2023

NICHD Division *of* Intramural Research

ANNUAL REPORT

NIH Eunice Kennedy Shriver National Institute
of Child Health and Human Development

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Board *of* Scientific Counselors

* nominee

Ursula Kaiser, MD, *Chair*
7/1/19 – 6/30/24
Endocrinology, Diabetes and Hypertension

Chief, Division of Endocrinology, Diabetes, and Hypertension
George W. Thorn, MD Distinguished Chair in Endocrinology
Director, Brigham Research Institute
Professor of Medicine, Harvard Medical School
Brigham and Women's Hospital

Carlos A. Bacino, MD, FACMG
7/1/22 – 6/30/27
Molecular and Human Genetics, Pediatrics

Vice Chair, Clinical Affairs
Department of Molecular and Human Genetics
Baylor College of Medicine
Chief, Genetics Service
Texas Children's Hospital

David R. Beier, MD, PhD*
7/1/21 – 6/30/26
Genomics

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

Distinguished Service Professor
Departments of Molecular and Human Genetics and
  Neuroscience
Baylor College of Medicine
Duncan Neurological Research Institute

P. Ellen Grant, MD
7/1/19 – 6/30/24
Newborn Medicine, Radiology

Professor of Radiology and Pediatrics, Harvard Medical
  School
Founding Director, Fetal-Neonatal Neuroimaging and
Developmental Science Center,
Endowed Chair in Neonatology,
Boston Children's Hospital

Mary C. Mullins, PhD
7/1/20 – 6/30/25
Developmental Biology

Professor, Vice Chair
Department of Cell and Developmental Biology Chair,
  Developmental, Stem Cell, and Regenerative Biology
  Graduate Program
Assistant Dean for Junior Faculty Advancement
University of Pennsylvania
Perelman School of Medicine

Errol Norwitz, MD, PhD, MBA
7/1/19 – 6/30/24
Obstetrics and Gynecology, Maternal-Fetal
Medicine

Professor of Obstetrics & Gynecology
Tufts University School of Medicine

Emily Oken, MD, MPH
7/1/22 – 6/30/27
Internal Medicine and Pediatrics

Professor
Department of Population Medicine
Harvard Medical School

Linda Overstreet-Wadiche, PhD
7/1/20 – 6/30/25
Neurobiology

Professor
Department of Neurobiology
University of Alabama at Birmingham School of Medicine

Ayyalusamy Ramamoorthy, PhD*
7/1/22 – 6/30/27
Biophysics, Structural Biology

Professor
Florida A&M University — Florida State University
National High Magnetic Field Laboratory
Department of Chemical and Biomedical Engineering

Laura E. Riley, MD
7/1/22 – 6/30/27
Maternal Fetal Medicine and Infectious
Disease

Given Foundation Professor and Chair
Obstetrician and Gynecologist-in-Chief
New York Presbyterian Hospital
Weill Cornell Medicine
Obstetrics and Gynecology

Yukiko Yamashita, PhD
7/1/22 – 6/30/27
Genomics

Member, Susan Lindquist Chair for Women in Science
Whitehead Institute for Biomedical Research
Professor, Department of Biology
Massachusetts Institute of Technology

Message *from the* Scientific Director

I am pleased to announce that the 2023 annual report of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Division of Intramural Research (DIR) is now available at *https://annualreport.nichd.nih.gov*. The report outlines our research endeavors and highlights our medical and scientific discoveries of the past year.

NICHD's intramural community comprises a broad array of basic, translational, and clinical researchers. We use a range of model systems to further knowledge of developmental, molecular, and cellular biology, neurosciences, structural biology, imaging, and biophysics. Investigators working with various animal models, including fruit flies, zebrafish, rats, and mice, are supported by multiple core services, from bioinformatics and imaging, to molecular genomics. Each investigator participates in one or more team-based and future-oriented *affinity groups*, which build on thematic interests while staying responsive to rapidly shifting scientific priorities and new knowledge.

The DIR researchers whose names appear in this publication remain committed to training the next generation of scientists and physician-scientists. They include tenure-track investigators, who joined us recently, as well as accomplished investigators, who continue to forge new scientific paths.

We invite you to review the report to learn about a colleague's current pursuits, or to identify a laboratory with which you may wish to collaborate or refer a student. The report is fully searchable to assist potential postdoctoral fellows, graduate students, clinical fellows, and others in navigating to the information they need.

I also encourage you to browse the report's *selection of our clinical trials* with an eye toward potential collaboration, made possible through the NIH U01 grant mechanism or the NIH Bench-to-Bedside and Back Program at the NIH Clinical Center. These programs could lead to our next great success, by helping us combine expertise and patient populations, while leveraging NIH infrastructure to address topics ranging from rare disorders to the most common problems affecting human health. Visit *https://ocr.od.nih.gov/new_u01/new_u01.html* and *https://ocreco.od.nih.gov/btb/btb_program.html* to learn more about these programs.

The drive and purpose of DIR, on behalf of the American public and the international community, is to lead research and training to understand human development, improve reproductive health, enhance the lives of children and adolescents, and optimize abilities for all. Share your ideas and proposals for collaborative initiatives with me at *mcbainc@mail.nih.gov*.

Sincerely yours,
Chris J. McBain, PhD
Scientific Director, NICHD, NIH
December 2023

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 support 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.

Chris J. McBain, PhD, *Scientific Director*
Sara K. King, *Chief of Staff*
Jessica Rigby, *Program Specialist*
Amaressa A. Abiodun, *Program Specialist*
Olga Cherkasova, *Administrative Support Specialist*

The BSC reviews site-visit reports, evaluations, and all other activities of the OSD. Each NICHD investigator is subject to a review at least once every four years, according to NIH policy (*NIH Sourcebook: Process for Reviewing NIH Intramural Science*). 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*.

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*

Veronica Harker, *Program Coordinator*

Katherine Lamb, *Program Coordinator*

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; and postbaccalaureate fellows and summer interns.

Our **TmT (Three-minute Talks) competition**, now in its ninth year, was held in conjunction with ten other institutes: NCATS, NIDCR, NHGRI, NEI, NIDDK, NIDCD, NIAID, NINDS, NIEHS, and NLM. Jong Park, postdoctoral fellow in the *Section on Vertebrate Organogenesis*, was the NICHD finalist and NIH 1st place winner.

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

An online **Annual Progress Review** for fellows, developed by the Office and launched in 2016, tracks scientific and career development and progress. As part of the assessments of investigators' mentoring, the reports by fellows are analyzed and provided to the site visits of the *Board of Scientific Counselors*. A new Individual Development Plan and Annual Progress Report system for intramural postbaccalaureate fellows was implemented in October 2023.

NICHD OFFICE OF EDUCATION

Activities, Programs, and Professional Development

LEADERSHIP AND MANAGEMENT
- Project management for scientists
- Organizational dynamics and supervisory skills
- Setting expectations and goals
- Improving lab communication

RESEARCH AWARDS
- Annual Fellows Award for Research Excellence (FARE)
- K99/R00, Pathway to Independence Award
- NIGMS Postdoctoral Research Associate (PRAT) Program
- National Research Service Award (NRSA), individual predoc and postdoc fellowships
- Intramural Research Fellowships for NICHD postdoc and clinical fellows

RESEARCH ETHICS
- An established training plan for all fellows
- 8-hour requirement, within the initial two years of training
- Completed by the fellow or facilitated by the mentor or Office of Education

TEACHING
- Skills workshops on curriculum design and active learning
- Six-week college teaching workshop series, each summer
- "Becoming an Effective Scientist" course for postbac fellows, an eleven-week lecture and journal club series taught by postdocs and clinical fellows

MEDICAL AND GRAD SCHOOL PREPARATION
- Individual consulting
- Mock interviews
- Shadowing opportunities

CAREERS
- Job interview preparation
- Alumni speakers and panels from various career paths
- Editing of job application materials (c.v., cover letter, resume, teaching/research statement)
- Guidance for IDPs/Annual Progress Reviews
- One-on-one advising and career counseling

WRITING AND PUBLISHING
- Editorial service
- Grant preparation
- Online classes for scientific writing
- Getting published: strategies and approaches

GRANTS
- Grant writing/review workshops
- Mock study sections
- Individualized coaching for the NIH application process
- NICHD Fellows Intramural Grants Supplement (FIGS) Award

ANNUAL FELLOWS RETREAT
- Postdocs, clinical fellows, graduate students retreat
- Off campus, one day event
- Current scientific topics, keynote addresses, fellow talks, posters, networking, and career events

SCIENCE COMMUNICATION
- Presentation skills (oral, poster, and chalk talks)
- Annual Three-Minute Talks (TmT) Competition
- Graduate student talks
- Individual sessions with a public speaking coach

NIH Eunice Kennedy Shriver National Institute of Child Health and Human Development

Any questions? Contact:
Dr. Erin Walsh
Director, Office of Education, NICHD, NIH
erin.walsh@nih.gov | 301-435-1104

For more information and resources, visit
fellows.nichd.nih.gov

LEFT TO RIGHT:
Veronica Harker; Katherine Lamb

In September 2023, the Division of Intramural Research (DIR) gave its **Mentor of the Year awards** to Ryan Dale, *Bioinformatics and Scientific Programming Core*, in the investigator category; and to Mor Alkaslasi, *Unit on Development of Neurodegeneration*, as fellow.

The **DIR Fellows' Exit Survey** was established in summer 2022 and is actively collecting data on trainee experience feedback and career outcomes, and also serves as an alumni database.

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*. In FY23, four DIR postdoctoral fellows were awarded the competitive NIH K99/Pathway to Independence Award, and two DIR fellows were accepted into the NIGMS PRAT Fellowship Program for their outstanding research proposals. Also, for NICHD, 11 Fellows Award for Research Excellence (FARE) awards were made for the 2023 (FY24) competition.

The **Fellows Intramural Grants Supplement (FIGS)** continues to recognize and stimulate grant applications among fellows, and we launched the 7th cycle for our Intramural Research Fellowships (IRFs), a new competitive internal funding opportunity for NICHD postdoctoral and clinical fellows. Its ultimate goal is to promote fellows' grant writing and to enhance their awareness of various components for an NIH grant application.

The Institute continues its **exchange program with INSERM** (the Institut National de la Santé et de la Recherche Médicale) in France, which provides a unique opportunity for US and French scientists to obtain postdoctoral training with French and US mentors, respectively. Since 2016, up to three second- and third-year medical students from **Santa Casa de São Paulo School of Medical Sciences** (Brazil) train with NICHD investigators for the **Future Researchers Program**.

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

The alumni group for our **NICHD Developing Talent Scholars program**, in its 13th year, added three new postbaccalaureate fellows in 2023. The Scholars program focuses on developing talent and supporting trainees' academic and career progression.

The DIR has continued its **collaboration with Howard University** for research, training, and mentoring opportunities. In the 2023 spring academic semester, we welcomed a third cohort of undergraduate student mentees from the College of Arts and Sciences into the Biology Secondary Mentors Program. Each student was paired with an NICHD Principal Investigator in the DIR, with whom they will work closely towards developing and executing a research project from their sophomore through their senior years of college. Our first cohort (who started in spring 2020) completed their senior theses and graduated in May 2023; they are now pursuing graduate and medical school training.

Postdoctoral fellows were also given the opportunity to organize and teach our **annual seminar series** for postbaccalaureate trainees, which entered its 17th year. For the 2023 series, Erin Walsh instructed sessions on the medical-school and graduate-school search and application processes. The 2023–2024 series will also provide sessions on biomedical career exploration and professional development, as well as opportunities for meeting and networking with scientists and physicians.

The **18th Annual Fellows Meeting** was held in Washington, DC, on October 27, 2023, and featured keynote speaker Dr. Guoyang Luo (Maternal Fetal Health and Obstetrics and Gynecology, INOVA Health System). The event also included a talk by NICHD Office of Health Equity Program Officer Dr. Erika Barr, and showcased oral and poster presentations by fellows, along with 10 career speaker discussions sessions. The program is developed and run by a fellows' steering committee, which was chaired by Thien Nguyen in 2023 (postdoctoral fellow, _Section on Translational Biophotonics_).

**The NICHD Connection** monthly newsletter continues its focus on mentoring, careers, and academic programs for young scientists, publishing its 161st issue in November 2023 and reaching all members of the intramural division and our alumni.

Contact
For further information, contact Dr. Erin Walsh (_erin.walsh@nih.gov_).

Office *of the* Clinical Director, NICHD

The NICHD Office of the Clinical Director (OCD) is responsible for research oversight, scientific review, and administration of the clinical research program within the Division of Intramural Research (DIR). The clinical research activities are coordinated with the Scientific Director and address diseases related to pediatrics and women's health.

The Institute's current clinical research portfolio includes clinical trials, natural history studies of rare diseases, and drug and device development. The clinical research program currently includes 68 protocols, conducted by 19 NICHD Principal Investigators and 170 associate investigators. Approximately half of the protocols include pediatric patients, many focusing on rare diseases. Fifteen percent (15%) of the protocols involve investigational drugs or devices.

Office of the Clinical Director: Professional, Administrative, and Biorepository Staff
Top: Catherine Gordon, Laverne Mensah, Karim Calis
Center: Meg Keil, Denise Phillips, Glynnis Vance
Bottom: Mahlet Asrat, Fathy Majadly, Loc Trinh

Catherine Gordon, MD, *Clinical Director*
Laverne Mensah, MD, *Deputy Clinical Director*
Meg Keil, PhD, CRNP, *Associate Director, Nursing and Protocol Navigation*
Karim Calis, PharmD, MPH, *Director of Clinical Research and Compliance*
An Dang Do, MD, PhD, *Assistant Clinical Investigator*
Cristina Tatsi, MD, *Assistant Clinical Investigator*
Alan DeCherney, MD, *Senior Investigator*
Veronica Gomez-Lobo, MD, *Senior Staff Clinician*
Tazim Dowlet-McElroy, MD, *Staff Clinician*
Jacqueline Maher, MD, *Staff Clinician*

(continued)

Current research areas include developmental disorders, healthy human development, developmental endocrinology, neurosciences, genetics, translational imaging, pediatric and reproductive endocrinology, lymphatic anomalies, infertility, fertility preservation, and population health. These clinical research activities are governed under the NIH Intramural Research Program (IRP) with patient participation in the NIH Clinical Center (CC). Delivery of quality and safe patient care in the NIH Clinical Center is vital in the context of NICHD's clinical research programs and services.

Partnerships and collaboration efforts, both between and within NIH institutes, and externally through research agreements with leading academic institutions nationally, and partnerships with industry, through technology transfer or cooperative research and development agreements, foster advancement of science and cutting-edge scientific research. Emphasis is placed on nurturing a clinical research environment that encourages creativity and on expanding the fundamental scientific knowledge of summer interns, postbaccalaureate research trainees, as well as postdoctoral and clinical fellows in various basic science and clinical disciplines. The aim of the various components of the Office of the Clinical Director are to achieve the mission of NICHD: "NICHD leads research and training to understand human development, improve reproductive health, enhance the lives of children and adolescents, and optimize abilities for all."

Contact

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

Samar Rahhal, MD, *Staff Clinician*
Denna Zeltser, MD, *Staff Clinician*
Craig Abbott, PhD, *Statistician*
Samah Agabein, MD, *Protocol Coordinator*
Glynnis Vance, *Protocol Coordinator*
Roberto Romero, MD, *Maternal Fetal Medicine*
Mahlet Asrat, MHM, *Program Analyst*
Lisa Ast, BA, RN, *Clinical Research Nurse*
Monique Bertschy, MVA, *Patient Care Coordinator*
Andrea Bowling, CRNP, *Nurse Practitioner*
Sheila Brady, CRNP, MSN, *Nurse Practitioner*
Alberta Derkyi, FNP, CRNP, MSN, *Nurse Practitioner*
Desiree Labor, MSN, CRNP, *Nurse Practitioner*
John Perreault, CRNP, *Nurse Practitioner*
Harinder Raipuria, DNP, FNP, *Nurse Practitioner*
Devora Stein, MSN, CRNP, *Nurse Practitioner*
Anna Dorsey, *Digital Imaging*
Kisha Jenkins, BSN, RN, *Clinical Nurse*
Lola Saidkhodjaeva, BSN, RN, *Clinical Nurse*
Sara Talvacchio, BSN, RN, *Clinical Nurse*
Harveen Kaur, MPH, *Clinical Research Coordinator*
Fathy Majadly, BS, *Patient Specimen Coordinator*
Denise Phillips, *Program Specialist*
Loc Trinh, *Research Chemical Engineer*

Clinical Trials *at* NICHD

NICHD's DIR leads numerous clinical protocols; visit *https://www.clinicaltrials.gov/search?term=nichd* for a complete listing of NICHD clinical trials. The following describes selected DIR clinical trials that are recruiting participants and provides contact information for the investigator(s) conducting those studies. For detailed information on all related research projects, please review the listed investigator's section of the annual report.

Developmental Endocrinology, Metabolism, Genetics, & Endocrine Oncology

» Patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma and paraganglioma. Contact **DR. KAREL PACAK** at *karel@mail.nih.gov* or **MS. ALBERTA DERKYI** and **MS. SARA TALVACCHIO** at *ppglgroup@mail.nih.gov*.

» Research on endocrine, genetic, and other pediatric disorders associated with endocrine and other tumors, which may affect the pituitary and other related organs. Contact **DR. CHRISTINA TATSI** at *christina.tatsi3@nih.gov* or 301-451-7170 or **MS. SAMAH AGABEIN** at *samah.agabein@nih.gov* or 301-451-7615.

» Research investigating the long-term effects of Cushing disease in childhood. Contact **DR. MEG KEIL** at *keilm@mail.nih.gov* or 301-435-3391.

» Study of the safety and efficacy of pegvisomant to treat children and adolescents with growth hormone excess, including those whose disease persists after surgical and/or radiation treatment, and those who are ineligible for those treatments. Contact **DR. CHRISTINA TATSI** at *christina.tatsi3@nih.gov* or 301-451-7170 or **MS. SAMAH AGABEIN** at *samah.agabein@nih.gov* or 301-451-7615.

» Studies on the role of genetics in the development of obesity. Contact **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-451-3783.

» Studies of pediatric disorders associated with a predisposition for obesity and diabetes, including Bardet-Biedl syndrome, Alström syndrome, Prader-Willi syndrome, leptin receptor deficiency, PCSK1 deficiency, and Pro-opiomelanocortin deficiency. Contact **DR. JACK YANOVSKI** at *yanovskj@mail.nih.gov* or 301-451-3783.

» Pharmacotherapy for excessive hunger and obesity in patients with Prader-Willi syndrome, Bardet-Biedl syndrome, and other rare disorders with known genetic causes. 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. 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.

» Clinical study evaluating the long-term safety and tolerability of Chronocort©, a modified-release form of hydrocortisone. Contact **DR. DEBORAH MERKE** at *dmerke@nih.gov* or **MS. ELIZABETH JOYAL** at *ejoyal@nih.gov*.

» Clinical trial to evaluate the efficacy of Crinecerfont©, a corticotropin-releasing factor type 1 receptor antagonist, for the treatment of congenital adrenal hyperplasia. Contact **DR. DEBORAH MERKE** at *dmerke@nih.gov* or **MS. LEE ANN KEENER** at *leeann.keener@nih.gov* or 240-858-9033.

» First-in-human gene therapy trial for congenital adrenal hyperplasia. Contact **DR. DEBORAH MERKE** at *dmerke@nih.gov* or **MS. ELIZABETH JOYAL** at *ejoyal@nih.gov*.

» Studies of genetic disorders related to altered cholesterol metabolism, including Smith-Lemli-Opitz syndrome (SLOS) and Niemann-Pick Disease, type C (NPC). For SLOS studies, contact **DR. FORBES PORTER** at *fdporter@mail.nih.gov* or **DR. SAMAR RAHHAL** at *samar.rahhal@nih.gov*. For NPC studies, contact **MS. DESIREE LABOR** at *desiree.labor@nih.gov* or **MR. DEREK ALEXANDER** at 301-827-0387.

» Studies of individuals with CLN3, or juvenile neuronal ceroid-lipofuscinosis/juvenile Batten disease, and

their family members. Contact **DR. AN NGOC DANG DO** at *an.dangdo@nih.gov* or **MS. KISHA JENKINS** at 301-594-2005.

» Studies of people with genetic disorders related to abnormal function of the creatine transporter gene and creatine transport deficiency. Contact **MR. JOHN PERREAULT** at 301-827-9235 or **MR. DEREK ALEXANDER** at 301-827-0387.

» Study of those with MEHMO (Mental disability, Epileptic seizure, Hypopituitarism/hypogenitalism, Microcephaly, Obesity) syndrome, or an eIF2-pathway related disorder, and their family members. Contact **DR. AN NGOC DANG DO** at *an.dangdo@nih.gov* or **MS. KISHA JENKINS** at 301-594-2005.

Lymphatic Disorders

» Studies of patients with suspected or confirmed disorders of the lymphatic system. Contact **DR. SARAH SHEPPARD** at *sarah.sheppard@nih.gov*, **MS. ANDREA BOWLING** at *Andrea.Bowling@nih.gov*, or the study team at *NICHD_LymphaticAnoma@mail.nih.gov*.

Maternal–Fetal Medicine & Translational Imaging

» *Studies to test and calibrate noninvasive optical imaging technology for functional brain imaging in healthy subjects.* This research investigates near infrared spectroscopy (NIRS) imaging techniques that will potentially improve the feasibility and reliability of using the system to meet the needs of populations for whom existing imaging systems are unsuitable. Functional NIRS (fNIRS) is an emerging noninvasive imaging technique that assesses brain function. Its measurements are based on local changes in cerebral hemodynamic levels (oxy- and deoxyhemoglobin) associated with brain activity. Neurovascular coupling leads to local changes in oxy- and deoxyhemoglobin levels that can serve as an indirect measure of brain activity. To probe brain activity-related cortical changes in oxy- and deoxyhemoglobin concentrations, researchers administer different tasks (such as the n-back, go-no go tests) to quantify spatial and temporal brain activity. Contact **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.

» *Studies of mirror neuron network (MNN) dysfunction as an early biomarker of neurodevelopmental disorder.* In this study, researchers use fNIRS combined with electroencephalography (EEG) to measure brain activity in the MNN, which is associated with the development of sophisticated social behaviors that emerge in typical infants. Researchers hope that modeling MNN development will uncover a sensitive measure for deviations in social communication development before clinical behavioral deficits can be detected. MNN activation is indicated through mu rhythm suppression using EEG. The first part of the study will determine whether MNN activation can be elicited in adult participants using a motor observation and a simultaneous execution paradigm of EEG/fNIRS systems. Researchers will apply advanced machine-learning methods on signal synchronicity to examine how the features from both signals relate to each other, and to help characterize brain function in the MNN. In the next step, researchers will measure the same signals in typically developing infants and infants at risk for developmental delays when they are between 9 and 12 months of age. At-risk infants will be evaluated again at 24 months of age to detect any deviations in their social communicative development. Examining their developmental status at 24 months in relation to their initial neural data will help determine whether MNN activation can predict developmental outcomes. Contact **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.

» *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, photoplethysmography [PPG] and temperature sensor) in measuring human vital signs. Later work will explore the device as a point-of-care method for screening and treatment response monitoring of those with an infectious respiratory illness, such as COVID-19 infection. The device will measure heart, respiratory, and tissue oxygenation parameters in healthy individuals at rest and during induced hypercapnia, breath holding, and paced breathing. Contact **DR. AMIR GANDJBAKHCHE** at *gandjbaa@mail.nih.gov*.

Pediatric and Adolescent Gynecology

» *Data collection study on pediatric and adolescent gynecology conditions.* This study will perform deep phenotyping and data collection of children and adolescents presenting with gynecologic conditions, including congenital anomalies. 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 evaluates possible mechanisms of follicle loss/dysfunction in children with Turner syndrome and classic galactosemia, and in adolescents recently diagnosed with primary ovarian insufficiency. Ovarian tissue will be cryopreserved and stored for participant's own future use. Contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

» *Biorepository for those who undergo ovarian tissue freezing before gonadotoxic therapy.* In this study, researchers are creating a databank of ovarian tissue obtained during ovarian tissue cryopreservation (OTC), which is performed as standard care for fertility preservation in individuals who will receive gonadotoxic therapy. The NIH will provide OTC as a clinical service and will request a portion of the tissue to use for research. Contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

» *Studies on androgen receptor sensitivity and implications for health and wellbeing: A natural history study of patients with androgen insensitivity.* Research on androgen-receptor genes and receptor abnormalities can improve care for those affected. It may also elucidate possible androgen receptor-mediated explanations of differences in physiology and health in other populations. Contact **DR. VERONICA GOMEZ-LOBO** at *veronica.gomez-lobo@nih.gov*.

» *Implications of maternal 45,X mosaicism as a secondary genomic finding following cell-free DNA sequencing during pregnancy.* This natural history study will identify health risks among people with 45,X mosaicism discovered during pregnancy. Mosaicism is a condition in which cells within the same person have a different genetic makeup. Sometimes, a type of mosaicism called 45,X may not be discovered until a person undergoes routine tests during pregnancy. This work seeks to expand knowledge about discovery of 45,X mosaicism during pregnancy and how it may affect long-term health. 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. Contact **DR. JOSHUA ZIMMERBERG** at *zimmerbj@mail.nih.gov* or **MS. HANG WATERS** at *watershn@mail.nih.gov*.

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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Clinical and research training under the NICHD–NIDDK–NIDCR 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 and metabolic diseases and diabetes. 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 National Military 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 (Lausanne)* 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.

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Pediatric Endocrinology Inter-Institute Training Program

The Fellowship in Pediatric Endocrinology is a three-year program, accredited by the Accreditation Council for Graduate Medical Education (ACGME). 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 National 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.

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 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

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(continued)

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.

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*.

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Publications

1. Szymczuk V, Boyce AM, Merchant N. Metaphyseal sclerosis in a child with a giant cell tumour treated with denosumab. *Lancet* 2023 402:e4.
2. Szymczuk V, Taylor J, Boyce AM. Craniofacial fibrous dysplasia: clinical and therapeutic implications. *Curr Osteoporos Rep* 2023 21:147–153.
3. Jee YH, Jumani S, Mericq V. The association of accelerated early growth, timing of puberty, and metabolic consequences in children. *J Clin Endocrinol Metab* 2023 108:e663–e670.
4. Zenno A, Brady SM, Faulkner LM, Ballenger KL, Fatima S, Yanovski JA. Circadian variation of serum leptin and adipose tissue changes in children. *Pediatr Obes* 2023 18:e12984.

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NICHD Pediatric and Adolescent Gynecology Training Program

The Pediatric and Adolescent Gynecology (PAG) program 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. Many of the conditions managed by pediatric gynecology are rare diseases and have been poorly studied, and PAG providers have become leaders in the field of fertility preservation in children and adolescents. The NICHD PAG program comprises fellowship training as well as laboratory and clinical research programs.

Fellowship

The NICHD Fellowship in Pediatric and Adolescent Gynecology (PAG) is a two-year fellowship program comprising faculty from The National Institute of Child Health and Human Development (NICHD) and Children's National Hospital (CNH). The fellowship is housed within NICHD at NIH, and the care of patients will take place at NIH and CNH.

The goal of this fellowship is to train graduates of an ACGME–accredited Obstetrics and Gynecology Residency in the gynecologic care of patients between birth and age 21. The fellow will learn the diagnosis and management of common problems in pediatric gynecology, as well as uncommon and more complex conditions, such as congenital reproductive anomalies and anorectal malformations, variations in sex characteristics (VSC), pediatric and adolescent pelvic masses and gynecologic malignancies, gynecologic disorders in medically complicated children, reproductive endocrinopathies, transgender care, fertility preservation, sexual abuse, and vulvar conditions. In addition, fellows will receive training in and support for research development. Our goal is that graduates will pursue careers that advance the field of pediatric and adolescent gynecology in all areas, including research, education, and patient care.

Our fellows will interact with fellows in reproductive endocrinology, infertility, and pediatric endocrinology within NICHD, as well as with

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Raghuveer Kavarthapu, PhD, *Staff Scientist*

Kirsten Das, MD, *Clinical Fellow*

Rama Kastury, DO, *Clinical Fellow*

Courtney Mascoe, MD, *Clinical Fellow*

Sreeramya Balasubramanian, BS, *Intramural Research Training Award Fellow*

Elizabeth Varghese, MD Candidate, *Medical Research Scholar Program*

Taylor Badger, MD Candidate, *Special Volunteer*

Hong Lou, MD, *Laboratory Manager/Biologist*

Maria De La Luz Sierra, MS, *Laboratory Manager*

Harveen Kaur, MPH, *Clinical Research Coordinator*

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

pediatric surgery, pediatric urology, and adolescent medicine fellows at CNH. Opportunities for collaboration with Women and Infant services fellows at MedStar Washington Hospital Center are also available. Such interaction nurtures a rich academic environment, which promotes excellence in clinical care, education, and research. Furthermore, our location in the Nation's capital allows fellows to become involved in advocacy through the American College of Obstetrics and Gynecology (ACOG).

The fellowship will entail 70% clinical work and 30% research, with rotational blocks dedicated to a fellow's research time. During clinical time at CNH, fellows will participate in general pediatric gynecology clinics, as well as in a variety of specialty clinics staffed by the PAG team. Multidisciplinary clinics are for the care of patients with fertility preservation, menorrhagia, VSC, Turner syndrome, anorectal malformations, gender development concerns, vulvar conditions, and complex endocrinology conditions. In addition, fellows will have opportunities to rotate with providers specializing in eating disorders and child abuse. At the NIH, fellows will participate in the consult service and assist in the gynecologic care and research for studies in McCune-Albright syndrome, congenital adrenal hyperplasia, Turner syndrome, Mullerian agenesis (MRKH), fertility preservation, and androgen insensitivity syndrome (AIS).

Research program

The research and laboratory program includes five IRB (institutional review board)–approved clinical protocols, which are conducted in the ambulatory, in-patient, and operating rooms of the NIH Clinical Center. In addition, laboratory space within the Clinical Center allows for experiments on ovarian tissue and other samples obtained from these clinical protocols. We also work closely with the core facilities within NICHD, including the *Molecular Genomics* and *Bioinformatics Cores*, and with NICHD's *Section on Epigenetics*.

The program includes research on fertility preservation in children, adolescents, and on rare conditions. Fertility preservation has emerged as an important aspect of cancer care in pediatric and adult populations. In December 2019, the American Society of Reproductive Medicine Committee Opinion on fertility preservation in patients undergoing gonadotoxic therapies stated that ovarian tissue cryopreservation (OTC) should no longer be considered experimental. However, there remain significant gaps in knowledge regarding OTC for fertility preservation in children and adolescents receiving gonadotoxic therapy, given that the fertility effect of gonadotoxic therapy as well as the fertility benefits of OTC cannot be ascertained until 5–30 years after therapy. Recently, there has been increased interest in fertility preservation in other populations, including individuals with Turner syndrome, galactosemia, transgender youth, differences in sex development, and in young women with recent premature ovarian insufficiency. Such individuals present further unknowns regarding the risks and benefits of OTC, the foremost being that they may have significantly lower populations of follicles than those planning to receive gonadotoxic therapy and may therefore not benefit from this technology. Furthermore, it is important to note that, although pregnancies have been achieved using this technology (in adults), much remains to be elucidated regarding ovarian histology, function, and mechanism of disease in the ovary, and ovarian-tissue cryopreservation has spurred new evaluation of the human ovary. The PAG program developed two protocols for ovarian-tissue cryopreservation in children and adolescents. The first is a protocol for ovarian-tissue cryopreservation for prepubertal children with Turner syndrome, individuals with Turner syndrome with Y chromosome material who undergo prophylactic gonadectomy, individuals with classic galactosemia, and for adolescents with recent primary ovarian insufficiency (POI), individuals with differences in sex development, and adolescents with diminished ovarian reserve. The second protocol for children who will undergo gonadotoxic therapy will allow us to collect tissue and data that will

inform the first protocol. In both protocols, we are performing oophorectomy for OTC and collecting a small portion of the ovary for research. Through these projects we are defining the components and anatomy of the ovary, evaluating the correlation of ovarian reserve markers with histologic findings, and attempting to elucidate crucial signaling pathways regulating follicle activation and loss, through collaborations with NICHD Core laboratories, using methods including RNA-seq and single-cell analytics.

Patients with PAG conditions (such as reproductive, endocrine, gynecologic tumors, skin conditions, menstrual abnormalities, and congenital anomalies of the reproductive tract) may provide exceptional gain of knowledge, which may advance research in pediatric and adolescent gynecology disorders, and their evaluation may catalyze the recognition of new disease processes and new research initiatives. Through the PAG conditions protocol, we are collecting deep phenotype data and specimens from PAG patients, thus creating a large database of PAG conditions, which will provide stimuli for new clinical research initiatives. One such condition, the androgen-insensitivity syndrome (AIS), is a state in which the body cannot sense the male hormones in blood or tissue. Because this is a rare condition, little is known about the risks and benefits of gonadectomy, optimal hormone replacement after gonadectomy, nor general health in individuals with these conditions. Furthermore, the androgen receptor is found in many tissues in the body, including skin, bone, muscle, and in the neurologic, immune, and metabolic systems. Through the PAG program Natural History study in individuals with AIS, we will provide information regarding health risks and optimal management of individuals with AIS, as well as elucidate the role of the androgen receptor.

Application information
Applications are submitted using the _common application form_ on the _North American Society for PAG (NASPAG) website_. They are accepted in July before the anticipated start date, and interviews are typically held in August or September before the start date. The 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. Mumford K, Hendricks S, Gomez-Lobo V. Should ovarian tissue cryopreservation in pediatric patients with Turner syndrome be limited to the research setting? _J Pediatric Adolesc Gynecol_ 2023 36(6):566–568.
2. Hartwick Das KJ, Hood C, Rutenberg A, Lobo VG. Pediatric and adolescent obstetric and gynecologic encounters in US emergency departments: a cross-sectional study. _Ann Emerg Med_ 2023 81(4):396–401.
3. Dwiggins M, Shimy J, Galloway LA, Hoefgen H, Patel V, Breech L, Gomez-Lobo V. Effects of ovarian tissue cryopreservation on primary ovarian insufficiency in girls undergoing bone marrow transplantation. _J Pediatric Adolesc Gynecol_ 2023 36(2):128–133.
4. Cohen A, Gomez-Lobo V, Willing L, Call D, Damle LF, D'Angelo LJ, Song A, Strang JF. Shifts in gender-related medical requests by transgender and gender-diverse adolescents. _J Adolesc Health_ 2023 72(3):428–436.
5. Finlayson C, Johnson EK, Chen D, Fechner PY, Hirsch J, Rosoklija I, Schafer-Kalkhoff T, Shnorhavorian M, Gomez-Lobo V. Fertility in individuals with differences in sex development: provider knowledge assessment. _J Pediatric Adolesc Gynecol_ 2022 35(5):558–561.

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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 100 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, 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

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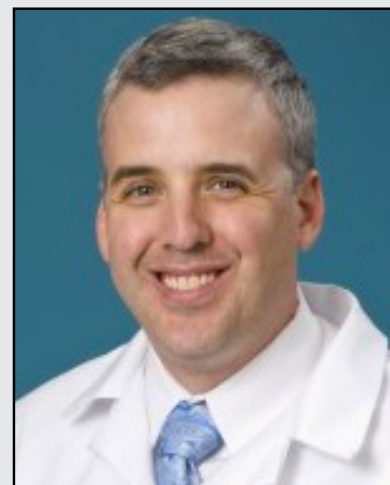
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Torie C. Plowden, MD, MPH, *Associate Program Director*

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given 20 months of protected research time. The curriculum includes 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.

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 through 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

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the *Society for the Study of Reproduction*. Moreover, fellows are encouraged to attend specialty meetings in their chosen interest areas.

Intracytoplasmic sperm injection

Publications

1. Tong ZB, Otsuka N, Tu W, Wei Q, DeCherney AH. P450 side-chain cleavage enzyme (P450-SCC) is an ovarian autoantigen in a mouse model for autoimmune oophoritis. *Reprod Sci* 2022 29(8):2391–2400.
2. Doyle N, Jahandideh S, Hill MJ, Widra EA, Levy M, Devine K. Effect of timing by endometrial receptivity testing vs standard timing of frozen embryo transfer on live birth in patients undergoing in vitro fertilization: a randomized clinical trial. *JAMA* 2022 328:2117–2125.
3. Devine K, Dolitsky S, Ludwin I, Ludwin A. Modern assessment of the uterine cavity and fallopian tubes in the era of high-efficacy assisted reproductive technology. *Fertil Steril* 2022 118:19–28.
4. Hewitt SC, Wu SP, Wang T, Ray M, Brolinson M, Young SL, Spencer TE, DeCherney A, DeMayo FJ. The estrogen receptor α cistrome in human endometrium and epithelial organoids. *Endocrinology* 2022 163:bqac116.
5. Eubanks AA, Nobles CJ, Mumford SL, Kim K, Hill MJ, Decherney AH, Sjaarda LA, Ye A, Radoc JG, Perkins NJ, Silver RM, Schisterman EF. The safety of low-dose aspirin on the mode of delivery: secondary analysis of the effect of aspirin in gestation and reproduction randomized controlled trial. *Am J Perinatol* 2022 39(6):658–665.

Collaborators

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- Saioa Torrealday, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
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Contact

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

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AMB Staff

Michelle Hudson, *Administrative Officer, Team Leader*

Lakeasha Mingo, *Administrative Officer, Team Leader*

Charlene Patrick, *Administrative Officer, Team Leader*

Sylvia Robinson, *Administrative Officer, Procurement Lead*

Gina Elmore, *Administrative Officer, Budget*

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Natacha Rene, *Administrative Officer*

Nancy Richman, *Administrative Officer*

Beverley Todd, *Administrative Officer*

Marlene Taulton, *Administrative Officer*

Brittany Corum, *Administrative Technician*

Barbara Hardy, *Administrative Technician*

Ashley Mason, *Budget Analyst*

Steven Norris, *Facilities Manager*

Jennifer Smith, *Procurement Technician*

Anju Vergheese, *Program Analyst*

John Burton, *Purchasing Agent*

Jax Chang, *Purchasing Agent*

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Roshonna Davis, *Purchasing Agent*

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Sherry Jones, *Purchasing Agent*

James Law, *Purchasing Agent*

David Shen, *Purchasing Agent*

Hanumanth Vishnuvajjala, *Purchasing Agent*

Brittney Corbin, *Program Specialist*

Kassie Nantz, *Program Specialist*

Tucker Brown, *Facilities Specialist*

Jessie Belcher, *Program Analyst*

Reem Adeeb, *Travel Technician*

Beza Solomon, *Purchasing Agent Trainee*

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, RAMB represents the interests of the NICHD Division of Intramural Research (DIR) on all aspects of animal research conducted within the Intramural Research Program (IRP). This includes animal facilities managed by NICHD as the lead institute, NICHD animals housed in other facilities, and all activities involving NICHD DIR–owned animals. The Branch specifically:

1. provides primary veterinary care;
2. advises the NICHD Scientific Director on animal care and use practices;
3. assures animal use within DIR is in accordance with applicable regulatory standards and guidelines;
4. coordinates intramural animal use, including appropriate animal model selection, support requirements, and Animal Study Proposal review;
5. advises the scientific staff on comparative medicine, Animal Study Proposal design, disease interference, and other factors that may complicate or invalidate research results;
6. implements and coordinates animal-health monitoring;
7. coordinates quarantine for incoming animals of unknown health status to prevent the introduction of agents pathogenic to humans or other animals;
8. coordinates a central ordering program for the NICHD;
9. provides administrative management of the NICHD Animal Care and Use Committee (ACUC); and
10. interfaces with organizations and institutions concerned with the ethical and humane care and use of animals in research.

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. RAMB staff and the NICHD 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

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

Julie Jacobs, *Animal Program Analyst*

Terrance McMeans, AA, *Rodent Facilities Manager*

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

Christopher Rishell, BS, *Aquatics Facilities Manager*

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

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, ACRF Animal Facility, and Building 49. The Building 6 SZF supports NICHD and NHGRI with 12,500 tanks with a total capacity of over 23,000 tanks. Additional aquatics facilities provide primary animal care and research support to over 180 tanks of *Xenopus* and over 95 tanks of *Astyanax* used by NICHD DIR researchers.

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 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 *schechj@mail.nih.gov* or visit *https://www.nichd.nih.gov/about/org/dir/osd/mt/ramb*.

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 the 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 Buildings 6, 49, and 35), who work directly with a subset of 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. We work closely with NICHD's *Molecular Genomics Core*, sharing sequencing data storage and infrastructure to streamline the process for our collaborators, and we host weekly meetings that are open to the entire Institute to explore and discuss trending bioinformatics topics.

Projects overview

In 2023, the BSPC worked on 88 projects, collaborating with principal investigators (PIs), fellows, staff scientists, and staff clinicians across 35 laboratories. Of these, 31 were new projects and 57 were carried over from the previous year. The projects included assays such as bulk RNA-seq, single-cell RNA-seq, spatially-resolved RNA-seq, ChIP-seq, whole-exome sequencing, whole-genome sequencing, DNA methylation, CUT&RUN, bulk ATAC-seq, and single-cell ATAC-seq, TRIP (Thousands of Reporters in Parallel) data, and long-read assembly. Some projects involved custom algorithm development and tool development, and many projects required integration with published studies.

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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*

Tyler Menold, BS, *Postbaccalaureate Fellow*

Ally Questell, BE, *Postbaccalaureate Fellow*

Lilly Shatford-Adams, BS, *Postbaccalaureate Fellow*

Alicia Evans, *Research Software Engineer*

Projects often begin with an in-depth discussion with researchers to understand the background and goals of the project. It is important for us to understand the underlying biology and details of the experimental design (when applicable) for each project, so that we can make the most informed analysis decisions. We then provide a prioritized plan for the first round of analysis and schedule the work. There are often several iterations of analysis as a project progresses. Each iteration may add more sophisticated analyses, new data generated by the lab, or integrate results with published data. As expected for a no-cost shared resource, the time it takes for one iteration on one project is highly dependent on the existing workload across all other projects that we are handling in the Institute.

After each iteration, we meet to discuss the results in detail. The meeting includes a walk-through of the results, the computational background, discussion of how to use and interpret the tables, figures, and other output, and recommendations for next steps. Depending on the 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. *Carnation* allows our collaborators to explore their bulk RNA-seq, and *cascade* allows them to explore single-cell RNA-seq (scRNA-seq) and spatially-resolved RNA-seq so they can 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. New this year is converting *cascade* to use an underlying high-performance data structure called AnnData, which helps scale to scRNA-seq experiments with hundreds of thousands of cells.

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 Posit 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 as well as documentation written by the BSPC and used in various projects.

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.

Core IT 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 our DIR research community, e.g., GraphPad Prism, Amira, DNASTAR Lasergene, MathWorks MATLAB, SnapGene, FlowJo, and Biorender, as well as cross-platform desktop, server, and application hosting in the Rock Spring and Bldg. 35 Data Centers. We also assist users in identifying, researching, and purchasing custom hardware configurations to match research instrument requirements.

The addition of a new Deputy Scientific Information Officer in 2023 enabled CSSC to expand support and collaboration. This included piloting backup and storage initiatives with NICHD's Information Resources Management Branch (IRMB), establishing routine meetings with NICHD's scientific technical support team, enhanced outreach to intramural investigators and to peers in other institutes, participation in NIH–wide working groups, improved handling of scientific software license management, and building capacity for intramural security.

Clinical informatics

In the past year, CSSC initiated a modernization effort, implementing multi-factor authentication, starting to shift from on-premises infrastructure to the cloud for CSSC non-clinical applications, and planning to upgrade the remaining hardware for the on-premises infrastructure.

CSSC continued to support and develop applications related to clinical and translational medicine, including the Clinical Trials Database (CTDB) project. Such informatics tools allow researchers to design, collect, and report clinical observations related to natural history

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Xinlian Liu, PhD, *Deputy Scientific Information Officer*

Asma Idriss, PMP, MS, *Program Manager*

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Sean Ivusic, MS, *Database Support Lead*

Kesa Koresko, MS, *Senior DB/BI Specialist*

Loc Vu, BS, *Lead Software Engineer*

Matt Breymaier, BS, *Senior Bio/Application Software Engineer*

Louis Battuello, PMP, BS, *Subject Matter Expert*

Nareg Bakirci, MS, *Website Developer*

Nicki Swan, BA, *Graphic Designer*

Rana Alneaimy, MD, *Documentation Specialist*

Audrey Harrell, MS, *Documentation Specialist*

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(continued)

and interval-based studies. The total number of protocols and research projects supported by the CTDB team for 15 NIH institutes increased to 751 studies. The Global Question Library expanded to over 283,000 research questions. Our software development group completed two CTDB releases. Features included improvements on the e-binder module, Forms module, and Samples module. We supported the Clinical Trial Survey System (CTSS), an application for patient self-reporting, servicing 84 active protocols. The team completed one CTSS release and, in its continuing effort to modernize, rolled out all 84 active CTSS redesigned websites. CTDB application also supports the _NICHD Office of Clinical Director (OCD)_ central biorepository and eligibility monitoring; the CTDB team supports NICHD OCD with customized report integrating with eligibility monitoring workflow. Through the global library in CTDB, several institutes are tracking research teams' CVs, trainings, and certificate documentation. In the past year, the CTDB team continued to support NICHD's DIPHR (Division of Population Health Research), and, after a multi-year effort building the Study of Pregnancy and Neonatal Health protocol, it was pushed to a pilot phase. Since this project's inception, data from CTDB supported over 1,500 NICHD publications.

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Jennifer Walling, MS, _Documentation Specialist_

Kelly Colligan, BS, _Information Specialist_

Kami Emanuel, _Information Specialist_

Breanna McGriff, BS, _Information Specialist_

Vida Bayat Mokhtari, MD, _Information Specialist_

George Tran, _Information Specialist_

Meinhart T. Vallar, BS, _Information Specialist_

Louis Battuello, BS, _Data SME_

Jeremy Swan, BS, _Web Developer_

Nicholas Piegari, BS, _Web Developer_

The database development and reporting team continued data integrations with other NIH institutes and the Clinical Center's BTRIS (Biomedical Translational Research Information System) and CRIS (Clinical Research Information System). The team worked with NHBLI CMRCoop (Cardiac Magnetic Resonance Cooperative) system and continued to work with various NIMH systems to incorporate data from these systems into the CTDB data-reporting environment. The team is working towards integrating the NIH Toolbox system used by investigators at NIMH. We continued supporting data marts as new reporting requirements appear and migrating data as needed. The team added extract data from NIH's Center for Information Technology's BTRIS to support investigators research in addition to migrating data into the data mart from various external research organizations (MedStar, Inova, John Hopkins, etc.). The team implemented scoring via the CTDB interface that automates the scoring for this instrument. The team successfully upgraded the Cognos reporting environment to IBM Cognos Cognos 11.2.2, and they plan to implement a new authentication mechanism for Cognos (OIDC). Additionally, the team has worked closely with various PIs across institutes to provide both management- and research-related reports for clinically related studies, publishing 510 reports in the past year. The team applied the latest patches to all production database environments to ensure continued uninterrupted services, and it monitors the successful completion of backup and data mart transformation services. Over the past year, the team planned and worked towards upgrading the hardware environment to a new Solaris host and Oracle 19c.

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 reports, and *Administrative Management Branch's (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 giving DIR Fellows a platform for providing feedback.

We continued to develop new features and improvements for the Package Tracking module used by the AMB, providing AMB staff real-time accuracy metrics for personnel and travel package compilation.

The team has also improved upon the Capital Equipment/Expenditure Request Tracking System, which allows users to efficiently submit requests through the review process, while giving administrative staff the ability to track requests through the workflow process. Additional features allow administrative staff to process and track requests after approval and funding. The project has been sufficiently well received to allow potential offerings to the NICHD extramural community as well as to the Office of the Director.

The CSSC team continued maintenance of Cost Tracker, an application that permits capturing, organizing, and reporting various expenses on a per-protocol basis. The work is done closely with the OCD to improve protocol cost vs. effectiveness and provides a protocol-cost estimator module.

The CSSC team continues to develop and support several feedback systems to support real-time customer satisfaction collection. These include surveys for the AMB, the OCD, laboratory administrative support staff, and NICHD's Administrative Services Branch. The system also offers more detailed feedback submissions periodically along with comprehensive response metrics than was previously possible. Along with application development, maintenance, and support, the CSSC team successfully migrated infrastructure and applications to Azure Cloud using NIH STRIDES (Science and Technology Research Infrastructure for Discovery, Experimentation, and Sustainability), one of the first organizations to do so.

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 Retreat, the DIR Annual Scientific Retreat, and the NICHD research labs and medical training programs. For intramural labs, we created scientific figures and illustrations for publication in medical and scientific journals. We supported the NICHD Office of Education by producing a monthly newsletter, *The NICHD Connection*, in collaboration with Intramural Fellows, the monthly *Scientific Director's Bulletin* for staff, and promotional posters and graphics for sponsored events. We continued maintaining websites for the NICHD DIR Annual Report and DIR Annual Fellows Retreat. The CSSC continued to provide a platform for conducting scientific review by the *Board of Scientific Counselors*, administrative intranet support, and business operations.

Additional Funding

- The Clinical Trials Database (CTDB) project receives funding from other NIH Intramural Institute or

Center programs, including NHLBI, NIMH, NIDCR, NIEHS, NIAMS, NINDS, CC, NINR, NIDCD, NHGRI, NCCIH, NIMHD, and NIDDK.

Collaborators

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- Maryland Pao, MD, *Clinical Director, NIMH, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, 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.

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.

Vincent Schram is the point person for light microscopy and data analysis and is also the team lead. Ling Yi is in charge of the histology/sample preparation unit. The Electron Microscopy (EM) branch of the Facility is staffed by Chip Dye. Tamás Balla is the scientific and administrative director of the core.

The MIC has an open-door policy with the NINDS Light Imaging Facility (LIF) in 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%.

Light microscopy

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

1. a Zeiss LSM 710 inverted for high-resolution confocal imaging;

Vincent Schram, PhD, _Acting Executive Director_

Tamás Balla, MD, PhD, _Scientific & Administrative Director_

Ling Yi, PhD, _Staff Scientist_

Louis (Chip) Dye, BS, _Research Assistant_

2. an LSM 900 equipped with an AiryScan detector;
3. a Zeiss 800 optimized for advanced tiling experiments;
4. a Zeiss 880 AiryScan with higher spatial resolution;
5. the Zeiss LSM 880 2-photon confocal was donated to an NICHD investigator, and replaced with a Leica Stellaris FLIM/STED system; and
6. a Nikon Spinning Disk/Total Internal Reflection Fluorescence (TIRF), equipped with advanced rotating TIRF capabilities.

The facility acquired a second automated slide scanner, a Zeiss Axioscan 7, to complement the existing Axioscan Z1. Both scanners are heavily used and free up hundreds of personnel hours for several research groups in the DIR. We continue to operate a high-end wide-angle fluorescence microscope for non-confocal imaging.

We provide image analysis services based on ImageJ, Zeiss Zen, Nikon Elements, and Bitplane Imaris. The MIC is not connected to the NIH network, so that users must move their data on removable drives.

The light microscopy branch of the MIC continues to rely on the following mode of operation: after an initial orientation, during which their project is researched by the staff and the best approach is decided upon, users receive hands-on training on the equipment and/or 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 section 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, in which end users perform their own processing and imaging. In the past 12 months, Chip Dye processed a total of 106 samples for morphology studies.

Dye continues to operate the automatic sample preparation device from Microscopy Solutions, which allows a larger volume of specimens to be processed. John Heuser, an expert in electron microscopy, continues to use the JEOL 1400 microscope and brings his extensive experience to the MIC.

Tissue preparation

Staffed by Ling Yi, the histology/sample preparation lab is a critical component of the MIC. Nineteen users were trained in person in rodent perfusion, cryopreservation, cryo-sectioning, immunofluorescence, and RNAscope. Perfusion and cryo-sectioning services were provided to twelve laboratories. Ling Yi invested heavily in implementing RNAscope applications in the MIC, to the point where it has become routine for many of the facility's users. She is currently working on optimizing tissue clearing methods by shortening the long incubation times these techniques require. She also developed several tutorial and white papers on sample preparation, in particular a series of videos on challenging rodent dissections.

Image analysis

As mentioned above, the MIC continues to provide high-end image processing based on ImageJ, Zeiss Zen, Nikon Element, and Bitplane Imaris. For difficult cases, in which conventional processing is not suitable, the

Nikon NIS-AI suite provides sophisticated tools for image restoration, segmentation, and feature extraction.

Collaborators

- John Heuser, PhD, *Section on Integrative Biophysics, NICHD, Bethesda, MD*
- Carolyn L. Smith, PhD, *Light Imaging Facility, NINDS, Bethesda, MD*

Contact

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

Molecular Genomics Core

With the goal of understanding the genetic changes and mechanisms underlying human diseases, the Molecular Genomics Core (MGC) supports NICHD investigators by providing next-generation deep sequencing and project data analysis.

Next–Generation sequencing and bioinformatics support

The MGC provides DNA and RNA sequencing services for genomic and genetic research to investigators within the NICHD. The MGC is currently operating with four sequencing machines. Most of our work is conducted on our high-capacity, production-scale machine: an Illumina NovaSeq 6000. 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 fourth sequencer is a Pacific Biosciences (PacBio) Sequel IIe (recently upgraded), 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 IIe permits mutation phasing, structural variant analysis, transposon location identification, nascent base modification reading, whole mRNA isoform sequencing, and other analyses that are not possible or practical with the other sequencers.

The MGC also operates a 10X Genomics Chromium Single Cell Controller and some related 10X instruments. 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 NovaSeq 6000 machine to generate thousands of cell-specific transcriptomes. Similarly, a multiome analysis can be performed in which ATAC libraries reading chromatin accessibility are derived from the same cells. The Visum platform and Cytassist controller allow for spatial resolution of transcriptomes in tissue slices.

Forbes D. Porter, MD, PhD, *Director, Molecular Genomics Core Facility*

Fabio Rueda Faucz, PhD, *Staff Scientist*

James R. Iben, PhD, *Staff Scientist*

Tianwei Li, PhD, *Staff Scientist*

Cameron Padilla, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

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 FY23, MGC sequenced 2,749 samples submitted as 187 projects across the full spectrum of sequencing types, generating 14.695 terabytes of sequencing data; the projects involved 40 NICHD Principal Investigators and collaborators from other Institutes. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support to 15 NICHD investigators across seven Affinity Groups. Our mission is to offer accurate and innovative sequencing and bioinformatic tools to facilitate research into the diagnosis, counseling, and treatment of hereditary disorders, and to support basic research that promotes understanding of human health and development.

Publications

1. Iben JR, Li T, Mattijssen S, Maraia RJ. Single-molecule poly(A) tail sequencing (SM-PATseq) using the PacBio Platform. *Methods Mol Biol* 2024 2723:285–301.
2. Piña JO, Raju R, Roth DM, Winchester EW, Chattaraj P, Kidwai F, Faucz FR, Iben J, Mitra A, Campbell K, Fridell G, Esnault C, Cotney JL, Dale RK, D'Souza RN. Multimodal spatiotemporal transcriptomic resolution of embryonic palate osteogenesis. *Nat Commun* 2023 14:5687.
3. Trivellin G, Daly AF, Hernández-Ramírez LC, Araldi E, Tatsi C, Dale RK, Fridell G, Mittal A, Faucz FR, Iben JR, Li T, Vitali E, Stojilkovic SS, Kamenicky P, Villa C, Baussart B, Chittiboina P, Toro C, Gahl WA, Eugster EA, Naves LA, Jaffrain-Rea ML, de Herder WW, Neggers SJ, Petrossians P, Beckers A, Lania AG, Mains RE, Eipper BA, Stratakis CA. Germline loss-of-function PAM variants are enriched in subjects with pituitary hypersecretion. *Front Endocrinol (Lausanne)* 2023 14:1166076.
4. Piña JO, Roth DM, Raju R, Winchester EW, Chattaraj P, Kidwai F, Faucz FR, Iben J, Padilla C, Cotney JL, D'Souza RN. Dkk2 interacts with Pax9 in palate mesenchyme to pattern and tune osteogenesis. *bioRxiv* 2023 doi:10.1101/2023.05.16.541037.
5. Boronat-Garcia A, Iben J, Dominguez-Martin E, Stopfer M. Identification and analysis of odorant receptors expressed in the two main olfactory organs, antennae and palps, of Schistocerca americana. *Sci Rep* 2022 12:22628.

Collaborators

- Jeffrey Baron, MD, *Section on Growth and Development, NICHD, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Michael Cashel, MD, PhD, *Section on Molecular Regulation, NICHD, Bethesda, MD*
- Rachel Caspi, PhD, *Immunoregulation Section, NEI, Bethesda, MD*
- Daniel Chertow, MD, PhD, *Critical Care Medicine, NIH Clinical Center, Bethesda, MD*
- Prashant Chittiboina, PhD, *Surgical Neurology Branch, NINDS, Bethesda, MD*
- Janice Chou, PhD, *Section on Cellular Differentiation, NICHD, Bethesda, MD*
- David J. Clark, PhD, *Section on Chromatin & Gene Expression, NICHD, Bethesda, MD*
- Robert J. Crouch, PhD, *Section on the Formation of RNA, NICHD, Bethesda, MD*
- An Ngoc Dang Do, PhD, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Mary Dasso, PhD, *Section on Cell Cycle Regulation, NICHD, Bethesda, MD*
- Rena N. D'Souza, PhD, *Section on Therapies for Craniofacial Disorders, NICHD, Bethesda, MD*
- Jeff Farrell, PhD, *Division of Developmental Biology, NICHD, Bethesda, MD*

- Benjamin Feldman, PhD, *Zebrafish Core, NICHD, Bethesda, MD*
- Veronica Gomez-Lobo, MD, *Pediatric & Adolescent Gynecology Program, NICHD, Bethesda, MD*
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- Matthias Machner, PhD, *Section on Microbial Pathogenesis, NICHD, Bethesda, MD*
- Richard Maraia, MD, *Section on Molecular and Cellular Biology, NICHD, Bethesda, MD*
- Joan C. Marini, MD, PhD, *Section on Heritable Disorders of Bone & Extracellular Matrix, NICHD, Bethesda, MD*
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- Keiko Ozato, PhD, *Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD*
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- Yun-Bo Shi, PhD, *Section on Molecular Morphogenesis, NICHD, Bethesda, MD*
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- Brant Weinstein, PhD, *Section on Vertebrate Organogenesis, NICHD, Bethesda, MD*

Contact

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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*

Oversight and support of client-specific projects

Over 2022–2023, Feldman engaged in research projects with four laboratories and directed the closure of the Core.

DEVER LAB (NICHD):
Translation of Distinct RNA Populations by the eukaryotic initiation factors Eif1 and Eif5. Feldman advised Dever and performed several microinjection experiments to explore differences in phenotypes resulting from ectopic expression of either Eif1 or Eif5 in zebrafish embryos and the possibility that restoration of a balanced Eif1/Eif5 ratio can ameliorate these phenotypes.

SACKETT LAB (NICHD):
Assessing Expression and Function of Zebrafish Alpha and Beta Tubulin Genes. The degree to which specific tubulin isotypes and/or their post-translational modification are essential for specific aspects of development in any organism remains a surprisingly open question. Feldman trained, and co-supervised two postbaccalaureate students through this ambitious project to systematically knock-out each zebrafish alpha and beta tubulin isotype in F0 embryos and determine how their absence affects early development. To date, this project has documented essential embryonic phenotypes for more than five tubulin genes.

GOLDEN LAB (NIDDK):

The Core previously generated a targeted amino acid alteration in the *cacna1c* gene. This year Feldman found that recessive mutants have profound developmental anomalies. Andy Golden, who was also collaborating with Harry Burgess, tragically passed away during the current reporting period. But characterization of this phenotype by Feldman and members of the *Burgess lab* is ongoing.

KEMPER LAB (NHLBI):

Function of zebrafish rca2.1. The Kemper lab is interested in zebrafish rca2.1s function, because it has certain similarities with human CD46 that are not found in the mouse genome. The Core previously generated two mutant rca2.1 alleles, revealing essential roles in growth and cardiac function. Phenotypic characterization is ongoing.

Independent research by the NICHD Zebrafish Core
STRATEGIES FOR CRISPR–CAS9–BASED HOMOLOGY-DIRECTED REPAIR (HDR)

Over the past several years, Feldman and NICHD Zebrafish Core Staff explored several approaches to generating zebrafish lines with amino-acid substitutions cognate to human disease alleles of interest and generated three such alleles in house: *atp7a*, *cacna1c* and *satb1.* This past year, Feldman worked to devise a less labor-intensive pipeline that will feature outsourcing of CRISPR–based design and reagent steps to In Vivo Biosciences, followed by microinjection and allele recovery in-house via high-throughput sequencing of extruded gametes from candidate carriers.

CRYOPRESERVATION AND *IN VITRO* FERTILIZATION OF ZEBRAFISH SPERM

Over the last year, Feldman, assisted by Felicia Benoit, has continued to focus on improving quality control measures to ensure viability of cryopreserved zebrafish lines and minimize variability in viability. This year, they developed an approach of pre-assessing the number and activity of sperm from individual males and only cryopreserving when yields exceeding two million active sperm are obtained.

Service
ACUC MEMBERSHIP

Feldman has served on the NICHD ACUC since 2015 and continued in this capacity this year, taking on the role of Alternate Chairperson, meeting monthly to evaluate and decide upon animal-study proposals, renewals and amendments, and ad hoc issues relevant to animal welfare.

Collaborators

- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Thomas Dever, PhD, *Section on Protein Biosynthesis, NICHD, Bethesda, MD*
- Andy Golden, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Claudia Kemper, PhD, *Laboratory for Complement and Inflammation Research, NHLBI, Bethesda, MD*
- Kenneth Olivier, MPH, MD, *Laboratory of Chronic Airway Infection, NHLBl, Bethesda, MD*
- Daniel Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*

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

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.

Ajay Chitnis, Head   *Ben Feldman (Core)*   *Brant Weinstein*
Harold Burgess   *Katherine Rogers*

Bone and Matrix Biology in Development and Disease

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

The current primary focus of the group is on translational studies of skeletal dysplasias and related bone disorders. We have identified and characterized molecular mechanisms of several novel skeletal development disorders in this spectrum. In addition to building on these successes and expanding our knowledge of

mechanisms and pathology of skeletal development, we are developing novel approaches to therapeutic intervention, which will be translated to clinical trials. We are expanding our research to extracellular matrix (ECM) development and pathology in other tissues and organs. We also anticipate expanding our research program toward studies of ECM disorders in placenta, cartilage, growth plate, and other tissues and organs that are involved in fetal health, prematurity, and early child growth and development.

Sergey Leikin, Head   *Rena D'Souza (NIDCR)*   *Joan Marini*

Cell and Structural Biology

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

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

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

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

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
An Dang Do
Catherine Gordon
Deborah Merke (CC)

Anil Mukherjee
Forbes Porter
Sarah Sheppard
Stanko Stojilkovic
Christina Tatsi
Jack Yanovski

NIH Inter-Institute Endocrinology Training Program
Pediatric Endocrinology Inter-Institute Training

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.

Pedro Rocha, Head
Judy Kassis

Paul Love
Todd Macfarlan

Keiko Ozato
Karl Pfeifer

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   *Bob Crouch*   *Roger Woodgate*
David Clark   *Mel DePamphilis*

Maternal–Fetal Medicine and Translational Imaging

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

Quantitative Imaging and Tissue Sciences (Basser) invents, develops, and translates novel *in vivo* microstructural and functional MRI methods designed to measure salient properties of the developing brain and assess and

characterize their changes in diseases and disorders. These novel quantitative imaging biomarkers are also used in neuroscience application to characterize brain network connectivity and dynamics, as well as brain tissue architectural organization.

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

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

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

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

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

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

Molecular Medicine

The **Molecular Medicine Group** brings together basic research programs that share the ultimate goal of developing treatments for human diseases through a better understanding of their pathophysiology. Main areas of research focus include the maintenance of iron homeostasis and mechanisms underlying neurodegeneration. At the intersection of our work, we hope to shed light on a group of rare diseases called neurodegeneration with brain iron accumulation or NBIA, which may also have broader implications for other types of related diseases.

Claire Le Pichon, Head   *Tracey Rouault*

Neurosciences

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

Tim Petros, Head	*Y. Peng Loh*	*Mark Stopfer*
Tamás Balla	*Chris McBain*	
Dax Hoffman	Jamie Morton (DIPHR)	

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   *Doreen Matthies*   *Alexander Sodt*
Sergey Bezrukov   Dan Sackett (SS)   *Joshua Zimmerberg*

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 (Training Program), Head   *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.

Lyme disease is a re-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. Yet, fundamental aspects of gene expression have not been studied extensively *B. burgdorferi*.

We recognize the importance of mapping RNA boundaries (their 5' and 3' ends) in bacteria, which is critical for gene annotation, the discovery of novel transcripts, and mechanistic characterization of genes. Several RNA-seq approaches have been developed to globally determine RNA boundaries: 5' RNA-seq identifies and distinguishes transcription starts sites (TSSs) from 5' processed ends; total RNA-seq sequences genes in their entirety; 3' RNA-seq captures termination events and identifies RNA 3' ends. As proof of concept, we first applied these sequencing approaches to the model organism *Escherichia coli* [Reference 1]. In doing so, we identified numerous *E. coli* 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 an *E. coli* small RNA encoded internal to an essential cell-division gene.

We next performed 5', total and 3' RNA-seq on RNA isolated from *B. burgdorferi* grown in culture [Reference 2]. This identified complex gene arrangements and operons, untranslated regions, and small RNAs. Remarkably, 63% of RNA 3' ends mapped upstream or internal to open reading frames (ORFs), including genes involved in the unique infectious cycle of *B. burgdorferi*. We hypothesize these RNAs result from premature termination and regulatory events such as *cis*-acting RNA regulation. Our findings uncovered an abundance of potential RNA regulators for future study in *B. burgdorferi*.

In a separate study, we compared identified 5' ends to transcriptionally

Philip P. Adams, PhD, *Head, Group on Gene Regulation in Bacterial Pathogens*

Ryan Fishman, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Emily Petroni, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Julia Silberman, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

Daniel Soliman, BS, *Postbaccalaureate Intramural Research Training Award Fellow*

active sequences on the *B. burgdorferi* genome during a mouse infection. 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. 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 3].

Ongoing work in our lab has been focused on characterization and the physiological roles of other *B. burgdorferi* genes, particularly regulatory RNAs, and regulatory networks, particularly those that regulate motility.
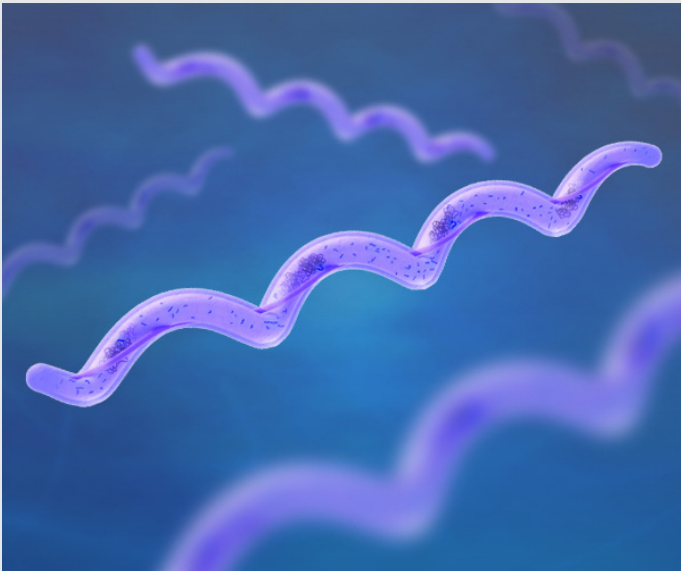
FIGURE 1. Rendering of the Lyme bacterium *Borrelia burgdorferi*, its genome, and various RNA transcripts

Additional Funding
- NICHD Early Career Award (2023) to Philip Adams, concluded

Publications
1. Adams PP, Baniulyte G, Esnault C, Chegireddy K, Singh N, Monge M, Dale RK, Storz G, Wade JT. Regulatory roles of Escherichia coli 5' UTR and ORF-internal RNAs detected by 3' end mapping. *eLife* 2021 10:e62438.
2. Petroni E, Esnault C, Tetreault D, Dale RK, Storz G, Adams PP. Extensive diversity in RNA termination and regulation revealed by transcriptome mapping for the Lyme pathogen Borrelia burgdorferi. *Nat Commun* 2023 14(1):3931.
3. Kuhn HW, Lasseter A, Adams PP, Flores Avile C, Stone BL, Akins DR, Jewett TJ, Jewett MW. BB0562 is a nutritional virulence determinant with lipase activity important for Borrelia burgdorferi infection and survival in fatty acid deficient environments. *PLoS Pathog* 2021 17(8):e1009869.

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Phosphoinositide Messengers in Cellular Signaling and Trafficking

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

Our research focuses 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. The lipid composition of cellular membranes is a major determinant of their biophysical properties and is unique to the different cellular organelles. 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 not only provides new strategies to fight various human diseases but also to intercept the life cycle of cellular pathogens, offering an alternative to antimicrobial drugs.

Metabolic routing maintains the unique fatty acid composition of phosphoinositides.

A unique feature of phosphatidylinositol (PI) and its phosphorylated PPIn derivatives is that they are highly enriched in poly-unsaturated arachidonic acid at the sn-2 position (stereospecific numbering, second carbon of glycerol) of the glycerol backbone, such that the stearoyl (C18:0)-arachidonoyl (C20:4) species is the predominant cellular form of PI. The metabolic processes responsible for regulating this enrichment are not fully understood, nor is it known what importance this unique side chain composition has for normal cell physiology. In this series of studies, we investigated the question as to whether PI synthesis uses a specific dedicated pool of the lipid precursor phosphatidic acid (PA) in the endoplasmic reticulum (ER). Given that the PA pools in the

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ER originate from different metabolic pathways, such as *de novo* synthesis or phospholipase C (PLC)– and phospholipase D (PLD)–mediated PA generation, it was important to determine whether PAs coming from all these sources are equally available for PI synthesis. We used a combination of approaches, all applied to a single cell line, to gain comprehensive information on the metabolic fates of PI precursors by following their side chain signature and matching their kinetics with bioluminescent resonance energy transfer (BRET)–based lipid measurements specifically within the plasma membrane (PM). We also performed lipidomics analyses and combined it with isotope labeling and pharmacological studies to identify differences in the handling of lipid intermediates with a specific fatty-acid composition that are involved in PPIn homeostasis. These studies concluded that metabolic routing of PA occurs at the ER and shows a clear preference for the stearoyl-arachidonoyl (38:4) species of diacylglycerol (DAG) as well as PA for conversion into PI, especially during the rapid recycling of breakdown products generated through PI(4,5)P$_2$ hydrolysis upon PLC activation within the PM. The significance of the studies is that by improving our understanding of the principles by which lipid precursors are segregated in the ER to serve various lipid-synthetic routes, it will be possible to selectively alter cellular lipid metabolism to interfere with lipid storage without affecting the membrane architecture of eukaryotic cells and organisms.

PI4K2A mutations in humans with developmental and epileptic–dyskinetic encephalopathy cause innate error in intracellular trafficking.

PI4K2A is one of the four PI 4-kinase enzymes that generates the lipid phosphatidylinositol 4-phosphate (PI4P) in cellular membranes. PI4K2A is located at the trans-Golgi network as well as on the surface of endosomes, providing PI4P primarily in the late endosomal/lysosomal compartment. Recent studies from our laboratory showed that in late endosomes PI4K2A is required for efficient fusion of autophagosomes and lysosomes. Other studies showed a critical role of PI4K2A in the repair of damaged lysosomes. Our group was approached by the clinical genetic group of Reza Maroofian. They identified two patients presented with developmental and epileptic-dyskinetic encephalopathy associated with corpus callosum dysgenesis, diffuse white matter volume loss, and hypoplastic vermis, as shown by neuroimaging. In addition, these patients showed neuro-developmental delay and recurrent infections with one of them dying at toddler age. Whole-exome sequencing revealed mutations in the *PI4K2A* gene. Our group performed functional studies recreating these mutations in PI4K2A and testing the properties of the protein in cells in which the *PI4K2A* gene was inactivated by CRISPR/Cas9 gene editing. The studies showed that the mutant enzyme lost its ability to localize to endomembranes and to catalyze the formation of PI4P in late endosomes. Understanding the consequences of PI4K2A defects in humans and their link to the clinical presentation will help us better understand the complexities of brain development and the identification of new means by which such disease conditions can be improved.

Calcium–prolactin secretion coupling in rat pituitary lactotrophs is controlled by PI4–kinase alpha.

Exocytosis is one of the most important membrane-remodeling events by which bioactive molecules, such as hormones and neurotransmitters, are rapidly secreted from cells to alter the function of other cells. The release of cargo from secretory vesicles requires their fusion with the plasma membrane (PM). Rapid Ca^{2+} elevation in the cytoplasm is the most commonly used signal to trigger this process, both in synaptic transmission and hormone release from endocrine cells. Phosphoinositide lipids (PPIns), in particular PI(4,5)P$_2$ enriched in the PM, are critical for membrane fusion event. In this collaborative study, led by the group of

Stanko Stojilkovic, we tested which PPIns control the exocytosis process in the cells of the pituitary gland. Single-cell RNA sequencing in cells obtained from rat pituitaries revealed the expression of several PI lipid kinases such as Pi4ka, Pi4kb, Pi4k2a, Pi4k2b, Pip5k1a, Pip5k1c, and Pik3ca, as well as Pikfyve and Pip4k2c, in at least 10% of lactotrophs cells, which are responsible for the secretion of prolactin (PRL). Using a pharmacological approach to specifically inhibit these enzymes, it was possible to show that PI4P made in the PM by PI4KA is critical for exocytosis without affecting the calcium signals, which trigger secretion. The experiments also showed that inhibition of the PI4KB enzyme, which generates PI4P in the Golgi, is dispensable for the exocytic step and revealed a key role of PI4KA–derived PI4P in the PM in calcium-secretion coupling in pituitary lactotrophs downstream of voltage-gated and PI(4,5)P$_2$–dependent calcium signaling. The studies identified a new type of regulation in the exocytic process, which is currently being further investigated.

Additional Funding

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Publications

1. Balla T, Gulyás G, Mandal A, Alvarez-Prats A, Niu Y, Kim YJ, Pemberton J. Roles of phosphatidylinositol 4-phosphorylation in non-vesicular cholesterol trafficking. _Adv Exp Med Biol_ 2023 1422:327–352.
2. Dafsari HS, Pemberton JG, Ferrer EA, Yammine T, Farra C, Mohammadi MH, Ghayoor Karimiani E, Hashemi N, Souaid M, Sabbagh S, Najarzadeh Torbati P, Khan S, Roze E, Moreno-De-Luca A, Bertoli-Avella AM, Houlden H, Balla T, Maroofian R. PI4K2A deficiency causes innate error in intracellular trafficking with developmental and epileptic-dyskinetic encephalopathy. _Ann Clin Transl Neurol_ 2022 9:1345–1358.
3. Kim YJ, Sengupta N, Sohn M, Mandal A, Pemberton JG, Choi U, Balla T. Metabolic routing maintains the unique fatty acid composition of phosphoinositides. _EMBO Reports_ 2022 23(7):e54532.
4. Kučka M, Gonzalez-Iglesias AE, Tomić M, Prévide RM, Smiljanic K, Sokanovic SJ, Fletcher PA, Sherman A, Balla T, Stojilkovic SS. Calcium-prolactin secretion coupling in rat pituitary lactotrophs is controlled by PI4-kinase alpha. _Front Endocrinol_ 2021 12:790441.
5. Gulyás G, Korzeniowski MK, Eugenio CEB, Vaca L, Kim YJ, Balla T. LIPID transfer proteins regulate store-operated calcium entry via control of plasma membrane phosphoinositides. _Cell Calcium_ 2022 106:102631.

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Structural and Chemical Biology

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 are of 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 enzymes or DHHC-PAT), so named because they contain a signature

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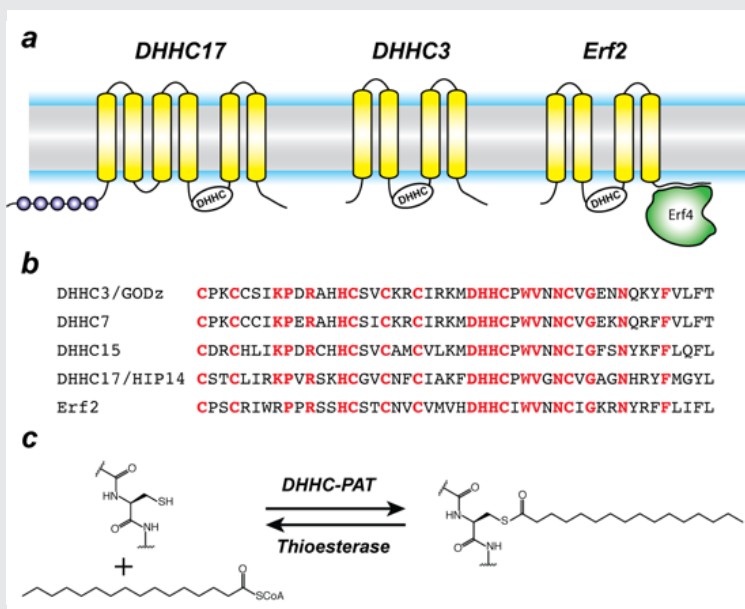Ryan Stoner, BS, *Postbaccalaureate Fellow*

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

a. The organization of three different DHHC-PATs is 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 (cysteine-rich domain) 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).

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 neuro-psychiatric 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 in 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.
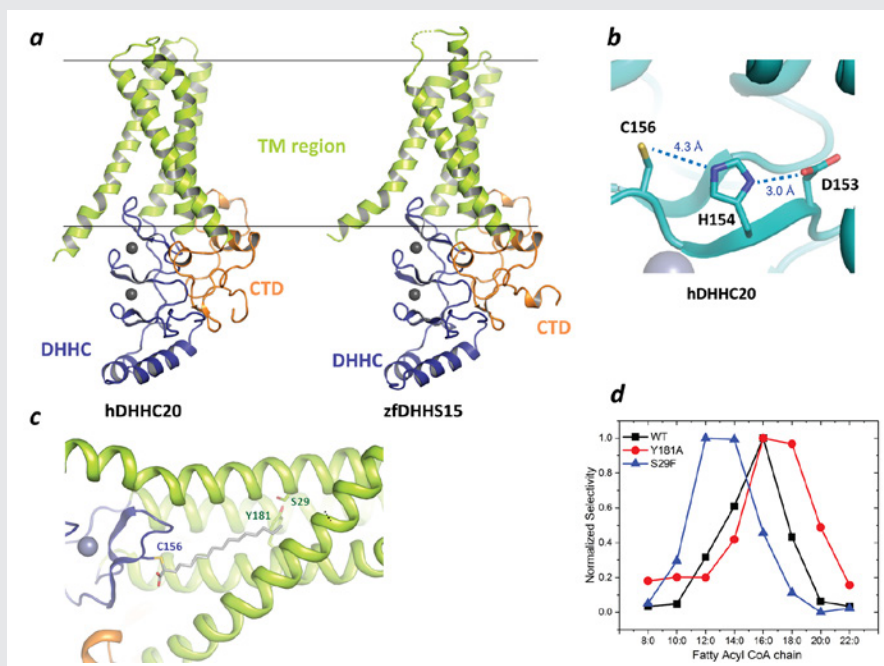
To advance the field, we earlier solved the high-resolution crystal structures of two members of the zDHHC family: human zDHHC20 (hDHHC20) and zebrafish zDHHC15 (Figure 2a), the first structures of any member of this family to be characterized. They reveal a tepee-like transmembrane-domain organization, 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, which is important because, although palmitate is the most prevalent fatty acid used by DHHC palmitoyltransferases, they can use fatty acyl–CoAs of other chain lengths, a property that varies between different members of the DHHC family.

An outstanding question in the field of protein S-acylation is the identity of the zDHHC enzymes for a given substrate. There are many substrates that are acted on by multiple zDHHC enzymes. With 23 zDHHC enzymes and more than 5000 substrates, the cellular zDHHC–substrate network is thus complex and the physiological
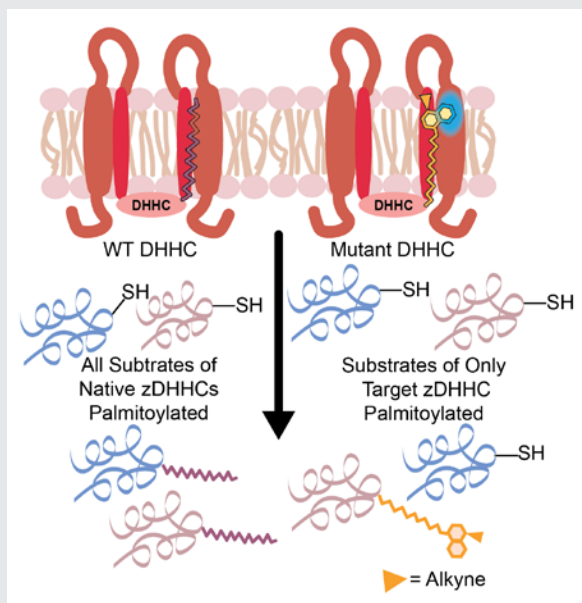
FIGURE 3. Principle of design and use of orthogonal acyl CoA-zDHHC pairs

All wild-type zDHHC enzymes can utilize palmitoyl CoA (*left*), but only the mutant of a specific zDHHC member can utilize the synthetic acyl CoA (*right*). In presence of the synthetic analog, only the substrates of this specific zDHHC become labelled with the analog, which are then identified with the use of the alkyne as a click chemistry handle.

substrates of each zDHHC enzyme remain unknown. Nevertheless, genetic strategies, such as knocking down or knocking out zDHHC enzymes, perturbs their cellular pool, and there is a dire need for a strategy that can be carried out at the native levels of zDHHC enzymes. Our previous structure-guided biochemical experiments demonstrated the feasibility of generating engineered zDHHC enzymes with altered fatty acyl–chain selectivity. Using this as a starting point, we generated an unnatural fatty acid with a click-chemistry handle and mutant human zDHHC20 (hDHHC20) and human zDHHC3 (hDHHC3) enzymes that act as orthogonal zDHHC/fatty acyl–CoA pairs. These led to the discovery of novel substrates of hDHHC3 and hDHHC20. They also led to the discovery, in collaboration with Mark Distefano´s lab, that the SERINC family of host restriction factors against pathogenic viruses such as HIV and SARS-CoV-2 have a conserved S-acylation site.

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 there Fe-S clusters are biosynthesized and iron is inserted into heme. Mitochondrial iron homeostasis plays a critical role in cellular iron homeostasis and in the overall physiology of the cell. In vertebrates, the only known major transporters of iron into mitochondria are mitoferrin-1 and mitoferrin-2, two homologous members of a large group of mitochondrial transporters known as the Mitochondrial Carrier family (Figure 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

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.
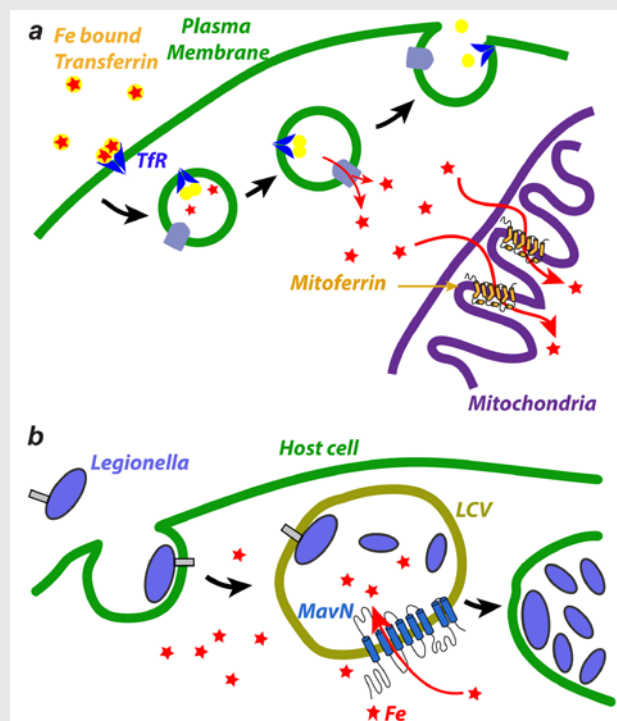
iron-transport assay starting from purified protein. In earlier work, we carried out heterologous purification, *in vitro* functional reconstitution, and mutational dissection of a vertebrate Mfrn1 (Figure 4), the first demonstration that Mfrn1 can indeed transport iron. Our studies provided the first 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). We are currently working towards understanding the detailed mechanism of iron recognition and transport by Mfrn1 and MavN.

Additional Funding
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Publications

1. Puthenveetil R, Auger SA, Gómez-Navarro N, Rana MS, Das R, Healy LB, Suazo KF, Shi ZD, Swenson RE, Distefano MD, Banerjee A. Orthogonal enzyme-substrate design strategy for discovery of human protein palmitoyltransferase substrates. *J Am Chem Soc* 2023 145(41):22287–22292.
2. Du G, Healy LB, David L, Walker C, Fontana P, Dong Y, Devant P, Puthenveetil R, Ficarro SB, Banerjee A, Kagan JC, Lieberman J, Wu H. ROS-dependent palmitoylation is an obligate licensing modification for GSDMD pore formation. *bioRxiv* 2023 2023.03.07.531538.
3. Raina R, Banerjee A. Architects of the membrane: structures of eukaryotic choline phosphotransferase 1 and choline/ethanolamine phosphotransferase 1. *Nat Struct Mol Biol* 2023 30(9):1247–1250.

4. Puthenveetil R, Gomez-Navarro N, Banerjee A. Access and utilization of long chain fatty acyl-CoA by zDHHC protein acyltransferases. *Curr Opin Struct Biol* 2022 77:102463.

5. Murphy RE, Banerjee A. In vitro reconstitution of substrate S-acylation by the zDHHC family of protein acyltransferases. *Open Biol* 2022 12(4):210390.

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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).

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

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. Less commonly, mutations in these genes cause excessive growth-plate chondrogenesis and therefore abnormally tall stature. Often the increased proliferation occurs in many tissues, producing a generalized overgrowth syndrome, which can include other medical problems such as developmental delay and elevated cancer risk.

For many children who are brought to medical attention for linear growth disorders, clinical, laboratory, and genetic evaluation fails to identify the underlying etiology. To discover new genetic causes of childhood growth disorders, we evaluated families with monogenic growth disorders using exome sequencing. When sequence variants
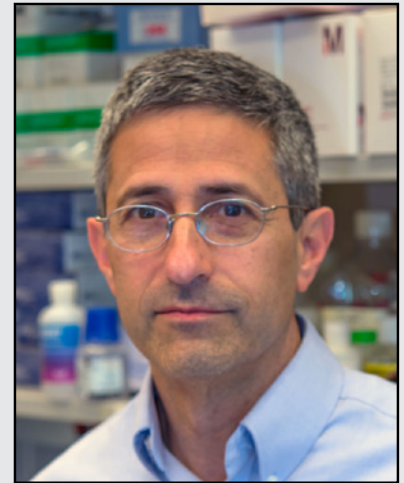
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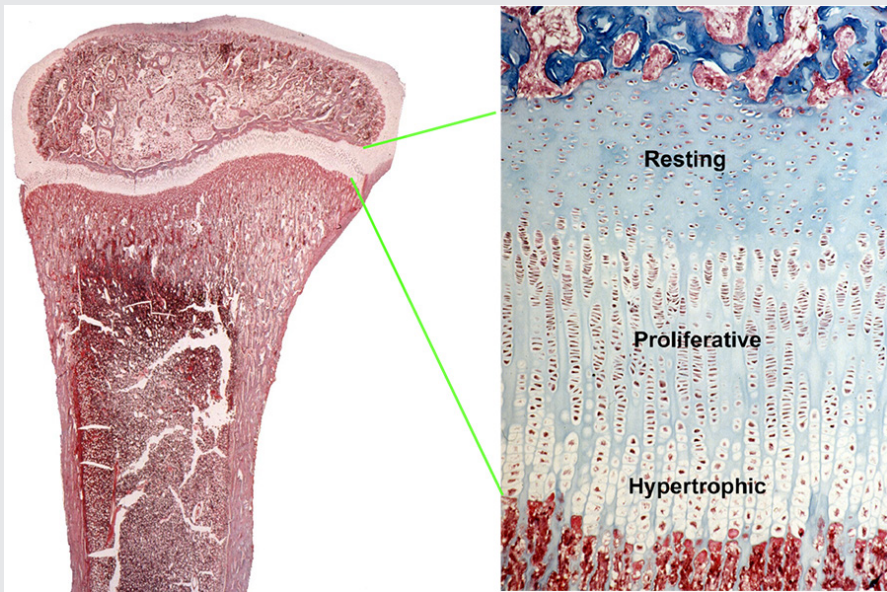Jacob Wagner, BS, *Postbaccalaureate Fellow*

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

that are likely to cause the disorder were identified, the variants and the genes in which they occur were 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.

Using this approach, we identified new causes of childhood growth disorders. We previously found that variants in *QRICH1*, a gene of unknown function, cause a chondrodysplasia attributable to impaired growth-plate chondrocyte hypertrophic differentiation. 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. Our group also discovered that variants in aggrecan (ACAN), a component of cartilage extracellular matrix, cause autosomal-dominant short stature with advanced skeletal maturation and that such patients tend to develop early-onset osteoarthritis.

We also studied a child with a complex skeletal dysplasia who presented with severe scoliosis, thickened calvarium, craniosynostosis, osteosclerosis of the clavicles and spine, and recurring fractures in the lower extremities. We discovered that the disorder was caused by a *de novo*, neomorphic mutation in *SP7*, which encodes a transcription factor required for differentiation of osteoblasts.

Recently, we have focused on disorders that cause excessive growth. We evaluated a patient with Weaver syndrome, an overgrowth disorder caused by variants in *EZH2*. *EZH2* encodes a histone methyltransferase and thereby acts as an epigenetic writer. We found that the *EZH2* variants responsible for Weaver syndrome cause a partial loss of enzymatic function.

We also studied a child with generalized overgrowth of prenatal onset (Figure 2). Exome sequencing identified a hemizygous frameshift variant in Spindlin 4 (*SPIN4*), with X-linked inheritance. Ablation of Spin4 in mice
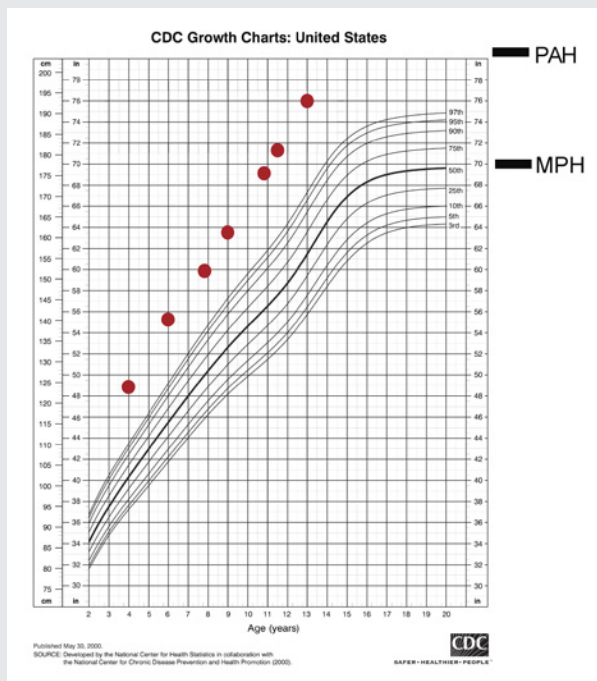
FIGURE 2. Growth chart of a child with overgrowth caused by a variant in SPIN4

recapitulated the human phenotype with generalized overgrowth, including increased longitudinal bone growth (Figure 3). We found evidence that SPIN4 binds specific histone modifications (Figure 4), promotes canonical WNT signaling, and inhibits cell proliferation *in vitro* and that the identified frameshift variant had lost all these functions. Growth-plate analysis revealed increased cell proliferation in the proliferative zone and an elevated number of progenitor chondrocytes in the resting zone. We also found evidence of decreased canonical Wnt signaling in growth-plate chondrocytes, providing a potential explanation for the elevated number of resting-zone chondrocytes. Taken together, our findings provide strong evidence that SPIN4 is an epigenetic reader that negatively regulates mammalian body growth, and that loss of SPIN4 causes an overgrowth syndrome in humans, expanding our knowledge of the epigenetic regulation of human growth.

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

Our group also studies the fundamental mechanisms governing childhood growth. Much of our work has focused on the growth plate. Growth at the growth plate is controlled by several interacting regulatory systems, involving endocrine, paracrine, extracellular matrix–related, and intracellular pathways.

FIGURE 3. Body weight (*left*) and sizes of several organs (*right*) are increased in mice lacking SPIN4.

Previously, our group studied growth-plate regulation by FGFs, BMPs, C-type natriuretic peptide, retinoids, WNTs, PTHrP/IHH, IGFs, estrogens, glucocorticoids, and microRNAs. We also found evidence that SOX9, a transcription factor, regulates the trans-differentiation of growth-plate chondrocytes into osteoblasts.

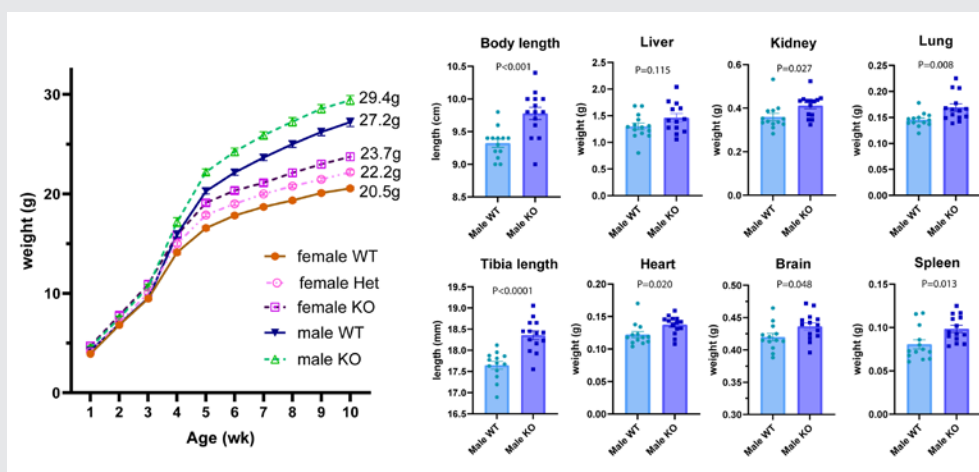We also investigated the mechanisms that cause bone growth to occur rapidly in early life but then to slow progressively with age and eventually cease. We found evidence that the developmental program responsible for the decline in growth-plate function plays out more slowly in larger bones than in smaller bones and that such differential aging contributes to the disparities in bone length and therefore to establishing normal mammalian skeletal proportions.

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 these disorders. Targeting such 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. We then created fusion proteins combining these cartilage-targeting antibody fragments with IGF-1, an endocrine/paracrine factor
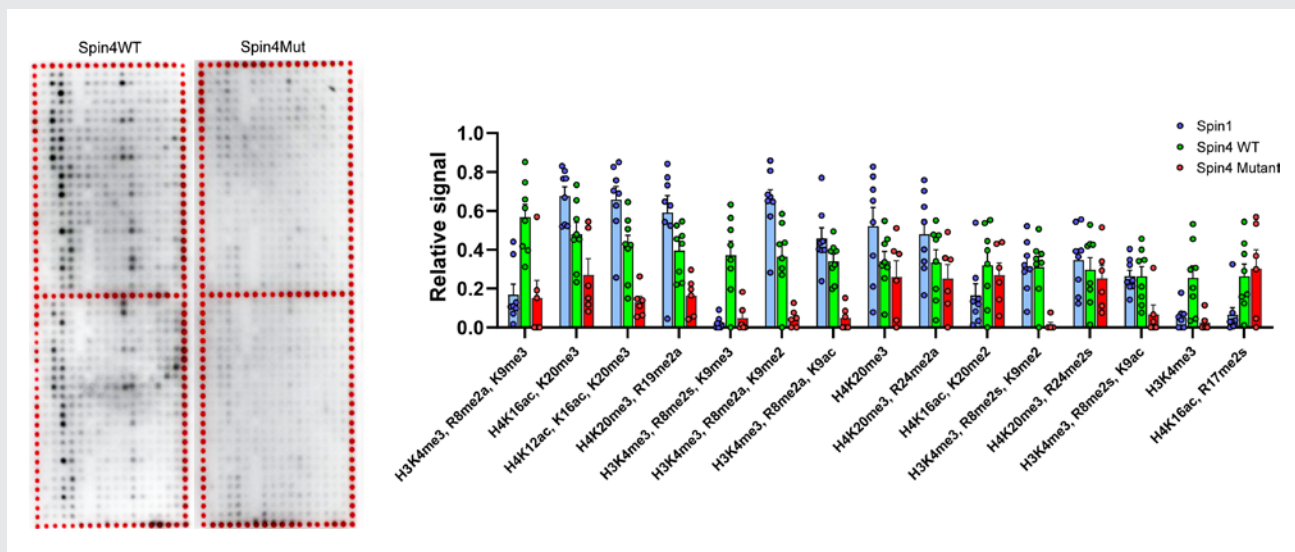
FIGURE 4. Spin4 binds to specific modified histones.

Histone peptide arrays were used to assess histone-binding properties of wild-type (WT) and mutant (Mut) SPIN4. Each spot on the array contains a peptide portion of a histone that has undergone specific post-translational modifications. Darker color indicates greater binding of the indicated Spin protein to that modified peptide. WT SPIN4 binds to specific modified histone peptides, while Mut SPIN4 showed substantially diminished binding. The box-and-whisker plot shows these data quantitatively.

that positively regulates chondrogenesis. Using a GH–deficient mouse model, we found that subcutaneous injections of such 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 with IGF-1 alone. Our findings provide proof of principle that targeting therapeutics to growth-plate cartilage can potentially improve treatment for childhood growth disorders.

We are currently working to optimize the efficacy of targeted IGF1 therapy. In other studies, we are 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.

Publications

1. Lui JC, Wagner J, Zhou E, Dong L, Barnes KM, Jee YH, Baron J. Loss of function variant in SPIN4 causes an X-linked overgrowth syndrome. *JCI Insight* 2023 8:8(9):e167074.
2. Lui JC, Raimann A, Hojo H, Dong L, Roschger P, Kikani B, Wintergerst U, Fratzl-Zelman N, Jee YH, Haeusler G, Baron J. A neomorphic variant in the transcription factor SP7 alters sequence specificity and causes a high-turnover bone disorder. *Nat Commun* 2022 13:700.
3. Lui JC, Baron J. Epigenetic causes of overgrowth syndromes. *J Clin Endocrinol Metab* 2023 online ahead of print.
4. Lui JC, Baron J. CNP-related short and tall stature: a close-knit family of growth disorders. *J Endocr Soc* 2022 6:bvac064.
5. Weiss B, Eberle B, Roeth R, de Bruin C, Lui JC, Paramasivam N, Hinderhofer K, van Duyvenvoorde HA, Baron J, Wit JM, Rappold GA. Evidence that non-syndromic familial tall stature has an oligogenic origin including ciliary genes. *Front Endocrinol (Lausanne)* 2021 12:660731.

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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 and transport 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. Inextricably connected with these activities is our study of water and its interactions with macromolecules and ions in biological media. Experimentally, we use water to probe tissue structure and function from nanometers to centimeters and from microseconds to lifetimes. Our armamentarium includes small-angle neutron scattering (SANS), small-angle X-ray scattering (SAXS), static light scattering (SLS), dynamic light scattering (DLS), atomic force microscopy (AFM), osmometry, and multi-dimensional nuclear magnetic resonance (NMR) relaxometry and diffusometry. A goal is to develop a self-consistent picture of system behavior across length and time scales. Armed with this understanding, we develop research tools that can be translated from bench-based quantitative methodologies to the bedside biomarkers to aid in diagnosis and therapy.

Our 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 produce 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 knowledge of physics, engineering, applied mathematics, imaging and computer sciences, as well as key insights gleaned from our tissue-sciences research, to discover, vet, 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). MRI is our imaging modality of choice because

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(continued)

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 deemed safe and effective for use with mothers, fetuses, and children in both clinical and research settings. Critical to this enterprise is our ability to follow water as it diffuses through complex media as a probe of its microstructure, and to assess water's interactions with biomolecules to identify distinct water compartments in tissues.

One of our translational goals, what we mean by "quantitative imaging," 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 and architecture, such as "imaging" the human connectome.

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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, throughout an imaging volume. Information derived from this quantity includes white-matter fiber-tract orientation, the orientationally averaged mean apparent diffusion constant (mADC) or mean diffusivity (MD), and other intrinsic scalar (invariant) quantities. Such imaging parameters have been used by radiologists and neuroscientists as non-invasive quantitative histological 'stains' that are obtained by probing endogenous tissue water *in vivo* without requiring any exogenous contrast agents or dyes. In neuro-radiology, the mADC is the most widely used diffusion imaging parameter to identify ischemic areas in the brain during acute stroke and to follow cancer patients' responses to therapy. With improvements in whole-body MRI, the mADC is becoming widely used to diagnose cancers, such as multiple myeloma, and assess disease progression and response 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 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 by neuroscientists to track white-matter pathways to help establish 'anatomical connectivity,' to help neurosurgeons plan brain surgeries and radiation oncologists plan radiation dosing, so as to spare 'eloquent' areas of the brain. Such advances in medical imaging also 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. Such features

FIGURE 1. Diffusion Tensor Distribution (DTD) MRI provides new quantitative biomarkers in the *in vivo* human brain.

Orientation heterogeneity in the brain and cerebellum are quantified by the fractional anisotropy (FA), microscopic FA (μFA), and V_{orient} measures. The color-coded primary eigenvector of the mean diffusion tensor from the direction-encoded color (DEC) map is provided for reference (*left column*). The μFA is uniform and higher in gray matter than in the FA. In white matter, loss in FA in regions with complex fiber architecture is recovered in the μFA image as shown, for example, using black arrows in the figure. V_{orient} is lower in coherent white matter fiber tracts and vice-versa, which explains the μFA findings observed in these regions. In gray matter, elevated values of V_{orient} were observed in cerebral cortex and cerebellar gray matter shown, using red and blue arrow respectively, indicating greater fiber incoherence.
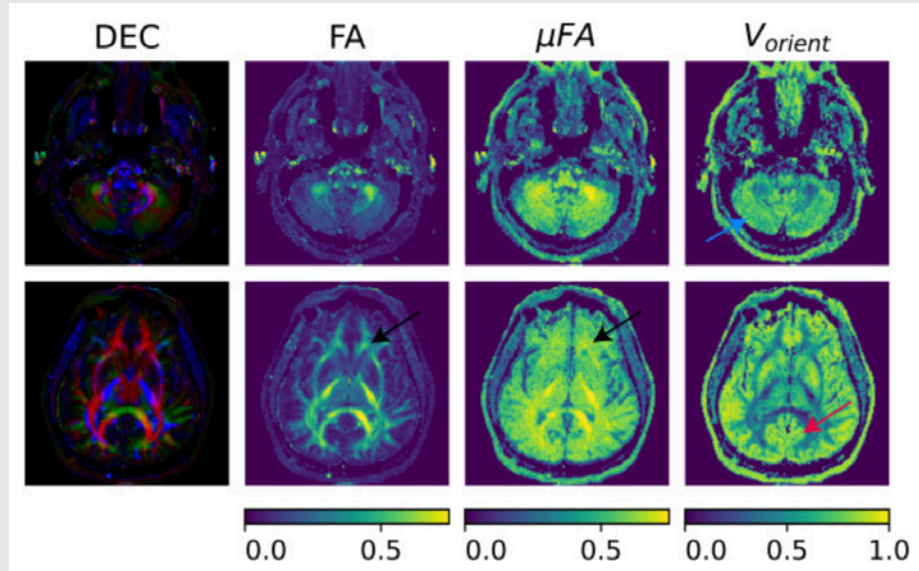
are generally orders of magnitude smaller than the imaging voxel, but remarkably can still be fleshed out using these advanced techniques. For example, we have been developing efficient means for performing 'k- and q-space MRI' in the living human brain, such as 'Mean Apparent Propagator' (MAP) MRI, an approach that can detect subtle microstructural and architectural features in both gray and white matter at micron-scale resolution. MAP MRI also subsumes DTI, as well as providing a bevy of new *in vivo* quantitative imaging biomarkers to measure and map. We also developed CHARMED MRI, which measures the average axon diameter (AAD), 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 is transmitted along axon bundles, and helps to determine the latencies or time delays of neural impulses 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. We also developed a companion mathematical theory to explain the observed ADDs in different fascicles, suggesting that they represent a trade-off between maximizing information flow and minimizing metabolic demands. We 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 distribution and cell shape.

Although gray matter appears featureless in the brain with DTI, 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 the brain's deep gray-matter. To target this tissue, we have been developing

FIGURE 2. Marmoset brainstem, cerebellum, and related structures

Mean Apparent Propagator (MAP) MRI data obtained in marmoset brainstem and cerebellum. (*A–C*) The mediolateral extent of the brainstem (midbrain, pons, and medulla) with the cerebellum in three sagittal Diffusion encoded color (DEC)–Fiber orientation distribution (FOD) images, spaced 0.6 mm, 1.2 mm, and 2.4 mm from the brain's midline. *Brainstem:* white letters indicate the gray matter regions or nuclei, and the reddish-white letters illustrate the major fiber tracts that run in different directions in the brainstem (see the color-coded spherical legend with directions at the top in *A*). Red, green, and blue indicate diffusion anisotropy along mediolateral (ML), rostrocaudal (RC), and dorsoventral (DV) directions, respectively. Three dashed lines passing through the sagittal sections indicate the coronal sections at the midbrain, pons, and medulla level (*D, E, F*). The dashed lines on the coronal sections indicate the location of sagittal images illustrated on the left (*A–C*). Other gray and white matter subregions rostral to the brainstem (e.g., thalamus and basal ganglia) are also included. *Cerebellum:* white letters indicate different cerebellar lobules, green letters show the names of cerebellar sulci and fissures, and white dashed lines show the sulci separating different lobules.
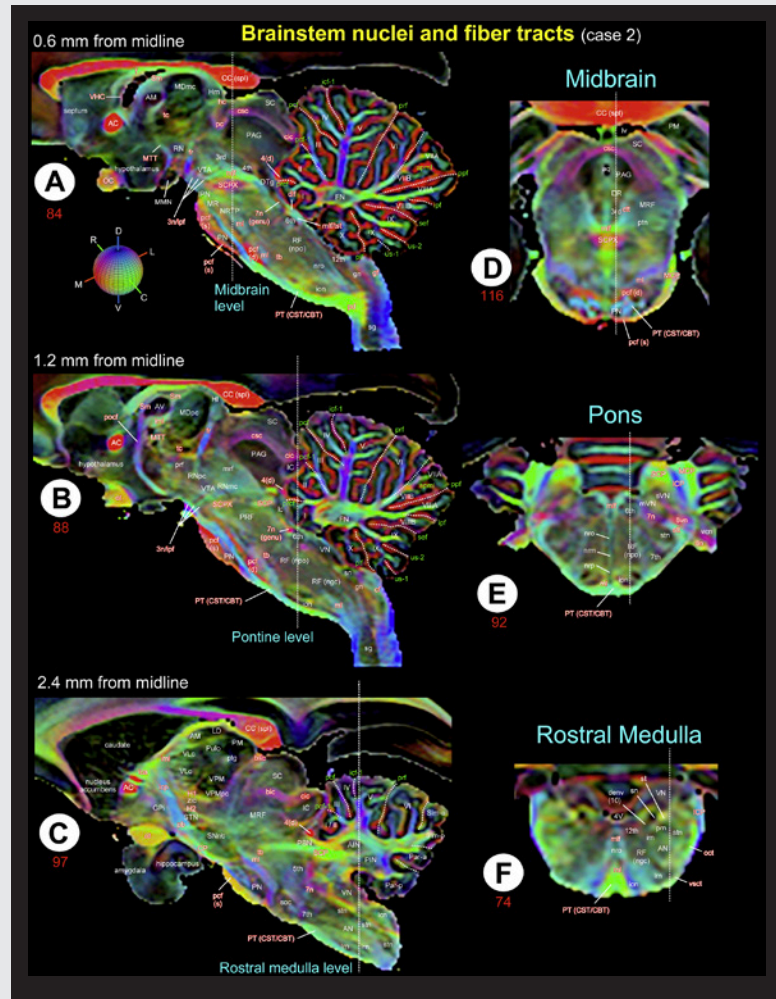
several non-invasive, *in vivo* methods to measure unique features of cortical gray-matter microstructure and architecture that are plainly visible in electron micrographs (EM) but currently invisible in conventional MRI. One example is diffusion tensor distribution (DTD) MRI, in which we use a normal tensor-variate distribution, which we were the first to propose, to characterize heterogeneity within complex tissue voxels. One of our long-term goals is to 'parcellate' or segment the cerebral cortex *in vivo* into its approximately 500 distinct cytoarchitechtonic areas using non-invasive imaging methods. To this end, we are developing advanced MRI sequences and analysis pipelines to assess variability in tissue properties in the cortex, and others that probe correlations among relaxivities and diffusivities of different water pools there. In the latter case, we use multi-dimensional MRI relaxometry– and diffusometry–based methods to study water mobility and diffusion in gray and white matter. 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. Along these lines, we made excellent progress in previous years developing radiological-pathological correlations between MR and neuropathological images of TBI tissue specimens as a way to identify potential quantitative imaging biomarkers of injury or inflammation that have the potential to detect TBI *in vivo*. We are in the process of migrating many of these methods both to pre-clinical and clinical applications.

FIGURE 3. Mean Apparent Propagator (MAP) MRI can delineate boundaries between cortical layers in macaque brain without having to use histological staining techniques.

The correspondence between cortical layers identified from histological images (*right column*) and MAP/DTI parameters (*left and middle columns*) in a representative region of area F4 in macaque brain. Cortical Layer 4 can be distinguished as a band of high diffusion anisotropy (*PA and FA*) with increased conspicuity as compared with multiple histological stains. In area F4, layer 4 shows high MD (mean diffusivity), low RTAP (return-to-the-axis probability), and a different NG (non-Gaussian) laminar pattern with bright and dark bands in layers 3, 4, and 5. PA, NG, and RTAP allow clear visualization of sublaminar structures within layer 3, e.g., 3a and 3b, and allow good differentiation between layers 5 and 6. Good conspicuity between gray matter and sub-cortical white matter. Scale bars are 1 mm.
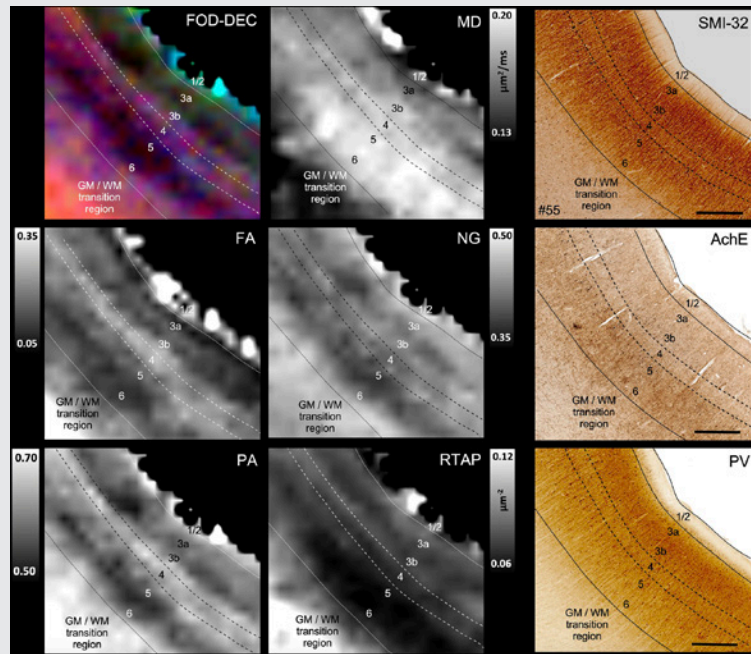
Quantitative MRI biomarker development

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.

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

previously 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 and other distance metrics, like the "earthmover's distance."

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 publishing, 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 collaborator Carlo Pierpaoli, who spearheaded 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*. A continuation of this effort to improve our ability to follow normal trajectories in pediatric brain development has been through a collaboration between the Bill and Melinda Gates Foundation and NICHD. Our role in this project is to develop new MRI pulse sequences and analysis pipelines to probe the state of different water populations in the developing brain. They have the potential to become quantitative imaging biomarkers suitable to follow pediatric brain development.

Traumatic Brain Injury (TBI) represents a significant public health challenge for our pediatric population, but also for 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 Military Traumatic Brain Injury Initiative (MTBI), 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 have been employing promising 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 MTBI, and to improve the correlation and integration of neuropathology and neuro-radiological imaging data, so as to speed the deployment of new MRI methods to assess TBI. We also partnered with MTBI 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 to migrate these imaging approaches to the clinic, 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 neuro-radiological methods. We are developing and testing various new MRI–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. One method we developed and believe has potential in this indication is

FIGURE 4. Changes in the T_2-MD multi-dimensional MR signature induced in confirmed astrogliosis

Maps of 2D spectra of subvoxel T_2-MD values reconstructed on a 16×16 grid of a representative (*A*) control and (*B*) injured subject, along with their respective Glial Fibrillary Acid Protein (GFAP) histological images (*top left* of each panel). (*C*) Magnified spectral region from the control case shows the clear separation of white (*yellow frame*) and grey (*teal frame*) matter according to their MD and T_2 values. (*D*) The same magnified spectral region as in *C* from the injured case shows that while the WM (white matter) and GM (grey matter) spectral information content is still clearly separable (*yellow and teal frames, respectively*), distinct spectral components can be seen on the grey–white matter interface (*purple frame*), which are qualitatively similar to the GFAP staining pattern of the sample. (*E*) T_2-MD spectra averaged across all subjects in WM, GM and GFAP–positive spatial regions of interest (*left* to *right*) and a superposition of the average spectra from the three regions of interest. It should be noted that the peak in normal-appearing WM was forced to align between subjects, but not in GM or in injured tissue.
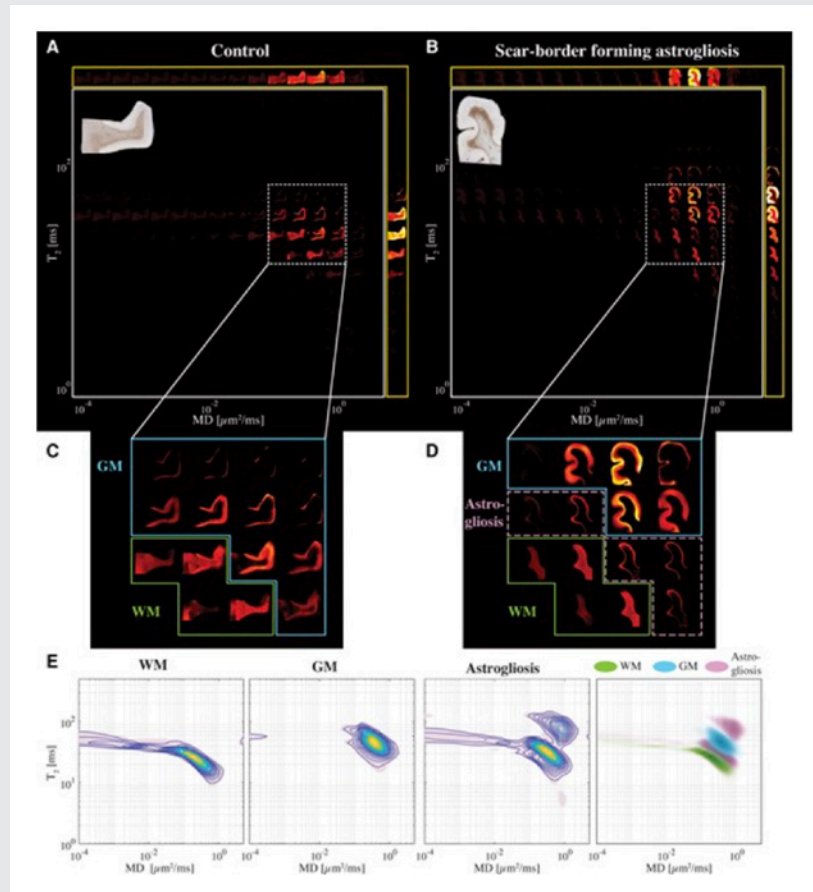
diffusion tensor distribution (DTD) MRI. We are currently testing the utility of this approach on Sara Inati's patient cohort.

Previously, we have been collaborating with *Roberto Romero* and Mark Haacke to develop novel fetal brain MR imaging applications. Currently, it is challenging to measure quantitative imaging biomarkers *in utero*, particularly with diffusion MRI, owing to large-scale fetal and maternal motion during the scanning session, and the difficulty in acquiring image volumes with a sufficient field-of-view, quality, and spatial resolution in a clinically feasible amount of time. Our lab has been developing novel approaches to address each of these critical issues. The closure of NICHD's Perinatology program in Detroit last fiscal year, however, has interrupted this research, which we hope to be able restart at another institution.

Biopolymer physics: water–ion–biopolymer interactions

Remarkably, despite their crucial role in biology, 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, SLS and DLS), which probe the structure and

FIGURE 5. Steady-state water exchange rate, measured by NMR, changes markedly during oxygen-glucose deprivation simulating stroke.

Comparisons between fixed, live (untreated), ouabain-treated, and post-70 min oxygen-glucose deprivation (OGD) of neonatal mouse spinal cord reveal how different treatments affect passive and active exchange rates, k. Bar graphs present the mean across all measurements (bar height), 95% CI of the mean (whiskers), and mean values from each sample (open circles). The exchange rate of live spinal cords (mean ± SD: $k=140\pm16s^{-1}$) is significantly greater than in fixed tissue ($k=87\pm10s^{-1}$), ouabain-treated ($k=36\pm11s^{-1}$), and spinal cord tissue after 70 min of OGD ($45\pm7s^{-1}$). Furthermore, the exchange rate of ouabain-treated spinal cords is significantly less than for fixed spinal cords. However, exchange rates are not significantly different between ouabain-treated spinal cords and spinal cords after 70 min OGD.
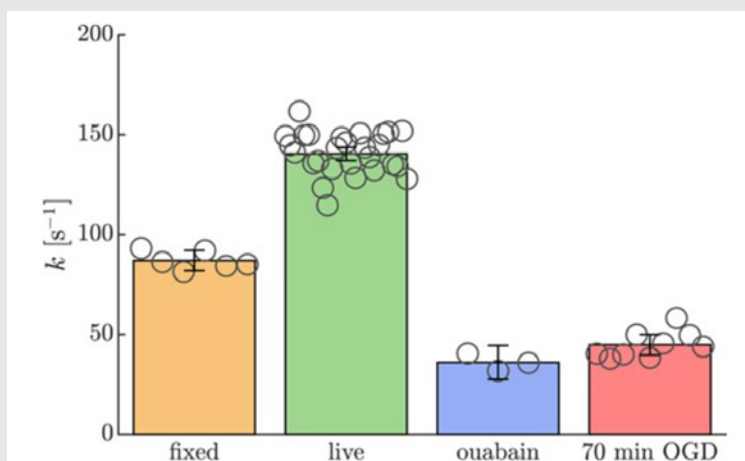
interactions over a broad range of length and time scales. Macroscopic swelling-pressure measurements provide information on the overall thermodynamic response of the system, while SANS, SAXS, SLS and DLS allow us to investigate biopolymers at molecular and atomic through supramolecular length scales 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 and continuum mechanics make it possible to design experiments to 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.

Offshoots of these basic studies have led to numerous novel MRI phantom designs to support our quantitative imaging program, including diffusion MRI phantoms, which we use to calibrate scanners 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 incorporated in the CaliberMRI phantom, enabling quantitative diffusion MRI studies at a myriad of clinical sites worldwide. Our colleagues at NIST Boulder have incorporated our polyvinylpyrrolidone (PVP) polymer into their own *diffusion MRI NIST standard*. We used various glass microcapillary array (GCA) geometries to mimic features of white matter pathways and to interrogate our AxCaliber and dPFG MRI models. We also developed a variety of NMR and MRI phantoms, such as a 3-D printed polymeric phantom, which possess various salient features of cell or tissue systems, such as microscopic anisotropy, providing data with which to test the validity of our models and experimental designs. We are also developing novel polymer gel phantoms to calibrate water exchange experiments designed to follow water moving between different microenvironments.

Measuring and mapping functional properties of extracellular matrix

Extracellular Matrix (ECM) is the tissue that holds organs together and mediates virtually all transport processes taking place in organs, such as the transport of charge, mass, momentum, and energy. Physiologically, one cannot underestimate its importance. We study interactions among the main ECM components, often using cartilage as a model system because it is aneural, avascular, and almost acellular. 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. As an example, understanding the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential to predicting its load-bearing and lubricating ability, which are 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 microgel particles, contributing to the improved dimensional stability and lubricating ability of the tissue. We further found that aggrecan is highly insensitive to changes in the ionic environment, particularly to divalent cations such as calcium, which is critical for maintaining the tissue's mechanical integrity and allowing aggrecan to serve as a calcium-ion reservoir in cartilage and bone.

More recently, 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 network or matrix. PAA mimics the proteoglycan phase (i.e., hyaluronic-aggrecan complexes), while PVA mimics the fibrous collagen network phase 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 osteo-arthritic human cartilage samples. Studies on these model composite hydrogels yield invaluable insights into how macromolecular factors (matrix stiffness, swelling pressure, fixed-charge density, etc.) affect 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 components of ECM (e.g., collagen and PGs) that are 'invisible' to MR 'visible' so as to predict the functional properties of the composite tissue, such as its load-bearing ability. An 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) now make it possible to detect the bound protons indirectly by transferring their magnetization first to the abundant free water protons surrounding them. It also enables us to estimate collagen content in tissue quantitatively. In previous 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. Recently, we have been developing molecular dynamics (MD)–based models of cartilage and cartilage ECM analogs in order to interpret our experimental findings in terms of molecular interactions and processes.

We have also been employing several novel MR methodologies to characterize ECM properties using our one-sided NMR systems to study water relaxation, diffusion, and exchange processes. Most recently, Velencia Witherspoon has been using these approaches to study the organization and structure of fascia. Our specialized NMR 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 an optical microscope provides.

Patents
1. Peter J Basser, Dan H Benjamini. Multi-dimensional spectroscopic NMR and MRI using marginal distributions external link. US Patent 11,415,652, 2022.
2. Magdoom Mohamed Kulam Najmudeen, Peter J. Basser, Michal E. Komlosh. Time efficient, multi-pulsed field gradient (MPFG) MRI without concomitant gradient field artifacts. US publication number: 20230266418, August 24, 2023

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

Publications
1. Avram AV, Saleem KS, Komlosh ME, Yen CC, Ye FQ, Basser PJ. High-resolution cortical MAP-MRI reveals areal borders and laminar substructures observed with histological staining. *NeuroImage* 2022 264:119653.
2. Saleem KS, Avram AV, Yen CC, Magdoom KN, Schram V, Basser PJ. Multimodal anatomical mapping of subcortical regions in Marmoset monkeys using high-resolution MRI and matched histology with multiple stains. *NeuroImage* 2023 281:120311.
3. Magdoom KN, Avram AV, Sarlls JE, Dario G, Basser PJ. A novel framework for in-vivo diffusion tensor distribution MRI of the human brain. *NeuroImage* 2023 271:120003.
4. Williamson NH, Ravin R, Cai TX, Falgairolle M, O'Donovan MJ, Basser PJ. Water exchange rates measure active transport and homeostasis in neural tissue. *PNAS Nexus* 2023 2(3):056.
5. Benjamini D, Priemer DS, Perl DP, Brody DL, Basser PJ. Mapping astrogliosis in the individual human brain using multidimensional MRI. *Brain* 2023 146:1212–1226.
6. Horkay F, Basser PJ, Geissler E. Ion-induced changes in DNA gels. *Soft Matter* 2023 19(28):5405–5415.

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Biophysics of Large Membrane Channels

We study membrane transport through mitochondrial and bacterial membrane proteins that form "large" beta-barrel channels. These channels, recognized as novel drug targets, are the gateways for metabolite exchange as well as the components of many toxins. Healthy cell functioning and development require effective communication between cells and cell organelles, which is facilitated by membrane channels. Our motivation is that learning the molecular mechanisms of channel operation is vital for developing new approaches to treat various diseases in which regulation of transport through ion channels plays a key role. The benchmark approach of our lab is channel reconstitution into planar lipid membranes, a technique that we complement with the physical theory of transport and different biophysical and cell biology methods. This allows us to develop both qualitative and quantitative understanding of the transport processes. Currently, we mostly focus on the VDAC (voltage-dependent anion channel from the outer membrane of mitochondria), but also on other beta-barrel channels such as MspA (major outer-membrane porin from *Mycobacterium smegmatis*), alpha-Hemolysin (toxin from *Staphylococcus aureus*), OmpF (general bacterial porin from *Escherichia coli*), LamB (sugar-specific bacterial porin from *Escherichia coli*), OprF (porin from *Pseudomonas aeruginosa*), translocation pores of *B. anthracis* (PA63); *C. botulinum* (C2IIa), and *C. perfringens* (Ib) binary toxins are also within the range of our interest. Though these large channels are responsible for drastically different biological functions, they have similar beta-barrel structural motifs and biophysical properties, so that their comparative analysis provides an in-depth understanding of the underlying transport mechanisms.

The channel-reconstitution technique allows us to investigate large beta-barrel channels under precisely controlled conditions and to study transport at the single-molecule level. We first isolate the corresponding channel-forming proteins from their host organisms (or refold recombinant proteins from inclusion bodies), purify them, and then insert them into planar lipid membranes for electrophysiological examination. Our main goal is to elucidate the physical principles of regulation of such channels under normal and pathological conditions. In our latest projects, we supplement our biophysical methods with imaging techniques to study the role of the VDAC in mitochondria during embryonic development. Given the location of the VDAC at

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the outer mitochondrial membrane, we also use the Seahorse real-time metabolic assay platform to study its role in mitochondrial function. Among other wet-lab methods, we take advantage of fluorescence correlation spectroscopy, bilayer overtone analysis, and confocal microscopy. Results, obtained through this rich combination of refined biophysical techniques with live-cell imaging and other cell-biology techniques, inspire new strategies and pharmacological approaches to effectively correct the deviant molecular interactions associated with pathology in disease and development.

Regulation of mitochondrial respiration by membrane-bound peripheral proteins with disordered polyanionic C-terminal domains

The crucial role of mitochondrial outer membrane (MOM) permeability in maintaining an efficient metabolite exchange between mitochondria and cytoplasm in normal respiration is well established, with the VDAC recognized as the key metabolite pathway and regulator in MOM. It is believed that the uniqueness of this relatively simple monomeric beta-barrel channel mainly arises from its crucial position at the interface between a mitochondrion and the cytosol. When reconstituted into lipid membranes, the VDAC responds to sufficiently large transmembrane potentials by transitioning to gated states in which ATP/ADP flux is reduced and calcium flux is increased. However, a major reason for uncertainty regarding the physiological role of the VDAC voltage gating for the regulation of MOM permeability is the source and magnitude of the outer membrane potential *in vivo*.

We found that two otherwise unrelated cytosolic proteins, tubulin, and alpha-synuclein (α-Syn), dock with the VDAC by a newly established mechanism, in which the transmembrane potential draws their disordered, polyanionic C-terminal domains into and through the VDAC channel, thus physically blocking the pore. Remarkably, for both tubulin and α-Syn, the blocked states are observed at much lower transmembrane potentials than VDAC–gated states with their bulk concentrations as small as 10 nanomoles. Therefore, in the

FIGURE 1. Increased sensitivity of VDAC (PDB ID: 3EMN) regulation to transmembrane potential in the presence of cytosolic regulators

Only a fraction of the outer membrane potential, relative to that necessary to induce channel voltage gating (*blue right-most curve*), is required for the effective control of VDAC–induced transport by tubulin (*red left-most curve*) and alpha-Syn (*green curve in the center*) at their bulk concentrations as small as 10 nanomolar. The changes in the transport properties of the channel in response to the outer membrane potential, i.e., decrease in metabolite flux and the concomitant increase in calcium flux, remain qualitatively the same in all three cases. The "effective gating charge," expressed as the steepness of the voltage-dependence curve, is the lowest for voltage gating and the highest for tubulin, with α-Syn in between.
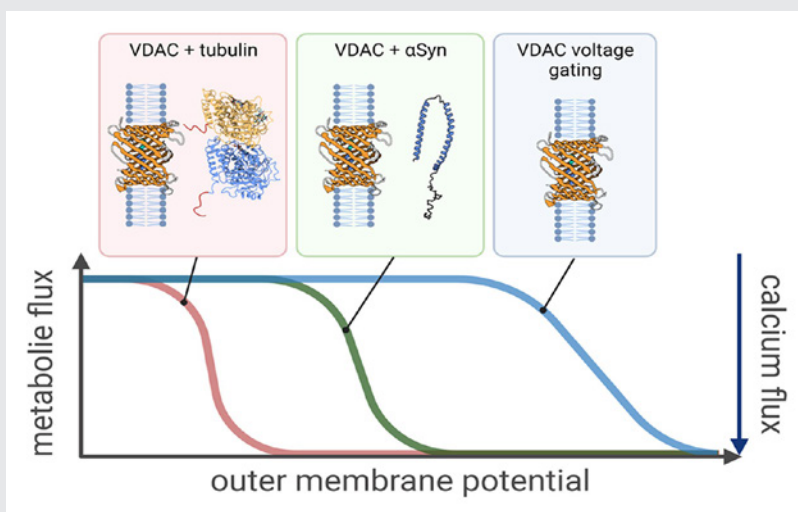
FIGURE 2. Brownian dynamics simulation cell

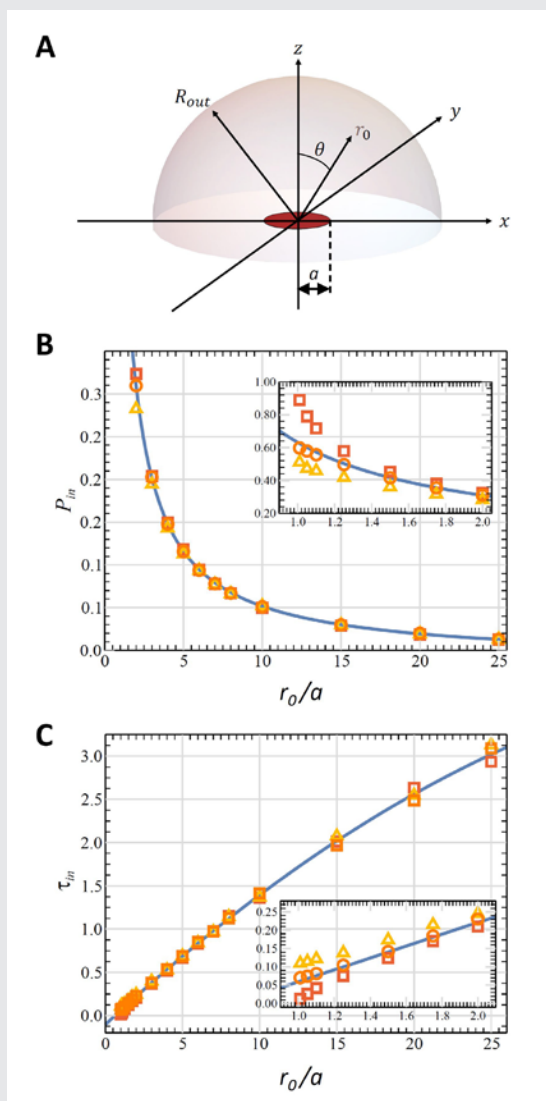(*A*) Notations: r_0 is the particle injection coordinate; R_{out} and a are radii of the absorbing sphere and disc on the surface.

(*B*) Results of simulations of splitting probability of the particle capture by the disk as a function of distance r_0 at different starting angles relative to the surface (triangles: starting point at 90^0; circles: at 30^0; and squares: starting on the reflecting surface, 0^0) vs. theory (*lines*) of the sphere approximation.

(*C*) Corresponding mean first-passage times vs. theory.

presence of these cytosolic docking proteins, the VDAC's sensitivity to transmembrane potential is dramatically increased. The features of the VDAC voltage-gated states relevant for bioenergetics (reduced metabolite flux and increased calcium flux) are preserved in the blocked states induced by either docking protein. The ability of tubulin and α-Syn to modulate mitochondrial potential and ATP production *in vivo* is now supported by many studies. The common physical origin of the interactions of both tubulin and α-Syn with the VDAC leads to a general model of a VDAC inhibitor, facilitates predictions of the effect of post-translational modifications of known inhibitors, and, because of its generality, points the way toward the development of novel therapeutics targeting the VDAC.

Trapping of single diffusing particles by a circular disk on a reflecting flat surface

Recent progress in cell biophysics (for example, in studies of chemical sensing and spatiotemporal cell signaling) poses new challenges to the statistical theory of trapping of single diffusing particles. We offered an analytical solution to one of them, namely, the trapping kinetics of single particles diffusing in a half-space bounded by a reflecting flat surface containing an absorbing circular disk. Our analysis is quite general and is thus relevant for various applications involving not only signal transduction problems in biology but operation of different kinds of chemical sensors. Signaling within and between cells is the key phenomenon underlying the development and functional behavior of cells and multicellular organisms. The so-called "spatiotemporal dynamic cell-signaling modeling" focuses on signaling processes both in individual cells and the spatial coupling between different cells within a tissue. As follows from the name, the efficiency and characteristic duration of signal transduction between cells are studied as functions of their relative spatial location. Researchers point out that the response strength could depend on a vector distance between the signal origin and
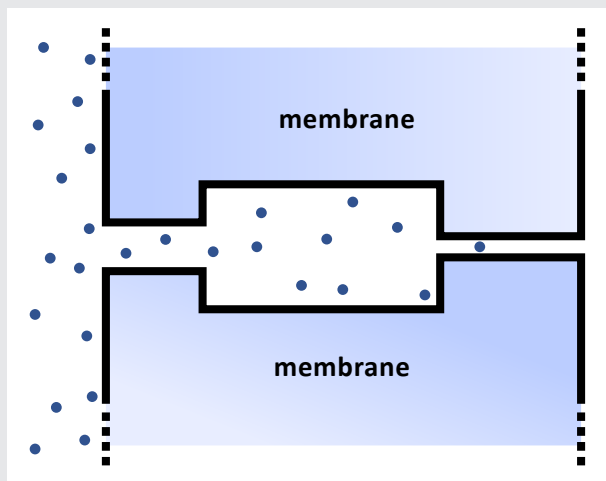
FIGURE 3. Three-segment channel with abrupt radius jumps

the receptor, rather than a scalar distance alone, thus exhibiting directional behavior. This trapping problem is essentially two-dimensional, and the question of the angular dependence of the kinetics on the particle starting point is highly nontrivial. We proposed an approximate approach to the problem that replaces the absorbing disk with an absorbing hemisphere of a properly chosen radius. The replacement makes the problem angular-independent and essentially one-dimensional. After the replacement, one can find an exact solution for the particle propagator (Green's function) that allows one to completely characterize the kinetics. Extensive testing of the theoretical predictions based on the absorbing hemisphere approximation against three-dimensional Brownian dynamics simulations shows excellent agreement between the analytical and simulation results when the particle starts sufficiently far from the disk. It works well for distances of the particle starting point from the disk center comparable with the disk diameter or larger. This condition reflects actual situations in multiple signaling pathways at the cell and organism levels.

Analytical theory for the permeability and diffusion resistance of porous membranes and its numerical test

Channels of cell and organelle membranes are often clustered, forming domains of tightly packed transport proteins. A well known example is the VDAC, the major gateway for metabolites and ions in the outer mitochondrial membrane. Indeed, electron micrographs of MOM fragments from *Neurospora crassa* and high-resolution atomic force imaging of MOM fragments from *Saccharomyces cerevisiae* show that, in both cases, VDAC beta-barrels form dense arrays of hundreds of channels. The characteristic distances between barrel centers are as small as 4 to 5 nm, which are comparable to the barrel outer diameter of about 3 nm. Estimates demonstrate that in these high-density domains, the VDAC protein occupies about 80% of the membrane surface. Previously, we considered the effect of clustering on particle trapping by absorbing disks and channel-facilitated transport, assuming that the size of the cluster is much smaller than the total membrane area. This year, we addressed a different problem. Specifically, we studied the case in which the channels are spread over the entire membrane, with a focus on the effects of channel "crowding" on membrane transport. We considered the transport of neutral solutes through porous flat membranes, driven by the solute concentration difference in the reservoirs separated by the membrane. Transport occurs through membrane channels, which are assumed to be non-overlapping, identical, straight cylindrical pores connecting the reservoirs. The key quantities characterizing transport are membrane permeability and diffusion resistance. Such transport problems, arising in very different contexts ranging from plant physiology and cell biology to chemical engineering, have been studied for more than a century. Nevertheless, an expression giving the permeability for a membrane of arbitrary thickness at arbitrary surface densities of the channel openings has been unknown. We have filled this gap and derived such an expression. Given that this expression involves

approximations, we compared its predictions with the results obtained from Brownian dynamics simulations and found excellent agreement between the two.

Publications

1. Rostovtseva TK, Bezrukov SM, Hoogerheide DP. Regulation of mitochondrial respiration by VDAC is enhanced by membrane-bound inhibitors with disordered polyanionic C-terminal domains. *Int J Mol Sci* 2021 22:7358.
2. Dagdug L, Berezhkovskii AM, Zitserman VY, Bezrukov SM. Effective diffusivity of a Brownian particle in a two-dimensional periodic channel of abruptly alternating width. *Phy Rev E* 2021 103:062106.
3. Skvortsov AT, Dagdug L, Berezhkovskii AM, Bezrukov SM. Blockage coefficient of cylindrical blocker and diffusion resistance of membrane channels. *Phys Fluids* 2023 35:011702.
4. Dagdug L, Berezhkovskii AM, Bezrukov SM. Trapping of single diffusing particles by a circular disk on a reflecting flat surface. Absorbing hemisphere approximation. *Phys Chem Chem Phys* 2023 25:2035–2042.
5. Skvortsov AT, Dagdug L, Hilder EF, Berezhkovskii AM, Bezrukov SM. Permeability and diffusion resistance of porous membranes: Analytical theory and its numerical test. *J Chem Phys* 2023 158:054114.

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Protein Sorting in the Endomembrane System

Our laboratory studies the molecular mechanisms underlying the sorting of transmembrane proteins (known as cargo) to various compartments within the endomembrane system of 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, cytotoxic granules), and various domains of the plasma membrane in polarized cells such as epithelial cells and neurons. The transport of cargo between these compartments is mediated by vesicular or tubular carriers that bud from a donor compartment, translocate through the cytoplasm, and fuse with an acceptor compartment. We study the molecular machineries that mediate these processes in the context of different intracellular transport pathways, including endocytosis, recycling from endosomes 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. Our fundamental research serves as a basis for explaining the pathogenetic mechanisms of protein traffic disorders, including the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS), hereditary spastic paraplegias (HSPs), and other neuro-developmental and early infantile neuro-degenerative disorders.

The adaptor protein chaperone AAGAB promotes assembly of the AP-4 complex.

Adaptor protein 4 (AP-4) is a heterotetrameric complex, composed of epsilon, beta4, mu4, and sigma4 subunits, that mediates export of transmembrane cargos, including autophagy protein 9A (ATG9A), from the TGN towards pre-autophagosomal structures (Figure 1). AP-4 has received particular attention in recent years because mutations in any of its subunits cause a complicated form of hereditary spastic paraplegia referred to as "AP-4–deficiency syndrome." This year, we reported that the alpha- and gamma-adaptin–binding protein (AAGAB, also known as p34) binds to and stabilizes AP-4 subunits, thus promoting complex assembly. The physiological importance of these interactions is underscored by the observation that AAGAB–knockout (KO) cells exhibit reduced levels of AP-4 subunits and accumulation of ATG9A at the TGN, like those in cells with mutations in AP-4–subunit
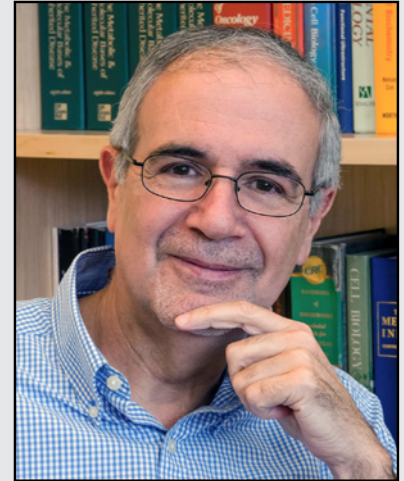
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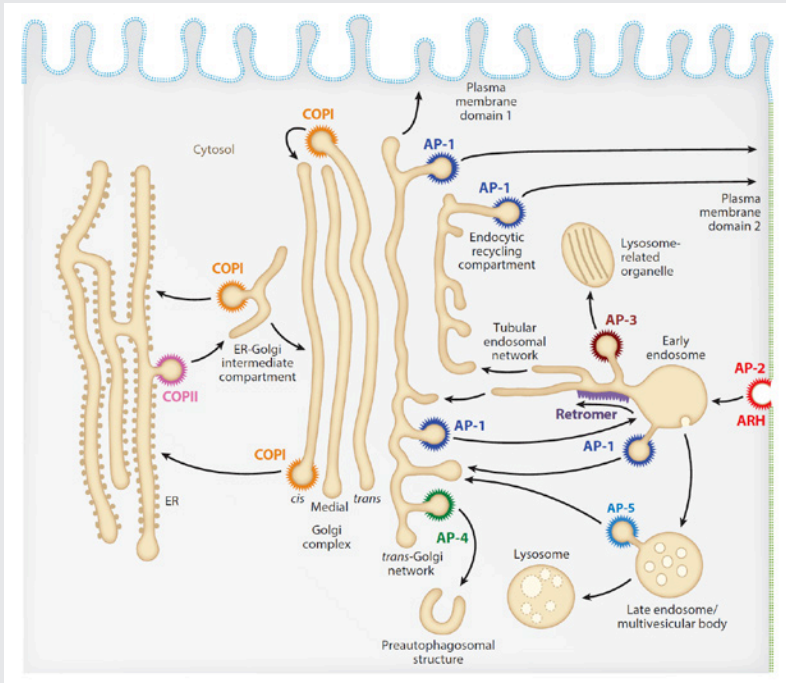
FIGURE 1. Schematic representation of the endomembrane system of eukaryotic cells showing the localization of coats involved in protein sorting

genes. These findings demonstrated that AP-4 assembly is not spontaneous but AAGAB–assisted, further contributing to the understanding of an adaptor protein complex that is critically involved in the development of the central nervous system.

Contribution to the development of intrathecal AAV9/AP4M1 gene therapy for hereditary spastic paraplegia 50 caused by mutations in the mu4 subunit of AP-4

We also contributed to the development of gene therapy for hereditary spastic paraplegia 50 (SPG50) in collaboration with Xin Chen, Steven Gray, and other colleagues. SPG50 is a rare childhood-onset neurological disorder caused by mutations in the *AP4M1* gene. Our laboratory demonstrated that infection of skin fibroblasts from patients having *AP4M1* mutations with an AAV2–AP4M1 vector rescued the assembly of the AP-4 complex and the export of ATG9A from the trans-Golgi network. Our collaborators then showed that intrathecal injection of a similar AAV9–AP4M1 vector had an acceptable safety profile in mice, rats, and non-human primates, and resulted in partial correction of phenotypic defects in AP4M1–KO mice, preclinical results that support an investigational gene transfer clinical trial to treat SPG50.

Architecture of the ESCPE-1 membrane coat: unveiling a key process of endosomal sorting

Intracellular recycling of membrane proteins plays a crucial role in re-using receptors, ion channels, and transporters. A critical player in this recycling machinery is the endosomal sorting complex for promoting exit 1 (ESCPE-1), responsible for rescuing transmembrane proteins from the endolysosomal pathway and transporting them to the trans-Golgi network and the plasma membrane. We collaborated with the laboratories of Aitor Hierro and Daniel Castaño-Díez to conduct biochemical, structural, and functional analyses into the

organization of ESCPE-1, revealing that the complex forms a coat with a single-layer architecture. Furthermore, we found that synergistic interactions between ESCPE-1 protomers, phosphoinositides, and cargo molecules lead to the global arrangement of amphipathic helices, driving the formation of recycling tubules.

Endolysosome fusion attenuates exosome secretion.

In previous research, we discovered an eight-subunit complex named BORC, which plays a crucial role in recruiting the small GTPase ARL8, kinesin motor proteins, and the tethering factor HOPS to late endosomes and lysosomes. This past year, we reported that the BORC–ARL8–HOPS axis is responsible for regulating exosome secretion. Exosomes are small vesicles that cells release to dispose of undegraded materials and facilitate intercellular communication. A major source of exosomes is intraluminal vesicles within multi-vesicular endosomes, which can undergo either exocytic fusion with the plasma membrane or fusion with lysosomes. The factors that determine these alternative fates, however, were previously unknown. Our findings showed that disrupting the BORC–ARL8–HOPS axis impairs endolysosomal fusion, preventing the delivery of intraluminal vesicles to lysosomes and thus increasing exosome secretion. Our findings additionally suggested that targeting the BORC–ARL8–HOPS pathway may be a promising strategy to enhance exosome yields for biotechnology applications.

Small GTPases coordinate HOPS−mediated tethering of late endosomes and lysosomes

The transportation of endocytosed cargoes to lysosomes relies on HOPS–dependent tethering of late endosomes to lysosomes prior to fusion. Although several proteins interact with HOPS, their exact localization and involvement in the tethering process remained unclear. To address this problem, we collaborated with Albert Haas and Andreas Jeschke to determine the order and functional interdependence of HOPS and its interacting proteins in cargo transport to lysosomes. Our findings revealed that the small GTPases RAB2A and RAB7 are associated with late endosomes, while the small GTPase ARL8 and the BORC complex localize to lysosomes. HOPS facilitates late endosome-lysosome fusion by bridging late endosomal RAB2A with lysosomal BORC–anchored ARL8. Additionally, we observed that RAB7 is not present at HOPS–dependent tethering sites, but promotes fusion by facilitating the movement of late endosomes via dynein.

ARF1−related disorder: unveiling pathophysiological mechanisms

ADP–ribosylation factor 1 (ARF1) is a small GTPase that plays a critical role in regulating membrane traffic at the Golgi apparatus and endosomes by interacting with various coat proteins and lipid-modifying enzymes. In collaboration with the laboratory of Tyler Pierson, we reported this past year a pediatric patient with an ARF1–related disorder caused by a monoallelic *de novo* missense variant (c.296 G > A; p.R99H) in the *ARF1* gene. The patient presented with developmental delay, hypotonia, intellectual disability, and motor stereotypies. Our functional analysis of the R99H–ARF1 variant protein showed that it was expressed at normal levels and correctly localized to the Golgi apparatus. However, the expression of this variant led to swelling of the Golgi apparatus and increased recruitment of coat proteins, as well as altered the morphology of recycling endosomes. Furthermore, protein interaction analyses indicated that R99H–ARF1 exhibited a stronger binding affinity to the ARF1–effector GGA3 than to wild-type ARF1, properties that suggested that the pathogenetic mechanism of the R99H–ARF1 variant involves constitutive activation, leading to Golgi and endosomal alterations. The study contributed to our understanding of the genetic basis of neuro-developmental disorders associated with ARF1 variants, shedding light on the pathophysiological mechanisms underlying these conditions.

Publications

1. Bonifacino JS. Getting where you want to go. *Mol Biol Cell* 2022 33:ae4.
2. Mattera R, De Pace R, Bonifacino JS. The adaptor protein chaperone AAGAB stabilizes AP-4 complex subunits. *Mol Biol Cell* 2022 33:ar109.
3. Lopez-Robles C, Scaramuzza S, Astorga-Simon EN, Ishida M, Williamson CD, Baños-Mateos S, Gil-Carton D, Romero-Durana M, Vidaurrazaga A, Fernandez-Recio J, Rojas AL, Bonifacino JS, Castaño-Díez D, Hierro A. Architecture of the ESCPE-1 membrane coat. *Nat Struct Mol Biol* 2023 30:958–969.
4. Shelke GV, Williamson CD, Jarnik M, Bonifacino JS. Inhibition of endolysosome fusion increases exosome secretion. *J Cell Biol* 2023 222:e202209084.
5. Chen X, Dong T, Hu Y, De Pace R, Mattera R, Eberhardt K, Ziegler M, Pirovolakis T, Sahin M, Bonifacino JS, Ebrahimi-Fakhari D, Gray SJ. Intrathecal AAV9/AP4M1 gene therapy for hereditary spastic paraplegia 50 shows safety and efficacy in preclinical studies. *J Clin Invest* 2023 133:e164575.
6. Ishida M, Otero MG, Freeman C, Sánchez-Lara PA, Guardia CM, Pierson TM, Bonifacino JS. A neurodevelopmental disorder associated with an activating de novo missense variant in ARF1. *Hum Mol Genet* 2023 32:1162–1174.
7. De Pace R, Bonifacino JS. Phagocytosis: Phagolysosome vesiculation promotes cell corpse degradation. *Curr Biol* 2023 33:R143–R146.
8. Schleinitz A, Pöttgen LA, Keren-Kaplan T, Pu J, Saftig P, Bonifacino JS, Haas A, Jeschke A. Consecutive functions of small GTPases guide HOPS-mediated tethering of late endosomes and lysosomes. *Cell Rep* 2023 42:111969.
9. Zhang Z, Venditti R, Ran L, Liu Z, Vivot K, Schürmann A, Bonifacino JS, De Matteis MA, Ricci R. Distinct changes in endosomal composition promote NLRP3 inflammasome activation. *Nat Immunol* 2023 24:30–41.
10. Jani RA, Di Cicco A, Keren-Kaplan T, Vale-Costa S, Hamaoui D, Hurbain I, Tsai FC, Di Marco M, Macé AS, Zhu Y, Amorim MJ, Bassereau P, Bonifacino JS, Subtil A, Marks MS, Lévy D, Raposo G, Delevoye C. PI4P and BLOC-1 remodel endosomal membranes into tubules. *J Cell Biol* 2022 221:e202110132.

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Neuregulin–ErbB and NMDA Receptor 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 behavioral and cognitive deficits in psychiatric disorders. We have been investigating whether 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. We use genetically modified NRG and ErbB4 mouse models, in combination with optogenetic, electrophysiological, behavioral, and molecular/cellular techniques to identify novel interactions between the NRG/ErbB4, glutamatergic, dopaminergic, and GABAergic signaling pathways associated with psychiatric disorders.

Our earlier studies demonstrated that NRG/ErbB4 signaling in GABAergic fast-spiking parvalbumin-positive (PV$^+$) interneurons regulates E-I balance, gamma oscillation network activity, and numerous behaviors relevant to psychiatric disorders. To understand how NRGs mediate their biological functions during brain development, we investigated how different NRG ligands are proteolytically processed and trafficked in neurons, using molecular, cellular, and genetic approaches. NRGs are synthesized as unprocessed pro-proteins (proNRGs) containing either a single or two transmembrane (TM) domains, and, contrary to dogma, we discovered that these two types of NRGs are processed and trafficked very differently. Single–TM NRGs are cleaved and shed from endoplasmic reticulum–plasma membrane (ER–PM) junctions on neuronal soma in an activity-dependent fashion in response to glutamate NMDA receptor (NMDAR) activation. By contrast, dual-pass TM NRGs are constitutively cleaved by BACE1 in the trans-Golgi network, sorted into axons by transcytosis, and then selectively retained at presynaptic glutamatergic terminals via juxtacrine trans-synaptic interactions with ErbB4 receptors on dendrites of GABAergic interneurons (Figure 1). These findings suggest that single-pass TM NRGs signal in paracrine mode at neuronal soma and proximal

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dendrites, whereas dual-pass TM NRGs signal in juxtacrine fashion from axons and presynaptic terminals by interacting with postsynaptic ErbB4 on GABAergic dendrites.

We are interested in how NRG/ErbB4 signaling would affect networks and behaviors associated with psychiatric disorders. The prefrontal cortex (PFC) is a site of convergence of long-range glutamatergic inputs, which integrates multiple modalities of information to produce goal-directed behaviors. GABAergic PV$^+$ interneurons coordinate pyramidal cell firing induced by this converging glutamatergic innervation to synchronize cortical networks that modulate goal-oriented behaviors, which are disrupted in psychiatric disorders. Interestingly, drugs that inhibit NMDAR activity (i.e., ketamine) alter E-I balance, increase gamma oscillation network activity, and disrupt behaviors associated with psychiatric disorders. Several lines of evidence suggest that NMDAR antagonists may disproportionally act on NMDARs expressed on PV$^+$ interneurons; however, there has been significant controversy as to whether adult PV$^+$ interneurons have functional NMDARs. Given the importance of PV$^+$ interneurons for cortical functions, and our prior findings that NRG/ErbB4 signaling inhibits NMDAR activity in GABAergic interneurons, we embarked on an investigation of the proportion of PFC adult PV$^+$ interneurons expressing functional NMDARs and their role in local network activity.

Presynaptic accumulation of NRG3 in central neurons is achieved by trans-synaptic retention: a novel mechanism for polarized axonal expression of proteins.

How stable axonal polarity is maintained remains a central question in neuroscience. We previously demonstrated that dual–TM proNRGs, comprising CRD-NRG1 type III and NRG3, are targeted to axons and accumulate at glutamatergic presynaptic terminals, where they signal in juxtacrine mode via postsynaptic ErbB4 receptors expressed at postsynaptic densities on GABAergic interneurons [Vullhorst, Ahmad *et al*. *J Neurosci* 2017; Figure 1]. Our new study aimed to understand how and where proNRG3 is cleaved by BACE1 and how the resulting biologically active NRG3 peptide is sorted and selectively retained in axons. For this study, an LOV (light-oxygen-voltage)–based optogenetic proNRG3 reporter (LA143–NRG3) was designed, which, in response to blue light, undergoes a conformational change that allows BACE1 to cleave proNRG3. Using LA143–NRG3, we

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

A dissociated hippocampal GABAergic interneuron showing pro–NRG2 surface puncta (*white*), clustered at ER–PM contacts, and processed NRG3 at presynaptic terminals (*green*) that are juxtaposed to postsynaptic ErbB4 receptors (*red*). Note the restricted distribution of pro–NRG2 on the soma and proximal dendrites that correspond to clusters of NRG2 at ER–PM junctions, as confirmed by electron microscopy (*inset*). In stark contrast to NRG2, NRG3 and ErbB4 staining overlap extensively, consistent with their trans-synaptic interactions at excitatory synapses onto inhibitory interneurons (see also Figure 2).
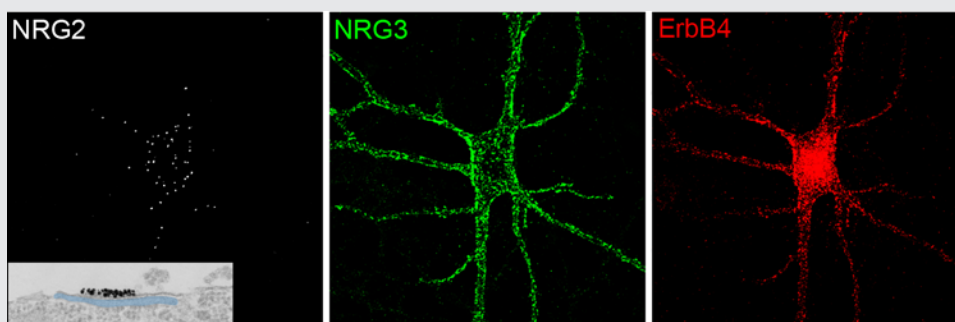
FIGURE 2. Diagram summarizing the results of our NRG3 trafficking studies

The diagram depicts the different steps of pro–NRG3 processing, which begin with its synthesis as a dual–TM protein in the endoplasmic reticulum, its cleavage, and sorting of the biologically active single–TM NRG3 ligand to axonal presynaptic terminals, where it is retained by virtue of its interaction with postsynaptic ErbB4 receptors. The three major steps of pro–NRG3 processing and axonal retention are:
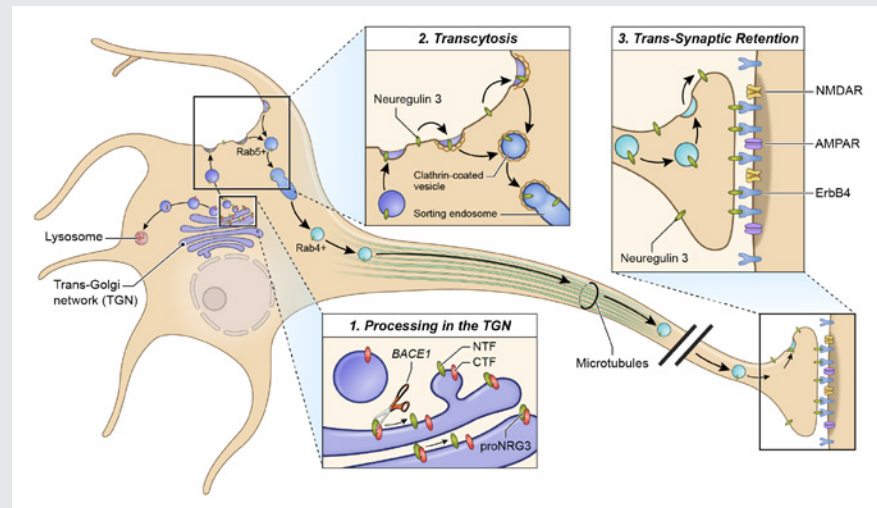
1. Unprocessed dual–TM pro-NRG3 requires cleavage by the protease BACE1 to release the biologically active single–TM NRG3 from the *trans*-Golgi network (TGN).

2. NRG3 then traffics to axons by transcytosis, a process that involves initial transport of NRG3 to the somatodendritic plasma membrane, re-endocytosis into Rab5–positive early endosomes, sorting, and anterograde axonal transport in Rab4–positive vesicles.

3. NRG3 is retained selectively at axonal terminals by virtue of its binding across the synapse to its cognate receptor ErbB4, which is expressed at glutamatergic postsynaptic densities on dendrites of GABAergic interneurons. We termed this mechanism, which is necessary for the polarized expression of NRG3 in axonal terminals, "trans-synaptic retention," and we propose that it accounts for the polarized expression of other neuronal transmembrane ligands and receptors in axons.

found that proNRG3 is retained in the TGN until cleaved by BACE1, whereupon mature NRG3 emerges on the somatodendritic plasma membrane and, by transcytosis, is re-endocytosed and anterogradely transported on Rab4⁺ (small GTPases involved in intracellular vesicle trafficking) vesicles into axons. Importantly, NRG3 accumulation at axonal presynaptic terminals is maintained by its continued trans-synaptic interaction with ErbB4 receptors expressed at postsynaptic glutamatergic synapses on GABAergic interneurons (Figure 2). We denote this novel mechanism "trans-synaptic retention" and propose that it can account for polarized axonal expression of other transmembrane ligands and receptors [Reference 1].

ER–PM junctions on GABAergic interneurons are organized by neuregulin 2/VAP interactions and regulated by NMDA receptors.

Two major unresolved questions we recently pursued were to understand, at a mechanistic level: (1) how proNRG2 clusters at ER–PM junctions; and (2) how proNRG2 dissociates from the junctions in response to NMDAR activation. We found that proNRG2 promotes the formation of ER–PM junctions in hippocampal GABAergic interneurons via interactions of its cytoplasmic tail with the ER–resident protein VAP. Interestingly, there are two stretches of amino acids in the intracellular cytoplasmic domain conserved between proNRG1 and proNRG2, denoted C- and D-boxes, which are required to stabilize proNRG2/VAP complexes during immunoprecipitation. Although the protein sequence of neither box conforms to known FFAT (acronym for two phenylalanines in an acidic tract) motifs, shown in other proteins to bind to VAP, the proNRG2 D-box

contains a track of acidic residues required for VAP binding and the C-box harbors a cryptic, phosphorylation-dependent VAP–binding site. Importantly, NMDAR activation stimulates dephosphorylation of Ser/Thr residues in the C-box and its dissociation from VAP, which reduces proNRG2 clustering at ER–PM junctions [Reference 2]. These observations are interesting because, although both proNRG2 and the potassium channel Kv2.1 are colocalized at ER–PM junctions and clustering at these sites is regulated by NMDA receptor activity, their modes of interaction with VAP differ (Figure 3). Based on these findings, we hypothesize that autocrine NRG2/ErbB4 signaling and Kv2.1 function synergistically as a homeostatic protective mechanism to downregulate GABAergic interneuron excitability during periods of strong excitatory activity and/or elevated extracellular glutamate levels, which would help protect these neurons from excitotoxicity.

Single-pass TM NRG2 in central neurons

Single-pass 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. Activation of excitatory glutamatergic NMDARs located on neuronal soma/dendrites promote calcium entry and activate phosphatases that dephosphorylate Ser/Thr residues in the proNRG2 intracellular region, resulting in the dissociation of proNRG2 from ER–PM junctions and ectodomain cleavage by the metalloproteinase ADAM10 [Vullhorst D, Buonanno A. *Mol Neurobiol* 2019;56:8345]. The released, biologically active NRG2 binds to and activates ErbB4 receptors expressed at postsynaptic excitatory glutamatergic synapses onto GABAergic interneurons, which in turn selectively downregulates NMDA excitatory currents [Vullhorst *et al*, *Nat Comm* 2015;6:7222]. We hypothesize that this bidirectional NMDAR–NRG2 (up)/ErbB4–NMDAR (down) signaling mode serves as a homeostatic mechanism that regulates the activity of GABAergic interneurons.

A remaining challenge was to understand how proNRG2 clusters at ER–PM junctions, a site where Kv2.1 potassium channels also accumulate via their interaction with VAP, and how NMDAR activation causes their dissociation from the junction. Using a combination of cell-biological and protein-biochemical approaches, we identified two conserved protein sequences (denoted C- and D-boxes) in the cytoplasmic tails of proNRG1 and proNRG2 that interact with the ER–resident protein VAP to promote the formation of ER–PM contact sites on the proximal somatodendritic region of GABAergic interneurons (Figure 3). Although the sequences of C/D-boxes do not conform to canonical FFAT VAP–binding sites, the proNRG2 D-box contains acidic residues required for VAP binding and the C-box harbors a cryptic, phosphorylation-dependent VAP binding site. Importantly, NMDAR activation stimulates C-box Ser/Thr dephosphorylation, dissociation of proNRG2 from VAP and dispersion of NRG2 from ER–PM junctions (Figure 3). Based on these findings, we hypothesize that Kv2.1 channels and bidirectional NRG2/ErbB4 autocrine signaling function synergistically as a homeostatic mechanism both to protect fast-spiking parvalbumin-positive interneurons from excitotoxic damage and to regulate the activity of GABAergic interneurons, which affect E-I balance and neuronal network activity associated with psychiatric-relevant behaviors disrupted in NRG2 and ErbB4 knockout mice [Reference 2].

Developmental, neurochemical, and behavioral analyses of ErbB4 Cyt-1 knockout mice

ErbB4 receptor transcripts are alternatively spliced to generate isoforms that either include (Cyt-1) or exclude (Cyt-2) exon 26, an exon that encodes a cytoplasmic domain that invests ErbB4 receptors with the ability to signal via the PI3K/Akt pathway rather than the MAPK pathway. To investigate the effects of germline (constitutive) and conditional (acute) deletions of the Cyt-1 exon, we generated and studied ErbB4–floxed (ErbB4-Cyt1fl/fl) mice because ErbB4 Cyt-1/2 isoforms had been only studied in cultured cells, and clinical
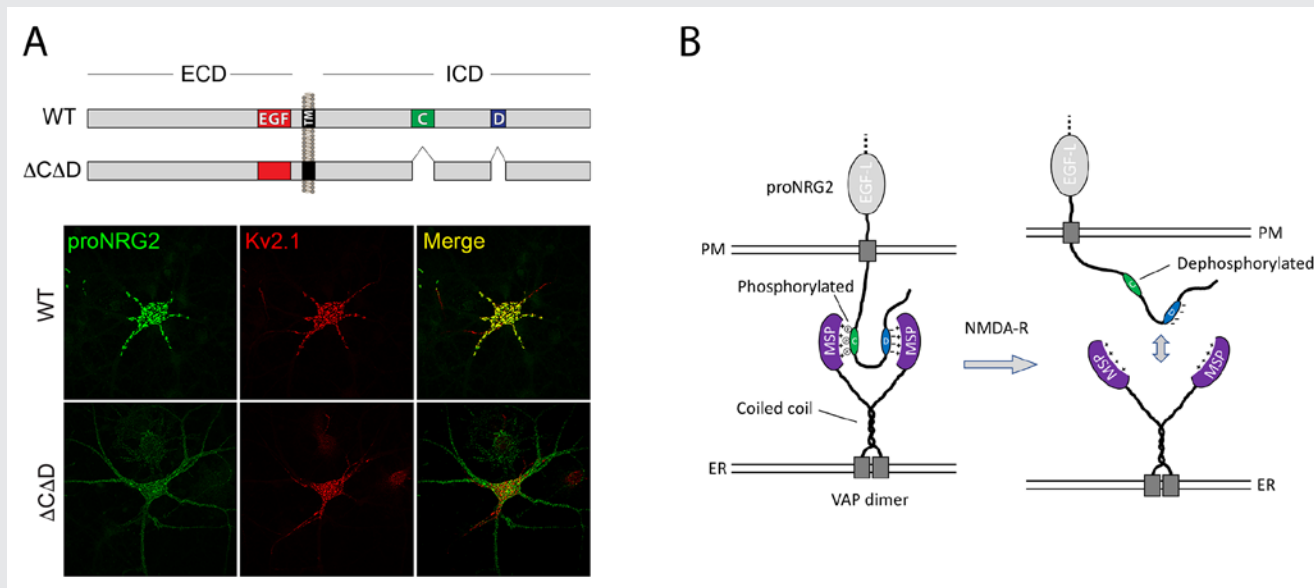
FIGURE 3. ProNRG2 clustering at ER–PM junctions is mediated by VAP binding and regulated by phosphorylation.

A. Cultured hippocampal neurons transduced with AAVs expressing wild-type (WT) proNRG2 (*top*) or proNRG2DCDD lacking the C- and D-boxes (*bottom*). WT NRG2 (*green*) forms large clusters at ER–PM junctions (revealed by Kv2.1 staining in *red*), which are located on the cell body and proximal dendrites. Removal of the VAP binding sites in proNRG2DCDD abolishes its accumulation at ER–PM junctions, causing it to broadly distribute throughout the neuronal plasma membrane.

B. Working model of proNRG2/VAP interactions. ProNRG2 cooperatively engages with a VAP dimer via its two low-affinity FFAT motifs in the C- and D-boxes. Interactions between the VAP major sperm protein (MSP) domain and the cryptic FFAT site in the C-box require Ser/Thr phosphorylation. Their dephosphorylation downstream of NMDAR activation promotes proNRG2 dissociation from VAP.

studies implicated ErbB4 Cyt-1 variants as schizophrenia risk factors. We found Cyt-1 knockouts (KOs) only encode ErbB4 Cyt-2 variants, but in contrast to ErbB4 null mice, GABAergic interneuron migration and number are unaltered in Cyt-1 KOs. Interestingly, basal extracellular dopamine (DA) levels are augmented in the medial prefrontal cortex of Cyt-1 mice, but they don't manifest the behavioral abnormalities we observed in mice lacking ErbB4 in DA neurons. To address the possibility that Cyt-2 variants compensate for the lack of Cyt-1 during development, we microinjected AAV (adeno-associated virus)–Cre into the DA–rich VTA (ventral tegmental area) of adult ErbB4-Cyt1fl/fl mice to acutely target exon 26. These conditional Cyt-1 KOs were found to exhibit behavioral abnormalities in the elevated plus maze and startle response, consistent with the idea that late exon 26 ablations may circumvent compensation by Cyt-2 variants. Our findings suggest that ErbB4 Cyt-1 function *in vivo* is important for modulating DA levels and for regulating behaviors in adult mice [References 3,4].

Pathway–specific contribution of parvalbumin interneuron NMDARs to synaptic currents and thalamocortical feedforward inhibition

The PFC is a site of convergence of long-range excitatory glutamatergic inputs, which integrates multiple

FIGURE 4. Pathway-specific contribution of PV+ interneuron NMDARs to synaptic currents and thalamocortical feedforward inhibition

Inhibitory fast-spiking GABAergic PV+ interneurons (*red*) in adult mouse PFC receive excitatory glutamatergic inputs from the contralateral PFC (cPFC; *orange*) and the ipsilateral thalamus (*green*). Optogenetic stimulation of either cPFC or thalamocortical inputs onto PV+ interneurons indicate that NMDARs contribute to the size and shape of excitatory synaptic currents at thalamic more than at cPFC synapses. By selectively knocking out NMDAR expression in adult inhibitory PV+ interneurons, we found that expression of these receptors at thalamic synapses of PFC PV+ interneurons are required for feedforward inhibition of pyramidal cells (PC).
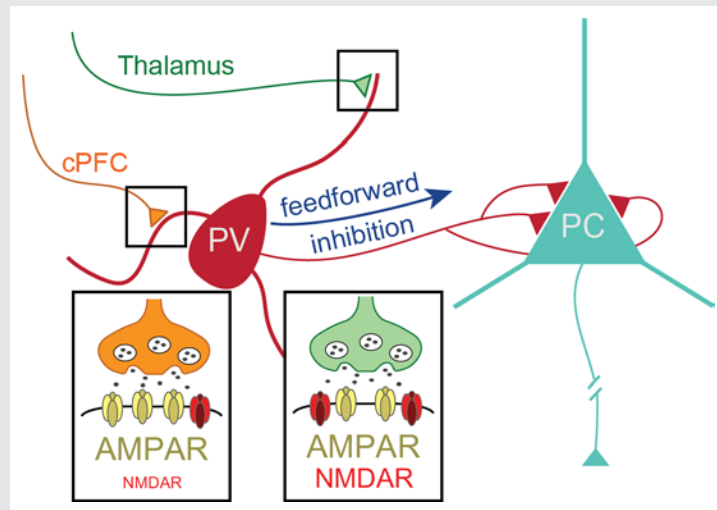
modalities of information to produce goal-directed behaviors, and drugs selectively targeting NMDA–type glutamate receptors alter PFC function and elicit numerous deficits associated with psychiatric disorders. Inhibitory GABAergic fast-spiking PV+ interneurons are uniquely suited to coordinate the firing of pyramidal neurons in response to these converging excitatory inputs, and to induce gamma oscillations in cortical networks that modulate behaviors and may be disrupted in several psychiatric disorders. Despite the importance of understanding how glutamatergic inputs onto PV+ interneurons affect network activity, behavior, and disease, when we began this project, there continued to be controversy as to whether both AMPA– and NMDA–type glutamate receptors or only AMPARs contribute to excitatory drive. We used a combination of molecular, electrophysiological, and optogenetic approaches, in combination with selective gene targeting techniques in PV+ interneurons, to resolve this long-standing controversy. We found that nearly 100% of PV+ interneurons in the adult medial PFC express transcripts encoding GluN1 and GluN2B, which assemble to form functional NMDARs. Importantly, by using selective optogenetic stimulation of corticocortical vs. thalamocortical inputs onto PV+ interneurons, we found that the relative synaptic NMDAR contribution to excitatory post-synaptic currents is pathway-specific. NMDARs contribute more at thalamocortical, rather than at corticocortical, synapses. We then demonstrated that PV+ interneuron NMDAR currents contribute to thalamus-mediated feedforward inhibition in PFC circuits (Figure 4). These findings, demonstrating a molecular and circuit-based mechanisms for cognitive impairment under conditions of reduced NMDAR function, represent an important conceptual advance, with implications for understanding the pathogenesis of psychiatric disorders [Reference 5].

Additional Funding

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Section on Molecular Neurobiology members who contributed to the work described in this Annual Report.

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Publications

1. Ahmad T, Vullhorst D, Chaudhuri R, Guardia CM, Chaudhary N, Karavanova I, Bonifacino JS, Buonanno A. Transcytosis and trans-synaptic retention by postsynaptic ErbB4 underlie axonal accumulation of NRG3. *J Cell Biol* 2022 221:e202110167.
2. Vullhorst D, Bloom MS, Akella N, Buonanno A. ER-PM junctions on GABAergic interneurons are organized by neuregulin 2/VAP interactions and regulated by NMDA receptors. *Int J Mol Sci* 2023 24(3):2908.
3. Erben L, Welday JP, Cronin ME, Murphy R, Skirzewski M, Vullhorst D, Carroll SL, Buonanno A. Developmental, neurochemical, and behavioral analyses of ErbB4 Cyt-1 knockout mice. *J Neurochem* 2022 161:435–452.
4. Erben L, Welday JP, Murphy R, Buonanno A. Toxic and phenotypic effects of AAV_Cre used to transduce mesencephalic dopaminergic neurons. *Int J Mol Sci* 2022 23(16):9462.
5. Lewis EM, Spence HE, Akella N, Buonanno A. Pathway-specific contribution of parvalbumin interneuron NMDARs to synaptic currents and thalamocortical feedforward inhibition. *Mol Psychiatry* 2022 27:5124–5134.

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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 neurodevelopmental 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.

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 study how

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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 for probing how gene mutations linked to neurodevelopmental disorders disrupt sensory processing. NMDA–receptor mutations have been linked to both autism-spectrum disorders and schizophrenia, and, accordingly, we demonstrated that zebrafish NMDA–receptor subunit mutants have both structural brain deficits and disrupted prepulse inhibition. Recently, we also identified neurons that mediate prepulse inhibition at intervals of less than 100 ms and confirmed previous reports that a GABAergic mechanism is involved. In ongoing experiments, we are now defining how such neurons suppress startle reflexes by examining their synaptic connections with, and molecular signaling to, central neurons that initiate startle responses. We are also defining connectivity from the auditory sensory ganglion in zebrafish (the statoacoustic ganglion), in order to elucidate pathways that transmit behaviorally relevant vibration and acceleration signals to central regions that process sensory information for startle behavior, balance, and eye movements (Figure 1).

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. We have

FIGURE 1. Morphological reconstruction of auditory sensory neurons in larval zebrafish

After sparse labeling, we traced more than 150 statoacoustic ganglion neurons to correlate patterns of connectivity with hair-cell patches to central projection targets. In this image, neurons are colored according to peripheral target. Central projections of neurons that share a peripheral target remain closely associated.
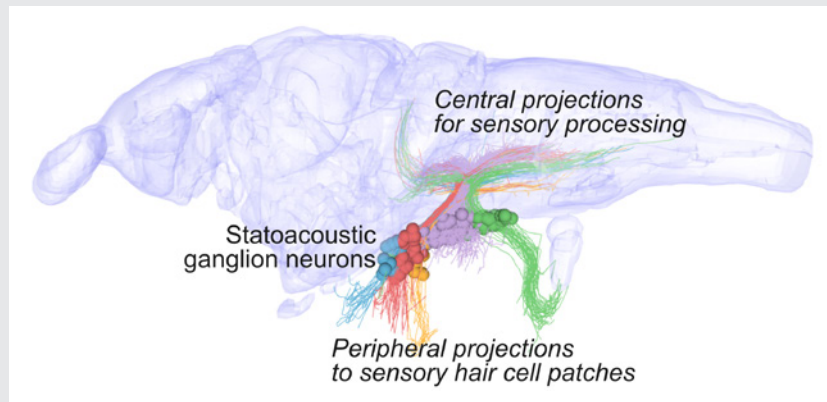
developed several paradigms in which the behavioral state of zebrafish is temporarily altered. Our strategy is to allow larvae to remain in the arena until their motor behavior achieves a stable state, then introduce a temporary perturbation to the environment and examine changes in behavior that persist on a time-scale of several minutes after the perturbation is removed (Figure 2).

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. 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 of Purkinje neurons in mammals also directly project to the parabrachial nucleus. The study identified, for the first time, a cellular pathway that mediates tonic immobility and suggests that the parabrachial complex has a deep evolutionary history in mediating defensive behavior [Reference 1].

A second paradigm that we used to study behavioral state control is flow-induced arousal. After exposure to a brief water flow stimulus, zebrafish larvae show elevated motor activity and sensory responsiveness that persists for several minutes after termination of water movement [Yokogawa T, Hannan MC, Burgess HA, *J Neurosci* 2012;32:15205]. We showed that the serotonergic raphe nucleus regulates sensory responsiveness during this state, but the neural basis for hyperactivity was not known. Recently, we screened Gal4 lines to identify neurons that are required for increased motor activity during flow-induced arousal, and assessed effects of pharmacological manipulations on this behavior. We are currently zeroing in on relevant neurons by selectively laser-ablating neurons in a transgenic line that labels neurons required for flow-induced arousal.

FIGURE 2. Paradigms for studying behavioral state control in zebrafish larvae

To study behavioral-state control, we developed behavioral paradigms in which motor activity shows a persistent change over a timescale of minutes following a perturbation to the environment. In tonic immobility (*left*), an intense vibration stimulus induced a reduction in movement that lasts for around a minute after the stimulus ceases. In flow-induced arousal (*right*), exposure to water flow induces elevated locomotor activity that continues for several minutes, even after water movement ceases.
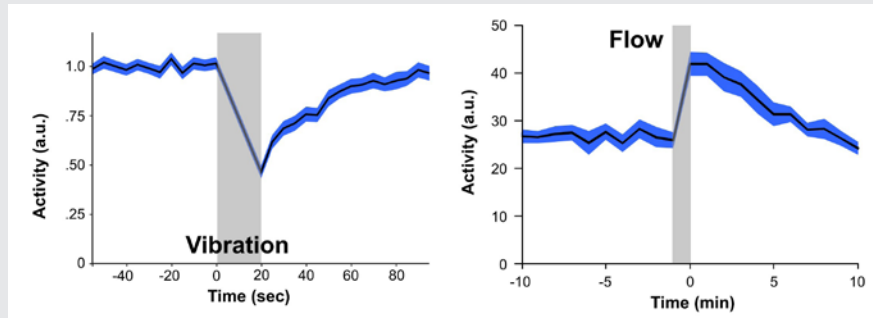
Zebrafish models of neurodevelopmental disorders

We collaborate widely with clinicians to generate and characterize zebrafish models for mutations discovered in humans (often through exome-sequencing) that are likely to have a neurodevelopmental origin. We use the CRISPR/Cas9 system to generate lesions in zebrafish genes that are homologous to those disrupted in the human disorders. We then apply behavioral analysis, transcriptomics, and voxel-based morphometry as part of a broad phenotyping strategy. To promote rigorous use of zebrafish neurological disease models, we wrote a critical review outlining advantages and limitations of the zebrafish system [References 2, 3].

Our work with brain morphometry arose from earlier studies in which we generated several hundred new Gal4 and Cre lines in order to provide genetic accessibility to neurons of interest. A unique feature of brain imaging in zebrafish is the ability to visualize the total architecture of the brain while simultaneously recording the position and morphology of every constituent labeled neuron. To make these transgenic lines 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. Then, in order to build a brain atlas, we optimized a protocol that permits highly precise brain registration [Reference 4].

We showed that such high precision of alignment permits statistically robust whole-brain analysis of neuronal composition and morphology in zebrafish mutant models, pinpointing brain regions with changes that are difficult to detect visually. The technique can be applied to almost any zebrafish neurodevelopmental model, thereby enabling robust and quantitative detection of subtle changes in brain structure or composition. We used this 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 a variety of neurodevelopmental disorders, including autism and intellectual disability. Through this work, we aim to provide insight into the fundamental molecular and cellular processes associated with each disorder.

Publications

1. Bhandiwad AA, Chu NC, Semenova SA, Holmes GA, Burgess HA. A cerebellar-prepontine circuit for tonic immobility triggered by an inescapable threat. *Sci Adv* 2022 8(39):eabo0549.
2. Burgess HA, Burton EA. A critical review of zebrafish neurological disease models – 1. The premise: neuroanatomical, cellular, and genetic homology, and experimental tractability. *Oxf Open Neurosci* 2023 kvac018.
3. Burton EA, Burgess HA. A critical review of zebrafish neurological disease models – 2. Application: functional and neuroanatomical phenotyping strategies and chemical screens. *Oxf Open Neurosci* 2023 kvac019.
4. Bhandiwad AA, Gupta T, Subedi A, Heigh V, Holmes GA, Burgess HA. Brain imaging and registration in larval zebrafish. *Methods Mol Biol* 2024 2707:141–153.

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Cell Fusion Stages in 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 better understanding of important fusion reactions to 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 intercellular fusion. Our general strategy is to combine in-depth analysis of the best characterized fusion reactions with comparative analysis of diverse less explored fusion reactions, which can reveal new kinds of fusion proteins and clarify the generality of emerging mechanistic insights. In our recent studies, we explored the mechanisms of osteoclast fusion in bone remodeling in *in vitro* models and in our new inducible explant model for dissecting osteoclast fusion and osteoclast-osteoblast coordination in fibrous dysplasia.

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Cell surface−bound La protein regulates the cell fusion stage of osteoclastogenesis.

Bone-resorbing osteoclasts are responsible for essential, life-long skeletal remodeling, and their dysfunction is a major contributor to bone diseases, affecting over 200 million individuals worldwide, including osteoporosis, fibrous dysplasia (FD), Paget's disease, and osteopetrosis. Multinucleated osteoclasts are formed by the successive fusion of mononucleated precursor cells, where each fusion event raises the bone-resorbing activity of osteoclasts. Despite the fundamental role of cell-cell fusion in osteoclast formation, the mechanisms of this fusion process in normal physiology and in disease remain to be fully understood. In our recent study [Reference 1], we discovered that osteoclastogenesis involves the lupus La protein (*SSB* gene product). La, also referred to as LARP3 or La autoantigen, is generally recognized as an abundant and ubiquitous, mostly nuclear

FIGURE 1. Cell-cell fusion stage in the formation of multinucleated osteoclasts is regulated by cells-surface phosphatidylserine (PS), PS–bound annexin A5 (Anx A5), and Anx A5–bound La protein [Reference 1].

Monocyte-to-osteoclast differentiation starts with a drastic reduction in the levels of the nuclear RNA chaperone La protein (shown as a green rectangular with C-terminal nuclear localization sequence [NLS] in red). As fusion begins, La reappears as a low molecular-weight species lacking NLS at the osteoclast surface, where it promotes fusion in a mechanism dependent on direct interactions between La and the PS–binding protein Anx A5, which anchors La to transiently exposed PS at the surface of fusing osteoclasts. When osteoclasts reach sizes (about 5–10 nuclei/cell) characteristic of mature multinucleated osteoclasts, the loss of cell-surface La and the reappearance of full-length La in the nuclei of mature osteoclasts are linked to cessation of cell fusion. We propose that coordinated changes in the surface levels of PS, Anx A5, and La in the differentiating osteoclasts act as on and off switches of their fusion activity.

RNA–binding protein. The best characterized function of nuclear La is to protect precursor tRNAs from exonuclease digestion through specific interactions between La's highly conserved, N-terminal La domain and the 3' ends of tRNA.

We found that the La protein has an additional function as an osteoclast fusion regulator. The differentiation of murine and human monocytes into multinucleated osteoclasts is accompanied by and depends on tightly choreographed changes in the steady-state level, post-translational modification, and cellular localization of La. In fusing osteoclasts, La is found at the surface of the cells, and the cell-surface associated La, rather than intracellular La, regulates osteoclast fusion. The changes in the expression of La and the functional role of its cell-surface form in osteoclastogenesis are unexpected in the context of the vast literature covering La's role in RNA biology. La is generally thought of as an abundant, ubiquitous, mostly phosphorylated RNA–binding protein largely confined to the nucleus in virtually all eukaryotic cell types. However, at the onset of osteoclastogenesis, M-CSF (macrophage colony stimulating factor)–derived precursors show a dramatic loss of La protein. In the following RANKL–induced (RANKL is a protein that controls bone resorption, regeneration, and modeling) stages of osteoclastogenesis, La reappears as a non-phosphorylated, proteolytically cleaved species in the cytoplasm and at the surface of the fusing osteoclast precursors. When the growth of osteoclasts slows, in the late stages of fusion, La is observed at its conventional molecular weight and nuclear localization. The rate of formation, the sizes of multinucleated syncytia, and the subsequent bone resorption activity of osteoclasts are regulated by cell-surface La protein. In fact, cell-surface La regulates osteoclast

functions by modulating the membrane fusion stage of osteoclast formation, not upstream differentiation processes. Lowering the amount of La by suppressing the steady-state level of its transcript, blocking its proteolytic processing, or inhibiting its activity with antibodies inhibits fusion. Conversely, increasing La's steady-state concentration by either overexpression or application of recombinant protein promotes fusion. In addition, the addition of α-La antibodies or recombinant La at the surface of osteoclasts inhibits and promotes synchronized osteoclast fusion, respectively. Importantly, the upregulation of cell-surface La and its involvement in osteoclast fusion have been observed for both primary human and murine osteoclasts, suggesting that La's role in regulating fusion is conserved in mammals.

The mechanisms by which cell-surface La regulates osteoclast fusion remain to be clarified. Given that La, on its own, initiates neither hemifusion nor fusion between bound membranes, it is unlikely that La directly catalyzes and/or drives membrane fusion. More likely, La recruits or stimulates other components of the osteoclast fusion complex. Our findings highlight La's association with the fusion regulator phosphatidylserine (PS)–binding protein Annexin A5 (Anx A5). Anx A5 has been implicated in several cell-cell fusion processes. In the case of osteoclast fusion, osteoclastogenic differentiation of human monocytes is associated with a strong increase in the amount of Anx A5 at the cell surface, and treatments suppressing the expression and activity of cell-surface Anx A5 inhibit synchronized osteoclast fusion. We found that recombinant La and Anx A5 directly interact, and that Anx A5 enriches La on membranes containing PS in a Ca^{2+}-dependent manner. These observations: (1) explain how La, a soluble protein, associates with cell membranes; (2) connect La function in osteoclastogenesis with the non-apoptotic PS exposure signaling pathway that is thought to trigger osteoclast fusion; (3) and, in combination with the previously reported dependence of osteoclast fusion on cell-surface PS and Anx A5, shed additional light on how osteoclasts employ PS to initiate the assembly of a fusion complex between committed precursors.

In summary, our work demonstrates that La, a key protein in the RNA biology of eukaryotic cells, lives a second life at the surface of osteoclasts, where it moonlights as a master regulator of osteoclast membrane fusion (Figure 1). We suggest that, in this new, highly specific role on the surface of fusing osteoclasts, La may present a promising target for treatment of bone diseases stemming from perturbed bone turnover. Considering that mononucleated osteoclasts do resorb bones, blocking La–dependent osteoclast fusion is expected to have more subtle and selective effects on bone resorption than blocking the upstream formation of osteoclast precursors with α-RANKL antibodies. Cell-surface La is accessible for cell-impermeable drugs. Furthermore, the only known function of cell-surface La is its newly identified role in regulating osteoclast fusion. Surface La's specificity may minimize off-target effects. The future development of safe and effective reagents targeting cell-surface bound La may lead to novel antiresorptive therapies for osteoporosis, mechanistically orthogonal to the existing approaches.

An inducible explant model of osteoclast–osteoprogenitor coordination in exacerbated osteoclastogenesis

Precise coordination of resorption of the bones by osteoclasts (OCs) and formation of the bones by osteoblasts (OBs) is critically important for maintaining our skeleton throughout our lifespan. Disruption of the coordination of OC and OB functions leads to skeletal diseases. Given that the paucity of simple, activatable, biologically relevant models of osteoclast-osteoblast coordination has hindered our understanding of the osteoblast-osteoclast crosstalk, we developed an inducible *ex vivo* model of osteoclast-osteoblast progenitor coordination [Reference 2]. Our experimental system utilizes a conditional, tetracycline-inducible mouse model of fibrous

FIGURE 2. *Ex vivo* FD marrow explants as an *ex vivo* model of osteoclast formation and osteoclast-osteoprogenitor coordination [Reference 2]

Doxycycline induction elicits osteoprogenitor expression of $Gα_s^{R201C}$ and release of the receptor activator of NF-κB ligand (RANKL). RANKL binding initiates osteoclastogenesis and the formation of multinucleated, bone-resorbing osteoclasts. Osteoclasts release extracellular vesicles (OC EVs) that correlate with pre-osteoblast proliferation. Peach cells are of the monocyte-to-osteoclast lineage; blue cells are of the skeletal stem-cell-to-osteoblast lineage.
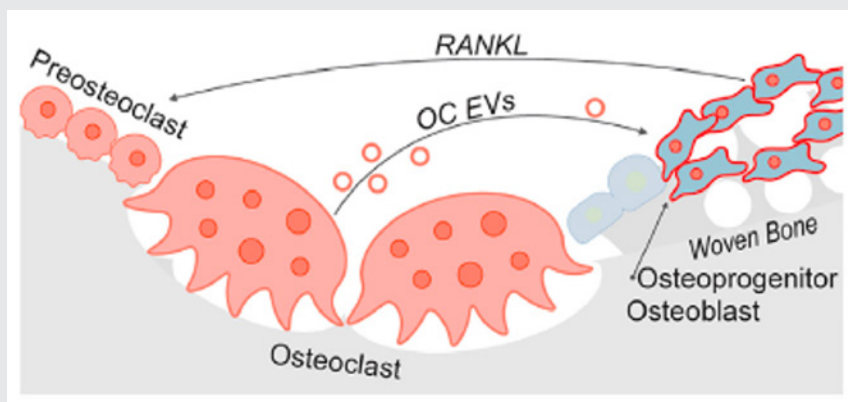
dysplasia (FD). Induction of the $Gα_s^{R201C}$ mutation in osteoprogenitors by treating the cells in bone marrow explants with doxycycline activates the release of osteoclastogenic factors from osteoprogenitors, which, in a RANKL (receptor activator of NF-kB ligand)–dependent manner, elicits the differentiation and fusion of neighboring preosteoclasts. In turn, multinucleated osteoclasts promote osteoprogenitor proliferation by releasing soluble coupling factors and RANKL–positive extracellular vesicles (Figure 2). Our model condenses to days the time course of excessive osteoclast formation typical of FD and many other bone diseases and thus facilitates exploring the mechanism of underlying molecular mechanisms. Although the cellular make-up of the explant cultures described and the biological activities represented are complex, employing this model is relatively simple. The training required to master the required dissections is minimal, we observe high phenotypic reproducibility between cultures and for at least four passages within individual cultures, and the cost in maintaining and implementing this model of osteoclast/osteoprogenitor biology is modest, as the only media additive required for activation is doxycycline. We expect this model to expedite the investigation of cell-cell fusion, osteoclast-osteoblast progenitor coordination, and extracellular vesicle signaling during bone remodeling and to offer a powerful tool for evaluating signaling cascades and novel therapeutic interventions in osteoclast-linked skeletal disease.

Additional Funding

Publications

1. Whitlock JM, Leikina E, Melikov K, de Castro, LF, Mattijssen S, Maraia RJ, Collins MT, Chernomordik LV. Cell surface-bound La protein regulates the cell fusion stage of osteoclastogenesis. *Nat Commun* 2023 14:616.
2. Whitlock JM, de Castro, LF, Collins MT, Chernomordik LV, Boyce AM. An inducible explant model of osteoclast-osteoprogenitor coordination in exacerbated osteoclastogenesis. *iScience* 2023 26:106470.

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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 (*Danio rerio*) 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 primordium'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 influence the number and size of epithelial rosettes in the migrating zebrafish posterior Lateral Line primordium.

Protoneuromasts are formed within the migrating primordium, starting from its trailing end, as clusters of cells apically constrict and form epithelial rosettes. Their formation is promoted by fibroblast growth factor (Fgf)–signaling centers, which form periodically in the wake of a shrinking Wnt–active domain that inhibits epithelial rosette formation and progressively shrinks toward the leading end of the primordium (Wnt and Fgf pathways are signaling pathways).

However, the precise number and size of epithelial rosettes is not strictly dependent on a pre-pattern of Fgf–signaling activity, as it is broadly influenced by the balance of mechanical interactions that promote or oppose formation of epithelial rosettes. When chemokine-dependent migration of leading cells is compromised, the resulting slowing of the primordium is accompanied by the fusion of epithelial 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 mechanics-based model, in which local interactions associated with apical constriction and cell adhesion promote aggregation, while tension along the length of the primordium, influenced by the relative efficacy of leading and trailing cell migration, opposes such aggregation. We described the development of a computational Cellular Potts model, which allowed us to explore how the relative speed of leading versus trailing cells, as well as changes in cell adhesion and mechanical coupling, differentially regulated by Wnt and Fgf signaling, can influence the pattern of neuromast formation and deposition by the migrating primordium. Our studies illustrate how signaling and mechanics cooperate to coordinate self-organization of morphogenesis in the migrating primordium.

Sox2 helps stabilize neuromasts prior to deposition, defining a final key step in the self-organization of neuromast formation in the migrating posterior Lateral Line primordium.

Neuromasts, the mechanosensory organs of the Lateral Line system are periodically formed and deposited by the migrating pLLp from its trailing end, as it migrates under the skin from the near the ear to the tip of the tail, to spearhead formation of the posterior Lateral line system in zebrafish. Understanding how they are formed and deposited in a robust and reproducible way serves as a model for understanding more broadly the steps that determine the reproducible self-organization of organ systems during development.

The pLLp forms as a group of about 140 cells separates from an epithelial placode located behind the ear. Prior to the initiation of migration, cells in the primordium are characterized by broad, relatively uniform Wnt activity. However, as the primordium begins to migrate caudally toward a source of chemokine signals secreted by the horizontal myoseptum, Wnt activity becomes polarized such that it is highest at the leading end of the primordium and lowest at its trailing end. Such polarization provides the context for subsequent patterning events within the primordium that coordinate both polarized migration of the primordium along a relatively uniform stripe of chemokine expression and for the periodic formation and deposition of neuromasts.

Protoneuromast formation is initiated in Fgf signaling domains that are periodically established in a trailing zone of the migrating primordium in response to Fgfs produced by Wnt active cells in a leading zone. Local promotion of Wnt activity, coupled with longer range inhibition of Wnt by Fgf–dependent Dkk1b (a protein that inhibits Wnt) expression, is thought to constitute a reaction diffusion system that determines self-organization of Fgf signaling centers, which initiate formation of nascent protoneuromasts. As previously formed protoneuromasts at the trailing end mature, they produce Wnt–signaling inhibiting factors, which contribute to progressive shrinking of the Wnt system. Progressive restriction of an initially broad Wnt signaling domain to a smaller leading zone allows new Fgf signaling–dependent protoneuromasts to form in the wake of the shrinking Wnt system.

While initial studies focused on Dkk1b as the primary inhibitor of Wnt signaling produced by newly formed protoneuromasts, a question arose about what inhibits Wnt signaling in the most mature protoneuromasts at the trailing end of the primordium, where *dkk1b* is not expressed. We showed that Sox2 (a transcription

factor that maintains pluripotency and neurogenesis) is expressed in nascent and maturing protoneuromasts in a pattern that is complementary to domains with Wnt signaling activity. Furthermore, Sox2 functions in a partially redundant manner with Sox1a and Sox3 to inhibit Wnt signaling. This helps keep Wnt activity restricted to a progressively smaller leading zone, which we show is essential for effective maturation of trailing protoneuromasts and the timely deposition of stable neuromasts.

Fgfs secreted by Wnt–active cells in a leading zone determine Fgf signaling activity in an adjacent trailing zone, which initiates protoneuromast formation by promoting expression of factors that promote epithelialization of cells and their apical constriction to form epithelial rosettes. Furthermore, Fgf signaling promotes expression of the transcription factor *atoh1a*, which gives cells the potential to become sensory hair cell progenitors, while lateral inhibition mediated by Notch (promotes proliferative signaling during neurogenesis) signaling ensures center-biased *atoh1a* expression, and sensory hair cell progenitor fate becomes restricted to a central cell in the nascent protoneuromast. However, as the protoneuromast matures in a trailing zone of the primordium, it becomes increasingly separated from leading Wnt–active cells, which are the source of Fgfs that initiated its formation. As a result, effective maturation requires a switch to a new regulatory network that ensures sustained Fgf activity in the maturing protoneuromast, independent of the Fgfs from the leading Wnt active zone that initiated its formation. This is achieved by the central Atoh1a–expressing sensory hair cell progenitor, which becomes a new source of Fgfs that sustains Fgf signaling in surrounding protoneuromast cells. In addition, the central Atoh1a–expressing cell determines expression of Notch ligands such as DeltaD, which activate Notch in neighboring cells to promote expression of factors that ensure stabilization of epithelial rosette morphology. Importantly, however, as the Atoh1a–expressing cell in the maturing proneuromast becomes separated from leading Wnt–active cells, which are the source of Fgfs that initiate *atoh1a* expression, Atoh1a promotes *atoh1b* expression and sustains its own expression through autoregulation.

Our analysis of Sox2 function has now shown that this critical switch in the genetic regulatory network, where Atoh1a expression becomes self-sustaining through autoregulation with *atoh1b*, only occurs when Wnt activity is adequately suppressed in trailing cells by Sox2. Failure to adequately suppress and restrict Wnt activity to a leading zone prevents timely initiation of *atoh1b* expression in trailing protoneuromasts and failure to deposit mature neuromasts with stable epithelial rosettes. Together with past observations, our analysis of Sox2 function now defines three key steps in the periodic self-organization of neuromasts in the primordium: first, a step that polarizes Wnt activity in the primordium; second, a pattern-forming step that generates periodic Fgf–signaling centers in the context of polarized Wnt activity; and third, a step involving Sox2, which helps stabilize fate and morphology in neuromasts formed in the earlier pattern forming stage.

Although the steps outlined above are specific to the way the primordium develops, they define more broadly the sequence of events in the self-organization of development in many different contexts. A symmetry-breaking event that polarizes a tissue is a common first step in most developmental processes, which then provides the context for self-organizing mechanisms that determine patterning of cell fate, morphology, and cell migration within the tissue. Although self-organizing mechanisms could generate pattern spontaneously, independently of tissue polarization, their operation in the context of prior polarization ensures a more predictable reproducible outcome. Furthermore, while a chemical signaling–based reaction diffusion system has been described above as an example of a pattern forming system, pattern formation can operate at many scales with different modalities. For example, our previous studies showed that the number and size of rosettes in the primordium can also be understood to be an outcome of mechanical interactions between cells

with periodic aggregates forming as a consequence of a balance of forces, such as apical constriction, that pull cells together to form rosettes, while tension along the length of the primordium, associated with collective cell migration, pulls cells apart, opposing formation of rosettes. These modalities operate in parallel, interact, and typically work synergistically to determine robust reproducible outcomes. Patterns initiated by such self-organizing mechanisms are not necessarily stable and, as seen in the example of the lateral line primordium, are followed by changes in regulation that help consolidate and stabilize form and fate established in an earlier patterning phase.

Wnt–dependent BMP expression inhibits rosette formation at the leading end of the primordium.

The zebrafish pLLp migrates from the ear to the tip of the tail, periodically forming and depositing neuromasts. Protoneuromasts form as clusters of primordium cells, epithelialize, apically constrict, and form rosettes. Formation of epithelial rosettes is promoted by periodic formation of Fgf–signaling centers, starting from the trailing end of the primordium. Fgf signaling promotes rosette formation by promoting apical basal polarization of cells and by determining expression of factors such as the protein Shroom3 that promote apical constriction. Conversely, Wnt signaling, which dominates in the leading part of the primordium, inhibits rosette formation, in part by promoting expression of factors such as Dusp6 and Sef1, which prevent Wnt–active cells from responding effectively to Fgf. However, the observation that the growth factors Bmp 2b, 4, and 5 are expressed in the leading part of the primordium under the influence of Wnt signaling suggested that Bmp signaling, previously known to promote EMT (epithelial–mesenchymal transition), may contribute to maintenance of the quasi-mesenchymal morphology of leading cells and to inhibition of rosette formation.

Consistent with a role for Bmps in inhibiting the formation of rosettes, exposing the embryos to a BMP inhibitor, K02288, allowed cells in the leading zone to aberrantly form epithelial rosettes. However, although K02288 was characterized as a potent inhibitor of the type I Bmp receptor Alk2, aberrant rosette formation in leading cells was not observed in an Alk2 mutant, or following knockdown of Bmp5, the Bmp expressed in the primordium that was thought to signal via the Alk2. We reported that exposure to DMH2, another Bmp inhibitor with greater specificity for Alk3 and Alk6, reproduces the aberrant rosette formation originally seen with K02288, which has a limited ability to inhibit these Type I receptors as well. This observation suggested that inhibition of rosette formation in the leading zone was not determined by Bmp5 but instead by Bmp2b and Bmp4, also expressed in the primordium and known to preferentially signal via Alk3 and Alk6. Consistent with this interpretation, knockdown of Alk6 also allowed rosettes to form aberrantly in the leading zone. Conversely, induced ectopic expression of Bmp2b inhibited rosette formation, resulting in a broader leading zone without rosettes in the migrating primordium.

Inhibition of Bmp signaling with K02288 has no obvious effect on the pattern of Wnt and Fgf signaling in the primordium, suggesting that Bmp normally operates downstream of these signaling pathways to inhibit rosette formation in the leading zone. Despite the absence of any change in Fgf signaling, we found that inhibiting Bmp signaling with K02288 allows *shroom3*, previously thought to be regulated by Fgf signaling, to be aberrantly expressed closer to the leading end of the primordium. The mechanism by which Bmp signaling normally inhibits *shroom3* expression, and possibly other factors that promote rosette formation in the leading domain, downstream of Fgf signaling, in now under investigation. Together, these observations suggest that Wnt–dependent BMP expression inhibits rosette formation at the leading end of the primordium.

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Molecular Genetics of Heritable Human Disorders

We conduct research to delineate the pathophysiology of and develop novel therapies for 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, to translocate G6P from the cytoplasm into the ER lumen. To function, G6Pase-α couples with G6PT to form a 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 the long-term complications of hepatocellular adenoma/carcinoma (HCA/HCC) and renal disease. There is no cure for either GSD-Ia or GSD-Ib. The current dietary therapies have enabled GSD-I patients to maintain a normalized metabolic phenotype if strictly adhered to. However, the underlying pathological processes remain uncorrected, and HCA/HCC and renal disease still occur in metabolically compensated GSD-I patients. We generated animal models of GSD-Ia and GSD-Ib, which are being exploited to both delineate the disease more precisely and to develop new treatment approaches, including gene therapy. We also generated G6PC– and G6PT–expressing recombinant adeno-associated virus (rAAV) vectors and showed that rAAV vector–mediated gene argumentation therapies for GSD-Ia and GSD-Ib are safe and efficacious. Our rAAV–G6PC vector (US patent #9,644,216) technology was licensed to Ultragenyx Pharmaceutical Inc (Novato, CA), who launched a phase I/II clinical trial (NCT03517085) in 2018, now in phase III (NCT05139316). To explore alternative genetic technologies for GSD-I therapies, we have established formal collaborations under the CRADA (cooperative research and development agreement) 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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FIGURE 1. CRISPR/Cas9–based double-strand oligonucleotide (dsODN) insertion strategy corrects metabolic abnormalities in murine glycogen storage disease type Ia.

Molecular mechanism underlying hepatic autophagy impairment in GSD–Ib

Clinically, GSD-Ib patients manifest a metabolic phenotype of impaired blood glucose homeostasis and a long-term risk of hepatocellular adenoma/carcinoma (HCA/HCC). The etiology of HCA/HCC in GSD-Ib is unknown. Studies have shown that deficiency in autophagy, 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 stresses contributes 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.

To understand the pathways contributing to hepatocarcinogenesis in GSD-Ib, we hypothesized that impaired hepatic autophagy is a significant contributor. In this study, we showed that G6PT deficiency leads to impaired hepatic autophagy evident from attenuated expression of many components of the autophagy network, decreased 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 the active p-AMPK. Importantly, we showed that overexpression of either SIRT1 or LKB1 in the G6PT–deficient liver restored autophagy and SIRT1/FoxO3a and LKB1/AMPK signaling. The hepatosteatosis in the G6PT–deficient liver lowered SIRT1 expression. LKB1 overexpression reduced hepatic triglyceride levels, providing a potential link between LKB1/AMPK signaling upregulation and the increase in SIRT1 expression. In conclusion, downregulation of SIRT1/FoxO3a and LKB1/AMPK signaling underlies impaired hepatic autophagy, which may contribute to HCA/HCC development in GSD-Ib. Understanding this mechanism may guide future therapies [Reference 1].
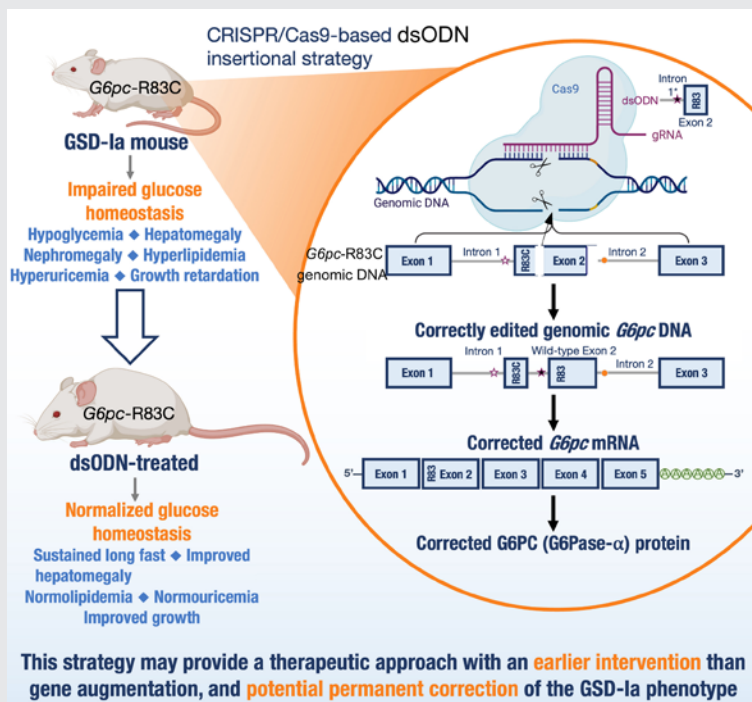
CRISPR/Cas9−based double strand oligonucleotide insertion strategy corrects metabolic abnormalities in murine glycogen storage disease type Ia.

GSD-Ia is a pediatric genetic disorder. The rAAV-G6PC vector used in the Phase III clinical trial for GSD-Ia (NCT05139316) is episomally expressed. Currently, there are insufficient clinical data to understand whether multi-decade episomal transgene expression can be maintained in the human liver at a therapeutic level. We therefore explored alternative genetic technologies for GSD-Ia therapy, such as CRISPR/Cas9−based gene editing. We previously generated a *G6pc*–R83C mouse strain carrying the prevalent pathogenic G6PC-p.R83C variant and showed that the *G6pc*–R83C mice exhibit the pathophysiology of impaired glucose homeostasis mimicking human GSD-Ia. In an initial exploration of CRISP/Cas-9–based editing using AAV to deliver the CRISPR reagents, we showed that a homology-directed repair strategy could correct the abnormal metabolic phenotype of neonatal *G6pc*–R83C mice.

Using the *G6pc*–R83C mice, we explored a CRISPR/Cas9–based double-strand DNA oligonucleotide (dsODN) insertional strategy that uses the non-homologous end-joining repair mechanism to correct the pathogenic p.R83C variant in *G6pc* exon-2. The strategy is based on the insertion of a short dsODN into *G6pc* exon-2 to disrupt the native exon, and to introduce an additional splice-acceptor site and the correcting sequence. When transcribed and spliced, the edited gene would generate a wild-type mRNA encoding the native G6Pase-α protein. The editing reagents, formulated in lipid nanoparticles (LNPs), were delivered to the liver. Mice were treated either with one dose of LNP-dsODN at age 4 weeks or with 2 doses of LNP-dsODN at age 2 and 4 weeks. The *G6pc*–R83C mice receiving successful editing expressed about 4% of normal hepatic G6Pase-α activity, maintained glucose homeostasis, lacked hypoglycemic seizures, and displayed a normalized blood metabolite profile. The outcomes are consistent with preclinical studies supporting previous gene-augmentation therapy, which is currently in clinical trials. This editing strategy may offer the basis for a therapeutic approach with an earlier clinical intervention than gene augmentation, with the additional benefit of a potentially permanent correction of the GSD-Ia phenotype [Reference 3].

Base editing corrects metabolic abnormalities and prevents hepatocarcinogenesis in murine GSD−Ia.

We explore the adenine base editor (ABE)–based technologies that enable a programmable conversion of A•T to G•C in genomic DNA for GSD-Ia therapy. 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, and can correct a pathogenic variant in its native genetic locus, leading to permanent, therapeutically effective long-term expression. This is a collaborative study with Beam Therapeutics, Cambridge, MA, under a CRADA.

The G6PC-p.R83C is the most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients, which contains a single G→A transition in the *G6PC* gene. We first generated a homozygous humanized (hu) R83C/R83C mouse strain, the huR83C mouse, by inserting the entire coding sequence of the human G6PC-p. R83C along with human *G6PC* 3'-UTR into exon 1 of the mouse *G6pc* gene at the ATG start codon. The insertion places the human transcript 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 showed that the huR83C 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 explored the efficacy of ABE to correct the G6PC-p.R83C variant in the

huR83C mice following systemic administration of editing reagents formulated in LNPs, and we monitored phenotypic correction up to 53 weeks of age. We showed that physiological levels of hepatic G6Pase-α activity with an editing efficiency up to about 60% could be restored in the edited huR83C mice. The edited mice maintained glucose homeostasis, survived long-term, and lacked hepatic tumors. While LNP–ABE failed to transduce the kidney, nephromegaly was improved in the edited mice. In summary, the ABE–mediated gene editing corrected a pathogenic G6PC variant in the native genetic locus, offering a permanent, non-inheritable, correction for GSD-Ia.

Inhibition of Wnt/–catenin signaling reduces renal fibrosis in murine glycogen storage disease type Ia.

Renal disease is a serious long-term complication for GSD-Ia. The early kidney manifestations of GSD-Ia are impaired renal gluconeogenesis, and nephromegaly caused by increased glycogen accumulation. The only therapies currently available to treat GSD-Ia are dietary therapies, which significantly alleviate metabolic abnormalities but only delay the onset of chronic kidney disease. The underlying pathological processes remain uncorrected, and glomerular hyperfiltration, hypercalciuria, hypocitraturia, and urinary albumin excretion still occur in metabolically compensated GSD-Ia patients. We previously showed that one mechanism underlying GSD-Ia nephropathy is fibrosis mediated by activation of the renin-angiotensin system (RAS).

Wnt/β-catenin (catenins are components of adherens junctions) signaling regulates the expression of a variety of downstream mediators implicated in renal fibrosis, including several genes in the RAS. Sustained activation of Wnt/β-catenin signaling is associated with the development and progression of renal fibrotic lesions, which can lead to chronic kidney disease. We examined the molecular mechanism underlying GSD-Ia nephropathy. Damage to kidney proximal tubules is known to trigger acute kidney injury (AKI) which can, in turn, activate Wnt/β-catenin signaling. We showed that GSD-Ia mice display AKI, which leads to activation of the Wnt/β-catenin/RAS axis. Renal fibrosis was demonstrated by increased renal levels of Snail1 (zinc finger transcriptional repressor), α-smooth muscle actin (α-SMA), and extracellular matrix proteins, including collagen-Iα1 and collagen-IV. Treating GSD-Ia mice with the CBP/β-catenin (CBP is a CREB-binding protein, where CREB is a cAMP response element–binding protein) inhibitor ICG-001 significantly reduced nuclear translocated active β-catenin and renal levels of renin, Snail1, α-SMA, and collagen-IV. The results suggest that inhibition of Wnt/β-catenin signaling may be a promising therapeutic strategy for GSD-Ia nephropathy [Reference 4].

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Publications

1. Gautam S, Zhang L, Lee C, Arnaoutova I, Chen HD, Resaz R, Eva A, Mansfield BC, Chou JY. Molecular mechanism underlying hepatic autophagy impairment in glycogen storage disease type Ib. *Hum Mol Genet* 2023 32:262–275.

2. Chou JY, Mansfield BC. Gene therapy and genome editing for type I glycogen storage diseases. *Frontiers Mol Med* 2023 3:1167091.
3. Samanta A, George N, Arnaoutova I, Chen HD, Mansfield BC, Hart C, Carlo T, Chou JY. CRISPR/Cas9-based double strand oligonucleotide insertion strategy corrects metabolic abnormalities in murine glycogen storage disease type Ia. *J Inherit Metab Dis* 2023 46(6):1147–1158.
4. Lee C, Pratap K, Zhang L, Chen HD, Gautam S, Arnaoutova I, Raghavankutty M, Starost MF, Kahn M, Mansfield BC, Chou JY. Inhibition of Wnt/β-catenin signaling reduces renal fibrosis in murine glycogen storage disease type Ia. *Biochim Biophys Acta Mol Basis Dis* 2023 1870(1):166874.

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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 just a packaging system for DNA in eukaryotic cells; it 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, which 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: (1) to 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, and whether they are functionally redundant; our studies so far indicate that each remodeling enzyme makes a different contribution to chromatin organization and is important because genes encoding subunits of some of these enzymes are often mutated in various cancers; (2) to test the hypothesis that nucleosomes control DNA accessibility and play a vital role in gene regulation by blocking promoters. We have developed a new method (qDA-seq) to obtain quantitative measurements of genome accessibility in nuclei and in living cells, using budding yeast and human or mouse cell lines as model systems.

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Measuring genome accessibility in living cells

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

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.
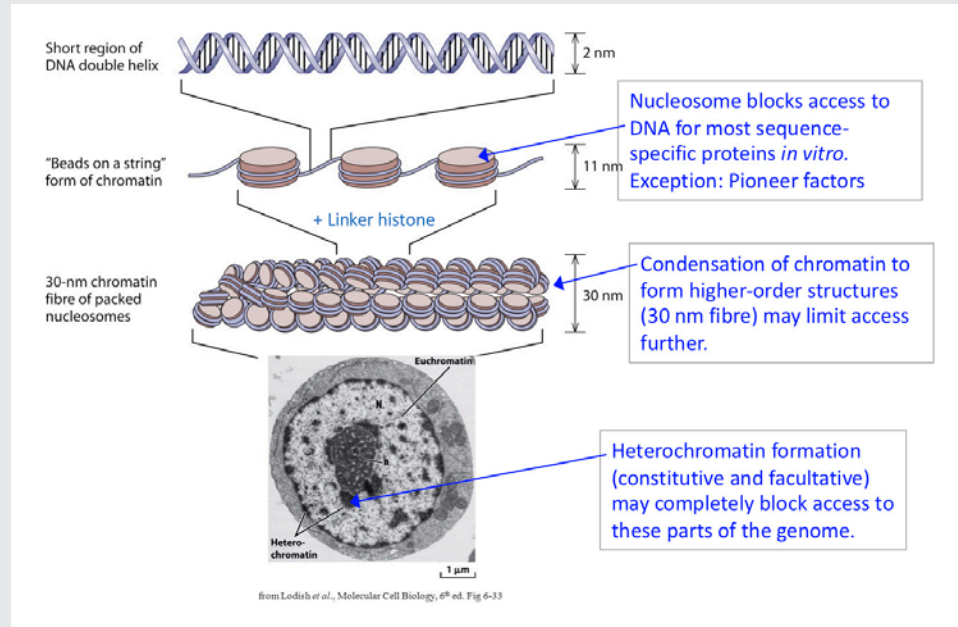
transcription. These events coincide with the removal of promoter nucleosomes to create a nucleosome-depleted region (NDR). This observation has led to the generally accepted model that promoter nucleosomes physically block transcript initiation, acting as repressors by preventing access to specific transcription factor–binding sites. The nucleosome is a highly stable structure containing tightly wound DNA, which is largely inaccessible to sequence-specific DNA–binding proteins. Activation occurs if sequence-specific 'pioneer' transcription factors (proteins that bind to nucleosomal sites with high affinity) are present, 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 either move nucleosomes along DNA, remove the histones altogether, or form arrays of regularly spaced nucleosomes. Examples include the SWI/SNF and RSC chromatin structure remodeling complexes, which remodel nucleosomes on genes and at promoters, and the CHD and ISWI remodeling complexes, some of which are involved in determining nucleosome spacing. The INO80C remodeling complex is unusual because it appears to have both properties. Human diseases have been linked to chromatin remodeling enzymes. For example, mutations in the hSNF5 subunit of the SWI/SNF complex are strongly linked to pediatric rhabdoid tumors, and the CHD remodelers have been linked to cancer and autism. Therapies and drugs aimed at epigenetic targets are being tested. Thus, a full understanding of chromatin structure and the mechanisms by which it is manipulated is vital.
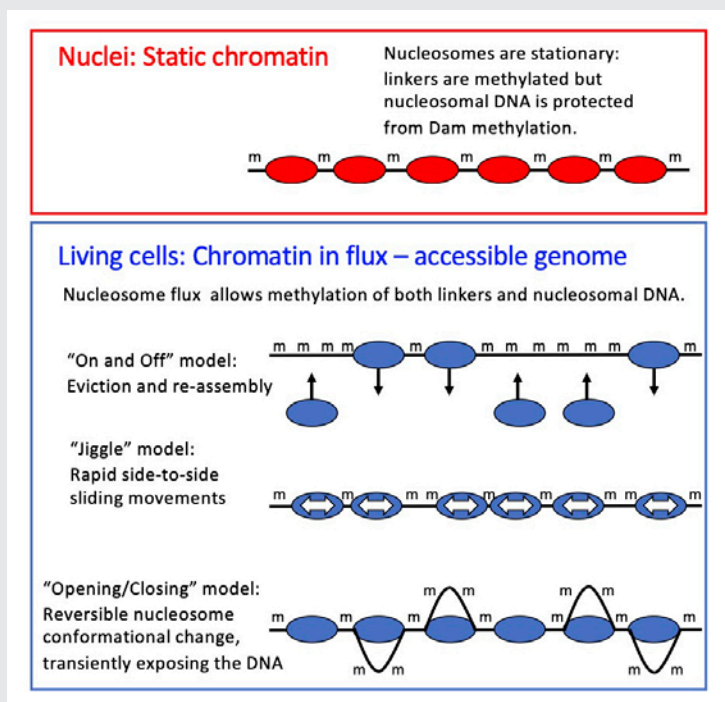
FIGURE 2. Chromatin flux model to explain the difference in genome accessibility in nuclei and living cells

We propose that nucleosomes are stationary in nuclei, protecting the DNA they contain (*top panel*). The Dam DNA methyltransferase only methylates the short stretches of linker DNA between regularly spaced nucleosomes. However, in living cells, Dam methylates virtually the entire genome (*bottom panel*), because the nucleosomes are in flux. Nucleosomes may be continuously removed and replaced, or shunted from side to side, or opened up, as shown, resulting in DNA exposure and methylation. We propose that the flux is generated by the competing activities of the various ATP–dependent chromatin remodelers (such as RSC, SWI/SNF, ISW1, and CHD1).

The general view is that nucleosomes and chromatin structure play a central role in gene regulation by restricting access to genomic DNA (Figure 1). Experiments measuring DNA accessibility in nuclei have established that nucleosomes block access to DNA (e.g., by MNase-seq, ATAC-seq, or qDA-seq). Studies from many groups have confirmed this observation and is stated as a fact in the introductions to most chromatin papers (including our own) and in reviews of the chromatin field. Indeed, the observation that nucleosomes block access to DNA is central to current models of gene regulation, as mentioned above. However, nuclei might not represent an accurate model for living cells, because chromatin dynamics may be "frozen" when cells are disrupted, which results in the loss of metabolites, such as the ATP required for remodeling.

To address this question, we are comparing the accessibility of DNA genome-wide in nuclei and in living yeast cells using DNA methylases. Remarkably, we find that the genome is globally accessible in living cells, unlike in nuclei. That is, nucleosomes do *not* block access to genomic DNA *in vivo*. We find that methylation by the Dam DNA methylase (which methylates A in the sequence GATC) is blocked by nucleosomes in isolated nuclei. However, expression of Dam using an inducible promoter results in methylation of the entire genome in living cells, with minimal interference from nucleosomes. Using a different DNA methylase, M.SssI (which methylates C in CG), together with Nanopore long-read sequencing, we showed that centromeric nucleosomes, unlike canonical nucleosomes, are exceptionally stable, protecting their DNA from methylation *in vivo*. We also observe that at least three ATP–dependent chromatin remodelers (RSC, ISW1, and CHD1) contribute to nucleosome dynamics *in vivo*, using a degron approach to detect nucleosome movements in living cells as remodelers are depleted.

Our data demonstrate that nucleosomes are in a continuous state of flux in living cells, but static in nuclei, presumably resulting from loss of critical factors during isolation. This flux may involve nucleosome sliding,

FIGURE 3. Mapping nucleosomes on single DNA molecules using methylation footprinting and PacBio long-read sequencing

The M.EcoGII DNA methyltransferase methylates adenine bases in DNA, converting them into 6-methyladenine (m6A).

(*A*) Nuclei are incubated with M.EcoGII, which methylates adenines in accessible DNA, including nucleosome-depleted regions at gene promoters and the short linkers between nucleosomes. Nucleosomal DNA is protected from methylation by the histones.

(*B*) After purification, genomic DNA is fragmented into molecules of several thousand base pairs, and sequenced using PacBio technology; m6A can be detected because the PacBio–sequencing polymerase pauses when it encounters m6A in the template strand. The result is a methylation map corresponding to accessible regions, which can be interpreted in terms of nucleosome footprints. Each mapped DNA molecule represents a stretch of DNA from a single cell. By comparing multiple molecules containing the same sequence, we detected extensive cell-to-cell variation in nucleosome positioning.

A. Treat chromatin in nuclei with M.EcoGII (adenine, A -> m6A)

M.EcoGII

nucleosome   linkers   Gene X

B. Purify genomic DNA with methylated accessible regions and PacBio sequencing

m = m6A

m   mmmmmm   m   m   m   m

unmethylated sequences = nucleosome footprints

PacBio long-read sequencing detects m6A in single DNA molecules
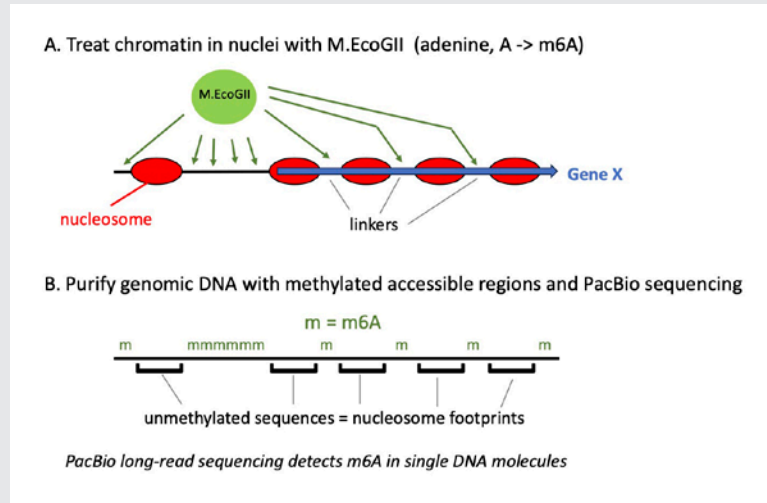
nucleosome removal and replacement, and/or nucleosome conformational changes, catalyzed by ATP–dependent chromatin remodelers (Figure 2). We propose that the various remodelers compete with one another *in vivo*, continually moving nucleosomes to different positions, resulting in a nucleosome flux that renders the yeast genome essentially transparent to transcription factors and other DNA–binding proteins. Our observations have profound implications for the chromatin field, requiring a re-examination of the roles of the chromatin remodelers in gene regulation, and of the extent to which packaging the genome into nucleosomes is actually repressive. A revised manuscript describing our data has been submitted. Currently, we are extending our studies to human cell lines.

Long-read sequencing methods, namely the PacBio and Nanopore platforms, are becoming increasingly popular, as the power of these new technologies is fully appreciated. In our own field of chromatin biology, epigenetic modifications such as m5C and m6A can now be detected directly in original (i.e., unamplified) multi-kilobase DNA molecules. A recent extension of this approach is the use of methylation footprinting, in which an exogenous DNA methylase is added to nuclei or chromatin, where it methylates accessible regions, such as nucleosome-depleted promoters and the linkers between nucleosomes, but not nucleosomal DNA, which it cannot access. The result is a methylation map of each molecule, to be interpreted in terms of footprints and accessible regions.

We performed methylation footprinting in budding yeast nuclei using M.EcoGII, an adenine-specific DNA methylase (Figure 3). The potential footprint resolution of this approach is very high because of the adenine density of DNA. We used PacBio long-read sequencing to detect m6A introduced by M.EcoGII, and we developed a pipeline to analyze the data. We discovered a number of critical, previously unreported, issues

concerning PacBio methylation footprinting data, which must be corrected before an accurate chromatin map can be obtained. Specifically, we observed low-limit methylation levels in the genomic DNA–positive controls, a wide range in the fraction of m6A from one DNA molecule to the next, and a strong local bias against methylation of AT–rich sequences and poly(A) runs. We also found that there is a high probability of observing a single m6A base within a nucleosome, breaking up the expected around 147 bp footprint. Our novel probability model resolves all of these critical interpretative problems. The pipeline output includes an IGV–ready bam file, which displays both called m6A bases, and our interpretation of the methylation pattern as accessible regions and nucleosomes in individual DNA molecules.

We used our data to investigate heterogeneity in chromatin structure around the transcription start sites of yeast genes. We found that nucleosome positioning on a specific gene varies widely from cell to cell, with only a small fraction of genes showing a similar ordered nucleosomal array in every cell, even though the cell population average is as expected from other techniques, such as MNase-seq. We quantify the degree of heterogeneity for every yeast gene using a novel correlation score. A revised manuscript describing our data has been submitted.

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

Ribonucleases H (RNases H) are considered essential enzymes in multicellular organisms, thereby placing the genes that encode the enzymes in the housekeeping category. 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 replication and RNA transcription. 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 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 the *RNASEH1* gene exhibit typical mitochondrial myopathy symptoms (neuromuscular disorder). We were the first to show that RNase H1 is essential for the maintenance of mitochondrial DNA. More than 1,000 proteins are targeted to mitochondria, many of which, when mutated, are known causes of mitochondrial myopathies. Mice deleted for the *Rnaseh1* gene arrest embryonic development at day 8.5 because 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. As many as 38 Mendelian genotypes may result in a type I interferonopathy, including mutations in each of the genes encoding the subunits of the heterotrimeric RNase H2, the hallmark of which is activation of the innate immune response.

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Differences between Class I and Class II RNases H

Over the years, many of our investigations focused on RNase H1. RNase H1 recognizes the 2'-OH of four consecutive ribonucleotides (rNMPs), 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 prokaryotes and eukaryotes, RNases H1 consist of a single polypeptide. In contrast, in eukaryotes RNase H2 is a complex of three distinct polypeptides but a single polypeptide in prokaryotes. The catalytic subunit of the hetero-trimeric RNase H2 of eukaryotes is similar in its primary amino-acid

FIGURE 1. Phenotypes of mice bearing a homozygous *Rnaseh2a* G37S mutation

sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave both RNA/DNA hybrids and a single ribonucleotide embedded in DNA, making an incision at the 5' of the ribonucleotide, or the transition from the ribonucleotide in the case of RNA–primed DNA synthesis (e.g., rrrr*rD*DDD in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide) [References 1 & 2].

Several types of RNA/DNA hybrid structures can be 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-strand 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 hybrids are substrates for class I and II RNases H. The third is uniquely recognized by type 2 RNases H, when the stretch of ribonucleotides embedded in DNA is less than four nucleotides long.

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 after 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, which 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 (Figure 1).

We obtained whole-genome sequence and RNA-seq data to search for genetic variants that account for the difference in viability between the mice that are perinatal lethal and those that are viable. Phenotypes of the viable mice include small size and white ventral spotting. Eye colobomas and kinky tails (vertebrae fusion) are found but are not 100% penetrant. These phenotypes are reminiscent of those associated with haploinsufficiency for ribosomal proteins of mouse models of Diamond Blackfan anemia, often described as a ribosomopathy.

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 called 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.

The embryonic lethality of mouse *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.

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 isoforms 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 the absence of mtDNA replication with lethality. We were curious to see the contribution effects in a system less complicated than 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 knock out the gene, using a CRE-lox method with the *Mb1* promoter–driving CRE (the *Mb1* gene encodes the Ig-α signaling subunit of the B cell antigen receptor). Transcripts of *Mb1* are initiated from the earliest stage of B cell development 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 that resting B cells were produced, although with a yield of half as many B cells as in 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 RNase H1 activity, no mtDNA, and their 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. We observed reductions in the following pathways: the cell-cycle, the immune system, DNA replication, RNA processing, and in mitochondria and ribosomes. The 50% yield of resting B cells 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*), which encodes basement-membrane glycoproteins, results in a 50% reduction of resting B cells compared with normal, the same reduction 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 difference between resting and stimulated KO mice are *Atf5*, *Gdf15*, *Atf3*, *Hspa9*, and *Ddit3*; *Atf5*, *Atf3*, and *Ddit3*, 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 for the presence of R-loops by DRIP-seq and, surprisingly, found no alteration in R-loops, indicating the lack of a role RNase H1 in the processing of these structures.

The RNA exosome and RNases H cooperate to suppress R−loop−mediated genome instability.

In addition to RNase H processing, there are other mechanisms to resolve R-loops and/or prevent their formation. The RNA exosome is a major 3'-5' RNA degradation, multi-subunit complex in eukaryotes, which eliminates cryptic and defective transcripts, preventing their engagement with DNA and suppressing R-loop formation.

In the yeast *S. cerevisiae*, we are studying the interaction and cooperation between RNases H and the RNA exosome in preventing R-loop–mediated genome instability. We found that over-expression of RNase H1 partially suppressed the growth defects of exosome-deficient mutants, suggesting that some of the problems in these cells are caused by harmful R-loops. We observed that cells defective in both RNase H and exosome activities are hypersensitive to the drug hydroxyurea, which induces replicative stress by reducing the cellular dNTP supply, and that replicative stress in these conditions is eliminated by expressing the RNase H2-RED variant that processes R-loops.

We are using a genetic system that correlates R-loop accumulation with homologous recombination to further elucidate the interplay between RNases H and the exosome in R-loop processing. In this system, yeast strains contain a fragment of the mouse switch Mu sequence (from the *IgH* locus) in an orientation that either facilitates or prevents R-loop formation. We found that RNAs with the fragment in both orientations accumulated in exosome-deficient mutants, indicating that these RNAs are targeted for degradation by the exosome. Moreover, strains with the fragment in the orientation that favors R-loop formation showed increased accumulation of RNA/DNA hybrids and recombination rates when both the exosome and RNases H were defective, compared with cells lacking only RNases H. We conclude that RNase H and the exosome pathways converge to promote genome stability by suppressing the harmful effects of R-loops.

Publications

1. Uehara R, Cerritelli SM, Hasin N, Sakhuja K, London M, Iranzo J, Chon H, Grinberg A, Crouch RJ. Two RNase H2 mutants with differential rNMP processing activity reveal a threshold of ribonucleotide tolerance in DNA for embryonic development. *Cell Rep* 2018 25:1135–1145.
2. Cerritelli SM, Iranzo J, Sharma S, Chabes A, Crouch RJ, Tollervey D, El Hage A. High density of unrepaired genomic ribonucleotides leads to Topoisomerase 1-mediated severe growth defects in absence of ribonucleotide reductase. *Nucleic Acids Res* 2020 48:4274–4297.
3. Liu Y, Rodriguez Y, Ross RL, Zhao R, Watts JA, Grunseich C, Bruzel A, Li D, Burdick JT, Prasad R, Crouch RJ, Limbach PA, Wilson SH, Cheung, VG. RNA abasic sites in yeast and human cells. *Proc Natl Acad Sci USA* 2020 117:20689–20695.
4. Watts JA, Grunseich C, Rodriguez Y, Liu Yaojuan, Li D, Burdick JT, Bruzel, Crouch RJ, Mahley RW, Wilson S, Cheung V. A common transcriptional mechanism involving R-loop and RNA abasic site regulates an enhancer RNA of APOE. *Nucleic Acids Res* 2022 50(21):12497–12514.

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Investigations of Cellular Stress in Development and Diseases

The overarching goal of the Unit is to build a foundation for a translational research program. The basic science component of the program will focus on investigating the regulation of the cellular integrated stress response (ISR) as (1) a mechanism for variable phenotypic expressions and (2) a potential therapeutic approach to relevant human diseases. The clinical component will focus on using rare, pediatric, neurogenetic conditions as models for the pursuits of investigator-initiated and sponsored interventional trials.

THE INTEGRATED STRESS RESPONSE (ISR)

ISR is an evolutionarily conserved process capable of inducing pro-survival or pro-apoptotic status in cells experiencing endoplasmic-reticulum (ER) and other stresses by activating either autophagy or apoptosis. Cellular stresses, such as unfolded protein accumulation, trigger the ISR through one of the four known eIF2α kinases. The integration occurs as Ser51–phosphorylated eIF2α inhibits the guanine nucleotide exchange activity of eIF2B, halts the formation of the translation initiation ternary complex, and effectively attenuates global mRNA translation. The reduced ternary complex formation paradoxically allows for increased translation of selected mRNAs, many of whose protein products are involved in determining cell fate and may be specific in the response to the instigating stress [Sonenberg N, Hinnebusch AG *Cell* 2009;136:731]. As an instigator or sequela, aberrant ISR is implicated in human disorders of metabolism (diabetes), growth (skeletal dysplasia, cancer) and neurologic processes (MEHMO, Down syndrome, Alzheimer's disease), amongst many others. Regulation of the ISR could provide therapeutic benefit.

CLN3 DISEASE

CLN3 disease (also known as Batten disease or juvenile neuronal ceroid lipofuscinosis) is a rare, fatal, pediatric, neurodegenerative, lysosomal disorder with no currently approved treatment. Syndromic CLN3 disease presentation includes vision loss, neurodevelopmental plateauing and decline, behavioral inflexibility and emotional lability, seizures, and motor dysfunction, as described previously (*OMIM 204200*) and also observed in a cohort of natural history study (*NCT03307304*) participants at the NIH. Accumulations of lipopigment consisting of carbohydrates, lipids, metal ions, and proteins (particularly mitochondrial ATP synthase subunit C [SCMAS]) form intracellular and lysosomal deposits that
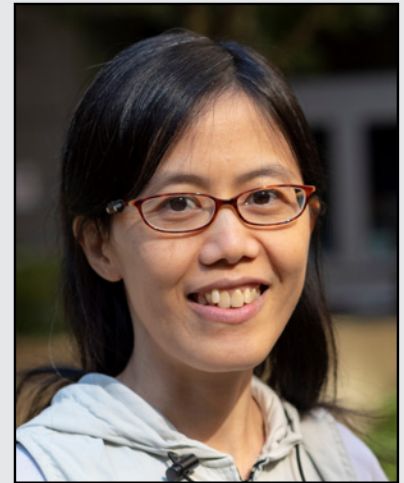
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autofluorescence under UV light and present as fingerprint patterns in electron microscopy. The affected *CLN3* gene encodes a 468–amino acid, transmembrane, ubiquitously expressed protein implicated in many cellular pathways, whose exact function is being defined [References 2, 3].

Findings from cells and flies implicate perturbed ISR following stress induction in the CLN3–deficient compared with wild-type (WT) controls. Reduced expression of WT CLN3 lowered SH-SY5Y cell (*in vitro* models of neurological function) viability following treatment with the antibiotic tunicamycin [Wu D *et al.* *Biochem Biophys Res Commun* 2014;447:115], and reduced *Drosophila* survival following exposure to chemical inducers of oxidative stress [Tuxworth RI *et al.* *Hum Mol Genet* 2011;20:2037]. Higher eIF2α phosphorylation and expression of the ER chaperone BiP/Grp78 in fibroblast cell lines from individuals with CLN3 disease than in WT controls followed treatment with NH_4Cl (Wei H *et al.* *Hum Mol Genet.* 2008;17:469). Disturbances in the lysosome autophagic pathway may also interconnect with the ISR through the transcription factor EB (TFEB) [Martina JA *et al.* *EMBO J* 2016;35:479; Burton TD *et al.* *J Biol Chem* 2020;295:7418]. TFEB is a widely studied autophagy regulator and a treatment target of interest for conditions involving lysosomes and neurodegeneration [Cortes CJ, La Spada AR. *Neurobiol Dis* 2019;122:83]. Cln3 mutant mice treated with trehalose, a disaccharide activator of TFEB, had improved intracellular lipopigment accumulation, brain weight, pain sensitivity, and survival [Palmieri M *et al.* *Nat Commun* 2017;8:14338].

MEHMO SYNDROME (MENTAL RETARDATION, EPILEPTIC SEIZURES, HYPOGONADISM/HYPOGENITALISM, MICROCEPHALY, OBESITY)

MEHMO syndrome is a very rare, X-linked recessive, pediatric, multisystemic, life-limiting condition with fewer than 100 affected males reported in the literature (*OMIM 300148*). Life expectancy in affected individuals ranges from infancy to early adulthood. The disease locus is located on the Xp21.1-p22.13 chromosomal band. Exome sequencing identified disease-associated variants in *EIF2S3*, the gene encoding the gamma subunit of the translation initiation factor eIF2, which holds a central role in the ISR, as demonstrated by pathologies and diseases associated with defective phosphorylation of the alpha subunit of eIF2 (eIF2α). Survival of affected individuals ranges from infancy to late teens. Diagnostic testing and disease-modifying therapies for MEHMO are not currently available.

Further understanding of the ISR in CLN3 disease and MEHMO syndrome models would provide insight into the underlying pathophysiology and inform novel therapeutic approaches.

The role of the integrated stress response in CLN3 (Batten disease) and the MEHMO syndrome

We continue to develop laboratory reagents, experimental models, and collaborations to address the overall objective of understanding the role of the integrated stress response in the human diseases CLN3 and MEHMO syndrome/eIF2-pathway related conditions. We are characterizing the cellular pathophysiology of the disease models, and their responses following exposure to stress conditions.

The CLN3 natural history study and biosample collection is ongoing. To date, our study contains the largest cohort of individuals with CLN3–related disorders extensively characterized and prospectively followed, with corresponding biosamples (cerebrospinal fluid [CSF], blood, urine, fibroblasts) collected. This (1) permits the development and assessment of outcome measures applicable for therapeutic trials (*NCT03307304*);

(2) builds the infrastructures for engagement in a sponsored Phase 1/2 study of miglustat therapy trial (*NCT05174039*), and in future investigator-initiated and sponsored interventional trials; and (3) provides the means for further CLN3 research through identification of disease-reflective biomarkers. Measurement of CSF and blood neurofilament light chain level, found to be elevated in individuals with CLN3 [Reference 1], is being applied in an ongoing *Phase 1/2 study of miglustat*. Additional candidate fluid [Reference 5] and imaging [Dang Do AN *et al*. *Mol Genet Metab* 2023;139:107584] markers may further the progress of CLN3 translational research.

Applying experiences from CLN3 translational research, we initiated a natural history study for individuals with MEHMO syndrome or eIF2 pathway–related disorders (*NCT06019182*) to characterize these conditions systematically and to establish concurrent collections of phenotypic data and biomaterials. The IRB–approved study is open for enrollment.

Additional Funding

- 2022 NICHD Early Career Award
- 2023 NICHD Scientific Director's Award

Publications

1. Dang Do AN, Sinaii N, Masvekar RR, Baker EH, Thurm AE, Soldatos AG, Bianconi SE, Bielekova B, Porter FD. Neurofilament light chain levels correlate with clinical measures in CLN3 disease. *Genet Med* 2021 23(4):751–757.
2. Brudvig JJ, Swier VJ, Johnson TB, Cain JC, Pratt M, Rechtzigel M, Leppert H, Dang Do AN, Porter FD, Weimer JM. Glycerophosphoinositol is elevated in blood samples from CLN3Δex7-8 pigs, Cln3Δex7-8 mice, and CLN3-affected individuals. *Biomark Insights* 2022 17:11772719221107765.
3. Laqtom NN, Dong W, Medoh UN, Cangelosi AL, Dharamdasani V, Chan SH, Kunchok T, Lewis CA, Heinze I, Tang R, Grimm C, Dang Do AN, Porter FD, Ori A, Sabatini DM, Abu-Remaileh M. CLN3 is required for the clearance of glycerophosphodiesters from lysosomes. *Nature* 2022 609:1005–1011.
4. Dang Do AN, Thurm AE, Farmer CA, Soldatos AG, Chlebowski CE, O'Reilly JK, Porter FD. Use of the Vineland-3, a measure of adaptive functioning, in CLN3. *Am J Med Genet A* 2022 188:1056–1064.
5. Dang Do AN, Sleat DE, Campbell K, Johnson NL, Zheng H, Wassif CA, Dale RK, Porter FD. Cerebrospinal Fluid Protein Biomarker Discovery in CLN3. *J Proteome Res* 2023 22:2493–2508.
6. Luckett A, Yousef M, Tifft C, Jenkins K, Smith A, Munoz A, Quimby R, Porter FD, Dang Do AN. Anesthesia outcomes in lysosomal disorders: CLN3 and GM1 gangliosidosis. *Am J Med Genet A* 2023 191:711–717.

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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 nucleus from the cytoplasmic compartment 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. 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 mis-regulated 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 goal is to define the biochemical roles of individual nucleoporins and understand how their dysregulation causes human disease.

Selective degradation of nucleo−cytoplasmic transport proteins

Understanding the activities of individual nucleoporins has been complicated by their multifaceted nature, abundance, and unusual stability. To overcome these issues, we employed strategies for selective and rapid degradation of individual proteins within human tissue-culture cells. 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 of AID–tagged

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proteins upon addition of the plant hormone auxin. We were successful in developing cell lines that allow conditional depletions of nucleoporins associated with different regions of the NPC.

Our recent investigations regarding the roles of nucleoporins aim to address three issues. First, we are studying the role of individual nucleoporins in NPC assembly and stability. Our results indicate that different regions of the NPC can persist independently after disruption of other structural domains, indicating that the NPC is surprisingly modular after it is assembled. Second, we are looking at the role of individual nucleoporins in different nuclear trafficking pathways. We are particularly interested in mRNA export that depends upon the transcription and export 2 (TREX2) complex in conjunction with the nucleoporin TPR. Third, we are analyzing the roles of nucleoporins in non-transport processes, such as mitotic cell-cycle signaling and chromosome segregation. 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.

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, and 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. Furthermore, near complete loss of the outer ring in NUP96–depleted cells did not cause collapse of the rest of the NPC, as demonstrated by immunostaining, live microscopy, and mass spectrometry. The 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. 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 both of them.

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. These 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. 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.

Functional analysis of TREX2 complex subunits and their individual roles in RNA export

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 (TREX2) complex, which plays a central role in bridging the transcription and export machinery. The GANP subunit of TREX2 localizes within the nucleus and associates with the NPC's nuclear basket, which protrudes from the nucleoplasmic face of the NPC. The TREX2 complex is highly conserved across eukaryotes; the mammalian TREX2 complex consists of GANP (*S. cerevisiae* homologue = Sac3), PCID2 (Thp1), ENY2 (Sus1), DSS1 (Sem1), and either CETN2 or CETN3 (Cdc31) proteins. In vertebrates, the nuclear basket comprises three nucleoporins, called NUP153, TPR, and NUP50. Our previous studies established that TPR interacts with GANP and plays a unique role in the export of TREX2–dependent mRNAs.

Our current research focuses on the roles of individual TREX2 complex subunits. We are particularly interested in the possibility that mRNAs may vary in the subset of TREX2 subunits that they utilize during processing and export. We are currently analyzing how individual TREX2 subunits contribute to mRNA transcript retention within the nucleus, thus shaping the cellular transcriptome, and how they work with nucleoporins to license RNA transcripts at the NPC for export.

Roles of nucleoporins during cell division

The process of chromosome segregation during cell division is essential to maintain genomic integrity. Defects in chromosome segregation lead to aneuploidy, a condition in which cells possess an abnormal number of chromosomes. Aneuploidy arising from mitotic divisions is a hallmark of many solid tumors, while aneuploidy arising during meiosis contributes to human pregnancy losses and genetic disorders, including Down's syndrome. A microtubule-based structure called the spindle mediates chromosome segregation during division. The spindle attaches to condensed chromosomes via a proteinaceous structure on the chromosomes called the kinetochore, allowing the accurate distribution of chromosomes into daughter cells at anaphase. Surprisingly, many proteins that contribute to the assembly of the kinetochore bind tightly to the NPC during interphase. Conversely, as cells undergo NPC disassembly and NE breakdown prior to mitosis, a large number of nucleoporins associate with kinetochores. We have ongoing studies that use AID-tagged cells to understand two aspects of these relationships.

First, proper chromosome attachment to spindles (biorientation) and their alignment on the metaphase plate are essential for accurate segregation. The Spindle Assembly Checkpoint (SAC) monitors binding of spindle microtubules to kinetochores. When chromosomes are not properly attached, the SAC generates a signal

that prevents anaphase onset. Three proteins that are essential for this signaling pathway (Mad1, Mad2, and p31) associate with NPCs during interphase through the nucleoporin TPR, an association that is widely conserved across eukaryotic species, but whose function is poorly understood. We are currently examining the biochemistry of their association, as well as its consequences for cellular function using the AID–tagging system. Specifically, we examined co-dependent relationships among Mad1, Mad2, p31, and TPR for NPC association by depleting individual proteins. We also ascertained that association among these proteins does not activate SAC signals prior to NE breakdown, arguing against previously proposed models for SAC control. We are currently testing the idea that SAC proteins play a role in controlling interphase NPC function or regulation of nuclear trafficking. Second, kinetochores form a large, crescent-shaped structure known as the fibrous corona, prior to spindle microtubule attachment; the Y-complex is strongly recruited to this structure. As previously demonstrated for other components of the fibrous corona, we found that the stability of Y-complex association to kinetochores is lost upon inhibition of Cyclin B/Cdc2, a key mitotic kinase. We are currently using AID lines to assess the role of the Y-complex in fibrous corona structure and stability.

Publications

1. Sundararajan S, Park H, Kawano S, Johansson M, Lama B, Saito-Fujita T, Saitoh N, Arnaoutov A, Dasso M, Wang Z, Clarke DJ, Azuma Y. Methylated histones on mitotic chromosomes promote Topoisomerase IIα function for high-fidelity chromosome segregation. *iScience* 2023 26:106743.
2. Bhat P, Aksenova V, Gazzara M, Rex EA, Aslam S, Haddad C, Gao S, Esparza M, Cagatay T, Batten K, El Zahed SS, Arnaoutov A, Zhong H, Shay JW, Tolbert B, Dasso M, Lynch KW, García-Sastre A, Fontoura BMA. Influenza virus mRNAs encode determinants for nuclear export via the cellular TREX-2 complex. *Nat Commun* 2023 14:2304.
3. Boukaba A, Liu J, Ward C, Wu Q, Arnaoutov A, Liang J, Pugacheva EM, Dasso M, Lobanenkov V, Esteban M, Strunnikov AV. Ectopic expression of meiotic cohesin generates chromosome instability in cancer cell line. *Proc Natl Acad Sci USA* 2022 119:e2204071119.
4. LaJoie D, Turkmen AM, Mackay DR, Jensen CC, Aksenova V, Niwa M, Dasso M, Ullman KS. A role for Nup153 in nuclear assembly reveals differential requirements for targeting of nuclear envelope constituents. *Mol Biol Cell* 2022 31:mbcE22050189.
5. Duan L, Zaepfel BL, Aksenova V, Dasso M, Rothstein JD, Kalab P, Hayes LR. Nuclear RNA binding regulates TDP-43 nuclear localization and passive nuclear export. *Cell Rep* 2022 40:111106.
6. Bley CJ, Nie S, Mobbs GW, Petrovic S, Gres AT, Liu X, Mukherjee S, Harvey S, Huber FM, Lin DH, Brown B, Tang AW, Rundlet EJ, Correia AR, Chen S, Regmi SG, Stevens TA, Jette CA, Dasso M, Patke A, Palazzo AF, Kossiakoff AA, Hoelz A. Architecture of the cytoplasmic face of the nuclear pore. *Science* 2022 376:6598.

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Regulation of Mammalian Cell Proliferation and Differentiation

Our research initially focused on mechanisms that restrict nuclear DNA replication during cell division to one complete copy of the genome during each of the trillions of cell divisions required for fertilized mammalian eggs to develop into adults. These studies led to discoveries in four areas. First, they elucidated the mechanisms by which trophoblast stem cells and megakaryoblasts are developmentally programmed to differentiate into nonproliferating polyploid cells via endoreplication, a process in which mitosis is bypassed and a second S-phase ensues. Second, they identified genes that are essential in non-malignant cells to prevent unscheduled endoreplication from promoting cancer, as well as genes essential to prevent normal cells from re-replicating their DNA more than once during a single S-phase. Third, these studies led to the discovery that induction of DNA re-replication during the same cell cycle can selectively kill cancer cells with little or no harm to normal cells. Our efforts to identify small molecules that could selectively induce DNA re-replication in cancer cells resulted in the discovery of a family of PIKFYVE phosphoinositide kinase inhibitors that can selectively kill autophagy-dependent cancer cells. Current research focuses on distinguishing sensitive cells from insensitive cells, the mechanism by which PIKFYVE inhibitors kill cancer cells, and the therapeutic potential of PIKFYVE inhibitors.

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PIP5K1C phosphoinositide kinase deficiency distinguishes PIKFYVE–dependent cancer cells from non–malignant cells.

Although PIKFYVE phosphoinositide kinase inhibitors can selectively eliminate PIKFYVE–dependent human cancer cells *in vitro* and *in vivo*, the basis for this selectivity has remained elusive. We showed that the sensitivity of cells to the PIKFYVE inhibitor WX8 is not linked to PIKFYVE expression, macroautophagic/autophagic flux, the BRAF[V600E] mutation, or ambiguous inhibitor specificity. PIKFYVE dependence results from a deficiency in the PIP5K1C phosphoinositide kinase, an enzyme required for the conversion of phosphatidylinositol-4-phosphate (PtdIns4P) into phosphatidylinositol-4,5-bisphosphate (PtdIns[4,5]P_2/PIP2), a phosphoinositide associated with lysosome homeostasis, endosome trafficking, and autophagy. PtdIns(4,5)P_2 is produced via two independent pathways: one requires PIP5K1C; the

other requires PIKFYVE and PIP4K2C to convert PtdIns3P into PtdIns(4,5)P$_2$. In PIKFYVE–dependent cells, low concentrations of WX8 specifically inhibit PIKFYVE *in situ*, thereby increasing the level of its substrate PtdIns3P, while suppressing PtdIns(4,5)P$_2$ synthesis and inhibiting lysosome function and cell proliferation. At higher concentrations, WX8 inhibits both PIKFYVE and PIP4K2C *in situ*, which amplifies these effects to further disrupt autophagy and induce cell death. WX8 did not alter PtdIns4P levels. Consequently, inhibition of PIP5K1C in WX8–resistant cells transformed them into sensitive cells, and overexpression of PIP5K1C in WX8–sensitive cells increased their resistance to WX8. The discovery suggests that PIKFYVE–dependent cancers could be identified clinically by low levels of PIP5K1C and treated with PIKFYVE inhibitors.

PIKfyve–specific inhibitors restrict replication of multiple coronaviruses *in vitro* but not in a murine model of COVID-19.

The ongoing COVID-19 pandemic has claimed more than 6 million lives and continues to test the world economy and healthcare systems. To combat this pandemic, the biological research community has shifted efforts to the development of medical countermeasures, including vaccines and therapeutics. However, to date, the only small molecules approved for the treatment of COVID-19 in the United States are the nucleoside analogue Remdesivir and the protease inhibitor Paxlovid, although several compounds have received Emergency Use Authorization and many more are currently being tested in human efficacy trials. One such compound, Apilimod, is being considered as a COVID-19 therapeutic in a Phase II efficacy trial. However, at the time of writing, there were no published efficacy data in human trials or animal COVID-19 models. We showed that, while Apilimod and other PIKfyve inhibitors have potent antiviral activity in various cell lines against multiple human coronaviruses, these compounds worsen disease in a COVID-19 murine model, when given prophylactically or therapeutically.

Selective elimination of pluripotent stem cells by PIKfyve–specific inhibitors

Inhibition of PIKfyve phosphoinositide kinase selectively kills autophagy-dependent cancer cells by disrupting lysosome homeostasis. We showed that PIKfyve inhibitors can also selectively eliminate pluripotent embryonal carcinoma cells (ECCs), embryonic stem cells, and induced pluripotent stem cells under conditions where differentiated cells remain viable. PIKfyve inhibitors prevented lysosome fission, induced autophagosome accumulation, and reduced cell proliferation in both pluripotent and differentiated cells, but they induced death only in pluripotent cells. The ability of PIKfyve inhibitors to distinguish between pluripotent and differentiated cells was confirmed with xenografts derived from 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 pluripotent cells. 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 vivo* as well as *in vitro*.

PIKFYVE inhibitors trigger interleukin-24–dependent termination of autophagy–dependent melanoma cells and tumors.

PIKFYVE phosphoinositide kinase–specific inhibitors disrupt lysosome homeostasis, thereby selectively terminating autophagy-dependent cancer cells *in vivo* as well as *in vitro* without harming the viability of non-malignant cells. To elucidate the mechanism by which PIKFYVE inhibition induces cell death, PIKFYVE–dependent melanoma cells were compared with PIKFYVE–independent foreskin fibroblasts. RNA sequence profiling suggested that PIKFYVE inhibitors upregulated an endoplasmic reticulum (ER) stress response involving interleukin-24 (IL24/mda-7) selectively in melanoma cells. Subsequent biochemical and genetic analyses

confirmed these results and extended them to tumor xenografts in which tumor formation and expansion were inhibited. IL24 gene expression was induced by the DDIT3/CHOP transcription factor, a component of the PERK (protein kinase R-like ER kinase)–dependent ER–stress response. Ectopic expression of the IL24 gene induced cell death in melanoma cells, but not in foreskin fibroblasts, whereas ablation of the IL24 gene in melanoma cells prevented death. Thus, unlike thapsigargin and tunicamycin, which induce ER stress indiscriminately, PIKFYVE inhibitors selectively terminated PIKFYVE–dependent melanoma by inducing IL24–dependent ER stress. Moreover, induction of cell death by a PIKFYVE inhibitor together with ectopic expression of IL24 protein was cumulative, thereby confirming the therapeutic potential of PIKFYVE inhibitors in the treatment of melanoma, the deadliest form of skin cancer.

Cell death and the p53 enigma during mammalian embryonic development

Twelve forms of programmed cell death (PCD) have been described in mammalian cells, but which of them occurs during embryonic development and the role played by the p53 transcription factor and tumor suppressor remains enigmatic. Although p53 is not required for mouse embryonic development, some studies conclude that PCD in pluripotent embryonic stem cells from mice (mESCs) or humans (hESCs) is p53–dependent, whereas others conclude that it is not. Given the importance of pluripotent stem cells as models of embryonic development and their applications in regenerative medicine, resolving this enigma is essential. Our review [Reference 1] reconciles contradictory results based on the facts that p53 cannot induce lethality in mice until gastrulation and that experimental conditions could account for differences in results with ESCs. Consequently, activation of the G2–checkpoint in mouse ESCs is p53–independent and generally, if not always, results in noncanonical apoptosis. Once initiated, PCD occurs at equivalent rates and to equivalent extents regardless of the presence or absence of p53. However, depending on experimental conditions, p53 can accelerate initiation of PCD in ESCs and late-stage blastocysts. In contrast, DNA damage following differentiation of ESCs *in vitro* or formation of embryonic fibroblasts *in vivo* induces p53–dependent cell cycle arrest and senescence.

Patent

Autophagy Modulators for Use in Treating Cancer: *US Patent 11,471,460,* October 18, 2022; for the WX8-family of PIKFYVE inhibitors described in: Sharma G, Guardia CM, Roy A, Vassilev A, Saric A, Griner LN, Marugan J, Ferrer M, Bonifacino JS, DePamphilis ML. A family of PIKFYVE inhibitors with therapeutic potential against autophagy-dependent cancer cells disrupt multiple events in lysosome homeostasis. *Autophagy* 2019;15(10):1694-1718.

Publications

1. Raj S, Jaiswal SK, DePamphilis ML. Cell death and the p53 enigma during embryonic development. *Stem Cells* 2022 40(3):227–238.
2. Chakraborty AR, Vassilev A, Jaiswal SK, O'Connell CE, Ahrens JF, Mallon BS, Pera MF, DePamphilis ML. Selective elimination of pluripotent stem cells by PIKfyve specific inhibitors. *Stem Cell Rep* 2022 17(2):397–412.
3. Logue J, Chakraborty AR, Johnson R, Goyal G, Rodas M, Taylor LJ, Baracco L, McGrath ME, Haupt R, Furlong BA, Soong M, Prabhala P, Horvath V, Carlson KE, Weston S, Ingber DE, DePamphilis ML, Frieman MB. PIKfyve-specific inhibitors restrict replication of multiple coronaviruses in vitro but not in a murine model of COVID-19. *Commun Biol* 2022 5(1):808.
4. Roy A, Chakraborty AR, Nomanbhoy T, DePamphilis ML. PIP5K1C phosphoinositide kinase deficiency distinguishes PIKFYVE-dependent cancer cells from non-malignant cells. *Autophagy* 2023 19(9):2464–2484.

5. Roy A, Chakraborty AR, DePamphilis ML. PIKFYVE inhibitors trigger interleukin-24 dependent termination of autophagy-dependent melanoma cells and tumors. *Mol Oncol* 2023; in press.

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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 (eukaryotic initiation factor 2), 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 the eIF2 subunit 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 rare 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) in select mRNAs.

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Molecular analysis of translation start–site selection stringency

A key interest of the lab is to study the regulation of translation start-site selection. While translation typically initiates at an AUG codon, the efficiency of initiation at a particular AUG codon is influenced by context nucleotides flanking the AUG codon and by levels of the factors eIF1 and eIF5. Interestingly, eIF1 and eIF5 auto- and trans-regulate the translation of their own mRNAs to control the levels of these factors in cells. We are exploiting such autoregulation to generate reporters to assess start-codon selection stringency, and we are also searching for mRNAs whose translation would be sensitive to changes in stringency. In a previous search of mammalian genes, we identified five homeobox (*Hox*) gene paralogs initiated by AUG codons

in conserved suboptimal context, as well as 13 *Hox* genes that contain evolutionarily conserved upstream open reading frames (uORFs) that initiate at AUG codons in poor sequence context [Reference 1]. Our collaborator Rachel Green mapped the 5' end of the *Hox* mRNAs, revealing that the mRNAs are much shorter than previously reported and lack proposed alternative translation elements. We found that the conserved uORFs inhibit *Hox* reporter expression and that altering the stringency of start-codon selection by overexpressing translation initiation factors eIF1 or eIF5 modulates the expression of *Hox* reporters. We also showed that modifying ribosome homeostasis by depleting a large ribosomal subunit protein or treating cells with sublethal concentrations of puromycin lowers the fidelity of start-codon selection. Given that the *Hox* genes encode developmental regulators of animal body plans, our studies reveal that alterations in start-codon selection stringency could control body plan formation in animals.

In parallel with these studies, we are collaborating with Jody Puglisi and colleagues to molecularly characterize the key processes in translation start-site selection including ribosomal scanning, AUG selection, and ribosomal subunit joining. Using single-molecule fluorescence spectroscopy, the Puglisi lab was able, for the first time, to directly track binding of the small 40S ribosomal subunit to an mRNA, scanning of the ribosome down the mRNA, and then joining of the 60S subunit. Their studies revealed that 40S binding to the mRNA is slow, whereas, once bound, the 40S ribosome scans rapidly. Interestingly, RNA hairpin sequences near start codons forced scanning ribosomes at start codons to move backward in the 5' direction, and *in vivo*, we showed that these secondary structures enhanced initiation at upstream near-cognate CUG or UUG start codons positioned 15-nucleotides before the stem-loop structure. Thus, RNA structures can influence the stringency of translation start-site selection [Reference 2].

The translation factor eIF5B is a GTPase required for the last step of translation initiation: the joining of the large 60S 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. Our ongoing studies with the Puglisi lab reveal that, in addition to promoting 60S subunit joining, eIF5B also controls a checkpoint that helps monitor the fidelity of translation start-site selection, a critical determinant in establishing the reading frame for translation on an mRNA.

Study of translational control by metabolite–sensing nascent peptides

Our search of genes with poor start codons identified several mRNAs containing noncanonical uORFs initiated by near-cognate start codons that differ from AUG by a single-nucleotide change or by AUG codons in poor context. One 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 are currently studying two additional examples of conserved uORFs that control translation in response to specific metabolites, and we hypothesize that similar mechanisms of nascent peptide recognition of the metabolite mediate the translational control.

Characterization of the MEHMO syndrome, an X–linked intellectual disability associated with mutations in translation initiation factor eIF2gamma

The human disease MEHMO syndrome is caused by mutations in the translation initiation factor eIF2gamma. We are characterizing yeast, mammalian cell, and mouse models of the MEHMO syndrome to better understand how the mutations impair eIF2 function and cause disease. 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, we showed that the mutations linked to the MEHMO syndrome impair eIF2 function, disrupt eIF2 complex integrity, and alter the stringency of translation start-codon selection. Over the past year, we have been characterizing additional novel *EIF2S3* mutations identified in patients with the MEHMO syndrome. Our studies aim to link genetic and biochemical properties of the broad clinical expressivity of the MEHMO syndrome.

In previous studies, we characterized induced pluripotent stem (iPS) cells derived from a patient with the MEHMO syndrome. Our studies revealed defects in general protein synthesis, constitutive induction of the integrated stress response (ISR), a cellular stress-response pathway that alters protein synthesis to mount an adaptive response, and hyper-induction of the ISR under stress conditions. The *EIF2S3* mutation also impaired neuronal differentiation by the iPS cells. We showed that 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* mutation, offering the possibility of therapeutic intervention for the MEHMO syndrome [Reference 3]. Recently, we generated a mouse model of the MEHMO syndrome, and we are currently characterizing the phenotypes and pathologies of the mouse to gain further insights into this rare disease and to identify potential new targets for therapeutic intervention.

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

Translation factor eIF5A is the sole cellular protein containing the unusual amino acid hypusine [N^e-(4-amino-2-hydroxybutyl)lysine]. We previously found that eIF5A promotes translation elongation and translation termination and that these activities are dependent on the 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. Using our *in vitro* reconstituted assay system, we also 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. Given that we previously found that eIF5A binds in the ribosome E site with the hypusine residue projecting toward the acceptor stem of the P-site tRNA, 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 studies, we are focusing on three areas:
1. identification of the function of hypusine;
2. elucidation of the role of eIF5A in controlling cellular polyamine levels; and
3. characterization of the fungal polyamine transporter Hol1.

To address the first aim, we are 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 of 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 and are now characterizing mutations in eIF5A that cause synthetic growth defects in cells lacking the hydroxylase. The mutations map to the ribosome-binding face of eIF5A and near to magnesium ions that coordinate eIF5A binding to the ribosome. 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.

Towards the second aim, we linked eIF5A to the regulation of polyamine metabolism in mammalian cells. 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. We identified a regulatory uORF in the leader of the *AZIN1* mRNA and found that high polyamine levels enhance translation initiation from the near-cognate start site of the uORF. Remarkably, this polyamine induction of uORF translation depends on the sequence of the encoded polypeptide, including a highly conserved Pro-Pro-Trp (PPW) motif, which causes polyamine-dependent pausing of elongating ribosomes. The polyamine-induced translation of the uORF blocks ribosome access to the *AZIN1* start codon and thereby inhibits synthesis of AZIN1.

In addition to elucidating the importance of the *cis*-acting amino acid motif in the uORF, 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. In ongoing studies, we have found that polyamine control of eIF5A function underlies the translational control of mRNAs encoding other regulators and enzymes in the polyamine biosynthetic pathway.

Regarding the third aim, we recently identified Hol1 as the high-affinity polyamine transporter in yeast [Reference 4]. Using ribosome profiling, we identified *HOL1* in the group of mRNAs whose translation was repressed in high polyamines. The Hol1 protein is a member of the drug-proton antiporter (DHA1) family of transporters, and 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. We identified a conserved uORF encoding the peptide MLLLPS in the leader of the *HOL1* mRNA, and we found that polyamine inhibition of eIF5A impairs translation termination at the Pro-Ser-stop (PS) motif of the uORF to repress Hol1 synthesis under conditions of elevated polyamines. Thus, polyamine transport, like polyamine biosynthesis, is under translational autoregulation by polyamines in yeast, highlighting the extensive control cells impose on polyamine levels. In ongoing studies, we are characterizing *HOL1* homologs in the pathogenic yeast *Candida albicans*, and our preliminary data indicate that polyamines are critical for *C. albicans* pathogenesis, raising the possibility that combined inhibition of Hol1 and polyamine synthesis might be an effective means to block growth of this pathogenic yeast.

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

Like its bacterial ortholog EF-G, the eukaryotic elongation factor 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 during our previous collaboration with Venki Ramakrishnan's lab (Cambridge, UK), we hypothesized 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 recently published studies [Reference 5], we found that diphthamide enhances translational fidelity.

Characterizing *Saccharomyces cerevisiae* mutants that lack diphthamide or that show synthetic growth defects in the absence of diphthamide, we found that loss of diphthamide increases -1 ribosomal frame-shifting at programmed frame-shifting sites in the HIV and SARS-CoV-2 viruses. In addition, using reporter assays we observed increased rates of frame-shifting at non-programmed sites during normal translation elongation. Ribosome profiling of yeast and mammalian cells lacking diphthamide revealed increased ribosomal drop-off during elongation with fewer ribosomes translating to the end of mRNAs. Interestingly, removal of out-of-frame stop codons restored ribosomal processivity on the ultralong yeast *MDN1* (gene encoding an essential ATPase required for ribosome biogenesis) mRNA. Our results reveal that loss of diphthamide impairs the fidelity of translocation during translation elongation, resulting in increased rates of ribosomal frame-shifting throughout elongation and leading to premature termination at out-of-frame stop codons. We propose that diphthamide, despite its non-essential nature in yeast or mammalian cells in culture, has been conserved throughout evolution to maintain the fidelity of translation elongation and block spurious frame-shifting events that would impair the production of native proteins and generate novel frame-shifted proteins that might be deleterious to the cell. Moreover, we propose that the beneficial effects of diphthamide on translational fidelity have ensured its retention during evolution despite its being a target for inactivation by bacterial toxins. Using reconstituted biochemical assays, we also showed that ADP–ribosylation of diphthamide by diphtheria toxin impairs protein synthesis by blocking the productive binding of eEF2 to elongating ribosomes.

Additional Funding

Publications

1. Ivanov IP, Saba JA, Fan CM, Wang J, Firth AE, Cao C, Green R, Dever TE. Evolutionarily conserved inhibitory uORFs sensitize Hox mRNA translation to start codon selection stringency. *Proc Natl Acad Sci USA* 2022 119:e2117226119.
2. Wang J, Shin BS, Alvarado C, Kim JR, Bohlen J, Dever TE, Puglisi JD. Rapid 40S scanning and its regulation by mRNA structure during eukaryotic translation initiation. *Cell* 2022 185:4474–4487.
3. Young-Baird SK, Lourenço MB, Elder MK, Klann E, Liebau S, Dever TE. Suppression of MEHMO syndrome mutation in eIF2 by small molecule ISRIB. *Mol Cell* 2020 77:875–886.
4. Vindu A, Shin BS, Choi K, Christenson ET, Ivanov IP, Cao C, Banerjee A, Dever TE. Translational autoregulation of the S. cerevisiae high-affinity polyamine transporter Hol1. *Mol Cell* 2021 81:3904–3918.
5. Shin BS, Ivanov IP, Kim JR, Cao C, Kinzy TG, Dever TE. eEF2 diphthamide modification restrains spurious frameshifting to maintain translational fidelity. *Nucleic Acids Res* 2023 51:6899–6913.

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Molecules and Therapies for Craniofacial and Dental Disorders

Embryonic development of the craniofacial complex requires tightly controlled molecular crosstalk between proliferating and differentiating cell networks to form the most intricate structures in the human body. During embryogenesis, perturbations in the environmental and/or genetic milieu can negatively affect craniofacial development. While considerable progress has been made in studying isolated genetic mutations leading to syndromic and non-syndromic craniofacial disorders, the broad molecular-genetic mechanisms driving morphogenetic events have yet to be sufficiently explored and understood. Such gaps in our understanding have restricted clinical treatment options for patients affected by common developmental anomalies, such as cleft palate or tooth agenesis. Thus, there is a strong biologic rationale for a thorough investigation of basic molecular mechanisms driving craniofacial structure morphogenesis, which may pave the way toward translatable therapeutic developments for patients.

The overarching goal of our research program is to conduct basic and translational studies of genetic and molecular mechanisms involved in craniofacial development, with the primary aim of unveiling novel regulatory molecules and putative patient-centric therapeutic solutions for craniofacial and dental disorders. Among the molecular pathways known to control craniofacial development is Wnt/β-catenin signaling. Wnts are also well known as upstream effectors of osteogenesis and odontogenesis. Our lab demonstrated, for the first time, the successful *in utero* correction of cleft palate defects in a *Pax9*−/− mouse genetic model by small-molecule neutralizing therapy targeting Wnt–antagonizing proteins. We are now actively investigating additional drug-delivery approaches and molecular targets for the modulation of Wnt signaling *in vivo* to permit targeted correction of both cleft palate and tooth agenesis. Our research group employs basic principles of developmental biology, next-generation sequencing, regenerative medicine, tissue engineering, and drug delivery models to identify and validate novel approaches to restore molecular equilibrium in genetic models of the highly relevant human diseases cleft palate and tooth agenesis. Actively fostering collaborations with both intramural and extramural investigators, from basic scientists, engineers, to clinicians, the lab aims to pioneer innovative approaches toward the treatment of patients

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affected by craniofacial disorders of development. Through these rigorous and robust preclinical, proof-of-principle studies, we hope to open up pathways toward novel clinical trials for the treatment of previously unpreventable disorders affecting the craniofacial complex.

Toward a novel *in utero* therapy for the prenatal cure of cleft palate in a mouse genetic model

Cleft palate (CP), together with cleft lip, is among the most common birth defects in humans, occurring in up to 1 in 500 live births. Such birth defects can inflict heavy physical, mental, psychosocial, and financial burdens on patients and their caregivers throughout life, often requiring many stages of complex surgical correction with varying success rates. Hence, there remains a substantial need for innovative approaches to alleviate the burden of postnatal care for these patients. While considerable progress has been made in studying isolated genetic mutations leading to syndromic and non-syndromic cleft disorders, the broad molecular-genetic mechanisms driving osteogenic differentiation from palatal shelf out-growth, elevation, and fusion events have yet to be sufficiently explored and understood. Such gaps in our understanding have restricted clinical treatment options for patients affected by cleft disorders. Given that prenatal molecular diagnostics have made CP identifiable earlier in gestation, the advancement of safe and efficacious interventions *in utero* to correct CP has become feasible.

The objective of our work is to investigate the molecular-genetic mechanisms driving palatal osteogenesis and to optimally manipulate key signaling environments *in utero* to promote CP correction in a mouse genetic model. If properly explored, such information can be applied to the development of novel therapeutics that can benefit individuals with isolated or syndromic CP defects, who face complex surgeries and the arduous burden of life-long care. The long-term goal of this research is to investigate the spatio-temporal molecular mechanisms driving osteogenic differentiation in normal palate development and in CP dysmorphogenesis,

FIGURE 1. Characterization of palate osteogenesis begins with fusion.

FIGURE 2. Wnt signaling drives palate osteogenesis and fusion.

using *Pax9*−/− as a model. Differential multiomic profiles of expression in spatial biological context will unveil the molecular framework for the development of novel therapeutic strategies to optimize signaling environments during development. The proposed research will test the following hypotheses: (1) Wnt signaling effector function is critical for osteogenesis of the embryonic palate; and (2) loss of the up-stream master regulator of Wnt signaling homeostasis, Pax9, results in the disruption of palatal osteogenesis via up-regulation of sclerostin (*Sost*), a potent inhibitor of Wnt signaling and bone formation (Figures 1, 2). The specific aims of this proposal are as follows: (1) to define the spatio-temporal transcriptomic profile of embryonic palate osteogenesis via unbiased stage-specific signature mapping of cell populations in the normal murine secondary palate; (2) to differentially compare epigenomic, proteomic, and gene expression signatures of Wnt–related osteogenesis in *Pax9*−/− CP; and (3) to pilot novel *in utero* drug delivery approaches and molecules, based on the molecular profiles observed *in situ*, for the prenatal cure of CP. The proposed research will add novel foundational knowledge of multiomic morphogenetic expression gradients of key Wnt signaling regulators within the embryonic palate and will propose an innovative therapeutic model whereby palatal clefts may be corrected *in utero*.

The proposed research will improve unbiased mechanistic understanding of palatal bone development and lead to the first preclinical study of intra-amniotic small-molecule and antibody-replacement drug delivery for targeted palatal osteogenesis. The validation of a translational *in utero* drug delivery system for reversal of single-gene CP disorders will lead to preventive and corrective prenatal therapeutic interventions in humans.

Profiles of Wnt pathway gene expression during tooth morphogenesis

Mouse and human genetic studies indicate key roles of the Wnt10a ligand in odontogenesis. Despite the advances, little is known about the temporo-spatial modulation of Wnt10a and whether its expression profiles explain the reciprocal epithelial-mesenchymal signaling events that drive morphogenesis and cell differentiation. We systematically compared the profiles of Wnt10a in developing murine molars and incisors
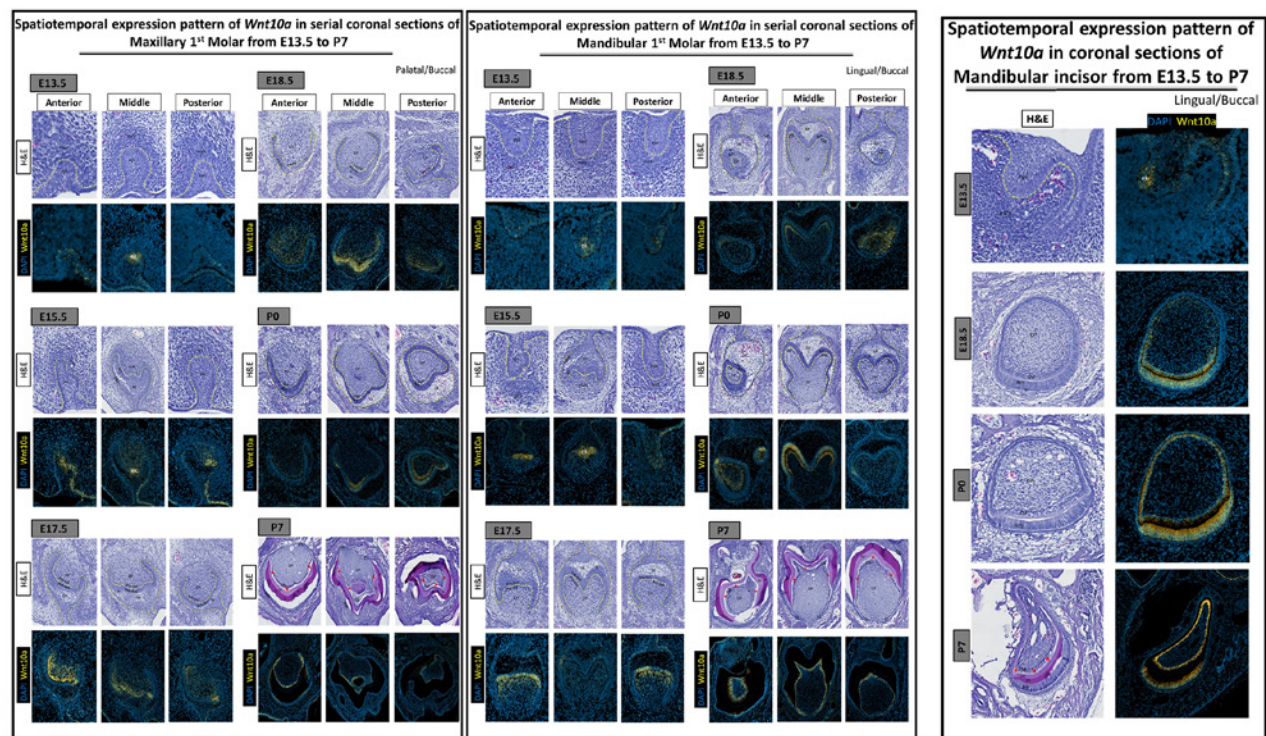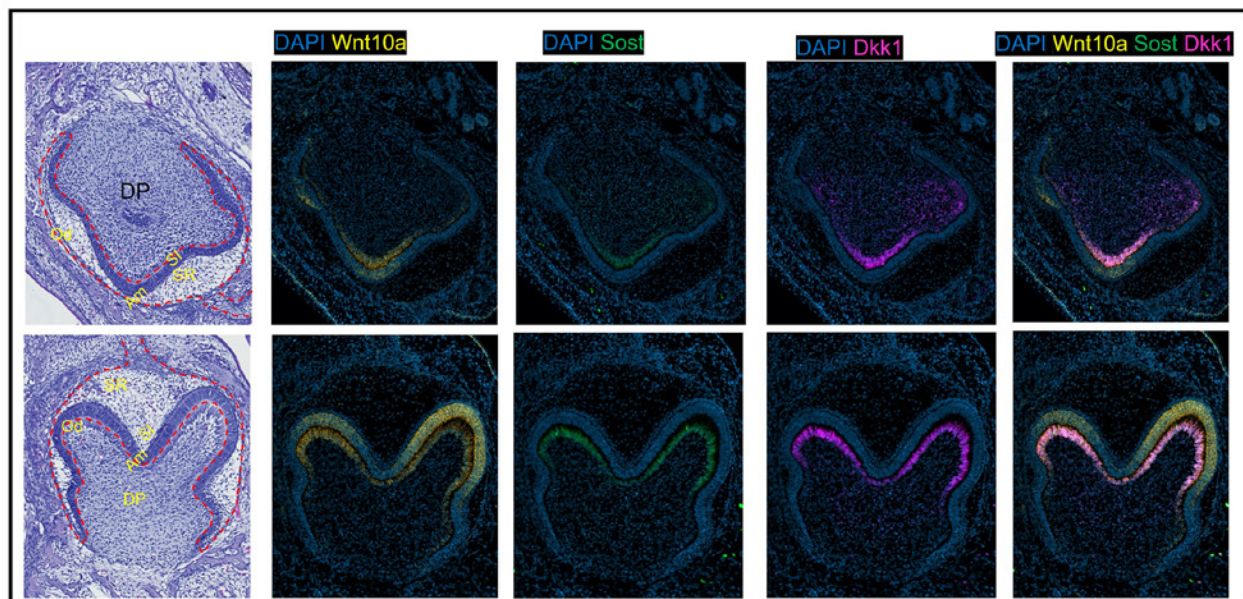
FIGURE 3. Wnt signaling drives palate osteogenesis and fusion.

DP: Dental papilla, SR: Stellate reticulum, SI: Stellate intermedium,, Od: Odontoblast, Am: Ameloblast
Note: All the data are not shown here
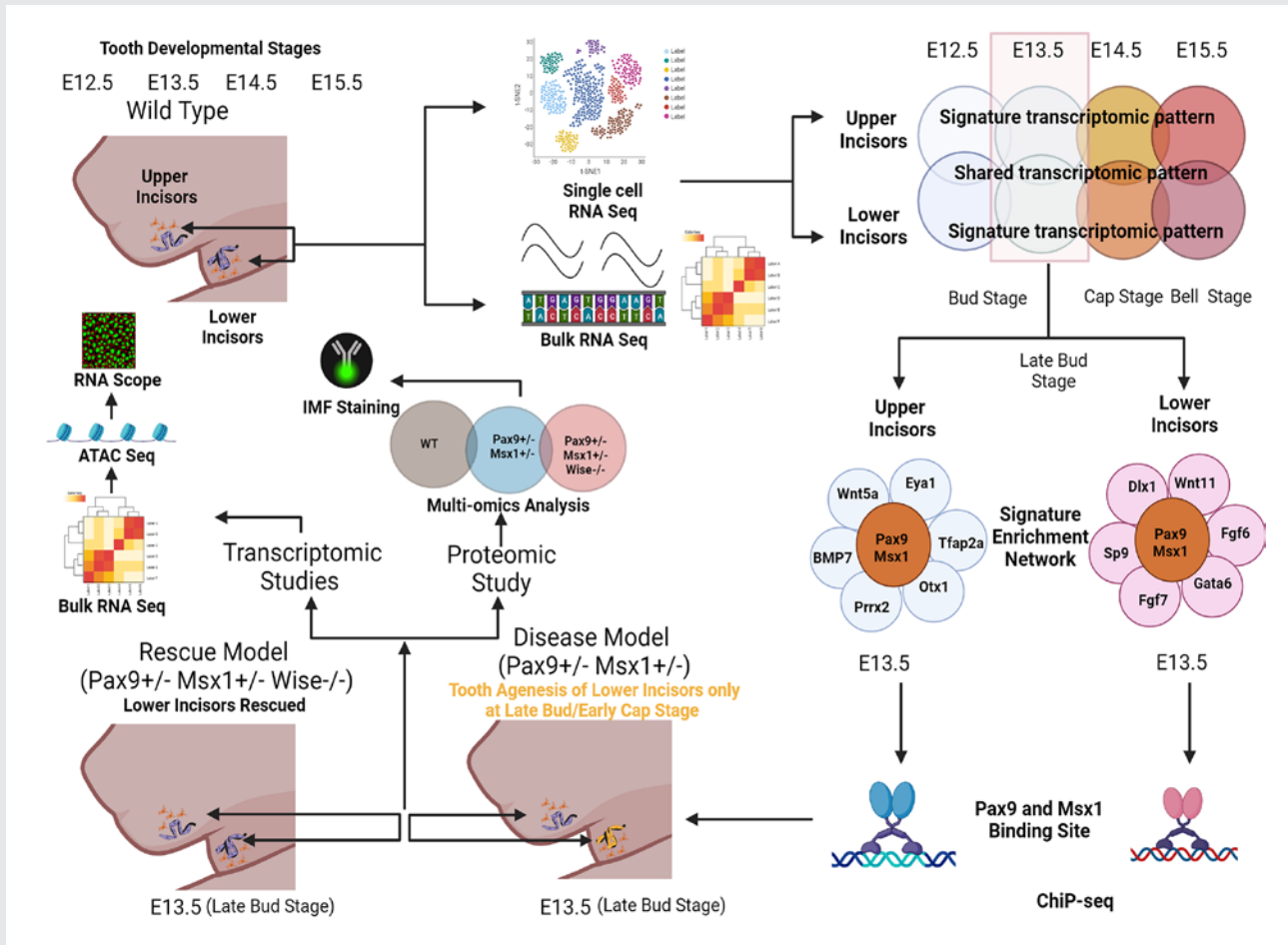
FIGURE 4. Wnt10a and Wnt modulators

FIGURE 5. *In vivo* molecular consequences of Sosdc1 deficiency on Pax9– and/or Msx1–dependent signaling events that control maxillary vs. mandibular incisors

with the Wnt signaling pathway inhibitors Sost and Dkk1, using multiplex *in situ* hybridization and single-cell RNA-sequencing (scRNA-seq). During tooth bud morphogenesis, Wnt10a transcripts were restricted to the epithelium, in contrast to the localization of Sost and Dkk1 to the dental mesenchyme. At E15.5, there was a marked shift of Wnt10a from dental epithelium to mesenchyme, while Sost and Dkk1 expression remained enriched in the mesenchyme. By E18.5 and P0 (postnatal day 0), Wnt10a expression coincided with the gradients of ameloblast and odontoblast differentiation from cusp to apical regions. Interestingly, Sost and Dkk1 co-expressed with Wnt10a in odontoblasts at these stages. At P7 and 14, following dentin and enamel mineralization, Wnt10a was confined to odontoblasts, while Wnt modulators were reduced or absent in the molars, but intense signals were continuously present in ameloblasts (Wnt10a) and odontoblasts (Wnt10a, Sost, and Dkk1) towards the proximal end of incisors near the cervical loop. scRNA-seq confirmed *in situ* expression signatures of target genes to clusters containing dental cells. These data provide cell type–specific insight into the role of Wnt signaling mediators during epithelial-mesenchymal interactions in odontogenesis. Our results provide a framework for future research on well timed therapies that target Wnt signaling for the reversal of tooth agenesis and for regenerating the dentin-pulp complex after injury.
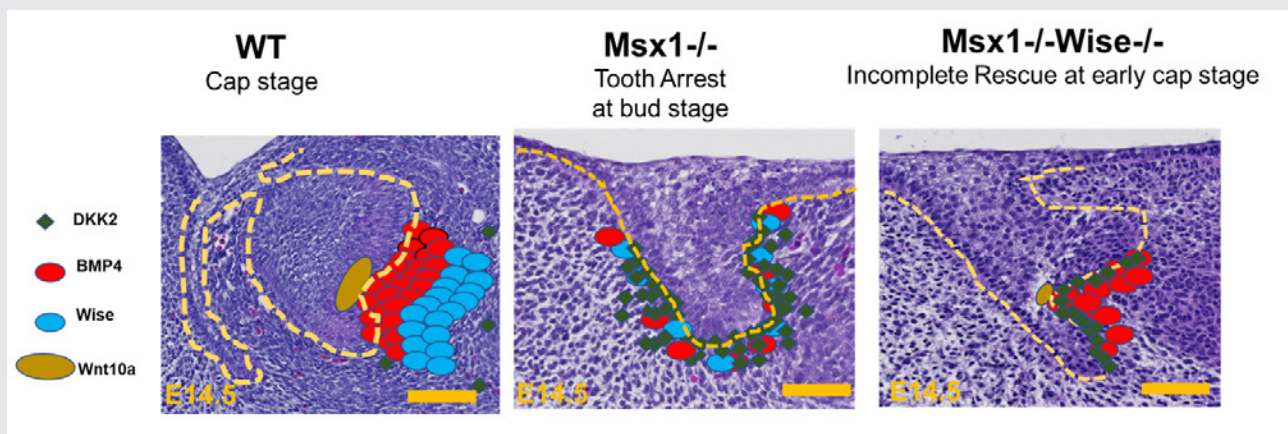
FIGURE 6. Genetic interaction between Msx1 and the Wnt signaling pathway during mandibular incisor development

In vivo molecular consequences of Sosdc1 deficiency on Pax9– and/or Msx1–dependent signaling events that control maxillary vs. mandibular incisors

Tooth agenesis is a developmental anomaly defined by the lack of one or more teeth (excluding third molars) resulting from to early failures in tooth formation. Several potential genes causing tooth agenesis have been studied. A familial autosomal dominant hypodontia (tooth agenesis) was demonstrated to be caused by a point mutation in the *MSX1* and *PAX9* genes. However, how MSX1 and PAX9 control the patterning of tooth agenesis requires further investigation. Interestingly, Pax9+/-/Msx1+/- mutant mice exhibit selective oligodontia (missing lower incisors only) and offer an excellent opportunity to study the Pax9– and Msx1–dependent tooth agenesis patterning. Concurrently, we and others have shown that the transcription factors Msx1 and Pax9 modulate the functions of Bmp and Wnt during odontogenesis in a mouse model. Interestingly, we found that transgenic inhibition of a known bifunctional BMP and Wnt antagonist, Sosdc1, was able to rescue the lower incisor in Pax9– and Msx1–deficient mice (Pax9+/-; Msx1+/-/Sosdc1-/-). However, how exclusively Msx1 and/or Pax9 interact with Sosdc1 in the mandibular incisor's domain vs. the maxillary incisor's domain requires further investigation. Therefore, our ongoing experiments (bulk RNA, multiplex *in situ* hybridization, and multiomics analysis) will explore the molecular consequences of Sosdc1 deficiency on Pax9– and/or Msx1–dependent signaling events in the maxillary vs. mandibular incisors domain.

Genetic interaction between Msx1 and the Wnt signaling pathway during mandibular incisor development

Tooth development is a distinct embryological process, which can be used as a model to study the multi-stage epithelial-mesenchymal interaction. The MSX1 transcription factor is essential in early tooth morphogenesis through bud-to-cap transitioning. Deleting the *Msx1* gene causes tooth arrest at the bud stage in mice. In mice deficient in *Msx1*, Bmp4 mRNA expression in the dental mesenchyme is abated at E13.5 and E14.5. Alternatively, deleting the Bmp and Wnt modulator Sosdc1 acts as an impetus to forming supernumerary teeth as a result of dysregulated Bmp and Wnt signaling activity in the rudimentary dental tissues. However, Msx1 interaction with Wnt modulators (Wise and/or Dkk2) in incisors has not been reported. Our multiplex

in situ hybridization results confirm the distinct interactions between Msx1 and the expression patterning of Wnt modulators (Sosdc1 and Dkk2) during lower incisor development (E13.5 and E14.5). Interestingly, at E14.5, Sosdc1 was found to be drastically downregulated in the Msx1$^{-/-}$ embryos. Alternatively, Dkk2 was found to be regulated differently than Sosdc1 in the lower incisiform of Msx1–deficient embryos (Msx1$^{-/-}$) at E14.5. Moreover, no alteration in the Dkk2 expression pattern at E14.5 was found in the lower incisiform of Msx1– and Sosdc1–deficient embryo (Msx1$^{-/-}$ Sosdc1$^{-/-}$) compared with Msx1$^{-/-}$ embryos. These data confirm that Msx1 is upstream of the Wnt modulator (Dkk2 and Sosdc1) and regulates Sosdc1 and DKK2 independently during the bud-to-cap stage transition.

Publications

1. Piña JO, Raju R, Roth DM, Winchester EW, Chattaraj P, Kidwai F, Faucz FR, Iben J, Mitra A, Campbell K, Fridell G, Esnault C, Cotney JL, Dale RK, D'Souza RN. Multimodal spatiotemporal transcriptomic resolution of embryonic palate osteogenesis. *Nat Commun* 2023 14:5687.
2. Raju R, Piña JO, Roth DM, Chattaraj P, Kidwai F, Faucz FR, Iben J, Mitra A, Campbell K, Fridell G, Dale RK, D'Souza RN. Profiles of Wnt pathway gene expression during tooth morphogenesis. *Front Physiol* 2023 14:1316635.
3. Reddy MS, D'Souza RN, Webster-Cyriaque J. A call for more oral health research in primary care. *JAMA* 2023 330:1629–1630.
4. Weintraub JA, Kaeberlein M, Perissinotto C, Atchison KA, Chen X, D'Souza RN, Feine JS, Ghezzi EM, Kirkwood KL, Ryder M, Slashcheva LD, Touger-Decker R, Wu B, Kapila Y. Geroscience: aging and oral health research. *Adv Dent Res* 2023 31:2–15.

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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 the cell type to adopt are controlled, and how 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 the most likely paths through gene expression that cells take during development [Farrell JA *et al, Science* 2018;360:eaar3131; Siebert S *et al*, *Science* 2019;365:eaav9314]. 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 with imaging, genetic, and classical embryological approaches to investigate the genetic control of cell specification and differentiation during vertebrate embryogenesis. We focus on zebrafish (*Danio rerio*) embryos as a model system in which to study these questions, because, among vertebrates, they are easy to culture, image, and manipulate, both embryologically and genetically.

Transcriptional diversity during zebrafish development

A critical step toward understanding the genetic programs that control cell specification and cell differentiation is to identify the different transcriptional cell types that are present during development, the genes expressed in each, and how their gene expression programs change over time in development. To this end, we generated a single-cell RNA-seq atlas spanning embryogenesis and early larval stages (62 timepoints from 3–120 hours post-fertilization), annotated over 300 cell types, and built a web portal (Daniocell) to enable

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other investigators to browse these data [Reference 3]. To better understand how cells acquire their cell type–specific features, we built a catalog of shared gene-expression programs that are re-used across many different tissues during development. We also identified transcriptional populations that are present for unusually long durations during development. We also performed focused analyses within a subset of tissues and uncovered the transcriptional profiles of several poorly characterized or unknown cell populations, including the pneumatic duct, distinct transcriptional subtypes of pericytes (a type of peri-vascular cell), *best4+* cells within the intestinal epithelium, and several layers of intestinal smooth muscle. We built developmental trajectories to describe the sequence of gene expression that likely gives rise to these cell types, and we used these trajectories to determine candidate regulatory factors that may drive their specification and which can be tested in future work.

In a separate project, in collaboration with the lab of Michal Rabani, we aimed to determine whether cell type–specific gene expression patterns could emerge from regulation of mRNA destruction as well as transcription. To do so, we investigated the destruction of maternally provided mRNAs that run early development, many of which are degraded at the maternal-to-zygotic transition, when the zygotic genome activates and cells begin transcribing their own mRNA. We combined 4sU RNA labeling with single-cell RNA-seq, enabling us to distinguish between maternally provided mRNA (which is unlabeled) and zygotically produced mRNA (which incorporates the 4sU label that we provide after fertilization) [Reference 4]. By profiling development at several time points, we built kinetic models that allow comparison of rates of maternal mRNA destruction between different genes and also between different cell types. Rates of destruction of maternal mRNAs vary widely, with transcription rates often being matched to produce constant mRNA levels. Maternal mRNA stability has long been known to differ in the primordial germ cells; most early germ cell–specific mRNAs are maternally deposited and specifically stabilized in that cell type. However, we found evidence that some maternal mRNAs are also stabilized in the enveloping layer, another cell type that differentiates early in zebrafish development.

Development and function of *best4+* cells

best4+ cells are a recently identified (2019) epithelial population in human intestines with undefined function and developmental program. The cells are dysregulated in disease: they are depleted in inflammatory bowel disease and their characteristic genes are upregulated in colorectal cancer patients. However, it is unclear whether these changes are causes or consequences of disease. We will use zebrafish as a model, where we and others recently identified homologs of *best4+* cells. We identified that zebrafish and human *best4+* cells have similar location, regionalization, and gene expression [Reference 3]. Zebrafish's advantages are: (1) their intestines are functionally similar to mammals but optically clear, facilitating experimental observation; (2) *best4+* cells are missing in popular rodent intestinal models; (3) experiments can be performed *in vivo*, in regionalized intestines with surrounding mesenchyme and smooth muscle, which produce many of the key signals that regulate the intestinal epithelium. We aim to: (1) determine the effects on the intestine of *best4+* cell loss by genetically ablating *best4+* cells; (2) determine the effects of inflammation on *best4+* cells; (3) build a gene-regulatory network (GRN) of intestinal epithelial specification; and (4) determine the developmental program that specifies *best4+* cells. Our results will help identify the function and role in disease of *best4+* cells and suggest how to modulate *best4+* cell function or cell number to potentially treat disease.

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 [Reference 1]; 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 [Satija *et al. Nat Biotechnol* 2015 May;33:495]. 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 the question as to 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.

Publications

1. Farrell JA, Wang Y, Riesenfeld SJ, Shekhar K, Regev A, Schier AF. Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science* 2018 360:eaar3131.
2. Siebert S, Farrell JA, Cazet J, Abeykoon Y, Primack A, Schnitzler C, Juliano CE. Stem cell differentiation trajectories in Hydra resolved at single-cell resolution. *Science* 2019 365:eaav9314.
3. Sur A, Wang Y, Capar P, Margolin G, Farrell JA. Single-cell analysis of shared signatures and transcriptional diversity during zebrafish development. *bioRxiv* 2023 10.1101/2023.03.20.533545.
4. Fishman L, Nechooshtan G, Erhard F, Revev A, Farrell JA, Rabani M. Single-cell temporal dynamics reveals the relative contributions of transcription and degradation to cell-type specific gene expression in zebrafish embryos. *bioRxiv* 2023 10.1101/2023.04.20.537620.
5. Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene expression data. *Nat Biotech* 2015 33:495–502.

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Nervous System Development and Plasticity

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 the 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 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. Furthermore, in response to the urgent call for NIH intramural scientists to devote effort to combating the world-wide SARS-Cov2 viral pandemic, our laboratory, in collaboration with others on the NIH campus and in Ecuador, developed a novel nanocellulose material that blocks SARS-Cov2 and HIV viral infection in cell culture.

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 is 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 myelin formation. Most recently, we determined that myelin thickness can be adjusted through a treadmilling process, which adds and removes layers of myelin from the sheath and alters nodes of Ranvier, where

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neural impulses are generated along axons. In studies on the visual system, our research shows that this process adjusts conduction velocity and improves functional performance by optimizing spike-time arrival at synapses. This new form of nervous system plasticity and learning is particularly important in childhood development, but it also operates in adulthood.

The discovery of activity-dependent myelin plasticity presents three major questions for this new field of research: 1) Synaptic plasticity is governed by spike timing–dependent plasticity learning rules (STDP), arising from Hebbian theory. What is the equivalent theory underlying myelin plasticity? 2) If myelin plasticity promotes information processing and learning by optimizing synchrony of spike time arrival at synaptic relay points, how can oligodendrocytes 'know' that optimal synchrony has been achieved? 3) Do changes in myelin accompany synaptic plasticity that is observed in well established experimental models? This year we obtained experimental and theoretical findings on all three of these major questions.

Learning is perhaps the most important function of childhood, and synaptic plasticity is fundamental to that process. Our research on synaptic plasticity is determining the molecular and cellular mechanisms that convert short-term into long-term memory. Similarly, 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, regulates 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 transmission 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 oscillation (brain waves), myelin-forming glia
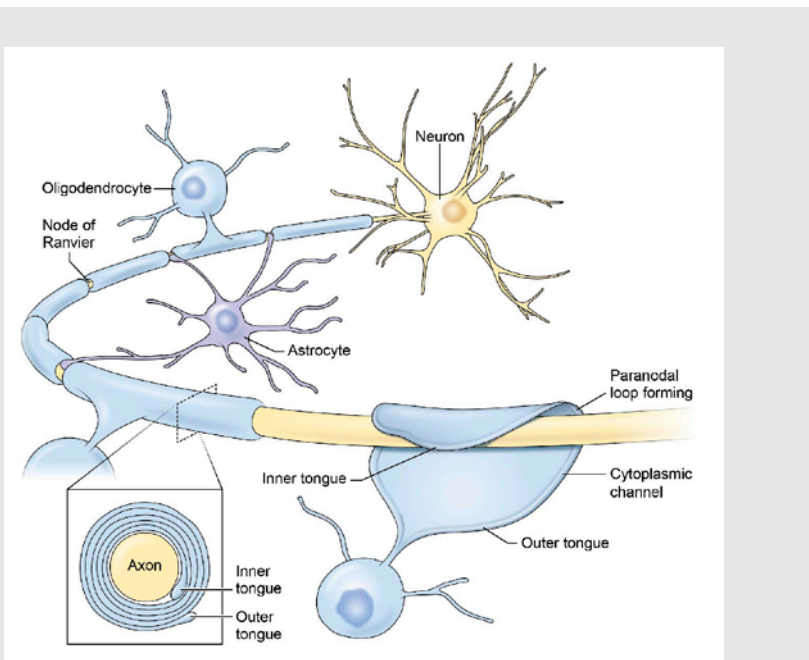

FIGURE 1. The structure of CNS myelin and nodes of Ranvier

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 a critical part of brain development, but the processes controlling myelination of appropriate axons are not well understood. Myelination begins in the late fetal period and continues throughout childhood and adolescence, but myelination of some brain regions is not complete until an individual's early twenties.

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, as, for example, in schizophrenia, epilepsy, dyslexia, and autism.

Our research shows that neurotransmitters are released not only at synapses but also along axons firing action potentials, to activate receptors on myelinating glia as well as astrocytes and other cells. 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. The process would be particularly important in the adolescent brain, where environmental experience during sensitive periods can have long-lasting effects on neural circuit development and behavior. The findings are also relevant to such demyelinating disorders as multiple sclerosis and to 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 and gene expression analysis, *in vivo* and in primary cell cultures.

MODIFICATION OF MYELIN STRUCTURE AND CONDUCTION VELOCITY BY ASTROCYTES

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. Conduction velocity in myelinated axons depends on the thickness of the myelin sheath and on the morphology

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 [Fields RD, Dutta DJ *Trends Neurosci* 2019;42:443].
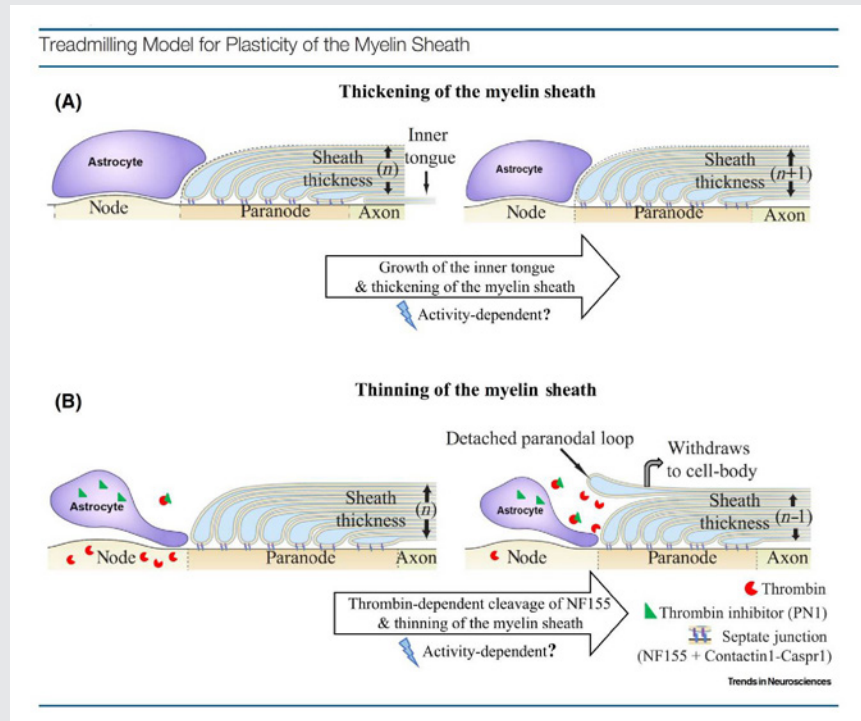
of the electrogenic nodes of Ranvier (periodic gaps in the myelin sheath acting as neural transmission relay points along axons). Our research shows that myelination of unmyelinated axons and the thickness of the myelin sheath can be increased in response to neural activity and environmental experience. 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). 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 uncovered 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.

THEORETICAL ADVANCES IN MYELIN PLASTICITY

Spike timing–dependent plasticity (STDP) learning rules govern activity-dependent changes in synaptic strength that underly learning and memory. An equivalent theory guiding myelin plasticity is lacking. Moreover, how oligodendrocytes situated along axons far from synaptic terminals could optimize synchrony of spike time arrival is unknown. In collaboration with our intramural colleagues in NICHD and NIMH, we put forth an oligodendrocyte myelin plasticity (OMP) model that addresses these questions. An oligodendrocyte myelinates multiple axons simultaneously through many slender cell processes extending from an individual cell, which rarely forms myelin on the same axon twice. We propose that this unusual morphology enables oligodendrocytes to sense the arrival of neural impulses at each of its cellular processes and to modify myelin to increase synchrony of action-potential firing among all axons within its domain. The process is extended serially through the chain of oligodendrocytes along the entire length of the axon. Our mathematical simulations show that the process results in greatly increased synchrony of spike time arrival at nerve terminals.

MYELIN PLASTICITY ACCOMPANYING WELL ESTABLISHED MODELS OF SYNAPTIC PLASTICITY

An implicit assumption in all prior research on synaptic plasticity is that neural-impulse transmission speed is fixed, and not altered by environmental experience. The discovery of myelin plasticity requires a re-examination of that assumption to determine whether or not myelin plasticity accompanies changes in synaptic plasticity. Studies on visual deprivation in kittens provided the fundamental understanding of how environmental experience and differences in the timing of neural impulses from the two eyes arriving on binocular neurons alters synaptic strength and connectivity. Using binocular and monocular visual deprivation in mice, in addition to monocular action-potential inhibition by transfecting a potassium channel into retinal ganglion neurons, we found that changes in myelin are evident in optic nerve and optic tract axons in a manner that is compatible with changes in synaptic strength. Thus, myelin plasticity contributes to, or could even drive, the changes in synaptic strength observed following these different types of visual deprivation.

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. Little is known about how neural firing patterns regulate gene expression. Our experiments are revealing that intracellular signaling and gene-regulatory networks 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 to measure 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 abundance significantly; nevertheless, the coordinated activity of gene-expression levels 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. We are exploring how chromatin structure is altered by neural impulse firing and how chromatin structure differs between neurons and glia.

Nanocellulose block of SARS-Cov2 and HIV infection

An important world-wide imperative is to develop methods to combat the SARS-Cov2 viral pandemic. In particular, new methods that have broad anti-viral action and are resistant to mutations in viruses that circumvent immunization are especially important. In response to the urgent need to develop methods for combatting Covid-19, our laboratory developed a new method to block infection of cells by SARS-CoV-2. We developed a compound that binds to both the SARS-CoV-2 and the HIV virus and that this prevents cellular infection. Nanocellulose derived from the ivory nut endosperm of a South American palm yields thin nanoparticles (from 1–5 nm width), which provide a high surface area for entrapment of microbes. We determined that the substance is a universal microbe binder, which prevents viral infection of cells in culture. The material can encapsulate the S protein of SARS-CoV-2, as well as whole SARS-CoV-2 and HIV-1 virions, thereby blocking infection of cells. These results were achieved through collaboration with our NIH intramural colleagues and Javier Carvajal Barriga of the Pontificia Universidad Católica del Ecuador, who conducted these experiments as a special volunteer in our laboratory.

Additional Funding

Publications

1. Pajevic S, Plenz D, Basser PJ, Fields RD. Oligodendrocyte-mediated myelin plasticity. *eLIFE* 2023 12:e81982.
2. Munyeshyaka M, Fields RD. Oligodendroglia are emerging players in several forms of learning and memory. *Commun Biol* 2022 5(1):1148.
3. Santos EN, Huffman WC, Fields RD. Effects of visual deprivation on remodeling of nodes of Ranvier in

optic nerve. *eNeuro* 2022 9(6):ENEURO.0194-22.2022.

4. Santos EN, Huffman WC, Fields RD. Recovery of node of Ranvier structure in optic nerve under visual deprivation. *Investigative Opthalmology and Visual Science* 2023; under review.

5. Carvajal-Barriga EJ, Fitzgerald W, Dimitriadis EK, Margolis L, Fields RD. Sulfated endospermic nanocellulose crystals prevent the transmission of SARS-CoV-2 and HIV-1. *Sci Rep* 2023 13:6959.

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Translational Biophotonics in Developmental Disorders and Diseases

Brain imaging and spectroscopy of developmental disorders

Utilizing functional near-infrared spectroscopy (fNIRS) in conjunction with complementary neuroimaging techniques, we investigated neural activity in two distinct cohorts: (1) a group of healthy volunteers; and (2) infants and children, both with and without developmental disorders. The primary aim of the first cohort was to validate fNIRS findings through comparison with previous results obtained from functional MRI (fMRI) and EEG studies, while the focus of the second cohort was to comprehensively explore the typical and atypical developmental trajectories in infants and children, including those at elevated risk for developmental disorders.

Before conducting fNIRS studies, we engaged in an exhaustive assessment of contemporary fNIRS findings pertaining to the tracking of neurodevelopmental trajectories in infants and children, encompassing those with or without developmental disorders [Reference 1].

In relation to the adult cohort, we successfully conducted a pilot study encompassing 30 healthy volunteers, aimed at assessing the viability of fNIRS for the study of the mirror neuron network (MNN). The culmination of our endeavors revealed compelling outcomes. Specifically, our findings highlight parietal regions, including the bilateral superior parietal lobule (SPL), bilateral inferior parietal lobule (IPL), the right supra-marginal region (SMG), and the right angular gyrus (AG) as potential candidate regions within the human MNN. Furthermore, we effectively demonstrated the paradigm's capacity to discern variations in subclinical levels of autistic traits, presenting an avenue for subsequent exploration in clinical populations characterized by challenges in action comprehension and representation, notably autism spectrum disorders (ASD) [Miguel *et al.*, *PloS One* 2021;16:e0253788]. Subsequent to this, a supplementary analysis utilizing a connectivity approach was undertaken [Nguyen *et al.*, *Brain Sci* 2021;11:397]. Notably, our findings revealed significant interplay between various brain regions during action execution, with distinct region-to-region connections within the left hemisphere apparent while participants executed actions with their right hand. These connections included interactions within the left precentral, left postcentral, left inferior parietal regions, as well as between the left supramarginal and left angular regions.

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(continued)

To comprehensively delineate the MNN utilizing concurrent EEG and fNIRS signals, we developed a multimodal, multiset data-fusion analysis, strategically leveraging the strengths of each neuroimaging modality, that is, the EEG's exceptional temporal resolution and the fNIRS's superior spatial resolution. In this pursuit, we harnessed the well established Mu suppression phenomenon as an indicator of MNN activation in EEG–related neural activity. By synergistically employing EEG and fNIRS, each modality contributing its unique attributes, we achieved a more refined understanding of MNN development. Specifically, we applied a structured sparse multiset canonical correlation analysis (ssmCCA) model to our merged dataset, revealing congruent activity patterns during both action execution and observation across designated regions of interest. These

Marc Bornstein, PhD, *Special Volunteer*

Han-Shin Hahn, PhD, *Special Volunteer*

Kosar Khaksari, PhD, *Special Volunteer*

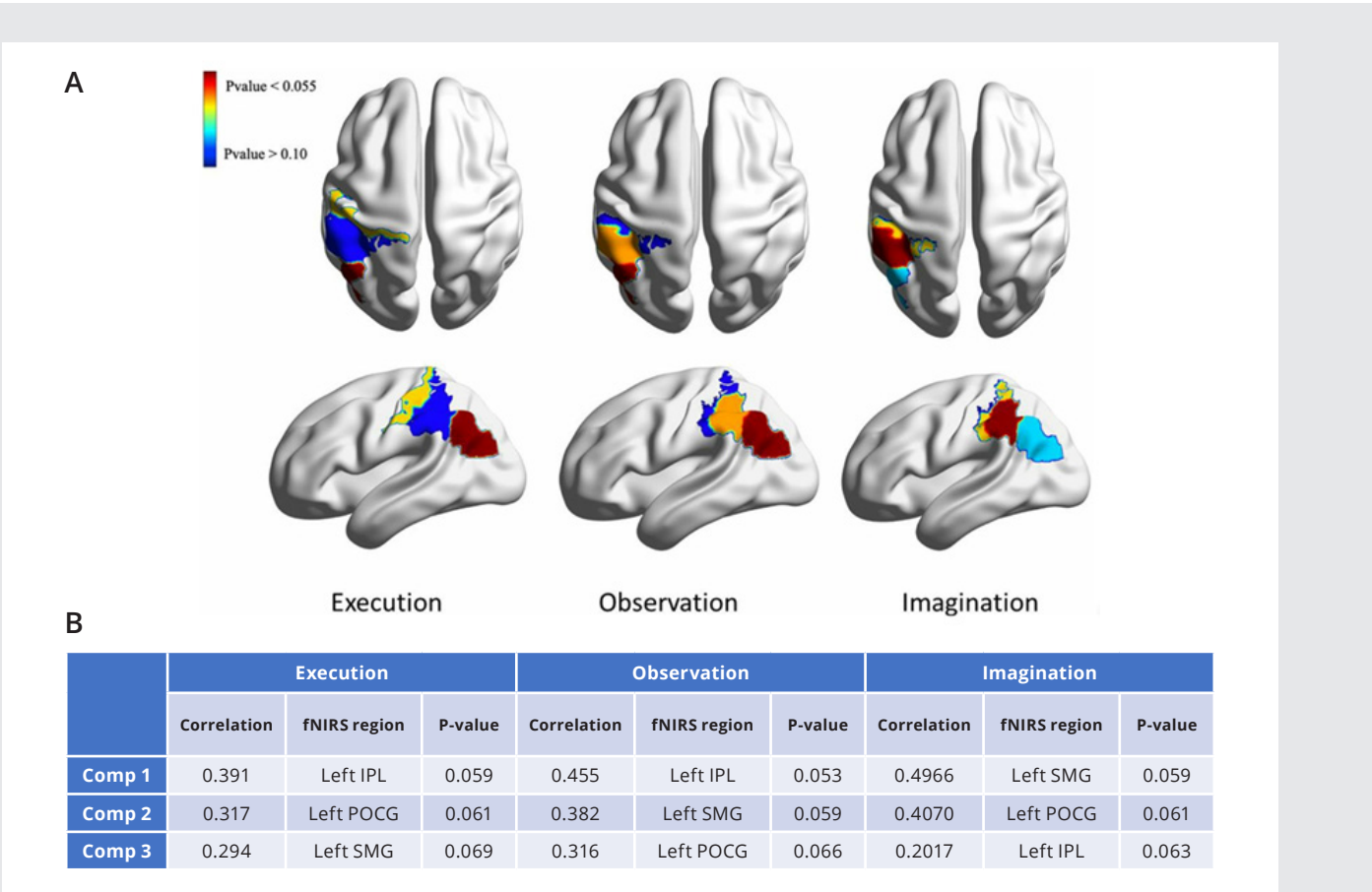Siamak Aram, PhD, *Guest Researcher*

	Execution			Observation			Imagination		
	Correlation	fNIRS region	P-value	Correlation	fNIRS region	P-value	Correlation	fNIRS region	P-value
Comp 1	0.391	Left IPL	0.059	0.455	Left IPL	0.053	0.4966	Left SMG	0.059
Comp 2	0.317	Left POCG	0.061	0.382	Left SMG	0.059	0.4070	Left POCG	0.061
Comp 3	0.294	Left SMG	0.069	0.316	Left POCG	0.066	0.2017	Left IPL	0.063

FIGURE 1. Cross-modality correlations and associated p-values during motor execution (ME), motor observation (MO), and motor imagery (MI) (*A*), and color-coded regions associated with execution, observation, and imagination (*B*).

The color bar refers to the p-values of the correlations in the region. Moderate correlations were found over the left inferior parietal lobe (IPL) (r=0.39; p=0.059), left posterior central gyrus (POCG) (r=0.38, p=0.061), and left supramarginal region (SMG) (r=0.29, p=0.069) during ME; over the left IPL (r=0.46; p=0.053), left SMG (r=0.38; p=0.059), and left POCG (r=0.32; p=0.066) during MO; and over the left SMG (r=0.50, p=0.059), left POCG (r=0.41; p=0.061), and left IPL (r=0.20, p=0.063) during MI.

results underscored heightened brain activity within the left hemisphere's paracentral, precentral, and inferior and superior parietal regions during action execution. This was the first report that fused distinct brain metrics (hemodynamic response function and electrical activity) characterize the MNN in the human brain [Dashtestani *et al. Sci Rep* 2022;12:6878]. Subsequent to our in-depth exploration of brain activity patterns during action execution and observation, we investigated the imagination condition in relation to both action execution and observation. The intrinsic value of introducing this dimension lies in the belief that motor cognitive abilities hinge on, and are cultivated through, the mechanisms of mental rehearsal and re-enactment of action execution. Our findings indicated the pivotal role of the left parietal inferior region as the primary contributor within the imagination condition. Concurrently, additional brain regions within the left hemisphere exhibited activation during the motor imagery task, mirroring the patterns witnessed during execution and observation conditions (Figure 1). Despite the relatively limited literature on action imagination, our outcomes harmonize with existing studies, validating the notion that both action observation and imagination stimulate the same sensory-motor cortical network that underpins the execution of the corresponding action. One plausible explanation for these consistent brain activity patterns across the three conditions is attributed to the internal rehearsal of action, reinforcing the intrinsic connection between observation, imagination, and execution [Reference 2].

COVID-19 point-of-care biosensor

At the beginning of the COVID-19 pandemic, our section proposed to develop a biosensor to monitor patients with respiratory infectious diseases such as COVID-19. We designed a wearable and wireless device, which can measure body temperature, chest movement, cardiac and respiratory functions, and tissue oxygenation parameters. We then conducted a clinical protocol entitled 'A Pilot Study to Evaluate a Noninvasive Multimodal Biosensing Device for Screening and Monitoring Response to Treatment of Infectious Respiratory Diseases' to evaluate performance of the device in healthy subjects at rest and during induced hypercapnia, breath holding, and paced breathing. Thirty-six volunteers were enrolled in the study, and measurements were performed on 26 participants. We are currently performing data analysis on the collected data.

In parallel with research at NIH, we were collaborating with Babak Shadgan to collect data on 24 healthy volunteers during normal breathing, restricted breathing, and rapid breathing through a clinical protocol. Tissue oxygenation levels and hemodynamics responses, including oxy-, deoxy-, and total hemoglobin, were measured using an NIRS device. The device consists of a multi-wavelength LED emitting light at 760 nm and 850 nm, and three photodiode detectors 3, 3.5, and 4 cm away from the LED. The NIRS device was placed over the participant's chest during data acquisition. In order to obtain signals similar to the breathing patterns experienced by COVID-19–infected patients, three breathing patterns, i.e., normal breathing, restricted breathing, and rapid breathing, were defined. In our section, two deep-learning classification algorithms for breathing patterns were developed based on convolutional neural network (CNN). The first algorithm is a classification model consisting of 113 layers. The residual concept was applied to prevent the vanishing gradient problem that occurs when the depth of the deep-learning model increases. 64-length (corresponding to 6.4 seconds) oxy-hemoglobin data signals were used to learn the classification model. We achieved a classification accuracy of 91.77% using this model [Reference 3].

The second model is an evolved version of the first model. The model improves classification accuracy by using additional compressed features that can form the data. We added an auto encoder model to generate compressed features of the data. The features compressed in the auto-encoder were fused with the features
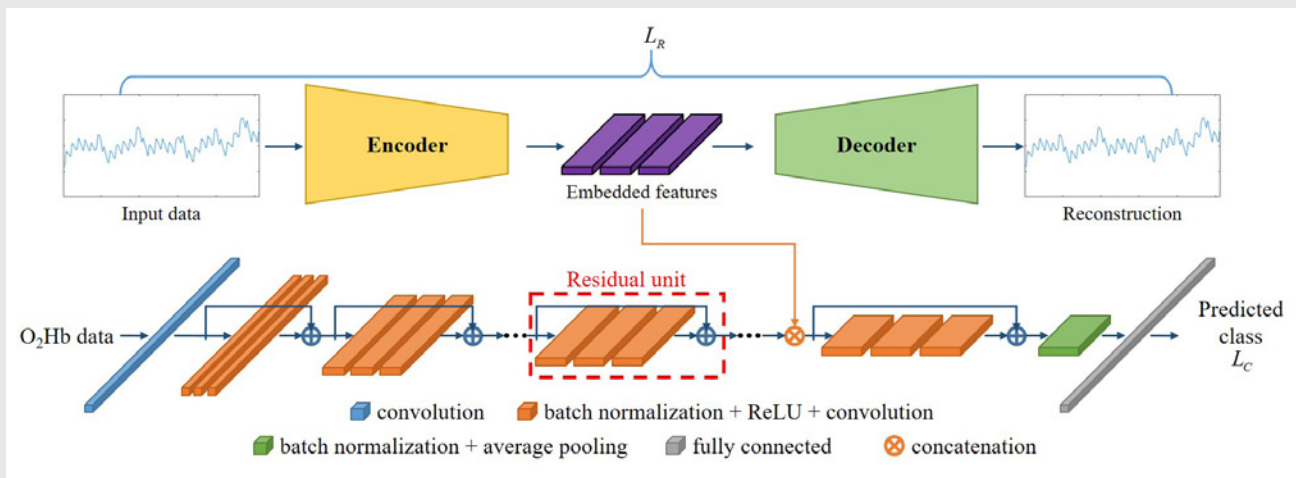
FIGURE 2. Architecture of deep learning model to classify different breathing patterns

Architecture of breathing pattern classification model with auto-encoder: The developed model consists of an auto-encoder network for generating compressed features and a classification network for data class classification. The auto encoder's encoder network is trained to compress data, and the decoder network is trained to reconstruct the input signal. The embedded features generated from the encoder are combined with the features generated from the classification network and then used to classify the classes.

generated by the first classification model (Figure 2). This model was trained on the 64-length oxy-hemoglobin data signals. We achieved an accuracy of 92.34% using this model.

Placenta oxygenation: from basics to point of care

Placental insufficiency is a condition in which the placenta fails to provide adequate nutrients or oxygen to the fetus, a condition that is associated with pregnancy complications such as fetal growth restriction, fetal death, preterm labor, and other complications. However, there is no available technology to assess placental condition. Our section proposed the use of NIRS to measure the placental oxygenation level, and then to utilize this parameter to evaluate the condition of the placenta. NIRS is a noninvasive optical technique to measure tissue hemodynamics, including oxy- and deoxy-hemoglobin concentration and tissue oxygenation. In a typical NIRS device, there is a light source, which shines NIR light into tissue, and a light detector, which detects reflected light. Based on the distinct absorption spectrum of oxy-hemoglobin and deoxy-hemoglobin in the NIR range, we can derive concentration of these two parameters, and hence tissue oxygenation. A major problem in the assessment of placental oxygenation using NIRS arises from the anatomical location of the placenta, which lies below skin, adipose tissue, and the uterine wall. Considering these tissues above the placenta, we designed several models of a wearable depth-resolving NIRS device, featuring 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. Our first model includes light sources with two wavelengths of 760 nm and 840 nm, which are sensitive to changes in blood oxy-hemoglobin and deoxy-hemoglobin. Using this model, under the protocol #090717MP4E, which was approved by the Wayne State University Human Investigations Committee Institutional Review Board (IRB), tissue oxygenation was measured in the placenta of 12 singleton pregnant women in their third trimester at the Center for Advanced

Obstetrical Care and Research of the Perinatology Research Branch, located at the Detroit Medical Center [Nguyen *et al.*, *Biomed Optic Exp* 2021;12:4119]. Five of these women had maternal pregnancy complications. Our preliminary results indicated a significantly lower tissue oxygen saturation level in the placenta of patients with complicated pregnancies (69.4% ± 6.7%) than in the placenta of their peers with normal pregnancies (75.0% ± 5.8%). After delivery, 10 of the 12 participants' placentas were delivered to the pathology department at the Detroit Medical Center to inspect for lesions. Five placentas were found to have chronic or acute lesions, four of which belonged to participants with maternal pregnancy complications. We further found that patients with lesion-free placentas presented a significantly higher placental oxygen saturation (74.2% ± 5.8%) than patients with lesions (68.7% ± 5.6%). The results suggest a relationship between the placental oxygen saturation and pregnancy complications and placental pathology. However, with our first model, we need to use data collected from two distances (e.g., 30 and 40 mm distances) to calculate placental oxygenation level. As a result, calculated placental oxygenation level was transabdominal.

In order to target different tissue depths, we developed a method called 'single source-detector separation' (SSDS), which uses one distance with three wavelengths to calculate tissue oxygenation level (StO_2) [Reference 4]. The method was tested using data from Monte-Carlo simulation with an average accuracy of 97%. We then performed StO2 measurements on seven healthy volunteers in the prefrontal cortex during a simulated hypercapnia test using a continuous wave (CW) NIRS device. The device consists of a light source and two photodetectors, which are 30 mm and 40 mm away from the light source. The cerebral oxygen saturation was calculated using both a conventional spatially resolved spectroscopy (SRS) approach, which uses the reflected intensities at both separations, and the SSDS approach, which employs the reflected intensities at either 30 mm or 40 mm separation. The SRS–based StO_2 calculation was similar to the value calculated from the SSDS method (average difference = 5.0% ± 1.1%). We submitted a provisional patent application for the SSDS method (E-037-2023-0-US-01).

In addition, we upgraded our first model wearable device so that light sources include three wavelengths: 735, 810, and 850 nm. The second model has a data acquisition rate of 20 Hz, which allows collection of high frequency signals such as maternal and fetal heart rates. An accelerometer was added to the second model

FIGURE 3. Data acquisition and data processing method to measure placental oxygenation level

Probing different tissue layers is critical to distinguish between maternal and placental oxygenation. Monte-Carlo methods are used to create a look-up table for this purpose.
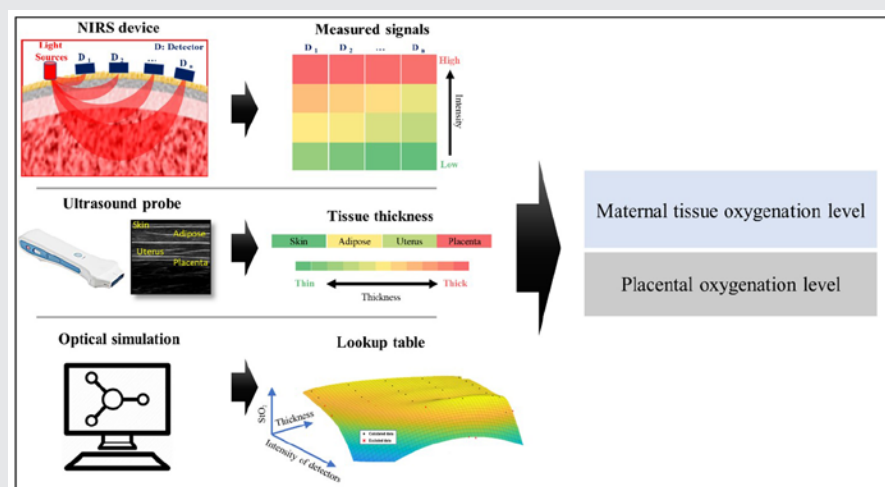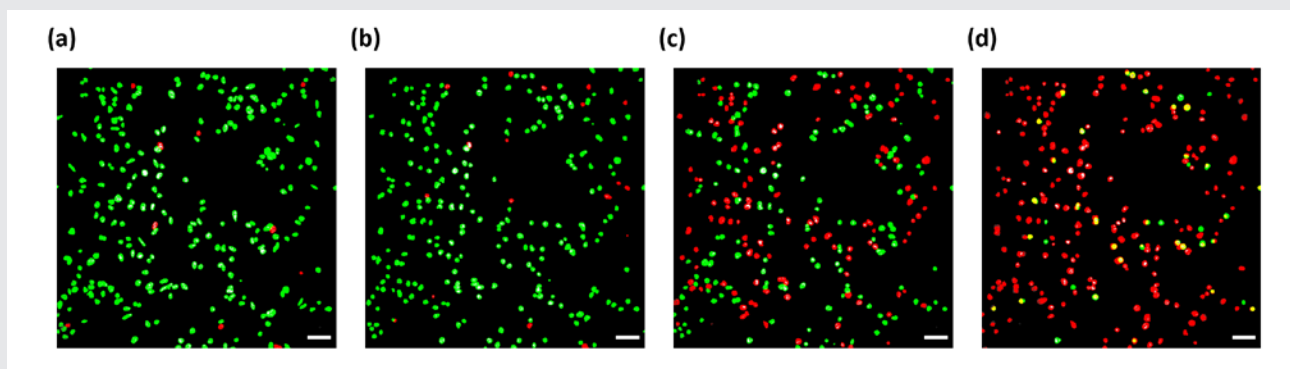
FIGURE 4. DFFOCT for cell viability assay

Assessment of cell viability state over by trained Logistic Regression model. After (*a*) 0-hour 15-minute, (*b*) 2-hour, (c) 4-hour, and (*d*) 6-hour time without CO_2 and maintained at 27°C. Green and red labels indicate live and dead cells, respectively. The inset white scale bar represents 100 mm.

to detect fetal movement. A provisional patent application was submitted for the second model (E-198-2022-0-US-01) (Figure 3). This model has been deployed to perform a one-time measurement of 24 pregnant women at their second and third trimesters at the Detroit Medical Center. Ten of the women in this cohort had maternal complications, including chronic hypertension, asthma, type II diabetes, renal failure with dialysis, and prolactinoma, and five had preeclampsia with severe features. The experimental procedure was performed in the same way as the previous measurement, where the participant lay down on the examination bed in a supine position. After delivery, the placentas of 22 participants were sent to a pathology laboratory to examine for lesions. Seventeen placentas had issues such as acute and/or chronic inflammatory lesions, acute funisitis and vasculitis, placental infarct, and lesions associated with maternal vascular malperfusion. In general, all 24 participants had either maternal complications, placental issues, and/or neonatal complications. The average oxygen saturation of the placenta of all participants was 68.9 ± 4.2%, which was similar to the oxygen saturation level found in the group of pregnant women with maternal complications and/or placental lesions in the first measurement.

We are in the process of designing an analysis algorithm that assesses the behavior of placental cells while taking into account different oxygen levels. The analysis is conducted in conjunction with the dynamic full-field optical coherence tomography (DFFOCT) system, simultaneously measuring placental oxygenation using the NIRS device. We are developing an algorithm designed to analyze changes in the dynamic activity (frequency and magnitude) of cellular organelle and derive a weighted mean frequency as a representative value. In our preliminary experiments, we evaluated cell viability using HeLa cells, a commonly employed immortalized human cell line in cell research. The approach we introduced is a label-free, non-invasive observation technique that permits precise quantitative analysis of cell dynamic activity without any impact on the cells themselves. Using this method, we effectively differentiated shifts in the survival status of HeLa cells by using parameters that characterize the cells' dynamic activity, with a focus on mean frequency [Park *et al*. *Biomed Optic Exp* 2021;112:6431]. Additionally, data obtained through DFFOCT is not biased by algorithmic models and has been successfully used in a variety of machine learning models, enabling highly accurate

cell viability determinations. Based on our findings, we believe that DFFOCT can serve as a valuable tool for assessing variations in the dynamic activities of placental cells in relation to oxygen saturation. We hypothesize that there is a relationship between the dynamic activity of placental cells and potential neurodevelopmental disorders. In our more recent research, we explored the dynamic activity of HeLa cells by subjecting them to varying oxygen concentrations within the range of 1% to 20%, while keeping other growth conditions constant, such as temperature and a 5% CO_2 environment (Figure 4). Our results reveal that the dynamic cellular movements observed at oxygen concentrations of 1% and 20% exhibit a significant difference of over 84%. We are currently analyzing the patterns of changes across different oxygen concentrations. In our future research, we plan to conduct a comparative analysis of dynamic activities in placental cells with a focus on oxygen levels, building upon the insights gained from our observations in HeLa cells (Figure 4).

Additional Funding

- Bench to Bedside Award 345 (2016): "Mirror neuron network dysfunction as an early biomarker of neurodevelopment" (ongoing)
- Human Placenta Project–NICHD (2016, ongoing)
- Scientific Director's Award

Publications

1. Su WC, Colacot R, Ahmed N, Nguyen T, George T, Gandjbakhche A. The use of functional near-infrared spectroscopy in tracking neurodevelopmental trajectories in infants and children with or without developmental disorders: a systematic review. *Front Psychiatry* 2023 14:121000.
2. Su WC, Dashtestani H, Miguel HO, Condy E, Buckley A, Park S, Perreault JB, Nguyen T, Zeytinoglu S, Millerhagen J, Fox N, Gandjbakhche A. Simultaneous multimodal fNIRS-EEG recordings reveal new insights in neural activity during motor execution, observation, and imagery. *Sci Rep* 2023 13:5151.
3. Park J, Mah AJ, Nguyen T, Park S, Ghazi Zadeh L, Shadgan B, Gandjbakhche AH. Modification of a conventional deep learning model to classify simulated breathing patterns: a step toward real-time monitoring of patients with respiratory infectious diseases. *Sensors* 2023 23:5592.
4. Nguyen T, Park S, Hill B, Gandjbakhche A. Single source-detector separation approach to calculate tissue oxygen saturation using continuous wave near-infrared spectroscopy. *IEEE Open J Engineering Med Biol* 2023 99:1–7.
5. Park S, Veluvolu V, Martin WS, Nguyen T, Park J, Sackett DL, Boccara C, Gandjbakhche A. Label-free, non-invasive, and repeatable cell viability bioassay using dynamic full-field optical coherence microscopy and supervised machine learning. *Biomed Optics Express* 2022 13:3187–3194.

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Predictors of Bone Health in Adolescents and Young Adults

The major aim of our lab is to understand adolescence factors that impact bone density and skeletal strength during the adult years. We are examining how modifiable factors such as nutrition and physical activity influence the development of peak bone mass, as well as variables such as skin pigmentation and an individual's genotype that are determined at birth. In both healthy youth and those with chronic disease, we are exploring the interrelationship between body composition, circulating hormones, and bone marrow adiposity and its effect on bone turnover and skeletal accrual.

A focus of our research is how physical and emotional health are compromised in adolescents and young women with premature ovarian insufficiency (POI). POI presents along a broad clinical spectrum. We are interested in both the presentation and causes of POI, including those seen in childhood cancer survivors, and ovarian dysfunction resulting from to autoimmune, metabolic, genetic/syndromic, and idiopathic (unknown) causes. We are conducting a natural history study to characterize numerous health outcomes and are launching a clinical trial to identify the optimal estrogen replacement regimen for adolescents and young women with this diagnosis. We are also employing novel tools to provide state-of-the-art assessments of bone density, body composition, and skeletal strength.

We are also interested in the skeletal phenotype associated with rare genetic diagnoses, some of which resemble or meet criteria for a skeletal dysplasia. Examples include progeria (Hutchinson-Gilford progeria syndrome), Ollier disease, and Maffucci syndrome.

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High-resolution peripheral computed tomography

High-resolution peripheral computed tomography (HRpQCT) is a bone assessment modality that affords measurements of the appendicular (peripheral) skeleton, as well as evaluation of bone microarchitecture and skeletal strength. As a non-invasive tool, it is ideal for obtaining measurements in the pediatric and adolescent population. Our lab is one of a relatively small number of centers that has an HRpQCT scanner. In a number of chronic disease models, we are examining the relation between failure load and other HRpQCT–derived outcomes and fracture risk.

Evaluations of bone marrow composition

Our team is using magnetic resonance (MR) imaging and spectroscopy to evaluate bone marrow fat, an outcome that is directly influenced by hormonal signals. We have studied bone marrow composition in adolescents with anorexia nervosa, and are employing this technique to examine the correlation between marrow fat and bone accrual in adolescents with inflammatory bowel disease and other pediatric clinical models. T1 maps and MR spectroscopy evaluations afford non-invasive means to evaluate bone marrow composition in children and adolescents.
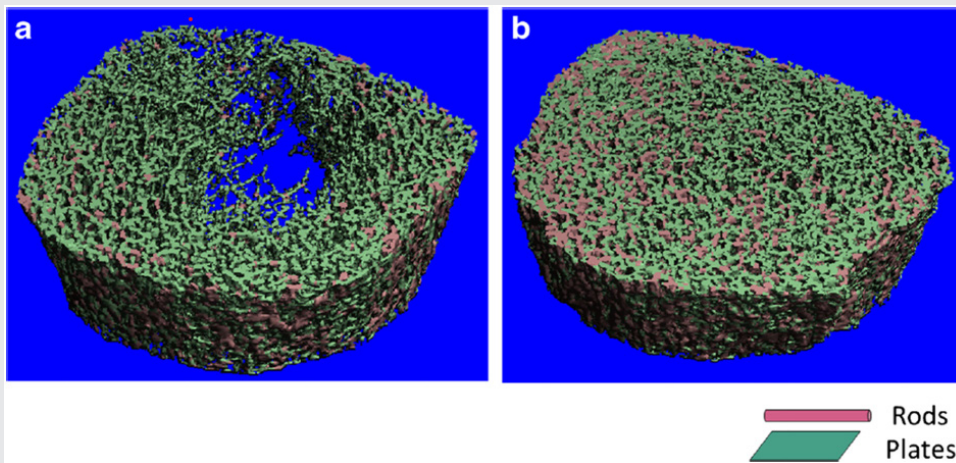

FIGURE 1. HRpQCT image

HRpQCT assessments (after trabecular segmentation) of the tibia in a 28 year-old woman with cystic fibrosis (a) and 28-year-old healthy female control participant (b).

Additional Funding

- U01HD107957-01 (Sobreira/Gordon), Delineation of the natural history of Ollier disease and Maffucci syndrome and investigation of their genetic bases

Publications

1. Wasserman HM, Kalkwarf HJ, Altaye M, Yolton K, Kanj RV, Gordon CM. Characterizing the adolescent premature ovarian insufficiency phenotype: a case control study. *J Pediatr Adolesc Gynecol* 2022 36:122–127.
2. Kanj RV, Ofei-Tenkorang NA, Altaye M, Gordon CM. Evaluation and management of primary ovarian insufficiency in adolescents and young adults. *J Pediatr Adolesc Gynecol* 2018 31:13–18.
3. Gordon CM, Cleveland RH, Baltrusaitis K, Massaro J, D'Agostino RB Sr, Liang MG, Snyder B, Walters M, Li X, Braddock DT, Kleinman ME, Kieran MW, Gordon LB. Extraskeletal calcifications in Hutchinson-Gilford progeria syndrome. *Bone* 2019 125:103–111.
4. Gordon RJ, Pappa HM, Vajapeyam S, Mulkern R, Ecklund K, Snapper SB, Gordon CM. Bone marrow adiposity in pediatric Crohn's disease. *Bone* 2022 162:116453
5. Pitts S, DiVasta AD, Gordon CM. Evaluation and management of amenorrhea. *JAMA* 2021 326:1962–1963.
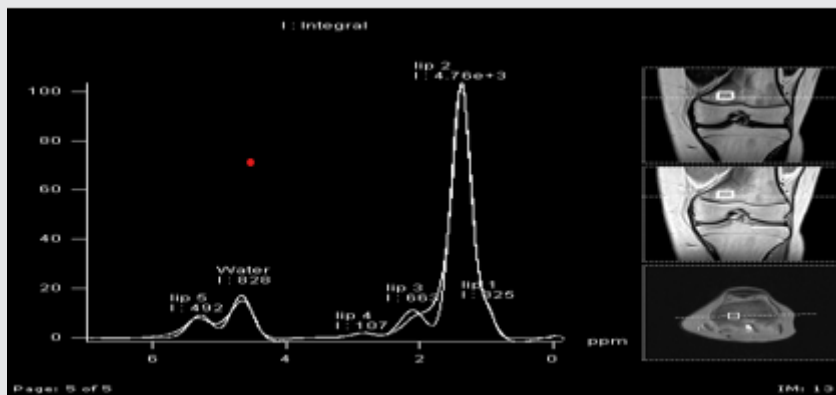
FIGURE 2. Magnetic resonance (MR) spectroscopy assessment of bone marrow composition

MR spectroscopy images from the left knee (distal femur) of an adolescent girl with anorexia nervosa

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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, and identify the RNA–binding proteins involved in these functions; (4) elucidate the regulation of Ded1, eIF4G, and Dhh1 functions in response to nutrient limitation or stress; (5) elucidate the roles of the yeast orthologs of eIF2A and eIF2D in eIF2–independent initiation of translation in stress conditions; and (6) elucidate the role of eIF4E–binding protein Eap1 in regulating mRNA decay and translation.

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We also analyze the regulation of amino acid–biosynthetic genes in budding yeast as a means to dissect 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 its induction 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 and preinitiation complex (PIC) assembly at Gcn4 target genes, their involvement in this process genome-wide, and the transcriptional consequences of defective nucleosome eviction or recruitment of the general transcription factor TATA–binding protein (TBP). 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 has been identified. 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); and the relative contributions of co-factors SAGA, TFIID, and Mot1 to TBP recruitment.

Differential requirements for P–stalk components in activating yeast protein kinase Gcn2 by stalled ribosomes during stress

A highly conserved response to amino acid starvation involves activation of the protein kinase Gcn2, which phosphorylates eukaryotic initiation factor 2, with attendant inhibition of global protein synthesis and increased translation of the yeast transcriptional activator GCN4. Gcn2 contains a domain related to histidyl-tRNA synthetase (HisRS–like domain), and a C-terminal ribosome-binding domain. Previous work indicated that Gcn2 is activated on translating ribosomes by uncharged tRNAs that accumulate in amino acid–starved cells and pair with the cognate codons in the empty ribosomal A, interacting directly with the HisRS–like domain to stimulate kinase activity. Gcn2 can also be activated by conditions that stall elongating ribosomes without reducing aminoacylation of tRNA, but it was unclear whether distinct molecular mechanisms operate in these two circumstances. We identified three regimes that activate Gcn2 in yeast by starvation-independent (SI) ribosome stalling, which leaves an empty A site on the stalled ribosome: (1) treatment with inhibitor tigecycline, which should stall ribosomes at all codons; (2) deleting the gene encoding tRNAArgUCC, which should stall ribosomes at AGG codons; and (3) depletion of translation-termination factor eRF1, which should stall ribosomes at stop codons. Subsequent genetic analysis demonstrated requirements for the HisRS–like and ribosome-binding domains of Gcn2, positive effectors Gcn1/Gcn20, and the tethering of at least one of two P1/P2 heterodimers of the 60S ribosomal P-stalk complex, for detectable activation by SI–ribosome stalling. Remarkably, no tethered P1/P2 proteins were required for strong Gcn2 activation by starvation for various amino acids, indicating that Gcn2 activation has different requirements for the P-stalk, depending on how ribosomes are stalled. We propose that accumulation of deacylated tRNAs in starved cells functionally substitutes for the P-stalk in binding to the HisRS–like domain for eIF2 kinase activation by ribosomes stalled with A sites.
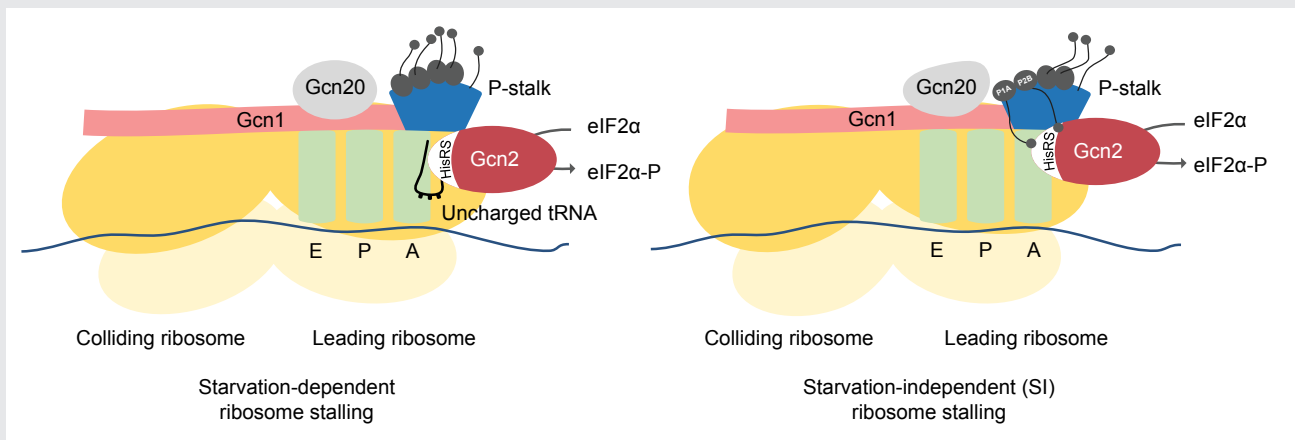
FIGURE 1. Differential requirements for P-stalk components in activating yeast protein kinase Gcn2 by stalled ribosomes during stress

Schematic model illustrating activation of protein kinase Gcn2 by starvation-dependent or -independent ribosome stalling.

Left: Ribosomes stall during translation with an empty A decoding site owing to insufficiency of the incoming cognate eEF1A/GTP/aminoacyl-tRNA ternary complex resulting from amino acid starvation. Uncharged tRNA binds to the HisRS–like domain of Gcn2, inducing a conformational change that stimulates eIF2 phosphorylation by the kinase domain.

Right: Ribosomes stall with an empty A-site owing to lack of the incoming cognate ternary complex, but, in the absence of accumulating uncharged tRNA, the P-stalk P1A-P2B heterodimer bound to 60S ribosomal protein uL10 interacts with the HisRS–like domain and induces the same conformational change needed for kinase activation.

Yeast mRNA decapping factors control mRNA abundance and translation to adjust metabolism and cell filamentation to nutrient availability.

Pat1 and helicase Dhh1 are conserved activators of the mRNA decapping enzyme Dcp1:Dcp2, are central to general mRNA decay, and were implicated in repressing translation in glucose-starved cells. Using ribosome profiling and RNA-seq analysis of *dhh1*, *pat1*, and *dhh1pat1* mutants cultured in rich medium, we identified hundreds of mRNAs up-regulated in a manner indicating cumulative repression by Pat1 and Dhh1. Although the environmental stress response (ESR) is mobilized in these mutants, involving increased expression of stress genes (iESR) and repression of ribosome production and translation factors (rESR), most up-regulated mRNAs are not iESR transcripts. CAGE (cap analysis of gene expression) analyses of capped mRNAs revealed enhanced accumulation of decapped intermediates for the up-regulated transcripts, and ChIP-seq analysis of RNA Pol II indicated decreased rather than increased transcription of the cognate genes, demonstrating that reduced decapping and 5'-3' pathway degradation drives transcript derepression in the mutants. The cumulative contributions of Dhh1 and Pat1 to mRNA decapping are consistent with their independent interactions with distinct segments of Dcp2 involved in its activation, and evidence for distinct decapping complexes containing Dhh1 or Pat1. Although previous work implicated Dhh1 and Pat1 in accelerating degradation of mRNAs enriched for slowly decoded codons, the mRNAs up-regulated in the mutants have average proportions of suboptimal codons. Pat1 and Dhh1 also collaborate to reduce the translational efficiencies (TEs) and protein production

of many mRNAs, including highly repressed mRNAs involved in cell adhesion or utilization of the poor nitrogen source allantoin. Pat1/Dhh1 also repress the abundance or TE of transcripts involved in oxidative phosphorylation (OXPHOS), catabolism of non-preferred carbon or nitrogen sources, or in autophagy. We obtained evidence for increased activity of the electron transport chain (ETC) of OXPHOS in the *dhh1* and *pat1* mutants, and elevated autophagic flux in the *pat1/dhh1* double mutant. As these genes/pathways are normally repressed in cells growing in rich medium replete, we concluded that Pat1 and Dhh1 function as post-transcriptional repressors of multiple pathways normally activated only during nutrient limitation.

Parallel analysis of the *dcp2* mutant led to the finding that, among the 1,300 mRNAs preferentially targeted for degradation by the decapping enzyme, 55% utilize Dhh1/Pat1 or decapping activators Scd6/Edc3 to promote decay, while the remainder employ the Upf factors that mediate nonsense-mediated mRNA decay (NMD). We also found that the *dcp2* mutation confers a broad reprogramming of translation, wherein well translated mRNAs exhibit increased TEs at the expense of poorly translated mRNAs, which we could attribute to increased competition for 43S PICs, given that *dcp2* cells contain elevated mRNA levels coupled with reduced ribosome abundance (owing to the ESR response). The increased mRNA/40S ratio and decreased 40S concentration should favor mRNAs with high rates of PIC recruitment at the expense of poorly initiated transcripts. As might be expected, the *dcp2* mutation up-regulates many of the same mRNAs required for respiration, utilization of poor carbon/nitrogen sources, and autophagy derepressed in the *pat1* and *dhh1* mutants, and confers elevated mitochondrial membrane potential and TCA cycle intermediates, indicating increased OXPHOS on glucose-rich medium. The *dcp2* mutant cells also resemble the decapping activator mutants in showing elevated expression of cell-adhesion proteins that function in forming pseudohyphae, and we observed increased filamentation of both *dcp2* and *pat1* mutant cells on rich medium. As filamentation is normally limited to starvation conditions and is viewed as a strategy for nutrient foraging, this phenotype supports the role of decapping factors in repressing pathways utilized primarily in starved cells.

Distinct functions of three chromatin remodelers in activator binding and preinitiation–complex (PIC) 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 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 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.

Differential requirements for Gcn5 and NuA4 HAT activities in the starvation–induced versus basal transcriptomes

Previously, we showed that elimination of Gcn5, the histone acetyltransferase (HAT) subunit in co-factor SAGA, did not fully impair nucleosome eviction at many starvation-induced genes, suggesting that Gcn5 might cooperate with other HATs in this process, similar to the functional cooperation we had identified for different CRs. The role of the HAT complex NuA4, responsible for most H4 and H2A acetylation in yeast, was of particular interest, as it was shown to be recruited to the Gcn4 target genes *ARG1* and *ARG4*. We examined the effects of disrupting the NuA4 complex, by eliminating its nonessential scaffold subunit Eaf1, on promoter nucleosome eviction and transcriptional activation at both starvation-induced and constitutively expressed genes. We also examined whether depleting Eaf1 from the nucleus (by anchor-away technology) confers defects in nucleosome eviction or transcription in cells lacking Gcn5 in order to evaluate whether NuA4 and Gcn5 make independent, additive contributions to these processes at particular genes *in vivo*. Our results revealed that NuA4 acts on par with Gcn5, and functions additively, in evicting and repositioning promoter nucleosomes, and in stimulating transcription, at starvation-induced genes. However, NuA4 is generally more important than Gcn5 in promoter nucleosome eviction, recruitment of the TATA-binding protein (TBP), and transcription at most other genes expressed constitutively in yeast. NuA4 also predominates over Gcn5 in stimulating TBP recruitment and transcription of genes categorized as principally dependent on the cofactor TFIID versus SAGA, except for the highly expressed subset encoding ribosomal proteins (RPs), where Gcn5 contributes strongly to PIC assembly and transcription. We found that both SAGA and NuA4 are recruited to promoter regions of starvation-induced genes in a manner that appears to be controlled by their HAT activities, and thus most likely act directly to promote transcription of these genes. Our findings reveal an intricate interplay between these two HATs in nucleosome eviction, PIC assembly, and transcription that differs between the starvation-induced and basal transcriptomes.

Publications

1. Gupta R, Hinnebusch AG. Differential requirements for P-stalk components in activating yeast protein kinase Gcn2 by stalled ribosomes during stress. *Proc Natl Acad Sci USA* 2023 120:e2300521120.
2. Vijjamarri AK, Niu X, Vandermeulen MD, Onu C, Zhang F, Qiu H, Gupta N, Gaikwad S, Greenberg ML, Cullen PJ, Lin Z, Hinnebusch AG. Decapping factor Dcp2 controls mRNA abundance and translation to adjust metabolism and filamentation to nutrient availability. *eLife* 2023 12:e85545.
3. Vijjamarri AK, Gupta N, Onu C, Niu X, Zhang F, Kumar R, Lin Z, Greenberg ML, Hinnebusch AG. mRNA decapping activators Pat1 and Dhh1 regulate transcript abundance and translation to tune cellular responses to nutrient availability. *Nucleic Acids Res* 2023 51:9314–9336.
4. Rawal Y, Qiu H, Hinnebusch AG. Distinct functions of three chromatin remodelers in activator binding and preinitiation complex assembly. *PLoS Genet* 2022 18:e1010277.
5. Zheng Q, Qiu H, Zhang H, Hinnebusch AG. Differential requirements for Gcn5 and NuA4 HAT activities in the starvation-induced versus basal transcriptomes. *Nucleic Acids Res* 2023 51:3696–3721.
6. Dever TE, Ivanov IP, Hinnebusch AG. Translational regulation by uORFs and start codon selection stringency. *Genes Dev* 2023 37:474–489.

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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. In humans, the hippocampus is essential for long-term memory 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.

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

KV4.2 COMPLEX REGULATION AND ITS ROLE IN COGNITIVE FLEXIBILITY

We recently identified a novel molecular cascade that regulates the

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FIGURE 1. Working model of neuronal activity or seizure-induced Kv4.2-DPP6 complex remodeling that may contribute to abnormal phenotypes in Angelman syndrome (AS) mice
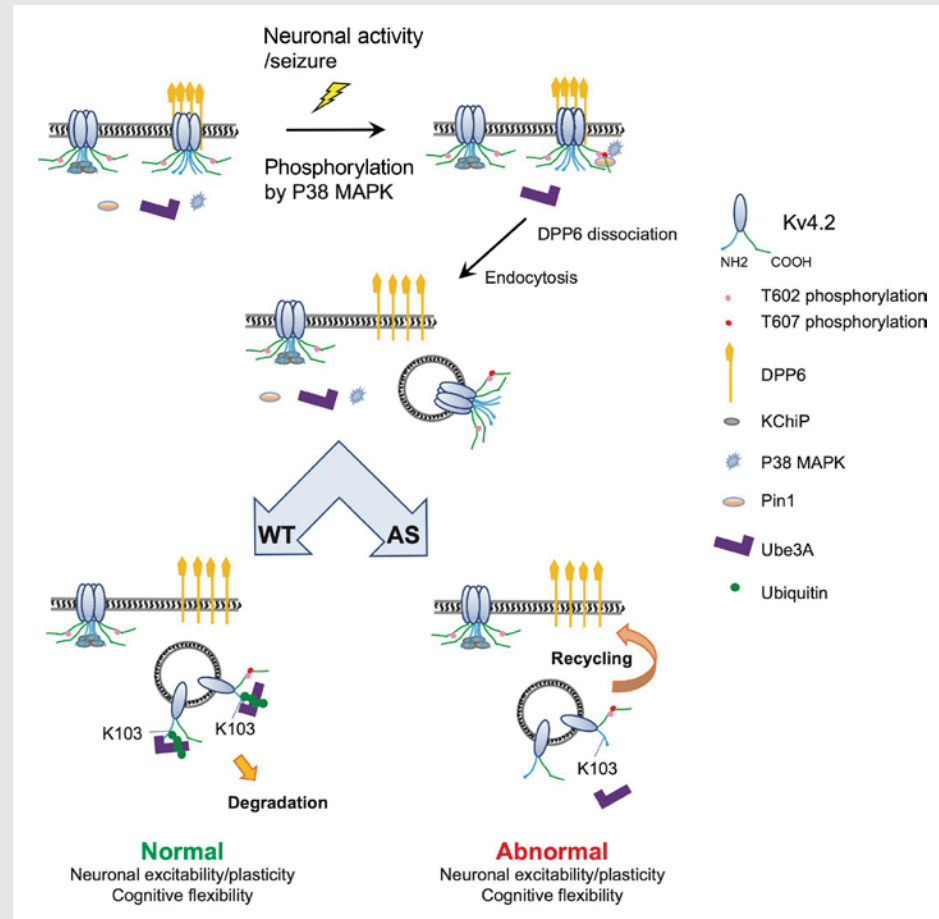
potassium channel Kv4.2's association with the auxiliary subunit DPP6 and membrane-surface expression in hippocampal neurons. The cascade is initiated by various activity patterns impinging on the neuron, triggering activation of p38 mitogen–activated protein kinase, which phosphorylates the C-terminal motif T607 in Kv4.2 in an activity-dependent manner. Phosphorylation by p38 initiates subsequent isomerization by a prolyl isomerase, Pin1, which selectively binds to and isomerizes phospho-Ser/Thr-Pro bonds. Pin1 is a ubiquitous isomerase that has been implicated in a growing number of nervous-system pathologies, including Alzheimer's disease, where it may protect against age-dependent neurodegeneration.

To address the role of the p38-Pin1-Kv4.2 in neuronal and neural circuit function, we developed a mutant knock-in mouse model with a Thr607-to-Ala substitution at the activity-induced p38 phosphorylation site (T607-to-A607; Kv4.2TA). The mutation significantly reduces p38 phosphorylation and Pin1 isomerization of this motif, and we observed impaired Kv4.2-DPP6 dynamics and loss of activity-induced internalization of Kv4.2 in such mice. Furthermore, we identified a reduction in intrinsic excitability of hippocampal CA1 pyramidal neurons using whole-cell patch clamp recordings in Kv4.2TA mice compared with wild-type (WT). The reduction in excitability can be traced to an increase in the density of Kv4.2–mediated outward K$^+$ current (A-current), supporting biochemical analysis that suggests loss of Kv4.2 internalization in the Kv4.2TA mice (increased surface Kv4.2). The hypo-excitability in individual neurons observed within the hippocampus of Kv4.2TA mice

extends to the circuit/network level, as we also identified reduced kainic acid–induced seizure intensity and progression in these mice.

Hypoactivity within the hippocampal circuit in Kv4.2TA mice may impact cognition. Perhaps most intriguingly, we found that Kv4.2TA mice exhibit normal initial learning and memory in the Morris Water Maze and Lever Press, two tests of hippocampal-dependent learning and memory. However, they exhibited better 'reversal' learning in both tests than did WT mice. The improvement in reversal learning indicates an enhancement in cognitive flexibility. Such data strongly support the idea that activity-dependent regulation of Kv4.2 plays an important role in cognitive flexibility, i.e., the ability to appropriately adjust one's behavior to a changing environment, which is impaired in various neuro-developmental disorders such as the autism-spectrum disorder.

Considering the finding that Kv4.2TA mice demonstrate enhanced cognitive flexibility, which to our knowledge represents the first mouse model exhibiting this phenotype, Cole Malloy is pursuing the mechanisms underlying this phenotype. We are focusing on potential differences in synaptic properties between WT and Kv4.2TA mice. Results to date have revealed a novel meta-plasticity mechanism in a Kv4.2 mouse model, which may provide insights into cognitive flexibility and which would be of interest in therapeutic design for treating neuro-developmental disorders characterized by impairments in cognitive flexibility.

KV4.2 K⁺ CHANNELS ARE A UBE3A SUBSTRATE AND CONTRIBUTE TO COGNITION IN ANGELMAN SYNDROME (AS).

Angelman syndrome (AS) is a severe, debilitating neuro-developmental disorder with an estimated incidence of 1 in 20,000. It is caused by loss of function of imprinted genes on human chromosome 15q11–13 or by mutations in the *Ube3A* gene, which resides in this region. Imprinting of this gene results in the exclusive expression of the maternal allele in hippocampal neurons and cerebellar Purkinje cells. Deficits of Ube3A lead to accumulation of its target proteins, which thus dysregulate neuronal function. Using a TAP-MS screen of Kv4.2–interacting proteins that we had previously developed, we identified Ube3A as a Kv4.2–binding protein. Follow-up biochemistry and cell-biology studies, led by Jiahua Hu, confirmed the interaction and demonstrated that Kv4.2–Ube3A binding is activity-dependent. We show that Ube3A binds to Kv4.2 at its N-terminus, and, using an *in vitro* ubiquitination assay, ubiquinates residue K103. Ubiquitination of a substrate by Ube3a usually causes the substrate degradation. We therefore examined whether Kv4.2 K103 ubiquitination affects the Kv4.2 protein level. The result showed that mutation of K103 significantly delayed protein loss compared with un-mutated Kv4.2 in response to AMPA treatment in cultured hippocampal neurons, suggesting that K103 is required for activity-induced Kv4.2 protein loss. In addition, we showed that Ube3A is associated with internalized Kv4.2, which complexes with the Kv4 auxiliary subunit DPP6.

To further study Kv4.2's role in AS, we imported a mouse model of AS in which Ube3A is deleted. We found that the Kv4.2 protein level and A-type K⁺ current are significantly elevated in the hippocampus of AS mice compared with WT littermates. Seizure or neuronal activity leads to Kv4.2 protein degradation. We examined whether Ube3A is required for Kv4.2 protein degradation. We found that seizure-induced Kv4.2 protein loss is abolished in AS, suggesting that seizure-induced Kv4.2 degradation requires Ube3A. Moreover, using patch-clamp electrophysiology, we found deficits in mEPSC frequency and spike-timing–dependent LTP (long-term potentiation) in AS mice. To further study the physiological function of Kv4.2 in AS, we generated CRE–dependent conditional Kv4.2 KO (knockout) mice and crossed them with Emx1-CRE mice to obtain conditional Kv4.2 KO mice (Kv4.2cKO). We then mated AS mice with Kv4.2cKO mice so that for Cole Malloy and Meghyn

Welch could examine whether electrophysiological deficits in AS mice can be rescued. Interestingly, deficits in mEPSC frequency and spike-timing–dependent LTP in AS mice were normal in AS/Kv4.2cKO mice. A behavioral test battery for mouse models of AS has been developed to assess phenotypes in the domains of motor performance, repetitive behavior, anxiety and to test drugs and novel Ube3A mutants. We examined the battery in WT littermates, AS mice, Kv4.2cKO mice, and AS/Kv4.2cKO DKO (double knockout) mice and found that locomotion and nesting behaviors can be partially rescued in the DKO mice. In learning and memory tests, AS mice showed impairments in initial learning and reversal learning in an operant reversal test. However, the deficits in AS mice in reversal learning can be rescued by DKO mice. These findings reveal a novel Ube3A–downstream pathway regulating plasticity and cognitive behaviors, and they provide potential targets for the treatment of AS.

DPP6 IMPACTS BRAIN DEVELOPMENT, FUNCTION, AND ALZHEIMER'S DISEASE/DEMENTIA.

In 2022, we reported [Reference 2] that DPP6–KO mice show enhanced neuro-degeneration associated with AD pathology. We also found that aging DPP6–KO mice display circadian dysfunction by home-cage tasks. To further study whether DPP6–KO mice have sleep disorders related to AD/dementia, we used an *in vivo* detection system by surgical implantation of HD-XO2 implantable telemetry, and recorded EEG/EMG/ activity from aging DPP6–KO mice brains. Electrophysiological data were collected for five days using Ponemah Physiology Platform software. We used NeuroScore software to analyze the sleep/wake time and perform power spectral analysis. From preliminary data, we found that 12-month-old DPP6–KO mice show less total sleep time, less slow-wave sleep duration, and more wake duration compared with WT.

To continue our examination of DPP6 function and its novel roles in preventing neuro-degeneration diseases such as AD/dementia, we are working on another *in vivo* assay of BioID (proximity-dependent biotin identification) by intra-cerebroventricular injection in neonatal mice with AAV(adeno-associated viral)-DPP6-BioID, to identify other proteins that can form dynamic DPP6–binding complexes, including those involved in transient interactions during cell trafficking as well as components of synaptic adhesion. Biotinylated proteins are isolated by affinity capture and identified by mass spectrometry. We found some interesting binding-partner candidates for further confirmation and functional study. These include, for example, the cell adhesion proteins that function in synapse maturation and enhancement and are involved in autism-spectrum disorders, schizophrenia, and neuro-degeneration diseases such as AD.

Publications

1. Murphy JG, Gutzmann JJ, Lin L, Hu J, Petralia RS, Wang YX, Hoffman DA. R-type voltage-gated Ca2+ channels mediate A-type K+ current regulation of synaptic input in hippocampal dendrites. *Cell Rep* 2022 38(3):110264.
2. Lin L, Petralia RS, Holtzclaw L, Wang YX, Abebe D, Hoffman DA. Alzheimer's disease/dementia-associated brain pathology in aging DPP6-KO mice. *Neurobiol Dis* 2022 174:105887.
3. Malloy C, Ahern M, Lin L, Hoffman DA. Neuronal roles of the multifunctional protein dipeptidyl peptidase-like 6 (DPP6). *Int J Mol Sci* 2022 23(16):9184.
4. Hu JH, Malloy C, Tabor GT, Gutzmann JJ, Liu Y, Abebe D, Karlsson RM, Durell S, Cameron HA, Hoffman DA. Activity-dependent isomerization of Kv4.2 by Pin1 regulates cognitive flexibility. *Nat Commun* 2020 11(1):1567.
5. Hu JH, Malloy C, Hoffman DA. P38 regulates kainic acid-induced seizure and neuronal firing via Kv4.2

phosphorylation. *Int J Mol Sci* 2020 21(16):5921.

6. Lin L, Petralia RS, Lake R, Wang YX, Hoffman DA. A novel structure associated with aging is augmented in the DPP6-KO mouse brain. *Acta Neuropathol Commun* 2020 8(1):197.

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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 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, Croc, 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 *invected-engrailed* (*inv-en*) 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 *inv-en* 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 by insulators that limit the size of the domain. We hypothesized that delimiting the size of a Polycomb domain contributes to the stability of both gene activation and repression, making gene expression reproducible and robust. We recently completed experiments showing that flanking the 79 kb transgene by insulator elements strengthens the expression of the transgene, providing evidence for our hypothesis. Thus, providing "ends" to the *inv-en* domain stabilizes both its "ON" and "OFF" transcriptional states.

We also recently completed an analysis of PcG protein binding, chromatin marks, and 3D chromatin structure of PcG target genes in the ON and OFF transcriptional states. One aspect of chromatin structure is the presence of loops between distant regulatory DNA. Our data show that PREs form loops with other PREs in both the ON and OFF transcriptional states. Further, our data also show that PREs loop with enhancers, data that are consistent with our genetic work that showed that PREs can facilitate enhancer-promoter communication and thus have been called promoter-tethering elements (PTEs).

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, inhibits chromatin remodeling, and compacts chromatin. 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 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

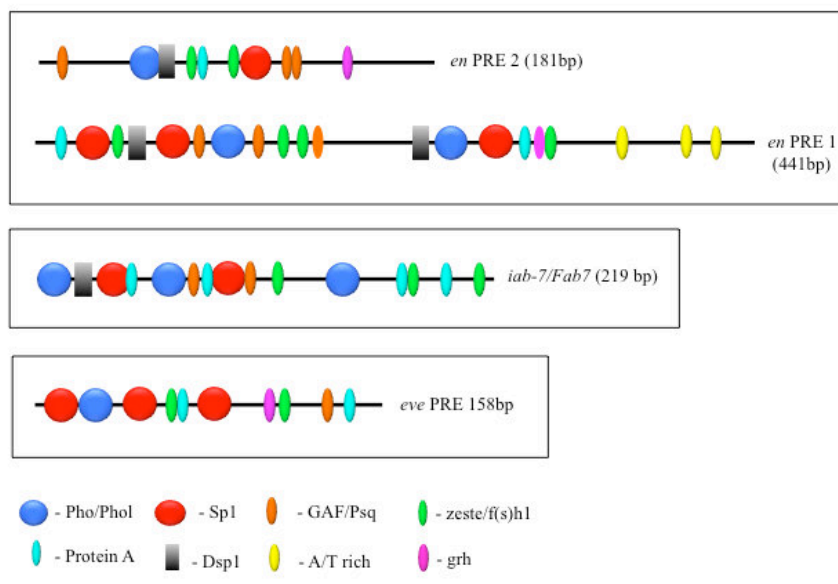**FIGURE 1. The order, number, and spatial arrangement of consensus-factor binding sites varies in different PREs.**

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

(Figure 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, Combgap, and recently collaborated on the characterization of the zinc-finger protein Crol as another PRE DNA–binding protein [Reference 2]. Clearly PREs are complex elements.

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 binding sites for a single PRE–binding protein can obliterate its ability to recruit PcG proteins and to repress gene expression. Thus, transcriptional silencing by a single PRE in a transgene requires the combinatorial activity of many DNA–binding proteins. We were interested to determine what happens when one of the PRE DNA–binding proteins (the 'recruiters') from the genome is removed, and we examined the effect on PcG recruitment genome-wide. We studied PcG binding genome-wide in mutants that lack the recruiters Spps or Pho [Reference 3]. We found that PcG recruitment to some PREs was completely disrupted, whereas recruitment of PcG proteins was hardly diminished at most PREs. Most PcG target genes, 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.

We took another approach to address the function of PRE DNA–binding proteins and chromatin environment in PRE function. Early data showed that mutation of Pho binding sites in PREs in transgenes abrogated the ability of those PREs to repress gene expression. In contrast, genome-wide experiments in *pho* mutants or by Pho knockdown showed that PcG proteins can bind to PREs in the absence of Pho. What could account for these differences? We directly addressed the importance of Pho binding sites in two *engrailed* (*en*) PREs at the endogenous locus and in transgenes [Reference 4]. Our results showed that Pho binding sites are required for PRE activity in transgenes with a single PRE. In a transgene, two PREs together lead to stronger, more stable repression and confer some resistance to the loss of Pho binding sites. Making the same mutation in Pho binding sites has little effect on PcG–protein binding at the endogenous *en* gene. Overall, our data support the model that Pho is important for PcG binding but emphasize how multiple PREs and chromatin environment increase the ability of PREs to function in the absence of Pho. This supports the view that many mechanisms contribute to PcG recruitment in *Drosophila*.

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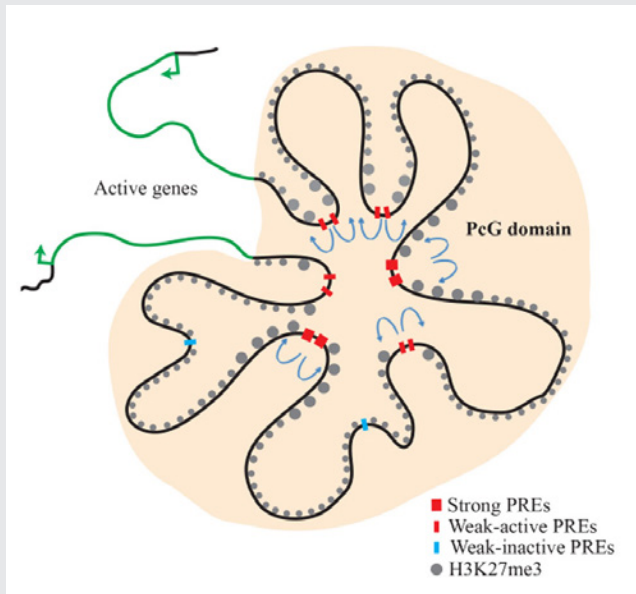
FIGURE 2. Model of the *inv-en* PcG domain in the transcriptionally silenced state

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 (Figure reprinted from Reference 3).

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 *inv-en* domain is not disrupted in either of these mutants.

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

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).
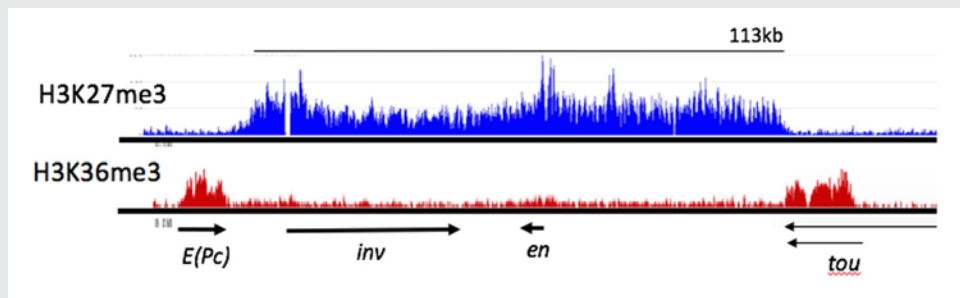
spreading of the H3K27me3 mark and stabilizes the repression of *inv* and *en* by PcG proteins. To test this assumption, we made a large transgene marked by HA–tagged Engrailed protein. A 79-kb *HA-en* transgene was able to correctly express En and completely rescue *inv-en* double mutants. We inserted 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 spreads 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. These 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. Our recent experiments show that adding insulator elements that block the spreading of H3K27me3 and the activity of the inv-en enhancers stabilizes the 79-kb *HA-en* transgene, making it behave more like the endogenous locus [Reference 5].

## Precise gene expression patterns are governed by a vast array of regulatory DNA.

Genes that control development are often used at different times and places in a developing embryo. Transcription of these important genes must be tightly regulated; therefore, these genes often have large arrays of regulatory DNA. In *Drosophila*, discrete fragments of DNA (enhancers) can be identified, which turn genes on in patterns in the early embryo. In cells in which the genes are transcriptionally ON, there are active modifications on chromatin, setting later enhancers in a transcription-permissive environment. In cells in which the genes are OFF, repressive chromatin marks keep later enhancers inactive. Our studies on the regulatory DNA of the *inv-en* gene have been highly informative.

Enhancers are often located tens or even hundreds of kb away from their promoter, sometimes even closer to the promoters of genes other than the one they activate. Several years ago, we showed that *en* enhancers can act over large distances, even skipping over other transcription units, choosing the *en* promoter over promoters 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-

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).
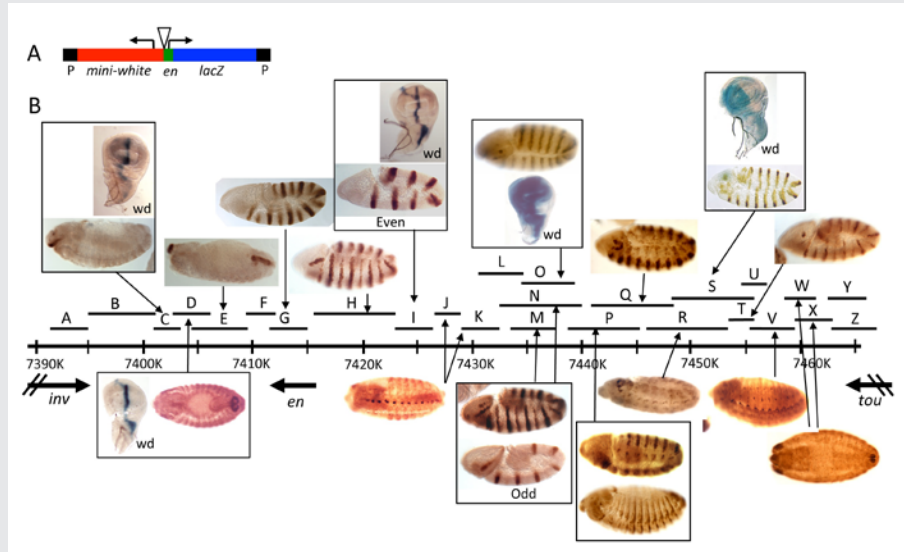
tethering element is required for the action of the imaginal disc enhancer (IDE); our data point to two partially redundant promoter-tethering elements. Third, the long-distance action of *en* enhancers requires a combination of the *en* promoter and sequences within or closely linked to the promoter-proximal 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 construct 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.
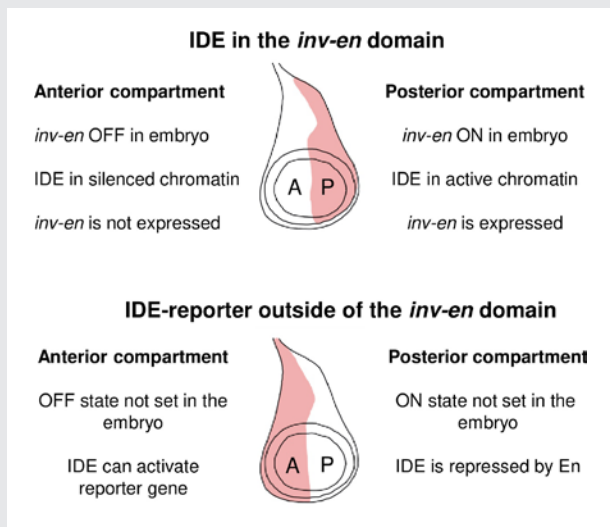
FIGURE 5. Model of how the *en* imaginal disc enhancers (IDEs) function inside and outside the *inv-en* domain

Diagrams of a wing disc with expression (*red shading*) in either the posterior (*P*) or anterior (*A*) compartment.

In recent work, we studied the activity of two *engrailed* imaginal disc enhancers (IDE) inside and outside the endogenous *inv-en* domain [Reference 5]. Inside the *inv-en* domain, IDEs drive expression of *inv* and en in the posterior compartment of imaginal discs (Figure 5). However, when the IDE is in a reporter gene located outside the *inv-en* domain, the reporter gene is expressed in the wrong part of the disc. We also showed that Engrailed itself binds to the IDE and represses its expression. Overall, our data show that the activity of enhancers can be greatly influenced by flanking regulatory DNA and the epigenetic state of its chromatin environment. In addition, Engrailed regulates its own expression level by binding to its own IDE.

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 the chromatin mark H3K27me3 in the genome. We conducted a genome-wide analysis of Polycomb boundaries in *Drosophila* larvae [Reference 6]. 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 6].

Why is important that H3K27me3 domains have ends? We addressed this question by adding boundaries to the ends of our 79kb engrailed transgene [Reference 5]. In this paper, we showed that adding a boundary to the transgene confined the activity of the *engrailed* enhancers and increased the activity of the 79kb transgene. It addition, the boundary element strengthened Polycomb silencing of the transgene, making it more resilient to reduced PcG function. Overall, our data showed that a boundary element strengthened both the ON and OFF transcriptional states of the 79kb engrailed transgene.

PREs can also act as promoter-tethering elements (PTEs).

In the OFF transcriptional state, PREs recruit PcG protein complexes including PRC2 that tri-methylates H3K27, forming large H3K27me3 domains. In addition, PREs make loops in chromatin and the looping strengthens silencing. In a genome-wide study in two difference cell types [Brown JL *et al. bioRxiv* 2023;11.02.565256], we addressed the question of what PcG proteins bind to PREs when PcG target genes are expressed, and whether PREs loop when these genes are ON. Our data show that the answer to this question is PRE–specific, but general conclusions can be reached. First, within a PcG–target gene, some regulatory DNA can remain covered

with H3K27me3 and PcG proteins remain bound to PREs in such regions. Second, when PREs are within H3K27ac domains, PcG binding decreases; however, this depends on the protein and PRE. The DNA–binding protein GAF, and the PcG protein Ph remain at PREs, even when other PcG proteins are greatly depleted. In the ON state, PREs can still loop with each other, but also form loops with presumptive enhancers. These data support the model in which, in addition to their role in PcG silencing, PREs can act as "promoter-tethering elements," mediating interactions between promoter-proximal PREs and distant enhancers. Further, our studies provide genetic evidence of the PTE activity of *engrailed* PRE2. Overall, our work shows the importance of PREs to both the ON and OFF transcriptional states.

Publications

1. Kassis JA, Kennison JA, Tamkun JW. Polycomb and Trithorax group genes in Drosophila. *Genetics* 2017 206:1699–1725.
2. Erokhin M, Brown JL, Lomaev D, Vorobyeva NE, Zhang L, Fab LV, Mazina MY, Kulakovskiy IV, Ziganshin RH, Schedl P, Georgiev P, Sun MA, Kassis JA, Chetverina D. Crol contributes to PRE-mediated repression and Polycomb group protein recruitment in Drosophila. *Nucleic Acids Res* 2023 51(12):6087–6100.
3. Brown JL, Sun M, Kassis JA. Global changes of H3K27me3 domains and Polycomb group protein distribution in the absence of recruiters Spps or Pho. *Proc Natl Acad Sci USA* 2018 115(8):E1839–E1848.
4. Brown JL, Price JD, Erokhin M, Kassis JA. Context-dependent role of Pho binding sites in Polycomb complex recruitment in Drosophila. *Genetics* 2023 224:iyad096.
5. Cheng Y, Chan F, Kassis JA. The activity of engrailed imaginal disc enhancers is modulated epigenetically by chromatin and autoregulation. *PLoS Genetics* 2023 19(11):e1010826.
6. De S, Gehred ND, Fujioka M, Chan FW, Jaynes JB, Kassis, JA. Defining the boundaries of Polycomb domains in Drosophila. *Genetics* 2020 216:689–700.

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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 caused by disruptions in collagen metabolism 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, 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 produce and secrete the massive amounts of type I procollagen needed to build the skeleton. Because 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. Cells use specialized ER chaperones to fold and stabilize the native conformation at body temperature. Secreted procollagen is converted to collagen and incorporated into stable collagen fibers before it denatures. Unincorporated molecules denature within several hours, followed by rapid proteolytic degradation. Up to 10–15% of procollagen is misfolded even under normal conditions, activating cell stress–response pathways responsible for degrading misfolded

molecules. As a result, osteoblasts 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 excessive cell stress caused by excessive accumulation of misfolded procollagen in the ER.

The most common hereditary cause of procollagen misfolding is a Gly substitution 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.

While we focus mostly on hereditary disorders affecting children, excessive procollagen misfolding may also 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. Nonetheless, the pathophysiology of misfolded procollagen accumulation in the ER remains poorly understood.

Cell biology of procollagen misfolding

To understand this pathophysiology, we are investigating how misfolded procollagen is recognized by the cell, why it accumulates in the ER, and how this accumulation affects a cell's function. In one approach, we are using live-cell imaging to study the synthesis, folding, trafficking, and degradation of fluorescently tagged procollagen. To facilitate these studies, we created several plasmids enabling transient expression of fluorescent procollagen in mouse and human cells. We also created several cell lines in which the fluorescent tag was inserted into the gene encoding endogenous pro-alpha2(I) chain. These new tools for live-cell imaging of procollagen have already been shared with dozens of laboratories in the USA and around the world.

Imaging of fluorescent procollagen enabled us to demonstrate that normally folded molecules are 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, such vesicles do not have a COPII coat nor do they contain HSP47, a collagen-specific ER chaperone, which 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. This 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.

In another approach, we are investigating the cell-stress response to procollagen misfolding caused by a Gly610-to-Cys substitution in the triple-helical region of pro-alpha2(I). We helped develop a mouse model of OI with this mutation (G610C mouse), which mimics the pathology found in a large group of patients with the same mutation. Our study of this model demonstrated that misfolding and accumulation of mutant procollagen in the ER of osteoblasts causes ER disruption, 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. For instance, 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 the malfunction associated with such accumulation play a significant role in OI pathophysiology. More detailed analysis of the ATG5 knockout effects confirmed that ERES microautophagy is the primary pathway of misfolded procollagen degradation in G610C osteoblasts. Furthermore, more recent studies revealed that accumulation of misfolded G610C procollagen in osteoblast ER activates transcription of 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 and Western blotting.

By combining the live-cell imaging, genetic, and biochemical analysis, we found that misfolding of procollagen in the ER of G610C osteoblasts activates the mitochondrial arm of the integrated cell-stress response (ISR) rather than the canonical unfolded protein response (UPR). Misfolded G610C molecules are not recognized and retained in the ER lumen by quality-control chaperones. Instead, they are retained at ERESs, blocking the exit of all secretory proteins from the ER into the secretory pathway. The resulting ER overcrowding activates the ISR without UPR as a result of disruption of ER–mitochondria contacts. The ISR is sufficient to stabilize G610C osteoblasts in a less efficient yet functional steady state, in which procollagen synthesis is reduced and degradation is increased. However, the same ISR may not be sufficient to prevent more severe ER disruption in other cells or other mutations, which may then cause misfolding of globular proteins and concomitant secondary UPR. For instance, we found that hypertrophic chondrocytes in the growth plate of the same G610C animals undergo a secondary UPR, which blocks their transition into osteoblasts and thereby limits longitudinal bone growth. Our discovery of mitochondrial involvement not only identified a new response pathway to protein misfolding in the ER but also opened up a new research direction. We are currently investigating how the ER–mitochondria contacts are disrupted and how the resulting mitochondrial dysfunction affects the cells.

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 cellular 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 studies on autophagy enhancement by a low-protein diet or intermittent fasting in G610C

mice revealed improved osteoblast differentiation and function, resulting in better bone quality, but we also observed stunted animal growth. We are thus evaluating other approaches that might provide the same benefits of autophagy enhancement without long-term nutrient deficiency.

In particular, we are testing drugs known to reduce ER disruption by enhancing secretion and autophagy of misfolded proteins (e.g., 4-phenylbutyrate [4PBA]) and drugs (e.g., integrated stress response inhibitor [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. However, although low-dose 4PBA treatment improved the function of hypertrophic chondrocytes and their conversion into osteoblasts in such mice, it did not improve 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 to 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 of OI and other skeletal dysplasias

Over the last several years, we participated 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, the 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 or 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 studied 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.

Given that cardio-pulmonary complications are the main cause of death in OI, we are presently focusing on understanding the causes of lung tissue pathology in patients and mouse models of OI. Our biggest breakthrough during the last year has been the demonstration of severe under-development (hypoplasia) of saccular structures in G610C mouse embryos caused by deficient lung inflation with amniotic fluid during fetal breathing movements. Deficient rib cage movements resulting from *in utero* rib fractures and deformities, as well as poor transmission of the forces generated by these movements to the saccular structures by weak collagen fibers, both contribute to insufficient saccular inflation and resulting lung hypoplasia. We are currently working with our clinical collaborators on identifying ways to confirm these observations in humans and raising the awareness in the clinical community that lung hypoplasia is to be expected in newborns with OI, as well as other forms of skeletal dysplasia that may affect the rib cage and lung collagen fibers. We recommend evaluation of such newborns for lung hypoplasia and the corresponding treatment by a pulmonologist, which may save many lives, given that lung hypoplasia is known to be responsible for 15–20% of neonatal death from all causes. Moreover, timely treatment may improve lung development during the neonatal period and infancy, preventing pulmonary complications, both in childhood and later in life.

Extracellular matrix pathology in tumors and fibrosis

Another aspect of our collagen metabolism pathology studies has been to characterize 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. However, homotrimers of three alpha1(I) chains are produced in carcinomas and some fibrotic tissues. 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. While we did not detect the type I collagen homotrimer synthesis we expected, we identified and characterized abnormal organization and mineralization of bone matrix as well as novel bone structures in mice with knockouts of various PKA subunits. 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 is crucial for understanding and treating collagen metabolism disorders, we are developing methods for characterizing cellular function in relation to ECM composition and structure. The methodology builds on our advances in high-definition infrared and Raman microspectroscopy, mRNA–based histology and histomorphometry, and combining different imaging and spectroscopic modalities for tissue sections.

In particular, we developed high-definition (HD) infrared imaging and Raman micro-spectroscopic methods, improving spectral reproducibility by up to two orders of magnitude, based on thermomechanical stabilization of the light path through tissue. The technology enabled us to uncover the causes of progressive cartilage degradation in a mouse model of diastrophic dysplasia caused by mutations in the S*LC26A2* sulfate transporter gene. It was essential for the analysis of abnormal collagen matrix deposition by CRTAP– and FKBP65–deficient cells. We used it in studies of bone structure and mineralization in the mouse models of the OI and PKA deficiencies described above and for establishing new approaches to characterizing bone formation *in vitro*. Beyond non-destructive characterization of ECM composition and structure in many of our studies, the technology proved useful for researchers from other NICHD and NIH laboratories. For instance, we assisted NIBIB scientists in characterizing a functionalized carbon-nanotube approach to the delivery of anticancer agents into cells that overexpress hyaluronate receptors.

We are presently working on integrating the HD infrared/Raman and histological ECM imaging modalities with cellular-function imaging based on *in situ* mRNA sequencing and fluorescent *in situ* mRNA hybridization. We have already combined all these modalities for proving formation of *bona fide* lamellar bone in osteoblast cultures and developing approaches to distinguishing this bone from other mineralized cell–ECM structures, which are generally more prevalent *in vitro*. We are now optimizing such structural and functional imaging for integrated characterization of the same tissue section, which would address the question of how the ECM structure and composition affect the function of adjacent cells and cell-cell interactions.

Publications

1. Gorrell L, Omari S, Makareeva E, Leikin S. Noncanonical ER–Golgi trafficking and autophagy of endogenous procollagen in osteoblasts. *Cell Mol Life Sci* 2021 78:8283.
2. Scheiber AL, Wilkinson KJ, Suzuki A, Enomoto-Iwamoto M, Kaito T, Cheah KS, Iwamoto M, Leikin S, Otsuru S. 4PBA reduces growth deficiency in osteogenesis imperfecta by enhancing transition of hypertrophic chondrocytes to osteoblasts. *JCI Insight* 2022 7:e149636.
3. Gorrell L, Makareeva E, Omari S, Otsuru S, Leikin S. Mitochondria, and ISR regulation by mt-HSP70 and ATF5 upon procollagen misfolding in osteoblasts. *Adv Sci (Weinh)* 2022 9:2201273.
4. Mertz EL, Makareeva E, Mirigian LS, Leikin S. Bone formation in 2D culture of primary cells. *JBMR Plus* 2023 7:e10701.
5. Dimori M, Fett J, Leikin S, Otsuru, S, Thostemson JD, Carroll JL, Morello R. Distinct type I collagen alterations cause intrinsic lung and respiratory defects of variable severity in mouse models of osteogenesis imperfecta. *J Physiol* 2023 601:355–379.

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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.

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 (induced pluripotent stem cells)–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 that such cells undergo during disease progression. Last 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

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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 amyotrophic lateral sclerosis (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 with those of vulnerable MN types across several time points in disease.

In parallel studies, we are developing and characterizing new models of juvenile onset motor neuron disease.

Publications

1. Wlaschin JJ, Donahue C, Gluski J, Osborne JF, Ramos LM, Silberberg H, Le Pichon CE. Promoting regeneration while blocking cell death preserves motor neuron function in a model of ALS. *Brain* 2023 146:2016–2028.
2. Enamorado M, Kulalert W, Han SJ, Rao I, Delaleu J, Link VM, Yong D, Smelkinson M, Gil L, Nakajima S, Linehan JL, Bouladoux N, Wlaschin J, Kabat J, Kamenyeva O, Deng L, Gribonika I, Chesler AT, Chiu IM, Le Pichon CE, Belkaid Y. Immunity to the microbiota promotes sensory neuron regeneration. *Cell* 2023 186:607–62.
3. Yadav A, Matson KJE, Li L, Hua I, Petrescu J, Kang K, Alkaslasi MR, Lee DI, Hasan S, Galuta A, Dedek A, Ameri S, Parnell J, Alshardan MM, Qumqumji FA, Alhamad SM, Wang AP, Poulen G, Lonjon N, Vachiery-Lahaye F, Gaur P, Nalls MA, Qi YA, Maric D, Ward ME, Hildebrand ME, Mery PF, Bourinet E, Bauchet L, Tsai EC, Phatnani H, Le Pichon CE, Menon V, Levine AJ. A cellular taxonomy of the adult human spinal cord. *Neuron* 2023 111:328–344.

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The Biological Impact of Transposable Elements

Long Terminal Repeat (LTR) retrotransposons are highly abundant and have evolved into ubiquitous families of elements that multiply through cycles of transcription, particle formation, reverse transcription, transport to the nucleus, and integration. Many families of LTR retrotransposons evolved envelope proteins, an addition that allows cell entry and transforms the elements into infectious retroviruses. This close relationship makes LTR retrotransposons ideal models for studying the molecular mechanisms responsible for retrovirus replication. The transposable elements (TEs) of model organisms, such as yeast, are particularly well suited to address the dynamics and impact of their replication. We study LTR retrotransposons of the fission yeast (*Schizosaccharomyces pombe*) to determine how integration sites are selected and to understand how patterns of integration impact the physiology of the cell. In past work, we found that integration of LTR retrotransposons in *S. pombe* alters gene expression and adapts cells to environmental stress. It is through selective adaptation that we believe TEs form gene-regulatory networks. In additional studies, we have adapted our methods of mapping large numbers of TE insertions to sequencing HIV-1 integration sites. To date our HIV-1 integration dataset represents the largest published study of positions and allows us to identify important mechanistic aspects of integration that previously have been neglected.

In humans, TEs represent 50% of genomic sequences. The dominant families of TEs are Long INterspersed Element-1 (LINE-1 or L1), which constitutes 17% of the genome, and Alu Short Interspersed Elements (SINEs), which are mobilized by L1 and constitute 10% of the genome. Given that TEs make up half of the human genome, it is not surprising that their regulatory features are abundant sources of tissue-specific promoter activity and are critical building blocks of gene-regulatory networks. Although the vast majority of TEs have lost mobility, each genome retains approximately 100 active copies. As a result, genome studies of human populations reveal many thousands of polymorphic TEs. Our goal is to determine the role of these genetic variants in health and disease.

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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 (N-acetyl transferase). 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.
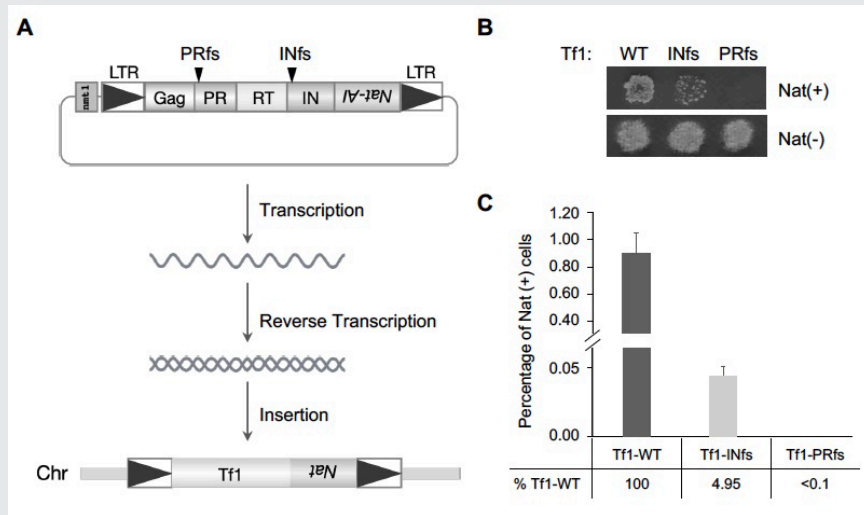
Identification of an integrase–independent pathway of retrotransposition

Despite the central role of integration in the propagation of retroviruses, important questions remain about residual insertions that occur in the absence of integrase (IN) activity. Mutations in the catalytic residues of HIV-1 IN produce residual infectious titers, typically with a 3- to 4-log reduction. However, in continuous cultures of HIV-1 lacking IN activity, insertion efficiency can be as high as 0.2–0.8% of a wild-type (WT) virus. These findings indicate that retroviruses possess a secondary, IN–independent pathway, which incorporates viral DNA into the host genome. Given that IN–independent infections could compromise the treatment of HIV-1 patients with IN inhibitors, it is important to identify the nature of this pathway.

LTR retrotransposons are important models of retroviruses because of their structural and mechanistic similarities. Tf1 and Tf2 are extensively characterized LTR retrotransposons with high integration activity in *S. pombe*. Studies of Tf1 expressed with genetic markers demonstrate that the Gag protein, protease (PR), reverse transcriptase (RT), and IN all contribute to transposition. Importantly, the resulting integration is directed to specific RNA pol II (RNA polymerase II) promoters by the DNA–binding factor Sap1. To identify a model system that can be used to study the mechanisms of IN–independent insertion, we measured the insertion of Tf1 lacking IN activity. We performed an insertion assay with Tf1 encoding a frameshift mutation at the start of IN (Tf1-INfs) that blocks expression of IN without altering RT expression or cDNA synthesis. We found Tf1-INfs retained 4.95% of the insertion activity of Tf1-WT [Reference 1], indicating that, in the absence of IN activity, Tf1 cDNA inserted into the host genome with surprising efficiency. Genome-wide insertion profiles of Tf1 lacking IN (Tf1-INfs) were significantly different from those of Tf1 expressing active IN. DNA logo analysis showed that the sequences downstream of the Tf1-INfs insertion sites had a prominent bias for the ATAAC nucleotide sequence, and upstream flanks showed a preference of CAA. Interestingly, the

downstream logo matches the sequence of the primer binding site (PBS), an 11 bp sequence retained after reverse transcription on the 3' end of the plus-strand cDNA. The CAA matches the last three base pairs of the polypurine tract (PPT), which is retained on the 3' end of the minus-strand cDNA. The PBS and PPT sequence preferences indicated that these single-stranded sequences contributed to insertion through homologous recombination (HR). If IN–independent insertions are directed to sites with homology to the PBS and PPT, we would expect that large numbers of insertions would occur at the 13 pre-existing copies of Tf2 that have PBS and PPT sequences identical to those of Tf1. By analyzing the raw downstream sequences, we found that approximately 70% of the IN–independent insertions occurred at homologous sequences within the pre-existing 5' LTRs of Tf2s. Whole-genome sequencing of these events revealed that the most common outcome of these insertions resulted in tandem copies of Tf1 and Tf2 elements.

Our data suggest that IN–independent insertion of Tf1 is likely mediated by a form of homologous recombination. To determine whether homologous recombination factors contribute to IN–independent insertion, we measured insertion frequencies of strains lacking *mre11, rad50, nbs1, rad51,* or *rad52* (genes encoding members of a complex that repairs double-strand DNA breaks). The results revealed that the insertions occurred through Rad52–dependent single-strand annealing (SSA), as Rad51 was dispensable. The *rad52*–R45A mutation, which specifically abolishes the SSA activity of Rad52, significantly reduced the frequency of Tf1-INfs insertions and resulted in dissociation of Rad52 from Tf1 cDNA. These data indicate that Rad52 plays a critical role in IN–independent insertions by binding to the ends of the cDNA, causing recombination with sequences similar to PBS and PPT.

The efficiency of HR–mediated IN–independent insertion of Tf1 raised questions about whether this pathway has a biological function. Our efforts to determine whether IN–independent events occur naturally showed that cultures with continuing expression of WT Tf1 produced insertions that were predominantly IN–independent [Reference 1]. These data demonstrate that Tf1 possesses two efficient insertion pathways, one relying on IN and the other being IN–independent but requiring Rad52. Significantly, we found in previously published data of HIV-1 IN–independent insertion sequences that five of 69 sites had strong similarity to the HIV-1 PBS. Together, our findings indicate that homology-dependent SSA provides a significant pathway of IN–independent insertion.

The role of LEDGF in transcription is intertwined with its function in HIV–1 integration.

HIV-1 integration occurs across actively transcribed genes, a specificity that is attributable to the interaction of host factor LEDGF (lens epithelium-derived growth factor) with integrase. Our understanding of HIV-1 integration is incomplete, in part because the cellular function of LEDGF is unclear. Although LEDGF was originally isolated as a co-activator that stimulates promoter activity in purified systems, this model is inconsistent with LEDGF–mediated integration across gene bodies and with data suggesting that LEDGF can regulate alternative splicing. To clarify the roles of LEDGF in transcription, we conducted RNA-seq. In the absence of LEDGF, 516 expressed genes were differentially expressed (a greater than 1.6-fold change), underscoring a significant role in gene expression. To examine the role of LEDGF in splicing, we analyzed genes that produce differentially expressed mRNA isoforms in the absence of LEDGF. The majority of these isoforms were expressed from different promoters, suggesting that the dominant function of LEDGF is to regulate promoter activity, not splicing. To determine how LEDGF regulates transcription, we measured H3K4me3 (a methylated histone) enrichment, a mark of active promoters. Cells lacking LEDGF had reduced

H3K4me3 at down-regulated genes and elevated levels in up-regulated genes. To evaluate the direct role of LEDGF in the expression of these genes, we contended with a long-standing problem in understanding HIV-1 integration, namely, there were no accurate maps of chromatin bound by LEDGF. Antibodies specific for LEDGF have not been successful in ChIP-seq experiments. By CRIPSR editing HEK293T cells, we scarlessly introduce a 3XFLAG tag to the 3' end of *PSIP1*, the native LEDGF gene. The resulting ChIP-seq experiments provided a high-resolution map of LEDGF–binding sites across the genome. Surprisingly for a protein that mediates integration across gene bodies, we observed pronounced peaks of LEDGF at the 5' end of transcription units that matched the peaks of H3K4me3 of active promoters. Significant reduction in H3K4me3 enrichment in LEDGF knockout (KO) cells at LEDGF–bound promoters indicated that LEDGF functions to regulate promoter activity. We also observed by ChIP-seq that levels of RNA Pol II at promoters were reduced in the absence of LEDGF. Efforts to understand how LEDGF is recruited to promoters tested the function of factors that bind to LEDGF, such as the histone methyltransferase MLL1. ChIP-seq showed that MLL1 peaks match the positions of LEDGF at promoters and, importantly, when levels of MLL1 are reduced with siRNA (small interfering RNA), the peaks of LEDGF at promoters are significantly reduced. Reduction of MLL1 also resulted in substantially lower levels of RNA Pol II Ser5 phosphorylation, a mark of active polymerase.

These experiments not only provided insight into the function of LEDGF in transcription but also revealed new aspects of how LEDGF directs integration. LEDGF possess an N-terminal PWWP domain, which is known to interact with histone H3K36me3, an epigenetic mark of active transcription. As a result, it is thought that the PWWP domain is responsible for directing LEDGF directly to the bodies of genes being actively transcribed. With ChIP-seq experiments, we mapped the chromatin binding of LEDGF lacking the PWWP domain and found that no changes occurred in binding locations. These results were surprising because crude measures of chromatin association indicated that PWWP was required for binding. In collaboration with Alan Engelman, we found that removal of the PWWP domain did not significantly alter the integration sites, supporting our finding that PWWP is not important for chromatin binding. Together with our studies on MLL1, these data support a model in which LEDGF is recruited to active promoters by MLL1 and subsequently travels across transcription units to effect HIV-1 integration. We are currently testing the role of MLL1 in integration by mapping insertion sites in cell culture.

Retrotransposon insertions associated with risk of neurologic and psychiatric diseases

Mental disorders affected about 970 million people worldwide in 2017. In 2020, 21% of adults in the United States suffered from some form of mental illness. Such diseases thus cause great social and economic burden. Studies of identical twins show that the heritability of diseases such as attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), bipolar disorder (BIP), and schizophrenia is extremely high, ranging from 74% to 81%. Because of 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.

TASs (trait-associated single-nucleotide polymorphisms [SNPs]) of GWAS are genetic tags identifying a genomic region that contains the causal mutation(s), which lead to increased disease risk. Limits on the design of GWAS

typically prevent such studies from identifying causal gene alleles. Thus, 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 readily alter gene expression 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. There may be functional consequences of common TE insertion variants that affect common disease risk. Some common polymorphic TEs have been implicated at disease loci detected by GWAS. Common polymorphic *Alu* (short transposable elements) insertions occur disproportionately near disease loci of GWAS, underscoring the fact that *Alu* insertions are potential causative variants.

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 [Reference 2]. 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. In all, we identified 10 polymorphic TEs to examine further as causal candidates because they were positioned in enhancer, promoter, heterochromatin, or transcribed sequences present in neurologic tissues.

We hypothesized that the polymorphic TEs have a causal relationship with risk of psychiatric and neurologic disorders by altering expression of genes in *cis*. For evidence of altered gene expression, we queried the Genotype-Tissue Expression (GTEx) database, which contains expression data for 948 donors across 54 tissues. GTEx readily identifies changes in tissue-specific gene expression associated with loci-specific genetic variation. SNPs in LD with a query gene are identified as eQTLs (expression quantitative trait loci) if the genetic loci with the variants are significantly associated with altered expression of a gene in a specific tissue. We found that 31 of the TASs linked to TEs were variants that are associated with changes in expression of one or more adjacent genes within regions of the brain.

Having identified a number of polymorphic *Alu* elements that are significantly associated with disease risk detected by GWAS and that are correlated with altered gene expression in neurologic tissues by eQTL

analysis, we developed a luciferase reporter assay to test whether the insert sequences in the context of flanking sequence can influence transcription activity. We measured the impact of candidate *Alu* and flanking sequences on the function of a minimal promoter in NCRM-1 (human neural stem cells). Of six candidate *Alu* insertions evaluated for their impact on promoter activity, we found that five significantly altered the expression of luciferase. Taken together, we identified 10 polymorphic TE insertions that are potential candidates on par with other variants for having a causal role in neurologic and psychiatric disorders.

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Publications
1. Li F, Lee M, Esnault C, Wendover K, Guo Y, Atkins P, Zaratiegui M, Levin HL. Identification of an integrase-independent pathway of retrotransposition. *Sci Adv* 2022 8:1–17.
2. Ahn H, Worman Z, Lechsinska A, Payer L, Wang T, Malik N, Li W, Burns K, Nath A, Levin HL. Retrotransposon insertions associated with risk of neurologic and psychiatric diseases. *EMBO Reports* 2023 24:1–17.

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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
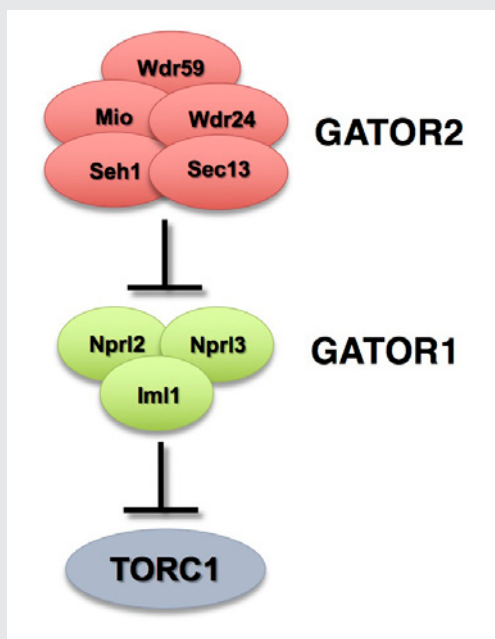
FIGURE 1. The GATOR complex regulates TORC1 activity.

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

starvation and 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), which inhibit TORC1 activity by inactivating the Rag GTPases. Notably, *Nprl2* and *Iml1* are tumor-suppressor genes, and mutations in *Iml1*, known as *DEPDC5* in mammals, are a leading cause of hereditary epilepsy.

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 *in vivo* functions of the GATOR1 and GATOR2 complexes in the model organism *Drosophila*.

The GATOR2 component Wdr59 promotes or inhibits TORC1 activity depending on cellular context.

We defined two novel functions for the GATOR2 component Wdr59. Studies performed in tissue culture cells, which often involve the rapid removal and subsequent return of amino acids to growth assays, have uniformly determined that all GATOR2 complex components are required for the activation of TORC1. In contrast, our whole animal studies in *Drosophila* have defined unique tissue-specific requirements for multiple individual GATOR2 subunits, including Mio, Seh1, and Wdr24. We determined that Wdr59, originally assigned to the GATOR2 complex based on studies performed in tissue culture cells, unexpectedly has a dual function in TORC1 regulation in *Drosophila*. We found that, in the ovary and the eye imaginal disc brain complex, Wdr59 inhibits TORC1 activity by opposing the GATOR2–dependent inhibition of GATOR1. Specifically, GATOR2 attenuates the binding of the GATOR1 to the RagA component of the Rag GTPase. Conversely, in the *Drosophila* fat body, Wdr59 promotes the accumulation of the GATOR2 components Mio and Wdr24 and is required for TORC1 activation. Similarly, in mammalian HeLa cells, WDR59 prevents the proteolytic destruction of these GATOR2 proteins. Consistent with reduced levels of the TORC1–activating GATOR2 complex, Wdr59KOs HeLa cells have reduced TORC1 activity, which is restored, along with GATOR2 protein levels, upon proteasome

FIGURE 2. Many 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 pathway dependent on the ubiquitin ligase HUWE1.

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.
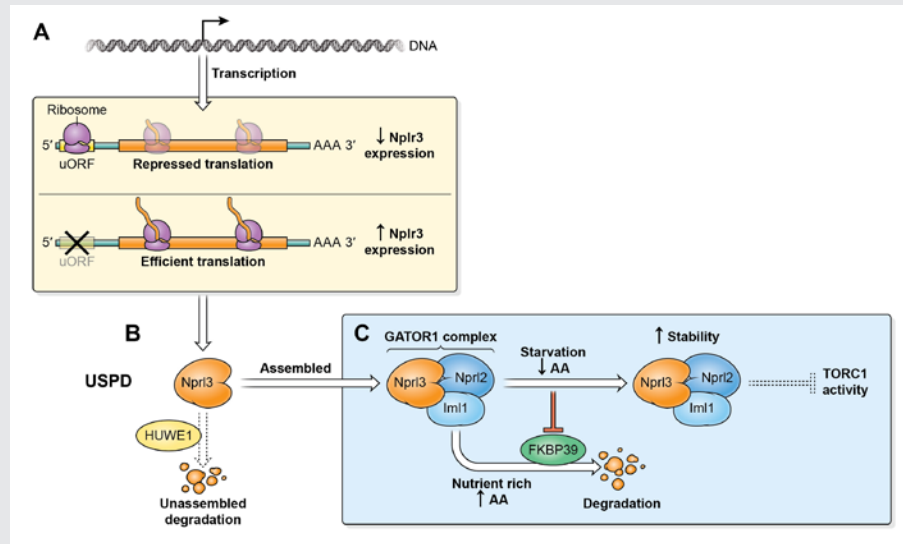
inhibition. Taken together, our data support the model in which the Wdr59 component of the GATOR2 complex functions to promote or inhibit TORC1 activity, depending on cellular or metabolic context. Importantly, our work on *Drosophila* challenges recent models of GATOR2 function based on structural analysis, which posit that the GATOR2 complex requires all core subunits, including Wdr59, to form a functional complex. Our studies broaden the understanding of the GATORTORC1 signaling axis in metazoans and highlight the complexity of metabolic regulation *in vivo*. The GATOR–TORC1 signaling pathway is frequently cited as a potential target of pharmaceutical intervention because of its role in cancer, neurodegeneration, and epilepsy. Thus, it is essential to have a full mechanistic understanding of the *in vivo* function of the GATOR complex in the regulation of TORC1 signaling and growth.

Many 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 uncovered novel mechanisms of Nprl3 regulation and identified an important role for *Drosophila* FKBP39 in the control of cellular metabolism and growth.

FIGURE 3. Model for the dual role of Wdr59 in TORC1 regulation

A. In wild-type animals, Wdr59 inhibits TORC1 activity upstream of the GATOR2 complex in the ovary and eye imaginal disc brain complex.

B. In *wdr59* mutants, GATOR2 increases its association with GATOR1, further inhibiting GATOR1 activity, and allowing for the increased activation of TORC1.

C. In the fat body of *Drosophila* and in mammalian HeLa cells, Wdr59 protects GATOR2 components from degradation by the proteasome.

D. In the absence of Wdr59 from the *Drosophila* fat body or from HeLa cells, GATOR2 components are destroyed by the proteasome, resulting in de-repression of GATOR1, increased interaction of GATOR1 with RagA, and concomitant reduction in TORC1 activity. Note that the GATOR1 complex regulates TORC1 activity by acting as a GAP (GTPase–activating protein) for the RagA component of the GTPase, which functions to activate TORC1.
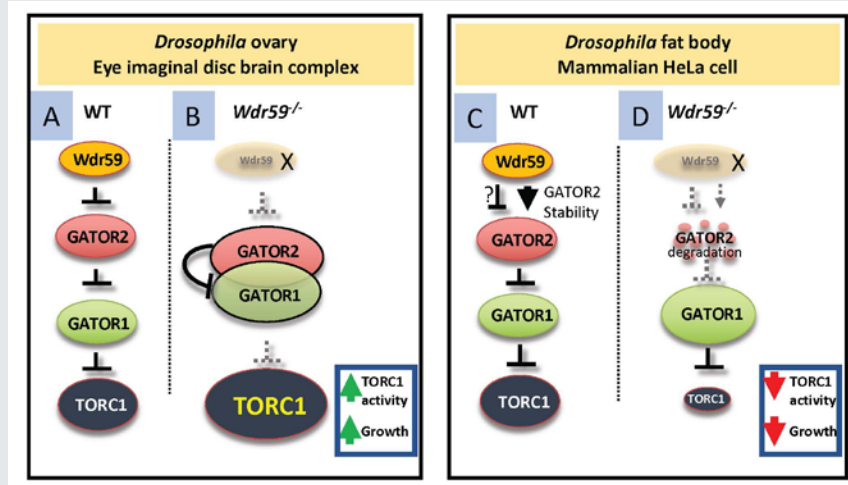
Publications

1. Wang ZH, Combs C, Zhao W, Zhang F, Knutson J, Lilly MA, Xu H. Mechanical stimulation from the surrounding tissue activates mitochondrial energy 1 metabolism in differentiating germ cells. *Dev Cell* 2023 58:2249–2260.
2. Zhang Y, Ting C-Y, Yang S, M. Lilly MA. Wdr59 promotes or inhibits TORC1 activity depending on cellular context. *Proc Natl Acad Sci USA* 2022 120:e2212330120.
3. Zhou Y, Guo J, Wang X, Cheng Y, Guan J, Barman P, Sun MA, Fu Y, Wei W, Feng C, Lilly MA, Wei Y. FKBP39 controls nutrient dependent Nprl3 expression and TORC1 activity in Drosophila. *Cell Death Dis* 2021 12(6):571.

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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 (pituitary corticotrope tumor cell line) cells, we demonstrated in both cases that POMC secretion via the constitutive secretory pathway was elevated. In double CPE–SgIII knock-down cells, elevated constitutive secretion of POMC and stimulated secretion of ACTH were perturbed. Thus, CPE mediates trafficking of POMC to the RSP; SgIII may play a compensatory role for CPE in POMC sorting to the RSP.

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

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anterograde vesicle movement to the plasma membrane for activity-dependent secretion. Additionally, 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 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 levels of ACTH vesicles at the terminus, and enhanced secretion of ACTH in AtT20 cells. Thus, our study 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 the 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 nonsense 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, 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. The mechanism of action of CPE/NF-alpha1 in neuroprotection in mouse and rat hippocampal neurons involves the activation of the ERK1/2 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]. 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, an interaction that was confirmed by co-immunoprecipitation and pulldown assays. Binding studies revealed a K_d of 13.82nM. Molecular dynamics studies followed by experimental validation indicated that CPE/NF-alpha1 interacts with 5-HTR1E via four salt bridges stabilized by several hydrogen bonds, and is independent of the serotonin binding pocket. Immunohistochemistry revealed co-localization of 5-HTR1E and CPE/NF-alpha1 on the surface of hippocampal neurons. Signal transduction studies showed that HTR1E–CPE/NF-alpha1 interaction activated the ERK1/2 (extracellular signal–regulated kinases)–CREB pathway via recruitment of beta-arrestin. This in turn activated the BCL2 pro-survival pathway. We showed that the 5-HTR1E–CPE/NF-alpha1 interaction mediated neuroprotection of human primary neurons against H_2O_2–induced cytotoxicity and glutamate-induced neurotoxicity. The findings indicate that CPE/NF-alpha1 interacts with 5-HTR1E to promote neuronal survival [Reference 3]. 5-HTR1E is only found in humans, primates, and guinea pig, but not in mice or rats. Work is

ongoing to identify a mouse receptor for CPE/NF-alpha1. Additionally, we showed that serotonin interacts with the 5-HTR1E receptor, simultaneously activating cAMP and ERK pathways in HEK293 (human embryonic kidney) cells, and that its expression is important for cell survival.

Examination of the pathway during stress *in vivo* revealed that, after mild chronic restraint stress (CRS) for one 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 but 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 (fibroblast growth factor 2). We demonstrated that mouse primary hippocampal neurons treated with CPE/NF-alpha1 increased FGF2 expression. Thus, another action of CPE/NF-alpha1 may be to increase FGF2, which is known to have neuroprotective effects. In summary, CPE/NF-alpha1 is a critical neurotrophin 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. 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 wild-type (WT) protein in the ER, causing ER stress, reduced 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. The 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 the gene for the first time with neurodegenerative disease and depression. We therefore explored whether CPE could rescue cognitive dysfunction and memory loss in AD mice. We injected AAV-CPE into the hippocampus of an AD mouse model (3xTg-AD) to determine its effect on AD pathology. AD mice injected with AAV-CPE at the pre-symptomatic stage (2 months old) did not develop AD pathology (including memory loss, neurodegeneration, tau hyperphosphorylation and increase in amyloid peptides Abeta 1–42) at 8 months of age. However, these pathologies, which are hallmarks of AD, were found in control AD mice injected with AAV-GFP. AAV-CPE treatment also down-regulated the expression of a microglia neuro-inflammatory protein, Card14, which may contribute mechanistically to the decreased microglia activation and

neuroinflammation compared with control AD mice. Additionally, expression of Plin4, a mitophagy inhibitor protein, was lowered. Thus, CPE-NF-alpha1 overexpressed in the hippocampus targets several regulatory components to prevent the development of AD pathology in AD mice [Reference 4]. CPE-NF-alpha1 gene therapy may be a promising approach for treating early Alzheimer disease patients.

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 the 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 5]. Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway [Reference 5]. 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 WT CPE/NF-alpha1, and two terminal-truncated isoforms of CPE/NF-alpha1-ΔN (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. Interestingly, CPE/NF-alpha1-ΔNs 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 neuro-development. We examined the effect of CPE/NF-alpha1 on E13.5 neocortex-derived neuro-spheres, 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 by 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.

Neurite outgrowth is key to the formation of synapses and the neural network during development. We found that CPE/NF-alpha1 prevented Wnt-3a inhibition of nerve growth factor (NGF)–stimulated neurite outgrowth in PC12 cells, a neuroendocrine cell line, and in cortical neurons. Moreover, CPE/NF-alpha1 augmented Wnt-5a–mediated neurite outgrowth. Thus, the interplay between NGF preventing neurite outgrowth, which is inhibited by Wnt-3a, and augmenting neurite outgrowth, which is mediated by Wnt-5a and CPE/NF-alpha1, could play an important role in regulating these positive and negative cues, which are critical for neurodevelopment. Analysis of the brain of 6- to 14-week-old *Cpe*–KO mice revealed poor dendritic pruning in cortical and hippocampal neurons, which could affect synaptogenesis.

We also studied the function of 40kD CPE/NF-alpha1-ΔN and showed that it is translocated from the cytoplasm into the nucleus of rat embryonic neurons. Overexpression of 40kD CPE/NFalpha1-ΔN in HT22 cells, a hippocampal cell line, resulted in an increase in the expression of IGF–binding protein2 (IGFBP2), <u>d</u>eath-<u>a</u>ssociated <u>p</u>rotein (DAP1), and Ephrin 1A mRNAs and proteins (receptor protein tyrosine kinases), which 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-ΔN–dependent manner in HT22 and mouse cortical neurons. Thus, 40kD CPE/NF-alpha1-ΔN functions to regulate expression of genes important in neurodevelopment [Reference 6]. Recent *in vivo* studies on a knock-in mouse model expressing CPE/NF-alpha1-ΔN but not CPE-WT revealed that some neurodevelopmental functions were normal in these KI mice, suggesting that CPE/NF-alpha1-ΔN plays an important role, independent of CPE-WT.

Publications

1. Xiao L, Sharma VK, Toulabi L, Yang X, Lee C, Abebe D, Peltekian A, Arnaoutova I, Lou H, Loh YP. Neurotrophic factor-a1, a novel tropin is critical for the prevention of stress-induced hippocampal CA3 cell death and cognitive dysfunction in mice: comparison to BDNF. *Translat Psychiatry* 2021 11:24.
2. Xiao L, Loh YP. Neurotrophic factor-α1/carboxypeptidase E functions in neuroprotection and alleviates depression. *Front Mol Neurosci* 2022 15:918852.
3. Sharma VK, Yang X, Kim K-S, Mafi A, Saiz-Sanchez D, Villanueva-Anguita P, Xiao L, Toulabi L, Inoue A, Goddard III W, Loh YP. Novel interaction between neurotrophic factor-α1/carboxypeptidase E and serotonin receptor, 5-HTR1E, protect human neurons against oxidative/neuroexcitotoxic stress via β-arrestin/ERK signaling. *Cell Mol Life Sci* 2021 79:24.
4. Xiao L, Yang X, Sharma VK, Abebe D, Loh YP. Hippocampal delivery of neurotrophic factor-α1/carboxypeptidase E gene prevents neurodegeneration, amyloidosis, memory loss in Alzheimer's Disease male mice. *Mol Psychiatry* 2023 8:3332–3342.
5. Cheng Y, Rodriguiz RM, Murthy SRK, Senatorov V, Thouennon E, Cawley NX, Aryal D, Ahn S, Lecka-Czernik B, Wetsel WC, Loh YP. Neurotrophic factor-alpha1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone. *Mol Psychiatry* 2015 20:744–754.
6. Xiao L, Yang X, Loh YP. Neurotrophic, gene regulation, and cognitive functions of carboxypeptidase E-neurotrophic factor-α1 and its variants. *Front Neurosci* 2019 13:243.

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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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We have continued our studies of the mechanism of translation initiation using our recently developed Rec-seq transcriptome–wide method. Rec-seq utilizes our fully reconstituted yeast translation initiation system and is able to simultaneously monitor the recruitment of each mRNA in the yeast transcriptome to the 43S ribosomal PIC. We completed experiments on the mode of action of the DEAD-box RNA helicase translation-initiation factor Ded1 using the Rec-seq system. We found that Ded1 promotes recruitment of mRNAs with long, structured 5'-UTRs. The set of mRNAs that are dependent on Ded1 for efficient translation initiation in the Rec–seq system highly overlap with the mRNAs previously identified as being hyper-dependent on Ded1 *in vivo* using ribosome profiling. Because the *in vitro* Rec–seq system isolates translation initiation from other cellular processes that occur *in vivo*, such as mRNA decay and mRNA localization, our results indicate that Ded1 directly influences translation initiation *in vivo*. Our data do not support a previously proposed model that Ded1 stimulates translation initiation by promoting read-through of start codons in 5'-UTRs to allow PICs to scan through and find the main start codons in mRNAs. Although Ded1 does enhance read-through of upstream start codons in 5'-UTRs in the Rec-seq system, the level of 5'-UTR translation in the absence of Ded1 and the amount Ded1 reduces the translation is much too small to account for the stimulatory effects of the factor on 48S PIC formation. We submitted a manuscript for publication describing this work. We also performed additional experiments to assess the effects of varying 40S ribosomal subunit concentration on 48S PIC formation transcriptome-wide. Our results appear to support a previously proposed model that limiting 40S subunit concentrations specifically disfavors translation of "weak" mRNAs that compete poorly for binding to the PIC compared with "strong," more competitive mRNAs.

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Exploring Genes and Signals Regulating Mammalian Hematopoiesis and New Approaches to Immunotherapy

Our research primarily 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. In newer studies, we are investigating whether modification of TCR signaling subunits can be used to enhance the tumoricidal activity of T cells for cancer treatment. 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 or RNA-seq. 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 are currently investigating the function of another Themis family member, Themis3, which is expressed only in the intestine. We found that a different, newly identified protein, Fbxl12, is important for regulating proliferation during early T cell development by coordinating pre–TCR and Notch signaling (the latter an important cell-signaling pathway).

Yet another area of investigation focuses on hematopoietic stem cells (HSCs) and early stages of T cell, B cell, and erythrocyte development. We began characterizing 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 in 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
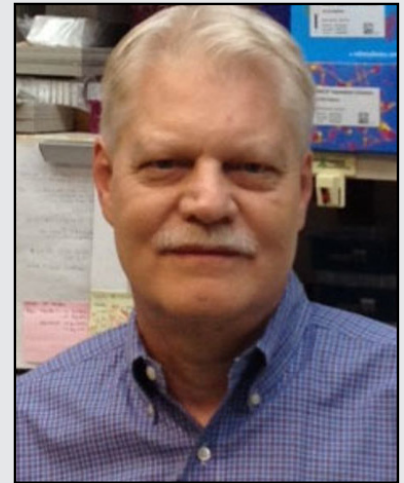
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therapeutic targets for the treatment of an aggressive form of childhood leukemia called <u>e</u>arly <u>T</u> progenitor <u>T</u> cell <u>a</u>cute <u>l</u>ymphoblastic <u>l</u>eukemia (ETP-T-ALL).

T cell antigen receptor (TCR) signaling in thymocyte development

Much of our research in the past has focused on the role of TCR signal transduction in thymocyte development. Signal transduction sequences, termed <u>i</u>mmuno-receptor <u>t</u>yrosine-based <u>a</u>ctivation <u>m</u>otifs (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 these 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 important 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
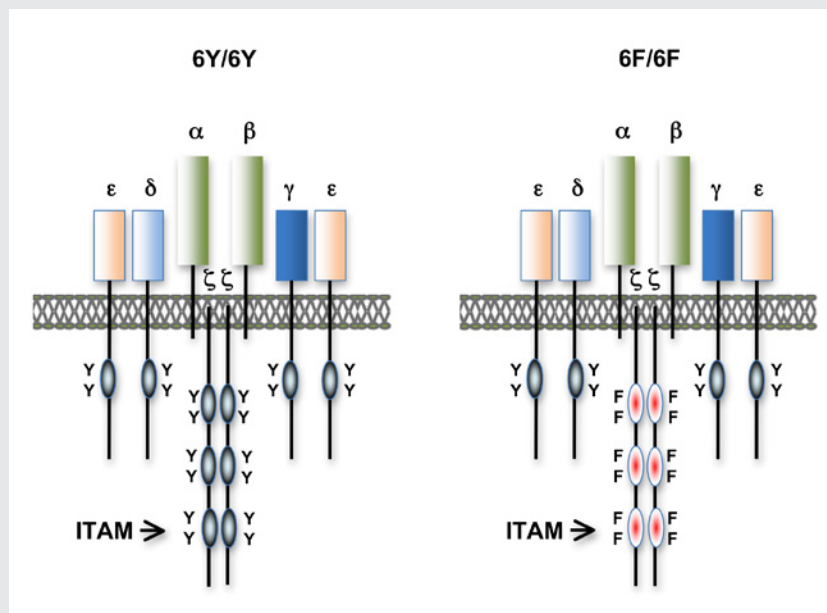

FIGURE 1. T cell antigen receptors expressed in 6Y/6Y and 6F/6F knock-in mice

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).
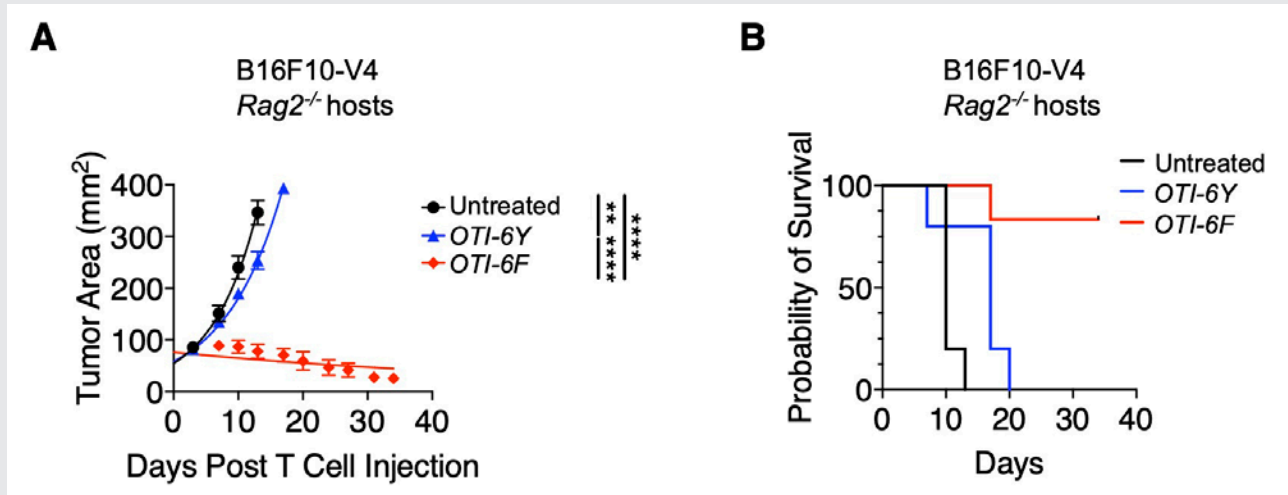
FIGURE 2. Expression of TCRs that include non-signaling (6F) CD3zeta subunits unexpectedly results in enhanced CD8 T cell–mediated tumor killing in response to low affinity antigens.

Rag2⁻ᐟ⁻ mice were injected with OTI TCR transgenic-6Y (OTI-6Y) or OTI-6F CD8 T cells 7 days after B16F10-V4 melanoma implantation. The OTI TCR binds with low affinity to a peptide from avian ovalbumin (V4). B16F10 melanoma cells were transduced to express V4 peptide as a ligand for the OTI TCR, serving as a pseudo-tumor antigen.

Left. Measurement of tumor size over time.

Right. Survival curves of experiment shown on left. N=5 biological replicates. Results shown are representative of 3 experiments. Data are represented as mean ± SD. **P < 0.01, ****P < 0.001.

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 some ITAMs perform more subtle, regulatory functions. To investigate this question further, 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 discovered an inhibitory role for zeta ITAM signaling in response to weak (low-affinity) antigens that was due to the recruitment of the inhibitory tyrosine phosphatase SHP-1 by mono-phosphorylated zeta ITAMs. Strikingly, inactivation of the zeta ITAMs resulted in enhanced TCR signaling and enhanced T cell effector functions when the TCR was 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 (Figure 2). These 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). Also, our results call for a revision of the current paradigm of TCR signaling, which assumes that all ITAMs perform only activating roles in TCR signaling, given that they demonstrate that, under certain conditions, zeta subunit ITAMs can inhibit TCR signaling.

Identification and characterization of TCR–tuning proteins that may serve as targets for immunotherapy

We extended our analysis of TCR–signaling subunits to other molecules that participate in or influence the TCR–signaling response. The cell-surface T cell protein CD5 negatively regulates TCR signaling and functions in thymocyte selection and in mature T cell responses. 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, including that encoding CD6, a surface receptor that is structurally similar to CD5, and we are validating their function as tuning molecules. Given that these 'tuning' 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, which are based on blocking the function of the induced inhibitory molecules PD-1 and CTLA-4. Experiments are under way to investigate this translational potential.

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 3). 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. In a collaboration with Richard Cornall, we also investigated the phenotype of Themis2 knockout mice generated in our lab (Themis2 is closely related to Themis1 but is expressed in B cells and myeloid cells instead of T cells). Our results identified an important role
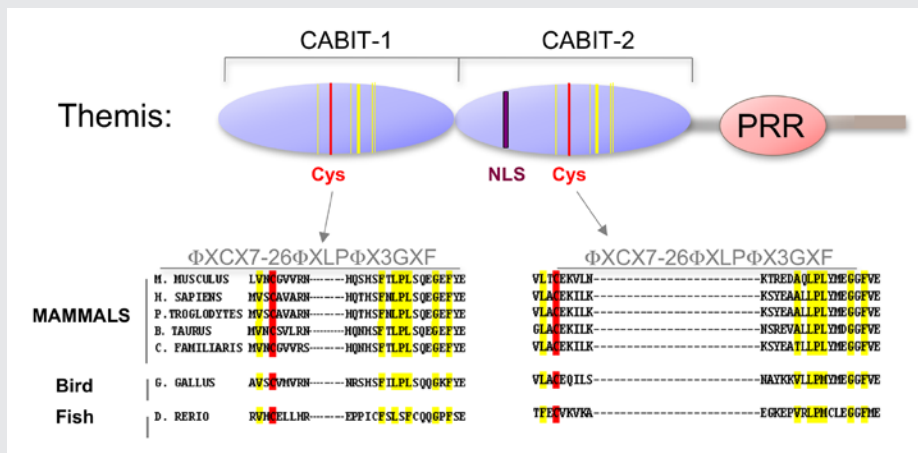
FIGURE 3. Themis is highly conserved in vertebrates.

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).

for Themis2 in facilitating B cell activation by low-avidity, but not high-avidity, B cell receptor (BCR)–antigen interactions.

More recently, 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 (cysteine-containing, all beta in Themis) module (Figure 3). Using *in vitro* 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 increased sensitivity of thymocytes to TCR signaling (Figure 4). 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, including the intestine-specific mouse protein Themis3, 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

FIGURE 4. Regulated expression of Themis during T cell development facilitates thymocyte positive selection.

Thymocytes are more sensitive to TCR signaling than mature T cells, exemplified by the fact that low-affinity self-ligands can activate thymocytes but are incapable of activating mature T cells. The relatively high expression of Themis in thymocytes vs. mature T cells contributes to establishing the sensitivity of thymocytes to TCR signaling. In thymocytes, where Themis is abundant, Themis binds to and inactivates the inhibitory tyrosine phosphatase, SHP-1, by promoting oxidation of the SHP-1 catalytic cysteine by Reactive Oxygen Species (ROS, including H_2O_2), resulting in stronger TCR signaling to weak self-ligands. In mature T cells, down-regulation of Themis results in a higher concentration of active (reduced) SHP-1, which can inhibit TCR signals to low affinity self-ligands, preventing autoimmunity.
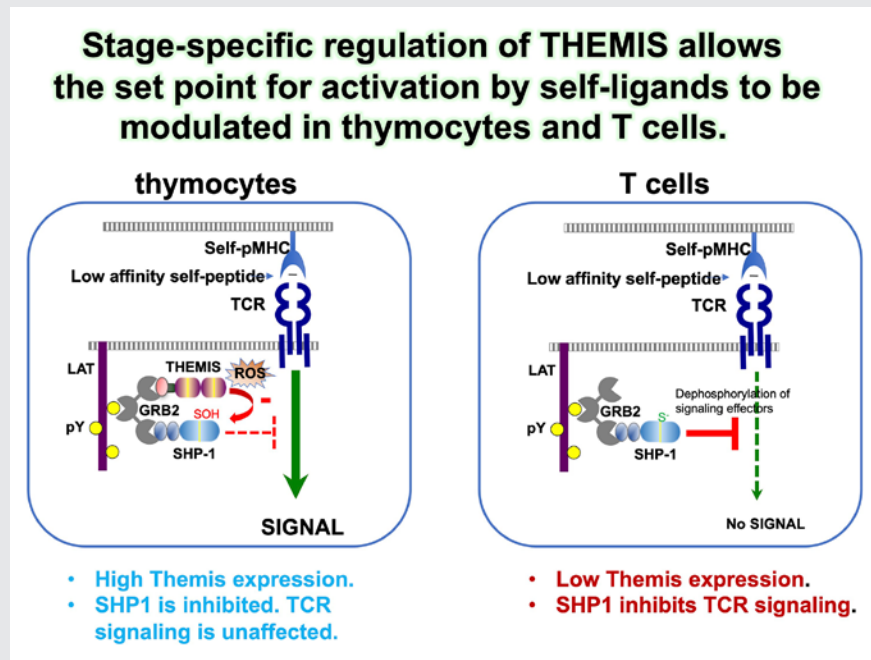
gene and which express a signaling complex called the pre–TCR). The proliferative burst that accompanies beta selection is estimated to result in a 100–200 fold expansion and is essential for further differentiation and for maximizing TCR diversity. Previous work showed that beta selection–associated 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 (worse than single deletion of Fbxl1 or Fbxl12), 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 5. 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.
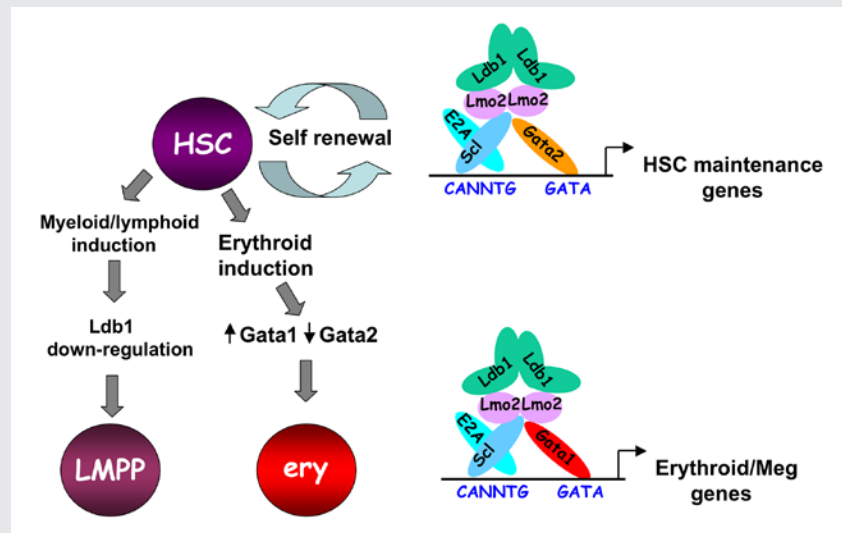
Role of Ldb1 transcription complexes in hematopoiesis and in T cell acute lymphoblastic leukemia (T-ALL)

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 by 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 5). Within the hematopoietic lineage, expression of *Ldb1* is highest in progenitor cells, which include hematopoietic stem cells (HSCs). We initially 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* in HSCs. We found that Ldb1 is not required for ESC maintenance but is for HSC maintenance. More recent data indicate that the loss of *Ldb1*$^{-/-}$ HSCs results from effects on differentiation rather than cell death. 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. Examination of the function of Ldb1 in cell lineages downstream of HSCs identified an essential function in the erythroid lineage but not in myeloid or lymphoid cells. Further, 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 5). 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.
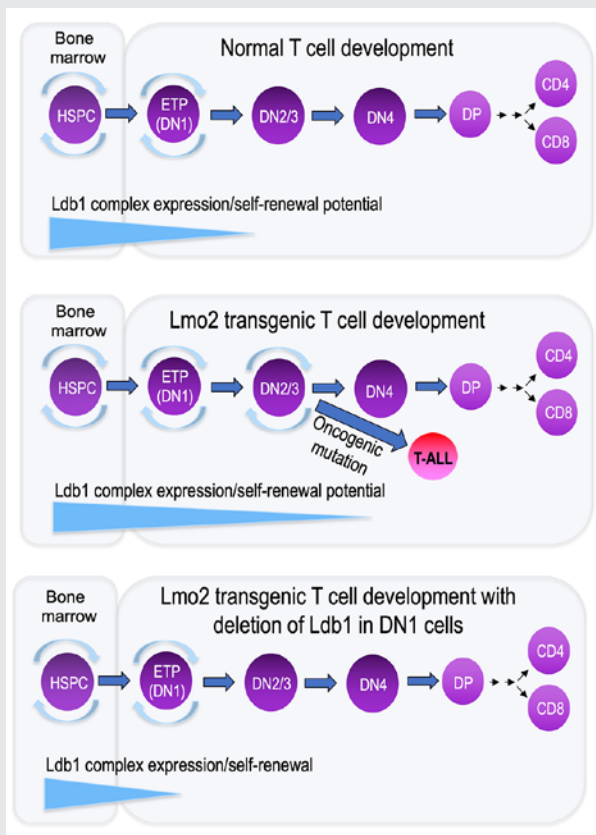
FIGURE 6. Deletion of Ldb1 prevents Lmo2–induced generation of T cell leukemia.

Failure to down-regulate the Ldb1 complex subunit and proto-oncogene Lmo2 in mice and humans results in induction of a severe form of T cell leukemia (ETP-T-ALL), characterized by prolonged expression of hematopoietic stem cell genes. Ldb1/Lmo2 multi-protein complexes are normally expressed in hematopoietic stem cells (HSCs) and function in the maintenance and self-renewal of HSCs (*upper panel*). Spontaneous mutations or retroviral insertions in humans or transgenic expression of Lmo2 in mice that cause sustained expression of Lmo2 in immature thymocytes promote abnormal self-renewal of thymocytes, rendering them sensitive to secondary oncogenic mutations, which result in induction of T-ALL (*middle panel*). Deletion of Ldb1 prevents induction of T-ALL in Lmo2 transgenic mice, identifying Ldb1 complexes as the cause of T-ALL in Lmo2–transgenic mice (*lower panel*).

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, one of the most informative being 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 do not over-express 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 self-renewal potential. *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, which 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 (Figure 6). Currently, we are addressing several key questions, including whether, as predicted, Ldb1, and by extension Ldb1 complexes, regulate expression of genes that govern the 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

Publications
1. Dutta A, Venkataganesh H, Love PE. Epigenetic regulation of T cell development. *Int Rev Immunol* 2023 42:82–90.
2. Choi S, Hatzihristidis T, Gaud G, Dutta A, Lee J, Arya A, Clubb LM, Stamos DB, Markovics A, Mikecz K, Love P. GRB2 promotes thymocyte positive selection by facilitating THEMIS-mediated inactivation of SHP1. *J Exp Med* 2023 220:e20221649.
3. Choi S, Lee J, Hatzihristidis T, Gaud G, Dutta A, Arya A, Clubb LM, Stamos DB, Markovics A, Mikecz K, Love PE. THEMIS increases TCR signaling in CD4+CD8+ thymocytes by inhibiting the activity of the tyrosine phosphatase SHP1. *Sci Signal* 2023 16:eade1274.
4. Lee JYM, Love PE. A simplified approach to evaluating T cell development by flow cytometry. *Methods Mol Biol* 2023 2580:131–149.
5. Gaud G, Achar S, Bourassa FXP, Davies J, Hatzihristidis T, Choi S, Kondo T, Gossa S, Lee J, Juneau P, Taylor N, Hinrichs CS, McGavern DB, François P, Altan-Bonnet G, Love PE. CD3ζ ITAMs enable ligand discrimination and antagonism by inhibiting TCR signaling in response to low-affinity peptides. *Nat Immunol* 2023 24(12):2121–2134.

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The Arms Race between Transposable Elements and KRAB-ZFPs and its Impact on Mammals

The central mission of the NICHD 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 proteins (KRAB-ZFPs)

Kruppel-associated box zinc-finger (ZF) proteins (KRAB-ZFPs) are rapidly evolving transcriptional repressors, which emerged in a common ancestor of coelacanths, birds, and tetrapods; they constitute the largest family of transcription factors in mammals (estimated to be several hundred in mice and humans). Each species has its own

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unique repertoire of KRAB-ZFPs, with some shared by closely related species and others specific to each species. Remarkably, there was an explosion of KRAB-ZFP genes in the earliest mammals, many of which have been retained by purifying selection, but the function of these (as well as the hundreds of species-restricted KRAB-ZFPs) have been largely unexplored. KRAB-ZFPs consist of an N-terminal KRAB domain, which 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 therefore began a systematic interrogation of KRAB-ZFP function as a potential adaptive repression system against ERVs.

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 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 (embryonic stem cell) 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. In contrast to the young (species-restricted) KRAB-ZFPs, we found that the older KRAB-ZFPs (that are conserved across mammals) bind to genetic loci that have themselves undergone regulatory innovations during evolution. By systematically studying these KRAB-ZFP genes and their targets, we are uncovering regulatory innovations unique to placental mammals.

CTCF barrier–breaking by ZFP661 promotes protocadherin diversity in mammalian brains.

Mammalian brains are larger and more densely packed with neurons than those of reptiles, but the genetic mechanisms underlying the increased connection complexity amongst neurons are unclear. The expression diversity of clustered protocadherins (Pcdhs), which is controlled by CTCF (CCCTC [DNA sequence]–binding factor) and cohesin, is crucial for proper dendritic arborization and cortical connectivity in vertebrates. We identified a highly conserved and mammalian-restricted KRAB-ZFP, ZFP661, that binds antagonistically at CTCF barriers at the *Pcdh* locus, preventing CTCF from trapping cohesin. ZFP661 balances the usage of Pcdh isoforms and increases Pcdh expression diversity. We demonstrated that loss of *Zfp661* causes cortical dendritic arborization defects and autism-like social deficits in mice. Our study reveals both a novel mechanism that regulates the trapping of cohesin by CTCF and a mammalian adaptation that promoted Pcdh expression diversity to accompany the expanded mammalian brain.

Dual histone methyl readers ZCWPW2 and ZCWPW1 connect PRDM9 to DNA double–strand breaks and their repair during meiotic recombination.

We also began a new exploration of the function of PRDM9, the most ancient KRAB-ZFP, which emerged in jawless fish and which 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
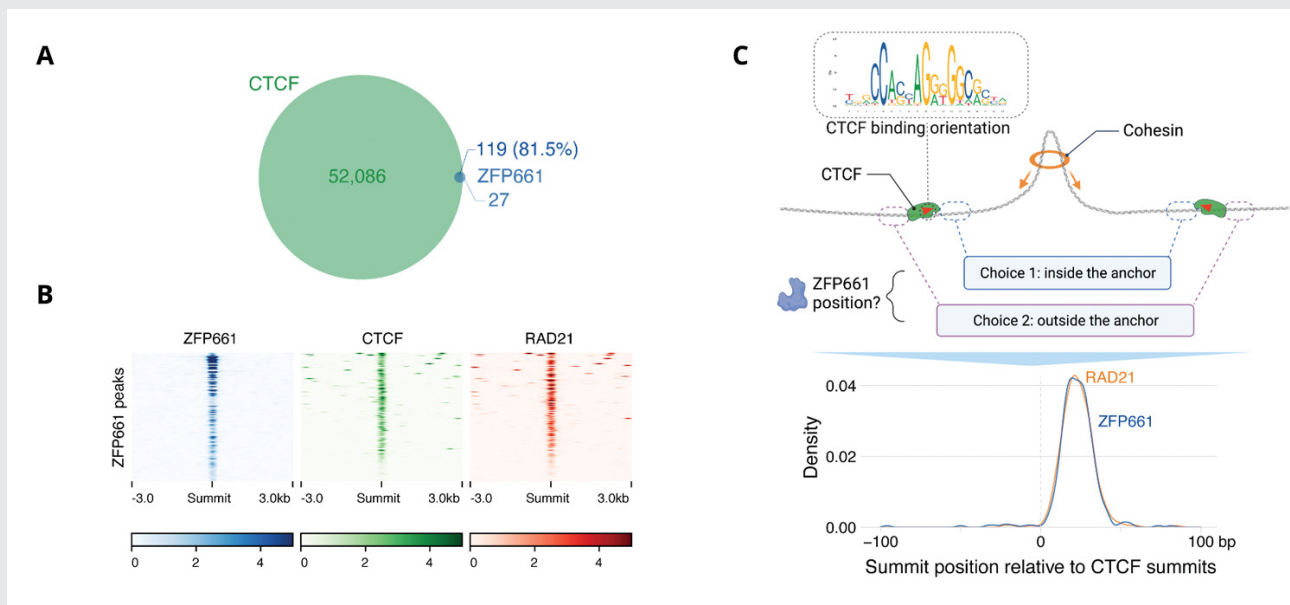
FIGURE 1. ZFP661 binds adjacent to a small subset of CTCF sites within loop anchors

A. ZFP661 binding peaks overlap with a small subset of CTCF binding peaks.

B. Heat maps of ChIP-seq signal across ZFP661 binding sites indicate strong overlap with CTCF and the cohesin subunit Rad21

C. ZFP661 binding is found adjacent to CTCF binding motifs, within loop anchors, at the same position at which cohesin is typically trapped.

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 (histone methyltransferase) activity that catalyzes dual trimethylation marks on histone H3 at lysine 4 and 36 (H3K4me3 and H3K36me3), both of whose activities are 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 first identified the dual histone methylation reader *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 for pairing homologous chromosomes in male 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. Since publishing these findings, we identified a *Zcwpw1* paralog, which was initially mis-annotated in the mouse genome, called *Zcwpw2*. Importantly, in the past year, we found that *Zcwpw2* is essential for both mouse meiosis and fertility in males and females, and that it is important for the efficient generation of double-strand breaks at hotspots relative to promoters. The studies have thus revealed a three-component system, comprising a rapidly evolving DNA–binding histone methyltransferase (PRDM9) and two dual histone methylation readers (ZCWPW2 and ZCWPW1), which play at least partially separable roles in mediating the PRDM9–dependent generation of DNA DSBs and their repair at meiotic recombination hotspots.

Additional Funding

Publications

1. Hoge C, de Manuel M, Mahgoub M, Okami N, Fuller Z, Banerjee S, Baker Z, McNulty M, Andolfatto P, Macfarlan TS, Schumer M, Tzika AC, Przeworski M. Patterns of recombination in snakes reveal a tug of war between PRDM9 and promoter-like features. *bioRxiv* 2023 07.11.548536.
2. Jin J, Ralls S, Wu E, Wolf G, Sun MA, Springer DA, Cosby RL, Senft AD, Macfarlan TS. CTCF barrier breaking by ZFP661 promotes protocadherin diversity in mammalian brains. *bioRxiv* 2023 *https://doi.org/10.1101/2023.05.08.539838*.
3. Xie G, Lee JE, Senft AD, Park YK, Jang Y, Chakraborty S, Thompson JJ, McKernan K, Liu C, Macfarlan TS, Rocha PP, Peng W, Ge K. MLL3/MLL4 methyltransferase activities control early embryonic development and embryonic stem cell differentiation in a lineage-selective manner. *Nat Genet* 2023 55(4):693–705.
4. Du C, Jiang J, Li Y, Yu M, Jin J, Chen S, Fan H, Macfarlan TS, Cao B, Sun MA. Regulation of endogenous retrovirus-derived regulatory elements by GATA2/3 and MSX2 in human trophoblast stem cells. *Genome Res* 2023 33(2):197–207.

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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 known 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). *L. pneumophila* mutants with a non-functional T4SS are degraded by macrophages (Figure 1), 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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these studies can help in the development of novel therapeutics aimed at treating or preventing Legionnaires' disease and related illnesses.

VpdC is a ubiquitin-activated phospholipase effector that regulates *Legionella* vacuole expansion during infection.

Like many other microbial pathogens, *Legionella* encodes a wide variety of phospholipases (at least 14), among them VpdC. Production of VpdC in the model organism *Saccharomyces cerevisiae* (Sc) strongly attenuated yeast growth, while VpdC(S338A), a mutant protein in which one of the residues predicted to be important for phospholipase activity had been mutated, had no impact on Sc viability, showing that VpdC was catalytically active in the context of eukaryotic cells. However, purified recombinant VpdC did not display any detectable phospholipase A2 (PLA2) activity *in vitro* unless an aliquot of lysate from COS-1 cells was added to the reaction, suggesting that the lysate contained an allosteric activator.

Pulldown studies from cell lysate paired with high-performance liquid chromatography-mass spectrometry (HPLC-MS) identified ubiquitin as an interaction partner for VpdC. Upon performing PLA2 assays *in vitro*, a mixture of both proteins exhibited robust PLA2 activity, while neither VpdC nor ubiquitin alone showed any detectable activity (Figure 2). Thus, ubiquitin was an allosteric activator of VpdC's PLA2 activity.

While examining the role of VpdC during *Legionella* growth in human U937 macrophages (the disease host), we made a curious discovery: unlike Lp02 (a common *Legionella* laboratory strain) lacking VpdC (Lp02DvpdC), which grew as efficiently as the parental strain Lp02 over 48 hours, the complemented strain Lp02DvpdC(pVpdC) showed a significant growth reduction (about 8-fold), which was not observed upon rescue
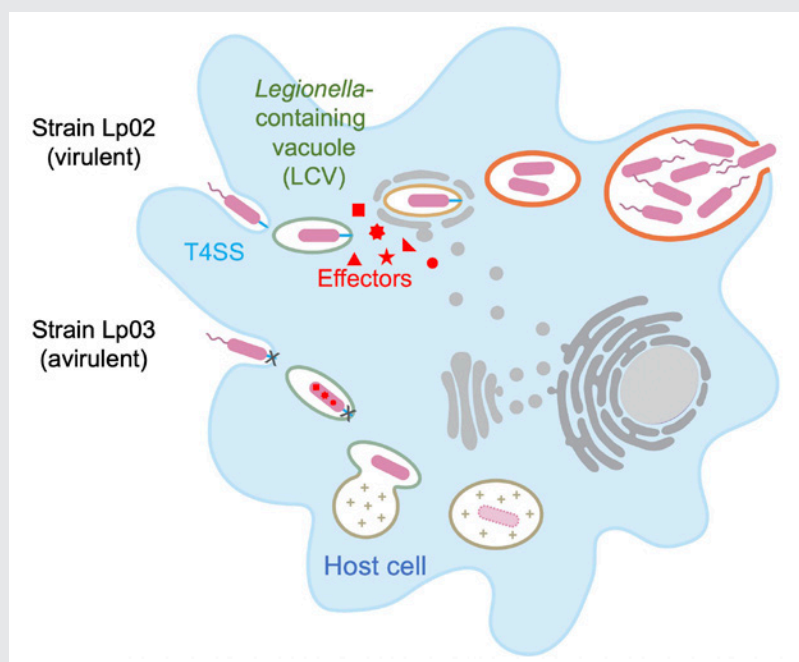
FIGURE 1. Intracellular replication cycle of *Legionella pneumophila*

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

**FIGURE 2. Lysophospholipids play an important role in regulating vacuole expansion during *Legionella* growth.**

(A) Ubiquitin stimulates the phospholipase A2 activity of VpdC *in vitro*.

(B) Upon infection of human macrophages, the effector protein VpdC from *Legionella* generates moderate levels of lysophospholipids within the membrane of its vacuole (LCV) to promote expansion. Overproduction of lysophospholipids (*right image*) prevents membrane acquisition and expansion of the LCV, trapping the bacteria (*labeled green in the micrographs*) within spatially confined vacuoles and reducing virulence. Scale bar, 5 μm.
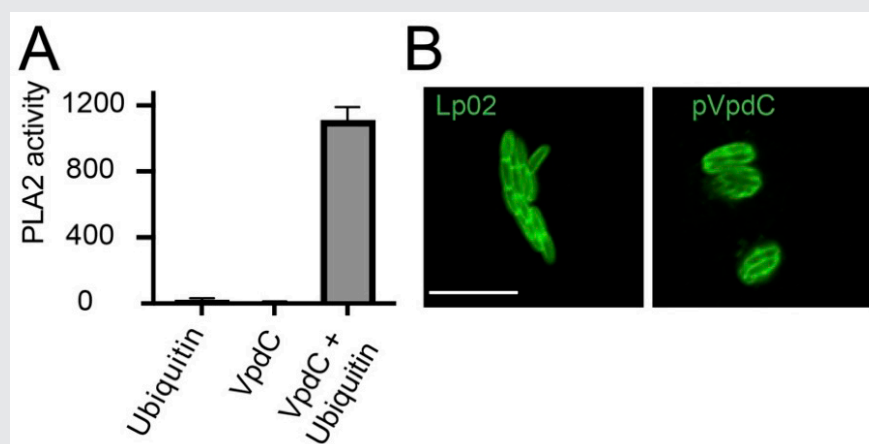
with catalytically inactive VpdC(S338A). Fluorescence microscopy revealed that LCVs of Lp02DvpdC(pVpdC) were considerably less spacious than those of Lp02 (Figure 2). The findings suggested that the augmented PLA2 activity of VpdC had impeded LCV expansion, likely by generating an excess of lyso-phospholipids (LPLs) which, through their inverted cone shape, are known to obstruct membrane fusion. Lipidomics analyses on infected U937 Mfs (a macrophage-derived cell line) confirmed that several types of LPLs, including lyso-PA, -PC, and -PE, were significantly more enriched in macrophages infected with Lp02DvpdC(pVpdC) than in cells challenged with Lp02DvpdC(pVpdC(S338A)). Our data support a model in which VpdC localizes to the surface of the LCV, where its PLA2 activity generates LPLs that, at physiological level, can promote fusion with surrounding membranes to promote gradual vacuole expansion. Augmented PLA2 activity upon VpdC overproduction by *Legionella* results in an increased LPL content within the LCV membrane, which blocked fusion, thus confining the bacteria to a spatially limited LCV. Our results show that the coordinated expansion of their vacuole is a critical virulence feature of *Legionella* and likely of other pathogens.

Identification of virulence-critical genes using CRISPR interference

A major obstacle in deciphering *Legionella* virulence mechanisms is that the deletion of effector-encoding genes, individually or even in groups, rarely results in detectable intracellular growth defects, a phenomenon that is attributed to the existence of a significant level of overlap in the effectors function and/or host targets, so that the loss of individual effectors is compensated for by the activity of synergistic or redundant effectors. Classical genetic approaches such as transposon mutagenesis or chemical mutagenesis, which target genes at random and often disrupt house-keeping genes essential for viability, have been mostly unsuccessful in identifying combinations of virulence-critical genes of *Legionella*, other than those encoding components of the essential Dot/Icm apparatus.

We created a novel gene-silencing tool in *Legionella* that harnesses the power of CRISPR interference (CRISPRi) to silence pairs or even entire groups of bacterial genes to systematically screen for combinations of virulence-critical effectors in this pathogen (Figure 3). CRISPRi requires the presence of three functional components: a
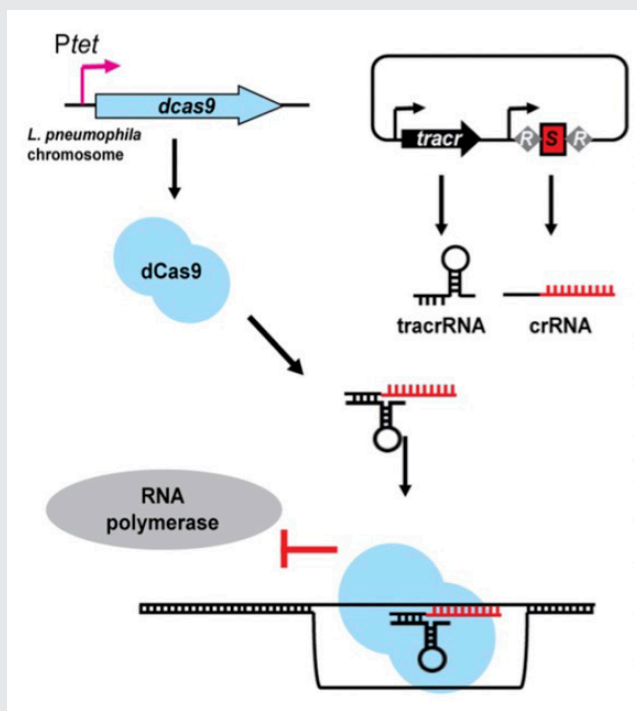
FIGURE 3. Concept of CRISPRi

The three components dCas9 (*blue*), tracrRNA (*black*), and crRNA (*red*) form a trimeric complex that binds to target genes via homologous base pairing and blocks transcription by RNA polymerase.

protein called dCas9, which is the nuclease-deficient form of the *Streptococcus pyogenes* Cas9 enzyme; a trans-activating CRISPR (tracr) RNA that interacts with both dCas9 and the third component of this system, the CRISPR (cr)RNA. crRNAs, together with the tracrRNA, specifically direct dCas9 through base-pairing to complementary target DNAs, thus imposing a road block that prevents gene expression by sterically precluding RNA polymerase from gaining access to the gene (Figure 3). In nature, CRISPR/Cas systems are found in the majority of bacterial and archaeal genomes, where they serve as a naturally occurring adaptive immune system. Fragments of foreign DNA, such as virus DNA, transposable elements, or plasmids, are degraded, and fragments are incorporated into the bacterial chromosome as a series of spacers separated by repeats. Such repeat-spacer-repeat arrays, which can contain dozens or even hundreds of unique spacers, serve as a memory of past infections and protect the bacteria from re-infection.

Given the natural multiplex (meaning multiple gene targets at a time) capability of CRISPRi, we reasoned that this system could be exploited in *Legionella* to simultaneously silence entire families of chromosomal genes simply by replacing the foreign DNA spacer sequences of CRISPR arrays with sequences complementary to the bacterium's own genes (Figure 3). The dCas9–encoding gene was integrated into the *Legionella* genome, while the tracrRNA– and crRNA–encoding sequences were provided on a plasmid, each under control of the anhydrous tetracycline-inducible tet promoter. Using biochemical and cell-based assays, we confirmed that our CRISPRi approach was tunable, specific, and functional during intracellular growth. Most importantly, it was multiplex-capable, allowing us to silence up to ten genes at a time in *Legionella*. Upon interrogation of nearly 200 effector-encoding genes using synthesized arrays of ten repeat-spacer-repeat elements, we discovered several combinations of genes that, when silenced simultaneously, led to profound growth defects of *Legionella* in human macrophages or amoeba, notably without compromising bacterial growth outside the host. Such newly discovered virulence genes represent promising new targets for therapeutic intervention.
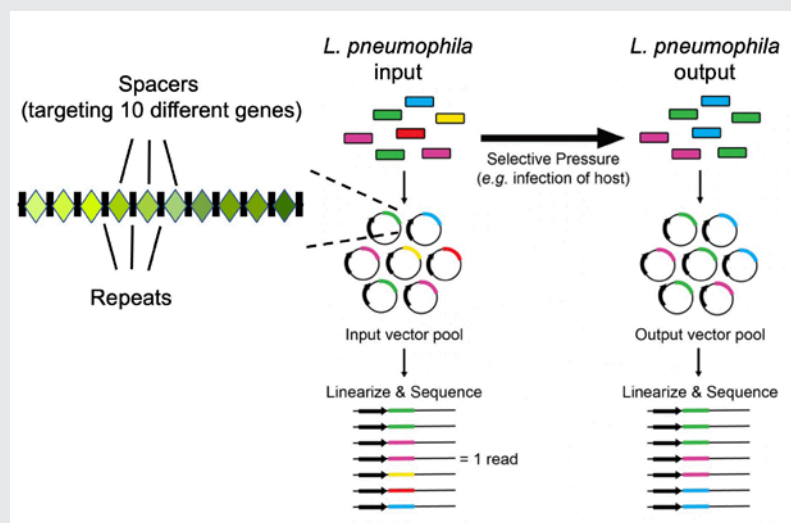
Design of a next generation randomized multiplex CRISPRi approach

A major limitation of the proof-of-concept CRISPRi approach described above was that it was not randomized, given that the 10-plex CRISPR arrays had to be commercially synthesized, meaning that they each targeted a predetermined group of ten specific *Legionella* genes. To bypass this limitation, we generated an improved

CRISPRi tool whereby the CRISPR arrays were self-assembled in a randomized (rather than predetermined) fashion from oligonucleotide pairs. We succeeded in assembling a collection of thousands of plasmids containing hundreds of unique combinations of arrays able to silence between two and eleven genes (average of 3.1 genes), serving as the foundation for the first multiplex, randomized CRISPR interference sequencing (MuRCiS) pipeline (Figure 4). We also designed a customized bioinformatics algorithm that monitored differences in array abundance in the output (post infection) vs input pool, where a reduction in the abundance of an array was indicative that it had silenced gene combinations indispensable for intracellular replication.

In a proof-of-concept study, we used MuRCiS to probe 44 highly conserved transmembrane domain (TMD)–containing effectors of *Legionella* for their importance during bacterial replication in both U937 macrophages (the disease host) and *Acanthamoeba castellanii* (the environmental host), and several gene combinations that were identified as vital have become the focus of our future research. The MuRCiS platform is applicable to other groups of gene families besides T4SS effectors and will undoubtedly identify more interesting biology related to *Legionella* pathogenesis.

The *Legionella pneumophila* effector DenR hijacks the host NRas proto-oncoprotein to downregulate MAPK signaling.

While studying intracellular trafficking of *Legionella* in human HT1080 cells, we discovered that the host protein N-Ras accumulates on LCVs (Figure 5). This was an unusual finding, given that Ras proteins, unlike GTPases of the Rab, Arf, Rho, and Ran subfamily, have not been shown to be targeted by *Legionella*. NRas is a member of the Ras subfamily of small GTPases. It transmits extracellular inputs from the plasma membrane (PM) along various signaling pathways to promote cell growth, proliferation, and survival. If mutated, Ras GTPases are responsible for 30% of all human cancers, with N-Ras mutations occurring in 15–20% of all melanomas.

We used site-directed mutagenesis to show that the two sites for lipidation, specifically farnesylation and S-acylation, within the C-terminal hypervariable region (HVR) of NRas are required for Ras trafficking to the

FIGURE 5. Model explaining how NRas recruitment dampens MAPK signaling from the PM while initiating alternative signaling from the LCV
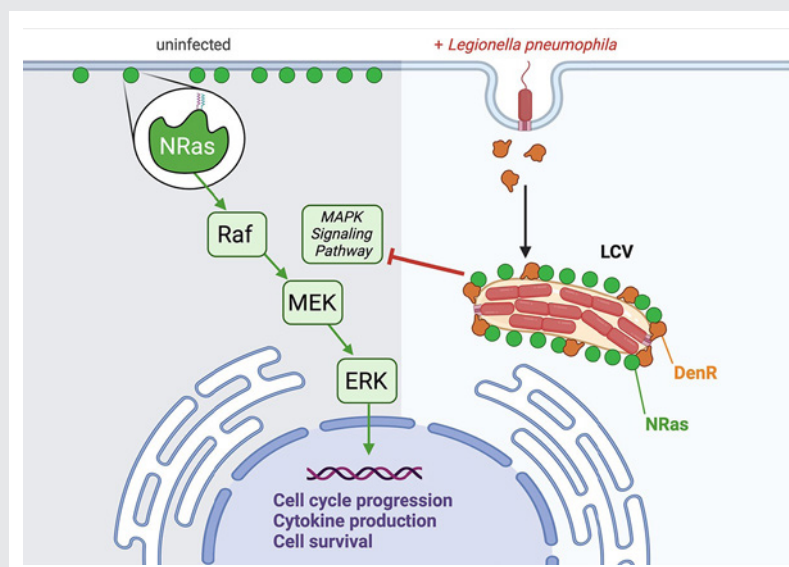
LCV. Using our multiplex CRISPRi tool described above, we found that silencing of a single gene, called *denR*, rendered *Legionella* defective for NRas recruitment (hence, denR). A strain with an in-frame deletion in *denR* (Lp02DdenR) phenocopied this recruitment defect and could be complemented with plasmid-encoded DenR. Using a translational beta-lactamase-reporter fusion, we verified that DenR was a *bona fide* T4SS effector. Co-precipitation assays from lysate of transiently transfected cells producing GFP-DenR and Halo-tagged NRas confirmed that the two proteins exist in a complex within eukaryotic cells. Lastly, upon production of GFP-DenR in transiently transfected HT1080 cells, we confirmed that DenR specifically localized to LCVs, as was to be expected for an effector that recruits NRas during infection.

To examine how redirecting NRas away from the PM and to the LCV affected cellular signaling, we performed comparative label-free global proteomics and phospho-proteomics on lysate collected from RAW264.7 macrophages challenged with either Lp02ΔdenR or the complemented strain Lp02ΔdenR(pdenR). We identified DenR–mediated alterations in several pathways known to be associated with Ras downstream signaling, most notably the well characterized mitogen-activated protein kinase (MAPK) signaling pathway (Figure 5). Using phospho-specific antibodies, we confirmed a reduction in the levels of activated phospho-MEK and phospho-ERK concomitant with increasing amounts of DenR.

In summary, these data provide evidence for a previously unrecognized involvement of Ras subfamily GTPases in *Legionella* pathogenesis, where DenR, by redirecting NRas away from the PM and to the LCV, dampens signaling cascades originating from the PM, most notably the MAPK pathway (Figure 5).

Additional Funding

Publications

1. Li X, Anderson DE, Chang Y-Y, Jarnik M, Machner MP. VpdC is a ubiquitin-activated phospholipase effector that regulates Legionella vacuole expansion during infection. *Proc Natl Acad Sci USA* 2022 119(48):e2209149119.
2. Ellis NA, Myers KS, Tung J, Ward AD, Johnston K, Bonnington KE, Donohue TJ, Machner MP. A randomized multiplex CRISPRi-Seq approach for the identification of critical combinations of genes. *bioRxiv* 2023; in press.
3. Lehman SH, Williamson C, Tucholski T, Ellis N, Bouchard S, Jarnik M, Allen M, Nita-Lazar A, Machner MP. The Legionella pneumophila effector DenR hijacks the host NRas proto-oncoprotein to downregulate MAPK signaling. *Cell Reports* 2023; in press.

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RNA Metabolism in Cell Biology, Growth, and Development

We study the biogenesis, processing, modification, and decay of tRNAs and mRNAs, and isoforms and fragments thereof, as well as some of their specific interacting proteins that contribute to cell proliferation, growth, and development during health and disease. Some of our studies focus on the La protein, which interacts with nuclear precursor tRNAs and other nascent transcripts synthesized by RNA polymerase III (Pol III) that are matured to small noncoding (nc)RNAs with a diversity of critical functions. Other studies focus on the La-related proteins (LARPs) 1 and 4, which interact mostly with cytoplasmic mRNAs, and the tRNA anticodon-loop modification enzyme known as tRNA-isopentenyltransferase-1 (TRIT1).

tRNAs are produced at over 10-fold higher molar levels than are ribosomes during cellular proliferation. La protein serves as a chaperone for pre-tRNAs, which must undergo proper folding, multiple processing, and enzymatic modifications prior to nuclear export. Many diseases are attributable to defective tRNA biogenesis and the consequent failure to support mRNA translation.

Pol 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 Pol III–transcribed genes. The nascent transcripts bear a copy of this terminator, U(n)U-3'OH, a recognition motif for the nuclear La protein, which binds in a sequence- and length-dependent manner. Notably, the 3'U(n) length–dependence of La binding is usually shorter than the minimal T-length required for efficient Pol III termination, the latter of which is 6, 5, and 4 Ts for *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe,* and humans, respectively. This suggests a La link with tRNA expression, a link that fits with human Pol III, which evolved a minimal 4T termination mechanism; the data suggest that this may direct some post-transcriptional events. Studies on development, structure, and its gene variants indicate that human Pol III evolved termination mechanisms to control gene-regulatory programs with greater intricacy.

The human La protein is a target of auto-antibodies in patients with chronic inflammatory diseases such as systemic lupus erythematosus, Sjögren's syndrome (SS) (La is also known as SS antigen-B, SSB), and
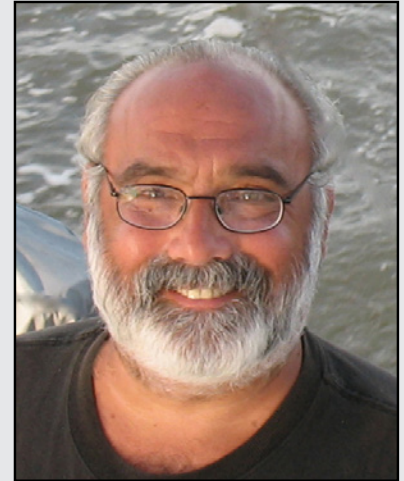
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FIGURE 1. The fission yeast *Schizosaccharomyces pombe* as a model organism

Red-white colony differentiation by tRNA–mediated suppression

neonatal lupus. Although La is active in other immune pathways, evidence for its primary involvement in autoimmunity is lacking. As noted above, La binds to POL III transcripts in all cells and serves as a chaperone during their nuclear maturation. La not only protects pre–tRNAs from 3' exonucleases, but also directs and temporally orders the first step in the 5' processing by the RNase P pathway. We propose a role for La 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. We are studying 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. Accordingly, some dual activity tRNA genes may serve as endogenous immune adjuvants or may contribute to autoimmunity. We are currently exploring these possibilities.

The rate of RNA production by Pol 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, as well as 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 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, Pol 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 minority subset modify both 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 that impair mt-DNA translation are uniquely associated 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 like that resulting from a nuclear gene mutation to a TME whose substrate is the mt-tRNA.

Numerous pathogenic alleles that encode defective subunits of Pol III cause hypomyelinating leukodystrophy (HLD), whose pathobiology is consistent with poor formation of axonal myelin sheaths rather than demyelination. Pol III produces several ncRNAs (non-coding RNAs) in addition to tRNAs. We suggest that another disruption mechanism of 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 mitochondrial function, with a 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 POL 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 to poly(A)–binding protein (PABP/PABPC1). LARP4 stabilizes mRNAs by opposing deadenylation of their poly(A) tails, substrates of Ccr4-Not deadenylase (a multi-protein 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.

It has been known for some time that human Pol III and associated factors are 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 Pol III structures provide insight into activities specific to the higher eukaryotic 17-subunit complex. First, the cancer-associated RPC7a Pol III subunit paralog (encoded by *POLR3G*) appeared to interfere with binding of the Pol III–negative regulator and tumor suppressor MAF1, whereas the RPC7b (POLR3GL) paralog subunit is enriched in cells programmed for differentiation (limited proliferation). Yeast Pol III has only one homolog. The second example involves the most striking feature of higher eukaryote-specific Pol 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 appears to have evolved activities that extend human Pol III beyond the housekeeping activities of its yeast counterpart. This fits with a view of hPol III in self vs. non-self surveillance

functions. Notable are Pol III Vault (Vt) ncRNAs (Vault is a hollow barrel-shaped ribonucleoprotein complex) with involvement in two activity types: innate immune surveillance, and differentiation vs. maintenance of undifferentiated states. Both Pol 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.

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

The Pol III multisubunit enzyme complex consists of 17 integral subunits, whereas Pols I and II consist of 14 and 12 respectively. However, during early vertebrate evolution, one of the Pol III subunits, POLR3G/RPC7, was duplicated, and both gene paralogs evolved as essential, such that present-day organisms use both. Thus, each molecule of cellular Pol III contains one or the other paralog, i.e., POLR3G or POLRGL, and this subunit can control Pol III–intrinsic activity and to direct cellular pathways toward differentiation-quiescence or proliferation-cancer type phenotypes.

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 Pol III to the correct start site. TFIIIB–Pol 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, which is required to drive translation during growth and development. In contrast to all other multisubunit RNA polymerases, termination and re-initiation by Pol III are functionally, if not physically, linked. POLR3G and POLRGL can control the extent to which Pol III may be sensitive to inhibition by the Pol III–negative regulator Maf1 and to recycling. Our laboratory developed *in vivo* and biochemical methods to examine the unique mechanisms used by Pol III. Hereditary mutations in Pol III cause hypomyelinating leukodystrophy (HLD), as well as defects in innate immunity. In addition to its being essential for cell proliferation, Pol III is also linked to aging.

Transcription termination delineates 3' ends of gene transcripts, prevents otherwise runaway Pol from intruding into downstream genes and regulatory elements, and enables release of the Pol for recycling. While other Pols require complex *cis* signals and/or accessory factors to accomplish these activities, eukaryotic Pol III does so autonomously with high efficiency and precision at a simple oligo(dT) stretch of 5–6 base pairs. A basis for this high-density *cis* information is that both the template and non-template strands of the Pol III terminator carry distinct signals for different stages of termination. High-density *cis* information is a feature of the Pol III system, which is also reflected in the dual functionalities of the tRNA promoters as both DNA and RNA elements. Furthermore, the TFIIF–like Pol III subunit C37 is required for this function of the non-template strand signal. Our results reveal the Pol 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. Pol III synthesizes all tRNAs in eukaryotes, and its derepression is associated with cancer. MAF1 is a conserved general repressor of Pol 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 $N2,N2$-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, Pol 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 Pol 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 Pol III) mutants with gain- and loss-of-termination phenotypes in *S. pombe*. We mapped 122 point mutation mutants to a recently solved 3.9 Å structure of the yeast Pol 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 Pol 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 is an unforeseen mechanism of Pol 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 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, 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 23 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 *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

1. Iben JR, Li T, Mattijssen S, Maraia RJ. Single-Molecule Poly(A) Tail Sequencing (SM-PATseq) Using the PacBio Platform. *Methods Mol Biol* 2024 2723:285–301.
2. Whitlock JM, Leikina E, Melikov K, De Castro LF, Mattijssen S, Maraia RJ, Collins MT, Chernomordik LV. Cell surface-bound La protein regulates the cell fusion stage of osteoclastogenesis. *Nat Commun* 2023 14:616.
3. Kozlov G, Mattijssen S, Jiang J, Nyandwi S, Sprules T, Iben JR, Coon SL, Gaidamakov SA, Noronha AM, Wilds CJ, Maraia RJ, Gehring K. Structural basis of 3'-end poly(A) RNA recognition by LARP1. *Nucleic Acids Res* 2022 50:9534–9547.
4. Khalique A., Mattijssen S, Maraia RJ. A versatile tRNA modification-sensitive northern blot method with enhanced performance. *RNA* 2022 28:418–432.
5. Mishra S, Hasan SH, Sakhawala RM, Chaudhry S, Maraia RJ. Mechanism of RNA polymerase III termination-associated reinitiation-recycling conferred by the essential function of the N terminal-and-linker domain of the C11 subunit. *Nat Commun* 2021 12:5900.

6. Mattijssen S, Kozlov G, Gaidamakov S, Ranjan A, Fonseca BD, Gehring K, Maraia RJ. The isolated La-module of LARP1 mediates 3' poly(A) protection and mRNA stabilization, dependent on its intrinsic PAM2 binding to PABPC1. *RNA Biol* 2021 18:275–289.

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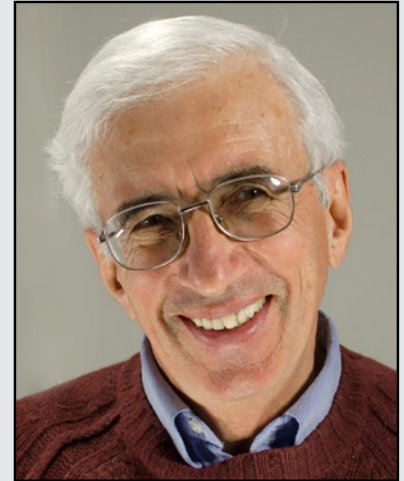
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Immune Activation and Viral Pathogenesis

The general goal of the Section is to understand the mechanisms of pathogenesis of human pathogens in the context of complex tissue microenvironments. In particular, we focused on the role of cytokines in viral pathogenesis. Our studies include extracellular vesicles (EVs) that mediate cell-cell communications and can carry cytokines. We found that EVs, including ones that carry cytokines, are altered in women experiencing pregnancy complications and can be used as biomarkers of such pathologies. Also, EVs in people with psycho-neurological symptom clusters are different from control ones. Both free cytokines and EV–associated cytokines are associated with the pattern of HIV infection and may determine the distinct course of HIV disease in women living with HIV undergoing anti-retroviral therapy. Our current studies are designed according to NICHD Strategic goals on reproductive health, healthy pregnancies, life-long wellness, and gender-based health disparities.

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Cytokines in HIV disease

Among the determinants of an effective pathogen immune responses, to viruses in particular, the complex network of cytokines plays an important role. Cytokines are protein messengers of intercellular communication between multiple immune and nonimmune cells. They are part of the language used by the innate and adaptive immune system to orchestrate an effective immune response to infectious pathogens. Although antiretroviral therapy (ART) efficiently suppresses HIV replication to undetectable levels, it does not fully restore immune functions. We investigated whether ART differentially affects the cytokine network in different body compartments. In addition to cytokines in blood plasma, our study focused on cytokines in semen, given that HIV transmission mostly occurs through sexual intercourse. We characterized the cytokine network in blood and semen in individuals longitudinally sampled before they began ART and after achieving suppression of HIV RNA, by measuring the concentrations of 34 cytokine/chemokines, using a multiplex bead-based assay. We used Partial Least Squares Discriminant Analysis (PLS-DA) to visualize the differences in cytokine patterns between the time points. Cytokines with VIP (variable importance in projection) scores (which reflect the difference in cytokines before and after antiretroviral therapy) exceeding 1 were deemed important for

predicting suppression status and were subsequently tested using Wilcoxon Signed Rank Tests. While PLS-DA projections in blood were fairly similar before and after viral suppression, they were very different in semen before and after ART. When tested individually, four cytokines were significantly different across time points in semen (MIG, IL-15, IL-7, I-TAC), and two in blood (MIG and IP-10). Our results identified specific changes in the cytokine networks in semen and blood as the immune system acclimates to chronic, suppressed HIV infection.

Distinct biological manifestations of sex-based differences have been described in people living with HIV. Women with HIV have lower plasma viral loads, especially during the early phases of infection, and a 1.6-fold higher risk of developing AIDS when accounting for viral load levels in chronic infection. Substantial differences in immune activation between men and women with HIV have been described, but the reasons for these differences have not been fully elucidated. One important factor may be the cytokine response evoked during infection. We investigated the sex differences in blood plasma of men and women with chronic HIV on suppressive ART, using Luminex, and investigated whether cytokines contribute to maintaining higher immune activation in women despite suppressive therapy with ART. We identified five cytokines that were significantly higher in women than in men, namely the pro-inflammatory chemokines CXCL1 (Gro-α), CCL5 (RANTES), CCL3 (MIP-1α), CCL4 (MIP-1β), as well as the T cell homeostatic factor IL-7. Thus, inflammatory cytokines remained higher in virologically suppressed women than in men living with HIV, suggesting that cytokines contribute to maintaining higher immune activation in women despite suppressive therapy with ART. Moreover, CCL3, CCL4, and CCL5, which are β chemokines that share the same receptor, CCR5, have been identified as strong HIV–suppressive factors produced by CD8 T cells. This observation could provide a possible explanation for the differences of viral loads previously reported between women and men. Overall, our results are consistent with the higher cellular activation observed in women. More studies are needed to better understand the underlying mechanisms that contribute to sex-based immune-cell regulation in people with HIV. Our study, together with others, reveals the importance of sex/gender–specific studies, which too often remain a neglected area of viral pathogenesis.

Although ART dramatically reduces HIV viral load and improves the life span and quality of life for most patients, people living with HIV are more likely to develop serious non–AIDS comorbidities, in part because of recurrent immune activation. The drivers of recurrent immune activation in both men and women living with HIV under ART remain to be fully understood. Cytomegalovirus (CMV) is considered to be one of the driving forces of persistent immune activation. Although the associations between CMV replication and systemic inflammation in people living with HIV during suppressive ART have been well documented, it is not clear whether CMV replication is associated with systemic immune activation already during the earliest phase of untreated HIV infection, and thus a potential target for early CMV intervention. We investigated whether genital CMV shedding contributed to systemic immune activation, as evaluated by the concentration of 34 blood cytokines in people living with HIV in the acute/early phase of HIV infection. Independent of CMV, we found that the concentrations of the chemokines IP-10, MIG, MCP-1, I-TAC 10, IL-16, and MIP-1β were modulated in the earliest phase of HIV infection compared with control individuals without HIV. In people with HIV, there was no difference in blood cytokines among CMV shedders vs non-shedders. Our results suggest that CMV shedding in the male genital tract is not the main driver of systemic immune activation in the early phase of HIV infection, in contrast with the later phase of HIV infection. Early ART initiation should remain the priority. Similar studies in women should reveal whether there is a sex-related difference in the role of CMV in early infection. Whatever the role of CMV is in HIV acquisition, our results on cytokine distribution may already explain why women progress faster to AIDS than men at a given viral load.

Extracellular vesicles as markers of complicated pregnancies

Extracellular vesicles (EVs) are released by all cells of the human body and report on the physiology of their cells of origin. Therefore, EVs can, in principle, report on the pathologic development in the organism. In particular, EVs can serve as biomarkers in complicated pregnancies. We found that the composition of EVs in maternal blood is significantly changed in the case of fetal death. Preliminary results found that 16 soluble cytokines, growth factors, and angiogenic factors are significantly different between maternal blood plasma of mothers experiencing fetal death and age-matched mothers without complications. Eight proteins were significantly different in the EV fractions, many of which were the same as the significant soluble proteins, but one protein, CD163, was significantly downregulated only in EV–associated form. Ongoing analysis will determine whether the EV–associated cytokines improve the prediction accuracy of poor outcomes, and future functional studies will focus on determining whether these EV–associated cytokines play distinct roles in pathology.

The cause of fetal death is not always known, but viral infection is a major cause of fetal mortality, in particular CMV infection. Congenital CMV (cCMV) infection is the most common congenital infection and is often associated with severe neurological disabilities or perinatal death. The prediction of neonatal status in the case of CMV infection is limited and therefore the identification of new prognostic markers in amniotic fluid that is sampled by amniocentesis for the diagnosis of fetal infection could improve timely prenatal assessment of infected fetuses. Among biological processes involved in the innate immunity, many cytokines are involved in the immune control of cCMV infection in fetuses. Earlier, we reported that EVs carry cytokines and that these cytokine-carrying EVs constitute a system of cytokine delivery to particular cells. EVs are present in many biological fluids, including amniotic fluid.

We studied whether cytokines measured in the amniotic fluid, in particular cytokines associated with EVs, can report on CMV infection in pregnancy. The study enrolled 80 pairs of women and fetuses/newborns, including 40 infected fetuses and 40 negative controls. Our data suggest that cCMV infection and related symptoms at birth are associated with changes in the immunological signature of the amniotic fluid. Four soluble pro-inflammatory mediators (IP-10, IL-18, ITAC, and TRAIL) and one mediated by EV (IP-10) were elevated in the case of cCMV infection. Among these proteins, five were related to symptoms at birth (IP-10 internal, IP-10 surface, IP-10 soluble, IL-18 soluble, and TRAIL soluble). Seven other cytokines, not related to cCMV infection, were significantly associated with symptomatic status at birth; therefore, a pattern for severe infection can be related with a specific increase in the presence and concentration of six mediators (IL-18 soluble, TRAIL soluble, CRP soluble, TRAIL surface, MIG internal, and RANTES internal). Thus, our data suggest that cCMV infection and its severity are associated with differential expression of cytokines, in particular EV–associated ones, in amniotic fluid at mid-gestation, and may thus serve as candidate biomarkers of severity in case of fetal infection diagnosed by CMV–PCR. Considering trafficking of EVs, EV–associated cytokines may also prove to be promising biomarkers in maternal blood, allowing less invasive tests than amniocentesis.

Extracellular vesicles as markers of psychoneurological symptom clusters

We investigated the associations between EV–associated and soluble cytokines with immune markers and symptom clusters in men with non-metastatic prostate cancer and women with breast cancer. Because extracellular vesicle EV–associated cytokines, both encapsulated and surface bound, have been associated with symptom severity, and may vary over the lifespan, they may be potential biomarkers to uncover underlying mechanisms of various conditions.

Psycho-neurological symptom clusters are co-occurring and interrelated physiological symptoms that may include cancer-related fatigue, pain, depressive symptoms, cognitive disturbances, and sleep disturbances. It is hypothesized that these symptoms share a common systemic pro-inflammatory etiology. We investigated the associations between extracellular vesicle EV–associated and soluble cytokines with immune markers and symptom clusters in men with non-metastatic prostate cancer. Our observational study included 40 men with non-metastatic prostate cancer at the start (T1) of external beam radiation therapy (EBRT) and three months post treatment (T2), as well as 20 men with non-metastatic prostate cancer on active surveillance (AS) seen at one time point.

Both EV–associated and soluble forms of the chemokine RANTES significantly correlated with the symptom cluster for EBRT at T1, whereas, at T2, soluble IFNα2, IL-9, and IL-17 correlated with the corresponding symptom cluster. For the AS group, soluble survivin (an inhibitor of apoptosis highly expressed in most cancers) correlated with psycho-neurological symptoms. Linking specific inflammatory cytokines with psycho-neurological symptom clusters in men receiving prostate cancer treatment can enhance our understanding of the underlying mechanisms of this phenomenon and aid in developing targeted interventions.

We also investigated the associations of soluble and EV–associated cytokine concentrations with distinct symptom profiles reported by 290 women with breast cancer. Patients were classified into older (60 years or more, *n*=93) and younger (less than 60 years, *n*=197) cohorts within two previously identified distinct symptom-severity profiles, which included pain, depressive symptoms, sleep disturbance, and fatigue (i.e., High Fatigue Low Pain and All Low). Results of this study suggest that levels of cytokine concentrations differ between EV and soluble fractions. Several EV and soluble pro-inflammatory cytokines had positive associations with depressive symptoms and fatigue within both age cohorts and symptom profiles. In addition, in the older cohort with the High Fatigue Low Pain symptom profile, EV GM-CSF (a white cell growth factor) concentrations were significantly higher than for the All Low symptom profile. These exploratory analyses provide new information on the association between cytokines and symptom profiles of older and younger cohorts. We found unique EV–associated cytokines in older patients and in specific symptom classes. These results suggest that EVs are potential biomarker discovery tools, as well as underlying distinct symptom class profiles, and may thus inform intervention trials and offer precision-medicine approaches.

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Publications
1. Ñahui-Palomino RA, Vanpouille C, Costantini PE, Margolis L. Microbiota–host communications: bacterial extracellular vesicles as a common language. *PLoS Pathog* 2021 17(5):e1009508.
2. Mercurio V, Fitzgerald W, Vanpouille C, Molodtsov I, Margolis L. Mechanisms of residual immune activation in HIV-1-infected human lymphoid tissue ex vivo. *AIDS* 2021 35:1179.
3. Sass D, Saligan L, Fitzgerald W, Berger AM, Torres I, Barb JJ, Kupzyk K, Margolis L. Extracellular vesicle associated and soluble immune marker profiles of psychoneurological symptom clusters in men with prostate cancer: an exploratory study. *Translat Psychiatry* 2021 11:440.
4. Vanpouille C, Wells A, Wilkin T, Mathad JS, Morris S, Margolis L, Gianella S. Sex differences in cytokine

profiles during suppressive antiretroviral therapy. *AIDS* 2022 36:1215.

5. Rawlings SA, Torres F, Wells A, Lisco A, Fitzgerald W, Margolis L, Gianella S, Vanpouille C. Effect of HIV suppression on the cytokine network in blood and seminal plasma. *AIDS* 2022 36:621.

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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 one for 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 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 the gene *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.

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Mechanism of rare forms of osteogenesis imperfecta

Type XIV OI is a recessive OI form caused by null mutations in *TMEM38B*, which encodes the ER–membrane intracellular cation channel TRIC-B. Previously, we showed that absence of *TMEM38B* alters calcium flux in the ER of OI patient osteoblasts and fibroblasts, which

further disrupts collagen synthesis and secretion. How the absence of *TMEM38B* affects osteoblast function is still poorly understood. We further investigated the role of *TMEM38B* in human osteoblast differentiation and mineralization [Reference 1]. *TMEM38B*–null osteoblasts showed altered expression of osteoblast marker genes and reduced mineralization. RNA-seq analysis revealed that cell-cell adhesion was one of the most downregulated pathways in *TMEM38B*–null osteoblasts, with further validation by real-time PCR and Western blot. Gap- and tight-junction proteins were also reduced by the absence of TRIC-B, both in patient osteoblasts and in calvarial osteoblasts of *Tmem38b*–null mice. Disrupted cell adhesion decreased mutant cell proliferation and cell-cycle progression. An important novel finding was that *TMEM38B*–null osteoblasts had elongated mitochondria with altered fusion and fission markers, MFN2 and DRP1. In addition, *TMEM38B*–null osteoblasts exhibited a significant increase in superoxide production in mitochondria, further supporting mitochondrial dysfunction. Together these results emphasize the novel role of TMEM38B/TRIC-B in osteoblast differentiation, affecting cell-cell proliferation, adhesion processes, gap- and tight-junction, cell-cycle, and mitochondrial function. We also collaborated with the Forlino laboratory on studies of the *TMEM38B*–null mouse that they generated. These studies showed that loss of TRIC-B function dysregulated SMAD signaling in bone. The impaired SMAD signaling detected in mutant mice was validated in OI patient osteoblasts, contributing to defects in osteoblast functioning [Reference 2].

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) 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 and whose bone had typical type VI histology, but whose *SERPINF1* sequences were normal [Reference 3]. Exome sequencing of 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. We generated a murine model for atypical type VI OI. Investigations published to date have focused on bone material properties and on the lacunar canalicular network [Reference 4]. When *Ifitm5*/BRIL p.S42L murine bone is embedded with rhodamine, it is apparent that canicular density is lower than in wild-type (WT) mice at all ages studied, a feature that is also seen in normal bone with aging, where it is associated with impaired signal propagation in response to loading. Second harmonic generation was used to examine the organization of collagen in bone. Although the *Ifitm5*/BRIL p.S42L mouse does not have a defect in collagen structure, collagen is massively disorganized in their bone. Recent investigations of the *Ifitm5*/BRIL p.S42L mouse and its bone biology revealed that both heterozygous and homozygous *Ifitm5*/BRIL p.S42L mice are non-lethal and subject to spontaneous fractures, showing reduced bone strength and markedly increased brittleness on bone mechanics. Although Bril is not directly involved with type I collagen, the bones of the mice are hypermineralized on BMDD (bone-mineralization density distribution), similar to other collagen-based OI forms, with a tripling of CaHigh (highly mineralized bone matrix) in cortical bone. Like OI type aVI patients, young heterozygous mice have normal serum PEDF levels but elevated serum alkaline phosphatase. However, other aspects of bone histology did not recapitulate findings in the patients. Bone osteoid was decreased, as

were the numbers of bone cells, both osteoblasts and osteoclasts, and bone formation rate, and the fish-scale lamellae were not detected under polarized light. Collagen secretion and cellular proliferation were increased in mutant osteoblasts, which is also atypical for OI. Second harmonic generation microscopy revealed disorganized matrix in male mice, similar to that detected among female mutants.

Type VI OI itself has been investigated in a knock-out murine model. We demonstrate that loss of PEDF delays osteoblast maturation as well as extracellular matrix (ECM) mineralization [Reference 5]. There was increased bone vascularization in KO mice, which correlated with elevated numbers of CD31($^+$)/Endomucin($^+$) endothelial cells, which are involved in coupling angiogenesis with osteogenesis. Global transcriptome analysis by RNA-seq of *Serpinf1*$^{(-/-)}$ mouse osteoblasts reveals that osteogenesis and angiogenesis are the biological processes most impacted by loss of PEDF. Intriguingly, TGF-β signaling is activated in type VI OI cells, and *Serpinf1*$^{(-/-)}$ osteoblasts are more sensitive to TGF-β stimulation than WT osteoblasts. These data suggest that the functional antagonism between PEDF and TGF-β pathways controls osteogenesis and bone vascularization and is implicated in type VI OI pathogenesis. The antagonism may be exploited for developing therapeutics for type VI OI.

The endoplasmic reticulum (ER)–resident procollagen 3-hydroxylation complex 3-hydroxylates type I collagen alpha1(I) chains. One member of the complex, cyclophilin B (CyPB), encoded by *PPIB*, is an ER–resident peptidyl-prolyl *cis-trans* isomerase (PPIase) and the major PPIase catalyzing collagen folding. Our group generated a *Ppib* knock-out (KO) mouse. We are studying the effect of absence of PPIB on the functioning of bone cells, both osteoblasts and osteoclasts, and the outcome for bone histomorphometry and correlating results with comparable studies in affected children.

In collaboration with Vorasuk Shotelersuk and Cecilia Giunta, we identified the first type of OI with X-linked inheritance. 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 3]. 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 has impaired RIP cleavage of the transcription factors OASIS, ATF6, and SREBP. The mutations in *MBTPS2* demonstrate that RIP plays a fundamental role in bone development. Recently, we have been investigating the differentiation of osteoblasts from two boys with type XVIII OI.

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 a former 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 measurements on their femora show significantly higher mineralization than in WT mice, which continues to increase in the high bone-mass mice (HBM), even after mineralization plateaus at six months in the WT mice. PINP and TRAP, serum markers of bone remodeling, are significantly elevated in such HBM. Osteocyte density is reduced, but the lacunar area is increased.

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 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 bisphosphonates. 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.

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 (a microtubule-destabilizing phosphoprotein), and cofilin-1 (an actin-modulating protein) in lethal pups. Reduced vimentin (an intermediate filament) can lead to cytoskeletal collapse, and increased stathmin and cofilin-1 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.

We are also investigating an important unexplained feature of OI and other dominant skeletal disorders: the variability of expression, that is, very different phenotypic severity even in patients from the same family. We are studying variability both in human osteoblasts and in murine models. Osteoblasts from individuals with the same mutation but different severity of OI are being compared for differentiation and mineralization. More severe phenotypes are associated with greater reduction in mineralization. In addition, the Brtl(Cys)(G349C) mouse is being compared with a Brtl(Ser)(G349S) mouse with a different substitution at the same residue. Surprisingly, the serine substitution results in severely reduced collagen secretion and a bone volume only 13% of WT, leading to a lethal phenotype.

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

Pulmonary issues are the most prevalent cause of morbidity and mortality in OI. We previously 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 in pulmonary function over time, 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. We have now published comprehensive pulmonary phenotyping results from a cohort of 37 individuals with OI evaluated at the NIH Clinical Center [Reference 6]. Lung function measurements, CT scans, and radiographic images from children and young adults with five different types of OI, predominantly the classical types III and IV OI, but also including the rare recessive types VI, VII, and XIV OI, were analyzed. We showed, for the first time, that arm span or ulnar lengths are comparable height surrogates for calculating pulmonary function testing (PFT) results in patients with OI. Most patients had restrictive lung disease even at this young age, accompanied by reduced gas exchange, pointing to parenchymal issues. In-depth analyses of CT scan images demonstrate a high prevalence of bronchial thickening at the level of small airways, which may be directly related to abnormal collagen or a secondary inflammatory response in OI. The functional impact of thickening of the walls of small bronchi is supported by reduced FEV25–75% (forced expiratory flow) air flow, which also measures small airways, in all patients with type III OI. In general, severity of pulmonary manifestations was more pronounced in patients with type III OI, which is consistent with overall severity of disease in patients with this disorder. We also found that decline in pulmonary function correlates with severity of scoliosis, supporting a role for extrinsic as well as intrinsic factors in OI lung disease.

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.

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 are placebo effects. Our recommendation is for treatment for two to three years, with subsequent follow-up of bone status. Our preliminary analysis of 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, 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 from each patient's two bone samples was subjected to whole-exome sequencing (WES), and sequences from each individual patient were compared. We identified two genes causing somatic mutations in melorheostotic lesions [Kang *et al*. *Nat Commun* 2018;9:1390; Kang *et al*. *Exp Med* 2020;217:e20191499]. Each gene was associated with one radiographic form of melorheostosis, and the bone lesions had distinct histology and mechanism along the TGFβ pathway.

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. Erythematous skin lesions overlying the affected bone are often mosaic for the MAP2K1 mutations and have increased vascularity [Kang *et al*. *Nat Commun* 2018;9:1390]. 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, a component of the canonical TGFβ pathway [Kang *et al*. *Exp Med* 2020;217:e20191499]. 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 osteoblast differentiation markers, and increased mineralization.

Melorheostotic bone from both MAP2K1–positive and SMAD3–positive patients showed two zones of distinct morphology [Fratzl-Zelman *et al. J Bone Miner Res* 2019;34:883-895]. In MAP2K1–positive melorheostosis, 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 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. Exploration of secreted cytokines and chemokines in the conditioned media of osteoblasts with a high variant allele frequency (VAF) of *MAP2K1* mutations revealed factors that stimulate angiogenesis, inhibit osteoblast differentiation, and increase osteoclastogenesis and inflammation. Thus, it is likely that mutant cells recruit non-mutant cells into the bone overgrowth via these and other secreted factors.

Publications

1. Jovanovic M, Mitra A, Besio R, Contento BM, Wong KW, Derkyi A, To M, Forlino A, Dale RK, Marini JC. Absence of TRIC-B from Type XIV OI osteoblasts alters cell adhesion and mitochondrial function – A multi-omics study. *Matrix Biol* 2023 121:127–148.
2. Besio R, Contento BM, Garibaldi N, Filibian M, Sonntag S, Shmerling D, Tonelli F, Biggiogera M, Brini M, Salmaso A, Jovanovic M, Marini JC, Rossi A, Forlino A. CaMKII inhibition due to TRIC-B loss-of-function dysregulates SMAD signaling in osteogenesis imperfecta. *Matrix Biol* 2023 120:43–59.
3. Jovanovic M, Guterman-Ram G, Marini JC. Osteogenesis Imperfecta: Mechanisms and signaling pathways connecting classical and rare OI types. *Endocr Rev* 2022 43(1):61–90.
4. Hedjazi G, Guterman-Ram G, Blouin S, Schemenz V, Wagermaier W, Fratzl P, Hartmann M, Zwerina J, Fratzl-Zelman N, Marini JC. Alterations of bone material properties in growing Ifitm5/BRIL p.S42 knock-in mice, a new model for atypical type VI osteogenesis imperfecta. *Bone* 2022 162:116451.
5. Kang H, Aryal Ac S, Barnes AM, Martin A, David V, Crawford S, Marini JC. Antagonism between PEDF and TGF-β contributes to type VI Osteogenesis Imperfecta bone and vascular pathogenesis. *J Bone Miner Res* 2022 37:925–937.
6. Gochuico BR, Hossain M, Talvacchio S, Zuo MXG, Barton M, Dang Do AN, Marini JC. Intrinsic and extrinsic lung abnormalities in children and young adults with osteogenesis imperfecta affect pulmonary function. *J Med Genet* 2023 60:1067–1075.

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High−Resolution Structural Biology of Membrane Protein Complexes in Their Native Environment

We are 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 the 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. 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, and 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).

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 diseases
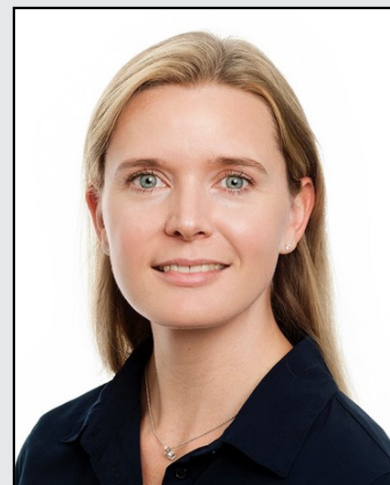
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Fei Zhou, PhD, *Scientist*

Louis Tung Faat Lai, PhD, *Visiting Postdoctoral Fellow*

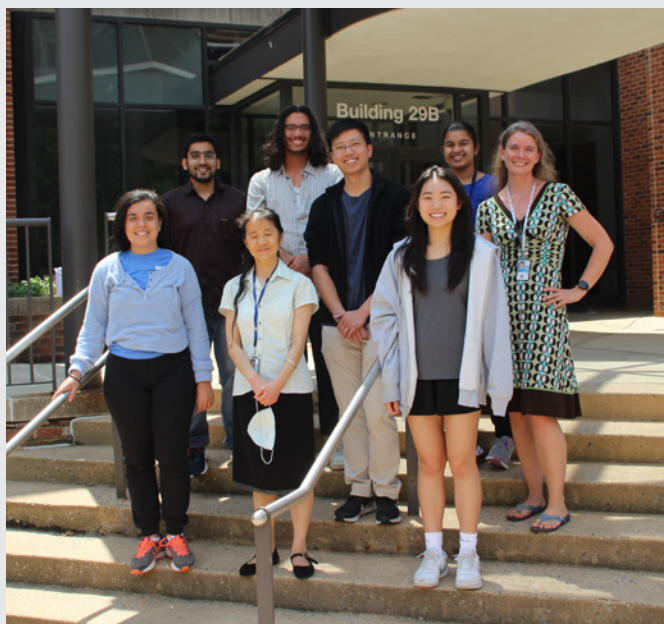Zaid Madni, PhD, *Visiting Postdoctoral Fellow*

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Group picture in front of NIH building 29B in August 2023

From left to right: Isadora Rocha De Abreu, Zaid Madni, Fei Zhou, Patrick O'Reilly, Louis Tung Faat Lai, Jasmin D. Wu, Jayashree Balaraman, Doreen Matthies

such 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 is expanding the research on magnesium channels, including looking at eukaryotic magnesium channels. He recently resolved high-resolution structures of the human inner mitochondrial membrane magnesium channel MRS2 and investigated its function and regulation. To further investigate the structure and mechanism of these channels, structural studies on synthetic and native nano-discs, as well as in liposomes, are planned.

Uptake of essential omega-3 fatty acids

Omega-3 fatty acids are considered essential because the human body cannot make their own and must obtain them through foods, such as algae, wild fish, walnuts, and flaxseeds. DHA in particular is enriched in the brain and is important for a healthy nervous system. Breastmilk and most infant formulas contain DHA, and deficiencies in this fatty acid have been linked to problems with learning and memory. To reach the brain, omega-3 fatty acids must pass through the blood-brain barrier via the lipid transporter MFSD2A. The transporter is essential for normal brain development and is targeted by the Zika virus, which can cause microcephaly (i.e., a smaller-than-expected head) when infections occur during pregnancy. Despite its importance, we did not know precisely how MFSD2A transports DHA and other omega-3 fatty acids. In the new study, our team provided five cryo-electron microscopy structures of zebrafish Mfsd2a, which is structurally similar to it human counterpart. The snapshots are the first to detail precisely how fatty acids move across the cell membrane. We identified three compartments in Mfsd2a that suggest distinct steps required to move and flip fatty acids through the transporter, as opposed to movement through a linear tunnel or along the surface of the protein complex. Our findings [Reference 3] promise to improve understanding of lipid transport across the blood-brain barrier and of disruptions in this process that can lead to birth defects or neurological conditions. The model also enables researchers to design drug molecules that are capable of directly reaching the brain. Overall, the findings provide key information on how MFSD2A transports omega-3 fatty acids into the brain and may enable researchers to optimize drug delivery via this route. The study also provides

FIGURE 1. Three-dimensional cryo-EM structure of the human inner mitochondrial membrane magnesium channel MRS2 [Reference 5]

The channel is shown from the side and as a cross-section embedded in a lipid bilayer. The intermembrane space of the mitochondria is shaded blue, and the mitochondrial matrix is shaded purple. Magnesium ions are small green spheres. Credit: Ethan Tyler, NIH Medical Arts; Louis Lai, Matthies Lab
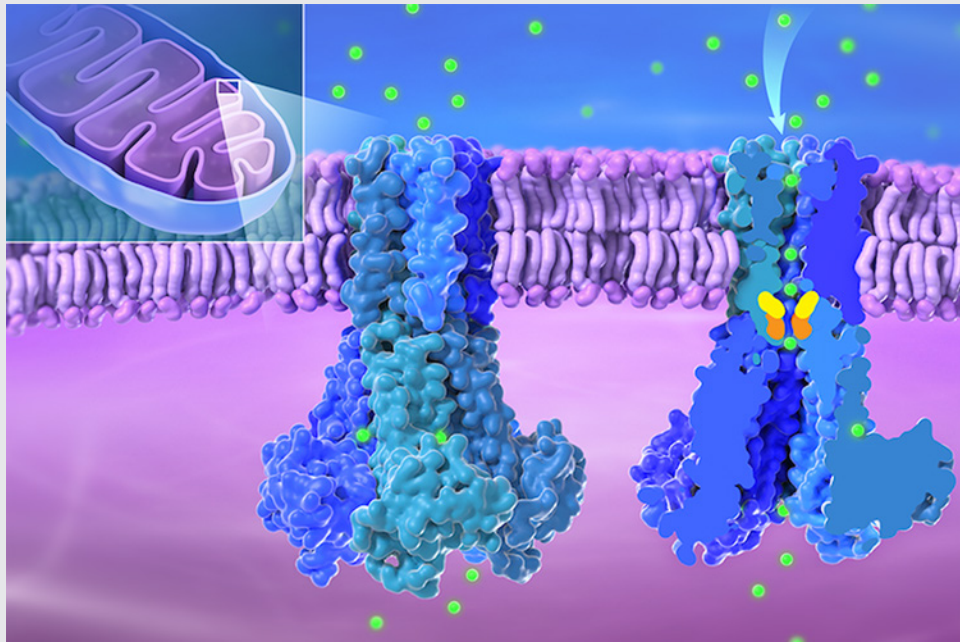
foundational knowledge for understanding how other members of this transporter family, called the major facilitator superfamily (MFS), regulate important cellular functions.
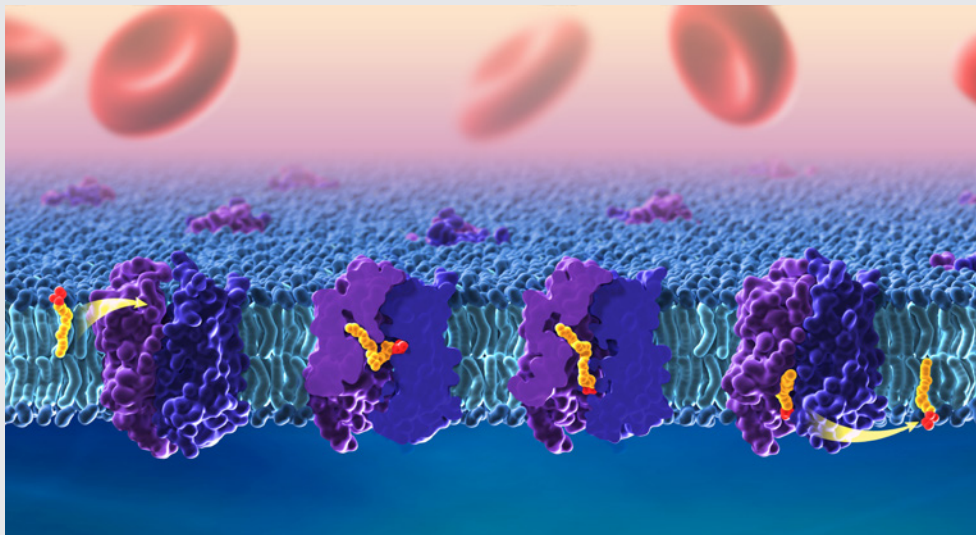
Collaborations

Our collaborations involve structural and computational studies on a variety of membrane-protein complexes, including transporters, channels, and receptors, 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

1. Petersen J, Lu J, Fitzgerald W, Zhou F, Blank PS, Matthies D, Zimmerberg J. Unique aggregation of retroviral particles pseudotyped with the delta variant SARS-CoV-2 spike protein. *Viruses* 2022 14:1024.
2. Ralhan I, Chang J, Moulton MJ, Goodman LD, Lee NYJ, Plummer G, Pasolli HA, Matthies D, Bellen HJ, Ioannou MS. Autolysosomal exocytosis of lipids protect neurons from ferroptosis. *J Cell Biol* 2023 222:e202207130.
3. Nguyen C, Lei H-T, Lai LTF, Gallenito MJ, Mu X, Matthies D, Gonen T. Lipid flipping in the omega-3 fatty-acid transporter. *Nat Commun* 2023 14:2571.
4. Prévost J, Chen Y, Zhou F, Tolbert WD, Gasser R, Medjahed H, Gottumukkala S, Hessell A, Rao VB, Pozharski E, Huang RK, Matthies D, Finzi A, Pazgier M. Structure-function analyses reveal key molecular determinants of HIV-1 CRF01_AE resistance to the entry inhibitor temsavir. *Nat Commun* 2023 14:6710.
5. Lai LTF, Balaraman J, Zhou F, Matthies D. Cryo-EM structures of human magnesium channel MRS2 reveal gating and regulatory mechanisms. *Nat Commun* 2023 14:7207.

FIGURE 2. Cryo-EM of the zebrafish lipid transporter Mfsd2a reveals stepwise lipid translocation.

The brain acquires essential omega-3 fatty acids through transport across the blood-brain barrier via the lipid transporter Mfsd2a. Cryo-EM of zebrafish Mfsd2a revealed different snapshots of its translocation pathway, detailing how lipids are flipped from the outer leaflet of the lipid bilayer and released in the inner leaflet. Credit: Ethan Tyler, NIH Medical Arts; Louis Lai, Matthies Lab, in collaboration with the Gonen Lab.

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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.

Background and significance

In hippocampus, GABAergic local circuit inhibitory INs account for about 10–15% of the total neuronal cell population. Despite being in the minority, this diverse neuronal population serves as a major determinant of all aspects of cortical circuit function and regulation. Within the hippocampus, INs have their cell bodies scattered across all major subfields, and the positioning of their somatodendritic arbors allows integration of input from a number of intrinsic and extrinsic afferent inputs. The axons of many IN subtypes largely remain local to the subfield housing their soma and dendrites; however, many form long-range projections that extend beyond their original location to ramify within both cortical and subcortical structures. Their

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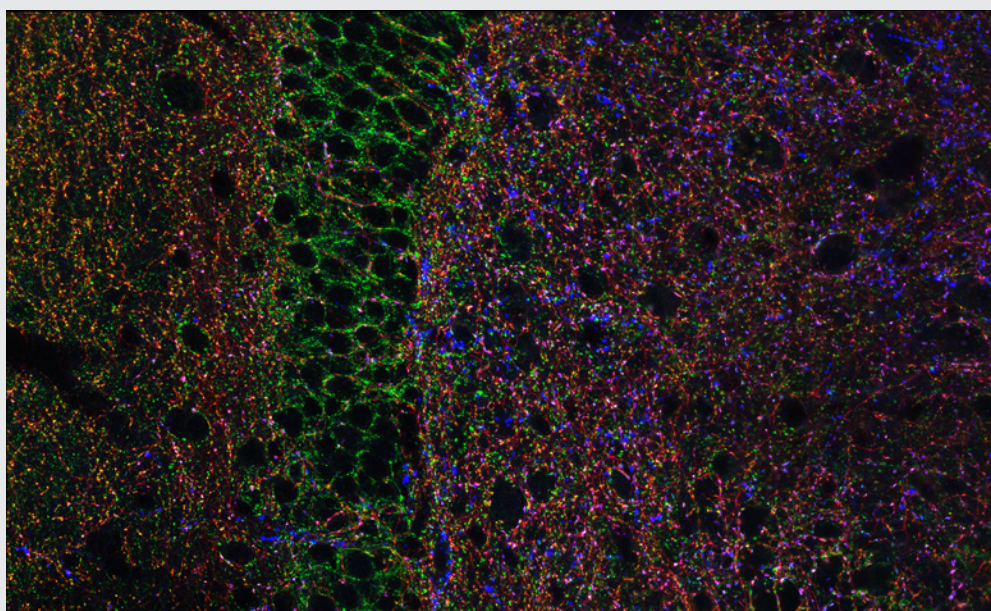Anna Vlachos, BSc, *Postbaccalaureate Fellow*

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.

axons target well defined narrow postsynaptic domains (i.e., soma and proximal dendrites) or can provide widespread input to large portions of target cell dendrites. This innervation of different postsynaptic cellular compartments ensures that virtually all domains of their principal neuron targets receive extensive coverage and, importantly, underscores that IN subtypes perform distinct roles in the hippocampal circuit. INs are primarily providers of inhibitory GABAergic synaptic input: a physiological role that utilizes Cl^- influx or K^+ efflux via cognate $GABA_A$ or $GABA_B$ receptor activation respectively, to transiently hyperpolarize or shunt the cell membrane away from action potential threshold. They play major roles in not only the regulation of single cell excitability, but provide well timed inhibitory input that dictates the temporal window for synaptic excitation, and subsequent action potential initiation, thus shaping the timing of afferent and efferent information flow. In addition, they harness and synchronize both local and distributed cortical circuits to facilitate oscillatory activity across broad frequency domains. Indeed, several developmentally regulated neural circuit disorders, such as epilepsy, schizophrenia, and autism, are likely associated with deficits in the numbers and function of distinct IN cohorts. For all of these reasons, INs have recently become the intense focus of investigators drawn from a wide variety of backgrounds.

In the past year, our research focused on three main aspects of IN function.
1. We continued our study of glutamatergic and GABAergic synaptic transmission made onto and from INs and their downstream targets, within the hippocampal and cortical formations.
2. We capitalized and expanded our research using genetic and viral approaches to examine the development of specific cohorts of medial- and caudal-ganglionic eminence–derived INs and their roles in both nascent and mature circuits.
3. As part of a multi-institute non-human primate consortium, we expanded our studies to consider evolutionary conservation or diversity of principal neuron and interneuron function, using tissue derived from rodent, non-human primates (macaque and marmoset) and, in collaboration with NINDS

FIGURE 2. MGE– and CGE–derived hippocampus interneurons

The figure shows the major cortical and hippocampal inhibitory interneuron subtypes derived from both the medial and caudal ganglionic eminences.

neurosurgery, surgically resected human hippocampal and cortical tissue. This multiparametric research approach to circuit development and function has been extremely fruitful and is a perfect example of why our research strategy is well suited for the intramural environment. Having the flexibility to pursue this line of research would not have been possible without the support and collaborative nature of the NIH intramural program.

Evolutionary conservation of hippocampal mossy fiber synapses

Computational models based primarily on rodent data predict that mossy fiber (MF) synaptic connections between hippocampal dentate gyrus granule cells (DGGCs) and CA3 pyramidal cells (PCs) are essential for encoding contextual memories. MF encoding of memory is considered to relate directly to a host of peculiar specialized structural/functional synaptic properties, including sparse innervation by large multi-release site terminals supporting a remarkable frequency-dependent dynamic range of transmission onto the most proximal dendrites of CA3 PCs. Though investigated in exquisite detail in model organisms, synapses, including MFs, have undergone relatively minimal functional interrogation in the human brain. To determine the translational relevance of rodent MF findings to the human brain, we evaluated the basic synaptic properties of MF connections within human hippocampal tissue resected for treatment of pharmaco-resistant epilepsy. Human MF transmission exhibits remarkably similar hallmark features to that of rodents, including AMPA–receptor dominated synapses with small contributions from NMDA and kainate receptors, large dynamic range with strong frequency facilitation, NMDA receptor–independent presynaptically expressed long-term potentiation, and strong cAMP sensitivity of presynaptic release. Moreover, serial array tomography electron microscopy confirmed evolutionary conservation of MF synapse ultrastructure. The astonishing congruence of the core features shared between rodent and human MF synapses argues that the basic properties of MF transmission reported in experimental animal models are also critical to human MF function. However, of

interest from the disease perspective, we observed a dramatic selective deficit in GABAergic inhibitory tone onto human MF postsynaptic targets, suggesting that unrestrained detonator excitatory drive contributes to circuit hyperexcitability in epilepsy.

Development of tools for evolutionary conservation

Over the last few years, we have begun to explore evolutionary conservation of both principal neuron and interneuron function from rodents through non-human primates to humans. This has necessitated the generation of tools that allow unequivocal identification of INs in species from which few studies exist. In two ongoing collaborations with the labs of Gordon Fishell and Matt Rowan, we developed and characterized several AAV (adeno-associated virus) vectors utilizing neuron type–specific regulatory transcriptional sequences (enhancer-AAVs), which have allowed us to overcome the limitations set by using mouse Cre-lines. The ability to precisely control transgene expression is essential for basic research and clinical applications. AAVs are non-pathogenic and can be used to drive stable expression in virtually any tissue, cell type, or species, but their limited genomic payload results in a trade-off between the transgenes that can be incorporated and the complexity of the regulatory elements controlling their expression. Resolving these competing imperatives in complex experiments inevitably results in compromises. We achieved this in compact vectors by integrating structural improvements of AAV vectors with innovative molecular tools. In a series of manuscripts, we illustrated the potential of this approach through a systematic demonstration of their utility for targeting cell types and querying their biology, using a wide array of genetically encoded tools. These enhancer viral tools were used in many of the studies described above and have proved of great utility in mouse, marmoset, macaque, and human tissue. Our hope is to continue this fruitful avenue of collaboration for future experimental use.

Additional Funding

- Connie Mackenzie Gray-Scott was funded by an NIH-Wellcome Trust Graduate Student award.

Publications

1. Pelkey KA, Vargish G, Pellegrini L, Calvigioni D, Chapeton J, Yuan X, Hunt S, Cummins AC, Eldridge MAG, Pickel J, Chittajallu R, Averbeck BB, Toth K, Zaghloul K, McBain CJ. Evolutionary conservation of hippocampal mossy fiber synapse properties. *Neuron* 2023 111(23):3802–3818.e5.
2. Mahadevan V, Mitra A, Zhang Y, Yuan X, Peltekian A, Chittajallu R, Esnault C, Maric D, Rhodes C, Pelkey KA, Dale R, Petros T, McBain CJ. NMDARs drive the expression of neuropsychiatric disorder risk genes within GABAergic interneuron subtypes in the juvenile brain. *Front Mol Neurosci* 2021 14:712609.
3. Xiao MF, Roh SE, Zhou J, Lucey B, Craig MT, Hays L, Jia M, Chien CC, Xu D, Zhou W, Talbot C, Weinberg D, Sawa A, Pelkey KA, McBain CJ, Savonenko A, Worley PF. A biomarker-authenticated model of schizophrenia implicating NPTX2 loss of function. *Sci Adv* 2021 7(48):eabf6935.
4. Mackenzie-Gray Scott CA, Pelkey KA, Caccavano A, Abebe D, Lai M, Black KN, Brown N, Trevelyan AJ, McBain CJ. Resilient hippocampal gamma rhythmogenesis and parvalbumin-expressing interneuron function before and after plaque burden in 5xFAD Alzheimer's disease model. *Front Synaptic Neurosci* 2022 14:857608.
5. Pouchelon G, Vergara J, McMahon J, Gorissen B. Lin J, Vormstein-Schneider D, Niehaus JL, Burbridge T, Wester JC, Sherer M, Fernandez-Otero M, Allaway K, Pelkey K, Chittajallu R, McBain CJ, Fan M, Nasse J,

Wildenberg J, Fishell G, Dimidschstein J. A versatile viral toolkit for functional discovery in the nervous system. *Cell Rep Methods* 2022 2:100225.

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Pathophysiology, Genetics, and Treatment of Congenital Adrenal Hyperplasia

In its most severe classic form, congenital adrenal hyperplasia (CAH) is a life-threatening, rare orphan disease that is part of the neonatal screen performed in all 50 U.S. states. In its mildest non-classic form, CAH is 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 smaller-than-normal amygdala, the emotion regulator of the brain, in CAH, providing insight into hormonal effects on the brain. We also found that approximately 15 percent of patients with CAH owing 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 and which we named CAH-X. Our studies over the years have had a major impact on the management of patients with CAH, including changing the recommended stress-dosing practices in the Endocrine Society Clinical Practice Guidelines and, more recently, establishing recommendations for the management of women with CAH during pre-conception, pregnancy, and postpartum. Central to our work is the study of new treatments, including a long-term trial testing sex hormone blockade in children, novel ways of replacing cortisol aimed at mimicking the normal circadian rhythm of cortisol secretion, and antagonists of the CRH (corticotropin-releasing hormone) type 1 receptor to suppress the drivers of excess androgen production to allow for lower-dose glucocorticoid therapy. 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. Through the study of a large cohort of these unique patients, there is a great potential to expand our understanding of hormonal effects on normal development and physiology, provide insights into gene regulation and expression, and develop and test new treatment approaches.

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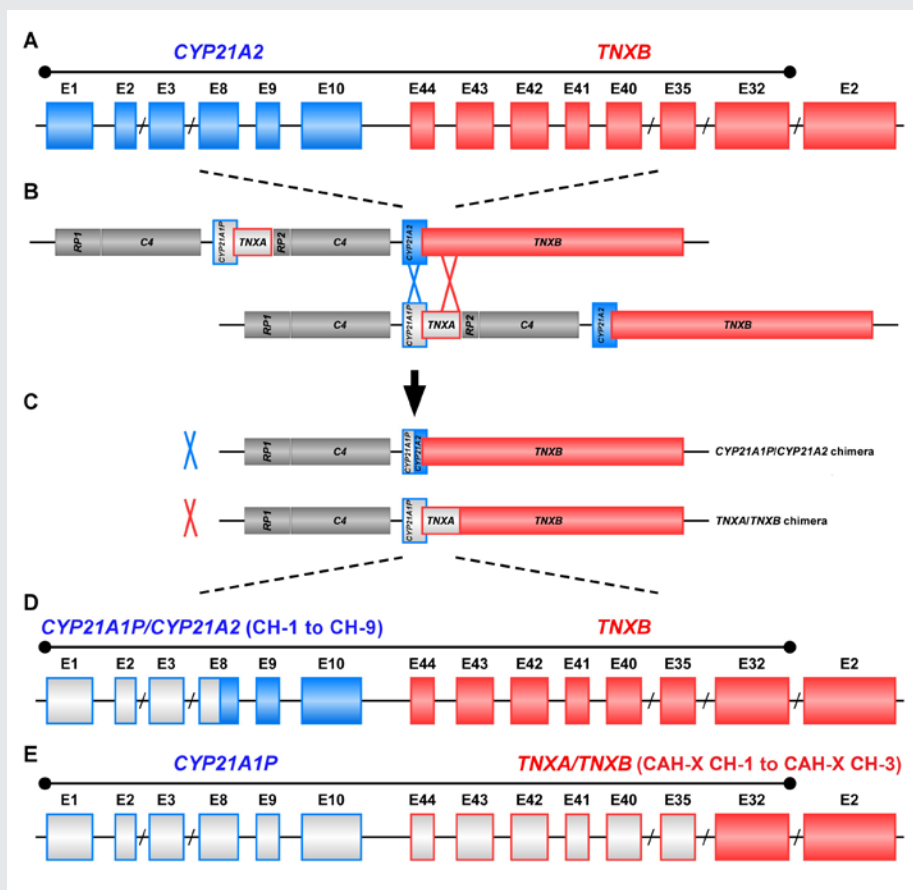
FIGURE 1. Schematic of *CYP21A1P/CYP21A2* and *TNXA/TNXB* chimeric genes

Formation of chimeric genes occurs as a result of misalignment of homologous genes during meiosis. Active genes are in solid colors; pseudogenes are in grey and are framed with the color of the corresponding functional gene. Representative chimeric genes are shown. In total, there are nine known *CYP21A1P/CYP21A2* chimeras (CH-1 to CH-9), and we identified three different types of *TNXA/TNXB* chimeras (CAH-X CH-1 to CAH-X CH-3) with different junction sites. Approximately 15 percent of patients with CAH owing to 21-hydroxylase deficiency carry at least one *TNXA/TNXB* chimera, resulting in hypermobility-type Ehlers-Danlos syndrome or CAH-X syndrome.

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, which 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 *TNXA*, a highly homologous pseudogene, 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 in 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 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 lab continues to investigate how *TNXB* contributes to the phenotype of CAH patients, and to identify novel chimeric genes.

To date, we have described 24 patients (19 families) with monoallelic CAH-X and three patients with biallelic CAH-X. It is now estimated that approximately 15% of patients with CAH resulting from 21-hydroxylase deficiency are affected by CAH-X. Overall, CAH-X patients have generalized joint hypermobility, sub-luxations, 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 and was recognized as an important additional molecular genetic testing platform by the European Molecular Genetics Quality Network, an international group of genetic methodology experts. We continue to refine and improve genetic testing methodology. This year we reported that pseudogene *TNXA* variants may interfere with the genetic testing of CAH-X.

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.

Longitudinal assessments of cardiovascular disease risk and metabolic morbidity

Our long-term natural history study has been critical for understanding disease-specific outcomes, and the long duration of our study has enabled us to analyze longitudinal data and disease trajectory. Longitudinal assessment of risk factors for cardiovascular disease (CVD) revealed higher prevalence of metabolic morbidity in patients with CAH than in the general population (National Health and Nutrition Examination Survey–NHANES) across the lifespan. Associations with treatment- and disease-related factors differed during childhood and adulthood.
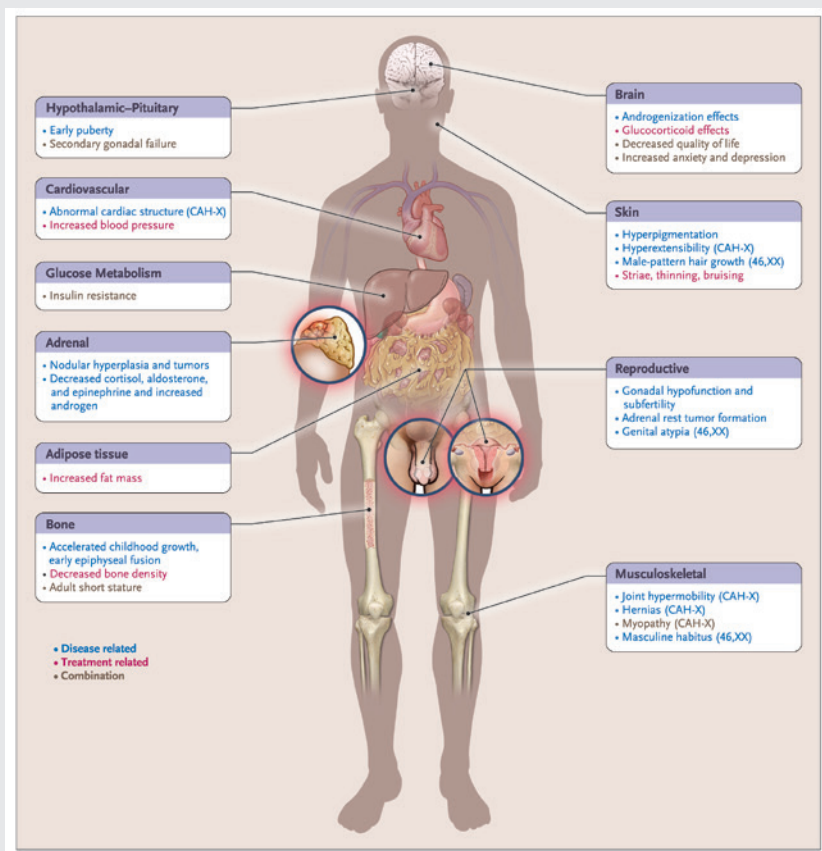
FIGURE 2. Clinical features of congenital adrenal hyperplasia

Disease-related manifestations are shown in blue, treatment-related manifestations are shown in red, and clinical manifestations related to both the disease and the treatment are shown in brown.

Prior studies of risk factors for cardiovascular disease and metabolic morbidity in CAH had been reported in cross-sectional studies with varied results. In this first longitudinal assessment of CVD risk factors, we identified the prevalence and age of onset of risk factors for CVD and metabolic morbidity, described risk factors over time, and evaluated these outcomes in relation to treatment- and disease-related characteristics in our large cohort of patients followed during childhood and into adulthood for an average of 18 years. We found that children with classic CAH have metabolic risk (obesity, hypertension, fasting hyperglycemia, and hypertriglyceridemia) starting at a young age (prior to 10), and that risks are distinctly different during adulthood. In particular, hypertension during childhood, but not adulthood, was associated with mineralocorticoid dosing. The study provided guidance regarding treatment practices in relation to metabolic and cardiovascular-disease risk.

The effect of GnRHa on bone health in CAH

Our study was the first clinical study to report the effect of GnRHa (gonadotropin-releasing hormone analog) on bone health in the CAH population. Reduced bone mineral density (BMD) is a known possible risk of delaying puberty, and our longitudinal data were especially valuable for evaluating the long-term effects of BMD into adulthood. Childhood GnRHa therapy to delay central puberty for an average of 4.5 years in patients with CAH did not affect BMD at attainment of adult height or during early adulthood when peak BMD occurs in healthy individuals. However, an overall reduction in BMD occurred during the second and third decades of life in our CAH cohort, regardless of GnRHa therapy, a possible effect of chronic supra-physiologic

glucocorticoids. Our study demonstrated that children with CAH who experience early puberty benefit from GnRHa treatment, as evidenced by a positive effect on adult height. The height gain for treated patients was 0.8 SD (approximately 2.5 inches) from predicted height at the start of puberty, which is clinically meaningful.

We found that GnRHa therapy can be used safely to delay central puberty in children with CAH and has a positive effect on adult height. Importantly, we also found that the CAH population is at risk for early-onset low bone mass, given the decline in z-scores observed over time and in early adulthood.

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 therefore 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 in 2021. 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. Our 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 under way.

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.

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 on 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.

Since the inception of our study on 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*).

Novel treatment approaches: hypothalamic–pituitary–adrenal axis suppression

Potential strategies to address the drivers of excess androgen production in CAH include suppression of the hypothalamic-pituitary-adrenal axis. Newly developed small-molecule antagonists of the corticotropin-releasing factor type 1 (CRF1) receptor antagonist are being studied. Successful phase 2 studies completed in 2021 led to multicenter, randomized, double-blind, placebo-controlled, long-term (52 weeks) clinical trials in adults with CAH (*NCT04490915*) to study the effect of CRF-1 antagonists on outcomes such as improved adrenal androgen control, as well as the potential of reducing glucocorticoid dose as an add-on therapy.

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- NIH U Grant: Abiraterone Acetate in Children with Classic 21-Hydroxylase Deficiency
- Cooperative Research and Development (CRADA) for a Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Safety and Efficacy of Crinecerfont in Adult Subjects with CAH, Followed by Open-Label Treatment

Publications

1. Mallappa A, Merke DP. Management challenges and therapeutic advances in congenital adrenal hyperplasia. *Nat Rev Endocrinol* 2022 18(6):337–352.
2. Maher JY, Gomez-Lobo V, Merke DP. The management of congenital adrenal hyperplasia during preconception, pregnancy, and postpartum. *Rev Endocr Metab Disord* 2022 24(1):71–83.
3. Lao Q, Zhou K, Parker M, Faucz FR, Merke DP. Pseudogene TNXA variants may interfere with the genetic testing of CAH-X. *Genes* 2023 14(2):265.
4. Torky A, Sinaii N, Jha S, El-Maouche, Desai J, El-Maouche D, Mallappa A, Merke DP. Cardiovascular disease risk factors and metabolic morbidity in a longitudinal study of congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2021 106(12):e5247–5257.

5. Flokas ME, Wakim P, Kollender S, Sinai N, Merke DP. Gonadotropin-releasing hormone agonist therapy and longitudinal bone mineral density in congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2023 dgad514.

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Childhood Neurodegenerative Lysosomal Storage Disorders

We conduct both basic and translational research into a group of hereditary childhood neurodegenerative lysosomal storage disorders (LSDs), called neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease. Loss-of-function mutations in 14 different genes (*CLN1–CLN14*) underlie various types of NCLs. Several years ago, we initiated investigations on CLN1 disease (INCL) caused by inactivating mutations in the *CLN1* gene, which encodes palmitoyl-protein thioesterase-1 (PPT1). PPT1 depalmitoylates *S*-palmitoylated proteins (constituents of ceroid). Numerous proteins, especially in the brain, require *S*-palmitoylation (also called *S*-acylation). It is the only reversible post-translational modification of proteins in which a saturated fatty acid (generally palmitate) 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. Dynamic *S*-palmitoylation (palmitoylation-depalmitoylation) requires coordinated actions of two types of enzymes with opposing functions. The enzymes that 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 or in the cytoplasm. Recently, several protein depalmitoylases, called ABHD17, were identified that catalyze the turnover of N-Ras (a GTPase signal-transduction protein).

The aim of our translational research has been 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 CLN1 disease led to a bench-to-bedside clinical trial to determine whether a combination of cysteamine bitartrate (Cystagon) and N-acetylcysteine is beneficial for patients with this disease. Using *Cln1*–knockout (*Cln1$^{-/-}$*) mice, which mimic human CLN1 disease, we discovered that PPT1 deficiency causes endoplasmic-reticulum (ER) and oxidative stresses, which, at least in part, causes neuronal death by apoptosis. During the past several years, we also
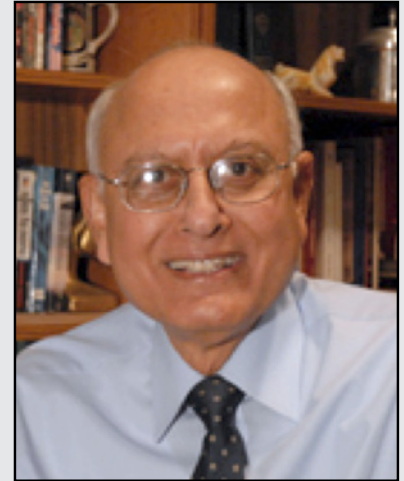
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delineated a mechanism by which PPT1 deficiency disrupts the recycling of synaptic vesicle (SV) proteins, which are essential for generating fresh SVs to replenish the SV pool size at the nerve terminals so as to maintain uninterrupted neurotransmission. We also discovered that ER and oxidative stress contribute to neuronal apoptosis and neuro-inflammation in CLN1 disease. Further, we found that PPT1 deficiency causes misrouting of the V0a1 subunit of v-ATPase (the proton pump on the lysosomal membrane), dysregulating lysosomal acidification, which causes elevated pH and thus adversely affects 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 method permits repeated evaluation of potential therapeutic agents in treated animals. Application of such methods in our clinical trial with CLN1 disease also allowed us to evaluate the progressive decline in brain volume and neurodegeneration. In collaboration with other investigators, we are conducting studies to determine whether electro-retinography can be used to assess the progressive retinal deterioration in *Cln1*^{−/−} as well as in *Cln1* knock-in (KI) mice, which carry the nonsense mutation in the *CLN1* gene commonly found in the CLN1–disease 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 as 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 neuropathology in this disease. These and related studies provide insight into the complex mechanisms of heritable disorders of neurodegeneration such as CLN1 disease, 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 CLN1 disease and may even be for CLN3 disease. More recently, we discovered that cathepsin D (CD) deficiency in lysosomes is a common pathogenic link between CLN1 disease and CLN10 disease. Our ongoing laboratory and translational investigations are attempting to advance our knowledge of CLN1, CLN3, and CLN10 disease.

Misrouting of Niemann Pick C1 hyperactivates mTORC1 by lysosomal cholesterol, contributing to CLN1 disease pathogenesis.

PPT1 is a depalmitoylating enzyme, which catalyzes the removal of fatty acids (generally palmitate) from *S*-palmitoylated proteins. Numerous proteins, especially in the brain, undergo *S*-palmitoylation, a post-translational modification of proteins by saturated fatty acids (generally palmitate). It confers hydrophobicity, increases membrane affinity, and promotes protein-protein interactions. Moreover, dynamic *S*-palmitoylation (palmitoylation-depalmitoylation) facilitates endosomal protein trafficking. Despite the discovery that inactivating mutations in the *CLN1* gene encoding PPT1 cause CLN1 disease, a clear picture of its pathogenic mechanism has not emerged for more than two decades. In addition to its degradative function, the lysosome plays a pivotal role in cholesterol homeostasis. It is the major cellular sorting station for dietary cholesterol. Cholesterol is transported to the late endosome/lysosome and is exported to diverse cellular compartments, including to the plasma membrane and the endoplasmic reticulum (ER). The Niemann Pick C1 (NPC1) protein, localized to the lysosomal limiting membrane, plays a critical role in sterol trafficking, and its inactivation causes the hereditary neurodegenerative lipid-storage disorder Niemann-Pick type C (NPC). Recently, it was reported that cells from patients with NPC have elevated cholesterol on the lysosomal limiting membrane,

FIGURE 1. Misrouting of Niemann-Pick C1 protein causes lysosomal cholesterol-mediated hyperactivation of mTORC1 kinase, contributing to neurodegeneration in CLN1 disease.

Endosomal trafficking of NPC1 in WT and *Cln1*⁻/⁻cells. In WT cells (*left panel*), trafficking of NPC1-protein is facilitated by dynamic *S*-palmitoylation on Cys97, requiring thioesterase activity of Ppt1; depalmitoylation of NPC1 allows handover of NPC1 from AP-2 to AP-3; the AP-3–bound NPC1 is then transported to the limiting membrane of late endosome/lysosome, where it mediates lysosomal cholesterol efflux. In *Cln1*⁻/⁻ cells (*right panel*) the lack of Ppt1 activity impairs dynamic *S*-palmitoylation of NPC1; consequently, NPC1 bound to AP-2 is not handed over to AP-3, and *S*-palmitoylated NPC1 is transported via recycling endosome to the plasma membrane instead of its normal location on lysosomal membrane; misrouting of NPC1 dysregulates lysosomal cholesterol homeostasis and results in cholesterol-induced mTORC1 activation, suppressed autophagy, and neurodegeneration in *Cln1*⁻/⁻ mice.
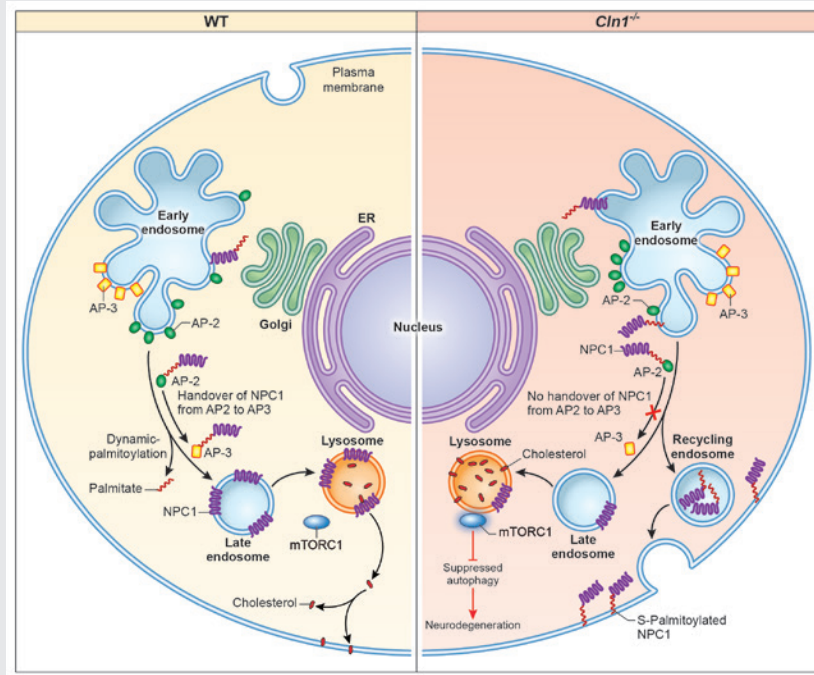
which mediates the activation of the mechanistic (mammalian) target of rapamycin complex 1 (mTORC1) protein kinase. The mTORC1 kinase integrates intracellular as well as environmental cues to regulate cell growth and metabolism. Aberrant activation of mTORC1 signaling negatively regulates autophagy, which is the principal pathway for lysosomal degradation and clearance of abnormal protein aggregates and damaged organelles. Remarkably, in all three types of autophagy, the lysosome plays pivotal roles in the degradation of cargo contained in the autophagosomes. Most notably, the dysregulation of autophagy has been implicated not only in the pathogenesis of common neurodegenerative diseases such as Alzheimer's and Parkinson's, but also in most of the LSDs, in which neurodegeneration is a frequent manifestation.

To understand the mechanism of CLN1 pathogenesis, we used the *Cln1*⁻/⁻ mouse. In the brain of these mice, total cholesterol levels have been reported to be significantly higher than those in their wild-type (WT) littermates. However, in that study the cholesterol levels in lysosomes were not measured. Thus, we first sought to determine the cholesterol levels in total homogenates of cortical tissues from 2-, 4- and 6-month-old WT and *Cln1*⁻/⁻ mice, as well as in lysosomal fractions from those tissues. We found that cholesterol levels in total lysates as well as in lysosomal fractions from cortical tissues of *Cln1*⁻/⁻ mice in all three age groups were significantly higher than those in their WT littermates. These results were further confirmed by confocal imaging in neurons from *Cln1*⁻/⁻ mice, which showed a substantially higher level of colocalization of Filipin III–stained cholesterol with lysosomes, which we stain with lysotracker red. Taken together, these results raised the possibility that, in the brain of *Cln1*⁻/⁻ mice, lysosomal cholesterol homeostasis is dysregulated. Cholesterol enters the cell in its esterified form packaged with lipoproteins via the low-density lipoprotein (LDL) receptor

(LDLR). Thus, dietary cholesterol enters the cell by a receptor-mediated pathway. Once within the cell, cholesterol esters are hydrolyzed by lysosomal acid lipase, liberating cholesterol, which is then transported to various cellular structures, including the ER and the plasma membrane. The Niemann-Pick C1 (NPC1) and NPC2 proteins mediate lysosomal cholesterol egress and import, respectively. Moreover, the lysosomal integral membrane protein (LIMP) 2/SCARB2 has also been reported to bind cholesterol and, like NPC1, transports cholesterol through a trans-glycocalyx tunnel, a membrane domain rich in glycoproteins and glycolipids. Under normal circumstances, the balance between the export and import maintains lysosomal cholesterol homeostasis. Thus, inactivating mutations in either the *NPC1* or *NPC2* gene dysregulate cellular cholesterol homeostasis, causing neurological dysfunction, leading to a fatal neurodegenerative Niemann-Pick type C disease.We found that NPC1 requires dynamic *S*-palmitoylation (palmitoylation-depalmitoylation) for trafficking to the lysosomal membrane. Intriguingly, in *Cln1*$^{-/-}$ mice, NPC1 mistargeting to the plasma membrane caused increased oxysterol-binding protein (OSBP) on lysosomal membrane, activating cholesterol-mediated mTORC1 signaling. Activated mTORC1 signaling suppressed autophagy, contributing to neurodegeneration. Importantly, treatment of *Cln1*$^{-/-}$ mice with OSW1, a pharmacological inhibitor of OSBP, suppressed mTORC1 activation, rescued autophagy, and ameliorated neuropathology. Our findings reveal a previously unrecognized role of CLN1/PPT1 in the pathogenesis of CLN1 disease and suggest that suppression of cholesterol-mediated activation of mTORC1 signaling may be a targetable pathway for CLN1 disease.

Impaired lysosomal Ca^{2+} homeostasis and neuropathology in a CLN1 disease model

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, which 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, 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 neuropathology in CLN1 disease.

We sought to determine the mechanism by which PPT1 deficiency impairs lysosomal degradative function and contributes to CLN1 disease 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 they suggest that the defect contributes to pathogenesis of CLN1 disease.

Loss-of-function mutations in the *CLN1* gene activates mTORC1 signaling in *Cln1*$^{-/-}$ mice.

Sensing of essential nutrients has emerged as an important function of the lysosome in coordinating cellular metabolism and growth. 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 such as autophagy. Whereas materials from extracellular sources are transported to the lysosome by endocytosis, those originating from intracellular sources are delivered by autophagy. Notably, activation of the mTORC1 pathway, situated at the crossroads of nutrient signaling, suppresses autophagy. The loss of autophagy in the central nervous system has been reported to cause neurodegeneration. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy, in all of which the lysosome plays a pivotal role in degrading the cargo contained in the autophagosomes. NCLs (Batten disease) mostly affect children and, despite the discovery more than two decades ago that the LSD CLN1 disease is caused by inactivating mutations in the *CLN1* gene, the mechanism of its pathogenesis has remained elusive. Children afflicted with CLN1 disease are phenotypically normal at birth, but by 6–18 months of age they manifest psychomotor retardation. Around two years of age, they develop complete retinal degeneration, causing blindness. At around four years of age, an isoelectric electroencephalogram (EEG) attests to a vegetative state. They remain in this condition for several more years before eventual death. These grim facts underscore an urgent need for understanding the mechanism underlying pathogenesis of CLN1 disease, which may facilitate the development of an effective treatment.

Palmitoyl-protein thioesterase-1 (PPT1), encoded by the *CLN1* gene, is a lysosomal depalmitoylating enzyme. Many proteins in the brain undergo *S*-palmitoylation (also called *S*-acylation), a post-translational modification in which a 16-carbon, saturated fatty acid (generally palmitate) is attached to specific cysteine residues in polypeptides via thioester linkage. *S*-palmitoylation confers hydrophobicity to soluble proteins, increases membrane affinity, promotes protein-protein interactions, and facilitates protein trafficking. Dynamic *S*-palmitoylation (palmitoylation-depalmitoylation) facilitates endosomal trafficking and the localization of many proteins, especially in the brain. While *S*-palmitoylation is catalyzed by palmitoyl acyltransferases (ZDHHC-PATs or simply ZDHHCs), depalmitoylation is mediated by thioesterases. Inactivating mutations in the *CLN1* gene causing PPT1 deficiency result in lysosomal accumulation of autofluorescent ceroid lipofuscin. When the ceroid lipofuscins are organized within lysosomes, they are called GRODS (granular osmiophilic deposits), a characteristic finding in neurons and other cell types from patients with CLN1 disease. The lysosomes are the dynamic regulators of the function of many proteins, especially in the brain, and their importance is underscored by the fact that impaired lysosomal function contributes to the pathogenesis of the LSDs. These inherited diseases are characterized by metabolic dysfunction, neurodegeneration, and shortened lifespan. Moreover, it has been suggested that the lysosomal pathway plays critical roles in many cellular functions,

including signaling in response to environmental cues. We reasoned that impaired dynamic *S*-palmitoylation of important proteins that are likely substrates of Ppt1 may impair their ability to traffic to their destination. This abnormality may result in varying impairment of functions of these proteins, cumulatively contributing to neurodegeneration.

We used tissues from the cerebral cortex of *Cln1⁻/⁻* mice; we used cortical tissues because, in our pilot study (*www.clinicaltrials.gov*; *NCT00028262*), MRI of the brain of children with CLN1 disease showed rapid degeneration of cortical tissues. We also used cultured cells from patients with CLN1 disease to determine whether the loss of CLN1/PPT1 causes aberrant activation mTORC1 signaling and suppresses autophagy. We found that, in the brain of *Cln1⁻/⁻* mice, Ppt1 deficiency caused aberrant activation of mechanistic (mammalian) target of rapamycin complex 1 (mTORC1), which suppressed autophagy, contributing to neurodegeneration. Emerging evidence indicates that sensing essential nutrients is an important function of the lysosome. Intriguingly, Ppt1 deficiency disrupted the lysosomal nutrient-sensing scaffold (LNSS) to which mTORC1 must attach to activate. Despite this defect, mTORC1 was activated by IGF1 via PI3K/Akt–mediated pathway. Importantly, treatment of *Cln1⁻/⁻* mice with pharmacological inhibitors of PI3K/Akt suppressed mTORC1 activation, restored autophagy and improved motor function. Our findings reveal a previously unrecognized mechanism by which *Cln1*/Ppt1 deficiency contributes to pathogenesis of CLN1 disease.

Defective ER–Golgi protein–trafficking contributes to ER stress in a CLN1 disease model.

We previously reported that in *Cln1⁻/⁻* mice endoplasmic reticulum (ER) stress contributes to neurodegeneration. However, the mechanism underlying ER stress in this disease has remained elusive. We sought to test a hypothesis that defective protein trafficking from the ER to the Golgi leads to excessive accumulation of proteins, causing ER stress in *Cln1⁻/⁻* mice. Newly synthesized proteins in the ER are transported to the Golgi via COPII (coat protein complex II) vesicles. Retrograde transport (Golgi to ER) is mediated by COPI vesicles. We found that the levels of five COPII vesicle–associated proteins (i.e., Sar1, Sec23, Sec24, Sec13, and Sec31) are significantly higher in the ER fractions of cortical tissues from *Cln1⁻/⁻* mice than in those from their WT littermates. Remarkably, all COPII proteins, except Sec13, undergo *S*-palmitoylation. Intriguingly, in *Cln1⁻/⁻* mice, ER–Golgi trafficking of Cln8-protein, mutated in Batten disease, is also disrupted. Moreover, Cln8-protein also undergoes *S*-palmitoylation and, in *Cln1⁻/⁻* mice, impaired dynamic *S*-palmitoylation of Cln8 along with several other COPII proteins disrupted ER–Golgi trafficking, causing excessive protein accumulation in the ER. We propose that defective ER–Golgi trafficking contributes to ER stress in this CLN1 disease model.

APOE–TREM2 signaling mediates neuroinflammation in a mouse model of CLN1 disease.

Neuro-inflammation contributes to neurodegeneration, and although neuro-inflammation has been reported in human CLN1 disease and in *Cln1⁻/⁻* mice, the precise underlying mechanism has remained largely unknown. Previously, we reported that in *Cln1⁻/⁻* mice, the activation of innate immune cells such as microglia and astrocytes mediate neuro-inflammation. We demonstrated that signaling via apolipoprotein E (APOE) and triggering its receptor expressed on myeloid cells 2 (TREM2) contribute to neuroinflammation in *Cln1⁻/⁻* mice. We found that the levels of *ApoE*– and *Trem2*–mRNA and protein levels are significantly higher in the brain of *Cln1⁻/⁻* mice than in those of their WT littermates. Consistent with these findings, the levels of transcription factor PU.1, which regulates ApoE expression, and Irf8, which controls Trem2 expression, requires *S*-palmitoylation for trafficking to the nucleus. Moreover, both Irf8 and PU.1 proteins required *S*-palmitoylation for trafficking

from the cytoplasm to the nucleus. Intriguingly, ApoE–Trem2 signaling in *Cln1*^{−/−} mouse brain mediated neuro-inflammation via the toll-like receptor 2 (TLR2)/MyD88/NF-kappa B signaling pathway. Our findings reveal a previously unrecognized role of *Cln1*/Ppt1 in ApoE–Trem2 signaling in mediating neuro-inflammation in this mouse model of CLN1 disease.

Publications

1. Sadhukhan T, Bagh MB, Appu AP, Mondal A, Zhang W, Liu A, Mukherjee AB. In a mouse model of INCL reduced S-palmitoylation of cytosolic thioesterase APT1 contributes to microglia proliferation and neuroinflammation. *J Inherit Metab Dis* 2021 44:1051–1069.
2. Mukherjee AB, Appu AP, Sadhukhan T, Casey S, Mondal A, Zhang Z, Bagh MB. Emerging new roles of the lysosome and neuronal ceroid lipofuscinoses. *Mol Neurodegener* 2019 14(1):4.
3. Sarkar C, Sadhukhan T, Bagh MB, Appu AP, Chandra G, Mondal A, Saha A, Mukherjee AB. Cln1-mutations suppress Rab7-RILP interaction and impair autophagy contributing to neuropathology in a mouse model of INCL. *J Inherit Metab Dis* 2020 43:1082–1101.
4. Sadhukhan T, Bagh MB, Sadhukhan S, Appu AP, Mondal A, Iben JR, Li T, Coon SL, Mukherjee AB. Ablation of microRNA-155 and neuroinflammation in a mouse model of CLN1-disease. *Biochem Biophys Res Commun* 2021 571:137–144.

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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 during transcriptional elongation, a defining feature of this variant. Other canonical core histones are deposited into nucleosomes during replication. For this reason, H3.3 is thought to be involved in epigenetic memory created after 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 but 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 against a wide array of pathogens (Figure 1A for its structure). 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.

BRD4 promotes cell–cycle progression by preventing DNA damage.

Cell proliferation depends on continuous rounds of cell-cycle progression, which are driven by sequential activation of transcription factors and other post-translational effectors. As stated above, chromatin-binding factor BRD4 is known to promote proliferation of

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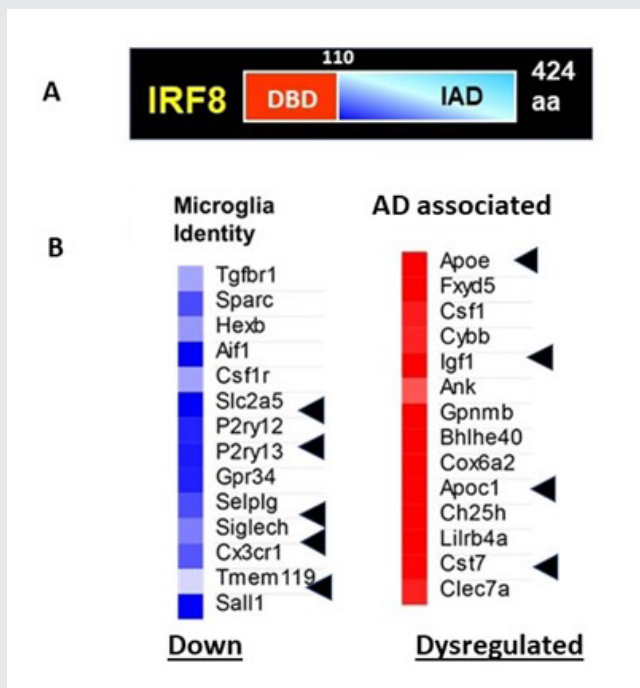
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FIGURE 1. IRF8 regulates microglia transcription.

A. IRF8 domain structure

B. IRF8 directs microglia identity and DAM genes (*right*). Many genes that define microglia are down-regulated in *Irf8* KO cells (*left*), while AD–associated genes are aberrantly expressed (*right*).

many cancers. BRD4 inhibitors (BETi) can arrest cancer growth (See Figure 4A for BRD4 structure). BETi, and BRD4 Protac inhibitors are thought to represent new therapeutic possibilities against cancer. However, the role of BRD4 in the proliferation of normal cells has remained elusive. We examined proliferation of embryonic fibroblasts from *Brd4* conditional knockout (KO) mice. Cell-cycle analysis of wild-type (WT) and *Brd4* KO cells showed that BRD4 is required for the transition from G0-G1, G1-S, and G2-M (Figure 4B). At the G2 to M stage, many *Brd4* KO cells underwent catastrophic mitotic failure, as chromosomes failed to align and segregate properly. 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 has been recently shown to be activated at S and necessary for G2-M passage. Our results indicate that BRD4 drives transcription of numerous cell cycle–regulated genes. Consistent with these results, BRD4 occupied numerous cell-cycle genes throughout all stages, as revealed by ChIP-seq analysis. BRD4 bound to these genes at all stages of the cell cycle, seen at the transcription start site (TSS) and gene body.

Remarkably, *Brd4* KO cells suffered from DNA damage at all stages of the cell cycle, which we found by extensive deposition of phospho (γ)-H2AX foci in the nucleus (Figure 4). H2AX is a variant histone H2, phosphorylated upon DNA damage through activation of the kinase ATM. Comet assay, another method to detect DNA damage, corroborated γH2AX results. DNA damage prompts DNA repair response by a series of enzymes and associated factors, and activates p53, a central factor that determines the downstream pathway that causes either cell-cycle arrest or apoptosis. We found that factors that regulate DNA–damage response are controlled by BRD4 for expression, including ATM/ATR, H2AX, and p53. These results indicate that BRD4 is critical for suppressing endogenous DNA damage, thus playing a major role in the maintenance of genome integrity.
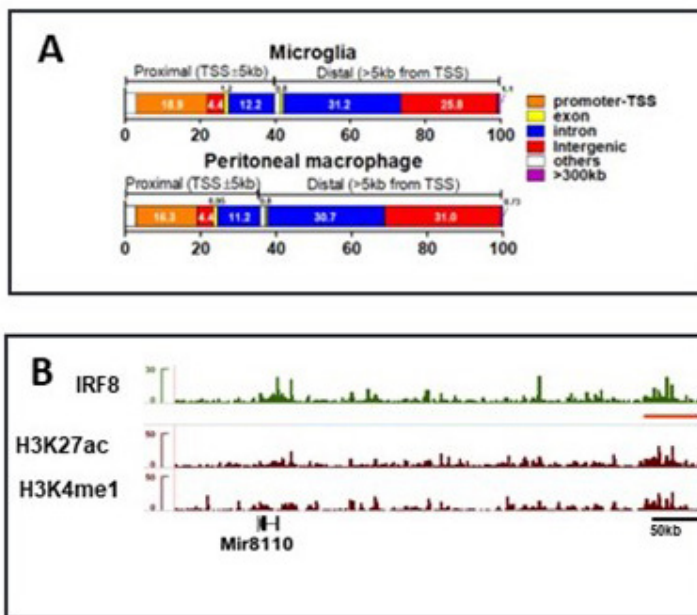
FIGURE 2. IRF8 binds to large enhancers in microglia.

A. IRF8 distribution in microglia and peritoneal macrophage genome

B. IRF8 binds as clusters colocalizing with H3k27ac and H3K4me1, markers for enhancers.

IRF8 sets microglia–specific epigenome structure and defines the transcriptome program.

Microglia are the sole cell type in the brain that protect from pathogen infection and restore neuronal injury. Microglia are also important for shaping neuronal development and cellular connections. Recent genome-wide SPN (single nucleotide polymorphism) analyses showed that genetic risk factors for Alzheimer's disease (AD) are either exclusively or most highly expressed in microglia, but not in neurons. Thus, it is thought that microglia play a central role in AD onset and progression. Microglia originate from the embryonic yolk sac as a progenitor, and then migrate into the embryonic brain, where they differentiate into functional microglia in the postnatal stage. In adult, microglia are distributed throughout the brain, including the cortex and hippocampus. Previous reports demonstrated that Spi1(PU.1) and IRF8 take part in early progenitor differentiation in the embryonic brain. However, IRF8's function in adult microglia was not fully understood. We examined microglia from adult brain in *Irf8* knockout (KO) mice. We found that *Irf8* KO microglia have abnormal morphology and do not express a number of microglia-specific surface markers. We then sorted microglia from adult WT and *Irf8* KO mice by FACS (fluorescence-activated cell sorting) and performed bulk and single-cell RNA-seq. Results revealed that, without *IRF8*, many genes that bestow microglia-specific properties were missing or downregulated, including those encoding 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 expressed in AD microglia were expressed in *Irf8* KO microglia (Figure 1B). In addition, we found that IRF8 is required for the expression of two transcription factors critical for adult microglia function, i.e., Sall1 and Batf3. 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 the number of harvestable microglia is low.
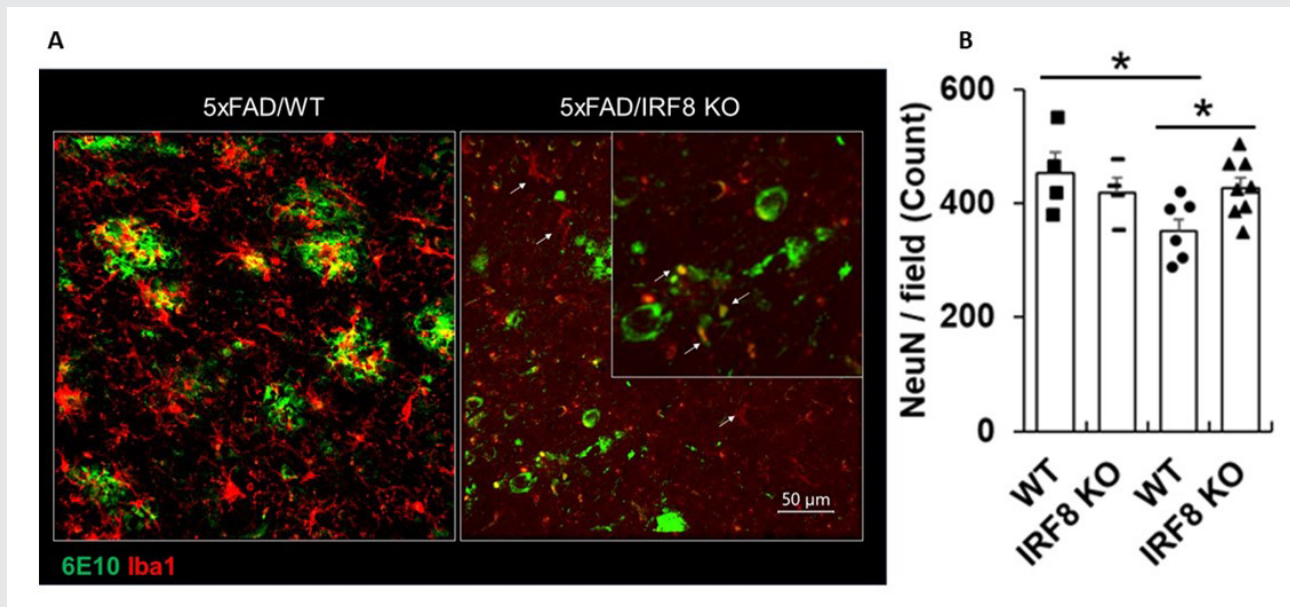
FIGURE 3. IRF8 modulates Alzheimer's disease progression in mice.

A. Large amyloid β plaques accumulate near microglia in 5xFAD brain, but amyloid plaques are smaller in *Irf8* KO 5xFAD brain (cortex). 6E10: polymerized amyloid plaques; Iba1: surface marker for microglia.

B. NeuN immunostaining quantification.

Modified Cut&Run assay 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 GAAA. In some enhancers IRF8 binding was closely clustered. Some of these sites were within the large stretched enhancers enriched in H3K27ac histone marks. Large enhancers are known to support transcription of genes essential for cell type–specific properties. IRF8 containing large enhancers neighbored genes essential for microglia, including *Sall1* and *Batf3*. Deletion of *Irf8* led to loss of large enhancers associated with microglia-identity genes. Furthermore ATAC-seq assay showed that IRF8 is important for setting open chromatin necessary for microglia's large enhancers. Our results clearly show that IRF8 directs the formation of the microglia-specific epigenome landscape. Consistent with this view, DNA methylome profiles revealed extensive changes in methylation associated with IRF8 binding.

Despite the blood-brain barrier, microglia respond to external immune stimuli such as bacterial lipopolysaccharides (LPS), which induce inflammatory genes. We found that IRF8 is required for the expression of many inflammatory genes induced by LPS in microglia. We next sought to determine whether IRF8 impacts the brain's health. Thus, we investigated whether IRF8 affects AD progression, using a mouse model of AD (5xFAD). 5xFAD transgenic mice accumulate plaques containing polymerized amyloid β peptides in the brain near microglia. Amyloid β plaques are thought to be the main cause of AD pathology, as they lead to neuronal death. We found that 5xFAD mice lacking the *Irf8* gene have smaller amyloid plaques in the cortex, indicating that IRF8 facilitates plaque formation in this model (Figure 3A). Given that IRF8 shapes microglia identity, our observations are in line with previous reports that microglia worsen AD pathogenesis in the 5xFAD model.
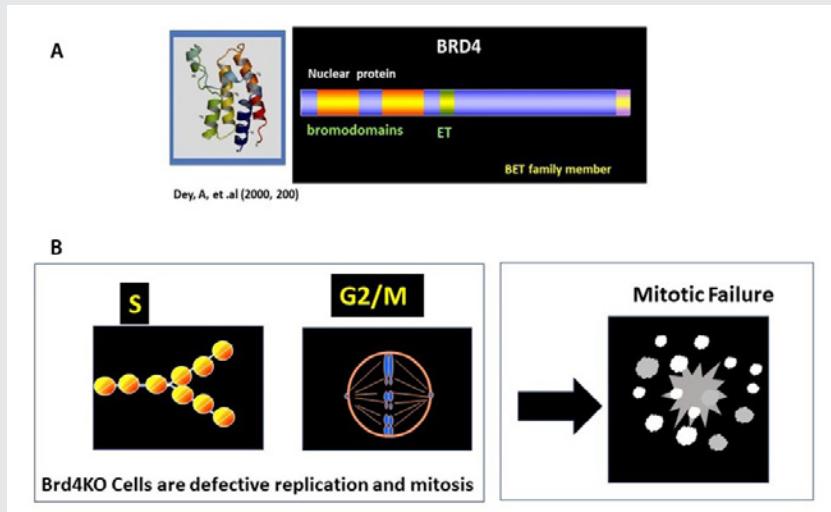
FIGURE 4. Structure and function of BRD4

A. BRD4 has two bromodomains and an ET (extra-terminal) domain.

B. BRD4 controls two fundamental events required for cell growth: replication, and mitosis. Brd4KO cells were defective in both and thus result in mitotic failure.
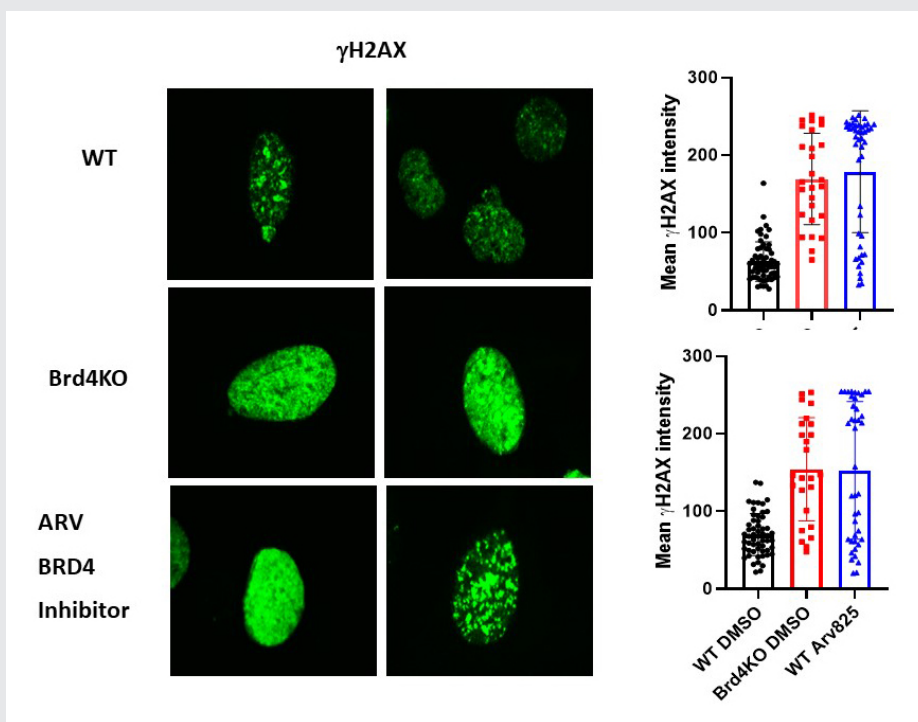

FIGURE 5. BRD4 limits endogenous DNA damage.

BRD4 depletion leads to DNA damage. WT cells, *Brd4* KO cells, and BRD4 inhibitor–treated WT cells were stained with antibody against g-H2AX. Increased DNA damage is verified by quantification on the right.

NeuN immunostaining found evidence of substantial neuronal loss in the one-year-old 5xFAD brain. However, NeuN immunoreactivity was more intense in Irf8KO 5xFAD brain, indicating reduced neuronal damage in the absence of Irf8 (Figure 3B). These results are consistent with the view that microglia can negatively impact AD pathogenesis.
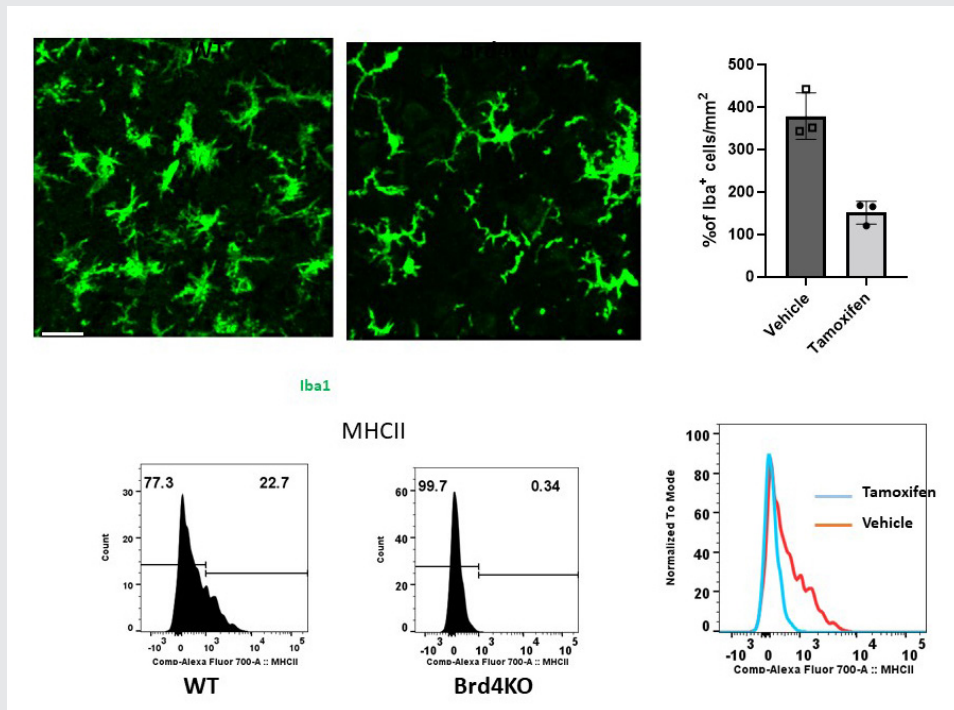
FIGURE 6. BRD4 promotes neuroinflammation in the mouse model of multiple sclerosis (EAE).

Brd4 KO microglia in EAE (experimental allergic encephalomyelitis). Microglia were visualized by Iba1 immunohistology. The numbers of microglia are reduced in *Brd4* KO brain (quantification on the right). MHCII expression is absent from *Brd4* KO microglia. *Brd4* deletion in microglia reduces EAE disease phenotypes.

BRD4 in microglia and neuroinflammation

To further study transcriptome and epigenome regulation of microglia, we began investigating the function of BRD4. We constructed mice in which *Brd4* is knocked out in microglia in adult brain (Brd4^f/f Cx3cr1Cre), where Brd4 is deleted after Tamoxifen injection. To study how BRD4 regulates neuro-inflammation, we analyzed a mouse model of multiple sclerosis: experimental allergic encephalomyelitis (EAE). EAE causes microglia to proliferate from the resident progenitor cells. These microglia, along with T cells and macrophages infiltrating from periphery, promote demyelination and neuronal damage, which results in paralysis. Brain histology revealed that the number of microglia is considerably reduced in the *Brd4* KO brain in EAE (Figure 5A). In addition, *Brd4* KO microglia exhibited aberrant morphology, with stunted extensions, and failed to express MHCII, a representative marker for active microglia (Figure 5B). Remarkably, mice with the microglial *Brd4* deletion had less demyelination, resulting in reduced tissue damage, and reduced paralysis. Neuro-inflammatory cytokines and chemokines such as Il1b were also lower in *Brd4* KO EAE microglia. These results indicate that BRD4 is required for microglia to detect incoming antigenic and inflammatory signals, interacting with and further stimulating infiltrated T cells. Our study revealed that BRD4 plays a central role in the pathogenesis of neuroinflammation. It will be of interest to clarify the role of BRD4 in microglia development and AD pathogenesis.

Publications

1. Milner JJ, Toma C, Quon S, Omilusik K, Scharping NE, Dey A, Reina-Campos M, Nguyen H, Getzler AJ, Diao H, Yu B, Delpoux A, Yoshida TM, Li D, Qi J, Vincek A, Hedrick SM, Egawa T, Zhou MM, Crotty S, Ozato K,

Pipkin ME, Goldrath AW. Bromodomain protein BRD4 directs and sustains CD8 T cell differentiation during infection. *J Exp Med* 2021 218:e20202512.
2. Nakasuji-Togi M, Togi S, Saeki K, Kojima Y, Ozato K. Herbal extracts that induce type I interferons through Toll-like receptor 4 signaling. *Food Nutr Res* 2022 66:5524. eCollection.
3. Kurotaki D, Kikuchi K, Cui K, Kawase W, Saeki K, Fukumoto J, Nishiyama A, Nagamune K, Zhao K, Ozato K, Rocha PP, Tamura T. Chromatin structure undergoes global and local reorganization during murine dendritic cell development and activation. *Proc Natl Acad Sci USA* 2022 119(34):e2207009119.
4. Kurotaki D, Kikuchi K, Cui K, Kawase W, Saeki K, Fukumoto J, Nishiyama A, Nagamune K, Zhao K, Ozato K, Rocha PP, Tamura T. Chromatin structure undergoes global and local reorganization during murine dendritic cell development and activation. *Proc Nat Acad Sci USA* 2022 34:e2207009119.
5. Wu T, Hou H, Dey A, Bachu M, Chen X, Wisniewski J, Kudoh F, Chen C, Chauhan S, Xiao H, Pan R, Ozato K. BRD4 directs mitotic cell division by inhibiting DNA damage. *bioRxiv* 2023 25:2023.07.02.547436.

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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 from the adrenal gland and from extra-adrenal paraganglia, respectively. 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.

Clinical and genetic aspects of pheochromocytoma and paraganglioma

Patients with germline *SDHD* pathogenic variants (encoding succinate dehydrogenase subunit D; i.e., paraganglioma 1 syndrome) are predominantly affected by head and neck paragangliomas (PGLs), which, in almost 20% of patients, might coexist with PGLs arising from other locations (e.g., adrenal medulla, para-aortic, cardiac or thoracic, and pelvic). Given the higher risk of tumor multifocality and bilaterality for pheochromocytomas and paragangliomas (PPGLs), more because of *SDHD* pathogenic variants than for their sporadic and other genotypic counterparts, the management of patients with *SDHD* PPGLs

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is clinically complex in terms of imaging, treatment, and management options. Furthermore, locally aggressive disease can be discovered at a young age or late in the disease course, which presents challenges in balancing surgical intervention with various medical and radiotherapeutic approaches. The axiom 'first, do no harm' should always be considered, and an initial period of observation (i.e., watchful waiting) is often appropriate to characterize tumor behavior in patients with these pathogenic variants. Such patients should be referred to specialized high-volume medical centers. Thus, we launched the first international consensus guideline on the management of PPGLs in these patients to help physicians with the clinical decision-making process when caring for patients with these tumors.

PPGLs have up to a 20% rate of metastatic disease, which cannot be reliably predicted. Our study prospectively assessed whether the dopamine metabolite methoxytyramine might predict metastatic disease, whether predictions might be improved using machine-learning models that incorporate other features, and how machine learning–based predictions compare with predictions made by specialists in the field. In this machine- learning modelling study, we used cross-sectional cohort data from the international trial, based in Germany, Poland, and the Netherlands, to prospectively examine the utility of methoxytyramine to predict metastatic disease in 267 patients with PPGLs and positive biochemical test results at initial screening. Another retrospective dataset of 493 patients with these tumors, enrolled under clinical protocols at National Institutes of Health (*00-CH-0093*) and the Netherlands (PRESCRIPT trial), was used to train and validate machine-learning models according to selections of additional features. The best performing machine-learning models were then externally validated using data for all patients in the international trial. For comparison, 12 specialists provided predictions of metastatic disease using data from the training and external validation datasets. Prospective predictions indicated that plasma methoxytyramine could identify metastatic disease at sensitivities of 52% and specificities of 85%. The best performing machine-learning model was based on an ensemble tree classifier algorithm that used nine features: plasma methoxytyramine, metanephrine, normetanephrine, age, sex, previous history of PPGL, location and size of primary tumors, and presence of multifocal disease. The model had an area under the receiver operating characteristic curve of 0·942 (95%) that was significantly larger than that of the best performing specialist (0.815) before and after (0.812) provision of *SDHB* variant data. Sensitivity for prediction of metastatic disease in the external validation cohort reached 83% at a specificity of 92%. We concluded that, although methoxytyramine has some utility for prediction of metastatic PPGLs, sensitivity is limited. Predictive value is considerably enhanced with machine-learning models that incorporate our nine recommended features. Our final model provides a preoperative approach to predict metastases in patients with PPGLs, and thereby guide individualized patient management and follow-up.

Recent advances in oncology-related immunotherapy, specifically in targeting of programmed cell death-1 (PD-1)/programmed death-ligand 1 (PD-L1) pathways, have identified a new treatment potential in a variety of tumors, including advanced and rare tumors. Only a fraction of patients being treated by immune checkpoint inhibitors have been shown to benefit from it, displaying a need for strategies that identify patients who are most likely to show a favorable response. Building on recent, promising outcomes in a clinical study of metastatic PPGL using pembrolizumab, a humanized IgG4κ monoclonal antibody targeting the PD-1/PD-L1 pathway, we examined PD-L1 and PD-L2 expression in relation to oncogenic drivers in our PPGL patient cohort to explore whether expression can predict metastatic potential and/or be considered a predictive marker for targeted therapy. We evaluated RNA expression in the NIH cohort of 48 patients with known genetic predisposition (sporadic; pseudohypoxia: *SDHB*, *VHL*, *EPAS1*, *EGLN1;* kinase signaling: *RET*, *NF1*) and six

normal medulla samples (NAM). For comparison, 72 PPGL samples from The Cancer Genome Atlas (TCGA) were used for analysis of gene expression based on the variant status (pseudohypoxia: *SDHB*, *VHL*, *EPAS1*, *EGLN1*; kinase signaling: *NF1*, *RET*). Expression of PD-L1 was higher in the PPGL cohort than in normal adrenal medulla, aligning with the TCGA analysis, whereas PD-L2 was not elevated. However, expression of PD-L1 was lower in the pseudohypoxia cluster than in the sporadic and the kinase-signaling subtype cluster, suggesting that sporadic and kinase-signaling cluster PPGLs could benefit from PD-1/PD-L1 therapy more than the pseudohypoxia cluster. Within the pseudohypoxia cluster, expression of PD-L1 was significantly lower in both *SDHB*– and non-*SDHB*–mutated tumors than in sporadic tumors. PD-L1 and PD-L2 expression were not affected by the metastatic status. We conclude that PD-L1 and PD-L2 expression in our cohort of PPGL tumors was not linked to metastatic behavior; however, the presence of PPGL driver mutation could be a predictive marker for PD-L1–targeted therapy and an important feature for further clinical studies in patients with PPGL.

Imaging of pheochromocytomas and paragangliomas

Adrenal neoplasms rarely occur in children. They can be diagnosed in the presence of endocrine, metabolic, or neurological problems, an abdominal mass, more rarely an adrenal incidentaloma, or in the context of an adrenal mass discovered in the evaluation of childhood cancer, including hematologic malignancy. According to standard medical practice, pediatric malignancies are almost always evaluated by ^{18}F-fluorodeoxyglucose positron emission tomography with computed tomography ([^{18}F]FDG PET/CT). Nuclear imaging using specific radiotracers is also an important tool for diagnosing and staging neuroblastoma, pheochromocytoma, hormone hypersecretion, or indeterminate adrenal masses. The Hippocratic oath "primum non nocere" encourages limitation of radiation in children per the ALARA concept (as low as reasonably achievable), but should not lead to the under-use of nuclear imaging because of the potential risk of inaccurate diagnosis or underestimation of the extent of disease. As in adults, nuclear imaging in children should be performed in conjunction with hormone evaluation and morphological imaging. In this imaging study, we summarized current knowledge focusing on imaging of adrenal neoplasms (including pheochromocytoma) in children.

In another study, we examined coronary arterial luminal diameter, vessel wall thickness (VWT), and plaque burden in PPGL patients and an age-matched population of healthy volunteers (HVs). Sixteen HVs and 16 PPGL patients participated in this NIH IRB–approved, HIPAA (Health Insurance Portability and Accountability Act)–compliant, prospective study. In patients with PPGLs, there was an approximate 55% reduction in luminal area without increased VWT. We concluded that PPGL patients have significantly reduced coronary luminal area, a likely consequence of catecholamine excess. The reduction in coronary luminal area, however, remained significant despite treatment with α-adrenoceptor blocking agents. Such findings suggest that the reduction in coronary arterial luminal area may be the result of excessive vasoconstriction as opposed to atherosclerosis or catecholamine-mediated arterial remodeling. Further investigation is needed, given that directed treatments such as with coronary artery vasodilators (such as with verapamil, already used to treat PPGL patients) may provide benefit.

Proteomic, metabolic, and immune aspects of pheochromocytoma and paraganglioma

New drug targets and proteins that would assist sensitive PPGL imagining could improve therapy and the quality of life of patients with PPGL, namely those with recurrent or metastatic disease. Using a combined proteomic strategy, we looked for such clinically relevant targets among integral membrane proteins (IMPs) upregulated on the surface of tumor cells and non-membrane druggable enzymes in PPGL. We conducted a

detailed proteomic analysis of 22 well characterized human PPGL samples and normal chromaffin tissue from adrenal medulla. A standard quantitative proteomic analysis of tumor lysate, which provides information largely on non-membrane proteins, was accompanied by specific membrane proteome–aimed methods, namely glycopeptide enrichment using lectin-affinity, glycopeptide capture by hydrazide chemistry, and enrichment of membrane-embedded hydrophobic transmembrane segments. The study identified 67 cell-surface integral membrane proteins strongly upregulated in PPGL compared with control chromaffin tissue. We prioritized the proteins based on their already documented direct role in cancer-cell growth or progression. Increased expression of the seven most promising drug targets (CD146, CD171, ANO1, CD39, ATP8A1, ACE, and SLC7A1) were confirmed using specific antibodies. Our experimental strategy also provided expression data for soluble proteins. Among the druggable non-membrane enzymes upregulated in PPGL, we identified three potential drug targets (SHMT2, ARG2, and autotaxin) and verified their upregulated expression. We concluded that the application of a combined proteomic strategy, recently presented as "Pitchfork," enabled quantitative analysis of both membrane and non-membrane proteome, and resulted in identification of 10 potential drug targets in human PPGL. Seven membrane proteins localized on the cell surface and three non-membrane druggable enzyme proteins were identified and verified as significantly upregulated in PPGL. All the proteins have been previously shown to be upregulated in several human cancers and to play direct role in cancer progression. Marked upregulation of these proteins, along with their localization and established direct roles in tumor progression, make these molecules promising candidates as drug targets or proteins for sensitive PPGL imaging.

The biochemical phenotype of PGLs is highly dependent on the underlying genetic background and tumor location. PGLs at extra-adrenal locations usually do not express phenylethanolamine *N*-methyltransferase (PNMT), the enzyme required for epinephrine production, which was explained by the absence of glucocorticoids. PGLs with pathogenic variants in Harvey rat-sarcoma viral oncogene homolog (HRAS) can occur in or outside the adrenal, but always synthesize epinephrine independently of localization. We characterized the signaling pathways through which pathogenic variants in HRAS influence PNMT expression. Catecholamines, cortisol, and transcriptional features of PGL tissues with known genetic background were analyzed. Genetically modified rat pheochromocytoma cells carrying pathogenic variants in *Hras* were generated and analyzed for regulation of *Pnmt* expression. Elevated epinephrine contents in PGLs with pathogenic variants in *Hras* were accompanied by an enrichment in mitogen-activated protein kinase (MAPK) signaling compared with PGLs with pathogenic variants in genes that activate hypoxia pathways. *In vitro*, *Hras* pathogenic variants exhibited elevated Pnmt expression and epinephrine biosynthesis through increased phosphorylation of SP1 via MAPK signaling. We provided a molecular mechanism that explains the pathogenic variant-dependent epinephrine-production of PGLs.

To understand prognostic immune-cell infiltration signatures in neuroendocrine neoplasms (NENs), particularly PPGL, we analyzed tumor transcriptomic data from The Cancer Genome Atlas (TCGA) and other published tumor transcriptomic data of NENs. We used CIBERSORT to infer immune-cell infiltrations from bulk tumor transcriptomic data from PPGLs, in comparison with gastroenteropancreatic neuroendocrine tumors (GEPNETs) and small-cell lung carcinomas (SCLCs). PPGL's immune signature was validated with NanoString Immune Panel in an independent cohort. Unsupervised clustering of the immune infiltration scores from CIBERSORT was used to find immune clusters. A prognostic immune score model for PPGLs and the other NENs were calculated as a linear combination of the estimated infiltration of activated CD8$^+$/CD4$^+$ T cells, activated NK cells, and M0 and M2 macrophages. In PPGLs, we found five dominant immune clusters, associated with M2 macrophages, monocytes, activated NK cells, M0 macrophages and regulatory T cells, and CD8$^+$/CD4$^+$ T cells respectively.

FIGURE 1. Immune signature of pheochromocytoma and paraganglioma (PPGL) in context of neuroendocrine neoplasms associated with prognosis

Clustering of immune cell infiltrations on TCGA (The Cancer Genome Atlas) PPGL samples:

a. Unsupervised clustering of PPGL according to immune cell infiltrations identifies 5 clusters. Cluster 1 (*red*) is enriched for macrophages and cell-cycle functions; cluster 2 (*gray*) is enriched for monocytes; cluster 3 (*green*) is enriched for chemokines associated with activated NK cells and mast cells; cluster 4 (*cyan*) is associated with regulatory T cells, TGF-beta, and macrophages; cluster 5 (blue) is associated with cytotoxic CD8+ T cells.

b. Mutual co-occurrence map for PPGL immune clusters and molecular subtypes. The mutual co-occurrence *p* values are calculated using Fisher's test for samples belonging to each immune cluster and molecular subtype of PPGL. PPGL patients belonging to *SDHx*–mutated subtype also belong to Immune cluster 1 (significant co-occurrence *p* < 0.05), and PPGL patients belonging to cortical admixture subtype also belong to Immune cluster 3 (significant co-occurrence *p* < 0.05).

c. The relative proportion of five immune clusters in known molecular subtypes of PPGL is shown by a stacked bar plot.
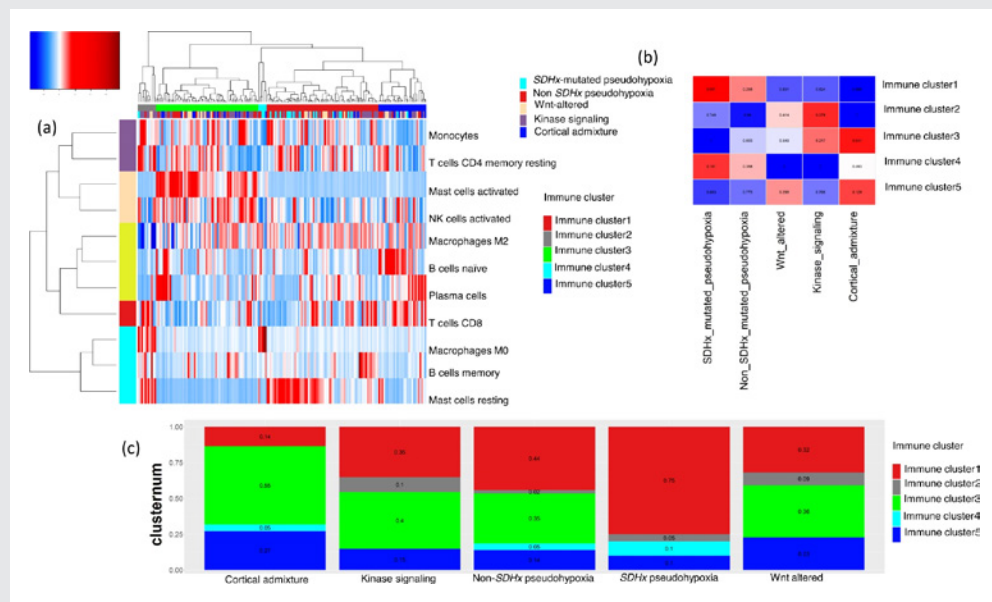
Non-metastatic tumors were associated with activated NK cells and metastatic tumors were associated with M0 macrophages and regulatory T cells. In GEPNETs and SCLCs, M0 macrophages and regulatory T cells were associated with unfavorable outcomes and features such as metastasis and high-grade tumors. The prognostic immune-score model for PPGLs and the NENs could predict non-aggressive and non-metastatic diseases. In PPGLs, the immune score was also an independent predictor of metastasis-free survival in a multivariate Cox regression analysis. We concluded that the transcriptomic immune signature in PPGLs correlates with clinical features such as metastasis and prognosis.

The mechanisms triggering metastasis in PPGLs are unknown, hindering therapeutic options for patients with metastatic tumors (mPPGL). By genomic profiling of a large cohort of mPPGLs, we showed that high mutational load, microsatellite instability, and somatic copy-number alteration burden are associated with *ATRX/TERT* gene mutations and are suitable prognostic markers. Transcriptomic analysis defines the signaling networks involved in the acquisition of metastatic competence and establishes a gene signature related to mPPGLs, highlighting CDK1 as an additional mPPGL marker. Immunogenomics accompanied by immunohistochemistry identifies a heterogeneous ecosystem at the tumor microenvironment level, linked to the genomic subtype and tumor behavior. Specifically, we defined a general immunosuppressive microenvironment in mPPGLs,

the exception being PD-L1–expressing *MAML3* (a transcription co-activator gene)–related tumors. Our study revealed canonical markers for risk of metastasis and suggests the usefulness of including immune parameters in clinical management for PPGL prognostication and identification of patients who might benefit from immunotherapy.

Therapeutic aspects of pheochromocytoma and paraganglioma

PPGLs with pathogenic mutations in the succinate dehydrogenase subunit B (SDHB) are associated with a high metastatic risk. Somatostatin receptor 2 (SSTR2)–dependent imaging is the most sensitive imaging modality for SDHB–related PPGLs, suggesting that SSTR2 expression is a significant cell-surface therapeutic biomarker of such tumors. We explored the relationship between SSTR2 immunoreactivity and SDHB immunoreactivity, mutational status, and clinical behavior of PPGLs and evaluated SSTR–based therapies in metastatic PPGLs. We conducted a retrospective analysis of a multicenter cohort of PPGLs at six specialized Endocrine Tumor Centers in Germany, The Netherlands, and Switzerland. Patients with PPGLs participating in the ENSAT registry were included. Clinical data were extracted from medical records, and immunohistochemistry (IHC) for SDHB and SSTR2 was performed in patients with available tumor tissue. Immunoreactivity of SSTR2 was investigated using Volante scores. The main outcome measure was the association of SSTR2 IHC positivity with genetic and clinical-pathological features of PPGLs. Of 202 patients with PPGLs, 50% were SSTR2–positive. SSTR2 positivity was significantly associated with SDHB– and SDHx–related PPGLs, with the strongest SSTR2 staining intensity in SDHB–related PPGLs. Moreover, SSTR2 expression was significantly associated with metastatic disease independent of SDHB/SDHx mutation status. In metastatic PPGLs, the disease control rate with first-line SSTR–based radionuclide therapy was 67% (n = 22, n = 11 SDHx), and with first-line "cold" somatostatin analogs 100% (n = 6, n = 3 SDHx). We concluded that SSTR2 expression was independently associated with SDHB/SDHx mutations and metastatic disease, and confirmed a high disease-control rate of somatostatin receptor–based therapies in metastatic PPGLs.

Animal model of pheochromocytoma and cell culture studies

Cancer immunotherapy has shown remarkable clinical progress in recent years. Although age is one of the biggest leading risk factors for cancer development, and older adults represent a majority of cancer patients, only a few new cancer immunotherapeutic interventions have been preclinically tested in aged animals. Thus, the lack of preclinical studies focused on age-dependent effect during cancer immunotherapy could lead to different therapeutic outcomes in young and aged animals and future modifications of human clinical trials. We compared the efficacy of previously developed and tested intra-tumoral immunotherapy, based on the combination of polysaccharide mannan, toll-like receptor ligands, and anti-CD40 antibody (MBTA immunotherapy), in young (six weeks) and aged (71 weeks) mice bearing experimental pheochromocytoma (PHEO). The results point out that, despite faster growth of PHEO in aged mice, MBTA intra-tumoral immunotherapy is an effective approach independent of age and could be one of the possible therapeutic interventions to enhance immune response to PHEO and perhaps other tumor types in aged and young hosts.

The study was conducted in collaboration with NCI scientists. Autologous tumor cell–based vaccines (ATVs) aim to prevent and treat tumor metastasis by activating patient-specific tumor antigens to induce immune memory. However, their clinical efficacy is limited. Mannan-BAM (MB), a pathogen-associated molecular pattern (PAMP), can coordinate an innate immune response that recognizes and eliminates mannan-BAM–labeled tumor cells. Toll-like receptor (TLR) agonists and anti-CD40 antibodies (TA) can enhance the immune response by activating antigen-presenting cells (APCs) to present tumor antigens to the adaptive immune

system. We investigated the efficacy and mechanism of action of rWTC–MBTA, an autologous whole tumor cell vaccine consisting of irradiated tumor cells (rWTC) pulsed with mannan-BAM, TLR agonists, and anti-CD40 antibody (MBTA), in preventing tumor metastasis in multiple animal models. The efficacy of the rWTC–MBTA vaccine was evaluated in mice using breast (4T1) and melanoma (B16-F10) tumor models via subcutaneous and intravenous injection of tumor cells to induce metastasis. The vaccine's effect was also assessed in a post-operative breast-tumor model (4T1) and tested in autologous and allogeneic syngeneic breast tumor models (4T1 and EMT6). Mechanistic investigations included immunohistochemistry, immunophenotyping analysis, ELISA, tumor-specific cytotoxicity testing, and T cell–depletion experiments. Biochemistry testing and histopathology of major tissues in vaccinated mice were also evaluated for potential systemic toxicity of the vaccine. The rWTC–MBTA vaccine effectively prevented metastasis and inhibited tumor growth in breast tumor and melanoma metastatic animal models. It also prevented tumor metastasis and prolonged survival in the post-operative breast tumor animal model. Cross-vaccination experiments revealed that the rWTC–MBTA vaccine prevented autologous tumor growth, but not allogeneic tumor growth. Mechanistic data demonstrated that the vaccine increased the percentage of antigen-presenting cells, induced effector and central memory cells, and enhanced CD4$^+$ and CD8$^+$ T cell responses. T cells obtained from mice that were vaccinated displayed tumor-specific cytotoxicity, as shown by enhanced tumor-cell killing in co-culture experiments, accompanied by increased levels of Granzyme B, TNF-α, IFN-γ, and CD107a in T cells. T cell–depletion experiments showed that the vaccine's anti-tumor efficacy depended on T cells, especially CD4$^+$ T cells. Biochemistry testing and histopathology of major tissues in vaccinated mice revealed negligible systemic toxicity of the vaccine. We concluded that the rWTC–MBTA vaccine demonstrated efficacy in multiple animal models through T cell–mediated cytotoxicity and has potential as a therapeutic option for preventing and treating tumor metastasis with minimal systemic toxicity.

Publications

1. Taïeb D, Wanna GB, Ahmad M, Lussey-Lepoutre C, Perrier ND, Nölting S, Amar L, Timmers HJLM, Schwam ZG, Estrera AL, Lim M, Pollom EL, Vitzthum L, Bourdeau I, Casey RT, Castinetti F, Clifton-Bligh R, Corssmit EPM, de Krijger RR, Del Rivero J, Eisenhofer G, Ghayee HK, Gimenez-Roqueplo AP, Grossman A, Imperiale A, Jansen JC, Jha A, Kerstens MN, Kunst HPM, Liu JK, Maher ER, Marchioni D, Mercado-Asis LB, Mete O, Naruse M, Nilubol N, Pandit-Taskar N, Sebag F, Tanabe A, Widimsky J, Meuter L, Lenders JWM, Pacak K. Clinical consensus guideline on the management of phaeochromocytoma and paraganglioma in patients harbouring germline SDHD pathogenic variants. *Lancet Diabetes Endocrinol* 2023 11:345–361.
2. Pamporaki C, Berends AMA, Filippatos A, Prodanov T, Meuter L, Prejbisz A, Beuschlein F, Fassnacht M, Timmers HJLM, Nölting S, Abhyankar K, Constantinescu G, Kunath C, de Haas RJ, Wang K, Remde H, Bornstein SR, Januszewicz A, Robledo M, Lenders JWM, Kerstens MN, Pacak K, Eisenhofer G. Prediction of metastatic pheochromocytoma and paraganglioma: a machine learning modelling study using data from a cross-sectional cohort. *Lancet Digit Health* 2023 5:e555–e559.
3. Calsina B, Piñeiro-Yáñez E, Martínez-Montes ÁM, Caleiras E, Fernández-Sanromán Á, Monteagudo M, Torres-Pérez R, Fustero-Torre C, Pulgarín-Alfaro M, Gil E, Letón R, Jiménez S, García-Martín S, Martin MC, Roldán-Romero JM, Lanillos J, Mellid S, Santos M, Díaz-Talavera A, Rubio Á, González P, Hernando B, Bechmann N, Dona M, Calatayud M, Guadalix S, Álvarez-Escolá C, Regojo RM, Aller J, Del Olmo-Garcia MI, López-Fernández A, Fliedner SMJ, Rapizzi E, Fassnacht M, Beuschlein F, Quinkler M, Toledo RA, Mannelli M, Timmers HJ, Eisenhofer G, Rodríguez-Perales S, Domínguez O, Macintyre G, Currás-Freixes M, Rodríguez-Antona C, Cascón A, Leandro-García LJ, Montero-Conde C, Roncador G, García-García JF, Pacak K, Al-

Shahrour F, Robledo M. Genomic and immune landscape of metastatic pheochromocytoma and paraganglioma. *Nat Commun* 2023 14:1122–1142.

4. Ghosal S, Vanova KH, Uher O, Das S, Patel M, Meuter L, Huynh TT, Jha A, Talvacchio S, Knue M, Prodanov T, Zeiger MA, Nilubol N, Taieb D, Crona J, Shankavaram UT, Pacak K. Immune signature of pheochromocytoma and paraganglioma in context of neuroendocrine neoplasms associated with prognosis. *Endocrine* 2023 79:171–179.

5. Vit O, Talacko P, Musil Z, Hartmann I, Pacak K, Petrak J. Identification of potential molecular targets for the treatment of cluster 1 human pheochromocytoma and paraganglioma via comprehensive proteomic characterization. *Clin Proteomics* 2023 20:39–55.

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Mechanisms Regulating Fate and Maturation of Forebrain GABAergic Interneurons

Proper brain function requires a balance between excitatory projection neurons and GABAergic inhibitory interneurons. Although interneurons constitute the minority (about 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. Interneurons are an extremely heterogeneous cell population, with distinct morphologies, connectivity, neurochemical markers, and electrophysiological properties. In fact, 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." Abnormal development and function of interneurons has been linked to the pathobiology of numerous neurological and psychiatric diseases, such as epilepsy, schizophrenia, and autism. Many genes implicated in brain disorders are enriched in young interneurons, and thus a thorough description of the cellular and molecular mechanisms regulating this diverse cell population is necessary to understand both normal development and disease models.

The lab focuses on understanding the how intrinsic genetic and epigenetic programs interact with the local brain environmental to generate this incredible diversity of interneuron subtypes. We take a multifaceted approach to this issue, utilizing both *in vitro* and *in vivo* approaches to identify candidate mechanisms that regulate interneuron fate decisions. We strive to develop cutting-edge techniques that will overcome the many challenges of studying interneuron development. Our ultimate goal is to discover genetic cascades and signaling mechanisms that direct interneuron differentiation and maturation during normal development and in disease states.

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Characterization of transcriptome diversity in radial glia cells throughout the embryonic forebrain

Radial glia cells (RGCs), located throughout the ventricular zone (VZ) of the developing nervous system, give rise to all neurons and glia.
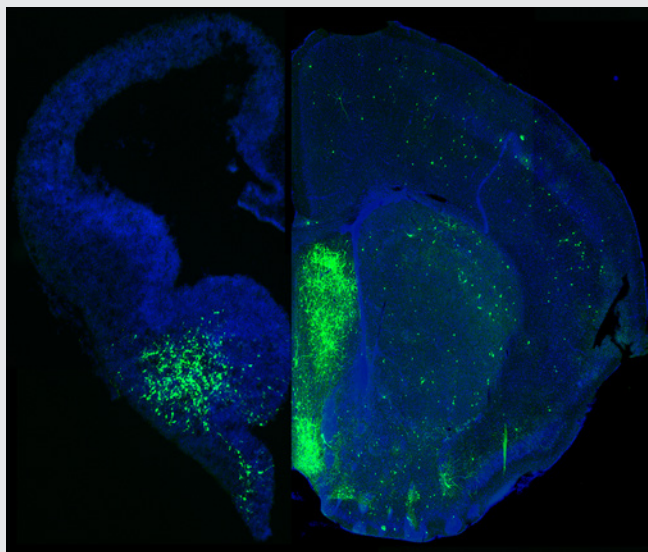
FIGURE 1. MGE–derived GABAergic cells populate many different brain regions.

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 a 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.

Early studies supported the hypothesis that RGCs are relatively homogenous and become progressively restricted over future cell divisions. Initial scRNA-seq studies on the GEs (ganglionic eminences) found that the first genetic signatures of mature interneuron subtypes appear in post-mitotic precursors, whereas very little transcriptional diversity was detected in VZ and SVZ (subventricular zone) progenitors. However, these studies were significantly underpowered for detecting potential transcriptional diversity in VZ neural progenitors because the GEs comprise primarily SVZ and mantle zone cells at these mid-embryonic ages. Transcriptionally heterogeneous VZ cell populations have been reported in other portions of the central nervous system, so we hypothesized that RGCs in the forebrain are more genetically heterogeneous than previously appreciated.

We performed scRNA-seq on the LGE, MGE, CGE (lateral, medial and caudal ganglionic eminences) and cortex from E12.5 and E14.5 mice to identify spatial and temporal genetic heterogeneity of VZ and SVZ neural progenitors, utilizing a mouse line with a destabilized VenusGFP protein driven by the *Nestin* promoter to enrich for RGCs. We identified significant transcriptional heterogeneity in VZ cells, with many genes specifically enriched in RGCs in the MGE, LGE or CGE. We verified many of these expression patterns using fluorescent *in situ* hybridization (FISH) and found that some genes were enriched in specific subdomains (e.g., dorsal vs. ventral) within each GE. Given that specific subdomains of the MGE preferentially generate distinct mature interneurons subtypes, our observation indicates that VZ cells already express spatially restricted genes that pattern the GE and likely are critical for future cell-fate decisions [Reference 1]. Also, the field currently lacks a strong Cre–driver to label and manipulate CGE–derived interneurons. We found that the *Igfbp5* gene (encoding insulin-like growth-factor binding protein 5) was specifically enriched in the CGE VZ, and we generated an *Igfbp5-Cre* mouse to explore whether this can be used to target CGE cells.

'Epigenome Atlas' of the embryonic mouse brain

While scRNA-seq experiments have greatly increased our understanding of the mature and developing brain, much remains to be discovered. Genome-wide association studies (GWAS) indicate that over 90% of disease-associated single-nucleotide polymorphisms (SNPs) are located outside coding regions, and many
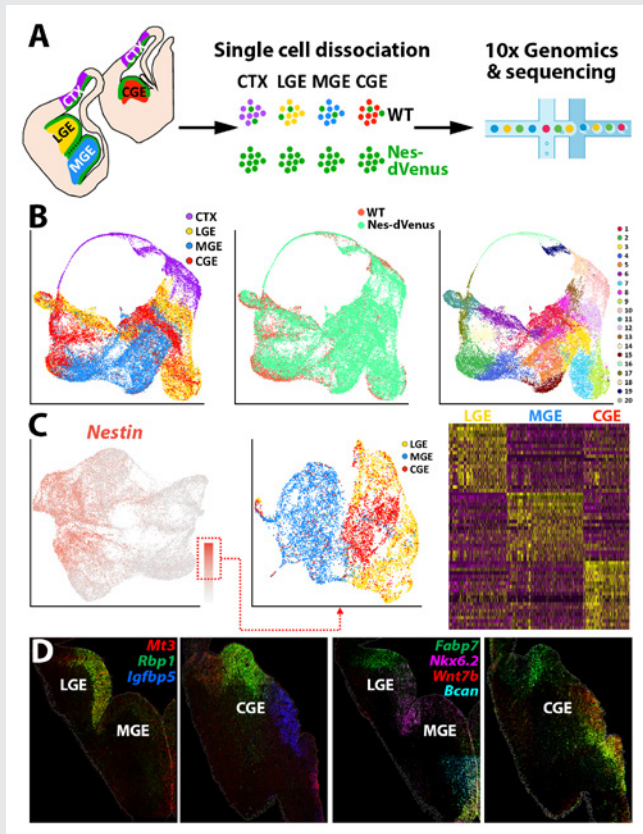
FIGURE 2. Transcriptional diversity in the ganglionic eminences of the embryonic mouse brain

A. Experimental paradigm to harvest cells from four distinct brain regions (MGE, LGE, CGE, cortex) from wild-type and Nestin-dVenus embryonic mouse brains for single-cell RNA sequencing.

B. UMAP plots of single cells categorized by brain region (*left*), mouse line (*middle*), or putative cell cluster (*right*).

C. High Nestin-expressing cells were extracted and repotted to identify differential gene expression between radial glia cells in the MGE, LGE, and CGE.

D. In situ hybridization confirmation of genes enriched in specific subdomains of the MGE, LGE, and CGE.

neurological disorders have been linked directly to SNPs in enhancer regions. Thus, a thorough characterization of the epigenomic landscape during neurogenesis is critical to understand normal brain development and potential disease etiologies. To this end, we performed the following set of experiments on the MGE, LGE, CGE, and cortex from E12.5 embryonic mouse brain: scRNA-seq, snATAC-seq to define chromatin accessibility at the single-cell level; CUT&Tag on histone modifications H3K4me3, H3K27ac, and H3K27me3 to identify active promoters, active enhancers, and closed chromatin, respectively; and Hi-C (a high-throughput genomic and epigenomic technique) and Capture-C to characterize higher-order chromatin structure and to increase confidence in promoter-enhancer interactions.

Our snATAC-seq data revealed significant differences in chromatin accessibility between brain regions. We used SnapATAC in combination with Cicero to identify candidate promoter-enhancer interactions, many of which were supported by H3K4me3 and H3K27ac data. Combining this with the scRNA data allowed us to follow the trajectory of promoter accessibility, enhancer accessibility, and RNA levels over time. Surprisingly, we found that RNA levels were downregulated prior to closing of promoters and enhancers, which has intriguing implications for how genes are regulated during development. The Hi-C data also revealed distinct chromatin organization between different embryonic brain regions. For example, the transcription factor Nkx2.1 is expressed in the MGE and is critical for all MGE–derived cells. There is a direct interaction between the *Nkx2.1* promoter and the *Mbip* locus in the MGE that is not present in the LGE, CGE, or cortex. Conversely, in these non–MGE regions, the *Nkx2.1* promoter interacts directly with the *Nkx2.9* and *Pax9* locus, whereas this interaction is not observed in the MGE. To our knowledge, this was the first study to explore differential epigenetic landscape in distinct
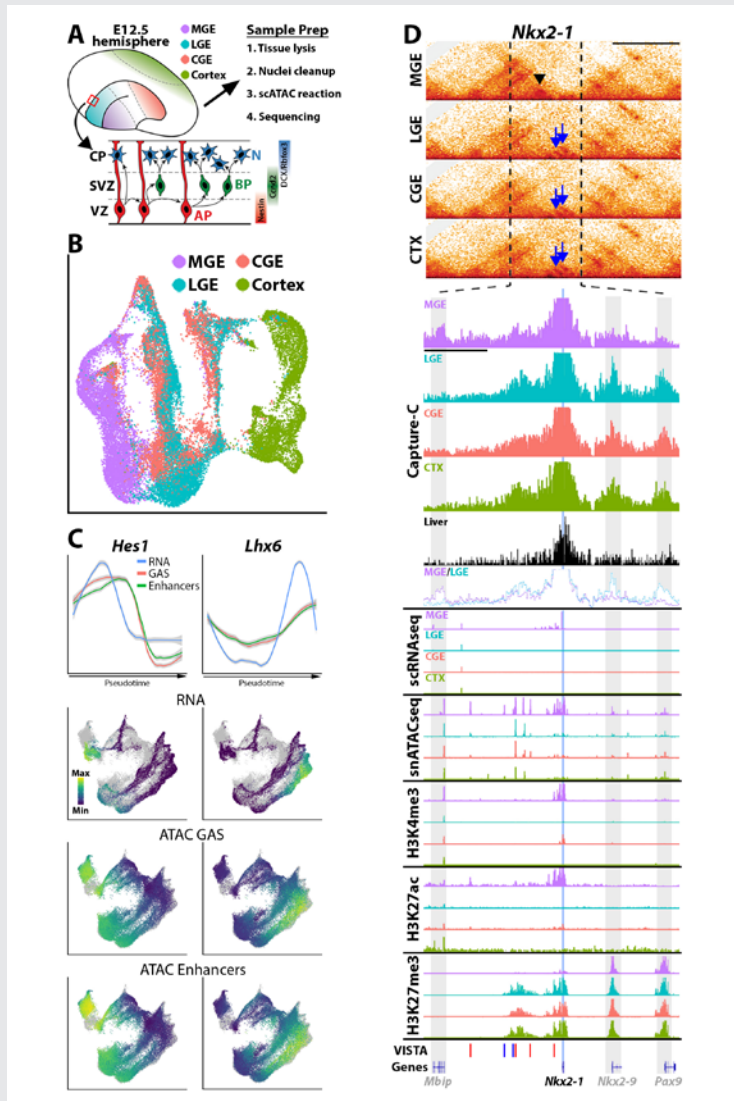
FIGURE 3. Epigenome atlas of the embryonic mouse brain

A. Schematic of snATAC-seq workflow and neurogenic cell types: apical progenitors (*APs*), basal progenitors (*BPs*), and neurons (*Ns*).

B. UMAP visualization of single nuclei clustered by brain region.

C. Top, graph depicting RNA levels (*blue*), promoter accessibility (*red*) and enhancer accessibility (*green*) for a gene whose expression decreases over time (*Hes1*) and increases over time (*Lhx6*). *Bottom*, UMAP plots depicting the RNA levels, promoter accessibility and enhancer accessibility in the RNA+ATAC integrated dataset for *Hes1* and *Lhx6*.

D. Combination of Hi-C, Capture-C, snRNA-seq, snATAC-seq, and histone modifications at the *Nkx2.1* locus. Note the MGE–specific interaction between the *Nkx2.1* promoter and the *Mbip* gene, and a non–MGE specific interaction between the *Nkx2.1* promoter and the *Nkx2.9* and *Pax9* genes.

regions of the embryonic mouse brain, and it highlights the importance of characterizing multiple epigenetic mechanisms *in vivo* because many of these differences would not have been observed using *in vitro* cell cultures [Reference 2].

We believe that this dataset represents a critical resource for the field, and to make this dataset publicly available in an easily searchable platform, we used the *UCSC Genome Browser platform*. Any investigator can search for a gene or loci of interest and view the RNA, ATAC, histone modification, Cicero interactions, and Hi-C data in four regions of the embryonic mouse brain.

We spend significant time developing efficient protocols to generate single-cell and single-nuclei suspensions from both embryonic and adult mouse brains. In addition to being critical for many of our own studies, our success with these procedures generated significant interest from other labs who were interested in performing single-cell sequencing experiments. This motivated us to publish an all-encompassing methods paper describing our protocols to generate single cell/nuclei suspensions from embryonic or adult mouse brains for numerous downstream applications [Reference 5].

Loss of Ezh2 in the MGE alters interneuron fate and function.

We were interested in exploring how genetic perturbations alter normal processes and regulate interneuron
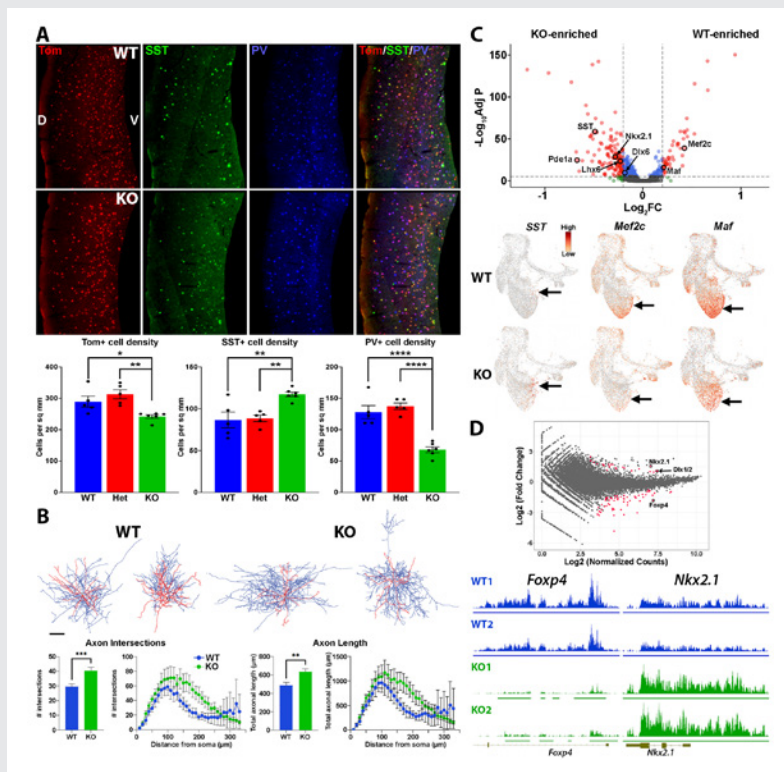
FIGURE 4. Loss of Ezh2 in the MGE affects interneuron fate and function.

A. Ezh2 KO mice have a reduction of MGE-derived cortical interneurons, with increased SST+ and decreased PV+ cells.

B. Fast-spiking PV+ cells display more complex axonal arbors in *Ezh2* KO mice.

C. Top, Volcano plot showing genes enriched in SST+ cells (*SST, Pde1a*) in *Ezh2* KO MGE and genes enriched in PV+ cells (*Mef2c, Maf*) in the WT MGE. *Bottom*, corresponding UMAP plots for *SST, Mef2c*, and *Maf*.

D. Top, MA plot showing genomic loci differential changes in H3K27me3 levels in the *Ezh2* KO mouse, as determined by CUT&Tag. *Bottom*, Trace showing H3K27me3 levels at genes more susceptible (*Foxp4*) or resistant (*Nkx2.1*) to loss of *Ezh2*.

fate. There is growing evidence that mutations in epigenetic machinery can lead to neuro-development disorders. One gene of interest was Enhancer of zeste homolog 2 (Ezh2), the primary methyltransferase of the polycomb repressive complex 2 (PRC2), which is critical for trimethylation of histone 3 lysine 27 (H3K27me3), resulting in gene repression. In humans, *EZH2* variants lead to the Weaver syndrome, a complex disease with varying degrees of intellectual disability. Loss of *Ezh2* in mice can lead to premature neuronal differentiation, migration defects, and changes in neuronal fate, but the role of *Ezh2* in forebrain interneurons had not been explored. To this end, we generated *Nkx2.1-Cre;Ezh2* conditional knockout (KO) mice to remove *Ezh2* from the MGE.

Loss of *Ezh2* reduced the overall number of MGE–derived interneurons in the cortex and caused a shift in fate with an increase in SST+ (somatostatin-positive) and a decrease in PV+ (parvalbumin-positive) interneurons. Similar shifts in cell fate were observed in the hippocampus and striatum. We did not observe altered cell fate when *Ezh2* was removed in postmitotic GE (ganglionic eminence) cells using *Dlx6a-Cre*, indicating that the critical function of Ezh2 occurs in cycling neural progenitors. In collaboration with Soohyun Lee, we characterized the electrophysiological (e-phys) properties of mutant cells in the cortex. We found no changes in the intrinsic e-phys properties of PV+ or SST+ cells in the cortex of KO mice. However, PV+ cells in the KO displayed more complex axonal arbors, with greater axonal length and arborization than in wild-type (WT) PV+ cells (Figure 4B). We believe this could be a form of compensation, given that one way to circumvent the loss of PV+ cells in the mutant would be for the surviving cells to have increased synaptic outputs and increased inhibition.

We performed the 10x Genomics Multiome assay (snRNA-seq and snATAC-seq) on the MGE of WT and KO mice. We found an increase in genes expressed by SST+ cells (*SST, Pde1a*) in the KO MGE, whereas genes predictive
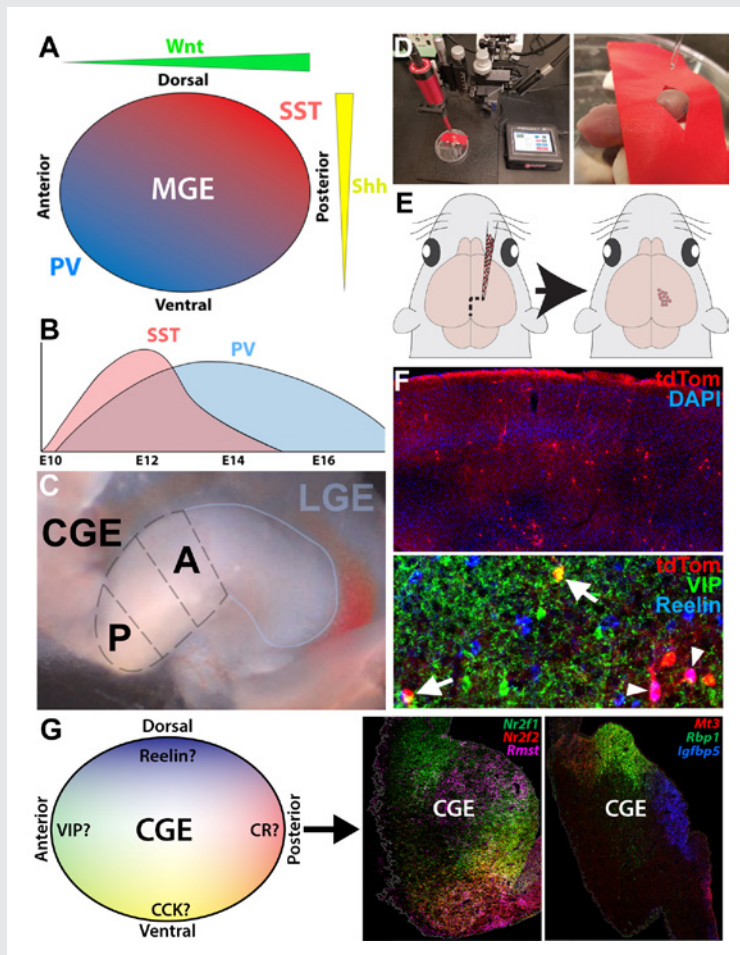
FIGURE 5. Cell transplants to determine the spatial-temporal relationship between CGE subdomains and mature interneuron fates

A-B. Spatial organization (*A*) and temporal birthdate (*B*) relating MGE progenitors to mature subtypes.

C. Dissection to harvest anterior (*A*) and posterior (*P*) CGE.

D. Injection setup.

E. Schematic of CGE cell injection.

F. P21 brain that was transplanted at P1 with E13.5 CGE cells from *Dlx6aCre;Ai9* mouse displaying VIP+/Tom+ (*arrows*) and Reelin+/Tom+ (*arrowheads*) cells.

G. Schematic of possible results to integrate with our previous scRNA-seq data.

of PV–fated interneurons (*Mef2c*, *Maf*) were enriched in WT MGE. We also observed concurrent shifts in the presence of Mef2c– and Maf–binding motifs in accessible regions in the snATAC-seq data. Thus, loss of *Ezh2* induces transcriptional and epigenetic changes in the embryonic MGE, which lead to shifts in mature interneuron fate. We also quantified changes in H3K27me3 levels at specific genomic loci via CUT&Tag. Despite a global reduction in H3K27me3 in *Ezh2* KO mice, we observed relative changes in H3K27me3 at specific loci. For example, H3K27me3 signal was nearly entirely depleted at the *Foxp4* locus, indicating that this region is extremely susceptible to loss of *Ezh2*. Conversely, the *Nkx2.1* locus displayed a relative increase in H3K27me3, meaning that it was significantly resistance to this epigenetic perturbation. The finding implies that genes critical for specific aspects of development (such as *Nkx2.1* in the MGE) may be evolutionarily resistant to epigenetic perturbations. In support of this concept, a similar finding was observed at the *Sox2* locus in our collaboration with Pedro Rocha, warranting further investigation of this concept [Reference 3].

Mechanisms regulating fate determination of CGE–derived interneurons

Research over the last 20 or so years has led to many insights into MGE development. However, our knowledge of mechanisms regulating CGE–derived interneurons lags significantly behind. The CGE contributes about 30%

of interneurons in mice, but humans and primates have a larger proportion of CGE–derived interneurons. Additionally, CGE progenitors are the primary source of tumors and cortical lesions in tuberous sclerosis complex (TSC), underscoring the importance of understanding development of CGE cells.

In previous studies, we transplanted fluorescent embryonic precursors from MGE subdomains into postnatal brains to determine the spatial and temporal origin of mature interneuron subtypes. The studies identified a spatial and temporal organization relating MGE progenitors to mature interneuron subtypes. Combining these insights with gene expression patterns has generated important insights into MGE development. Surprisingly, whether a similar organization occurs in the CGE has not been explored. We are harvesting anterior, posterior, ventral, and dorsal CGE cells (aCGE, pCGE, vCGE, dCGE) from *Dlx6aCre;Ai9* mice and transplanting these cells into the cortex of WT pups. We are harvesting CGE subdomains from two different ages (E13.5 and E15.5) to identify both temporal and spatial relationships between CGE progenitors and mature interneuron subtypes, as both location and birthdate play a role in fate determination within the MGE. In total, this constitutes eight different conditions (four CGE subdomains at two different time points). Brains are harvested at P30–35, sectioned and immunostained for CGE–specific interneuron markers (e.g., VIP, CCK, reelin, and calretinin) to correlate spatial and temporal origin with mature CGE–derived interneuron subtypes. Our preliminary cell counts from aCGE and pCGE transplants indicate that a spatial bias does exist in the CGE, with distinct CGE–derived interneuron subtypes preferentially arising from specific CGE subdomains. Our CGE spatio-temporal map will be integrated with our scRNA-seq datasets described above to identify candidate genes expressed in CGE subdomains related to mature subtypes.

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Publications

1. Lee DR, Rhodes C, Mitra A, Zhang Y, Maric D, Dale RK, Petros TJ. Transcriptional heterogeneity of ventricular zone cells in the ganglionic eminences of the mouse forebrain. *eLife* 2022 *https://doi.org/10.7554/eLife.71864*.
2. Rhodes CT, Thompson JJ, Mitra A, Asokumar D, Lee DR, Lee DJ, Zhang Y, Jason E, Dale RK, Rocha PP, Petros TJ. An epigenome atlas of neural progenitors within the embryonic mouse forebrain. *Nat Commun* 2022 13(1):4196.
3. Rhodes CT, Asokumar D, Sohn M, Naskar S, Elisha L, Stevenson P, Lee DR, Zhang Y, Rocha PP, Dale RK, Lee S, Petros TJ. Loss of Ezh2 in the medial ganglionic eminence alters interneuron fate, cell morphology and gene expression profiles. *bioRxiv* 2023 doi: *https://doi.org/10.1101/2023.09.06.556544*.
4. Chakraborty S, Kopitchinski N, Zuo Z, Eraso A, Awasthi P, Chari P, Mitra A, Tobias IC, Moorthy SD, Dale RK, Mitchell JA, Petros TJ, Rocha PP. Enhancers that activate target genes across CTCF boundaries increase phenotypic robustness. *Nat Genet* 2023 55(2):280–290.
5. Lee DR, Zhang Y, Rhodes CT, Petros TJ. Generation of single cell and single nuclei suspensions from embryonic and adult mouse brains. *STAR Protocols* 2023 4(1):1–22.

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Molecular Genetics of an Imprinted Gene Cluster on Mouse Distal Chromosome 7

Our lab is interested in epigenetic mechanisms that regulate gene expression in order to understand how the epigenome is established and then modified during development and how disruptions in the epigenome can lead to developmental disorders and disease. A simple definition of the epigenome is that it is the total array of heritable changes in chromosome structures that influence gene function. Known epigenetic mechanisms include DNA modifications (such as cytosine methylation), altering histone localization and chemical structures, and DNA looping. Our approach is to build and then characterize mouse models for human disorders in which the fundamental problem is disruption of the normal epigenome. By understanding disease progression in these models, we hope to learn about mechanisms for regulating the epigenome. But we also hope that clarifying the exact molecular defects in these specific diseases will identify new therapeutic targets. We are currently investigating two disease models. First, we are continuing our long-term and comprehensive analysis of maternal loss of imprinting (LOI) at the *H19/Igf2* locus. As detailed in the next paragraph, maternal LOI is a model for the Beckwith-Wiedeman syndrome. Second, we are generating and characterizing new mouse models that disrupt cohesin accumulation on the chromosomes. Altered cohesin patterns are the molecular cause for the Cornelia-de-Lange syndrome.

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. Imprinting represents the cleanest possible example of epigenetic regulation of gene expression, and therefore understanding imprinting is an excellent means to understand epigenetics. 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 *Igf2* is regulated by the *H19* ICR, which is located just upstream of the *H19*

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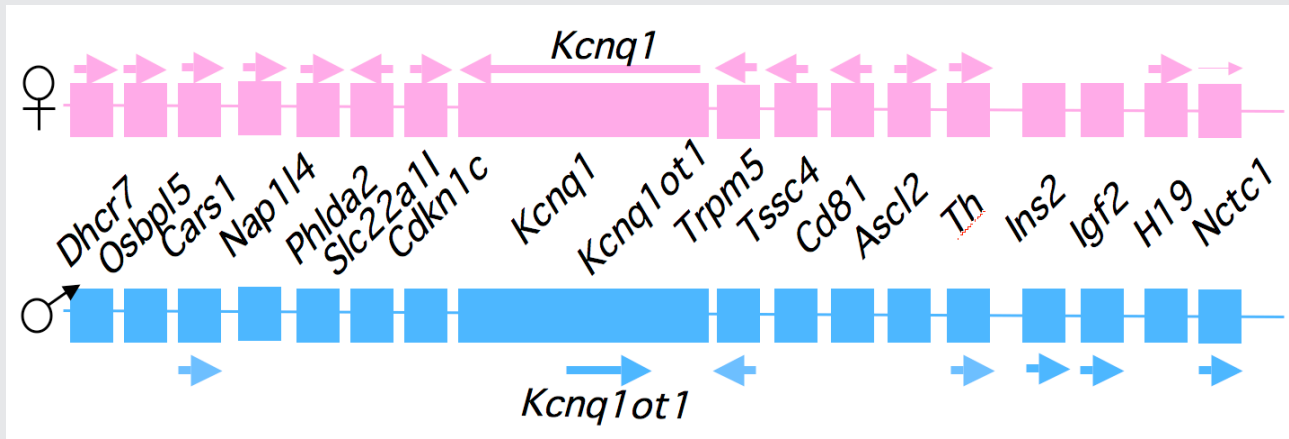Abucar Mohamed, BS, *Postbaccalaureate Fellow*

FIGURE 1. An imprinted domain on mouse distal chromosome 7

Maternal (*pink*) and paternal (*blue*) chromosomes are indicated. Horizontal arrows denote RNA transcription.

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.

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). Imprinting at the *Igf2/H19* locus depends on the 2.4 kb *H19* ICR, which lies between the two genes, just upstream of the *H19* promoter (Figure 2). On the maternal chromosome, binding of the CTCF protein, a transcriptional 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.
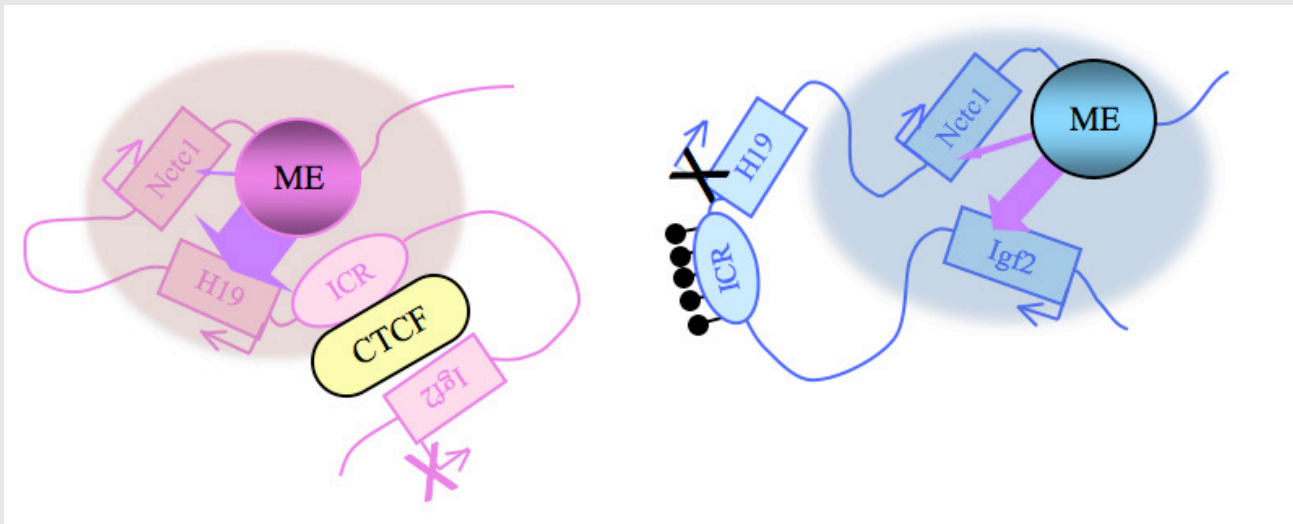
FIGURE 2. Distinct maternal and paternal chromosomal conformations at the distal 7 locus

Epigenetic modifications on the 2.4 kb ICR generate alternative 3D organizations across a large domain on paternal (*blue*) and maternal (*pink*) chromosomes and thereby regulate gene expression. ICR, imprinting control region; ME, muscle enhancer; filled lollipops, CpG methylation covering the paternal ICR.

The *H19* ICR is not only necessary but is also sufficient for genomic imprinting. To demonstrate this, we used knock-in experiments to insert the 2.4 kb element at heterologous loci and demonstrated its ability to imprint these regions. 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); the element 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.

FIGURE 3. The shared muscle enhancer (ME) directs RNAP binding and RNA transcription across the entire 150 kb locus.
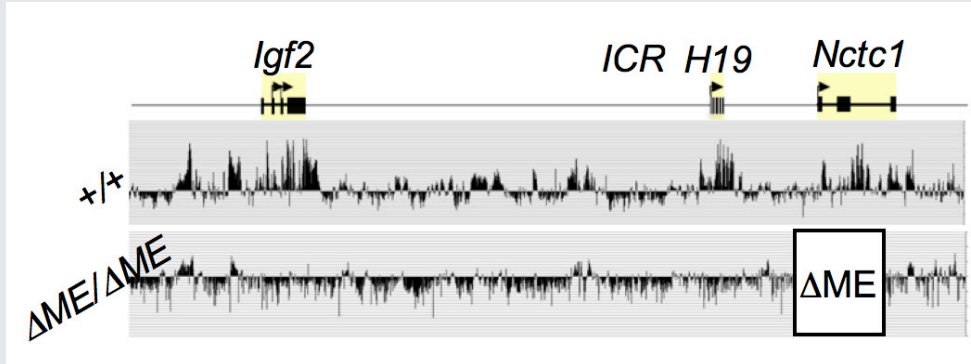
Molecular mechanisms for tissue-specific promoter activation by distal enhancers

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 and 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 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 between 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
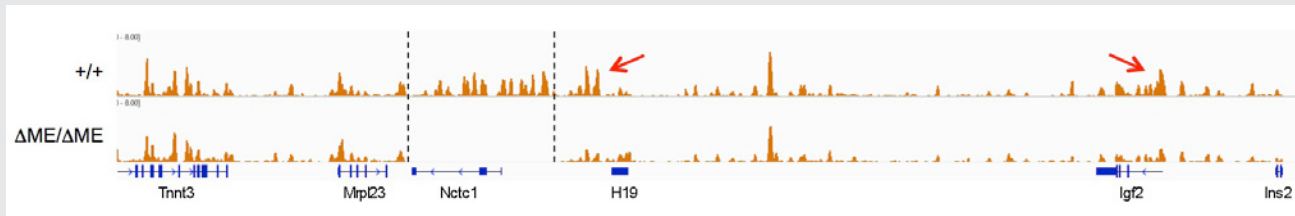
FIGURE 4. Chromatin patterns at the *Igf2/H19* locus are independent of enhancer activity.

Chromatin was isolated from wild-type and enhancer-deletion muscle cells, using antibodies against H3K4me1, and analyzed by DNA sequencing.

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.

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 Wig-1 (which is involved in the regulation of mRNA stability). 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

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.
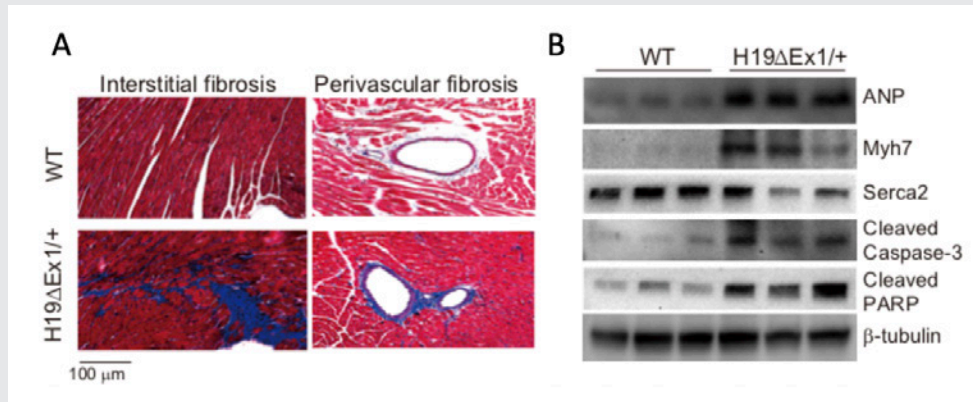
technology (ART) show increased incidence of BWS, which can be explained by increasingly frequent loss of *H19/Igf2* imprinting in such children. Moreover, the children show high frequency of cardiac dysfunction. Taken together, such findings suggest that abnormal expression of the *H19/Igf2* locus can 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* expression 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.

Loss of expression of *H19* is pathologic (Figure 5). Hearts show progressive heart disease, 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 for preventing 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 observed that disruption of the *let-7* microRNA binding sites on the H19 RNA results in the same myopathies as complete gene ablation (Figure 6).

FIGURE 6. Let-7 binding sites on H19 lncRNA are essential for normal heart function.

Mice carrying *H19* alleles that do not include let-7 binding sites are fibrotic (*panels A and B*), hypertrophic (*panel B*), and display protein expression profiles typical of cardiac failure.
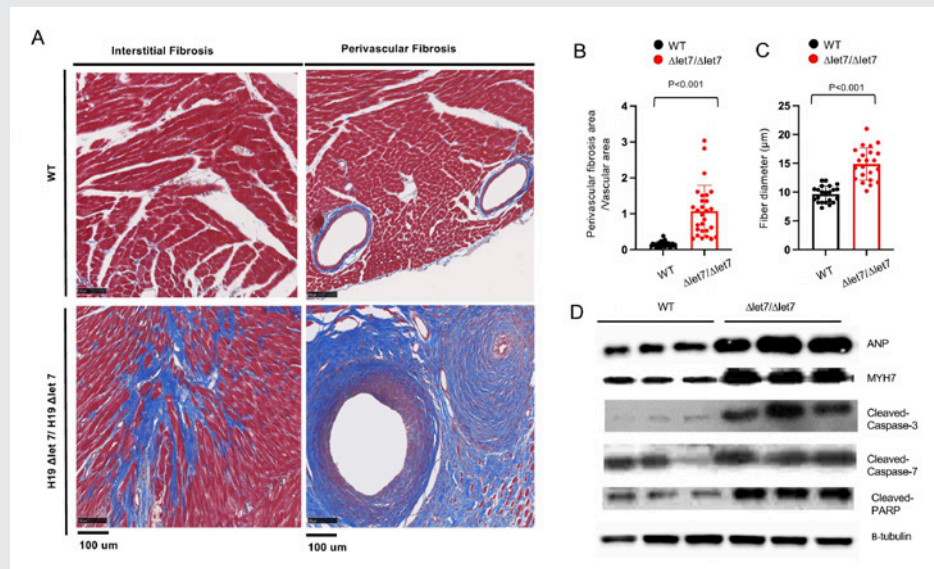
Function of H19 RNAs in muscle development

Studies in mice and humans indicate that *H19* gene function is critical for normal development and function of skeletal muscle. As described in the previous section, H19 precursor RNA is processed into two distinct products: 2.3 kb lncRNA and a small microRNA (mi675). We generated mouse models that specifically ablate one or the other. The *H19*^DeltaMIR allele generates an H19 lncRNA with a 40 bp deletion that is expressed at normal levels and maintains all H19 lncRNA functions that we tested. For example, the *H19*^DeltaMIR lncRNA is sufficient to prevent cardiomyopathy and cellular senescence in myoblasts. However, *H19*^DeltaMIR*H19*^DeltaMIR mice

FIGURE 7. *H19*–encoded microRNA mir675 is essential for muscle injury recovery.
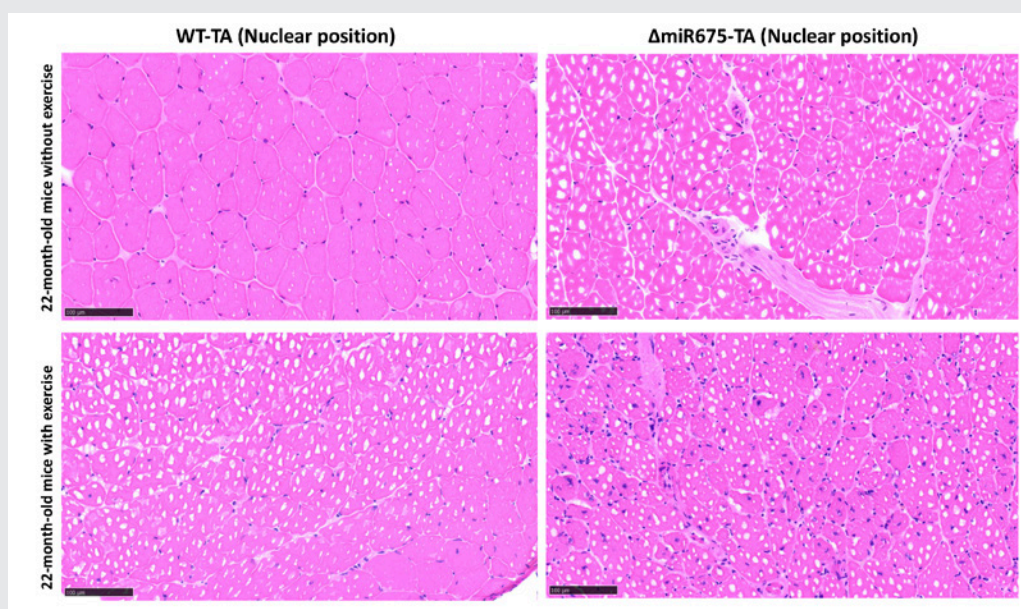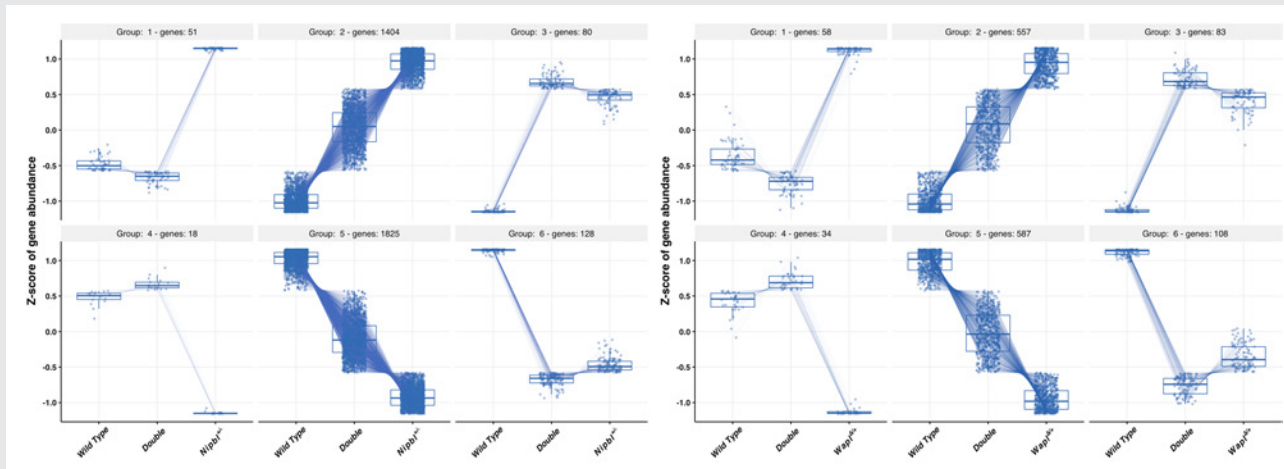
FIGURE 8. Rescue of transcriptome phenotypes in *Wapl⁺/⁻ Nipbl⁺/⁻* double heterozygotes

A. 94% of 3,506 genes dysregulated in *Nipbl⁺/⁻* embryonic brains are partially or fully rescued by reducing *Wapl* gene function.

B. Similarly, 87% of 1,427 genes dysregulated in *Wapl⁺/⁻* embryonic brains are rescued by reducing *Nipbl* function.

present defects in muscle development and muscle regeneration. Specifically, muscle and muscle fibers are significantly smaller in mutant mice, and mutant mice cannot recover efficiently from muscle injury (Figure 7). The latter phenotype is exacerbated by age, indicating that *H19* mutant mice are a model for sarcopenia. Current research focuses on identifying the molecular bases for these phenotypes.

Reducing *Wapl* dosage partially corrects embryonic growth and brain transcriptome phenotypes in *Nipbl⁺/⁻* embryos.

Cohesin rings interact with DNA and modulate expression of thousands of genes. Cohesin is loaded onto chromosomes by NIPBL (a protein that is required for the association of cohesin with DNA) and unloaded by the cohesin-release factor WAPL. Haploinsufficiency for *NIPBL* leads to a developmental disorder, the Cornelia-de-Lange syndrome (CdLS), that is modeled in *Nipbl⁺/⁻* mice. Key phenotypes in this mouse model include slow embryonic growth, dysregulation of thousands of genes, and perinatal lethality. Also in *Drosophila*, reduced *Nipbl* function causes developmental defects. Importantly, reducing *Wapl* and *Nipbl* function together alleviates many of the developmental defects. To determine whether this is true in mammals, we generated mice with novel *Wapl* hypomorph alleles and then compared the phenotypes of *Wapl⁺/⁺ Nipbl⁺/⁺* (wild-type), *Wapl⁺/⁻ Nipbl⁺/⁺* (*Wapl* hets), *Wapl⁺/⁺ Nipbl⁺/⁻* (*Nipbl* hets), and *Wapl⁺/⁻ Nipbl⁺/⁻* (double hets, i.e., two heterozygous gene pairs) littermates. Disruption of *Wapl* and *Nipbl* each lead to reduced growth and dysregulation of a (mostly) overlapping set of genes. Interesting, these phenotypes are not exacerbated but instead are partially rescued in the double mutant (Figure 8). The results suggest that *Nipbl/Wapl* balance (i.e., loading/unloading balance) is the critical factor in regulating cohesin function, and also suggest novel therapeutic approaches for CdLS patients.

Publications

1. Kean CM, Tracy CJ, Mitra A, Rahat B, Van Winkle MT, Gebert CM, Noeker JA, Calof AL, Lander AD, Kassis JA, Pfeifer K. Decreasing Wapl dosage partially corrects embryonic growth and brain transcriptome phenotypes in Nipbl+/- embryos. *Sci Adv* 2022 8:eadd4136.
2. Park K-S, Rahat B, Lee HC, Noeker J, Mitra A, Kean CM, Knutsen RH, Springer D, Gebert CM, Kozel BA, Pfeifer K. Cardiac pathologies in mouse loss of imprinting models are due to misexpression of H19 long noncoding RNA. *eLife* 2021 10:e67250.
3. Flores DJ, Duong T, Brandenberger LO, Mitra A, Shirali A, Johnson JC, Springer D, Noguchi A, Yu ZX, Ebert SN, Ludwig A, Knollmann BC, Levin MD, Pfeifer K. Conditional ablation and conditional rescue models for Casq2 elucidate the role of development and of cell-type specific expression of Casq2 in the CPVT2 phenotype. *Hum Mol Genet* 2018 27:1533–1544.
4. Park K-S, Mitra A, Rahat B, Kim B, Pfeifer K. Loss of imprinting mutations define both distinct and overlapping roles for misexpression of IGF2 and of H19 lncRNA. *Nucleic Acids Res* 2017 45:12766–12779.
5. Mitra A, Park K-S, Pfeifer K. Circular RNAs and competing endogenous (ceRNA) networks. *Transl Cancer Res* 2018 7:S624–628.

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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 from 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 study Niemann-Pick disease type C (NPC), as well as Juvenile Batten disease caused by pathogenic variants of *CLN3* (CLN3 disease). 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 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, CLN3, and NPC1. In collaboration with NCATS (the National Center for Advancing Translational Sciences), our research group has been involved in a multicenter trial of creatine transporter deficiency.

Inborn errors of cholesterol synthesis
SMITH–LEMLI–OPITZ SYNDROME (SLOS)

SLOS is an autosomal recessive, multiple-malformation syndrome characterized by dysmorphic facial features, cognitive impairment, hypotonia, poor growth, and various structural anomalies of heart, lungs, brain, limbs, gastrointestinal tract, and genitalia. The SLOS phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die as result of multiple major

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(continued)

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 delta7-reductase gene (*DHCR7*) and demonstrated mutations of the gene in SLOS patients. Together with others, we have so far identified over 100 mutations in *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

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(Not shown: Shikha Salhotra)

FIGURE 1.

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

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.

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. We are also working to identify cerebrospinal-fluid biomarkers that may provide insight into neurological dysfunction.

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, Dana's Angels Research Trust, and Together Strong. We have enrolled over 135 individuals with NPC1 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

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 (glial fibrillary acidic protein) and IBA1 (ionized calcium binding adaptor molecule 1) are used to detect astrogliosis and microgliosis, respectively. Nuclei are stained with Hoechst 3342.

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 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 are studying the efficacy of cyclodextrin to ameliorate liver disease in infants with NPC. We are now collaborating with several groups in order to advance gene therapy.

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 sequences 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.
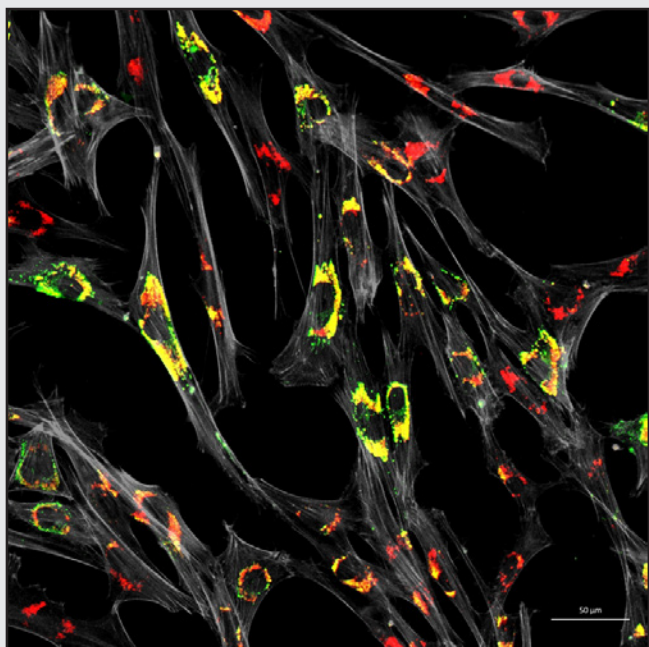
FIGURE 3. Accumulation of unesterified cholesterol in NPC1 patient fibroblasts

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*).

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 reduce 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 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 be efficiently 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 <u>s</u>olute <u>c</u>arrier <u>f</u>amily <u>6</u> Member <u>8</u>, 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
- SOAR NPC
- FireFly Fund
- NICHD Scientific Director's Award

Publications

1. Francis KR, Ton AN, Xin Y, O'Halloran PE, Wassif CA, Malik N, Williams IM, Cluzeau CV, Trivedi NS, Pavan WJ, Cho W, Westphal H, Porter FD. Modeling Smith-Lemli-Opitz syndrome with induced pluripotent stem cells reveals a causal role for Wnt/beta-catenin defects in neuronal cholesterol synthesis phenotypes. *Nat Med* 2016 22:388–396.
2. Ory DS, Ottinger EA, Farhat NY, King KA, Jiang X, Weissfeld L, Berry-Kravis E, Davidson CD, Bianconi S, Keener L, Rao R, Soldatos A, Sidhu R, Walters KA, Xu X, Thurm A, Solomon B, Pavan WJ, Machielse BN, Kao M, Silber SA, McKew JC, Brewer CC, Vite CH, Walkley SU, Austin CP, Porter FD. Intrathecal 2-hydroxypropyl-β-cyclodextrin decreases neurological disease progression in Niemann-Pick disease, type C1: an ad-hoc analysis of a non-randomized, open-label, phase 1/2 trial. *Lancet* 2017 390(10104):1758–1768.
3. Cougnoux A, Yerger JC, Fellmeth M, Serra-Vinardell J, Martin K, Navid F, Iben J, Wassif CA, Cawley NX, Porter FD. Single cell transcriptome analysis of Niemann-Pick disease, type C1 cerebella. *Int J Mol Sci* 2020 21(15):5368.
4. Solomon BI, Smith AC, Sinaii N, Farhat N, King MC, Machielse L, Porter FD. Association of miglustat with swallowing outcomes in Niemann-Pick disease, type C1. *JAMA Neurol* 2020 77(12):1564–1568.
5. Agrawal N, Farhat NY, Sinaii N, Do AD, Xiao C, Berry-Kravis E, Bianconi S, Masvekar R, Bielekova B, Solomon B, Porter FD. Neurofilament light chain in cerebrospinal fluid as a novel biomarker in evaluating both clinical severity and therapeutic response in Niemann-Pick disease type C1. *Genet Med* 2023 25:100349.

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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.

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**FIGURE 1. Representative image of the lab's research**

We combine imaging techniques in both fixed and living cells with sequencing-based genomic techniques that assess DNA–DNA interactions.

A. Hi-C and CTCF ChIP-Seq of GM1278 cells, which allow characterization of chromatin structure and identification of binding sites of an important architectural protein.

B. dCAS9 MCP-EGFP and PCP-CHERRY live imaging of the *Igh* and *Akap6* loci. The mouse embryo is an unparalleled system in mammalian biology for understanding how tissue-specific gene expression is achieved.

C. Whole-mount *in situ* hybridization for patterning markers in mid and late gastrulating embryos.

D. Tetraploid aggregation with GFP (green fluorescent protein)–labeled ES (embryonic stem) cells allows generation of fully ES cell–derived embryos.
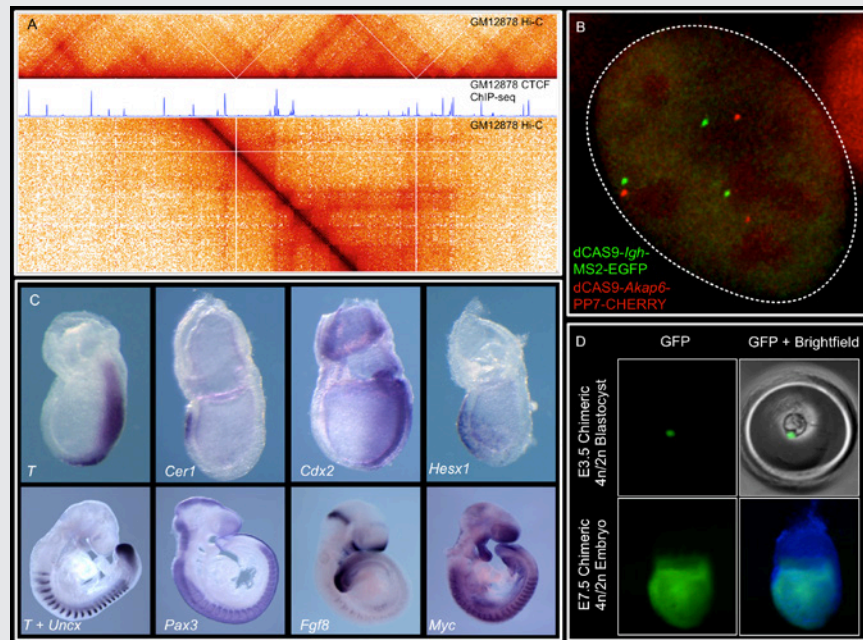
Following fertilization and within a few cell divisions, the first cell lineages are established and different gene-expression programs are put into action. In mammals, the result is the formation of the blastocyst, a structure that contains three different cell types, each with a defined differentiation 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 (clustered regularly interspaced short palindromic repeats) screens. Ultimately, we will fully characterize *in vivo* candidates identified this way in order to stringently determine their impact on gene regulation during mammalian development.

Publications

1. Chakraborty S, Kopitchinski N, Zuo Z, Eraso A, Awasthi P, Chari R, Mitra A, Tobias IC, Moorthy SD, Dale RK,

Mitchell JA, Petros TJ, Rocha PP. Enhancer-promoter interactions can bypass CTCF-mediated boundaries and contribute to phenotypic robustness. *Nat Genet* 2022 1–12.

2. Thompson JJ, Lee DJ, Mitra A, Frail S, Dale RK, Rocha PP. Extensive co-binding and rapid redistribution of NANOG and GATA6 during emergence of divergent lineages. *Nat Commun* 2022 13:4257.
3. Kurotaki D, Kikuchi K, Cui K, Kawase W, Saeki K, Fukumoto J, Nishiyama A, Nagamune K, Zhao K, Ozato K, Rocha PP, Tamura T. Chromatin structure undergoes global and local reorganization during murine dendritic cell development and activation. *Proc Natl Acad Sci USA* 2022 119:e2207009119.
4. Xie G, Lee J, Senft AD, Park Y, Chakraborty S, Thompson JJ, Liu,C, Macfarlan TS, Rocha PP, Peng W, Ge K. MLL3/MLL4 methyltransferase activities control early embryonic development and embryonic stem cell differentiation in a lineage-selective manner. *Nat Genet* 2023 55(4):693–705.
5. Rhodes CT, Thompson JJ, Mitra A, Asokumar D, Lee DR, Lee DJ, Zhang Y, Jason E, Dale RK, Rocha PP, Petros TJ. An epigenome atlas of neural progenitors within the embryonic mouse forebrain. *Nat Commun* 2022 13(1):4196.

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Decoding Developmental Signaling in Vertebrate Embryos

To create the different tissues required in healthy adults, embryos must activate fate-specifying genes in a variety of stereotyped patterns (Figure 1A), a process that is mediated by signaling molecules that spread though embryonic tissues. It is thought that signaling levels, dynamics, and combinations 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 'decoded' 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 use molecular optogenetics approaches that offer tunable, reversible experimental manipulations with excellent temporal (seconds) and spatial (subcellular) resolution. Using the microscopy-friendly zebrafish embryo as a vertebrate model system, our lab harnesses established optogenetic approaches and develops new ones to understand how cells decode signaling during embryogenesis.

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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 (transcytosis), or be confined to the producing cells themselves. We will develop optogenetic tools to probe how extracellular diffusion and transcytosis, among others, affect signaling-molecule distribution, and we will 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.
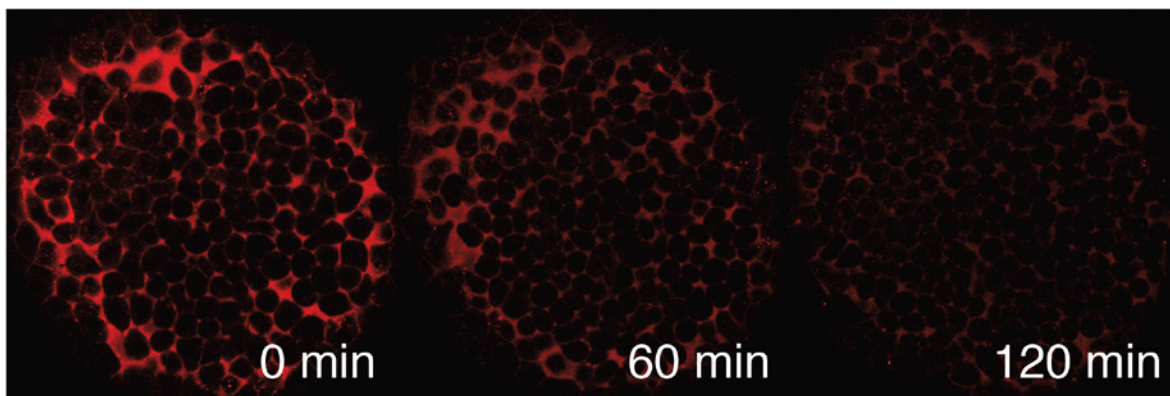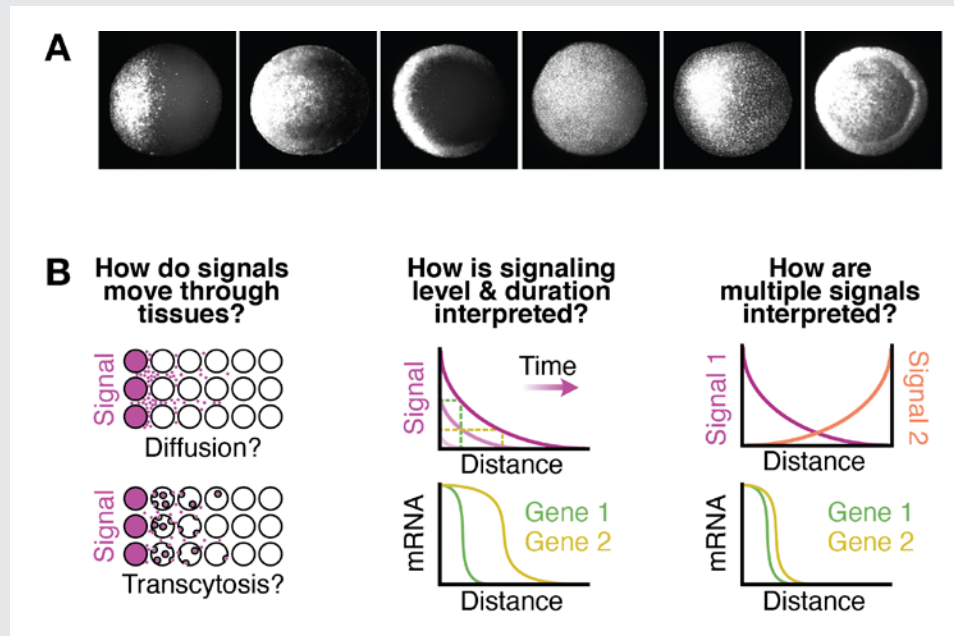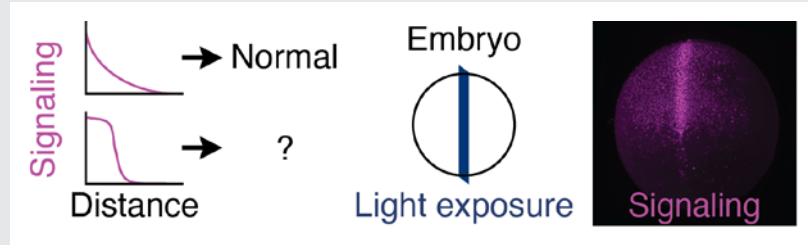
B. Outstanding developmental questions

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. We investigate how different genes respond to these inputs and we seek to determine the input/output relationship between signaling and gene expression during early vertebrate embryogenesis. To achieve this, we are developing 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 explain how the diverse gene expression patterns needed to produce healthy adults are robustly generated.

FIGURE 4.

Orthogonal opto-signaling experiment
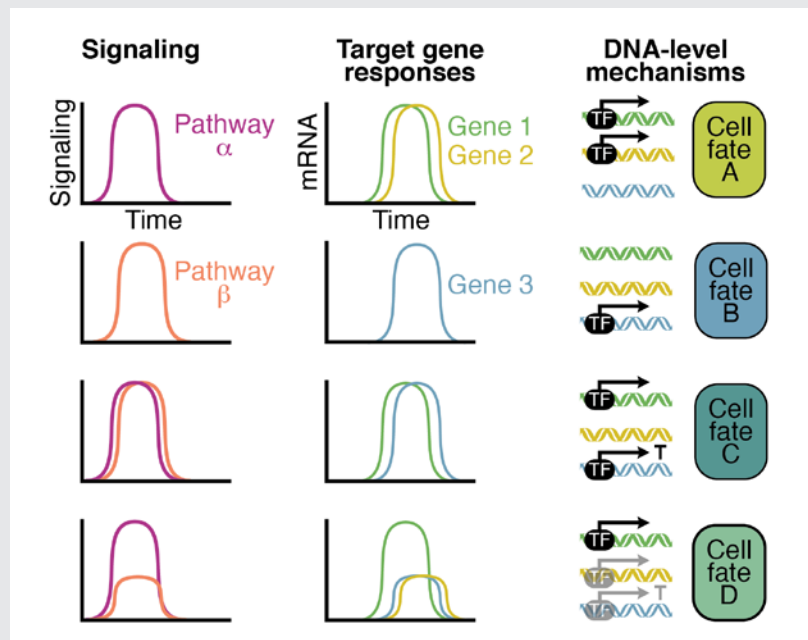
Publications

1. Saul AJ, Rogers CE, Garmendia-Cedillos M, Pohida T, Rogers KW. Optogenetic signaling activation in zebrafish embryos. *J Vis Exp* 2023 10.3791/65733.
2. Rogers KW, ElGamacy M, Jordan BM, Müller P. Optogenetic investigation of BMP target gene expression diversity. *eLife* 2020 e58641.
3. Rogers KW, Müller P. Optogenetic approaches to investigate spatiotemporal signaling during development. *In: Gradients and Tissue Patterning. Small S, Briscoe J, eds,* 2020 37–77.
4. Rogers KW, Lord ND, Gagnon JA, Pauli A, Zimmerman S, Aksel DC, Reyon D, Tsai SQ, Joung JK, Schier AF. Nodal patterning without Lefty inhibitory feedback is functional but fragile. *eLife* 2017 e28785.
5. Pomreinke AP, Soh GH, Rogers KW, Bergmann JK, Bläßle AJ, Müller P. Dynamics of BMP signaling and distribution during zebrafish dorsal-ventral patterning. *eLife* 2017 e25861.

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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, and two-thirds of all preterm births occur after the onset of preterm labor. The cost of prematurity in the United States alone was estimated at $26 billion per year in 2007. Therefore, important goals are to understand the mechanisms of disease responsible for preterm birth and fetal injury and to improve the prediction and prevention of preterm birth. The Pregnancy Research Branch proposed that preterm parturition is a syndrome caused by multiple pathologic processes [Romero R *et al*. *Science* 2014;345:760]. The Branch developed methods for the rapid diagnosis of intra-amniotic infection/inflammation and showed that such pathologic processes can be treated successfully. In addition, the current approach to predict and prevent spontaneous preterm birth in clinical obstetrics is based on the work of the Branch (the PREGNANT trial and subsequent meta-analyses). The research team continues to study the physiology of pregnancy and parturition to inform studies of spontaneous preterm labor, and, in particular, the use of high-dimensional, post-genomic tools, such as transcriptomics, proteomics, and the analysis of parturition at single-cell resolution.

Imaging is a powerful instrument for scientific discovery and has changed the practice of obstetrics and maternal-fetal medicine. The single most important step that has made fetal medicine a discipline is the transformation of the fetus from an invisible to a visible subject through the use of imaging techniques, in particular, ultrasound. The technology has allowed the definition of fetal anatomy, biometry, and growth as well as the study of physiologic parameters, e.g., cardiac function, fetal sleep, and breathing. We use different imaging modalities to examine the diagnosis of anomalies and obstetrical syndromes. Such modalities include ultrasound (2-dimensional, 3-dimensional), magnetic resonance imaging, and optical methods. We have also utilized other imaging techniques to study the human placenta.

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A new taxonomy of obstetrical disease based on clinical symptoms and the results of placental pathology

The current taxonomy of obstetrical disease refers to preeclampsia, preterm labor, preterm premature rupture of the membranes, small-for-gestational-age fetus, fetal death, etc. Each complication

of pregnancy is named based on symptoms and signs, but this does not provide etiological information. We propose that a new taxonomy of obstetrical syndromes, which incorporates information derived from placental pathology, would facilitate the discovery of biomarkers, which are tools to predict, diagnose, and monitor response to therapy. This study was conducted to examine whether sub-classification of obstetrical syndromes, according to the presence or absence of placental lesions, would improve the performance of biomarkers.

We reported the results of a retrospective case cohort study based on 4,006 pregnant women. The case cohort included 1,499 patients from the parent cohort. Pregnant patients were classified into a control group of patients who delivered at term without pregnancy complications (n=540) and a series of cases including patients with: (1) preterm labor with intact membranes (n=203); (2) preterm PROM (premature rupture of membranes) (n=112); (3) preeclampsia (n=230); and (4) small for gestational age (n=334). Serial samples of maternal plasma were assayed for placental growth factor (PlGF) and the soluble VEGF receptor Flt-1 (7,560 samples). Placentas were examined by pathologists masked to the clinical outcome to assess the presence or absence of lesions reflecting maternal vascular malperfusion. We compared the profile of PlGF and sFlt-1, and its ratio between controls and each obstetrical syndrome with and without subclassification of cases according to the presence or absence of maternal vascular lesions of malperfusion.

We found that: (1) when obstetrical syndromes are classified based on the presence or absence of placental lesions of maternal vascular malperfusion, significant differences in the mean plasma concentrations of PlGF, sFlt-1, and the PlGF/sFlt-1 ratio between cases and controls emerge earlier in gestation; (2) the strength of association between an abnormal PlGF/sFlt-1 ratio and the occurrence of obstetrical syndromes increases when placental lesions of maternal vascular malperfusion are present; and (3) the PlGF/sFlt-1 ratio at 28 to 32 weeks of gestation is abnormal in patients who subsequently delivered as a result of preterm labor with intact membranes and in those with preterm premature rupture of the membranes if both groups have placental lesions of maternal vascular malperfusion. The association is not significant in patients with these obstetrical syndromes who do not have placental lesions. We propose that a new taxonomy of obstetrical disorders informed by placental pathology will facilitate the discovery and implementation of biomarkers, as well as the prediction and prevention of such disorders.

Preeclampsia at term: the identification of two clusters based on angiogenic and anti–angiogenic markers with different clinical characteristics and pregnancy outcomes

Preeclampsia is a major cause of maternal and neonatal death. Most cases of preeclampsia occur at term. An anti-angiogenic state is a mechanism of disease in this syndrome, and maternal plasma concentrations of angiogenic and anti-angiogenic factors such as PlGF and sFlt-1, respectively, have been used for risk assessment (now approved by the FDA). The role of the PlGF/sFLT-1 ratio is mainly in early onset disease. Our study was conducted to determine the prevalence and clinical significance of abnormal angiogenic and anti-angiogenic factors in preeclampsia at term. We found that, while 90% of cases of early preeclampsia had an abnormal angiogenic profile, only 50% of women with preeclampsia at term had such abnormal profile. Patients with preeclampsia at term with an abnormal PlGF/sFLT-1 ratio were more likely to be nulliparous, had a higher rate of maternal and neonatal complications, and were more likely to have placental lesions of malperfusion than those without an abnormal profile. Patients with a normal angiogenic profile have a higher frequency of chronic hypertension and obesity than those with an abnormal profile. These observations have implications for the understanding of preeclampsia at term, prediction of late onset preeclampsia, and its clinical management.

The vaginal microbiota of pregnant women varies with gestational age, maternal age, and parity.

Intra-amniotic infection is a major cause of spontaneous premature labor and delivery, and is present in 25% of spontaneous preterm births. Such infections are largely subclinical in nature and thought to result from microorganisms ascending from the vagina. Changes in the microbial ecosystem or microbiota are believed to predispose to ascending infection. The study was undertaken to characterize the vaginal microbiota of pregnant women who delivered at term without complications, using sequence-based microbiologic techniques (16s). We performed a longitudinal study that included 474 women and 1,862 samples of vaginal fluid from a predominantly African American cohort. Each patient had 3–4 samples collected between 8–38 weeks of gestation. The general pattern was that the composition of the vaginal microbiota remains or transitions to a state of *Lactobacillus* dominance. We found that the vaginal microbiota changed as a function of gestational age, maternal age, and parity. Network analyses revealed dynamic associations among specific bacterial taxa within the vaginal ecosystem, which shift through the course of pregnancy. The study provides a robust foundational understanding of the vaginal microbiota in pregnancy and lays the groundwork for further investigation of the microbiota prior to spontaneous premature labor.

Fetal intelligent navigation is superior to manual navigation to examine the fetal heart with ultrasound.

Congenital heart disease (CHD) is the most common birth defect by organ system, and the leading cause of infant morbidity and mortality related to birth defects. In 2013, hospital costs exceeded $6 billion to care for children with CHD. All pregnancies should undergo prenatal sonographic screening for cardiac defects, given that up to 90% of cases occur in the absence of risk factors. Moreover, there is evidence that prenatal diagnosis of specific cardiac anomalies improves the survival after surgery, and long-term neurocognitive function and outcome. The overall prenatal detection rate for congenital heart disease with ultrasound remains suboptimal. To address these issues, the PRB successfully developed a novel method known as Fetal Intelligent Navigation Echocardiography (FINE) to interrogate fetal sonographic cardiac volume datasets. The method allows the automatic display of nine standard fetal cardiac views required to diagnose most cardiac defects. The FINE method simplifies examination of the fetal heart and reduces operator dependency. Virtually all ultrasound examinations use manual navigation to examine the fetal heart. We conducted a study to compare the performance of manual and intelligent navigation (FINE) of the fetal heart by non-expert sonologists. This prospective observational study included ten sonologists who underwent formal training on both navigational methods. Subsequently, we tested the ability of participants to obtain nine cardiac views from five STIC (spatiotemporal image correlation) volumes of normal fetal hearts (19–28 gestational weeks) using such methods. The following parameters were determined for both methods: (1) success rate of obtaining nine cardiac views; (2) mean time to obtain nine cardiac views per sonologist; and (3) maximum number of cardiac views successfully obtained for each STIC volume.

All fetal cardiac images were obtained from 100 STIC volumes (50 for each navigational method) and reviewed by an expert in fetal echocardiography. Compared with manual navigation, FINE had a significantly: (1) higher success rate for obtaining eight (excluding the abdomen view) appropriate cardiac views (92–100% vs. 56–88%); (2) shorter mean time (minute:seconds) to obtain nine cardiac views (2:11 ± 0:37 vs. 15:49 ± 7:44); and (3) higher success rate for obtaining all nine cardiac views for a given STIC volume (86% vs. 14%).

We conclude that when performed by non-expert sonologists, intelligent navigation (FINE) had a superior

performance than manual navigation of the fetal heart. Specifically, FINE obtained appropriate fetal cardiac views in 92–100% of cases.

Syndecan−1, a biomarker for fetal growth restriction

The identification of fetal growth disorders is an important priority in obstetrics, given that they increase the risk of perinatal morbidity and mortality, as well as adult disease. A subset of small-for-gestational age (SGA) infants are growth-restricted, which is often attributed to placental insufficiency. Syndecan-1, a product of the degradation of the endothelial glycocalyx, has been proposed as a biomarker of endothelial damage in various pathological conditions. During pregnancy, there is a "specialized" form of the glycocalyx, the "syncytiotrophoblast glycocalyx," which covers the placental villi.

We conducted a study to determine whether maternal plasma syndecan-1 concentrations can be used as a biomarker for fetal growth restriction. A cross-sectional study was performed to include 130 women with normal pregnancy and 50 pregnant women who delivered SGA neonates. Doppler velocimetry of the uterine artery and umbilical artery was performed in women with SGA fetuses at the time of diagnosis. Venipuncture was performed within 48 hours of Doppler velocimetry and plasma concentrations of syndecan-1 were determined by a specific and sensitive immunoassay.

The results showed that the mothers with pregnancies complicated with an SGA fetus had a significantly lower mean plasma concentration of syndecan-1 than those with an appropriate for gestational age fetus. This difference was attributed to fetal growth restriction, as the mean plasma syndecan-1 concentration was significantly lower only in the group of women with SGA fetuses with an abnormal umbilical- and uterine artery–Doppler velocimetry compared with controls. Among women with SGA fetuses, those with abnormal umbilical- and uterine artery–Doppler findings had a significantly lower mean plasma syndecan-1 concentration. A plasma syndecan-1 concentration of less than 850 ng/mL had a positive likelihood ratio of 4.4 and a negative likelihood ratio of 0.24 for the identification of a mother with an SGA fetus with abnormal umbilical artery–Doppler velocimetry. The results of the study suggest that plasma syndecan-1 could be used as a biomarker to identify fetal growth restriction.

Publications

1. Motomura K, Romero R, Plazyo O, Garcia-Flores V, Gershater M, Galaz J, Miller D, Gomez-Lopez N. The alarmin S100A12 causes sterile inflammation of the human chorioamniotic membranes as well as preterm birth and neonatal mortality in mice. *Biol Reprod* 2021 1494–1509.
2. Garcia-Flores V, Romero R, Xu Y, Theis KR, Arenas-Hernandez M, Miller D, Peyvandipour A, Bhatti G, Galaz J, Gershater M, Levenson D, Pusod E, Tao L, Kracht D, Florova V, Leng Y, Motomura K, Para R, Faucett M, Hsu CD, Zhang G, Tarca AL, Pique-Regi R, Gomez-Lopez N. Maternal-fetal immune responses in pregnant women infected with SARS-CoV-2. *Nat Commun* 2022 1–20.
3. Pique-Regi R, Romero R, Garcia-Flores V, Peyvandipour A, Tarca AL, Pusod E, Galaz J, Miller D, Bhatti G, Para R, Kanninen T, Hadaya O, Paredes C, Motomura K, Johnson JR, Jung E, Hsu CD, Berry SM, Gomez-Lopez N. A single-cell atlas of the myometrium in human parturition. *JCI Insight* 2022 1–21.
4. Oh KJ, Romero R, Kim HJ, Jung E, Gotsch F, Suksai M, Yoon BH. A role for intraamniotic inflammation in threatened midtrimester miscarriage. *Am J Obstet Gynecol* 2022 1.e1–1.e13.
5. Romero R, Jung E, Chaiworapongsa T, Erez O, Gudicha DW, Kim YM, Kim JS, Kim B, Kusanovic JP, Gotsch F,

Taran AB, Yoon BH, Hassan SS, Hsu CD, Chaemsaithong P, Gomez-Lopez N, Yeo L, Kim CJ, Tarca AL. Toward a new taxonomy of obstetrical disease: improved performance of maternal blood biomarkers for the great obstetrical syndromes when classified according to placental pathology. *Am J Obstet Gynecol* 2022 615.e1–615.e25.

6. Purandare N, Kunji Y, Xi Y, Romero R, Gomez-Lopez N, Fribley A, Grossman LI, Aras S. Lipopolysaccharide induces placental mitochondrial dysfunction in murine and human systems by reducing MNRR1 levels via a TLR4-independent pathway. *iScience* 2022 25:105342.

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Regulation of Mammalian 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 cluster cofactors. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) regulate the expression of numerous proteins involved in iron metabolism. In iron-depleted cells, IRP 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 a cytosolic aconitase in iron-replete cells. IRP2 is homologous to IRP1 but undergoes iron-dependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration, phenotypes that are all likely caused by functional iron deficiency. Using this mouse model of neurodegeneration, we found that the antioxidant Tempol works by activating the latent IRE–binding activity of IRP1, which partially restores normal iron homeostasis. 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 described and reported a case series of three unrelated patients who develop neonatal and progressive neurodevelopmental delays attributable to complete loss of IRP2 function ($IREB2^{-/-}$), to microdeletions, or to missense mutations.

Our ongoing work on iron-sulfur cluster biogenesis has led to new insights into how mammalian iron-sulfur clusters are synthesized and transferred to specific recipient proteins [Maio N, Rouault TA. *Trends Biochem Sci* 2020;45:411-426]. Several human diseases are now known to be caused by deficiencies in the iron-sulfur cluster biogenesis machinery. We developed an anti-sense 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 proposed an algorithm that facilitates discovery of previously unrecognized mammalian iron-sulfur proteins, work that led us to suggest that there are hundreds of previously unrecognized mammalian iron-sulfur proteins. Identification of iron-sulfur cofactors will lead to breakthroughs in several research areas

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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, which 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. We are working to develop an antiviral therapy that will be effective against many coronaviral infections by inactivating viral replication.

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 neurodegeneration; analyses of gene expression and iron status at various stages of disease are ongoing. Dietary supplementation with Tempol mitigates neurodegeneration; the treatment appears to work by recruiting the IRE–binding activity of IRP1. We found that motor neurons were the most adversely affected neurons in *Irp2*⁻/⁻ mice and that neuronal degeneration accounted for the gait abnormalities. In collaboration, we identified two *IRP2*⁻/⁻ patients who suffered from severe neurodegenerative disease in infancy and died before adolescence or were bed-ridden. A third infant with choreoathetosis established IRP2 deficiency as a cause of infantile and childhood neurodevelopmental disease.

We discovered that a transcript of the iron exporter ferroportin that lacks the IRE at its 5' end is important in intestinal iron uptake. It allows ferroportin to permit iron to cross the duodenal mucosa in iron-deficient animals and also to prevent 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 (HIF2α) translation in the renal interstitium through the IRE–IRP system. We confirmed that overexpression of HIF2α 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 HIF2α 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 HIF2α 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. We are evaluating results from a large experiment on transcript expression in macrophages after red cell phagocytosis.

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, and 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 the 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 a 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. Also, we have discovered more rare diseases that result from mutations in the mammalian biogenesis machinery, some of which predominantly affect cytosolic iron sulfur biogenesis.

We identified a tripeptide motif, LYR, in many apoproteins that are recipients of nascent iron-sulfur clusters. The co-chaperone HSC20 (also known as HSCB) binds to HSPA9, its partner HSP70–type chaperone, and the chaperone complex binds to ISCU bearing a nascent iron-sulfur cluster that is delivered 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, to 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. 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 clusters to many recipient proteins in the cell.

Using informatics, we identified several potential iron sulfur proteins encoded by SARS-CoV-2. We demonstrated 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. The helicase encoded by the SARS-CoV-2 mRNA contains a cubane iron sulfur cofactor in a domain known as the zinc-binding domain, which also ligates two zinc atoms. 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. Other coronaviruses also require iron sulfur cofactors for function, including the original SARs and MERs, and likely, three causes of the common cold. We are actively pursuing studies and treatments for coronaviral disease based on these insights. We are studying a related coronavirus, OC43, which causes the common cold and can be studied safely. Our discovery that multiple iron sulfur cofactors are present in the SARS-CoV-2 multimeric replication complex implies that viral exploitation of host iron sulfur biogenesis machinery and consumption of reducing equivalents is an important mechanism for viruses to tap into the energy stored in the cytosol of mammalian cells.

Additional Funding

Publications

1. Maio N, Lafont BAP, Sil D, Li Y, Bollinger JM, Krebs C, Pierson TC, Linehan WM, Rouault TA. Fe-S cofactors in the SARS-CoV-2 RNA-dependent RNA polymerase are potential antiviral targets. *Science* 2021 373:236–241.
2. Maio N, Cherry S, Schultz DC, Hurst BL, Linehan WM, Rouault TA. TEMPOL inhibits SARS-CoV-2 replication and development of lung disease in the Syrian hamster model. *iScience* 2022 25:105074.
3. Maio N, Saneto RP, Steet R, Sotero de Menezes MA, Skinner C, Rouault TA. Disruption of cellular iron homeostasis by IREB2 missense variants causes severe neurodevelopmental delay, dystonia and seizures. *Brain Commun* 2022 23:1–14.
4. Porras CA, Rouault TA. Iron homeostasis in the CNS: an overview of the pathological consequences of iron metabolism disruption. *Int J Mol Sci* 2022 23:4490.
5. Maio N, Raza MK, Li Y, Zhang DL, Bollinger Jr.J.M, Krebs C, Rouault TA. An iron-sulfur cluster in the zinc-binding domain of the SARS-CoV-2 helicase modulates its RNA-binding and -unwinding activities. *Proc Natl Acad Sci USA* 2023 120:e2303860120.

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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 is designed to mediate rapid and efficient transmission of signals across the synaptic cleft. Crucial to this function is the ability of a synapse to change its properties, so that it can optimize its activity and adapt to the status of the cells engaged in communication and/or to the larger network comprising them. Consequently, synapse development is a highly orchestrated process coordinated by intercellular communication between the pre- and postsynaptic compartments and by neuronal activity itself. Our long-term goal is to elucidate the molecular mechanisms that regulate assembly of functional synapses during development and their fine-tuning during maturation, plasticity, and homeostasis. We use a comprehensive set of approaches that include genetics, biochemistry, molecular biology, cell biology super-resolution imaging, and electrophysiology recordings in live animals and in reconstituted systems. In recent studies, we also utilized single-cell RNA sequencing (scRNA-seq) methodologies to describe various populations of neurons.

Because of its many advantages, we use the *Drosophila* 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 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. The basic discoveries of our laboratory

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makes in the fly will serve our overarching goal of understanding how chemical synapses are assembled and sculpted during development and homeostasis.

Cellular diversity in the *Drosophila* third instar larval ventral cord revealed by single-cell transcriptomics

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 postsynaptic glutamate receptors. 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.

The nervous system has a bewildering array of cells with massive diversity. The diversity of cell identity translates into functional diversity, with different types of cells working in concert to perform different tasks. We wish to understand how this diversity develops and how is it harnessed for function. A highly choreographed division of labor ensures that the nervous system functions properly. However, the number of different cell types involved and how we define these cells and their contributions are still open questions.

The fruit fly nervous system is a powerful model system for finding order in this complexity. Much has been learned by studying subsets of neurons and/or circuits one by one. Defining neuronal diversity feature by feature has been informative but has yielded limited information. A wealth of specific information has been collected, but the general themes are hard to discern, and it is impossible to tell what is missing. Single-cell RNA-seq approaches promise to fill this gap. The *Drosophila* larval ventral nerve cord (VNC) is the ideal system in which to conduct such work, because it is complex enough to sustain coordinated locomotor behaviors but also small enough to enable thorough analyses. Following a first wave of neurogenesis, the fly embryos hatch into the first instar larval stage with a central nervous system (CNS) consisting of two brain lobes and a VNC, with an estimated 10,000 cells. The reactivation of neuroblasts during the larval stage allows the VNC to expand to over 100k cells as the larva progresses to adult stages. The crawling larva and the flying adult have different food preferences, different predators, and use dramatically different sensory modalities and locomotor circuits to perform their specific behaviors. Our questions are what the relationship is, if any, between the nervous system of the larva and adult and how many of the cells born during larval stages are added to augment larval function and how many are made to serve dedicated, non-larval functions in the adult animal. Do larval and adult neurons have common features and if yes, how do they relate to their specific functions? Is the nature of glia diversity similar or different from neurons? Which features are idiosyncratic to a given cell and which ones are indicative of general principles of circuit development and function? In the past years we addressed some of these questions by scRNA-seq analyses of the larval VNC. During this process, we adapted and developed new protocols for dissociating single cells from fly larvae, then assembled a custom multistage analysis pipeline that integrates modules contained in different R packages to ensure flexible, high-quality RNA-seq data analysis. This work was conducted with support from the NICHD Genomics Core and in collaboration with Steve Coon, Fabio Faucz, and James Iben.

In brief, we dissected third instar larvae VNCs, dissociated the cells, and sequenced over 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. We found that only 40% of the larval VNC cells (13,120) are mature and could fulfill larval functions. The other 60% cells (17,920) are dedicated to setting
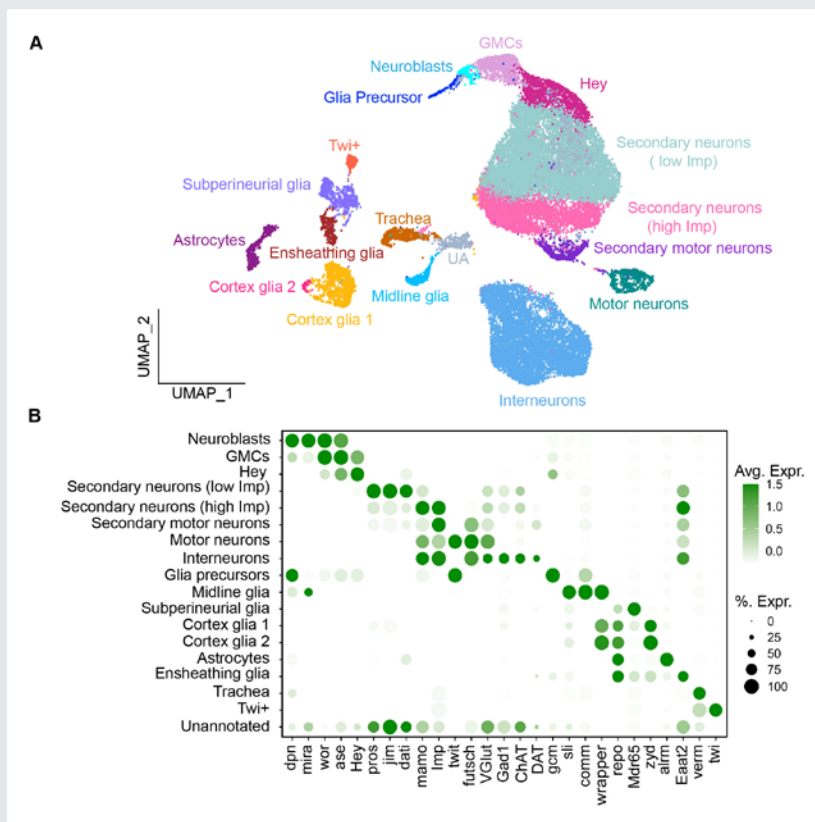
FIGURE 1. Major cell types in third instar larval VNC

A. Two-dimensional representation (UMAP; uniform manifold approximation and projection) of 31,040 cells from five different control sets. Each dot represents a single cell. Each cell type was assigned based on expression of known markers and illustrated by unique color.

B. Dot plot of marker genes for each major cell type. The dot size represents the percent of cells that express each gene of interest (GOI) within the cluster. The color intensity reflects average expression level.

up the VNC cell populations needed later, during adult stages. These immature cells include neural progenitors called neuroblasts (NBs), and their progenies that will become the future adult neurons. Using defining genetic markers, we captured a developmental trajectory that included neuroblasts (marked by the presence of *dpn, mira, wor*, and *ase*), their immediate progeny ganglion mother cells (GMCs) (*wor, ase*), newborn neurons (*Hey*), and immature secondary neurons at two distinct developmental stages, first with lower levels of *Imp* (*jim, dati, pros*, and low *Imp*) and second with high levels of *Imp* expression (*mamo* and high *Imp*) (Figure 1).

Two clusters, identified by specific motor neuron (MN) markers (*futsch, VGlut, lbm, Proc, rhea*), illustrate the essence of the larval VNCs divide: one population (primary larval MNs) is metabolically active and expresses all the components of active, functional synapses while a second population (secondary MNs) is metabolically and functionally silent. Upon finding their target muscles, primary MNs receive retrograde, muscle-derived BMP (bone morphogenetic protein) signals, which modulate transcription and coordinate synapse development. Indeed, primary MNs express BMP target genes such as *twit* (*target of wit*); in contrast, immature secondary MNs show no *twit* expression, suggesting they lack retrograde BMP signaling at this stage. A large cluster of putative interneurons contains heterogenous neurons that are metabolically and functionally active during third instar larvae and express distinct neurotransmitter markers (*VGlut, ChAT, Gad1*, and *DAT*). We also identified many glia subtypes, including a glial precursor (marked by *gcm*), midline glia (*sli, comm, wrapper*), subperineurial glia (*Mdr65*), cortex glia (*wrapper, zyd*), astrocytes (*alrm*), and ensheathing glia (*Eaat2*). Besides glia and neurons, we also identified a cluster of trachea cells, characterized by the expression of *verm*.
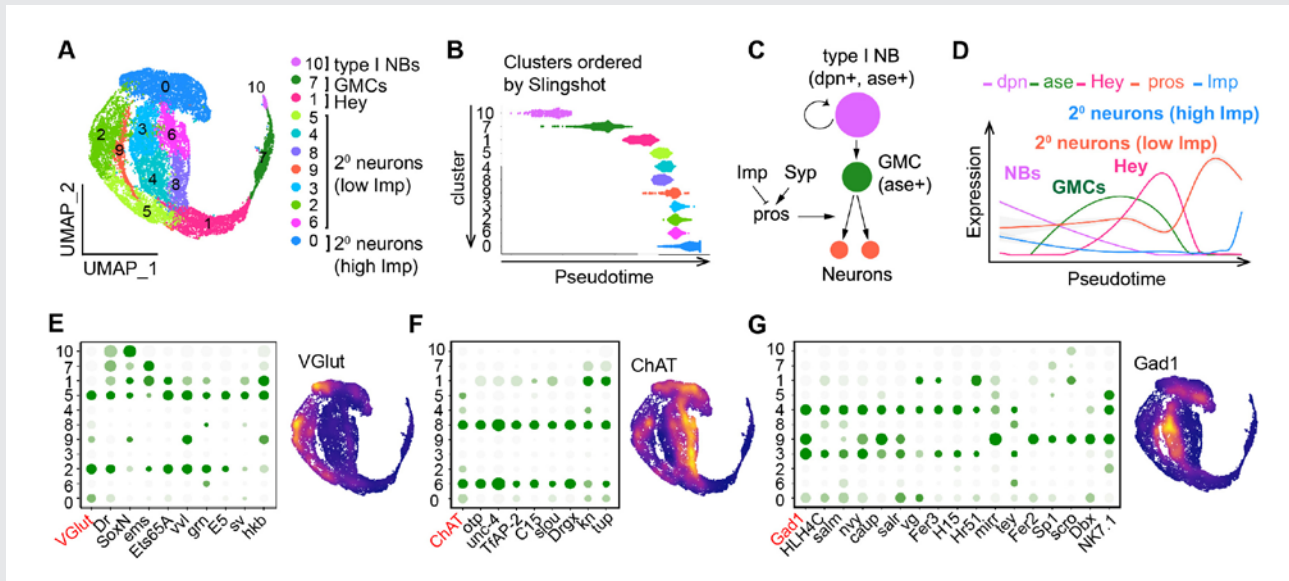
FIGURE 2. A developmental progression from neuroblasts to immature secondary neurons

A. UMAP plot of developmental trajectory clusters reveals five major developmental states.

B. Trajectory clusters ordered along a pseudotime calculated by Slingshot;

C. Classic diagram of developmental progression from type I neuroblasts to newborn neurons;

D. Pseudo-temporal expression of established markers for the five major stages;

E–G. Dot and density plots illustrating that the larval secondary neurons are already committed to different cell fates, including neurotransmitter identities.

These initial assessments indicate the richness of our atlas and its capacity to distinguish among different cell types, cell states, and functional characteristics within the larval VNC.

A developmental trajectory of secondary neurons

The high proportion of immature secondary neurons (60% of the total) in the third instar larval VNC and their apparent organization into a developmental trajectory prompted us to examine whether some of these neurons are already specified and have acquired their cell identities. We re-clustered these cells, applied different pseudotime algorithms, including slingshot, diffusion map, and monocle 2, and found that all algorithms placed these cell clusters into a pseudotime trajectory with excellent correlation scores (Figure 2). We found that peak expression for five previously described cell identifiers (*dpn, ase, Hey, pros, Imp*), which follow the progression from type I NBs to secondary neurons, aligned perfectly within this developmental trajectory. In our dataset, each of the cell populations along the trajectory are extremely well defined; this provides new insights in the respective cell functions.

By third instar larval stage, secondary neurons have already acquired cell-fate determinants and have committed to distinct neurotransmitter identities (Figure 2). We found that immature neuron clusters can be

FIGURE 3. Most adult neurons acquire cell-fate identity during larval stages.

A. tSNE plot showing reference hemilineages from the published adult VNC RNA-seq dataset; each hemilineage was annotated based on already known markers.

B. tSNE plot of re-clustered larval secondary neurons (without NBs, GMCs, or Hey populations);

C. tSNE plot indicating predicted larval VNC hemilineages, using the adult dataset as reference;

D. Correlogram comparing adult reference hemilineages and larval secondary neuron sub-clusters (as shown in *B*).

grouped unambiguously based on (1) distinct neurotransmitter markers such as *vGlut* (vesicular glutamate transporter), *Chat* (choline acetyltransferase) or *Gad1* (glutamic acid decarboxylase, an enzyme involved in GABA synthesis) and (2) different combinations of transcription factors. For example, glutamatergic neurons in clusters 5 and 2 are enriched in *Ets65A, HLH3B, aop*, and *E5*; GABAergic neurons in clusters 3, 4, and 9 express *HLH4C*, *sp1*, *exex*, *salr*, and *scro*; finally, cholinergic neurons in clusters 8 and 4 are enriched in *slou, unc-4, acj6*, and *kn*. These observations indicate that the secondary neurons within the developmental trajectory are already committed to different cell identities.

Larval and adult postembryonic neurons share common features

To examine what proportion of these cell identities is maintained in the adult stages and whether the development of the adult VNC involves profound changes that alter the identities of these already committed cells, we compared the transcriptional profiles of secondary neurons from larval VNC with the adult VNC neurons, as derived from the published dataset. First, we used unsupervised clustering algorithms to classify the cells in adult VNC and manually annotated these cells into known hemilineages based on their cell identities (Figure 3). To capture more diversity within the larval secondary neurons, we sub-clustered these populations using more principal components (PCs) (45 total). Using the adult hemilineages as a reference dataset, we then combined two machine-learning algorithms (support vector machine and random forest) included in the ELeFHAnt R package, to uncover any similarity between postembryonic neurons in larval and adult VNCs. Remarkably, these algorithms predicted that transcriptional profiles of 20 out of 21 adult hemilineages have high similarities with discrete subclusters of secondary larval neurons (predicted larval

hemilineages). The remaining hemilineage, lin15B, was defined as adult MNs and corresponds to a well defined larval cluster outside the trajectory cells, called here secondary MNs.

To confirm the identity between predicted larval hemilineages and reference adult hemilineages, we examined their differential gene expression. We found that expression of marker genes in each hemilineage is strikingly similar between adult and larval stages, starting with combinatorial arrays of transcription factors, which are the cell-fate determinants for individual hemilineages. The larval and adult clusters are also perfectly matched in their neurotransmitter identities and even in the signaling networks they engage. We searched for additional hemilineages within the adult VNC dataset and uncovered at least three new lineages with highly similar gene expression. Furthermore, when cells within each hemilineage were highlighted within UMAP (uniform manifold approximation and projection) plots, we noted that, with maybe one exception (lin13B), all hemilineages follow the developmental trajectory described above and include newborn and progressively differentiating neurons. Such distributions are consistent with larval hemilineages being actively engaged in a second wave of neurogenesis.

In contrast to adult lineages, larval secondary neurons have low levels of glycolysis-related transcripts, indicative of metabolically low or inactive neurons. Critical synaptic components are not expressed in larval secondary neurons, indicating that these neurons are immature, with no functional synapses. These include *Rbp*, encoding a presynaptic scaffold essential for neurotransmitter release, *Sif*, encoding a Rho-family GTPase involved in the reorganization of the actin cytoskeleton within the axonal terminals during synaptogenesis, *Para*, encoding an essential alpha subunit of the voltage-gated sodium channels, and *Comt*, encoding an ATPase required for the maintenance of neurotransmitter release. In the absence of any of these essential transcripts, there is no synaptic activity, either because synapses do not form properly or there is no action potential or sustained neurotransmitter release. Therefore, during larval stages, many adult neurons are born, acquire their cell identities, and differentiate, but halt their maturation until later developmental stages.

Most of these larval secondary neurons will mature as adult interneurons. In addition, the larval VNC employs a large population of mature, primary interneurons (born during the first wave of neurogenesis) that coordinate larval functions. We subclustered these primary interneurons into 48 clusters and found that each cluster was defined by unique combinations of transcription factors. Interestingly, the primary interneurons have very limited similarities with the secondary interneurons (born during the second wave of neurogenesis), which will function during adult stages. We reason that the limited similarities observed may primarily reflect differences between immature and actively connected neurons.

Our larval atlas offers unique insights into neurogenesis and into the strategies and signaling networks utilized for generation of the adult VNC. It also provides a high-resolution characterization of the larval VNC, capturing primary neurons, glia, and the functional landscape that coordinates larval behavior.

Versatile nanobody–based approach to image, track, and reconstitute functional synaptic proteins *in vivo*

Synaptic proteins, in particular the transmembrane proteins, are notoriously difficult to track and study because of their low abundance and high density of functional domains. Often, studies using transgenic lines that express selective tagged isoforms/variants cannot generate definitive conclusions because of heterologous promoters and overexpression artifacts. To facilitate structure-function studies and accomplish

reliable detection of low-abundant synaptic proteins in different tissues, we turned to a recently described cell-biology tool, the ALFA system. The system consists of a synthetically designed epitope tag of only 14 amino acids, the ALFA tag (AT), with no homology in the animal kingdom, and a nanobody (NbALFA) that binds to ALFA–tagged proteins with picomolar affinities. The high affinity of ALFA tag/NbALFA binding and the intrabodies capabilities of NbALFA (that is binding ALFA–tagged proteins when expressed in living cells) have prompted the development of a variety of *in vitro* cell-biology applications, from super-resolution to live detection of tagged proteins.

To probe whether this methodology is suitable for *in vivo* application, we chose a case-study protein, *Drosophila* Neurexin-1 (Nrx-1). Neurexins are key adhesion proteins that coordinate extracellular and intracellular synaptic assembles. Neurexins are also very difficult to tag and study because of their low abundance, multiple extracellular functional modules, and intracellular protein-protein binding domains and docking motifs. The proteins are crucial for synapse assembly and function; however, the role of some of their domains (for example the C-terminal PDZ–binding motifs; PDZ domains are primarily involved in anchoring receptor proteins to the cytoskeleton) has been only inferred from *in vitro* studies. Guided by phylogenetic analysis and secondary structure prediction, we generated ALFA–tagged Nrx-1 variants, including an endogenously tagged *Nrx-1*^{AT} allele, which is indistinguishable from the wild-type control, and a *Nrx*$^{dPDZ-AT}$ allele that resembles the *Nrx-1*^{null} mutant. Using a combination of classic genetics and cell biology and electrophysiology approaches, we found that *Nrx-1*^{AT} NMJs have normal morphology and function, whereas the *Nrx*$^{dPDZ-AT}$ mutants have smaller NMJs with much reduced basal neurotransmission, reminiscent of *Nrx-1*^{null} mutant. Similar to untagged Nrx-1, endogenously edited Nrx-1^{AT} localizes at presynaptic sites; remarkably, the ALFA system enabled detection of endogenous Nrx-1^{AT} in only one immunohistochemistry step using the monovalent binder NbALFA conjugated to two fluorophores (FluoTag-X2 anti-ALFA). These data confirm the expectation that the ALFA system ensures high-affinity binding, linear (monovalent) signals with respect to target molecule, with no amplification by polyclonal secondaries, and virtually no background in animal tissues.

To test whether the ALFA system could facilitate compartment-specific detection of tagged proteins *in vivo*, we built genetically encoded NbALFA-mScarlet cytosolic chimera (*UAS-Nb-mScarlet* transgenes) and examined their subcellular distribution relative to ALFA–tagged and untagged Nrx-1. In the presence of untagged Nrx-1, Nb-mScarlet accumulated in the neuron soma; in the presence of Nrx-1-AT, Nb-mScarlet became restricted to near the neuronal membranes. This result indicates that the AT/NbALFA binding re-directs the cytoplasmic Nb-mScarlet to the synaptic terminals, where Nrx-1-AT resides. These multi-component complexes could also be followed *in vivo*, en route to the synaptic terminals: we detected mobile mScarlet–labeled vesicles in animals expressing *UAS-Nrx-1-AT* but not in untagged Nrx-1 controls. The estimated velocities of vesicles were 0.24± 0.03 mm/s (n=27) away from the ventral cord and 0.20± 0.10 mm/s (n=35) towards the ventral cord. Recent studies reported that Rab2–marked vesicles, originating from the trans-Golgi network, enable the trafficking of presynaptic components at the fly NMJ. Upon labeling the transport vesicles observed for Rab2, we found that *Drosophila* Nrx-1 co-migrates with Rab2–positive vesicles to synaptic terminals.

In addition, we found that the PDZ–binding motif is key to Nrx-1 *in vivo* surface expression and synaptic localization: the *Nrx*$^{dPDZ-AT}$ variant was trapped in the ER, unable to traffic to the cell surface (Figure 4). This explains why the *Nrx*$^{dPDZ-AT}$ allele had NMJ defects similar to the *Nrx-1*^{null} mutant. Given that the ALFA system is very compact and has high binding affinity, both inside and outside the cells, we next asked whether this system could deliver the missing PDZ–binding motif in *trans*, facilitating the reconstitution of functional
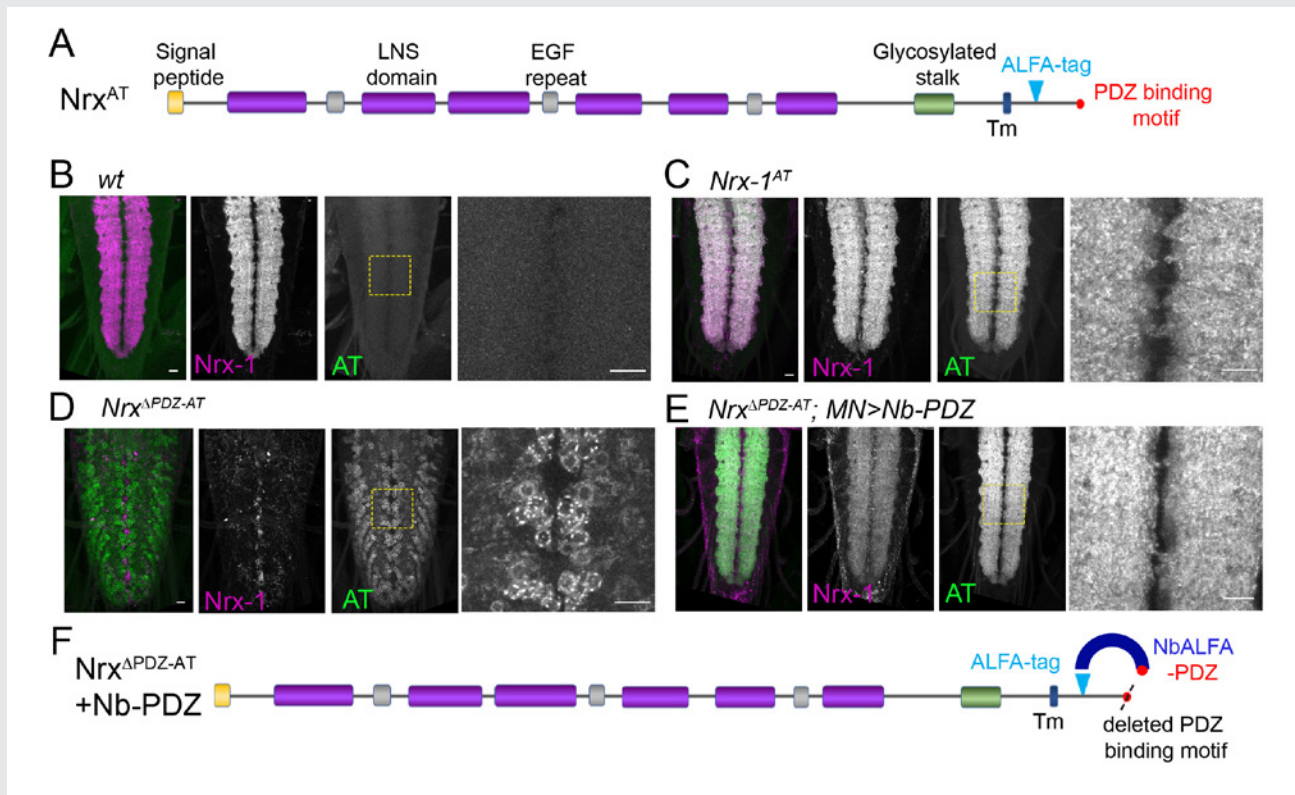
FIGURE 4. ALFA system as a versatile split-system for structure-function analyses of proteins *in vivo*

A. Domain organization of the Drosophila Nrx-1 with the ALFA-tag insertion site marked.

B–E. Confocal images of larval VNCs of the indicated genotypes labeled for ALFA tag (*green*) and Nrx-1 (*magenta*). The images were acquired with the same confocal microscopy settings and scaled equally for direct visual comparison. The edited Nrx-1^{AT} shows the expected Nrx-1 distribution (*C*).

The absence of a PDZ–binding motif (domains that are primarily involved in anchoring receptor proteins to the cytoskeleton) disrupts the subcellular distribution of Nrx$^{dPDZ-AT}$ (*D*). Addition of the PDZ–binding motif in *trans* restores the cell-surface location and normal synaptic distribution of reconstituted Nrx-1 (Nrx$^{dPDZ-AT}$/Nb-PDZ) (*E*).

F. Diagram for the in *trans* reconstitution of Nrx-1 (Nrx$^{dPDZ-AT}$ + Nb-PDZ). The missing PDZ–binding motif of Nrx$^{dPDZ-AT}$ is delivered in *trans* via genetically encoded NbALFA-PDZ–binding motif chimera (Nb-PDZ).

Nrx-1. To this end, we generated genetically encoded NbALFA-PDZ–binding motif chimera (*UAS-Nb-PDZ*) and expressed it in the *Nrx*$^{dPDZ-AT}$ neurons. The resulting animals were viable and fertile and had normal NMJ morphology and function, indicating that a PDZ–binding motif provided *in trans* fully restored the synaptic localization and function of Nrx$^{dPDZ-AT}$.

The ability to use the ALFA system as a split system to reconstitute and track functional proteins *in vivo* opens up a new realm of possibilities for functional studies in specific cells/tissues and during defined developmental windows. We anticipate that the methodology will pave the way towards dissecting functional domains of complex proteins *in vivo*.

Publications

1. Vicidomini R, Serpe M. Local BMP signaling: a sensor for synaptic activity that balances synapse growth and function. *Curr Topics Dev Biol* 2022 50:211–246.
2. 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.
3. 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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Cellular and Molecular Mechanisms of Lymphatic Disorders

The primary goal of our translational research group is to develop efficacious therapies for patients with complex lymphatic anomalies. To do this, we seek to understand the molecular etiologies of these complex lymphatic malformations, how the molecular etiologies alter molecular signaling, and how this affects the cellular mechanisms regulating normal lymphatic development. Ultimately, these answers will allow us to develop novel therapies.

Complex lymphatic anomaly is a term that encompasses four different complex lymphatic malformations: central conducting lymphatic anomaly (CCLA); generalized lymphatic anomaly (GLA); Kaposiform lymphangiomatosis (KLA); and Gorham Stout disease (GSD). Patients suffer from symptoms such as pleural effusions, pericardial effusions, ascites, and bone lesions, which can cause significant morbidity and even death. Currently, there is only one medication approved for patients with complex lymphatic anomalies caused by *PIK3CA,* a gene mutation known known to cause lymphatic malformations. Similar, precision-medicine approaches are needed for patients with other complex lymphatic anomalies.

Research in our lab will combine patient studies and genomics with the zebrafish model to identify novel therapies. The zebrafish model allows for us to manipulate the genetics rapidly to create patient-based models, image the developing vasculature, understand cellular dynamics *in vivo*, and perform drug screening.

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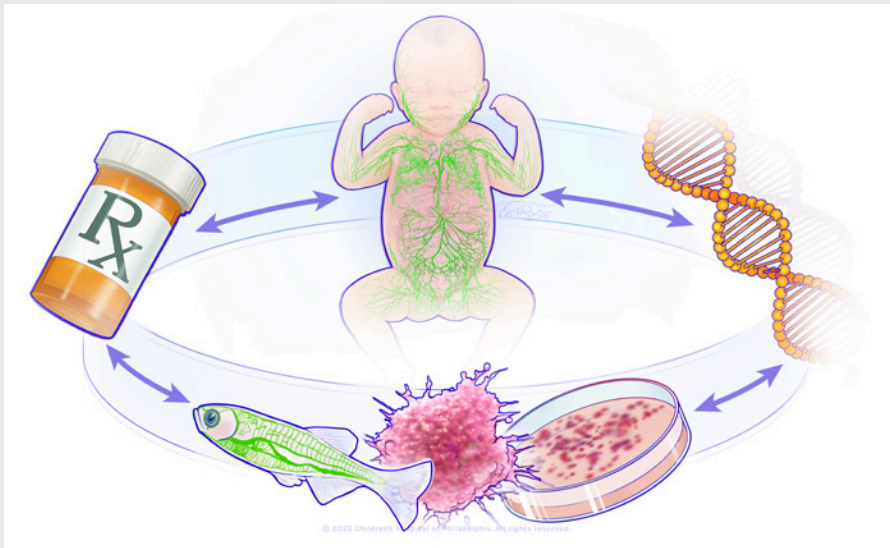
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Natural history study of lymphatic disorders

Our group launched a prospective natural history study for individuals with lymphatic anomalies to systematically evaluate the disease phenotypes and long-term outcomes. This will allow us to provide improved prognostication to families, establish screening/monitoring guidelines, determine best practices for genetic diagnosis, and explore fertility outcomes for those on long-term medication management. The study will allow us to identify novel end-points for future clinical trials.

Genotype–phenotype correlations in central conducting lymphatic anomaly

Central conducting lymphatic anomalies (CCLA) occur when there

FIGURE 1. A bedside-to-bench precision medicine program for lymphatic anomalies

The figure shows a circle with the major components of the research in the lab: a child with abnormal lymphatics, a DNA molecule, a representation of a zebrafish model and organoid model, and a pill bottle. This represents a bedside-to-bench-to-bedside program using organoid and zebrafish to model patient's lymphatic anomalies and develop therapies that will be translated back to the patients.

is a disruption of central lymphatic flow, resulting in complications such as non-immune fetal hydrops, chylothorax, chylous ascites, protein-losing enteropathy, other effusions, or lymphedema. The heterogeneity of CCLA complicates diagnosis, treatment, and prognostication. Understanding the molecular etiology of a patient's disease can improve medical care, including novel treatment strategies. However, few genetic causes have been identified for CCLA. Clinical geneticists use distinct facial features to assist in diagnosis of rare disorders. Given the recent advances in lymphatic imaging, we sought to understand whether we could use features identified by dynamic contrast magnetic resonance imaging for diagnosing CCLA. We discovered that only about a quarter of patients with CCLA have an underlying genetic diagnosis that can be identified by routine clinical evaluation. We also demonstrated that germline RASopathies (group of genetic syndromes caused by germline mutations in genes that encode components or regulators of the Ras/mitogen-activated protein kinase pathway), mosaic KRASopathies (a disorder caused by a somatic pathogenic variant in KRAS), *PIEZO1* (a gene that encodes a mechanically activated ion channel that links mechanical forces to biological signal)–related lymphatic dysplasia, and Trisomy 21 have distinct central lymphatic flow phenotypes. In recent published work [Reference 2], we developed novel techniques such as the use of cfDNA and identified novel causes for CCLA and other complex lymphatic anomalies. We also collaborated on the spectrum of complex lymphatic anomalies caused by *PIK3CA*.

Cellular and molecular mechanisms of lymphatic disorders

We published work [Reference 1] demonstrating that activating variants in *KRAS* can drive lymphatic malformations in the zebrafish, which can be treated with MEK (kinase that phosphorylates mitogen-activated protein kinase) inhibitors. We identified several genetic causes of CCLA. We are evaluating these novel potential causes to understand their effect on the cellular and molecular mechanisms driving lymphatic development and identify new therapies.

FIGURE 2. Clinical imaging of lymphatic anomalies according to genotype

T2 space and dynamic contrast MR (magnetic resonance) lymphangiography (DCMRL) from seven different genotypes, illustrating lymphatic conduction abnormalities.

A. Mosaic *BRAF* (p.Val600Glu): T2 space shows significant edema in the intercostal, mesentery, and liver lymphatics (*left panel*) (*arrows*) that correlates with abnormal perfusion patterns on intrahepatic DCMRL (*right*). Also note the abnormal lymphatic thoracic vessels with absence of a normal thoracic duct (*BRAF* encodes a serine/threonine kinase involved in cell growth and survival).

B. Mosaic *KRAS* (p.Gly12Asp): There is edema on T2 space within the mediastinum and lungs (*arrows*). Patient also with cystic right kidney (*asterisk*). Intrahepatic DCMRL demonstrates correlation with mediastinal, pulmonary, and supraclavicular edema, with perfusion of dilated lymphatic structures. Of note, this patient has a central thoracic duct (*arrow heads*), but it was not patent to the venous circulation on ultrasound contrast imaging.

C. Noonan syndrome (*PTPN11* p.Gln510His): T2 space imaging demonstrating mediastinal and intercostal edema predominately. With intranodal DCMRL, these areas correlate with abnormal perfusion (*arrows*). Again, note there is no central thoracic duct, but persistent pulmonary and intercostal perfusion.

D. Trisomy 21: T2 space imaging with edema in the supraclavicular and superior mediastinal lymphatics (*arrows*). On intrahepatic DCMRL, there is retrograde flow into retroperitoneal lymphatics, intercostal, mediastinal, pulmonary, and supraclavicular perfusion (*arrows*). There is a patent thoracic duct that courses to the left venous angle (*arrowhead*).

E. PIEZO1: T2 space shows bilateral pleural effusions and pulmonary and retroperitoneal edema (*arrows*). Intrahepatic DCMRL shows extensive flow to the hepatic capsular lymphatics, with extension into the mediastinum and pulmonary lymphatics (*arrows*). There is also retrograde flow into the retroperitoneal lumbar and mesenteric lymphatics. A small thoracic duct can be seen coursing to the left venous angle (*arrow head*), patent on follow-up imaging.

F. Gaucher's disease Type III: T2 space notable for ascites. Intrahepatic DCMRL shows retrograde perfusion to retroperitoneal lumbar lymphatics and mesentery (*arrows*). The thoracic duct is mildly dilated and tortuous as it courses to the left venous angle (*arrowhead*).

G. Andersen's disease: T2 space imaging with significant ascites, pleural effusions, and anasarca (*arrows*). With intranodal DCMRL, there is extensive dermal perfusion and dilated retroperitoneal lymphatics. A thoracic duct is present and mildly dilated and tortuous (*arrowhead*). Figure from Liu M, Smith CL, Biko DM *et al*, *Eur J Hum Genet* 2022;30:1022.
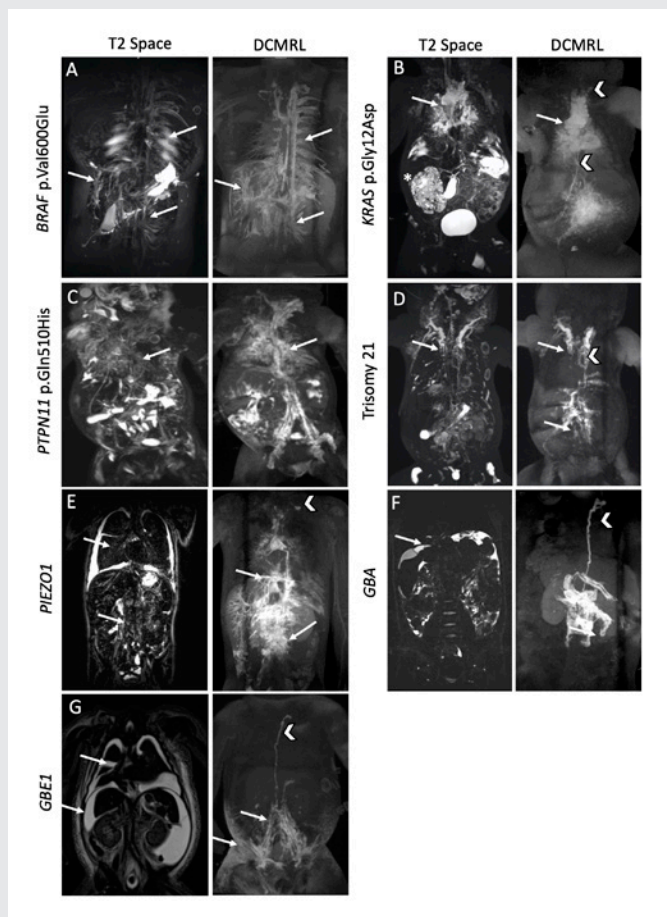
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Publications

1. Sheppard SE, March ME, Seiler C, Matsuoka LS, Kim SE, Kao C, Rubin AI, Battig MR, Khalek N, Schindewolf E, O'Connor N, Pinto E, Priestley JR, Sanders VR, Niazi R, Ganguly A, Hou C, Slater D, Frieden IJ, Huynh T, Shieh JT, Krantz ID, Guerrero JC, Surrey LF, Biko DM, Laje P, Castelo-Soccio L, Nakano TA, Snyder K, Smith CL, Li D, Dori Y, Hakonarson H. Lymphatic disorders caused by mosaic, activating KRAS variants respond to MEK inhibition. *JCI Insight* 2023 8(9):e155888.
2. Li D, Sheppard SE, March ME, Battig MR, Surrey LF, Srinivasan AS, Matsuoka LS, Tian L, Wang F, Seiler C, Dayneka J, Borst AJ, Matos MC, Paulissen SM, Krishnamurthy G, Nriagu B, Sikder T, Casey M, Williams L, Rangu S, O'Connor N, Thomas A, Pinto E, Hou C, Nguyen K, Pellegrino da Silva R, Chehimi SN, Kao C, Biroc L, Britt AD, Queenan M, Reid JR, Napoli JA, Low DM, Vatsky S, Treat J, Smith CL, Cahill AM, Snyder KM, Adams DM, Dori Y, Hakonarson H. Genomic profiling informs diagnoses and treatment in vascular anomalies. *Nat Med* 2023 29(6):1530–1539.
3. Nriagu BN, Williams LS, Brewer N, Surrey LF, Srinivasan AS, Li D, Britt A, Treat J, Crowley TB, O'Connor N, Ganguly A, Low D, Queenan M, Drivas TG, Zackai EH, Adams DM, Hakonarson H, Snyder KM, Sheppard SE. Microcystic lymphatic malformations in Turner syndrome are due to somatic mosaicism of PIK3CA. *Am J Med Genet A* 2023 doi: 10.1002/ajmg.a.63385.
4. Grenier JM, Borst AJ, Sheppard SE, Snyder KM, Li D, Surrey LF, Al-Ibraheemi A, Weber DR, Treat JR, Smith CL, Laje P, Dori Y, Adams DM, Acord M, Srinivasan AS. Pathogenic variants in PIK3CA are associated with clinical phenotypes of kaposiform lymphangiomatosis, generalized lymphatic anomaly, and central conducting lymphatic anomaly. *Pediatr Blood Cancer* 2023 e30419.
5. Zarowin D, Heymann WR, Yan AC, Treat J, Sheppard SE. Segmental vasoconstricted patches with a border of telangiectasia. *Pediatr Dermatol* 2023 40(3):565–567.
6. Bolli A, Nriagu B, Britt AD, Toole AD, Treat J, Srinivasan A, Sheppard SE. Mosaic pathogenic variants in AKT3 cause capillary malformation and undergrowth. *Am J Med Genet A* 2023 191(5):1442–1446.

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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 pseudo-tetraploid *Xenopus laevis* and diploid *Xenopus tropicalis*, two highly related species that offer unique but complementary advantages. The control of this developmental process by TH offers a paradigm to study gene function in postembryonic organ development. During metamorphosis, different organs undergo vastly different changes. Some, like the tail, undergo complete resorption, while others, such as the limb, are developed *de novo*. The majority of the larval organs persist through metamorphosis but are dramatically remodeled to function in a frog. For example, tadpole intestine is a simple tubular structure consisting primarily of a single layer of larval epithelial cells. During metamorphosis, through specific larval epithelial cell death and *de novo* development of the adult epithelial stem cells followed by their proliferation and differentiation, it is transformed into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis both *in vivo*, by using genetic approaches or hormone treatment of whole animals, and *in vitro* in organ cultures offer an excellent opportunity to (1) study the developmental function of TH receptors (TRs) and the underlying mechanisms *in vivo* and (2) identify and functionally characterize genes that are critical for organogenesis, particularly, the formation of the adult intestinal epithelial stem cells, during postembryonic development in vertebrates [Reference 1]. A major recent focus has been to make use of the TALEN and CRISPR/Cas9 technologies to knock out the endogenous genes for functional analyses. In addition, the recent improvements in *Xenopus tropicalis* genome annotation allow us to carry out RNA-seq and chromatin-immunoprecipitation (ChIP)-seq analyses at the genome-wide level. They also allow us to adapt single-cell sequencing technology to study how TH induces cell transformations during vertebrate development. Thus, in recent years, we have focused our research on the diploid *Xenopus tropicalis*. 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.
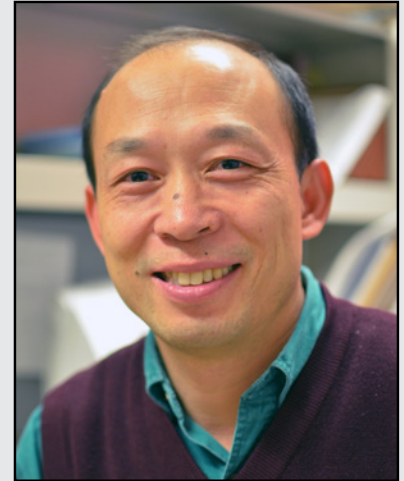
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FIGURE 1. Effects of TR double KO on developmental rate. TR double KO leads to premature initiation of metamorphosis but slows metamorphic progress and causes lethality at the metamorphic climax.

A. TR double KO animals take a shorter time to reach the onset of metamorphosis (stage 54), indicating accelerated pre-metamorphic 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 stage 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 able 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, *trα⁻/⁻trβ⁺/⁺*, *trα⁻/⁻trβ⁺/⁻*, and *trα⁻/⁻trβ⁻/⁻*, was thus obtained and plotted. Note that no double knockout tadpoles developed to stage 62 and that a single copy of *trβ⁺/⁻* was sufficient for the animal to complete metamorphosis and develop into a reproductive adult.
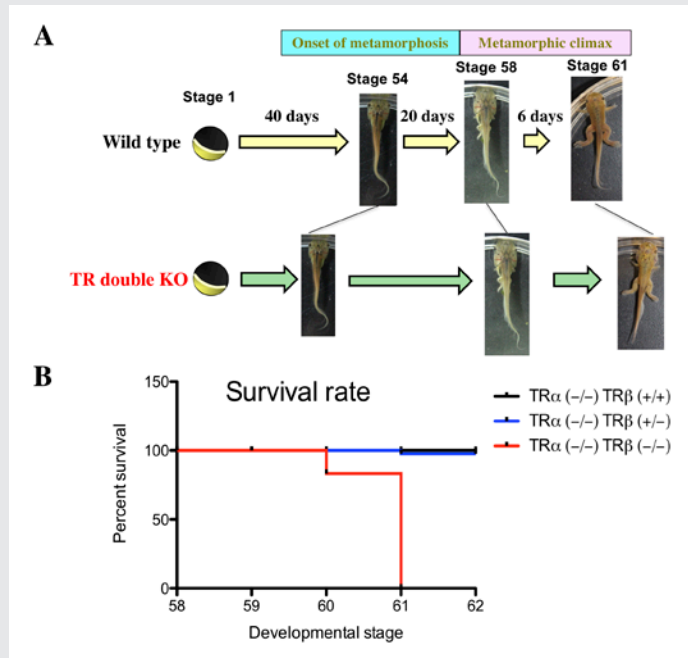
Organ–specific effects on target binding due to knocking out thyroid hormone receptor *Xenopus* metamorphosis

There are two TR genes, *TRα* and *TRb*, in all vertebrates. We previously generated *TRα* knockout (*Xtr.thra*^tmshi^) tadpoles and showed that TRα is important for TH–dependent intestinal remodeling and hindlimb development, but not tail resorption, during metamorphosis. To investigate the underlying molecular basis, we earlier used chromatin immunoprecipitation-sequencing (ChIP-seq) to identify genes bound by TR in the intestine and hindlimbs of pre-metamorphic wild-type and *Xtr.thra*^tmshi^ tadpoles with or without TH treatment. We have now carried out similar analyses on the tail and compared the findings with those in the intestine and hindlimb [Reference 1]. We found that the tail had far fewer genes bound by TR or affected by *TRα* knockout. Bioinformatics analyses revealed that, among the genes bound by TR in wild-type but not *Xtr.thra*^tmshi^ organs, fewer gene-ontology (GO) terms or biological pathways related to metamorphosis were enriched in the tail than in the intestine and hindlimb; the difference likely underlies the drastic effects of *TRα* knockout on the metamorphosis of the intestine and hindlimb but not the tail. Thus, TRα has tissue-specific roles in regulating TH-dependent anuran metamorphosis by directly targeting the pathways and GO terms important for metamorphosis.

Comparative analysis of transcriptome profiles reveals distinct and organ–dependent genomic and nongenomic actions of thyroid hormone in *Xenopus tropicalis* tadpoles.

TH is essential for development and organ metabolism in all vertebrates. TH has both genomic and nongenomic effects on target cells. While much has been learnt on its genomic effects via TRs during vertebrate development,

mostly through TR–knockout and knockin studies, little is known about the effects of TH on gene expression in animals in the absence of TR. By using the recently generated TR double-knockout (TRDKO) *Xenopus tropicalis* animals, we compared the effects of TH on global gene expression in tadpole tissues in the presence or absence of TR [Reference 2]. We carried out RNA-seq analyses on gene expression in tadpole tail and intestine of wild-type and TRDKO tadpoles with or without TH treatment. We observed that removing TRs reduced the number of genes regulated by TH in both organs. Gene Ontology (GO) analysis revealed that TH affected distinct biological processes and pathways in wild-type and TRDKO tadpoles. Many GO terms were enriched among genes regulated in wild-type tissues and are likely involved in mediating the effects of TH on metamorphosis, e.g., those related to development, stem cells, apoptosis, and cell cycle/cell proliferation. However, such GO terms and pathways were not enriched among TH–regulated genes in TRDKO tadpoles. Instead, in TRDKO tadpoles, GO terms and pathways related to "metabolism" and "immune response" were highly enriched among TH–regulated genes. We further observed strong divergence in the TR–independent, nongenomic effects of TH in the intestine and tail. Our data suggest that TH has distinct and organ-dependent effects on gene expression in developing tadpoles. The TR–mediated effects are consistent with the metamorphic changes, in agreement with the fact that TR is necessary and sufficient to mediate the effects of TH on metamorphosis. TH appears to have a major effect on metabolism and immune response via TR–independent nongenomic processes [Reference 2].

Thyroid hormone receptor knockout prevents the loss of *Xenopus* tail regeneration capacity at metamorphic climax.

Animal regeneration is the natural process of replacing or restoring damaged or missing cells, tissues, organs, and even the entire body to full function. Studies in mammals have revealed that many organs lose regenerative capacity soon after birth when TH level is high. This suggests that TH plays an important role in organ regeneration. Intriguingly, plasma TH level peaks during amphibian metamorphosis, which is very similar to postembryonic development in humans. In addition, many organs, such as heart and tail, also lose their regenerative ability during metamorphosis, making frogs a good model in which to address how the organs gradually lose their regenerative ability during development and what roles TH may play in this process. Early tail-regeneration studies have been done mainly in the tetraploid *Xenopus laevis* (*X. laevis*), which does not lend itself easily to gene knockout studies. We used the highly related but diploid anuran *X. tropicalis* to investigate the role of TH signaling in tail regeneration with gene knockout approaches. We discovered that *X. tropicalis* tadpoles could regenerate their tail from pre-metamorphic stages up to the climax stage 59, whereupon they lose regenerative capacity as tail resorption begins, just as is observed for *X. laevis*. To test the hypothesis that the TH–induced metamorphic program inhibits tail regeneration, we used TR double-knockout (TRDKO) tadpoles lacking both *TRa* and *TRb*, the only two receptor genes in vertebrates, for tail regeneration studies [Reference 3]. Our results showed that TRs were not necessary for tail regeneration at any stage. However, unlike wild-type tadpoles, TRDKO tadpoles retained regenerative capacity at the climax stages 60/61, likely in part by increasing apoptosis during the early regenerative period and enhancing subsequent cell proliferation. In addition, TRDKO animals had higher levels of amputation-induced expression of many genes important for tail regeneration, compared with the non-regenerative wild-type tadpoles at stage 61. The high level of apoptosis in the remaining uncut portion of the tail, as wild-type tadpoles undergo tail resorption after stage 61, appeared to also contribute to the loss of regenerative ability. For the first time, our findings revealed evolutionary conservation in the loss of tail regeneration capacity at metamorphic climax between *X. laevis* and *X. tropicalis*. Our studies with molecular and genetic approaches demonstrated that the TR–mediated, TH–induced gene regulation program is responsible not only for tail resorption but also for the loss of tail

regeneration capacity [Reference 3]. Further studies using the model should uncover how TH modulates the regenerative outcome, and should offer potential new avenues for regenerative medicines for human patients.

Competitive PCR with dual fluorescent primers enhances the specificity and reproducibility of genotyping animals generated from genome editing.

Targeted genome editing is a powerful tool for studying gene function in almost every aspect of biological and pathological processes. The most widely used genome editing approach is to introduce engineered endonucleases or CRISPR/Cas system into cells or fertilized eggs to generate double-strand DNA breaks within the targeted region, leading to DNA repair through homologous recombination or non-homologous end joining (NHEJ). DNA repair through the NHEJ mechanism is an error-prone process, which often results in point mutations or stretches of indels (insertions and deletions) within the targeted region. Such mutations in embryos are germline-transmissible, thus providing an easy means to generate organisms with gene mutations. However, point mutations and short indels are difficult to genotype, often requiring labor-intensive sequencing to obtain reliable results. We developed a single-tube competitive PCR assay with dual fluorescent primers that allows simple and reliable genotyping. While we used *Xenopus tropicalis* as a model organism, the approach should be applicable to genotyping of any organism.

Upregulation of the protooncogene *Ski* by thyroid hormone in the intestine and tail during *Xenopus* metamorphosis

TH affects frog metamorphosis through TH receptor (TR)–mediated regulation of TH response genes, where TR forms a heterodimer with RXR (9-*cis* retinoic acid receptor) and binds to TH response elements (TREs) in TH response genes to regulate their transcription. To study how TH regulates intestinal stem-cell development and/or proliferation, we previously identified many putative direct TH response genes in *Xenopus tropicalis* tadpole intestine by using ChIP (chromatin immunoprecipitation)-on-chip assays. Among them is the proto-oncogene *Ski*, which encodes a nuclear protein with complex functions in regulating cell fate. We showed that *Ski* is upregulated in the intestine and tail of pre-metamorphic tadpoles upon TH treatment, and its expression peaks at stage 62, the climax of metamorphosis [Reference 4]. We further discovered a TRE in the first exon that can bind to TR/RXR *in vitro* and mediate TH regulation of the promoter *in vivo*. These data demonstrate that *Ski* is activated by TH through TR binding to a TRE in the first exon during *Xenopus tropicalis* metamorphosis, implicating a role of *Ski* in regulating cell fate in this process.

Liver development during *Xenopus tropicalis* metamorphosis is controlled by TH activation of WNT signaling.

Many mammalian organs and tissues, including erythrocytes, mature into their adult forms during postembryonic development when plasma TH level peaks, resembling amphibian metamorphosis. TR mutations/deletions can cause hematopoietic dysfunction, suggesting that TH plays a role in erythropoiesis during development. We recently generated TR double knockout (TRDKO) *Xenopus tropicalis* as a model in which to study TH function during postembryonic development. Our analyses of TRDKO tadpoles during metamorphosis revealed that they exhibited characteristics similar to human iron-deficiency anemia. Given that the liver is the hematopoietic organ, our finding suggests a defect in liver development in TRDKO tadpoles. We analyzed liver metamorphosis in wild-type and TRDKO tadpoles and found that wild-type liver metamorphosis involved increased cell proliferation, hepatocyte hypertrophy, and activation of urea-cycle gene expression, a key feature of adult/mature liver in vertebrates [Reference 5]. Interestingly, the TRDKO liver

FIGURE 2. Intestinal metamorphosis involves the formation of clusters of proliferating, undifferentiated epithelial cells at the climax.

Tadpoles at pre-metamorphic stage 54 (*A*), climax, stage 62 (*B*), and the end of metamorphosis, stage 66 (*C*) 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 pre-metamorphosis (*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.
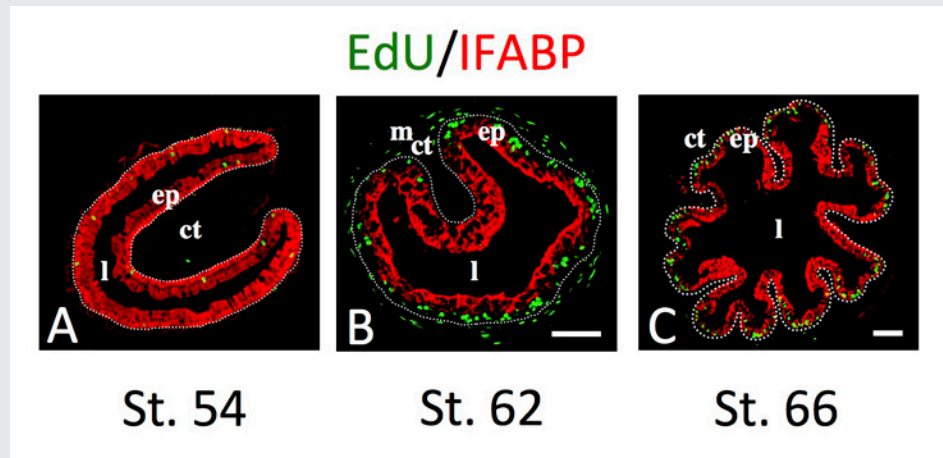
had developmental defects such as reduced cell proliferation and failure to undergo hepatocyte hypertrophy or activate the expression of urea-cycle genes. To reveal the molecular pathways regulated by TH during liver remodeling, we performed RNA-seq analysis and found that TH activated the canonical Wnt pathway in the liver. Wnt11 was particularly activated in both fibroblasts and hepatic cells and, in turn, likely acted to promote stem-cell development and/or proliferation and maturation of hepatocytes [Reference 5]. Our findings also resemble those from studies on liver regeneration in mammals. Thus, analyses of liver metamorphosis have the potential to bring new insights not only into how TH regulates liver development but are also a potential means to improve liver regeneration.

The l–type amino acid transporter 1 (LAT1) in hypothalamic neurons in mice maintains energy and bone homeostasis.

To regulate cellular processes, TH has to be actively transported into cells, a process that is mediated by several different types of transporters. One of our previously identified TH–response genes in *Xenopus* intestine, LAT1, encodes the light chain of a heterodimeric system L type of TH transporter, which also transports several amino acids. Interestingly, LAT1 is highly upregulated at the climax of metamorphosis in the tadpole intestine, coinciding with the formation and rapid proliferation of adult intestinal stem cells. Further, we found that LAT1 was also highly expressed in the mouse intestine during the neonatal period when the mouse intestine matured into the adult form, a process that appears also to involve TH–dependent formation and/proliferation of adult intestinal stem cells. In a collaborative study, we generated a mouse line with the *LAT1* gene floxed, which allows conditional knockout of *LAT1* upon expression of the Cre recombinase. We are currently analyzing the effect of *LAT1* knockout specifically in the mouse intestine by expressing Cre under the control of the intestinal epithelial-specific villin promoter. In another collaborative study, we discovered

LAT1 (also known as *Slc7a5)* expression in hypothalamic neurons, which regulate body homeostasis by sensing and integrating changes in the levels of key hormones and primary nutrients (amino acids, glucose, and lipids). Importantly, we found that LAT1 in hypothalamic leptin receptor (LepR)–expressing neurons was important for systemic energy and bone homeostasis. We observed LAT1–dependent amino acid uptake in the hypothalamus, which was compromised in a mouse model of obesity and diabetes. Mice lacking LAT1 (encoded by *Slc7a5*) in LepR–expressing neurons exhibited obesity-related phenotypes and higher bone mass. *Slc7a5* deficiency caused sympathetic dysfunction and leptin insensitivity in LepR–expressing neurons before obesity onset. Importantly, restoring *Slc7a5* expression selectively in LepR–expressing ventromedial hypothalamus neurons rescued energy and bone homeostasis in mice deficient in *Slc7a5* in LepR–expressing cells. We found that the mechanistic target of rapamycin complex-1 (mTORC1) is a crucial mediator of the LAT1–dependent regulation of energy and bone homeostasis. These results suggest that the LAT1–mTORC1 axis in LepR–expressing neurons controls energy and bone homeostasis by fine-tuning sympathetic outflow, thus providing *in vivo* evidence of amino-acid sensing by hypothalamic neurons in body homeostasis.

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Publications

1. Tanizaki Y, Zhang H, Shibata Y, Shi Y-B. Organ-specific effects on target binding due to knocking out thyroid hormone receptor α during Xenopus metamorphosis. *Dev Growth Differ* 2023 65:23–28.
2. Wang S, Shibata Y, Tanizaki Y, Zhang H, Yan W, Fu L, Shi Y-B. Comparative analysis of transcriptome profiles reveals distinct and organ-dependent genomic and nongenomic actions of thyroid hormone in Xenopus tropicalis tadpoles. *Thyroid* 2023 33:511–522.
3. Wang S, Shibata Y, Fu L, Tanizaki Y, Luu N, Bao L, Peng Z, Shi Y-B. Thyroid hormone receptor knockout prevents the loss of Xenopus tail regeneration capacity at metamorphic climax. *Cell Biosci* 2023 13(1):40.
4. Fu L, Liu R, Ma V, Shi Y-B. Upregulation of protooncogene Ski by thyroid hormone in the intestine and tail during Xenopus metamorphosis. *Gen Comp Endocrinol* 2022 328:114102.
5. Tanizaki Y, Wang S, Zhang H, Shibata Y, Shi Y-B. Liver development during Xenopus tropicalis metamorphosis is controlled by T3-activation of WNT signaling. *iScience* 2023 26:106301.

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Modeling the Biophysics of Cellular Membranes

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, both with 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.

The unexpected influence of cholesterol on fusion/fission pore dynamics

Cellular membranes are the medium by which cellular protein machinery is transported to and from the plasma membrane. Patches of membrane are constantly reshaped by the cell into spherical transport vesicles. In this simulation study [Reference 1], we used a special-purpose-built computer resource ("Anton 2") to test the effect of cholesterol dynamics on the shape and stability of membrane pores that are high-energy intermediates in the process of reshaping. We found that cholesterol is strongly excluded from the highly curved neck of the fusion pore. This is intuitively consistent with its tendency to thicken membranes, but inconsistent with its strong preference for the high curvature of the pore interior. We resolved this discrepancy with a modification to elasticity theory for cholesterol. In our model, cholesterol has a strong collapsing effect on fusion pores, favoring endocytosis. This may be one clue as to why cholesterol is enriched so strongly in the plasma membrane of human cells and what can go wrong in development if cholesterol levels are insufficient.

Membrane stiffness is strongly influenced by lipid lateral dynamics.

In a joint theory-experimental collaboration with the Dimova lab, we analyze the dynamic relaxation of giant unilamellar vesicles, a model system for biological membranes. We predicted that the relaxation timescales of mixtures of two lipids (with very different elastic properties) would be strongly influenced by slow diffusion, a prediction that was borne out in the experiment. The nanometer-scale implication is that lipids dynamically redistribute to regions of the membrane with shape that they favor, and thus, that mixtures of lipids nearly always tend to be softer than simpler bilayers. The membranes of living cells have hundreds of unique lipids, suggesting that lipid diversity is contributing to the unique mechanical properties of biological membranes.

Publications

1. Beaven AH, Sapp K, Sodt AJ. Simulated dynamic cholesterol redistribution favors membrane fusion pore constriction. *Biophys J* 2023 122:2162–2175.
2. Sapp K, Aleksanyan M, Kerr K, Dimova R, Sodt AJ. Kinetic relaxation of giant vesicles validates diffusional softening in a binary lipid mixture. *Phys Rev E* 2023 107(5–1):054403.
3. Soubias O, Sodt AJ, Teague WE, Hines KG, Gawrisch K. Physiological changes in bilayer thickness induced by cholesterol control GPCR rhodopsin function. *Biophys J* 2023 122:973–983.
4. Jimenez-Munguia I, Beaven AH, Blank PS, Sodt AJ, Zimmerberg J. Interferon-induced transmembrane protein 3 (IFITM3) and its antiviral activity. *Curr Opin Struct Biol* 2022 77:10246.

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Signaling and Secretion in Neuroendocrine Cells

The Section investigates cell-signaling cascades, gene expression, and secretion in hypothalamic and pituitary cells, with special emphasis on interactions between electrical events at the plasma membrane and receptor-controlled pathways. Specifically, we address how these neuroendocrine cells use ion channels and G protein–coupled receptors (GPCRs) as signaling platforms for efficient information processing. For this purpose, we characterize both natural and recombinant receptors and channels cloned from neuroendocrine cells. In the past, our work has focused on voltage- and ligand-gated ion channels, the cell type–specific patterns of electrical activity and channels involved, the physiological relevance of such activity, and the crosstalk between GPCRs and ion channels. Our current work focuses on age-, sex-, and tissue structure–specific gene expression, signaling and secretion, heterogeneity of secretory cells that reflect their postnatal genesis, and cell type–specific exocytic pathways. Ongoing and proposed projects include the use of transgenic and conditional knockout mouse models, and the research depends in part on the use of equipment at NICHD's *Microscopy and Imaging Facility* and *Molecular Genomics Core*.

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Single-cell RNA sequencing (scRNA-seq) of pituitary cells

We continued investigations on genes expressed in mammalian pituitary cells and their role in cell signaling and function. We contributed to the work of our collaborator Prashant Chittiboina on scRNA-seq studies of human hormone-producing pituitary adenomas causing Cushing's disease. The analysis included over 25,000 cells and identified a Cushing's disease adenoma transcriptomic signature, as compared with adjacent normal cells, with validation by bulk RNA-seq, DNA methylation, qRT-PCR, and immunohistochemistry. Cushing's disease adenoma cells include a subpopulation of proliferating, terminally differentiated corticotrophs. In Cushing's disease adenomas, we found recurrent promoter hypomethylation and transcriptional upregulation of PMAIP1 (encoding proapoptotic BH3-only bcl-2 protein noxa), but paradoxical noxa downregulation. Using primary Cushing's disease adenoma cell cultures and a corticotroph-enriched mouse cell line, we found that selective proteasomal inhibition with bortezomib stabilizes noxa and induces apoptosis, indicating its utility as an anti-tumor agent [Reference 1].

FIGURE 1. Common and female-specific effects of DKO on the hypothalamic kisspeptin-secreting neurons

(*A–D*) Kisspeptin immunoreactivity in the rostral periventricular region of the third ventricle. Pattern of kisspeptin immunoreactivity of WT (*A*) and DKO males (*B*), and WT (*C*) and DKO females (*D*).

(*E–H*) Kisspeptin immunoreactivity in the arcuate nucleus. Patterns of kisspeptin immunoreactivity of WT (*E*) and DKO males (*F*) and WT (*G*) and DKO females (*H*). Horizontal bars of 200 µm apply to all panels. Arrows indicate representative fiber density in the rostral periventricular region of the third ventricle (RP3V) (*A–D*) and the arcuate nucleus (*E–H*).

(*I*) Quantification of kisspeptinergic cell bodies (*left panel*) and fiber densities (*right panel*) in the RP3V region of WT and DKO females whose representative matched sections are shown in *C–D*.

(*J*) Quantification of kisspeptinergic fiber densities in the arcuate nucleus of WT and DKO males (*left panel*) and females (*right panel*), whose representative matched sections are shown in *E–F* and *G–H*, respectively.
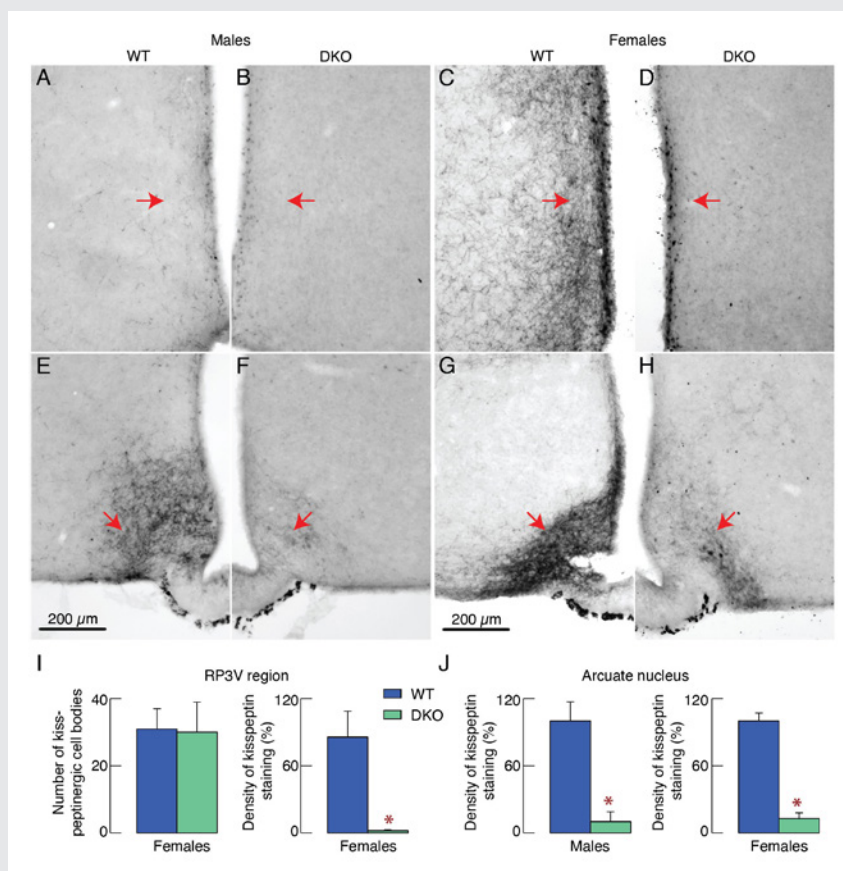
Our recent experiments on transcriptome profiles of secretory and non-secretory cell types using scRNA-seq of freshly dispersed pituitary cells revealed the presence of six hormone-producing cell types: melanotrophs, corticotrophs, gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs. We also identified four non-hormonal cell types: folliculostellate cells (FSCs), pituicytes, vascular pericytes, and endothelial cells. Initially, we characterized cell type– and sex-dependent transcriptome profiles of rat anterior pituitary cells. More recently, we summarized scRNA-seq and immunohistofluorescence analyses of adult female rat pituitary with a focus on transcriptomic profiles of non-hormonal cell types. Samples obtained from whole pituitaries and separated anterior and posterior lobe cells contained all expected resident pituitary cell types and a lobe-specific subpopulation of vascular cells. FSCs and pituicytes expressed *S100B*, *ALDOC*, *EAAT1*, *ALDH1A1*, and *VIM* genes and proteins, as well as several other astroglial marker genes, some common and some cell type–specific. We also found that the *SOX2* gene and protein are expressed in 15% of pituitary cells, including FSCs, pituicytes, and a fraction of hormone-producing cells, arguing against its stem-cell specificity. FSCs comprised two Sox2–expressing subclusters: FSC1 contained more cells but lower genetic diversity, while FSC2 contained proliferative cells, shared genes with hormone-producing cells, and expressed genes consistent with stem-cell niche formation, regulation of cell proliferation, and stem-cell pluripotency, including the Hippo and Wnt pathways. FSC1 are randomly distributed in the anterior and intermediate lobes, while FSC2 are

**FIGURE 2. Reduced LH levels in the pituitary gland of DKO animals**

(*A*) Western blot analysis of LHbeta (LHB) of male (*top panel*) and female (*bottom panel*) pituitaries in WT and DKO mice. The data shown are representative from five experiments.

(*B* and *C*) Pituitary LH content in pituitary tissue (*B*) and cultured pituitary cells (*C*) from WT and DKO mice assessed by ELISA.

(*D*) Basal LH release by cultured pituitary cells during a four-hour incubation.

(*E*) Time course of GnRH–stimulated LH release in cultured pituitary cells.

(*F*) Pituitary LH content (*left panel*) and serum LH concentration (*right panel*) 90 minutes after the intraperitoneal injection of saline (*S*) or 0.5 μg buserelin acetate (*B*), a GnRH receptor agonist, in WT and DKO mice. The bars shown are mean ± SEM values; number of replicates are indicated above the bars; asterisks denote P < 0.01 between pairs. The circles indicate mean values from duplicate determination.
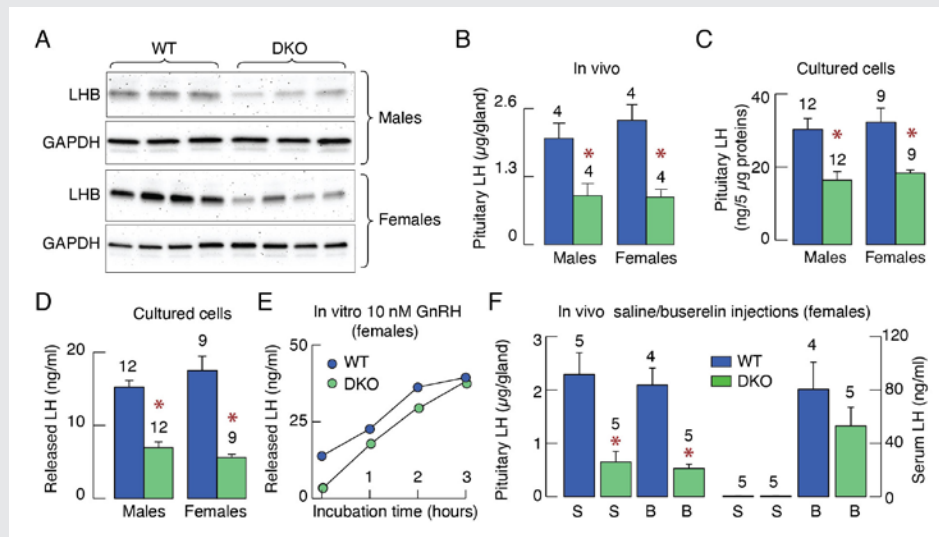
localized exclusively in the marginal zone between the anterior and intermediate lobes. These data indicate that FSCs are specialized anterior pituitary–specific astroglia, with FSC1 representing differentiated cells with transcriptomes consistent with classical FSC roles and FSC2 exhibiting additional stem cell–like features [Reference 2].

Roles of protein tyrosine phosphatase receptor N and N2 genes in mice reproduction

All pituitary hormone–producing cells express common genes associated with secretory functions, such as the sister genes encoding regulated endocrine-specific protein 18, *Resp18*, and the protein tyrosine phosphatase receptor genes *Ptprn* and *Ptprn2*, as well as *Chga*, *Chgb*, *Scg2*, *Snap25*, and *Uchl1* genes. Unlike cell type–specific hormone and hormone-receptor genes, the roles of these common genes are not well characterized. Our recent studies confirmed that simultaneous knockout of the neuroendocrine marker genes *Ptprn* and *Ptprn2* causes infertility in female mice while males are fertile. To elucidate the mechanism of the sex-specific roles of *Ptprn* and *Ptprn2* in mouse reproduction, we further analyzed the effects of their double knockout (DKO) on the hypothalamic-pituitary-gonadal axis. In DKO females, we observed delayed puberty and a lack of ovulation, complemented by changes in ovarian gene expression and steroidogenesis. In contrast, testicular gene expression, steroidogenesis, and the development of reproductive organs were not significantly affected in DKO males. However, hypothalamic *Gnrh1* and *Kiss1* gene expression was reduced in DKO females and males. In parallel, we detected a significant reduction in the density of immunoreactive GnRH and kisspeptin fibers in the hypothalamic arcuate nucleus of DKO females and males. Female-specific immunoreactivity of

the neuromodulator kisspeptin in the rostral periventricular region of the third ventricle was also reduced in DKO females but not in DKO males (Figure 1). Furthermore, in both sexes, pituitary luteinizing hormone (LH) beta gene expression and LH level, as well as follicle-stimulating hormone beta gene and gonadotropin-releasing hormone (GnRH) gene were reduced, while the calcium-mobilizing and LH secretory actions of GnRH were preserved (Figure 2). These data indicate a critical role of *Ptprn* and *Ptprn2* in kisspeptin–GnRH neuronal function and sexual dimorphism and in the threshold levels of GnRH required to preserve reproductive functions [Reference 3]. Ongoing experiments on this topic focus on the physiological status of anterior pituitary corticotrophs and intermediate lobe–located melanotrophs of DKO mice.

PI(4,5)P2–dependent and –independent roles of PI4P in pituitary cell function

In collaboration with Tamás Balla's group, we are also studying the functions of three phosphoinositides, PI4P, PI(4,5)P2, and PI(3,4,5)P3, in cell signaling and exocytosis, focusing on hormone-producing pituitary cells. PI(4,5)P2, which acts as a substrate for phospholipase C, plays a key role in the control of pituitary cell functions, including hormone synthesis and secretion. PI(4,5)P2 also acts as a substrate for class I PI3-kinases, leading to the generation of two intracellular messengers, PI(3,4,5)P3 and PI(3,4)P2, which act through their intracellular effectors, including Akt. PI(4,5)P2 can also influence the release of pituitary hormones, acting as an intact lipid to regulate ion channel gating and concomitant calcium signaling, as well as the exocytic pathway. Recent experiments also showed the expression of several PI lipid kinase genes, including *Pi4ka, Pi4kb, Pi4k2a, Pi4k2b, Pip5k1a, Pip5k1c,* and *Pik3ca*, as well as *Pikfyve* and *Pip4k2c*, in pituitary lactotrophs, which are responsible for the secretion of prolactin, a hormone that controls lactation. Using a pharmacological approach to specifically inhibit these enzymes, we showed that PI4P, made in the plasma membrane by PI4KA, is critical for exocytosis, without affecting the calcium signals that drive secretion. Our experiments also indicate that inhibition of the PI4KB enzyme, which generates PI4P in the Golgi, is dispensable for the exocytic step. These experiments revealed a key role of PI4KA–derived PI4P in the plasma membrane in calcium-secretion coupling in pituitary lactotrophs downstream of voltage-gated and PI(4,5)P2–dependent calcium signaling [Reference 4].

Our recently published study on this topic focused on the role of PI4KA in gonadotroph function by knocking out this enzyme in cells expressing the GnRH receptor. Knockout mice were infertile, reflecting underdeveloped gonads and reproductive tracts, and lack of puberty. The number and distribution of hypothalamic GnRH neurons and *Gnrh1* expression in postnatal knockouts were not affected, while *Kiss1*/kisspeptin expression was elevated. Knockout of PI4KA also did not alter embryonic establishment and neonatal development or function of the gonadotroph population. However, during the postnatal period, there was a progressive loss of expression of gonadotroph-specific genes, including *Fshb*, *Lhb*, and *Gnrhr*, accompanied by low synthesis of gonadotropins, but not of other pituitary lineage–specific genes and their hormones. The postnatal gonadotroph population also progressively declined, reaching approximately one third of that observed in controls at 100 days of age. In these residual gonadotrophs, GnRH–dependent calcium signaling, and calcium-dependent membrane potential changes were lost, but intracellular administration of inositol-1,4,5-trisphosphate rescued this signaling. These results indicate that PI4KA plays a key role in the postnatal development and maintenance of a functional gonadotroph population [Reference 5]. Ongoing experiments on this topic focus on the role of PI4KA in the postnatal development and function of pituitary lactotrophs and hypothalamic GnRH–secreting neurons.

Publications

1. Asuzu DT, Alvarez R, Fletcher PA, Mandal D, Johnson K, Wu W, Elkahloun A, Clavijo P, Allen C, Maric D, Ray-Chaudhury A, Rajan S, Abdullaev Z, Nwokoye D, Aldape K, Nieman LK, Stratakis C, Stojilkovic SS, Chittiboina P. Pituitary adenomas evade apoptosis via noxa deregulation in Cushing's disease. *Cell Rep* 2022 40:111223.
2. Fletcher PA, Smiljanic K, Prévide RM, Constantin S, Sherman AS, Coon SL, Stojilkovic SS. The astroglial and stem cell functions of adult rat folliculostellate cells. *Glia* 2023 7:205–228.
3. Sokanovic SJ, Constantin S, Lamarca Dams A, Mochimaru Y, Smiljanic K, Bjelobaba I, Previde RM, Stojilkovic SS. Common and female-specific roles of protein tyrosine phosphatase receptors N and N2 in mice reproduction. *Sci Rep* 2013 13(1):355.
4. Stojilkovic SS, Balla T. PI(4,5)P2-dependent and -independent roles of PI4P in the control of hormone secretion by pituitary cells. *Endocrinol (Lausanne)* 2023 14:1118744.
5. Constantin S, Sokanovic SJ, Mochimaru Y, Smiljanic K, Sivcev S, Previde RM, Wray S, Balla T, Stojilkovic SS. Postnatal development and maintenance of functional pituitary gonadotrophs is dependent on PI4-kinase A. *Endocrinology* 2023 164(12):doi:10.1210/endocr/bqad168.

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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 vertebrates, 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 us to target 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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Olfactory receptor neurons generate multiple response motifs, increasing coding space dimensionality.

Odors provide many types of important information about the environment and are characterized by their chemical compositions and concentrations. The tens or hundreds of thousands of detectable odorant molecules come in many different shapes, sizes, and charge distributions, requiring the brain to generate a high-dimensional description. Adding to this complexity, odorants often travel in chaotic and turbulent plumes comprising odorized pulses separated by clean air. The structures of odor pulses within a plume are determined

by factors such as wind speed, environmental features such as hills, trees, or buildings, and distance from the source. Therefore, the timing of odorants reaching a sensor can convey important information about the surroundings. To make use of this information, olfactory systems must generate high-dimensional representations of information about an odorant's chemical composition and delivery timing.

The process begins when odorants, binding to olfactory receptor neurons, trigger bursts of action potentials, providing the brain with its only experience of the olfactory environment. To better characterize the response dynamics of these neurons, we made hundreds of extracellular recordings from locust antennae *in vivo*, while presenting odor pulses individually or in regular trains, or as realistic, chaotic odor plumes. The recordings revealed something novel: rather than falling into a continuum of patterns, odor-elicited responses of olfactory receptor neurons clustered neatly into four distinct motifs, each defined by a reliable temporal profile that we called excitatory, delayed, offset, and inhibitory motifs. Different odorants could elicit different response motifs from a given olfactory receptor neuron, a novel property we termed motif switching. Further, each motif underwent its own form of sensory adaptation when activated by repeated odor pulses; for example, when repeatedly activated, excitatory responses significantly decreased, but offset responses significantly increased.

The response motifs we found *in vivo* seemed likely to contribute to the processing of olfactory information. To test this idea rigorously, we built a computational model based on our observations. Our model revealed that organizing responses into multiple motifs provides substantial benefits for classifying odors and processing complex odor plumes: each motif contributes uniquely to encode different aspects of the plume's composition and structure. Multiple motifs and motif switching further improve odor classification by expanding the dimensionality of the coding space. Our model demonstrated that these response features could provide benefits for olfactory navigation, including measuring and encoding the distance to an odor source.

Our work provides a new perspective on the first stage of olfactory coding, revealing a fundamental way receptor neurons can begin the process of extracting many important features from a sensory stream.

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, these roles are performed by relatively few neurons, which can be interrogated efficiently, revealing fundamental principles of olfactory coding. GABAergic neurons play critical roles in mediating the sparsening of olfactory neural representations. With intracellular recordings and a new large-scale biophysical model, which includes tens of thousands of neurons and spans multiple layers of processing, we focus on the locust's Giant GABAergic Neuron, GGN, to test ideas about how feedback inhibition creates a sparse odor representation in a higher order brain center.

The GGN's giant structure is likely an important factor in its function. It receives excitatory input from all 50,000 Kenyon cells in the mushroom body's α lobe and, in turn, provides inhibitory feedback to all Kenyon cells 400–500µm away in the calyx. The GGN is a huge neuron, but does not generate spikes, raising questions about how its far-flung regions interact. In addition to its large size, along its path from the α lobe to the calyx, its initially thick processes divide at myriad branch points into thin fibers. Cable theory applied to neurons predicts that a passive voltage signal within such a structure will attenuate dramatically. To test whether this giant neuron has the biophysical capacity to perform its suggested function of carrying effective signals
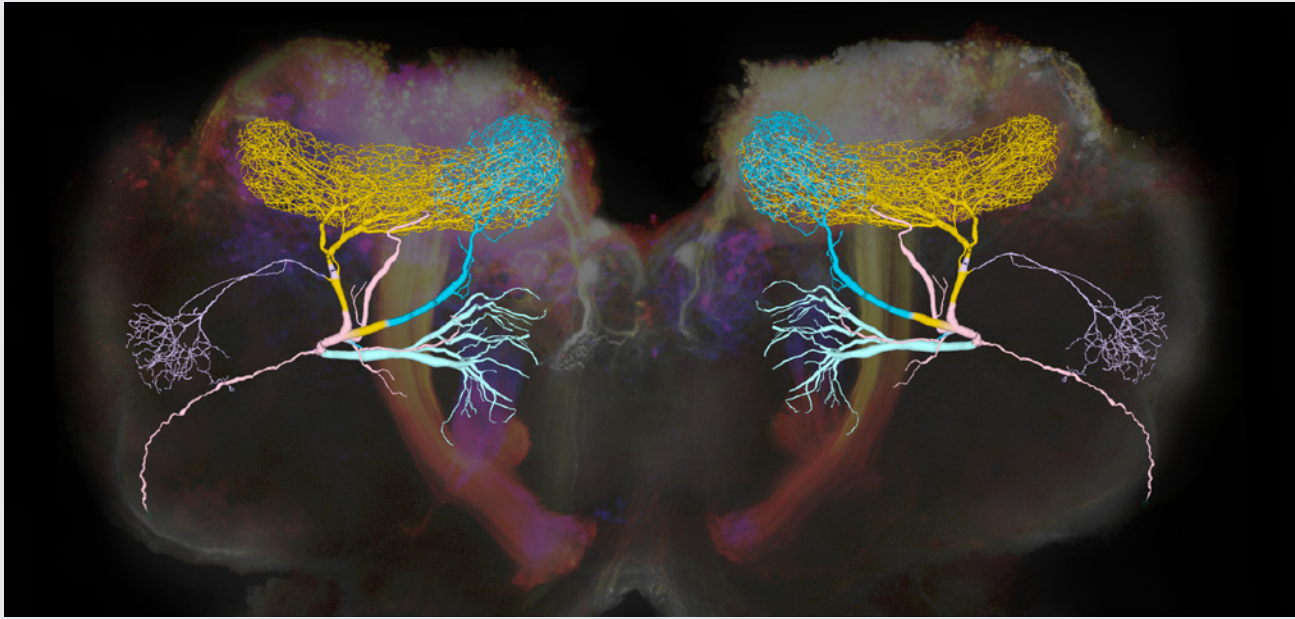
FIGURE 1. Giant GABAergic neurons regulate olfactory responses in the locust brain.

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.

passively from the α lobe to distant points in the calyx, we made dozens of recordings from the GGN *in vivo*, filled it with dye, and reconstructed it into a realistic computational model to characterize signal attenuation through its structure. Our model showed that, although electrical signals undergo substantial attenuation throughout its structure, signals in the GGN's calyceal branches appear strong enough to provide global inhibition to Kenyon cells.

To further understand the network determinants of the GGN's responses to odors, we recorded from it *in vivo* while delivering odors to the animal, and then used our large-scale model to investigate the types of network activity needed to generate the patterns we had observed in the GGN *in vivo*. We identified several novel features in the olfactory network. Surprisingly, our model predicted that a small portion of the notoriously quiet Kenyon cells must respond to odors with relatively high spike rates. We tested this prediction *in vivo* with patch-clamp recordings from many Kenyon cells while presenting odors to the animal's antenna. Indeed, we confirmed that the predicted portion of hyperactive Kenyon cells really exists. We also documented novel, complex response patterns not previously observed in the GGN, including periods of hyperpolarization, that vary with the odorant. Our model predicts that this behavior is likely driven by a novel, yet unknown odor-activated pathway. Together, the results of our *in vivo* recordings and large-scale computational modeling provide a more complete understanding of how different parts of the olfactory system interact.

Identification and analysis of odorant receptors expressed in two main olfactory organs: antennae and palps of a model organism, the locust *Schistocerca americana*

Olfaction allows animals to detect, identify, and discriminate among hundreds of thousands of odor molecules present in the environment. It requires a complex process to generate the high dimensional neural representations needed to characterize odorant molecules, which have different sizes, shapes, and electrical charges, and are often organized into chaotic and turbulent odor plumes. Understanding the anatomical organization of the olfactory system at cellular and molecular levels has provided important insights into the coding mechanisms underlying olfaction, and studies performed in insects have contributed substantially to our knowledge of odor processing. Further, mechanisms that allow the olfactory system to generate representations for odors have been shown to be widely conserved among very divergent species. Extending our understanding of olfaction requires knowledge of the molecular and structural organization of the olfactory system.

Odor sensing begins with olfactory receptor neurons (ORNs), which express odorant receptors (ORs). In insects, ORNs are housed, in varying numbers, in olfactory sensilla. Because the organization of ORs within sensilla affects their function, it is essential to identify the ORs they contain. Using RNA sequencing, we identified 179 putative ORs in the transcriptomes of the two main olfactory organs, antenna and palp, of the locust *Schistocerca americana*. Quantitative expression analysis showed that most putative ORs (140) are expressed in antennae, while only 31 are in the palps. Further, one OR was detected only in palps, and seven are expressed differentially by sex. An *in situ* analysis of OR expression revealed at least six classes of sensilla in the antenna. A phylogenetic comparison of OR–predicted protein sequences revealed homologous relationships among two other Acrididae species. Our results provide a foundation for understanding the organization of the first stage of the olfactory system in *S. americana*, a well studied model for olfactory processing.

Development of the olfactory system

The sense of smell is essential for survival throughout the lifespan of most animals, from the moment of birth. Studying the olfactory system as an animal develops can reveal fundamental aspects of information processing by networks of neurons as they grow and interact with the environment. The study of relatively simple animals such as locusts has proved to be a useful strategy for answering basic questions about olfaction.

Many animals, including humans, display innate preferences for some odors, but the neural mechanisms underlying these preferences are poorly understood. To investigate the encoding of innate preferences, it is necessary to establish a model system that allows both clear behavioral demonstrations of olfactory preferences and tractable analyses of neural mechanism. Thus, using locusts that had just hatched, we designed and used an open-field arena to provide only olfactory cues to guide navigation choices, and we used our own Argos tracking software to quantify the movements of naïve hatchlings. We found that newly hatched locusts navigated toward, and spent more time near, the odor released by a typical locust food, wheat grass, than humidified air. Notably, in similar tests, we found that hatchlings avoided moderate concentrations of major individual components of the wheat grass odor, 1-hexanol and hexanal diluted in mineral oil relative to control presentations of unscented mineral oil. Hatchlings were neither attracted nor repelled by a lower concentration of 1-hexanol but were moderately attracted to a low concentration of hexanal.

Our behavioral results provide the first unambiguous demonstration that fresh-from-the-egg locust hatchlings have a strong, innate preference for the odor of wheat grass (a blend of many components), but the valence

of the blend's individual components may be different and may change depending on the concentration. Our results provide an essential entry point for an analysis of neural mechanisms underlying innate sensory preferences.

As locusts grow from hatchling to adulthood, they acquire experience with the olfactory environment, and their body size increases tenfold while their brain size almost triples. Understanding how these changes affect the processing of olfactory information requires a rigorous investigation of the structure and function of developing locusts.

We therefore conducted an analysis of the structure and function of the first stages in the olfactory pathway in newly hatched and adult locusts by making electrophysiological recordings from their neurons to characterize their responses to odors and filling them with dye to visualize their morphologies. Our quantitative comparisons indicate that, although smaller in size, hatchling olfactory neurons are functionally and structurally like those of adults. With electroantennograms elicited by a broad panel of odors, we determined that the tuning of the olfactory receptor neuron population is similar in hatchlings and adults. Furthermore, with patch-clamp recordings, we found that local and projection neurons of the antennal lobe respond to odors with quantitatively similar complex, synchronous, oscillatory activity patterns in hatchlings and adults.

Notably, we found that odor-elicited neural oscillations in hatchlings gradually increase in frequency as the locusts grow. Based on our earlier computational modeling results, we hypothesized that this frequency change could be explained by increased odor-elicited excitatory drive, which accrues as new olfactory receptor neurons are added to growing antennae. To test this hypothesis, we successively removed segments containing olfactory receptor neurons from the adult antenna while recording local field potentials from the mushroom bodies. Consistent with our hypothesis, removing antennal segments significantly reduced the frequency of odor-elicited neural oscillations only on the side of the brain ipsilateral to the clipped antenna.

Overall, our developmental analysis shows that locusts hatch with a fully formed olfactory system, which structurally and functionally resembles that of the adult, despite its small size and lack of prior experience with olfactory stimuli.

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 labeled 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 labeled 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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Publications

1. Ray S, Aldworth Z, Stopfer M. Feedback inhibition and its control in an insect olfactory circuit. *eLife* 2020 9:e53281.
2. Gupta N, Singh SS, Stopfer M. Oscillatory integration windows in neurons. *Nat Commun* 2016 7:13808.
3. Reiter S, Campillo Rodriguez C, Sun K, Stopfer M. Spatiotemporal coding of individual chemicals by the gustatory system. *J Neurosci* 2015 35:12309–12321.
4. Ray S, Stopfer M. Argos: a toolkit for tracking multiple animals in complex visual environments. *Methods Ecol Evolution* 2022 133:585–595.
5. Aldworth ZN, Stopfer M. Insect neuroscience: filling the knowledge gap on gap junctions. *Curr Biol* 2022 32(9):R420–R423.

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Regulatory Small RNAs and Small Proteins

The group currently has two main interests: identification and characterization of small noncoding RNAs (sRNAs), 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 being found to have important regulatory roles in organisms ranging from bacteria to humans.

Identification and characterization of small regulatory RNAs

During the past 20 years, we have carried out several different systematic screens for small regulatory RNAs in *Escherichia coli*. The screens included computational searches for conservation of intergenic regions and direct detection after size selection or co-immunoprecipitation with RNA–binding proteins. 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 1]. This work showed that sRNAs are encoded by diverse loci including sequences overlapping mRNAs.

A major focus for the group has been to elucidate the functions of the small RNAs that we and others identified. Early on, we showed that the OxyS RNA, whose expression is induced in response to oxidative stress, acts to repress translation through limited base-pairing with target mRNAs. We discovered that OxyS action is dependent on the Sm-like Hfq protein, which acts as a chaperone to facilitate OxyS RNA base pairing with its target mRNAs. Follow up studies allowed us to learn more about the mechanism by which the Hfq protein facilitates base pairing through multiple RNA binding domains [Reference 2]. We also started to explore the role of ProQ, a second RNA chaperone in *E. coli*, and, by comparing the sRNA–mRNA interactomes by deep sequencing, found that ProQ and Hfq have overlapping as well as competing roles in the cell. It is likely that still other RNA–binding proteins such as KH domain proteins are involved in small RNA–mediated regulation [Reference 3].

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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. 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 found that an sRNA derived from the 3' UTR (untranslated region) of the *glnA* mRNA, encoding glutamine synthetase, impacts *E. coli* growth under low nitrogen conditions by modulating the expression of genes that affect carbon and nitrogen flux [Reference 4]. We recently discovered four UTR–derived sRNAs (UhpU, MotR, FliX, and FlgO), whose expression is controlled by the flagella sigma factor σ[28] (*fliA*), and reported that MotR and FliX modulate the timing of flagella synthesis in *E. coli* [Reference 5], work that illustrated how sRNA–mediated regulation can overlay a complex network, enabling nuanced control of flagella synthesis. A recent collaborative study of *Vibrio cholerae* revealed that the QrrX RNA controls quorum sensing dynamics and biofilm formation [Reference 6]. As more and more sRNAs encoded by 5' or 3' UTRs, or that are internal to coding sequences, are being found, our observations raise the possibility that phenotypes currently attributed to protein defects are the result of deficiencies in unappreciated regulatory RNAs.

One interesting recent observation is that some small RNAs have dual functions in that they act by both base pairing and by encoding a small, regulatory protein. For example, we discovered the Spot 42 RNA also encodes a 15-amino acid protein (denoted SpfP) [Aoyama JJ, Raina M, Zhong A, Storz G. Dual-function Spot 42 RNA encodes a 15-amino acid protein that regulates the CRP transcription factor. *Proc Natl Acad Sci USA* 2022;119:e21198661197]. Overexpression of just the small protein from a Spot 42 derivative deficient in base-pairing activity, or just the base-pairing activity from a Spot 42 derivative with a stop codon mutation, both prevented growth on galactose, revealing that the small protein and the small RNA impact the same pathway. Copurification experiments showed that SpfP binds to the CRP (cAMP receptor protein) transcription factor, affecting the kinetics of induction when cells are shifted from glucose to galactose medium. Thus, the small protein reinforces the feedforward loop regulated by the base-pairing activity of the Spot 42 RNA. As a second example, we found a 164–nucleotide RNA previously shown to encode a 28–amino acid protein (denoted AzuC) also base pairs with the *cadA* (lysine decarboxylase involved in maintaining pH homeostasis) and *galE* (encoding UDP-glucose 4-epimerase) mRNAs to block expression [Raina M, Aoyama JJ, Bhatt S, Paul BJ, Zhang A, Updegrove TB, Miranda-Ríos J, Storz G. Dual-function AzuCR RNA modulates carbon metabolism. *Proc Natl Acad Sci USA* 2022;119:e21179301198]. Interestingly, AzuC translation interferes with the observed repression of *cadA* and *galE* by the RNA, and base pairing interferes with AzuC translation, demonstrating that the translation and base-pairing functions compete. We hypothesize that many more dual-function RNAs remain to be discovered and suggest that they can be exploited to control gene expression at many levels [Aoyama JJ, Storz G. Two for one: regulatory RNAs that encode small proteins. *Trends Biochem Sci* 2023;48:1035-1043].

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 a broadly conserved RNA structure motif, the *yybP–ykoY* motif, found in the 5' UTR of the *mntP* gene encoding a manganese exporter, directly binds manganese, resulting in a conformation that liberates the ribosome-binding site.

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. Furthermore, there is limited evidence that proteins are synthesized from annotated and predicted short ORFs (open reading frames). Although these proteins have largely been missed, the few small proteins that have been studied in detail in bacterial and mammalian cells were shown to have important functions in regulation, signaling, and cellular defenses [Gray T, Storz G, Papenfort K. Small proteins; big questions. *J Bacteriol* 2022;204:e0034121]. 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 *E. coli* genome. We tested expression of these predicted as well as previously annotated small proteins by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and assaying for synthesis using immunoblot assays. The approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids are synthesized. We also carried out a complementary approach based on genome-wide ribosome profiling of ribosomes arrested on start codons to identify many additional candidates; the presence 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.

Many of the initially discovered proteins were predicted to consist of a single transmembrane alpha-helix and were found to be in the inner membrane. Interestingly, despite their diminutive size, small membrane proteins display considerable diversity in topology and insertion pathways. Additionally, systematic assays for the accumulation of tagged versions of the proteins showed that many small proteins accumulate under specific growth conditions or after exposure to stress.

We are using the tagged derivatives and information about synthesis and subcellular localization, along with many of the approaches the group used to characterize the functions of small regulatory RNAs, to elucidate the functions of the small proteins. The combined approaches are beginning to give insights into how the small proteins act in *E. coli*. For example, we discovered the 49–amino acid inner membrane protein AcrZ, whose synthesis is increased in response to noxious compounds such as antibiotics and oxidizing agents, associates with the inner membrane AcrB component of the AcrAB–TolC multidrug efflux pump. Mutants lacking AcrZ are sensitive to many, but not all, of the antibiotics transported by AcrAB–TolC as the result of AcrZ effects on the conformation of the AcrB drug-binding pocket. We also found that synthesis of the 42–amino acid protein MntS is repressed by high levels of manganese by the MntR transcription factor. The lack of MntS leads to reduced activities of manganese-dependent enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese and bacteriostasis under manganese-rich conditions. These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP. Additionally, we showed that the 31–amino acid inner membrane protein MgtS, whose synthesis is induced by very low magnesium by the PhoPQ two-component regulatory system, acts to raise intracellular magnesium levels and maintain cell integrity upon magnesium depletion. Upon development of a functional tagged derivative of MgtS, we found that MgtS interacts with MgtA to increase the levels of this P-type ATPase magnesium transporter under magnesium-limiting conditions. Correspondingly, the effects of MgtS upon magnesium limitation are lost in an *mgtA* mutant, and MgtA
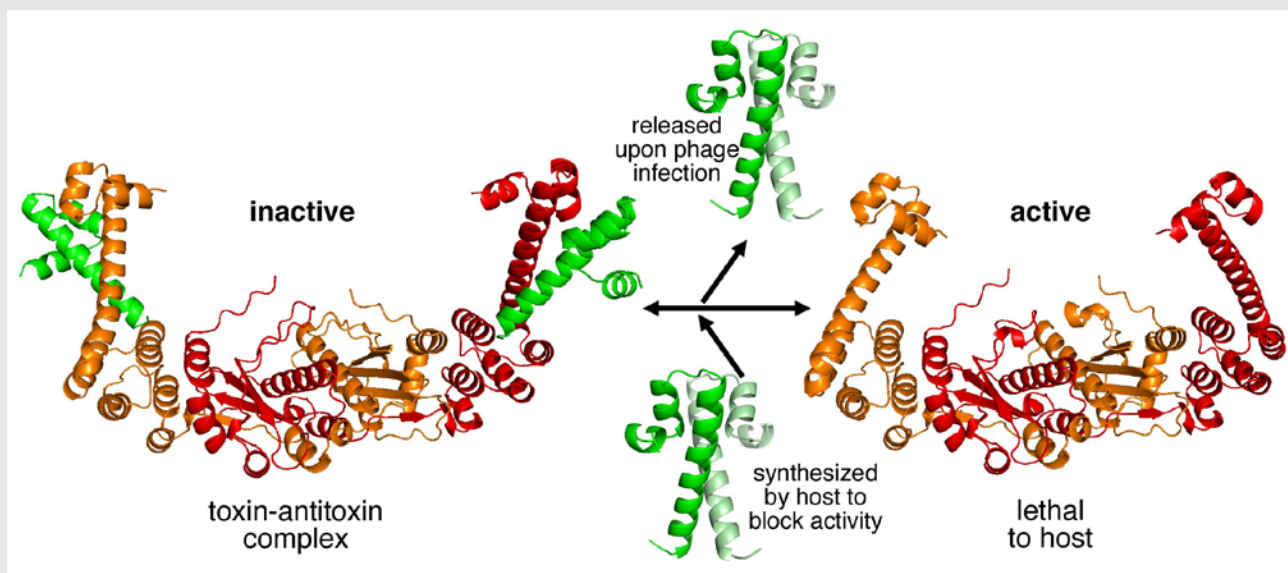
FIGURE 1. Model for functions of RpnL and RpnS proteins

In normally growing cells, the RpnS proteins, synthesized from a translation initiation site internal to the rpnL coding sequences, associate with and block the DNA endonuclease activities of the longer RpnL proteins. Upon phage infection, the RpnS proteins dissociate, and the RpnL proteins constitute an antiphage defense. The structure of the RpnS dimer (*green*) was solved by X-ray crystallography, while the structures of the RpnL-RpnS tetramer (*left*) and RpnL dimer (*right*) were predicted with AlphaFold–Multimer.

overexpression can suppress the mgtS phenotype. MgtS stabilization of MgtA provides an additional layer of regulation of this tightly controlled magnesium importer. Unexpectedly, 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.

The ribosome profiling used to identify the intergenic-encoded small proteins revealed that there is significant translation initiation within larger open reading frames in the *E. coli* genome. All five *E. coli* genes encoding Rpn (recombination-promoting nuclease) proteins have such an internal translation site. We showed that the small, highly variable Rpn C–terminal domains (RpnS), which are translated separately from the full-length proteins (RpnL), directly block the activities of the toxic full-length RpnL proteins, constituting a novel toxin-antitoxin system [Zhong A, Jiang X, Hickman AB, Klier K, Teodoro GIC, Dyda F, Laub MT, Storz G. Toxic antiphage defense proteins inhibited by intragenic antitoxin proteins. *Proc Natl Acad Sci USA* 2023;120:e2307382120]. The crystal structure of RpnAS revealed a dimerization interface encompassing helix that can have four amino acid repeats, whose number varies widely among strains of the same species. Consistent with strong selection for the variation, we documented that plasmid-encoded RpnP2L protects *E. coli* against certain phages, indicating that the RpnL–RpnS represent a novel antiphage defense system (Figure 1). We propose that many more intragenic-encoded small proteins that serve regulatory roles remain to be discovered in all organisms.

The ribosome profiling also revealed that some regulatory RNAs encode a small protein and are thus dual-function RNAs. As mentioned above, we documented the 109–nucleotide Spot 42 RNA, one of the best

characterized base-pairing small RNAs (sRNAs) in *E. coli* encodes a 15–amino acid protein (denoted SpfP), which binds to the global transcriptional regulator CRP. The binding blocks the ability of CRP to activate specific genes, impacting the kinetics of induction when cells are shifted from glucose to galactose medium. Thus, the small protein reinforces the feedforward loop regulated by the base-pairing activity of the Spot 42 RNA. Another 164–nucleotide RNA was previously shown to encode a 28–amino acid, amphipathic-helix protein (denoted AzuC). We discovered that the membrane-associated AzuC protein interacts with GlpD, the aerobic glycerol-3-phosphate dehydrogenase, and increases dehydrogenase activity [Raina M, Aoyama JJ, Bhatt S, Paul BJ, Zhang A, Updegrove TB, Miranda-Ríos J, Storz G. Dual-function AzuCR RNA modulates carbon metabolism. *Proc Natl Acad Sci USA* 2022;119:e2117930119]. Overexpression of the RNA encoding AzuC results in a growth defect in glycerol and galactose medium. The defect in galactose medium was still observed for a stop codon mutant derivative, consistent with the second base-pairing role for the RNA. Interestingly, the MgtS protein mentioned above is encoded divergent from the MgrR small regulatory RNA, which is also important for bacterial adaptation to low magnesium. We constructed synthetic dual-function RNAs comprising MgrR and MgtS. Such constructs allowed us to probe how the organization of the coding and base-pairing sequences and the distance between the two components 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.

Our work, along with related findings by others in eukaryotic cells, supports our hypothesis that small proteins are an overlooked but important class of proteins, which we continue to study.

Additional Funding

Publications
1. Petroni E, Esnault C, Tetreault D, Dale RK, Storz G, Adams PP. Extensive diversity in RNA termination and regulation revealed by transcriptome mapping for the Lyme pathogen Borrelia burgdorferi. *Nat Commun* 2023 14:3931.
2. Kavita K, Zhang A, Tai CH, Majdalani N, Storz G, Gottesman S. Multiple in vivo roles for the C-terminal domain of the RNA chaperone Hfq. *Nucleic Acids Res* 2022 50:1718–1733.
3. Olejniczak M, Jiang X, Basczok MM, Storz G. KH-domain proteins: another family of bacterial RNA matchmakers? *Mol Microbiol* 2022 117:10–19.
4. Walling LR, Kouse AB, Shabalina SA, Zhang H, Storz G. A 3' UTR-derived small RNA connecting nitrogen and carbon metabolism in enteric bacteria. *Nucleic Acids Res* 2022 50:10093–10109.
5. Melamed S, Zhang A, Jarnik M, Mills J, Silverman A, Zhang H, Storz G. σ28-dependent small RNA regulation of flagella biosynthesis. *eLife* 2023 12:RP87151.
6. Huber M, Lippegaus A, Melamed S, Siemers M, Wucher BR, Hoyos M, Nadell C, Storz G, Papenfort K. An RNA sponge controls quorum sensing dynamics and biofilm formation in Vibrio cholerae. *Nat Commun* 2022 13:7585.

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Evaluation of Hypothalamic and Pituitary Disorders

The goal of our research is to phenotype patients with rare hypothalamic and pituitary disorders and to identify biomarkers for disease diagnosis and of presentation and progression. We are also continuing to investigate the genetic mechanisms underlying pituitary tumorigenesis, which, along with novel biomarkers, will ultimately provide new therapeutic targets.

Phenotyping of patients with hypothalamic and pituitary disorders

Hypothalamic and pituitary tumors are rare disorders, which may present with hormone excess or hormone deficiencies. Hormone dysregulation or mass effects may lead to various complications. For example, patients with hypercortisolemia manifest with, among others, growth stagnation, obesity, and hyperglycemia. Under the protocol *97-CH-0076*, we are evaluating patients with pituitary disorders/adenomas/tumors. We are collecting clinical and biochemical data, as well as research biospecimens, to advance our knowledge of the genetic etiology, pathophysiology, presentation, diagnosis, and treatment of pituitary disorders. Previous results from this protocol have assisted in evaluation and management of these patients.

Molecular genetics of pituitary disorders

We continue our investigation into the genetic mechanisms of pituitary tumorigenesis. Previously, in collaboration with Constantine Stratakis, we reported that chromosomal aberrations may lead to more aggressive behavior of pituitary tumors. We are currently offering genetic testing for all patients with proven pituitary tumors, in a collaboration with the National Institute of Allergy and Infectious Diseases (NIAID). Additionally, we continue to pursue studies to understand novel candidate gene function and effect in pituitary tumors.

Survivors of pediatric Cushing's disease

Meg Keil is leading a research protocol on the long-term effects of pediatric hypercortisolemia (*19-CH-0051*) to identify residual clinical and biochemical findings after cure of pediatric Cushing's syndrome. Patients are eligible for the protocol if they were diagnosed with

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ACTH–secreting pituitary adenomas (Cushing's disease) before the age of 21 and are in biochemical remission. Patients are studied at five-year intervals until 20 years after cure. Anthropometric, clinical, biochemical, imaging, and other data are collected.

Immunologic effects of hypercortisolemia

Glucocorticoids have many effects on the immune system, which makes them one of the most commonly prescribed medications. Patients with ACTH–secreting pituitary adenomas present with endogenous hypercortisolemia and are a model for the evaluation of the immune effects of glucocorticoids, especially in the pediatric population, where other confounding co-existing morbidities are rare. We started our investigation on the immune effects of hypercortisolemia by looking at the complete blood count (CBC) of these patients, and we reported that simple inflammatory biomarkers can be used for the diagnosis of these patients. We further collaborated with Sergio Rosenzweig's group to describe specific cytokine patterns that may be involved in the pathophysiology of glucocorticoid-related immunosuppression. Luis Franco's group is currently conducting a study to further characterize the immune phenotype of our patients.

Therapeutic approaches for patients with rare sellar/suprasellar disorders

Gigantism is a condition characterized by excessive growth hormone (GH) secretion, which is commonly caused by GH–secreting pituitary adenomas, or by other rare disorders of dysregulated GH secretion. Although surgery remains the first line of treatment for these patients, up to 50% of patients will not achieve remission and will require further medical treatment or radiation. Currently, all therapeutic options we have for children derive from studies in adults and selected case reports. Under the protocol *19-CH-0071*, we study the safety and efficacy of pegvisomant, a GH–receptor antagonist, in children and adolescents with growth hormone excess, a protocol that is open to enrolment.

Publications

1. Piña JO, Faucz FR, Padilla C, Floudas CS, Chittiboina P, Quezado M, Tatsi C. Spatial transcriptomic analysis of pituitary corticotroph tumors unveils intratumor heterogeneity. *medRxiv* 2023 2023.08.04.23293576.
2. Nguyen MH, Zhang W, Pankratz N, Lane J, Chitiboina P, Faucz FR, Mills JL, Stratakis CA, Tatsi C. Exploratory study of the association of genetic factors with recovery of adrenal function in Cushing disease. *J Endocr Soc* 2023 7(6):bvad046.
3. Wurth R, Rescigno M, Flippo C, Stratakis CA, Tatsi C. Inflammatory biomarkers in the evaluation of pediatric endogenous Cushing syndrome. *Eur J Endocrinol* 2022 186(4):503–510.
4. Tatsi C, Bompou ME, Flippo C, Keil M, Chittiboina P, Stratakis CA. Paediatric patients with Cushing disease and negative pituitary MRI have a higher risk of nonremission after transsphenoidal surgery. *Clin Endocrinol (Oxf)* 2021 95:856–862.
5. Tatsi C, Saidkhodjaeva L, Flippo C, Stratakis CA. Subclinical hemorrhage of ACTH-secreting pituitary adenomas in children and adolescents changes their biochemical profile. *J Endocr Soc* 2022 6:bvac080.

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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 diseases.

Optical characterization of vascular health in sickle cell disease

Sickle cell disease (SCD) is an inherited hemoglobinopathy that disproportionately impacts minority populations in the United States and affects about 100,000 individuals. The substitution of valine by glutamic acid on the 6th amino acid of beta globin results in an abnormal hemoglobin variant (HbS), which polymerizes once deoxygenated and alters both the structure and function of red blood cells (RBCs), distorting them into a 'sickle' shape. Sickled RBCs have one sixth of the lifespan of normal RBCs, resulting in chronic hemolytic anemia; they are also rigid and can obstruct microvascular blood flow, causing recurring, unpredictable cycles of acute vaso-occlusive pain, commonly referred to as vaso-occlusive crises (VOC). Recurrent VOCs lead to hypoxia-reperfusion injury and, together with the intravascular hemolysis, promote inflammation and vascular endothelial damage. The systemic vasculopathy affects many organs, leading to cardiovascular complications, chronic pain, and cerebral and kidney impairment. Given that current treatment options are not universally effective, there is a significant, unmet need for new technologies that can quantitatively characterize SCD physiology and provide new insights for optimizing therapeutic impact in SCD patients.

Given the impairments to microvascular flow and endothelial dysfunction associated with SCD, advanced quantitative NIRS (near-infrared spectroscopy) is an attractive candidate to provide comprehensive hemodynamic evaluations in point-of-care settings. We are optically characterizing tissue composition, metabolism, and perfusion in several SCD studies, led by our collaborators Swee Lay Thein, Arun Shet, and Courtney Fitzhugh.

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- [NCT04610866](#) – Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of Long-term Mitapivat Dosing in Subjects With Stable Sickle Cell Disease: An Extension of a Phase I Pilot Study of Mitapivat
- [NCT05604547](#) – Exploring Near Infrared Spectroscopy (NIRS) Technologies for Assessment of Muscle Physiology, Tissue Oxygenation, and Blood Flow in Patients With Sickle Cell Disease (SCD)
- [NCT04514510](#) – Fixed Dose Flavonoid Isoquercetin on Thrombo-Inflammatory Biomarkers in Subjects With Stable Sickle Cell Disease
- [NCT05213572](#) – Observational Study to Deeply Phenotype Major Organs in Sickle Cell Disease After Curative Therapies

Our group's role is to assess the sensitivity of various optical devices to hemodynamic changes induced by SCD treatments and to evaluate whether the changes correlate with what is observed from blood chemistry. Our latest preliminary findings are noted in Figure 1.

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 the patient to stop breathing involuntarily for ten seconds or more throughout the night during sleep. When breathing stops, the oxygen level in the blood falls, sometimes waking the sleeper. Pediatric obstructive sleep apnea (POSA) can be particularly concerning, with several associated morbidities, which can have long-term effects extending into adulthood, including adverse changes in cardiovascular, metabolic, and developmental health. Onset usually occurs between ages 2–8, as tonsils reach their peak growth. Unfortunately, pediatric OSA remains largely under-diagnosed because of a lack of education about symptoms and the limited availability of sleep-medicine physicians. Early diagnosis and treatment are imperative for preventing many of these morbidities.

FIGURE 1. Monitoring sustained change due to Mitapivat treatment

We performed an optical hemodynamic assessment for each patient for a total of eight study visits over the span of a year, including at baseline prior to drug initiation. NIRS probes were affixed to the forearm and forehead to collect hemodynamic information characteristic of brain and skeletal muscle while performing a brachial cuff occlusion challenge. Over the first six months, we observed an increase in forehead StO_2 (tissue oxygen saturation) from 56% to 60% and forearm StO_2 from 59% to 63%; blood Hb levels exhibited a similar increase, going from 8.5 g/dL to 9.8 g/dL; this increase was sustained over the second six months for all three parameters. Average forehead StO_2 remained +3.6% above baseline, while forearm StO_2 remained +1.7% above baseline at the end of a year.
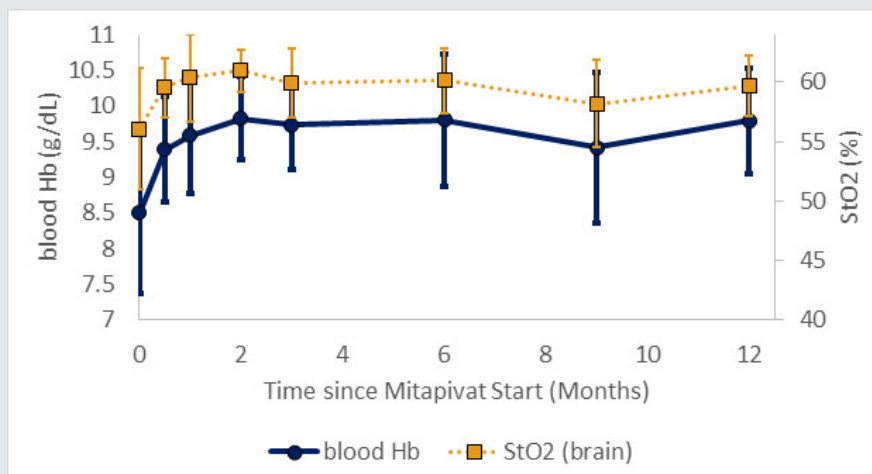
NIRS (near-infrared spectroscopy) is a non-invasive technology well suited for measuring cerebral hemodynamics during sleep. NIRS uses near-infrared light to penetrate human tissue and measure changes in oxygenation. Extensive literature documents the utility of NIRS in sleep studies of both normal and sleep-disordered breathing (SDB). In normal sleep, NIRS has been used to investigate cerebral hemodynamics in sleep stages and transitions between stages. For sleep apnea applications, NIRS can detect reductions in cerebral oxygenation, as well as respiration, heart rate, and blood flow changes that occur due to apnea. Previous work in NIRS sleep analysis showed that transient rises in cerebral deoxyhemoglobin are a prominent feature of apneas and hypopneas, and autoregulatory mechanisms have been shown to fail to prevent hypoxia during severe obstructive events. NIRS is uniquely suited to capture such breathing-induced cerebrovascular disturbances with high sensitivity and temporal resolution.

NCT05052216 — DEVELOPMENT OF A WEARABLE POINT OF CARE MONITORING DEVICE FOR PEDIATRIC OBSTRUCTIVE SLEEP APNEA

In collaboration with Ashura Buckley and the NIMH Sleep and Neurodevelopment Service (SNS), we are currently enrolling children aged 3–12 to investigate how optical signals change throughout sleep by comparing physiological signals derived from NIRS with data from polysomnography. More than 40 children have participated in this study to date, and preliminary data analysis is ongoing.

Vascular diseases driven by genetic alterations and COVID infection

The study and management of diseases with unknown vascular phenotypes is challenging. Given the importance of the vascular network to every organ system, understanding the extent and severity of vascular complications is necessary in order to develop effective treatments. Monogenic vascular diseases are characterized by a single genetic mutation, which can have deleterious effects on protein structure, function, or synthesis, and can be associated with severe complications, with high mortality and morbidity rates. While each individual disease is rare, these diseases can encompass a wide array of vascular phenotypes affecting any part of the body. Infectious diseases are another pathway for vascular complications, which can either manifest directly by viral/bacterial infection of the endothelial lining or indirectly as a result of damage triggered by inflammatory responses to the pathogen.

For this project, we aim to develop multi-modal NIRS assessment methods that are sensitive to the hemodynamic impairments of two patient cohorts: (1) patients with rare monogenic vascular disease; and (2) patients recovering from COVID-19. This consists of the selection of both the appropriate optical modality and measurement challenge to characterize the afflicted area. Our goal is to perform comprehensive clinical evaluations of patients recruited into the study, in order to better understand the disease pathology, heterogeneity of symptoms within the disease population, and progression of the various, rare vascular diseases over time. In collaboration with the Translational Vascular Medicine Branch (TVMB) at the NHLBI, three specific disease cohorts will be explored: ACDC (arterial calcification due to CD73 deficiency), CADASIL (cerebral autosomal dominant arteriopathy and leukoencephalopathy), and COVID-19.

NCT03538639 — VASCULAR DISEASE DISCOVERY PROTOCOL

ACDC is characterized by progressive vascular calcification, typically affecting arteries of the lower limbs, and manifests clinically as debilitating lower extremity pain resulting from the lower limb claudication and ischemia. For the ACDC cohort, we will evaluate vascular reactivity and endothelial function in the lower limbs using a hyperthermia challenge with an optical probe combining diffuse reflectance spectroscopy (DRS)

FIGURE 2. Breath hold dynamics of a CADASIL patient and healthy volunteer

Representative tracing of cerebral oxyhemoglobin ($[O_2Hb]$) concentration measured with time-domain NIRS during a breath hold in a healthy volunteer (*black*) and a patient with CADASIL (*red*). During the breath hold, there is a slight increase in $[O_2Hb]$ in the healthy volunteer, which corresponds to vasodilation in response to the hypercapnia that develops. In contrast, the CADASIL patient shows minimal $[O_2Hb]$ change, which could suggest impaired vascular reactivity. The $[O_2Hb]$ rate of change in the healthy volunteer was higher than in the CADASIL patient: 0.033 µM/s (*dashed line*) vs 0.01 µM/s (*solid line*). Prior literature suggested that cerebrovascular reactivity could be a predictive test of disease progression. To date, 16 patients have been enrolled in the study; data analysis is ongoing.
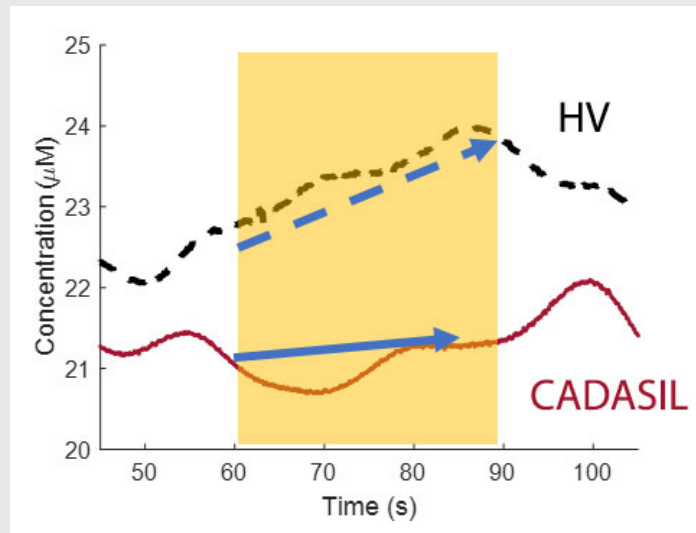
and laser doppler flowmetry (LDF) (Perimed AB, Sweden) to evaluate vascular reactivity in skin. The probe measures hemodynamic parameters such as hemoglobin (Hb) concentration, tissue oxygen saturation (StO2), and tissue perfusion. Measures of vascular reactivity and endothelial function in the lower limbs can provide a rapid and direct method for assessing the extent of severity.

NCT05072483 — NATURAL HISTORY STUDY OF CADASIL

CADASIL is a monogenic small-vessel disease that affects the arteries in the brain. Clinical manifestations include migraines, recurrent strokes, and progressive white matter degeneration. Current diagnosis for CADASIL is through MRI imaging of the white matter, genetic testing, or through family history. Previous literature reported impaired cerebrovascular reactivity detected by MRI and transcranial doppler. NIRS is an attractive alternative method for assessing cerebrovascular reactivity because of its compact size and penetration depth. For the CADASIL cohort, optical probes will be affixed to the forehead and forearm to acquire information from the prefrontal cortex and skeletal muscle respectively. The optical measurement consists of a five-minute baseline period followed by a five-minute brachial cuff occlusion and a five-minute recovery period. A series of three end-exhalation breath holds follow the recovery period; patients will be asked to exhale and hold their breaths for either 30 seconds or for as long as they can tolerate. We anticipate that these assessments of cerebrovascular compared with skeletal muscle reactivity could provide a predictive test of disease progression.

NCT04595773 — COVID-19 - CHRONIC ADAPTATION AND RESPONSE TO EXERCISE (COVID-CARE): A RANDOMIZED CONTROLLED TRIAL

While COVID-19 is mainly perceived as a respiratory illness, there is also significant evidence of vascular complications, especially in those with severe symptoms and long-term effects. In collaboration with NHLBI's TVMB and the NIH Clinical Center's Rehabilitation Medicine Department, we have performed optical hemodynamic assessments in a cohort of patients recovering from COVID. The measurements are one

component of a larger randomized clinical study evaluating the efficacy of targeted exercise intervention to improve recovery from COVID. Patients recruited for this study are randomized into either a control or treatment arm. Patients in the treatment arm undergo a ten-week exercise regimen after their baseline visit, followed by a follow-up visit. Patients in the control arm continue with their typical daily routine for ten weeks after the baseline visit and return for a follow-up visit; patients then begin the ten-week exercise regimen followed by another follow-up visit. Patients recruited into the study undergo a brachial cuff occlusion and also participate in a set of breath-holding exercises. In a separate measurement session, patients undergo an exercise test during which optical measures are acquired before and after exercise. As the study is ongoing, analysis is currently limited to comparing optical measures to established clinical metrics. A more comprehensive analysis will be performed upon conclusion of the study.

Continuous blood pressure and vascular monitoring technologies for optimizing maternal health

Pre-eclampsia is a pregnancy-specific disorder responsible for more than 70,000 maternal deaths and 500,000 fetal deaths worldwide every year. While high blood pressure is the main clinical phenotype, pre-eclampsia can trigger several hemodynamic changes, including increased vascular stiffness and reduced vascular reactivity. While delivery can resolve most symptoms, they can persist postpartum and continue to impact the mother. Early diagnosis combined with treatment remains key to improving patient outcomes. However, the transition from gestational hypertension to mild pre-eclampsia to severe pre-eclampsia is a dynamic process; early diagnosis would ideally necessitate continuous, heightened surveillance. There is a need for technologies that can easily monitor the hemodynamic state of the mother and aid in the detection and risk stratification of pre-eclampsia.

Affixed transmission speckle analysis (ATSA) is a promising candidate to measure blood flow, vascular stiffness, and pulse transit time in a compact form-factor. To recover blood flow information, ATSA sends coherent light through a peripheral digit to measure intensity fluctuations caused by moving red blood cells. The high acquisition speed of ATSA enables the recovery of a pulsatile waveform originating from blood flow, referred to as the speckle plethysmograph (SPG). While previous work with pulse transit time evaluated the feasibility of PPG–based approaches, the SPG offers better signal quality than the photoplethysmograph (PPG), with SPG estimations of heart rate variability having improved accuracy over PPG estimations.

NCT05554315 — SPECKLE PLETHYSMOGRAPHY PULSE TRANSIT TIME AS A MARKER OF BLOOD PRESSURE CHANGES

Launched earlier this year, this healthy volunteer protocol seeks to investigate ATSA/SPG for cuff-free blood pressure estimation. Participants are asked to undergo a number of challenges designed to perturb blood pressure while being monitored by PPG and SPG sensors alongside ECG and a continuous noninvasive arterial pressure monitor.

Optical techniques for the evaluation of brain metabolism in neurocognitive disorders

Neurocognitive diseases, which can have devastating effects, often affect how the brain uses oxygen. While imaging techniques capable of assessing cerebral blood flow and metabolism, such as FDG-PET (18-fluorodeoxyglucose positron emission tomography), have been used to evaluate disease severity and monitor progression, these modalities are resource-intensive and may use radiation, which make them

FIGURE 3. Qualitative comparison between SPG and PPG during vasoconstriction

Left. Normalized SPG (speckle plethysmograph) during baseline (*solid black line*) and cold pressor test (*dashed gray line*).

Right. Normalized PPG (photoplethysmograph) during baseline (*solid blue line*) and cold pressor test (*dashed blue line*). The SPG waveform exhibits much higher signal quality, which is preserved during the cold pressor test. The cold pressor test triggers a sympathetic activation that leads to vasoconstriction, which severely impacts the PPG waveform.
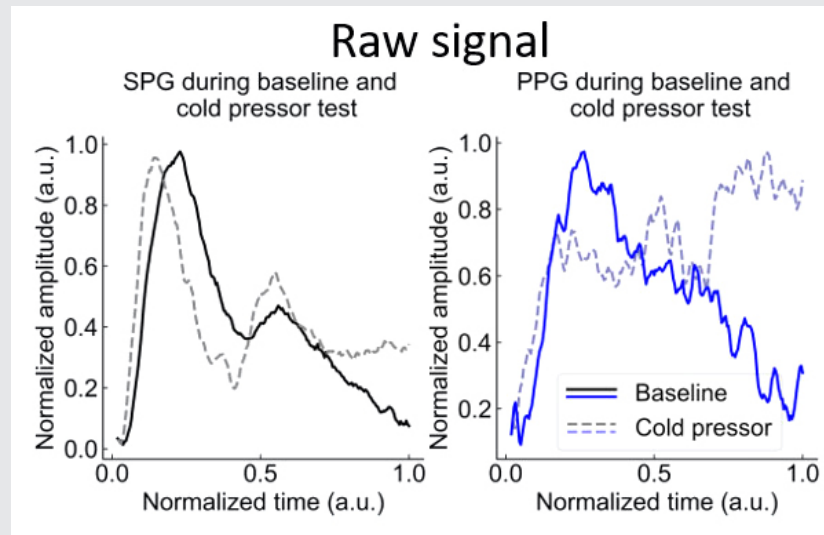
unsuitable for researching these disorders in children. Given these limitations, there is a paucity of data regarding brain metabolic function in childhood neurocognitive disorders. New methods are needed to non-invasively characterize these disorders and improve evaluation of disease severity, progression, and therapy monitoring.

Diffuse correlation spectroscopy (DCS) is an emerging non-invasive optical technique that uses the light fluctuations of moving particles to quantify blood flow deep in tissue. When coupled with advanced NIRS methods that characterize tissue absorption and oxygenation, the total metabolic rate of oxygen consumption can be recovered. In collaboration with Forbes Porter, Samar Rahhal, and Amir Gandjbakhche, we are exploring the feasibility of using NIRS and DCS to provide a non-invasive, non-sedative method for functional brain imaging of neurocognitive disorders. A pilot study was launched this year that uses these technologies to study a number of disorders that are extensively followed by NICHD clinicians: Niemann-Pick disease type C1 (NPC1), Smith Lemli Opitz syndrome (SLOS), and Juvenile Neuronal Ceroid Lipofuscinosis (CLN3).

[NCT05642221](#) — PILOT STUDY OF THE USE OF FUNCTIONAL NEAR–INFRARED SPECTROSCOPY (FNIRS) COMBINED WITH DIFFUSE CORRELATION SPECTROSCOPY (DCS) IN NEUROCOGNITIVE DISEASE AS COMPARED TO HEALTHY NEUROTYPICAL CONTROLS.
To date, over 30 subjects have been enrolled in this study. While preliminary results are limited, they support findings of hypometabolism in NPC patients that have been previously reported.

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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 growth, patterning, and morphogenesis, regulation of vascular integrity, assembly of the lymphatic system, and the roles of novel vascular-associated cells.

In addition to our work on vessel development, as a second major effort, 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 during development and regeneration, using a novel "EpiTag" epigenetic reporter line and genetic screens for tissue-specific vertebrate epigenetic regulators.

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

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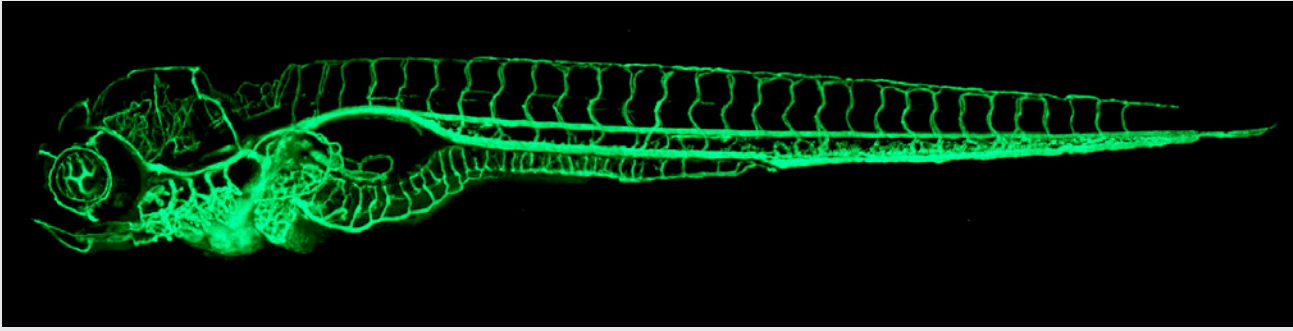Jean Prosper Santiago, BS, *Postbaccalaureate Fellow*

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.
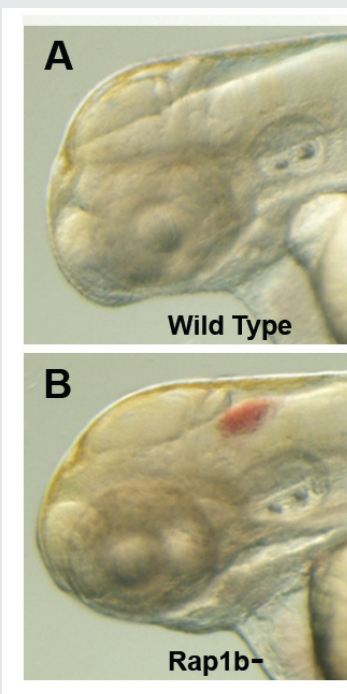

FIGURE 2. Intracranial hemorrhage (ICH) in the developing zebrafish

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*).

insights into mechanisms of blood vessel formation have come from studies in model organisms, including the zebrafish. We are carrying out several projects using fish, described below.

NEW TOOLS FOR EXPERIMENTAL ANALYSIS OF VASCULAR DEVELOPMENT

We generate novel transgenic lines for visualizing different types of endothelial and perivascular cells, and for driving gene expression or performing molecular profiling of mRNAs in these cell populations.

GENETIC ANALYSIS OF VASCULAR DEVELOPMENT

We have identified novel mutants affecting vascular development in our transgene-assisted forward-genetic screens and are characterizing the phenotypes and molecular basis for several of the mutants.

ANALYSIS OF VASCULAR SPECIFICATION, PATTERNING, MORPHOGENESIS, AND FUNCTION

We are studying the development and function of several vascular beds, including the vasculature of the gills, the fish equivalent of the mammalian lungs, which contains unique endothelial cell populations with important roles in gas exchange.

Regulation of vascular integrity

We are using the zebrafish to understand the cellular and molecular mechanisms responsible for proper vessel morphogenesis, for the generation and maintenance of vascular integrity, and for proper repair of the vasculature after injury. Disruption of vascular
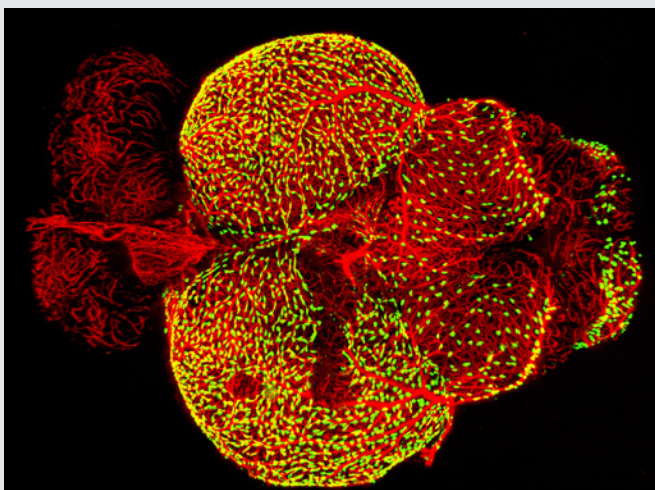
FIGURE 3. Novel perivascular cells on the zebrafish brain

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.

integrity is associated with hemorrhagic stroke, a severe and debilitating form of stroke associated with high morbidity and mortality. Meningeal vascular dysfunction is also associated with neuro-cognitive deficits and neurodegenerative disease. Vessels play important roles in supporting wound healing and recovery and regeneration of tissues and organs after injury, including the skin. Many recent insights into molecular mechanisms regulating the vasculature in health and disease have come from studies in model organisms such as the zebrafish. We are pursuing several related projects.

GENES REGULATING VASCULAR INTEGRITY

We have used forward-genetic screens to identify mutants that disrupt cranial vascular integrity in the zebrafish (Figure 2), and next-gen sequencing methods to identify the defective genes from these mutants, including *GDF6* (encoding growth differentiation factor 6,) and *RHOA* (encoding a small GTPase protein), uncovering their roles in vascular integrity and angiogenesis.

REVASCULARIZATION AFTER CUTANEOUS INJURY

Vascular regrowth and remodeling are critical for proper wound healing, and defective vessel growth in cutaneous wounds is associated with delayed wound repair and/or chronic open wounds susceptible to infection. We developed a new zebrafish model of vascular regrowth and repair after cutaneous injury in adult zebrafish, and are using this model to understand the cellular and molecular changes that lead to defective vascular responses to injury and poor wound healing in aging or diabetes, with an eye toward uncovering new therapeutic targets to promote improved revascularization and healing in these contexts.

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 the tissue's importance, the cell types present, and its function and embryonic origins are still not well understood. We recently discovered and characterized zebrafish fluorescent granular perithelial cells (FGPs), a novel endothelium-derived perivascular cell population closely associated with meningeal blood vessels, which likely plays a critical role in meningeal function (Figure 3). As discussed further below, we also discovered
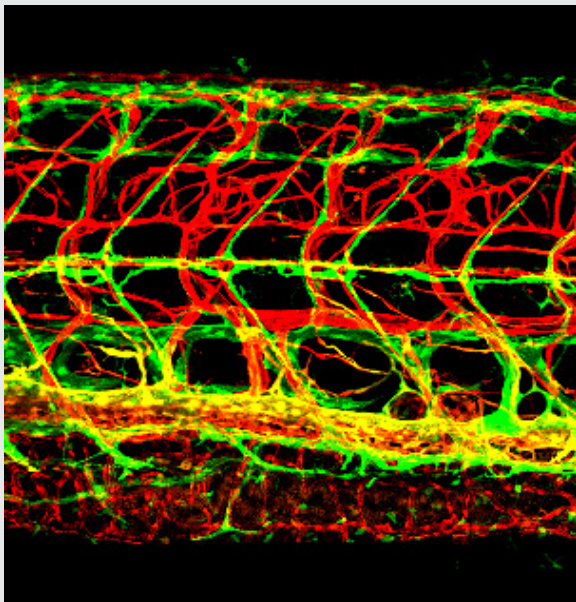
FIGURE 4. Novel lymphatic vascular reporter

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.

a *bona fide* meningeal lymphatic vascular network in the zebrafish. We are currently carrying out additional anatomical and molecular studies to understand the structure and cellular composition of the zebrafish meninges and the function of FGPs, meningeal lymphatics, and other novel meningeal cell populations in health, injury, and disease.

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 mammalian model organisms. Our studies demonstrated that zebrafish possess a lymphatic system with 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 have further studied the zebrafish lymphatic system through several projects.

1. We generated transgenic lines that permit specific visualization, tissue-specific molecular profiling, and functional manipulation of lymphatic vessels, and we are using these transgenic animals to further characterize lymphatic development and function (Figure 4).
2. We characterized and studied novel microRNAs expressed in the lymphatic endothelium and how these small regulatory RNAs influence lymphatic gene expression and lymphatic development.
3. We discovered a previously unreported lymphatic network in the dural meninges of the zebrafish (Figure 5). Like similar recently discovered meningeal lymphatics surrounding the mammalian brain, the zebrafish network likely plays a critical role in maintaining homeostasis and protecting the brain from
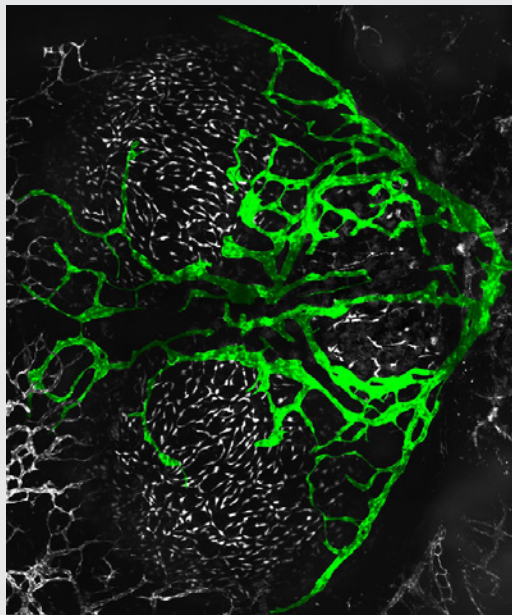
FIGURE 5. Adult zebrafish meningeal lymphatic network

Confocal image of the dorsal head of a *Tg(mrc1a:egfp)^y251^, casper* (transparent) adult zebrafish, with mrc1a+FGPs and superficial lymphatics in grey and intracranial meningeal lymphatics pseudo-colored green. Anterior is to the left, right is up.

mechanical trauma and infection, and we are studying these critical vessels and their role in immune cell interaction and trafficking in homeostasis and following traumatic brain injury (TBI), using new zebrafish models of meningeal TBI that we developed.

By combining the genetic and experimental tools available in the zebrafish with the ability to perform high-resolution microscopic imaging of vascular structures in living animals, our studies are providing important new insights into the origins and growth of the lymphatic system and molecular mechanisms that are critical both during lymphatic development and in adult lymphatic pathologies.

Epigenetics of development

We are using the genetically and experimentally accessible zebrafish and Mexican tetra *(Astyanax mexicanus)* models to uncover the molecular basis for organ- and tissue-specific epigenetic regulation during development and regeneration through several 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 performed the first large-scale F3 genetic screen in a vertebrate to identify recessive mutants in regulators of epigenetic gene silencing or activation (Figure 6). Among other mutants, the screen yielded epigenetic regulators of liver development, pharynx development, and arterial differentiation, and we are currently pursuing follow-up studies on these mutants.

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 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
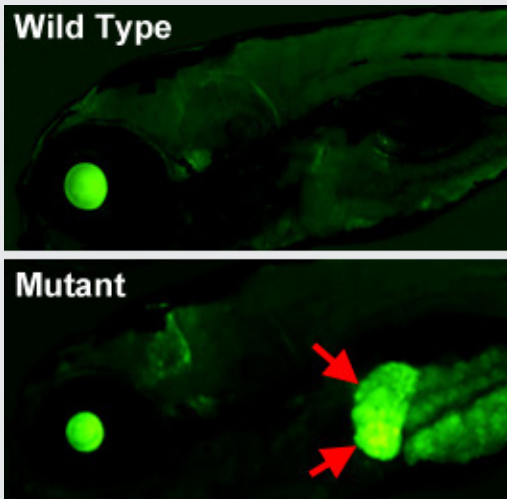
FIGURE 6. An epigenetic silencing mutant in the zebrafish

Lateral views of the head and anterior trunk of a wild-type (*top*) and tissue-specific epigenetic silencing mutant (*bottom*) zebrafish. The mutation 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.

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 molecular targets and, potentially, to the design of personalized, epigenetic-based therapies to alleviate the enormous burden of diabetic vasculopathy.

EPIGENETICS OF VASCULAR CHANGES IN CAVEFISH

In addition to eye and pigment loss and other adaptations, *Astyanax* cavefish (Figure 7) have unusual vascular and metabolic adaptations that allow them to survive hypoxic conditions, chronic and long-term food deprivation, and cold. As we recently showed for the loss of eyes, changes in epigenetic regulation may
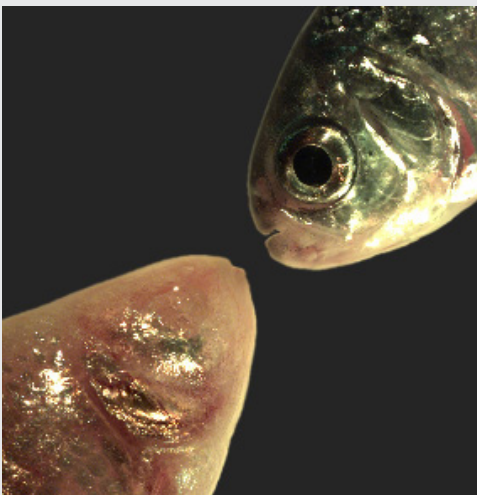

FIGURE 7. Mexican tetra cave- and surface fish

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 lab suggest that altered DNA methylation and resulting coordinated changes in the expression of large sets of genes have helped drive at least some of this rapid evolutionary change.

underlie the rapid evolution of cavefish vascular and metabolic adaptations. We are generating new transgenic tools that will allow us to use imaging and transcriptional and epigenetic profiling to investigate differences in vascular endothelial cells in cavefish compared with surface morphs of *Astyanax.* We will follow up on these findings to elucidate how differential epigenetic signatures influence divergent vascular endothelial behavior in surface and cave morphs.

Additional Funding

Publications

1. Pillay LM, Yano JJ, Davis AE, Butler MG, Ezeude MO, Park JS, Barnes KA, Reyes VL, Castranova D, Gore AV, Swift MR, Iben JR, Kenton MI, Stratman AN, Weinstein BM. In vivo dissection of Rhoa function in vascular development using zebrafish. *Angiogenesis* 2022 25:411–434.
2. Paulissen SM, Castranova DM, Krispin SM, Burns MC, Menéndez J, Torres-Vázquez J, Weinstein BM. Anatomy and development of the pectoral fin vascular network in the zebrafish. *Development* 2022 149:dev199676.
3. Castranova D, Samasa B, Venero Galanternik M, Gore AV, Goldstein AE, Park JS, Weinstein BM. Long-term imaging of living adult zebrafish. *Development* 2022 149:dev199667.
4. Olayinka O, Weinstein BM, Jung H-M. Development and structure of the lymphoid system. In: *Clinical Immuno-Oncology. J. E. Niederbuber, Ed. Elsevier Press, New York* 2022 95–103.
5. Tamaoki J, Maeda H, Kobayashi I, Takeuchi M, Ohashi K, Gore A, Bonkhofer F, Patient R, Weinstein BM, Kobayashi M. LSD1 promotes the egress of hematopoietic stem and progenitor cells into the bloodstream during the endothelial-to-hematopoietic transition. *Dev Biol* 2023 501:92–103.
6. Greenspan LJ, Ameyaw K, Castranova D, Mertus CA, Weinstein BM. Live imaging of cutaneous wound healing in zebrafish. *J Invest Dermatol* 2023 doi: 10.1016/j.jid.2023.10.015.

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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.

Prokaryotic studies

STRAND SPECIFICITY OF RIBONUCLEOTIDE EXCISION REPAIR IN *E. COLI*

In *Escherichia coli,* replication of both strands of genomic DNA is carried out by a single replicase: DNA polymerase III holoenzyme (pol III HE). However, in certain genetic backgrounds, the low-fidelity TLS polymerase, DNA polymerase V (pol V), gains access to undamaged genomic DNA, where it promotes elevated levels of spontaneous mutagenesis preferentially on the lagging strand. As part of a collaboration with scientists at the Polish Academy of Sciences in Warsaw, Poland, we employed active-site mutants of pol III (pol III alpha_S759N) and pol V (pol V_Y11A) to analyze ribonucleotide incorporation and removal from the *E. coli* chromosome on a genome-wide scale under conditions of normal replication, as well as SOS induction. Using a variety of methods tuned to the specific properties of these polymerases (e.g., analysis of *lacI* mutational spectra, *lacZ* reversion assay, HydEn-seq, and alkaline gel electrophoresis), we

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presented evidence that repair of ribonucleotides from both DNA strands in *E. coli* is unequal. While RNase HII plays a primary role in leading-strand ribonucleotide excision repair (RER), the lagging strand is subject to other repair systems (RNase HI and, under conditions of SOS activation, also nucleotide excision repair). Importantly, we suggested that RNase HI activity can also influence the repair of single ribonucleotides incorporated by the replicase pol III HE into the lagging strand.

IDENTIFICATION OF AN INHIBITOR OF LEXA CLEAVAGE

As antibiotic resistance has become more prevalent, the social and economic impacts are increasingly pressing. Indeed, bacteria have developed the SOS response, which facilitates the evolution of resistance under genotoxic stress. The transcriptional repressor LexA plays a key role in this response. Mutation of LexA to a non-cleavable form that prevents the induction of the SOS response sensitizes bacteria to antibiotics. Achieving the same inhibition of proteolysis with small molecules also increases antibiotic susceptibility and reduces acquisition of drug resistance. Previous attempts to develop inhibitors have investigated 1,2,3-triazole molecules binding to the hydrophobic cleft, and boronic acids that covalently bound to Ser-119. Neither of these resulted in any molecules progressing to preclinical trials. In collaboration with scientists at the Queensland Institute of Technology in Brisbane, Australia, we found that the cleavage site region (CSR) of the LexA protein is a classical Type II beta-turn, and that published 1,2,3-triazole compounds mimic the beta-turn. Based on this, we took a dual approach to the identification of a novel proteolytic inhibitor. We docked generic covalent molecule libraries and a β-turn–mimetic library to the LexA C-terminal domain using molecular modelling methods in FlexX and CovDock. The 133 highest scoring molecules were screened for their ability to inhibit LexA cleavage under alkaline conditions and the top molecules were then tested using a RecA–mediated counter assay, research that led to the discovery of an electrophilic serine warhead that can inhibit LexA proteolysis, reacting with Ser-119 via a nitrile moiety. Our studies therefore present a starting point for hit-to-lead optimization, which could lead to inhibition of the SOS response and prevent the acquisition of antibiotic resistance.

CHARACTERIZATION OF THE MYCOBACTERIAL MUTASOME

A DNA damage–inducible mutagenic gene cassette has been implicated in the emergence of drug resistance in *Mycobacterium tuberculosis* during anti-tuberculosis (TB) chemotherapy. However, the molecular composition and operation of the encoded "mycobacterial mutasome," minimally comprising DnaE2 polymerase and ImuA' and ImuB accessory proteins, remain elusive. As part of a large international collaboration led by our collaborator Digby Warner, we exposed mycobacteria to DNA–damaging agents and observed that DnaE2 and ImuB co-localize with the DNA polymerase III beta subunit (beta clamp) in distinct intracellular foci. Notably, genetic inactivation of the mutasome in an *imuB* mutant containing a disrupted beta clamp–binding motif abolishes ImuB–beta clamp focus formation, a phenotype recapitulated pharmacologically by treating bacilli with griselimycin, as well as in biochemical assays in which this beta clamp–binding antibiotic collapses pre-formed ImuB–beta clamp complexes. These observations established the essentiality of the ImuB–beta clamp interaction for mutagenic DNA repair in mycobacteria and identified the mutasome as a target for adjunctive therapeutics designed to protect anti–TB drugs against emerging resistance.

Publications

1. Łazowski K, Faraz M, Vaisman A, Ashton NW, Jonczyk P, Fijalkowska IJ, Clausen AR, Woodgate R, Makiela-Dzbenska K. Strand specificity of ribonucleotide excision repair in Escherichia coli. *Nucleic Acids Res* 2023 51(4):1766–1782.

2. Schuurs ZP, McDonald JP, Croft LV, Richard DJ, Woodgate R, Gandhi NS. Integration of molecular modelling and in vitro studies to inhibit LexA proteolysis. *Front Cell Infect Microbiol* 2023 13:1051602.

3. Gessner S, Martin ZA, Reiche MA, Santos JA, Dinkele R, Ramudzuli A, Dhar N, de Wet TJ, Anoosheh S, Lang DM, Arron J, Chew TL, Herrmann J, Müller R, McKinney JD, Woodgate R, Mizrahi V, Venclovas Č, Lamers MH, Warner DF. The mycobacterial ImuA'-ImuB-DnaE2 mutasome: composition and recruitment in live cells. *Elife* 2023 12:e75628.

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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 endo-phenotypes 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 have obesity or are in the "pre-obese" state, permitting 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 these approaches will improve our ability to predict which children are at greatest risk for obesity and its comorbid conditions and will lead to more targeted, etiology-based prevention and treatment strategies for pediatric obesity.

Genetic factors important for childhood body–weight regulation

To identify gene variants affecting body composition, we have been examining polymorphisms in genes involved in the leptin signaling pathway. 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 the 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
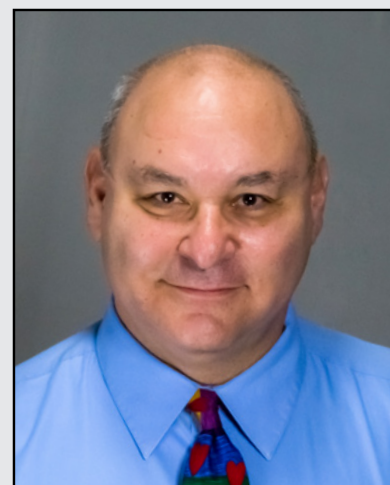
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mass and higher plasma levels of insulin and leptin than unaffected or heterozygous children and appear to eat more at laboratory test meals (Figure 1). *In vitro* studies subsequently found that signal transduction and protein expression were significantly lower for the double-mutant (DM) *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 (WT) and the human DM MC3R. Using homozygous knock-in mouse models replacing murine *Mc3r* with WT human (*MC3R*$^{hWT/hWT}$) and DM (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 MC3R plays in stem-cell fate, and how variations in *Mc3r* may alter signaling of several downstream signaling pathways. Using tissue-specific knockout and reactivation models, we are also studying the importance of hepatic and adipose tissue MC3R for whole body homeostasis.

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Physiology, metabolism, and psychology of childhood body-weight regulation

Our studies are directed at understanding the physiological, psychological, and metabolic factors that place children at risk for undue weight gain. As part of these studies, we examined how best to measure 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 previously found that leptin is an important predictor of weight gain in children and identified children with hyperleptinemia and proximal leptin-signaling pathway 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 have led to recent explorations of other syndromes associated with obesity that may cause dysregulation of leptin signaling, including WAGR (Wilms tumor-aniridia genitourinary anomalies and mental retardation), Bardet-Biedl, and Alström syndromes [Reference 1]. Our 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, negative affective states such as depression and anxiety, food reinforcement and food-related inhibitory control [Reference 2], alexithymia, executive functioning, and LOC eating. Some recent

FIGURE 1. Study of energy intake, 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).
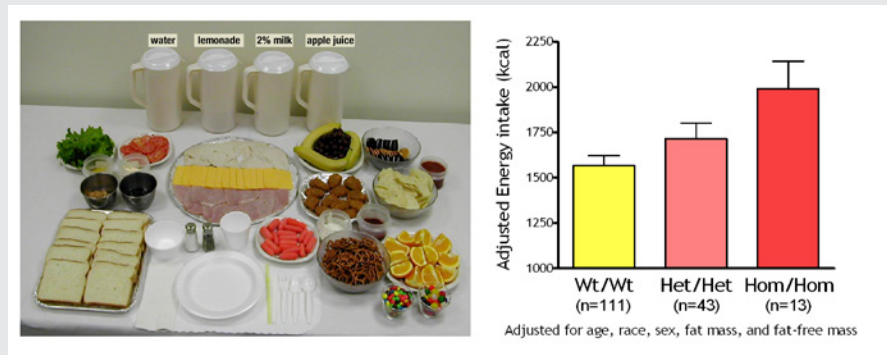
initiatives have targeted insulin resistance in girls at high risk for type 2 diabetes because of obesity and a family history of diabetes.

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors are also associated with adiposity in children and abnormalities in metabolism. We have found that certain eating patterns may predict future weight gain in children at risk for obesity: children reporting binge-eating behaviors such as LOC over eating gained, on average, an additional 2.4 kg of weight per year compared with non-binge-eating children. Our data also suggest that children endorsing binge eating consume more energy during meals. Actual intake during buffet meals averaged 400 kcal more in children with binge eating, but despite their greater intake, such children reported shorter-lived satiety than children without binge-eating episodes. The ability to consume large quantities of palatable foods, especially when coupled with 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 have significantly higher serum leptin and are at significantly greater risk for worsening of components of the metabolic syndrome compared

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*).
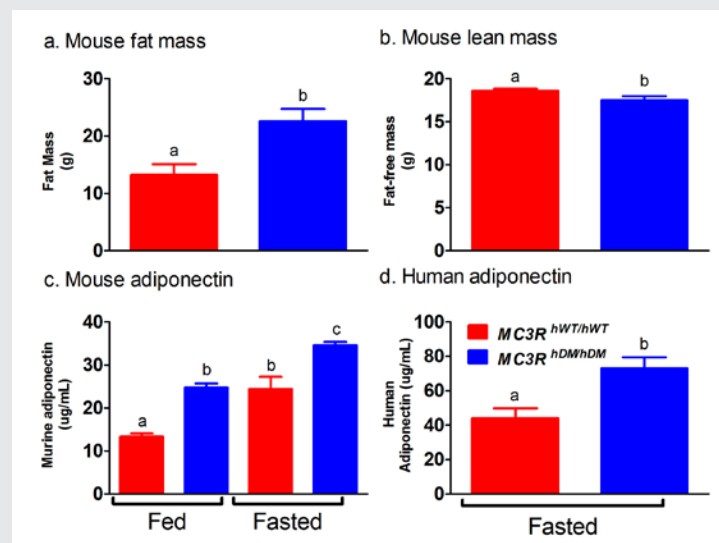
FIGURE 3. Loss of control (LOC) eating and metabolic complications in a longitudinal study

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 ($P = 0.02$).
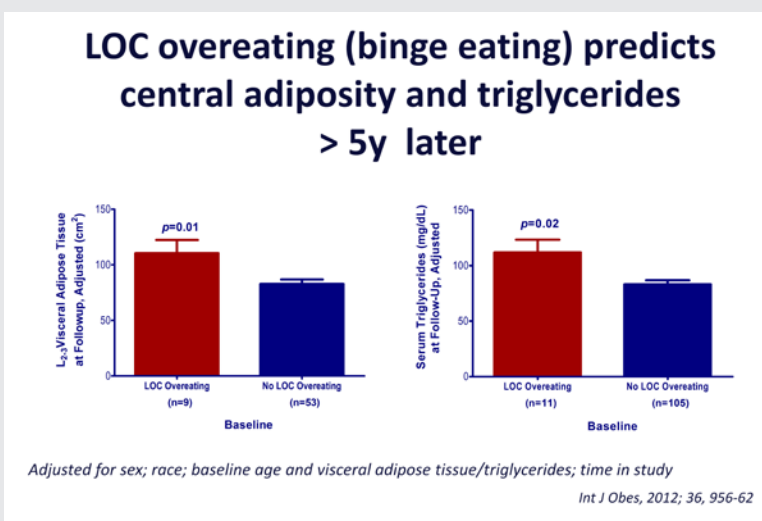
with those without LOC episodes, even after adjusting for adiposity and other relevant covariates. Our data also suggest that anxiety symptoms may interact with LOC eating to become an important co-factor for excessive weight gain among children. These data also suggest that interventions targeting disordered 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.

We study normal-weight children and adolescents, children who already have obesity, and the children of parents with obesity who do not have obesity, in order to determine the factors that are most important for development of the complications of obesity in youth. We examine body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, energy expenditure [Reference 3], 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 are following 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) and in a 5-day parallel groups design, we found that glucose homeostasis was markedly improved in children with overweight or obesity who engaged in moderate activity for just three minutes every half hour, versus remaining sedentary.

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

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*).
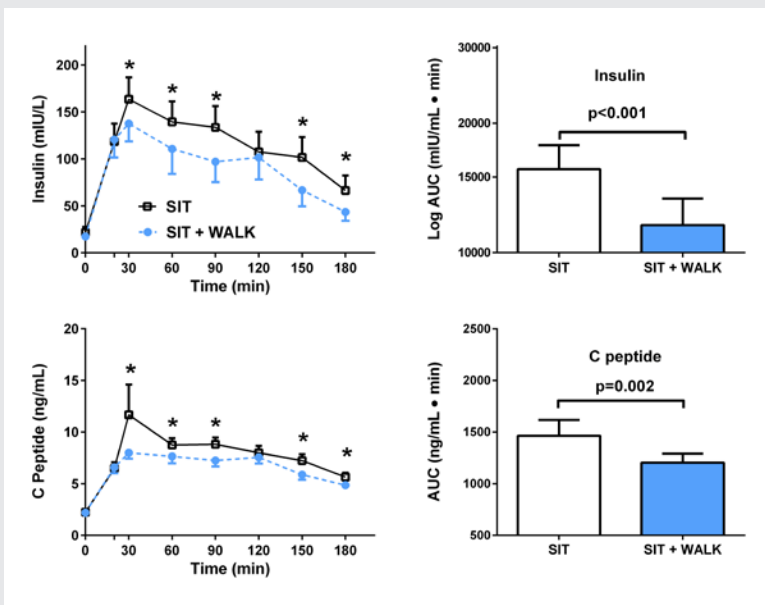

excessive body weight. We completed a randomized controlled trial to examine the mechanism by which metformin may affect the body weight of younger children who have hyperinsulinemia and are therefore at risk for later development of type 2 diabetes. Compared with placebo-treated children, those randomized to metformin significantly decreased BMI, BMI-Z score, and body fat mass. Serum glucose and HOMA-IR (homeostasis model assessment of insulin resistance) also declined significantly more in metformin-treated than in placebo-treated children. A second study examined the prevention of weight gain using interpersonal therapy (IPT) versus a control health education program (HE) in adolescents reporting LOC eating behaviors. At three-year follow-up, baseline social-adjustment problems and trait-anxiety moderated outcome. Among girls with high self-reported baseline social-adjustment problems or anxiety, IPT was associated with the steepest declines in BMIz compared with HE. For adiposity, girls with high or low anxiety in HE and girls with low anxiety in IPT experienced gains, while girls in IPT with high anxiety stabilized. Parent reports yielded complementary findings. The results have stimulated ongoing research to examine how anxiety may stimulate energy intake. 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, 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 1-year change. An ongoing study, based on lab data finding links between attentional biases to high-palatability foods in children with obesity, investigates whether adolescents' attentional biases can be retrained. We also studied the effects of modulating the leptin signaling pathway with the melanocortin agonist setmelanotide in patients with proximal signaling defects (patients with Bardet Biedl syndrome) [Reference 1]. Most recently, we completed another study of specific pharmacotherapy for patients with Prader-Willi syndrome, using diazoxide choline extended release [Reference 4]. These latest trials are examples of precision medicine approaches to treat obesity. We also completed a novel randomized controlled pilot trial of colchicine to ameliorate the inflammation of obesity and thus improve its complications

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.
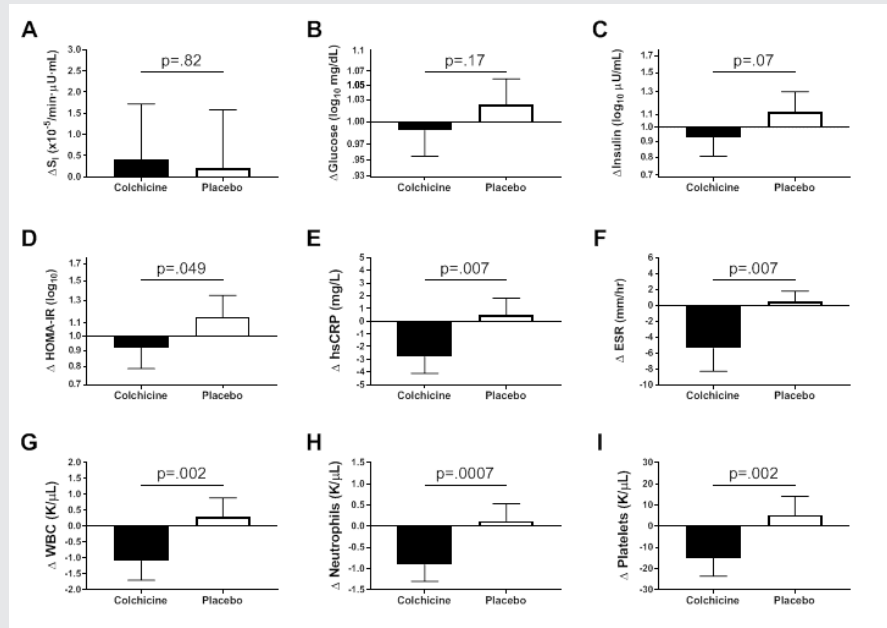
[Reference 5]. Adults with obesity and metabolic syndrome, but who did not have diabetes, were randomized to colchicine 0.6 mg or placebo capsules twice daily for three months. Compared with placebo, colchicine significantly reduced C-reactive protein and erythrocyte sedimentation rate (Figure 5). The 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. Our group is 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

Publications

1. Haqq A, Chung WK, Dollfus H, Haws R, Martos-Moreno GA, Poitou C, Yanovski JA, Mittleman RS, Yuan G, Forsythe E, Clément K, Argente J. Efficacy and safety of setmelanotide, a melanocortin-4 receptor agonist, in patients with Bardet-Biedl syndrome and Alström syndrome: a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial with an open-label period. *Lancet Diabetes Endocrinol* 2022 10(12):859–868.

2. Loch LK, Tanofsky-Kraff M, Parker MN, Haynes HE, Te-Vazquez JA, Bloomer BF, Lazareva J, Moursi NA, Nwosu EA, Yang SB, Turner SA, Brady SM, Bowling AI, Chen KY, Yanovski JA. Associations of food reinforcement and food-related inhibitory control with adiposity and weight gain in children and adolescents. *Physiol Behav* 2023 266:114198.
3. Speakman JR, de Jong JMA, Sinha S, Westerterp KR, Yamada Y, Yanovski JA, Schoeller DA, Wong WW. Total daily energy expenditure has declined over the last 3 decades due to declining basal expenditure not reduced activity expenditure. *Nat Metab* 2023 5(4):579–588.
4. Miller JL, Gevers E, Bridges N, Yanovski JA, Salehi P, Obrynba KS, Felner EI, Bird LM, Shoemaker AH, Angulo M, Butler MG, Stevenson D, Abuzzahab J, Barrett T, Lah M, Littlejohn E, Matthew V, Cowen NM, Bhatnagar A. Diazoxide choline extended-release tablet in people with Prader-Willi syndrome: a double-blind, placebo-controlled trial. *J Clin Endocrinol Metab* 2023 108(7):1676–1685.
5. Patel TB, Levine JA, Elizondo DM, Arner BE, Jain A, Saxena A, Lopez-Ocasio M, Dagur PK, Famuyiwa O, Gupta S, Sarrafan-Chaharsoughi Z, Biancotto A, McCoy JP, Demidowich AP, Yanovski JA. Immunomodulatory effects of colchicine on peripheral blood mononuclear cell subpopulations in human obesity: data from a randomized controlled trial. *Obesity (Silver Spring)* 2023 31(2):466–478.

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Membrane Interactions Underly Viral Spike Protein Porations, Microvesicle Assembly Sites on Umbilical Cord Endothelial Cells, and Glioblastoma Cell Migration.

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, i.e., 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.

Extracellular vesicles that secrete exosomes from internal multivesicular bodies

Signaling between cells, mediated by secreted membrane-enclosed organelles called extracellular vesicles (EVs), is a widespread form of intercellular communication, evolutionarily conserved from bacteria to plants and animals. Cells load EVs with a range of bioactive cargos, including lipids, membrane proteins, adhesion proteins, cytoskeletal elements, enzymes, signaling molecules, and nucleic acids. Once released into the extracellular milieu, EVs can signal locally or travel long distances in body fluids, such as blood, lymph, cerebrospinal fluid, amniotic fluid, to act on remote tissue targets. Upon reaching recipient cells, specific interactions between EVs and target cells promote binding and uptake by pinocytosis, phagocytosis, endocytosis, or direct fusion with the plasma membrane. EV–mediated intercellular signaling is a ubiquitous mechanism occurring under physiological and disease states. Plasma EVs are proving to be sensitive biomarkers of numerous disease states, such as cancer, and can be obtained by a liquid biopsy. Furthermore, EVs are being developed as vehicles for delivery of therapeutic agents. Despite these important physiological functions and medical utilities, much remains to be discovered about the biosynthesis of EVs.

It is generally viewed that EVs fall into two categories based on their site of biogenesis. Microvesicles arise at the plasma membrane,

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by outward budding and pinching off directly from the cell surface. In contrast, exosomes are of endosomal origin, produced when intraluminal vesicles (ILVs) contained in multivesicular bodies (MVBs) are exocytosed upon MVB fusion with the plasma membrane. Endothelial cells form the endothelium, a single cell–thick lining of the blood and lymphatic vessels, which controls the exchange of oxygen and nutrients between the vessel contents and underlying tissues. In their role on the 'front lines' of vessels, exposed to circulating body fluids, endothelial cells release a significant proportion of the EVs found in blood. EVs released by the endothelium contribute to its role in supporting vascular homeostasis, which includes maintenance of the anti-thrombogenic surface of the vessels (blood fluidity) and vasodilation, inhibition of inflammation, cell survival, and angiogenesis. To gain a better understanding of the mechanisms and structural aspects of EV release from endothelial cells under pro-angiogenic but non-inflammatory conditions, we used thin-section electron microscopy (EM) to examine HUVECs (human umbilical vein endothelial cells), and look for structural features consistent with microvesicle budding from the plasma membrane and exocytic release of exosomes from MVBs. Cells were preserved by ultra-fast freezing, which is optimal for capturing fast events such as exocytosis, and processed by a freeze-substitution protocol optimized for plasma membrane enhancement.

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In thin sections, groups of protrusions were observed on the otherwise smooth HUVEC plasma membrane that were often branched and contained vesicular organelles, including MVBs with ILVs. Beyond cell peripheries, vesicles that contained MVB–like vesicles were observed, suggesting that they were microvesicles that had pinched off from the protrusions, diffused, and occasionally adhered to the coverslip. Serial sections through the presumptive microvesicles on the coverslip confirmed that they were not connected to cells by cellular extensions and that ILV–like vesicles were within the MVB–like vesicles. Further examination revealed omega figures, the structural hallmarks of exocytosis, occurring between MVB–like vesicles inside the microvesicles and their limiting membrane. On occasion, such omega figures contained small vesicles that were identical to ILVs. These observations support the notion that microvesicles containing many membrane compartments (referred to as multi-compartmented microvesicles, or MCMVs, a newly discovered class of microvesicles that bud from cellular protrusions clustered on the plasma membrane of HUVECs) pinch off from MVB–containing protrusions at specialized sites on the cell surface. MCMVs contain MVBs that apparently can release exosomes after transiting away from the parent cell.

The cellular membrane protrusions contain vesicular cargo that, when compared with cytoplasmic organelles, could be identified as MVBs with ILVs, endosomes (round, tubular, and clathrin-coated), ER, and mitochondria. Serial sections showed that the protrusions are a few hundred nanometers to 1 micron thick and in some cases extended up in the Z axis relative to the coverslips for more than approximately 1200 nm, beyond the scope of the serial sections analyzed. Protrusions were often branched and intermingled. At branch points and connections to the cell, the protrusions often became constricted to thin necks of 65–200 nm. Exploration of the coverslip surface between cells revealed MCMVs that were immobilized on the coverslip and contained MVB–like vesicles containing ILV–like vesicles, in addition to other vesicle types. The direct observation of omega figures joining membranes of internal vesicles of MCMVs with the peripheral membrane of MCMVs, and the many images of ILV–like vesicles in and immediately adjacent to the fusion pore of omega figures, and associated with the periphery of MCMVs, together suggest to us that ILVs can be released from MCMVs, a function akin to the exocytosis of ILVs from cells. Given that this could only be possible if MCMVs contain
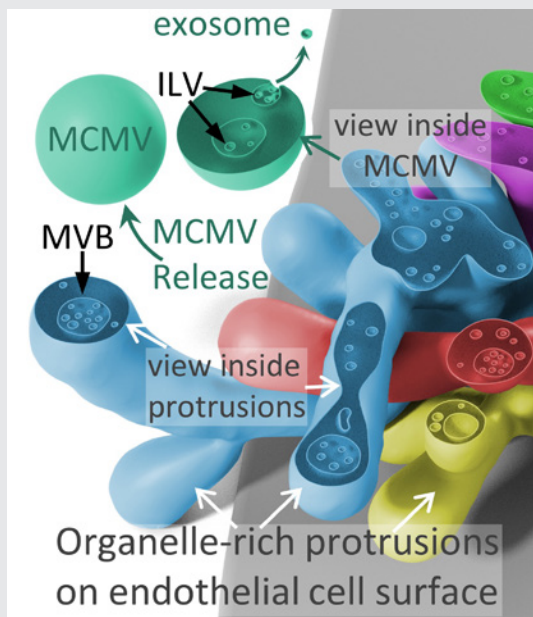
FIGURE 1. Artistic representation based on data sets of a protrusion site on a cultured endothelial cell and omega figures on an MCMV a distance from a cell

(*a*) Depiction of several protrusions clustered on a cultured endothelial cell surface, and a nearby pinched off MCMV (multi-compartmented microvesicles) floating in the extracellular milieu. A slice plane in the *en face* orientation relative to the coverslip is represented by the dashed line.

(*b*) A view at a slightly different angle shows that the protrusions are often branching.

(*c*) A slice through the protrusions and the MCMV shows internal round and tubular vesicular organelles, including MVBs containing ILVs, inside the protrusions and the MCMV. An omega figure on the MCMV–limiting membrane indicates exosome secretion from the MCMV.

compartments akin to MVBs of cells, such a hypothesis would be unique to MCMVs. Preservation, by fast-freezing, of omega figures on the MCMV–limiting membrane showed a lack of any membrane coat. Thus, these omega figures are not the result of any coat-mediated endocytosis such as those mediated by clathrin or caveolin. It also seems unlikely, but not impossible, that ILV–sized vesicles are captured and internalized into MCMVs from the relatively vast volume of culture medium. Taken together, these observations suggest a novel pathway by which a subset of exosomes are released from a transiting MCMV after pinching off from a protrusion on the HUVEC surface.

A challenge for the study of EVs has been the isolation of EV subpopulations. Though possessing different sites of origin, microvesicles and exosomes share overlapping size ranges, molecular compositions, and densities, rendering biochemical enrichment and characterization of EV subsets a challenge. Our findings suggest that part of the difficulty may arise from EVs that consist of exosomes inside of microvesicles. Their presence could go unrecognized or be misinterpreted as apparent overlap in biophysical properties. Moreover, attempts to separate microvesicles from exosomes may prove futile when MCMVs are both. Of note, the *in vitro* cultivated HUVECs used in this study differ in some respects from tissue vascular endothelium, and future studies are needed to explore these findings in more a physiological system. The glycocalyx of HUVECs may differ from those in tissue, and an ILV could have more trouble traversing the glycocalyx of a tissue cell once released. Also, the vascular endothelium in tissue is not proliferating, whereas HUVECs in culture are exposed to several angiogenic growth factors in the medium and replicate many features of endothelial cells undergoing an angiogenic response. Angiogenic responses are known to alter the production and contents of endothelial-derived EVs, and EVs produced by endothelial cells can regulate angiogenic responses. The EV production we studied may be relevant to their role in angiogenesis. Furthermore, the HUVECs analyzed were sub-confluent to better observe potential sites of EV biogenesis on the cell peripheries; possibly, release of MCMVs occurs in response to a wound-like state to influence wound healing. Future studies are needed to determine whether MCMVs are released from confluent endothelial cells *in vivo*.

In summary, we have described a domain of protrusions extending from the plasma membrane of HUVECs that contain membrane-bound organelles including MVBs. MCMVs bud from the protrusions and contain vesicular compartments, including MVBs that can fuse with the MCMV–limiting membrane and release exosomes. This implies that the function of MCMVs is signaling rather than removal of cellular material, as has been proposed for exophers (membrane-bound EVs that are released by budding out of cells into the extracellular space) and migrasomes (EVs that are formed in migrating cells and mediate extracellular communication). To be functional as signaling entities, EVs must deliver messages, in the form of bioactive molecules, to recipient cells. Packaging cargos inside multiple layers of membrane, rather than a unilamellar carrier, could shield EV contents from degradation in the extracellular space, enabling them to voyage farther before being released from the MCMV or taken up into recipient cells. Multiple layers of membrane could also help vesicle contents avoid lysosomal degradation in the recipient cytoplasm and/or reach the nucleus. Additionally, grouping many vesicles of related signaling molecules into a single EV could deliver contents as a component kit, rather than relying on coincidental arrival of components in separate EVs, at the right place and in the right quantities, allowing for more efficient signaling. MCMVs can be evaluated as a new type of organelle-containing microvesicle, and a potential source of exosome release that occurs remotely from the parent cell, adding new considerations to when, where, and how EVs are assembled and released from the endothelium and potentially other cells and tissues.

Planar aggregation of the influenza viral fusion peptide alters membrane structure and hydration, promoting poration.

For all enveloped viruses, one or more glycoproteins on the surface of the viral membrane mediate the fusion of the envelope with the cell membrane for transport of the viral genome to the target cell cytoplasm, bringing about infection. In the first electron-microscopy visualizations of purified viral spike proteins from rabies, rubella, influenza, and other viruses, a striking similarity between spikes from different viruses was the assembly of these purified viral spike proteins into aggregates, termed rosettes, as their hydrophobic transmembrane domains (TMDs) aggregated. Most enveloped viruses enter their target cells via the endocytic pathway, where the viral envelope spike protein encounters an acidic pH. For the influenza virus spike protein hemagglutinin (HA), acidic pH activation of HA is necessary and sufficient for triggering fusion of the viral envelope with a variety of target membranes, including receptor-doped phospholipid bilayers. In the absence of their TMDs, activation of isolated soluble ectodomains of HA led anew to fresh rosettes of those trimers. The N-terminal domain of HA2 is responsible for this second aggregation of HA ectodomains; it is a short amphiphilic N-terminal sequence that became known as the fusion peptide (FP). As with the first rosette of HA, this second rosette formation is considered a consequence of the hydrophobic effect: the hydrophobic surface formed by one side of the FP would avoid water via association with the hydrophobic surface of another FP. The influenza FP comprises the N-terminal 21 amino acids of HA2, located within the HA ectodomain (at neutral pH), proximal to the HA trimer surface but near the TMD. At low pH, the FP is found in the target membrane, as evidenced by hydrophobic photolabeling. The FP is required for infection *in vivo* and membrane fusion *in vitro* and is featured in all hypotheses on HA–mediated fusion, although there is little agreement to date on structural, compositional, and mechanistic data on its exact role. Given that the FP is a highly conserved region of the influenza virus genome across many different subtypes of influenza virus, and a universal feature of enveloped viral fusion proteins, determining the FP's role in infectivity and membrane fusion is critical to finding variant-independent immunogens and pan-viral therapeutics to ameliorate morbidity and mortality.

In the first paper reporting this discovery, a study of the membrane mechanisms by which the influenza virus can disrupt a target membrane, we established that FPs underly this disruption: in target membranes, a reversible pore forms upon addition of FP in the absence of virus or even the rest of HA [Reference 2]. In molecular dynamics simulations crafted to understand the chemistry by which FPs act, a third kind of rosette emerged: the aggregation of FP via their lateral side chains (not their hydrophobic surfaces) into FP microdomains that displace lipids in the cis leaflet. This aggregated structure locally thinned the bilayer and significantly increased the probability of water entry. A new model is proposed to explain our data based on a tilting of FPs towards each other to further thin the remaining lipids immediately under even an FP dimer. For larger aggregates, this more hydrated, thinner membrane structure replaces the lipid bilayer in a small domain wherein a lipidic pore can form.

A second paper on the membrane mechanisms by which the influenza virus can disrupt a target membrane [Reference 3] described how the fusion peptide (FP) domain is necessary for the fusogenic activity of spike proteins in a variety of enveloped viruses, allowing the virus to infect the host cell; it is the only part of the protein that interacts directly with the target membrane lipid tails during fusion. There are consistent findings of poration by this domain in experimental model membrane systems, and, in certain conditions, the isolated FPs can generate pores. We used molecular dynamics simulations to investigate the specifics of how these FP–induced pores form in membranes with different compositions of lysolipids (derivatives of lipids resulting from hydrolytic removal of an acyl chain) and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine). The simulations show that pores form spontaneously at high lysolipid concentrations via hybrid intermediates, where FP aggregates in the cis leaflet tilt to form a funnel-like structure that spans the leaflet and locally reduces the hydrophobic thickness, which must be traversed by water to form a pore. By restraining a single FP within an FP aggregate to this tilted conformation, pores can be formed in membranes of lower lysolipid content, including pure POPC, on the 100-ns timescale, much more rapidly than in unbiased simulations in bilayers with the same composition. The pore formation pathway is similar to the spontaneous formation at high lysolipid concentrations. Depending on the membrane composition, the pores can be metastable (as seen in POPC) or lead to membrane rupture.

Perivascular invasion of primary human glioblastoma cells in organotypic human brain slices: human cells migrating in human brain

Glioblastoma (GBM) is the most common and lethal adult primary brain tumor. Despite aggressive surgery, followed by standard-of-care chemo- and radiation therapy, median overall survival is only 14.6 months from the time of diagnosis. One significant feature of GBM is its highly invasive behavior, a feature that makes complete surgical resection virtually impossible, leading to inevitable recurrence and death. GBM cells undergo several biological modifications, including cellular volume changes, cytoskeletal modification, protein secretion, and development of functional multicellular network structures to actively invade the brain through the perivascular space, meninges, and white matter tracts. GBM invasion into brain tissue is influenced by many factors, including extra-cellular matrix (ECM) modification, the immune system, chemo-attractants, communication with other central nervous system (CNS) cells, and glucose and oxygen concentrations.

The brain-tumor microenvironment provides adequate extracellular matrix (ECM) components, cellular interactions, and mechanical properties to foment the invasion of tumor cells into healthy brain. Individual roles that microenvironment components play in the invasion characteristics of human GBM cells are studied in isolation, such as with the wound-healing assay, the microliter-scale migration assay, the spot assay,

and the transwell migration assay. However, these systems do not attempt to model the totality of the brain microenvironment. Novel 3D-bioprinted models and microfluidics are designed to mimic the brain's cytoarchitecture and to study cell-to-cell interactions, but they lack the specific ECM components and microenvironment of the human brain. While animal systems do utilize a living brain microenvironment *in vivo*, they do not represent the human brain microenvironment, and they are not always practical in terms of time, cost, and availability. Moreover, animal models do not accurately represent human cellular interactions, given the striking differences between human and rodent brain tissue architecture.

We evaluated the perivascular migration of human GBM cells in a human-derived *ex vivo* organotypic model, a model that allows one to see and study the movement of human cancer cells in human brain and their cellular interactions with the healthy brain cytoarchitecture. Brain viability after surgical excision and maintenance in the laboratory lasts for 14 days, ample time to study many aspects of GBM invasion. Live tissue fluorescence microscopy of brain slices inoculated with labeled GBM–derived cells directly demonstrated how GBM cells infiltrate the brain parenchyma, interacting with the surrounding microenvironment, as they move. After initiation of infiltration, inoculated GBM cells moved at a basal speed; their speed increased significantly when GBM cell processes contacted other structures such as blood vessels. Subsequently, GBM cells rapidly moved onto the surface of these structures, and then migrated along the structures at a speed slower than basal. The model can be used to experimentally manipulate and analyze GBM cell migration in human brain tissue *ex vivo*. It provides the closest representation of human brain cancer cell migration outside a living subject, and the list of potential applications is long, including studies of chemotherapeutics, mitogens, cell signaling pathways, and drug testing.

METHODS TO STUDY HUMAN GBM CELL INVASION AND MIGRATION

The strategies that have been traditionally used to study GBM cell invasion include 2D alternatives such as cell-culture surface manipulation, extracellular matrix substrates such as matrigel, and biomaterial scaffolds. Slightly more complex, quasi 3D models have been recently developed to study brain cancer migration; however, they all lack many aspects of the human brain parenchyma, as stated above. The use of human brain tissue sections offers the unique possibility of observing cell invasion in a more relevant microenvironment. Similar *ex vivo* approaches have been used to study glioma cell migration in rat brain pre-implanted with the glioma cell line C6 and then prepared as organotypic slices. Mammalian models *in vivo* include mice, rats, and pigs, but rely on non-human tissue. Studying human cell migration within non-human tissue is suboptimal for many reasons, including the need for immunocompromised hosts, the lack of human cell interactions, and the lack of human-specific ultrastructural differences. Organotypic tissue models are well established; they do require maintaining tissue *ex vivo* (outside of the organism's body). Typically, this maintenance relies on the air-medium interface, whereby tissue explants are placed on a membrane at the interface between air and medium. Viability requires diffusion of substrates from the medium to the tissue explant. The model has primarily been described for rodent-based tissues; human-derived organotypic models are relatively uncommon. In previous work, our tumor organotypic model was maintained reproducibly for two weeks without significant change in tissue cyto-architecture, based on immunohistochemistry and electron microscopy. We have generalized this model to make use of the non-invaded adult human cortex to study human GBM cell migration and interactions *ex vivo*.

GBM INVASION

Promptly upon implantation, GBM cells direct their migration towards blood vessels in a specifically oriented

way, as determined by their persistence of directionality. Previous work demonstrated the attraction of glioma cells towards endothelial cells or endothelial-conditioned media. However, the identification of the extracellular signals that might regulate this initial attraction towards blood vessels is still a matter of investigation. The interplay between tumoral cells within the tumor microenvironment likely contributes significantly to tumor progression and resistance to therapy. Our model presents an opportunity to study these phenomena in human tissue. Similarly, it permits the study of molecules that allow GBM cell invasion through the brain parenchyma, such as matrix metalloproteases.

GBM INVADE HUMAN BRAIN TISSUE SECTIONS USING BLOOD VESSELS AS PATHS.

One reason for the preference of glioma cells for blood vessels could be a mechanistic one: they offer a path of least resistance for migration through an otherwise tight brain parenchyma. Another explanation may be that biological cues secreted or expressed by endothelial and pericyte cells could attract glioma cells. We observed that GBM–derived cells have a biased migration pattern towards blood vessels once placed directly on the surface of an organotypic slice. These cells increase their speed as they approach and make initial contact with a blood vessel and then slow down, once they fully attach to and follow its projection. These observations may indicate that human GBM cells have an intrinsic tropism towards blood vessels. They also suggest that when GBM cells lose their niche, migration increases until they find a new niche in the vicinity of the blood vessels. This property may be important because, following surgery, remaining GBM cells that are left in the resection cavity or surgical margin might lose their niche, causing them to accelerate their migration into less invaded brain parenchyma until a new niche is contacted. Preventing accelerated migration following surgery may require new therapeutic interventions.

In conclusion, we described an *ex vivo* organotypic explant model of human GBM invasion into human brain tissue. We demonstrated that GBM cells start migrating in the search of new perivascular niches and change their migratory dynamics once they contact blood vessels [Reference 4].

Publications

1. Petersen JD, Mekhedov EM, Kaur S, Roberts DD, Zimmerberg J. Endothelial cells release microvesicles that harbour multivesicular bodies and secrete exosomes. *J Extracell Biol* 2023 2:e79.
2. Rice A, Haldar S, Wang E, Blank PS, Akimov SA, Galimzyanov TR, Pastor RW, Zimmerberg J. Planar aggregation of the influenza viral fusion peptide alters membrane structure and hydration, promoting poration. *Nat Commun* 2022 13:7336.
3. Rice A, Zimmerberg J, Pastor RW. Initiation and evolution of pores formed by influenza fusion peptides probed by lysolipid inclusion. *Biophys J* 2023 122:1018–1032.
4. Ravin R, Suarez-Meade P, Busse B, Blank PS, Vivas-Buitrago T, Norton ES, Graepel S, Chaichana KL, Bezrukov L, Guerrero-Cazares H, Zimmerberg J, Quiñones-Hinojosa A. Perivascular invasion of primary human glioblastoma cells in organotypic human brain slices: human cells migrating in human brain. *J Neurooncol* 2023 164:43–54.
5. Petersen JD, Lu J, Fitzgerald W, Zhou F, Blank PS, Matthies D, Zimmerberg J. Unique aggregation of retroviral particles pseudotyped with the delta variant SARS-CoV-2 spike protein. *Viruses* 2022 14:1024.
6. Pfeiffer A, Petersen JD, Falduto GH, Anderson DE, Zimmerberg J, Metcalfe DD, Olivera A. Selective immunocapture reveals neoplastic human mast cells secrete distinct microvesicle- and exosome-like populations of KIT-containing extracellular vesicles. *J Extracell Vesicles* 2022 11:e12272.

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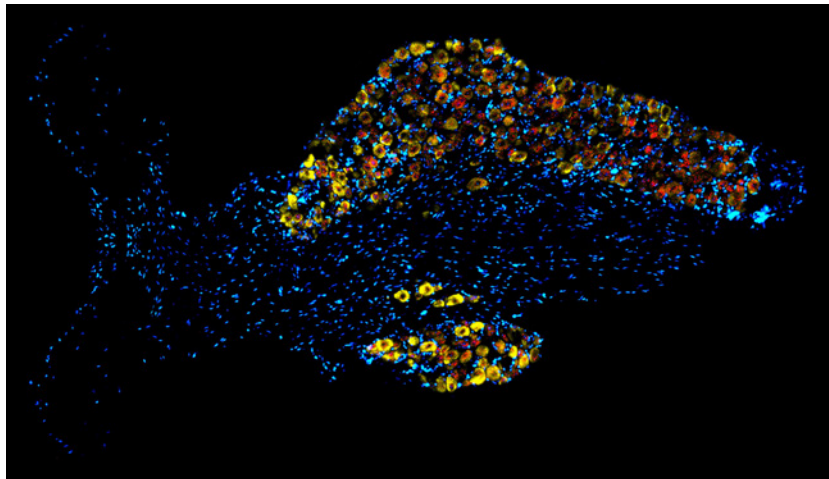
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Colophon

About the Cover Image

"The RNA-FISH:" Multiplexed
Fluorescence in situ hybridization
(FISH) of a section of a dorsal root
ganglia from an adult mouse

Image by Bruno Siqueira Mietto, PhD,
Rouault and *Le Pichon* laboratories.

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