, surrounded by support cells that 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 the lateral line's 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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Signaling and mechanics interact to determine the number and size of protoneuromasts in the migrating primordium.

The zebrafish pLLp migrates under the skin, periodically forming and depositing neuromasts. Neuromasts are formed sequentially within the migrating primordium, starting from its trailing end as clusters of cells apically constrict and form epithelial rosettes. Periodic formation of such protoneuromasts is coordinated by interactions between the Wnt and Fgf signaling pathways. Wnt activity dominates in a leading domain, where it inhibits Fgf signaling and the formation of epithelial

rosettes, while periodic formation of Fgf signaling centers in the wake of a shrinking Wnt system determines sequential reorganization of clusters of cells to form epithelial rosettes. While Fgf signaling may indeed seed the reorganization of cells to form epithelial rosettes, their precise number and the spacing of epithelial rosettes appears ultimately to be determined by a balance of forces promoting and opposing aggregation of cells to form these rosettes. Migration of cells in the leading Wnt–active domain is determined by chemokine signaling, while migration of trailing cells is determined by Fgf signaling. When chemokine-dependent migration is compromised, trailing cells, whose migration is dependent on Fgf signaling, keep moving, while leading cells, whose movement is dependent on chemokine signaling, slowdown. This results in local concertina-like buckling or compaction of the primordium and the fusion of trailing epithelial rosettes with leading rosettes to form fewer larger rosettes. However, such fusion is not observed when Fgf signaling, responsible for migration of trailing cells, is inhibited to slow primordium migration. These observations can be accounted for by a simple mechanics-based model of the migrating primordium, where local adhesive interactions promote aggregation, while tension along the length of the primordium, which pulls cells apart and is determined by the relative efficacy of leading chemokine-dependent and trailing Fgf–dependent collective cell migration, opposes such aggregation. In this manner, the migrating primordium provides a simple platform to visualize and understand how the interplay between signaling and mechanics determines emergent patterns of morphogenesis during development.

How does the dynamic behavior of individual cells determine tissue scale behavior in the migrating primordium?

Individual cells interact with their neighbors, change shape, and move to determine collective migration of the pLLp as protoneuromasts are periodically formed and deposited. In the past, we examined how various signaling systems, including the Wnt, Fgf, Notch, BMP, and chemokine pathways, affect morphogenesis of epithelial rosettes and specification of a central sensory hair-cell progenitor during the sequential formation of protoneuromasts in the migrating primordium, the collective migration of cells in the primordium, and the pace at which protoneuromasts are periodically formed and deposited by the migrating primordium. However, as it is technically difficult to examine the dynamic behavior of individual cells, our understanding has typically been limited to understanding these events at the tissue scale. However, in 2020, when physical work in the laboratory initially halted and then resumed at a much slower pace, Damian Dalle Nogare used the opportunity to develop a python-based pipeline to segment individual cells from high-resolution time-lapse imaging data sets obtained with DiSPIM (Dualview Inverted Selective Plane Illumination Microscope) imaging. The pipeline has now been used to segment and track all the cells in a migrating primordium. Using these data, we can reconstruct in three dimensions and over time the entire complement of cells in the pLLp and quantify and characterize various parameters associated with the diversity of cells in the primordium. We can also analyze the way in which cells interact during migration and morphogenesis of this tissue. These data have allowed us to confirm a prediction of our previous work, that superficial cells of the pLLp behave in a more fluid manner than the underlying rosette cells, undergoing more neighbor rearrangements than the more static neuromast-forming cells. Going forward, the pipeline will allow us to analyze with high precision the effect of manipulations that interfere with the normal development of the primordium, such as mutants and chemical inhibitors of signaling.

Additional Funding

- DIR Director's Award FY21–22
- Developing Talent Award to Rachel Dansereau 2020–2022

Publications

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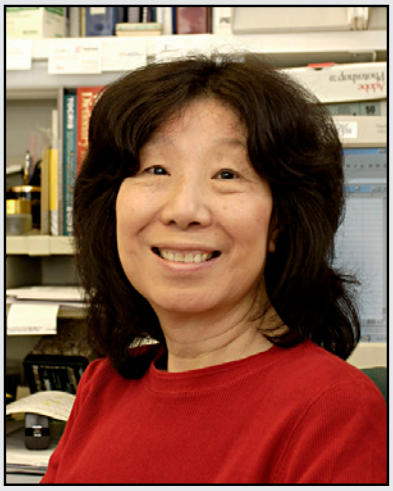
Molecular Genetics of Heritable Human Disorders

We conduct research to delineate the pathophysiology of and develop novel therapies for the two major type I glycogen storage disease (GSD-I) subtypes, GSD-Ia and GSD-Ib. GSD-Ia is caused by a deficiency in the liver/kidney/intestine-restricted glucose-6-phosphatase-α (G6Pase-α or G6PC) and GSD-Ib is caused by a deficiency in the ubiquitously expressed glucose-6-phosphate transporter (G6PT or SLC37A4). G6Pase-α is an endoplasmic reticulum (ER)–transmembrane protein that regulates intracellular glucose production by catalyzing the hydrolysis of G6P to glucose and phosphate. The active site of G6Pase-α faces into the ER lumen and depends on G6PT, another ER transmembrane protein, and translocates G6P from the cytoplasm into the lumen. To function, G6Pase-α must couple with G6PT to form a functional G6Pase-α/G6PT complex, which maintains interprandial glucose homeostasis. GSD-Ia and GSD-Ib patients manifest a common metabolic phenotype of impaired glucose homeostasis and severe long-term complications of hepatocellular adenoma/carcinoma (HCA/HCC) and renal disease. There is no cure for GSD-Ia and GSD-Ib. We generated animal models of both disorders, which are being exploited to both delineate the disease more precisely and develop new treatment approaches, including gene therapy.

We also generated several efficacious G6Pase-α– 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, effective, and appropriate for clinical trials. In 2018, our GSD-Ia rAAV vector technology was licensed to Ultragenyx Pharmaceutical Inc. (Novato, CA), which is currently undertaking a phase I/II clinical trial for GSD-Ia. We have begun to explore alternative genetic technologies for GSD-I therapies. We have established formal collaborations under cooperative research and development agreements (CRADAs) with CRISPR Therapeutics (Cambridge, MA) and Beam Therapeutics (Cambridge, MA) to evaluate the efficacy of CRISPR/Cas9–based and adenine base editor (ABE)–based gene editing systems, respectively, to correct gene-specific *G6PC* mutations in animal models of GSD-Ia.



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Correction of metabolic abnormalities in a mouse model of glycogen storage disease type Ia by CRISPR/Cas9–based gene editing

The rAAV-co-G6PC vector used in the current phase I/II clinical trial is episomally expressed, and its long-term durability of expression in

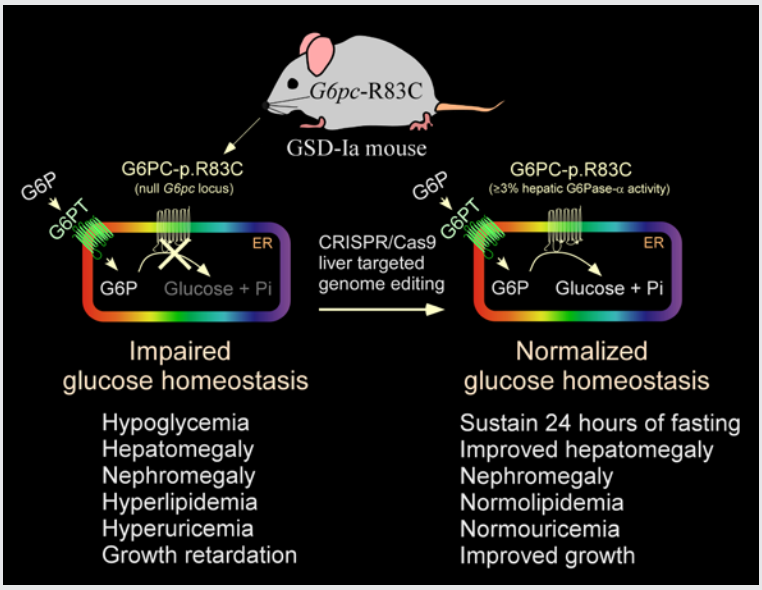

FIGURE 1. Correction of metabolic abnormalities of the GSD-Ia mice by CRISPR/Cas9–based gene editing

humans is currently being established. We therefore sought to explore the use of the CRISPR/Cas9 technology to correct a pathogenic GSD-Ia variant in its native genetic locus. The most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients is G6PC-p.R83C, representing 32% of diseased alleles. Using the CRISPR/Cas9–based gene editing technology, we generated a GSD-Ia mouse disease model, the *G6pc*-R83C mouse, which is homozygous for the G6PC-p.R83C mutation, and we showed that these mice manifest impaired glucose homeostasis mimicking that of human GSD-Ia. We then used a CRISPR/Cas9–based gene editing system to treat newborn *G6pc*-R83C mice and showed that the treated mice grew normally to age 16 weeks without hypoglycemia seizures. The treated *G6pc*-R83C mice, expressing 3% or more of normal hepatic G6Pase-α activity, maintained glucose homeostasis, displayed normalized blood metabolites, and could sustain 24 hours of fasting. Taken together, we have developed a second-generation therapy in which *in vivo* correction of a pathogenic G6PC-p.R83C variant in its native genetic locus could lead to potentially permanent, durable, long-term correction of the GSD-Ia disorder [Reference 1].

Gene therapy for GSD–Ia

We generated four efficacious *G6PC* gene transfer rAAV vectors for GSD-Ia gene therapy: rAAV-G6PC that expresses the wild-type G6PC, rAAV-co-G6PC that expresses a codon-optimized (co) G6PC, rAAV-G6PC-S298C that expresses a G6PC-S298C variant with increased efficacy, and rAAV-co-G6PC-S298C. Using the rAAV-G6PC and rAAV-co-G6PC vectors, we showed that rAAV–treated *G6pc*$^{-/-}$ mice that express 3% or more of normal hepatic G6Pase-α activity maintain glucose homeostasis and show no evidence of HCA/HCC. Our GSD-Ia rAAV vector technology was commercially licensed to Ultragenyx Pharmaceutical, which has launched a phase I/II clinical trial for GSD-Ia using the rAAV-co-G6PC vector. The initial dose-escalation trial is currently in progress and no safety issues have been reported to date.

The rAAV-co-G6PC vector contains a 20% change in the native *G6PC* coding sequence. While routinely used in clinical therapies, codon-optimized vectors may not always be optimal. To develop alternative approaches to

increase the potency of the *G6PC* gene-transfer vector, we generated the rAAV-G6PC-S298C and rAAV-co-G6PC-S298C vectors; the latter combines codon optimization with the S298C variant in the same construct. In a short-term (4–week) study using *G6pc*$^{-/-}$ mice, we showed that the efficacy of the rAAV-G6PC-S298C and rAAV-co-G6PC-S298C vectors were 3– and 5–fold higher than that of the rAAV-G6PC vector. We then undertook a 65–76–week study to examine the longer-term efficacy of these vectors. We infused four separate groups of *G6pc*$^{-/-}$ mice, each with one of the rAAV vectors and examined phenotypic correction of the mice at age 65–76 weeks. We showed that hepatic G6Pase-α activities in rAAV-co-G6PC–, rAAV-G6PC-S298C–, and rAAV-co-G6PC-S298C–treated *G6pc*$^{-/-}$ mice were 2.2–, 2.3–, and 6.2–fold higher, respectively, than that in rAAV-G6PC–treated mice. The rAAV-G6PC-S298C vector, which contains a 2% change in the native *G6PC* coding sequence, provides equal or greater efficacy to the codon optimization approach, offering a valuable alternative vector for clinical translation in human GSD-Ia. The rAAV-co-G6PC-S298C vector is three times as efficient as the rAAV-co-G6PC vector currently used in the clinical trials and thus offers a vector of choice for clinical translation in human GSD-Ia [References 2, 3].

Evaluation of the adenine base editor–based gene-editing system to correct a pathogenic G6PC mutation in a humanized mouse model of GSD-Ia

We are exploring the adenine base editor (ABE)–based technologies, which enable a programmable conversion of A•T to G•C in genomic DNA for GSD-Ia therapies. The ABE system works in both dividing and non-dividing cells, is reported to produce virtually no indels or off-target editing in the genome, can correct a pathogenic variant in its native genetic locus, and can lead to permanent, therapeutically effective long-term expression. This is a collaborative study with Beam Therapeutics, Cambridge, MA, under a CRADA.

As mentioned above, the G6PC-p.R83C is the most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients. It contains a single G→A transition in the *G6PC* gene. We first generated a homozygous humanized (h) R83C/R83C (hR83C) mouse strain by inserting the entire coding sequence of the human G6PC-p.R83C cDNA along with human *G6PC* 3'-UTR into exon 1 of the mouse *G6pc* gene at the ATG start codon. This insertion places the human cDNA under the control of the native mouse *G6pc* promoter/enhancer. The mouse *G6pc* gene is disrupted by a premature STOP codon created in the mouse *G6pc* exon 1. We first showed that hR83C mice manifest impaired glucose homeostasis characterized by growth retardation, hypoglycemia, hyperlipidemia, hyperuricemia, hepatomegaly, and nephromegaly, mimicking the abnormal metabolic phenotype of human GSD-Ia. We then treated newborn hR83C mice with lipid nanoparticles encompassing the guide RNA and mRNA encoding ABE (LNP-ABE) and showed that the treated mice grew normally to age 8 weeks without hypoglycemia seizures. The LNP-ABE–treated hR83C mice expressed significant levels of hepatic G6Pase-α activity with an editing efficiency up to about 60% and displayed normalized blood metabolite profiles. Taken together, our data demonstrate the potential of base editing to correct the G6PC-p.R83C mutation and the abnormal GSD-Ia phenotype.

Molecular mechanism underlying hepatic autophagy impairment in GSD-Ib

As stated above, GSD-Ia and GSD-Ib patients manifest a common phenotype of impaired glucose homeostasis and long-term risk of HCA/HCC. The etiology of HCA/HCC in GSD-I is unknown. Autophagy is an evolutionarily conserved, degradative process that produces energy and building blocks through lysosomal degradation of intracellular proteins and organelles in times of nutrient deprivation and environmental stress. Studies have shown that a deficiency in hepatic autophagy can contribute to hepatocarcinogenesis. Autophagy can be regulated positively by sirtuin 1 (SIRT1), AMP–activated protein kinase (AMPK), and forkhead box O (FoxO)

transcription factor family members. In the liver, AMPK is activated via phosphorylation of the AMPK α-subunit at residue T172 by the liver kinase B-1 (LKB1), a serine/threonine kinase which is also a tumor suppressor. We showed that G6Pase-α deficiency impairs hepatic autophagy through downregulation of SIRT1/FoxO3a signaling. We hypothesized that, in GSD-Ib, G6PT deficiency leads to hepatic autophagy deficiency.

Using mouse models of GSD-Ib, we showed that G6PT deficiency leads to impaired hepatic autophagy, evident from attenuated expression of many components of the autophagy network, impaired autophagosome formation, and reduced autophagy flux. The G6PT–deficient liver displayed impaired SIRT1 and AMPK signaling, along with reduced expression of SIRT1, FoxO3a, LKB1, and p-AMPK. Hepatic overexpression of either SIRT1 or LKB1 in the G6PT–deficient liver restored autophagy and SIRT1/FoxO3a/LKB1/AMPK signaling. The hepatosteatosis in GSD-Ib mice reduced SIRT1 expression. LKB1 overexpression reduced hepatic triglyceride levels, linking LKB1/AMPK signaling to SIRT1 expression. Taken together, our data show that G6PT deficiency leads to impaired autophagy in GSD-Ib primarily by downregulating SIRT1/FoxO3a/LKB1/AMPK signaling [Reference 1].

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Chromatin Remodeling and Gene Activation

Aberrant gene regulation is the basis of many disease states. Our main objective is to understand how genes are activated for transcription in the context of chromatin structure. Chromatin is not solely a packaging system for DNA in eukaryotic cells, but also participates in gene regulation. The structural subunit of chromatin is the nucleosome, which contains nearly two turns of DNA coiled around a central core histone octamer. Nucleosomes are generally quite regularly spaced along the DNA, like beads on a string. Gene regulation involves either attenuation of the inherently repressive properties of nucleosomes to facilitate gene expression, or enhancement of those properties to ensure complete repression. These events are choreographed by DNA sequence–specific transcription factors (activators and repressors) and chromatin-remodeling complexes. The latter can be divided into two groups: histone- or DNA–modifying enzymes that implement the "epigenetic code", and ATP–dependent remodeling machines that move or displace nucleosomes. We are exploiting and developing high-throughput technologies to obtain genome-wide maps of nucleosomes, chromatin-remodeling complexes, and RNA polymerase II in budding yeast to determine how chromatin organization is affected when genes are activated. The current objectives of our yeast studies are to: (1) determine the roles of the various chromatin-remodeling complexes (RSC, SWI/SNF, ISW1, ISW2, CHD1, Ino80C) in chromatin organization and gene expression; why there are so many different remodelers; whether they are functionally redundant, our studies so far indicating that each remodeling enzyme makes a different contribution to chromatin organization; (2) test the hypothesis that nucleosomes control DNA accessibility and play a vital role in gene regulation by blocking promoters (Figure 1). We are extending our studies of chromatin remodeling from yeast to higher organisms.

Many human diseases have been linked to chromatin-remodeling enzymes and epigenetic modifications. For example, mutations in the gene encoding the hSNF5 subunit of the SWI/SNF ATP–dependent chromatin-remodeling complex are strongly linked to pediatric rhabdoid tumors. The CHD (chromatin helicase DNA binding) class of ATP–dependent remodelers has also been linked to cancer and autism. Cancer therapies and drugs aimed at epigenetic targets are being tested. A full understanding of chromatin structure and the mechanisms by which it is manipulated is therefore of vital importance.

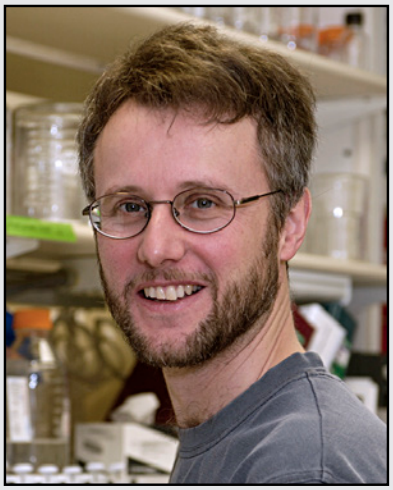

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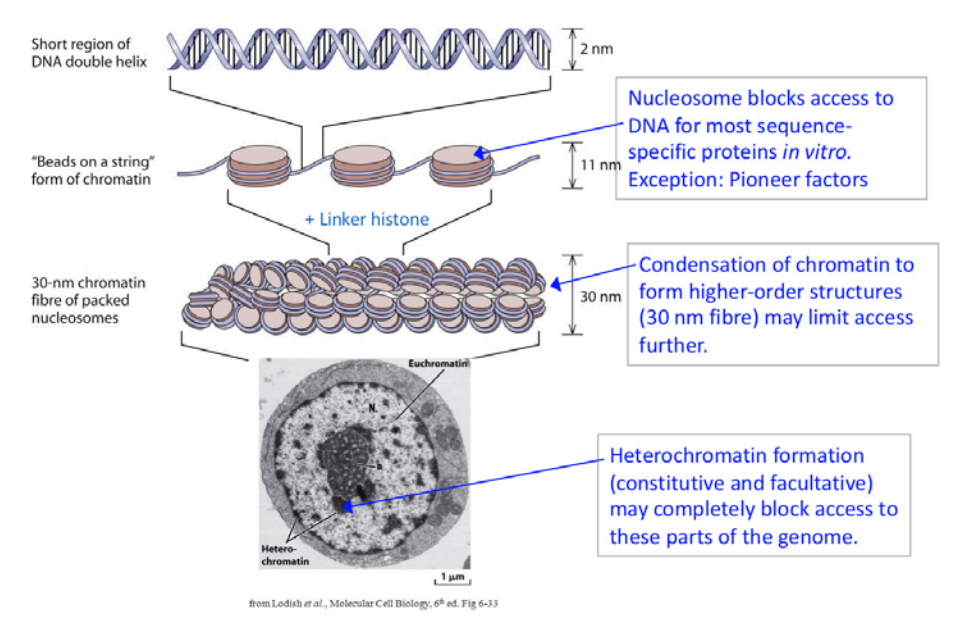
FIGURE 1. DNA packaging in the nucleus: to what extent does chromatin compaction limit access to the DNA?



DNA is packaged into the nucleus by histones. The basic structural subunit of chromatin is the nucleosome core, which contains about 147 bp of DNA wrapped nearly twice around a central octamer of core histones. Nucleosomes are regularly spaced along the DNA like beads on a string; the intervening DNA is called the linker DNA and is bound by linker histone (H1). The beads-on-a-string fiber spontaneously condenses into a heterogeneous fiber of about 30 nm width. Genomic regions rich in repetitive elements form constitutive heterochromatin in all cells, in which the chromatin fiber is even more condensed. Facultative heterochromatin is formed on genes that should be permanently silent in a specific differentiated cell type. Heterochromatin is densely packed and darkly staining in the electron micrograph shown here. Euchromatin is less condensed (*light staining*) and contains active genes. We are interested in determining to what extent chromatin limits DNA accessibility. Figure adapted from Chereji *et al*. *Genome Res* 2019;29:1985-1995.

Chromatin remodeling and 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 and transcription, events that coincide with the removal of promoter nucleosomes to create a nucleosome-depleted region (NDR). This observation has led to the generally accepted model that promoter nucleosomes physically block transcript initiation, acting as repressors by preventing access to specific transcription factor binding sites. The nucleosome is a highly stable structure containing tightly wound DNA that is largely inaccessible to sequence-specific DNA–binding proteins. Activation occurs if sequence-specific 'pioneer' transcription factors are present (proteins that bind to nucleosomal sites with high affinity), and/or if 'classical' transcription factors, which are normally blocked by nucleosomes, recruit ATP–dependent chromatin remodelers to move or evict promoter nucleosomes, thus facilitating initiation complex formation.

The ATP–dependent chromatin remodelers variously move nucleosomes along DNA, remove the histones altogether, or form arrays of regularly spaced nucleosomes. Examples include the SWI/SNF and RSC complexes, which remodel nucleosomes on genes and at promoters, and the CHD and ISWI complexes, some of which are involved in determining nucleosome spacing. The INO80C complex is unusual because it has both properties. All these remodeling enzymes have human homologs.

Our activities this year were severely curtailed by the pandemic, but we were still able to complete two projects. In the first, we focused on understanding the roles of the two forms of the ISW1 complex in yeast [Reference 1]. Two different ATP–dependent nucleosome-spacing enzymes are required to establish regular arrays of phased nucleosomes near transcription start sites of yeast genes: Isw1 and Chd1. Cells lacking both Isw1 and Chd1 have extremely disrupted chromatin, such that the nucleosomes are poorly spaced and poorly positioned relative to the transcription start site. We had shown previously that such chromatin disorganization is at least partly attributable to a propensity in the double mutant to form close-packed dinucleosomes near the beginnings of genes (i.e., two nucleosomes jammed close together with no intervening linker DNA). Reaching a mechanistic understanding of the roles of the different remodelers is complicated by the fact that the Isw1 ATPase subunit occurs in two different remodeling complexes: ISW1a (composed of Isw1 and Ioc3) and ISW1b (composed of Isw1, Ioc2, and Ioc4). We constructed yeast strains with various combinations of deletions in the Ioc2/3/4 subunits and determined their chromatin organization using MNase-Seq, which provides a detailed nucleosome map for each strain. We discovered that ISW1b is primarily responsible for setting nucleosome spacing and resolving close-packed dinucleosomes, whereas ISW1a plays only a minor role. ISW1b and Chd1 make additive contributions to dinucleosome resolution, such that neither enzyme is capable of resolving all dinucleosomes on its own.

Given that the Ioc4 subunit of ISW1b binds preferentially to the histone H3-K36me3 mark, which is mediated by the Set2 H3-K36 methyltransferase and associated with active transcription, we tested whether loss of Set2 phenocopies loss of Ioc4. We found that dinucleosome levels are higher in cells lacking Set2, as is true for cells lacking Ioc4 (ISW1b), but, unlike *ioc4*-null cells, *set2*-null cells exhibit only a weak effect on nucleosome spacing. We propose that Set2–mediated H3-K36 trimethylation is important for ISW1b–mediated dinucleosome separation which, in turn, may be important for facilitating the passage of RNA polymerase II through the nucleosomes. We conclude that the nucleosome spacing and dinucleosome-resolving activities of ISW1b and Chd1 are critical for normal global chromatin organization, whereas ISW1a plays little or no role in chromatin organization at the global level. Thus, ISW1b is responsible for the effects of Isw1 on global chromatin structure, whereas the role of ISW1a is still unclear.

Global histone–surface accessibility in yeast indicates a uniformly loosely packed genome

Almost all methods designed to probe global chromatin structure measure DNA accessibility to nucleases (e.g. MNase-Seq and ATAC-Seq) or employ chemical crosslinking (e.g. ChIP-Seq and HiC). In a collaboration with Jeff Hayes, we developed a method to measure genome-wide accessibility of histone protein surfaces within nucleosomes by assessing reactivity of engineered cysteine residues with biotin-maleimide (BM), a thiol-specific reagent [Reference 2]. We determined the accessibility of three different histone residues by mutating them to cysteine in the appropriate yeast histone gene and then treating purified nuclei with BM. The chromatin is digested to mono-nucleosomes, streptavidin is used to purify modified nucleosomes, and the nucleosomal DNA is subjected to paired-end sequencing. Sequence data for modified nucleosomes are compared with data for input nucleosomes. In the first case, we measured the accessibility of the histone mutant H2B-S116C. The cysteine might be expected to be accessible, unless the chromatin is fully condensed, preventing BM modification. We found that it is generally accessible throughout the genome, suggesting that this histone surface is not usually obscured by tight nucleosome packing. In the second and third cases, we investigated the accessibility of cysteine residues buried inside the nucleosome, which might be exposed if the nucleosome is conformationally altered during transcription, as suggested by studies from other labs:

H3-S102C, located at the H2A–H2B dimer/H3–H4 tetramer interface, and H3-A110C, located at the H3–H3 interface. However, we found that neither of these internal sites are significantly exposed. In conclusion, our data are consistent with a global, relatively decondensed chromatin structure, containing nucleosomes that do not generally undergo major conformational changes.

Role of the ARID1A subunit of the mouse SWI/SNF complex in gene regulation.

In a collaboration with Tom Owen-Hughes, we investigated the effects on gene regulation of depleting mouse cells of ARID1A, a critical subunit of the SWI/SNF ATP–dependent chromatin-remodeling complex. We found that loss of ARID1A results in a re-organization of chromatin structure around pluripotent transcription factor binding sites and a redistribution of the EP300 coactivator [Reference 3]. At enhancers, loss of EP300 correlates with reduced gene expression, whereas gain of EP300 correlates with increased gene expression. Surprisingly few genes are affected directly by loss of EP300, but cumulative indirect effects eventually phenocopy pre-malignancy.

Publications

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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.

Patients with mutations in *RNASEH1* exhibit a typical mitochondrial myopathy phenotype (muscular). 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.

Differences between Class I and Class II RNases H

Many of our investigations over the years focused on RNase H1. RNase H1 recognizes the 2'-OH of four consecutive ribonucleotides, while the DNA strand is distorted to fit into a pocket of the enzyme. Thus, the enzyme requires more than one ribonucleotide for cleavage of RNA in RNA/DNA hybrids. In both eukaryotes and prokaryotes, RNases H1 consist of a single polypeptide. In contrast, RNase H2 is a complex of three distinct polypeptides in eukaryotes but a single polypeptide in prokaryotes. The catalytic subunit of the hetero-trimeric RNase H2 of eukaryotes is similar in its primary amino-acid sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave both RNA/DNA hybrids and a single ribonucleotide 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) [References 1 & 2].

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Several types of RNA/DNA hybrid structure 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 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* (which encodes a nuclease), 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. Damaged DNA that finds its way into the cytoplasm can be sensed by the cGAS protein producing the small molecule cGAMP, which interacts with the Sting protein, an important protein for the DNA–sensing in the 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 (interferon-stimulated genes). 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 among 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 in yeast producing the RNase H2^{RED} the 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 strains 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 [References 1 & 2]. 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 [Reference 2]. Interestingly, the *Rnaseh2*^{RED} mice die around the same time as the *Rnaseh2b–KO* mice. Therefore, lethality of the KO and RED RNase H2 mouse strains may be caused by increased rNMPs in genomic DNA. *Rnaseh2a*$^{G37S/RED}$ embryos also arrest at approximately the same stage as *Rnaseh2a*$^{RED/RED}$ embryos because of better association of RNase H2^{RED} than RNase H2^{G37S} with DNA 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.

Our studies on RNase H2-RED have permitted us and others to assign specific substrates to each of the two activities and determine which functions are related to various phenotypes seen when RNase H2 is absent [References 1–3]. One of our goals is to produce an RNase H2 with robust incision at single rNMPs in DNA but with poor RNA/DNA hybrid cleavage (hybrid-defective [HD]) to complement the RNase H2-RED enzyme. We have identified amino-acid changes that appear to produce such RNase H2-HD and are in the early stages of studies in both cell cultures and mice.

Abasic substrates

Incorporated rNMPs embedded in DNA could be converted to abasic sites in which the flanking dNMPs would be connected by a ribose phosphate rather than an rNMP. In collaboration with the Storici and Tell groups, we examined the abilities of eukaryotic RNases H2 to cleave substrates containing a single ribose abasic (only sugar phosphate) site in duplex DNA. Prokaryotic RNases HII, but not eukaryotic RNases H2, can recognize and cleave at rNMPs in duplex DNA. Little was known about abasic sites in RNA until, in collaboration with Vivian Cheung's group, we discovered that abasic sites are present in RNAs of yeast and human cells, and likely in all organisms. The abasic sites are located in or near R-loops. The Cheung group had previously shown that MPG (N-methylpurine DNA glycosylase) and APE1 (apurinic/apyrimidinic endonuclease 1) interact with R-loops. MPG is a glycosylase that removes the base of DNA, and, as reported in our papers with Cheung, or RNA, which in turn can be cleaved by the APE1. Abasic sites in DNA are repaired by *Ape1* excision using the complementary DNA strand as template for repair. RNAs have no template to correct for the absence of a base. The association of abasic sites with R-loops is intriguing and suggests that RNAs complexed with DNA are protected from RNase H activities, similar to stable R-loops present at transcription start sites of highly transcribed genes. In theory, the DNA of R-loops could serve as templates for repair of the abasic site, in which case the abasic site would survive longer than abasic sites in single-stranded RNA, giving the appearance of association of abasic sites with R-loops. However, binding of MPG and APE1 suggests that R-loops might be good substrates for MPG to generate the abasic sites.

Loss of RNase H1 in early B cell development activates the mitochondrial unfolded protein response without affecting the nuclear R–loops.

We made a knockout of the mouse *Rnaseh1* gene and discovered that two proteins of RNase H1 are produced from a single mRNA by a leaky scanning method for differential translation. One protein is localized to the nucleus and a second is targeted to mitochondria. Nuclear DNA replication begins at fertilization, with mitochondrial DNA (mtDNA) beginning amplification several days later. We observed early embryonic death shortly after mtDNA replication should have begun, thereby linking absence of mtDNA replication with death. We were curious to see the contribution effects in a system less complicated then embryonic development. We chose mouse B cell development because (1) B cells are not required for viability when mice are housed in a germ-free environment; (2) B cell development occurs in only a few rounds of cell duplication; (3) resting B cells are in G0, providing a population of cells that respond together when stimulated; (4) many useful tools for analyses and manipulation are available; and (5) RNase H has potential known substrates in B cell development.

We generated an *Rnaseh1* conditional KO mouse strain in which we can specifically knocked out the gene using a CRE-lox method with the *Mb1* (the *Mb1* gene encodes the Ig-α signaling subunit of the B cell antigen receptor) promoter–driving CRE. Transcripts of *Mb1* are initiated from the earliest stage of B cell and persist until plasmacytes are formed. B cells develop to the resting stage, at which point they can be stimulated to undergo isotype switching by class switch recombination (CSR), ultimately producing circulating antibodies. We found that *Mb1-CRE* KO of the *Rnaseh1* gene resulted in little or no circulating antibodies but did produce resting B cells, although yielding half as many B cells as did wild-type (WT) mice. Stimulation of these B cells initiated transitioning from G0 to G1 phase of the cell cycle, but essentially they never entered S-phase. The resting B cells had no intact *Rnaseh1* gene, no mtDNA, the cells had no RNase H1 activity, and mitochondria exhibited abnormal morphology. We performed RNA-Seq analyses of resting and 24 h–stimulated mutant and WT B cells to discover genes related to loss of mtDNA and/or a nuclear DNA–damage response. Pathways that exhibited reductions were the cell-cycle, the immune system, DNA replication, mitochondrion, RNA processing, and ribosomes. The 50% yield of resting B cell in the KO strain must occur during cell amplification in bone marrow. The loss of RNase H1 was initiated just prior to cell amplification and might limit the number of cell cycles. It is also possible that defects affecting the time of residence of the B cells in the bone marrow niche are affected. Loss of the Nidogen1 gene (*NID1*) results in reduction of resting B cells to 50% of normal, the same as in our KO mice. We noticed a significant difference between WT and mutant resting B cells for the *NID1* transcripts.

The list of genes with the highest fold difference between resting and stimulated KO mice are *Atf5*, *Gdf15*, *Atf3*, *Hspa9*, and *Ddit3*. *Atf5*, *Atf3*, and *Ddit3* are all hallmarks of the unfolded mitochondrial response (UPRmt). The activation of the UPRmt indicates that loss of mtDNA takes precedence over nuclear DNA damage response, just as we observed in embryonic development when the *Rnaseh1* gene was deleted in the male and female gametes. We checked the presence of R-loops by DRIP-Seq and, surprisingly, found no alteration in R-loops indicating the lack of RNase H1 in processing these structures.

Ataxic mouse model of lissencephaly–5

R-loops are three-stranded structures in which RNA loops out a DNA strand by annealing to the non-displaced DNA strand. It has been proposed that triplet expansion disease could result from improper realigning when the RNA is removed. We were particularly interested to see a mouse with severe ataxia appear in a mating with mice bearing mutations in the *Rnaseh1* gene and wished to understand the origin of the phenotype in

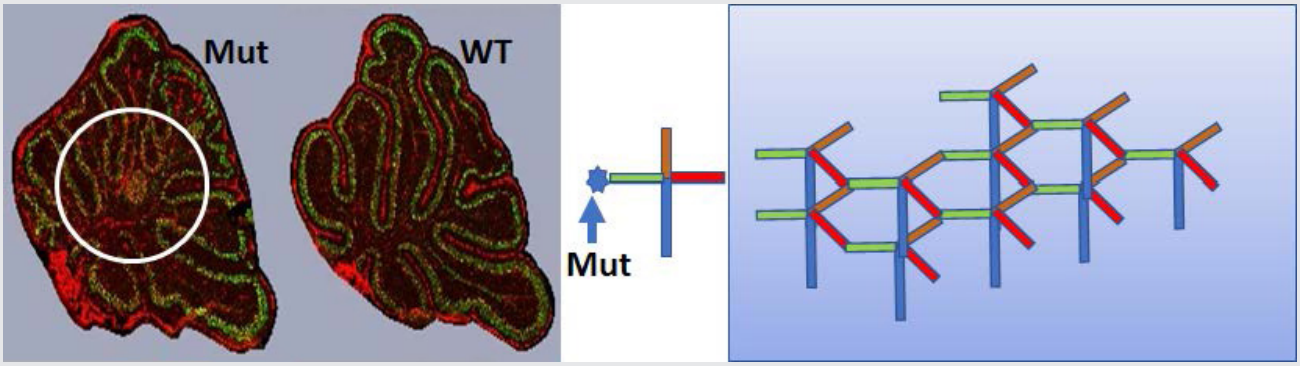

FIGURE 1. Ser248Leu in mouse *Lamb1*

Day P7 Cerebellum sections stained with Calbindin (*green*) and anti-Lamb1 (*red*). Immunofluorescent images: *left Lamb1* homo; *right* WT. The circle highlights clear mobility defects in the mutant mouse's cerebellum in addition to the abnormal lobe morphology. The cartoon is described in the text.

this mouse. A point mutation in the *Lamb1* gene (which encodes the basement-membrane protein laminin subunit β1) rather than a triplet-expansion was discovered as the cause for the cerebellar ataxia. Mutations in the *LAMB1* gene of human patients are known causes of lissencephaly-5, a neuronal migration disorder with characteristic protrusions of neurons. Lamb1 is a part of a complex of three similar proteins, a1, b1, and c1, that interact with dystroglycans and are part of congenital muscular dystrophies. One model for assembly of the complexes (Figure 1, right-hand panel) posits that these basement-membrane proteins interact in an umbrella-like manner with the C-terminal coiled-coiled regions intertwined (*blue*) and the N-terminal domain of each protein interacting with one or more adjacent a1 (*red*), b1(*green*), c1 (*orange*) complexes forming an external canopy. The coiled-coiled region forms a handle of the umbrella [McKee KK, *et al*., *Matrix Biology* 2021;98:49-63]. The mutation we found in Lamb1 is in the N-terminal loop of Lamb1, where an exposed serine has been substituted with a leucine. Structural studies could be used to support a role for the ser in protein-protein interactions. We performed multiple H&E staining and immunofluorescent studies (Figure 1, left-hand panel), which showed abnormal cerebellar lobe folding when the Lamb1 Ser-Leu mutation was homozygous but not when heterozygous. Lobes VI and VII were the most disordered, with an unusual area, possibly associated with the fastigial nucleus and/or poorly migrated components of lobes VI and VII. Reduced Lamb1 immunofluorescence signal was seen throughout the cerebellar sections in both *Lamb1* heterologous and homozygous mutants. The structural integrity of the basement membrane and a normal gate can be maintained with what appears to be 50% of normal a1, b1, c1 laminin. We expect that this interesting mouse will become a model for human lissencephaly-5.

Increased incorporation of ribonucleotides in yeast DNA leads to genomic instability in the absence of RNases H.

The cellular concentration of deoxyribonucleoside triphosphates (dNTPs), the building blocks of DNA, are tightly controlled during the cell cycle to allow the replication and repair of genomic DNA. Ribonucleotide reductase (RNR) is a key enzyme, which converts ribonucleotides to deoxyribonucleotides and regulates dNTP/rNTP ratios. Cellular levels of dNTPs are much lower than those of rNTPs (ribonucleoside tri-phosphates) but increase when cells enter S-phase to facilitate genomic DNA synthesis. In addition, DNA polymerases (Pol) have active

sites that select against the bulky rNTP residues in favor of dNTPs, which have a far better fit. To increase the incorporation of rNMPs in the DNA of *Saccharomyces cerevisiae*, we reduced the dNTP pools by depleting Rnr1, the major catalytic subunit of RNR. We observed that when dNTP pools were reduced, RNase H1 and RNase H2 were required for viability. We suggested that, under the conditions of replicative stress created by low dNTP concentrations, R-loops that accumulate in the absence of both RNase H1 and RNase H2 could become insurmountable impediments to the progression of the replication fork, inducing fork collapse and genomic instability. We further increased rNMPs incorporation in genomic DNA by depleting Rnr1 in strains that harbor DNA Pol mutants that have higher propensity to incorporate rNMPs in genomic DNA. Under such conditions, RNase H2 became essential. In addition to R-loop processing, RNase H2 initiates the ribonucleotide excision repair (RER) process, which efficiently removes single rNMPs in genomic DNA. The lethality of the triple mutant lacking RNase H2 in the DNA Pol mutant depleted of Rnr1 could be reversed in the absence of topoisomerase (Top1). We concluded that when RER is defective, Top1 processes rNMPs in DNA in a mutagenic way, inducing genome instability. When a threshold of single genomic rNMPs is exceeded in cells with limited dNTP pools and without RER, the Top1–mediated DNA damage results in severe growth defects and lethality.

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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 34 proteins called nucleoporins (Figure 1). Beyond nucleo-cytoplasmic 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 centers on 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 took a multifaceted approach toward this question, using both CRISPR–based strategies in mammalian cells and fly (*Drosophila melanogaster*) developmental genetics.

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Selective degradation of nucleo–cytoplasmic transport proteins and their interphase function

Understanding the activities of individual nucleoporins has been complicated by their multifaceted nature, abundance, and unusual

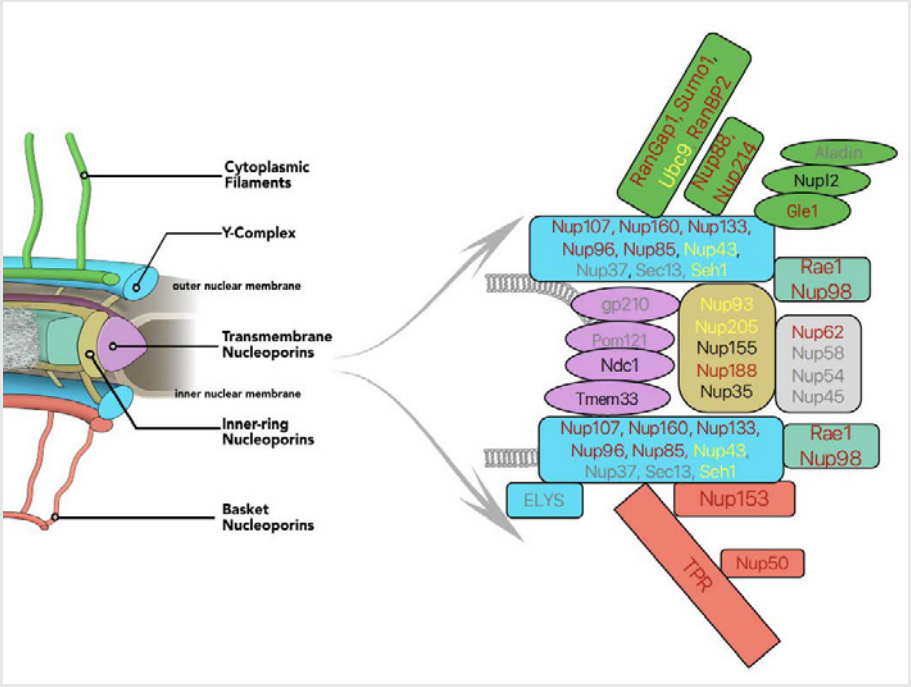


FIGURE 1. Auxin-induced degron (AID) tagging of nucleoporins

Left. Model of nuclear pore structure with cytoplasmic filaments (*green*) oriented upward and nucleoplasmic basket (*reddish brown*) oriented downward. Other domains of the NPC as indicated.

Right. 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: *reddish brown*: successful tagging; *black*: in progress; *yellow*: tagging achieved but cell-line quality issues; *grey*: tagging unsuccessfully attempted.

stability. To overcome these issues, we employed strategies for 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 promotes 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 recent findings regarding the roles of nucleoporins during interphase address three issues. First, we investigated 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 investigated 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 are able to differentiate the roles of individual nucleoporins and can specifically show that the TPR protein has a unique and important role in nuclear mRNA export via the transcription-export-2 (TREX-2) complex (see also below). Third, we investigated how organization of the nuclear transport machinery impacts development. In particular, we found that an evolutionarily controlled mechanism for association of Ran's GTPase–activating protein (RanGAP) is critical for the development of *Drosophila*. Defining the mechanism through which individual nucleoporins contribute to each of these processes will allow us to better design future experiments examining nucleoporin function in human development and disease.

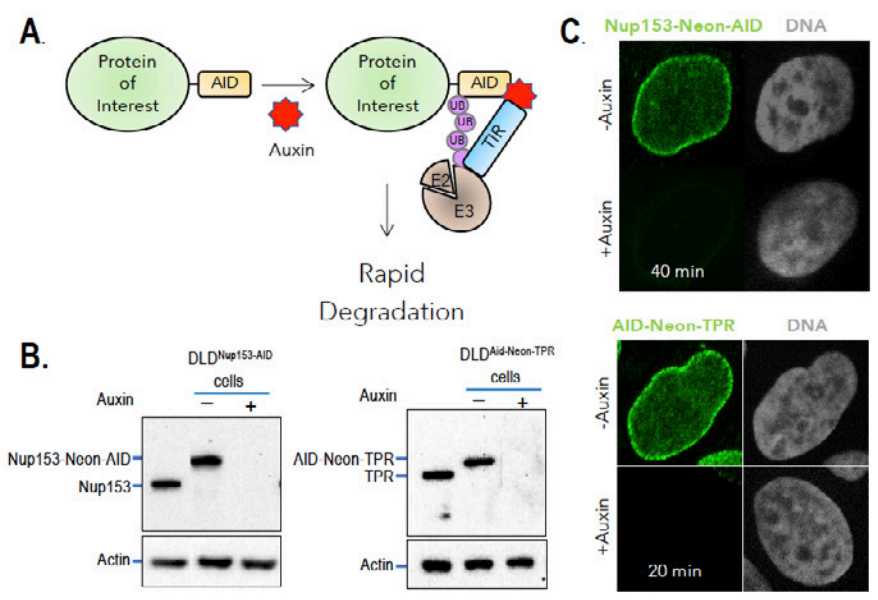


FIGURE 2. Auxin-induced degradation of AID–tagged nucleoporins

A. TIR1–expressing cells ubiquitinate and degrade proteins tagged with 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*).

Analysis of nucleoporins demonstrates that the NPC is a highly modular structure.

NPCs are built from many copies of roughly 34 distinct nucleoporins. Models of the NPC depict it as a composite of several sub-domains, which have been named the outer rings, inner ring, cytoplasmic fibrils, and nuclear basket. The outer-ring domains of the NPC are formed from the Y-complex, which contains nine core nucleoporins (SEH1, SEC13, NUP37, NUP43, NUP85, NUP96, NUP107, NUP133, and NUP160), with a tenth subunit (ELYS) required for chromatin recruitment. Other nucleoporins (NUP205, NUP188, NUP155, NUP93, NUP35) form the inner-ring structures. The distinct roles of individual nucleoporins and their functional interactions remain poorly understood. Moreover, NPCs undergo a disassembly-reassembly cycle during mitotic division, and a lack of tools for acute manipulation of individual nucleoporins has therefore precluded the study of their roles in maintaining structures within pre-existing pores without complications from disruption of NPC assembly.

We added AID tags and fluorescent moieties by homozygously targeting gene loci encoding Y-complex and inner-ring nucleoporins. Auxin addition resulted in a rapid loss of the targeted proteins in each case, without degradation of other nucleoporins. We anticipated that loss of any Y-complex member should result in complete destabilization of the outer rings. While this was true after depletion of NUP96 or NUP107, the loss of other Y-complex members surprisingly left the outer-ring lattice in place. The findings suggest that the outer-ring structure is remarkably resistant to perturbations once it is fully assembled and show that its members are not of equivalent importance in sustaining its stability. Importantly, near-complete loss of the outer ring in NUP96–depleted cells did not cause collapse of the rest of the NPC, as demonstrated through immunostaining, live microscopy, and mass spectrometry. Particularly, the remarkable persistence of inner-ring nucleoporins indicated resilience of the NPC structure. Interestingly, depletion of the inner-ring nucleoporin NUP188 caused an NPC disassembly that was opposite to the profile after NUP96 depletion: inner-ring components were extensively displaced, while the components of the cytosolic fibrils, outer ring, and basket were largely unaffected. Also, there was a global reduction of almost all nucleoporins upon loss of NUP93. High-resolution

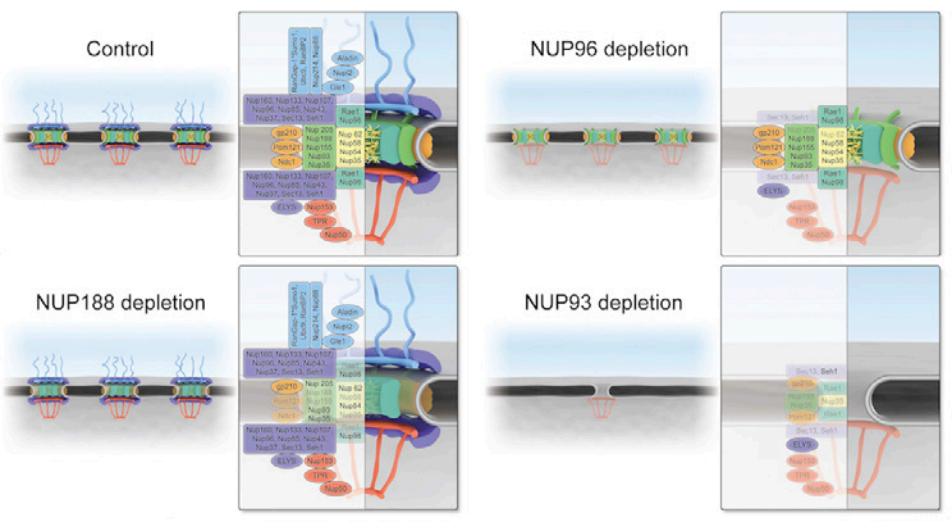


FIGURE 3. Schematic representations of NPC structure upon loss of NUP188, NUP96, or NUP93

scanning electron microscopy of residual NPCs after depletion of NUP96 or NUP93 further confirmed the status of these structures. Together, our results indicate that the inner and outer rings of the NPC form distinct and independent structures, and that NUP93 serves as an NPC lynchpin essential for them both (Figure 3).

After depletion of the inner ring or outer rings, we tested whether the residual structures remained functional for the import and export of a model nuclear transport substrate. Remarkably, there were only minimal changes in both nuclear import and export rates upon loss of NUP96 or NUP188. However, NUP93 depletion caused a complete block in nuclear transport in both directions, confirming that global disruption functionally disabled NPCs. Together, the results indicate that persistent inner-ring or outer-ring structures could still act as conduits for vectoral nuclear trafficking and that these modules can support independent and redundant trafficking routes. Removal of both sets of structures forecloses all nuclear trafficking. Notably, the persistence of functional pores lacking a subset of canonical nucleoporins suggests that terminally differentiated cells might retain substantial nuclear trafficking even with divergent NPC composition. Differentiated cells might thus customize function through altered NPC composition, potentially modulating specific trafficking pathways or aspects of NPC activity such as gene regulation and post-translational protein modifications.

Roles of nucleoporins in gene expression and RNA trafficking

A series of evolutionarily conserved complexes are co-transcriptionally recruited to nascent mRNAs, facilitating their processing as well as escorting them to and through the NPC, actions that are functionally linked; a failure to perform any of them during mRNA biogenesis directly impacts both upstream and downstream events. A key player in mRNA maturation is the transcription and export 2 (TREX-2) complex. Loss of the TREX-2 complex leads to defects in mRNA export, similar to the phenotype observed after loss of the major mRNA export receptor NXF1. The GANP subunit of TREX-2 localizes within the nucleus and associates with the NPC's nuclear basket, which protrudes from the nucleoplasmic face of the NPC. In vertebrates, the nuclear basket comprises three nucleoporins (BSK-NUPs) called NUP153, TPR, and NUP50. BSK-NUPs have been implicated in numerous processes beyond protein import and export, including chromatin remodeling, control of gene expression, and protein modification, as well as mRNA processing and export.

It has been difficult to analyze discrete NPC functions in the absence of vertebrate BSK-NUPs; knockout of their genes is deleterious for organisms, and their depletion by RNAi requires extended incubations, potentially allowing the emergence of secondary phenotypes from prolonged NPC disruption or defective post-mitotic NPC re-assembly. We used the auxin-mediated degradation system (AID) to untangle the functions of individual BSK-NUPs in both nuclear basket architecture and gene expression. We found that NUP153 and TPR bound to the NPC independently of each other and that loss of individual BSK-NUPs did not destabilize the NPC. We further found that TPR, but not NUP153 or NUP50, tethers the TREX-2 complex to the NPC. Loss of NUP50, NUP153, or TPR led to unique transcriptomic responses. Importantly, transcriptomic signatures after loss of TPR were more pronounced and similar to changes upon the loss of either the GANP subunit of the TREX-2 complex or of the RNA–export receptor NXF1. Moreover, similar to the case of NXF1 or GANP, loss of TPR led to retention of both upregulated and downregulated mRNA transcripts within the nucleus.

Taken together, these data support a unique role of TPR in transcription regulation and mRNA export through the TREX-2 complex. The findings both support our hypothesis that individual nucleoporins have distinct and non-redundant cellular functions, and they demonstrate the utility of the AID system to analyze their unique roles within cells.

Impact of Ran pathway organization beyond nuclear trafficking: development and mitosis

The Ran GTPase is a critical regulator of nuclear trafficking in eukaryotic cells. Ran's cytoplasmic GTPase–activating protein, RanGAP, and its chromatin-bound nucleotide exchange factor, RCC1, establish compartmental patterns for Ran's nucleotide-binding status: GTP–bound Ran (Ran-GTP) is abundant in the nucleus while GDP–bound Ran (RanGDP) is abundant in the cytosol. The asymmetry drives nucleo-cytoplasmic trafficking through controlled cargo binding and release by karyopherins, a family of RanGTP–binding transport receptors.

In multicellular organisms, RanGAP is tethered to the cytoplasmic side of the NE, but the functional consequences of its localization had not been clarified. To investigate the importance of RanGAP association with the NE, we used human tissue-culture cells and *Drosophila*. In mammals, RanGAP tethering is mediated by its covalent conjugation to SUMO1, a small ubiquitin-like protein. We made human tissue-culture cells in which RanGAP was neither SUMOylated nor localized to the NE. Surprisingly, the cells showed neither obvious changes in viability nor substantial defects in nucleo-cytoplasmic transport of a model substrate. In *Drosophila*, we found that SUMOylation of RanGAP did not control its association with the NE. Rather, we identified a specific region within the nucleoporin dmRanBP2 (*Drosophila melanogaster* RanBP2) that directly binds to dmRanGAP, tethering it to the NPC (Figure 4). A dmRanBP2 mutant lacking this region showed no apparent growth defects during larval stages, but arrested at the early pupal stage. The developmental arrest was rescued by a direct fusion of dmRanGAP to the dmRanBP2 mutant, indicating that recruitment of dmRanGAP to dmRanBP2 per se was necessary for the pupal ecdysis sequence during development. Collectively, the results indicate that, while the localization of dmRanGAP to the NE is widely conserved in multicellular organisms, the targeting mechanisms are not. Furthermore, the localization appears to be critical for developmental processes rather than for viability at the cellular level. We continue to explore the developmental consequences of releasing the interaction of dmRanGAP to dmRanBP2.

We have a long-standing interest in the process of chromosome segregation. The Ran GTPase pathway and nucleoporins promote chromosome segregation through their important roles in spindle assembly and cell-

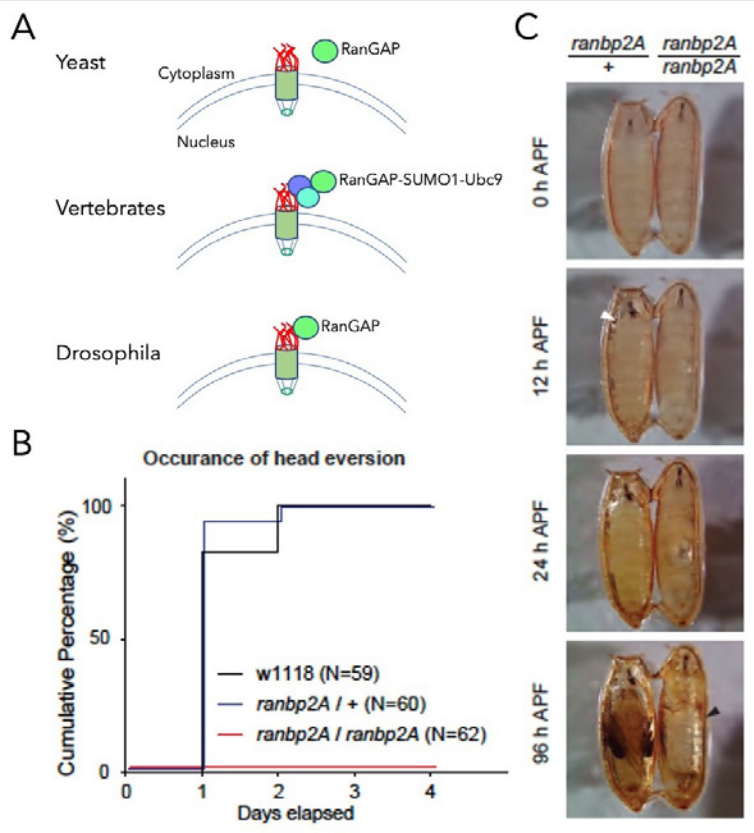


FIGURE 4. Anchorage of dmRanGAP to dmRanBP2 is required for development.

A. Schematic representation of RanGAP association to NPCs. In yeast, RanGAP (*green*) is not bound to the NPC (*top*). In vertebrates, RanGAP binds to RanBP2 (*red*) in a complex that also contains Ubc9 (*aqua*) and SUMO1 (*blue*, *middle*). In flies, dmRanGAP binds directly to dmRanBP2 (*bottom*).

B. Cumulative step histogram of head eversion event during pupal stage. Late 3rd instar larvae (5–day old) were collected on day 0. While head eversion occurred robustly in controls (both *w1118* and heterozygous *ranbp2A* flies), no head eversion event was observed in homozygous *ranbp2A* flies.

C. Behavior analysis of pupal ecdysis. Heterozygous or homozygous *ranbp2A* flies were collected at the white pupal stage (APF: after puparium formation). The homozygous *ranbp2A* fly failed to undergo head eversion (*white arrowhead*) and air bubble translocation after 24 hours, and the air bubble occupied most of the pupa (*black arrowhead*) after 96 hours.

cycle progression. After mitotic NE breakdown, RCC1 generates RanGTP near chromosomes, while Ran distal to chromosomes is GDP–bound, directing spindle assembly through spatially regulated release of spindle assembly factors (SAFs) from karyopherins. To segregate chromosomes accurately, RanGTP distribution must be tightly regulated, both spatially and temporally. Defects in chromosome segregation lead to aneuploidy, a condition in which cells possess an abnormal number of chromosomes. The error-prone nature of Ran–dependent spindle assembly is particularly important during meiosis, potentially contributing to human pregnancy losses and genetic disorders, including Down's syndrome. Moreover, aneuploidy arising from mitotic divisions is a hallmark of many solid tumors.

RanBP1 is a RanGTP–binding protein that forms a stable heterotrimeric complex with Ran and RCC1 *in vitro* (RRR complex), inhibiting RCC1's nucleotide exchange activity. We previously reported that RRR complex formation determines RCC1's partitioning between its chromatin-bound and soluble forms in embryonic systems, where RCC1 levels are very high, as well as specifically inhibiting the activity of soluble RCC1. Somatic cells have less soluble RCC1 during mitosis, raising questions about whether RRR complex formation is important after early development. To investigate the mitotic role of the RRR complex after early development, we examined whether RanBP1 or a related protein called RanBP3 might be important for controlling mitotic RanGTP gradients within somatic cells. We systematically varied RanBP1 and RanBP3 levels in HCT116 or DLD1 cell lines through overexpression or fusion with AID tags. Consistent with earlier reports, RanBP1 was dispensable for interphase import or export of a model substrate, while RanBP3 appears to facilitate

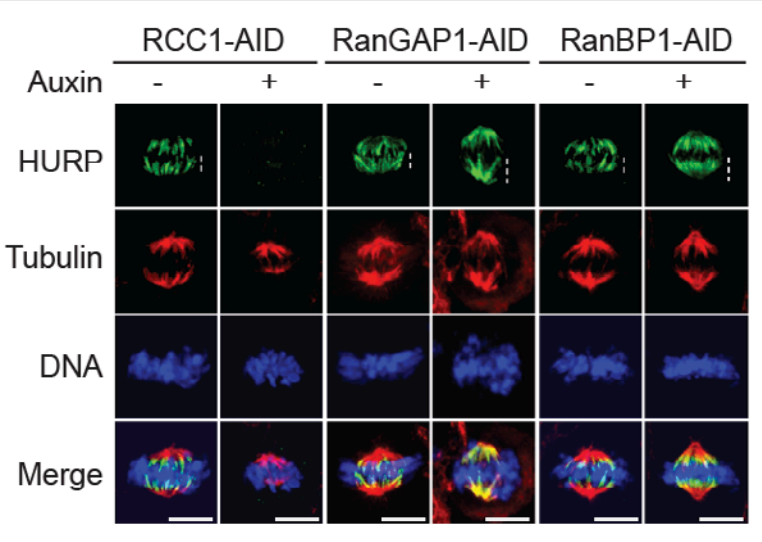


FIGURE 5. Regulation of mitotic Ran–GTP gradients by the RRR complex

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.

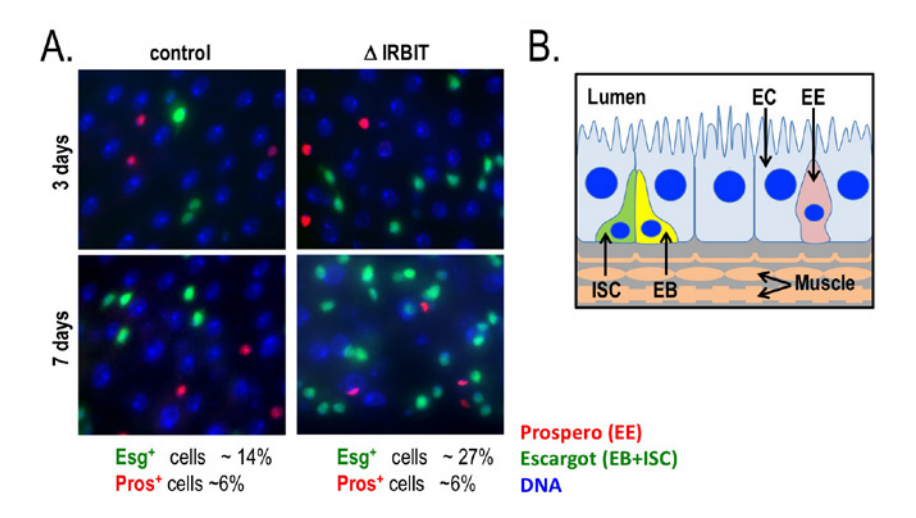
nuclear export via the Crm1 karyopherin. Within mitosis, altering RanBP1 levels substantially altered RCC1 dynamics on metaphase chromosomes, while altering RanBP3 levels did not. Moreover, we found dramatic re-localization of the SAF hepatoma up-regulated protein (HURP, a component of the spindle-assembly pathway) during metaphase in direct correspondence with changes in RCC1 dynamics (Figure 5), showing that RanGTP levels and SAF activity near chromosomes correlate with altered RCC1 behavior. Analogous to findings in embryonic systems, the data indicate an important mitotic role in human somatic cells for RanBP1 in controlling RCC1 dynamics and determining the accurate spatial distribution and magnitude of Ran-GTP gradients, thus ensuring correct execution of Ran–dependent mitotic events.

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) for DNA synthesis. We further found that mammalian tissue-culture cells show altered cell-cycle progression and genome stability in the absence of IRBIT, and that the mechanism is conserved between humans and *Drosophila*. Therefore, in collaboration with Mihaela Serpe and Brian Oliver, we used flies as a model organism to understand the role of IRBIT in development and tissue homeostasis.

In situ hybridization showed *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 6B): 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 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.

FIGURE 6. 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 (*red*, to identify EE cells) and Escargot (*green*, to identify 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.

We examined IRBIT's role in the midgut by generating an *IRBIT* null fly (*IRBIT*$^{-/-}$) (Figure 6A). 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 associated with inflammation and aging. The phenotypes are fully rescued through expression of full-length IRBIT; 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 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, which can be reversed by specifically inhibiting RNR within progeny cells. Collectively, our findings showed that RNR suppression by IRBIT is an important mechanism, directing 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 re-replicate their nuclear genome before cell division, the result is DNA damage, mitotic catastrophe, and programmed cell death (apoptosis). On rare occasions, specialized cells can duplicate their genome several times without undergoing cell division (termed endocycles), but when this occurs, it generally results in terminally differentiated polyploid cells, which are viable but no longer proliferate. However, as we age, the ability to regulate genome duplication diminishes, resulting in genome instability, which allows genetic alterations that can result in promiscuous cell division, better known as cancer.

Our research program focuses on one question: how can we selectively destroy cancer cells with little or no harm to non-malignant cells.

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Cell cycle arrest and apoptosis are not dependent on p53 prior to p53–dependent embryonic stem cell differentiation

Previous efforts to determine whether or not the transcription factor and tumor-suppressor protein p53 is required for DNA damage–induced apoptosis in pluripotent embryonic stem cells (ESCs) produced contradictory conclusions. To resolve this issue, we used $p53^{+/+}$ and $p53^{-/-}$ ESCs to quantify time-dependent changes in nuclear DNA content; annexin-V binding; cell permeabilization; and protein expression, modification, and localization. The results revealed that doxorubicin (Adriamycin [ADR]) concentrations 10 to 40 times lower than commonly used in previous studies induced the DNA damage–dependent G2-checkpoint and completed apoptosis within the same time frame, regardless of the presence or absence of p53, p21, or PUMA (p53–upregulated modulator of apoptosis). Increased ADR concentrations delayed initiation of apoptosis in $p53^{-/-}$ ESCs, but the rates of apoptosis

remained equivalent. Similar results were obtained by inducing apoptosis with either staurosporine inhibition of kinase activities or WX8 (chemical that binds specifically to PIKfyve phosphoinositide kinase) disruption of lysosome homeostasis. Differentiation of ESCs by the LIF (a member of the interleukin-6 family of cytokines) deprivation revealed p53–dependent formation of haploid cells, increased genomic stability, and suppression of the G2-checkpoint. Minimal induction of DNA damage now resulted in p53–facilitated apoptosis, but regulation of pluripotent gene expression remained p53–independent. Primary embryonic fibroblasts underwent p53–dependent total cell-cycle arrest (a prelude to cell senescence), and p53–independent apoptosis occurred in the presence of 10-fold higher levels of ADR, consistent with previous studies. Taken together, the results reveal that the many roles of p53 in cell-cycle regulation and apoptosis are first acquired during pluripotent stem cell differentiation [Reference 1].

Efficacy of a small-molecule inhibitor of the transcriptional cofactor PC4 in prevention and treatment of non-small-cell lung cancer

The human positive coactivator 4 (PC4) was originally identified as a multi-functional cofactor capable of mediating transcription activation by diverse gene- and tissue-specific activators. Recent studies suggest that PC4 might also function as a novel cancer biomarker and therapeutic target for different types of cancers. siRNA (small interfering RNA) knockdown studies indicated that down-regulation of PC4 expression could inhibit tumorigeneicity of the A549 non-small-cell lung cancer tumor model in nude mice. We showed that AG-1031, a small molecule identified by high throughput screening, can inhibit the double-stranded DNA–binding activity of PC4, more effectively than its single-stranded DNA–binding activity. AG-1031 also specifically inhibited PC4-dependent transcriptional activation *in vitro* using purified transcription factors. AG-1031 inhibited proliferation of several cultured cell lines derived from non-small-cell lung cancers (NSCLC) and growth of tumors that formed from A549 cell xenografts in immune-compromised mice. Moreover, pre-injection of AG-1031 in these mice not only reduced tumor size, but also prevented tumor formation in 20% of the animals. AG-1031–treated A549 cells and tumors from AG-1031–treated animals showed a significant decline in the levels of both PC4 and VEGFC, a key mediator of angiogenesis in cancer. On the other hand, all tested mice maintained constant weight during animal trials. The results demonstrated that AG-1031 has potential as a therapy for PC4–positive NSCLC [Reference 2].

Developmental acquisition of p53 functions

Remarkably, the p53 transcription factor, referred to as “the guardian of the genome,” is not essential for mammalian development. Moreover, efforts to identify p53–dependent developmental events have produced contradictory conclusions. Given the importance of pluripotent stem cells as models of mammalian development, and their applications in regenerative medicine and disease, resolving these conflicts is essential. We attempted to reconcile disparate data into justifiable conclusions predicated on reports that p53–dependent transcription is first detected in late mouse blastocysts, that p53 activity first becomes potentially lethal during gastrulation, and that apoptosis does not depend on p53. Furthermore, p53 does not regulate expression of genes required for pluripotency in ESCs; it contributes to ESC genomic stability and differentiation. Depending on conditions, p53 accelerates initiation of apoptosis in ESCs in response to DNA damage, but cell-cycle arrest as well as the rate and extent of apoptosis in ESCs are p53–independent. In embryonic fibroblasts, p53 induces cell-cycle arrest, to allow repair of DNA damage, and cell senescence, to prevent proliferation of cells with extensive damage [Reference 3].

A p53 enigma in mammalian embryonic development

Of the eleven forms of programmed cell death (PCD) in mammals, only apoptosis and autophagy have been reported during embryonic development, and efforts to determine whether or not the p53 transcription factor

and tumor suppressor promotes PCD during embryogenesis have produced contradictory conclusions. Some studies conclude that the DNA damage response in pluripotent ESCs is p53–dependent whereas others conclude that it is not. Given the complexities of PCD, the importance of pluripotent stem cells as models of embryonic development and their applications in regenerative medicine, resolving the role of p53 in PCD in pluripotent cells is essential. We reconciled contradictions based on the fact that p53 cannot induce lethality during embryogenesis until gastrulation, and that differences in experimental conditions could account for differences in conclusions. The DNA–damage G2–checkpoint in ESCs is p53–independent. However, depending on conditions, p53 can accelerate initiation of apoptosis in ESCs, but once initiated, apoptosis occurs at equivalent rates and to equivalent extents regardless of the presence or absence of p53. In differentiated embryonic cells, DNA damage results in p53–dependent cell cycle arrest and senescence [Reference 4].

Selective elimination of pluripotent stem cells by PIKfyve–specific inhibitors

Inhibition of the PIKfyve phosphoinositide kinase selectively kills autophagy-dependent human cancer cells by disrupting lysosome homeostasis. We showed that PIKfyve inhibitors can also selectively kill human pluripotent embryonal carcinoma cells (ECCs) derived from teratocarcinomas, as well as pluripotent ESCs under conditions in which differentiated cells remain viable. PIKfyve–specific inhibitors prevented lysosome fission, induced autophagosome accumulation, and reduced cell proliferation to varying extents in each of these cell types, but they induced cell death only in pluripotent stem cells. Cell death followed disruption of autophagy and occurred via a non-canonical form of apoptosis, as demonstrated by a concomitant loss of ATP, binding of annexin-V, membrane permeabilization, and loss of DNA that did not involve caspase-3 cleavage. The ability of PIKfyve inhibitors to distinguish between pluripotent cancer stem cells and differentiated cells was confirmed with xenografts derived from human ECCs. Pretreatment of ECCs with the PIKfyve–specific inhibitor WX8 suppressed their ability to form teratocarcinomas in mice, and intraperitoneal injections of WX8 into mice harboring teratocarcinoma xenografts selectively eliminated the pluripotent cells. The differentiated cells continued to proliferate, but at a reduced rate. The results provide a proof-of-principle that PIKfyve–specific inhibitors can selectively eliminate pluripotent stem cells *in vitro* and *in vivo* [Reference 5].

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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, as well as 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 the factor plays in polyamine-regulated gene-specific translational control mechanisms, and we are characterizing metabolite control of translation via non-canonical upstream open reading frames (uORFs) 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 with altered function of eIF2. In previous studies, we showed that the MEHMO syndrome (named based on the 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. Using genetic and biochemical techniques in yeast models of human MEHMO–syndrome mutations, we previously characterized several mutations that impair eIF2 function, disrupt

eIF2 complex integrity, and alter the stringency of translation start-codon selection. Our collaborators have linked the EIF2S3 mutations with variable levels of motor delay, microcephaly, ID, epilepsy, central obesity, and diabetes, thus revealing a broad genetic spectrum and clinical expressivity of the MEHMO syndrome [References 1,2].

Using induced pluripotent stem (iPS) cells derived from a patient with the MEHMO syndrome mutation *EIF2S3-I465Sfs*4*, we observed a general reduction in protein synthesis and constitutive induction of the integrated stress response with elevated expression of a translational regulatory response typically associated with eIF2alpha phosphorylation, including heightened expression of the transcriptional activators ATF4 and CHOP and the protein phosphatase–regulatory subunit GADD34. Under stress conditions, hyperactivation of the integrated stress response in the mutant iPS cells triggered apoptosis. Upon differentiation into neurons, the mutant cells exhibited reduced dendritic arborization [Reference 3].

We propose that the mutations in eIF2gamma impair the efficiency and fidelity of protein synthesis, and that such altered control of protein synthesis underlies the MEHMO syndrome. Interestingly, addition of the drug ISRIB, an activator of the eIF2 guanine nucleotide exchange factor, rescued the cell-growth, translation, and neuronal-differentiation defects associated with the *EIF2S3-I465Sfs*4* mutation, offering the possibility of therapeutic intervention for the MEHMO syndrome [Reference 3]. Our current efforts are aimed at characterizing additional novel *EIF2S3* mutations linked to the MEHMO syndrome, testing whether ISRIB can suppress the phenotypes associated with other MEHMO mutations, and generating a mouse model of the MEHMO syndrome.

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

The translation factor eIF5A is the sole cellular protein containing the unusual amino acid hypusine [N^{ϵ}-(4-amino-2-hydroxybutyl)lysine]. In previous studies, we showed that eIF5A promotes translation elongation and that such activity depends on its hypusine modification. Moreover, using *in vivo* reporter assays and *in vitro* translation assays, we showed that eIF5A in yeast, like its bacterial homolog EF-P, is especially critical for the synthesis of proteins containing runs of consecutive proline residues. In collaboration with Rachel Green, we reported that eIF5A functions globally to promote both translation elongation and termination. Moreover, using 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. Together with Marat Yusupov, we found that eIF5A binds in the ribosome E site to the hypusine residue projecting toward the acceptor stem of the P-site tRNA. Based on these findings, we propose that eIF5A and its hypusine residue function to reposition the acceptor arm of the P-site tRNA to enhance reactivity towards either an aminoacyl-tRNA, for peptide bond formation, or a release factor, for translation termination.

In ongoing experiments, we are further investigating 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 the formation of hypusine. In contrast to the essential deoxyhypusine synthase, which catalyzes the first step in hypusine formation, the *LIA1* gene, encoding the hydroxylase, is non-essential in yeast. We identified mutations in eIF5A that cause synthetic growth defects in cells lacking the hydroxylase. Interestingly, the mutations map to the ribosome-binding face

of eIF5A. 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 an unusual upstream open reading frame (uORF) in the leader of the *AZIN1* mRNA is critical for the regulation. The uORF lacks a canonical 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 uORF. Remarkably, polyamine induction of uORF translation depends on the sequence of the encoded polypeptide, including a highly conserved Pro-Pro-Trp (PPW) motif that causes polyamine-dependent pausing of elongating ribosomes.

We proposed that, under low polyamine conditions, many scanning ribosomes bypass the near-cognate start codon of the uORF without initiating and then translate *AZIN1*. Under high polyamine conditions, ribosomes elongating on the uORF pause on the PPW motif. The paused ribosome serves as a roadblock to subsequent scanning ribosomes that bypass the near-cognate start codon. The resulting queue of scanning ribosomes behind the paused elongating ribosome positions a ribosome near the start site of the uORF, providing greater opportunity for initiation at the weak start site. This induction of uORF translation reinforces the inhibition of *AZIN1* synthesis.

In further studies on the *AZIN1* regulatory mechanism, we identified eIF5A as a sensor and effector for polyamine control of uORF 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. 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.

In recent studies examining translational control by polyamines, we identified the yeast high-affinity polyamine transporter [Reference 5]. Using ribosome profiling, we identified mRNAs whose translation was sensitive to changes in polyamine levels. One of the mRNAs encoded a member of the drug-proton antiporter (DHA1) family of transporters called Hol1. We showed that *HOL1* was required for yeast growth under limiting polyamine conditions and for high-affinity polyamine uptake by yeast. Together with Anirban Banerjee's lab, we showed that purified Hol1 transports polyamines. The leader of the *HOL1* mRNA contains a highly conserved uORF encoding the peptide MLLLPS*. We found that polyamine inhibition of the translation factor eIF5A impairs translation termination at the Pro-Ser-stop (PS*) motif of the uORF to repress Hol1 synthesis under conditions of elevated polyamines. Our findings reveal that polyamine transport, like polyamine biosynthesis, is under translational autoregulation by polyamines in yeast, highlighting the extensive control cells impose on polyamine levels.

Translational control by metabolite–sensing nascent peptides

In ongoing studies, we searched for additional mRNAs containing noncanonical uORFs. One such candidate was identified in plants in the mRNA encoding GDP-L-galactose phosphorylase (GGP), a control enzyme in the vitamin C biosynthetic pathway. Using reporter assays in mammalian cells and, *in vitro*, using rabbit reticulocyte lysates, we revealed that a uORF–like element in the GGP mRNA mediates translational control by vitamin C. We propose that interaction of vitamin C with the GGP uORF 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 uORF and prevents ribosome access to the GGP ORF. We hypothesize that the mechanism by which a paused elongating ribosome promotes initiation at an upstream weak start site via ribosome queuing may underlie the control of translation of other mRNAs, especially those whose translation is derepressed by conditions that impair ribosome loading.

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

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 most eukaryotes and archaea, a conserved histidine residue at the tip of eEF2 is post-translationally modified to diphthamide through the action of seven non-essential proteins. The function of diphthamide and the rationale for its evolutionary conservation are not well understood. The name diphthamide is derived from diphtheria, a disease of the nose and throat caused by the bacterium *Corynebacterium diphtheriae*. Infections with *C. diphtheriae* can lead to respiratory distress and even death; however, an effective vaccine is available. The bacterium expresses a toxin that ADP–ribosylates the diphthamide residue, leading to inactivation of eEF2. Several additional bacterial pathogens, including *Pseudomonas aeruginosa* and *Vibrio cholerae*, express distinct toxins that also modify the diphthamide residue and inactivate eEF2.

Based on a cryo-electron microscopy structure of eEF2 bound to the yeast 80S ribosome obtained by our collaborators in Venki Ramakrishnan's lab, we hypothesize that diphthamide has at least two functions: first, to disrupt the decoding interactions of rRNA with the codon-anticodon duplex in the ribosomal A site; and second, to help chaperone the codon-anticodon interaction as the A-site tRNA is translocated to the P site. In ongoing studies, we are further exploring the role of diphthamide in promoting the accuracy and efficiency of translation elongation. Our preliminary data indicate that loss of diphthamide impairs ribosome processivity during elongation as a result of increased levels of frameshifting and translation termination at out-of-frame stop codons. Such increased frameshifting in yeast and mammalian cells lacking diphthamide occurs at both programmed frameshifting sites in the HIV and SARS-CoV-2 viruses and throughout translation elongation at non-programmed sites. We propose that diphthamide, despite its non-essential nature in yeast, has been conserved throughout evolution to maintain the fidelity of translation elongation and block spurious frameshifting events that would impair the production of the native proteins and generate novel frameshifted proteins that might be deleterious to the cell.

Molecular analysis of eIF5B and a translational fidelity checkpoint at subunit joining

The translation factor eIF5B is a GTPase required for the last step of translation initiation: joining of the large ribosomal subunit to the small subunit poised on the start codon of an mRNA. The eIF5B binds to the 40S

subunit and collaborates in the correct positioning of the initiator Met-tRNA$_i^{Met}$ on the ribosome in the later stages of translation initiation, gating entrance into elongation. Working with collaborators in California, New York, and Spain, we helped show that eIF5B plays an important role in translation start-site selection, ensuring high fidelity in this process, which establishes the reading frame for translation on an mRNA.

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Regulation of Hormone Receptors and Gonadal Genes

The Section investigates the regulatory mechanism(s) involved in the progression of spermatogenesis and the control of Leydig cell function. We investigate novel gonadotropin-regulated genes relevant to the progression of testicular gametogenesis, Leydig cell function, and other endocrine processes. Our studies concentrate on the function and regulation of the gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), a member of the DEAD–box family of RNA helicases discovered, cloned, and characterized in our laboratory, which is essential for the progress of spermatogenesis [Reference 1]. The various functions of GRTH/DDX25 provide fertile ground for the development of a non-hormonal male contraceptive.

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Gonadotropin–regulated testicular RNA helicase (GRTH)

GRTH is present in Leydig cells (LCs) and meiotic (pachytene spermatocytes) and haploid germ cells (round and elongated spermatids). Male null mice lacking GRTH are sterile owing to azoospermia resulting from the failure of round spermatids to elongate. We demonstrated GRTH's participation in the nuclear export/transport of specific mRNAs, the structural integrity of the chromatoid body (CB, an organelle prevalent in round spermatids [RS] and found to be key for the progress of spermatogenesis), storage/processing of relevant mRNAs, and their transit/association to the actively translating polyribosomes, where it may regulate translational initiation of genes. GRTH is the only DEAD-box family member regulated by hormones. *GRTH* transcription is stimulated in LCs by LH (luteinizing hormone)/cAMP through direct actions of the androgen (A)/A receptor (AR) (autocrine), and in germ cells in paracrine fashion through the AR in Sertoli cells. The upstream region of the *GRTH* gene directs its expression in germ cells and downstream in LCs. Through these regions, A/AR exerts its direct (endogenous) regulation of the *GRTH* gene in the LC, and indirectly in germ cells.

We identified functional binding sites for the germ-cell nuclear factor (GCNF), which are present in RS and spermatocytes (SP), and its regulation by A/AR 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

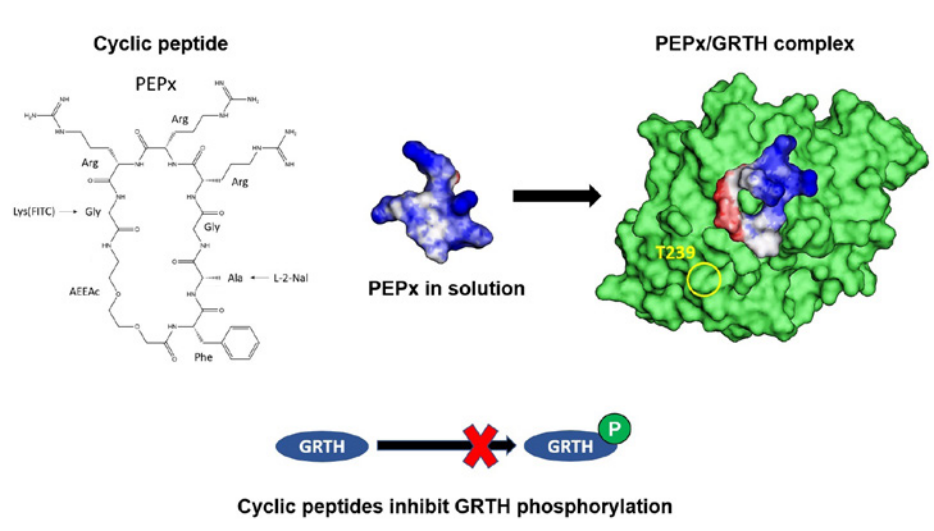
cells through GCNF as an A–regulated trans-factor that controls transcription/expression of GRTH (Kavarthapu & Dufau, *Mol Endocrinol* 2015;29:1792). These findings provide a connection between androgen action and two relevant germ-cell genes (*GRTH* and *GCNF*), which are essential for the progress of spermatogenesis, and we established their regulatory interrelationship.

Our early studies revealed that when the missense mutation of R to H at aa 242 of GRTH, found in 5.8% of 100 azoospermic patients, was transfected in COS1 cells, it caused loss of the 61 kDa phospho-species (pGRTH) with preservation of the 52 kDa non-phospho form (Tsai-Morris *et al*., *Mol Human Reprod* 2007;13:887). The finding provided an avenue to elucidate the function of pGRTH in spermatogenesis. We generated humanized mutant *GRTH* knock-in (KI) mice. The mutant mice are sterile, with reduction on testicular size, they lack sperm with arrest at step 8 of round spermatids (RS), and exhibit complete loss of the pGRTH species with preservation of the nuclear 52 kDa form [Reference 2]. The mouse model allows us to study 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), while the cytoplasmic functions, including shuttling of messages, storage in the CB, and translational events all requiring pGRTH, are absent. We observed a marked reduction in the size of the CB in RS and lack of pGRTH in the CBs. Germ-cell apoptosis was present in pachytene spermatocytes (PS) and in RS. In contrast to KO, KI showed no changes in miRNA biosynthesis, excluding participation of pGRTH as a transcriptional regulator of the microprocessor complex (which consists of Drosha, a miRNA–processing enzyme and its cofactor the RNA–binding protein DCGR), affecting pri-miRNA (primary microRNA) formation and indicative of the participation of non-phospho GRTH in such processes. In KI mice there is loss of chromatin-remodeling and related proteins, including TP2 (transition protein 2, a testis-specific DNA–condensing protein), PRM2 (a protamine that facilitates DNA condensation), and TSSK6 (a testis-specific serine/threonone-protein kinase, which is involved in the compaction of DNA during spermatogenesis). Significant reductions in 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.

In recent studies, we elucidated the GRTH phospho-site at a threonine (T239), which is 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 pointed to the amino acids that formed the GRTH/protein kinase alpha (PKA) interface, solvent accessibility, and H-bonding. These include, in addition to the core residues T239 and R242, amino acids E165, K240, and D237 [Reference 3]. We demonstrated the relevance of these residues by disrupting amino acids using site-directed mutagenesis (single or double mutations), which caused reduction or abolition of the pGRTH at T239. The pGRTH form is the cytoplasmic species that was demonstrated as essential for the progress of spermatogenesis beyond step 8 of RS and for viable sperm formation. It is important to note that the deleterious effects on GRTH phosphorylation caused by the mutations did not result from changes in PKA–catalytic binding affinity but rather in consequential structural changes that can affect PKA catalytic efficiency. Studies based on the abolition of the phospho-form provide the basis for drug design, such as for discovery of a reversible chemical inhibitor for use as male contraceptive.

During the past year, we determined that cyclic peptides that fit the shallow pocket of the GRTH/DDX5/PKA interphase are preferred compounds to block GRTH phosphorylation and amenable for use in the development of an oral male contraceptive [Reference 4] (Figure 1). In this regard, these cyclic peptides (PEP0, PEP1, and PEP2) have been designed and synthesized as promising therapeutic agents. PEP1 and PEP2, revealed by fluorescein isothiocyanate (FITC), showed effective internalization in COS-1 cells and seminiferous

FIGURE 1. Cyclic peptides inhibit GRTH phosphorylation.



Peptide x (*left*) ([1]FAGRRRG[7]) represents one of the simplest cyclic peptides that accommodates stringent conditions and the main elements of the pharmacophore. The molecular scaffold provided the basis for the design of cyclic peptides (CPs) that bind to GRTH. A computational multistage cyclization procedure predicted the binding modes at the interphase. The simulations indicated that, in solution, the CPs are quite flexible, an attribute that permits adaptation to the GRTH/PKA interphase (*center*, *right*) to exert its inhibitory function on the phosphorylation of GRTH at T239 (*yellow circle*, *right*). Binding of the CPs to GRTH was confirmed by thermal stabilization of non-phospho–GRTH and increased efficiency in FRET assay [Reference 4].

tubules after a 4-hour treatment. We observed a dose-dependent inhibitory effect on GRTH phosphorylation in a COS-1 stable cell line expressing GRTH, with significant reduction in pGRTH protein observed after 8–16 hour treatments. CETSA (cellular thermal shift assay) showed compound binding resulting in thermal stabilization of the soluble non–pGRTH protein when compared with control peptides. Increased efficiency in a fluorescence resonance energy transfer (FRET) assay revealed the interaction of the cyclic peptide with GRTH. Exposure of a culture of seminiferous tubules to these compounds resulted in significant inhibition of the pGRTH protein species. Similar results were obtained with the compound that lacks FITC. Taken together, effective internalization and targeted reduction in the expression of pGRTH by cyclic peptides provide a promising angle to develop effective compounds for use as a non-hormonal male contraceptive.

Also, work in transcriptome profiles of germ cell isolated form GRTH–KI mice vs. those of wild type (WT) mice, using the RNA-Seq technique, has provided further insights linking phospho-GRTH to histone ubiquitination and acetylation essential for chromatin compaction and spermatid elongation during spermiogenesis [Reference 5].

We also initiated studies on the role of phosphorylated GRTH in the storage of messages in the CB. We observed absence of pGRTH from the CB of GRTH KI mice (with an insertion of the R242H human mutation that abolishes GRTH phosphorylation at T239 and spermatogenesis). RNA-Seq analysis of mRNA isolated from the CB revealed that the abundance of 947 genes was reduced and that of 474 genes increased [Reference 5]. Transcripts related to spermatid development, differentiation, and chromatin remodeling were reduced; in contrast those encoding factors involved in RNA transport, regulation, surveillance, and transcriptional and translational regulation were increased in the CB of KI mice, which was validated by qPCR. Transcripts

of several initiation factors (eIF4e, 4ebp2, 3land 3m), together with mRNAs related to the 60S subunit (RpL101/RPlp0), were increased and accumulated in CB, mRNAs that could not be transported from the CB to polyribosomes for translation; instead, they remain stored in the CB in KI mice owing to loss of pGRTH [Reference 6]. Our studies demonstrated the importance of phospho–GRTH in the maintenance of the structure of the CB and its role in the storage and stability of germ cell–specific mRNAs during spermiogenesis.

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Transcriptional Control of Cell Specification and Differentiation

Animals consist of a collection of cells with diverse shapes, structures, and functions, a diversity that is rebuilt from scratch by every embryo. The genetic programs that direct the process are the central mystery of developmental and regenerative biology. We are interested in how decisions about what cell type to adopt are controlled, and what genetic programs direct the morphological and functional specialization of different cells.

The single-cell revolution in developmental biology has given us new access and new tools to address these questions. I previously developed high temporal-resolution single-cell RNA sequencing approaches to identify transcriptional trajectories, i.e., the 'highways' or most likely paths through gene expression that cells take during development. From such data, we were able to identify the sequence of genes expressed by individual cell types during early development, which provides insight into the genetic programs that regulate cells' choice of cell type and then their downstream functional transformations at a wider breadth than was previously achievable. Work in the lab focuses on more deeply exploring such processes, using the approaches we developed. Our lab combines single-cell genomics, imaging, genetic, and classical embryological approaches to investigate the genetic control of cell specification and differentiation during vertebrate embryogenesis. We focus on zebrafish embryos as a model system to study these questions, because among vertebrates, they are easy to culture, image, and manipulate, both embryologically and genetically.

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Genetic underpinnings of cell differentiation

Once a cell has been specified, it must acquire the particular morphology and functionality of its cell type through the process of differentiation, a process that is driven by cell type–specific expression of differentiation genes and often results in dramatic changes in basic cell-biological processes. We aim to identify those genes that drive differentiation and understand their regulation. To do this, we have generated a single-cell RNA-Seq atlas spanning embryogenesis and early larval stages. We aim to find the cascade of genes expressed during differentiation of every cell type, determine their membership in functional gene modules (groups of genes that work together),

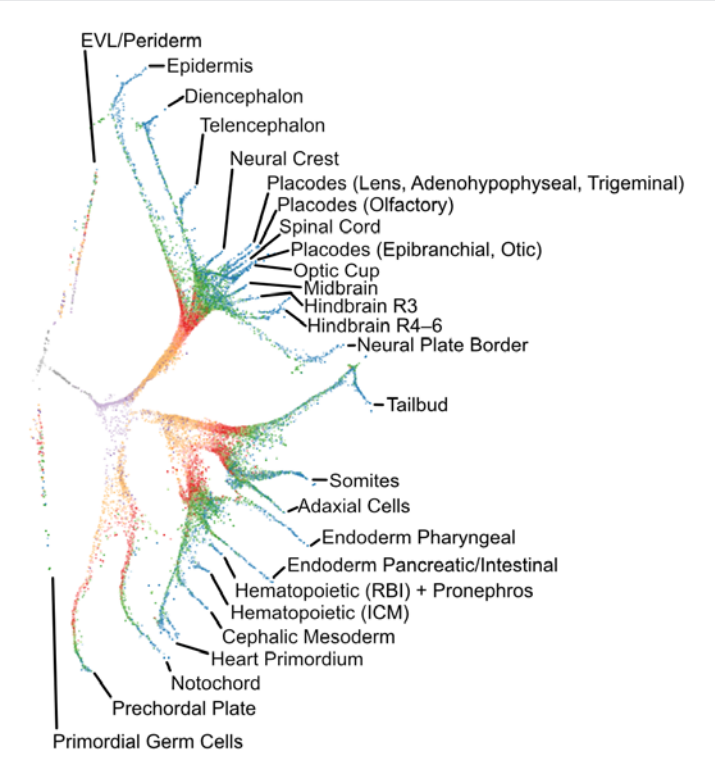


FIGURE 1. Transcriptional trajectories in early zebrafish development

Single-cell transcriptomes were isolated from zebrafish embryos at 12 developmental stages spanning 3–12 hours post-fertilization. The branching transcriptional trajectories, which represent the gene-expression events that give rise to 25 different differentiated cell types, were then reconstructed using URD, a simulated diffusion-based computational reconstruction method, which is software that we developed and published in 2018 [Reference 1].

and associate them with that cell type's cell-biological transformations during differentiation. The studies will permit comparisons of differentiation-gene deployment across cell types to understand the reuse of differentiation programs during development.

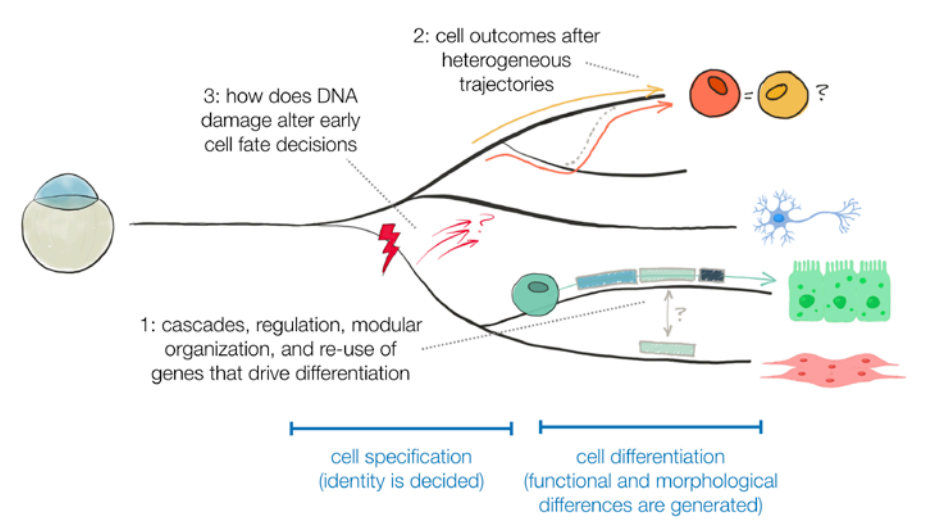
Consequences of heterogeneous developmental trajectories

Distinct cell types can arise through many developmental trajectories or developmental histories. We and others have observed refinement at the boundaries between groups of cells specified to become different tissues; at such boundaries, some cells switch from one specification state to another. We use the axial mesoderm as a model and seek to understand (1) what drives cell-type switching; (2) the long-term consequences for a cell that switched; and (3) the mechanisms that assist in successful switching.

Effect of environmental insults on developmental choices

During early embryogenesis, a field of equipotent cells are instructed to initiate different gene expression programs by external developmental signals and cell-intrinsic cues. We recently observed that cells that experience DNA damage in early zebrafish embryos initiate an unusual transcriptional response during a very limited window in development. Moreover, most damaged cells are not eliminated but appear to be excluded from contributing to some tissues in the animal, which suggests that responding to DNA damage may affect cells' choices during development and which raises a question of how that occurs. We are investigating (1) the fate of cells in early development that experience DNA damage; (2) the role this unusual transcriptional response plays; and (3) what drives the bias in damaged cells' future cell type.

FIGURE 2. Overview of ongoing research projects



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Nervous System Development and Plasticity

The healthy development of the brain and cognition in children is central to the mission of the NICHD. 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 central nervous system [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 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.

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Learning is perhaps the most important function of childhood. Our research is determining the molecular and cellular mechanisms that convert short-term into long-term memory. If functional experiences produce lasting effects on brain development and plasticity, specific genes must be regulated by specific patterns of impulse firing. We are determining how the pattern of neural impulses, i.e., the neural information code, regulate specific genes controlling development and plasticity of neurons and glia.

Nervous system plasticity by activity-dependent myelination

The fundamental cellular mechanism of learning, memory, and neural plasticity is synaptic plasticity, in which the strength of synaptic transmission or the number of synapses is modified by experience. Our laboratory has a long-standing interest in synaptic plasticity, but our current interest is to explore new, non-synaptic mechanisms involved in these processes. In particular, we are investigating how changes in neural impulse conduction velocity contribute to learning and plasticity, and the involvement of glial cells that form the myelin sheath in this new form of plasticity. Our research indicates that, by modifying conduction velocity to optimize the timing of neural impulse arrival at relay points in neural networks, and by influencing the phase and frequency of neural oscillations, myelin-forming glia participate in learning, neural plasticity, and nervous system development in accordance with functional activity and experience.

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.

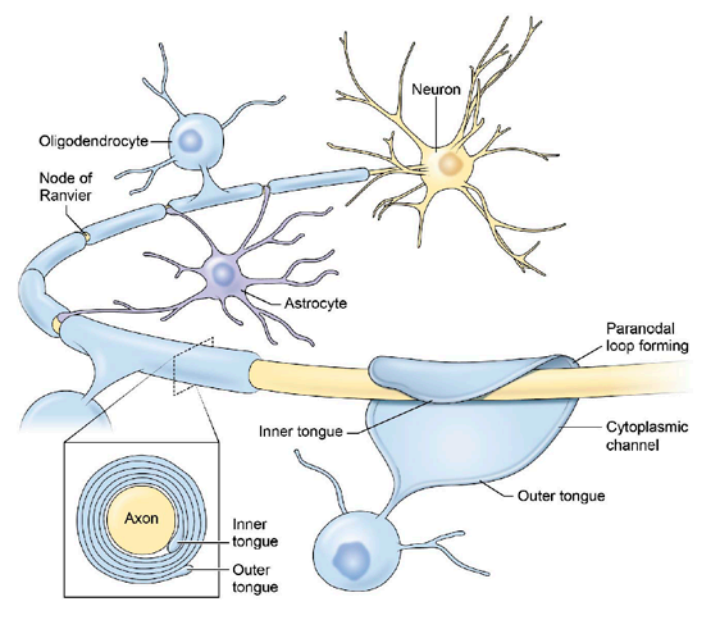
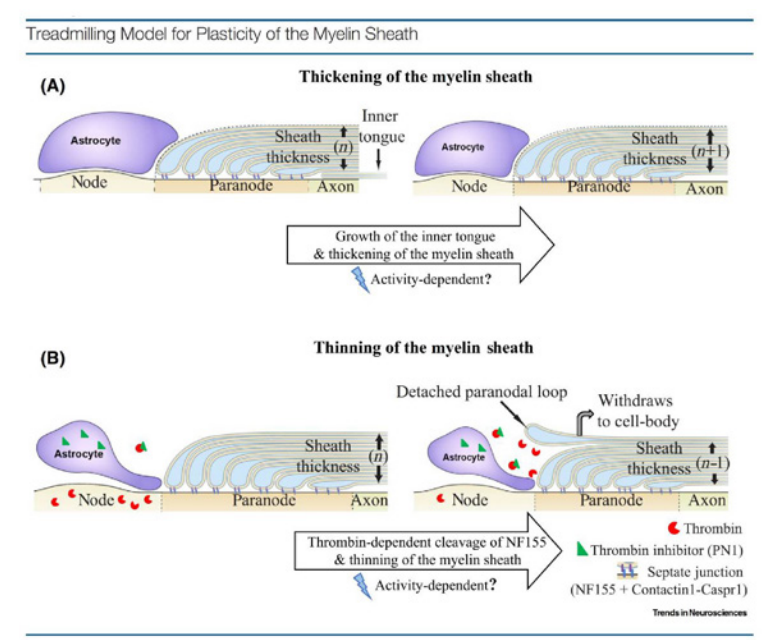


FIGURE 1. The structure of CNS myelin and nodes of Ranvier

Traditionally myelin has been viewed in terms of conduction failure after damage (for example in multiple sclerosis), but we are exploring how changes in myelin driven by functional activity affect the timing of neural-impulse arrival at synaptic relay points, which is critical for information processing and synaptic activity. In addition, the frequency, phase, and amplitude coupling of oscillations in the brain (brainwaves) requires appropriate impulse conduction velocity, which is influenced by myelination. Many neurological and psychological dysfunctions can develop when optimal neural synchrony of spike-time arrival and neural oscillations are disturbed, for example, in schizophrenia, epilepsy, dyslexia, and autism.

FIGURE 2. Treadmilling model for plasticity of the myelin sheath



The speed of neural impulse transmission is altered by changes in myelin structure. The thickness of the myelin sheath in CNS axons is determined by two opposing processes: one (*A*) that adds additional wraps of myelin to the axon, and the other (*B*) that removes the outer layer, thereby increasing and decreasing impulse conduction velocity, respectively. New layers of myelin are added beneath the overlaying layers by expansion of the inner tongue of myelin. Myelin is attached to the axon at the paranodal region flanking the node of Ranvier via septate junctions, consisting of neurofascin 155 on myelin interacting with the contactin1-caspr1 complex on the axon. Cleavage of neurofascin 155 by thrombin (*red*) can break this interaction, resulting in detachment of the outer paranodal loop from the axon, and withdrawal of the outer layer of myelin, which increases nodal gap length and reduces myelin sheath thickness; both effects slow conduction velocity. Perinodal astrocytes at the nodes of Ranvier regulate the process by secreting thrombin inhibitors (*green triangle*), such as protease nexin1. The treadmilling process helps achieve optimal conduction velocity in individual axons [Reference 4].

Our research shows that, to activate receptors on myelinating glia as well as on astrocytes and other cells, neurotransmitters are released not only at synapses but also along axons firing action potentials. The recipient cells in turn release growth factors, cytokines, and other molecules that regulate myelination, proliferation, and 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 electrically inactive axons, thus regulating myelination of axons and neural circuit function according to functional experience, which would be particularly important in the adolescent brain, for example, 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 re-myelination after axon injury.

Myelin in the PNS is formed by a different type of glial cell: the Schwann cells. Our research is also investigating how neural impulse activity can influence myelination in the PNS, and the results indicate that different

mechanisms are responsible from those operating on CNS myelin. These studies involve optogenetics, gene expression analysis, *in vivo* and in primary cell cultures.

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 (gaps in the myelin sheath) along axons. Our research showed that myelination of unmyelinated axons and the thickness of the myelin sheath can be increased in response to neural activity and environmental experience. Prior to our research, myelin structure was believed to be static, and there was no known mechanism that could reduce the thickness of the mature myelin sheath (except in the context of pathology). However, 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, 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 for myelin disruption and repair.

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. Given that long-lasting changes in the nervous system require regulated gene expression, appropriate patterns of neural impulse firing driving neural plasticity must control transcription of specific genes, a fundamental question central to the processes of experience-dependent plasticity during development and learning. However, little is known about how neural firing patterns regulate gene expression. 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.

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 for 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 the 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 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 question, 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, indicated 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 our colleague 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 (micrococcal nuclease) 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 such 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.

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Translational Biophotonics in Developmental Disorders and Diseases

Brain imaging and spectroscopy of developmental disorders

We use functional near infrared spectroscopy (fNIRS) to study brain activity in two distinct lines of research: (1) developmental trajectories of cognitive abilities in children; and (2) validity of fNIRS using cognitive tasks previously evaluated using fMRI. For the developmental studies, we continued the collaboration with Audrey Thurm and Nathan Fox on the mirror neuron network (MNN) in infants. The MNN is associated with the development of sophisticated social behaviors that emerge in typical human infants (e.g., complex imitation, decoding emotional states). Modeling MNN development will create a sensitive measure of deviations in social-communication development before clinical behavioral deficits can be detected. MNN activation has already been associated with mu wave suppression using EEG. To investigate the MNN we used EEG (with high temporal resolution) in conjunction with fNIRS, which provides a more precise spatial resolution of neural activity based on hemodynamic activation. Three manuscripts were published from this work and one manuscript is under review. We conducted a review on the current fNIRS literature examining the action-observation network (AON) [Reference 1]. The review assessed and critiqued the methodological and analytic approaches that have been used to study the action-observation network in healthy adults using fNIRS. This paper was an important preparatory step in planning our empirical approach for our mirror-neuron project and contributed to the field by encouraging other researchers to consider integrating fNIRS measurement into these types of studies.

We also demonstrated the feasibility of using fNIRS to measure the neural correlates of action-observation and action-execution regions during a live task [Reference 2]. Our results indicated that the parietal regions, including bilateral superior parietal lobule (SPL), bilateral inferior parietal lobule (IPL), right supra-marginal region (SMG), and right angular gyrus (AG), share neural activity during action observation and action execution. Our findings confirm the applicability of fNIRS to the study of the AON and lay the foundation for future work with developmental and clinical populations.

We used a functional connectivity approach to investigate functional connectivity in the MNN and assess relationship between MNN

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connectivity and subclinical autistic traits in neuro-typical adults [Reference 3]. In this study, we examined functional connectivity for action execution, action observation and explored MNN connectivity by identifying regions with greater connectivity in both conditions. Our results showed that during the action execution, while a participant was performing an action with the right hand, several region-to-region connections within the left hemisphere were related (connections within the left precentral, left postcentral, left inferior parietal and between the left supramarginal and left angular regions). In addition, we found five significant connections that overlapped between the two conditions: connections within the right precentral, right supramarginal, left inferior parietal, left postcentral, and between the left supramarginal and left angular regions. These connections were considered as potential candidates for mirror neuron network. Interestingly, we also found that individuals with higher subclinical autistic traits present higher connectivity in both action-execution and action-observation conditions. The results support the correlation between MNN connectivity and subclinical autistic traits can be used in future studies to investigate MNN in a developing population with autism spectrum disorder.

For the multimodal analysis we developed structured sparse multiset canonical correlation analysis (ssmCCA) to perform EEG-fNIRS data fusion. MCCA is a generalization of CCA to more than two sets of variables which is commonly used in medical multimodal data fusion. However, mCCA suffers from multi-collinearity, high dimensionality, unimodal feature selection, and loss of spatial information in results interpretation. A limited number of participants (small sample-size) is another problem in mCCA, leading to overfitted models. We adopted graph-guided (structured) fused LASSO (sparse) penalty to mCCA to conduct feature selection, incorporating structural information amongst variables (i.e., brain regions). Benefitting from concurrent recordings of brain hemodynamic and electrophysiological responses, the proposed ssmCCA finds linear transforms of each modality such that the correlation between their projections is maximized. Our analysis of 21 right-handed subjects indicated that the left inferior parietal region was active during both action execution and action observation. Our findings provide new insights into the neural correlates of AON which are more fine-tuned than the results from each individual EEG or fNIRS analysis and validate the use of ssmCCA to fuse EEG and fNIRS datasets. Moreover, comparing the data fusion and unimodal results, the findings from our data fusion analysis are more specific, pointing to the left inferior parietal lobe as the region that presents the highest covariation between EEG and fNIR signals during an AON paradigm. A paper related to EEG-fNIRS data fusion is currently under review. Taken together, the studies derived from our pilot study validate the MNN live paradigm to study infants at risk of neuro-developmental disorders, namely autism. We were unable to recruit infant participants in the past year owing to the COVID-19 pandemic but plan to start recruitment during the current year. Once we have tested the paradigm in a subset of typically developing infants, we will start recruiting infants at risk.

We continued our collaboration with Andrea Gropman to examine developmental deficits in children with urea cycle disorders (UCD), specifically ornithine transcarbamylase deficiency (OTCD), which is characterized by presence of hyperammonia (HA). HA is known to cause impairments in executive function and working memory. Monitoring OTCD progression and investigating neurocognitive biomarkers can become critical in examining the underlying brain function in OTCD. Using fNIRS we examined the hemodynamics of the prefrontal cortex

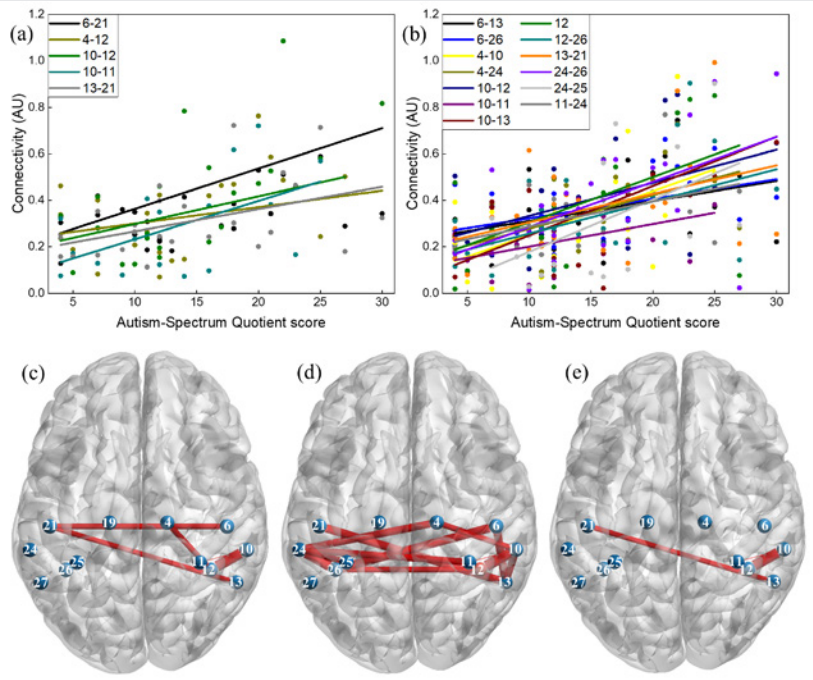

FIGURE 1. Correlations between functional connectivity and AQ score

(*a*) Five connections with a significant correlation coefficient during action-execution; (*b*) 13 connections with a significant correlation coefficient during action-observation; and brain maps showing the location of connections with significant correlation coefficients (*c*) during action-execution; (*d*) during action-observation; (*e*) during both conditions.

(PFC) in an OTCD population and in fraternal twins with and without OTCD. Results revealed a distinction in left PFC activation between controls and patients with OTCD, where controls showed higher task related activation increase while performing the Stroop task (Anderson *et al.*, *Front Neurol* 2020;11:809). Subjects with OTCD also exhibited a bilateral increase in PFC activation. We quantified the hemodynamic variations in total-hemoglobin, while twins performed the N-Back Working Memory task. Our preliminary results showed that the sibling with OTCD had higher variations in a very low frequency band than did the control sibling, possibly owing to the effect of HA (Anderson *et al.*, *Front Neurol* 2020;11:809). Functional connectivity (FC) analysis also revealed lower interhemispheric FC in an OTCD sibling as the task load increased.

As part of our ongoing fNIRS calibration protocol, two articles were published. The first [Reference 3] reported simultaneously collected fNIRS from the PFC and high-frequency heart rate variability (HF-HRV, as derived from electrocardiogram) to examine the connection between prefrontal activation and parasympathetic nervous system activity (as measured HF-HRV) during a behavioral flexibility task (the go/no-go task). The relationship between these measures was previously described by the neuro-visceral integration model (Thayer & Lane, *J Atten Disord* 2000;61:201); however, so far, no study had examined such measures simultaneously. We collected data from 38 healthy adult controls at rest and during the go/no-go task. We then compared the time course of HF-HRV and prefrontal fNIRS activation over the baseline period with that of the task period to determine whether they were related over time. Results indicated that, at rest, HF-HRV is negatively related to prefrontal activation, consistent with previous studies that had collected HF-HRV and brain activity during separate resting state sessions (Allen B *et al*., Psychophysiology 2015;52:277). The results support the tenets of the neuro-visceral integration model and the utility of fNIRS in future studies to examine the model and its relation to cognitive functions. The second publication [Reference 5] presented a secondary data analysis on the go/no-go task used in the above study to examine prefrontal connectivity during a simple

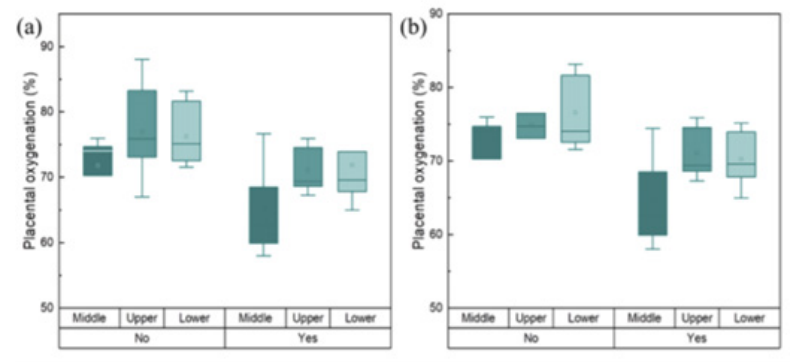

FIGURE 2. Transabdominal placental oxygenation levels at different measurement positions above the placenta

(*a*) Pregnancy with maternal complications (yes, n = 5 at each position) and no complications (no, n = 7 at each position); (*b*) placenta with lesions (yes, n = 5 at each position) and without lesions (no, n = 5 at each position).

go/no-go task and an emotional go/no-go task. We found that stronger connectivity was associated with better performance on the simple task in both males and females; however, stronger connectivity was only associated with better performance on the emotional go/no-go in males. The findings are relevant to how the brain may function differently during emotional behavior inhibition in males than in females and will be further explored.

COVID-19 point-of-care biosensor

The COVID-19 pandemic is challenging the medical community to develop biosensors to detect patients with early signs of COVID-19 infection. Consequently, we developed and tested a multimodal sensing device for monitoring parameters related to physiological changes in respiratory infections. The device consists of a near-infrared spectroscopy (NIRS) device, a photoplethysmography (PPG) device, and three sensors for temperature sensing. NIRS is a non-invasive method for measuring blood hemoglobin concentrations using a near-infrared (NIR) light source. PPG is another optical method to detect changes in blood volume at the microvascular level. Respiratory function and cardiac parameters can be obtained from NIRS and PPG signals, respectively. The device's NIRS sensor differs from conventional NIRS sensors in that it uses a three-wavelength NIR source (730 nm, 810 nm, and 850 nm) with three source-detector distances of 2 cm, 3 cm, and 4 cm. The study is a collaboration with Bruce Tromberg's Section and with Babak Shadgan. We started to recruit subjects at the NIH through a clinical study. At the University of British Columbia, 20 subjects have been studied so far. Preliminary data showed significant differences in respiratory parameters detected by the device between normal, healthy breathing, and breathing that simulated what is observed in patients with a respiratory infection.

Placenta oxygenation: from basics to point of care

The placenta plays an essential role in the health of both mother and fetus. An abnormal placenta is associated with pregnancy complications such as preeclampsia, fetal growth restriction, fetal death, preterm labor, and other complications. NIRS is an optical method for non-invasive measurement of blood oxygenated and deoxygenated hemoglobin and tissue oxygenation in deep tissue layers such as brain, muscle, and placenta. However, studies examining placental oxygenation levels have yielded conflicting results, discrepancies that may be the result of unknown placental scattering coefficients used for oxygenation calculations or differences in patient populations. A major problem in the assessment of placental oxygenation using NIRS arises from the anatomical location of the organ. Taking into account the anatomical location of the maternal placenta (e.g., skin, adipose tissue, uterine wall), we designed a new wearable depth-resolving NIRS device featuring

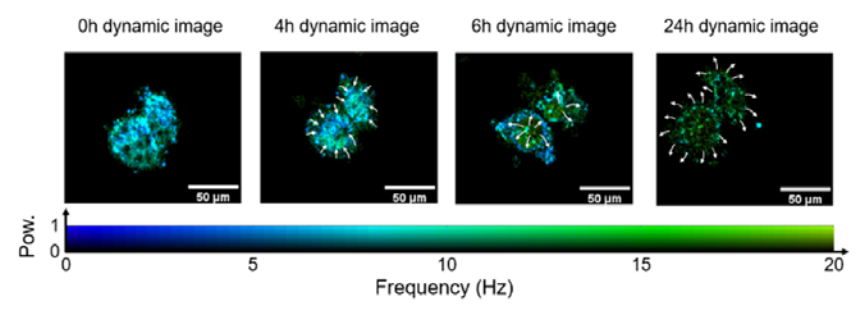

FIGURE 3. Dynamic and structural changes in HeLa cells over time.

The frequency component with the dominant dynamic motion of the scatterers is prominently displayed in the image. The intracellular migration of main dynamic active elements is indicated by white arrows. The power density was normalized.

six source-detector distances in the range of 10–60 mm. Distinct source and detector distances scan different tissue depths to help distinguish between placental and maternal oxygenation. The device also uses two light sources with wavelengths of 760 nm and 840 nm so that it is sensitive to changes in blood oxyhemoglobin and deoxyhemoglobin. We evaluated the performance of the NIRS device by observing changes in the optical properties of a placental-mimicking phantom at a depth of 25 mm. In addition, to evaluating the accuracy and validating the performance of the custom NIRS device, we performed *in vivo* oxygen measurements on two human subjects using a wearable NIRS device in various parts of the body, including arms, calves, and abdomen. We also compared the wearable NIRS device with a time-domain NIRS system (TRS-41 system, Hamamatsu Photonics, Japan) on different parts of the body, including the arms, calves, and abdomen to evaluate the accuracy and validate the performance. We found an average error of 2.7% ± 1.8% between the two devices/systems, an agreement between the measurements of the wearable NIRS device and the well established TRS system that validates the high accuracy of the device in *in vivo* tissue oxygenation measurements.

We then used the NIRS device to measure placental oxygenation *in vivo* in 12 volunteers from the Advanced Obstetrics and Gynecology Research Center located at Detroit Medical Center (DMC). Measurements were performed at three locations: upper, middle, and lower parts of the placenta. Of the 12 subjects, five had maternal pregnancy complications such as short cervix, hypertension, or polyhydramnios. Eleven of the 12 participants delivered at the DMC. After delivery, the placenta of 10 of 11 participants was transferred to the DMC pathology department to examine the lesion. Chronic or acute lesions were found in five placentas, four of which were from participants with maternal pregnancy complications. The level of placental oxygenation was calculated using the intensity of backscattered light at the appropriate light source-detector separation. For each patient, three oxygenation levels at the three measurement sites were used: the upper, middle, and lower parts of the placenta. Our results suggest a possible relationship between placental oxygenation levels and pregnancy complications and placental pathology. However, the sample size used in this study is small (12 participants). We will conduct further studies to include more subjects. Results from this study have been published [Reference 4]. We are performing Monte Carlo simulations on the four-layer model to better understand the experimental results and to create theoretical indicators. Simulations are based on thickness and scattering and absorption coefficients of all maternal layers (dermis, adipose, uterus/rectus) and placenta. Based on Monte Carlo's random walk theory and experimental results, we are attempting to develop a system that can analyze the characteristics of light scattering and propagation in the mother's maternal layers and more clearly measure the oxygenation levels of the mother's placenta.

Also, we are developing an analysis algorithm that evaluates the behavior of placental cells in consideration of various oxygen levels using the dynamic full-field optical coherence tomography (DFFOCT) system in parallel with measurement of placental oxygenation using the NIRS device. We are developing an algorithm designed to analyze changes in the dynamic activity (frequency and magnitude of cells) within a cell and calculate a mean frequency representing a weighted frequency. As a preliminary experiment, we evaluated the viability status of HeLa cells, an immortal human cell line widely used in cell research, from alive to dead. With the developed algorithm, the dynamic activity of cells was quantitatively and clearly distinguishable according to changes in their viability status [Reference 6]. From these results, we believe that DFFOCT can be used to analyze changes in cellular dynamic activity depending on the nutrient and oxygen saturation contained in placental cells. We hypothesize that there is a relationship between the dynamic activity of placental cells and potential neuro-developmental disorders. For the next study, we will analyze the dynamic activity of the placenta cells, taking into account various oxygen levels in the placenta. To this end, we plan to create a special sample chamber to control and maintain the oxygen concentration in the cells while conducting the experiment.

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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 (Met-tRNA$_i$) base-paired to the AUG start codon. The Met-tRNA$_i$ 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 eIF1 and eIF1A, which induce an open conformation of the 40S and rapid TC binding in a conformation suitable for the scanning of successive triplets entering the ribosomal P site (P-out), and by eIF4F and other RNA helicases, such as Ded1 and its paralog Dbp1, 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 Met-tRNA$_i$ 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 the functions of eIF1, eIF5, eIF3, and 40S ribosomal proteins in TC recruitment and start-codon recognition; (2) identify distinct functions of the RNA helicases eIF4A (and its cofactors eIF4G/eIF4B), Ded1, and Dbp1, and of the poly(A)–binding protein (PABP) in mRNA activation, 48S PIC assembly, and scanning *in vivo*; (3) uncover the mechanisms of translational repression and regulation of mRNA abundance by the repressors Scd6, Pat1, the helicase Dhh1, and the mRNA–decapping enzyme Dcp2; (4) elucidate the regulation of Ded1, eIF4G, and Dhh1 functions in response to nutrient limitation or stress; (5) elucidate the *in vivo* functions of yeast eIF2D orthologs and of the MCT-1/DENR complex in 40S ribosome recycling at stop codons and reinitiation in 3' untranslated regions *in vivo*; and (6) elucidate the roles of yeast orthologs of eIF2A and eIF2D in eIF2–independent initiation of translation in stress conditions.

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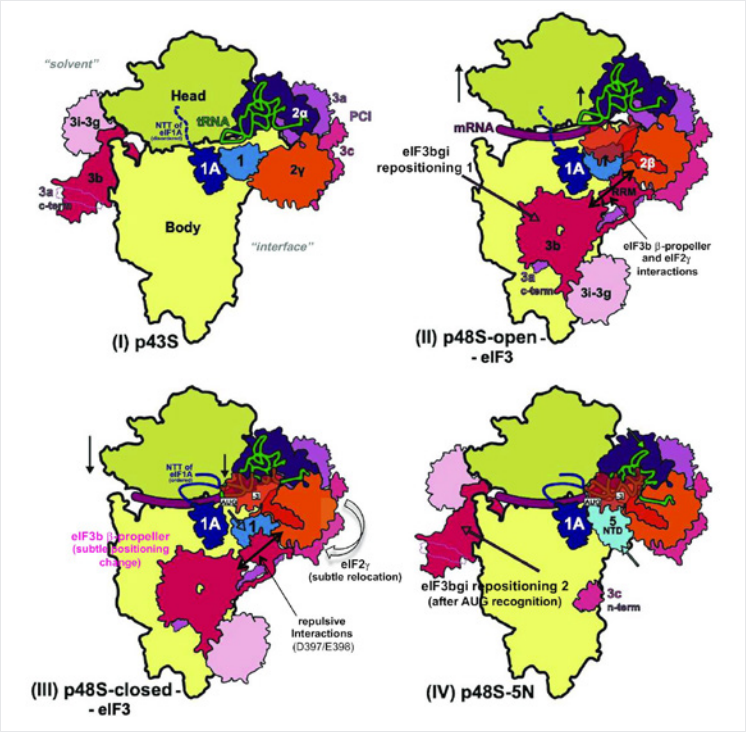
We also analyze the regulation of amino acid–biosynthetic genes in budding yeast as a means of dissecting fundamental mechanisms of transcriptional control of gene expression. During amino acid limitation, transcription of such genes is coordinately induced by the activator Gcn4 as the result of induction of Gcn4 at the translational level. The eviction of nucleosomes that occlude promoter DNA sequences and block access by RNA polymerase is thought to be a rate-limiting step for transcriptional activation. Previous studies implicated certain histone chaperones, ATP–dependent chromatin-remodeling complexes, or histone acetyltransferase (HAT) complexes in eviction of promoter nucleosomes at certain yeast genes, but it is unclear whether these co-factors function at Gcn4 target genes. Our aim is to elucidate the full set of co-factors that participate in promoter nucleosome eviction at Gcn4 target genes, their involvement in this process genome-wide, and the transcriptional consequences of defective nucleosome eviction. Functional cooperation among the chromatin-remodeling complexes SWI/SNF, RSC, and Ino80, as well as the HAT complexes SAGA, NuA4, NuA3, and Rtt109/Asf1, in these processes are under study. We 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, as well as the roles of co-transcriptional histone methylation, nucleosome reassembly, and mRNA decay enzymes in controlling their synthesis and abundance. We are also probing mechanisms involved in the asymmetric transcriptional induction of genes belonging to pairs of divergently oriented genes where only one gene responds to Gcn4 binding at the shared upstream activation sequences (enhancer).

eIF2α interactions with mRNA control accurate start–codon selection by the translation preinitiation complex.

Comparison of previous cryo-EM structures of 48S PICs in open scanning-conducive, or closed arrested conformations revealed interactions unique to the closed complex between Arg residues R55 and R57 of domain 1 of the α-subunit of eIF2 (eIF2α-D1) with mRNA nucleotides 5' of the AUG codon, including the –3 residue of the 'Kozak' context (the Kozak consensus sequence is a nucleic-acid motif that functions as the protein-translation initiation site in most eukaryotic mRNA transcripts). We showed that substitutions of R55 and R57 reduce recognition of the poor-context AUG codon for *SUI1* mRNA (encoding eIF1) and also UUG start codons in Sui$^-$ cells (the Ssu$^-$ phenotype). We further showed that the R55G-R57E Ssu$^-$ substitutions destabilize TC binding to 48S PICs reconstituted with mRNA with a UUG start codon in the *in vitro* reconstituted system, in the manner expected from specific destabilization of the closed complex at a near-cognate codon. Interestingly, residue R53 of eIF2α-D1 interacts with rRNA residues exclusively in the open complex; we found that the R53E substitution enhances initiation at UUG codons (the Sui$^-$ phenotype) and the poor-context *SUI1* AUG, and also confers the Gcd$^-$ phenotype, indicating constitutively depressed translation of *GCN4* mRNA, which results from slow recruitment of the TC to scanning 40S subunits engaged in re-initiation on this mRNA *in vivo*. In the reconstituted system, R53E stabilized TC binding to UUG complexes while simultaneously reducing the on rate of TC loading, all in the manner predicted for specific destabilization of the open complex and shift towards the closed state. We conclude that distinct interactions of eIF2α-D1 with the rRNA or mRNA stabilize first the open, and then the closed, conformation of the PIC to regulate the accuracy and efficiency of start codon selection *in vivo* [Reference 1].

FIGURE 1. Model depicting reversible repositioning of the eIF3b/eIF3i/eIF3g/eIF3a-C-term module between the solvent-exposed and subunit-interface surfaces of the 40S at the onset of scanning and following AUG recognition



Schematics showing the two different locations observed for the eIF3b/eIF3i/eIF3g/eIF3a-C-term (eIF3 b/i/g/a) module on either the solvent or subunit surfaces of the 40S subunit in the following partial (p) initiation complexes: (*I*) p43S, (*IV*) py48S-eIF5N and (*II*/*III*) open or closed py48S complexes, respectively. The red arrows in (*II*) and (*IV*) depict the direction of the movement of the b/i/g/a module from the solvent surface to the subunit interface of the 40S subunit upon 43S PIC attachment to mRNA and formation of the open, scanning conformation of the 48S PIC (Repositioning 1), and then back to the solvent surface after AUG recognition, complete accommodation of Met-tRNA$_i$ and eIF1 dissociation from the 48S complex (Repositioning 2). Also indicated are upward or downward movements of the 40S head relative to the body (*single-headed black arrows*), additional interactions (*two-headed black arrows*) and subtle conformational changes (*grey arrows* or *red type*) of different eIF3 elements, tRNA, the 40S head and eIF2γ at the subunit interface accompanying transition from the open to closed states of the 48S complex on AUG recognition (*II*)-*III*); followed by repositioning of the eIF3b/eIF3i/eIF3g/eIF3a-Cterm module (eIF3bgi) to the solvent side of the 40S following AUG recognition, eIF1 dissociation, and replacement of eIF1 with the eIF5 NTD near the P-site (*IV*). Adapted from Reference 4.

Large-scale movement of eIF3 domains during translation initiation modulate start-codon selection.

In our previous cryo-electron microscopy (cryo-EM) reconstructions of yeast 48S preinitiation complexes (PIC), the eIF3 subcomplex (dubbed the b/i/g/a module), comprising the eIF3b subunit C-terminal domain (CTD), eIF3i, the eIF3g N-terminal domain (NTD), and an extended helical segment of eIF3a-CTD, is located near the decoding center at the 40S subunit interface, interacting with eIF1, eIF2γ, eIF3c, and the 40S itself and appearing to lock the mRNA into the 40S binding cleft. The b/i/g/a module is found at this location in both the open and closed conformations of the PIC, which are thought to depict scanning and initiation conformations, respectively, with certain contacts restricted to only the open or closed state. Surprisingly, the b/i/g/a module was found at a dramatically different position on the solvent-exposed 40S surface in our more recent py48S-5N complex, where the eIF5–NTD replaces eIF1 on the 40S subunit in a later stage of the initiation pathway. We hypothesized that, following 43S PIC attachment to mRNA, the eIF3 b/i/g/a module relocates from the solvent side to the subunit interface of the open py48S complex to help prevent PIC drop-off from mRNA during scanning, that certain of its contacts at the interface surface are remodeled on AUG recognition, and that on dissociation of eIF1 and attendant loss of eIF3b–RRM (RNA recognition motif) interaction with eIF1, the b/i/g/a module relocates to the solvent side of the 40S to allow binding of the eIF5–NTD in place of eIF1 on the 40S platform. Examining eIF3b–CTD substitutions designed to disrupt interactions of its β-propeller or RRM

with eIF2γ, eIF1, or eIF3c found uniquely at the interface surface revealed that those conferring the strongest phenotypes increased discrimination against near-cognate UUG start codons (the Ssu$^-$ phenotype). Binding assays confirmed the interaction of the eIF3b–RRM with eIF3c, found exclusively at the 40S subunit interface, in a manner perturbed by one such Ssu$^-$ substitution at a predicted contact with eIF3c. Interestingly, strong Ssu$^-$ phenotypes were also observed for eIF3b substitutions that perturb eIF3b interaction made exclusively at the solvent-exposed surface of the 40S subunit. The findings suggest that interactions of the b/i/g/a module with certain initiation factors at the subunit interface acts primarily to stabilize the closed conformation of the PIC on start-codon recognition, that relocation of the module back to the solvent interface is required to finalize start-codon selection, and that these interactions are crucial for the ability to utilize non-cognate initiation codons *in vivo*.

Amino acid residues of 40S protein uS5/Rps2 at the mRNA entry channel enhance initiation at suboptimal start codons *in vivo*.

The ribosomal protein uS3/Rps3 is positioned at the solvent side of the 40S near the mRNA entry channel. We showed previously that substituting uS3/Rps3 residues that contact mRNA preferentially destabilizes the closed conformation of the PIC, reducing initiation at both UUG codons and at AUG start codons that reside in suboptimal 'Kozak' sequence context. Particular residues of uS5/Rps2 make distinct mRNA contacts at the 40S entry channel and also interact with rRNA elements that communicate with the 40S decoding center. We found that uS5/Rps2 substitutions V121D and I125K resemble the previously characterized uS3/Rps3 substitutions in suppressing initiation at UUG codons as well the poor-context AUG start codons in *SUI1* mRNA or an elongated form of upstream open-reading frame 1 of *GCN4* mRNA (el.-uORF1). Interestingly, the uS5/Rps2 substitutions D78A, Q89K, and K119A suppress UUG initiation but do not reduce initiation at the poor-context AUG codons, and thus appear to be specific for suppressing near-cognate initiation. Q94D and T96K diminish initiation at the poor-context AUG codons of *SUI1* and el.-uORF1, and they efficiently suppress UUG initiation only when the UUG resides in poor sequence context. Thus, the latter two residues appear to act mainly in discriminating against poor Kozak context. The findings suggest that different uS5/Rps2 residues are involved in distinct mechanisms of discrimination against different features of poor initiation sites *in vivo*.

eIF4A and eIF4E interactions with distinct residues of the Ded1 N–terminus stimulate Ded1 function in translation initiation *in vivo*.

Binding of eIF4F to the mRNA cap structure enhances recruitment of the 43S PIC to the 5'end and subsequent scanning of the 5'UTR. Ded1 physically interacts with eIF4A and the eIF4G subunit of eIF4F, and eIF4A and eIF4G can both stimulate unwinding of a model RNA substrate by Ded1 *in vitro*. Previously, we showed that the Ded1 C-terminal domain (CTD) and its two interacting domains in eIF4G, dubbed RNA2 and RNA3, and the Ded1 N-terminal domain (NTD) that interacts with eIF4A, all enhance Ded1 stimulation of 48S PIC assembly in the reconstituted *in vitro* system. Ded1 also interacts with eIF4E; however the binding sites for eIF4A and eIF4E in the Ded1–NTD were unknown. By substituting blocks of conserved residues in the Ded1–NTD, we found that alanine replacements of residues 21–27 and 51–57 reduce Ded1 binding to eIF4A *in vitro*, impair association between native Ded1 and eIF4A in cell extracts, and reduce cell growth, bulk translation initiation, and translation of Ded1–hyperdependent reporter mRNAs harboring stem-loop insertions. Overexpressing eIF4A diminished the growth defects for each single substitution, but not for the 21–27/51–57 double substitution, which is null for eIF4A binding, supporting the importance of Ded1–NTD/eIF4A interaction in cells. Substituting the non-overlapping residues 59–65 and 83–89 reduced Ded1–NTD binding to eIF4E *in vitro*, as well as Ded1–eIF4E association in extracts, and conferred reduced translation of the Ded1–hyperdependent reporters.

Combining all four NTD substitutions conferred an additive growth defect indistinguishable from deletion of the NTD, suggesting that eIF4A/eIF4E binding is the key *in vivo* function of the Ded1 NTD. Deleting the Ded1–CTD impairs growth only when combined with NTD substitutions, implying that the Ded1–CTD interaction with eIF4G is dispensable when Ded1 can interact with eIF4A and eIF4E. In the reconstituted system, the Ded1 NTD substitutions that eliminate eIF4A binding reduce the maximal rate of 48S PIC assembly on a Ded1–dependent mRNA harboring a 5'UTR SL (stem loop), and also increase the amount of Ded1 required to achieve the half-maximal rate (K1/2). Disruption of the Ded1–NTD/eIF4E interaction has a similar effect of elevating the Ded1 K1/2 for 48S assembly. The findings support the notion that Ded1 NTD interactions with eIF4A and eIF4E stabilize a Ded1–eIF4E–eIF4G–eIF4A quaternary complex that enhances Ded1's ability to resolve secondary structures in 5'UTRs [Reference 2].

Reprogramming of translation in yeast cells impaired for ribosome recycling favors short, efficiently translated mRNAs.

In eukaryotes, formation of the 43S preinitiation complex (PIC), containing initiator Met-tRNA$_i$ bound to the small ribosomal subunit, is a rate-determining step of translation initiation. Ribosome recycling after translation termination produces free 40S subunits needed to reassemble 43S PICs for new initiation events. Yeast mutants lacking orthologs of mammalian eIF2D (Tma64), and either MCT-1 (Tma20) or DENR (Tma22), are broadly impaired for 40S recycling; however, it was unknown whether the defect alters the translational efficiencies (TEs) of mRNAs. Based on previous experiments, it was also possible that Tma64/eIF2D can substitute for eukaryotic initiation factor 2 (eIF2) in recruitment of Met-tRNA$_i$ during initiation. Consistent with impaired initiation, the *tma64Δtma20Δ* mutant exhibits reduced assembly of bulk polysomes. Ribosome profiling of this mutant reveals a marked reprogramming of translational efficiencies, wherein translation of the most efficiently translated ('strong') mRNAs tends to be elevated, whereas translation of 'weak' mRNAs generally declines. Profiling of the *tma64Δ* single mutant reveals none of the hallmarks of impaired 40S recycling nor changes in translation efficiencies, suggesting that the defects found in *tmaΔΔ* cells are associated with defective ribosome recycling rather than loss of eIF2D function in Met-tRNA$_i$ recruitment. Remarkably similar translational re-programming was seen on reducing 43S PIC assembly by inducing phosphorylation of eIF2 or by decreasing total 40S subunit levels by depleting Rps26, without affecting ribosome recycling. Moreover, the *tmaΔΔ* mutation specifically impaired translation of mRNAs with cap-proximal secondary structures that are expected to impede PIC attachment. Our findings suggest that strong mRNAs outcompete weak mRNAs in response to 43S PIC limitation achieved in various ways at the step of 43S PIC recruitment, in accordance with mathematical modeling of how translational efficiencies of different groups of mRNAs are altered by reduced ribosome abundance. They also have important implications for understanding changes in translation occurring in human ribosomopathies in which 40S subunit levels are diminished.

Distinct functions of three chromatin remodelers in activator binding and preinitiation complex assembly

The nucleosome-remodeling complexes (CRs) SWI/SNF, RSC, and Ino80C cooperate in evicting or repositioning nucleosomes to produce nucleosome-depleted regions (NDRs) at the promoters of many yeast genes induced by amino acid starvation. We analyzed mutants lacking the CR catalytic subunits for binding of the transcriptional activator Gcn4 and recruitment of TATA–binding protein (TBP) during preinitiation complex (PIC) assembly. RSC and Ino80 enhance Gcn4 binding to UAS (upstream activation sequence) elements in NDRs upstream of many promoters as well as to unconventional binding sites within nucleosome-occupied coding sequences; and SWI/SNF contributes to UAS binding when RSC is depleted. All three CRs are actively recruited

by Gcn4 to most UAS elements and appear to enhance Gcn4 binding by reducing nucleosome occupancies at the binding motifs, indicating a positive regulatory loop. SWI/SNF acts unexpectedly in wild-type (WT) cells to prevent excessive Gcn4 binding at certain UAS elements, which might involve transient nucleosome sliding that does not alter steady-state nucleosome occupancies. All three CRs also stimulate TBP recruitment, at least partly by reducing nucleosome occupancies at TBP binding sites, with SWI/SNF acting preferentially at the most highly expressed Gcn4 target genes. RSC and Ino80 function more broadly than SWI/SNF to stimulate TBP recruitment at most constitutively expressed genes, including ribosomal protein genes, whereas SWI/SNF acts preferentially at a distinct subset of highly expressed genes. Our findings point to a complex interplay among the three CRs in evicting promoter nucleosomes to regulate activator binding and stimulate PIC assembly.

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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 the CA1 pyramidal neuron, one of its principal cell types. Each of these cells 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

ISOMERASE REGULATION OF POTASSIUM CHANNEL TRAFFICKING AND FUNCTION

The transient voltage-gated K^+ current (IA), mediated by Kv4.2 in CA1

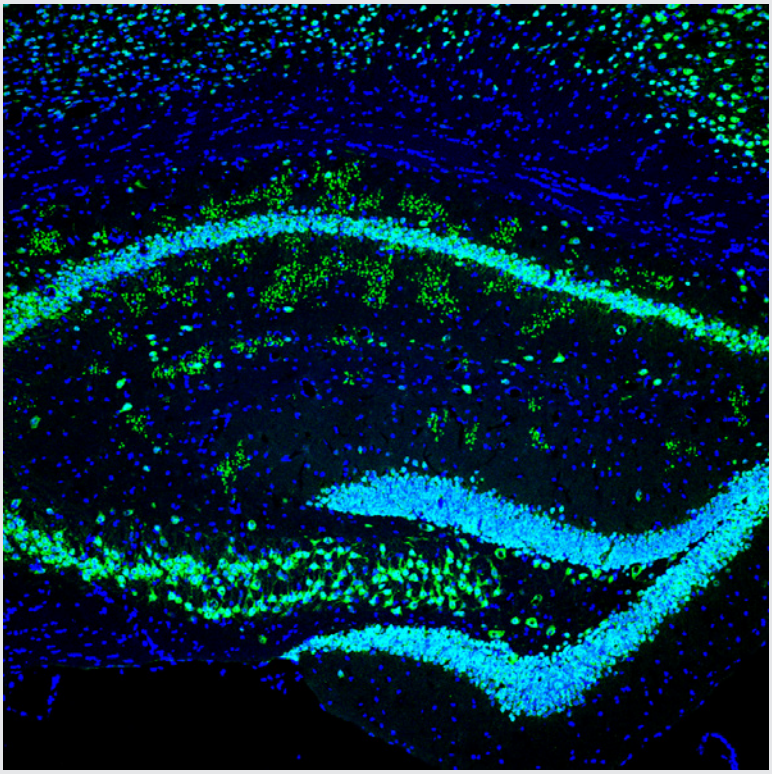

FIGURE 1. Aging DPP6–KO mice have a novel structure with characteristics of abnormal presynaptic terminals and amyloid plaques in hippocampus.

Green staining NeuN, Blue DAPI

hippocampal pyramidal neurons, regulates dendritic excitability, synaptic plasticity, and learning. We recently identified a novel molecular cascade initiated by the activation of p38 kinase and subsequent Pin1–dependent isomerization of a C-terminal motif (T607) in Kv4.2 that triggers dissociation from its auxiliary subunit DPP6, a reduction IA, and an increase in neuronal excitability. Pin1 is a prolyl isomerase that selectively binds to and isomerizes phospho-Ser/Thr-Pro (pSer/Thr-Pro) bonds. Mis-regulation of Pin1 plays an important role in a growing number of pathological conditions including Alzheimer's disease, where it may protect against age-dependent neurodegeneration. We identified Pin1 as a Kv4.2–binding partner via a TAP-MS pulldown assay. Subsequent biochemical studies revealed that Pin1–Kv4.2 binding is direct and via the canonical Pin1–binding motif. Stimuli including seizure induction and exposure to enriched, novel environments increased Kv4.2 phosphorylation at the Pin1 binding site T607 by p38 MAPK in the mouse cortex and hippocampus. Using biochemical and electrophysiological techniques, we showed that Pin1 activity is required for the dissociation of the Kv4.2–DPP6 complex and that this action alters neuronal excitability. To investigate the consequences of this cascade on behavior and neuronal physiology, we used CRISPR-Cas9 techniques to generate a knockin mouse in which the isomerase binding site is specifically abolished (Kv4.2TA). The mice are viable and appear normal, although the activity-dependent dissociation of the Kv4.2–DPP6 complex is impaired.

Cole Malloy used patch-clamp electrophysiology in pyramidal cells of hippocampal slices from Kv4.2TA and wild-type (WT) mice to decipher the role of p38-Pin1–mediated regulation of Kv4.2 on neuronal excitability. He found that Kv4.2TA cells displayed lower action potential (AP) firing than in WT cells in response to somatic current injections. The reduced excitability can be traced to increased Kv4.2–mediated current in Kv4.2TA cells in outside-out somatic patches. Pharmacological block of both p38 kinase and Pin1 in WT recapitulated

the impact of the mutation on neuronal firing properties and IA, confirming the specificity of the cascade underlying such effects.

To detect how these alterations in neuronal physiology may manifest in behavioral changes, Jiahua Hu performed a battery of tests probing seizure susceptibility and learning and memory capability. In response to IP kainic acid injection, Kv4.2TA mice exhibited lower seizure intensity over an hour-long period than did WT mice. The reduced seizure intensity also could be recapitulated in WT mice with pharmacological block of p38 kinase and Pin1. We have therefore identified a novel signaling cascade that can be a target for therapeutic intervention to mitigate seizure intensity in epilepsy by reducing Kv4.2 downregulation.

Kv4.2TA mice exhibit normal initial learning and memory in the Morris Water Maze. However they exhibited better 'reversal' learning in the Morris Water Maze than did WT mice. 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. Considering the finding that Kv4.2TA mice exhibit enhanced cognitive flexibility, ongoing experiments are investigating potential differences in synaptic properties between WT and Kv4.2TA mice. Collectively, such experiments will reveal the cellular mechanisms underlying the reversal learning phenotype in Kv4.2TA mice and will provide further insight into mechanisms impacting cognitive flexibility.

CA^{2+} REGULATION OF POTASSIUM CHANNEL FUNCTION

Jonathan Murphy found that Ca^{2+} entry mediated by the voltage-gated Ca^{2+} channel subunit Cav2.3 regulates Kv4.2 function both in a heterologous expression system and endogenously in CA1 pyramidal neurons through Ca^{2+} binding to auxiliary subunits known as K^+ channel–interacting proteins (KChIPs). KChIPs are calcium-sensing molecules containing four EF-hands, which are dysregulated in several diseases and disorders including epilepsy, Huntington's disease, and Alzheimer's disease. He characterized a KChIP–independent interaction between Cav2.3 and Kv4.2 using immunofluorescence colocalization, coimmunoprecipitation, electron microscopy, (FRAP (fluorescence recovery after photobleaching), and FRET (fluorescence resonance energy transfer). We found that Ca^{2+} entry via Cav2.3 increases Kv4.2–mediated whole-cell current, which is attributable in part to an increase in Kv4.2 surface expression. In hippocampal neurons, pharmacological block of Cav2.3 reduced whole-cell IA. We also found a reduction in whole-cell IA in Cav2.3 knockout (KO) mice mouse neurons with a loss of the characteristic dendritic IA gradient. Furthermore, the Cav2.3–Kv4.2 complex was found to regulate the size of synaptic currents and spine Ca^{2+} transients. The results reveal an intermolecular Cav2.3–Kv4.2 complex impacting synaptic integration in CA1 hippocampal neurons. To directly test whether the binding interaction of Cav2.3 and Kv4.2 is required for Cav2.3 regulation of Kv4.2 function, we have worked toward developing tools to disrupt the Cav2.3–Kv4.2 interaction while sparing the expression of Cav2.3.

The KChIP protein, but not mRNA expression, has been shown to be reduced in Kv4.2 KO mouse brains, suggesting increased KChIP protein degradation in the absence of Kv4.2. We hypothesized that KChIP protein degradation depends on binding to Kv4.2 and that there is increased KChIP protein degradation in the absence of Kv4.2. We aimed to elucidate the undetermined molecular mechanism of KChIP protein degradation and its effect on Kv4.2 protein levels and function. While a member of the Section, former postbaccalaureate fellow Joe Krzeski identified the pathway through which KChIP is degraded and a novel function for KChIP regulation of Kv4.2. Jiahua Hu generated a conditional Kv4.2 KO mouse using CRISPR-Cas9 techniques. Joe Krzeski injected

AAV-CRE-GFP virus into the CA1 in hippocampus of the conditional Kv4.2 KO mice. We found that the Kv4.2 protein level is significantly reduced in the CRE–positive area. Interestingly, the KChIP protein level is also significantly reduced in the same area. These data suggest that KChIP protein can be dynamically regulated by Kv4.2 expression. Krzeski identified a conserved lysine residue that can be ubiquitinated. Further studies will elucidate the mechanism of KChIP degradation and its regulation by Kv4.2. A mechanistic understanding of KChIP protein degradation is important, as it may lead to new therapeutic strategies to treat diseases in which KChIPs are dysregulated.

DPP6 IMPACTS BRAIN DEVELOPMENT AND BEHAVIOR.

We previously showed that the Kv4 auxiliary subunit DPP6 has a novel function in regulating dendritic filopodia formation and stability, affecting synaptic development and function (Lin *et al*., *Nat Commun* 2013;4:2270). Recently, using immunofluorescence and electron microscopy, in a project lead by Lin Lin, we discovered a novel structure in hippocampal area CA1 that was significantly more prevalent in DPP6–KO mice than in WT mice of the same age and that such structures were observed earlier in development in DPP6–KO mice. The novel structures appeared as clusters of large puncta that colocalized NeuN, synaptophysin, and chromogranin A. Electron microscopy revealed that the structures are abnormal, enlarged presynaptic swellings filled with mainly fibrous material with occasional peripheral, presynaptic active zones forming synapses. We found diagnostic biomarkers of Alzheimer's disease present in abnormal levels in DPP6–KO mice, including accumulation of amyloid and amyloid precursor protein (APP) in the hippocampal CA1 area and a significant increase in the expression of hyper-phosphorylated tau. The amyloid and phosphorylated tau pathologies were associated with neuroinflammation characterized by activation of microglia and astrocytes. We also found that activated astrocytes and microglia were significantly increased in DPP6–KO brain sections. We showed that DPP6–KO mice display circadian dysfunction, a common symptom of Alzheimer's disease. Taken together, the results indicate that DPP6–KO mice show symptoms of enhanced neurodegeneration reminiscent of Alzheimer's disease associated with a novel structure resulting from synapse loss and neuronal death. We continue to investigate DPP6 in neurodegeneration.

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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 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

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expression. We found that there are subtle differences in the gene expression program of the 79-kb transgene and the endogenous locus. Polycomb domains are flanked by active genes or insulators that limit the size of the domain. We hypothesize that delimiting the size of a Polycomb domain contributes to the stability of both gene activation and repression, making gene expression reproducible and robust. Our current experiments are designed to test this hypothesis.

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 distinct 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

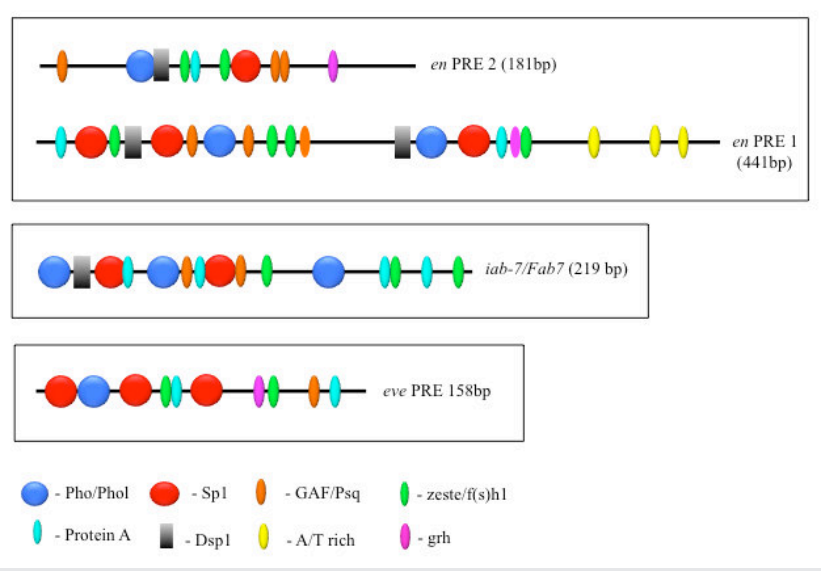


FIGURE 1. The order, number, and spatial arrangement of consensus-factor binding sites varies in different PREs.

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.

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.

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 PRE-like DNA fragments? 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 PRE–like DNA fragments can silence expression when included in a transgene. Our results so far argue against this, i.e., these DNA fragments do not function as PREs in a transgene silencing assay. Such results show that we do not yet fully understand what constitutes a PRE.

The role of PREs at the *en* gene

The *Drosophila engrailed (en)* gene encodes a homeodomain protein that plays an important role in the development of many parts of the embryo, including formation of the segments, nervous system, head, and gut. By specifying the posterior compartment of each imaginal disc, *en* also plays a significant role in the development of the adult. Accordingly, *en* is expressed in a highly specific and complex manner in the developing organism. The *en* gene exists in a gene complex with *invected* (*inv*), an adjacent gene; *inv* encodes a protein with a nearly identical homeodomain; *en* and *inv* are co-regulated and express proteins with largely redundant functions. Unlike *en*, *inv* is dispensable for *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 gain-of-function ectopic expression. Unexpectedly, when we made a 1.5kb deletion removing PRE1 and PRE2, flies were viable and had a partial loss-of-function phenotype in the wing. Similarly, deletion of *inv* PREs yielded viable flies with no mis-expression of *en* or *inv*. Importantly, the H3K27me3 *en/inv* domain is not disrupted in either of these mutants [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,

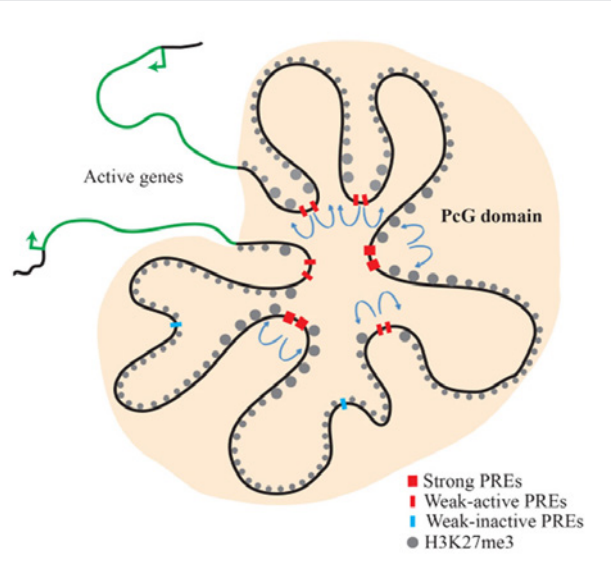


FIGURE 2. Model of the *inv-en* PcG domain in the transcriptionally silenced state

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).

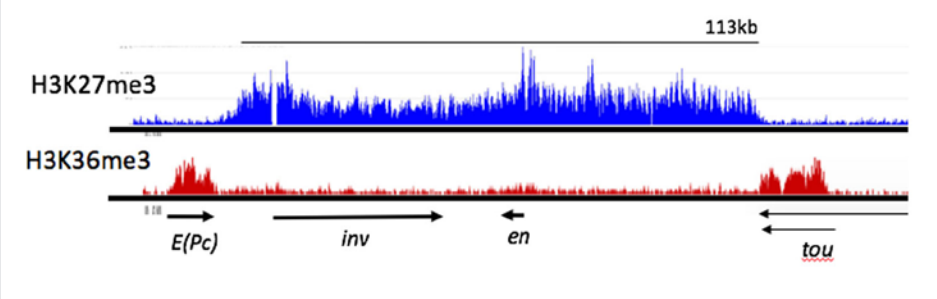
the flies are viable, and there is no mis-expression of *en* or *inv* in embryos or larvae. The question arises as to how PcG proteins are recruited to the *inv-en* domain in the absence of these PREs. We performed chromatin-immunoprecipitation followed by Next-Gen sequencing (ChIP-seq) on the PcG proteins Pho and Polyhomeotic (Ph). The data showed that, in addition to the large Pho/Ph peaks at the known PREs, there are many smaller Pho/Ph peaks within the *inv/en* domain. We found that those peaks may also function as PREs. Thus, rather than a few PREs, there are many PREs controlling *inv-en* expression, and some may act in tissue-specific ways [Reference 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. Also, 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.

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

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 do not express *inv* or *en*. Data from Reference 4.

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 enhancers that drive *engrailed* expression in stripes 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 *inv* gene (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) depends 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. Our results show there are at least two imaginal disc enhancers that behave redundantly to control Inv and En expression in the endogenous *inv-en* domain.

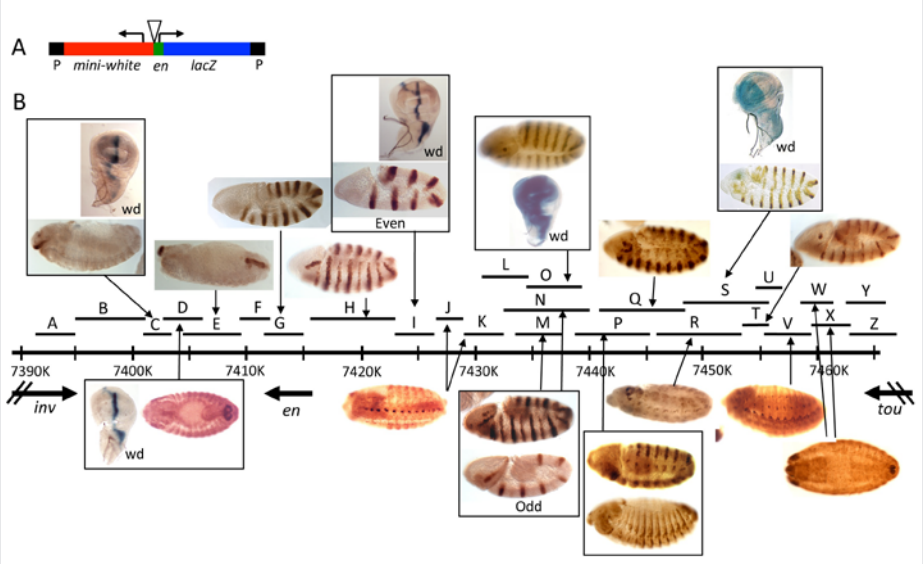


FIGURE 4. Enhancers of the *invected* and *engrailed* genes

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.

Defining the ends of Polycomb domains in *Drosophila*

Actively transcribed genes flank many Polycomb domains, and previous genomic studies showed that inhibition of transcription using chemical inhibitors leads to a spreading of H3K27me3 in the genome. We conducted a genome-wide analysis of Polycomb boundaries in *Drosophila* larvae [Reference 5]. We found six different types of Polycomb-domain boundaries, including those made by insulator proteins, and actively transcribed genes. The *inv-en* Polycomb domain is flanked by two actively transcribed genes, *E(Pc)* and *tou* (Figure 3). Insertion of a transcriptional stop within the *tou* gene causes an extension of the H3K27me3 mark to the point of active transcription. We also suggest that active transcription limits the range of *inv-en* enhancers and that promoter specificity is important for *inv-en* enhancer activity [Reference 5]. Currently, we are working to add actively transcribed genes, as well as, separately, to add insulators to delimit the ends of the 79-kb transgene. We hypothesize that adding boundaries will increase the accuracy and robustness of the expression of the transgene.

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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 that form 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 alpha1(I) chains and one alpha2(I) chain and 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 the molecular mechanisms of ECM disorders involving abnormal collagen metabolism, identify treatment targets, and bring this knowledge to clinical research and practice.

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Procollagen folding and its role in ECM disorders

Osteoblasts and fibroblasts produce and secrete the massive amounts of type I procollagen needed to build the skeleton and other tissues. 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, above 35°C, the conformation of natively folded human procollagen is less favorable than the unfolded one. 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 that are responsible for degradation of misfolded molecules and which force the cell to always function in a high-stress mode. Our findings indicate that one of the key pathophysiological mechanisms of OI and other hereditary type I collagen–metabolism disorders is cell stress caused by excessive accumulation of misfolded procollagen in the ER.

While we focus mostly on hereditary disorders affecting bone and other tissue development in children, excessive procollagen misfolding of nonhereditary origin is also likely. Our data suggest that such misfolding should occur upon changes in the ER associated with environmental factors, inflammation, aging, etc. It is likely to contribute to fibrosis, cancer, age-related osteoporosis, and many other common ailments. However, molecular mechanisms underlying misfolded procollagen accumulation in the ER and its consequences for the cell remain poorly understood.

The most common hereditary cause of 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. Their pathophysiology is one of the key topics of our studies. For instance, our studies on OI patients with over 50 different Gly substitutions revealed several structural regions within the collagen where such mutations might be responsible for distinct OI phenotypes. One such region is the first 85–90 amino acids at the N-terminal end of the triple helix, mutations in which prevent normal N-propeptide cleavage. Incorporation of the uncleaved molecules into collagen fibrils leads to distinct OI/EDS with hyperextensibility and joint laxity.

Cell biology of procollagen misfolding

Our current research focuses on the cell biology of procollagen misfolding. In one approach, we use 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 (ERESs) that are 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 ERESs. Instead, transport-vesicle formation depends on COPI coat assembly and HSP47 release at distal regions of ERESs, potentially explaining unusual skeletal pathologies caused by mutations in HSP47, COPI, and COPII coat proteins. Misfolded procollagen is retained at ERESs, resulting in a COPII–dependent modification of ERES membranes by ubiquitin and autophagic machinery. We discovered that such ERESs are then directly engulfed by lysosomes and degraded, delineating a new ERES micro-autophagy pathway.

Rerouting of ERES–loaded cargo from secretion to micro-autophagy may have wide implications. It is likely to be a general rather than a collagen-specific phenomenon, considering the known COPII coat involvement in both protein secretion and degradation. The hypothesis is currently under investigation in our and several collaborating laboratories. From clinical and translational perspectives, our findings may explain why patients with mutations in different COPII proteins have distinct pathologies in the development of bone, cartilage, and other tissues.

To gain these and other insights, we are expanding the toolbox for studies of collagen metabolism pathology. In particular, we recently created an osteoblast cell line in which the endogenous pro-alpha2(I) chain has a fluorescent tag and Flp–recombinase target sites for replacing the tag with other peptides. We demonstrated that the cells produce bone-like ECM, enabling us to perform live-cell imaging of endogenous procollagen, confirm its trafficking pathways, and identify that misfolded procollagen is detected at ERESs rather than in the ER lumen, resulting in activation of non-canonical cell stress pathways.

In another approach, we are investigating 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 with the same mutation. 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 therefore investigating the mechanism of this stress and its role in pathology by altering how the cells adapt to it and by examining cell-stress response pathways activated by the mutation. Specifically, we reduced autophagic degradation of ERESs containing misfolded procollagen by osteoblast-specific knockout of ATG5 (autophagy-related factor 5). Increased bone pathology caused by the resulting additional accumulation of misfolded procollagen in the ER confirmed our hypothesis that osteoblast cell stress and malfunction associated with such accumulation play a significant role in OI pathophysiology. Next, our studies revealed that accumulation of misfolded G610C procollagen in osteoblast ER activates transcription of the integrated stress response genes (e.g., *Ddit3*, *Eif4ebp1*, and *Nupr1*) but not of the canonical ER stress transducers *Atf4* and *Hspa5*, suggesting non-canonical cell stress and identifying its transducers as *Atf5* and *Hspa9* paralogs of *Atf4* and *Hspa5.* We validated these findings by bulk, single-cell, and spatially resolved RNA sequencing as well as by *in situ* RNA hybridization. We are currently investigating the cell-stress pathways activated by these transducers, aiming to identify potential therapeutic targets for alleviating the cell stress, improving osteoblast function, and thereby reducing the severity of bone pathology. More recently, by combining the live-cell imaging, genetic, and biochemical analysis, we found that the unusual cell-stress response in G610C osteoblasts is triggered by recognition of the misfolded procollagen at ERESs rather than by ER–lumen chaperones responsible for activating the canonical unfolded protein response (UPR). Blockage of ERESs by misfolded procollagen results in accumulation of all secretory proteins in the ER lumen and ER dilation, which is not initially accompanied by the UPR. Increased protein accumulation in the ER of cells not capable of handling it eventually leads to misfolding of other proteins and triggering the UPR as a secondary response. In G610C mice, most osteoblasts avoid an UPR by reducing procollagen synthesis and entering a less functional state. In contrast, hypertrophic chondrocytes in the growth plate of the same animals do trigger a secondary UPR, which blocks their transition into osteoblasts and thereby limits longitudinal bone growth (probably because they produce less type I collagen and a much larger fraction of other proteins).

New approaches to analysis and treatment of ECM 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 the treatment of cell malfunction caused by procollagen misfolding in cases that do not involve pathogenic mutations.

To pursue the 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 testing approaches to reducing misfolded procollagen load in bone cells. For instance, our studies on the effects of reduced and increased ATG5 expression confirmed *in vivo* degradation of misfolded procollagen by osteoblasts via ERES micro-autophagy, but we also found that this pathway is only moderately affected by ATG5, necessitating a search for other therapeutic targets. We are therefore testing drugs known to reduce ER disruption by enhancing secretion and autophagy of misfolded proteins (e.g., 4-phenylbutyrate or 4PBA) and drugs (e.g., ISRIB) known to reduce the impact of accumulating misfolded proteins on overall protein synthesis. We found that 4PBA reduces bone pathology in a zebrafish model of OI and in G610C mice. In such mice, however, low-dose 4PBA treatment improved the function of hypertrophic chondrocytes and their conversion into osteoblasts but not the function of osteoblasts, probably because it alleviated secondary UPR more efficiently than the primary non-canonical cell stress. At the same time, a higher-dosage treatment is challenging because 4PBA is very rapidly metabolized and therefore difficult to deliver in a sustainable fashion to bone cells. We are therefore exploring other drugs, alternative delivery methods, and other approaches.

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 with which to characterize 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 several years, 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 learning this approach, we hope that it will soon be adapted not only for research but also for 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), or 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 post-translational modification of procollagen and deposition of collagen matrix by a different mechanism. It remains unclear why some FKBP65 mutations cause severe OI with joint contractures (Bruck'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, which had significantly reduced thermal stability owing to under-hydroxylation 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 conducted translational studies of OI caused by missense mutations in type I collagen that are not substitutions of obligatory Gly residues. For instance, we found that substitutions of Y-position arginine (Y-Arg) residues in the Gly-X-Y triplets within the collagen triple helix cause procollagen misfolding and accumulation in the ER to almost the same extent as Gly substitutions, likely because Y-Arg enhances collagen triple-helix stability and promotes triple-helix folding through binding of HSP47.

Presently, we are examining the molecular mechanism underlying OI caused by mutations in the ER–membrane stress receptor CREB3L1/OASIS. Bulk and single-cell RNA sequencing, qPCR, and our new mRNA–based histopathology assay suggest a crucial role CREB3L1 in type I procollagen synthesis or export from the ER. However, much work remains to be done before we understand how CREB3L1 mutations cause bone pathology.

Extracellular matrix pathology in tumors and fibrosis

Another aspect of our collagen metabolism pathology studies has been characterization of the 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 this 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., those 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 anti-inflammatory drug treatments in such 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 imaging and mapping of tissues

Given that tissue analysis plays a 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 micro-spectroscopic infrared and Raman imaging of tissues and cell cultures provides important information about the chemical composition, organization, and biological reactions inaccessible by traditional histology. By resolving the problem of light-path variations with passive thermomechanical stabilization, we developed high-definition (HD) infrared imaging and Raman micro-spectroscopic 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 to our current studies of bone structure and mineralization in the mouse models of the 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 *SLC26A2* sulfate transporter gene, deficient sulfate uptake by chondrocytes, and resulting under-sulfation of glycosaminoglycans in cartilage matrix. For instance, we found that chondroitin under-sulfation 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 under-sulfation and the rate of its synthesis across the growing epiphyseal cartilage and built a mathematical model for the sulfation pathway, predicting treatment targets for sulfation-related chondrodysplasias and genes that might contribute to the juvenile idiopathic arthritis recently associated with single-nucleotide polymorphisms in *SLC26A2*. 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) that is being developed by our collaborators.

As a test of the technology and its important translational application, we are presently working on combining our advances in mRNA–based and micro-spectroscopy–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, but yet poorly understood features of the disease. Spatially resolved imaging of mRNA at a single-cell level and mRNA sequencing are enabling us to identify the progression of growth-plate chondrocytes through differentiation steps and expression of different ECM components at these steps. Spatially resolved micro-spectroscopic 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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From Axon Damage to Disease: Common Pathways in Neurodegeneration

Our work is dedicated to advancing our understanding of common molecular and cellular mechanisms of neurodegeneration, with the ultimate goal of developing treatments for neurodegenerative diseases and even preventing them. The hypothesis driving our work is that common mechanisms are responsible for neurodegeneration during development and in aging. One focus is on mechanisms of stress-response pathways in neurons, such as the evolutionarily conserved axon-damage signaling pathway under the control of DLK (dual leucine zipper kinase; MAP3K12). Another theme is to understand fundamental differences between vulnerable and resilient populations of neurons in models of acute injury and in chronic disease. The lab uses the mouse and human iPSC–derived neurons as model systems.

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Elucidation of mechanisms of axon damage signaling in human neurons

DLK is an essential player in the axonal response to neuronal injury. It promotes axon degeneration, neuronal cell death, and regeneration, depending on the neuronal cell type. To elucidate regulators and substrates of DLK function, about which relatively little is known, we study DLK localization, trafficking, and interactors in a human iPSC–derived neuron model (i3neurons; Fernandopulle MS *et al*., *Curr Protoc Cell Biol* 2018;79: e51). Importantly, very few studies have examined DLK function in human neurons despite DLK inhibitors being considered in clinical trials. Almost all we know comes from studies in model organisms (worm, fly, mouse).

Understanding fundamental differences between vulnerable and resilient populations of spinal motor neurons in disease

We are individually profiling transcriptomes of spinal-cord motor neurons in healthy mice and disease models to track the transcriptomic alterations such cells undergo during disease progression. This year, we published a single-cell transcriptomic atlas of adult mouse spinal motor neurons [Reference 1]. Previously, very few spinal motor neurons (MNs) had been resolved at the single-cell level, both because they are relatively rare among all spinal cells, and because they do not survive single-cell isolation protocols well. Our success relied on two strategies: (1) enriching for spinal MNs using a Chat-Cre line; and (2) capturing

single nuclei, which are more robust than whole cells. We were able to collect single-nucleus RNA sequencing data from 16,000 cholinergic nuclei, define the full heterogeneity of these neurons at the single-cell level, and provide a comprehensive transcriptomic description of the lower MNs that selectively degenerate in ALS and other motor neuron diseases. We observed three main classes of skeletal MNs: alpha, gamma, and a third type potentially corresponding to beta MNs. Within each skeletal MN class, we identified previously uncharacterized subtypes corresponding to anatomical and functional specializations. The data from this study can be browsed at *http://www.spinalcordatlas.org* and will soon also be available at *https://seqseek.ninds.nih.gov*.

Having laid this important groundwork, we are currently obtaining data from mouse models of motor neuron disease and will compare the transcriptomes of resilient versus vulnerable MN types across several time points in disease.

Additional Funding

- NICHD SD Award 2020–2021

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The Biological Impact of Transposable Elements

Long terminal repeat (LTR)–retrotransposons constitute a significant fraction of eukaryotic genomes and have produced a large share of their genetic diversity. Their infectious counterparts, the retroviruses, are wide-spread pathogens of vertebrates, which evolved over 450 million years ago. LTR–retrotransposons and retroviruses propagate through a unique cycle in which an RNA intermediate is reverse-transcribed into cDNA copies that are inserted into host chromosomes by integrases (IN). The integration of cDNA results in dysregulation and outbreaks of neoplastic disease in a wide range of species, including salmon and koala bear, and in the case of the worldwide pandemic of AIDS, integration of HIV-1 results in acute loss of immune cells and tragic rates of mortality. Potent inhibitors of IN–strand transfer activity are a frontline component of antiretroviral treatment of AIDS patients, confirming that integration is a central feature of HIV-1 replication.

Despite the central role of integration, important questions remain about residual infection that occurs in the absence of IN activity. Mutations in HIV-1 IN of the catalytic residues produce residual infectious titer, typically with a 3 to 4-log decrease. However, in continuous cultures of HIV-1 lacking IN activity, insertion efficiency can be as high as 0.2–0.8% of the wild-type virus. Consistent with the studies of HIV-1, a distantly related retrovirus, murine leukemia virus (MLV), also possesses residual insertion activity when IN is inactivated. Such results indicate that retroviruses possess a secondary, IN–independent pathway, which incorporates viral DNA into the host genome. Given that IN–independent infections may compromise the treatment of HIV-1 patients with IN inhibitors, it is critical to identify the nature of this pathway.

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Identification of an integrase–independent pathway of retrotransposition

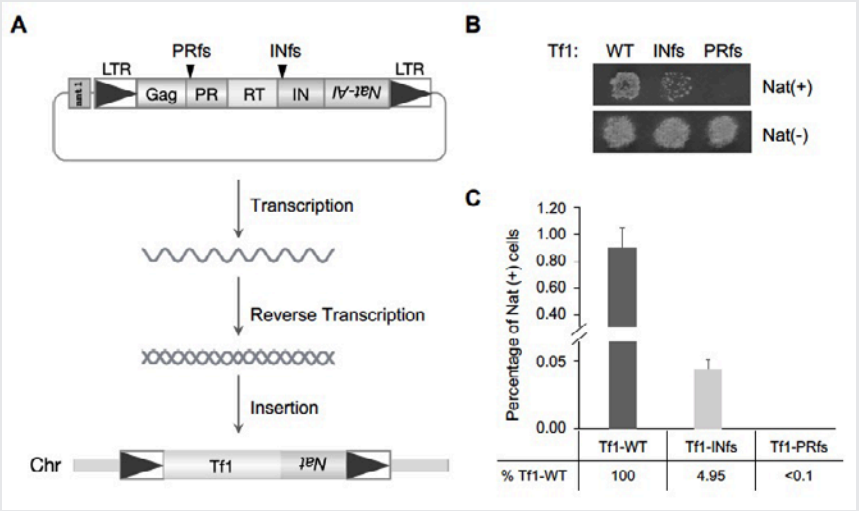
As a result of their structural similarities, LTR retrotransposons have been widely used as important models for studying the pathogeny of retroviruses. Tf1 and Tf2 are extensively characterized LTR retrotransposons with high integration activity in *Schizosaccharomyces pombe* [Reference 1]. We found that Tf1 retains 5% insertion activity in the absence of IN, which allowed us to study the mechanistic underpinnings of the process (Figure 1). With high-throughput sequencing, we found that insertions of Tf1 lacking IN (Tf1-INfs)

FIGURE 1. Tf1 insertion takes place in the absence of integrase.

A. The diagram shows the strategy of monitoring Tf1 retrotransposition. A drug-resistant gene, *nat*, with artificial intron (*nat*-AI) is introduced into Tf1, and the integration of Tf1 into host chromosomes allows cells to grow on plates containing Nat. The black arrows indicate the frame shift (fs) sites of PR and IN respectively. LTR: long terminal repeat; PR: protease; RT: reverse transcriptase; IN: integrase; WT: wild-type.

B. Growth phenotypes of Tf1-WT, Tf1-INfs, and Tf1-PRfs on medium containing Nat after inducing Tf1 expression.

C. Quantitative transposition analysis Tf1-WT, Tf1- INfs, and Tf1-PRfs.



occurred at sites that possessed homology to the primer-binding site (PBS) and poly purine tract (PPT), whose sequences serve as RNA primers of reverse transcription and that are copied into single stranded DNAs at the 3' ends of the cDNA. Notably, we found that in previously published data of HIV-1 lacking IN activity insertions sites can have strong similarity to the PBS, indicating that this process maybe widespread among retroviruses.

Additional analysis revealed that a substantial fraction of Tf1–INfs insertions occurred adjacent to pre-existing retrotransposons, resulting in tandem structures that expand to serve as reservoirs of active elements. A genetic screen revealed that IN–independent insertions were mediated by Rad52 (a member of the homologous recombination pathway, important for maintenance of genome integrity). Mutations in *rad52* showed that the Tf1 insertions result from single-strand annealing (SSA), a non-canonical form of homologous recombination mediated by Rad52 that is independent of Rad51. Surprisingly, we discovered that wild-type Tf1 can switch from IN–dependent to this IN–independent pathway of insertion depending on culture conditions. Taken together, we demonstrated that there are two efficient insertion pathways of cDNA, one relying on IN while the other is IN–independent but requires Rad52–mediated SSA.

Dense transposon integration reveals that 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

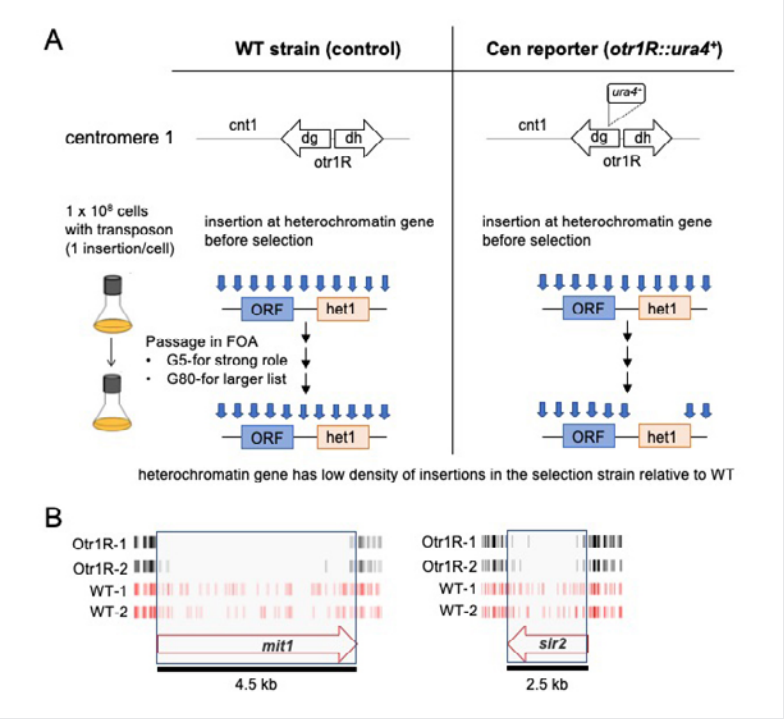


FIGURE 2. Dense maps of transposable element integration identify genes important for heterochromatin at centromere repeats.

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).

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 to 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 to single-cell eukaryotes [References 2–5].

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 [Reference 2]. 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.

We identified other RNA–processing factors 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-deltaC) by removing 38 amino acids that, based on the Hermes insertions, were not important for viability. Iss1-deltaC 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 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-deltaC mutation to evaluate changes in expression and transcription termination genome-wide. RNA-Seq data revealed that Iss1-deltaC 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-immunoprecipitation 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-deltaC 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 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-deltaC mutation caused significant increases in the 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 not obtainable with other screens.

Retrotransposon insertions associated with risk of neurologic and psychiatric diseases

Neurologic and psychiatric disorders affect 25% of the world population. Given the complexity of the mammalian nervous system, the genetic and cellular etiology of such diseases remains largely unclear. Progress in genetic methodology has provided the potential to identify mechanisms that underlie the diseases. One approach that has successfully identified important disease loci is genome-wide association

studies (GWAS). However, in the cases of neurologic and major psychiatric disorders, GWAS have identified large numbers of loci, each associated with small increases in risk. Importantly, there is extensive overlap of the loci that contribute to major psychiatric disorders, indicating that related molecular mechanisms may underlie distinct clinical phenotypes.

SINGLE-NUCLEOTIDE POLYMORPHISMS (SNPS) IDENTIFIED BY GWAS WITH THE HIGHEST DISEASE ASSOCIATION

Trait associated SNPs (TASs) are genetic tags identifying a genomic region that contains the causal mutation(s) leading to the disease risk. Limits on the design of GWAS typically prevent such studies from identifying causal gene alleles. Determining causal variants remains the most challenging and rate-limiting, but also the most important step in defining the genetic architecture of diseases. The vast majority of GWAS TASs lie in intergenic or intronic regions and therefore do not alter coding sequence. For such SNPs to be causal they would likely have regulatory effects on transcription. Structural variants such as rearrangements, copy number variants, and transposable element (TE) insertions constitute a substantial and disproportionately large fraction of the genetic variants found to alter gene expression.

In humans, the dominant families of TEs are long interspersed element-1 (LINE-1 or L1) and Alu elements, which are short interspersed elements (SINEs) and are mobilized by L1. TEs alter gene expression particularly easily because they have evolved various sequences that act on enhancers. Given that TEs make up approximately 45% of the human genome, it is not surprising that their regulatory features are abundant sources of tissue-specific promoter activity.

Relatively recent TE insertions can proliferate in the population and become common alleles. The 1000 Genomes Project described genetic variation of diverse human populations by sequencing whole genomes of 2,504 individuals. The extensive survey of genetic variation detected 17,000 polymorphic insertions of TEs, which have the potential to alter gene expression and affect common-disease risk. Some TEs have been implicated at disease loci detected by GWAS.

Given the difficulty in identifying genetic variants responsible for neurologic and psychiatric disorders and the regulatory capacity of TEs, we tested whether polymorphic TEs are potential causative variants of such diseases. We analyzed 593 GWAS of neurologic and psychiatric diseases, which in total reported 753 TASs. From the 17,000 polymorphic TEs, we found that 76 were in linkage disequilibrium (LD) with TASs, indicating that the TEs were among the variants with the potential to be causative. We extended our analysis by evaluating each candidate TE for a role in altering expression of proximal genes. In one approach we determined whether polymorphic TEs could disrupt regulatory sequences, as annotated with the epigenomic data of the NIH Roadmap Epigenomics Consortium. Ten of the TE candidates were located in regions of chromatin with active regulatory function in neurologic tissues. We also tested whether the polymorphic TEs were significantly associated with altered expression of proximal genes. By analyzing multi-tissue expression data from GTEx (Genotype-Tissue Expression project), we found that 31 of the TASs linked to TEs were expression-quantitative trait loci (eQTLs, loci that seek to identify genetic variants that affect the expression of one or more genes) for adjacent genes, showing correlation with altered expression within regions of the brain. These expression data, together with epigenetic and eQTL analyses, indicate that polymorphic TE insertions are important candidates for causing disease risk for Parkinson's disease, schizophrenia, and amyotrophic lateral sclerosis, on par with other variants at these loci.

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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 in 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

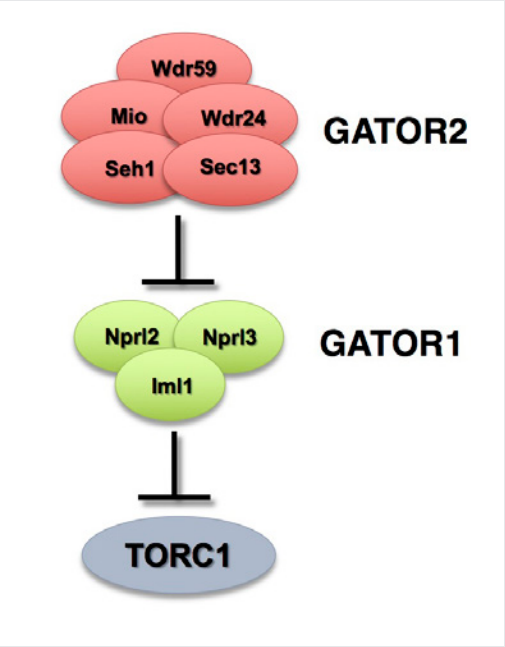


FIGURE 1. The GATOR complex regulates TORC1 activity.

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 past year, we used molecular, genetic, and cell-biological approaches to define the role of the GATOR complex in the regulation of meiotic progression and genomic stability during oogenesis. Moreover, we used oogenesis as a model system to define a novel mechanism of TORC1 inhibition in response to both amino acid and growth factor restriction.

Multiple independent pathways converge on Nprl3 to regulate TORC1 activity in *Drosophila*.

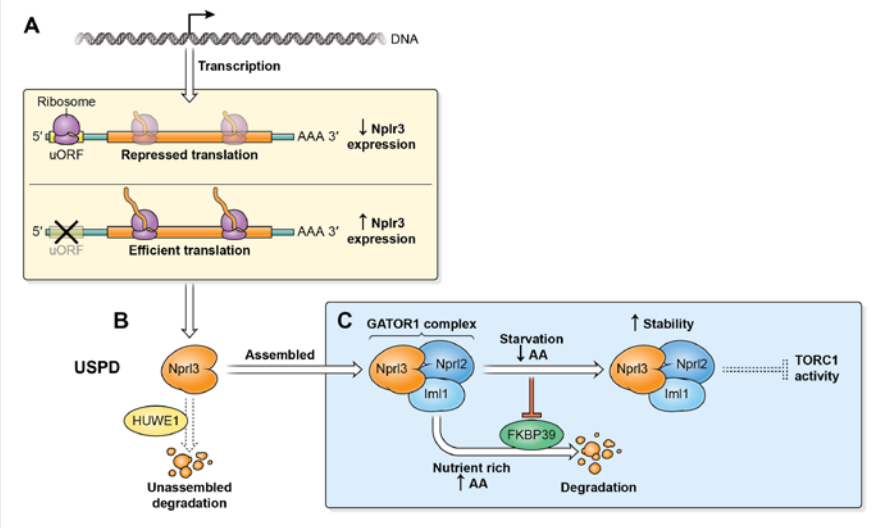
In collaboration with the laboratory of Youheng Wei, we characterized several pathways that regulate the expression of the GATOR1 component Nprl3 in *Drosophila* (Figure 2). We determined that the stability of Nprl3 is impacted by the Unassembled Soluble Complex Proteins Degradation (USPD) pathway. In addition, we found that FK506 binding protein 39 (FKBP39)–dependent proteolytic destruction maintains Nprl3 at low levels in nutrient-replete conditions. Nutrient starvation abrogates the degradation of the Nprl3 protein and rapidly promotes Nprl3 accumulation. Consistent with a role in promoting the stability of a TORC1 inhibitor, mutations in *fkbp39* reduced TORC1 activity and increased autophagy. We also demonstrated that the 5'UTR of *nprl3* transcripts contain a functional upstream open-reading frame (uORF) that inhibits main ORF translation. In summary, our work has uncovered novel mechanisms of Nprl3 regulation and identifies an important role for *Drosophila* FKBP39 in the control of cellular metabolism and growth.

FIGURE 2. Multiple pathways regulate the levels of the TORC1 inhibitor Nprl3.

A. The *nprl3* mRNA contains a functional uORF that reduces Nprl3 translation.

B. Nprl3 forms the trimeric GATOR1 complex with the proteins Nprl2 and Iml1. When not assembled into the GATOR1 complex, Nprl3 is degraded via a HUWEI–dependent pathway.

C. In nutrient-replete conditions, FKBP39 associates with Nprl3 and promotes its degradation. Upon amino acid starvation, the FKBP39–dependent destruction of Nprl3 is blocked, and the increased levels of GATOR1 result in reduced TORC1 activity.



A unified model of TORC1 regulation: the Rag GTPase regulates the dynamic behavior of TSC downstream of both amino-acid and growth-factor restriction.

The dysregulation of the metabolic regulator TORC1 contributes to a wide array of human pathologies. Tuberous sclerosis is a rare multi-organ genetic disorder affecting 1 in 6,000 newborns a year. Mutations in components of the tuberous sclerosis complex (TSC) result in the hyperactivation of 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 GATOR1 subcomplex 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. The GATOR2 complex opposes the activity of GATOR1.

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 controlling 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 lysosomes. Moreover, by using a photoconvertible protein–tagged TSC2, we determined that the rapid association of TSC2 to lysosomes is accompanied by its rapid dissociation in *wdr24*$^{-/-}$ cells. 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 (Figure 3).

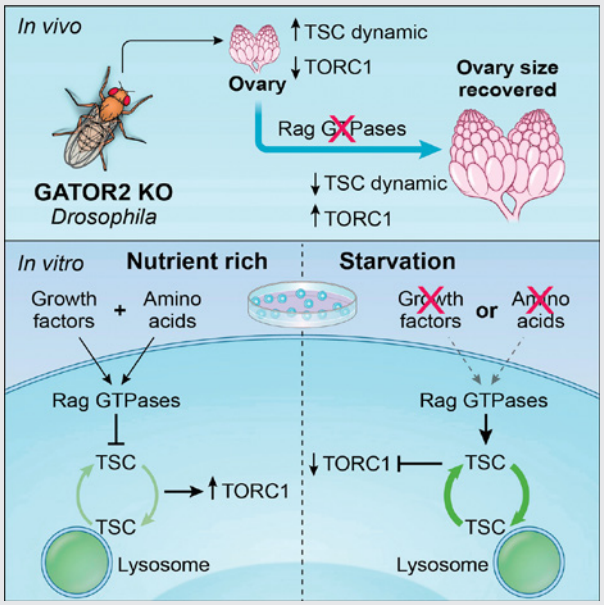


FIGURE 3. Integrated model of TORC1 regulation

The Rag GTPase integrates information from the amino-acid and growth-factor signaling pathways to control the activation of the TORC1 inhibitor TSC by regulating its lysosomal-cytosolic exchange rate.

Currently, there are two working models for the role of TSC in the inhibition of TORC1 activity. The first model posits that TSC lies exclusively downstream of the PI3K-AKT growth-factor signaling pathway, while the second model proposes that TSC is a critical downstream effector of both the growth-factor signaling and amino acid–sensing pathways. Our findings on the function of the GATOR2 complex are consistent with the second model, which implicate different nucleotide states of the Rag GTPase in the recruitment of TORC1 versus TSC to lysosomes in response to amino-acid starvation. Moreover, we determined that the Rag GTPase, which was previously thought to exclusively function in amino-acid sensing, regulates the recruitment of TSC to lysosomes in response to growth-factor restriction. Thus, our data support a model in which both the amino acid–sensing pathway and growth factor–signaling pathway converge on the Rag GTPase to recruit TSC to lysosomes in response to inhibitory signals. Notably, we found that, both in HeLa cells and *Drosophila*, the Rag GTPase promotes the rapid exchange of TSC between the lysosome and cytosol in response to negative inputs. Moreover, the rate of exchange mirrors TSC function, with depletions of the Rag GTPase blocking TSC lysosomal mobility and rescuing TORC1 activity. Demonstrating further integration of the amino acid–sensing and growth factor–signaling pathways, we showed that the GATOR2 complex acts upstream of the Rag GTPase to promote both the activating phosphorylation of AKT and the AKT–dependent inhibitory phosphorylation of TSC2.

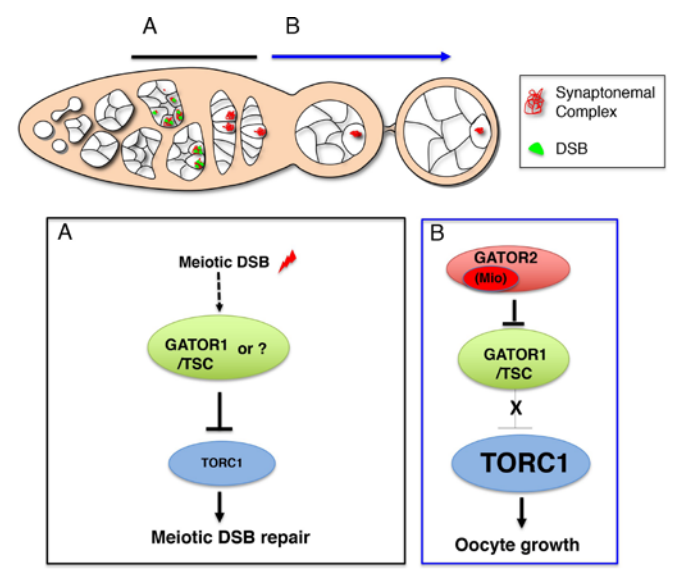
The GATOR complex regulates an essential response to meiotic double-stranded breaks.

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 past year, we expanded our examination into 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

FIGURE 4. Working model for the role of the GATOR complex in the response to meiotic DSBs

A. After ovarian cysts enter meiosis, meiotic DSBs function to activate and/or maintain a GATOR1/TSC–dependent pathway to ensure low TORC1 activity in early prophase of meiosis I. Low TORC1 activity promotes the timely repair of meiotic DSBs. Currently, whether meiotic DSBs directly activate the GATOR1/TSC pathway or an alternative pathway that works in concert with, or in parallel to, GATOR1/TSC is not known.

B. Subsequently, the GATOR2 component Mio is required to attenuate the activity of the GATOR1/TSC pathway, thus allowing for increased TORC1 activity and the growth and development of the oocyte in later stages of oogenesis.



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, 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 levels of TORC1 activity, exhibit many phenotypes consistent with the mis-regulation 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. 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 mis-regulation 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*. The results raise the possibility that GATOR1–mediated downregulation of TORC1 activity may be a common feature of the early meiotic cycle in many eukaryotes.

Genotoxic stress has been implicated in the deregulation of retrotransposon expression in several organisms, including *Drosophila*. In line with these studies, we found 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.*

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Neurosecretory Proteins in Neuroprotection and Neurodevelopment

Mechanism of sorting, transport, and regulated secretion of neuroproteins

The intracellular sorting of pro-neuropeptides and neurotrophins to the regulated secretory pathway (RSP) is essential for processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of proopiomelanocortin (POMC, also known as pro-ACTH/endorphin), proinsulin, and brain-derived neurotrophic factor (BDNF) to the RSP. Our studies showed that these pro-proteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum (ER) to the *trans*-Golgi network (TGN). In the TGN, the pro-proteins are sorted into 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 pro-proteins.

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

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

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vesicles interact with dynactin and the microtubule-based motors KIF1A/KIF3A to effect anterograde vesicle movement to the plasma membrane for activity-dependent secretion. Additionally, in collaboration with Joshua Park, we showed that another player, snapin, binds directly to the cytoplasmic tail of CPE and connects with the microtubule motor complex, which consists of kinesin-2 and kinesin-3, to mediate the post-Golgi anterograde transport of POMC/ACTH vesicles to the process terminals of AtT20 cells for secretion. Knockdown of snapin reduced stimulated ACTH secretion, while protein kinase A (PKA) activation by forskolin significantly increased the interactions of kinesin-2 and kinesin-3 with CPE and the levels of ACTH vesicles at the terminus and enhanced secretion of ACTH in AtT20 cells. Thus, our study has uncovered a novel complex consisting of the CPE cytoplasmic tail snapin–kinesin-2 and -3, which mediates anterograde transport of ACTH/POMC vesicles to the process terminals for secretion in a PKA–dependent manner 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

Several null and homozygous non-sense mutations in the CPE (also known as neurotrophic factor-alpha1, NF-alpha1) gene were identified in seven children and a young adult woman from five different families. They display clinical features that include childhood onset obesity, type 2 diabetes, intellectual disabilities, hypogonadotrophic hypogonadism, and infantile hypotonia, indicating the importance of CPE in human disease. To study the physiological functions of CPE/NF-alpha1 *in vivo,* we generated a *Cpe* knock-out (KO) mouse. The KO mouse exhibited obesity, infertility, and diabetes. Further analysis of *Cpe*–KO mice in the

Morris water maze and by object-preference tests revealed defects in learning and memory, and depressive-like behavior in the forced swim test. Electrophysiological measurements showed a defect in the generation of long-term potentiation in hippocampal slices. We discovered that a major cause of the neurological defects in such mice was the loss of CA3 neurons in the hippocampus after weaning stress. Hippocampal neurons in the CA3 region are enriched in CPE. *Cpe*–KO mice showed normal hippocampal cytoarchitecture at three weeks of age just before weaning, indicating that the defect was not a developmental problem. Rather, we hypothesized that the degeneration of the CA3 neurons was 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. The hypothesis was supported by the finding that treatment with carbamazepine, an anti-epileptic drug, prior to weaning prevented the stress-induced degeneration of the CA3 neurons in the *Cpe*–KO mice. Hence, CPE/NF-alpha1 is important for the survival of CA3 neurons during severe stress. To determine whether the neuroprotective effect of CPE/NF-alpha1 depends on the BDNF–TrkB pathway (Trk: tropomyosin receptor kinase), we treated mice with ANA12, a trkB inhibitor. Interestingly, downregulation of the BDNF–TrkB pathway had no detrimental effect on the survival of the CA3 neurons after the weaning stress paradigm, unlike the *Cpe*–KO mice, which showed complete degeneration, suggesting that CPE/NF-alpha1 is more critical than BDNF in protecting CA3 neurons from severe stress–induced cell death [Reference 1].

Furthermore, we 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 of CPE/NF-alpha1 is independent of its enzymatic activity [Reference 1]. We showed that CPE/NF-alpha1 (E342Q), either overexpressed or applied externally to cultured hippocampal or cortical neurons, protected the 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 bound specifically to the cell surface of HT22 cells, an immortalized hippocampal neuronal cell line, in a saturable manner, suggesting the existence of a receptor. K235a, a Trk 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 CPE/NF-alpha1 likely uses a different class of receptors than those of the Trk family or FGFRs. We then screened a human GPCR (G protein–coupled receptor) library using CPE/NF-alpha1 as a ligand and identified the serotonin receptor, 5-HTR1E, as a binding partner. 5-HTR1E is only found in humans, primates and guinea pig but not in mice or rats. Thus work is ongoing to identify a mouse receptor for CPE/NF-alpha1.

The mechanism of action of CPE/NF-alpha1 in neuroprotection in rodent hippocampal neurons involves the activation of the ERK1/2 (extracellular signal–regulated kinase) signaling pathway during stress, which then leads to enhanced expression of a pro-survival mitochondrial protein, BCL2, inhibition of caspase 3 activation, and promotion of neuronal survival [Reference 2]. Indeed, we were able to demonstrate that interaction of CPE/NF-alpha1 with 5-HTR1E activated the ERK pathway via beta arrestin, which then led to increased BCL2 expression and neuroprotection of human neurons against oxidative and neurotoxic stress. Furthermore, this CPE/NF-alpha1–mediated neuroprotection pathway can be 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 1 hour per day for seven days, mice showed significantly elevated levels of CPE/NF-alpha1 mRNA and protein, as well as the anti-apoptotic protein Bcl2, in the hippocampus. *In situ* hybridization studies indicated particularly elevated CPE/NF-alpha1 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 pro-apoptotic protein, after dexamethasone (a synthetic glucocorticoid) treatment. 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, and degeneration of the CA3 neurons. Furthermore, CRS also elevated expression of the signaling protein FGF2. We demonstrated that mouse primary hippocampal neurons treated with CPE/NF-alpha1 increased FGF2 expression. Thus, another action of CPE/NF-alpha1 may be by increasing FGF2, which is known to have neuroprotective effects. In summary, CPE/NF-alpha1 is a critical neurotropin for protecting CA3 neurons against stress-induced cell death via the Erk–Bcl2 signaling pathway.

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 3]. Our search of 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. Overexpression of CPE-QQ into neuroblastoma cells indicated that the mutated protein aggregates with the WT protein in the ER, causing ER stress, decreased Bcl2 levels, and neuronal cell death. We 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, as there is 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 in 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. These studies thus 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 3].

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 4]. Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway [Reference 4]. Interestingly, 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), which encode 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 5]. 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 that CPE/NF-alpha1 plays a role in neurodevelopment. We examined the effect of CPE/NF-alpha1 on E13.5 neocortex-derived neurospheres, which contain stem cells and neuro-progenitors. Application of recombinant CPE/NF-alpha1 reduced the number and size of the neuro-spheres formed, suggesting inhibition of proliferation and maintenance of the 'stemness' of the stem cells in the neuro-spheres. CPE/NF-alpha1 down-regulated the wnt pathway in the neuro-spheres, 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 neuro-spheres 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 neuro-spheres 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 in 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 6].

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 neuro-endocrine 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 the expression of IGF binding protein2 (IGFBP2), death-associated protein (DAP1), and Ephrin 1A mRNAs, proteins and an mRNA 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 5]. 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 is to elucidate the molecular mechanisms underlying the initiation phase of protein synthesis in eukaryotic organisms. We use the yeast *Saccharomyces cerevisiae* as a model system and employ a range of approaches, from genetics to biochemistry to structural biology, in collaboration with Alan Hinnebusch's and Tom Dever's labs and several other research groups around the world.

Eukaryotic translation initiation is a key control point in the regulation of gene expression. It begins when an initiator methionyl tRNA (Met-tRNAi) is loaded onto the small (40S) ribosomal subunit. Met-tRNAi binds to the 40S subunit as a ternary complex (TC) with the GTP–bound form of the initiation factor eIF2. Three other factors, eIF1, eIF1A, and eIF3, also bind to the 40S subunit and promote the loading of the TC. The resulting 43S 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, which is conducive to scanning to a closed one, which is not. At this stage, eIF2•GDP dissociates from the PIC, and eIF1A and a second GTPase factor, eIF5B, coordinate joining of the large ribosomal subunit to form the 80S initiation complex. In a process that appears to result in conformational reorganization of the complex, eIF5B hydrolyzes GTP and then dissociates along with eIF1A.

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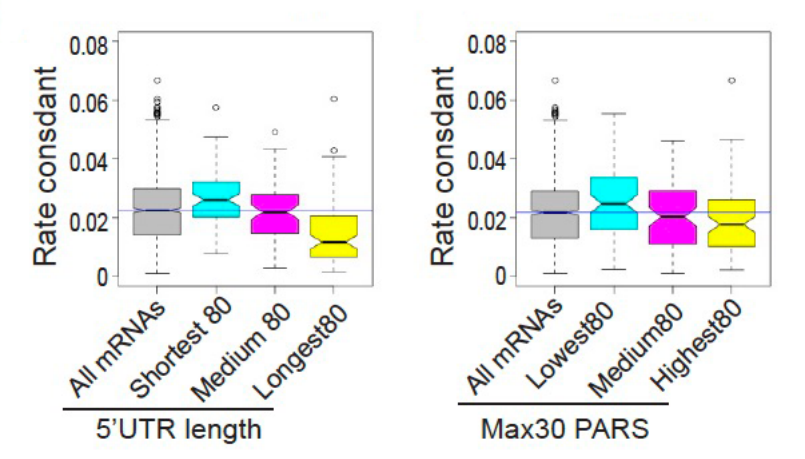

FIGURE 1.

Figure showing the correlation between rate constants for mRNA recruitment, measured using the RecSeq platform and 5'-UTR length (left) or structure (right). PARS is a measure of RNA structure.

In 2021, we continued to make progress on implementation of the RecSeq platform that we had developed to allow measurement of translation initiation rates transcriptome-wide in a fully reconstituted *in vitro* system. We used the system to measure the rate constants for recruitment of most yeast mRNAs, something that has never been done before. Our data indicate that the length of the 5'-untranslated region (5'-UTR) and the amount of structure in the 5'-UTR both inversely correlate with the rate of mRNA recruitment. We also compiled a large amount of data about the role of the RNA helicase initiation factor Ded1 in the mRNA recruitment process using this system. Ded1 preferentially accelerates the rate of recruitment of mRNAs with long, structured 5'-UTRs, consistent with data from *in vivo* experiments from Alan Hinnebusch's lab. We expanded these studies to probe the roles of the factor eIF4A and test the effects of competition among mRNAs for limiting 40S ribosomal subunits in the system. Such studies are significant because of debates in the field around whether certain pathologies (e.g., ribosomopathies) affect gene expression by altering translation of certain mRNAs as a result of differential competition among mRNAs that are inherently well or poorly translated, or because ribosomes lacking or containing different components (e.g., ribosomal proteins) have different inherent specificities for certain mRNAs owing to ribosome-mRNA interactions ("specialized ribosomes"). The studies are breaking new ground by allowing us to actually measure rates of mRNA recruitment to 40S ribosomes transcriptome-wide under defined conditions in a system in which we can isolate translation initiation from other cellular processes, such as transcription and mRNA decay.

The pandemic slowed progress by our group, and a senior member of the lab (Jagpreet Nanda) departed to take a position as a Scientific Review Officer at NICHD, but despite these challenges we have progressed significantly. We have now hired a new member of the lab, Meizhen Hou, who has exceptional experience in protein expression and purification and will thus be a great asset to the lab and the NICHD IRP. We also expect to have a postbaccalaureate student joining the lab this summer, after her graduation from Wellesley College. This will bring staffing in the lab to two biologists and a postbaccalaureate.

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Publication

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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 non-signaling 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 also investigate whether modification of TCR–signaling subunits can be used to enhance the tumoricidal activity of T cells. Using gene profiling, we seek 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, such molecules may also be targets to enhance anti-tumor activity. We also investigate the function of new T cell–specific proteins that we identified by subtraction library screening. Our studies 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 early 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, which revealed a critical function for Ldb1 as a key subunit of multimeric DNA–binding complexes in controlling the self-renewal/differentiation cell-fate decision in HSCs. Current work, which involves genome-wide screening by RNA-Seq and ChIP-Seq, 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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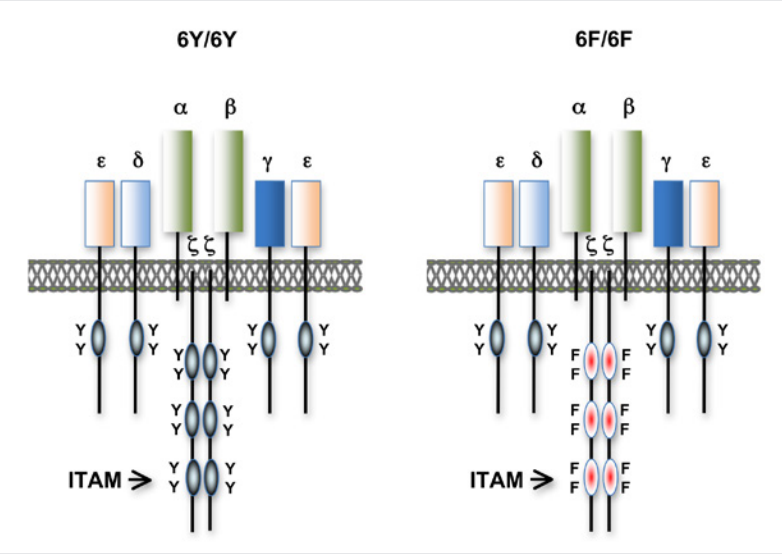


FIGURE 1. T cell antigen receptors expressed in 6Y/6Y and 6F/6F knock-in mice

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).

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 sequences, termed immuno-receptor tyrosine-based activation motifs (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 non-identical, 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 auto-reactive 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 necessary 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 knock-in 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 such mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. We confirmed that the knock-in zeta locus functions as predicted. We next evaluated the effect of late 'switching' from 6Y zeta to 6F zeta in mature T cells generated with wild-type 6Y zeta containing TCRs and found that the phenotype was identical to germline inactivation of zeta ITAMs, demonstrating that compensation does not explain the mild phenotype of zeta 6F mice. Unexpectedly, we also discovered an inhibitory role for zeta ITAM signaling in response to weak (low-affinity) antigens. Strikingly, inactivation of the zeta ITAMs resulted in enhanced TCR signaling and enhanced T cell effector functions when the TCR is engaged by low-affinity ligands, but zeta ITAMs contributed positively to signaling by high-affinity ligands. This revealed a dual (activating and inhibitory) function for zeta ITAMs in TCR signaling depending on the affinity of the TCR–ligand interaction. Given that most tumor-specific antigens are low affinity and that this property limits current TCR–based approaches to tumor immunotherapy, we explored the effect of zeta ITAM inactivation on T cell tumoricidal activity. Notably, we found that inactivation of zeta ITAMs markedly enhanced T cell tumoricidal activity against low-affinity tumor antigens. Such experiments should provide information relevant to the design of engineered tumor antigen–specific TCRs and possibly chimeric antigen receptor T cells (CAR T cells), which are currently configured to express zeta ITAM signaling module(s).

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, 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 cancer or 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.

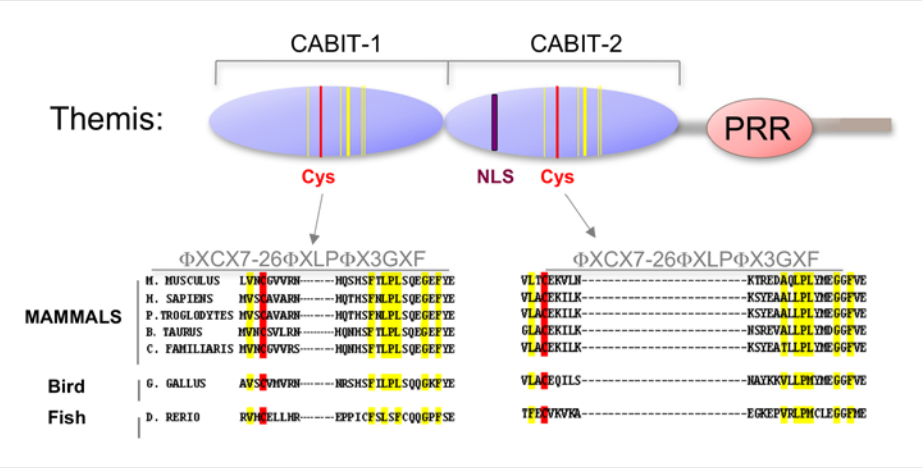


FIGURE 2. Themis is highly conserved in vertebrates.

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).

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 knock-down cell lines, Themis1 knock-out 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. First, 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 is equivalent to that of Themis1 for restoring normal T cell development in *Themis1*$^{-/-}$ mice, thus demonstrating functional redundancy of Themis1 and Themis2. Second, we generated retroviruses encoding domain-deletion mutants of Themis1, infected *Themis1*$^{-/-}$ bone marrow progenitors, and made bone marrow chimeras to determine which regions of Themis1 are important for *in vivo* function. We found that the Themis1 proline-rich sequence (PRS), which mediates binding to the signaling protein Grb2, was required for *in vivo* function, as assessed by rescue of the developmental block in *Themis1*$^{-/-}$ thymocytes, but that the CABIT (cysteine-containing, all beta in Themis)–domain cysteines are not essential. Third, 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.

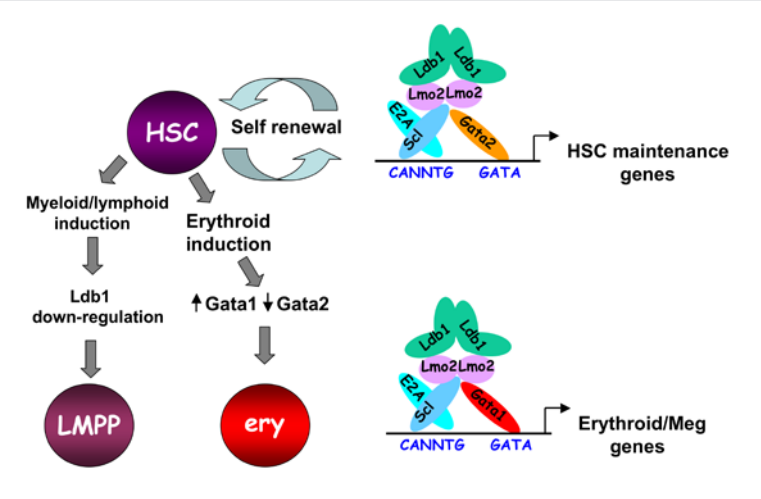
In the past few years, we focused on determining the molecular function of Themis1. Themis1, Themis2, and a large family of related metazoan proteins contain a novel globular domain of unknown function called the CABIT module (see above and Figure 2). Using cell transfection and biochemical and protein-assay 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 several 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 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 metazoan CABIT–containing proteins (which number in the hundreds). Our ongoing studies are focusing on further characterization of CABIT proteins and determining their role in development and their possible involvement in human disease.

Role of the F-box protein Fbxl12 in thymocyte development

A major aspect of the T cell maturation process is the precise regulation of cell proliferation. Rather than being a shared property among all or most developing thymocytes, proliferation is strictly limited to two stages during early development. The initial proliferative phase is driven by thymus-expressed cytokines, the second coincides with 'beta selection' (i.e., is initiated in cells that have productively rearranged the TCRbeta chain and express a signaling complex called the pre–TCR). The proliferative burst that accompanies beta selection is estimated to result in a 100–200 fold expansion and is essential for further differentiation and for maximizing TCR diversity. Previous work showed that beta selection–associated proliferation requires concurrent signals by the pre–TCR and Notch receptors, 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, a defect that was rescued by co-deletion of Cdkn1b. We identified a new F-box protein, Fbxl12, that is highly expressed in thymocytes. We found that Fbxl12 also functions as an SCF E3 ligase subunit, which, like Fbxl1, directs Cdkn1b degradation. The phenotype of Fbxl12–deficient mice generated in our lab was strikingly similar to that of 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.

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 complexes, 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.



Role of Ldb1 transcription complexes in hematopoiesis and in T cell acute lymphoblastic leukemia

The 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 Lyl1 or Tal1 and Gata1 or Gata2) to form multi-molecular transcription complexes (Figure 3). Within the hematopoietic lineage, expression of *Ldb1* is highest in progenitor cells, which include hematopoietic stem cells (HSCs). *Ldb1*–null (*Ldb1*$^{-/-}$) mice die between day 9 and 10 of gestation, preventing us from directly studying the impact of loss of Ldb1 on fetal or adult hematopoiesis. We investigated the role of Ldb1 in hematopoiesis by following the fate of *Ldb1*$^{-/-}$ embryonic stem cells (ESCs) in mouse blastocyst chimeras and by conditional, stage-specific deletion of *Ldb1*. Significantly, *Ldb1*$^{-/-}$ ESCs were capable of generating HSCs, which could give rise to both myeloid and lymphoid lineage cells; however, the number of *Ldb1*$^{-/-}$ HSCs gradually diminished at later stages of development. Following adoptive transfer of fetal liver hematopoietic progenitor cells, *Ldb1*$^{-/-}$ HSCs were rapidly lost, indicating a failure of self-renewal or survival. More recent data indicate that the loss of *Ldb1*$^{-/-}$ HSCs results from differentiation rather than cell death. Although expressed in ESCs, Ldb1 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 for maintaining 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 Ldb1 complexes in HSCs contain the transcription factor Gata2, whereas Ldb1 complexes in erythroid progenitors 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 as 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 hypothesized 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 are also determining the subunit structure and binding sites of Ldb1 complexes expressed in *Lmo2-tg* thymocytes. We anticipate that our results will provide insights into the mechanisms controlling T-ALL oncogenesis in humans and may thus provide new therapeutic avenues for the treatment of this devastating pediatric disease.

Additional Funding

- NICHD Director's Award

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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 to 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 sequences that contributed to mammalian-specific traits including the evolution of the placenta. Our primary interest is to explore the impact of such 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) on 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 of our research to explore whether KZFPs play broader roles in genome regulation, beyond gene silencing, and how such functions impact mammalian development and evolution.

Kruppel-associated box zinc-finger (ZF) 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, and they constitute 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 and a larger fraction specific to each species. Despite

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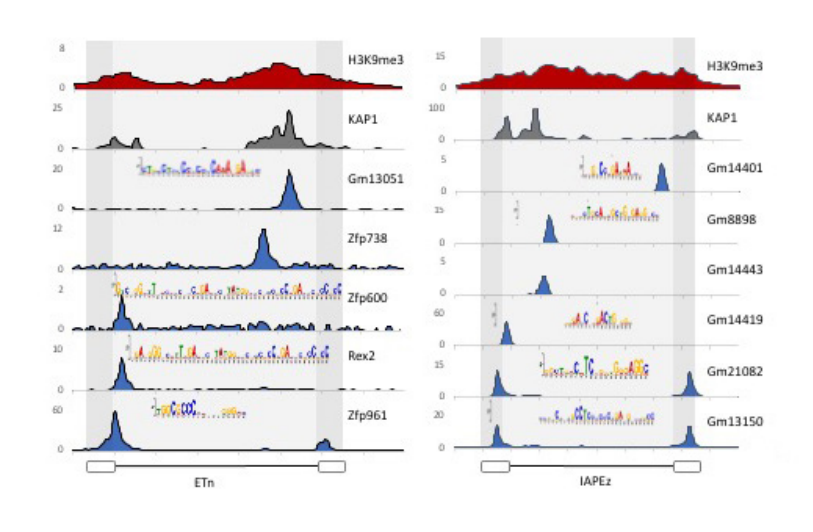

FIGURE 1. KRAB-ZFPs bind redundantly to the active retrotransposons ETn and IAP.

ChIP-Seq track for indicated KRAB-ZFPs displayed across a consensus sequence of ETn and IAP retrotransposons. ChIP-Seq was performed by expressing epitope-tagged KRAB-ZFPs in F9 ECs or ESCs and ChIP'ing with antibodies that recognize the epitope.

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 ZF 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 ZF 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 initially 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 functions *in vivo* to repress other ERVs that utilize 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 conditional alleles and rescue experiments, we determined that ZFP809 activity was required in development to initiate silencing, but not in somatic cells to maintain silencing. The studies 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 began a systematic analysis of KRAB-ZFPs using a medium-throughput ChIP-Seq screen and functional genomics of KRAB-ZFP clusters and individual KRAB-ZFP

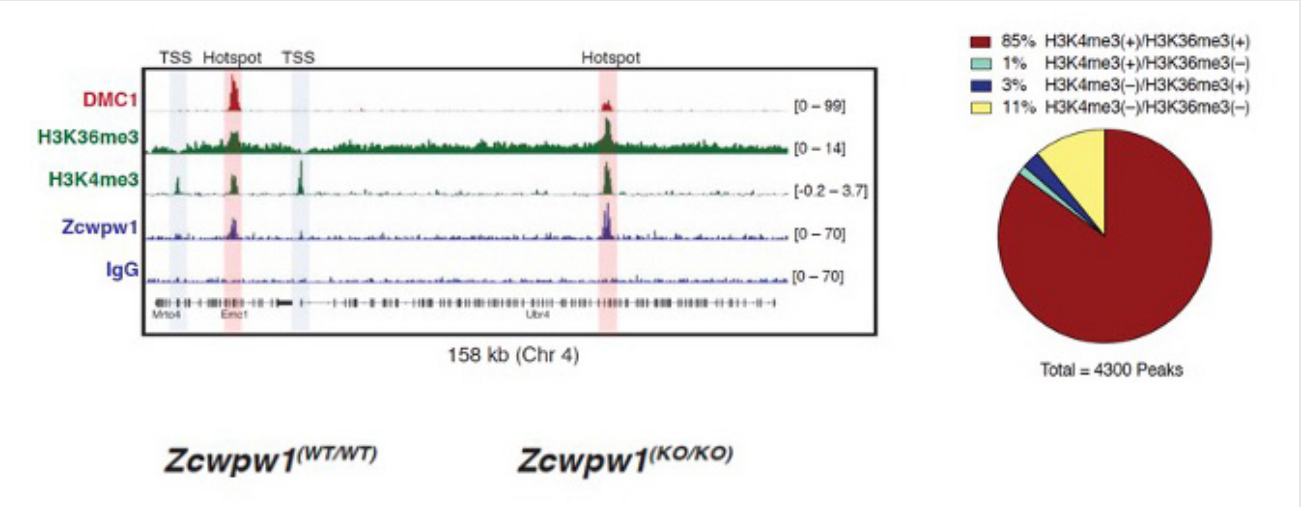

FIGURE 2. ZCWPW1 binds to meiotic recombination hotspots in spermatocytes harboring dual H3K4me3 and H3K36me3 marks.

ChIP-Seq or Cut & Run was performed with indicated antibodies in mouse spermatocytes, and read pileup is displayed across a region on Chr 4. The pie chart at the right displays percentage of ZCWPW1 peaks that overlap peaks of either H3K4me3, H3K36me3, or both marks.

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, 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 to or repress ERVs. One such KRAB-ZFP, ZFP568, plays an important role in silencing a key developmental gene that may have played a critical role in the onset of viviparity in mammals. Using ChIP-Seq and biochemical assays, we determined that ZFP568 is a direct repressor of a placental-specific isoform of the *Igf2* gene called *Igf2-P0*. Insulin-like growth factor 2 (Igf2) is the major fetal growth hormone in mammals. We demonstrated that loss of *Zfp568*, which causes gastrulation failure, or mutation of the ZFP568 binding site at the *Igf2-P0* promoter, cause inappropriate *Igf2-P0* activation. We also showed that the 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 they identify an additional layer of transcriptional control of a key growth factor regulating fetal and placental development. In a 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 ZFs bound to the *Igf2-P0* binding site, which reveals several non-canonical

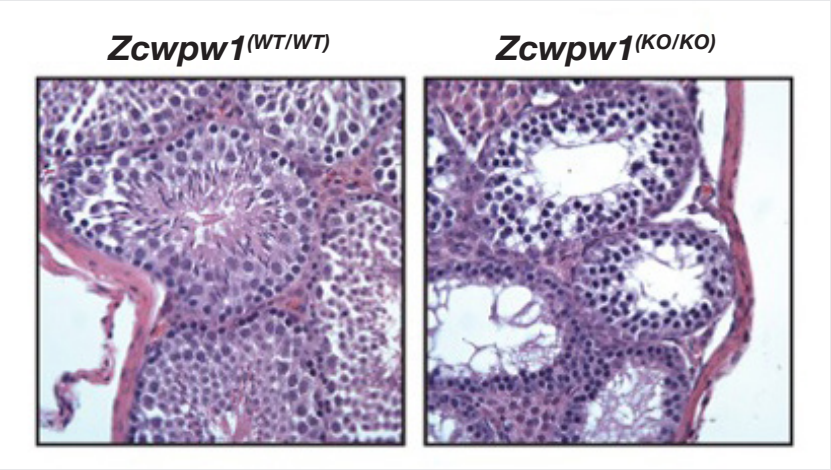

FIGURE 3. *Zcwpw1* KO mice are azoospermic.

H&E staining of adult testes from WT or *Zcwpw1* knockout (KO) mice. Sperm are completely lacking in *Zcwpw1* mutants due to defective meiotic double-strand-break repair.

ZF-DNA contacts, highlighting the ability of individual ZFs to change confirmation depending upon ZF context and DNA structure. 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.

We began a new exploration of the function and mechanism of PRDM9, the most ancient KRAB-ZFP, which emerged in jawless fish and plays a highly specialized role in meiotic recombination (MR). MR generates genetic diversity in sexually reproducing organisms and ensures proper synapsis and segregation of homologous chromosomes in gametes. Errors in MR that lead to mis-segregation of chromosomes are a leading cause of miscarriage and childhood disease. MR is initiated by programmed double-strand breaks (DSBs) in DNA that are distributed non-randomly at thousands of specific 1–2 kb regions called hotspots. In most mammals, hotspots are defined by PRDM9, a protein that contains a rapidly evolving DNA–binding ZF array and a specialized HMT activity that catalyzes dual trimethylation marks on histone H3 at lysine 4 and 36 (H3K4me3 and H3K36me3), whose activities are both required for hotspot specification. *Prdm9* loss-of-function causes sterility in mice, and PRDM9 mutations have been associated with male infertility in humans. In species lacking *Prdm9*, including yeast, plants, and birds, hotspots are located in H3K4me3–rich regions at gene promoters. Thus, the emergence of PRDM9 during evolution reshaped the MR landscape by relocating DSBs away from promoters to chromatin sites bound by the rapidly evolving PRDM9, which allowed for rapid interspecies hotspot diversification. We set out to address whether other factors, in addition to PRDM9, are required to 're-engineer' hotspot selection and how the DNA break and repair machinery is recruited to sites marked by PRDM9. We identified the dual histone methylation readers *Zcwpw1,* which co-evolved with and is tightly co-expressed with *Prdm9*. Using a mouse model, we found that ZCWPW1 is an essential meiotic recombination factor required for efficient repair of PRDM9–dependent DSBs and pairing homologous chromosomes in males mice. However, ZCWPW1 is not required for the initiation of DSBs at PRDM9 binding sites. Our results indicate that the evolution of a dual histone methylation writer (PRDM9) and reader (ZCWPW1) system in vertebrates remodeled genetic recombination hotspot selection from an ancestral static pattern near genes towards a flexible pattern controlled by the rapidly evolving DNA–binding activity of PRDM9.

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Virulence Mechanisms of Microbial Pathogens

Our main research goal is to define mechanisms by which pathogenic bacteria subvert the human host defense and cause disease. In parallel, we investigate whether these mechanisms may be manipulated for preventative and/or therapeutic purposes. 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 our 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 objective is to obtain detailed mechanistic insight into *L. pneumophila* effectors by investigating their biological role at molecular, cellular, and structural levels. Knowledge obtained from

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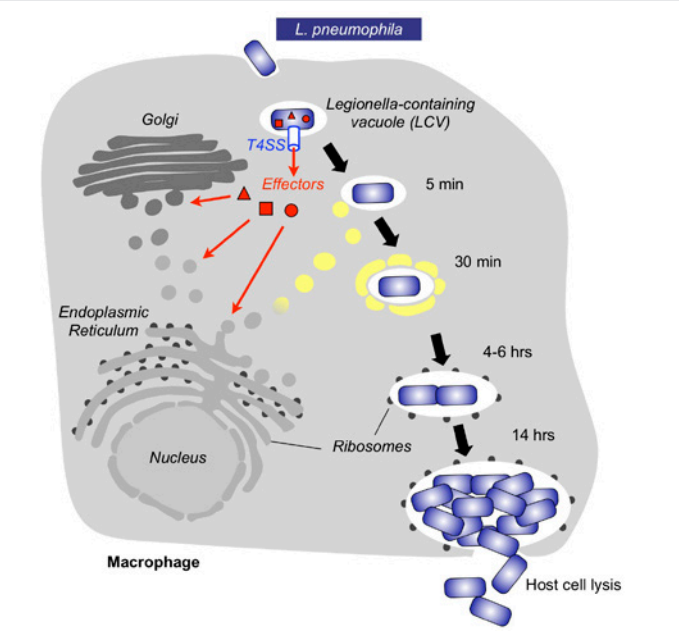


FIGURE 1. Intracellular replication cycle of *Legionella pneumophila*

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

these studies can help in the development of novel therapeutics aimed at treating or preventing Legionnaires' disease and related illnesses.

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

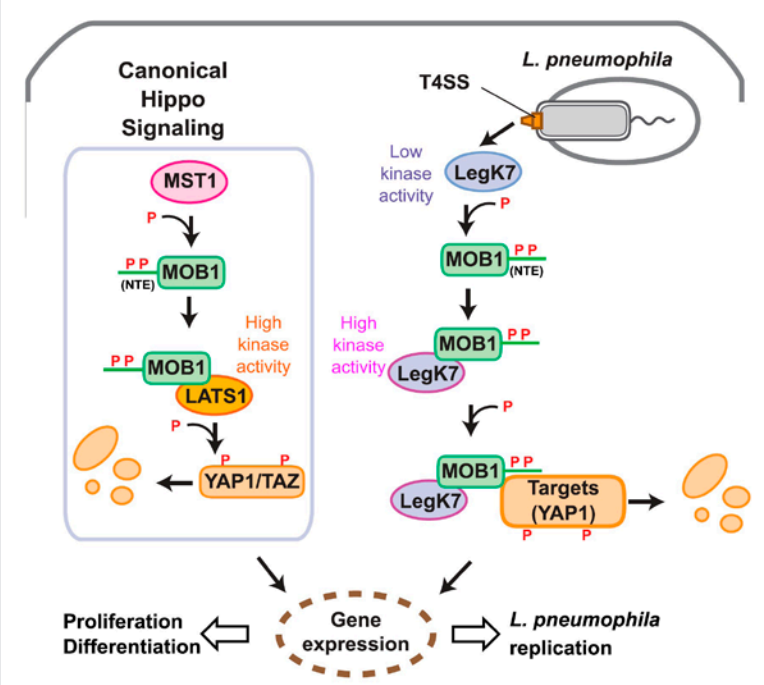
Microbial pathogens manipulate host-cell signaling pathways by encoding molecular mimics of protein kinases. Kinases catalyze target phosphorylation by attaching a phosphate group onto side chains of substrate proteins, preferentially serine, threonine, or tyrosine residues, thereby altering the activity, localization, or stability of their substrates. Protein phosphorylation is one of the most abundant and most important post-translational modifications in living cells, and mammals encode hundreds of kinases that target thousands of substrate proteins. To promote infection, many pathogens, including bacteria and eukaryotic parasites, encode effector kinases that are translocated into the host cytosol, where they alter signaling cascades, suggesting that kinase mimicry is a commonly used strategy to exploit host cell function.

Using structural prediction to identify cryptic catalytic domains within *L. pneumophila* effectors, we discovered that the protein LegK7 contains a central domain (residues 183–462) that has folding homology to eukaryotic protein kinases. Using a specific labeling technique, we experimentally confirmed that LegK7 indeed exhibits kinase activity *in vitro*. Upon probing a human protein microarray platform with LegK7, we identified MOB1 as a direct substrate of LegK7 (Figure 2). 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 molecularly mimicking the host Hippo kinase (MST1 in mammals), the key regulator of pathway activation. LegK7, like Hippo/MST1, phosphorylates the scaffolding protein MOB1 on two residues, which triggers a

FIGURE 2. *Legionella* LegK7 manipulates the host cell Hippo signaling pathway.

Cartoon comparing canonical Hippo signaling (*left*) with signaling by *Legionella* LegK7 (*right*). In the canonical Hippo pathway, the Hippo kinase MST1 phosphorylates the N-terminal extension (NTE) of MOB1A on two tyrosine residues, T12 and T35, to promote formation of the MOB1A–LATS1 kinase complex. The activated MOB1A–LATS1 kinase complex phosphorylates the transcriptional regulators YAP1/TAZ, which results in degradation of YAP1/TAZ and, consequently, expression of genes involved in cell proliferation or differentiation.

During infection, *L. pneumophila* translocates the effector kinase LegK7 into host cells. Like MST1, LegK7 directly phosphorylates T12 and T35 on the NTE of MOB1A. LegK7 then interacts directly with phosphorylated MOB1A and becomes highly active. Active LegK7 uses MOB1A to recruit downstream substrates, possibly YAP1, for further phosphorylation and manipulates the conserved Hippo pathway to promote infection.



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 permissive to *L. pneumophila* intracellular growth. Thus, a conserved *L. pneumophila* effector kinase exploits the Hippo pathway to promote bacterial growth and infection (Figure 2) [Reference 1].

Legionella LegK7 exploits the host scaffold protein MOB1A for allostery and substrate phosphorylation.

The finding that LegK7 targets MOB1A and manipulates its function points toward an unexpected role for the Hippo pathway in microbial pathogenesis. Given that LegK7 appears to functionally mimic MST1/2, it became important to more thoroughly investigate this host-pathogen interaction and to determine, at a molecular level, how LegK7 exploits a signaling pathway that is engrained within the developmental program of eukaryotic cells.

Using *in vitro* kinase reconstitution assays, we showed that MOB1A, in addition to being a substrate of LegK7, functions as an allosteric activator of LegK7's kinase activity. A crystallographic analysis of the LegK7–MOB1A complex revealed that the N-terminal half of LegK7 is structurally similar to eukaryotic protein kinases, and that MOB1A, like the mammalian kinase LATS1/2, directly binds to the LegK7 kinase domain (Figure 3). Substitution of interface residues critical for complex formation abrogated allosteric activation of LegK7 both *in vitro* and

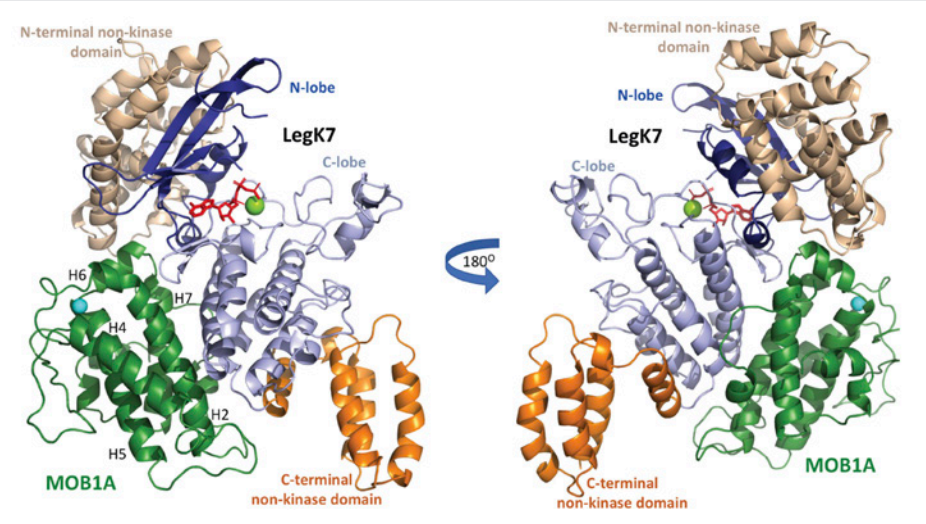


FIGURE 3. Crystal structure of the LegK7–MOB1A complex

Cartoon representation of the LegK7(11–530)–MOB1A33 complex. MOB1A33 (*green*) contacts predominantly the kinase domain of LegK7 with additional contacts to the N-terminal domain. Flanking N- (*wheat*) and C-terminal domains of LegK7 (*orange*); N lobe of the kinase domain (*blue*); C lobe (*slate*); adenosine monophosphate (AMP)-PNP (*red, in stick representation*); Mn^{2+} (*green sphere*).

within cells and diminished MOB1A phosphorylation. We also provided evidence that LegK7 forms a complex with MOB1A in order to use its N-terminal extension as a binding platform for the recruitment of downstream substrates, so that their phosphorylation can occur more efficiently.

Together, our findings suggest that LegK7 has evolved into a functional chimera of MST1/2 and LATS, and that it combines features from both of the core kinases of the Hippo pathway in order to exploit the downstream signaling cascade. We propose that, by using this strategy, *L. pneumophila* has developed the ability to survive within a wide range of diverse host species, including humans [Reference 2].

A multiplex CRISPR interference tool for virulence gene interrogation in an intracellular pathogen

Clustered, regularly interspaced short palindromic repeats (CRISPR)-Cas gene editing technologies have recently arisen as a mechanism for both fast and targeted gene manipulation in a variety of systems. In the simplest CRISPR-Cas system, Type II, only a single protein known as Cas9 is required for crRNA–guided DNA cleavage, making it the most developed genetic tool. In the absence of target cleavage, catalytically inactive dCas9 imposes transcriptional gene repression by sterically precluding RNA polymerase activity at a given gene to which it was directed by CRISPR (cr)RNAs. This gene-silencing technology, referred to as CRISPR interference (CRISPRi), has been employed in various bacterial species to interrogate genes, mostly individually or in pairs. To our knowledge, no group has fully exploited or probed the natural multiplex capability of CRISPR repeat/spacer arrays as a gene silencing tool in bacterial systems.

We thus developed a multiplex (MC) CRISPRi platform in the pathogen *L. pneumophila* capable of silencing up to ten genes simultaneously (Figure 4). We provided proof-of-concept that this novel platform can be used not only during growth in axenic media but also during macrophage infection, where it reproduced known intracellular growth phenotypes. Importantly, by placing the crRNA–encoding spacer in positions further downstream within the array, the degree of gene silencing was titratable. In contrast, when combined with a boxA element (a short DNA motif that antagonizes premature transcript termination in bacteria by recruiting

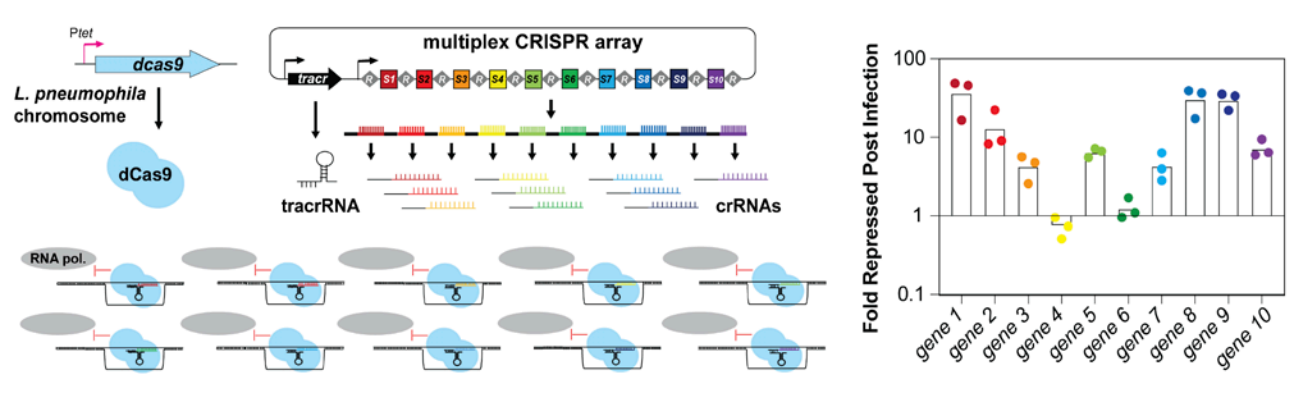


FIGURE 4. Concept of multiplex CRISPR interference (CRISPRi)

A series of repeats, R, and spacers, S1–S10, are expressed as a single precursor crRNA. Upon processing, individual crRNAs come together with a tracrRNA and dCas9 to simultaneously target ten unique genes for silencing. Fold repression of target genes 1–10 compared with a control strain was determined using qPCR.

the anti-termination factor Nus), our 10-plex CRISPR array had the potential to silence up to ten unique genes simultaneously (Figure 4), making it a powerful tool to study even synergistic genetic interactions [Reference 3].

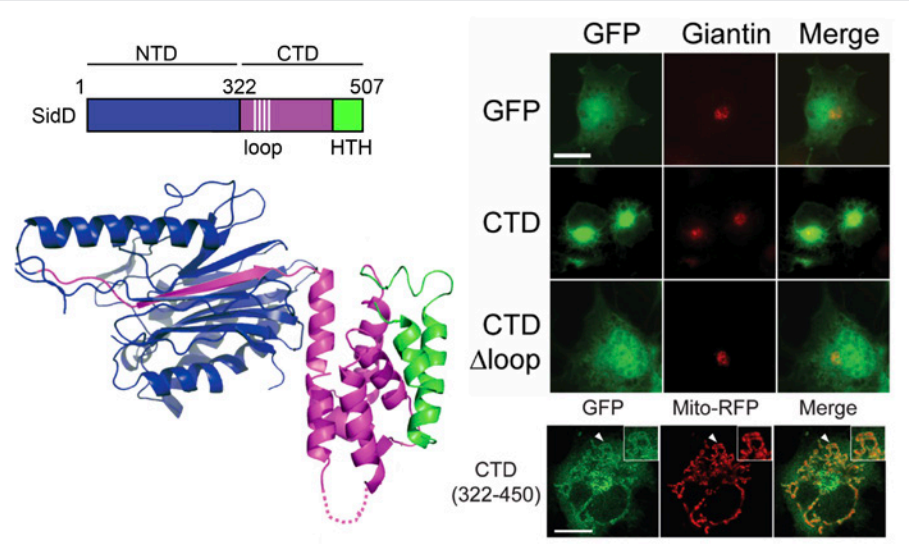
Looking forward, the multiplex CRISPRi approach we developed holds the promise of one day probing functional overlap amongst the hundreds of *L. pneumophila* effectors. Not only can genes be silenced in bulk groups during infection, but the mobility of our single plasmid–based CRISPRi platform allows for easy transfer of MC constructs into a variety of *Legionella* mutant-strain backgrounds to directly assess redundancy, presuming they have been equipped with a copy of dCas9.

Structural insight into the membrane-targeting domain of the *Legionella* deAMPylase SidD

AMPylation, the post-translational modification with adenosine monophosphate (AMP), is catalyzed by effector proteins from a variety of pathogens. *L. pneumophila* is thus far the only known pathogen that, in addition to encoding an AMPylase (SidM), also encodes a deAMPylase, called SidD, that reverses SidM–mediated AMPylation of the host-cell vesicle transport GTPase Rab1; deAMPylation is catalyzed by the N-terminal phosphatase-like domain of SidD. The structure of the C-terminal domain and its biological role had remained unclear.

We determined the crystal structure of full length SidD, including the previously uncharacterized C-terminal domain (Figure 5). We found that the C-terminal domain assumed a novel fold not yet described in other proteins, which is critical for membrane localization of SidD within host cells. Notably, the localization depended on two structural elements within the C-terminal domain: a flexible loop composed of hydrophobic amino acid residues, which represents a general membrane-targeting determinant; and a two-helix bundle at the C-terminal end that functions as the specificity determinant. Deletion of either of these elements interfered with the correct localization of SidD within mammalian cells. Moreover, we found that, during growth in mouse macrophages, a *L. pneumophila* strain producing SidD without the loop phenocopied a *L. pneumophila* strain producing no SidD at all, thus revealing that membrane targeting of SidD via its C-terminal domain is a critical

FIGURE 5. SidD membrane localization requires the C-terminal domain.



A. Domain organization of SidD with the N-terminal domain (NTD, *blue*) and C-terminal domain (CTD, *magenta*). The hydrophobic loop (*dashed line*) is unstructured, indicative of intrinsic flexibility. Numbers indicate amino acid positions.

B. Intracellular localization of CTD variants. Transiently transfected COS-1 cells producing either GFP (control) or GFP-CTD variants were fixed and stained using an antibody directed against the Golgi marker protein giantin (*middle*). Merged images of SidD proteins (*green*) and giantin (*red*). Scale bar: 10 μm.

C. CTD(322–450) localizes to mitochondria membranes. Transiently transfected COS-1 cells coproducing GFP-CTD(322–450) and Mito-RFP (a mitochondria marker) were chemically fixed, and the fluorescence signal was examined by confocal microscopy. Arrowheads indicate the position of membranes magnified in the insets. Scale bar: 10 μm.

prerequisite for its ability to catalyze Rab1 deAMPylation during *L. pneumophila* infection. Future studies will reveal whether additional effectors from *L. pneumophila* or related pathogens use a similar membrane-targeting strategy within host cells [Reference 4].

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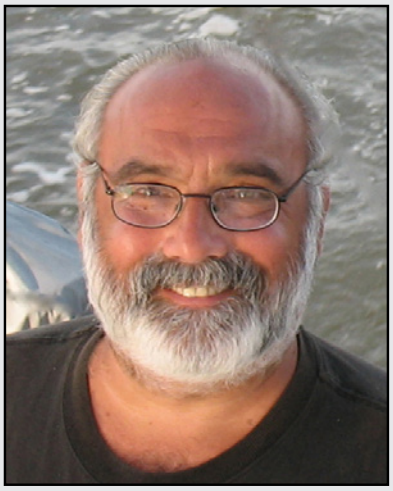
RNA Metabolism in Cell Biology, Growth, and Development

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. tRNAs are produced at over 10-fold higher molar levels than are ribosomes during cellular proliferation. After synthesis by RNA polymerase III (RNAP III, also known as Pol III), post-transcriptional tRNA processing steps and modifications occur prior to nuclear export and additional modifications. Failure to support programmed mRNA translation is evident by developmental and other diseases attributable to defective tRNA biogenesis.

RNAP III synthesizes high levels of tRNA by a conserved process of transcription termination–associated reinitiation, relevant to development and cancer. Termination occurs within a short tract of T residues in the non-template (NT) DNA strand at the ends of RNAP III–transcribed genes. The nascent transcripts bear a copy of this terminator, (U)UU-3'OH, a recognition motif for the nuclear La protein, which binds in a sequence- and length-dependent manner. Notably, 3'U(n) length-dependence of La binding was found to be one residue shorter than the minimal T-length required for efficient RNAP III termination, the latter of which is 6, 5, and 4 Ts for *S. cerevisiae*, *S. pombe,* and human, respectively. This suggests a La link with tRNA expression, a link that fits with human (h)RNAP III that evolved a minimal 4T termination mechanism, and data suggest that this may direct some post-transcriptional events. Studies on development, structure, and its gene variants indicate that hRNAP III evolved beyond housekeeping via tRNA expression, to more intricate control of gene-regulatory programs.

The human La protein is a target of auto-antibodies in patients with chronic inflammatory disease such as systemic lupus erythematosus, Sjögren's syndrome (SS) (La is also known as SS antigen-B, SSB), and neonatal lupus. Although La is active in other immune pathways, evidence of its primary involvement in autoimmunity is lacking. As noted above, La binds to RNAP III transcripts in all cells and serves as a chaperone during their nuclear maturation. La not only protects pre-tRNAs from 3' exonucleases, it directs and temporally orders the first step in the 5' processing by RNase P pathway. We propose a role for La



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in a novel pathway, different from tRNA maturation. Our data suggest that La is a determinant for a subset of pre-tRNAs (genes) to enter an alternative pathway to specific activation of the type-I interferon (IFN) response. Accordingly, some dual-activity tRNA genes may serve as endogenous immune adjuvants or may contribute to autoimmunity. We are currently exploring these possibilities.

We also work on La-related proteins (LARP) 1 and 4, which directly bind to 3' poly(A) and poly(A)–binding protein (PABP) to modulate mRNA stability. LARP4 mRNA itself bears a coding determinant that can sense tRNA levels, with potential for translation homeostasis. A larger theme is that translational fidelity via tRNAs, to meet mRNA demands, is a key element of health and disease. Our goal is to develop knowledge and potential for translational research.

A VIEW OF TRNA SYNTHESIS, PROCESSING, MODIFICATION, AND MRNA ACTIVITY

A major part of our work centers on processes involved in tRNA biogenesis and metabolism and on their function in translation. The rate of RNA production by RNAP III was shown to affect the efficiency by which pre-tRNAs acquire the common $m^{2,2}G26$ modification, which increases tRNA activity. Disrupted tRNA biogenesis, attributable to gene variants, leads to developmental and other diseases, and to neurodegeneration and intellectual impairment. About 120 modifications occur on tRNAs, of which about 40 have been documented for human cytoplasmic (cy-) tRNAs and several others for human mitochondrial (mt-) tRNAs. Many tRNA–modification enzymes (TME) are multisubunit, and some composite modifications require several gene products. Thus, mutations in numerous TMEs cause disease.

Critical modifications to tRNA anticodon loops fine-tune base pairing for optimal decoding and wobble decoding of synonymous codons, which is an important component of a tunable system of high-fidelity cy-translation. It is important to note that the 61 sense codons for 20 amino acids (aa) are decoded by cy-tRNAs, which collectively carry only about 45 anticodons, a feature of tRNA genes, named anticodon-sparing, that is widely conserved, although the number and identity of the 'missing' anticodons differ among kingdoms, anti-correlated with wobble-base modifications. In humans, about 15 standard codons must be wobble-decoded by cy-tRNAs whose activities are controlled by anticodon modifications. Thus, RNAP III transcription of tRNA genes is only one level on which the translation of a range of cy-mRNAs with biased codon content can be regulated. While this constitutes a nuclear-cytoplasmic translation system with much potential for intricate regulation, the complexities of overlapping/redundant decoding activities also create potential for translation infidelity in cases of faulty modification or tRNA pool imbalance.

All TMEs are nuclear-encoded, and a modest subset modify cy- and mt-tRNAs, with potential to synchronize translation in both compartments. Distinct decoding rules apply in mitochondria, in part because each mt-DNA encodes only one tRNA for each of 18 amino acids. Synonymous codons occupy mt-mRNAs but are not distinguished by different tRNAs, as is the case for cy-mRNAs. For example, while four codons each for Ala, Gly, Pro, Thr, and Val are decoded by three cy-tRNAs, a single mt-tRNA for each must wobble-decode its four cognate codons. Furthermore, these mt-tRNAs use unmodified U34 as their wobble base, whereas cy-tRNA wobble bases are part of an elaborate modification system, especially for U34. Each mt-tRNA is critical; point mutations impair mt-translation with unique association with oxidative phosphorylation diseases, whereas most nuclear tRNA genes are buffered by multiple copies; a known exception is a unique, single-copy, brain-specific tRNA. Yet, some diseases caused by a mutation in a mt-tRNA exhibit a phenotype similar to that resulting from a nuclear gene mutation to a TME whose substrate is the mt-tRNA.

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

Red-white colony differentiation by tRNA–mediated suppression

Mechanisms linking tRNA homeostasis and neurologic disease may vary. One model is that nerve tissue is highly susceptible to low-fidelity translation, leading to protein aggregation and altered proteostasis, as observed with TME deficiencies (also in yeast). We showed that La deletion from mouse brain soon after birth perturbed tRNA processing and initiated inflammation and neurodegeneration. La is a general pre-tRNA factor that acts redundantly with TMEs, and thus tRNA homeostasis is likely to be disrupted by its deficiency or dysfunction.

Numerous pathogenic alleles that encode defective subunits of RNAP III cause a type of leukodystrophy known as hypomyelinating leukodystrophy (HLD), whose pathobiology is consistent with poor formation of axonal myelin sheaths rather than demyelination. While RNAP III produces several ncRNAs (non-coding RNAs) other than tRNAs, HLDs and disorders of RNAP III–associated factors are considered tRNA-opathies that disrupt translation. We proposed models in which tRNA imbalances may alter cell type–specific mRNA translation profiles. Even subtle tRNA imbalances applied across mRNA populations may challenge proper translation in the developing CNS. Changes in the rate of RNAP III output can not only alter relative tRNA levels but also their modification status in a way that can differentially impact their decoding activities. Curiously, codon use by brain-specific genes is more conserved than by other tissue-specific genes, and function-related genes are intolerant of variation in synonymous codons. Other models suggest that deficiency in brain-specific RNAP III transcripts as well as tRNAs contribute to hypomyelinating leukodystrophy and other CNS–specific phenotypes.

We suggest that another disruption mechanism to a tRNA biogenesis–CNS network is also possible. As TME defects often manifest as neurological, reflecting tissue reliance on mitochondria, and because cy- and mt-translation are synchronized including by tRNA modifications and other factors, disruption of general tRNA homeostasis may impair mt-function with pathophysiologic contribution to disease, including in developing oligodendrocytes.

mRNA levels are determined in significant part by levels of cognate tRNAs, from yeast to human. In addition, certain mRNAs are particularly sensitive to tRNA levels. The LARP4 mRNA has a tract of about 70 codons with a very poor match to cellular tRNA levels. Thus, unprogrammed changes in tRNA output (e.g., genetic mutation in RNAP III) could shift mRNA stabilities and translation efficiencies in unpredictable ways with uncertain outcomes.

Translation of LARP4 mRNA produces LARP4, which binds to poly(A) and also to poly(A)–binding protein (PABP/PABPC1). LARP4 stabilizes mRNAs by opposing deadenylation of their poly(A) tails, substrates of Ccr4-Not deadenylase (a multiprotein complex that functions in gene expression in the nucleus, where it regulates transcription, and in the cytoplasm, where it associates with translating ribosomes and RNA processing bodies). Potential for regulation is that Ccr4-Not monitors mRNA–ribosomes for codon–tRNA match. For ribosomal

protein–encoding mRNAs stabilized by LARP4, this supports a working model in which LARP4 mRNA senses tRNA levels and relays this by producing LARP4 to regulate ribosome biogenesis, perhaps with LARP1.

Human RNAP III and associated factors have been known to be dysregulated in cancer. More recently, functionally related mRNAs favoring proliferative or differentiation states were shown to be biased in synonymous codons and in the cognate tRNAs differentially expressed in those cells. Strikingly, the first hRNAP III structures provide insight into activities specific to the higher eukaryotic 17–subunit complex. First, the cancer-associated RPC7a subunit paralog (encoded by *POLR3G*) appeared to interfere with binding of the RNAP III–negative regulator and tumor suppressor MAF1, whereas the RPC7b (POLR3GL) paralog subunit is enriched in cells programmed for differentiation (limited proliferation). Yeast RNAP III has only one homolog. The second example involves the most striking feature of higher eukaryote–specific RNAP III, the multi-domain expansion of RPC5 and hypothesized associated higher eukaryote promoter–type specificity, and its link to termination-reinitiation recycling.

MECHANISTIC CONTROL OF NCRNA GENES THAT APPEAR TO HAVE EVOLVED ACTIVITIES THAT EXTEND HRNAP III BEYOND THE HOUSEKEEPING ACTIVITIES OF ITS YEAST COUNTERPART

This fits a view of hRNAP III in self vs. non-self surveillance functions. Notable are RNAP III Vault (Vt) ncRNAs with involvement in two activity types, in innate immune surveillance, and differentiation vs. maintenance of undifferentiated states. Both RNAP III Vt and snaR (small nuclear factor 90–associated RNA) ncRNAs are processed to miRNAs that exert downstream effects on mRNA profiles and/or differentiation/cancer. Our proposal includes the study of a dual-activity tRNA gene(s) for which La is a key determinant of whether the nascent 4T–terminated transcripts are directed to tRNA maturation or to an alternate pathway of innate immune activation.

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

The RNAP III multisubunit 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 (promoter elements of tRNA genes) and recruits TFIIIB to direct RNAP III to the correct start site. TFIIIB–RNAP III complexes appear highly stable and demonstrate great productivity in supporting the many cycles of initiation, termination, and re-initiation necessary to produce the more than 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 re-initiation by RNAP III are functionally, if not physically, linked. Our laboratory 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 (HLD), 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 non-template strands of the RNAP III terminator carry distinct signals for different stages of termination. High-density *cis* information

is a feature of the RNAP III system that is also reflected 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 non-template strand signal. Our results reveal the RNAP III terminator to be an information-rich control element. While the template strand promotes destabilization via a weak oligo(rU:dA) hybrid, the non-template strand provides distinct sequence-specific destabilizing information through interactions with the C37 subunit.

Control of the differential abundance or activity of tRNAs can be an important determinant of gene regulation. RNAP III synthesizes all tRNAs in eukaryotes, and its derepression is associated with cancer. MAF1 is a conserved general repressor of RNAP III under the control of TOR (target of rapamycin), which acts to integrate transcriptional output and protein-synthetic demand toward metabolic economy. We used tRNA–Hydro-Seq (hydrolysis-based tRNA sequencing) to document that little change occurred in the relative levels of different tRNAs in *maf1* mutated cells. By contrast, the efficiency of *N*2,*N*2-dimethyl G26 ($m^{2,2}$G26) modification on certain tRNAs was reduced in response to *maf1* deletion and associated with anti-suppression, which we validated by other methods. Overexpression of Trm1 (tRNA dimethyl transferase), which produces $m^{2,2}$G26, reversed *maf1* anti-suppression. The model that emerges is that competition by elevated tRNA levels in *maf1-delta* cells leads to $m^{2,2}$G26 hypo-modification resulting from limiting Trm1, thus reducing the activity of suppressor tRNASerUCA (UCA is the anticodon for serine) and accounting for anti-suppression. Consistent with this, RNAP III mutations associated with HLD reduce tRNA transcription, increase $m^{2,2}$G26 efficiency, and reverse anti-suppression. Extending this more broadly, a reduction in tRNA synthesis by treatment with rapamycin leads to increased $m^{2,2}$G26 modification, a response that is conserved among highly divergent yeasts and human cells [Arimbasseri AG *et al*., *PLoS Genetics* 2015;11:e1005671].

The ability of RNAP III to efficiently recycle from termination to re-initiation 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.

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. LARP4 emerged later in evolution, and we found it to be an mRNA–associated cytoplasmic factor associated with poly(A)–binding protein C1 (PABPC1, PABP). LARP4 uses two regions to bind to PABPC1. We showed that the N-terminal domain (NTD, amino acids 1–286) of LARP4, consisting of an N-terminal region (NTR, amino acids 1–111) followed by two tandem RNA–binding motifs known as an 'La module' (111–285), exhibits preferential binding to poly(A). The NTR contains a unique PAM2w motif that binds to the MLLE (a peptide-binding domain) of PABP. The group of our collaborator Maria

Conte showed that the N-terminal region (NTR) itself is responsible for most of the poly(A) binding and that, moreover, this involves conserved residues unique to the PAM2w of LARP4. The La module is flanked by a different motif on each side, each independently interacting 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 (TNFα); 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. Working with researchers in the NICHD Molecular Genomics Core facility, we developed a single-molecule, high-throughput nucleotide-resolution poly(A)–tail sequencing method referred to as SM-PAT-Seq, which yielded insights into LARP4 function and mechanism. LARP4 is a global factor involved in mRNA poly(A) length homeostasis and appears to effect mRNA stabilization by opposing the action of deadenylases when poly(A) tails are short.

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 the 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 *S. pombe*, the human La protein can replace the tRNA–processing/maturation function of Sla1p, the *S. pombe* equivalent of the La protein. Moreover, in *S. pombe*, 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 the system to study transcription by RNAP III, post-transcriptional processing, and tRNA modifications by conserved enzymes that produce tRNA isopentenyl-adenosine-37 and dimethyl-guanosine-26 (*N*2,*N*2-dimethyl G26, $m^{2,2}G26$).

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

A major interest of ours is to decipher 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, which 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], which may be important because ribosome production is regulated during growth and development, and because the potential circuit involving LARP4 control by tRNA could be an important point of control.

Publications

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Immune Activation and Viral Pathogenesis

The general goal of the Section for Intercellular Interactions is to understand the tissue-pathogenic mechanisms of human pathogens and the role of extracellular vesicles in such processes. We focused on the pathogenesis of the human immunodeficiency virus 1 (HIV-1) and on its co-pathogens. We found that extracellular vesicles (EVs) facilitate residual immune activation of HIV–infected human lymphoid tissue after viral replication is suppressed by antiviral therapy. In contrast, EVs released by vaginal microbiota bind to HIV-1 virions, preventing viral transmission. Thus, EVs play an important role in HIV infection and may serve a new target for therapy. Also, we also continue a project on SARS-CoV-2 pathogenesis.

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Extracellular vesicles support residual HIV–triggered immune activation after HIV suppression.

Improper immune activation accompanies many human pathologies, including mental diseases, abnormal pregnancies, and HIV disease. In particular, immune activation is considered to be the driving force of HIV-1 disease, resulting in premature age-related diseases, such as cardiovascular disease or dementia. HIV–triggered immune activation continues for years, even when HIV-1 replication has been successfully suppressed by antiviral therapy (ART). Neither the causes nor the mechanisms of persistent systemic immune activation in HIV-1–infected patients under antiretroviral therapy are known. However, the residual immune activation is a major contributor to the early development of several pathologies in such patients. We addressed this problem in an *ex vivo* system of human lymphoid tissues that faithfully reflects many aspects of pathogenesis of various viruses *in vivo*. The goal of our work was to investigate the mechanisms of sustained immune activation in this system. Upon HIV infection, the *ex vivo* tissues become immuno-stimulated, faithfully reflecting the *in vivo* infection. We treated the infected tissues with several of the antivirals used for patients, in particular the protease inhibitor ritonavir (RTV) and the nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine (AZT), in combination with lamivudine (3TC) and nevirapine (NVP, nonNRTI). All these compounds completely suppressed HIV replication. However, despite viral suppression, the tissues remained immune-activated, again reflecting the *in vivo* situation with ART–

treated HIV–infected individuals. To understand the mechanisms underlying this phenomenon, we tested the following working hypotheses in this system.

1. Immune activation is the result of the pro-inflammatory effects of certain antiretroviral drugs themselves. To test this, we compared cytokines released by *ex vivo* human lymphoid tissues under ART with those released by donor-matched untreated controls. However, ART did not lead to a significant increase in cytokines throughout the culture period.
2. It is conceivable that an HIV–triggered upregulation of cytokines could stimulate immune cells to produce a long-term cascade of other cytokines, even after HIV-1 is suppressed. To investigate this possibility, we simulated this situation by treating uninfected *ex vivo* lymphoid tissues with a cocktail of cytokines that are upregulated in HIV-1 infection. However, tissue exposure to an exogenous cytokine combination did not result in a significant and sustained increase of cytokines.
3. HIV-1 infection reactivates endogenous viruses, in particular human herpes viruses (HHVs), which may continue to replicate and induce immune activation after HIV is suppressed. To test this hypothesis, we quantified, by droplet digital PCR, HHVs 2–7 in tissues infected with HIV-1 and treated with ART. The only HHV that was upregulated upon HIV infection was cytomegalovirus. However, the upregulation was no longer significant after ART was applied.
4. HIV-1 proteins that continue to be released, in spite of ART, activate the immune system. To test this hypothesis we treated *ex vivo* tissues with gp120, Tat, or Nef at a concentration that is comparable to that present in the viral inoculum used in our experiments. However, none of these tested HIV–encoded proteins induced any significant sustained cytokine upregulation.

Thus, our results did not support any of the four hypotheses. We therefore went on to propose that defective HIV-1 virions or extracellular vesicles carrying HIV components may trigger immune activation. We treated HIV-1 with aldrathiol-2 (AT-2), a compound that inactivates the virus but preserves virion morphology and its ability to interact with cell receptors. We inoculated tissues with AT-2–inactivated virions in the same amount as infectious virus. Repeated AT-2 HIV-1 exposure of tissues significantly upregulated cytokines. Even a single exposure of tissue to AT-2–inactivated HIV triggered sustained, significant immune activation.

We also applied EVs isolated from tonsil supernatants to the tissues. We found that EVs from tissues productively infected with HIV-1, and also from tissues in which HIV-1 replication was suppressed by ART, induced significant, sustained upregulation of cytokines. EVs isolated from control uninfected tissues or from uninfected tissues treated with ART did not produce a significantly sustained increase in cytokines compared with control untreated tissues.

In conclusion, the *ex vivo* lymphoid tissue system allowed the investigation, under laboratory-controlled conditions, of possible mechanisms involved in persistent immune activation in HIV-1 patients under ART. Our results with lymphoid tissue indicate that the mechanisms of sustained immune activation in these patients may include the presence of defective (replication-incompetent) virions and of EVs. These elements constitute potential therapeutic targets to combat the progression of various pathologies in HIV–infected individuals after HIV-1 itself has been successfully suppressed.

Vaginal bacteria–released extracellular vesicles in HIV infection

The vaginal microbiota of healthy reproductive-age women is generally dominated by *Lactobacillus* species. *Lactobacilli* are health-promoting microorganisms as they are involved in maintaining vaginal homeostasis by

preventing overgrowth of pathogenic and opportunistic organisms. In particular, *Lactobacilli* have been reported to protect against vaginal transmission of HIV, although the mechanisms of protection remain unclear. Earlier, we established an *ex vivo* system of human cervico-vaginal tissue culture that recapitulates the features of HIV transmission and found in the system that several strains of *Lactobacillus* inhibited HIV-1 transmission.

We continued this work by investigating the mechanisms of this phenomenon, in particular the role of extracellular vesicles (EVs). EVs are released by both gram-negative and gram-positive bacteria. Like mammalian cells, bacterial EVs contain components from their mother cells and, despite a huge difference between bacterial and mammalian cells in size, structure, metabolism, and general physiology, the EVs that bacteria release are essentially of the same size. They carry diverse bioactive molecules, including proteins, nucleic acids, lipids, and metabolites. Although once thought to be useless cell debris, it is now clear that bacterial EVs are major players in important aspects of bacterial virulence, host immunomodulation, communication with other cells, survival, and other phenomena. Bacterial EVs have been implicated in bacteria-bacteria and bacteria-host interactions, promoting health or causing various pathologies.

After demonstrating for the first time that vaginal *Lactobacilli* isolated from the vaginas of healthy women (*L. crispatus*, *L. gasseri*) released nano-sized EVs similar to those released by *Lactobacillus* strains of gastrointestinal origin, such as *L. casei*, *L. rhamnosus*, *L. reuteri*, and *L. plantarum*, we investigated whether *Lactobacillus*-derived EVs are capable of inhibiting HIV-1 infection. Lymphocytic cell lines as well as human cervico-vaginal and tonsillar tissues *ex vivo* were infected with HIV-1 and treated with EVs from four different strains of *Lactobacilli* isolated from the vagina of healthy women. The choice of these *Lactobacillus* strains (*L. crispatus* BC3, *L. crispatus* BC5, *L. gasseri* BC12, and *L. gasseri* BC13) was based on our previous report on the anti-HIV-1 activity of these bacteria in human tissues *ex vivo*.

We found that EVs released by *L. crispatus* BC3 and *L. gasseri* BC12 largely protected human tissues *ex vivo* and T cells from HIV-1 infection. The HIV-1–inhibitory effects of EVs from *L. crispatus* BC3 or *L. gasseri* BC12 were dose-dependent. At the highest concentration used in our study there were about 1000 EVs per HIV-1 target cell. At this concentration, EVs were not cytotoxic, as evaluated with three different techniques (propidium-iodide-based assay, flow cytometry, and the MTT assay, which assesses cell viability). Thus, inhibition of HIV-1 infection was not the result of EV–induced cell death.

Not all *Lactobacilli*-released EVs inhibited HIV-1 infection: EVs from *L. crispatus* BC5 or *L. gasseri* BC13 did not. The inhibitory activity of EVs from *L. crispatus* BC3 and *L. gasseri* BC12 was therefore related to their composition, as the same number of EVs were used for all strains. Proteomic analysis showed that EVs that inhibited HIV-1 replication differ from those that did not, in terms of several proteins, namely enolase 2, 60 kDa chaperonin, elongation factor Tu, ATP synthase gamma chain, foldase protein PrsA 1, ATP synthase subunit delta, and triosephosphate isomerase. We identified several bioactive molecules in HIV-1–inhibiting EVs, in particular several enolases derived from *Lactobacillus* that were shown to inhibit the adherence of *Neisseria gonorrhoeae* to epithelial cells; also, bifidobacterial enolase, a cell-surface receptor for human plasminogen, was involved in the interaction with human host cells; the elongation factor Tu was shown to play an important role in the attachment of *Lactobacillus johnsonii* to human intestinal cells and mucins.

Whether the inhibition of HIV-1 entry is the result of the action of one or of a combination of several of these bioactive molecules acting synergistically remains an open question. Also, they may act only when associated

with EVs. For these reasons, we next tested the effect of EVs as a whole in a cellular model of HIV-1 entry using TZM-bl cells. TZM-bl cells contain integrated reporter genes for firefly luciferase and *E. coli* β–galactosidase under the control of an HIV-1 long-terminal repeat, permitting sensitive and accurate measurements of infection at the entry/attachment level. We showed that viral attachment/entry to TZM-bl cells was inversely proportional to the concentration of bacterial EVs. Another set of experiments performed in the T cell line MT-4 confirmed that EVs directly inhibit HIV-1 attachment to cells. Thus, the anti–HIV-1 effect of *Lactobacillus*-derived EVs is mediated by the reduction in viral entry/attachment to the target cells, which appears to be related to direct alteration of HIV-1 virions by bacterial EVs. We found that virions pretreated with EVs released by *L. crispatus* BC3 and *L. gasseri* BC12, but not by *L. crispatus* BC5 or *L. gasseri* BC13, were no longer recognized by PG9, an antibody that specifically binds to functional trimeric gp120. Bacterial EVs from *L. crispatus* BC3 and *L. gasseri* BC12 interfere with the accessibility of viral Env, thus explaining the HIV-1 inhibition observed in cell lines and in human tissues *ex vivo*.

In summary, pretreatment of cells with bacterial EVs did not affect HIV-1 infection. However one treats cells with EVs and remove the vesicles, cells are as ineffaceable by the intact virus, as are control cells, i.e., viruses become defective and do not infect cells. Thus, bacterial EV–mediated HIV-1 inhibition is the consequence of EVs affecting the infectivity of virions rather than cell functions. In other words, of two participants of the infection, cells and viruses, EVs affect the latter. If confirmed *in vivo*, the finding may lead to new strategies to prevent male-to-female sexual HIV-1 transmission, for example by use of EVs derived from symbiotic bacteria.

SARS CoV–2 pathogenesis *ex vivo*

We continued our project on SARS-CoV-2 approved by the NIH Committee.

Investigating the mechanisms of SARS-CoV-2 tissue pathogenesis *in vivo* requires the development of an adequate system of human tissue culture under laboratory-controlled conditions. We developed such a system. Specifically, blocks of human lung tissue are cultured at the air-liquid interface on collagen sponges, and their cytoarchitecture is maintained for 14 days. Flow cytometry of cells from these blocks confirmed the viability of several cell types, including macrophages and other leukocytes, endothelial cells, and epithelial cells, and it also confirmed that the expression of the ACE-2 receptor, which the virus uses to invade a cell, is maintained. Histology revealed well preserved structural elements. Inoculation of these blocks with SARS-CoV-2 resulted in sustained viral replication and viral release into the culture medium. Flow cytometry identified infected cells, a finding that will be confirmed by immunohistochemistry. Analysis of culture medium from SARS-CoV-2–infected lung explants by multiplexed bead-based assays reveals that many cytokines are upregulated upon infection. The majority of the up-regulated cytokines are the same as those up-regulated *in vivo.*

Also, we used retroviral-based SARS-CoV-2 pseudoviruses with GFP and RFP protein reporters to study SARS-CoV-2 viral entry into target cells. Pseudoviruses were designed to express the SARS-CoV-2 spike (S) protein as well as other viral structural proteins, such as nucleocapsid (N), envelope (E), and membrane (M), in different combinations. Such one-cycle viruses infected 293T cell lines expressing ACE-2, and entry could be inhibited with neutralizing antibodies against the S protein. We found that M, N, and E proteins did not significantly affect the ability of the viruses to enter cells. In contrast, mutations in S protein that were identified *in vivo* changed the efficiency of pseudoviruses to enter cells. The United Kingdom B.1.1.7 variant infected 2.6 times more cells than pseudoviruses with wild-type S protein, and the South African variant B.1.351 infected 1.6 times more cells. Competition experiments between the pseudovariants is in progress and will reveal more

information about how mutations in the S protein can affect entry in the setting of mixed viral populations.

To address pseudovirus binding to ACE-2 and to anti-S antibodies, we also designed a cell-free system. Using the magnetic nanoparticle (MNP) system that we had developed for analysis of HIV virions, we bound ACE2-Fc (Fc domain of human IgG linked to ACE-2) or anti-S antibodies to MNPs and evaluated the ability of pseudoviruses with S mutations to bind. The studies provide an alternate method of evaluating S mutations.

Furthermore, we demonstrated that primary human trophoblasts, isolated from placenta and shown to express ACE-2 and TMPRSS2, are susceptible to entry of SARS-CoV-2 pseudoviruses. We showed that the virus is able to enter the trophoblasts, as measured by presence of p24 retrovirus core protein inside the cells. However, the GFP reporter is rarely seen in these cells, indicating that restriction factors present in the trophoblasts prevent SARS-CoV-2 infection, implying that the placental anti-viral defense against SARS-CoV-2 likely involves post-entry processing.

In summary, all three systems, SARS-CoV-2–infected lung tissue *ex vivo*, pseudovirus cell infection of cells, and virus binding to nanoparticles-coupled SARS-CoV-2 cell receptors, can be used to study different aspects of viral pathogenesis and can be transformed into a platform to evaluate the efficiency of viral entry with S protein mutations, as well as for testing potential antivirals.

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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 to apply this knowledge to patient treatment. We identified several key genes causing recessive and X-linked OI. Discoveries of defects in collagen modification 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, post-translational modification, or processing cause recessive OI. Our challenge now is to understand the cellular and biochemical mechanisms of recessive OI. We 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, the skeletal matrix of OI, and the effects of pharmacological therapies. Our clinical studies involve both children with the more prevalent types III and IV OI, as well as those with the rare recessive forms, 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 radiographic patterns of either 'dripping candle wax' or endosteal bone overgrowth. We recently identified mosaic mutations in the oncogene *MAP2K1* as the cause of 'dripping candle wax' melorheostosis and somatic mutations in *SMAD3* as the cause of endosteal melorheostosis. In each gene, the causative mutations occur at a hot spot and result in gain of function. 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 lack serum PEDF and have elevated alkaline phosphatase (ALPL)

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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 [Reference 1]. 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 the encoded protein BRIL (an osteoblast-specific, mineralization-modifying, IFITM–like membrane 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 occur in connected cellular pathways that affect 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 in components of the collagen P3H (prolyl 3-hydroxylase) complex causes recessive OI [References 1,2]. For type VIII OI, we investigated bone and osteoblasts, demonstrating that P3H1 is the unique enzyme responsible for collagen 3-hydroxylation. Bone histomorphometry revealed patches of increased osteoid. Quantitative backscattered electron imaging (qBEI) showed increased mineralization of cortical and trabecular bone, as in other OI types. We also generated a murine model in which the P986 site was substituted with Ala and could not be hydroxylated. Such mice showed only defects in cross-linking but not the symptoms of recessive OI [Reference 2], demonstrating a role for the substrate modification in bone matrix integrity, while a non-functional complex led to development of full recessive OI symptoms.

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 (1). 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 in 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 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 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 [Reference 1]. 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, which enter the nucleus and activate gene transcription. Mutant S2P protein is stable but has impaired RIP cleavage of the transcription factors 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 [Lindahl et al., *Hum Mutat* 2011;32:598]. 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 development, we developed a knock-in murine model with a *COL1A1* (the gene encoding pro-alpha1 type I collagen chain) cleavage site mutation. Bone collagen fibrils showed a 'barbed-wire' appearance consistent with the presence of the processing intermediate pC-collagen, which 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 their femora show significantly higher mineralization than in wild-type (WT) mice, which continues to increase in the high bone-mass mice (HBM) even after in the WT mice mineralization plateaus at 6 months. PINP and TRAP, serum markers of bone remodeling, are significantly elevated in such HBM. Osteocyte density is reduced but the 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 are present in about 6% of OI patients. Our investigation of the biochemical consequences of C-propeptide mutations in comparison with collagen helical mutations revealed both intra- and extracellular differences. Immunofluorescence microscopy indicated that procollagen with C-propeptide defects was mis-localized to the ER lumen, in contrast to the ER–membrane localization of normal procollagen and to helical mutations. Furthermore, 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 in cleavage assays with purified C-proteinase, consequences that 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 type IV OI features. Brtl has provided important insights into the mechanism of OI and its treatment.

We collaborated with Kenneth Kozloff's group to investigate a potential anabolic therapy, sclerostin antibody (Scl-AB), which stimulates osteoblasts via the canonical Wnt pathway. Scl-AB stimulated bone formation in

young Brtl mice and increased bone mass and load-to-fracture. Treatment with Scl-AB caused no detrimental change in Brtl bone material properties. Nano-indentation studies indicated unchanged mineralization, unlike the hyper-mineralization induced by bisphosphonate treatment. In addition, Scl-AB was successfully anabolic in adult Brtl mice, and may thus 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 cessation of Scl-AB treatment preserved the anabolic gains from Scl-AB. Alternatively, a single low dose of bisphosphonate concurrent with Scl-AB treatment facilitated the anabolic action of Scl-AB by increasing the availability of trabecular surfaces for new bone formation. 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 of neural foramina was seen. The anti-sclerostin antibody is now entering clinical trials for pediatric OI from two pharmaceutical companies.

Brtl mice provided important information on 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. Decreased vimentin (an intermediate filament) can lead to cytoskeletal collapse, and increased stathmin (a regulatory factor that promotes microtubular disassembly) and cofilin-1 (an inducer of actin depolymerization) work in concert to disrupt cytoskeletal cellular functions. The alterations affected osteoblast proliferation, collagen deposition, integrin, and TGF-beta signaling. The data suggest that cytoskeletal elements present novel OI treatment targets. Another potential novel treatment may be 4-PBA, a chemical chaperone. When the 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.

Natural history and bisphosphonate treatment of children with types III and IV OI

We 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 in lung volumes and flow rates. The decline was worse in the 36 children with scoliosis but also occurred in 20 participants without scoliosis, who had declining function with restrictive disease, suggesting that pulmonary dysfunction of OI is attributable to a primary defect related to abnormal collagen in the lung. Pulmonary issues are the most prevalent cause of morbidity and mortality in OI; patients should seek anticipatory evaluation.

Although short stature is a cardinal feature of OI, OI–specific growth curves were not previously available. 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. The data show that gender and OI type, but not the specific mutant collagen chain, have significant effects on height in OI. A pubertal growth spurt is generally absent or blunted in types III/IV OI. The BMI 50th and 95th centile curves are distinctly shifted above respective CDC curves in both genders. 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.

We published a collaborative study on the effect of stress-shielding by large diameter rods on diaphyseal bone in lower extremity long bones, in comparison to unrodded bone in the same individual. Utilization of large-diameter rods unweights bone. After approximately two years, there is diaphyseal atrophy of the rodded bone, compromising bone strength. Rod replacement with a small diameter rod is a difficult surgery and requires a period of intensive rehabilitation, but the diaphysis can recover in pediatric bone.

We examined the effect of OI genotype and clinical phenotype on adiposity and resting energy expenditure in children and young adults with OI [Reference 3], comparing them with healthy controls of matched age and BMI. The fat mass percent differed only for those patients with non-collagenous mutations, in whom it was increased vs. matched controls. The same subgroup of OI patients had a decrease in resting energy expenditure, which may contribute intrinsically to their adiposity.

Our trial of bisphosphonate in children with types III and IV OI was the first randomized controlled bisphosphonate trial for OI in the United States. It examined direct skeletal and secondary gains reported in uncontrolled trials. We found increased BMD (bone mineral density) Z-scores and improved vertebral geometry. Vertebral BMD improvement tapered off after two years' treatment. Our treatment group did not experience fewer long-bone fractures, coinciding with equivocal improvement in fractures in other controlled trials. Our trial did not support claims for improved ambulation level, lower-extremity strength, or pain alleviation, suggesting these were placebo effects. 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 sometimes has a hyperpigmented, vascular lesion. Because attempts to identify germline mutations causing melorheostosis were unsuccessful, we proposed that somatic mutations were causative. Our collaborative team (with Tim Bhattacharyya 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.

DNA extracted from each patient’s two bone samples was subjected to whole-exome sequencing (WES); sequences from each individual patient were compared, and secondarily compared among the set of patients. We identified two genes causing somatic mutations in melorheostotic lesions [References 4, 5]. Each gene was associated with one of the radiographic forms of melorheostosis, and the bone lesions had distinct histology and mechanism along the TGFβ pathway. In affected but not unaffected bone or blood, eight of the 15 patients had somatic mutations for *MAP2K1* (dual-specificity mitogen-activated protein kinase 1), located in two adjacent residues of the negative regulatory domain and that would be expected to increase MEK1 (meiotic chromosome axis–associated kinase) activity. Increased MAPK activity along the non-canonical TGFβ pathway

leads to increased phosphorylation and activation of ERK1/2 (ERK: extracellular signal-regulated kinase), accounting for the mosaic pattern of increased p-ERK1/2 in osteoblasts on immunohistochemistry of affected bone. Osteoblasts cultured from affected bone constitute two populations with distinct p-ERK1/2 levels, as demonstrated by flow cytometry, enhanced ERK1/2 activation, and elevated cell proliferation. Erythematous skin lesions overlying the affected bone are often mosaic for the MAP2K1 mutations and have increased vascularity [Reference 5]. Our data show that the *MAP2K1* oncogene is important in human bone formation, and they implicate MAP2K1 inhibition as a potential treatment avenue for melorheostosis.

Four patients were determined to have causative somatic mutations in SMAD3 [Reference 4], a component of the canonical TGFβ pathway. SMAD3 phosphorylation was increased in affected bone, and downstream target genes of TGFβ signaling had elevated expression. The mutations were associated with an endosteal radiographic pattern. Cultured osteoblasts from affected bone exhibited reduced proliferation *in vitro*, increased expression of osteoblast differentiation markers, and increased mineralization. However, the constitutive activation of the SMAD3 dampened the activity of BMP2, because addition of BMP2 to culture media decreased osteoblast differentiation and mineralization *in vitro*. Bone lesions with SMAD3 mosaicism did not show increased cellularity or osteoid accumulation and were more highly mineralized.

Melorheostotic bone from both MAP2K1–positive and SMAD3–positive patients showed two zones of distinct morphology. In MAP2K1–positive melorheostosis [Reference 5], the inner osteonal zone is intensely remodeled and has increased osteoid. The zone is covered by an outer zone containing compact multi-layered lamellae. The remodeling zone has low bone mineralization and high porosity, reflecting high vascularity. The lamellar portion is less mineralized than the remodeling zone, indicating a younger tissue age. Nano-indentation was not increased in the lamellar zone, indicating that the surgical hardness of this bone reflects its lamellar structure. We propose that the genetically induced deterioration of bone micro-architecture in the remodeling zone triggers a periosteal reaction. Our current interests are to investigate communication between mutant and non-mutant cells in the affected bone, and to understand the mechanism of the SMAD3 mutation using a recently generated murine model.

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High-Resolution Structural Biology of Membrane Protein Complexes in Their Native Environment

The Matthies lab is interested in the structure and function of membrane protein complexes in their native lipid membrane environment to understand their mechanism and the influence of their immediate surrounding and how these affect human health and disease. A cell contains many different lipid membranes with various lipid contents and distributions, which are very important for a membrane's morphology and function. However, very little is understood about how these various micro-environments are formed and maintained and how they influence the structure and function of membrane proteins. Studying membrane protein complexes in their native biological membrane is therefore required.

We use a combination of molecular biology, biochemistry, and biophysical methods to study molecular transport across membranes with a focus on how the immediate native environment influences the structure and function of membrane proteins, but also how proteins and lipids shape and functionalize a lipid membrane.

Cryo-electron microscopy (Cryo-EM) is one of the main structural-biology methods of the lab. Using single-particle Cryo-EM, we solve high-resolution structures of membrane proteins in artificial environments such as in detergent micelles and lipid nano-discs. But we are extending this approach to studying membrane proteins in their native environment, using native lipid nano-discs, membrane fractions in forms of vesicles, and intact cells and tissues, using a combination of correlative light and electron microscopy techniques, including cryo-fluorescent microscopy, cryo-focused ion beam-scanning electron microscopy (Cryo-FIBSEM), Cryo-EM, and cryo-electron tomography (Cryo-ET).

Doreen Matthies, PhD, *Head, Unit on Structural Biology*

Louis Tung Faat Lai, PhD, *Visiting Postdoctoral Fellow*

Munazza Shahid, PhD, *Visiting Postdoctoral Fellow*

Fei Zhou, PhD, *Scientist*

Structural and functional investigation of chemokine receptors in their role in recurrent miscarriage

Recurrent miscarriage (RM) is usually defined as the loss of three or more consecutive pregnancies prior to the 20th week of gestation and affects approximately 1% of women of reproductive age. The cause of 50% of cases of RM is unknown, but there is evidence supporting immune causes, more specifically, that a T helper (Th) 1–

type response is associated with the pathogenesis of RM. Women who suffer RM show elevated ratios of Th1 (CXCR3 and CCR5) to Th2 (CCR3 and CCR4) chemokine receptors. A Th1–type reaction in the materno-fetal interface mainly triggers an inflammatory response, while a Th2–type reaction typically promotes growth of trophoblastic cells, which is beneficial for the successful maintenance of a pregnancy. To work towards a treatment to prevent pregnancy loss in women with RM, Munazza Shahid will study the structure and function of chemokine receptors alone and in complex with their ligands and other interaction partners.

Structure and function of magnesium channels

Magnesium (Mg^{2+}) is the most abundant divalent cation inside cells, with an average Mg^{2+} concentration of about 20 mM, most of it bound to proteins and ATP. Magnesium plays an essential role in cellular physiology, acting as a cofactor for more than 600 enzymes, including protein kinases, ATPase, exonucleases, and other nucleotide-related enzymes. Deficiency in Mg^{2+} is associated with such diseases as muscular dysfunction, bone wasting, immunodeficiency, cardiac syndromes, and neuronal disorders. The bacterial magnesium channel CorA is a homo-pentameric channel, which forms a symmetric closed state at normal to high concentrations of magnesium, with magnesium-binding sites between protomers as well as near the membrane pore. At low magnesium concentrations, the channel undergoes an asymmetric opening, which is likely to be caused by the destabilization of protomer interactions when magnesium ions dissociate from their binding site. Louis Lai will expand the research on magnesium channels, including looking at eukaryotic magnesium channels. To investigate the structure and mechanism of these channels, structural studies in synthetic as well as native nano-discs as well as in liposomes are planned.

Structural determination of the full-length SARS-CoV-2 spike protein and drug development

COVID-19 caused by the SARS-CoV-2 virus has posed a global threat since it was first identified at the end of 2019. The rapid development of vaccines has helped counteract the rapid spread of COVID-19. However, vaccines for children under the age of 12 have only just been approved. More children have been infected by more contagious variants of the virus, and the recent surge in COVID-19 cases has put an unprecedented pressure on the pediatric health care system. The SARS-CoV-2 spike protein is responsible for the initial binding of the virus to the ACE2 receptor on human cells. Better understanding of the function and structure of the spike protein is critical for primary prevention and for the development of a vaccine and therapeutic treatments to combat the COVID-19 pandemic. Structures of the spike protein's soluble ectodomain have been determined, but the full-length spike, including its membrane domain, has not been well studied. We are working towards determining the structures of full-length spike protein complexes and identifying the key vaccine- and drug-binding interfaces in order to develop treatments that block viral entry into human cells with high efficiency and specificity, and which are also safe for children. Fei Zhou has successfully cloned and expressed the full-length spike protein, and we are working towards high-resolution structural determination of different variants and complexes.

Collaborations

Our collaborations involve structural and computational studies on a variety of membrane-protein complexes, including transporters, channels, and receptors, as well as viral spike proteins in different cellular compartments, virus-like-particles (VLP), SARS-CoV-2 accessory membrane proteins, extracellular vesicles, and lipid transport across cells, as well as novel detergents and polymers to gently extract membrane-protein complexes from their native lipid environment for high-resolution structural studies.

Publications

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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 gamma-aminobutyric acid (GABA) onto a variety of targets. Distinct cohorts of INs regulate sub- and supra-threshold 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 the 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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Aberrant sorting of hippocampal complex pyramidal cells in type I lissencephaly alters topological innervation.

Layering has been a long-appreciated feature of higher-order mammalian brain structures, but the extent to which it plays an instructive role in synaptic specification remains unknown. We examined the formation of synaptic circuitry under cellular heterotopia (which occurs when neurons do not migrate properly during the early development of the fetal brain) in the hippocampal CA1 region, using a mouse model of the human neuro-developmental disorder type I lissencephaly. We identified calbindin-expressing principal cells that are mis-positioned under cellular

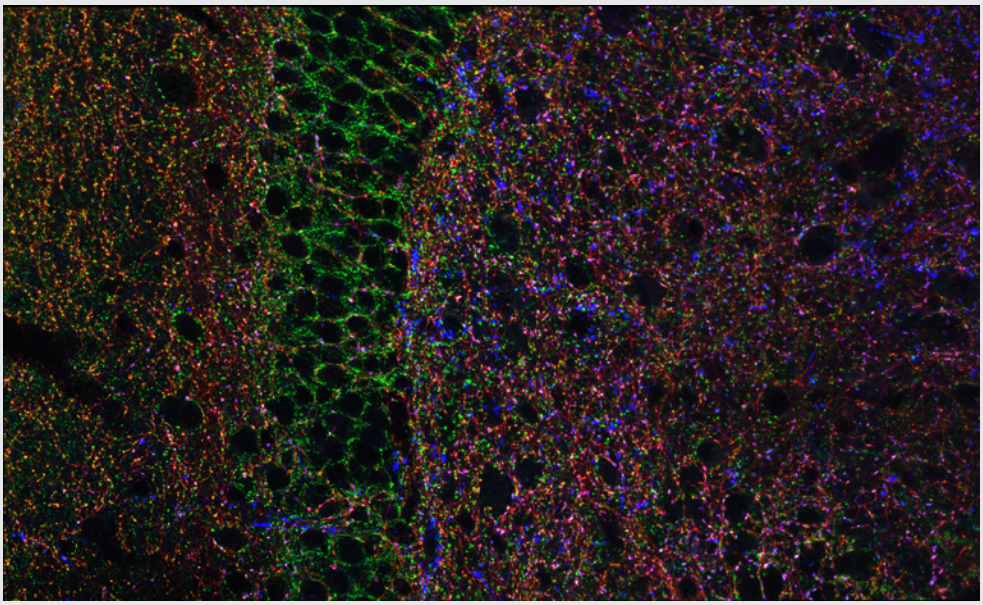

FIGURE 1. Hippocampal GABAergic terminals

Three major types of GABAergic terminal are shown in the hippocampal dentate gyrus (*red, blue, and green punch*). The black negative space shows the cell bodies of principal cells innervated by the GABAergic terminals.

heterotopia. Ectopic calbindin-expressing principal cells develop relatively normal morphological features but stunted intrinsic physiological features. Regarding network development, a connectivity preference for cholecystokinin-expressing interneurons to target calbindin expressing principal cells is diminished. Moreover, *in vitro* gamma oscillatory activity is less synchronous across heterotopic bands, and mutants are less responsive to pharmacological antagonism of cholecystokinin-containing interneurons. With this study, we hope to aid not only in our understanding of how cellular networks form but highlight vulnerable cellular circuit motifs that might be generalized across disease states.

Emergence of non-canonical parvalbumin-containing interneurons in hippocampus of a murine model of type I lissencephaly

Type I lissencephaly is a neuronal migration disorder caused by haploinsuffiency of the *LIS1 gene* and is characterized in humans by agyria, mis-lamination of brain structures, developmental delays, and epilepsy. We investigated the impact of the *LIS1* mutation on the cellular migration, morphophysiology, microcircuitry, and genomics of mouse hippocampal CA1 parvalbumin-containing inhibitory interneurons (PV+INTs). We found that wild-type (WT) PV+INTs consist of two physiological subtypes (80% fast-spiking [FS], 20% non-fast-spiking [NFS]) and four morphological subtypes (basket, axo-axonic, bistratified, radiatum-targeting). We also discovered that cell-autonomous mutations within interneurons disrupt morphological development of PV+INTs, which results in the emergence of a non-canonical “intermediate spiking (IS)” subset of PV+INTs. In the GlobalLis mutant mouse (a mouse model of lissencephaly), IS/NFS cells become the dominant PV+INT subtypes (56%) and the percentage of FS cells shrinks to 44%. We also found that IS/NFS cells are prone to entering depolarizing block, causing them to temporarily lose the ability to initiate action potentials and control network excitation, potentially promoting seizures. Also, single-cell nuclear RNA-Seq of PV+INTs revealed several mis-regulated genes related to morphogenesis, cellular excitability, and synapse formation.

NMDAR–mediated transcriptional control of gene expression in the specification of interneuron subtype identity.

Medial ganglionic eminence (MGE)–derived parvalbumin $(PV)^+$, somatostatin $(SST)^+$, and neurogliaform (NGFC)–type cortical, and hippocampal interneurons have distinct molecular, anatomical, and physiological properties. However, the molecular mechanisms regulating their diversity remain poorly understood. Using a single-cell transcriptomics, we showed that the obligate NMDA–type glutamate receptor (NMDAR) subunit gene *Grin1* mediates subtype-specific transcriptional regulation of gene expression in MGE–derived interneurons, leading to altered subtype identities. Notably, MGE–specific conditional *Grin1* loss results in a systemic downregulation of diverse transcriptional, synaptogenic, and membrane excitability regulatory programs. Such widespread gene-expression abnormalities mirror aberrations that are typically associated with neuro-developmental disorders, particularly schizophrenia. Our study thus provides a road map for the systematic examination of NMDAR signaling in interneuron subtypes, revealing potential MGE–specific genetic targets that could instruct future therapies for psychiatric disorders.

Intrinsic electrophysiological properties predict variability in morphology and connectivity among striatal parvalbumin-expressing Pthlh cells.

Determining the cellular content of the nervous system in terms of cell types and the rules of their connectivity represents a fundamental challenge to the neurosciences. The recent advent of high-throughput techniques, such as single-cell RNA-Seq, has allowed for greater resolution in the identification of cell types and/or states. Although most of the current neuronal classification schemes comprise discrete clusters, several recent studies have suggested that, perhaps especially within the striatum, neuronal populations exist in continua with regard to both their molecular and electrophysiological properties. Whether these continua are stable properties, established during development, or whether they reflect acute differences in activity-dependent regulation of critical genes is currently unknown. We set out to determine whether gradient-like molecular differences in the recently described *Pthlh*–expressing inhibitory interneuron population, which contains the *Pvalb*–expressing cells, correlate with differences in morphological and connectivity properties. We showed that morphology and long-range inputs correlate with a spatially organized molecular and electrophysiological gradient of Pthlh interneurons, suggesting that the processing of different types of information (by distinct anatomical striatal regions) has different computational requirements.

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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 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 to 15 percent of patients with CAH due to 21-hydroxylase deficiency have a contiguous gene-deletion syndrome resulting in connective tissue dysplasia and a hypermobility-type 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 such 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 CAH have impairment of cortisol production and are therefore at risk for life-threatening adrenal crises. Management of illness episodes aims to prevent such crises. We evaluated rates of illnesses and associated factors in our large cohort of patients with adrenal insufficiency attributable to CAH, who were followed prospectively for many years at the NIH Clinical Center and received repeated glucocorticoid stress-dosing education. We performed a longitudinal analysis of approximately 2,300 visits from 156 CAH patients over 23 years. 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. We found that children who had elevated plasma renin activity were more likely to have an emergency room visit, and, for all ages, those who had elevated plasma renin activity were more likely to have an adrenal crisis. Gastrointestinal and upper respiratory tract infections were the two most common precipitating events for adrenal crises and hospitalizations across all ages. Life-threatening adrenal crises 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. Our results highlight the importance of epinephrine and aldosterone deficiencies in playing a role in risk of adrenal crisis.

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 to prevent life-threatening adrenal crisis with hypoglycemia. Our new age-appropriate guidelines aim to reduce adrenal crises and prevent hypoglycemia, particularly in children. Our suggestions were incorporated into the Endocrine Society Clinical Practice Guideline for Congenital Adrenal Hyperplasia.

Genotype–phenotype studies of CAH-X

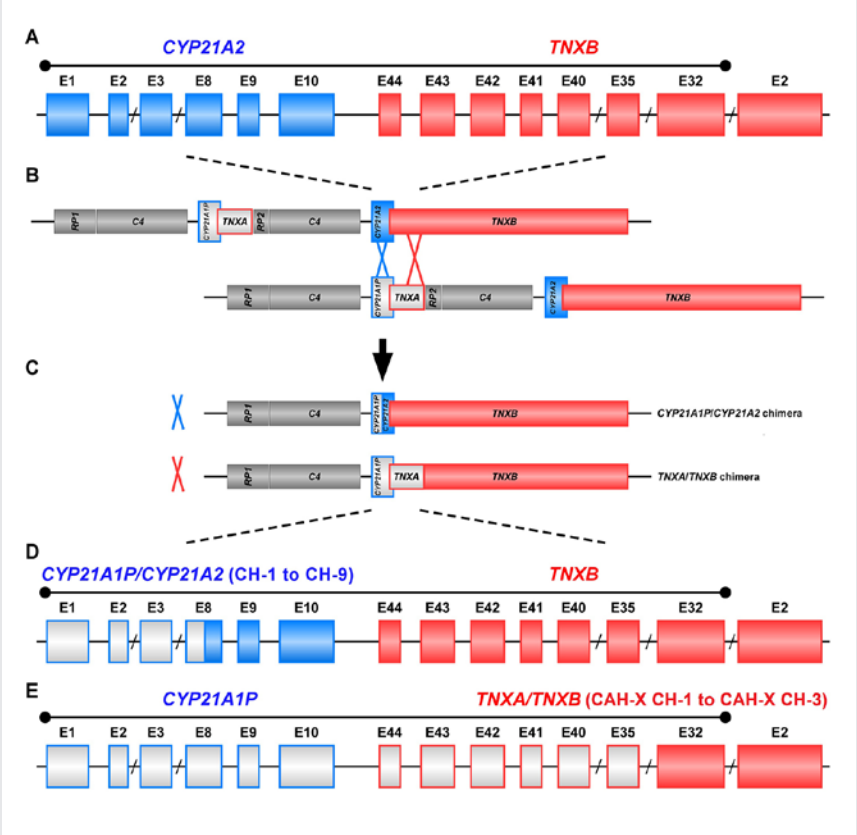
CAH is most commonly caused by 21-hydroxylase deficiency. The gene encoding 21-hydroxylase, *CYP21A2*, and a highly homologous pseudogene, *CYP21A1P*, map to the short arm of chromosome 6 within the human leukocyte antigen histocompatibility complex. The deleterious sequence in the *CYP21A1P* pseudogene 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 the 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 phenomenon 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; such 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 the study, the term CAH-X was coined to describe the subset of CAH patients who display an Ehlers-Danlos syndrome phenotype resulting from 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, a region of the genome that 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

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

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



deletes *CYP21A2* and therefore represents a CAH disease–causing allele. We described three unique types of *TNXA/TNXB* chimera (CH): CAH-X CH-1 renders the gene nonfunctional, resulting in reduced dermal and serum TNX expression; CAH-X CH-2 alters protein structure; and CAH-X CH-3 is predicted to reduce protein folding energy. Our laboratory continues to investigate how *TNXB* contributes to the phenotype of CAH patients.

To date, we have described 24 patients (19 families) with monoallelic CAH-X and three patients with biallelic CAH-X. Approximately 10 to 15 percent of patients with CAH resulting from 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. In 2019, we developed a PCR–based, high-throughput, cost-effective assay that accurately identifies CAH-X. The assay had 100% sensitivity and 99.2% specificity.

The study of the 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.

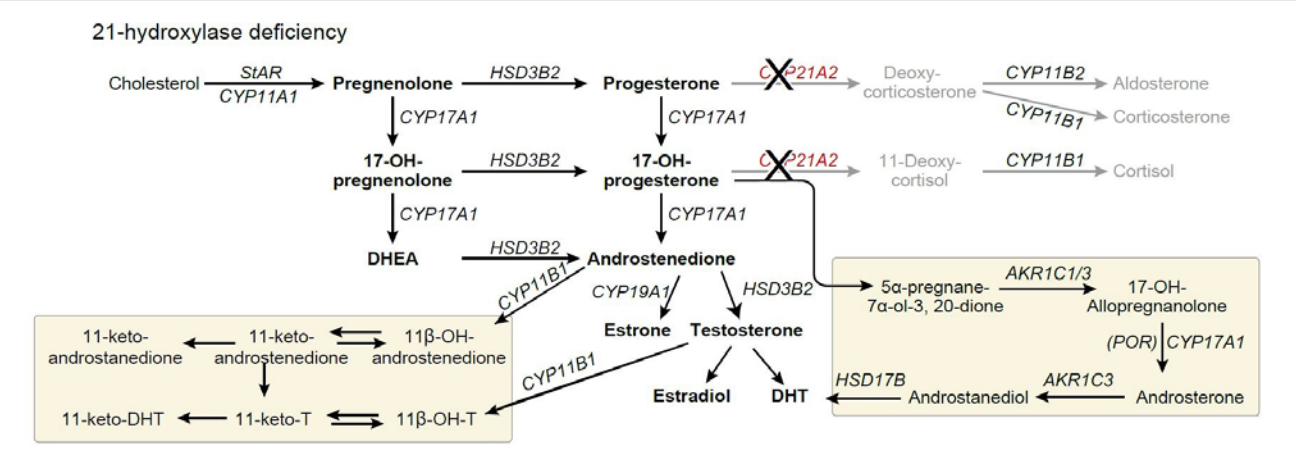


FIGURE 2. Classic and alternative steroidogenesis pathways leading to adrenal androgen production

In 21-hydroxylase deficiency, elevations of 17α-hydroxyprogesterone and androstenedione can activate alternative steroidogenic pathways (*yellow boxes*).

Pathophysiology of tumor formation

Patients with CAH are at risk for tumor formation, with the common development of adrenal tumors and adrenal rest tumors (ARTs). ARTs are extra-adrenal-like masses with similarities to adrenocortical tissue. Testicular ARTs (TARTs) are commonly observed in men with CAH, often cause infertility, and are easily detected by ultrasound. The etiology of TARTs and factors contributing to their origin and progression are not completely clear. Some studies support the concept that these benign tumors arise from pluripotent progenitor cells or from cells that are adrenal in origin and which descend with the testis during embryogenesis.

Understanding the pathogenesis and functional features of tumor formation is essential in developing treatment strategies. We performed the first study describing the structural morphology of the cells residing in adrenals from patients with CAH in comparison with ARTs, and we also performed gene expression studies. We found that CAH–affected adrenal glands and ARTs have similar expression profiles and morphology, demonstrating mostly zona reticularis characteristics and lymphocytic infiltration, suggesting a common origin that is similarly affected by the abnormal hormonal milieu. The study provided a comprehensive characterization of CAH–affected adrenals and ARTs in relation to control tissues, thus providing insight into disease-specific tissue transformation. In addition, immune system modulators may play a role in tumor formation in CAH.

New and improved biomarkers of CAH

The diagnosis and management of CAH has been limited by inadequate biomarkers. Several pitfalls were identified in the use of 17α-hydroxyprogesterone (17-OHP) and androstenedione, the traditional biomarkers used for disease management. 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. Adrenal-derived 11-oxygenated androgens have emerged as potential new biomarkers, given that traditional biomarkers are subject to variability and are not adrenal-specific, contributing to management challenges. We found that steroids synthesized with the participation of 11beta-hydroxylase (11-oxygenated

C19 steroids) are abundant in patients with classic CAH resulting from 21-hydroxylase deficiency, and correlate well with long-term disease control and disease-specific comorbidities (e.g., increased adrenal volume, TARTs, menstrual irregularity, hirsutism) (Figure 2).

With our collaborators Richard Auchus and Adina Turcu, we compared traditional and 11-oxygenated androgens in patients with nonclassic (mild) CAH resulting from 21-hydroxylase deficiency and patients with symptoms of hyperandrogenism from other causes. Patients with nonclassic CAH present with clinical manifestations of hyperandrogenism, features that are shared with other disorders of androgen excess. In particular, the clinical phenotype of women with nonclassic CAH is similar to the more common polycystic ovary syndrome. The diagnosis of nonclassic CAH is based on serum 17-OHP and usually requires dynamic testing with synthetic ACTH (cosyntropin). We found that 11-oxygenated C19 steroids are disproportionately elevated compared with conventional androgens in nonclassic CAH, and that steroid panels can accurately diagnose nonclassic CAH in unstimulated blood tests.

In a retrospective analysis of approximately 2,800 laboratory assessments obtained as part of Natural History Study of CAH at the NIH Clinical Center, we found discrepant 17-OHP and androstenedione in 469 (17%) of laboratory assessments. Of these, 403 (86%) had elevated 17-OHP with androstenedione in reference range. Using frozen serum, we evaluated the utility of 11-oxygenated C19 steroids in the setting of inconclusive conventional biomarkers. We found that 11-hydroxytestosterone provided the best discrimination between poor and good clinical control. We continue to explore the utility of these newly described steroids in the diagnosis and management of CAH.

Novel treatment approaches: sex steroid blockade and inhibition

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 (two-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 of the effect of an antiandrogen (flutamide) and an aromatase inhibitor (letrozole), as well as reduced hydrocortisone dose vs. conventional treatment, on adult height is near completion; 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 the 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 17α-hydroxylase, a key enzyme required for testosterone synthesis, in a multicenter Phase 1/2 study in prepubescent children (*NCT02574910*).

In 2020, we reported for the first time the use of nevanimibe, an orally administered ACAT1/sterol O-acyltransferase 1 (SOAT1) inhibitor (nevanimibe), as adjuvant therapy for CAH. The enzyme is the gatekeeper for the esterification of cholesterol, a necessary step for adrenocortical steroid biosynthesis. This proof-of-concept study showed that short-term (two-week) use of nevanimibe at various doses reduced 17α-hydroxyprogesterone, a biomarker of adrenal androgen production. Gastrointestinal-related side effects were common (30%). A longer-duration study was terminated following an interim data review.

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 have been studying is circadian cortisol replacement in patients with CAH.

In 2016, we successfully replaced cortisol in a physiologic manner through the use of a pump usually used to deliver insulin. A programmed 24-hour infusion of hydrocortisone was delivered subcutaneously for six months to eight patients with adrenal insufficiency resulting from 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 their 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.

Our group was the first to study circadian cortisol replacement in CAH patients with the use of a modified-release formulation of hydrocortisone, (MR-HC, 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 MR-HC yielded improved disease control throughout the day, using a lower hydrocortisone dose equivalent. Successful completion of this phase 2 study, 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. 122 Adults with classic CAH completed the phase 3 study. The primary endpoint, 17α-hydroxyprogesterone 24-hour area under the curve standard deviation, did not differ between the two groups; however, improved biochemical control of CAH was observed in the morning and early afternoon in those receiving the MR-HC compared with standard treatments. Sustained benefits with decreased dosage were observed in 18 months extension. Based on these data, MR-HC is now licensed in the UK and Europe. Patients who were enrolled in the phase 3 study are continuing MR-HC therapy in a long-term follow-up study, and future US studies are being planned.

Studies of circadian cortisol replacement 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.

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Childhood Neurodegenerative Lysosomal Storage Disorders

The Section on Developmental Genetics conducts both basic and translational research into a group of the most common childhood neurodegenerative lysosomal storage disorders (LSDs), called neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease. These diseases mostly affect children, and there is no curative treatment for any of the NCLs. Mutations in at least 14 different genes (called *CLNs*) underlie various forms of NCL. The *CLN1*, *CLN2*, *CLN5*, *CLN10*, and *CLN13* genes encode soluble lysosomal enzymes; the *CLN4* and *CLN14* encode peripherally associated cytoplasmic proteins; the *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.* The infantile NCL (INCL), a fatal neurodegenerative LSD, is caused by inactivating mutations in the *CLN1* gene. *CLN1* encodes a lysosomal depalmitoylating enzyme called palmitoyl-protein-thioesterase-1 (PPT1). Our investigations focus on understanding the molecular mechanisms of pathogenesis underlying INCL (CLN1 disease), juvenile NCL (JNCL: CLN3 disease), and congenital NCL (CNCL: CLN10 disease). Interestingly, all NCL types share some common clinical features such as epileptic seizures, progressive psychomotor decline, and visual impairment resulting from retinal degeneration. The pathologic features include intracellular accumulation of auto-fluorescent material, neuro-inflammation, and cortical atrophy. Such patients also have a shortened lifespan.

Several years ago, we initiated investigations on INCL. Numerous proteins, especially in the brain, require *S*-palmitoylation (also called *S*-acylation), a reversible post-translational modification in which a 16-carbon, saturated fatty acid (generally palmitic acid) is attached to specific cysteine residues in polypeptides via thioester linkage. While *S*-palmitoylation plays important roles in membrane anchorage of soluble proteins, protein-protein interactions, protein trafficking, and protein stability, such lipid-modified proteins must also be depalmitoylated for recycling or degradation and clearance by lysosomal hydrolases. Thus, dynamic *S*-palmitoylation (palmitoylation-depalmitoylation), just as phosphorylation-dephosphorylation, regulates the function of many proteins, especially in the brain. Dynamic *S*-palmitoylation requires coordinated actions of two types of enzyme with opposing functions. The enzymes that

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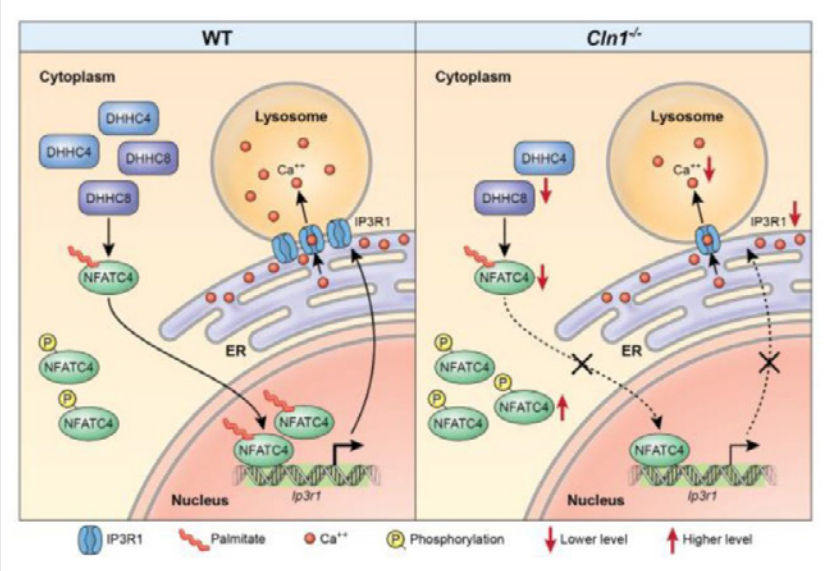
FIGURE 1. Dysregulation of lysosomal calcium homeostasis in a mouse model of INCL

Schematic representation showing how *S*-palmitoylation of NFATC4 may regulate IP3R1 expression thereby controlling lysosomal Ca^{2+}-homeostasis, which is dysregulated in INCL mice. In the *Cln1*$^{-/-}$ mouse brain, the levels of two palmitoyl acyl transferase enzymes (i.e., ZDHHC4 and ZDHHC8), enzymes that catalyze *S*-palmitoylation of NFATC4, are reduced. As a result of their reduced levels in *Cln1*$^{-/-}$ mouse brain, *S*-palmitoylation of NFATC4 is suppressed, preventing its trafficking to the nucleus, which dysregulates *Ip3r1* expression, thereby, suppressing the transport of Ca^{2+} from the ER to the lysosome. Low lysosomal Ca^{2+} suppresses the catalytic activities of Ca^{2+}-dependent lysosomal acid hydrolases, causing storage of *S*-acylated proteins in lysosomes, which contributes to INCL pathogenesis.



catalyze *S*-palmitoylation are palmitoyl acyltransferases (PATs), which are zinc-finger proteins with a common DHHC (Asp-His-His-Cys) motif, and they are called ZDHHC PATs or simply ZDHHCs. The mammalian genome encodes a family of 23 ZDHHC PATS. Similarly, the palmitoyl thioesterases, which depalmitoylate *S*-acylated proteins, are localized either in the lysosomes like PPT1 or in the cytoplasm like acyl-protein thioesterase-1 (APT1). Recently, several protein depalmitoylases called ABHD17 were identified, which catalyze the turnover of N-Ras (a GTP-ase signal-transduction protein).

PPT1 catalyzes the cleavage of the thioester linkage of *S*-palmitoylated proteins, which is vitally important because such lipid-modified 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 as the mechanism of INCL pathogenesis. However, the precise molecular mechanism underlying INCL pathogenesis has remained elusive for more than two decades. Children afflicted with INCL are normal at birth but, by 11 to 18 months of age, 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 translational research is to apply the knowledge gained from our basic 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 synaptic-vesicle (SV)

proteins, which are essential for generating fresh SVs to replenish the SV pool size at the nerve terminals so as to maintain uninterrupted neurotransmission. We also discovered that ER and oxidative stress contribute to neuronal apoptosis and neuro-inflammation in INCL. Further, we found that PPT1 deficiency causes mis-routing 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 non-invasive 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 electro-retinography can be used to assess the progressive retinal deterioration in *Cln1*$^{-/-}$ as well as in *Cln1*–knockin (KI) mice, which carry the nonsense mutation in the *CLN1* gene commonly found in the INCL patient population in the US. Moreover, we discovered that the blood-brain barrier is disrupted in *Cln1*$^{-/-}$ mice and that this 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. The compound is currently undergoing preclinical evaluation for the approval of an IND by the FDA. Intriguingly, we discovered that in *Cln1*$^{-/-}$ mice the lysosomes contain insufficient amounts of PPT1 protein and PPT1–enzymatic activity, contributing to neuro-pathology in this disease. These and related studies provide insight into the complex mechanisms of heritable disorders of neurodegeneration such as CLN1 disease (INCL) as well as CLN3 disease (JNCL) and identify several potential therapeutic targets. Our results suggest that thioesterase-mimetic small molecules such as NtBuHA are potential therapeutics for INCL and may even be for JNCL. More recently, we discovered that cathepsin D (CD) deficiency in lysosomes is a common pathogenic link between CLN1 disease and CLN10 disease (CNCL). Our ongoing laboratory and translational investigations are attempting to advance our knowledge of INCL, JNCL, and congenital NCL (CNCL) diseases.

Cln3 mutations significantly reduce lysosomal Ppt1–protein and Ppt1–enzyme activity.

Because PPT1 deficiency causes lysosomal accumulation of autofluorescent ceroid, leading to INCL, and intracellular accumulation of ceroid is a characteristic of all NCLs, a common pathogenic link for these diseases has been suggested. It has been reported that CLN3 mutations suppress the exit of the cation-independent mannose 6-phosphate receptor (CI-M6PR) from the *trans*-Golgi network (TGN). Because CI-M6PR transports soluble proteins such as PPT1 from the TGN to the lysosome, we hypothesized that CLN3 mutations may cause lysosomal PPT1 insufficiency, thus 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 the Ppt1 protein and Ppt1–enzyme activity and progressively accumulate autofluorescent ceroid. Furthermore, in JNCL fibroblasts the V0a1 subunit of v-ATPase, which regulates lysosomal acidification, is mis-localized to the plasma membrane instead of to its normal location on lysosomal membrane. The defect dysregulates lysosomal acidification, as we previously reported in *Cln1*$^{-/-}$ mice. Our findings uncover a previously unrecognized role of CLN3 in lysosomal homeostasis and suggest that CLN3 mutations causing lysosomal Ppt1 insufficiency may at least in part contribute to JNCL pathogenesis.

Impaired lysosomal Ca^{2+} homeostasis contributes to pathogenesis in INCL mice.

The lysosome is an organelle long known for mediating degradation and clearance of cellular waste. In recent years, it has become evident that it is a highly dynamic structure that also plays important roles in cell metabolism in response to environmental cues. Impaired lysosomal degradative function leads to a family of about 60 inherited LSDs. Dysregulation of cellular Ca^{2+} homeostasis is reported to play important roles in the pathogenesis of several human diseases, including the LSDs. Defective lysosomal Ca^{2+} homeostasis has also been reported to impair autophagy. In most of the LSDs, defective autophagy leads to neurodegeneration.

The ER is the major Ca^{2+}repository in the cell, and Ca^{2+} plays a key regulatory role in autophagy. It is an intracellular degradative process that requires Ca^{2+}–dependent lysosomal hydrolases for the degradation and clearance of the cargo contained in the autophagosomes. Lysosomal Ca^{2+}homeostasis is mediated by inositol 3-phosphate receptor 1(IP3R1)–mediated transport of Ca^{2+} from the ER to the lysosome. It has also been reported that selective interaction of IP3Rs with the ER–lysosome contact sites is required for the delivery of Ca^{2+} to the lysosome. Moreover, antagonists of IP3Rs rapidly and completely block lysosomal Ca^{2+} refilling. Interestingly, IP3R1 has been reported to undergo *S*-palmitoylation for regulating Ca^{2+} flux in immune cells. Furthermore, disruption of Ca^{2+} homeostasis may dysregulate neurotransmitter release, contributing to neurodegeneration. Autophagy is impaired by dysregulation of Ca^{2+}homeostasis in many LSDs including in *Cln1*$^{-/-}$ mice. We sought to test the hypothesis that *CLN1* mutations dysregulate lysosomal Ca^{2+} homeostasis and suppress the catalytic activities of Ca^{2+}–dependent lysosomal hydrolases, which impair the degradation of undigested cargo in autophagosomes, causing neuro-pathology in INCL.

We sought to determine the mechanism by which PPT1 deficiency impairs lysosomal degradative function and contributes to INCL pathogenesis. We found that in *Cln1*$^{-/-}$ mice low levels of IP3R1 dysregulate lysosomal Ca^{2+} homeostasis. Intriguingly, the transcription factor NFATC4, which regulates *IP3R1* expression, required *S*-palmitoylation for trafficking from the cytoplasm to the nucleus. We identified two palmitoyl acyltransferases, ZDHHC4 and ZDHHC8, which catalyzed *S*-palmitoylation of NFATC4. Notably, in *Cln1*$^{-/-}$mice, reduced ZDHHC4 and ZDHHC8 levels markedly lowered *S*-palmitoylated NFATC4 (active) in the nucleus, which inhibited *IP3R1* expression, thereby, dysregulating lysosomal Ca^{2+} homeostasis. Consequently, Ca^{2+}–dependent lysosomal enzyme activities were markedly suppressed. Impaired lysosomal degradative function impaired autophagy, which caused lysosomal storage of undigested cargo. Importantly, IP3R1 overexpression in *Cln1*$^{-/-}$ mouse fibroblasts ameliorated this defect. Our results reveal a previously unrecognized role of *Cln1/Ppt1* in regulating lysosomal Ca^{2+} homeostasis and suggest that the defect contributes to INCL pathogenesis.

Dysregulation of lysosomal acidification contributes to neurodegeneration in INCL mice.

In eukaryotic organisms, the lysosome is the primary organelle for intracellular digestion. It contains enzymes that require an acidic pH for optimal degradative function. Thus, lysosomal acidification is of fundamental importance for the degradation of macromolecules of intra- and extracellular origin that are delivered to the lysosome, and its dysregulation contributes to pathogenesis in virtually all LSDs, including NCLs. Furthermore, defective regulation of lysosomal pH has also been reported in common neurodegenerative diseases such as Alzheimer's and Parkinson's. However, despite intense studies, the precise mechanism(s) underlying defective lysosomal acidification in these diseases has remained elusive. Lysosomal acidification is regulated by vacuolar ATPase (v-ATPase), a multi-subunit protein complex consisting 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, which regulates lysosomal acidification.

Two thioesterases are cytosolic (APT1 and APT2) and two (PPT1 and PPT2) are localized to the lysosome. Dynamic*S*-palmitoylation (palmitoylation–depalmitoylation) requires the coordinated action of these two groups of enzymes with opposing functions (i.e., ZDHHCs and PPTs), which maintains the steady-state membrane localization and function of numerous important proteins, especially in the brain. By catalyzing depalmitoylation, thioesterases also facilitate recycling or degradation of proteins that undergo *S*-palmitoylation.

We tested a hypothesis that one or more subunits of v-ATPase require *S*-palmitoylation for endosomal sorting, trafficking, and reversible assembly of V1/V0 on the lysosomal membrane, which is essential for regulating lysosomal pH, and that Ppt1 deficiency disrupts v-ATPase activity, impairing its proton-transport function, thereby dysregulating acidification of lysosomal lumen. Our results show that the lysosomal membrane–anchored V0 sector isoform a1 (V0a1) subunit of v-ATPase indeed undergoes *S*-palmitoylation, which is required for its sorting and trafficking to the lysosomal membrane. The process appears to be defective in Ppt1–deficient *Cln1*$^{-/-}$ mice. Notably, we demonstrated that treatment of these mice with the thioesterase (Ppt1)–mimetic small molecule NtBuHA restores near normal v-ATPase activity and rescues the defective lysosomal acidification phenotype. The results demonstrate the potential of NtBuHA as a drug target for INCL.

Cholesterol- and IGF1–mediated mTORC1 signaling contribute to INCL neuro-pathology.

The lysosome has long been considered the terminal organelle for degradation. However, emerging evidence has, remarkably, changed our understanding of the lysosome as a terminal degradative organelle to a critical mediator of fundamental metabolic processes. Signals from nutrients such as glucose, amino acids, fatty acids, and cholesterol are integrated by the lysosome, turning the cellular events from anabolic to catabolic processes, including autophagy. Whereas materials from extracellular sources are transported to the lysosome via endocytosis, materials originating from intracellular sources are delivered by autophagy. Autophagy is a critical process, dysregulation of which is implicated in many LSDs. It has been suggested that the autophagy-lysosomal pathway plays critical roles in many cellular functions, including signaling in response to environmental cues. Dysregulation of autophagy also underlies pathogenesis of common neurodegenerative diseases such Alzheimer's and Parkinson's. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. In all three types the lysosome plays a pivotal role in the degradation of cargo contained in autophagosomes.

The mechanism(s) underlying neurodegeneration in various LSDs has not been clearly defined. The mechanistic target of rapamycin (mTOR), a serine/threonine kinase, is a master regulator of cellular growth and metabolism. It is activated by cues from nutrients and growth factors as well as by cholesterol. Activation of mTOR signaling, which impairs autophagy, underlies both neuro-psychiatric and neurodegenerative disorders. We sought to determine whether Ppt1 deficiency in *Cln1*$^{-/-}$ mice mediates aberrant activation of mTORC1 signaling, suppressing autophagy and contributing to neuro-pathology in this mouse model of INCL. We found that several constituent proteins (e.g., vATPase and Lamtor1) of the lysosomal nutrient-sensing scaffold (LNSS) and the lysosomal cholesterol exporter, Niemann-Pick Complex 1 (NPC1) protein, require *S*-palmitoylation for lysosomal targeting. However, in *Cln1*$^{-/-}$ mice, lack of Ppt1 caused mis-targeting of these proteins to the plasma membrane instead of their normal location on lysosomal membrane. We found that, despite the disruption of the LNSS, aberrant

activation of mTORC1 signaling occurred via insulin-like growth factor 1 (IGF1)– and cholesterol-mediated pathways. Importantly, pharmacological inhibition of these pathways significantly suppressed mTORC1 signaling and ameliorated the dysregulation of autophagy, implicating these agents as potential drug targets for INCL.

Ablation of microRNA–155 does not ameliorate neuro-inflammation in INCL mice.

The INCL–mimicking *Cln1*$^{-/-}$ mice manifest progressive neuro-inflammation, contributing to neurodegeneration. However, the underlying mechanism of neuro-inflammation in INCL and in Cln1$^{-/-}$ mice has remained elusive. Previously, it had been reported that microRNA-155 (miR-155) regulates inflammation, and miR profiling in the *Cln1*$^{-/-}$ mouse brain showed that the level of miR-155 was upregulated. Thus, we sought to determine whether ablation of miR-155 in *Cln1*$^{-/-}$ mice suppresses neuro-inflammation in such mice. Towards this goal, we generated *Cln1*$^{-/-}$*/miR-155*$^{-/-}$ double knockout (KO) mice and evaluated the inflammatory signatures in the brain. We found that the brains of double KO mice manifest progressive neuro-inflammatory changes virtually identical to those found in *Cln1*$^{-/-}$ mice. We conclude that ablation of miR-155 in *Cln1*$^{-/-}$ mice does not alter the neuro-inflammatory trajectory in INCL mouse model.

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Gene Regulation in Innate Immunity

The laboratory is interested in chromatin and gene regulation in innate immunity. We study the role of three nuclear factors, histone H3.3, BRD4, and IRF8. Histone H3.3 is a variant histone that is incorporated into nucleosomes along with transcriptional elongation, an unusual but defining feature of the variant. Most other histones are deposited into nucleosomes during replication. For this reason, H3.3 is thought to be involved in epigenetic memory created by transcription, although experimental evidence for memory formation/maintenance is scant. BRD4 is a bromodomain protein of the BET family, expressed broadly in many cells, from early embryos to adults. Through the bromodomain, BRD4 binds to acetylated histones, not unacetylated histones. BRD4 is thus called a 'chromatin reader,' a type of regulatory factor capable of conveying epigenome information to gene expression. Furthermore, BRD4, binds to the elongation factor complex P-TEFb through the C-terminal domain, and drives transcription of many genes by driving RNA polymerase II to move through the gene body, generating nascent mRNA. Many recent reports point out that BRD4 promotes growth of cancer cells, including various blood cancers, by mediating the formation of super-enhancers involved in cell-cycle progression. As we reported in 1990, IRF8 is a DNA–binding transcription factor that plays an essential role in innate resistance to a wide array of pathogens (IRF8's structure is shown in Figure 1A). IRF8 is expressed mostly in cells of the myeloid lineage, including monocytes/macrophages, dendritic cells, and microglia. IRF8 is strongly induced when stimulated by interferons (IFN). In addition, it is upregulated when myeloid cells encounter pathogen-derived molecules and agents produced by stress. In turn, IRF8 activates many genes important for host resistance. IRF8–induced genes include those involved in autophagy and lysosome-mediated pathogen clearance. IRF8 does so by binding to small DNA motifs present in promoter and enhancer regions of the target genes.

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IRF8 sets microglia-specific epigenome structure and defines the transcriptome program.

Microglia are the sole cell type in the brain that protect against pathogen infection. They originate from the embryonic yolk sac as progenitors, which then migrate into the embryonic brain, where they differentiate

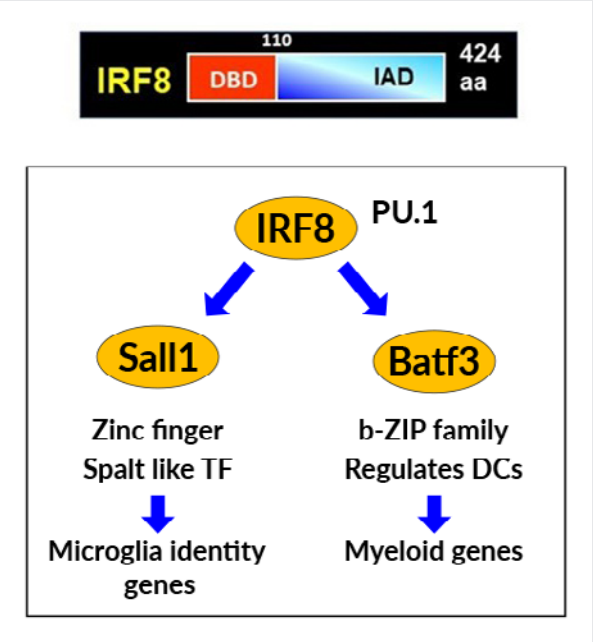

FIGURE 1. IRF8 installs a microglia-specific transcription cascade.

A. IRF8 structure. It has the DNA–binding and regulatory domains in N- and C-terminal regions.

B. IRF8 drives expression of Sall1 and Batf3, two transcription factors critical for microglia.

into functional microglia in the postnatal stage. In the adult, microglia are distributed throughout the brain including in the cortex and hippocampus. Early reports demonstrated that the regulatory transcription factors Spi1(PU.1) and IRF8 take part in progenitor differentiation, but the role of IRF8 in adult microglia has not been fully understood. To this end, we examined microglia from *Irf8* knockout (KO) mice and found that they have an abnormal morphology and do not express several microglia-specific surface markers. Using fluorescence activated cell sorting (FACS), we then sorted microglia from adult WT and *Irf8* KO mice and performed bulk and single-cell RNA-Seq. Results revealed that, without IRF8, many genes that give microglia-specific properties were missing or downregulated, including cell-surface markers such as *P2ry12*, *Iba1*, *Cx3cr1*, and *Ccr5*. On the other hand, some of IFN–stimulated genes (ISGs) and disease-associated microglia (DAM) genes were aberrantly expressed in *Irf8* KO microglia. In addition, we found that IRF8 is required for the expression of two transcription factors critical for adult microglia, i.e., Sall1 and Batf3 (Figure 1B). Our results show that IRF8 directs a transcriptional cascade that defines the microglia transcriptome program. It was important to determine the DNA sites in the microglia genome to which IRF8 binds, information that is missing in the literature. This was technically difficult, because microglia yields were low. Nevertheless, modified Cut&Run assay (Cut&Run or cleavage under targets and release using nuclease, a method to analyze protein interactions with DNA) provided reproducible IRF8–binding profiles. Our data showed that IRF8 binds mostly over distant enhancer regions, located upstream and downstream of its target genes (Figure 2). IRF8–binding sites were strongly enriched with DNA motifs containing the GAAA nucleotide sequence. In some enhancers IRF8 binding was closely clustered. Some of these sites were within the large 'super-enhancers' enriched with H3K27ac histone marks. Super-enhancers support transcription of genes essential for cell-type specific properties. IRF8–containing super-enhancers neighbored genes essential for microglia, including Sall1 and Batf3. Deletion of *Irf8* led to loss of super-enhancers associated with microglia identity genes, including Sall1 and Batf3. Furthermore the ATAC-Seq assay (assay for transposase-accessible chromatin using sequencing) found that IRF8 is important for setting open chromatin necessary for microglia super-enhancers. Along with

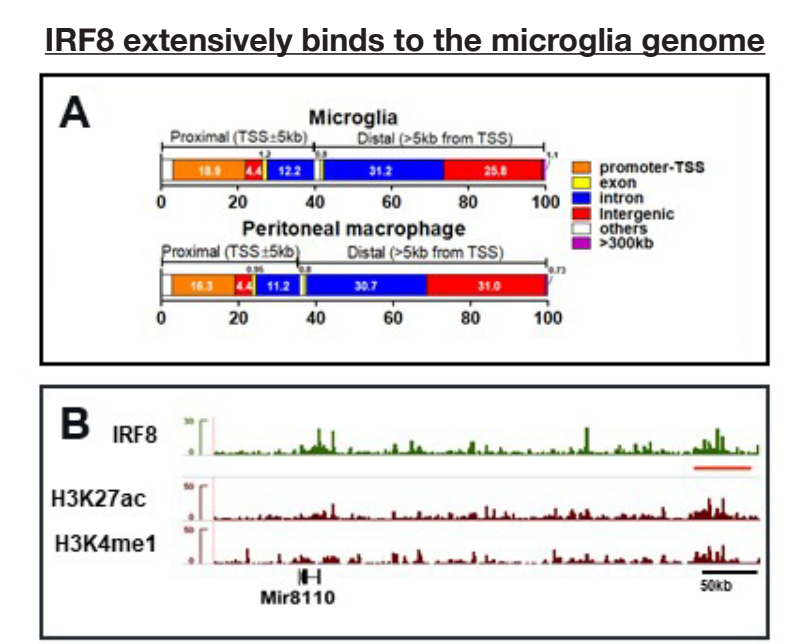

FIGURE 2. IRF8 localizes to microglia super-enhancers.

A. IRF8 binds to intergenic and introgenic regions of microglia. Comparison with peripheral myeloid cells.

B. IGV track example on the Sall1 locus. IRF8 binding closely coincides with the super-enhancer marks H3K27ac and H3K4me1.

extensive changes in histone modification patterns in *Brd4* KO cells, our results clearly show that IRF8 directs the formation of microglia-specific epigenome landscape. Consistent with this view, DNA methylome profiles revealed extensive changes in CpG methylation patterns, indicating that IRF8 regulates overall CpG–island methylation patterns.

In a recently published study on a mouse model of Alzheimer's disease (AD), it was suggested that IRF8 is involved in the progression of the disease. We investigated an AD mouse model (5FAD) with and without the *Irf8* gene. Our transcriptome and other analyses indicate that IRF8 has dual roles and can promote AD pathogenesis in a complex manner.

BRD4 promotes cell–cycle progression by preventing DNA damage.

Cell proliferation depends on continuous rounds of cell-cycle progression and is driven by sequential activation of transcription factors and by other post-translational effectors. The chromatin-binding factor BRD4 is known to promote the proliferation of many cancers, and BRD4 inhibitors (BETi) can arrest cancer growth (see Figure 3A for BRD4 structure). BETi thus offer a new possibility for anti-cancer therapy. However, the role of BRD4 in controlling proliferation of normal cells has remained elusive. We examined the role of BRD4 in normal cell growth by testing cells from *Brd4* conditional knockout (KO) mice. Cell-cycle analysis of wild-type (WT) and Brd4 KO fibroblasts showed that BRD4 is required for transition from G0-G1, G1-S, and G2-M (Figure 3B). At the G2 to M stage, many Brd4 KO cells underwent catastrophic mitotic failure, as chromosomes failed to align and segregate properly, leading to apoptosis. Transcriptome analysis found that many cell cycle–regulated genes were markedly downregulated in *Brd4* KO cells, including several histone genes at S phase, as well as the G2/M master regulators FOXM1 and ATM/ATR. FOXM1 is a transcription factor of the forkhead family and promotes transcription of many G2/M genes. ATM/ATR are kinases previously known to be involved in DNA–damage repair. ATR/ATM were recently shown to play a role at the G2-M transition in growing cells. Our

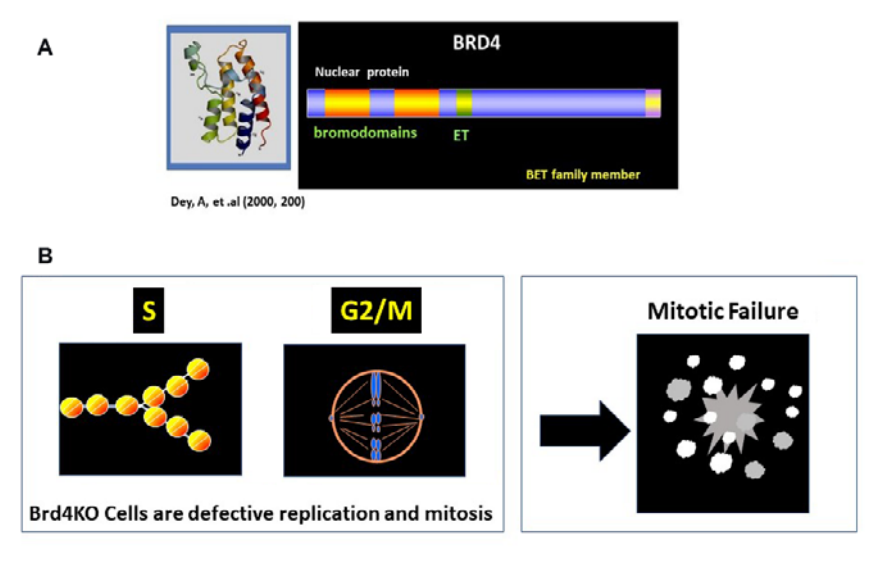

FIGURE 3. BRD4 drives cell-cycle progression.

A. Structure of BRD4. It has two bromodomains and the ET domain at the C-terminus.

B. S and G2/M progression depends on BRD4. DNA and histone replication and as well as mitotic cell division depend on BRD4. Brd4KO cells undergo catastrophic mitotic failure.

results indicate that BRD4 drives transcription of numerous cell cycle–regulated genes. Consistent with these results, BRD4 occupies numerous cell-cycle genes throughout all stages, as revealed by ChIP-Seq analysis. BRD4 binds to these genes at all stages of the cell cycle, which can be seen at the transcription start site (TSS) and gene body. Together, BRD4 orchestrates the entire cell-cycle program in normal and cancer cells.

BRD4 AND DNA DAMAGE

Recent studies highlight that BETi can arrest cancer cell growth not only by inhibiting transcription of cell-cycle genes but by inflicting DNA damage. Such reports put forward a new role for BRD4 beyond chromatin reading and transcription. We tested whether Brd4KO cells, like BETi–treated cells, suffer DNA damage. Brd4KO cells indeed showed a marked increase in phospho-H2AX foci in the nucleus (Figure 4). H2AX is a variant histone H2 phosphorylated upon DNA damage, a highly reliable marker for DNA damage. Phospho-H2AX foci thus represent the site of DNA damage. Increased DNA damage in Brd4KO cells was confirmed by R-loop formation and nuclear disintegration assays. S phase cells displayed more DNA damage than did G1 cells, suggesting that DNA damage may be associated with defective DNA/histone replication in Brd4KO cells. Thus, in the absence of BRD4, cell cycle progression without external stress causes DNA damage. DNA damage is known to occur upon oxidative stress, as it causes single-strand and double-strand DNA breaks. Our results show that BRD4 prevents DNA damage by mechanisms and pathways that have yet to be deciphered. It will be important to further study how BRD4 inhibits DNA damage or facilitate repair.

Histone H3.3 and HIRA in innate immunity

HISTONE H3.3 AND TRANSCRIPTIONAL MEMORY

We have been interested in the role of histone H3.3, as this histone is implicated in transcriptional memory. Previous work showed that H3.3 localizes to actively transcribed and bivalent regions. The process is mediated by the H3.3–specific histone chaperone HIRA. H3.3 also localizes to heterochromatin regions including the telomere regions, where transcription is silenced. The latter process is mediated by another chaperone, ATRX. Thus, H3.3 localization is thought to change dynamically according to changes in transcriptional activity.

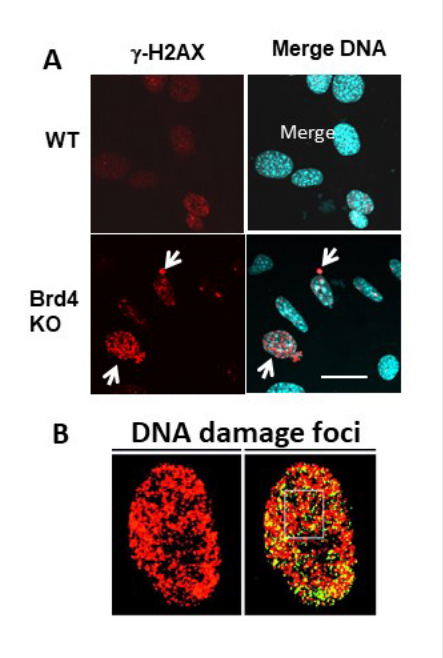

FIGURE 4. BRD4 controls endogenous DNA damage.

Brd4KO cells have increased DNA damage, as detected by gamma H2AX nuclear foci.

However, genome-wide dynamics of H3.3 distribution has not been well deciphered, particularly in cells *in vivo*. To this end, we generated a mouse model to readily localize H3.3 in various *in vivo* cells, constructing knock-in mice carrying HA–tagged H3.3 for both H3.3 genes (*H3f3a* and *H3f3b*). With this model, we showed that H3.3 is present in all adult tissues tested, including various immune cells. We plan to study H3.3 distribution in hematopoietic stem cells (HSCs) and myeloid-lineage cells, including monocytes/macrophages and microglia.

Our previous study showed that H3.3 is robustly deposited on many genes after interferon (IFN) stimulation, which creates transcriptional memory for IFN response. To investigate the biological significance of IFN–induced H3.3 deposition, we constructed another set of mice to conditionally knockout H3.3. Currently, we are testing IFN memory in the bone marrow (BM)–derived macrophage (BMDM) model. First, we found that deletion of H3.3 did not prevent development of phenotypically normal macrophages, indicating that H3.3 is partly dispensable for myeloid progenitors to differentiate into macrophages. To our surprise, H3.3–deleted macrophages expressed a subset of IFN–stimulated genes even without IFN stimulation. Our results point to the idea that H3.3 directs a complex regulatory process affecting fundamental innate immunity. Cut&Run analysis found that H3.3 deletion globally alters of histone modification patterns, indicating a previously unknown role for H3.3 in regulating epigenome structure and transcription.

HIRA CONFERS CHROMATIN ACCESSIBILITY ON HEMATOPOIETIC STEM CELLS AND GUIDES ENTIRE HEMATOPOIETIC LINEAGE DEVELOPMENT.

Because HIRA deposits histone H3.3 specifically in the genic regions, this histone chaperon is likely to play a role in the defining epigenome landscape and transcriptome programs, including transcriptional memory. We constructed *Hiraf/f* mice carrying Vav-Cre in which the *Hira* gene is deleted in HSCs. It is of note that HIRA is deleted in DiGeorge syndromes, where an over 11 Mb DNA stretch of Chr. 22 (containing HIRA) is deleted. Patients with DiGeorge syndrome manifest varied abnormalities, some including immunodeficiency and thrombocytopenia. Our analysis found that HIRA is essential for the development and maintenance of BM HSCs, in that long-term (LT) HSCs, which provide self-renewal and progenitor differentiation capacity, were virtually absent from *Hira* KO mice. In addition, *Hira* KO mice were deficient in all three blood lineages, erythroid, myeloid, and lymphoid cells, leading to anemia, thrombocytopenia, and severe immunodeficiency (Figure 5). While some T cells were found in peripheral lymphoid organs, including the thymus and lymph nodes, mature B cells were nonexistent in *Hira* KO mice. Adoptive transfer experiments verified the defects in BM LT-HSC. However, fetal hematopoiesis was normal in *Hira* KO mice, although fetal HSCs lacked reconstitution capacity. Transcriptome analysis revealed that HIRA is required for the expression of many

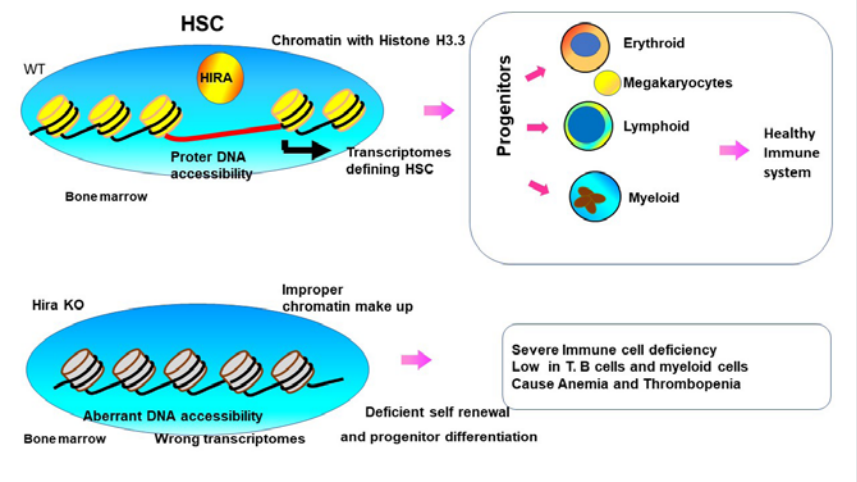

FIGURE 5. HIRA, histone H3.3 chaperone, directs hematopoietic stem cell (HSC) development.

HSCs have two essential functions, to self-renew and differentiate into mature hematopoietic cells. Analysis of *Hira* KO mice reveals that acquisition and maintenance of these functions are dependent on HIRA. RNA-Seq and ATAC-Seq data show that HIRA sets the epigenome landscape and transcription programs that enable HSC to gain and execute HSC function.

transcription factors and signaling molecules critical for HSCs. ATAC-Seq analysis revealed that HIRA is required for establishing HSC–specific DNA accessibility, including to the SPI1/PU.1 sites. Our study demonstrates that HIRA creates a chromatin environment essential for HSCs to carry out self-renewal and differentiation activity (Figure 5).

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Diagnosis, Localization, Pathophysiology, and Molecular Biology of Pheochromocytoma and Paraganglioma

Pheochromocytomas (PHEOs) and paragangliomas (PGLs) are rare but 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, collectively known as PPGLs, result from the overproduction and 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 or false-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, PPGLs 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 a predisposition to develop PPGLs and the expression of various neurochemical phenotypes and malignant potentials, including therapeutic responses and appropriate follow-up.

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Clinical and genetic aspects of pheochromocytoma and paraganglioma

Current biomarker tests for PHEOs/PGLs are technically complex or limited. We assessed the diagnostic utility of a neuroendrocrine tumor–specific 51-marker gene blood assay (NETest) in patients with PHEOs/PGLs (n = 81), including ten pediatric patients, and age-/gender-matched controls (n = 142) using a prospective case:control (1:2) analysis. We measured mRNA (qPCR), and results were scaled from 0 to 100 (upper limit of normal < 20). NETest accuracy for PHEO/PGL diagnosis was 100%. PHEO/PGL scores were 70 ± 3 vs 8.5 ± 1 in controls. Diagnostic metrics were 94% accurate, 100% sensitive,

FIGURE 1. Detection of *SUCLG2* variants in PHEO/PGL patients

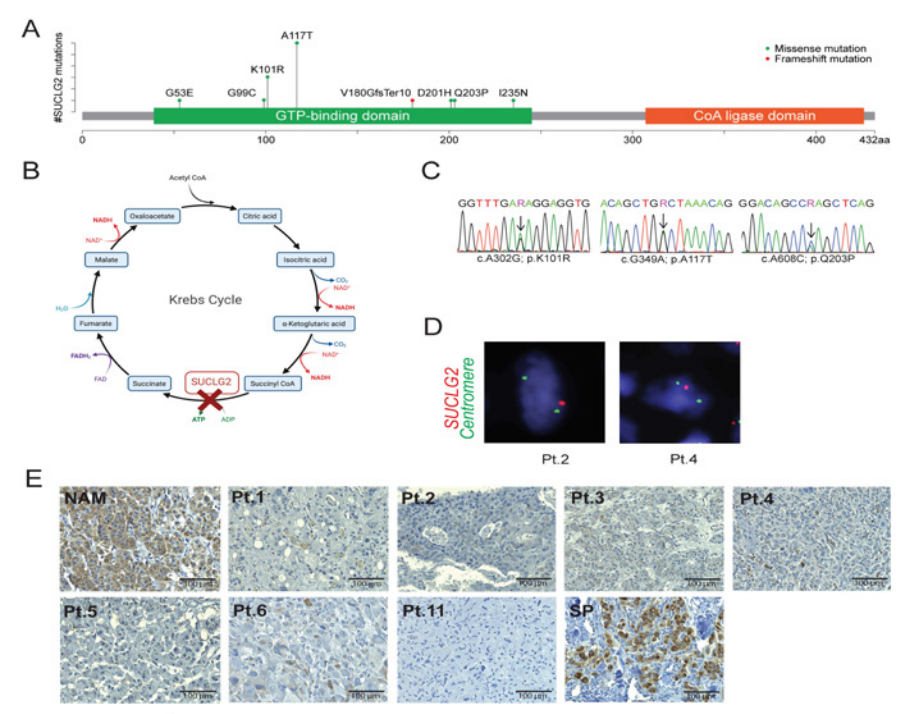
A. Schematic illustration of *SUCLG2* germline variants in the GTP–binding site.

B. SUCLG2 position in Krebs cycle.

C. Sanger sequencing showing *SUCLG2* germline variants in patient leukocyte DNA.

D. FISH assay showing apparent heterozygous deletion of *SUCLG2* in tumor specimens.

E. Immuno-histochemistry analysis revealing a substantial decrease in SUCLG2 level in PPGL with *SUCLG2* germline variants compared with that in normal adrenal medulla (NAM) and sporadic PHEO/PGL (SP). Pt: patient; NAM: normal adrenal medulla; SP: sporadic PHEO/PGL.



and 92% specific. Imaging correlation with ^{68}Ga-PET-SSA was 100%. NETest levels in PHEOs (n = 26) were significantly elevated and mixed in PHEOs/PGLs (n = 5). Adrenal-derived tumors (n = 30) exhibited significantly higher scores than did extra-adrenal–derived tumors. Cluster 2 tumors exhibited significantly higher NETest levels (n = 4: 92 ± 2) than did cluster 1 tumors (n = 35: 69 ± 4). Overall regulatory-pathway analysis identified significantly higher RAS-RAF, metastatic, pluripotential, neural, and secretory gene cluster levels in PHEOs than in PGLs. Cluster 2 PHEOs/PGLs exhibited significantly higher levels of growth-factor signaling genes than did cluster 1. The PHEOs/PGLs in the pediatric cohort (n = 10) were all NETest-positive and exhibited a gene-expression profile spectrum analogous to adults. Circulating NET transcript analysis thus identifies PHEOs/PGLs with 100% efficacy and is likely to have clinical utility in the diagnosis and management of PHEO/PGL patients.

PHEOs/PGLs are tumors with frequent mutations in genes linked to the tricarboxylic acid cycle (Krebs cycle). Until now, no pathogenic variant has been found in succinyl-CoA ligase (SUCL), an enzyme that provides substrate for succinate dehydrogenase (SDH; mitochondrial complex II; CII) and a known tumor suppressor in PHEO/PGL. A cohort of 352 subjects with apparently sporadic PHEOs/PGLs underwent genetic testing using a panel of 54 genes developed at the National Institutes of Health, including the SUCLG2 subunit of SUCL. Gene deletion, succinate levels, and protein levels were assessed in tumors where possible. To confirm the possible mechanism, we used a progenitor cell line, hPheo1, derived from a human pheochromocytoma, and ablated and re-expressed SUCLG2. We found eight germline variants in the GTP–binding domain of SUCLG2 in 15 patients (15 of 352, 4.3%) with apparently sporadic PHEO/PGL. Analysis of SUCLG2–mutated tumors and SUCLG2–deficient hPheo1 cells revealed absence of SUCLG2 protein, a reduction in the level of the SDHB subunit of CII, and faulty assembly of the complex, resulting in aberrant respiration and elevated succinate

accumulation. Our study suggests SUCLG2 as a novel candidate gene in the genetic landscape of PHEO/PGL. Large-scale sequencing may uncover additional cases harboring SUCLG2 variants and provide more detailed information about their prevalence and penetrance.

Approximately 20% of patients diagnosed with a PHEO/PGL carry a germline mutation in one of the SDHx genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*), which encode the four subunits of the SDH enzyme. When a pathogenic SDHx mutation is identified in an affected patient, genetic counseling is proposed for first-degree relatives. So far, there is no consensus on optimal initial evaluation and follow-up of individuals who are asymptomatic but might carry SDHx mutations. Thus, we established an international consensus algorithm of clinical, biochemical, and imaging screening at diagnosis and during surveillance for both adults and children. An international panel of 29 experts from 12 countries was assembled, and the Delphi method (which consists of several rounds of written questionnaires that allow experts to give their opinions) was used to reach a consensus on 41 statements. The Consensus Statement covers a range of topics, including age of first genetic testing, appropriate biochemical and imaging tests for initial tumor screening and follow-up, screening for rare SDHx–related tumors, and management of elderly people who have an SDHx mutation. This Consensus Statement focuses on the management of asymptomatic SDHx mutation carriers and provides clinicians with much needed guidance. The standardization of practice will facilitate prospective studies in the near future.

Imaging of pheochromocytomas and paragangliomas

Targeted radionuclide therapies (TRT) using ^{131}I-metaiodobenzylguanidine (^{131}I-MIBG) and peptide-receptor radionuclide therapy (^{177}Lu or ^{90}Y) represent several of the therapeutic options in the management of metastatic/inoperable pheochromocytoma/paraganglioma. Recently, high-specific-activity ^{131}I-MIBG therapy was approved by the FDA, and both ^{177}Lu-DOTATATE and ^{131}I-MIBG therapy were recommended by the National Comprehensive Cancer Network guidelines for the treatment of metastatic pheochromocytoma/paraganglioma. However, a clinical dilemma often arises in the selection of TRT, especially when a patient can be treated with either type of therapy based on eligibility for MIBG and somatostatin receptor imaging. To address this problem, we assembled a group of international experts, including oncologists, endocrinologists, and nuclear-medicine physicians with substantial experience in treating neuroendocrine tumors with TRTs, to develop consensus and provide expert recommendations and perspectives on how to decide between these two therapeutic options for metastatic/inoperable pheochromocytoma/paraganglioma. Reference 3 summarizes the survival outcomes of the available TRTs, discusses personalized treatment strategies based on functional imaging scans, addresses practical issues, including regulatory approvals, and compares toxicities and risk factors across treatments. Furthermore, it discusses the emerging TRTs.

Recent professional society guidelines for radionuclide imaging of sporadic pheochromocytoma (PHEO) recommend ^{18}F-FDOPA as the radiotracer of choice, deeming ^{68}Ga-DOTATATE and FDG to be second- and third-line agents, respectively. An additional agent, ^{18}F-FDA, remains experimental for PHEO detection. A paucity of research has performed head-to-head comparison among these agents. Therefore, we performed an intra-individual comparison of ^{68}Ga-DOTATATE PET/CT, FDG PET/CT, ^{18}F-FDOPA PET/CT, ^{18}F-FDA PET/CT, CT, and MRI in visualization of sporadic primary PHEO. The prospective study enrolled patients referred with clinical suspicion for sporadic PHEO. Patients were scheduled for ^{68}Ga-DOTATATE PET/CT, FDG PET/CT, ^{18}F-FDOPA PET/CT, ^{18}F-FDA PET/CT, whole-body staging CT (portal venous phase), and MRI, within a three-month period. Analysis included only patients with histologically confirmed PHEO on resection. We included 14 patients (8 women, 6 men; mean age, 52.4±16.8 years) with PHEO. Both ^{68}Ga-DOTATATE PET/CT and FDG PET/CT were completed in all

14 patients, ^{18}F-FDOPA PET/CT in 11/14, ^{18}F-FDA PET/CT in 7 of 14, CT in 12 of 14, and MRI in 12 of 14 patients. Findings from this small intra-individual comparative study support ^{18}F-FDOPA PET/CT as a preferred first-line imaging modality in evaluation of sporadic PHEO. In summary, the study provides data supporting current guidelines for imaging evaluation of suspected PHEO.

The aim of the next study was to identify quantitative MR biomarkers in head and neck PGLs (HNPGLs). Sixty HNPGLs were included from 50 patients. The control group consisted of 30 parapharyngeal space lesions (27 patients), which included nerve sheath tumors (n = 12) and metastatic lymph nodes (n = 18) from squamous cell carcinomas. The study identified a multi-parametric signature for disease subtyping, providing a strong impetus for switching from qualitative to quantitative analysis of deep soft-tissue tumors of the neck.

Immune and metabolic aspects of pheochromocytoma and paraganglioma

Immunotherapy has become an essential component in cancer treatment. Discovery of such tumor-specific epitopes through tumor sequencing has revolutionized patient outcomes in many types of cancers that were previously untreatable. However, the majority of solid metastatic cancers, such as PHEO, are resistant to this approach. Therefore, understanding immune cell composition in primary and distant metastatic tumors is important for therapeutic intervention and diagnostics. Combined mannan-BAM, TLR ligand, and anti-CD40 antibody-based intra-tumoral immunotherapy (MBTA therapy) previously resulted in the complete eradication of murine subcutaneous PHEO and demonstrated a systemic antitumor immune response in a metastatic model. We further evaluated this systemic effect using a bilateral PHEO model, performing MBTA therapy through injection into the primary tumor and using distant (non-injected) tumors to monitor size changes and detailed immune cell infiltration. MBTA therapy suppressed the growth not only of injected but also of distal tumors and prolonged the survival of MBTA–treated mice. Our flow-cytometry analysis showed that MBTA therapy led to increased recruitment of innate and adaptive immune cells in both tumors and the spleen. Moreover, adoptive $CD4^+$ T cell transfer from successfully MBTA–treated mice (i.e., subcutaneous PHEO) demonstrates the importance of such cells in long-term immunological memory. In summary, the study unravels further details on the systemic effect of MBTA therapy and its use for tumor and metastasis reduction or even elimination.

We further extended the MBTA therapy to other tumors and applications in close collaboration with NCI investigators. For example, emerging evidence is demonstrating the extent of T cell infiltration within the tumor micro-environment and thus has favorable prognostic and therapeutic implications. Hence, immuno-therapeutic strategies that augment the T cell signature of tumors hold promising therapeutic potential. Recently, immuno-therapy based on intra-tumoral injection of MBTA demonstrated promising potential to modulate the immune phenotype of injected tumors, including PHEO. The strategy promotes the phagocytosis of tumor cells to facilitate the recognition of tumor antigens and induce a tumor-specific adaptive immune response. Using a syngeneic colon carcinoma model, we demonstrated MBTA's potential to augment $CD8^+$ T cell tumor infiltrate when administered intra-tumorally or subcutaneously as part of a whole tumor cell vaccine. Both immuno-therapeutic strategies proved effective in controlling tumor growth, prolonged survival, and induced immunological memory against the parental cell line. Collectively, our investigation demonstrates MBTA's potential to trigger a potent anti-tumor immune response. We also reviewed the most promising glioblastoma vaccination strategies to contextualize the MBTA vaccine. By reviewing current evidence using translational tumor models supporting MBTA vaccination, we evaluated the underlying principles that

validate its clinical applicability. We also showed the translational potential of MBTA vaccination as a potential immunotherapy in glioblastoma, along with established surgical and immunologic cancer treatment paradigms.

Therapeutic aspects of pheochromocytoma and paraganglioma

PHEOs/PGLs are characterized by a unique molecular landscape that allows their assignment to clusters depending on underlying genetic alterations. With around 30–35% of Caucasian patients (a lower percentage in the Chinese population) showing germline mutations in susceptibility genes, PHEOs/PGLs have the highest rate of heritability among all tumors. A further 35–40% of Caucasian patients (a higher percentage in the Chinese population) are affected by somatic driver mutations. Thus, around 70% of all patients with PHEOs/PGLs can be assigned to one of three main molecular clusters with different phenotypes and clinical behavior. Krebs cycle/VHL/EPAS1–related cluster 1 tumors tend to have a noradrenergic biochemical phenotype and require very close follow-up because of the risk of metastasis and recurrence. In contrast, kinase signaling–related cluster 2 tumors are characterized by an adrenergic phenotype and episodic symptoms, with a generally less aggressive course. The clinical correlates of patients with Wnt signaling–related cluster 3 tumors are currently poorly described, but aggressive behavior appears likely. In a review [Reference 2], we explored and explained why cluster-specific (personalized) management of PHEO/PGL is essential to ascertain clinical behavior and prognosis and guide individual diagnostic procedures (biochemical interpretation, choice of the most sensitive imaging modalities), and personalized management and follow-up. Although cluster-specific therapy of inoperable/metastatic disease has not yet entered routine clinical practice, we suggest that informed personalized genetic-driven treatment should be implemented as a logical next step. The review published guidelines and expert views within each cluster for a coherent individualized patient management plan.

The risk factors for severe COVID-19 are diverse, yet closely resemble the clinical manifestations of catecholamine excess states (e.g., hypertension, cardiovascular disease, immune dysregulation, and hyperglycemia), suggesting a possible common basis for disease. Unfortunately, severe illness (e.g., respiratory failure, compromised cardiac function, and shock) incurred by COVID-19 hinders the direct study of catecholamines in these patients, especially among those on multiple medications or those on adrenaline or noradrenaline infusions, or both. PHEOs/PGLs are tumors that secrete catecholamines, namely adrenaline and noradrenaline, often in excess. PHEOs/PGLs are well studied disease processes in which the effects of catecholamines are easily discernible, and therefore their potential biochemical and physiological influences in patients with COVID-19 can be explored. Because catecholamines are expected to have a role in patients with critical illness, patients on vasopressor infusions, and patients who sustain some acute and chronic physical stresses, the challenges involved in the management of catecholamine excess states are directly relevant to the treatment of patients with COVID-19. In a Personal View article type, we discussed the complex interplay between catecholamines and COVID-19, and the management of catecholamine excess states, while referencing relevant insights derived from the study of PHEO/PGL.

Germline variants in approximately 20 PHEO/PGL susceptibility genes are found in about 40% of patients, half of which are found in the genes that encode SDH. Patients with SDH subunit B (*SDHB*)–mutated PHEO/PGL exhibit a higher likelihood of developing metastatic disease, which can be partially explained by the metabolic cell reprogramming and redox imbalance caused by the mutation. Reactive oxygen species (ROS) are highly reactive molecules involved in many important signaling pathways. A moderate level of ROS production can help regulate cellular physiology; however, an excessive level of oxidative stress can lead to tumorigenic processes, including stimulation of growth factor–dependent pathways and the induction of genetic instability.

Tumor cells effectively exploit antioxidant enzymes in order to protect themselves against harmful intracellular ROS accumulation, which highlights the essential balance between ROS production and scavenging. Exploiting ROS accumulation can be used as a possible therapeutic strategy in ROS–scavenging tumor cells. We focused on the role of ROS production in PHEO and PGL, predominantly in *SDHB*–mutated cases. We discussed potential strategies and approaches to anticancer therapies by enhancing ROS production in these difficult-to-treat tumors.

Animal model of pheochromocytoma and cell culture studies

We previously identified the syndrome of multiple paragangliomas and pheochromocytomas, duodenal somatostatinoma, and polycythemia resulting from post-zygotic *EPAS1 (HIF2A)*-gain-of-function mutations (also called Pacak-Zhuang syndrome). The mutations, located in the oxygen-degradation domain (ODD) of hypoxia-inducible factor-2a (HIF-2a), have been shown to impair hydroxylation by prolyl hydroxylase domain–containing protein 2 (PHD2) and subsequent association with the von Hippel-Lindau (VHL) protein. In such a situation, degradation of HIF-2a is impaired, resulting in its stabilization, prolonged activation, lack of response to normal or increasing oxygen tension, and activation of the transcription of many genes participating in tumorigenesis. Recently, in collaboration with NCI investigators, we developed transgenic mice with a gain-of-function *Epas1*^{A529V} mutation (corresponding to human *EPAS1*^{A530V}), which demonstrated elevated levels of erythropoietin and polycythemia, a reduced urinary metanephrine-to-normetanephrine ratio, and increased expression of somatostatin in the ampullary region of duodenum. The findings demonstrate the vital roles of *EPAS1* mutations in development of the syndrome and the great potential of the *Epas1*^{A529V} animal model for further pathogenesis and therapeutics studies. The model is also being used to study other malformations in animals as well as to match them with those seen in our patients (neurological, vascular, and ocular malformations) as described below.

Patients referred to the NIH for new, recurrent, and/or metastatic PHEO/PGL were confirmed for the EPAS1 gain-of-function mutation; imaging was evaluated for vascular malformations. We evaluated the *Epas1*^{A529V} transgenic syndrome mouse model, corresponding to the mutation initially detected in the patients (*EPAS1*^{A530V}), for vascular malformations via intravital 2-photon microscopy of meningeal vessels, terminal vascular perfusion with Microfil silicate polymer and subsequent intact *ex vivo* 14T MRI and micro-CT, and histologic sectioning and staining of the brain and identified pathologies. Further, we evaluated retinas from corresponding developmental time points (P7, P14, and P21) and the adult dura via immunofluorescent labeling of vessels and confocal imaging. We identified a spectrum of vascular malformations in all nine syndromic patients and in all our tested mutant mice. Patient vessels had higher variant allele frequency than adjacent normal tissue. Veins of the murine retina and intracranial dura failed to regress normally at the expected developmental time points. The findings add vascular malformation as a new clinical feature of the EPAS1 gain-of-function syndrome.

Currently, the precise role of HIF2α in the predisposition to metastatic disease remains unclear. To assess such a role, we combined gene manipulations in PHEO cell lines with retrospective analyses of patient data and gene-expression profiling in tumor specimens. Among 425 patients with PPGLs identified with mutations in tumor-susceptibility genes, those with tumors resulting from activation of pseudohypoxic pathways had a significantly higher frequency of metastatic disease than those with tumors resulting from activation of kinase-signaling pathways, even without inclusion of patients with mutations in SDHB. Three out of nine (33%) patients with gain-of-function mutations in HIF2α had metastatic disease. In cell-line studies, elevated

expression of HIF2α enhanced cell proliferation and led to increased migration and invasion capacity. Moreover, HIF2α expression in HIF2α–deficient cells resulted in increased cell motility, diffuse cluster formation, and emergence of pseudopodia, indicating changes in cell adhesion and cytoskeletal remodeling. In a mouse liver–metastasis model, Hif2a enhanced the metastatic load. Transcriptomics data revealed alterations in focal adhesion and extracellular matrix–receptor interactions in HIF2α–mutated PHEOs/PGLs. Our translational findings demonstrate that HIF2α supports pro-metastatic behavior in PHEOs/PGLs, although other factors remain critical for subsequent transition to metastasis. We identified *LAMB1* (which encodes laminin subunit beta 1, an extracellular matrix glycoprotein) and *COL4A2* (which encodes six subunits of type IV collagen) as new potential therapeutic targets for HIF2α–driven PHEOs/PGLs. Identified HIF2α downstream targets might open a new therapeutic window for aggressive HIF2α–expressing tumors.

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Mechanisms Regulating GABAergic Cell Development

The incredible diversity and heterogeneity of interneurons was observed over a century ago, with Ramón 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 such as 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 our 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

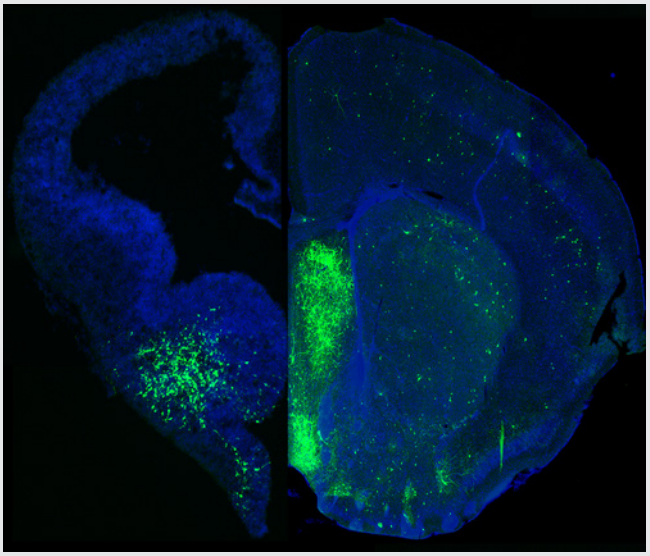


FIGURE 1. MGE–derived GABAergic cells populate many different brain regions.

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, which 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.

We 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 are coordinated to regulate initial fate decisions of MGE progenitors. To this end, we performed a comprehensive single-cell RNA sequencing (scRNA-Seq) analysis of ventricular zone (VZ) and subventricular zone (SVZ) cells throughout the mouse embryonic forebrain. This allows us to compare gene expression profiles both between distinct brain regions [MGE, lateral ganglionic eminence (LGE), caudal ganglionic eminence (CGE), cortex)] and within specific subdomains of these regions (dorsal vs. ventral LGE).

Characterization of the epigenetic landscape during embryonic neurogenesis

In multicellular organisms, cells are genetically homogenous but structurally and functionally heterogeneous as a result of differential gene expression, which 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

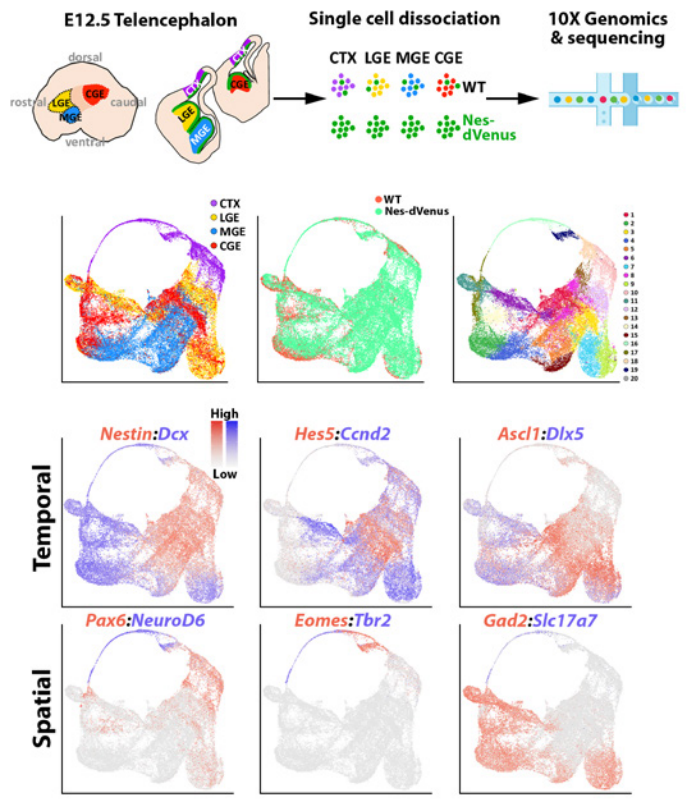


FIGURE 2. Single-cell sequencing in the embryonic mouse forebrain

Top row. Experimental paradigm to harvest cells from four distinct brain regions (MGE, LGE, CGE, cortex) from WT and Nestin-dVenus embryonic mouse brains for single-cell RNA-sequencing.

Middle row. UMAP plots of single cells categorized by brain region (*left*), mouse line (*middle*), or putative cell cluster (*right*).

Bottom rows. UMAP plots depicting gene expression profiles enriched in different stages of neural progenitors (*top*) or spatial domains (*bottom*).

structure in many stem-cell and developmentally critical processes. Previous scRNA-Seq 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. The 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 genome-wide association studies (GWAS) map to non-coding regions, implying that epigenetic regulation of gene expression may underlie some disease etiologies.

We performed single-cell assay for transposase-accessible chromatin with sequencing (scATAC-Seq) in combination with Cut&Tag, also known as cleavage under targets and tagmentation, and Capture-C, a high-throughput approach to analyze *cis* interactions, in order to define the ground truth chromatin state in distinct embryonic brain regions. We are currently expanding on this knowledge to determine how such chromatin and epigenetic organization is disrupted in various gene mutations.

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

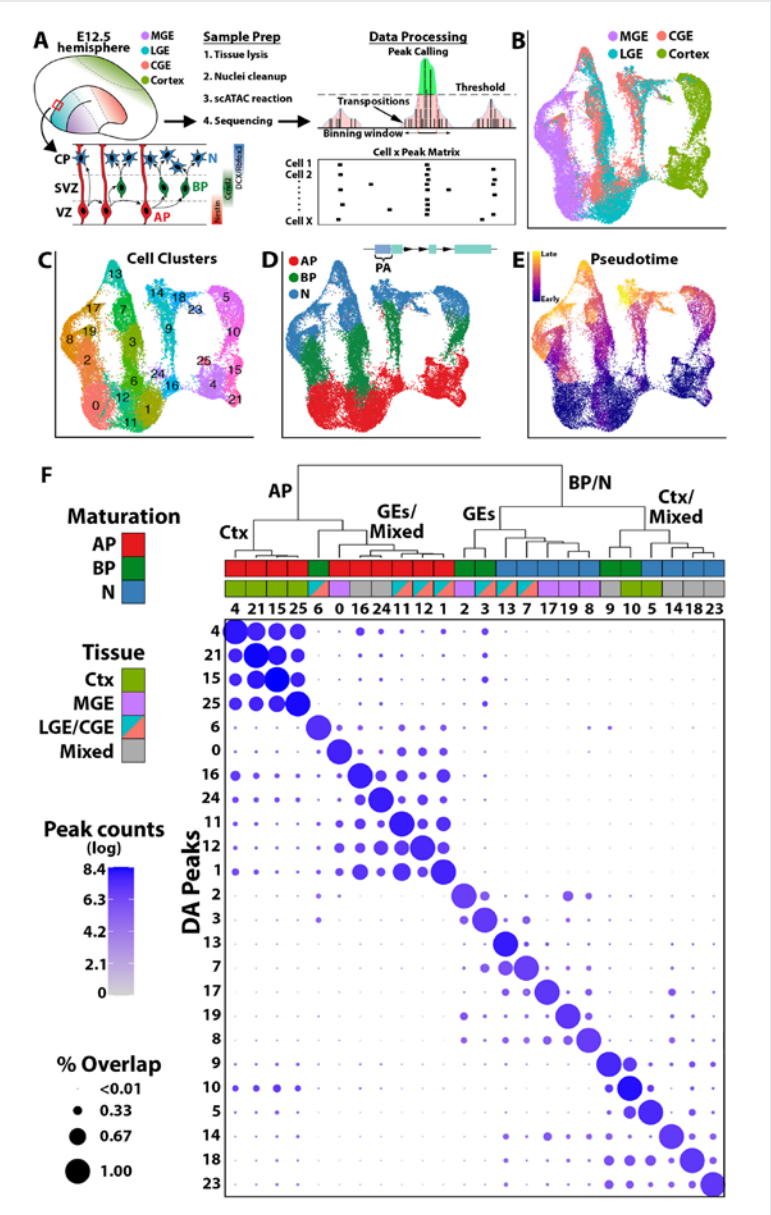


FIGURE 3. snATAC-Seq in distinct regions of the mouse embryonic forebrain

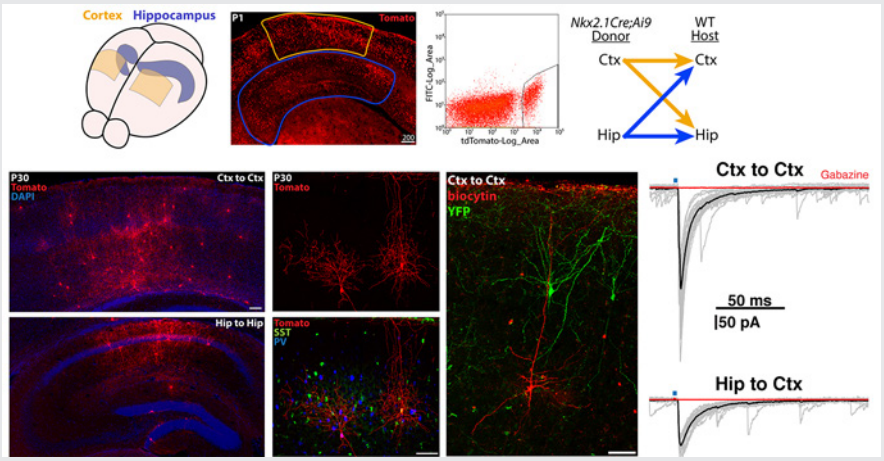
A. Schematic of snATAC-Seq (single-nucleus analysis of transposase-accessible chromatin using sequencing) workflow and neurogenic cell types: apical progenitors (APs), basal progenitors (BPs) and neurons (Ns).

B–E. UMAP visualization of single nuclei clustered by brain region (*B*), SLM (*C*), neurogenic cell type (*D*), and pseudotime (*E*). In (*D*), PA = promoter accessibility, representing reads mapping within 2 kb upstream of TSSs.

F. Embryonic snATAC-Seq dot plot of differentially accessible peaks (DA peaks) for each cluster. Dot diameter indicates the percent of DA peaks from one cluster (column cluster labels) that are detectable in any other cluster (row cluster labels). Color intensity represents the total DA peak count per cluster. Hierarchical clustering was performed using correlation distance and average linkage.

interneuron subtypes varies significantly between different brain regions. Numerous experiments indicate that general interneuron classes, e.g., PV^{+}– or SST^{+}–expressing interneurons, are determined as cells become post-mitotic during embryogenesis. However, 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 alternative hypothesis is that immature interneurons are already genetically hardwired into definitive subgroups, and that the environment more passively sculpts the maturation of these cells.

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.

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.

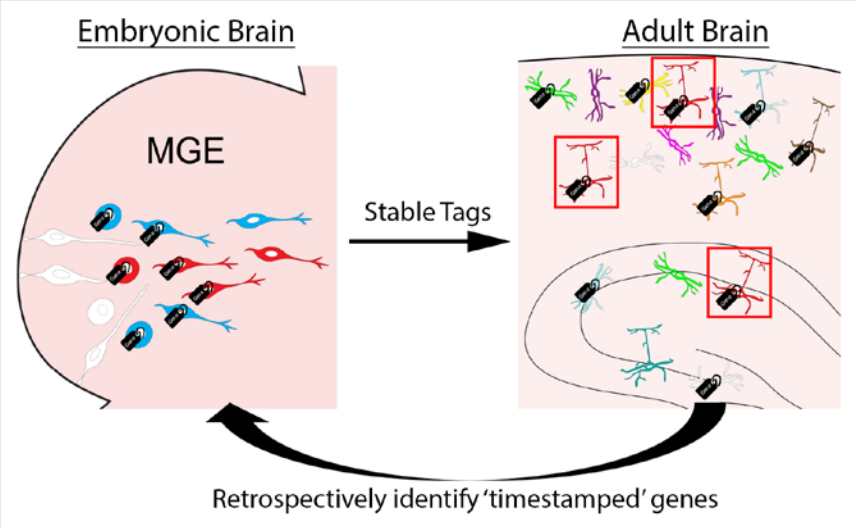
Novel approach to identifying genetic cascades underlying interneuron fate decisions

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, such 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 separate interneuron progenitors from other cell types. Additionally, many markers that define mature interneuron subtypes are not expressed embryonically, and thus the class-defining markers are not helpful for studying MGE progenitors.

In an ideal scenario, we would like to identify actively transcribed genes in MGE progenitors undergoing fate decisions while retaining the capacity to identify whether such cells become PV– or SST–expressing interneurons in the postnatal brain. To this end, we are developing a spatially and temporally inducible form

FIGURE 5. Timestamp of actively transcribed genes during development for future analysis

The goal of this approach is to label actively transcribed genes with stable methylation tags during embryogenesis as progenitors are undergoing initial fate decisions in the MGE. We can then harvest specific interneuron subtypes in the adult brain using various transgenic mouse lines. Retrospective identification of an actively transcribed gene during embryogenesis will provide us with candidate fate-determining genes for specific interneuron subtypes.



of DNA adenine methylase identification (DamID), which will allow us to label the transcriptome of MGE progenitors. Once we have the tools to distinguish between specific interneuron cell types, labeled cells can be harvested at maturity. 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 *H19* ICR, which is located just upstream of the *H19* promoter. We showed that the molecular function of the *H19* ICR is to organize the region into alternative 3D structures. In humans, epigenetic mutations that disrupt *H19* ICR function result in loss of monoallelic expression. Mutations in the paternal *H19* ICR lead to loss of *Igf2* expression and biallelic (2X) *H19* expression and are associated with the Russell-Silver syndrome. Mutations in the maternal *H19* ICR 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 mis-expression 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.

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Alternative long-range interactions between distal regulatory elements establish allele-specific expression at the *Igf2/H19* locus.

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 *H19* ICR, which lies between the two genes, just upstream of

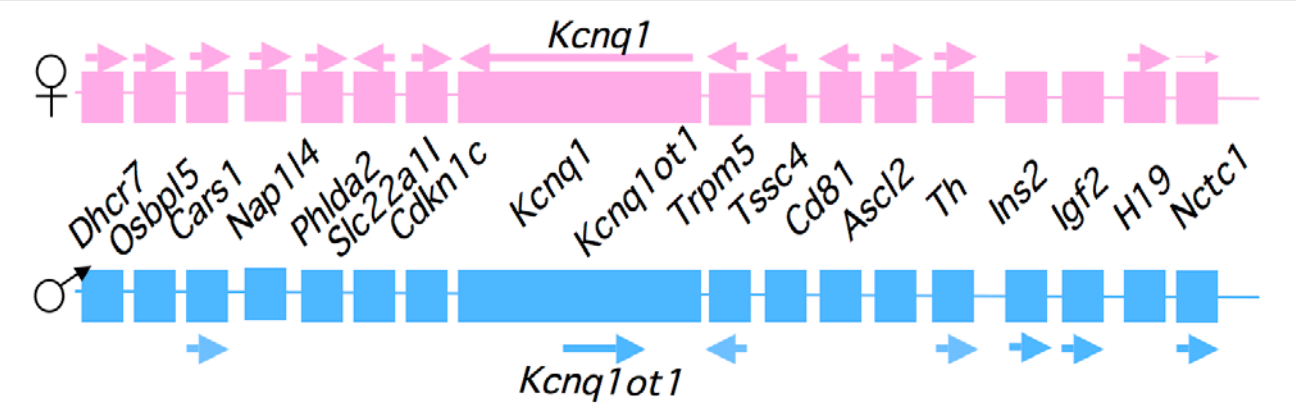


FIGURE 1. An imprinted domain on mouse distal chromosome 7

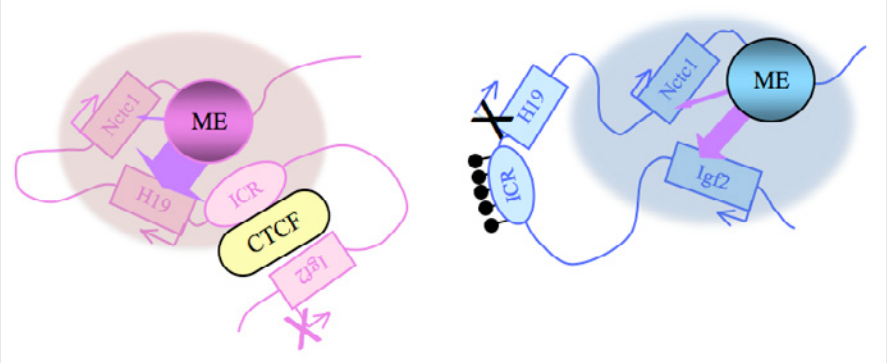
Maternal (*pink*) and paternal (*blue*) chromosomes are indicated. Horizontal arrows denote RNA transcription.

the *H19* promoter (Figure 2). On the maternal chromosome, binding of the CTCF protein, a transcriptional repressor, to the *H19* ICR establishes a transcriptional insulator that organizes the chromosome into loop structures that bring the *H19* promoter into contact with downstream enhancers but exclude the *Igf2* promoter from these enhancer interactions. 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

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.



imprint these regions. Furthermore, 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 the 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 spliced, polyadenylated long noncoding RNA (lncRNA), which 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 (*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.

The lncRNA, encoded by *Nctc1*, is an essential element of the muscle enhancer, as demonstrated by transient transfection analyses that define a 300–bp element that is both necessary and sufficient for maximal enhancer activity. However, stable transfection and mouse mutations indicate that this core element is not sufficient for enhancer function in a chromosomal context. Instead, the *Nctc1* promoter element is also essential; the *Nctc1* RNA itself is not required (at least in *trans*). Instead mutational analysis demonstrates that it is *Nctc1* transcription through the core enhancer that is necessary for enhancer function. Curiously, the *Nctc1*

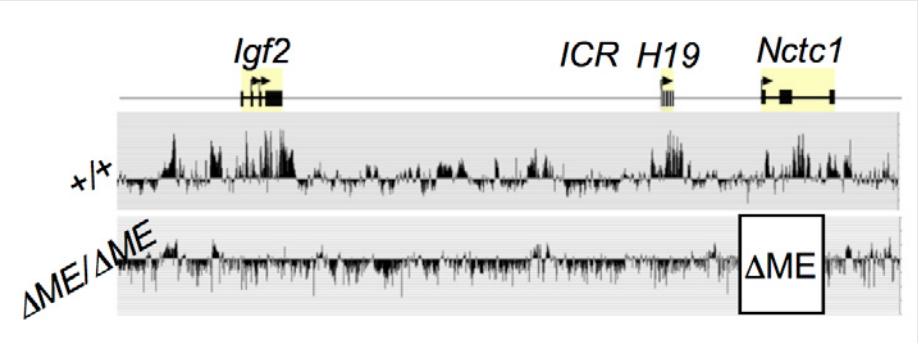


FIGURE 3. The shared muscle enhancer (ME) directs RNAP binding and RNA transcription across the entire 150 kb locus.

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. To demonstrate this, we used ChIP-on-chip to analyze RNAP localization on chromatin prepared from wild-type and 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 only at specific localized sites but across the entire domain. The results support a facilitated tracking model for enhancer activity.

RNAP binding at 'real' genes and across the intergenic regions is qualitatively different. To demonstrate this, we used naturally occurring single-nucleotide polymorphisms (SNPs) to investigate allelic differences in binding of RNAP and activation of gene expression in wild-type cells and in cells carrying enhancer deletions or insulator insertion mutations. RNAP binding across the *Igf2* and *H19* genes is both enhancer-dependent and insulator-sensitive; that is, a functional insulator located between an enhancer and its regulated gene prevents RNAP binding and likewise prevents RNA transcription. Across the intergenic regions, RNAP binding and RNA transcription are similarly enhancer-dependent (see above). However, intergenic RNAP binding and transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as 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, because 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 against 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.

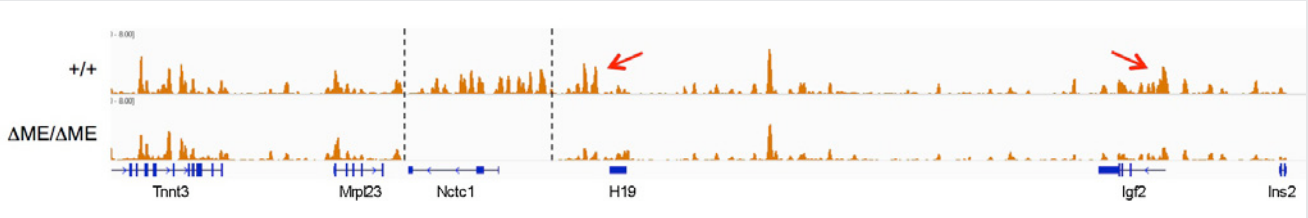


FIGURE 4. Chromatin patterns at the *Igf2/H19* locus are independent of enhancer activity.

Chromatin was isolated from wild-type and enhancer-deletion muscle cells, using antibodies against H3K4me1, and analyzed by DNA sequencing.

Functions of *H19* lncRNA in regulating cell-cycle progression and senescence

To determine the biochemical functions of *H19* lncRNA, we use *in vitro* models including primary myoblasts, C2C12 myoblasts, and NIH3T3 cells. Abrupt depletion of *H19* by either siRNA or cre-induced recombination of *H19*–floxed alleles results in increased p21 RNA (p21 is a cyclin-dependent kinase inhibitor involved in cell-cycle arrest) and peptide, and such increased p21 activity in turn prevents cell-cycle progression and induces cellular senescence. *H19* lncRNA regulation of p21 is at the level p21 mRNA stability and translation efficiency and occurs via the p21 3'-UTR. Genetic and biochemical analyses suggest that H19 lncRNA facilitates interactions between p21 mRNA and Wig1. Current experiments focus on identifying the molecular mechanisms for these regulatory actions.

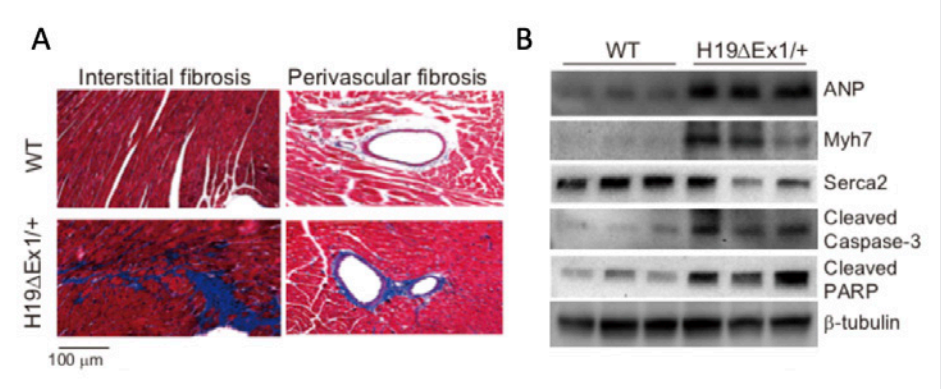
Functions of *H19* lncRNA in regulating cardiac development

The Beckwith-Wiedemann 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 such children. Moreover the children show high frequency of cardiac dysfunction. Taken together, such findings 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 elevated levels of circulating IGF2 peptide, which super-activates 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, i.e., 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.

FIGURE 5. Cardiac disease in *H19*-deficient mice

Mice lacking *H19* are hypertrophic, fibrotic (*panel A*), display protein expression profiles typical of cardiac failure (*panel B*), and show aberrant function on echocardiograms.

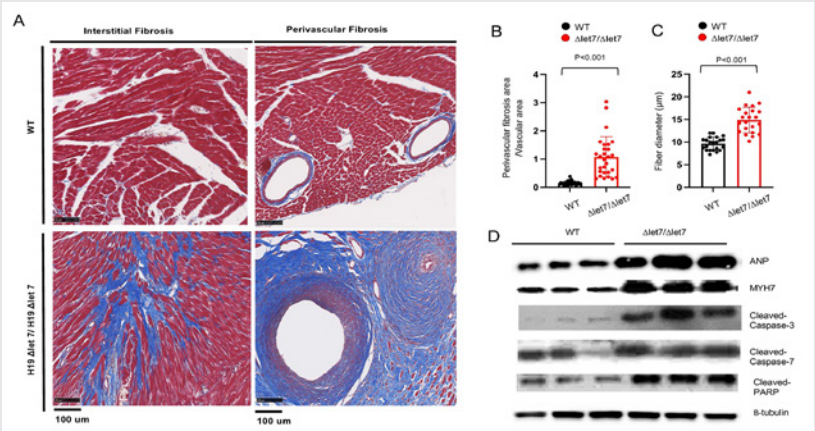


Loss of expression of *H19* is pathologic (Figure 4). Hearts show progressive heart disease, as manifested by hypertrophy, increased fibrosis, expression of cardiac failure markers, and reduced and abnormal heart function, as measured by echocardiography. *H19* expression in hearts is restricted to endothelial 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 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 notion that *H19* regulates the cell fate of endothelial cells, and future experiments aim to identify the underlying molecular mechanisms.

The *H19* gene does not encode a protein. Rather H19 RNA is the functional gene product. The *H19* precursor RNA is processed into two final products: a 2.3 kb lncRNA and microRNA 675. To determine which *H19* RNA is essential to prevent cardiomyopathy, we developed two new mouse strains that selectively disrupt accumulation of the lncRNA or the miRNA. Disruption of the lncRNA is necessary and sufficient to induce cardiac disease. To understand the molecular bases for *H19* lncRNA function, we generated alleles that disrupt specific domains. We observe that disruption of *let7* miRNA binding sites on the H19 RNA results in the same myopathies as complete gene ablation (Figure 6).

FIGURE 6. Let7 binding sites on H19 lncRNA are essential for normal heart function.

Mice carrying H19 alleles that do not include let7 binding sites are fibrotic (*panels A and B*), hypertrophic (*panel B*), and display protein expression profiles typical of cardiac failure.



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Rare Genetic Disorders of Cholesterol Homeostasis and Lysosomal Diseases

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 the Smith-Lemli-Opitz syndrome (SLOS), lathosterolosis, desmosterolosis, X-linked dominant chondrodysplasia punctata type 2 (CDPX2), and the CHILD syndrome. We also undertake complementary basic and clinical research efforts to study Niemann-Pick disease type C (NPC) and Juvenile Batten disease caused by pathogenic variants of *CLN3.* Both NPC and CLN3 are lysosomal diseases that result in progressive neurodegeneration. Our research group uses basic, translational, and clinical research approaches with the ultimate goal of developing and testing therapeutic interventions for these rare genetic disorders. Our basic research uses induced pluripotent stem cells (iPSC)–derived 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. We have large cross-sectional and longitudinal collections of biomaterial from individuals whose disease course and phenotype are known, and such samples can be used for both for biomarker discovery and validation. Therapeutic trials have been conducted for SLOS and NPC1. A therapeutic trial for CLN3 disease is being initiated. Also, 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

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phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die as result of multiple major malformations, while mild SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is the result of 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*$^{T93M/delta3–5}$ and 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 iPSCs from SLOS patients demonstrated a defect in neurogenesis, which results from inhibition of Wnt signaling owing to a toxic effect of 7-DHC.

2021 Research Group

Research Group Members (left to right)

Top row: Samar Rahal, Denny Porter, Chris Wassif, Niamh Cawley, An Dang Do

Middle row: Andrea Munoz, Nicole Farhat, Derek Alexander, Kisha Jenkins, Andrea Gutierrez Maria, Insung Kang, Dorie Scordilis

Bottom row: Tristan Furnary, Rachel Luke, Andrew Smith, Kyli McKee, Katie Scott

FIGURE 1.

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

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 125 SLOS patients. The studies are now focused on defining the phenotype of older adolescents and young adults with SLOS.

A laboratory observation that SLOS fibroblasts can develop an NPC–like cellular phenotype with endolysosomal storage of unesterified cholesterol is leading to development of a therapeutic trial. This is a prime example of how our research group translates basic research to clinical research.

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.

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 enrolled over 130 NPC1 patients in a longitudinal Natural History trial. The goals of the trial are to identify (1) a blood-based diagnostic/screening test, (2) biomarkers that can be used as tools to facilitate development and implementation of therapeutic trials, and (3) clinical symptoms/signs that may be used as efficacy outcome measures in a therapeutic trial.

Currently, the average time from first symptom to diagnosis, the 'diagnostic delay,' in our cohort of NPC patients is on the order of four to five years. In collaboration with Daniel Ory, we found elevated levels of non-enzymatically produced oxysterols in NPC1 patients. Testing for oxysterols or bile acid derivatives has now become a standard method of diagnosis, and they are a potential biomarker that may be used to follow therapeutic interventions. We are now involved in a collaboration to implement newborn screening for NPC.

In addition to our Natural History study, we completed a randomized, placebo-controlled, cross-over trial to investigate the safety and efficacy of *N*-acetyl cysteine (NAC) in NPC1. The goal was to determine whether NAC treatment would reduce oxidative stress and subsequently lower levels of the non-enzymatically produced oxysterols. 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

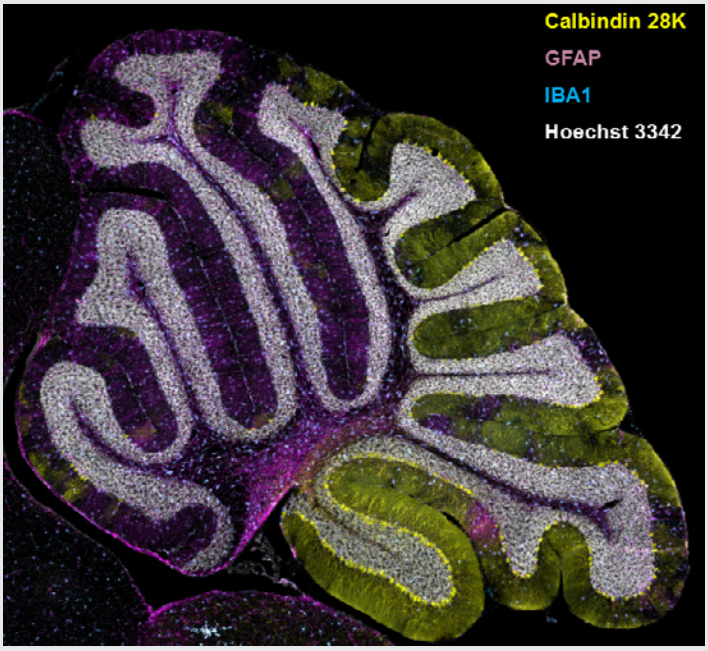

FIGURE 2. Gliosis in NPC1 mouse cerebellum

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.

Program of NCATS, we completed a phase 1/2a therapeutic trial of lumbar intrathecal cyclodextrin (VTS-270, adrabetadex) therapy in NPC1. We participated in a multicenter, international phase 2b/3 of adrabetadex and investigated the safety and efficacy of combined intrathecal and intravenous adrabetadex. In collaboration with investigators at St. Louis Children's Hospital, we study the efficacy of cyclodextrin to ameliorate liver disease in infants with NPC.

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. In collaboration with investigators from NHGRI and Scripps, we are obtaining genomic sequence on a large cohort of well phenotyped individuals with NPC1. Utilizing novel machine learning/artificial intelligence techniques, we are attempting to gain insight into genetic modifiers of the NPC1 phenotype.

Development of NPC– induced pluripotent stem cell lines that can be efficiently differentiated into neurons has permitted us to initiate a number of studies in collaboration with NCATS to find drugs/genes that decrease endolysosomal cholesterol storage in NPC1 neurons.

CLN3 Disease

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

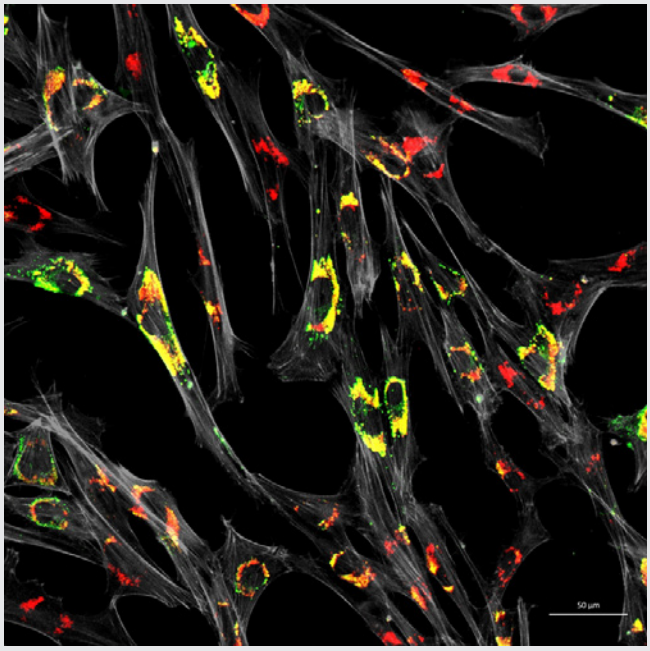

FIGURE 3. Accumulation of unesterified cholesterol in NPC1 patient fibroblasts

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*).

underlying these disorders as well as to develop therapeutic interventions. A major effort in our laboratory is to identify biomarkers that provide insight into CLN3 pathology and facilitate therapeutic trials. A diagnostic metabolite, especially one that could be used in a newborn screen, would be a major advance in the field.

To complement our clinical research, we are also studying CLN3 in our laboratory. We have developed an induced pluripotent stem cell line that can efficiently be induced to form CLN3–deficient neurons. The neurons are being used in genome-wide CRISPRi screens to identify genes that modify the CLN3 neuronal phenotype. The induced pluripotent stem cells (iPSC)–derived neurons will also be used to screen for potential therapeutic drugs.

Creatine transport deficiency

In collaboration with NCATS, we initiated a natural history trial of creatine transport deficiency (CTD). CTD is an X-linked disorder arising from mutation of *SLC6A8* (which encodes solute carrier family 6 Member 8, a sodium- and chloride-dependent creatine transporter). Individuals with CTD manifest significant developmental delay and have frequent seizures. The work on CTD is a multicenter trial conducted in collaboration with NCATS and Ultragenyx. 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. A major clinical finding of this natural history trial was the detailed characterization of prolonged electrocardiographic QTc in many of the individuals with CTD, which has led to specific clinical recommendations.

Additional Funding

- Ara Parseghian Medical Research Fund, University of Notre Dame
- Together Strong NPC Foundation
- NCL-Stiftung Award

- Ultragenyx CRADA
- Mandos Health CRADA
- Amicus CRADA
- Beyond Batten Disease Foundation CRADA
- NICHD Scientific Director's Award
- NICHD Strategic Plan Award
- Dana's Angels Research Trust

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

Our lab seeks to understand cell-lineage differentiation, gene regulation, and how non-coding DNA elements and the 3D architecture of chromosomes contribute to such 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.

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 to understand how such processes occur *in vivo* and how their regulation dictates cell identity and cell-fate decisions in mammals.

To do so, we combine the robustness of mouse-genome editing and genetics with cutting-edge sequencing-based genomic techniques such as ATAC-Seq (assay for transposase-accessible chromatin using sequencing), ChIP-Seq (chromatin-immuno-precipitation DNA-sequencing), and Hi-C (high-throughput chromosome conformation capture technique), 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 that 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 they impact future developmental decisions.

Following fertilization and within a few cell divisions, the first cell lineages are established and different gene-expression programs are

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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 such questions, we will employ several high-throughput technologies that we have established in the lab, in combination with genome-wide CRISPR screens. Ultimately, we will fully characterize *in vivo* candidates identified this way to stringently determine their impact on gene regulation during mammalian development.

Additional Funding

- DIR Scientific Director's Award

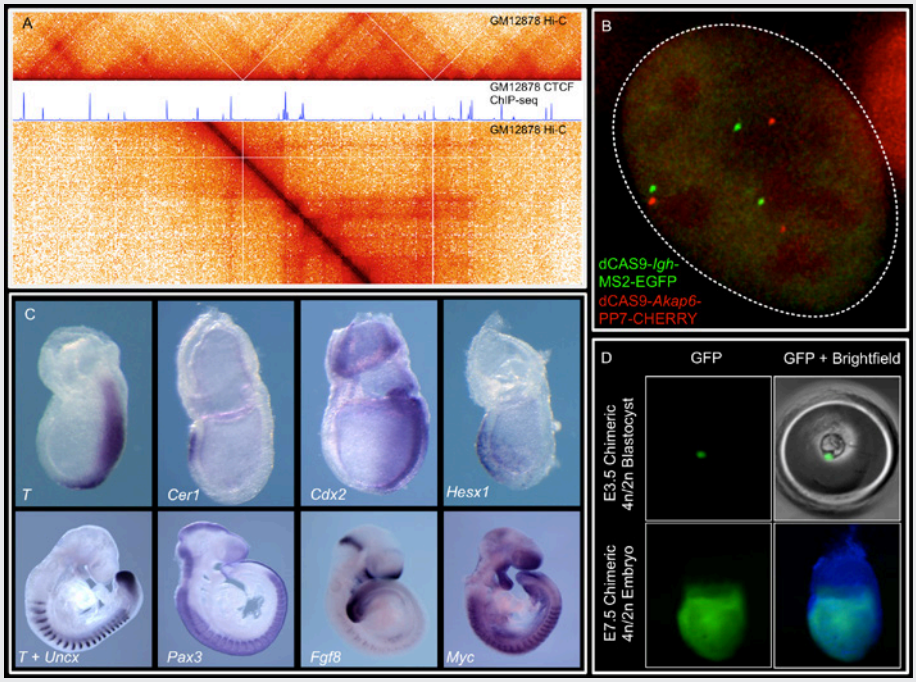
Representative image of the lab's research

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 (green fluorescent protein)–labeled ES (embryonic stem) cells allows generation of fully ES cell–derived embryos.

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Signaling Interpretation during Vertebrate Embryogenesis

To create the different tissues required in healthy adults, embryos must activate fate-specifying genes in a variety of stereotyped positions (Figure 1A), which is mediated by signaling molecules that spread though embryonic tissues. It is thought that signaling levels and dynamics regulate gene expression, and that position-specific signaling differences underlie the diverse gene expression patterns required for normal development. However, it is not clear what signaling features are 'read out' by genes and how those features are converted into differential gene expression during vertebrate embryogenesis.

We investigate how signaling molecules spread through embryonic tissues, how signaling levels and dynamics are decoded, and how many pathways cooperate to pattern the body plan (Figure 1B). To directly examine these processes, we need precise *in vivo* experimental manipulations that are difficult to achieve with conventional genetic and pharmacological methods. In contrast, molecular optogenetic approaches can offer tunable, reversible manipulation of biological processes with excellent temporal (seconds) and spatial (subcellular) resolution, and such approaches have been robustly demonstrated both *in vitro* and *in vivo*. Using the microscopy-friendly zebrafish embryo as a vertebrate model system, our lab will harness established optogenetic approaches and develop new ones to investigate patterning mediated by conserved signaling pathways.

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William K. Anderson, BS, *Research Technician*

Catherine E. Rogers, BS, *Postbaccalaureate Fellow*

How do signaling molecules move through tissues?

The distribution of signaling molecules within developing tissues helps determine patterns of gene expression. Competing models have been proposed to explain how signaling-molecule distributions are established: signals may diffuse away from producing cells through the extracellular space, move through cells (trans-cytosis), or be confined to the producing cells themselves. We will develop optogenetic tools to probe how factors, including extracellular diffusion and trans-cytosis, affect signaling molecule distribution and use these with *in vivo* methods, including FRAP (fluorescence recovery after photobleaching) and FDAP (fluorescence decay after photoactivation) (Figure 2), to directly measure signaling molecule mobility and stability. This will help determine how signaling-molecule distribution is regulated during zebrafish embryogenesis.

What information is encoded in signaling gradients?

Signaling gradients are found in developing tissues from the fly wing precursor to the mammalian neural tube. The classic morphogen model proposes that the precisely graded distribution of signaling is important because genes are activated by different signaling levels. Alternatively, a simple signaling asymmetry may suffice to pattern tissues in some contexts. The relatively subtle signaling perturbations required to distinguish between these models can be difficult to achieve *in vivo*. We will develop optogenetic approaches and use

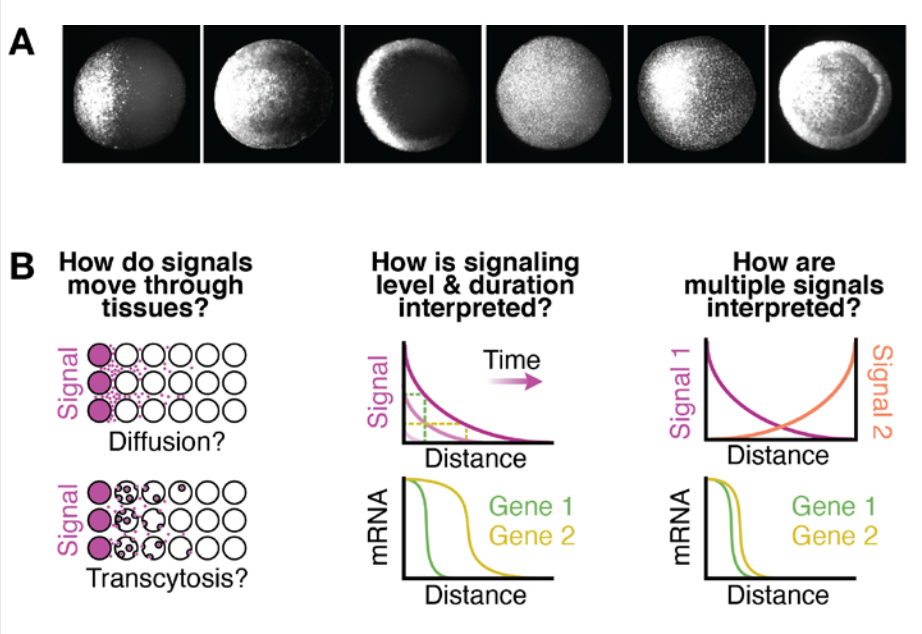


FIGURE 1.

A. Gene expression in the zebrafish embryo;

B. outstanding developmental questions

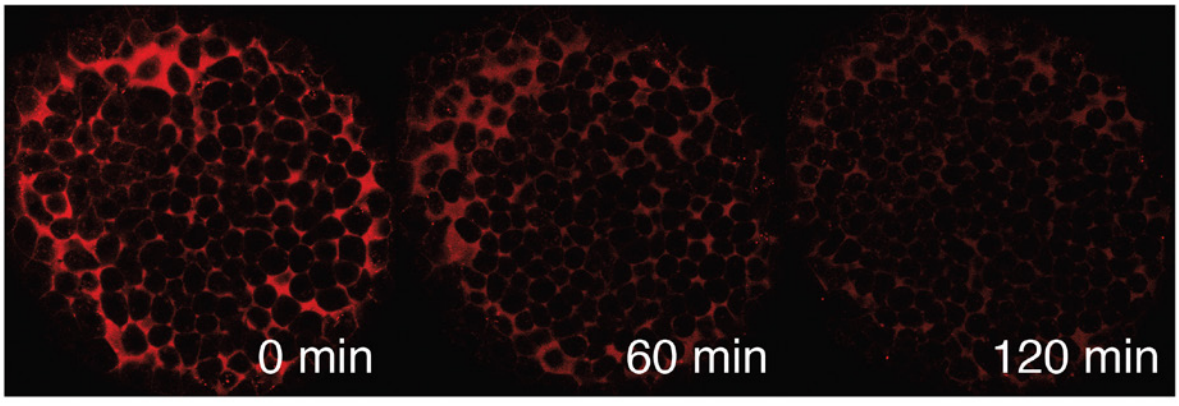
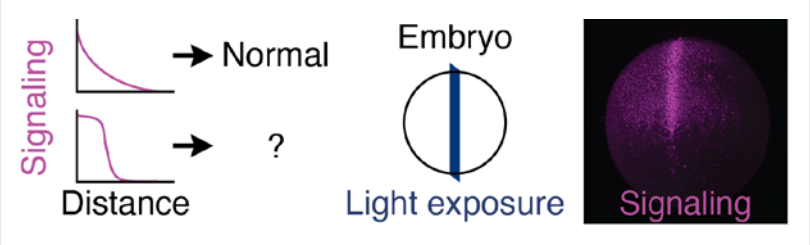


FIGURE 2.

Measuring signaling-molecule stability *in vivo* using FDAP

FIGURE 3.

Spatial gradient manipulation



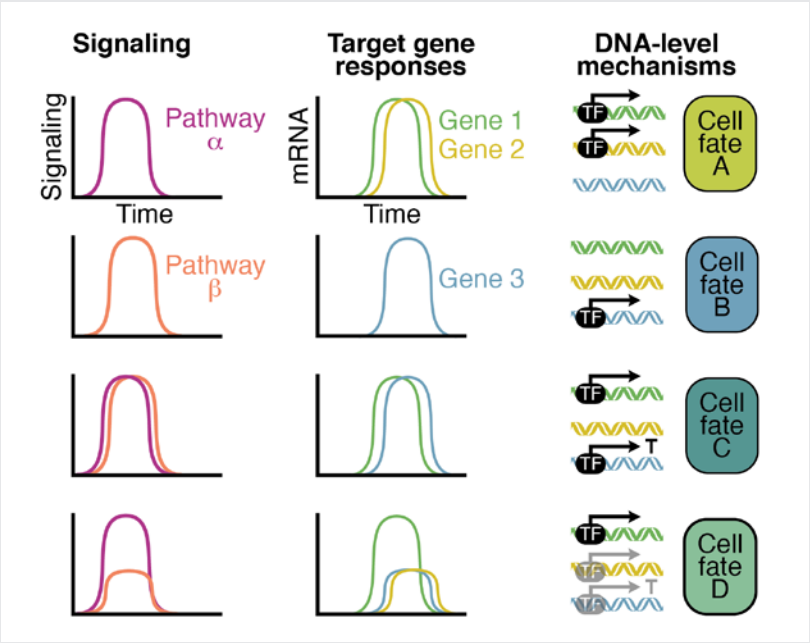
them with a digital micromirror device to introduce novel signaling distributions in zebrafish embryos and assess patterning consequences (Figure 3). This will determine the spatiotemporal signaling requirements for normal tissue patterning during early zebrafish development.

How are signaling levels, dynamics, and combinations interpreted in the embryo?

Cells in developing tissues experience a variety of signaling levels and dynamics, as well as simultaneous signaling from several pathways. How do different genes respond to these inputs? We seek to determine the input/output relationship between signaling and gene expression during early vertebrate embryogenesis. To achieve this, we will develop orthogonal optogenetic tools to manipulate signaling levels and dynamics in zebrafish embryos. We will characterize gene responses and investigate the DNA–level mechanisms responsible for differential responses (Figure 4). This will help elucidate which features of signaling encode information and help explain how the diverse gene expression patterns needed to produce healthy adults are robustly generated.

FIGURE 4.

Orthogonal opto-signaling experiment



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

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. The cost of prematurity in the U.S. alone is estimated to be $26 billion per year. An important goal is to understand the mechanisms of disease responsible for spontaneous preterm birth and fetal injury and to develop methods for the prediction and prevention of preterm birth. The Perinatology Research Branch (PRB) 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 the Branch is to study intra-amniotic infection and inflammation, vascular disorders, maternal anti-fetal rejection (chronic inflammatory lesions of the placenta), cervical disease, and a decline in progesterone action.

However, as has been the case for many clinical and translational research enterprises, an additional focus of ours the last 18 months has been on determining the extent to which the SARS-CoV-2 infection is influencing and potentially exacerbating previously identified causes of preterm birth and other pregnancy complications. We reported that SARS-CoV-2 infection is strongly associated with preeclampsia and preterm birth. Furthermore, SARS-CoV-2 infection in the mother, even in asymptomatic cases, induces unique maternal and fetal inflammatory responses at the maternal-fetal interface (placental tissues) and in the neonate (umbilical cord blood).

In addition to spontaneous preterm birth, 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 permits the definition of fetal anatomy, biometry, growth, and the study of physiologic parameters, such as cardiac function, fetal sleep, and breathing.

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Chief, Perinatology Research Branch

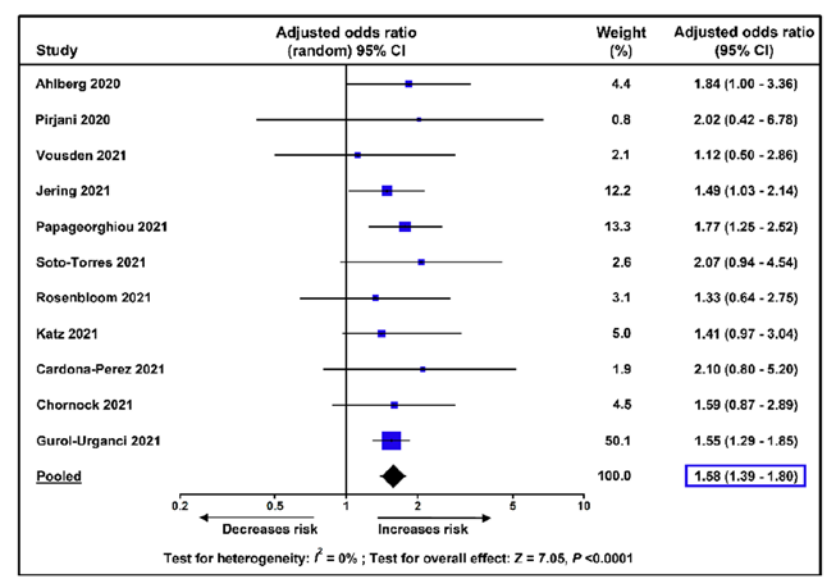

FIGURE 1.

Meta-analysis of adjusted odds ratios for the association between SARS-CoV-2 infection during pregnancy and preeclampsia

SARS–CoV–2 infection during pregnancy and the risk of preeclampsia

Preeclampsia, a multisystem syndrome that complicates about 5% of pregnancies, is one of the leading causes of maternal mortality worldwide, accounting for approximately 14% of all maternal deaths. During the current pandemic, epidemiological studies have shown that pregnant women with SARS-CoV-2 infection have a significantly higher risk of maternal death, admission to the intensive care unit, preterm birth, and stillbirth than those without SARS-CoV-2 infection. Hence, we performed a systematic review and meta-analysis to assess whether SARS-CoV-2 infection during pregnancy also increases the risk of preeclampsia. A total of 28 studies that included 790,954 pregnant women, among which 15,524 were diagnosed with SARS-CoV-2 infection, met the inclusion criteria. Overall, meta-analyses of unadjusted and adjusted risk estimates showed that pregnant women with SARS-CoV-2 infection had a significantly higher risk of preeclampsia than did pregnant women without SARS-CoV-2 infection. Moreover, there was a significant increase in the odds of preeclampsia with severe features, such as eclampsia and HELLP syndrome, among pregnant women with SARS-CoV-2 infection, as compared with those without the infection. Both asymptomatic and symptomatic SARS-CoV-2 infections significantly increased the odds of preeclampsia, although it was higher among patients with symptomatic illness than among those with asymptomatic illness (Figure 1). We concluded that SARS-CoV-2 infection during pregnancy is associated with higher odds of preeclampsia and hypothesized that this relationship may be causal. The findings of our study have clear implications for patient care, public health policy, and future research.

SARS–COV–2 and the subsequent development of preeclampsia and preterm birth: evidence of a dose response relationship supporting causality

Pregnant women infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have a worse clinical outcome than non-pregnant women infected with SARS-CoV-2. Such adverse outcomes include admission to the intensive care unit, use of invasive mechanical ventilation, and even death. Case series, systematic reviews, and meta-analyses showed an association between SARS-CoV-2 infection and a higher

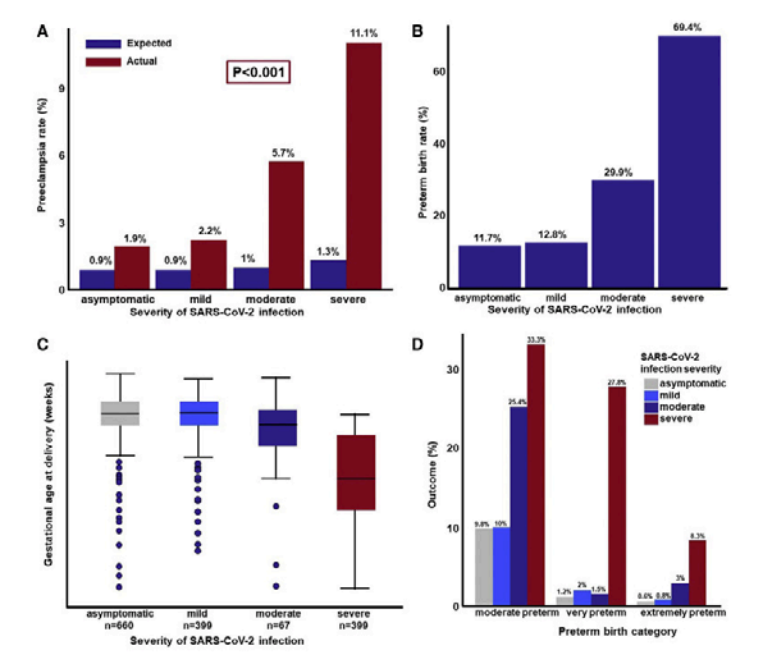

FIGURE 2. Association between SARS-CoV-2 infection severity and pregnancy outcomes

A. Expected and observed rates of preeclampsia in women with SARS-CoV-2 infection.

B. Observed rates of preterm birth in women with SARS-CoV-2 infection who had a live neonate.

C. Gestational age at delivery in women with SARS-CoV-2 infection who had a live neonate.

D. Rate of moderate, very, and extreme preterm birth as a function of the severity of SARS-CoV-2 infection.

risk for developing preeclampsia; yet, causality needs to be determined. To investigate causality between SARS-CoV-2 infection during pregnancy and preeclampsia, we conducted a retrospective observational study based on data from 14 National Health Service (NHS) maternity hospitals in the United Kingdom, where our collaborators are located. Our study cohort included 1,223 pregnant women with a positive SARS-CoV-2 PCR test. One of the Bradford Hill criteria to assess causality is the existence of a dose-response relationship between exposure and the outcome of interest, in this case, the severity of SARS-CoV-2 infection and the likelihood of developing preeclampsia. The severity of infection with SARS-CoV-2 was defined as asymptomatic, mild, moderate, or severe, and its effect on the rate of preeclampsia and preterm birth was assessed using robust Poisson regression models and chi-square tests for trend. The observed rate of preeclampsia diagnosed at or after SARS-CoV-2 infection was higher than expected (Figure 2A). Patients with severe COVID-19 had a five-fold greater risk of preeclampsia than asymptomatic patients. The relative risk of developing preeclampsia in women with moderate or severe COVID-19 was 3.3-fold higher than in those with asymptomatic/mild infection. The monotonic relationship between the severity of COVID-19 and the risk of developing preeclampsia was significant according to a test for trend. There was also a dose response relationship between the severity of SARS-CoV-2 infection and the risk and severity of preterm birth (Figure 2B-D). In conclusion, the study provides evidence that the more severe the infection with SARS-CoV-2, the greater the risk of preeclampsia and preterm birth. SARS-CoV-2 infection can lead to endothelial dysfunction, intravascular inflammation, proteinuria, activation of thrombin, and hypertension, which are all features of preeclampsia. Therefore, it is likely that SARS-CoV2 infection during pregnancy causes preeclampsia.

Maternal and fetal immune responses in pregnant women infected with SARS–CoV–2

To date, over 118,000 pregnant women in the United States have been infected with SARS-CoV-2, the virus responsible for the coronavirus disease 2019 (COVID-19). Pregnant women with SARS-CoV-2 infection are at a

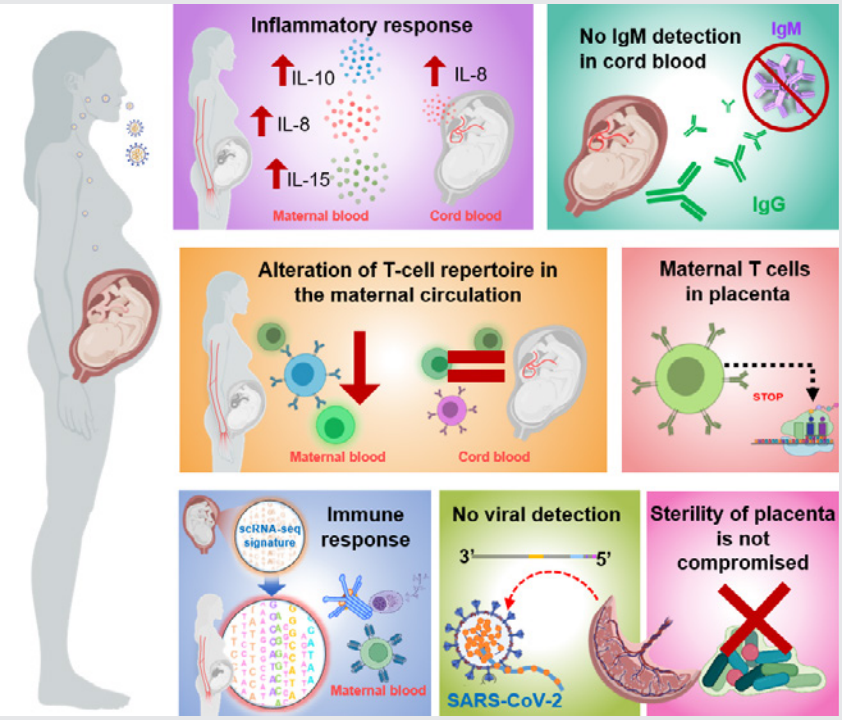

FIGURE 3. Maternal and fetal immune responses in pregnant women infected with SARS-CoV-2

Overall study design showing the detection of SARS-CoV-2 IgM/IgG, multiplex cytokine assays, immuno-phenotyping, single-cell transcriptomics, and viral RNA and protein detection that were applied to investigate host immune responses in pregnant women infected with SARS-CoV-2.

high risk for severe/critical disease and mortality as well as preterm birth. Therefore, we investigated the host immune responses in pregnant women infected with SARS-CoV-2, even if they were asymptomatic. The studies comprise a multidisciplinary approach including the detection of SARS-CoV-2 IgM/IgG, multiplex cytokine assays, immunophenotyping, single-cell and bulk RNA sequencing, and viral RNA and protein detection (Figure 3), together with the assessment of the microbiome diversity and histopathology of the placenta, to characterize the maternal-fetal immune responses triggered by SARS-CoV-2 during pregnancy. We gathered evidence showing that SARS-CoV-2 infection during pregnancy primarily induced unique inflammatory responses in the circulation and at the maternal-fetal interface, with the latter being governed by maternal T cells and fetal stromal cells. SARS-CoV-2 infection during pregnancy is also associated with a mild cytokine response in the fetal circulation (i.e., umbilical cord blood) without compromising the T-cell repertoire or initiating IgM responses. Moreover, bulk RNA sequencing of maternal and cord blood revealed that SARS-CoV-2 infection differentially impacts the transcriptome of the mother and neonate, and correlation analyses between bulk RNA-Seq blood data and single-cell placental data, indicated that maternal and neonatal transcriptomic changes are partly shared with those in the placental tissues. Importantly, SARS-CoV-2 has not been detected in the placental tissues, nor was the sterility of the placenta compromised by maternal viral infection. The study provides insight into the maternal-fetal immune responses triggered by SARS-CoV-2 and further emphasizes the rarity of placental infection.

Crowdsourcing assessment of maternal blood multi-omics for predicting gestational age and preterm birth

Identification of pregnancies at risk of preterm birth, the leading cause of newborn deaths, remains challenging given the syndromic nature of the disease. We reported a longitudinal multi-omics study coupled

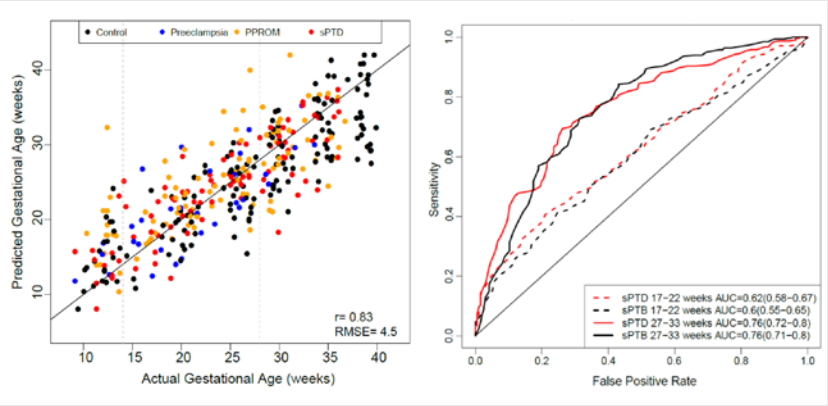

FIGURE 4. Prediction of gestational age and preterm birth using maternal blood omics data

The figure shows blood RNA–based predicted gestational ages in normal and complicated pregnancies versus the actual values (*left*), and sensitivity of plasma protein models as a function of false positive rate for prediction of preterm birth based on samples collected at 17–22 and 27–33 weeks in asymptomatic women (*right*). sPTB: spontaneous preterm birth; sPTD; spontaneous preterm birth with intact membranes; PPROM: preterm, prelabor rupture of membranes.

with a crowdsourcing initiative (DREAM challenge) to develop predictive models of gestational age and spontaneous preterm birth. The computational challenge was based on previously published and new data the Branch generated, while incentives for participating in the challenge were funded by Wayne State University and the March of Dimes. The findings indicate that whole-blood gene expression predicts ultrasound-based gestational ages at blood draw in normal and complicated pregnancies. Based on samples collected before 33 weeks in asymptomatic women, our analysis suggests that gene-expression changes preceding preterm prelabor rupture of the membranes are consistent across time points and cohorts, and involve leukocyte-mediated immunity. Models built from plasma proteomic data predict spontaneous preterm delivery with intact membranes with higher accuracy and earlier in pregnancy than transcriptomic models (Figure 4). The work therefore identified RNAs and proteins in the maternal circulation predictive of spontaneous preterm birth in asymptomatic patients that can be further evaluated in larger targeted studies. Early prediction of women at risk could enable preventive strategies. Moreover, a byproduct of the study is a suite of methods and software tools for longitudinal omics data that can be applied to the study of other adverse pregnancy outcomes to identify biomarkers. These methods are available at *https://www.synapse.org/pretermbirth*.

The amniotic fluid cell-free transcriptome in spontaneous preterm labor

Amniotic fluid cell-free RNA was shown to reflect physiological and pathological processes in pregnancy, but its value in the prediction of spontaneous preterm delivery is unknown. We therefore profiled cell-free RNA in amniotic fluid samples collected from women who underwent transabdominal amniocentesis after an episode of spontaneous preterm labor and subsequently delivered within 24h or later in gestation. Expression of known placental single-cell RNA-Seq signatures was quantified in amniotic fluid cell-free RNA and compared between the groups. We applied random forest models to predict time-to-delivery after amniocentesis. We found 2,385 genes differentially expressed in samples of women who delivered within 24 h of amniocentesis compared with gestational age-matched samples from women who delivered 24 h after amniocentesis. Genes with cell-free RNA changes were associated with immune and inflammatory processes related to the onset of labor, and the expression of placental single-cell RNA-Seq signatures of immune cells was increased with imminent delivery. Transcriptomic prediction models captured these effects and predicted delivery within 24 h of amniocentesis (Figure 5). The results may inform the development of biomarkers for spontaneous preterm birth.

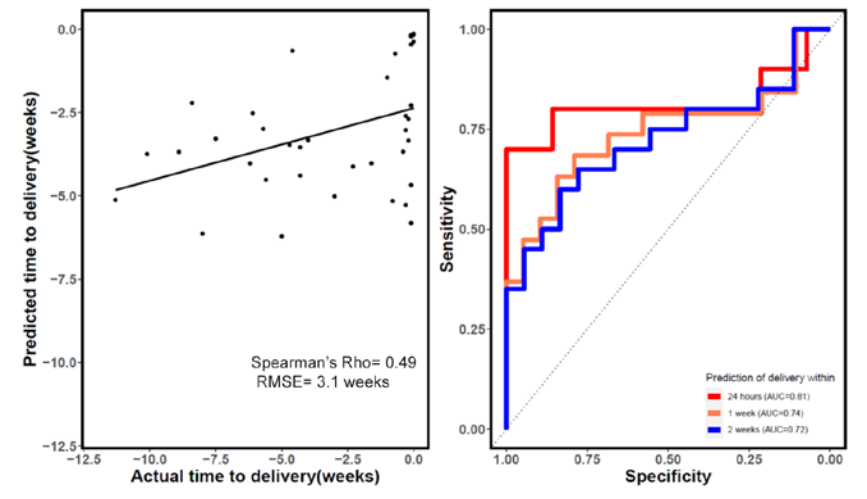

FIGURE 5. Prediction of time to delivery after an episode of preterm labor

The figure shows amniotic fluid cell free RNA–predicted time from amniocentesis to delivery versus actual time to delivery (*left*) and sensitivity versus specificity of RNA–based prediction of imminent delivery (within 24h, 1 week, or 2 weeks) (*right*).

Clinical chorioamnionitis

Clinical chorioamnionitis at term is considered the most common infection-related diagnosis in labor and delivery units worldwide. The syndrome affects 5–12% of all term pregnancies and is a leading cause of maternal morbidity and mortality as well as neonatal death and sepsis. We found that: (1) intra-amniotic infection (defined as the combination of microorganisms detected in amniotic fluid and an elevated IL-6 concentration) was present in 63% of cases; (2) the most common microorganisms found in the amniotic fluid samples were *Ureaplasma* species, followed by *Gardnerella vaginalis*; (3) sterile intra-amniotic inflammation (elevated IL-6 in amniotic fluid but without detectable microorganisms) was present in 5% of cases; (4) 26% of patients with the diagnosis of clinical chorioamnionitis had no evidence of intra-amniotic infection or intra-amniotic inflammation; (5) intra-amniotic infection was more common when the membranes were ruptured than when they were intact (78% vs. 38%); (6) the traditional criteria for the diagnosis of clinical chorioamnionitis had poor diagnostic performance in identifying proven intra-amniotic infection (overall accuracy, 40–58%); (7) neonatal bacteremia was diagnosed in 4.9% of cases; and (8) a fetal inflammatory response defined as the presence of severe acute funisitis was observed in 33% of cases. These observations have implications for the diagnosis, treatment, and optimal management of the neonate born to mothers with the most common infection in obstetrics.

Resolution of acute cervical insufficiency after antibiotics

Cervical insufficiency generally refers to a condition in which there is mid-trimester cervical dilatation or protruding chorioamniotic membranes in the absence of uterine contractions. The condition is a risk factor for mid-trimester spontaneous abortion or early preterm birth, and is associated with adverse neonatal outcomes. Both intra-amniotic infection and inflammation, ascertained by amniocentesis, have been identified in patients with cervical insufficiency, and are poor prognostic factors. A subset of patients with intra-amniotic inflammation will have no demonstrable microorganisms detected via cultivation or molecular methods, and therefore represent cases of sterile intra-amniotic inflammation. Amniotic fluid sludge (free-floating hyperechogenic material within the amniotic fluid in close proximity to the uterine cervix) identified on sonography is a biomarker for intra-amniotic infection and inflammation. Recent evidence suggests that intra-amniotic infection, as well as sterile intra-amniotic inflammation, can be treated successfully using

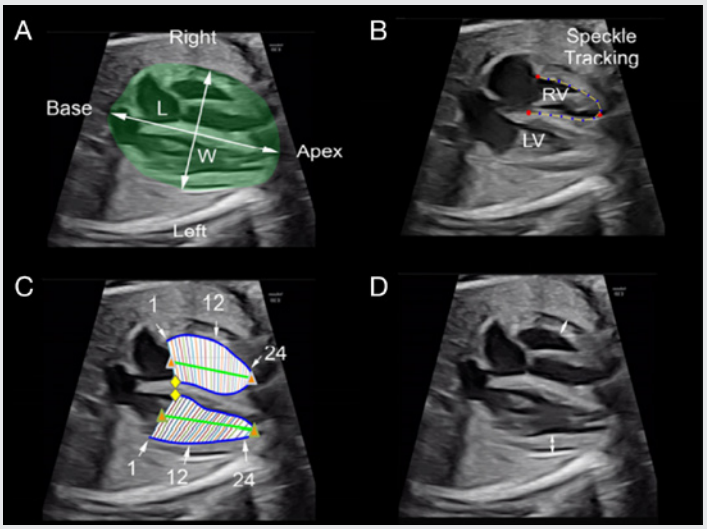

FIGURE 6. Measurements of the 4CV, RV, and LV

A. End-diastolic length (*L*) and width (*W*) of the 4CV measured at the point of the greatest length and width.

B. Speckle-tracking contour from the RV at end diastole.

C. Twenty-four–segment transverse widths and ventricular length computed from the speckle-tracking analysis of each ventricular chamber. Numbers identify segments 1, 12, and 24.

D. Measurements of ventricular wall thickness (*double arrows*) from the midsection of the RV and LV. 4CV: four chamber view; RV: right ventricle; LV: left ventricle.

antimicrobial agents. We reported a unique case in which administration of antibiotics in the presence of mid-trimester cervical insufficiency, sterile intra-amniotic inflammation, and amniotic fluid sludge was associated with resolution of the cervical findings (as demonstrated on both sonographic and speculum examination). The patient successfully underwent elective cesarean delivery at 36 2/7 weeks of gestation. The case illustrates that antibiotic therapy may be effective despite the presence of several high-risk pregnancy conditions, and that successful outcome is possible.

Cardiac measurements of size and shape in fetuses with absent or reversed end-diastolic velocity of the umbilical artery and perinatal survival and severe growth restriction before 34 weeks' gestation

The purpose of this study was to evaluate the end-diastolic size and shape of the 4-chamber view as well as the right ventricle (RV) and left ventricle (LV) in growth-restricted fetuses before 34 weeks' gestation with absent or reversed end-diastolic velocity of the umbilical artery and to compare the results between those with perinatal deaths with those who survived the neonatal period. Of the 49 fetuses, there were 13 perinatal deaths (27%) and 36 (63%) neonatal survivors. Measurements that were unique for neonatal survivors were an increased RV apical transverse width and decreased measurements of the following: LV and RV widths, LV and RV areas, as well as RV sphericity indices (Figure 6). We concluded that fetuses with a smaller RV and LV size and area and those with a globular-shaped RV were at significantly lower risk for perinatal death.

Additional Funding

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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 neuro-degeneration, all the likely the result of functional iron deficiency. Biochemically and using expression arrays, we studied, in *Irp2*$^{-/-}$ mice, the mechanisms that lead to anemia and neuro-degeneration with motor neuron loss. We are using this mouse model of neuro-degeneration to identify compounds that can prevent neuro-degeneration; 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 hypothesized 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, work that led us to

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suggest 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. In 2021, we discovered that SARS-CoV-2 contains iron-sulfur cofactors in its replicase that can be inactivated by treatment with the stable nitroxide Tempol, resulting in attenuation of infection in tissue culture and also in Golden Syrian hamster models.

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 of 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 the two essential iron-sensing proteins IRP1 and IRP2. IRPs bind to IREs when iron levels are depleted, resulting in either inhibition of translation of ferritin mRNA and of other transcripts that contain an IRE in the 5' untranslated regions (UTR) or stabilization of the transferrin receptor mRNA and possibly other transcripts that contain IREs in the 3' UTR. The IRE–binding activity of IRP1 depends on the presence of an iron-sulfur cluster (see “Mammalian iron-sulfur cluster biogenesis” below). IRP2 also binds to IREs in iron-depleted cells but, unlike IRP1, in iron-replete cells it is selectively ubiquitinated and then degraded by the proteasome.

To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we initially observed no abnormalities in iron metabolism associated with loss of IRP1 function. *Irp2*$^{-/-}$ mice develop a progressive neurologic syndrome characterized by gait abnormalities and axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. *Irp2*$^{-/-}$ animals develop iron-insufficiency anemia and erythropoietic protoporphyria. In animals that lack IRP1, IRP2 compensates for loss of IRP1's regulatory activity in most cell types, but we discovered several cell types and accompanying phenotypes in which *Irp2* expression cannot be sufficiently increased to compensate. Animals that lack both IRP1 and IRP2 die as early embryos. The adult-onset neurodegeneration of adult *Irp2*$^{-/-}$ mice is exacerbated when one copy of *Irp1* is also deleted. *Irp2*$^{-/-}$ mice offer a unique example of spontaneous adult-onset, slowly progressive neuro-degeneration; analyses of gene expression and iron status at various stages of disease are ongoing. Dietary supplementation with Tempol prevents neuro-degeneration; 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 neuro-degenerative 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 iron-deficient animals in permitting iron to cross the duodenal mucosa and in preventing developing erythroid cells from retaining high amounts of iron. 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 the 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 by derepressing hypoxia-inducible factor 2-alpha (HIF2a) translation in the renal interstitium through the IRE–IRP system. We confirmed that overexpression of HIF2a drives production of erythropoietin and polycythemia in a mouse model of Chuvash polycythemia (an autosomal recessive form of erythrocytosis, which is endemic in patients from Chuvashia, an autonomous republic within the Russian Federation), 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, transplants that were successful. We then discovered that the 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 that this represents an underdiagnosed and often misdiagnosed rare human disease.

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.

The iron-sulfur protein IRP1 is related to mitochondrial aconitase, a citric acid cycle enzyme; it 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 the 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 co-chaperone HSC20 binds to HSPA9, its partner HSP70–type chaperone, and the chaperone complex binds to ISCU bearing a nascent iron-sulfur cluster and to iron-sulfur cluster–recipient proteins. We identified several direct iron-sulfur–recipient proteins in a yeast two-hybrid assay, using HSC20 as bait. By studying one known iron-sulfur recipient, succinate dehydrogenase subunit B (SDHB), we discovered that several LYR motifs of the SDHB primary sequence engage the iron-sulfur 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 informatics, we predicted that amino levulinic acid dehydratase (ALAD), a heme-biosynthetic enzyme, is a previously unrecognized iron-sulfur protein, and we identified more unrecognized iron-sulfur proteins by using the LYR motif to analyze candidate proteins.

Using informatics, over-expression of candidate proteins, and iron detection with 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. 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.

We discovered that the intermediary scaffold protein NFU1 acquires its iron-sulfur clusters from ISCU2 and the iron-scaffold assembly protein ISCA1 to form a cubane iron-sulfur cluster that is delivered directly to lipoic acid

synthase. We are working to shed light on the complex use of such secondary iron-sulfur scaffold proteins to deliver iron-sulfur cluster to many recipient proteins in the cell.

We discovered that the SARs CoV-2 replicase Nsp12 ligates two cubane iron sulfur clusters, one of which is needed for primer extension, whereas the other is needed for full assembly formation. Using Tempol to degrade the iron sulfur clusters, we stopped viral replication in tissue culture and greatly mitigated COVID19 disease in a Syrian Golden hamster model.

Additional Funding

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Mechanisms of Synapse Assembly and Homeostasis

The purpose of our research is to understand the mechanisms of synapse development and homeostasis. The chemical synapse is the fundamental nervous-system communication unit that connects neurons to one another and to non-neuronal cells, and it is designed to mediate rapid and efficient transmission of signals across the synaptic cleft. The transmission forms the basis for the biological computations that underlie and enable our complex behavior. Crucial to the 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 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 to a set point after perturbations. We address the molecular mechanisms underlying these processes using a comprehensive set of approaches that include genetics, biochemistry, molecular biology, super-resolution imaging, and electrophysiology recordings in live animals and in reconstituted systems.

Because of its many advantages, we study these events in a powerful genetics system, *Drosophila melanogaster*, and 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. Importantly, the fly NMJ relies entirely on kainate-type receptors, a family of ionotropic glutamate receptors that impact synaptic transmission and neuronal excitability in the mammalian central nervous system but

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remain poorly understood. The *Drosophila* NMJ can thus be used to analyze and model defects in the structural and physiological plasticity of glutamatergic synapses, which are associated with a variety of human pathologies, from learning and memory deficits to autism. *Drosophila* has long served as a source of insight into human genetics, development, and disease, and the basic discoveries of our laboratory makes in the fly are likely to serve our overarching goal of understanding how chemical synapses are assembled and sculpted during development and homeostasis.

Synapse assembly

The first step in the assembly of synapses involves recruitment of synaptic components at the proper site. Prior to the motor neuron arrival, the ionotropic glutamate receptors (iGluRs) form small, nascent clusters on the muscle, which are distributed in the vicinity of future synaptic sites. Neuron arrival at its target muscle triggers the 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 absolutely 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, associations with various postsynaptic density (PSD) scaffolds, and, importantly, their channel properties. iGluRs assembled from different subunits have strikingly different biophysical properties, and their association with different auxiliary subunits increases such diversity even further. In flies as in humans, synapse strength and plasticity are determined by the interplay between different postsynaptic iGluRs subtypes. At the fly NMJ, the type-A and type-B iGluRs consist of four different subunits: either GluRIIA or GluRIIB, plus GluRIIC, GluRIID and GluRIIE. In addition, a presynaptic autoreceptor, which contains the KaiRID (kainate receptor ID) subunit, controls basal neurotransmission.

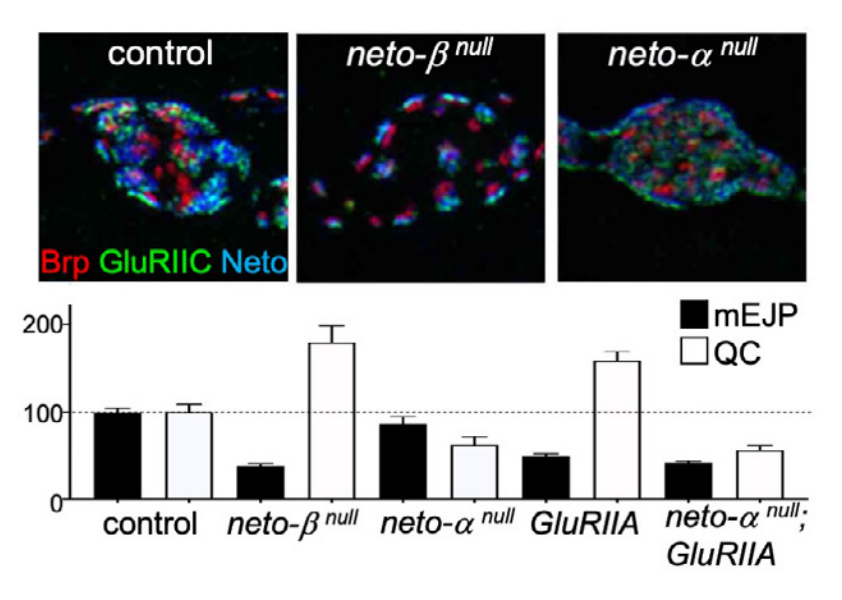
We previously discovered that an obligatory auxiliary protein, Neto, is absolutely required for iGluR clustering and NMJ functionality. Our investigations uncovered essential roles for Neto during synapse development and strongly support the notion that trafficking of both postsynaptic iGluR subtypes and the presynaptic KaiRID–containing autoreceptor, their synaptic recruitment and stabilization, and their function are tightly regulated by Neto. Our results further indicate that the fly Neto isoforms (α and β) directly engage iGluRs as well as other intracellular and extracellular proteins to selectively regulate the distribution of iGluR subtypes, the recruitment of postsynaptic proteins, and the organization of postsynaptic structures. Given that iGluRs gating properties control the distribution and trafficking of these receptors *in vivo*, Neto could influence the synaptic recruitment of iGluRs by simultaneously controlling several steps in receptor trafficking and clustering and/or receptor function.

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 were shown to modulate the properties of selective iGluRs, in particular the kainate-type receptors. *Neto1/Neto2* double-knockout mice have defects in long-term potentiation and learning and memory, but the underlying mechanisms are extremely difficult to study owing to the low abundance of the kainate receptor channels and the small currents they

FIGURE 1. Neto isoforms have distinct roles in PSD organization and NMJ function.

Upper panels. Structure illumination (3D-SIM) images of control, *neto-β*^{null}, and *neto-α*^{null} synaptic boutons labeled for Brp (*red*), a presynaptic scaffold, GluRIIC (*green*), a share postsynaptic iGluR subunit, and Neto (*blue*).

Lower panels. Quantification of mEJP amplitude and quantal content (QC) values normalized to control captures a robust presynaptic compensatory response at *neto-β*^{null} and *GluRIIA*–mutant NMJs. In contrast, neurotransmitter release is reduced at *neto-α*^{null} terminals (as indicated by reduced QC and no significant reduction of mEJP amplitude), and there is no compensatory response upon further removal of the GluRIIA subunit.



elicit. Phylogenetic analyses indicated that both postsynaptic and presynaptic glutamate receptor subunits at the *Drosophila* NMJ belong to the kainate sub-family. Therefore, the fly NMJ, an essential synapse, is probably the best genetic system in which to probe the repertoire of both kainate receptors and Neto-type functions. Furthermore, using live imaging, we showed that Neto clusters at nascent NMJs at the time when iGluRs begin to accumulate and cluster. Also, Neto and iGluRs depend on each other for trafficking and stabilization at synaptic sites. Thus, mutants lacking any essential, shared iGluR subunits or Neto are completely paralyzed and die as embryos, with the iGluRs scattered as small aggregates away from the neuronal arbor. Mutants with suboptimal levels of Neto may live to adulthood, but they exhibit a wide range of behavioral deficits throughout development because of structural and functional synaptic defects, including reduced synaptic receptors, increased levels of extra-junctional iGluRs, and a series of PSD deficits. Our studies demonstrate that Neto engages the iGluRs on the cell membrane and together traffic 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.

Neto-mediated intracellular interactions sculpt postsynaptic iGluR fields.

Type-A and type-B receptor complexes differ in their trafficking to the synapses, subsynaptic localization, and synaptic responses; at synapses with both type-A and type-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, a postsynaptic contribution that 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 instead incorporate type-B. 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.

Evidence in support of the model comes from our studies on Neto-β, the predominant Neto isoform at the larval NMJ. 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-β*^{null} synapses have reduced iGluRs synaptic clusters (Figure 1), in particular the type-A subtype, which reflects the drastic reduction in Neto net levels. However, a *neto-β*^{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, a short Neto-β, that cannot recruit type-A stabilizers, clusters type-B receptors. The finding suggests 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 the synaptic accumulation of iGluRs. Instead, 3D-SIM analysis captured the enlarged receptor fields, which appeared to fill the small *neto-α*^{null} boutons (Figure 1). Muscle overexpression of a *neto-α* transgene fully rescued the PSD size of *neto-α*^{null} synapses. Thus, Neto-α limits the size of the postsynaptic receptor fields but has no detectable role in the organization of presynaptic specializations.

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 via synapse-specific recruitment of GluRIIA. Robust homeostatic mechanisms keep synapses within an appropriate dynamic range such 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, which is referred to as presynaptic homeostatic potentiation (PHP).

The drastic reduction in synaptic iGluRs (primarily GluRIIA) at *neto-β*^{null} NMJs causes reduced mini frequency and amplitudes (mEJP), but such NMJs have normal evoked potentials owing to increased QC. 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. 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, as well as compensatory decrease in QC (triggered by neuronal overexpression of vGlut). Loss of presynaptic Neto-α renders such NMJs unable to express PHP but has no effect on homeostatic depression. Specifically, 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, 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 kainate receptor, KaiRID, has been implicated in the control of basal neurotransmission and the expression of PHP, we examined whether Neto-α modulates KaiRID synaptic distribution and function. We found that Neto-α controls neurotransmitter release in a KaiRID–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 (ΔCTD) can reach the presynaptic terminal and rescue the basal neurotransmission defects of *neto-α*^{null}, but it cannot restore the PHP. Our studies demonstrate that the intracellular part of Neto-α functions as a bona fide effector of PHP. The low-abundant Neto-α appears to be recruited and/or stabilized at synapses by the presynaptic KaiRID. The finding challenges our current assumption that auxiliary subunits 'assist' iGluRs and provides an exquisite example of an auxiliary protein that performs a key synaptic function with assistance from iGluRs.

Genetic interactors of *neto*

Our previous studies demonstrated that Neto enables trafficking of iGluRs at synaptic locations and the formation of stable clusters. At the same time Neto recruits postsynaptic components and controls postsynaptic differentiation. As Neto does not contain any catalytic domain, Neto functions via binding to iGluRs and other interaction partners. To identify proteins that interact with Neto and control synapse assembly at the developing NMJ, we conducted a series of biochemical and genetic screens. To screen for *neto* genetic interactors, we took advantage of a *neto* hypomorph, *neto*^{109}, with drastically reduced NMJ function and synaptic structures, which exhibits locomotor deficits and partial lethality. The allele expresses less than 10% of the normal levels of Neto and has impaired PSD structures with much diminished synaptic iGluRs. Given that 50% of *neto*^{109} hypomorphs die developmentally, 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 control the development of NMJ. In a screen of 418 overlapping deficiencies on chromosomes 2 and 3 (Bloomington Df kit) and also candidate interactors, we found and confirmed, firstly, known NMJ modulators such as Glass bottom boat (Gbb, a BMP ligand with critical roles during NMJ development), secondly, known components of synapse homeostasis, such as KaiRID, the presynaptic autoreceptor subunit, but thirdly, we also uncovered new players at the NMJ, such as Tenectin (Tnc), an integrin ligand required for NMJ development. Additional candidates revealed by this screen are currently being tested and examined for their potential role in synapse development and homeostasis.

Synapse maturation and homeostasis

Transsynaptic signaling modulates synapse assembly but also synapse maturation and developmental plasticity. How these signals monitor synaptic strength and relay this information to pre- and postsynaptic compartments is one of the central problems in neuroscience. Several families of developmentally important signaling molecules, including Wnt, TGF-β, and neurotrophins, have been implicated in sculpting synaptic junctions. Understanding the underlying mechanisms has been hindered by the complexity of these signaling networks, which are transduced by a web of transcriptional programs and local functions that govern multiple processes during neural development. For example, BMP signaling is critical for NMJ growth and neurotransmitter release and has been implicated in synapse homeostasis via unknown mechanisms. BMP signals via (1) a canonical pathway, which activates transcriptional programs with distinct roles in the structural and functional development of the NMJ in response to accumulation of phosphorylated Smad (pMad, an intracellular transducer of the BMP pathway) in motor neuron nuclei; and (2) a noncanonical, Mad–independent pathway, which connects synaptic structures to microtubules to regulate synapse stability. Intriguingly, pMad also accumulates at synaptic locations, but the biological relevance of this phenomenon remained a mystery for over a decade.

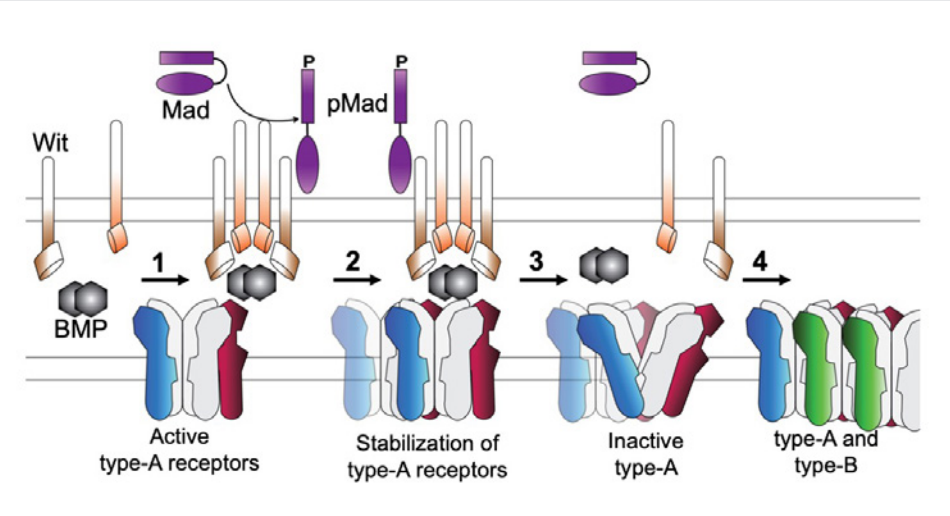


FIGURE 2.

Model for type-A receptor synaptic stabilization via transsynaptic interactions with local BMP signaling complexes

In previous work we discovered that postsynaptic type-A iGluRs, in their active state, together with Neto, trigger accumulation of pMad at active zones, the site of neurotransmitter release. We found that synaptic pMad marks a novel, local BMP pathway, genetically distinguishable from all other known BMP signaling cascades. This novel pathway does not contribute to NMJ growth but instead appears to provide a positive feedback loop that modulates the postsynaptic distribution of type-A and type-B receptors as a function of synapse activity.

Type-A receptors are the first to arrive at a nascent synapse; they form the 'core' of the receptor field and are surrounded by type-B receptors. Incorporation of type-A receptors in stable synaptic complexes depends on channel 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. First, postsynaptic type-A receptors, in their active form, trigger accumulation of presynaptic pMad at active zones; secondly, genetic manipulations that disrupt pMad accumulation at the active zones trigger a reduction of synaptic type-A receptors, creating a positive feedback loop that stabilizes synaptic type-A receptors as a function of their activity.

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 2, step 1). 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, the complexes function as ‘accumulation centers’ for stabilizing type-A receptors at nascent synapses (step 2). Dissociation of these local complexes terminates further incorporation of type-A receptors and allows for recruitment of type-B subtypes, which mark mature synapses (steps 3–4). Importantly, the local BMP/BMPR complexes share limiting components with the other BMP signaling pathways, which control NMJ growth and synaptic stability, suggesting that neurons may use the novel BMP pathway to monitor synapse activity and then coordinate NMJ growth with synapse maturation and stabilization.

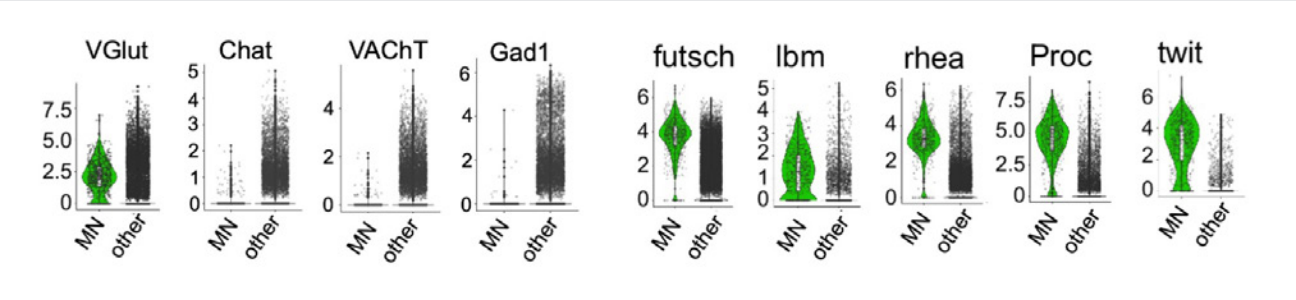


FIGURE 3. Violin plots of transcripts enriched in larval glutamatergic motor neurons

The motor neuron (MN) cluster contains exclusively glutamatergic neurons, which express high levels of the vesicular glutamate transporter VGluT. The MNs do not express choline acetyltransferase (Chat), vesicular acetyl choline transporter (VAchT), or glutamic acid decarboxylase 1 (Gad1).

Additional MN–specific enriched transcripts include those of *futsch* (encoding a microtubule-binding protein involved in the formation of synaptic boutons at the neuromuscular junctions), *lbm* (tetraspanin implicated in axon guidance), *rhea* (or talin, essential for integrin function), *Proc* (neurohormone), and *twit* (target of BMP signaling pathway).

Cellular diversity in the *Drosophila* third instar larval ventral cord revealed by single-cell transcriptomics

To learn how motor neurons respond to different BMP signaling modalities or compensate for various postsynaptic perturbations, we performed single-cell RNA sequencing (scRNA-Seq) of larval ventral nerve cords (VNCs), the fly equivalent of the mammalian spinal cord. However, motor neurons represent a relatively small fraction (less than 2%) of the cells within the larval VNC. Without a larval VNC atlas available, it was practically impossible to recognize the motor neuron–specific transcriptomes and to begin comparing them under different conditions/genotypes. First, we had to assemble a larval VNC atlas that captures and characterizes the cellular diversity within the *Drosophila* third instar larvae. To accomplish this goal, we adapted and developed new protocols for dissociating single cells from fly larvae, and then assembled a custom multistage analysis pipeline that integrates modules contained in different R packages to ensure flexible, high-quality RNA-Seq data analysis. The work was conducted in collaboration with Steve Coon and James Iben.

We dissected third instar larvae VNCs, dissociated the cells, and sequenced about 31,000 high-quality single cells. Using un-supervised clustering algorithms, we clustered the cells into distinct populations. We then assigned the populations to specific cell types using known markers. Through a series of reiterative processes, we first identified eight different glia subtypes, each with distinct metabolic pathways. Secondly, based on the expression of neuroblast genes and the temporal determinant genes, we revealed a developmental trajectory leading from neural precursors to newborn neurons. We also detected novel differentially expressed genes along this trajectory. Thirdly, we identified over 40 types of clearly differentiated interneurons, each expressing unique combinations of transcription factors and neurotransmitters (*VGlut, Gad1, VAChT, DAT).* We found that most interneuron subtypes express multiple GPCRs (G protein–coupled receptors) and cell-recognition molecules, suggesting that many sensory modalities converge onto single cells to elicit specific motor functions/behaviors. We also identified a large motor neuron (MN) cluster (Figure 3), in which all cells express *VGlut* (encoding a glutamate transporter) but not the other neurotransmitter transporters or

markers (*Gad1, VAChT, DAT*) (Figure 3). As expected, the MN transcriptomes are enriched in *futsch* (encoding a microtubule-binding protein involved in the formation of synaptic boutons at the neuromuscular junctions), *proctolin* (*proc*, encoding a neurohormone that modulates NMJ function through unknown mechanisms) and *target of wit* (*twit*, a BMP transcriptional target previously implicated in NMJ function).

To increase our ability to examine the heterogeneity within the MN cluster, we marked the MNs with a *twit-Gal4* promoter (and *UAS-nls-GFP*), dissociated VNCs from *twit-GFP* third instar larvae, and FACS–sorted the GFP–positive MNs. With this enrichment we were able to generate high quality scRNA-seq data for over 1,200 MNs. This reasonably large pool of MNs was then subdivided in 28 clusters. Several of the clusters are very well isolated and correspond to neurosecretory cells (expressing orcokinin, leukokonin or GPa), type II MNs (expressing Tdc2 and Vmat) and type III/peptidergic neurons (expressing the neurohormone bursicon, and the neuropeptide crustacean cardioactive peptide, CCAP). Our dataset reveals new markers for these types of neurons. For example, the AMPA–type glutamate receptor subunits GluRIA and GluRIB are primarily expressed in type II and type III neurons, suggesting that the modulatory activity of these neurons is regulated by glutamatergic input.

Drosophila has two type I MNs: tonic I-b neurons (with large synaptic boutons), which innervate single, dedicated muscles; and phasic I-s neurons, with small boutons and innervation spanning up to 7–8 muscles. Each larval hemisegment contains two I-s MNs, one projecting dorsally and one ventrally, and about 30 type I-b MNs, organized in distinct bundles that innervate subsets of body-wall muscles. Among the 28 MN clusters, two adjacent ones correspond to I-s MNs; they both express *DIP-alpha* (encoding a cell-surface molecule of the immunoglobulin superfamily), with the transcription factor gene *eve* marking the dorsally projecting I-s cluster. We found that *anachronism* (*ana*, encoding a secreted glycoprotein) specifically marks dorsally projecting I-s and confirmed this restricted expression using an *ana-CRIMIC* line. We also recognized different I-b MN bundles, projecting dorsally, ventrally, or laterally, such as the SNc bundle marked by the *tey* transcript (encoding a transcription factor). Interestingly, we found that all type I MNs show high *CG11155* expression; *CG11155* is predicted to code for a kainate-type glutamate receptor subunit. To search for the role of CG11155 during MNs development and function, we used CRISPR/Cas9 and RNAi to generate null mutants and tissue-specific knock downs. We found that CG11155 functions in MNs to ensure normal basal neurotransmission; in the absence of CG11155, basal neurotransmission is reduced to half that of control. This loss-of-function phenotype is reminiscent of the KaiRID loss-of-function defects. Given that iGluRs function as heterotetramers, usually dimers of dimers, our data suggest that KaiRID and CG11155 may be two subunits of the presynaptic autoreceptor that controls basal neurotransmission at the larval NMJ.

Our studies on the transcriptomes of larval MNs together with the assembly of a larval VNC atlas have already uncovered new molecules critical for synapse development and function and will provide a valuable resource for future studies on neuronal development and behavior.

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Publications

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2. Nguyen TH, Han TH, Newfeld SJ, Serpe M. Selective disruption of synaptic BMP signaling by a Smad mutation adjacent to the highly conserved H2 helix. *Genetics* 2020 216:159–175.
3. Nguyen TH, Vicidomini R, Choudhury S, Coon SL, Iben J, Brody T, Serpe M. Single-cell RNA sequencing analysis of the Drosophila larval ventral cord. *Curr Protoc* 2021 e38.
4. Vicidomini R, Nguyen TH, Choudhury S, Brody T, Serpe M. Assembly and exploration of a single cell atlas of the Drosophila larval ventral cord. Identification of rare cell types. *Curr Protoc* 2021 e37.
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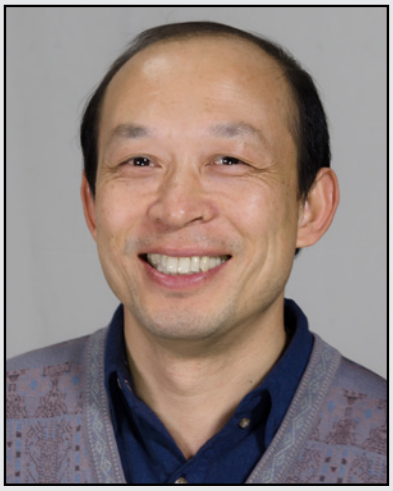
Thyroid Hormone Regulation of Vertebrate Postembryonic Development

The laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during postembryonic development, a period around birth in mammals when plasma TH levels peak. The main model is the metamorphosis of *Xenopus laevis* and *X. tropicalis*, two highly related species that offer unique but complementary advantages. The control of 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, it is transformed into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles, by a process that involves specific larval epithelial cell death and *de novo* development of the adult epithelial stem cells, followed by their proliferation and differentiation. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both *in vivo*, by using genetic approaches or hormone treatment of whole animals, and *in vitro,* in organ cultures, offer an excellent opportunity, first, to study the developmental function of TH receptors (TRs) and their underlying mechanisms *in vivo* and, second, to identify and functionally characterize genes that are critical for organogenesis, in particular, for 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 for functional analyses. In addition, the recent improvements in *X. tropicalis* genome annotation allow us to carry out RNA-Seq and chromatin-immunoprecipitation (ChIP)-Seq analyses at the genome-wide level. We also complement our frog studies by investigating the genes found to be important for frog intestinal stem cell development in the developing mouse intestine by making use of the ability to carry out conditional knockout.



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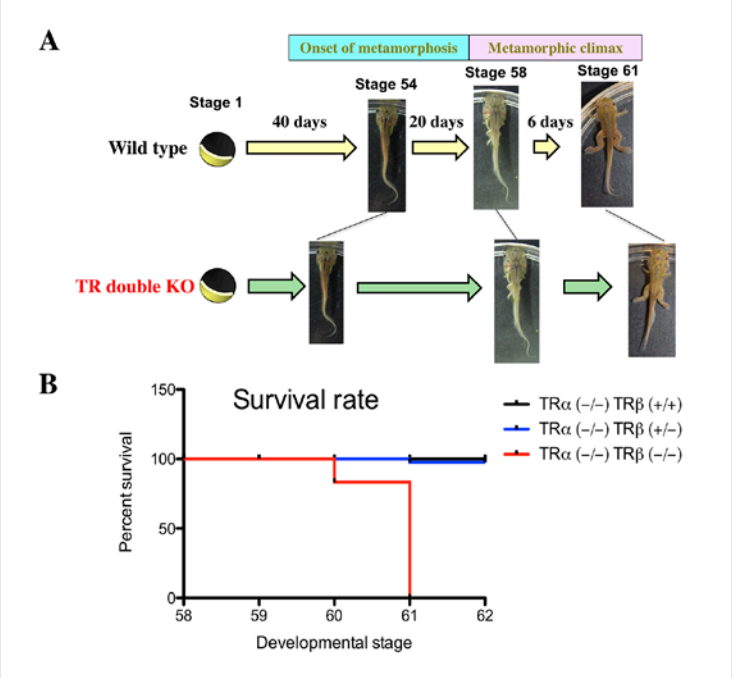


FIGURE 1. Effects of TR double KO on developmental rate. TR double KO leads to premature initiation of metamorphosis but slows metamorphic progress and causes lethality at the metamorphic climax.

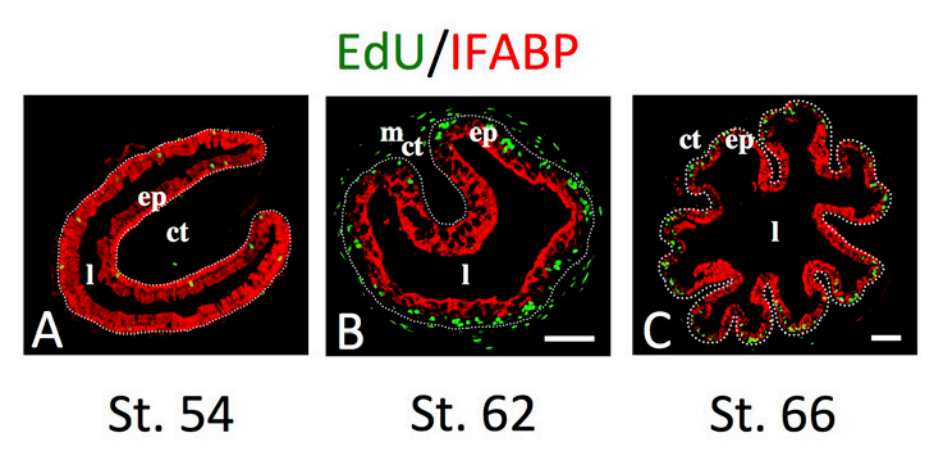
A. TR double KO animals take a shorter time to reach the onset of metamorphosis (stage 54), indicating accelerated premetamorphic development. Once metamorphosis begins, the KO animals take longer to reach the beginning of metamorphic climax (stage 58) and also develop more slowly during the climax stages, between stages 58 and 61. The length of each indicates the relative time needed for development between two adjacent stages.

B. Tadpoles without any TR die during the climax of metamorphosis. The tadpoles of mixed genotypes at stage 58 were allowed to develop to stage 62 and were genotyped at stage 62 or when they died during this developmental period. The survival rate for each of the three genotypes, $tr\alpha^{-/-}tr\beta^{+/+}$, $tr\alpha^{-/-}tr\beta^{+/-}$, and $tr\alpha^{-/-}tr\beta^{-/-}$, was thus obtained and plotted. Note that no double knockout tadpoles developed to stage 62 and that a single copy of $tr\beta^{+/-}$ was sufficient for the animal to complete metamorphosis and develop into a reproductive adult.

Analysis of *trα*–knockout tadpoles reveals that the activation of the cell-cycle program is involved in TH–induced larval epithelial cell death and adult intestinal stem cell development during *Xenopus tropicalis* metamorphosis.

We recently knocked out the TR genes *trα* and *trβ*, individually or both, in *X. tropicalis* and analyzed the knockouts' effect on tadpole development and the metamorphosis of various organs. Of interest is intestinal remodeling, which involves near-complete degeneration of the larval epithelium through apoptosis. Concurrently, adult intestinal stem cells are formed *de novo* and subsequently give rise to the self-renewing adult epithelial system, resembling intestinal maturation around birth in mammals. We observed that both *trα* and *trβ* play important roles in intestinal remodeling. To understand the underlying molecular mechanism, we recently studied the function of endogenous Trα in the tadpole intestine by using knockout animals and RNA-Seq analysis [Reference 1]. We observed that removing endogenous Trα caused defects in intestinal remodeling, including drastically reduced larval epithelial cell death and adult intestinal stem cell proliferation. Using RNA-Seq on intestinal RNA from pre-metamorphic wild-type and *trα*–knockout tadpoles treated with or without TH for one day and prior to any detectable TH–induced cell death and stem-cell formation in the tadpole intestine, we identified over 1,500 genes regulated by TH treatment of the wild-type but not of *trα*–knockout tadpoles. Gene ontology and biological-pathway analyses revealed that, surprisingly, these Trα–regulated genes were highly enriched with cell cycle–related genes, in addition to genes related to stem cells and apoptosis. Our findings suggest that Trα–mediated TH–activation of the cell-cycle program is involved in larval epithelial cell death and adult epithelial stem-cell development during intestinal remodeling. We

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 the end of metamorphosis (*C*, stage 66) were injected with 5-ethynyl-2'-deoxyuridine (EdU) one hour before sacrifice. 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.

carried out a comprehensive gene-expression analysis in the notochord during metamorphosis using RNA-Seq analyses of whole tail at stage 60 before any noticeable reduction in tail length, whole tail at stage 63 when the tail length is reduced by about one half, and the rest of the tail at stage 63 after removing the notochord. This allowed us to identify many notochord-enriched, metamorphosis-induced genes at stage 63 [Reference 1]. Future studies on these genes should help determine whether they are regulated by trβ and play any roles in notochord regression.

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 showed 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* 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.

A role of endogenous histone acetyltransferase steroid hormone receptor coactivator 3 (Src3) in TH signaling during *Xenopus* intestinal metamorphosis

We showed previously that, during metamorphosis, liganded TR recruits coactivator complexes that include Src3, which is a histone acetyltransferase, to TH–responsive promoters. To investigate the functions of endogenous coactivators such as Src3 during metamorphosis, we generated *X. tropicalis* animals lacking a functional *src3* gene and analyzed the resulting phenotype [Reference 2]. While removing *src3* had no apparent effect on

external development and animal gross morphology, the *src3*$^{-/-}$ tadpoles displayed a reduction in the acetylation of histone H4 in the intestine comparing with that in wild-type animals. Furthermore, the expression of TR target genes was also reduced in *src3*$^{-/-}$ tadpoles during intestinal remodeling. Importantly, intestinal remodeling during natural and TH–induced metamorphosis was inhibited/delayed in *src3*$^{-/-}$ tadpoles and included reduced adult intestinal stem-cell proliferation and apoptosis of larval epithelial cells. Our results thus demonstrate that, during intestinal remodeling, Src3 is a critical component of the TR–signaling pathway *in vivo*.

Evolutionary divergence in tail regeneration between *X. laevis* and *X. tropicalis*

Tissue regeneration is of fast-growing importance in the development of biomedicine, particularly in organ replacement therapies. Unfortunately, many human organs cannot regenerate. As many tadpole organs can, the auran *X. laevis* has been used as a model to study regeneration. In particular, the tail, which consists of many axial and paraxial tissues, such as spinal cord, dorsal aorta, and muscle, commonly present in vertebrates, can fully regenerate when amputated at late embryonic stages and at most of the tadpole stages. Interestingly, between stage 45, when feeding begins, and stage 47, the pseudo-tetraploid *X. laevis* tail cannot regenerate after amputation. This period, termed the "refractory period," has been known for about 20 years. The underlying molecular and genetic bases are unclear, in part because it is difficult to carry out genetic studies in this pseudo-tetraploid species. The availability of the highly related but diploid *X. tropicalis* offers an opportunity to study the molecular and genetic mechanisms of tail regeneration during the refractory period. We compared tail regeneration between *X. laevis* and *X. tropicalis* and found, surprisingly, that *X. tropicalis* lacked the refractory period [Reference 3]. Further molecular and genetic studies, more feasible in this diploid species, should reveal the basis for this evolutionary divergence in tail regeneration between two related species and facilitate the understanding of how tissue regenerative capacity is controlled. In addition, it is well known that many tadpole tissues lose their regenerative capacity during metamorphosis, suggesting a role for TH and TR in regeneration. Making use of our recently generated *tr*–knockout animals, we also plan to investigate whether and how TH and TR regulate tissue regeneration. Such studies should have important implications for human regenerative medicine.

The TR is essential for larval epithelial apoptosis and adult epithelial stem-cell development but not for adult intestinal morphogenesis during *X. tropicalis* metamorphosis.

We recently generated TR double-knockout (TRDKO) *X. tropicalis* animals and reported that TR is essential for the completion of metamorphosis. Furthermore, TRDKO tadpoles are stalled at the climax of metamorphosis before eventual death. To investigate the underlying defects resulting from TRDKO, we analyzed the intestine at the climax of metamorphosis in wild-type and TRDKO animals at the climax of metamorphosis [Reference 4]. We showed that the TRDKO intestine lacked larval epithelial cell death and adult stem-cell formation/proliferation during natural metamorphosis. Interestingly, TRDKO tadpole intestine displayed premature formation of adult-like epithelial folds and muscle development. In addition, TH treatment of premetamorphic TRDKO tadpoles failed to induce any metamorphic changes in the intestine. Furthermore, RNA-Seq analysis revealed that TRDKO altered the expression of many genes in biological pathways, such as Wnt signaling and the cell cycle, that likely underlie the inhibition of larval epithelial cell death and adult stem-cell development caused by removing both TR genes. Our data suggest that liganded TR is required for larval epithelial cell degeneration and adult stem-cell formation, whereas unliganded TR prevents precocious adult tissue morphogenesis such as smooth muscle development and epithelial folding in the intestine.

TH directly activates mitochondrial fission process 1 (*mtfp1*) gene transcription during adult intestinal stem-cell development and proliferation in *X. tropicalis.*

TH functions by regulating target-gene expression through TRs. Thus, identification and characterization of TH target genes are essential toward understanding how TH regulates adult intestinal stem-cell development during metamorphosis. We previously identified many candidate TR target genes during *X. tropicalis* intestinal metamorphosis, a process that involves apoptotic degeneration of most of the larval epithelial cells and *de novo* development of adult epithelial stem cells. Among such putative TR target genes is mitochondrial fission process 1 (*mtfp1*), a nuclear-encoded mitochondrial gene. Our recent studies showed that *mtfp1* gene expression peaked in the intestine during both natural and TH–induced metamorphosis when adult epithelial stem-cell development and proliferation took place. Furthermore, *mtfp1* contained a TH–response element (TRE) within the first intron, which was bound by TR to mediate TH–induced local histone H3K79 methylation and RNA polymerase recruitment in the intestine during metamorphosis. Additionally, we demonstrated that the *mtfp1* promoter could be activated by TH in a reconstituted frog oocyte system *in vivo* and that the activation is dependent on the intronic TRE. The findings suggest that TH activates the *mtfp1* gene directly via the intronic TRE and that *mtfp1* in turn facilitates adult intestinal stem-cell development/proliferation by affecting the mitochondrial fission process.

The protein arginine methyltransferase 1 regulates cell proliferation and differentiation in adult mouse adult intestine.

Adult stem cells play an essential role in adult organ physiology and tissue repair and regeneration. While much has been learnt about the property and function of various adult stem cells, the mechanisms of their development remain poorly understood in mammals. Earlier studies suggested that the formation of adult mouse intestinal stem cells takes place during the first few weeks after birth, the postembryonic period when plasma TH levels are high. Furthermore, deficiency in TH signaling leads to defects in the adult mouse intestine, including reduced cell proliferation in the intestinal crypts, where stem cells reside. Our earlier studies had shown that the protein arginine methyltransferase 1 (PRMT1), a TR coactivator, is highly expressed during intestinal maturation in the mouse, resembling its upregulation during intestinal metamorphosis. Furthermore, we previously showed that Prmt1 is important for adult intestinal stem-cell development and/or proliferation during *Xenopus* metamorphosis. To determine whether PRMT1 has a conserved role in adult stem-cell development, we analyzed the expression of PRMT1 by immunohistochemistry and studied the effect of tissue-specific knockout of *Prmt1* in the intestinal epithelium [Reference 5]. We showed that PRMT1 was highly expressed in the proliferating transit-amplifying cells and crypt-base stem cells. By using a conditional knockout mouse line, we demonstrated that the expression of *Prmt*1 in the intestinal epithelium is critical for the development of the adult mouse intestine. Specific removal of PRMT1 in the intestinal epithelium resulted, surprisingly, in more elongated adult intestinal crypts with increased cell proliferation. In addition, epithelial cell migration along the crypt-villus axis and cell death on the villus were also increased. Furthermore, there were more Goblet cells and fewer Paneth cells in the crypt, while the number of crypt-base stem cells remained unchanged. Our finding that *Prmt*1 knockout increases cell proliferation is surprising, considering the role of PRMT1 in TH signaling and the importance of TH for intestinal development, and suggests that PRMT1 likely regulates pathways in addition to TH–signaling to affect intestinal development and/or homeostasis, thus affecting cell proliferating and epithelial turnover in the adult.

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- Awards from the China Scholarship Council (CSC) to Drs. Shouhong Wang and Lusha Liu

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1. Tanizaki Y, Shibata Y, Zhang H, Shi Y-B. Analysis of thyroid hormone receptor a knockout tadpoles reveals that the activation of cell cycle program is involved in thyroid hormone-induced larval epithelial cell death and adult intestinal stem cell development during *Xenopus tropicalis* metamorphosis. *Thyroid* 2021 31:128–142.
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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 physics to the 'big' processes that happen in life. Our lab 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. Our group is making a sustained effort to validate our simulation findings by applying neutron scattering techniques. This year, our lab initiated new collaborations on the basis of our previous years' work developing methodology for predicting the scattering signal from our simulations.

The projects use the NIH computing resources, including the Biowulf 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.

New methodology probes the energy of complex lipid structures related to membrane fusion.

The work required to form a vesicle from the surface of the cell or an organelle is challenging to predict. Yet a model of this process is essential to understand many biological processes, including intracellular traffic and viral entry and exit.

A lipid bilayer is somewhere between an elastic sheet and the surface of a puddle. Like specks of dust on the surface of a puddle, lipids and proteins move along the surface. Yet like a sheet of rubber, the surface itself resists folding. It is not clear whether forming a vesicle from a sheet of lipids is more like covering a ball with a flat rubber sheet (impossible without cutting the sheet) or covering the ball with water (easy).

In the simulations described in our recent paper [Reference 1], we use advanced molecular simulation techniques to detect whether particular lipids are more stable composing vesicles on tubes, on sheets, or on exotic structures more closely resembling large cellular organelles. The study opens a path to determining how the chemistry of the hundreds of lipids in the cell determines the possible shapes biological machinery can create.

Separating individual lipids' contributions to membrane reshaping events

Membrane biophysicists have tested and modeled the effect of lipid chemistry on membrane shapes for decades. Studies have identified how specific lipids localize to high curvature and support membrane fusion and fission. Underlying these studies have been conflicting assumptions about how individual lipids act. Do they work locally, rearranging on the nanometer scale to cooperate with the cellular machinery that reshapes membranes? Or do they work globally, determining only whether the entire membrane is stiff or soft?

In our recently published study [Reference 2], we described the development of a novel theoretical analysis of how lipids shift and move on an undulating membrane, which we simulated on the high-performance computing resources at the NIH. Two results emerged: first, as determined by head-group chemistry, lipids act locally, allowing them to support very small, highly curved structures like membrane pores; second, lipids with saturated (well ordered) tail chemistry tended to prefer curving with a particular wavelength. This was unexpected and is a plausible mechanism for the interesting shapes and patterns that form from complex lipid mixtures.

The results of the study represent a significant opportunity to better understand how lipid gradients inside the cell promote or disinhibit vesicle traffic, which depends on the work required to reshape membranes.

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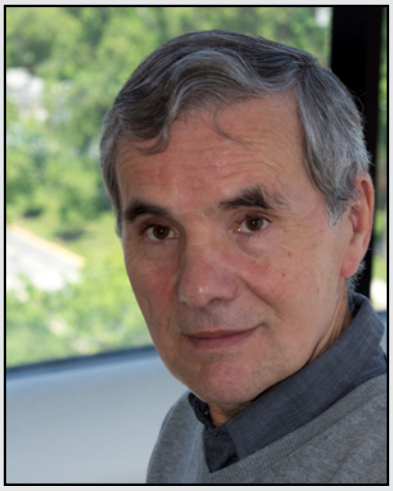
Signaling and Secretion in Neuroendocrine Cells

The main goal of the research in our Section is to examine cell-signaling cascades, gene expression, and hormone secretion in neuroendocrine cells from the hypothalamus and pituitary gland during development. We place special emphasis on the characterization of individual cells, using fluorescence imaging, patch-clamp recordings, simultaneous membrane potential/calcium and current/calcium recordings, electrophysiological and imaging recordings of single-cell exocytic events, single-cell RNA sequencing (scRNA-Seq), and single-cell quantitative reverse transcription polymerase chain reaction (RT-PCR). Our recent and ongoing work has focused on signaling, transcription, and secretion in the pituitary gland specific for age, sex, and tissue structure; pituitary cell heterogeneity, reflecting their postnatal gene expression; the role of 1-phosphatidylinositol 4-kinases and protein receptor tyrosine N2 phosphatase type in postnatal proliferation and maintenance of pituitary lineages; and cell-specific electrical activity and exocytic pathways. Current and proposed studies depend in part on the use of equipment in NICHD's *Microscopy and Imaging Core Facility* and the *Molecular Genomics Core Facility*.



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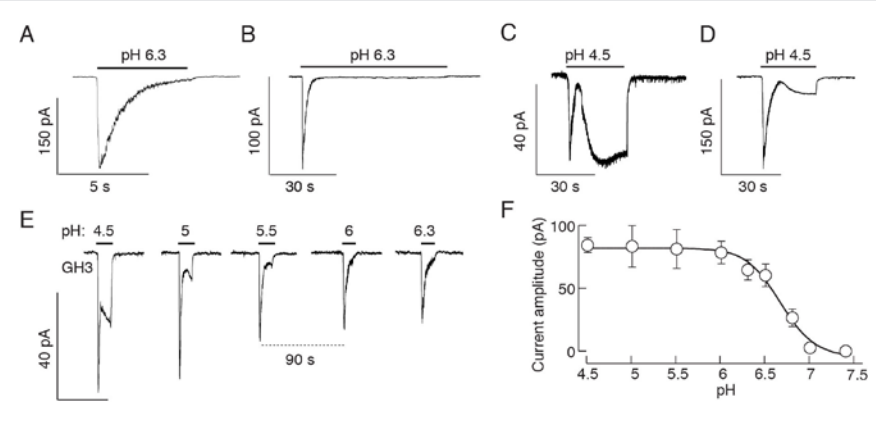
Proton sensing by pituitary cells

Extracellular protons act as orthosteric ligands for proton-sensing G protein–coupled receptors (GPRs) and acid-sensing ion channels (ASICs), as well as allosteric regulators of other GPRs and channels. Until now, the expression of these receptors and channels and their roles in pituitary-cell functions have not been systematically investigated. We recently systematically evaluated the expression of proton-sensing receptors and channels in rat pituitary cells. The main focus in our work was on ASICs encoded by a family of five genes, generating seven subunits: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC4, and ASIC5. Our RT-PCR and scRNA-Seq analyses indicate that four mRNAs were expressed in rat anterior pituitary glands (ASIC1a, ASIC1b, ASIC2b, and ASIC4), whereas GH3 cells (a rat anterior pituitary cell line) express ASIC1a and ASIC2b only. We also provided, to our knowledge, novel information about cell type–specific expression of ASIC mRNAs, proteins, and currents. Our scRNA-Seq and double immunostaining analyses demonstrated the expression of ASIC1 mRNA and proteins in all secretory cell types. In contrast, *Asic1* expression was detected in less than 5% of folliculostellate cells

FIGURE 1. Effects of a drop in extracellular pH on current responses in immortalized GH3 cells



A–D. Patterns of proton-activated currents in voltage-clamped single cells: a biphasic response composed of a transient spike phase and a sustained small-amplitude plateau phase during long-term stimulation with pH 6.3 (*A* and *B*), and two-current responses to pH 4.5 (*C* and *D*) in different cells.

E and F. Proton-concentration dependence of the peak amplitudes of fast and slow current responses; representative traces from a single cell during repeated proton application (*E*) and mean ± SEM values derived from 5–10 single-cell recordings per dose (*F*). Traces shown are representative of 6 to 10 cells per experiment.

(FSCs), and ASIC1 immunoreactivity was detected only in about 3% of these cells. *Asic2* was expressed in FSCs, gonadotrophs, thyrotrophs, and somatotrophs, but not in corticotrophs or lactotrophs. *Asic4* was exclusively expressed in lactotrophs. Consistent with these findings, we detected a proton-induced current with the biophysical and pharmacological characteristics of ASICs in normal and immortalized pituitary, i.e., a fast-developing current activated by a drop in pH, which rapidly desensitized and was abolished by removal of bath sodium and application of amiloride. The current was observed in corticotrophs, thyrotrophs, somatotrophs, and GH3 cells. The pH profile of the ASIC–like current, with an EC50 pH-value of 6.5–6.7 in normal and immortalized pituitary cells, is consistent with data in the literature in other cell types. We also observed the ASIC–like current in gonadotrophs and lactotrophs, but only in a small fraction of such cells.

We also detected another proton-activated current in secretory cells, with a profile distinct from ASIC–mediated currents; the current developed slowly and deactivated rapidly, was not blocked by amiloride, nor did it desensitize during prolonged stimulation or show rundown of current during repetitive stimulation. The slow-developing current was present in all secretory cell types, but the amplitude was significantly higher in gonadotrophs and lactotrophs not exhibiting ASIC current. We used an electrophysiological protocol, with pH ranging between 7.4 and 4.5, to separate ASIC from slow-developing currents. The ASIC current was activated by changes in pH from 7.4 to 6, whereas the slow-activating current required a higher proton concentration, a pH ranging between 5.5 and 4.5. In further contrast to ASIC currents, the slow-developing current was not abolished by total replacement of bath sodium with NMDG (*N*-methyl-D-glucamine), with only a minor reduction in current amplitude under those conditions. Our investigations further showed that the current did not reverse when the holding potential was dropped below the potassium equilibrium potential and that the current was not abolished when bath sodium was replaced by calcium, nor was it abolished by removal of bath calcium. The results suggest that the slow-developing current could be mediated by cation non-specific channels. Further studies are needed to identify these channels.

Our scRNA-Seq and immunocytochemical analyses also confirmed the expression of the *GPR68* gene in non-excitable FSCs of the anterior pituitary, as previously reported, but not in native gonadotrophs, in contrast to

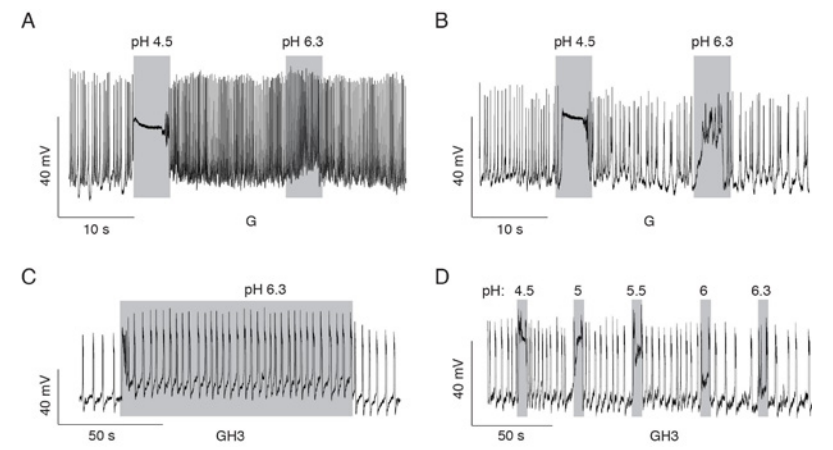


FIGURE 2. Patterns of proton-induced changes in electrical activity in spontaneously firing single pituitary cells

A and *B*. Gonadotrophs expressing slow current (*A*) and both currents (*B*).

C and *D*. GH3 cells expressing both currents. A rapid high depolarization and a sustained low depolarization of sufficient amplitude to increase the frequency of firing of action potentials (*C*) and proton-concentration dependence of the sustained low depolarization (*D*).

a previous report describing the expression and role of this receptor in immortalized mouse gonadotrophs. We also reported for the first time, to our knowledge, that the endothelial cells of pituitary portal capillaries express the *GPR4* gene, which may contribute to the proposed active role of the vasculature in pituitary function [Reference 1].

Pituitary TSH stimulates corticotrophs and melanotrophs.

It is well established that thyroid-stimulating hormone (TSH) regulates thyroid hormone synthesis and release from the thyroid gland by activating TSH receptors (TSHRs) expressed in thyroid follicular cells. Several extra-thyroidal expression sites of TSHRs have also been reported, including in the normal and adenomatous human pituitary, and receptors were identified in normal and immortalized FSCs. It has also been suggested that TSHR autoantibodies can suppress intrapituitary TSH levels independently of circulating thyroid hormone levels, suggesting that the receptors are functional. Using cultured rat pituitary cells and double immunocytochemistry, we showed, for the first time, the presence of immunoreactive TSHR in the sister cells corticotrophs and melanotrophs. The receptors were functional, as shown by a TSH–induced rise in cAMP production at an amplitude sufficient to allow the release of this messenger into the extracellular medium. The function of the receptor was further confirmed in single-cell calcium measurements in the identified corticotrophs and melanotrophs. We also showed that, in static cultures and perfused pituitary cells, TSH stimulates ACTH and beta endorphin secretion.

In general, the rise in cytosolic calcium concentration in corticotrophs and melanotrophs could be coupled to stimulation of *Pomc* gene (gene responsible for proopiomelanocortin production) expression and/or activation of the regulated exocytotic pathway. However, in our experiments, we did not observe changes in *Pomc* expression. *In vitro* TSH was effective in 2 to 200 nM concentration range, which is consistent with measurements of TSH levels in rat portal blood of around 360 nM. We also observed concentration dependence of cAMP production and hormone secretion in this concentration range. Several lines of our investigations confirmed that the stimulation of calcium influx in corticotrophs and melanotrophs bathed in calcium-containing medium reflects the coupling of TSHR to the Gs signaling pathway (which activates the cAMP pathway) and cAMP–dependent facilitation of voltage-gated calcium influx. Furthermore, our results argue against the role of protein kinase A in these processes. Further studies are needed to identify

channels modulated by the Gs signaling pathway independently of protein kinase A. In general, cAMP can regulate excitability of pituitary cells and calcium-dependent hormone secretion directly by activating hyperpolarization-activated cyclic nucleotide–gated channels, which have been identified in immortalized mouse corticotrophs. cAMP could also regulate excitability and calcium influx indirectly through EPAC (exchange factor directly activated by cAMP), whose role in CRH (corticotropin-releasing hormone)–induced signaling via a cAMP–dependent mechanism has been described in corticotroph cell lines. These results indicate that TSH is a potential paracrine modulator of anterior pituitary corticotrophs and melanotrophs, controlling the exocytotic but not the transcriptional pathway in a cAMP/calcium influx–dependent manner [Reference 3].

Down-regulation of testicular steroidogenesis during experimental autoimmune encephalomyelitis

Multiple sclerosis is an autoimmune, neurodegenerative disease of the central nervous system. This chronic inflammatory disease is more common in women than in men, but clinical progression tends to be faster and more severe in men. Studies show that low testosterone levels in the prenatal period, puberty, and adulthood increase the risk for multiple sclerosis in men. It has also been shown that low serum testosterone levels can be detected in up to 40% of multiple sclerosis patients. Such patients may experience secondary hypogonadism and poorer clinical outcomes, including a faster decline in cognitive abilities. Similarly, we and others have shown reduced serum testosterone levels during experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. However, the possible causes of declining testosterone levels in EAE animals or in patients with multiple sclerosis have not yet been elucidated. Testosterone's anti-inflammatory, neuroprotective, and promyelinating effects have been well documented using a variety of animal models and cell cultures. Moreover, testosterone is considered to be a protective factor in autoimmune diseases, including multiple sclerosis and EAE. It is therefore of great importance to clarify the events that lead to a decrease in testosterone in autoimmunity.

To address this issue, we studied the expression profile of the testicular steroidogenic pathway and evaluated testicular responsiveness to *in vivo* application of the placental gonadotropin hCG (human chorionic gonadotropin) and the GnRH (gonadotropin releasing hormone) analog buserelin acetate, during EAE. We observed no changes in the number of interstitial cells in EAE animals, but the expression of the insulin-like 3 gene was reduced at the peak of the disease, implying that the Leydig cell's functional capacity was affected. Consistent with this finding, the expression of most steroidogenic enzyme genes and proteins was reduced during EAE, including StAR, CYP11A1, CYP17A1, and HSD3B. No signs of testicular inflammation were observed. Recovery of steroidogenesis was observed after injection of hCG, the placental gonadotropin, or buserelin acetate, a gonadotropin-releasing hormone analogue, at the peak of EAE. Together, our results are consistent with the hypothesis that impaired testicular steroidogenesis originates upstream of the testes and that low serum LH is the main cause of decreased testosterone levels during EAE [Reference 4].

Calcium–prolactin secretion coupling in pituitary lactotrophs is controlled by PI4-kinase alpha.

Endocrine pituitary cells secrete hormones using both calcium signaling pathways: spontaneous voltage-gated calcium influx and receptor-controlled IP3 (inositol trisphosphate)–dependent calcium mobilization coupled with facilitated voltage-gated calcium influx. Pituitary lactotrophs, which are the subject of the present study, exhibit spontaneous plateau-bursting of electrical activity, resulting in calcium transients that have

sufficient amplitude to trigger basal prolactin (PRL) secretion. In such cells, the physiological mechanism for control of PRL release is via dopamine suppression of basal secretion. Furthermore, thyrotropin-releasing hormone triggers IP3–dependent calcium mobilization from intracellular stores and induces additional hormone secretion. However, the contribution of phosphoinositides beyond the role of PI(4,5)P2 supporting IP3 production in pituitary cell exocytosis remains poorly understood, which reflects the difficulties in up- or down-regulating the expression of enzymes that control phosphoinositide levels in cultured pituitary cells as a result of the resistance of these cells to conventional transfection procedures. The use of immortalized pituitary cells, such as GH3 lacto-somatotrophs, is also limited, because these cells predominantly secrete hormones in a constitutive manner.

This prompted us to use a pharmacological approach in this initial study on the role of phosphoinositides in calcium-secretion coupling in cultured pituitary lactotrophs, focusing on PI4P, PI(4,5)P2, and PI(3,4,5)P3. Recently, we analyzed the contribution of phosphatidylinositol kinases (PIKs) to calcium-driven prolactin (PRL) release in pituitary lactotrophs: PI4Ks, which control PI4P production; PIP5Ks, which synthesize PI(4,5) P2 by phosphorylating the D-5 position of the inositol ring of PI4P; and PI3KCs, which phosphorylate PI(4,5) P2 to generate PI(3,4,5)P3. We used common and PIK–specific inhibitors to evaluate the strength of calcium-secretion coupling in rat lactotrophs. We analyzed gene expression by scRNA-Seq and qRT-PCR analysis; intracellular and released hormones were assessed by radioimmunoassay and ELISA; and single-cell calcium signaling was recorded by Fura 2 imaging. Single-cell RNA-Seq revealed the expression of *Pi4ka*, *Pi4kb*, *Pi4k2a*, *Pi4k2b*, *Pip5k1a*, *Pip5k1c*, and *Pik3ca* genes, as well as *Pikfyve* and *Pip4k2c* genes, in lactotrophs. Wortmannin, a PI3K and PI4K inhibitor, but not LY294002, a PI3K inhibitor, blocked spontaneous action potential–driven PRL release with a half-time of about 20 min when applied in 10 μM concentration, leading to accumulation of intracellular PRL content. Wortmannin also inhibited increase in PRL release by high potassium, the calcium-channel agonist Bay K8644, and calcium-mobilizing thyrotropin-releasing hormone without affecting accompanying calcium signaling. GSK-A1, a specific inhibitor of PI4KA, also inhibited calcium-driven PRL secretion without affecting calcium signaling and PRL gene expression. In contrast, PIK93, a specific inhibitor of PI4KB, and ISA2011B and UNC3230, specific inhibitors of PIP5K1A and PIP5K1C, respectively, did not affect PRL release. These experiments revealed a key role of PI4KA in calcium-secretion coupling in pituitary lactotrophs downstream of voltage-gated and PI(4,5)P2–dependent calcium signaling [Reference 5].

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Chemosensory Coding and Decoding by Neuron Ensembles

All animals need to know what is going on in the world around them. Brain mechanisms have thus evolved to gather and organize sensory information to build transient and sometimes enduring internal representations of the environment.

Using relatively simple animals and focusing primarily on olfaction and gustation, we combine electrophysiological, anatomical, behavioral, computational, optogenetic, and other techniques to examine the ways in which intact neural circuits, driven by sensory stimuli, process information. Our work reveals basic mechanisms by which sensory information is transformed, stabilized, and compared, as it makes its way through the nervous system.

We use three species of insects, each with specific and interlocking experimental advantages, as our experimental preparations: locusts, moths, and fruit flies. Compared with the vertebrate, the insect nervous system contains relatively few neurons, most of which are readily accessible for electrophysiological study. Essentially intact insect preparations perform robustly following surgical manipulations, and insects can be trained to provide behavioral answers to questions about their perceptions and memories. Ongoing advances in genetics permit targeting specific neurons for optogenetic or electrophysiological recording or manipulations of activity. Furthermore, the relatively small neural networks of insects are ideal for tightly constrained computational models that test and explicate fundamental circuit properties.

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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

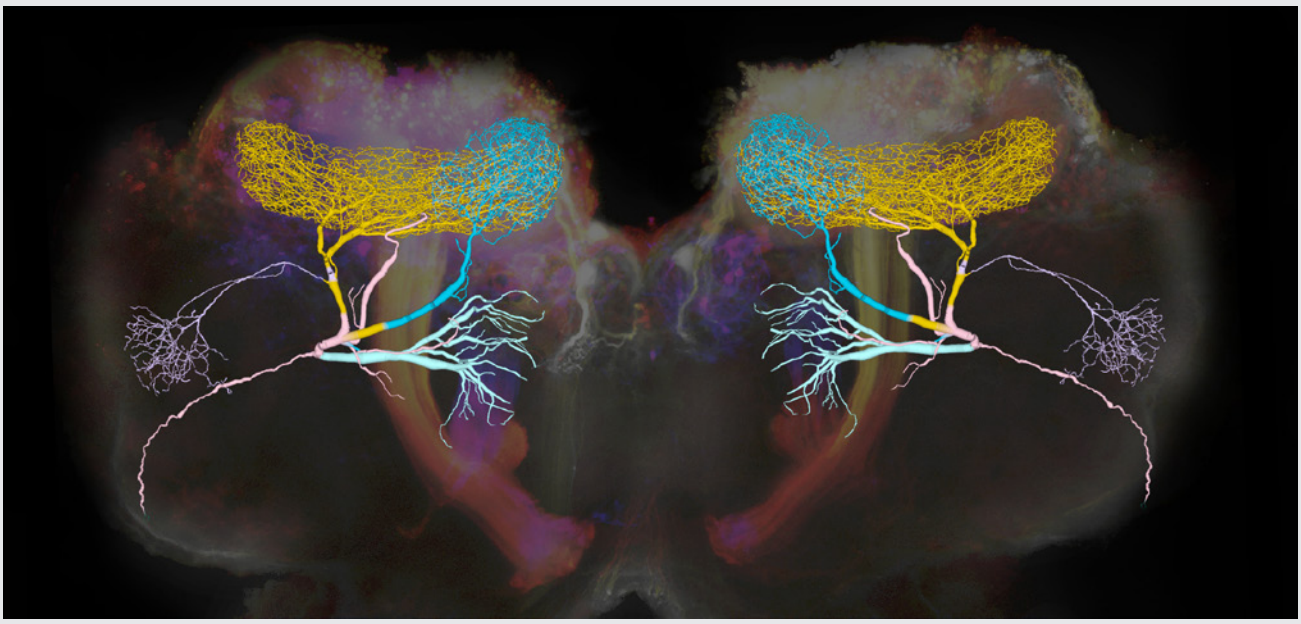

FIGURE 1. Giant GABAergic neurons regulate olfactory responses in the locust brain.

The composite image shows the structure of a compartmental computational model of the giant GABAergic neurons (GGNs) superimposed on dextran-dyed mushroom bodies in the locust brain. Different branches of GGN are shown in different colors. GGNs, only one on each side of the brain, regulate the firing of tens of thousands of olfactory neurons through feedback inhibition.

types undergo different forms of adaptation during lengthy or repeated stimuli. A computational analysis showed that such diversity of odor-elicited spiking patterns helps the olfactory system efficiently encode odor identity, concentration, novelty, and timing, particularly in realistic environments.

Feedback inhibition and its control in an insect olfactory circuit

Inhibitory neurons play critical roles in regulating and shaping olfactory responses in vertebrates and invertebrates. In insects, such roles are performed by relatively few neurons, which can be interrogated efficiently, revealing fundamental principles of olfactory coding. With electrophysiological recordings from the locust and a large-scale biophysical model, we analyzed the properties and functions of the giant GABAergic neuron (GGN), a unique neuron that plays a central role in structuring olfactory codes in the locust brain (see Figure 1). Analysis of our *in vivo* recordings and simulations of our model of the olfactory network suggest that the GGN extends the dynamic range of Kenyon cells (high-order neurons in a brain area analogous to the vertebrate piriform cortex, which fire spikes when the animal is presented with an odor pulse), which leads us to predict the existence of a yet undiscovered olfactory pathway. Our analysis of GGN–intrinsic properties, inputs, and outputs, *in vivo* and *in silico*, reveals basic new features of this critical neuron and the olfactory network that surrounds it. Together, results of our *in vivo* recordings and large-scale realistic computational modeling provide a more complete understanding of how different parts of the olfactory system interact.

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, i.e., 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, as well as controlled stimulus manipulations, we directly tested the idea in the locust olfactory system. We focused on the responses of Kenyon cells. We found that inputs arriving in Kenyon cells sum most effectively in a preferred window of the oscillation cycle. With a computational model, we established that the non-uniform structure of noisy activity in the membrane potential helps mediate the process. Further experiments performed *in vivo* demonstrated that integration windows can form in the absence of inhibition and in a broad range of oscillation frequencies.

Our results establish that cyclic integration windows can be formed from very few ingredients, i.e., 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.

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 exception is gustation. Gustatory coding by the nervous system is thought to be relatively simple, i.e., 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: identification and characterization of small noncoding RNAs; and identification and characterization of small proteins of less than 50 amino acids. Both small RNAs and small proteins have been overlooked because they are not detected in biochemical assays, and the corresponding genes are 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.

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Identification and characterization of small regulatory RNAs

During the past 20 years, we carried out several different systematic screens for small regulatory RNA genes in *Escherichia coli*, which showed that small RNAs are encoded by diverse loci, including sequences overlapping mRNAs [Reference 1]. The screens included computational searches for conservation of intergenic regions and direct detection after size selection or co-immunoprecipitation with the RNA–binding protein Hfq. Most recently, we have been using deep sequencing approaches to map the 5' and 3' ends of all transcripts to further extend our identification of small RNAs in a range of bacteria species [Reference 2]. The work uncovered several small RNAs encoded entirely within open reading frames (ORFs), including an RNA internal to an essential cell-division gene, which was found to have an independent function as a sponge that blocks the activity of another small RNA.

A major focus for the group was to elucidate the functions of the small RNAs that we and others had 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 also explored the role of ProQ, a second RNA chaperone in *E. coli* and, by comparing the small RNA–mRNA interactomes by deep sequencing, we found that Hfq and ProQ have overlapping as well as competing roles in the cell [Reference 3]. In organisms that do not have Hfq or ProQ, other proteins such as KhpA and KhpB bind to small RNAs and may have similar chaperone roles [Reference 4].

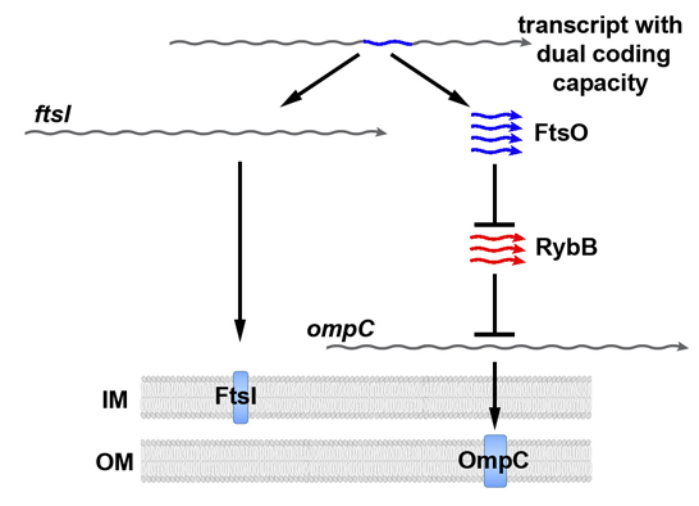

FIGURE 1. ORF–internal small RNA FtsO acts as a sponge of the RybB base-pairing small RNA.

Model showing how the same DNA sequence can encode two different gene products. The ftsI mRNA encodes the essential FtsI cell division protein found in the inner membrane (IM). The transcript also encodes the FtsO small RNA (*blue*), which blocks the activity of the RybB base-pairing small RNA (*red*). When not blocked by FtsO, RybB down-regulates the synthesis of outer membrane (OM) porins such as OmpC.

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 5]. For example, we showed that the Spot 42 RNA, whose levels are highest when glucose is present, plays a broad role in catabolite repression by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse non-preferred carbon sources. Similarly, we discovered that a Sigma(E)–dependent small RNA, MicL, transcribed from a promoter located within the coding sequence of the *cutC* gene, represses synthesis of the lipoprotein Lpp, the most abundant protein in the cell, to oppose membrane stress. We found that the copper-sensitivity phenotype previously ascribed to inactivation of the *cutC* gene is in fact derived from the loss of MicL and elevated Lpp levels. As more and more small RNAs encoded by 5' or 3' UTRs or internal to coding sequences are being found, our observations regarding MicL raise the possibility that other phenotypes currently attributed to protein defects are attributable to deficiencies in unappreciated regulatory RNAs.

In addition to small RNAs that act via limited base pairing, we have been interested in regulatory RNAs that act by other mechanisms. For instance, early work showed that the 6S RNA binds to and modulates RNA polymerase by mimicking the structure of an open promoter. In another study, we discovered that the yybP-ykoY motif, a broadly conserved RNA structure motif found in the 5' UTR of the *mntP* gene encoding a manganese exporter, directly binds to manganese, resulting in a conformation that liberates the ribosome-binding site.

Further studies to characterize other Hfq– and ProQ–binding RNAs and their physiological roles and evolution, as well as regulatory RNAs that act in ways other than base pairing, are ongoing.

Identification and characterization of small proteins

In our genome-wide screens for small RNAs, we found that a number of short RNAs actually encode small proteins. The correct annotation of the smallest proteins is one of the biggest challenges of genome annotation, and there is limited evidence that proteins are synthesized from annotated and predicted short ORFs. Although

these proteins have largely been missed, the few small proteins that have been studied in detail in bacterial and mammalian cells have been shown to have important functions in regulation, signaling, and cellular defenses [Reference 6]. 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. This approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids are synthesized. We also 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. These studies, together with the work of others, documented that *E. coli* synthesize over 150 small proteins [Hemm MR, Weaver J, Storz G. *EcoSal Plus* 2020;9:doi:10.1128/ecosalplus.ESP-0031-2019].

Many of the initially discovered proteins were predicted to consist of a single transmembrane alpha-helix and, by biochemical fractionation, were found 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 of the levels of tagged derivatives 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 are employing many of the approaches the group has used to characterize the functions of small regulatory RNAs to elucidate the functions of the small proteins. The combined approaches are beginning to yield insights into how the small proteins are acting in *E. coli*. For example, we discovered the 49–amino acid inner membrane protein AcrZ (formerly named YbhT), whose synthesis is increased in response to noxious compounds such as antibiotics and oxidizing agents, associates with the AcrAB-TolC multidrug efflux pump, which confers resistance to a wide variety of antibiotics and other compounds. Co-purification experiments, two-hybrid assays, and suppressor mutations showed that AcrZ interacts directly with the inner-membrane protein AcrB. Mutants lacking AcrZ are sensitive to many, but not all, the antibiotics transported by AcrAB-TolC. The differential antibiotic sensitivity suggests that AcrZ enhances the ability of the AcrAB-TolC pump to export certain classes of substrates. Detailed cryo-EM and mutational studies recently showed that AcrZ and cardiolipin cooperate to allosterically modulate AcrB activity [Du D *et al*., *Structure* 2020;28:625-634].

Two small proteins act to increase the intracellular levels of manganese and magnesium. We found that synthesis of a 42–amino acid protein, now denoted MntS, is increased under low magnesium conditions, which eliminates repression by MntR. The lack of MntS leads to decreased activities of manganese-dependent enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese and bacteriostasis under manganese-rich conditions. These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP. 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 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.

A limited number of transcripts encoding both a small protein and possessing base-pairing activity have been identified and denoted dual-function RNAs. However, given that few have been characterized, little is known about the interplay between the two regulatory functions. Interestingly, MgtS is encoded differently from the MgrR small regulatory RNA, which is also important for bacterial adaptation to low magnesium. To investigate the competition between protein-coding and base-pairing activities, we constructed synthetic dual-function RNAs comprised MgrR and MgtS [Aoyama JJ, Raina M, Storz G. *J Bacteriol* 2021; 204(1):JB0034521]. These constructs allowed us to probe how the organization of components and the distance between the coding and base-pairing sequences contribute to the proper function of both activities of a dual-function RNA. By understanding the features of natural and synthetic dual-function RNAs, future synthetic molecules can be designed to maximize their regulatory impact.

This work, together with our ongoing studies of other small proteins and related findings by others in eukaryotic cells, supports our hypothesis that small proteins are an overlooked class of regulators.

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Molecular Genetics of Endocrine Tumors and Related Disorders

In the project that was started in the late 1990s, our approach has been 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. 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 non-pigmented 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/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

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(continued)

tissues. Ongoing work with collaborating NCI laboratories aims to clarify the role of PDE in the predisposition to these tumors. However, it is clear from these data 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, which is caused by somatic mutations in the *GNAS* gene (encoding the G protein–stimulatory subunit alpha [$G_s\alpha$]). 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 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

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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 investigating and confirming 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 pathways interacting with cAMP/PKA in the adrenal cortex. 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.

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 (DNA-Seq). As part of the clinical protocols, much clinical research consists mostly of observations of new associations, description of novel applications or modifications, and improvements in older diagnostic methods, tests, or imaging tools, 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, which 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.

As the era of rare diseases gene discovery comes to an end, with more than half of them now molecularly elucidated, and in excess of 30 genetic associations studied by the Section's researchers over the last 25 years, the Section follows the shift that was first proposed in 2016: the clinical protocols that focused on Carney complex (1995–2020), adrenal hyperplasia (2000–2020), and other adrenal disorders were closed in 2020, as their main goals were accomplished (i.e., elucidation of the main genetic causes leading to these tumors). We replaced these studies with disease-specific, new molecular treatment trials (e.g., Pegvisomant® treatment of children with gigantism, supported by Pfizer, Inc.) and research on common disorders that relate to the

rare diseases and their molecular pathways that we have been investigating (e.g., clinical and molecular characteristics of primary aldosteronism in Black individuals, a collaborative program with the University of Michigan, Ann Arbor and others).

In the laboratory, too, our emphasis is on the identification of molecular targets that may lead to clinical trials that we will implement at the NIH CRC. We have already identified compounds in both our *PRKACA* and *GPR101* screening efforts. We are now characterizing the compounds in the laboratory and, soon, in animal models. The work would not have been done without access to NIH's Center for Advancing Translational Sciences (NCATS) and its Chemical Genomics Center (now Early Translation Branch or ETB [www.ncats.nih.gov/etb]), as well as several international collaborators.

Carney complex (CNC) genetics

We 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). 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 other diseases

We are investigating the functional and genetic consequences of *PRKAR1A* mutations in cell lines from a variety of tumors. We measure both cAMP and PKA activity in the cell lines, along with the expression of the other subunits of the PKA tetramer. 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.

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 AD *et al.*, *J Intern Med* 2019;285:215] 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

FIGURE 1.

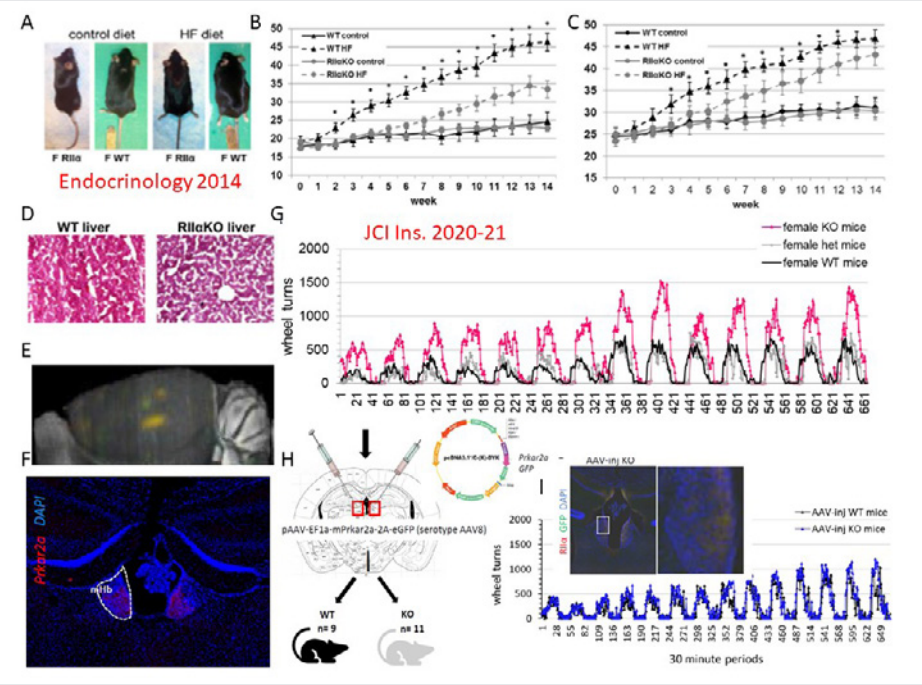
A. After a high-fat diet (HFD), *Prkar2a* knockout (KO) mice were leaner than wild-type (WT).

B, *C* & *D*. Female and male KO mice remained leaner as they aged and did not develop a fatty liver after a HFD.

E & *F*. *Prkar2a* expression was mapped to the medial habenula (MHb) and, in part, the leaner phenotype was the result of reduced HFD intake.

G. Female (shown) and male KO mice (not shown) run more than twice as much as WT during home cage running wheel access.

H & *I*. Direct injection of *Prkar2a* into the MHb reduced voluntary running* to the levels of WT and restored sucrose preference.



*Voluntary running activity was graphed in bins of 30 minutes over a two-week period.

in additional phenotypes. An example of the ongoing work using PKA–subunit animal models is the work on the Prkar2a mouse model (Figure 1). Ongoing work with several animal crosses is investigating various aspects of PKA subunit functions and the possible involvement of cAMP–pathway perturbations in several pathophysiologic and/or disease-related states.

The *Prkar2a*$^{-/-}$ mouse is involved in motivation to exercise and has preference for certain foods.

The habenula (Hb) is a bilateral, evolutionarily conserved epithalamic structure connecting forebrain and midbrain structures, which has gained attention for its roles in depression, addiction, rewards processing, and motivation. Of its two major subdivisions, the medial Hb (MHb) and lateral Hb (LHb), MHb circuitry and function are poorly understood compared with those of the LHb. *Prkar2a* encodes the cAMP–dependent protein kinase (PKA) regulatory subunit IIa (RIIa), a component of the PKA holoenzyme, which is at the center of one of the major cell-signaling pathways conserved across systems and species. Type 2 regulatory subunits (RIIα, RIIβ) determine the subcellular localization of PKA, and, unlike other PKA subunits, *Prkar2a* shows minimal brain expression except in the MHb. We previously showed that RIIα-knockout (*RII*α-KO) mice resist diet-induced obesity. More recently, we reported that *RII*α-KO mice consume less palatable, 'rewarding' foods and are more motivated for voluntary exercise. *Prkar2a* deficiency led to decreased habenular PKA enzymatic activity and impaired dendritic localization of PKA catalytic subunits in MHb neurons. Re-expression

of *Prkar2a* in the Hb rescued this phenotype, confirming differential roles for *Prkar2a* in regulating the drives for palatable foods and voluntary exercise. Our findings show that, in the MHb, decreased PKA signaling and dendritic PKA activity reduce motivation for palatable foods, while enhancing the motivation for exercise, a desirable combination of behaviors (Figure 1).

Genes encoding phosphodiesterase (PDE) in endocrine and other tumors

In patients who do not exhibit CNC or have *PRKAR1A* mutations but present 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. We have now identified variants in PRKAR1B and PRKACB predisposing to adrenal tumors, in addition to PRKACA, ARMC5, and of course PRKAR1A.

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, which is caused by Xq26.3 genomic duplication and characterized by early-onset gigantism resulting from excessive GPR101 function and consequent GH excess. To find additional patients with this disorder, we collaborated with a group 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 identifying 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

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- 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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Point of Care and Wearable Biophotonics for Characterizing Tissue Composition and Metabolism

By advancing models, methods, and devices that utilize the interaction of light with biological tissue, we strive to develop non-invasive techniques that can help guide therapy and aid in clinical decision making. The techniques are used to perform real-time quantitative measurements of clinically relevant information, including tissue blood flow, oxygen extraction, and body/tissue composition. Our research seeks to move such technologies from 'bench to bedside,' where they can be applied to clinical problems, including vascular and metabolic disease.

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Non-invasive optical imaging technology for characterization of tissue hemodynamics and composition

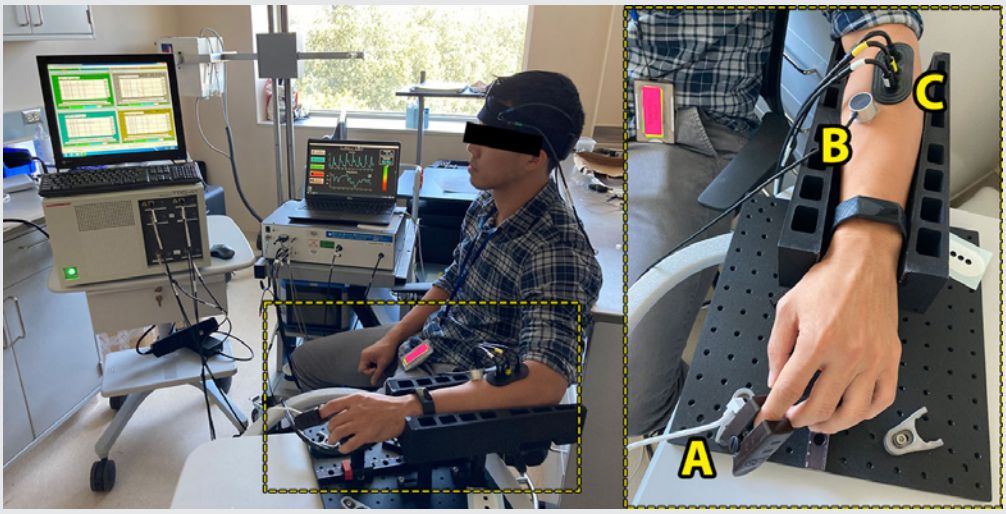
Aberrations in tissue hemodynamics (i.e., blood flow, oxygenation, and oxygen metabolism) and tissue composition are observed in a wide variety of diseases, including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders. In all such conditions, techniques that can characterize tissue hemodynamics and composition can improve strategies for early diagnosis, screening, and treatment-response monitoring.

There are several clinically accepted ways to assess cardiovascular function, specifically pertaining to tissue metabolism and composition. Techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have been used for measuring blood flow through tissue. Additionally, MRI and dual X-ray absorptiometry (DXA) can be used to determine body composition and distinguish between lean and soft tissue. While the techniques can provide comprehensive cardiovascular assessments, they are also time- and resource-intensive and can only be accessed in a hospital setting. As a result, patients typically undergo such assessments only after the onset of severe symptoms. At-home technologies, while more accessible to the general public, are limited to relatively crude cardiovascular assessments such as heart rate, oxygen saturation, and blood pressure. As alterations in vascular health occur gradually over time, there is a need for technologies that can comprehensively assess tissue hemodynamics and composition at the point of care.

To characterize tissue hemodynamics or composition in portable form factors, near-infrared spectroscopy (NIRS) techniques have emerged as

FIGURE 1. Sickle cell disease (SCD) measurement setup

Optical imaging setup for the SCD study. Several NIRS modalities are deployed for assessing hemodynamic changes during the course of treatment. The bounding box (*right*) features a closer view of the optical probes that capture hemodynamics in the fingertip (*A*), skin (*B*), and skeletal muscle (*C*).



lower-cost and non-invasive alternatives. Techniques such as diffuse optical spectroscopic imaging (DOSI) or spatial frequency domain imaging (SFDI) can quantify the concentration of hemoglobin, water, and bulk lipids. In addition to the compositional information that can be obtained, continuous measurements with such techniques can also assess tissue hemodynamics in terms of the delivery and consumption of oxygen by observing changes in oxy- and deoxy-hemoglobin concentration. While the techniques employ similar principles to measure similar information, they differ in terms of the field of view and the depth of tissue interrogated. Another subset of NIRS techniques can quantify blood flow, which is necessary to more accurately characterize tissue metabolic activity. Technologies such as laser speckle imaging (LSI), laser Doppler flowmetry (LDF), and diffuse correlation spectroscopy (DCS) all measure fluctuations in intensity caused by light-scattering events from moving particles such as red blood cells, in order to provide quantitative measure of blood flow.

These diverse optical technologies are promising candidates to provide more comprehensive assessments of tissue metabolism and composition in a range of different measurement configurations and enable our group to select optical modalities that best suit a given clinical context. We are currently working on translating the biophotonics technologies into a clinical setting and characterizing the tissue composition and metabolism in several patient cohorts with various disease states.

We are performing the current studies in collaboration with various groups in the NIH Clinical Center to evaluate changes in tissue metabolism and composition in response to different therapeutic interventions:

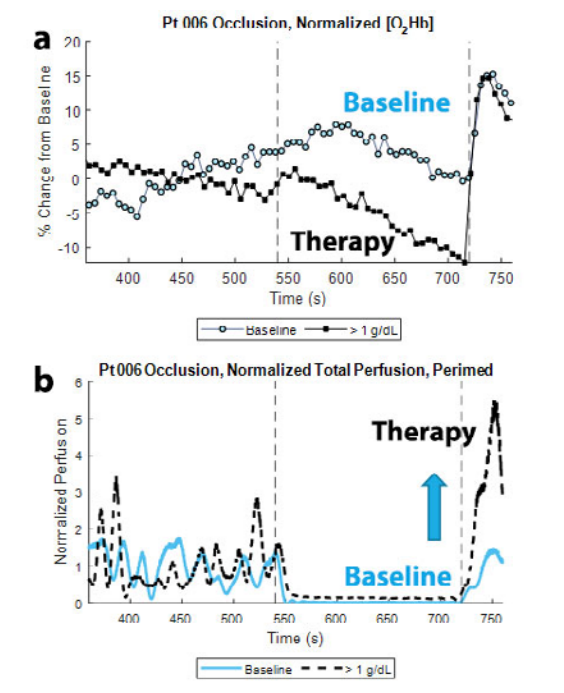
NCT04595773 – COVID-19 – CHRONIC ADAPTATION AND RESPONSE TO EXERCISE (COVID-CARE): A RANDOMIZED CONTROLLED TRIAL

The study, led by Leighton Chan, will evaluate whether a rehabilitation exercise program can help people recovering from COVID-19. Standard clinical cardiovascular-functions tests to evaluate recovery will be compared with optically obtained biomarkers.

FIGURE 2. Occlusion response at baseline and during therapy

a. Comparison of skeletal muscle O_2Hb response at the baseline visit and the first study visit in which the patient had a greater than 1 g/dL increase in blood Hb level. Both responses have been normalized by their respective mean O_2Hb concentration during the three-minute baseline time for ease of comparison. During the response visit, we can observe a larger hyperemic response in the O_2Hb trace, which can be quantified as the magnitude of the difference between the O_2Hb levels at the end of the occlusion and at the peak of the hyperemic response (ΔO_2Hb).

b. Comparison of the blood perfusion data acquired from diffuse reflectance spectroscopy (DRS) in the fingertip during the baseline and response visits. As was observed in the skeletal muscle data, we can observe a greater hyperemic response during the response visit. The data begin to suggest that there is an observable change in the vascular reactivity as a result of the Mitapivat treatment.



NCT03538639 – VASCULAR DISEASE DISCOVERY PROTOCOL

Led by Manfred Boehm, the study follows individuals with diseases of the heart and/or blood vessels. Optical signals will be characterized in vascular disease and compared with standard clinical health tests.

Optical characterization of vascular health in sickle cell disease

Given the impairments to microvascular flow and endothelial dysfunction associated with sickle cell disease (SCD), advanced quantitative NIRS is an attractive candidate to provide comprehensive hemodynamic evaluations in point-of-care settings. There are limited studies evaluating NIRS devices in SCD applications. We are optically characterizing tissue composition, metabolism, and perfusion in a cohort of SCD patients being treated with Mitapivat, a pyruvate kinase activator. While on Mitapivat, we expect improvement in the patients' red blood cell metabolism, which reduces the occurrence of hemoglobin (Hb) sickling and also improves their patients' survivability. This can be observed in blood chemistry as an increase in blood hemoglobin levels in addition to a reduction in hemolytic markers such as lactate dehydrogenase (LDH) and reticulocyte count.

Our group is assessing the sensitivity of various optical devices to hemodynamic changes induced by these treatments and evaluating whether the changes correlate with what is observed from blood chemistry. The study is being performed in collaboration with the NHLBI sickle cell branch led by Swee Lay Thein. Following an initial baseline visit, patients are started on the study drug and return for regular, follow-up visits according to a pre-set schedule. NIRS measurements are acquired at each of these visits, including at the baseline visit. For the NIRS component of the study, hemodynamics from the skin, fingertip, and skeletal muscle tissue compartments are non-invasively acquired by multiple NIRS modalities during a brachial cuff occlusion measurement (Figure 1). In this observational, proof-of-concept study, we showed that it is feasible to perform all the different measurements within an hour, with minimal discomfort to the subjects.

Patients enrolled in the study undergo a brachial cuff occlusion at each study visit. Prior literature cited the reactive hyperemic response following the end of a brachial cuff occlusion as an index of endothelial function, using modalities such as peripheral arterial tonometry. A larger hyperemic response is indicative of better endothelial function. Figure 2 summarizes some of the optical data during an occlusion from a SCD patient who exhibited an expected response to the Mitapivat treatment. Figure 2a compares the skeletal muscle oxyhemoglobin (O_2Hb) response at the baseline visit and the first study visit in which the patient had a more than 1 g/dL increase in blood Hb level. Both responses were normalized by their respective mean O_2Hb concentration during the three-minute baseline time for ease of comparison. During the response visit, we can observe a larger hyperemic response in the O_2Hb trace, which can be quantified as the magnitude of the difference between the O_2Hb levels at the end of the occlusion and at the peak of the hyperemic response (ΔO_2Hb). Figure 2b compares the blood perfusion data acquired from diffuse reflectance spectroscopy (DRS) in the fingertip during the baseline and response visits. As was observed in the skeletal muscle data, we can observe a greater hyperemic response during the response visit, which we can quantify in terms of the area under the curve (AUC) of the respective curves. The data suggest that there is an observable change in the vascular reactivity as a result of the Mitapivat treatment.

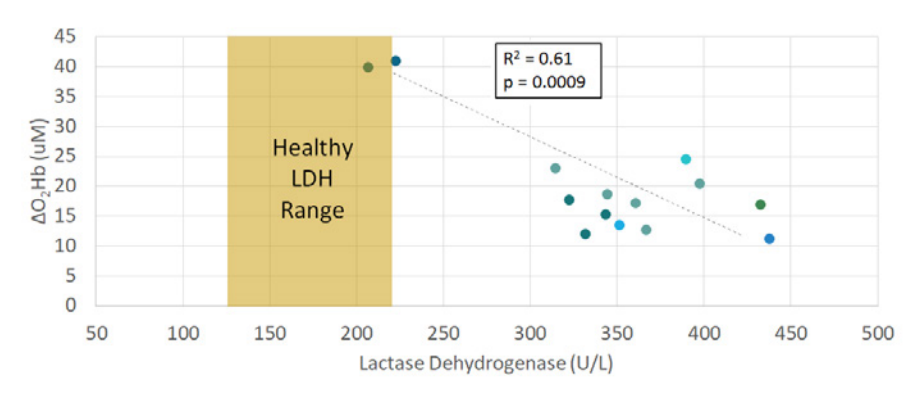
Figure 3 compares the FD-NIRS–derived optical hyperemic response to the LDH levels taken from blood draws at each available study visit (n = 14 visits). LDH has also been reported as a potential marker of nitric oxide (NO) bioavailability, which is a key component of endothelial function. In this initial dataset, there is a moderate, negative correlation ($R^2 = 0.61$) between the ΔO_2Hb and LDH levels, which would be expected, given that the lower LDH levels would signify greater bioavailability of NO and therefore improved endothelial function. As the study continues, we will incorporate additional data to further verify our findings.

Development of a wearable point-of-care monitoring device for pediatric obstructive sleep apnea

Obstructive sleep apnea (OSA) is the most common type of sleep apnea, in which the blockage of the airway causes breathing to stop involuntarily for ten seconds or more throughout the night during sleep. When

FIGURE 3. Lactate dehydrogenase vs. delta-oxyhemoglobin

LDH from blood draws is compared to the FD-NIRS–derived hyperemic response for n=14 study visits. LDH is a potential marker of nitric oxide (NO) bioavailability, which is a key component of endothelial function. In this initial dataset, there is a moderate, negative correlation between the ΔO_2Hb and LDH levels, which would be expected as lower LDH levels would signify greater bioavailability of NO and therefore improved endothelial function.



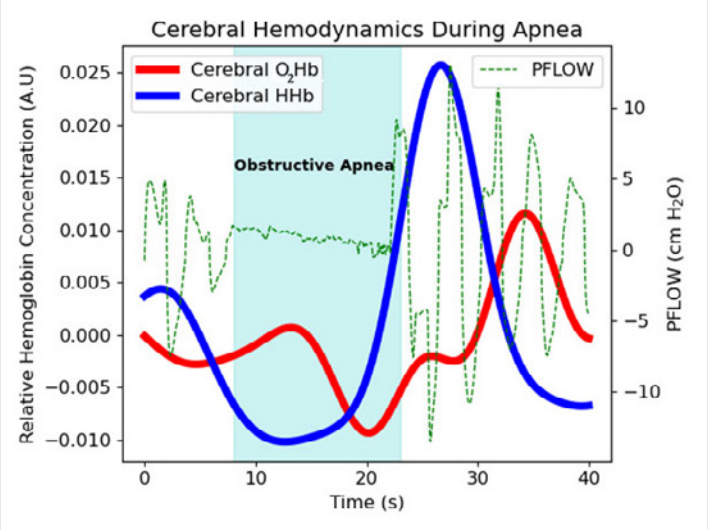


FIGURE 4. Cerebral hemodynamics during apnea

Cerebral oxyhemoglobin (O_2Hb), deoxyhemoglobin (HHb), and nasal pressure during an obstructive apnea event. During apnea, cerebral HHb increases, reaching its maximum at the end of the event. As breathing resumes, cerebral HHb falls and O_2Hb increases, signifying recovery from the hypoxia.

breathing stops, the oxygen level in the blood can drop to harmfully low levels. Pediatric obstructive sleep apnea (POSA) can be especially concerning, with several associated morbidities that can have long-term effects extending into adulthood, including adverse changes in cardiovascular, metabolic, and developmental health. Unfortunately, POSA remains largely under-diagnosed owing to a lack of education about symptoms and limited availability of sleep-medicine physicians. Early diagnosis and treatment are imperative to prevent many of the morbidities. Polysomnography (PSG), the current standard of care, is expensive, cumbersome, and resource-intensive. At-home sleep apnea tests (HSAT) are less expensive but are equally uncomfortable, prone to user error, and are less effective in children. There is a pressing need for an accurate, robust, unobtrusive alternative for monitoring POSA in the home.

NIRS is a non-invasive optical technique that can provide direct examination of tissue hemodynamics using near-infrared light in the range of 700 to 1000 nm. Compared with other well established brain imaging modalities, such as fMRI and PET, the technique has a higher temporal resolution (in order of milliseconds), and provides additional physiological information, including heart rate variability and respiration rate. Also, NIRS devices are smaller and can be built into a compact, inexpensive form factor, which is advantageous for ease-of-use and accessibility. NIRS instruments can tolerate subject motion to a greater extent than can fMRI. Such features make the technique ideally suited for studying children, particularly those with problems such as attention-deficit hyperactivity disorder (ADHD), which can make keeping still for long periods of time highly challenging. The relationships between NIRS parameters and traditional measurements taken during a sleep study are still largely unknown. NIRS could provide better prediction of health outcomes and a greater understanding of brain hypoxia during OSA events.

Initial measurements using the platform were obtained on adults recruited at the UC Irvine sleep center by Ruth Benca and Rami Khayat. Figure 4 shows NIRS signals for oxy- (O_2Hb) and deoxy-hemoglobin (HHb) during a sample apnea event. The most pronounced feature is the rise in HHb during the interruption in respiration (nasal air flow obtained by PSG is shown in green). Based on this feature, we created an automated optical index by counting the number of HHb peaks occurring per hour. Sleep sessions were separated into

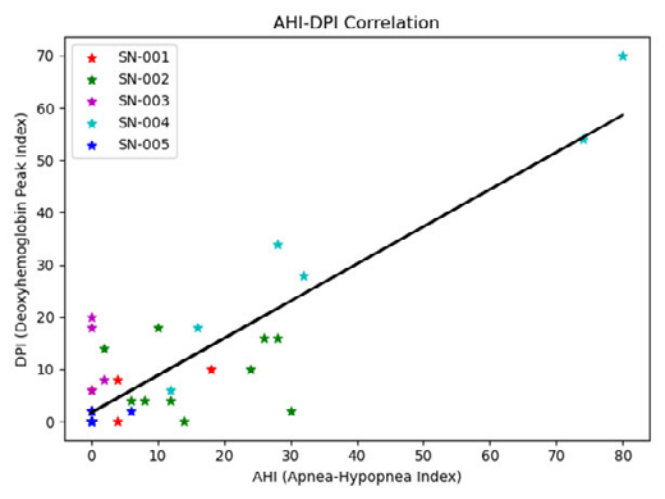


FIGURE 5. AHI–DPI correlation

Scatterplot of DPI (deoxyhemoglobin peak index) vs. AHI (apnea hypopnea index) for contiguous thirty-minute intervals for each patient (separated by color) with the line of best fit (*black*).

30 minute increments over which our optical index and the number of apnea and hypopnea events per hour (AHI) were determined. Apnea and hypopnea events are manually scored by a sleep technician following the sleep study. The Pearson's correlation coefficient, a measure of the strength of linear association, was 0.88, indicating a strong correlation between our NIRS–based parameter and the traditional sleep metric (Figure 5).

The IRB protocol (*NCT05052216*) "Development of a Wearable Point of Care Monitoring Device for Pediatric Obstructive Sleep Apnea" was recently approved and will begin enrolling subjects at the NIH Clinical Center early next year in collaboration with Amir Gandjbakhche and Ashura Buckley. We will continue to investigate how optical signals change throughout sleep by comparing physiological signals derived from NIRS with data from PSG and how to develop future optical technologies to provide better patient care that can be used in a home setting. We will test the potential of NIRS technology to diagnose POSA and examine the response of the brain and tissue during apnea/hypopnea events.

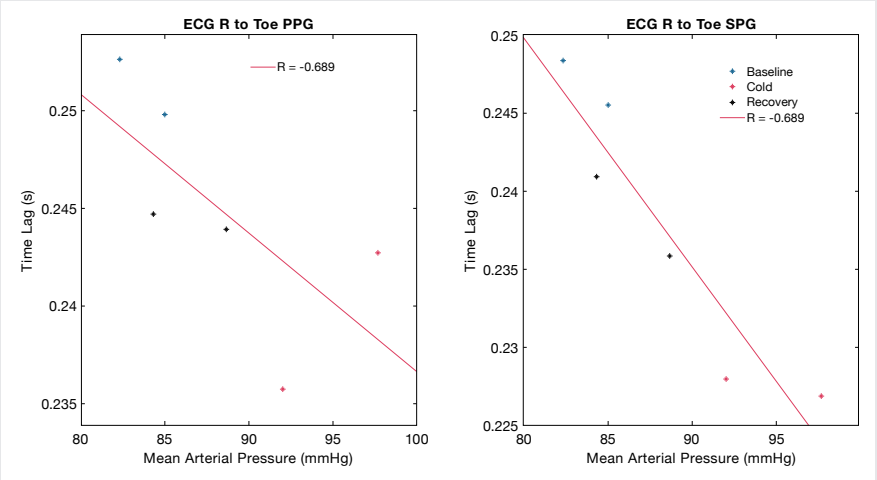
Wearable laser-speckle imaging techniques

We are currently working to advance a modified form of laser-speckle imaging (LSI) known as affixed transmission speckle analysis (ATSA). The wearable device uses a single coherent light source and a camera sensor to capture two types of pulsatile waveform data, a waveform that is known as the photoplethysmographic (PPG) waveform, which is related to changes in blood volume, and the speckle plethysmographic (SPG) waveform, which is related to changes in blood flow. These related waveforms contain important information regarding cardiovascular health, with PPG commonly used in pulse oximetry as well as heart rate and heart-rate variation measurements. More advanced analyses of these signals can potentially aid in evaluating cardiovascular disease and produce markers of vascular stiffness. Additionally, the signals have the potential to act as a surrogate for continuous blood-pressure monitoring. Our lab is working to improve the ATSA technology by improving the accuracy and stability of the device over a larger range of flow speeds.

Blood pressure (BP) is one of the key markers of cardiovascular health, signaling the proper flow of blood from the heart to the body. While cuff-based methods for determining BP are reliable for standard clinical settings, they can be time-limited, allowing only intermittent measurements that are not easily integrated

FIGURE 6. Pulse transit time vs. blood pressure

Comparison of PTT (pulse transit time) using conventional PPG (photoplethysmography) (*left*) and SPG (speckle plethysmography) (*right*) during a cold pressor test. PTT based on SPG displays a higher correlation with measured blood pressure.



into everyday life. A continuous method of assessing BP would be highly beneficial for monitoring patients after any kind of medical intervention and could serve as a valuable predictor of future cardiovascular events. Recently, several approaches have evaluated pulse transit time (PTT) measurements as a means of estimating BP continuously. The techniques commonly employ ECG coupled with PPG waveforms and measure the time taken for an arterial pulse pressure wave to travel from the aortic valve to a peripheral site. SPG has been demonstrated to have better signal quality than PPG, which makes it an attractive candidate to evaluate in this context. Figure 6 shows preliminary data comparing PTT measurements based on PPG and SPG during a cold pressor test. The cold pressor test induces vasoconstriction and an increase in blood pressure by lowering the ambient temperature around a peripheral limb. While both methods display an inverse relationship with blood pressure, PPG PTT (Figure 6 left, R = -0.689) displays a more moderate correlation than does SPG PTT (Figure 6 right, R = -0.938). A study to further investigate SPG PTT as a marker for cuff-free continuous monitoring of BP is currently being developed.

Development of a multimodal biosensor for screening and monitoring infectious diseases

The ongoing COVID-19 pandemic has been a taxing challenge to healthcare systems worldwide. A surge in cases can threaten to overwhelm hospitals' personnel and resources. The wide range and varying severities of COVID-19 symptoms further add to the challenge of managing the disease. While some cases of COVID-19 are mild and require no further intervention, more severe cases can involve major cardiorespiratory symptoms that could require a ventilator. Rapid, point-of-care tests are valuable for identifying patients who are infected with the virus; however, they do not serve as indicators for patient outcomes. A compact method to monitor patient health status and predict patient outcomes could improve our understanding of physiological impact of COVID-19 and alleviate potential strain on hospital resources.

The current standard of care to monitor patient health status revolves around vital-sign parameters such as heart rate, arterial oxygen saturation, respiration rate, and temperature. Some reports have examined personal health devices such as the Apple Watch, Oura Ring, and WHOOP, and the continuous health data they all collect, in order to develop models to identify symptomatic patients infected with COVID-19. However, the work still

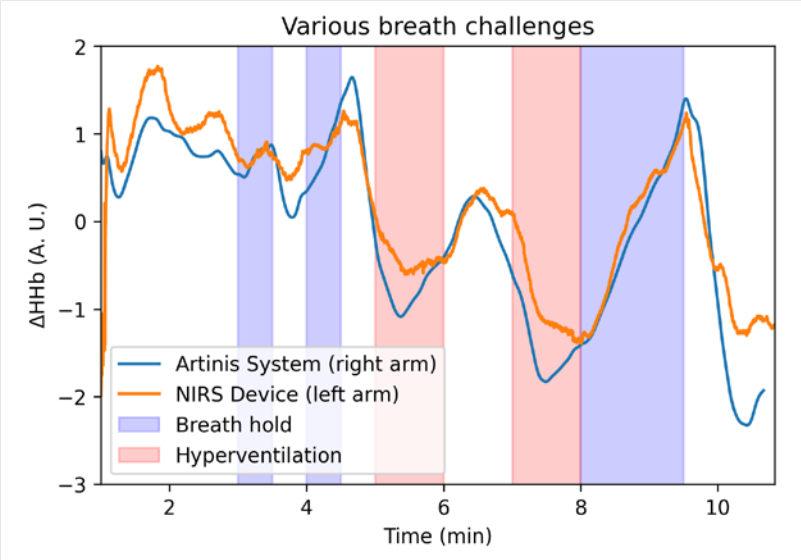


FIGURE 7. Validation of biosensor with a commercial NIRS device

Comparison of deoxyhemoglobin changes during breath hold and hyperventilation challenges as measured by the biosensor and a commercial NIRS system.

relies on the basic vital-sign parameters mentioned above, which only represent a small subset of information on how the body is functioning. Given the growing evidence of the negative impact of COVID-19 on the cardiovascular system, especially the microvasculature, characterization of microvascular health could further improve these predictive models.

NIRS is uniquely positioned to provide distinct information about the delivery and consumption of oxygen in compact form factors. Tissue oxygen saturation, which describes the oxygenation of blood at the tissue and capillary level, is one such metric that can offer valuable insight into microvascular function. In contrast, arterial oxygen saturation, which is normally reported with pulse oximetry, describes the oxygenation of blood coming from the arteries and is therefore an indicator only of oxygen supply. With a sufficiently high sampling rate, the PPG waveform can also be acquired, which enables NIRS to collect the same vital sign parameters as those collected by commercially available wearables.

We developed a multi-modal optical biosensor capable of real-time, continuous monitoring of tissue oxygenation in addition to vital-sign parameters such as heart rate, respiration rate, and temperature. Figure 7 demonstrates the performance of the biosensor in measuring deoxyhemoglobin changes during respiratory challenges compared with a commercial NIRS device. A pilot study to characterize performance of the biosensor in healthy adults has been approved (*NCT05035420*) and will soon start to enroll subjects. We believe that the device has the potential to provide continuous assessment of microvascular health, which can supplement current patient-monitoring tools and aid in the prediction of patient outcomes.

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- ”Development of a Wearable Point of Care Monitoring Device for Pediatric Obstructive Sleep Apnea,” NICHD Director’s Award, NIH IRP

Publications

1. Lam J, Hill B, Quang T, Amelard R, Kim S, Yazdi H, Warren R, Cutler K, Tromberg B. Multi-modal diffuse optical spectroscopy for high-speed monitoring and wide-area mapping of tissue optical properties and hemodynamics. *J Biomed Opt* 2021 26:085002.
2. Warren R, Bar-Yoseph R, Hill B, Reilly D, Chiu A, Radom-Aizik S, Cooper D, Tromberg B. Diffuse optical spectroscopic method for tissue and body composition assessment. *J Biomed Opt* 2021; submitted.

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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 that both types of vessels play in pathologies such as 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, which are readily accessible to observation and experimental manipulation, features that permit observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific defects (Figure 1). 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.

As a second major effort in addition to our work on vessel development, we are 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, using a novel 'EpiTag' epigenetic reporter line and the first large-scale genetic screen for tissue-specific epigenetic regulators in a vertebrate organism.

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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

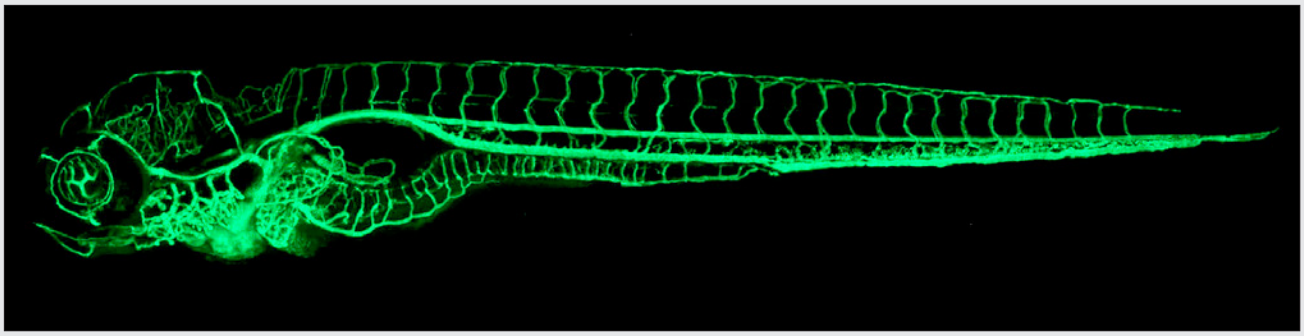

FIGURE 1. The zebrafish vascular system

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.

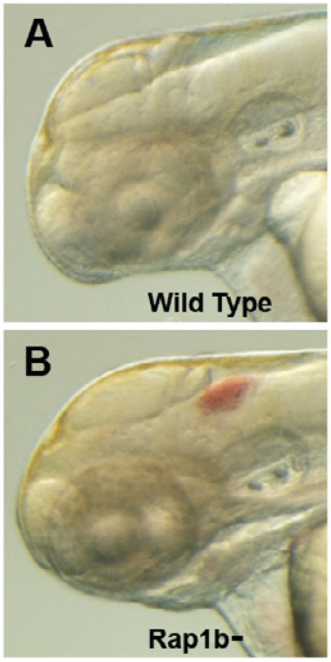

FIGURE 2. Intracranial hemorrhage (ICH) in the developing zebrafish

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*).

studies in model organisms, including the zebrafish. Using the fish, we are carrying out several related projects, which are described below.

NEW TOOLS FOR EXPERIMENTAL ANALYSIS OF VASCULAR DEVELOPMENT

We generate novel transgenic lines for visualizing different types of endothelial perivascular cells and for driving gene expression or performing molecular profiling of mRNAs and microRNAs in these 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 the 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

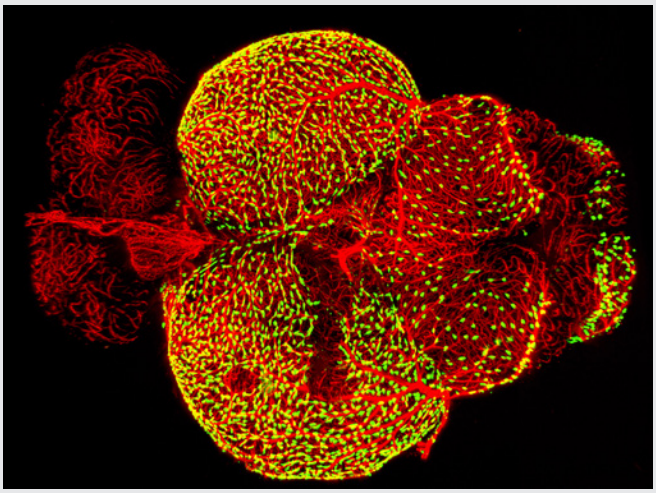

FIGURE 3. Novel perivascular cells on the zebrafish brain

Confocal micrograph of fluorescent granular perithelial cells (FGPs, *green*) adhering to the outside of meningeal blood vessels (*red*) 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.

and debilitating form of stroke associated with high morbidity and mortality. Meningeal vascular dysfunction is also associated with neuro-cognitive deficits and neuro-degenerative 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

With forward-genetic screens we 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), which result 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 its function and embryonic origins are still not well understood. We recently discovered and characterized fluorescent granular perithelial cells (FGPs) in the zebrafish, a novel endothelium-derived perivascular cell population closely associated with meningeal blood vessels, 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.

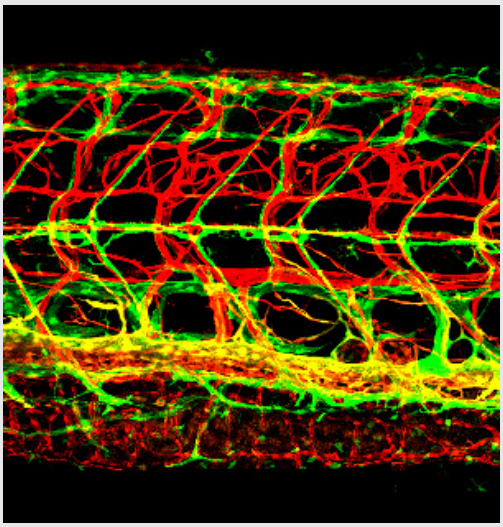

FIGURE 4. Novel lymphatic vascular reporter

Lateral view confocal image of the trunk of a 12 dpf (days post-fertilization) *Tg(kdrl:cherry); Tg(mrc1a:egfp)* double-transgenic zebrafish with red fluorescent blood vessels and green fluorescent lymphatics. See Jung HM, *et al*. *Development* 2017;144:2070 for additional details.

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 ground-breaking 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 purposes 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, and tissue-specific molecular profiling of developing lymphatic vessels and are using these transgenic animals to further 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 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.

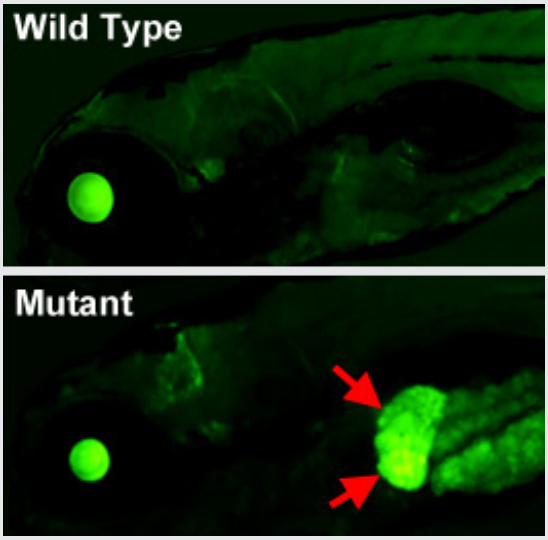

FIGURE 5. An epigenetic silencing mutant in the zebrafish

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, which permits dynamic, tissue-specific visualization of epigenetic silencing in living animals.

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.

FORWARD–GENETIC SCREEN FOR EPIGENETIC REGULATORY FACTORS

Genetic screens carried out in *Drosophila* and the nematode *Caenorhabditis 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 5).

MOLECULAR MEDIATORS OF GLYCEMIC MEMORY IN DIABETIC VASCULOPATHY

The global burden of diabetes has risen dramatically, with projections that more than 600 million adults will be affected by 2030. Micro- and macrovascular complications in patients with diabetes are the major causes of cardiovascular mortality, renal failure, blindness, and non-traumatic amputations. Diabetes-related complications can emerge even many years after the blood sugar level levels have been brought under control, a phenomenon known as 'glycemic memory.' Although the cause of the phenomenon remains to be elucidated, epigenetic alterations in endothelial cells (ECs) may be responsible for the perdurance of diabetic vascular effects. We are using the zebrafish as an *in vivo* model to examine whether short-term exposure to hyperglycemia results in persistent transcriptomic and epigenomic changes in endothelial cells, even after return to normo-glycemic conditions. We identified several genes with significantly altered endothelial transcription and methylation levels during hyperglycemia that persist during the memory phase. We are currently carrying out further investigation of these 'glycemic memory loci' by a variety of methods. Unveiling the epigenetic and transcriptomic landscape of glycemic memory in ECs may lead to better identification of

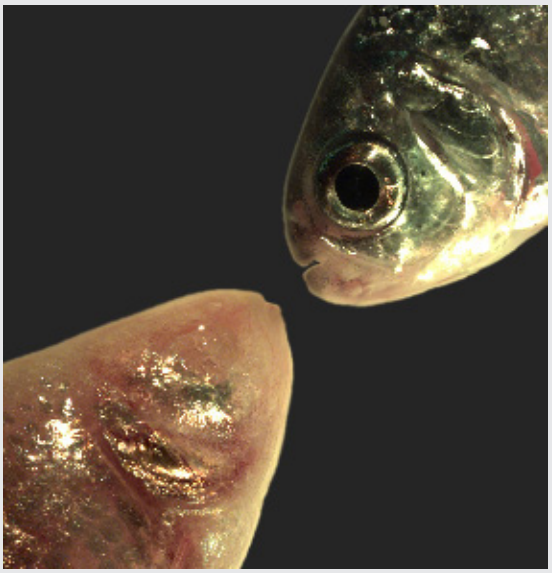

FIGURE 6. Mexican tetra cave- and surface fish

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.

molecular targets and, potentially, to the design of personalized, epigenetic-based therapies to alleviate the enormous burden of diabetic vasculopathy.

EPIGENETIC REGULATION OF FAT AND MUSCLE DEVELOPMENT IN CAVEFISH

In addition to eye and pigment loss and other adaptations, *Astyanax* cavefish (Figure 6) have extreme and unusual metabolic adaptations that allow them to survive chronic and long-term food deprivation, including excess fat deposition, altered liver function, and resistance to metabolic disease. We hypothesize that, in a similar manner to loss of eyes, changes in epigenetic gene regulation may also underlie cavefish metabolic adaptations. We are using single-cell profiling to investigate differences in adipocytes and other cell types in the muscles (where in cavefish there are large amounts of stored fat) and livers of cavefish and surface fish. We are also performing whole-genome bisulfite sequencing and RNA-Seq from surface and cavefish muscles and livers to identify differentially expressed and methylated genes. We will follow up on these findings to elucidate how differential DNA methylation influences fat metabolism and obesity.

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- NICHD Intramural Research Fellowship (to M. Rahmani)

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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 trans-lesion 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 (pol IV/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 studies

The *Escherichia coli dnaE* gene encodes the alpha-catalytic subunit (pol III alpha) of DNA polymerase III, the cell's main replicase. Like all high-fidelity DNA polymerases, pol III possesses stringent base and sugar discrimination. The latter is mediated by a so-called “steric gate” residue in the active site of the polymerase that physically clashes with the 2'-OH of an incoming ribonucleotide. Our structural modeling data suggests that H760 is the steric gate residue in *E. coli* pol III alpha. To understand how H760 and the adjacent S759 residue help maintain genome stability, we generated DNA fragments in which the codons for H760 or S759 were systematically changed to the other nineteen naturally occurring amino acids, and we attempted to clone them into a plasmid expressing pol III core (alpha, theta, and epsilon). Of the possible 38 mutants, only 9 were successfully sub-cloned: 3 with substitutions at H760 and 6 with substitutions at S759. Three of the plasmid-encoded alleles, S759C, S759N and S759T, exhibited mild to moderate mutator activity and were moved onto the chromosome

for further characterization. The studies revealed altered phenotypes regarding deoxyribonucleotide base selectivity and ribonucleotide discrimination. We believe that these are the first *dnaE* mutants with such phenotypes to be reported in the literature [Reference 1].

In a collaboration with Myron Goodman, we explored the role of the beta-sliding processivity clamp on the efficiency of *E. coli* DNA polymerase V (pol V), catalyzed DNA synthesis on undamaged DNA, and during translesion DNA synthesis (TLS). Primer elongation efficiencies and TLS were strongly enhanced in the presence of beta. The results suggest that the beta-sliding clamp may have two stabilizing roles: its canonical role in tethering the polymerase at a primer-3'-terminus, and a possible second role in inhibiting pol V ATPase, so as to reduce the rate of mutasome-DNA dissociation [Reference 2].

We extended our prokaryotic ribonucleotide excision repair (RER) studies in a collaboration with Anders Clausen. Ribonucleotides are frequently incorporated into DNA and can be used as a marker of DNA replication enzymology. To investigate on a genome-wide scale, how *E. coli* pol V accesses undamaged chromosomal DNA during the SOS response, we mapped the location of ribonucleotides incorporated by steric-gate variants of pol V across the entire *E. coli* genome. To do so, we used strains that were deficient in ribonucleotide excision repair (delta rnhB) and deficient in pol IV DNA polymerase, that constitutively express all SOS–regulated genes [*lexA*(Def)] and constitutively "activated" RecA* (*recA730*). The strains also harbor two steric gate variants of *E. coli* pol V (Y11A or F10L) or a homolog of pol V (pol V_{R391}-Y13A). Ribonucleotides are frequently incorporated by the pol V-Y11A and pol V_{R391}-Y13A variants, with a preference for the lagging strand. In contrast, the pol V-F10L variant incorporates fewer ribonucleotides, and no strand preference was observed. We observed sharp transitions in strand specificity at the replication origin (oriC), while a gradient was observed at the termination region. To activate RecA* in a $recA^+$ strain, we treated the strains with ciprofloxacin and mapped the location of the incorporated ribonucleotides genome-wide. Again, the pol V-Y11A steric gate variant exhibited a lagging strand preference. Our data are therefore consistent with a specific role for pol V in lagging strand DNA synthesis across the entire *E. coli* genome during the SOS response [Reference 3].

When subcloned into low-copy-number expression vectors, *rumAB*, encoding pol V_{R391} ($RumA'_2$ B), is best characterized as a potent mutator that gives rise to high levels of spontaneous mutagenesis *in vivo*. This is in dramatic contrast to the poorly mutable phenotype when $polV_{R391}$ is expressed from the native 88.5 kb R391, suggesting that R391 expresses *cis*-acting factors that suppress the expression and/or the activity of $polV_{R391}$. Indeed, we recently discovered that $SetR_{R391}$, an ortholog of lambda cI repressor, is a transcriptional repressor of rumAB. Our studies revealed that $CroS_{R391}$, an ortholog of lambda Cro, also serves as a potent transcriptional repressor of rumAB. Levels of RumA are dependent upon an interplay between $SetR_{R391}$ and $CroS_{R391}$, with the greatest reduction in RumA protein levels observed in the absence of $SetR_{R391}$ and the presence of $CroS_{R391}$. Under these conditions, $CroS_{R391}$ completely abolishes the high levels of mutagenesis promoted by $polV_{R391}$ expressed from low-copy-number plasmids. Furthermore, deletion of $croS_{R391}$ on the native R391 results in a dramatic increase in mutagenesis, indicating that $CroS_{R391}$ plays a major role in suppressing $polV_{R391}$ mutagenesis *in vivo*. Inactivating mutations in $CroS_{R391}$ therefore have the distinct possibility of increasing cellular mutagenesis, which could lead to the evolution of antibiotic resistance of pathogenic bacteria harboring R391 [Reference 4].

Eukaryotic studies

Maintaining the genomic integrity of cells is vital, as alterations in the genetic code can result in deregulation of cellular function, malignant transformation, or cell death, which can lead to a variety of disorders, including

neurological degeneration, premature aging, developmental defects, and cancer. To prevent genetic alterations, cells employ a range of genome-stability pathways, which allow for the accurate metabolism of the DNA, as well as for any DNA errors or damage to be rapidly repaired. Post-translational modifications play an essential role in the signaling, activation, and coordination of the genome stability pathways. The reversible ubiquitination of proteins is one such essential modification. Ubiquitination is mediated by a cascade of E1, E2, and E3 ubiquitin enzymes, which covalently attach the 8.5 kDa ubiquitin protein onto a substrate molecule, while de-ubiquitinating enzymes (DUBs) can edit or remove ubiquitin modifications.

Human DNA polymerase iota (pol iota) was discovered by scientists in our lab two decades ago, yet its cellular function remains enigmatic. As part of our ongoing research on pol iota, we previously reported that the enzyme is ubiquitinated at over 27 individual sites in the 715–amino acid protein. In collaborative studies with Irina Bezsonova, we have now identified ubiquitin-specific protease 7 (USP7) as the enzyme that de-ubiquitinates pol iota. This is particularly interesting, as USP7 has recently emerged as a key regulator of ubiquitination in the genome stability pathways because of its extensive network of interacting partners and established roles in cell-cycle activation, immune responses, and DNA replication. USP7 is also deregulated in many cancer types, where deviances in USP7 protein levels are correlated with cancer progression.

USP7 contains of an N-terminal tumor necrosis receptor associated factor (TRAF)–like domain, a catalytic domain, and five C-terminal ubiquitin-like domains (UBLs). While the catalytic domain mediates the enzymatic function of the protein, the TRAF–like and UBL domains are essential for substrate specificity and enzymatic activation. These functions are mediated by protein-binding sites, located on TRAF and the first and second UBL domain (UBL1–2). Interestingly, while all other characterized USP7 substrates bind to one or the other protein-binding sites, our studies with DNA polymerase iota revealed that a novel USP7 substrate interacts with both domains. Using biophysical approaches and mutational analysis, we characterized both interfaces and demonstrated that bipartite binding to both USP7 domains is required for efficient DNA polymerase iota de-ubiquitination. Taken together, our data established a new bipartite mode of USP7–substrate binding [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. The alarming rise in body weight has likely occurred because the current environment affords easy access to energy-dense foods and requires less voluntary energy expenditure. However, such an environment leads to obesity only in those individuals whose body weight–regulatory systems are not able to control body adiposity with sufficient precision in our high calorie/low activity environment, which suggests there are subgroups in the U.S. with a uniquely high susceptibility to weight gain under the prevailing environmental conditions. Our primary goal is to elucidate the genetic underpinnings of the metabolic and behavioral endophenotypes that contribute to the development of obesity in children. Using our unique longitudinal cohorts of children 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 intensively study genetic variants that impair gene function. We expect that such 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. Such genes include the leptin receptor (*LEPR*), genes that appear to alter leptin receptor signal transduction such as those that are part of the BBSome (a protein complex of seven proteins), 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 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 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 lower length and fat-free mass than *MC3R*$^{hWT/hWT}$. *MC3R*$^{hDM/hDM}$ mice do not have increased adipose tissue inflammatory-cell infiltration or greater expression of inflammatory markers despite their greater fat mass. Serum adiponectin is elevated 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 do *MC3R*$^{hWT/hWT}$ *MSCs*. *MC3R*$^{hDM/hDM}$ thus impacts nutrient partitioning to generate increased adipose tissue that appears metabolically healthy. These data confirm the importance of MC3R signaling in human metabolism and suggest a previously unrecognized role for the MC3R in adipose tissue development. Ongoing studies continue to improve our understanding of the phenotype of such mice. We are investigating a novel role for 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 (Reference 1), we are also studying the importance of hepatic and adipose tissue MC3R for whole body homeostasis.

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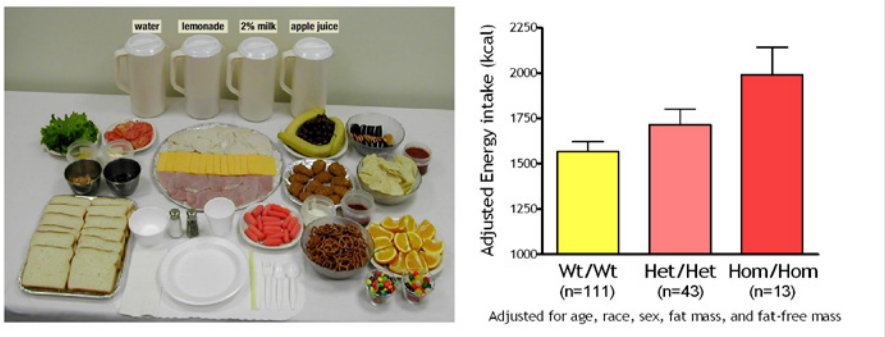
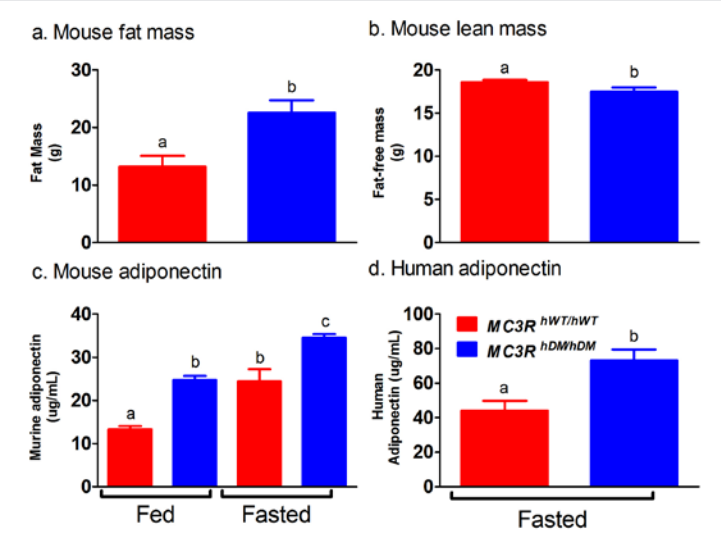


FIGURE 1. Energy intake studied using free-access buffet meals of palatable foods

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*).



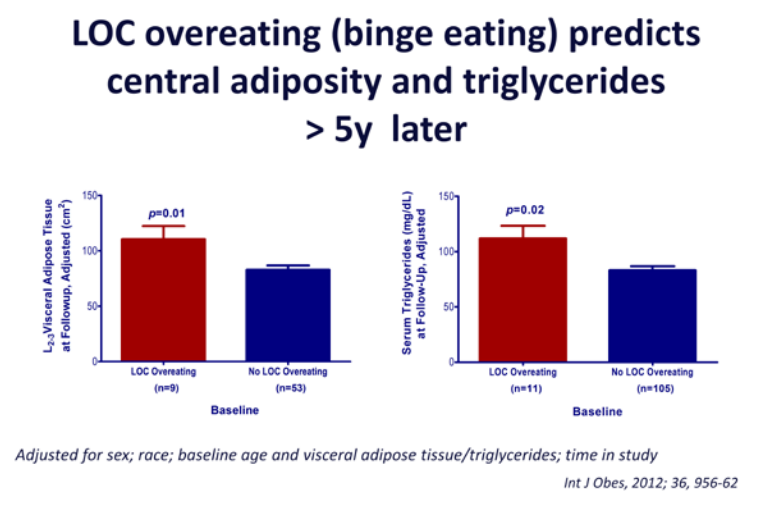
Physiology, metabolism, and psychology of childhood body-weight regulation

Our studies are directed at understanding the physiological, psychological, and metabolic factors that place children at risk for undue weight gain. As part of these studies, we examined how best to measure eating-related psychopathology, insulin sensitivity, changes in body composition, energy intake, and energy expenditure in children, and we studied the short- and long-term stability of the components of the metabolic syndrome. We had previously found that leptin is an important predictor of weight gain in children, and we identified children with hyperleptinemia and leptin-receptor mutations. We also found hyperleptinemia to be out of proportion with body fat mass in children with psychological loss of control (LOC) over eating. Such data suggest the importance of leptin resistance as a factor stimulating weight gain and led to recent explorations of other syndromes associated with obesity that may cause dysregulation of leptin signaling, including WAGR, Bardet-Biedl (Reference 2), and Alström syndromes. Current studies are directed at understanding additional genetic, physiological, and psychological factors that place children at risk for undue weight gain, including humoral factors, sleep, food cravings (Reference 3), negative affective states such as depression and anxiety, weight-based teasing, alexithymia, executive functioning, and LOC eating. Some recent initiatives have targeted insulin resistance in girls at high risk for type 2 diabetes because of obesity and a family history of diabetes.

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors also are associated with adiposity in children and abnormalities in metabolism. We found that binge-eating behaviors may predict future weight gain in children at risk for obesity: children reporting binge-eating behaviors such as LOC 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 reduced 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 eating had higher serum leptin and are at significantly greater risk for

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 the 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).



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. These data also suggest that interventions targeting disordered eating behaviors may be useful in preventing excessive fat gain in children prone to obesity and have led to trials of preventative strategies related to binge eating.

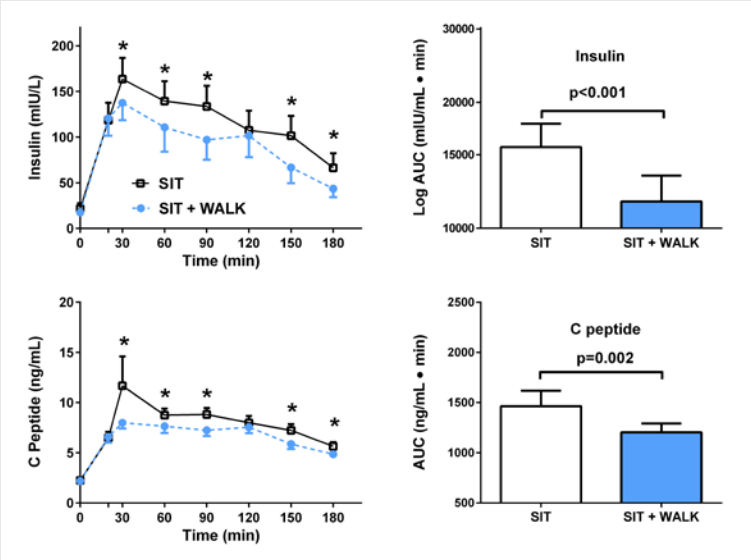
In order to determine the factors that are most important for the development of the complications of obesity in youth, we study normal-weight children and adolescents, children who are already obese, and the children of obese parents who themselves are not obese. We examine body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, energy expenditure (Reference 4), 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 are also studied. We follow children 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 better in children with overweight or obesity who engaged in moderate activity for just three minutes every half hour than in those who remained sedentary.

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

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*).



metformin may affect the body weight of younger children who have hyperinsulinemia and are therefore at risk for later development of type 2 diabetes. Those randomized to metformin decreased BMI, BMI-Z score, and body fat mass to a significantly greater extent than did placebo-treated children. Serum glucose and HOMA-IR (homeostatic model assessment for insulin resistance) were also significantly lower in metformin-treated than in placebo-treated children.

A second study compared prevention of weight gain using interpersonal therapy (IPT) with a control health education program (HE) in adolescents reporting LOC 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 was associated with the steepest declines in BMIz compared with HE. For adiposity, girls with high or low anxiety in HE and girls with low anxiety in IPT experienced gains, while girls in IPT with high anxiety stabilized. Parent reports yielded complementary findings. Such results have stimulated ongoing research to examine how anxiety may stimulate energy intake. We also published preliminary data from a third study examining IPT approaches in younger children, finding good tolerability for such a program.

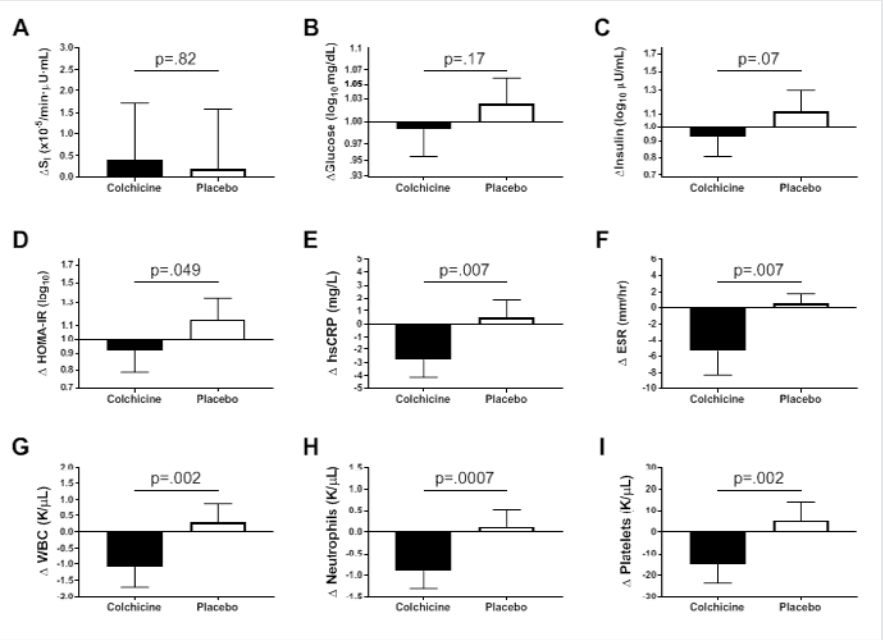
A fourth study examined whether reducing depressive symptoms could ameliorate insulin resistance in adolescents at risk for type 2 diabetes. Among girls with greater (moderate) baseline depressive symptoms (N = 78), those in cognitive behavioral therapy (CBT) developed significantly lower two-hour insulin than those in HE. Additional metabolic benefits of CBT were seen for this subgroup in *post hoc* analyses of post-treatment to one-year change.

An ongoing study based on lab data finding links between attentional biases to high-palatability foods in children with obesity examines 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 in patients with Bardet Biedl syndrome

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.



(Reference 2). 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 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 the metabolic syndrome, but who did not have diabetes, were randomized to 0.6 mg colchicine or placebo capsules twice daily for three months. Compared with placebo, colchicine significantly reduced C-reactive protein and erythrocyte sedimentation rate (Figure 5). The significant changes in homeostatic model assessment of insulin resistance, fasting insulin, and glucose effectiveness suggested metabolic improvements in the colchicine compared with the placebo group. We are now conducting a larger, adequately powered study 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: Colchicine for Treatment of Metabolic Syndrome. 2020–2021
- 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 diazoxide choline sustained release tablets in patients with Prader-Willi syndrome and hyperphagia. 2018–2020
- NIH Clinical Center "Bench-to-Bedside" Award: Liraglutide in Adolescents with Obesity After Sleeve Gastrectomy. 2021–2022

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The Biophysics of Protein–Lipid Interactions in Influenza and Coronavirus, Malaria, and Muscular Dystrophy

Eukaryotic life must create the many shapes and sizes of the system of internal membranes and organelles that inhabit the variety of cells in nature, membranes that must remodel for cells to repair damaged plasmalemma and deal with infectious agents such as viruses and parasites. Such basic membrane mechanisms must be highly regulated and highly organized in various hierarchies in space and time to allow the organism to thrive despite environmental challenges, genetic instability, unpredictable food supply, and physical trauma. We are using our expertise and the techniques we perfected over the years to address various biological problems that have in common the underlying regulation or disturbance of protein/lipid interactions. The overall goal of this project is to determine the physico-chemical mechanisms of membrane remodeling in cells.

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Identification of inhibitors of coronaviral entry

Some of the most dangerous human and animal pathogens, in particular coronavirus, influenza virus, and human immunodeficiency virus (HIV), are enveloped viruses. For these viruses, receptor binding and entry are accomplished by a single viral envelope protein (termed the spike or fusion protein), the structural changes in which trigger the remodeling and merger of the viral and target cellular membranes. We are focusing our continuing research on the physiology of deadly viruses and parasites towards fighting the COVID-19 pandemic, in areas closest to our expertise: helping to produce drugs against COVID-19 and producing viral-like particles of SARS-CoV-2. While some coronaviruses are endemic and cause only a common cold, others, those like SARS-CoV-2, are highly pathogenic and cause COVID-19. Previously the coronavirus strain SARS-CoV caused a severe acute respiratory syndrome outbreak in Asia in 2003, and during 2013 the Middle East respiratory syndrome (MERS) emerged with clinical symptoms similar to SARS, and the causative agent was named MERS-CoV. Many clinical trials are under way in search of treatments for COVID-19. Anti-inflammatory drugs such as corticosteroids are being used, and some treatments have received emergency use authorization by the FDA, such as monoclonal antibody therapy and remdesivir. However, more effective therapies are needed to combat the COVID-19 pandemic.

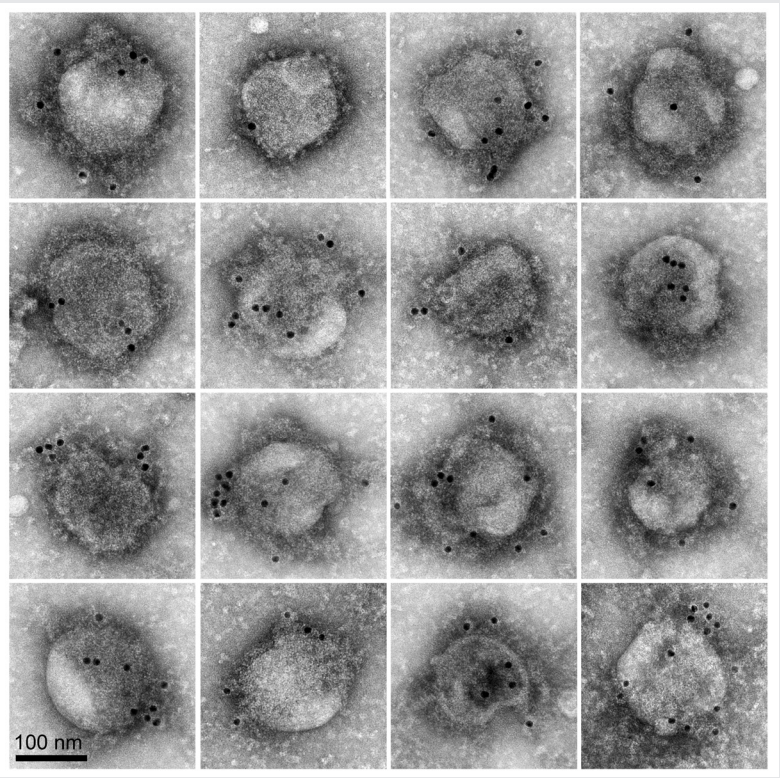

FIGURE 1. SARS-CoV-2 virus entry particles

Negative stain microscopy image shows a montage of "SARS-CoV-2 viral entry mimics," or pseudotyped particles (PPs). The PPs are membrane-encased, virus-sized spheres decorated with the SARS-CoV-2 spike protein. PPs are non-infectious, so can be safely used to study how the spike protein mediates binding to and entry into host cells and to identify compounds that block spike-mediated viral entry in high-throughput drug screening assays. The spike proteins appear as a dense fuzz surrounding the 150 nm–diameter particles and are labeled with 10-nm immunogold (*black dots*).

To combat COVID-19, high throughput screening (HTS) of thousands of candidate compounds that may block or reduce SARS-CoV-2 infection of target tissues is a powerful tool in drug discovery. However, the SARS-CoV-2 pathogen requires a biological safety level 3 (BSL-3), whereas most HTS facilities are only BSL-2. To overcome this obstacle and meet the desperate need for HTS of compounds with SARS-CoV-2 antiviral activity, an HTS screening assay was developed using pseudotyped particles (PP). PP serve as mimics for viral binding and entry into target cells, are BSL-2–compatible, and can be used for HTS. PP contain viral-envelope proteins such as the spike protein (S protein), which mediates viral entry into target cells, but carry a fluorescence-based reporter gene instead of the viral genome, and thus display the necessary viral coat proteins for host-receptor and membrane interactions without the capacity for replication. Similar PP–based BSL-2 viral entry assays have been successfully applied to HTS campaigns against the Ebola, influenza, and human immunodeficiency viruses (HIV). In a collaboration with scientists at NCATS, we used negative-stain electron microscopy and immunogold labeling to evaluate the structure of S proteins and quantify their distribution on PP that were used in their HTS assays for compounds with anti-SARS-CoV-2 activity. The technique was used countless times over this year to continuously monitor quality control for the PP for various SARS-CoV-2 S protein variants, as well as control PP. The PP were created by co-transfection of a plasmid of the S protein of a coronavirus and a plasmid for the Gag protein of a murine leukemia virus. For measurement of S–mediated entry, luciferase was included in the PP; successful target-cell entry was measured as luciferase activity. In this way, our colleagues identified inhibitors of S–mediated cell entry in a high throughput screen of an approved drugs library with SARS-S and MERS-S pseudotyped particle entry assays. Six compounds were discovered (cepharanthine, abemaciclib, osimertinib, trimipramine, colforsin, and ingenol) to be broad-spectrum inhibitors of S–mediated entry. The work could contribute to the development of effective treatments against the initial stage of viral

infection and provide mechanistic information that might aid in the design of new drug combinations for clinical trials for COVID-19 patients. After further testing in a SARS-CoV-2 live-virus cytopathic-effect (CPE) assay and removing cytotoxic compounds, six out of seven entry inhibitors were able to rescue the CPE of SARS-CoV-2 infection, indicating the utility of these PP entry assays.

New assays for enveloped viral fusion

Survival of the SARS-COV-2 virus particle for person-to-person transmission and its ability to fuse with the host cell membrane are highly dependent on the lipid composition of the viral envelope. Determining the lipid composition and physical properties of attenuated virus and virus-like particles (VLPs) will permit the study of membrane stability and fusogenicity under physiological conditions. Our goal is to give the scientific community a VLP to efficiently study virus infection in widely available non-BL2 laboratory space and yield enough lipid for analysis. Viral fusion is a critical step in the entry pathway of enveloped viruses and remains a viable target for antiviral exploration. The current approaches to studying fusion mechanisms include ensemble fusion assays, high-resolution cryo-transmission electron microscopy (TEM), and single-molecule fluorescence-based methods. While these methods have provided invaluable insights into the dynamic events underlying fusion processes, they come with their own limitations, which often include extensive data and image analysis in addition to experimental time and technical requirements. Our work proposes the use of the spin-spin T2 relaxation technique as a sensitive bioanalytical method for the rapid quantification of interactions between viral fusion proteins and lipids in real time. In the study, new liposome-coated iron oxide nanosensors (LIONs), which mimic as magnetic-labeled host membranes, are reported to detect minute interactions between the membrane and hemagglutinin (HA), influenza's fusion glycoprotein. The influenza fusion protein's interaction with the LION membrane is detected by measuring changes in the sensitive spin-spin T2 magnetic relaxation time using a bench-top nuclear-magnetic resonance (NMR) instrument. More data is gleaned from including the fluorescent dye DiI into the LION membrane. In addition, the effects of environmental factors on protein-lipid interaction that affect fusion, such as pH, time of incubation, trypsin, and cholesterol, were also examined. Furthermore, using VLPs we demonstrated the efficacy and sensitivity of the spin-spin T2 relaxation assay in quantifying similar protein/lipid interactions with more native configurations of HA. Shorter domains derived from HA were used to start a reductionist path to identify the parts of HA responsible for the NMR changes observed. Also, the known fusion inhibitor Arbidol was employed in our spin-spin T2 relaxation-based fusion assay to demonstrate the application of LIONs in real-time monitoring of this aspect of fusion for evaluation of potential fusion inhibitors.

Mechanisms of membrane electro-poration

Living cells are open non-equilibrium systems. To exist, a cell requires precisely controlled maintenance of gradients in the chemical potential between the extracellular environment, the cytoplasm, and the lumen of organelles, of many constituents. The amphiphilic nature of lipid molecules, self-assembling into lipid bilayers, provides an extremely low permeability barrier to both electrolytes and large non-electrolytes. For the processes of life, a continuous exchange of matter must occur across all membranes. For example, uptake of large molecules and compounds from the outside occurs via endocytosis, phagocytosis, and macro- and micro-pinocytosis, secretion occurs via exocytosis, and intracellular protein trafficking via transport vesicles between endoplasmic reticulum, Golgi apparatus, endosomes, and lysosomes. The movement of such membrane-bound cargo dictates membrane recycling, or cells and organelles would be incapable of maintaining their volumes and shapes. These ubiquitous and multifarious events, plus the accommodation of lipid bilayers to membrane proteins and transient pores, all require the lipid bilayer to change its topology. Physical models of such topological changes, essential to life, must take into account the energy of membrane deformations within the

framework of an adequate theory of elasticity. Although transport of molecules into cells via electro-poration is a common biomedical procedure, its protocols are often based on trial and error. Despite a long history of theoretical effort, the underlying mechanisms of cell-membrane electro-poration have not been sufficiently elucidated, in part, because many independent fitting parameters need to link theory with experiment.

In this project, we investigate whether the electro-poration behavior of a reduced cell membrane is consistent with time-resolved, atomistic, molecular dynamics (MD) simulations of phospholipid bilayers responding to electric fields. For pore formation giant unilamellar vesicles (GUVs) were used to avoid solvent and tension effects, and transport kinetics were measured by the entry of the impermeant fluorescent dye calcein. Because the timescale of electrical pulses needed to restructure bilayers into pores is much shorter than the time resolution of current techniques for membrane transport kinetics measurements, we measured the lifetimes of lipid bilayer electro-pores using systematic variation of the initial MD simulation conditions, whereas GUV transport kinetics were detected in response to a nanosecond-timescale variation in the applied electric pulse lifetimes and interpulse intervals.

Molecular transport after GUV permeabilization induced by multiple pulses is additive for interpulse intervals as short as 50 ns but not for 5–ns intervals, consistent with the 1050– ns lifetimes of electro-pores in MD simulations. Although the results were mostly consistent between GUV and MD simulations, the kinetics of ultrashort, electric field–induced permeabilization of GUVs were significantly different from published results in cells exposed to ultrashort (6 and 2 ns) electric fields, suggesting that cellular electro-poration involves additional structures and processes. The experimental data are consistent with the hypothesis that calcein-permeable lipid electro-pores in GUVs are created within a few nanoseconds and that most are annihilated within a few tens of nanoseconds, consistent with molecular simulations, but in contrast to the typical persistent electro-permeabilization (many seconds to minutes) observed in living cells. Furthermore, the magnitudes of the increases in intravesicular dye concentration are much smaller with nanosecond-pulsed electric fields than those observed in the presence of the pore-forming peptide melittin or exposure to influenza virus at a low pH. Nanosecond bipolar pulse cancellation, a phenomenon recently described in cells, was not observed in GUVs. The absence of persistent electro-permeabilization and nanosecond bipolar cancellation of GUVs suggests that the electro-permeabilization of cells involves structures and processes that go beyond transport through lipid pores and that models of electro-poration must be modified accordingly.

Determining the structure of the placental malaria vaccine candidate VAR2CSA

Women become more susceptible to malaria infection during pregnancy despite pre-existing immunity acquired from childhood, causing substantial risk of severe outcomes for the mother and her offspring. Placental malaria is caused by the accumulation of *Plasmodium falciparum*–infected erythrocytes in the placenta of pregnant women, resulting in high rates of maternal anemia, low birth weight, stillbirth, and spontaneous pregnancy loss. Each year, up to 200,000 infant deaths and 10,000 maternal deaths globally are attributed to malaria infection in pregnancy. The surge in deaths can be traced to the development of parasite resistance to the affordable drug chloroquine. Artemisinin, a newer, powerful, and affordable antimalarial drug, appeared promising at first, but resistant strains now abound. Vaccines have proven very difficult to produce, but focusing on women provides a strong basis for the development of vaccines to prevent placental malaria, because women naturally acquire resistance to placental malaria over successive pregnancies.

P. falciparum expresses a family of proteins, referred to as erythrocyte membrane protein 1 (PfEMP1), that are translocated to the surface of the infected erythrocyte to enable adherence to different host organs and to evade the host immune response. VAR2CSA (parasite-encoded variant surface antigens binding to chondroitin sulfate A), the leading placental malaria vaccine candidate, is a member of the PfEMP1 family that specifically binds to the syncytiotrophoblast surface receptor chondroitin sulfate A. The interaction facilitates placental sequestration of malaria parasites, leading to placental malaria. Given its large size (including a 310–kDa extracellular domain), production of VAR2CSA protein for vaccine development and scientific study has proved to be challenging. Furthermore, the highly polymorphic nature of the extracellular domain of VAR2CSA in parasite isolates may hinder the development of a strain-transcending vaccine. Lastly, vaccine-induced and naturally acquired immunity may differ in important ways that need to be carefully examined. CSA (chondroitin sulfate A) is presented by diverse cancer cells, and specific targeting of cells by VAR2CSA may become a viable approach for cancer treatment. We determined the Cryo-EM structures of the full-length ectodomain of VAR2CSA from *P. falciparum* strain NF54 in complex with CSA, and VAR2CSA from a second *P. falciparum* strain FCR3. VAR2CSA is composed of a stable core flanked by a flexible arm. CSA traverses the core domain by binding within two channels, and CSA binding does not induce major conformational changes in VAR2CSA. The CSA–binding elements are conserved across VAR2CSA variants and are flanked by polymorphic segments, suggesting immune selection outside the CSA–binding sites. The work thus provides paths for developing interventions against placental malaria and cancer.

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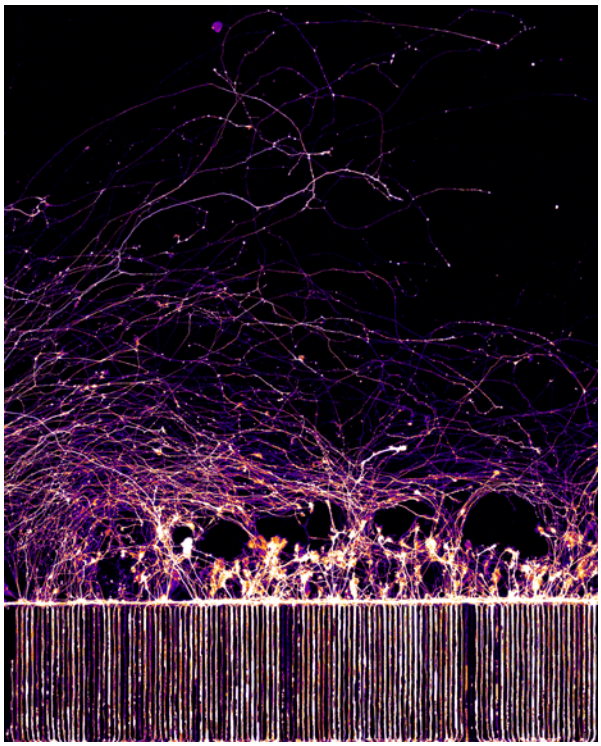
Colophon

About the Cover Image

This image shows human neuronal axons growing on microfluidic chambers. Using these devices, we can start to understand key biological processes unique to human neuronal axons, such as how they grow or why they degenerate.

Image created by Jorge Gomez-Deza, PhD, of the *Le Pichon lab*.



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