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Organizers

Leadership

Richard Smith
USA

Hela Azaiez
Tunisia

National Organizing and Advisory Committee

Kevin Booth
Boston

John Brigande
Portland

David Corey
Boston

Stefan Heller
Stanford

Maryna Ivanchenko
Boston

Cynthia Morton
Boston

Ulrich Mueller
Baltimore

Allen Ryan
San Diego

Peter Steyger
Omaha
International Organizing and Advisory Committee

Mike Bowl  
UK

Aziz El Amraoui  
France

Hannie Kremer  
The Netherlands

Saber Masmoudi  
Tunisia

Miguel Moreno-Pelayo  
Spain

Hossein Najmabadi  
Iran

Jakob Neef  
Germany

Christine Petit  
France

Karen Steel  
UK

Shin-ichi Usami  
Japan

Ambroise Wonkam  
South Africa

Huijin Yuan  
China

Local Organizing Committee

Dan Eberl  
Iowa

Bernd Fritzsch  
Iowa

Bruce Gantz  
Iowa

Steve Green  
Iowa

Marlan Hansen  
Iowa
The University of Iowa is pleased to host

**The Molecular Biology of Hearing and Deafness Conference**

in Iowa City, May 24 - 27, 2022

The Molecular Biology of Hearing and Deafness is a conference series that has been held successfully since 1992. Its goals are to:

- **Promote** research in the emerging areas of molecular biology in the field of auditory sciences;
- **Highlight** progress and anticipate new growth areas in the molecular biology of hearing and deafness by inviting select scientists doing cutting-edge research to present keynote lectures;
- **Encourage** engagement by trainees and young scientists by soliciting oral and poster presentations related to the auditory system and offering opportunities to meet and interact with senior investigators through a speed-dating event;
- **Facilitate** interaction and collaboration between laboratories engaged in molecular auditory research, as well as between basic scientists, translational scientists and clinicians with related interests in the molecular biology and molecular genetics of deafness through multiple interactive panels and social events linked to poster presentations;
- **Foster** translational research that can lead to novel diagnostic and therapeutic strategies for the treatment of hearing disorders;
- **Enable** trainees and scientists from under-represented groups to attend and foster interest in careers in the molecular biology of hearing and deafness;
- **Provide** outreach to patients and patient advocacy groups for education in the genetics of deafness and hearing research.

We are committed to providing a COVID-safe environment for the conference in 2022. To keep our community safe, wearing a mask is required in all indoor events and activities. A proof of COVID-19 vaccination is required to register for the conference.
Conference Venue

The conference will be held at MacBride Hall Auditorium

17 N Clinton St, Iowa City, IA 52240

~7min walking from The Graduate Hotel
Invited Speakers

Peter Barr-Gillespie    Zheng-Yi Chen    Stefan Heller

Thomas Friedman    Lisa Goodrich    Andrew Groves

Eri Hashino    Christine Petit    Karen Steel
Panelists

Allen Ryan - University of California San Diego
Ambrose Wonkam - John Hopkins
Angelika Doetzhofer - Johns Hopkins University School of Medicine
Aziz El Amraoui - Institut de l'Audition - Institut Pasteur
Bernd Fritzsch - The University of Iowa
Cynthia Morton - Brigham and Women's Hospital
David Corey - Harvard Medical School
Doris Wu - NIDCD
Ellen Reisinger - University of Tuebingen
Hainan Lang - Medical University of South Carolina
Hannie Kremer - Radboud University Medical Center
Hela Azaiez - University of Iowa
John Brigande - Oregon Hearing Research Center
Kevin Booth - Harvard Medical School
Katie Kindt - NIDCD
Mark Warchol - Washington University School of Medicine
Matt Kelly - NIDCD
Miguel Moreno-Pelayo - Hospital Ramón y Cajal-IRYCIS-CIBERER
Mike Bowl - UCL Ear Institute
Peter Steyger - Creighton University
Sandrine Marlin - Hôpital Necker, APHP; Institut Imagine
Tejbeer Kaur - Creighton University School of Medicine
Ulrich Mueller - Johns Hopkins University
Xue Liu Zhong - University of Miami
Yehoash Raphael - University of Michigan
Travel Award Recipients

Amandine Jarysta
The Jackson Laboratory
Harbor, Maine, United States

Benjamin Shuster
University of Maryland School of Medicine
Baltimore, Maryland, United States

Carlos Aguilar
MRC Harwell Institute
Oxford, United Kingdom

Cong Tian
Creighton University
Omaha, Nebraska, United States

Cybel Mehaweij
Lebanese American University
Beirut, Lebanon

Dominika Ozioło
Institute of Physiology and Pathology of Hearing
Warsaw, Poland

Elisa Martelletti
King’s College
London, England

Elvis Twumasi Aborgye
University of Ghana
Ghana, West Africa

Grethy Lopez
Pontificia Universidad Javeriana
Bogota, Colombia

Isabel Aristizábal-Ramírez
University of Kentucky
Lexington, Kentucky, United States

Jeffery Rumschlag
Medical University of South Carolina
Charleston, South Carolina, United States

Kathleen Guilliam
University of Maryland
Baltimore, Maryland, United States

Kevin Rose
University of Maryland
Baltimore, Maryland, United States

Lara Kamal
Tel Aviv University
Tel Aviv, Israel

Marie Valenie Roche
University of Miami
Miami, Florida, United States

Melissa McGovern
Baylor College of Medicine
Houston, Texas, United States

Samuel Mawuli Adadey
University of Cape Town
Cape Town, South Africa

Sandra de Haan
Karolinska Institute / NIH
Stockholm, Sweden

Stephanie Mauria
Boston Children’s Hospital & Harvard Medical School
Boston, Massachusetts, United States

Suraj Chakravarthi
National Centre for Biological Sciences
Karnataka, India

Tuo Shi
University of Southern California
Los Angeles, California, United States

Vignesh Rathinavelpani Anandavel
Creighton University
Omaha, Nebraska, United States

Wen Du
Harvard Medical School/Mass Eye and Ear
Cambridge, Massachusetts, United States

Xufeng Qiu
Johns Hopkins University
Baltimore, Maryland, United States

Yacouba Dia
University Cheikh Anta Diop (Ucad)
Dakar, Senegal
Program

MBHD will be a three-day conference. Each half-day block will include two thematic areas, with each thematic area built on a TED-style talk to review the current state of knowledge and knowledge gaps to be addressed. TED-style talks are delivered without notes, from memory, although they are not spontaneous; rather, they are scripted and carefully rehearsed. The speaker is never behind a lectern and the supporting slides are highly visual with images of exceptional quality. The theme will not focus on the invited scientist’s personal research experience but rather lay the foundation for the subsequent talks in the session by including representative data and discoveries from all research scientists who have moved the area forward. TED-talk speakers should educate, inspire, motivate and captivate the audience.

The invited talks that follow TED-style talks will be 10-minute talks thematically linked to the specific topic.

Selected sessions will include panel discussions to engage the audience, facilitates dialogue and to promote interactivity
## Session 1

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:00 - 14:20</td>
<td>Richard Smith, MBHD Chair</td>
<td></td>
<td>Welcome and Opening Remarks</td>
</tr>
<tr>
<td>14:20 - 14:40</td>
<td>Tom Friedman</td>
<td>Scientist - Academia</td>
<td>Naughty Noncoding Variant or Nicely in Disequilibrium?</td>
</tr>
<tr>
<td>14:40 - 15:10</td>
<td>Mustafa Tekin</td>
<td>Scientist - Academia</td>
<td>Characterization of MINAR2 as a Novel Autosomal Recessive Deafness Gene</td>
</tr>
<tr>
<td>14:55 - 15:10</td>
<td>Jonathan Lin</td>
<td>Scientist - Academia</td>
<td>Mutations in the Unfolded Protein Response Regulator Atf6 Cause Sensorineural Hearing Loss and Impairment of the Organ of Corti</td>
</tr>
<tr>
<td>15:10 - 15:25</td>
<td>Miles Klimara</td>
<td>Medical resident</td>
<td>De Novo Mutations are a Common Cause of Genetic Hearing Loss</td>
</tr>
<tr>
<td>15:25 - 15:40</td>
<td>Elvis Twumasi Aboagye</td>
<td>Graduate Student</td>
<td>Age Estimates of GJB2-p.(Arg143Trp) Founder Variant in Hearing Impairment in Ghana, suggest Multiple Independent Origins across Populations</td>
</tr>
</tbody>
</table>

### The Expanding Genetic and Genomic Landscapes of Deafness

Moderators: Hannie Kremer & Hela Azaiez

## Session 2

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:00 - 16:20</td>
<td>Peter Barr-Gillespie</td>
<td></td>
<td>Fifty Years of Hair-Cell Mechanotransduction</td>
</tr>
<tr>
<td>16:20 - 16:35</td>
<td>Brian McDermott</td>
<td>Scientist - Academia</td>
<td>Asymmetric Mechanotransduction by Hair Cells of the Zebrafish Lateral Line</td>
</tr>
<tr>
<td>16:35 - 16:50</td>
<td>Nicolas Grillet</td>
<td>Scientist - Academia</td>
<td>LOXHD1 is required for Mechanotransduction and localizes at the Lower Tip-Link insertion point</td>
</tr>
<tr>
<td>16:50 - 17:05</td>
<td>Xufeng Qiu</td>
<td>Postdoctoral Trainee</td>
<td>The Tetraspan LHFPL5 Establishes Force Sensitivity of the Mechanotransduction Channel of Cochlear Hair Cells</td>
</tr>
<tr>
<td>17:05 - 17:20</td>
<td>Isabel Aristizábal-Ramírez</td>
<td>Graduate Student</td>
<td>Deafness-associated Variant of the Calcium and Integrin-Binding Protein 2 (CIB2) Slows Down Force Transmission to the Mechanotransducer Channels in Mammalian Auditory Hair Cells</td>
</tr>
<tr>
<td>17:20 - 17:35</td>
<td>Marcos Sotomayor</td>
<td>Scientist - Academia</td>
<td>Towards an Atomistic Model of the Inner-Ear Transduction Apparatus</td>
</tr>
</tbody>
</table>

### Coffee Break: 15:40 - 16:00

### Panel Discussion

17:35 - 18:00  Richard SMITH, Thomas FRIEDMAN, Hannie KREMER, Hela AZAIEZ, Peter BARR-GILLESPIE, David COREY, Ulrich MUELLER

### Reception at The Museum of Natural History
### Session 3

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:00 - 8:20</td>
<td>Andrew Groves</td>
<td></td>
<td>Molecular Biology of Cochlear Development: Problems, Puzzles and Paradoxes in Making the World’s Best Hearing Aid</td>
</tr>
<tr>
<td>8:20 - 8:35</td>
<td>Sandra De Haan</td>
<td>Graduate Student</td>
<td>Effects of Jag1-Mediated Notch Activation in Inner Ear Patterning: Insights from the Nodder Mouse Model for Alagille Syndrome</td>
</tr>
<tr>
<td>8:35 - 8:50</td>
<td>Angelika Doetzlhofe</td>
<td>Scientist - Academia</td>
<td>The RNA-binding protein TRIM71 Regulates Pro-Sensory Cell Behavior in the Mammalian Cochlea</td>
</tr>
<tr>
<td>8:50 - 9:05</td>
<td>Suraj Chakravarthy</td>
<td>Graduate Student</td>
<td>The Role of Ezrin and Radixin Proteins in “Blooming” of Nascent Stereocilia in Auditory Hair Cells</td>
</tr>
<tr>
<td>9:05 - 9:20</td>
<td>Benjamin Perrin</td>
<td>Scientist - Academia</td>
<td>A Novel Population of Short Actin Filaments at Stereocilia Tips and a Tip-Down Widening Mechanism</td>
</tr>
<tr>
<td>9:20 - 9:35</td>
<td>Basile Tarchini</td>
<td>Scientist - Academia</td>
<td>Heterotrimeric G Protein Signaling Regulates the Graded Height Architecture of the Stereocilia Bundle</td>
</tr>
<tr>
<td>9:35 - 9:50</td>
<td>Botond Banfi</td>
<td>Scientist - Academia</td>
<td>A Novel Regulator of Hair Cell Maturation Enhances ATOH1 Activity in the Hearing Organ</td>
</tr>
</tbody>
</table>

### Session 4

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:20 - 10:40</td>
<td>Lisa Goodrich</td>
<td></td>
<td>Diversity of Otic Mesenchyme Cells During Cochlear Development</td>
</tr>
<tr>
<td>10:40 - 10:55</td>
<td>Kevin Rose</td>
<td>Graduate Student</td>
<td>Opposing Gradients of Retinoic Acid and Sonic Hedgehog Signaling Specify the Tonotopic Axis in the Murine Cochlea</td>
</tr>
<tr>
<td>10:55 - 11:10</td>
<td>Joerg Waldhaus</td>
<td>Scientist - Academia</td>
<td>Defining the Developmental Trajectory of Prosensory Cells in Human Inner Ear Organoids at Single-Cell Resolution</td>
</tr>
<tr>
<td>11:10 - 11:25</td>
<td>Yoshitomo Ueda</td>
<td>Postdoctoral Trainee</td>
<td>Transcriptomic Differences between Adult Mouse Cochlear and Vestibular Hair Cells</td>
</tr>
<tr>
<td>11:25-11:40</td>
<td>David He</td>
<td>Scientist - Academia</td>
<td>Development of Neuronal Phenotypes in the Spiral Ganglion</td>
</tr>
<tr>
<td>11:40-11:55</td>
<td>Matthew Kelly</td>
<td>Scientist - Academia</td>
<td>The Deafness Variation Database (DVD)</td>
</tr>
</tbody>
</table>

**Panel Discussion**

11:55-12:20 Andrew GROVES, Lisa GOODRICH, Doris WU, Bernd FRITZSCH, Angelika DOETZLHOFER & Matt KELLY

**Lunch:** 12:30 - 14:00 at Iowa Memorial Union

### Session 5

**Poster Social and Speed-Dating at the Main Lounge, Iowa Memorial Union**

### Workshops

**16:00 - 17:00**

The Deafness Variation Database (DVD)

**17:00 - 18:30**

The gene Expression Analysis Resource (gEAR)

**Free Night**
### DAY 3: Thursday May 26th

<table>
<thead>
<tr>
<th>Session 6</th>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>8:00 - 8:20</td>
<td>Karen Steel</td>
<td></td>
<td>A Thousand Ways to Lose Your Hearing</td>
</tr>
<tr>
<td></td>
<td>8:20 - 8:35</td>
<td>Miguel Angel Moreno-Pelayo</td>
<td>Scientist - Academia</td>
<td>Insights into the Pathophysiology of DFNA44 Hearing Loss Associated with CCDC50 Variants</td>
</tr>
<tr>
<td></td>
<td>8:35 - 8:50</td>
<td>Yehree Kim</td>
<td>Scientist - Academia</td>
<td>Auditory Phenotype and Histopathologic Findings of a Mutant Nlrp3 Expression Mouse Model</td>
</tr>
<tr>
<td></td>
<td>8:50 - 9:05</td>
<td>Isabelle Roux</td>
<td>Scientist - Academia</td>
<td>Interaction of SLC26A4 with Adaptor Protein (AP-2) Complex in Mitochondria-Rich Cells of the Endolymphatic Sac</td>
</tr>
<tr>
<td></td>
<td>9:05 - 9:20</td>
<td>Maria Lachgar-Ruiz</td>
<td>Graduate Student</td>
<td>Exploring the Pathological Mechanisms of miR-96 Mutations in the Inner Ear</td>
</tr>
<tr>
<td></td>
<td>9:20 - 9:35</td>
<td>Sheryllanne Newton</td>
<td>Postdoctoral Trainee</td>
<td>Neuroplastin Genetically Interacts with Cadherin 23 and Np55 is Sufficient for Cochlear Hair Cell Function and Hearing</td>
</tr>
</tbody>
</table>

**Pathological Mechanisms Underlying Deafness (1)**
Moderators: Sandrine Marlin & Amal Abu Rayan

<table>
<thead>
<tr>
<th>Session 7</th>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:00 - 10:15</td>
<td>Ben Warren</td>
<td>Scientist - Academia</td>
<td>Age and Noise Affect Distinct Physiological Processes in the Auditory System</td>
</tr>
<tr>
<td></td>
<td>10:15 - 10:30</td>
<td>Ben Gansemer</td>
<td>Postdoctoral Trainee</td>
<td>Neurodegeneration in the Cochlea After Hair Cell Loss is Due to an Immune Response</td>
</tr>
<tr>
<td></td>
<td>10:30 - 10:45</td>
<td>Allen F Ryan</td>
<td>Scientist - Academia</td>
<td>The Role of Middle Ear Epithelial Cells in the Resolution of Otitis Media</td>
</tr>
<tr>
<td></td>
<td>10:45 - 11:00</td>
<td>Bernd Fritzsch</td>
<td>Scientist - Academia</td>
<td>Neurons and Hair Cells Require Lmx1a/b for Normal Brainstem Development</td>
</tr>
<tr>
<td></td>
<td>11:00 - 11:15</td>
<td>Mark Rutherford</td>
<td>Scientist - Academia</td>
<td>Central and Peripheral Auditory Function in APP/PS1 Mice</td>
</tr>
<tr>
<td></td>
<td>11:15-11:30</td>
<td>Elisa Martelletti</td>
<td></td>
<td>Reversal of Hearing Loss in Spsn2 Mutant Mice</td>
</tr>
</tbody>
</table>

**Pathological Mechanisms Underlying Deafness (2)**
Moderators: Aziz El AMRAOUI & Katie KINDT

<table>
<thead>
<tr>
<th>Panel Discussion</th>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11:30 - 12:00</td>
<td>Karen STEEL, Mike BOWL, Aziz EL AMRAOUI, Sandrine MARLIN, Katie KINDT, Allen RYAN, Miguel MORENO-PELAYO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Session 8

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:00 - 14:20</td>
<td>Stefan HELLER</td>
<td></td>
<td>VICs - Very Important Cells</td>
</tr>
<tr>
<td>14:20 - 14:35</td>
<td>Allison Coffin</td>
<td>Scientist - Academia</td>
<td>Ototoxic Potential of COVID-19 Therapeutics</td>
</tr>
<tr>
<td>14:35 - 14:50</td>
<td>Jinkyung Kim</td>
<td>Postdoctoral Trainee</td>
<td>In Vivo Real-Time Imaging Reveals Megalin As Aminoglycoside Transporter Into Cochlea Whose Inhibition Is Otoprotective</td>
</tr>
<tr>
<td>14:50 - 15:05</td>
<td>A. Catalina Velez-Ortega</td>
<td>Scientist - Academia</td>
<td>TRPA1 Activation in Non-Sensory Supporting Cells Contributes to the Regulation of Cochlear Sensitivity after Acoustic Trauma</td>
</tr>
</tbody>
</table>

**Hair cell damage, repair and regeneration**

Moderators: Yehoash RAPHAEL and Tejbeer KAUR

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:05 - 15:20</td>
<td>Nesrine Benkafadar</td>
<td>Postdoctoral Trainee</td>
<td>Protease-Activated Receptor is Essential for Mitotic Basilar Papilla Hair Cell Regeneration</td>
</tr>
<tr>
<td>15:20 - 15:35</td>
<td>Melissa McGovern</td>
<td>Postdoctoral Trainee</td>
<td>Regeneration of Hair Cells in the Mature Mouse Cochlea Following Reprogramming with Atoh1, Gfi1, and Pou4F1</td>
</tr>
</tbody>
</table>

**Coffee Break: 15:35 - 16:00**

### Session 9

<table>
<thead>
<tr>
<th>Time</th>
<th>SPEAKER</th>
<th>Position</th>
<th>TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:00 - 16:20</td>
<td>Zheng-Yi Chen</td>
<td></td>
<td>VIDEO - A New Era in Precision Medicine for Genetic Hearing Loss</td>
</tr>
<tr>
<td>16:20 - 16:35</td>
<td>Michael Anne Gratton</td>
<td>Scientist - Academia</td>
<td>Sparsentan Protects from Hearing Loss, Improves Kidney Function &amp; Prolongs Lifespan in Alport Mice with Developed Pathology</td>
</tr>
<tr>
<td>16:35 - 16:50</td>
<td>Danielle Lenz</td>
<td>Scientist - Industry</td>
<td>Evaluating miR-Target Sites as a Strategy to Allow AAV Vector-based De-targeting of Gene Expression in the Inner Ear</td>
</tr>
<tr>
<td>16:50 - 17:05</td>
<td>Kathrin Kusch</td>
<td>Scientist - Academia</td>
<td>Characterization of Promoter Expression in Type 1 Spiral Ganglion Neurons in vitro and in vivo</td>
</tr>
<tr>
<td>17:05 - 17:20</td>
<td>Osama Tarabichi</td>
<td>Medical Resident</td>
<td>Development of novel helper dependent adenoviral vectors for inner ear gene therapy</td>
</tr>
<tr>
<td>17:20 - 17:35</td>
<td>Olga Shubina-Oleinik</td>
<td>Postdoctoral Trainee</td>
<td>Dual Vector Gene Therapy Restores Hearing in a Mouse Model of DFNB16 Hearing Loss</td>
</tr>
</tbody>
</table>

**Molecular Therapies for Deafness**

Moderators: Ellen REISINGER & Maryna Ivanchenko

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>17:35 - 18:00</td>
<td>Stefan HELLER, Yehoash RAPHAEL, Ellen REISINGER, Xue Liu ZHONG, Marc WARCHOL, Tejbeer KAUR</td>
<td></td>
<td>Panel Discussion</td>
</tr>
</tbody>
</table>

**BBQ dinner and square dancing at The Celebration Farm 19:00 - 22:00**
## Session 10

### Time  
### SPEAKER  
### Position  
### TITLE

**8:00 - 8:20**  
Christine Petit  
Hearing Medicine in The Light of Scientific Advances: Dream or Reality?

**8:20 - 8:35**  
Ryan Carlson  
Postdoctoral Trainee  
Genetic and Phenotypic Heterogeneity of Childhood-Onset Hearing Loss and Implications for Success of Cochlear Implants

**8:35 - 8:50**  
Erik de Vrieze  
Scientist - Academia  
Development of a Genetic Therapy for DFNA21 using Allele-specific Antisense Oligonucleotides

**8:50 - 9:05**  
Ellen Reisinger  
Scientist - Academia  
A Dual-AAV Approach with Human Otoferlin cDNA Rescues Hearing in Otof-Knock-Out Mice for at Least 10 Months

**9:05 - 9:20**  
Maryna Ivanchenko  
Scientist - Academia  
Mini-PCDH15 Gene Therapy Rescues Hearing in a Mouse Model of Usher Syndrome Type 1F

### Translational and Precision Medicine for Deafness

Moderators: Cynthia MORTON and Kevin BOOTH

### Day 4: Friday May 27th

**Panel Discussion**  
Christine PETIT, Eri HASHINO, Cynthia MORTON, Ambroise WONKAM, John BRIGANDE, Peter STEYGER, Hainan LANG, Kevin BOOTH

**11:00- 11:30**  
Lunch: 12:35 - 14:00 at the Museum of Natural History

**12:15 - 12:30**  
Richard Smith and Hela Azaiez, MBHD Chairs

**Poster Blitz and Award Ceremony**  
11:30-12:15
Session 1

The Expanding Genetic and Genomic Landscapes of Deafness

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<td>14:40 - 14:55</td>
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<td>15:25 - 15:40</td>
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Naughty Noncoding Variant or Nicely in Disequilibrium?

Thomas B. Friedman

Laboratory of Molecular Genetics, Section on Human Genetics, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Maryland 20892

Hundreds of pathogenic protein-coding variants of numerous genes are firmly associated with human deafness. For many of these genes, a variety of mouse models of the orthologous genes have been engineered or variants identified in mutagenesis screens. These valuable deaf mice have been used to identify pathogenic mechanisms as well as to study wild-type gene functions necessary for hearing. However, many large human families segregating deafness as a recessive trait have been ascertained but no biallelic pathogenic variants have been identified in the annotated human exome. There are likely exons of well-studied genes that are not yet annotated and could harbor “missing” pathogenic variants. A second possibility is that some of the yet to be identified pathogenic variants are located in cis-acting regulator elements governing cell type, quantity or temporal gene expression. For this second possibility, how does one validate the potential pathogenicity of such a noncoding variant, distinct from a benign variant in disequilibrium but pointing to the causal closely-linked variant? Each human has plenty of inherited benign noncoding variants and in each generation, there is a new crop of de novo variants. Why is it so difficult to confirm a noncoding variant as causally connected with a human disorder? This topic will be discussed using noncoding variants of the human HGF gene associated with DFNB39 and three Hgf mouse models, as an example of how this issue was resolved for one gene.
Characterization of MINAR2 as a Novel Autosomal Recessive Deafness Gene

Guney Bademci¹, María Lachgar-Ruiz²,³,⁴, Mangesh Deokar⁵,⁶, Mohammad Faraz Zafeer⁷, Clemer Abad⁷, Muzeyyen Yildirim Baylan⁸, Neil J. Ingham², Jing Chen², Claire J. Sineni⁷, Nirmal Vadgama⁹, Ioannis Karakikes⁹, Shengru Guo⁷, Duygu Duman¹⁰, Nitu Singh⁶, Gaurav Harlalka⁵, Shirish P. Jain⁵, Barry Chioza¹¹, Katherina Walz¹,⁷, Karen P. Steel², Jamal Nasir¹², Mustafa Tekin¹,⁷,¹³*

¹) Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami Miller School of Medicine, Miami, FL, 33136, USA; ²) Wolfson Centre for Age-Related Diseases, King’s College London, London, SE1 1UL, UK; ³) Servicio de Genética, Hospital Universitario Ramón y Cajal, IRYCIS, Carretera de Colmenar 17 km 9.100, 28034 Madrid, Spain; ⁴) Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), 28034 Madrid, Spain; ⁵) Rajarshi Shahu College of Pharmacy, Malvihir, Buldana, India; ⁶) Oriental College of Pharmacy and Research, Oriental University, Indore, MP, India; ⁷) John P. Hussmann Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, FL 33136, USA; ⁸) Department of Otorhinolaryngology, Faculty of Medicine, Dicle University, Diyarbakir, 21200 Turkey; ⁹) Cardiovascular Institute and Department of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, CA, 94305, USA; ¹⁰) Department of Audiology, Faculty of Health Sciences, Ankara University, Ankara, 06100, Turkey; ¹¹) RILD Building, Wellcome Wolfson Centre, University of Exeter Medical School, Exeter, UK; ¹²) Molecular Biosciences Research Group, Faculty of Health and Society, University of Northampton, UK 2; ¹³) Department of Otolaryngology, University of Miami Miller School of Medicine, Miami, FL, 34 33136, USA

Discovery of deafness genes and elucidation of their functions have substantially contributed to our understanding of hearing physiology and its pathologies. Via exome and genome sequencing, we identified variants in MINAR2, membrane integral NOTCH2-associated receptor 2, in four families diagnosed with autosomal recessive non-syndromic deafness. MINAR2 is a recently annotated gene with limited functional understanding. We detected three MINAR2 variants, p.Trp48*, p.Arg138Valfs*10, and p.Lys131Asn, in 13 individuals with congenital or prelingual-onset severe to profound sensorineural hearing loss. We subsequently showed that the p.Lys131Asn variant disrupts a splice donor site leading to a shift in the reading frame. We demonstrate that Minar2 is expressed in the mouse inner ear, with the protein localizing mainly in the hair cells, spiral ganglia, the spiral limbus, and the stria vascularis. Mice homozygous for a Minar2 mutation (Minar2tm1b) present with rapidly progressive sensorineural hearing loss associated with a reduction in outer hair cell stereocilia in the shortest row. Via in vitro studies, we demonstrate that MINAR2 suppresses NOTCH2. We conclude that MINAR2 is essential for hearing in humans and mice and its disruption leads to sensorineural hearing loss. Progressive hearing loss observed in mice and in some affected individuals and as well as relative preservation of hair cells provides an opportunity to interfere with hearing loss using genetic therapies.
Mutations in the Unfolded Protein Response Regulator Atf6 Cause Sensorineural Hearing Loss and Impairment of the Organ of Corti

Eun-Jin Lee1,2,3, Eduardo Chavez4, Kyle Kim1,2, Korina J. Steinbergs1,2, Lance A. Safarta1,2, Hyejung Min1,2, Allen F. Ryan4, Jonathan H. Lin1,2,3*

1 Pathology, Stanford University School of Medicine, Stanford, CA, United States; 2 Ophthalmology, Stanford University School of Medicine, Stanford, CA, United States; 3 VA Palo Alto Healthcare System, Palo Alto, CA, United States; 4 Departments of Surgery and Neuroscience, University of California San Diego and Veterans Administration Medical Center, La Jolla, CA, United States

Activating transcription factor 6 (Atf6) is a key regulator of the unfolded protein response (UPR) and is required for endoplasmic reticulum (ER) function and protein homeostasis in metazoan cells. Patients carrying loss-of-function \textit{ATF6} disease alleles develop the cone dysfunction disorder achromatopsia. Some of these patients also report hearing loss. Here, we investigated auditory function of patients homozygous for \textit{ATF6} disease alleles and in \textit{Atf6}−/− mice. In the mice, we further examined the cochlear histological and molecular consequences of loss of \textit{Atf6} function.

Auditory phenotypes of 4 patients homozygous for \textit{ATF6} disease alleles p.Arg324Cys or p.Tyr567Asn were analyzed by audiogram. 2-week- and 2-month-old male and female WT (\textit{Atf6}+/+) and \textit{Atf6}−/− mice on a C57BL/6J background were used to determine the role of \textit{Atf6} in the cochlea. Immunohistochemistry was performed on cochlear wholemounts with an antibody against MYO7A and phalloidin. Immunoreactive cells were quantified using image-processing software (Leica-application suitex, Photoshop). The hearing sensitivity of mice was assessed by auditory brainstem response (ABR) thresholds.

All 3 siblings homozygous for the \textit{ATF6} loss-of-function mutation encoding p.ARG324Cys (age: 49, 51, 52) and one patient with homozygosity for the \textit{ATF6} mutation encoding p.Tyr567Asn (age 14) showed neurosensory hearing loss in both ears. In 2-month-old \textit{Atf6}−/− mice, ABR thresholds were significantly higher ($p <0.0001$) compared to WT mice. There were no gender differences. In 2-week-old \textit{Atf6}−/− mice, no ABR threshold differences were seen. \textit{Atf6}−/− mice showed loss of outer-hair cells at 2 months ($p=0.001$). In addition, inner-hair cell stereocilia were disorganized.

Our findings implicate \textit{ATF6} as a novel regulator of cochlear function. We suggest that impaired \textit{ATF6} increases ER stress and undermines organ of Corti function. Our findings indicate that \textit{ATF6} disease variants cause sensory defects in both eye and ear.
De Novo Mutations are a Common Cause of Genetic Hearing Loss

Miles J. Klimara, Carla Nishimura, Donghong Wang, Diana L. Kolbe, Amanda M. Schaefer, William D. Walls, Kathy L. Frees, Richard J.H. Smith, and Hela Azaiez

1. Molecular Otolaryngology and Renal Research Laboratories, Department of Otolaryngology, University of Iowa, Iowa City, IA 52242, USA
2. Department of Otolaryngology – Head and Neck Surgery, University of Iowa, Iowa City, IA 52242, USA

De novo mutations (DNMs) are a well-recognized cause of genetic disorders. The contribution of DNMs to hearing loss (HL) is poorly characterized. We hypothesized that DNMs are an underappreciated etiology of HL, and leveraged our cohort of individuals with HL to assess their contribution.

Targeted genomic enrichment (TGE) and massively parallel sequencing (MPS) were used for genetic testing of all exons and flanking intronic sequences of known deafness-associated genes. Segregation analysis was performed in all families in which a potentially causative variant was detected in the proband. Familial testing results and systematic review of previous DNM reports in the Deafness Variation Database (DVD), PubMed and ClinVar were reviewed to characterize the rate, distribution, and spectrum of DNM.

DNMs were detected in 10% (24 of 238) of probands for whom testing of both parents was completed. DNMs were detected in 15 deafness-associated genes, including 9 novel variants in ACTG1 (n = 2), AIFM1, ATP2B2, CDH23, GATA3, MITF, MYO6, and NR2F1. DNMs in MITF were most common (21% of all DNMs), followed by GATA3 (13%), STRC (13%), and ACTG1 (8%). Review of novel and previously reported DNMs revealed a gene-specific de novo mutational spectrum and variability in the contribution of DNM to pathogenic variation.

DNMs are a common cause of genetic HL, and must be considered in all cases of sporadic HL. Gene-specific variability in the contribution of DNM to genetic HL necessitates the development of gene-specific criteria for interpretation of variants with de novo origin.
Age estimates of GJB2- p.(Arg143Trp) Founder Variant in Hearing Impairment in Ghana, Suggest Multiple Independent Origins Across Populations

Elvis Twumasi Aboagye 1,2*, Samuel Mawuli Adadey 1,2, Kevin Esoh 2, Mario Jonas 2, Carmen de Kock 2, Lucas Amenga-Etego 1, Gordon A. Awandare 1, and Ambroise Wonkam 2,3

1 West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, LG Box 54, Ghana; etaboagye@st.ug.edu.gh; smadadey@st.ug.edu.gh; lucasmenga@gmail.com; and gawandare@ug.edu.gh
2 Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa;
3 McKusick-Nathans Institute and Department of Genetic Medicine, John Hopkins University School of Medicine, Baltimore, MD 21205, USA; awonkam1@jhmi.edu

Gap junction protein beta 2 (GJB2) (connexin 26) variants are commonly implicated in non-syndromic hearing impairment (NSHI). In Ghana, the GJB2 variant p.(Arg143Trp) is the largest contributor to NSHI and has a reported prevalence of 25.9% in affected multiplex families. To date, in the African continent, GJB2- p.(Arg143Trp) has been reported only in Ghana. Using Whole Exome sequencing data from 32 individuals from 16 families segregating NSHI, and 38 unrelated normal hearing controls with the same ethnolinguistic background, we investigated the date and origin of p.(Arg143Trp) in Ghana using linked markers. With a Bayesian linkage disequilibrium gene mapping method, we estimated GJB2-p.(Arg143Trp) to have originated about 9,625 years (385 generations) ago in Ghana. Haplotype analysis comparing data extracted from Ghanaian and those from the 1000 Genomes project revealed that GJB2-p.(Arg143Trp) is carried on different haplotype backgrounds in Ghanaian and same founder variant reported in Japan, as well as among populations of European ancestry, lending further support to the multiple independent origins of the variant. In addition, we found substantial haplotype conservation in the genetic background of Ghanaian individuals with biallelic GJB2-p.(Arg143Trp), compared to GJB2-p.(Arg143Trp)-negative group with normal hearing from Ghana, suggesting a strong evolutionary constraint in this genomic region in Ghanaian population that are GJB2-p.(Arg143Trp) homozygous. The present study evaluates the age of GJB2-p.(Arg143Trp) to 9,625 years, and supports the multiple independent origin of this variant in multiple global populations.
# Session 2

## Molecular Basis of Mechanotransduction Machinery

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<td>Fifty Years of Hair-Cell Mechanotransduction</td>
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<td>16:20 - 16:35</td>
<td>Brian McDermott</td>
<td>Scientist - Academia</td>
<td>Asymmetric Mechanotransduction by Hair Cells of the Zebrafish Lateral Line</td>
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<td>16:35 - 16:50</td>
<td>Nicolas Grillet</td>
<td>Scientist - Academia</td>
<td>LOXHD1 is required for Mechanotransduction and localizes at the Lower Tip-Link insertion point</td>
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<td>Xufeng Qiu</td>
<td>Postdoctoral Trainee</td>
<td>The Tetraspan LHFPLS Establishes Force Sensitivity of the Mechanotransduction Channel of Cochlear Hair Cells</td>
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<td>17:05 - 17:20</td>
<td>Isabel Aristizábal-Ramírez</td>
<td>Graduate Student</td>
<td>Deafness-associated Variant of the Calcium and Integrin-Binding Protein 2 (CIB2) Slows Down Force Transmission to the Mechanotransducer Channels in Mammalian Auditory Hair Cells</td>
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<td>17:20 - 17:35</td>
<td>Marcos Sotomayor</td>
<td>Scientist - Academia</td>
<td>Towards an Atomistic Model of the Inner-Ear Transduction Apparatus</td>
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Fifty Years of Hair-Cell Mechano transduction

Peter Barr-Gillespie
Oregon Health & Science University, Portland, OR, USA

A unique feature of the inner ear is its enigmatic mechanotransduction mechanism, which is responsible for transducing sound or head movements into electrical signals that are transmitted to the brain. The essential role of hair cells in responding electrically to mechanical stimuli was recognized more than fifty years ago, and I divide the subsequent experimental approaches to the transduction apparatus into three major eras. First was the Model Systems Era, where convenient preparations were identified that allowed detailed investigation of mechanotransduction with electrophysiological and biophysical methods. This era led to the development of simple, predictive models for the function of mechanotransduction. The next era was the Mouse Cochlea Era, where investigators developed an understanding of the unique properties of inner and outer hair cells in the mouse, chosen for the ability of its genome to be modified. Work during this era highlighted how distinct mammalian cochlea hair cells are from those of nonmammalian vertebrates, and also how different inner and outer hair cells of the cochlea are. Finally, we are presently in the middle of the Molecular Reconstruction Era, where the molecular composition and three-dimensional arrangement of the transduction apparatus is being worked out. During this era, the mouse is a principal model system, albeit not the only one. Many key components of the transduction apparatus have been identified, including the transduction channel pore, and increasing focus is being directed at determining how all of these components interact to form the sensitive transduction apparatus. A true atomic understanding of the molecular basis of mechanotransduction still eludes us, but we are getting ever so much closer.
Asymmetric Mechanotransduction by Hair Cells of the Zebrafish Lateral Line

Kayla Kindig1,2, Ruben Stepanyan1,3, Katie Kindt4, and Brian M. McDermott Jr.1,2,3,5,*

1Department of Otolaryngology–Head and Neck Surgery, Case Western Reserve University School of Medicine, Cleveland, Ohio, 44106, United States of America; 2Department of Biology, Case Western Reserve University, Cleveland, Ohio, 44106, United States of America; 3Department of Neurosciences, Case Western Reserve University School of Medicine, Cleveland, Ohio, 44106, United States of America; 4Section on Sensory Cell Development and Function, National Institute on Deafness and other Communication Disorders, Bethesda, Maryland, 20892 United States of America; 5Department of Genetics and Genome Sciences, Case Western Reserve University School of Medicine, Cleveland, Ohio, 44106, United States of America.

In the lateral line system, detection of water motion is conducted by neuromast organs, fundamental units that are arrayed on a fish’s surface. Each neuromast contains hair cells, specialized mechanoreceptors that convert mechanical stimuli, in the form of water movement, into electrical signals. The orientation of hair cells’ mechanosensitive structures ensures that the opening of mechanically-gated channels is optimal when deflected in a single direction. In each neuromast organ, hair cells have two opposing orientations, enabling bi-directional detection of water movement. Interestingly, Tmc2b and Tmc2a proteins that constitute the mechanotransduction channels in neuromasts distribute asymmetrically so that Tmc2a is expressed in hair cells of only one orientation. Here, using both in vivo recording of extracellular potentials and calcium imaging of neuromasts, we demonstrate that hair cells of one orientation have larger mechanosensitive responses. The associated afferent neurons that innervate neuromast hair cells faithfully preserve this functional difference en route to the brain. Moreover, Emx2, a transcription factor required to create hair cells with opposing orientations, is necessary to establish this functional asymmetry within neuromasts. Remarkably, genetic removal of Tmc2a abolishes this functional asymmetry. Overall, our work indicates that oppositely oriented hair cells employ different proteins to alter mechanotransduction in order to sense water motion.
LOXHD1 is required for Mechanotransduction and localizes at the Lower Tip-Link insertion point

Pei Wang¹, Katharine Miller¹, Enqi He¹, Sid Dhawan¹ & Nicolas Grillet¹,*

¹Department of Otolaryngology-Head and Neck Surgery, Stanford University, 240 Pasteur drive, Stanford, CA, USA

We investigate the molecular function of the LOXHD1 gene that we previously linked to an autosomal-recessive form of hearing loss in mice and humans (DFNB77) (Grillet N., AJHG, 2009). LOXHD1 encodes a protein made of 15 PLAT (Polycystin/Lipoxygenase/Alpha-Toxin) domains, known in other proteins to bind lipid and proteins. LOXHD1 is expressed selectively by hair cells and required for their function as assessed by ABR and DPOAE. The molecular function of LOXHD1 is unknown.

In two mouse mutants carrying mutations in the exon encoding the 10th PLAT repeat, inner hair cells present mechanotransduction currents of similar amplitude to controls at P7. Still, they are suddenly reduced by 95% at P11. Despite this strong MET phenotype, the hair bundle organization is not affected at this age, and the number of tip-links has not decreased. The upper tip-link protein Harmonin and the lower tip-link protein LHFPL5 are still properly localized at P11 in these mutants (Trouillet et al., 2021). Our results suggest that when LOXHD1 is mutated, at least some components of the mechanotransduction machinery are still maintained, but the channels are poorly activatable.

We will review these data and present new ones obtained from newly generated Loxhd1 mouse alleles: First, an allele where the entire gene has been deleted (160 kb) to prevent splicing isoform compensation; and secondly, a tagged knock-in allele that demonstrates that LOXHD1 localizes at the lower tip-link insertion point.

In conclusion, LOXHD1 is a new player in the hair cell mechanotransduction process. Our lab aims at defining in detail its role in this process.
The Tetraspan LHFPL5 Establishes Force Sensitivity of the Mechano-transduction Channel of Cochlear Hair Cells

Xufeng Qiu, Xiaoping Liang, Christopher Cunningham, Jose P. Llongueras, Guihong Peng and Ulrich Müller

The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

The mechanoelectrical transduction (MET) channel of cochlear hair cells is located in hair cells near tip links, the fine extracellular filaments that are thought to gate the MET channel. TMC1/2, TMIE, LHFPL5 and CIB2/3 are essential components of the MET channel complex. TMC1/2 and TMIE bind to each other to form a functional ion channel complex and mutations in each of these proteins affect channel pore properties. CIB2/3 binds to TMC1/2 and serves as an auxiliary subunit that regulates MET channel localization and function. The function of LHFPL5 in the MET complex has remained elusive. Here we show that LHFPL5 is critical to regulates the response for the MET channel to mechanical force, including the regulation of optimal channel activation and maximal force sensitivity. Using structure-based predictions and mutational analysis, we have identified domains critical for the function of LHFPL5 in regulating MET channel activity. Our studies thus provide insights into the gating mechanisms of the MET channel in hair cells and define a crucial role for LHFPL5 in establishing the channels force-sensitivity.
Deafness-associated Variant of the Calcium and Integrin-Binding Protein 2 (CIB2) Slows Down Force Transmission to the Mechanotransducer Channels in Mammalian Auditory Hair Cells

Isabel Aristizábal-Ramírez* 1, Arnaud P.J. Giese2, Abigail K. Dragich1, K. Sofia Zuluaga-Osorio1, Julie Watkins1, Shadan Hadi1, Saima Riazuddin2, Zubair M. Ahmed2, Gregory I. Frolenkov1

1Department of Physiology, University of Kentucky, Lexington, KY, 40536, USA
2Department of Otorhinolaryngology Head & Neck Surgery, University of Maryland, Baltimore, MD, 21201, USA

Calcium and integrin-binding protein 2 (CIB2) is essential for mechano-electrical transduction (MET) in mammalian auditory hair cells. We and others showed that CIB2 interacts with TMC1/2 and is required for TMC1/2 localization to the stereocilia bundle (Giese et al., 2017; Liang et al., 2021). Since CIB2 also binds to whirlin (Riazuddin et al., 2012), it may provide a Ca2+-dependent link between the plasma membrane and the actin core of stereocilium. To test this, we generated a mouse strain carrying the deafness-associated p.R186W Cib2 variant that does not disrupt CIB2-TMC1/2 interaction and results in decreased but still measurable MET currents in young postnatal cochlear hair cells. Using a custom piezo-driven probe that deflects stereocilia bundles in young postnatal cochlear hair cells. Using a custom piezo-driven probe that deflects stereocilia bundles in <30 μs, we determined that MET currents in Cib2R186W/R186W hair cells have “slow” but no “fast” adaptation. Surprisingly, the time constant of MET current activation in Cib2R186W/R186W mice is about three-fold slower than in control littermates and it does not depend on the amplitude of the MET current. These data suggest that p.R186W variant slows force transmission to the MET channels. We next estimated hair bundle stiffness by deflecting stereocilia with a fluid jet and measuring these deflections with high-speed camera. The results showed that p.R186W variant decreased hair bundle stiffness. Finally, we examined ultrastructural changes at the tips of stereocilia with different electron microscopy techniques and found that the lower tip-link density is disrupted in Cib2R186W/R186W mutants, while second row stereocilia are over-elongated. This over-elongation cannot be explained by known MET-dependent remodeling of stereocilia. We concluded that CIB2 is involved in establishing proper mechanical connections of the MET channels at the tips of stereocilia.

Supported by NIDCD/NIH (R01 DC012564 to Z.A. and G.F.)
Towards an Atomistic Model of the Inner-Ear Transduction Apparatus

Marcos Sotomayor*

The Ohio State University

At the foundation of vertebrate hearing and balance is the process of mechanotransduction, in which forces from sound and head movements are transduced into electrochemical signals in the inner ear. Mechanotransduction takes place in inner-ear hair cells and involves tip-link filaments that pull on ion channels to trigger sensory perception. Each tip link is made of cadherin-23 (CDH23) and protocadherin-15 (PCDH15) proteins, while the pore forming subunits of the transduction ion channel are formed by transmembrane channel-like proteins (TMCs) 1 and 2, likely coupled to accessory units formed by calcium- and integrin-binding proteins (CIBs) 2 and 3, the transmembrane inner-ear expressed protein TMIE, and the tetraspan membrane protein of hair-cell stereocilia TMHS (also known as LHFPL5). Here we present structural, biochemical, and computational studies aimed at elucidating the molecular mechanisms underlying function of the hair-cell transduction apparatus components. Data from X-ray crystallography, small-angle X-ray scattering, analytical ultracentrifugation experiments and low-resolution cryo-EM along with AlphaFold 2 predictions allowed us to build atomistic models of the entire tip link. Similarly, we used AlphaFold 2 models and nuclear magnetic resonance data to build TMC protein models in complex with CIBs suggesting a ‘clamp-like’ architecture involving TMC cytosolic domains. Molecular dynamics simulations of these models and of TMHS coupled to PCDH15 provide additional insights into possible mechanisms underlying the role of membrane tension in TMC gating. These data and models are providing a rigorous molecular view of mechanotransduction in normal and impaired hearing and balance.
# Session 3

## Molecular Biology of Cochlear Development

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<td>8:00 - 8:20</td>
<td>Andrew Groves</td>
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<td>Molecular Biology of Cochlear Development: Problems, Puzzles and Paradoxes in Making the World’s Best Hearing Aid</td>
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<td>8:20 - 8:35</td>
<td>Sandra De Haan</td>
<td>Graduate Student</td>
<td>Effects of Jag1-Mediated Notch Activation in Inner Ear Patterning: Insights from the Nodder Mouse Model for Alagille Syndrome</td>
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<td>8:35 - 8:50</td>
<td>Angelika Doetzhofer</td>
<td>Scientist - Academia</td>
<td>The RNA-binding Protein TRIM71 Regulates Pro-Sensory Cell Behavior in the Mammalian Cochlea</td>
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<td>8:50 - 9:05</td>
<td>Suraj Chakravarthy</td>
<td>Graduate Student</td>
<td>The Role of Ezrin and Radixin Proteins in “Blooming” of Nascent Stereocilia in Auditory Hair Cells</td>
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<td>9:05 - 9:20</td>
<td>Benjamin Perrin</td>
<td>Scientist - Academia</td>
<td>A Novel Population of Short Actin Filaments at Stereocilia Tips and a Tip-Down Widening Mechanism</td>
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<td>9:20 - 9:35</td>
<td>Basile Tarchini</td>
<td>Scientist - Academia</td>
<td>Heterotrimeric G Protein Signaling Regulates the Graded Height Architecture of the Stereocilia Bundle</td>
</tr>
<tr>
<td>9:35 - 9:50</td>
<td>Botond Banfi</td>
<td>Scientist - Academia</td>
<td>A Novel Regulator of Hair Cell Maturation Enhances ATOH1 Activity in the Hearing Organ</td>
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The mammalian cochlea grows out from the ventral portion of the embryonic inner ear. Cochlear progenitor cells have already received some patterning information before this outgrowth begins, but the mechanism of how this information is translated into forming distinct domains of the cochlear duct remains a mystery. Different regions of the cochlear duct exit the cell cycle at different times, with organ of Corti progenitors in particular showing an unusual uncoupling of cell cycle exit (in the apical-basal direction) from differentiation into hair cells and supporting cells (in a basal-apical direction). Although the signals responsible for forming hair cell and supporting cells are beginning to be identified, much remains to be discovered about how particular types of hair cells (inner versus outer hair cells) and supporting cells (e.g. Deiters’ versus pillar cells) arise in the correct place at the correct time. Although the development of non-sensory regions of the cochlea such as the lateral wall and Reissner’s membrane have received less attention than the organ of Corti, they are absolutely essential for cochlear function, and an investigation of their development is long overdue. My presentation will examine some of these questions and highlight additional interesting but unsolved questions in cochlear development.
Effects of Jag1-Mediated Notch Activation in Inner Ear Patterning: Insights from the Nodder Mouse Model for Alagille Syndrome.

Sandra de Haan*1,2, Matthew W. Kelley2, Emma R. Andersson1.

1. Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden
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Proper auditory function requires a precisely ordered mosaic pattern of mechanosensitive inner hair cells (IHCs), outer hair cells (OHCs) and subtypes of associated supporting cells (SCs) within the Organ of Corti. This pattern is established by a series of cell fate decisions mediated, in part, by Notch signalling, starting from early pro-sensory domain induction followed by inhibitory cues specifying HCs versus SCs.

Defects in Notch signalling lead to patterning defects that include extra IHCs and fewer OHCs, and subsequent hearing deficits, as exemplified by mice with mutations in the Notch ligand Jagged1 (Jag1), and patients with JAG1-disorder Alagille syndrome, a multisystem disorder that includes hearing loss. However, research into the role of Jag1 in cochlear development has been 1) limited to conditional and heterozygous loss of function models due to late embryonic lethality of Jag1 germline mutants and 2) incomplete, due to a predominant focus on late embryonic HC phenotypes, while Jag1, like Notch target genes, is expressed prior to sensory fate specification and remains expressed in SCs postnatally.

In this study, we aim to identify the roles of Jag1-mediated Notch activation in inner ear development by using immunohistochemistry and RNAscope to characterize changes in cochlear development in Nodder (Jag1Ndr/Ndr) mice, which are viable in the homozygous condition and recapitulate Alagille syndrome. Nodder mice display profound hearing loss and patterning defects that include an increased number of IHCs and associated SCs, fewer OHCs and associated SCs and atypical OHC-like cells located in the IHC-compartment that share morphological and molecular characteristics with OHCs.

Our data highlights the importance of Jag1 in several aspects of cochlear development and we are in the process of generating single cell sequencing data of our Nodder mice to provide mechanistic insights in Jag1-mediated pattern formation and to identify potential regenerative strategies to combat hearing deficits.
The RNA-binding Protein TRIM71 Regulates Pro-Sensory Cell Behavior in the Mammalian Cochlea

Xiaojun Li¹, Prathamesh T Nadar Ponniah¹, Charles Morgan¹, Lale Evsen¹, Kris Kahle², Waldemar Kolanus³, Angelika Doetzlhofer¹*

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The stereotyped cellular arrangement of mechano-sensory hair cells (HCs) and supporting cells (SCs) within the mammalian cochlea is safeguarded by a highly unusual pattern of terminal mitosis and differentiation. Although, the genes that regulate cell cycle exit and differentiation of HC and SC progenitors (pro-sensory cells) have been largely identified, how these distinct events are timed and coordinated relative to one another remains under active investigation. Here, we identify the RNA-binding protein TRIM71 as an essential regulator of auditory pro-sensory cell behavior. The evolutionary conserved Trim71 gene recently emerged as an essential regulator of embryonic development; however, its role in cochlear development is unaddressed. We found Trim71 expression peaked during early otic development, and persisted in cochlear pro-sensory cells, but rapidly declined upon their differentiation. To stage specifically delete Trim71, we administered doxycycline to Trim71 floxed mice carrying a tetracycline-inducible cre transgene. We found that doxycycline administration starting between E8.5 and E10.5 resulted in premature cell withdrawal and differentiation of auditory pro-sensory cells and caused hearing deficits in the mid to high frequency range. Similar defects were observed in Trim71 mutant mice (Trim71^{R595H/Δ}), in which one allele harbors a point mutation in TRIM71’s RNA-binding domain found in patients with congenital hydrocephaly and hearing loss. RNA-sequencing of E13.5 control and Trim71 mutant cochlear epithelia revealed that TRIM71 inhibits the expression of pro-differentiation genes, including Inhba (Activin A) and Tgfbr2, suggesting that TRIM71 maintains pro-sensory cells in a proliferative and undifferentiated state by antagonizing TGF-β type signaling. Consistent with such role, we found that inner ear-specific deletion of Inhba and Tgfbr1 delayed pro-sensory cell cycle withdrawal and differentiation. However, Inhba Tgfbr1 double mutants had normal hearing and investigations are ongoing to address the basis of high frequency hearing deficits in Trim71 mutant mice.
The Role of Ezrin and Radixin Proteins in “Blooming” of Nascent Stereocilia in Auditory Hair Cells

Suraj Ranganath Chakravarthy* and Raj K Ladher

National Centre for Biological Sciences, Tata Institute for Fundamental Research, GKV PO, Bellary Road, Bangalore, India,

Stereociliary development occurs in multiple steps. These have been well-characterised by Tilney et.al. in chick and Kaltenbach et.al. in golden hamster. Based on these studies, stereocilia formation starts an initial bloom of actin microvilli. These then elongate, staircase pattern is formed, and excess microvilli resorbed to give rise to a mature stereociliary bundle. In this study, using Scanning electron microscopy and super-resolution microscopy, I have characterised hair bundle development in the mouse organ of Corti from embryonic day (E) 14.5 to Postnatal (P) 12. These stages provide a comprehensive account of hair bundle initiation at E15.5 in IHCs, establishment of staircase patterning by P2 in Inner hair cells (IHCs) and Outer hair cells (OHCs), and resorption of extra microvilli by P11 in OHCs. To understand the initiation of the stereocilia, we examined the molecular mechanisms that may promote “microviliogenesis”. Using light microscopy, we examined the expression of ezrin and radixin. We find that they are expressed in the apical plasma membrane from E15.5 onwards with radixin and ezrin coexpressing in microvilli-like immature stereociliary bundle of IHCs. ERM (Ezrin, Radixin and Moesin) proteins have been found to link plasma membrane to actin filaments. We have perturbed ERM proteins using small molecule inhibitors in organotypic cultures of mouse organ of Corti and chick basilar papilla. We have used electron microscopy and super-resolution microscopy to observe the effects of these inhibitors. Following ezrin inhibitor treatment, we observed that there was a stage dependant perturbation in planar cell polarity of hair bundles. In contrast, radixin inhibitor treated explants showed specific morphological changes such as reduction in number and fusion of immature stereocilia at early embryonic stages of mouse and chick. Our findings suggest specific roles of the ERM proteins Ezrin and Radixin in initiation of immature stereocilia.
A Novel Population of Short Actin Filaments at Stereocilia Tips and a Tip-Down Widening Mechanism

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Hair cell stereocilia sizes and shapes are dictated by its actin core, which is comprised of parallel actin filaments (F-actin) that are oriented with their fast-growing barbed ends oriented towards stereocilia tips and their slower-growing pointed ends oriented towards bases. F-actin in the stereocilia core is highly stable, but actin at stereocilia tips turns over more rapidly. Here, we provide evidence that the dynamic actin at tips includes short actin filaments (tip filaments) that are separate from the core filaments and suggest that these tip filaments contribute to stereocilia growth. We identified tip filaments by probing permeabilized postnatal mouse cochlear tissue with purified, exogenous His-tropomodulin1 (His-TMOD1). Tropomodulins are well-characterized proteins that bind pointed ends of actin filaments, but not barbed ends. His-TMOD1 labeled the tips of stereocilia in all rows before postnatal day 6 (P6), with labeling declining until P9. Since actin in the stereocilia core have only their barbed ends at stereocilia tips, we propose that the pointed ends detected by the His-TMOD1 probe are short actin filaments that are not part of the core. Tip filament levels are highest when stereocilia are widening, suggesting they may contribute to this aspect of stereocilia growth. Correspondingly, transient overexpression of EGFP-actin changed the distribution of pointed ends so they were more evident in the stereocilia shaft, which is consistent with actin filaments adding to the core in a tip-down fashion. In addition, live-cell imaging revealed that overexpressed EGFP-actin initially localized to stereocilia tips, but then extended down the stereocilia shaft over time. Super-resolution imaging of fixed samples showed that the newly expressed EGFP-actin signal surrounded the stable, preexisting F-actin core. Together, these data suggest a tip-down widening mechanism where tip filaments may initiate actin polymerization and create new parallel F-actin at the periphery of the stereocilia core.
Heterotrimeric G Protein Signaling Regulates the Graded Height Architecture of the Stereocilia Bundle

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¹The Jackson Laboratory, Bar Harbor, ME. ²Department of Medicine, Tufts University, Boston, MA.

In hair cells, mechanosensory stereocilia are organized in rows of graded heights, a slanted architecture essential for auditory function. At row 1 only, the Elongation Complex trafficked to stereocilia tips by Myosin 15 is augmented by an atypical G protein module: inhibitory G protein alpha (GNAI) bound to the GPSM2 scaffold. GPSM2-GNAI boosts enrichment of the Elongation Complex, conferring row 1 its tallest identity. GPSM2 keeps GNAI in a GDP-bound state, but how GPSM2-GNAI is generated and relates to heterotrimeric G protein signaling is not known. Here we discover that heterotrimeric G protein signaling is the source of the GPSM2-GNAI complex, and thus an upstream cue conferring distinct stereocilia identities across rows. We first show that a negative regulator of heterotrimeric G protein signaling, Regulator of G protein Signaling 12 (RGS12), is a new deafness protein. RGS12 is asymmetrically enriched at the lateral hair cell junction, where it colocalizes with the positive regulator DAPLE. In Rgs12 mouse mutants, GPSM2-GNAI cannot localize to the bare zone, the apical membrane region sitting between the lateral junction and row 1 stereocilia. Instead, GPSM2-GNAI accumulates at the base of stereocilia in all rows, producing severe defects mimicking Gpsm2 and Gnai mutants. Interestingly, GPSM2 and RGS12 both have GoLoco motifs that bind and sequester GDP-bound GNAI, but GPSM2 can outcompete RGS12. Upon FRAP, GNAI is recovered at the lateral junction before the bare zone. Together, our results suggest that DAPLE and RGS12 dissociate G protein heterotrimers at the lateral hair cell junction and generate free GNAI(GDP). GNAI(GDP) is then transferred to the adjacent apical membrane with GPSM2, where polarization at the bare zone limits GPSM2-GNAI trafficking to row 1 stereocilia. GPSM2-GNAI is thus a heterotrimeric G protein derivative polarized by RGS12 to impart asymmetry to the forming stereocilia bundle and enable auditory function.
A Novel Regulator of Hair Cell Maturation Enhances ATOH1 Activity in the Hearing Organ

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1Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA.

ATOH1 is an essential transcription factor for the differentiation of pro-sensory cells to hair cells (HCs). Ectopic expression of ATOH1 results in trans-differentiation of greater epithelial ridge (GER) cells to HC-like cells in the immature cochlea. Other cochlear cells are less readily reprogrammed by ATOH1, indicating that certain potentiators of ATOH1 activity are expressed predominantly in pro-sensory and GER cells. The identification of these potentiators is necessary for improving the rational design of HC-regenerating approaches that are based on Atoh1 gene delivery. Here we show that the transcription factor TOX is a previously unrecognized potentiator of ATOH1 activity and a crucial regulator of cochlear HC maturation. Tox is upregulated in maturing HCs in parallel with Atoh1. Tox is also expressed in the GER in mice until the end of the first postnatal week. Genetic inactivations of TOX by gene trap insertion (Toxgt/gt) and exon deletion (ToxΔ/Δ) cause severe stereocilium defects, postnatal loss of cochlear HCs, and deafness. In Toxgt/gt mice, removal of the gene trap specifically in immature HCs rescues HCs and restores hearing. In the organ of Corti of newborn ToxΔ/Δ mice, the expression levels of hundreds of genes are abnormal. The ‘abnormally low expression’ group includes many known target genes of ATOH1. Consistent with a notion of functional association between ATOH1 and TOX, deletion of one Atoh1 allele (Atoh1+-/-) accelerates dramatically the loss of inner HCs in ToxΔ/Δ mice but not in control (Tox+/+) mice. Lastly, in GER cultures from 1-week old wild-type mice, ectopic co-expression of ATOH1 and TOX results in more HC-like cells as compared to ectopic expression of ATOH1 alone. Together, our data reveal that TOX is a crucial regulator of HC maturation and a candidate for use in HC regeneration-inducing gene cocktails.
# Session 4

## TranscriptOME Profiling of the Auditory System

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<td>10:00 - 10:20</td>
<td>Lisa Goodrich</td>
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<tr>
<td>10:20 - 10:35</td>
<td>Kevin Rose</td>
<td>Graduate Student</td>
<td>Diversity of Otic Mesenchyme Cells During Cochlear Development</td>
</tr>
<tr>
<td>10:35 - 10:50</td>
<td>Joerg Waldhaus</td>
<td>Scientist - Academia</td>
<td>Opposing Gradients of Retinoic Acid and Sonic Hedgehog Signaling Specify the Tonotopic Axis in the Murine Cochlea</td>
</tr>
<tr>
<td>10:50 - 11:05</td>
<td>Yoshitomo Ueda</td>
<td>Postdoctoral Trainee</td>
<td>Defining the Developmental Trajectory of Prosensory Cells in Human Inner Ear Organoids at Single-Cell Resolution</td>
</tr>
<tr>
<td>11:05-11:20</td>
<td>David He</td>
<td>Scientist - Academia</td>
<td>Transcriptomic Differences between Adult Mouse Cochlear and Vestibular Hair Cells</td>
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<tr>
<td>11:20-11:35</td>
<td>Matthew Kelly</td>
<td>Scientist - Academia</td>
<td>Development of Neuronal Phenotypes in the Spiral Ganglion</td>
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<td>11:35-12:15</td>
<td>Andrew GROVES, Lisa GOODRICH, Doris WU, Bernd FRITZSCH, Angelika DOETZLHOFER &amp; Matt KELLY</td>
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Diversity of Otic Mesenchyme During Cochlear Development

Kevin P. Rose1,2, Paige Brooks3, Beatrice Milon1,3, Ori Zalzman4, Mansa Gurjar3, Thomas Coate3, and Ronna Hertzano1,2,5

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2 Program in Molecular Medicine, University of Maryland School of Medicine, Baltimore, MD, 21201, USA
3 Department of Biology, Georgetown University, Washington, District of Columbia, 20007, USA
4 Department of Otorhinolaryngology Head and Neck Surgery, University of Maryland School of Medicine, Baltimore 21201, USA
5 Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore 21201, USA

The cochlea is made up of a large heterogenous group of cells, all working in harmony to convert mechanical stimuli into electrical signals resulting in the perception of sound. One such cell type are the otic mesenchyme cells, the most numerous cell type within the cochlea during development, essential for maturation of normal hearing in both human and mouse. Otic mesenchyme cells differentiate into many key cochlear cell types, including fibrocytes of the lateral wall and spiral limbus, osteoblasts/casts of the modiolar bone, and the tympanic border cells of the basilar membrane. There are many human deafness genes directly related to cochlear mesenchyme including *Pou3f4*, which causes a multitude of different abnormalities affecting many key cochlear processes. This leads us to the hypothesis that otic mesenchyme are not a homogenous cell type but are spatially, transcriptionally and functionally distinct during cochlear development. In this study, we performed scRNA-seq to characterize otic mesenchyme cells at multiple timepoints (E15.5 and P7) during cochlear development. Analyses were performed using the R packages Seurat, Monocle, and Cellchat. Validation of scRNA-seq results were completed via immunohistochemistry. We show that indeed otic mesenchyme can be divided into four transcriptionally distinct subpopulations, each with unique marker genes that reveal each population is also spatially distinct at both E15.5 and P7. Cell-cell communication analyses identify the paracrine signaling pathways that influence the development of surrounding cell types such as the spiral ganglion neurons and stria vascularis cell types. Finally, pseudotime analysis elucidates key transcriptional regulators in each otic mesenchyme subpopulation during maturation. In conclusion, otic mesenchyme are heterogenous in nature and fully understanding their role in cochlear development allows for a more focused approach in studying the development of the endocochlear potential, role of the basilar membrane and tympanic border cells, axonal guidance, and modiolar ossification.
Opposing Gradients of Retinoic Acid and Sonic Hedgehog Signaling Specify the Tonotopic Axis in the Murine Cochlea

Shuze Wang\textsuperscript{1,2}, Saikat Chakraborty\textsuperscript{1}, Yujuan Fu\textsuperscript{3}, Mary P. Lee\textsuperscript{1}, Jie Liu\textsuperscript{2}, Joerg Waldhaus\textsuperscript{1*}

\textsuperscript{1}Department of Otolaryngology–Head and Neck Surgery, Kresge Hearing Research Institute, University of Michigan, Ann Arbor, Michigan, USA. \textsuperscript{2}Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, Michigan, USA

In the mammalian auditory system, frequency discrimination depends on morphological and physiological properties of the organ of Corti that gradually change along the longitudinal (tonotopic) axis of the organ. For instance, hair cell size, hair bundle length, and intracellular calcium buffer concentration change gradually along the longitudinal axis. Together, these factors have an impact on parameters like membrane capacitance, adaptation kinetics, and resting potential, and therefore shape the receptor potential and tuning properties of the individual hair cell. At the molecular level, those frequency-specific characteristics are mirrored in gene expression gradients, which in turn require tonotopic patterning of the cochlea. However, the molecular mechanisms specifying tonotopic identity remain poorly understood. To investigate the molecular mechanisms patterning the organ of Corti along the frequency axis, we reconstructed the embryonic cochlear duct in 3D-space from single-cell gene expression data at two developmental timepoints. These analyses resulted in the hypothesis that morphogens rather than a time-related mechanism confer spatial identity in the cochlea. Subsequently, retinoic acid signaling was identified to form a gradient in the cochlear floor. Utilizing cochlear explants, we observed that the retinoic acid signaling cascade is functional in the cochlear floor at E14.5. Further experiments indicated that sonic hedgehog signaling is involved in shaping the retinoic acid gradient via transcriptional regulation of the retinoic acid degrading enzyme \textit{Cyp26b1}. These findings suggest that retinoic acid and sonic hedgehog form opposing morphogen gradients, whereby retinoic acid patterns the base and sonic hedgehog the apex of the murine cochlea.
Defining the Developmental Trajectory of Prosensory Cells in Human Inner Ear Organoids at Single-Cell Resolution

Yoshitomo Ueda, Takashi Nakamura, Jing Nie, Alexander J. Solivais, John R. Hoffman, & Eri Hashino

Department of Otolaryngology-Head and Neck Surgery, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA.

The sensory epithelia in the inner ear contain mechanosensitive hair cells and supporting cells. Both cell types arise from SOX2-expressing prosensory cells, but the mechanisms underlying the diversification of these cell lineages are poorly understood. SOX2 is a unique protein as its expression is maintained in the sensory epithelia throughout the developmental stages while surrounding neuronal and periotic mesenchymal cells are devoid of SOX2 expression. We established a SOX2-2A-ntdTomato human embryonic stem cell line using CRISPR/Cas9 and using this new reporter line, investigated spatiotemporal changes in SOX2 expression in human inner ear organoids. To determine the transcriptional trajectory of prosensory cells, single-cell RNA sequencing (scRNA-seq) analysis was carried out with SOX2-positive cells isolated from inner ear organoids at various time points between differentiation days 20 and 60. Differential expression between cell types was determined using DESeq2, and the results were passed to iDEA, a platform for gene set enrichment analysis, where differentially expressed genes were compared to gene sets taken from the Molecular Signatures Database (MSigDB). The R Package Monocle3 was used to identify the developmental trajectory of the SOX2-positive cells, revealing the expression dynamics of key genes driving inner ear development. Our preliminary analysis suggests that hair cells arise primarily from supporting cells, rather than multipotent transitional cells in organoids. Moreover, gene sets associated with ion channel- and ion transporter-related processes are enriched in supporting cells vs. prosensory cells, whereas ones associated with Wnt signaling-related processes are enriched in hair cells vs. supporting cells. We also found a set of genes upregulated early in hair cells. These findings provide valuable insights into how prosensory cells give rise to hair cells and supporting cells during human inner ear development, and may provide a clue to promote hair cell regeneration from resident supporting cell populations in individuals with profound hearing loss.
Transcriptomic Differences between Adult Mouse Cochlear and Vestibular Hair Cells

Zhenhang Xu, Huizhan Liu, Shu Tu, Caroline Pass, Sarath Vijayakumar, Jian Zuo, and David Z He*

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The auditory and vestibular sensory epithelia in the mammalian inner ear contain mechanosensitive hair cells (HCs). HCs transduce mechanical stimuli into electrical activity. Although HCs in the adult cochlear and vestibular end organs are similar, they have substantially different morphology and physiology. The molecular mechanisms underlying their differences are still poorly understood. We used single-cell RNA-seq (scRNA-seq) to examine the transcriptomes of adult cochlear and vestibular HCs (CHCs and VHCs). Sensory epithelia from cochleae and vestibular end organs were dissected out from 10-week-old CBA/J mice. Dissociated cells were then run through 10x Genomics scRNA sequencing platform for preparing the cDNA Libraries. Libraries were sequenced on an Illumina NextSeq and scRNA-seq data were analyzed and clustered using Seurat. A minimum of three biological replicates were included for each group. RNAscope was used for gene expression validation. We collected and sequenced ~20,282 single cells from cochleae and 11,444 cells from vestibular end organs. 413 and 394 cells meet the criteria of outer and inner HCs, respectively, based on their unique gene expression profiles. Similarly, 231 and 257 are regarded as type I and type II VHCs, respectively. The analysis identified both common and unique genes in different HC types. The top 50 differentially expressed genes include Ocm, Gata3, Slc7a14, Lbh, Dnm3 and Kcnj13 in CHCs and Sox2, Spp1, Adam11, Rbp1, Ccer2 and Wfdc2 in VHCs. We generated Kcnj13 conditional knockout mice to examine the role of KCNJ13 in HCs and show that the mice have progressive hearing loss at adult age. Our analysis provides insights into the genes and molecular pathways underlying differences between adult CHCs and VHCs.
Development of Neuronal Phenotypes in the Spiral Ganglion

Tessa R. Sanders and Matthew W. Kelley*

Laboratory of Cochlear Development, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Maryland.

Afferent neurons in the cochlear spiral ganglion (SG) are the primary relays between mechanosensory hair cells and the auditory brainstem. Anatomical, physiological, and molecular studies have identified four distinct neuronal subtypes within the ganglion. Type I SG neurons, which form unipolar synapses with inner hair cells, can be subdivided into three classes, IA, IB, and IC, based on spontaneous firing rate, location of terminal synapses, and gene expression. Type II SG neurons, which form 5 to 10% of the total SG population, make up the fourth subtype. The presence of all four phenotypes is thought to be necessary for normal auditory function, however the developmental processes that specify each unique neuronal identity are unknown.

To characterize the development of SG neuronal subtypes, neurons, associated glia and mesenchyme were dissected from mice at embryonic days 14 (E14), E16, E18 or P1. Ganglia from each age were dissociated to a single cell suspension and single cell RNA sequencing was performed using the 10X Genomics Chromium Platform and an Illumina Nextseq. Neurons from each time point were identified based on gene expression and re-clustered using Seurat. An initial review of the data indicated that all four SG neuronal subtypes are present by P1, indicating that the specification occurs embryonically. Pseudotime analysis using Slingshot identified a bifurcating lineage in which immature SG neurons initially split into Type 1A/II and Type 1B/C precursors between E14 and E16 followed by subsequent secondary splits between E16 and E18 to give rise to all four phenotypes prior to birth. Regulon analysis using SCENIC identified candidate transcription factors for each of the lineage bifurcations, including Tle4 and Sox9, which may act to direct developing SG neurons along specific lineages. Future experiments will test candidate transcription factors using a combination of conditional mouse mutants, anatomical analyses and single cell RNAseq.
## Session 6

### Pathological Mechanisms Underlying Deafness (1)

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<td>8:00 - 8:20</td>
<td><strong>Karen Steel</strong></td>
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<td>A Thousand Ways to Lose Your Hearing</td>
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<tr>
<td>8:20 - 8:35</td>
<td><strong>Miguel Angel Moreno-Pelayo</strong></td>
<td>Scientist - Academia</td>
<td>Insights into the Pathophysiology of DFNA44 Hearing Loss Associated with CCDC50 Variants</td>
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<tr>
<td>8:35 - 8:50</td>
<td><strong>Yehree Kim</strong></td>
<td>Scientist - Academia</td>
<td>Auditory Phenotype and Histopathologic Findings of a Mutant Nlrp3 Expression Mouse Model</td>
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<td>8:50 - 9:05</td>
<td><strong>Isabelle Roux</strong></td>
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<td>Interaction of SLC26A4 with Adaptor Protein (AP-2) Complex in Mitochondria-Rich Cells of the Endolymphatic Sac</td>
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<td>9:05 - 9:20</td>
<td><strong>Maria Lachgar-Ruiz</strong></td>
<td>Graduate Student</td>
<td>Exploring the Pathological Mechanisms of miR-96 Mutations in the Inner Ear</td>
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<td>9:20 - 9:35</td>
<td><strong>Sherylanne Newton</strong></td>
<td>Postdoctoral Trainee</td>
<td>Neuroplastin Genetically Interacts with Cadherin 23 and Np55 is Sufficient for Cochlear Hair Cell Function and Hearing</td>
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A Thousand Ways to Lose Your Hearing

Karen P Steel

King’s College London

Hearing impairment is highly heterogeneous. Over 700 genes have already been reported to underlie deafness and each one has a different molecular mechanism leading to dysfunction, in addition to the varied environmental impacts. The resulting phenotypes can be grouped according to the developmental stage affected (early inner ear morphogenesis, postmitotic differentiation, maintenance of auditory function during the lifespan) or to the site of the primary lesion. Sites of lesion can include the middle or outer ear, the bony labyrinth, the lateral wall of the cochlear duct, the cochlear vasculature, sensory hair cells, supporting cells of the organ of Corti, neurons and their synapses with hair cells, or sites in the central auditory pathways. Hearing impairment can be found associated with other features as part of a syndrome or can be non-syndromic. A single gene can have many different mutations in different individuals leading to different outcomes; for example, MYO7A mutations can lead to Usher syndrome, non-syndromic deafness, early onset or late onset sensory loss. Recent studies of the genomic basis of age-related hearing loss have highlighted the involvement of several genes already known to underlie early-onset childhood deafness. It is a complex landscape.

Given the very broad range of disease mechanisms, it is curious that so much attention has focussed on hair cells and so little to other cell types involved in deafness. It is often said that hair cell degeneration is the major cause of hearing loss in the human population, but is there any evidence for this? Correlation is not the same as causation. Hair cells do seem to be particularly fragile, but degeneration is usually the end result of cochlear dysfunction. Would it be helpful to think about repair and restoration of cochlear function before hair cells die? Is there a window of opportunity for repair before the secondary degeneration of hair cells? Can we develop tools to identify the primary site-of-lesion to apply the appropriate treatment during this window? Can we develop generic treatments for, say, boosting strial function or encouraging reconnection of wayward neurites, to provide affordable diagnostics and treatments for groups of pathologies? Is it only hair cells that matter? Some of these questions will be discussed.
Insights into the Pathophysiology of DFNA44 Hearing Loss Associated with CCDC50 Variants

Matías Morín1, María Lachgar-Ruiz1,2, Elisa Martelletti2, Neil J Ingham2, Lorenzo Preite2, Luciana Santos Serrão de Castro1, Karen P Steel2, Miguel Ángel Moreno-Pelayo1*

1Servicio de Genética, Hospital Universitario Ramón y Cajal, IRYCIS and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Carretera de Colmenar km 9.100, 28034 Madrid, Spain. 2Wolfson Centre for Age-Related Diseases, King’s College London, Guy’s Campus, London SE1 1UL, UK

CCDC50 maps to the DFNA44 locus and encodes Ymer protein, an effector of epidermal growth factor (EGF)-mediated cell signaling. We previously reported that the c.866_873dup mutation in CCDC50 cause progressive non-syndromic hearing loss, producing the mutant protein (p.(Phe292Hisfs*37)) aberrantly accumulated in the perinuclear area of NIH 3T3 transfected cells. In this study we have expanded the mutation spectrum of DFNA44 hearing loss in Spain by identifying a novel mutation in CCDC50 after screening 111 familial cases with our custom OTO-NGS-V1 gene panel. To gain insight into the pathological mechanism associated with CCDC50 mutations an additional set of 6 artificial Ymer mutants sharing different parts of the protein tail were created by site-directed mutagenesis and the cellular distribution was assessed by immunohistochemistry in transfected NIH 3T3 cells. Moreover, to study the role of CCDC50 in the inner ear we have generated a Ccdc50tm1b mouse mutant and performed Auditory Brainstem Response (ABR) recordings in the animals.

The novel frameshift mutation (c.828_858del; p.(Asp276Glufs*40)) produces an aberrant protein, 39 amino acids longer than the wildtype. Pairwise alignment revealed that p.(Asp276Glufs*40) protein tail was identical to the one generated by former mutant p.(Phe292Hisfs*37) and led to a similar altered distribution pattern in NIH3T3, suggesting that the effect of the mutation might be mediated through the aberrant protein tail. Interestingly, in vitro cellular studies revealed that only the mutants containing the six amino acid sequence CLENGL as part of their aberrant protein tail showed perinuclear aggregates of Ymer. Moreover, Ccdc50tm1b homozygous and heterozygous mutant mice showed normal ABR thresholds up to 6 months old, suggesting the hearing loss is not due to a loss of function effect. These findings taken in aggregate may suggest that mutations in CCDC50 exert their effect through a dominant-negative or a gain of function mechanism mediated by the CLENGL motif.
The pathogenesis of hearing loss in autoinflammatory disorders due to activation of the inflammasome remains incompletely understood. Previously no animals expressing mutant Nlrp3 survived to an age when hearing evaluation was possible due to embryonic lethality. We aimed to establish a novel mouse model that manifests quantifiable hearing loss with other syndromic features due to alteration of Nlrp3 and investigate the audiologic and histopathologic phenotype in the cochlea to clarify how the genetic alterations of NLRP3 could induce autoinflammatory hearing loss.

To induce inner ear expression of the mutant Nlrp3, Nlrp3D301NneoR mice were bred with Gfi1Cre knock-in mice for conditional mutant Nlrp3 activation. Hearing thresholds were measured. Hematoxylin-eosin sections of the cochlea was examined under light microscopy. Immunohistochemical analyses of cochlear whole-mount preparations and frozen sections were performed.

We established a mouse model that manifests quantifiable hearing loss due to Nlrp3 alteration. ABR recordings of Nlrp3D301NneoR/Gfi1Cre mice exhibited severe to profound hearing loss at P20. There was overexpression of mutant Nlrp3, and mutant Nlrp3 expression was noted in the spiral prominence, the outer sulcus region (Claudius cells and outer sulcus cells), the organ of Corti, the inner sulcus, and the spiral ganglion neurons in the cochlea. The hematoxylin-eosin sections of Nlrp3D301NneoR/Gfi1Cre mice cochleae at P12 exhibited a disorganized organ of Corti between the outer hair cells/supporting Dieter’s cells and basilar membrane compared with the wild type mice, leading to a collapsed Nuel’s space. This morphologic feature gradually returned to normal by P15.

We report a mutant Nlrp3 overexpression mouse model (Nlrp3D301NneoR/Gfi1Cre) that shows overexpression of Nlrp3 in the cochlea, a transient developmental lag and severe hearing loss. We expect that this mouse line, which models human autoinflammatory hearing loss, could provide a valuable tool to elucidate the underlying pathogenic mechanism of inflammasome activation-mediated hearing loss.
Interaction of SLC26A4 with Adaptor Protein (AP-2) Complex in Mitochondria-Rich Cells of the Endolymphatic Sac

Hyun Jae Lee¹, Cristina Fenollar-Ferrer¹,², Kevin Isgrig³, Ya-Xian Wang⁴, Juleh Eide¹, Keiji Honda¹, Wade W. Chien³,⁵, Ronald S. Petralia⁴, Juan S. Bonifacino⁶, Andrew J. Griffith¹,⁷, Isabelle Roux¹*  

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Pathogenic variants in SLC26A4 are a common cause of sensorineural hearing loss associated with enlargement of the vestibular aqueduct (EVA). SLC26A4 encodes an anion exchanger, SLC26A4 (pendrin) expressed in epithelial cells of the inner ear, kidney and thyroid. SLC26A4 is enriched in the apical region of mitochondria-rich cells of the endolymphatic sac, where it transports chloride and bicarbonate, and is necessary for endolymph reabsorption and inner ear morphogenesis. To gain insights into SLC26A4 regulation and functional network, we used yeast two-hybrid screenings to define the SLC26A4 interactome (Hybrigenics Services, France). The µ2 subunit of the adaptor protein 2 (AP-2) complex was identified as a possible partner of SLC26A4. AP-2, a key component required for clathrin-mediated endocytosis, could contribute to the regulation of SLC26A4 surface expression.

Confocal microscopy demonstrated that both SLC26A4 and AP-2 immunolabeling are concentrated and colocalized in the apical region of the endolymphatic sac epithelium. Immunogold electron microscopy showed that SLC26A4 is localized along the microvilli of the apical-luminal surface of mitochondria-rich cells and is also associated with clathrin-coated vesicles near the basal regions of these microvilli.

The interaction between the carboxy-terminal domain of SLC26A4 and the µ2 subunit of AP-2 was validated in yeast cells using two-hybrid assays, and in HeLa cells using nanoscale pull-down assays. Using both these assays and computational structural modeling, we identified amino acid residues in SLC26A4 and the µ2 subunit that are necessary for this interaction to occur, and modeled the docking of these two proteins.

We are currently testing how changes in clathrin-mediated endocytosis and µ2 expression influence SLC26A4 surface expression in the endolymphatic sac epithelium. If by modulating the interaction between SLC26A4 and µ2 we can increase SLC26A4 retention at the plasma membrane, this could be a novel approach to treatment of hearing loss caused by SLC26A4 insufficiency.
Exploring the Pathological Mechanisms of miR-96 Mutations in the Inner Ear

María Lachgar-Ruiz*, Morag A Lewis¹, Jing Chen¹, Francesca Di Domenico¹, Matías Morín², Sergio Fernández Peñalver², Miguel Ángel Moreno-Pelayo², Karen P Steel¹

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The microRNA miR-96 is important for hearing, as it acts as a transcriptional regulator in the inner ear and coordinates hair cell maturation. Point mutations in the seed region of miR-96 cause dominant progressive hearing loss in humans and mice. Here, we present two mouse mutants carrying two point mutations identified as underlying progressive hearing loss in humans (Mir96s403 and Mir96s1334). This study aims to determine the underlying pathological mechanisms in the inner ear.

Auditory brainstem response (ABR) measurements, scanning electron microscopy (SEM) and immunolabelling of pre- and post-synaptic components were used to determine the onset of hearing impairment, the hair bundles' morphology, and look for synaptic defects, respectively. We performed RNAseq of the organ of Corti and RT-qPCR to determine how the different mutations affect the gene expression profile. We are currently using several approaches such as gene set enrichment analysis (GSEA) to construct the miR-96 regulatory network.

Our results indicate that the two mutations lead to different physiological, structural and transcriptional phenotypes. Homozygotes of both mouse lines exhibit profound hearing loss, but only Mir96s1334 heterozygous mice have a mild progressive hearing loss. Structural analyses showed hair cell degeneration and misshapen hair bundles in both mutants, with Mir96s1334 mice being more severely affected. Moreover, Mir96s403 homozygotes show a reduction in the number of inner hair cell synapses. The structural phenotype of Mir96s1334 mice is more severe than that of Mir96s403 mice, consistent with the audiological features displayed by humans carrying those mutations. However, the lack of hearing impairment in Mir96s403 heterozygotes in contrast to the human findings might indicate that mutant miR-96 is acquiring different targets in mice and humans. Identifying the critical pathways underlying hearing impairment will ultimately allow the pharmacological modulation of the miR-96 regulatory network for the prevention or delay of hearing loss.
Neuroplastin Genetically Interacts with Cadherin 23 and Np55 is Sufficient for Cochlear Hair Cell Function and Hearing

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Mammalian hearing involves the mechanoelectrical transduction (MET) of sound-induced fluid waves in the cochlea. Essential to this process are the specialised sensory cochlear cells, the inner (IHCs) and outer hair cells (OHCs). While genetic hearing loss is highly heterogeneous, understanding the requirement of each gene will lead to a better understanding of the molecular basis of hearing and also to therapeutic opportunities for deafness. The Neuroplastin (Nptn) gene, which encodes two protein isoforms Np55 and Np65, is required for hearing, and homozygous loss-of-function mutations that affect both isoforms lead to profound deafness in mice. Here we have utilised several distinct mouse models to elaborate upon the spatial, temporal, and functional requirement of Nptn for hearing. While we demonstrate that both Np55 and Np65 are present in cochlear cells, characterisation of a Np65-specific mouse knockout shows normal hearing thresholds indicating that Np65 is functionally redundant for hearing. In contrast, we find that Nptn-knockout mice have significantly reduced maximal MET currents and MET channel open probabilities in mature OHCs, with both OHCs and IHCs also failing to develop fully mature basolateral currents. Furthermore, comparing the hearing thresholds and IHC synapse structure of Nptn-knockout mice with those of mice that lack Nptn only in IHCs and OHCs shows that the majority of the auditory deficit is explained by hair cell dysfunction, with abnormal afferent synapses contributing only a small proportion of the hearing loss. Finally, we show that continued expression of Neuroplastin in OHCs of adult mice is required for membrane localisation of Plasma Membrane Ca²⁺ ATPase 2 (PMCA2), which is essential for hearing function. Moreover, Nptn haploinsufficiency phenocopies Atp2b2 (encodes PMCA2) mutations, with heterozygous Nptn-knockout mice exhibiting hearing loss through genetic interaction with the Cdh23ahl allele. Together, our findings provide further insight to the functional requirement of Neuroplastin for mammalian hearing.
# Session 7

## Pathological Mechanisms Underlying Deafness (2)

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Karen STEEL, Mike BOWL, Aziz EL AMRAOUI, Sandrine MARLIN, Katie KINDT, Allen RYAN, Miguel MORENO-PELAYO
Age and Noise Affect Distinct Physiological Processes in the Auditory System

Ben Warren*, Alix Blockley, Daisy Ogle, Charlie Woodrow, Fernando Montealegre-Z

Biological and mechanical systems, whether by their overuse or their aging, will inevitably fail. Hearing provides a poignant example of this with noise-induced and age-related hearing loss. Both noise-induced and age-related hearing loss is not unique to humans, however, and is experienced by all animals in the face of wild and eclectic differences in ear morphology and operation.

In humans the cumulative effect of noise-induced hearing loss is largest during middle life. As old age approaches age-related hearing loss dominates so that hearing thresholds are similar – irrespective of how many rock concerts you attended. Is this pattern of hearing loss unique to humans? Does it result from mammalian specialisms? Our data suggests not as we found the same “middle-aged” pattern of hearing loss in an insect, the desert locust.

We exploited the high experimental throughput of the desert locust to thoroughly quantify several deafness-associated changes: auditory receptor number, electrophysiological properties and function of auditory receptors, auditory nerve thickness, lymph potential, supporting cell number. We used a combination of electrophysiological approaches, morphological analysis and biomechanical measurements of the tympanum. Each part of the auditory system, measured here, shares homologous function across animal auditory systems. We found components and regions in the insect ear are specifically affected by noise, others by age and still others are compounded by both. We believe this complexity is due to the cell-specific molecular control processes that operate in all auditory systems, including mammals.
Neurodegeneration in the Cochlea After Hair Cell Loss is Due to an Immune Response

Steven Green, Ben Gansemer*, Muhammad Taifur Rahman, Adrianna Caro

Departments of Biology and Otolaryngology, University of Iowa

Destruction of cochlear hair cells by aminoglycoside antibiotics leads to gradual death of the spiral ganglion neurons (SGNs) that relay auditory information from hair cells to the brain. To investigate the causes of this neurodegeneration, we assessed transcriptomic changes in the rat spiral ganglion following hair cell destruction. Rats were deafened by daily kanamycin injection from postnatal day 8 (P8) through P16. Spiral ganglia were dissected for RNA extraction at P32 – when SGNs are beginning to degenerate – and P60 – by which time about half of the SGNs have died. Prominent among the upregulated genes are those involved in both innate and adaptive immune responses. This is supported by our observation of increased abundance of activated macrophages as well as lymphocytes in the deafened rat spiral ganglia. We also used a Pou4f3^DTR mouse (“DTR mice”) in which a human diphtheria toxin receptor is inserted into the Pou4f3 locus and expressed in hair cells (mice provided by Dr. Ed Rubel, University of Washington). In DTR mice, hair cells are completely destroyed following neonatal (P5) injection of diphtheria toxin. As in the rat model, SGN death was associated with an increased number of macrophages and lymphocytes in the spiral ganglion.

Treatment of deafened rats with anti-inflammatory agents dexamethasone or ibuprofen, respectively, prevented or reduced SGN degeneration. Ibuprofen and dexamethasone also reduced macrophage activation. CRISPR-Cas9-mediated knockout of complement component 3 in rats (rats provided by Dr. Feng Lin, Cleveland Clinic) did not reduce SGN death. In contrast, knockout of MHCII components in the DTR mouse deafness model effectively prevented SGN loss. Haploinsufficiency of MHCII in heterozygotes partially prevented SGN loss. Our results support a critical role of the immune system, particularly the adaptive immune system, in SGN degeneration after cochlear hair cell loss.
The Role of Middle Ear Epithelial Cells in the Resolution of Otitis Media

Ryan F. Allen*, Arwa Kurabi

University of California San Diego

According to the WHO, otitis media (OM) is the world’s leading cause of disabling hearing loss, accounting for 50% of cases primarily in developing countries but also in underserved populations of developed nations. Understanding OM resolution is critical to developing new treatments as alternatives to antibiotics, which foster resistant bacterial strains, and surgery which is unavailable to much of the world’s population. While immunocytes are clearly critical in the middle ear (ME) infection response, the roles of other cell types are less clear. In this study we focused on the ME epithelium.

Single-cell RNA-Seq documented gene expression in >24 cell types present in the MEs of mice, during a complete episode of OM induced nontypeable Haemophilus influenzae (NTHi). This included five classes of epithelial cells.

All ME epithelial cells exhibited robust innate immune responses to infection, including expression of innate immune receptors, chemokines, cytokines and antibacterial compounds. Many genes were regulatory targets of Irf7, an interferon response transcription factor strongly upregulated in epithelial cells early in OM, even though interferons were not expressed. The immune response was paralleled by production of compounds that protect epithelia from inflammatory damage, including cytokine receptor antagonists and protease inhibitors. Irf7 expression declined during OM, while expression of its negative regulator Irf3 increased. In addition to innate immunity, hyperplasia and tissue remodeling genes were strongly regulated, especially in epithelial basal and intermediate cells.

Epithelial cells play a major role in the ME defense against bacterial infection. There is significant regulation of this epithelial innate immune response by the IRF7/IRF3 system. Almost all of genes recognized as targets of this system in other tissues were regulated in ME epithelial cells, in contrast to other cell types. Pharmacological manipulation of these IRFs could enhance bacterial clearance and reduce ME tissue pathogenesis.
Neurons and Hair Cells Require Lmx1a/b for Normal Brainstem Development

*Bernd Fritzsch, Karen L Elliott, Gabriela Pavlinkova, Victor V Chizhikov & Ebenezer N Yamoah

University of Iowa, Dept of Biology & Dept of Otolaryngology, Iowa City, IA, USA; Institute of Biotechnology, Czech Academy of Sciences, Vestex, Czechia; Department of Anatomy and Neurobiology, Tennessee Health Science, Memphis, TN, USA; Department of Physiology and Cell Biology, University of Nevada, Reno, NV, USA

The vertebrate auditory system, consisting of the inner ear hair cells, projections from the spiral ganglion, and brainstem nuclei, is essential for the detection of sound and vestibular sensation. It is believed that the evolution of complex systems, such as the auditory system, depends on duplicated sets of genes that evolved in ancestral vertebrates. The contribution of duplicated genes to auditory system development, however, is poorly understood. We describe that Lmx1a and Lmx1b, which originate from the invertebrate Lmx1b-like gene, redundantly regulate development of multiple principal components of the mammalian auditory system. Combined, but not individual, loss of Lmx1a/b eliminated the auditory inner ear organ of Corti and the spiral ganglion, which was preceded by a diminished expression of their critical regulator Pax2. Innervation of the remaining inner ear vestibular organs revealed unusual sizes and was more affected compared to Lmx1 single-gene mutants. Individual loss of Lmx1 genes did not disrupt brainstem auditory nuclei or inner ear central projections. Combined loss of Lmx1a/b, however, eliminated excitatory neurons in cochlear nuclei, and also eliminates the expression of a master regulator, Atoh1, in their progenitors in the lower rhombic lip. Finally, in Lmx1a/b double mutants, vestibular afferents aberrantly projected to the roof plate in the absence of the choroid plexus. This phenotype was associated with altered expression of Wnt3a, a secreted ligand of the Wnt pathway that regulates pathfinding of inner ear projections. Thus, Lmx1a/b are required for development of the mammalian inner ear, inner ear central projections, and cochlear nuclei.
Here we asked if mice with pathological amyloidogenesis exhibit accelerated age-related hearing loss (AHL) or neurodegeneration in the cochlea and/or brain stem. These APP/PS1-dE9 transgenic (Tg) mice overexpress Abeta, with plaques by 6 months and cognitive impairment by 12 months, and they lack Cdh23-related AHL mutations. Like humans in cognitive decline, these mice have reduced pre-pulse inhibition (PPI) of the acoustic startle reflex (ASR), and this impairment of sensorimotor gating was correlated with higher levels of Abeta40 in the hippocampus. We hypothesized that hearing loss could explain a reduced PPI. We found amyloid precursor protein (APP) and B-secretase in the inner ear, and we detected Abeta aggregates near synapses between inner hair cells and spiral ganglion neurons. We measured levels of Abeta40 and 42 in temporal bones with ELISA, using APPKO as control. We detected low levels in WT and higher levels in the temporal bones of Tg mice. Cochlear ribbon synapse numbers appear to be similar in Tg mice and their non-carrier siblings. ABR waveforms and thresholds are nearly identical at 6, 12-14, and 16-17 months. This was verified with a replication cohort. Next, with APP/PS1-Jukcer mice on C57BL/6 ApoE4KI background, we injected pathogenic tau from human cadavers into the left hippocampus and cortex at 4 months followed by sleep deprivation for 2 months and testing at 6 months. ABR thresholds and wave-1 were similar comparing the Jucker + and – mice, regardless of sleep deprivation, tau injection, or ApoE background. In conclusion, we found no afferent auditory dysfunction in these mice, suggesting that familial AD amyloid mutations and our other manipulations were not sufficient to cause hearing loss in mice. This suggests involvement of extra-auditory brain regions in the altered sensorimotor gating phenotype of reduced PPI of the ASR.
Reversal of Hearing Loss in Spns2 Mutant Mice

Elisa Martelletti (*), Neil J. Ingham and Karen P. Steel

Wolfson Centre for Age-Related Diseases, King’s College London, Guy’s Campus, London SE1 1UL, UK

Spinster homolog 2, Spns2, is a sphingosine-1-phosphate (S1P) transporter, and Spns2tm1a mutant mice were previously described by our group (Chen et al., 2014) showing a rapidly progressive hearing loss associated with a decline in endocochlear potential (EP). As EP appears to develop normally at first in mutants, we considered ways of restoring it to normal levels after the onset of hearing loss. In this study, we asked if progressive hearing loss can be reversed after it has developed as a proof-of-concept.

We used a genetic approach to initiate expression of the Spns2 gene, using tamoxifen injection to activate Flp recombinase which recognises FRT sites in the Spns2tm1a allele, leading to restoration of Spns2 gene activity. Tamoxifen was injected at 4 different ages (Postnatal day (P)14, P17, P21 and P28) and ABRs were recorded at intervals before and after injection up to 8 weeks old when the EP was also measured.

By comparing pre and post tamoxifen ABR thresholds in the same mouse, we observed that ABR thresholds are recovered at 6, 12 and 18 kHz in the injected mice at P14 and P17. Some improvement was found at 12kHz with injection as late as P28. EP levels at 8 weeks old were generally higher in mutants injected at younger ages than in those injected at P21 or P28, and lower 12 kHz ABR thresholds correlated with higher EP levels. Hair cell degeneration was observed in mice injected at P28, but not in mice injected at P14.

Overall, our results show that hearing loss due to the Spns2 mutation can be reversed. This study provides a proof of concept that certain forms of hearing loss can be reversed after the loss has occurred, which is important support for the development of new treatments for humans.
## Session 8

**Hair Cell Damage, Repair and Regeneration**

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<td>14:00 - 14:20</td>
<td>Stefan Heller</td>
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<tr>
<td>14:20 - 14:35</td>
<td>Allison Coffin</td>
<td>Scientist - Academia</td>
<td>Ototoxic Potential of COVID-19 Therapeutics</td>
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<td>14:35 - 14:50</td>
<td>Jinkyung Kim</td>
<td>Postdoctoral Trainee</td>
<td>In Vivo Real-Time Imaging Reveals Megalin As Aminoglycoside Transporter Into Cochlea Whose Inhibition Is Otoprotective</td>
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<td>14:50 - 15:05</td>
<td>A. Catalina Velez-Ortega</td>
<td>Scientist - Academia</td>
<td>TRPA1 Activation in Non-Sensory Supporting Cells Contributes to the Regulation of Cochlear Sensitivity after Acoustic Trauma</td>
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<td>15:05 - 15:20</td>
<td>Nesrine Benkafadar</td>
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<td>Protease-Activated Receptor is Essential for Mitotic Basilar Papilla Hair Cell Regeneration</td>
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<td>15:20 - 15:35</td>
<td>Melissa McGovern</td>
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Ototoxic Potential of COVID-19 Therapeutics

Allison B. Coffin¹,²*, Emily Dale¹, Emilee Doppenberg¹, Forrest Fearington¹, Tamasen Hayward¹, Jordan Hill¹, Olivia Molano¹

¹College of Arts and Science, Washington State University Vancouver
²Department of Integrative Physiology and Neuroscience, Washington State University Vancouver

COVID-19 drugs are rapidly being developed as the biomedical community rushes to bring much-needed therapies to market, with over 900 drugs and drug combinations currently in clinical trials. While this pace of drug development is necessary, it also comes with increased risk of producing therapies with significant side-effects. One likely side-effect of some COVID-19 drugs is hearing loss, yet hearing is not assessed during clinical trials. We used the zebrafish lateral line, an established model for drug-induced hair cell damage, to assess the ototoxic potential of seven drugs in clinical trials for treatment of COVID-19. We found that ivermectin, lopinavir, imatinib, and ritonavir were significantly toxic to lateral line hair cells. By contrast, neither remdesivir nor dexamethasone caused damage, nor did the antibiotic azithromycin, despite previous reports of hair cell toxicity. Pharmacological inhibition of the mechanotransduction channel attenuated damage caused by lopinavir and ritonavir but did not alter imatinib or ivermectin toxicity. While ivermectin is not recommended by the FDA for treating COVID-19, many people have chosen to take ivermectin without a doctor’s guidance and subsequently have been hospitalized. Therefore, we assessed the ototoxic potential of ivermectin in vivo in rats. One week after baseline auditory brainstem response (ABR) recordings, we injected rats each day for 10 days with a clinically relevant ivermectin dose (0.2 mg/kg). We then recorded post-treatment ABRs after a 3-week recovery period. In contrast to our zebrafish assays, ivermectin did not cause a threshold shift in rats, although there may be subtle morphological damage. Future experiments will examine ototoxicity of additional drugs in both our zebrafish and rat models. Considering the large number of COVID-19 therapies in clinical trials, our research can help identify drugs with the fewest side-effects and determine which therapies warrant audiometric monitoring.
In Vivo Real-Time Imaging Reveals Megalin as Aminoglycoside Transporter Into Cochlea Whose Inhibition is Otoprotective

Jinkyung Kim1* and Anthony J. Ricci1,2

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2Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305, USA

Aminoglycosides (AGs) are commonly used antibiotics that cause deafness through the irreversible loss of cochlear sensory hair cells (HCs). How AGs enter the cochlea and then target HCs remains unresolved. Here, we performed time-lapse multicellular imaging of cochlea in live adult hearing mice via a chemo-mechanical cochleostomy. The in vivo tracking revealed that systemically administered Texas Red–labeled gentamicin (GTTR) enters the cochlea via the stria vascularis and then HCs selectively. GTTR uptake into HCs was completely abolished in transmembrane channel-like protein 1 (TMC1) knockout mice, indicating mechanotransducer channel-dependent AG uptake. Blockage of megalin, the candidate AG transporter in the stria vascularis, by binding competitor cilastatin prevented GTTR accumulation in HCs. Furthermore, cilastatin treatment markedly reduced AG-induced HC degeneration and hearing loss in vivo. Together, our in vivo real-time tracking of megalin-dependent AG transport across the blood–labyrinth barrier identifies new therapeutic targets for preventing AG-induced ototoxicity.
TRPA1 Activation in Non-Sensory Supporting Cells Contributes to the Regulation of Cochlear Sensitivity after Acoustic Trauma

A. Catalina Vélez-Ortega1*, Ruben Stepanyan1, Stephanie E. Edelmann1, Sara Torres-Gallego1, Channy Park2, Desislava A. Marinkova1, Joshua S. Nowacki1, Kelvin Y. Kwan3, Ghanshyam P. Sinha1, David P. Corey3, Gregory I. Frolenkov1

1University of Kentucky, 2UCLA and 3Harvard Medical School

TRPA1 channels are sensors for noxious stimuli in a subset of nociceptive neurons. In the inner ear, TRPA1 is also expressed in the sensory hair cells as well as in several types of non-sensory supporting cells. Given that Trpa1−/− mice exhibit normal hearing, balance, and hair cell mechanotransduction, the function of TRPA1 channels in the inner ear remains unknown. Here, we evaluated TRPA1-mediated responses in different cell types within the organ of Corti. Hensen’s cells exhibited the most robust and long-lasting responses after stimulation with 4-HNE, an endogenous TRPA1 agonist produced by lipid peroxidation. These Ca2+ responses often propagated to other supporting cells and were accompanied by prominent shape changes in Deiters’ and pillar cells. Noise exposure is known to increase the oxidative stress in the cochlea, generating 4-HNE throughout several days. Therefore, we hypothesized that 4-HNE generation after acoustic overstimulation would lead to TRPA1-initiated changes of the supporting cell shape that would alter the geometry of the organ of Corti and modify cochlear amplification. To test this, we exposed young adult mice to mild noise that generates largely temporary shift of hearing thresholds and evaluated the recovery of auditory function over time. Consistent with our hypothesis, we found longer-lasting inhibition of cochlear amplification in wild type mice than in Trpa1−/− littermates. In contrast, severe noise exposure caused greater permanent threshold shifts in wild type mice compared to Trpa1−/− littermates. Our results indicate that the non-sensory supporting cells of the hearing organ detect tissue damage via the activation of TRPA1 channels and subsequently modulate cochlear amplification through active cell shape changes. This novel mechanism of cochlear regulation protects the organ of Corti after mild/moderate acoustic trauma but could have deleterious effects after severe noise exposure.

Supported by NIDCD/NIH (R01DC009434 to G.I.F) and HHF (2018-ERG to A.C.V).
Protease-Activated Receptor is Essential for Mitotic Basilar Papilla Hair Cell Regeneration

Nesrine Benkafadar¹,²,*, Amanda Janesick¹,², Mirko Scheibinger¹,², Angela H. Ling³, Taha A. Jan³, and Stefan Heller¹,²

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Loss of auditory hair cells due to genetic predisposition, aging, noise, and ototoxic drugs results in permanent hearing loss. However, hair cell loss in the hearing organs of non-mammals is temporary because supporting cells act as facultative stem cells that can proliferate and regenerate hair cells. The mechanism of how the normally mitotically quiescent avian basilar papilla orchestrates a coordinated regenerative program is unknown.

Towards unraveling the mechanisms of avian hair cell regeneration, we have conducted single-cell RNA sequencing after infusing a single dose of sisomicin into the posterior semicircular canal of 7-days-old chickens at specific time points. We computationally reconstructed the temporal trajectory of gene expression changes in supporting cells upon sisomicin-induced hair cell demise. We focused on the earliest detectable changes in responding supporting cells. The major constituents of the identified candidate signaling pathway that potentially initiates hair cell regeneration were functionally assessed in vivo using pharmacological approaches.

Our results show that supporting cells display distinct gene expression profiles changes as early as 12h post sisomicin infusion when the first sign of DNA fragmentation in hair cell nuclei is detected. In situ validation confirmed the upregulation of distinct genes in early responding supporting cells. We identified a specific signaling pathway associated with proteolytic activation of the receptor F2RL1 followed by a matrix metalloprotease (MMP)-mediated cascade involving EGF receptor activity, MAP kinase signaling, and STAT3 phosphorylation that ultimately leads to upregulation of several transcription factors and proliferative hair cell regeneration. We hypothesize that sensing extracellular proteolytic activity is a key trigger of supporting cell proliferation.

We provide functional evidence showing that the key components of the proposed mechanism are essential for mitotic hair cell regeneration in the avian hearing organ. Our findings inform novel therapeutic strategies aimed at reversing hearing loss in mammals.
Regeneration of Hair Cells in the Mature Mouse Cochlea Following Reprogramming with Atoh1, Gfi1, and Pou4f3

Melissa M. McGovern1, Sumana Ghosh3, Ken Y. Nguyen1, Bradley J. Walters3, and Andrew K. Groves1,2

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2 Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas
3 Department of Neurobiology and Anatomical Sciences, The University of Mississippi Medical Center, Jackson, Mississippi

Hair cells in the mature mammalian cochlea do not regenerate and any hair cell loss is permanent. Neonatal cochlear cells respond to the ectopic expression of the hair transcription factor Atoh1 by differentiating into hair cells. In the mature cochlea, however, Atoh1 alone does not induce the conversion of supporting cells into hair cells. Moreover, in neonatal mice as well as non-mammalian species, the regenerative ability of supporting cells depends on the removal of regulator signaling from HCs. It is possible that mature hair cells regulate mature supporting cells, and that the loss of the hair cells can improve the response to reprogramming factors.

We targeted the ROSA locus to conditionally express Gfi1, Atoh1, and Pou4f3 (Rosa-GAP). When combined with Lfng-CreER, Rosa-GAP expresses the transcription factors in supporting cells. We ablated hair cells in Lfng-CreER::Rosa26-GAP mice by using the Pou4f3DTR mouse. For acute damage, reprogramming and hair cell killing were induced simultaneously at 3 weeks of age. For chronic damage, hair cell killing was induced at 3 weeks of age followed by reprogramming at 6 weeks of age.

Following both acute and chronic HC damage, regeneration was observed throughout the length of the cochlea with inner and outer HCs identifiable by location. Examination of regenerated hair cell-like cells following acute damage revealed neural connections and phalloidin labeled disorganized stereocilia bundles that were also detected via SEM. Further work is ongoing to characterize regenerated hair cells and investigate the expression profile of these cells.

Currently, the best therapeutics for hearing loss are hearing aids and cochlear implants. While these enable users to re-gain some hearing, they provide incomplete recovery. Understanding the response of supporting cells to hair cell death and reprogramming provides insight into the molecular pathways regulating supporting cell identity.
## Session 9

### Molecular Therapies for Deafness

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<td>16:00 - 16:20</td>
<td>Zheng-Yi Chen</td>
<td></td>
<td><strong>A New Era in Precision Medicine for Genetic Hearing Loss</strong></td>
</tr>
<tr>
<td>16:20 - 16:35</td>
<td>Michael Anne Gratton</td>
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<td>Sparsentan Protects from Hearing Loss, Improves Kidney Function &amp; Prolongs Lifespan in Alport Mice with Developed Pathology</td>
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<tr>
<td>16:35 - 16:50</td>
<td>Danielle Lenz</td>
<td>Scientist - Industry</td>
<td>Evaluating miR-Target Sites as a Strategy to Allow AAV Vector-based De-targeting of Gene Expression in the Inner Ear</td>
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<td>16:50 - 17:05</td>
<td>Kathrin Kusch</td>
<td>Scientist - Academia</td>
<td>Characterization of Promoter Expression in Type 1 Spiral Ganglion Neurons in vitro and in vivo</td>
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<td>17:05 - 17:20</td>
<td>Osama Tarabichi</td>
<td>Medical Resident</td>
<td>Development of novel helper dependent adenoviral vectors for inner ear gene therapy</td>
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<td>17:20 - 17:35</td>
<td>Olga Shubina-Oleinik</td>
<td>Postdoctoral Trainee</td>
<td>Dual Vector Gene Therapy Restores Hearing in a Mouse Model of DFNB16 Hearing Loss</td>
</tr>
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<td>17:35 - 18:00</td>
<td>Zheng-Yi CHEN, Stephan HELLER, Yehoash RAPHAEL, Ellen REISINGER, Xue Liu ZHONG, Marc Warchol, Tejbeer KAUR</td>
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A New Era in Precision Medicine for Genetic Hearing Loss

Zheng-Yi Chen

Eaton-Peabody Laboratories, Massachusetts Eye & Ear Infirmary; Department of Otolaryngology-Head and Neck Surgery, Harvard Medical School

Major progress has been made in the identification of over one hundred genes responsible for genetic hearing loss. In the last few decades, we have witnessed the surge of precision medicine in the forms of gene therapy, genome editing, anti-sense oligos and siRNA that promise to bring unprecedented treatments for genetic hearing loss, for which no FDA approved drug is available. Gene therapy, especially by AAV, has been successfully implemented in numerous mouse models of human recessive genetic hearing loss with a rescue effect that can be robust and sustained. Genome editing technology has been developed to treat primarily dominant hearing loss by liposomal-RNP and AAV delivery. Increasingly, sophisticated editing technologies such as base editing has been expanded to treat genetic hearing loss. Delivery tools including different AAVs that target the inner ear have also been identified.

Despite the progress, the road to precision medicine for hearing loss and the clinic is still filled with obstacles. One of the major challenges is to ascertain if congenital genetic deafness, which accounts for the majority of genetic hearing loss, is amendable to intervention, as the human inner ear is fully developed in uterus and we lack the information on the availability of the affected cell types in the human inner ear. For AAV delivery, the field is impacted by the limited AAV serotypes that can efficiently and specifically target diverse inner ear cell types, as well as the insert size limitation. Further, we still do not know how the results in mouse models can be successfully translated into human patients.

For translational approaches to have a better chance of success in clinic, the initial target on hearing loss with delayed onset and progressive loss should be a priority. In addition to existing AAV vectors, new modifications could identify additional AAVs with tropisms for more inner ear cell types. Other non-AAV viral vectors such as adenovirus and lentivirus, as well as non-viral delivery methods including nanoparticles should be studied to build a new toolbox to overcome the limitations of AAV. Increasing understanding of the biology behind hearing loss may offer new opportunities, such as exon skipping by editing or construction of mini-genes, to develop as new strategies to rescue hearing. Interventions that result in hearing rescue in fully mature inner ear will further increase the confidence that a similar approach may be applied to humans. Given the multiple programs that are advancing toward clinical trials, we can expect one of the most exciting times in the application of precision medicine for the treatment of genetic hearing loss.
Sparsentan Protects from Hearing Loss, Improves Kidney Function & Prolongs Lifespan in Alport Mice with Developed Pathology

Michael Anne Gratton¹, Dominic Cosgrove¹, Daniel Meehan¹, Denise Vosik¹, Jacob Madison¹, Duane Delimont¹, Gina Samuelson¹, Diana Jarocki², Radko Komers³, Brendan Smyth¹, Mai Nguyen³, and Celia Jenkinson³

¹Boys Town National Research Hospital, Omaha, NE; ²Washington University, St. Louis, MO; ³Travere Therapeutics, Inc., San Diego, CA

There are no approved therapies for Alport syndrome (AS) and none in development target hearing loss. In AS, endothelin type A receptor (ETAR) activation is an important trigger for renal and cochlear pathologies. Our previous work showed Sparsentan administered to COL4A3⁻/⁻ mice prevented increases in proteinuria, hearing loss (HL), stria vascularis, and renal structural dysmorphology. Whether these effects translate into preservation of kidney function, increased lifespan, and protection from HL in mice with developed cochlear and renal pathology is unknown.

The auditory brainstem response assessed hearing and cochlear ability to tolerate a metabolically stressful noise at 8-8.75 weeks (W) of age in wild-type and AS mice treated with vehicle (V) or Sparsentan 120 mg/kg from 5W. Sparsentan levels in cochlear lateral wall (LW) tissue were determined at 9W after 5W dosing. In renal studies, AS mice were gavaged daily with Sparsentan starting at 4, 5, 6, or 7W. Glomerular filtration rate (GFR) was measured at 9W in mice dosed from 4W and glomerulosclerosis was evaluated at 10W in kidney sections stained for fibronectin.

Sparsentan initiated at 5W improved post noise thresholds with significant prevention of HL at 16 and 24 kHz. Sparsentan crossed the blood/labyrinth barrier into LW tissue at levels expected to antagonize both receptor targets. Sparsentan initiated at 4W abrogated GFR decline at 9W compared to AS-V mice and provided significant protection from glomerulosclerosis at 10W. Sparsentan extended median lifespan even when dosing began in mice with detectable glomerulosclerosis.

Sparsentan prevented HL in AS mice likely through direct effects on strial ETAR and AT1R. Maintenance of GFR and lifespan extension occurs in mice with developed strial and renal structural changes. If these results are translated successfully into the clinic, Sparsentan may offer a novel treatment for protecting hearing and delaying progression of stria vascularis and glomerulosclerosis.
Evaluating miR-Target Sites as a Strategy to Allow AAV Vector-based De-targeting of Gene Expression in the Inner Ear

Richard Churchill¹, Danielle R. Lenz ¹, Shimon Francis ¹, Hao Chiang¹, Yukako Asai¹, Junaid Syed¹, Pascal Schamber¹, Kenyaria Noble¹, and Robert Ng¹

¹Akouos, Inc., Boston, MA

MicroRNAs are short, single-stranded, non-coding RNA molecules with a key role in negative regulation of gene expression. Each mature microRNA binds to its specific target site to mediate translational repression or mRNA degradation. The target-site sequence is specific to a microRNA, but any gene can include coding information for multiple target sites to be regulated by numerous microRNAs.

In the development of AAV gene therapy vectors, a goal is to generate safe and efficacious product candidates that confer transgene expression where it is needed to have the desired effect, while limiting transgene expression where it may not be well tolerated. In recognition of this transgene-dependent need, the potential use of microRNA target site (miR-TS) incorporation in AAVAnc80 vectors was explored for de-targeting transgene expression in different cochlear cell types.

Using previously published datasets from neonate mouse cochleae, multiple microRNAs with differential expression patterns in cochlear cells were identified. Their respective target sites were then incorporated in an AAVAnc80-delivered transgene, together with a ubiquitous promoter, and the transgene expression in vitro and ex vivo was evaluated in search of distinct expression profiles.

Using an in vitro model, expression of transgene mRNA and protein in the absence of the target sites was demonstrated. Decreased transgene levels were observed once a target site was included. Using a cochlear explant model, miR-TS that abolished expression in both hair cells and supporting cells were identified. Sequences that promoted hair cell de-targeting while preserving supporting cell expression were also identified.

Transgene de-targeting may be beneficial for AAV vectors when expression may not be well tolerated in a subset of cells. The work described here identified several miR-TS that enable differential expression in cochlear cells. Future in vivo evaluation of various miR-TS combinations will guide the selection of regulatory sequences for selective, physiologically relevant transgene expression.
Characterization of Promoter Expression in Type 1 Spiral Ganglion Neurons

*in vitro and in vivo*

Kathrin Kusch*1,2, Dominik Simon Botermann1,2, Lennart Roos1,3,4,7, Tabea Quilitz1,2, Lena Lindner1,7, Alica Blenkle1,7, Christian Wrobel1,7, Tobias Moser1,3,4,6,7

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4Cluster of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC), University of Göttingen, Göttingen, Germany.
5Advanced Optogenes Group, Institute for Auditory Neuroscience and InnerEarLab, University Medical Center Göttingen, Germany, 37075, Göttingen, Germany.
6Auditory Neuroscience and Optogenetics Group, German Primate Center, 37077 Göttingen, Germany.
7Department of Otorhinolaryngology, University Medical Center Göttingen, 37075 Göttingen, Germany.

Spiral ganglion neurons (SGNs) inside the cochlea play a pivotal role in hearing and are subdivided in type 1 (T1) and type 2 (T2) SGNs. Stimulation of T1 SGNs via optogenetic tools is a promising technique to restore hearing. However, strong and SGN type specific expression of a transgene is still demanding. Therefore, the characterization of potential promoters that drive target gene expression in T1 SGNs is essential.

In order to achieve this goal, RNAseq data (umgear.org) were screened for genes specifically and highly expressed in T1 SGNs, corresponding promoter elements were cloned, and their *in vitro* activity was analyzed by luciferase assay. Successfully evaluated promoters were used to drive transgene expression of f-Chrimson, a red-light-activated channelrhodopsin, in murine cochleae after early postnatal adenovirus associated virus injection. Promotor function was examined *in vivo*, by functional response to optogenetic stimulation (optically evoked auditory brainstem response measurement, oABR) followed by validation of cell type specific f-Chrimson expression by histology. Preliminary data show that the investigated promoters are capable to drive transgene expression sufficient to evoke oABRs. Additionally, histological analysis revealed cell type specific expression of the transgene in SGNs.

Taken together, our data indicate that we can specifically target SGNs *in vivo* and this will further support the development of optogenetic hearing restoration.
Hearing loss is the most common sensory disorder worldwide and most often occurs from dysfunction within the inner ear sensory organ. Viral vector mediated gene therapy has emerged as a promising biologic strategy to address underlying molecular mechanisms of hearing loss. Adeno-associated virus (AAV) is a commonly studied vector for inner ear gene therapy. However, the packaging capacity of AAV is small (~4.8 kb), limiting its use in applications requiring delivery of large or multiple transgenes. Helper-dependent adenovirus (HdAd) is a viral vector that has a large packaging capacity (~37 kb). We have found that HdAd Type 5 transduces multiple cell types in the mouse cochlea but with a low efficiency. The adenovirus fiber knob protein mediates attachment of the virus to specific receptors on the cell surface. One strategy to improve transduction efficiency and/or target subpopulations of cells is to engineer vectors with alternative fiber knob proteins, which can alter tropism. We sought to develop chimeric HdAd vectors and evaluate their ability to transduce cochlear tissues in mouse models.

HdAd 5 and HdAd 5/35 vectors with CAG promoters were developed. Vector was injected in adult wild type mice and humanized CD46+ transgenic mice by two approaches: 1) round window with canal fenestration and 2) direct injection into scala media. Immunostaining and Confocal microscopy of organ of Corti whole mounts were performed to study transduction patterns 7 days post-injection.

Multiple cell types were transduced using HdAd5 and chimeric vectors in both mouse models. Transduction was most notably seen in the spiral ligament, peri- lymphatic lining, modiolar region and supporting cells.

Transduction of various cell types of the inner ear is feasible with HdAd- based vectors at 7-days post injection. Studies are ongoing to further characterize transduction, safety, and stability of HdAd in the inner ear.
Dual Vector Gene Therapy Restores Hearing in a Mouse Model of DFNB16 Hearing Loss

Olga Shubina-Oleinik1*, Carl Nist-Lund1, Courtney French2, Shira Rockowitz2, Eliot Shearer1,2 and Jeffrey R. Holt1,3

Departments of 1Otolaryngology & 3Neurology, Boston Children’s Hospital and Harvard Medical School, Boston, MA 02115, USA 2 Children’s Rare Disease Cohort Initiative, Boston Children’s Hospital, Boston, MA

Hearing loss (HL) affects an estimated 430 million people worldwide, with a significant fraction due to genetic mutations. The second common form of genetic HL results from mutations in the STRC gene, which encodes protein stereocilin. STRC mutations cause progressive, mild/moderate autosomal recessive HL (DFNB16). Based on our analysis of the heterozygous carrier frequency of 1.8% in a cohort of ~1,200 normal hearing subjects, we estimate that ~2.3 million patients worldwide may carry biallelic pathogenic STRC mutations and suffer from DFNB16. To model DFNB16, we generated Strc knockout mice (StrcΔ/Δ) using CRISPR/Cas9. The StrcΔ/Δ mice lacked distortion product optoacoustic emissions (DPOAEs) and had elevated auditory brainstem response (ABR) thresholds, indicating severe HL. Because stereocilin protein couples adjacent stereocilia in outer hair cell (OHC) bundles, and the tips of OHC bundles to the overlying tectorial membrane, we suspect the HL phenotype in StrcΔ/Δ mice was due to OHC dysfunction. To develop a gene therapy approach to replace defective stereocilin in OHCs we used synthetic dual AAV9-PHP.B vectors to deliver the split Strc coding sequence into inner ears of StrcΔ/Δ mice. Immunostaining with anti-stereocilin antibodies performed four weeks post-treatment revealed that cochleas excised from StrcΔ/Δ mice injected with dual AAVs had robust recovery of STRC expression in OHCs, proper STRC localization at the tips of OHC bundles and recovery of OHC bundle morphology. We found that recombination of full-length STRC led to significant hearing recovery at four weeks of age, in some cases close to wild-type levels. DPOAEs were improved with average thresholds of 40 dB and ABR thresholds were as low as 30 dB in some cases. The data demonstrate that our dual AAV strategy is an efficient gene replacement therapy in mice, suggesting the approach could potentially be translated for the benefit of patients who are affected by STRC mutations.
## Session 10

### Translational and Precision Medicine for Deafness

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<td><strong>Christine Petit</strong></td>
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<td>Hearing Medicine in The Light of Scientific Advances: Dream or Reality?</td>
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<td>8:20 - 8:35</td>
<td><strong>Ryan Carlson</strong></td>
<td>Postdoctoral Trainee</td>
<td>Genetic and Phenotypic Heterogeneity of Childhood-Onset Hearing Loss and Implications for Success of Cochlear Implants</td>
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<td>8:35 - 8:50</td>
<td><strong>Erik de Vrieze</strong></td>
<td>Scientist - Academia</td>
<td>Development of a Genetic Therapy for DFNA21 using Allele-specific Antisense Oligonucleotides</td>
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<td>8:50 - 9:05</td>
<td><strong>Ellen Reisinger</strong></td>
<td>Scientist - Academia</td>
<td>A Dual-AAV Approach with Human Otoferlin cDNA Rescues Hearing in Otof- Knock-Out Mice for at Least 10 Months</td>
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<tr>
<td>9:05 - 9:20</td>
<td><strong>Maryna Ivanchenko</strong></td>
<td>Scientist - Academia</td>
<td>Mini-PCDH15 Gene Therapy Rescues Hearing in a Mouse Model of Usher Syndrome Type 1F</td>
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Hearing Medicine in The Light of Scientific Advances: Dream or Reality?

Christine PETIT

Institut Pasteur, Université Paris Cité, Inserm UA06, Institut de l’Audition, Collège de France, Paris, France

We will see how the spectacular progress of the last 30 years in our understanding of the molecular mechanisms underlying the physiology of the hearing system — principally as concerns the peripheral auditory system — and its pathophysiology, has led to expectations of the emergence of hearing medicine. We will analyze the advances already made in the domain of diagnosis, the problems that remain to be resolved and the conditions of implementation for these new diagnostic tests. We will take the hearing impairments covered by the term “auditory neuropathy spectrum disorders” (ANSD) and related disorders as an example. Finally, we will discuss precision medicine in the field of hearing, focusing on various gene therapy approaches.

The recent work discussed is supported by Fondation pour l’Audition (FPA IDA05), Ile de France (DIM Thérapie génique), PRESAGE (ANR-21-CE14-0075), RHU AUDINNOVE (ANR-18-RHUS-0007).
Genetic and Phenotypic Heterogeneity of Childhood-Onset Hearing Loss  
and Implications for Success of Cochlear Implants

*Ryan Carlson¹,², Tom Walsh¹, Jessica Mandell¹, Amal Abu Rayyan¹, David Horn²,³,⁴, Henry Ou³,⁴, Kathleen Sie³,⁴,  
Lisa Mancl⁵, Jay Rubinstein²,³,⁴, Mary-Claire King¹,².

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Surgery, University of Washington School of Medicine, Seattle, Washington; ⁴Division of Pediatric Otolaryngology-Head  
and Neck Surgery, Seattle Children’s Hospital, Seattle, Washington; ⁵Center on Human Development and Disability, University  
of Washington Medical Center, Seattle, Washington

In the US, most childhood-onset hearing loss is genetic and highly heterogeneous with more than 120  
causal genes and thousands of different causal alleles known. Cochlear implants are the management of choice  
for many children with hearing loss, and their success may be associated with the cause of the hearing loss. In a  
cohort of 449 children from 407 families treated for hearing loss at Seattle Children’s Hospital or the University of  
Washington between 2019 and 2021, we evaluated the genetic causes of hearing loss and how these causes might  
contribute to cochlear implant success. Genomic DNA was evaluated by targeted sequencing of 191 genes and by  
structural variant analysis. Longitudinal audiologic testing was used to evaluate severity and progression of  
hearing loss, and longitudinal speech perception testing was used to evaluate success of cochlear implants. A  
genetic cause of hearing loss was found for 53% (213/407) of families, with causal mutations in 49 different genes.  
Audioprofiles over time revealed gene-dependent and allele-dependent variation in severity, affected  
frequencies, and progression with age. Hearing loss was significantly progressive for children with hearing loss  
due to mutations in MYO6, OTOA, SLC26A4, TMPRSS3, or severe loss-of-function mutations in GJB2. Cochlear  
implant success was high overall, with 89% of implanted patients scoring at least 60% on adult-level speech  
perception tests. Even so, level of success varied significantly by genotype (ANCOVA P < 0.0001). Cochlear implants  
benefitted children with hearing loss regardless of cause, but level of speech perception after implant varied by  
causal gene. Therefore, management of childhood-onset hearing loss can benefit from cochlear implantation  
undertaken in the context of genetic diagnosis.

Supported by research grants from the Bloedel Foundation, the Ben B. Cheney Foundation, and by NIH/NIDCD  
5R01DC011835.
Development of a Genetic Therapy for DFNA21 using Allele-specific Antisense Oligonucleotides

Suzanne E. de Bruijn, Ronald J.E. Pennings, Erwin van Wijk, Hannie Kremer, Erik de Vrieze*

DFNA21 is a type of dominantly inherited (mainly) adult-onset hearing loss. The 12-nucleotide deletion c.1696_1707del in the RIPOR2 gene is the only variant associated with DFNA21 up to this day. Previously, this c.1696_1707del RIPOR2 variant was reported to be the most frequent cause of inherited adult-onset HL in Northwest Europe. There are strong indications that the RIPOR2 deletion acts via a dominant, non-haploinsufficiency disease mechanism. Mutant RIPOR2 is aberrantly localized in the stereocilia of murine auditory hair cells, suggesting a toxic gain-of-function effect. Both humans and mice carrying heterozygous loss-of-function alleles do not display hearing loss. This implies that inhibiting the synthesis of mutant RIPOR2 protein, by selectively degrading the (pre)-mRNA transcribed from the mutant allele, can alleviate the negative consequences of mutant RIPOR2 on auditory function. In this study, gapmer antisense oligonucleotides (ASOs) were designed to specifically target mutant RIPOR2 transcripts for degradation by the endogenous RNase H1 enzyme. The molecular efficacy of the ASOs was validated in DFNA21 patient-derived fibroblast and HEK293T cells. This revealed a lead ASO molecule that was able to significantly reduce mutant RIPOR2 transcript levels (up to ~90% in HEK293T cells), whilst leaving the level of wildtype RIPOR2 mRNA intact. Additionally, western blot analyses showed that the decrease in mutant RIPOR2 transcripts leads to a marked decrease in mutant protein synthesis. Wildtype RIPOR2 protein levels were also affected by the mutant-specific ASO, albeit to a lesser extent. This suggests that a cleavage-independent mechanism interferes with translation of the wildtype transcript. Lowering the ASO concentration resolved this, and chemical ASO modifications are being investigated for their ability to reduce the translation inhibition of wildtype RIPOR2 transcripts. With the proven safety of ASOs in humans, and rapid advancements in inner ear drug delivery, our in-vitro studies indicate that ASOs offer a promising treatment modality for DFNA21.
A Dual-AAV Approach with Human Otoferlin cDNA Rescues Hearing in

*Otof*\(^{-}\) Mice for at Least 10 Months

Hanan Al-Moyed\(^1\), Sophia Schweickhardt\(^2\), Xanthoula Smyrnakou-Biedenbänder\(^2\), Robert Ng\(^3\), Michelle D. Valero\(^3\), Manny Simons\(^3\), Ellen Reisinger*\(^{1,2}\)

\(^1\)Molecular Biology of Hearing and Deafness Research Group, University of Goettingen, Germany
\(^2\)Gene Therapy for Hearing Impairment Research Group, University of Tuebingen Medical Center, Germany
\(^3\)Akouos Inc, Boston, USA

Adeno-associated viruses (AAVs) are preferred vectors for gene therapy, since they are non-pathogenic and exhibit only low immunogenicity. To restore hearing in recessively inherited forms of hearing impairment, recombinant AAVs are applied to deliver the cDNA of the missing protein to target cells in the inner ear. After transduction, viral genomes assemble to form long episomal concatemers (head-to-tail multimers) in nuclei of target cells. The limited cargo capacity of AAVs of approximately 4.7 kb can be overcome by splitting the coding sequence to two (or more) AAVs. In co-transduced cells, the viral genomes form hetero-multimers, and by splicing of intersecting viral genome sequences, a full-length mRNA is generated. In an initial exploration, we demonstrated that a dual-AAV (DNA hybrid) approach was suitable to transduce inner hair cells (IHCs) of otoferlin gene knock-out (*Otof*\(^{-}\)) mice with the 6 kb full-length mouse otoferlin cDNA (Al-Moyed et al., 2019). In follow-up work, we tested a dual-AAV approach with AAV2/Anc80, this time encoding human otoferlin, which restored hearing in *Otof*\(^{-}\) mice. We found the mean ABR thresholds in response to click stimuli, absent in this model without intervention, to be ~50 dB SPL at 1 month of age, approximately three weeks after vector delivery. At the age of 10 months, these animals displayed click-evoked ABR thresholds of ~40 dB SPL, on average, suggesting that the restoration of auditory function is very stable for at least 10 months. ABR wave amplitudes were unchanged over time. We present here results of histologic analyses from *Otof*\(^{-}\) mice transduced with AAV2/Anc80 from distinct timepoints following administration. In conclusion, dual-AAV mediated expression of human otoferlin in murine IHCs continues for months, and persistently restores auditory function in otherwise deaf *Otof*\(^{-}\) mice.
Mini-PCDH15 Gene Therapy Rescues Hearing in a Mouse Model of Usher Syndrome Type 1F

Maryna V Ivanchenko1*, Daniel M Hathaway1,2, Alex J Klein1, Olga Strelkova2, Pedro De-la-Torre2, Xudong Wu1, Eric M Mulhall1, Kevin T Booth1, Cole W Peters1, Corey Goldstein1, Marcos Sotomayor3, Artur A Indzhykulian2, David P Corey1

1Department of Neurobiology, Harvard Medical School
2Department of Otolaryngology-Head and Neck Surgery, Massachusetts Eye and Ear, Harvard Medical School
3Department of Chemistry and Biochemistry, The Ohio State University

Mutations in PCDH15 cause Usher syndrome type 1F, manifesting as profound deafness and lack of balance at birth, and as blindness developing over several decades. Similarly, mice lacking PCDH15 are deaf and have a severe balance deficit. Gene addition to cochlea or retina is an attractive therapeutic strategy, however the PCDH15 coding sequence, at 5.8 kb, is too large for a single AAV. The extracellular domain of PCDH15 contains 11 link-like “EC” repeats which convey tension to the transduction channel. We hypothesized that some EC repeats may not be essential for function and that a “mini-PCDH15” lacking some of them could be functional and encoded in a single AAV capsid.

Based on the atomic structure of the PCDH15 extracellular domain, we engineered eight mini-PCDH15s, deleting up to five EC repeats. HEK293 cells were transfected with mini-PCDH15 genes in culture. Immunofluorescence microscopy and immunogold SEM showed that all PCDH15s went to the cell surface like full-length controls, and they mediated cell aggregation by binding to CDH23-expressing cells.

Pcdh15 conditional knockout mice are deaf at P30 and have degenerated hair bundles. Knockout mice were injected at P1 through the RWM with AAV9-PHP.B encoding a mini-PCDH15. With immunofluorescence and immunogold SEM, strong mini-PCDH15 signal was detected at the tips of stereocilia in cochlear hair cells, with gold beads labeling tip links. SEM also showed robust rescue of hair bundle morphology and presence of tip links on stereocilia. FM1-43 labeling at P30 demonstrated rescue of mechanotransduction in inner and outer hair cells, comparable to that of normal hearing littermates. At P30, hearing thresholds were preserved almost to wild-type thresholds.

A PCDH15 mini-gene strategy is a promising approach for therapeutic gene delivery to the human inner ear. Because structural demands on PCDH15 are likely greater in the cochlea than in the retina, these results are also encouraging for treatment of the progressive blindness in Usher 1F.
## Session 11

### Emerging Themes and Methods

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Emerging Technologies in Hearing Research

Eri Hashino

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Intense investigation in human genetics over the past few decades has led to impressive achievements in the identification of genetic mutations responsible for congenital and progressive inner ear disorders. Targeted gene inactivation in mouse and zebrafish models has recapitulated inner ear phenotypes arising from these genetic mutations. However, there is still a significant gap in our understanding of genetic predispositions to human pathological conditions. In addition, the paucity and inaccessibility of human inner ear tissues have hampered progress in identifying and testing therapeutic treatment options. To overcome these limitations in the existing models, new model systems and approaches, including transgenic primates, chimeric organs, stem cell-derived organoids and in vivo engineering approaches, have been developed. I will overview these emerging technologies and discuss in depth recent progress in the human inner ear organoid model that now contains cochlear cell types and is amenable to genetic/chemical/tissue engineering explorations.
Wnt Signaling Promotes Cell Caudalization and Inner Ear Differentiation in Mouse Stem Cell-Derived Organoids

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The inner ear is derived from the otic placode, one of numerous cranial sensory placodes that emerges from the pre-placodal ectoderm (PPE) along its anterior-posterior axis. However, the molecular dynamics underlying how the PPE is regionalized are poorly resolved. We used stem cell-derived inner ear organoids to investigate the effects of Wnt signaling on otic placode development and found that modulating Wnt signaling significantly increased inner ear organoid induction efficiency and reproducibility. Alongside single-cell RNA sequencing, our data reveal that the canonical Wnt signaling pathway contributes PPE regionalization and, more specifically, medium Wnt levels induce 1) expansion of the caudal neural plate border (NPB), 2) gene signatures of the posterior PPE and otic placodes, and 3) a suitable caudal microenvironment for otic specification. Our data further suggest direct transitions from the caudal NPB to the pPPE and demonstrate that the inner ear organoid system mirrors an in vivo developmental path.
Determining the Molecular Mechanisms of Inner Ear Sensory Neuron Guidance to Direct a Novel Approach of Ear Transplantation

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Loss of inner ear hair cells or sensory neurons results in hearing and/or balance defects. Current attempts at biologically targeted hearing restoration focus mostly on hair cells and their innervation by sensory neurons, with less emphasis on how these neurons reestablish correct central connections after regeneration. We investigated the cell-specific role and expression patterns of two genes, *Fzd3* and *Neurod1*, on inner ear afferent central pathfinding to guide our novel approach of ear transplantation. These genes have been shown to play a conserved role in axon guidance. Here, conditional knockout of *Fzd3* or *Neurod1* in mouse inner ear afferents revealed a cell-autonomous role in central axon guidance. Overexpression of *Neurod1* in inner ear afferents also resulted in an aberrant central phenotype, suggesting that level of gene expression may contribute to central guidance. We investigated levels of gene expression across the cochlea and found an apex to base gradient of expression exists for *Neurod1*. Similarly, *Fzd3* expression in embryonic wild type spiral ganglion neurons showed a comparable gradient. Conditional knockout of *Neurod1* resulted in a reduced *Fzd3* expression in spiral ganglion neurons, highlighting a relationship between these genes in the ear. Knowing that these expression gradients were established around the time inner ear neurons were reaching the hindbrain during normal development, we transplanted early embryonic otic vesicles in in frogs, chickens, and mice. Transplanted ears could develop various inner ear structures, such as canals and ducts, as well as hair cells and neurons. In addition, sensory neurons could navigate to proper hindbrain targets and make functional connections. Collectively these data suggest that *Fzd3* and *Neurod1* are acting cell-autonomously to guide inner ear afferents and that ear replacement strategies may function as a novel therapeutic approach.
Macrophages Promote Repair of Damaged Ribbon Synapses and SGN Survival Following Noise-Induced Cochlear Synaptopathy

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Prolonged exposure to noise causes rapid loss of ribbon synapses between inner hair cells and spiral ganglion neurons (SGNs), termed as cochlear synaptopathy. As a consequence, slow and progressive SGN loss is prevalent. Our previous study has shown an immediate migration of local macrophages into the damaged synaptic regions and spontaneous synaptic repair following TTS-like synaptopathic noise trauma. Nevertheless, the precise role of macrophages in ribbon synapse degeneration and/or repair remains elusive. In this study, cochlear resident macrophages were eliminated by targeting colony stimulating factor 1 receptor (CSF1R), using PLX5622 chow and macrophages were repopulated in the cochlea lacking macrophages after synaptopathic noise exposure by PLX5622 withdrawal and replacement with control chow. Lack of macrophages did not influence the degree of loss of ribbon synapses at 1-day post noise exposure, compared to noise damaged cochlea with intact macrophages. At 30-days post noise exposure, in the presence of intact macrophages partial synaptic repair was observed, however such synaptic repair was absent in the cochlea lacking macrophages. Remarkably, macrophage repopulation in the noise damaged cochlea partially repaired the damaged ribbon synapses and increased the density of the postsynaptic AMPA receptor subunit GluA2. Such synaptic repair positively correlated with partial recovery of auditory brainstem response (ABR) thresholds and peak 1 amplitudes at suprathreshold sound levels. Notably, cochlea with intact and repopulated macrophages facilitated SGN survival after noise exposure however, increased SGN loss was evident in the absence of macrophages. Together, these data imply that macrophages neither prevent nor aggravate synaptic loss but are necessary and sufficient to facilitate the recovery of elevated hearing thresholds, repair damaged IHC-ribbon synapses and promote SGN survival after synaptopathic noise exposure. Our data represents a novel and clinically feasible approach to promote synaptic regeneration in noise-damaged cochlea and to treat hidden hearing loss.
Initial Phenotypic Characterization of a Rhesus Macaque Model of Usher Syndrome Type 1B

Martha Neuringer1, Junghyun Ryu2, Jon D. Hennebold2, John P. Statz2, William Chan2, Cathy Ramsey2, Fernanda C. Burch2, J. Beth Kempton3, Edward V. Porsov3, Lauren Renner1, Benjamin J. Burwitz4, Carol B. Hanna2, John V. Brigande*3

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Usher Syndrome is the leading cause of deaf-blindness in developed countries. The time of onset and severity of auditory, vestibular, and vision phenotypes predicate clinical classification. Genetic testing for mutations in the nine genes known to cause Usher Syndrome complements diagnosis. Type I is the most severe form characterized by congenital, profound hearing loss or deafness; congenital balance abnormalities; and aberrant night vision by early adolescence and substantial vision loss by middle age. Mutations in the unconventional myosin motor protein gene, myosin-VIIa (MYO7A), are the most frequent cause of Type I disease accounting for ~50% of cases. Inner ear and retinal development, physiology, and function in the rhesus macaque closely models that in humans. We sought to create a nonhuman primate model of MYO7A deficiency to better understand Type I pathogenesis and define safe gene therapies that restore sensory function. Exon 3 of MYO7A was targeted by injection of two single guide RNAs and Cas9 mRNA into zygotes 16 hr post-fertilization. Laser trophectoderm biopsy and genotyping of blastocysts identified embryos with MYO7A mutations for transfer to surrogate dams. DNA from cheek, skin, and peripheral blood monocytes was sequenced to confirm neonate genotype. Auditory brainstem responses (ABR), distortion product otoacoustic emissions (DPOAE), and retinal multimodal imaging were used to assess hearing and retinal structure. In November 2021, a compound heterozygous infant was born that harbored 1bp and 63bp deletions in exon 3. Pure tone stimuli failed to elicit ABR or DPOAE responses at 28 and 56 days. The infant has gait abnormalities, musculoskeletal instability, and hangs upside down for excessive periods. At 4 months, local retinal degeneration was seen temporal to the optic nerve, including disruption of photoreceptor layers and thinning of the outer nuclear layer. The CRISPR/Cas9-edited MYO7A infant has pathophysiological traits consistent with Usher Syndrome Type I.
Poster Session

The official poster session is on Wednesday, May 25th from 2-5 pm at the Iowa Memorial Union (125 N Madison St, Iowa City, IA 52245). We also encourage all attendees to view posters during lunch breaks and in their free time.

You are expected to be at your poster during your assigned time slot to present your work and answer questions. Assigned time slots and poster numbers will be available 2 weeks before the meeting.

Poster Awards: Several awards ($500 each) will be given to best posters by junior scientists or trainees. There will also be a "People's Choice" award for best poster by a trainee.

Posters will be judged by the organizing committee and awards will be presented at the closing ceremony.

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Awardees of best posters by a trainee will also be given the opportunity to present their work as a Poster Blitz. Poster Blitz will be a 1-minute talk (maximum). Only 1 slide is allowed for a Poster Blitz.

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Session 1

The Expanding Genetic and Genomic Landscapes of Deafness
Auditory Development of Patients with Genetically Determined Hearing Loss

Oziębło Dominika 1*, Obrycka Anita 2, Skarżyński Henryk 3, Oldak Monika 1

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Every year, approximately 1-6/1000 children are born with severe to profound hearing loss (HL) and for this group of patients cochlear implantation (CI) is the treatment of choice. The aim of our study was to analyse the auditory development of DFNB1-negative CI patients.

The study group (n=51) was recruited from patients with profound prelingual HL that were negative for DFNB1 locus pathogenic variants and had no environmental HL risk factors.

In all probands exome sequencing (WES) was performed and followed by bioinformatics analysis. Family segregation analysis and detection of the CEVA haplotype were performed using standard Sanger sequencing. Copy number variants were validated with qPCR. Evaluation of patients auditory development was performed with the LittlEARS questionnaire (LEAQ) in three subsequent intervals – at the time of cochlear implant activation as well as in 5th and 9th month after CI.

Causative variants were identified in 72.5% of patients (37/51). The majority of them are localized in the MYO15A (n=7), PAX3 (n=5), MITF (n=4), TMC1 (n=4) and CDH23 (n=3) genes. More than half (28/54) of the identified HL causative variants have not been associated with HL before. The auditory development of the studied children was the most dynamic in the first 5 months after CI and slowed down between the 5 and 9 months of using the device. No differences were observed between the auditory development of patients with an identified and unknown genetic causes of HL.

Obtained results show a high heterogeneity of genetic HL causes in the population of Polish DFNB1-negative cochlea-implanted patients. All tested children were good candidates for CI as their HL causative genetic variants are localized in genes preferentially expressed in the cochlea.

Supported by NCN grant: 2017/27/N/NZ5/02369
Autosomal dominant hearing loss (ADHL) is the second most common form of inherited HL with an onset usually after the first decade of life. It affects mainly high frequencies and progresses over time. Autosomal-dominant genes are responsible for about 20% of cases of hereditary non-syndromic deafness, with 51 different genes identified to date.

In this study, 105 families with a vertical inheritance pattern of hearing impairment underwent targeted next-generation sequencing (NGS) using a cochlea-specific HL multi-gene panel (237 genes). Genomic DNA was isolated from peripheral blood samples or buccal swabs of available family members. Prior to NGS, environmental HL risk factors and DFNB1 locus (GJB2 and GJB6) related hearing impairment had been excluded in all probands. Presence of the selected probably pathogenic variants and their segregation with HL within the family were confirmed by standard Sanger sequencing.

Genetic cause of ADHL was identified in 43.8% (46/105) of the examined families. Among the 46 identified HL variants only 26% (12/46) have been previously reported and the remaining 74% are novel (34/46). We identified missense variants (27/46; 58.7%), splice site variant (9/46; 19.5%), stop-gain variants (5/46; 10.9%) as well as frameshift variants (5/46; 10.9%).

Among the most common causative genes were MYO6 (n=8), TBC1D24 (n=5), KCNQ4 (n=4), GSDME (n=4), POU4F3 (n=4) and WFS1 (n=4). Pathogenic variants causative of HL in the SLC44A4, NLRP3, LMX1A, FGFR3, CD164, GRHL2, TMC1, COCH, ATP2B2 and CEACAM16 genes were detected in single families.

Our custom panel has demonstrated good diagnostic performance. Considering frequent identification of novel genetic variants it is necessary to perform thorough clinical examination and variant segregation analysis with ADHL in all available family members. In the largest families without genetic diagnosis of HL linkage analysis and whole genome sequencing will be performed.

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Pathogenicity of CLIC5 and SLC12A2 Variants Associated with Hearing Impairment in two African families

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We previously reported CLIC5 and SLC12A2 variants in two families from Cameroon and Ghana, segregating non-syndromic hearing impairment (NSHI). In this study, with a mammalian expression construct using site-directed mutagenesis, in vitro biological assays were performed to further functionally investigate the pathogenicity of CLIC5 [c.224T>C; p.(L75P)] and SLC12A2 [c.2935G>A; p.(E979K)] variants. HEK-293 cells were transfected with either the wild-type or mutant constructs and western blotting was performed to examine the differential expression, while confocal microscopy was used to study differences in protein localization and impact on cellular morphology. Compared to wild-type, both the CLIC5 as well as SLC12A2 variant proteins were overexpressed. The mutant CLIC5 protein appears as aggregated perinuclear bodies while the wild-type protein was evenly distributed in the cytoplasm. Furthermore, the wild-type CLIC5 transfected cells formed filopodia-like structures which were absent in the CLIC5 mutant expressing and control cells. On the other hand, the wild-type SLC12A2 transfected cells had an axon-like morphology which was not observed in the mutant transfected and control cells. A comprehensive literature review aimed at summarizing the contributions of these two genes to hearing impairment (HI), as well as protein network analyses, revealed that CLIC5 can interact with at least eight proteins at the base of the stereocilia. No published literature on SLC12A2 protein-protein interaction was found. This study has generated novel biological data associated with the pathogenicity of targeted variants in CLIC5 and SLC12A2, found in two African families, and therefore expands our understanding of their pathobiology in HI.

Keywords: CLIC5; SLC12A2; hearing impairment pathobiology; Africa.
Genomic analysis of inherited hearing loss in the Palestinian population

Amal Aburayyan*, Lara Kamal, Zippora Brownstein, Hashem Shahin, Grace Rabie, Ryan Carlson, Suleyman Gulsuner, Karen B. Avraham, Tom Walsh, Mary-Claire King, Moien N. Kanaan

University of Washington

Genomic analysis and characterization of inherited hearing loss for an entire population provides valuable information that will aid in its clinical management. We characterized the genetic basis of hearing loss in 2,198 participants from 491 Palestinian families from the West Bank and Gaza, using panel-based genomic DNA sequencing, followed by segregation analysis in families and functional assessment. We obtained genetic diagnoses for 68% (312/458) of families with non-syndromic hearing loss, and for 76% (25/33) of families with syndromic hearing loss. Variants that were pathogenic due to altered transcription were identified for 24% (80/337) of solved families. Damaging effects of these variants were confirmed by analysis of patient RNA for genes expressed in blood. Genetic heterogeneity was striking with respect to both genes and alleles: The 337 solved families harbored 143 different mutations in 48 different genes. A particular interesting founder allele was a non-coding variant of MYO15A that destroys the splice site of a cochlear-specific exon not included on reference sequence databases. A second interesting founder allele was a nonsense mutation specific to the only isoform of PCDH15 critical to hearing. We hypothesize that a portion of unsolved hereditary hearing loss cases may be attributable to variants in non-coding, regulatory regions of the genome. Historically, consanguineous marriages have been common in the Palestinian population. We estimate that in our cohort, ~58% of hearing loss is due to consanguinity. Consanguinity in this community is decreasing rapidly, in parallel with widespread education of young women, and we anticipate that incidence of inherited hearing loss will correspondingly decrease. Meanwhile, genetic diagnoses for hearing loss enable parents to anticipate possible progression or syndromic effects and give the opportunity to use preimplantation genetic diagnosis to prevent deafness in future children.
Clinical exome sequencing identifies a novel OTOF splice-site mutation in a UAE family with hearing loss

Maria Asaad & Abdelaziz Tlili

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Hereditary hearing loss is the most frequent neurosensorial disease. The autosomal recessive nonsyndromic form is considered as one of the most common monogenic diseases. It is characterized by high allelic and locus heterogeneities that make its diagnosis a challenging task. NGS-based techniques have revolutionized the discovery of novel genes and mutations, particularly for diseases with high genetic heterogeneity. In this study, clinical exome sequencing revealed a new splice-site mutation in a UAE family with hereditary hearing loss. Moreover, Sanger sequencing and PCR-RFLP confirmed the presence of this variant in the affected family, its uniqueness/rarity and suggested its pathogenic effect in the studied family. In addition, and in order to perform a functional study at mRNA level, we cloned both normal and mutant alleles in the pSPL3 vector. Finally, this study expands our knowledge of the spectrum of non-syndromic hearing loss mutations in the UAE population. It also reinforces the powerfulness of next generation sequencing in the diagnosis of genetic disorders with genetic heterogeneity.
Investigating the Role of Epigenetic Mechanisms in Presbycusis

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Presbycusis or Age-Related Hearing Loss (ARHL) is characterized by a bilateral, progressive sensorineural hearing loss. The development of ARHL is multifactorial, entailing both extrinsic and intrinsic factors. Genetic predisposition to hearing loss account for a significant proportion of the variations between ARHL patients. Consequently, presbycusis could stem from unclarified epigenetic susceptibility, however, there is a scarcity of information on the precise involvement of aberrant epigenetic regulation to ARHL.

The objective of this study is to examine whether DNA methylation mediated silencing could be a risk factor associated with Presbycusis. The inclusion criteria were elderly patients aged 40 years and above with greater than 30 dB HL hearing loss (bone conduction pure tone average [PTA] of frequencies 500, 1000, 2000, and 4000 Hz.

Hearing measurements were used to determine the audioprofiles; the audiometric patterns that were more frequent in the cohort study are “High frequency Steeply Slopping” or HFSS (33%), “High frequency Gently Slopping” or HFGS (31%) and “FLAT” (27%) while the other patterns were less prevalent. The Illumina Infinium® Methylation EPIC BeadChip has been used to identify regions with aberrant levels of methylation across genomes from 16 patients.

Our preliminary data show a significant association between DNA methylation levels in several deafness-associated genes and the hearing thresholds. A PCR-based bisulfite DNA methylation detection assay has been used as a validation method to confirm methylation levels at specific gene locus in presbycusis patients’ samples.

The extent of involvement of epigenetic modifiers into hearing loss is minimally understood nevertheless aberrant DNA methylation and its impact on gene expression have been associated with diverse biological processes. By interrogating methylation status across the genome at single-nucleotide resolution, our study will help establish the association between audiometric patterns and methylation status in deafness-related genes.
Big Data to Therapy: Precision Medicine for the Deaf

Lara Kamal1,*, Zippora Brownstein1, Tal Koffler-Brill1, Shahar Taiber1, Yazeed Zoabi2, Rachel Katz3, Tal Patalon3, Noam Shomron2, Karen B. Avraham1

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Precision medicine has become the optimal strategy to provide patients with early diagnosis and tailored treatments at the opportune time. The field of hearing loss is ideal for integrating a precision medicine approach, since it is the most common sensorineural disorder worldwide, and is genotypically and phenotypically heterogeneous, with over 150 deafness-related genes identified to date. The recent advances in next generation sequencing over the past decade have facilitated rapid gene discovery; however, about half of inherited deafness cases in our populations still remain unsolved. We are conducting a large-scale study of the hearing-impaired population in Israel, utilizing KSM (Kahn-Sagol-Maccabi) TipaBiobank of 60,635 anonymized samples. NGS is being performed on almost 1200 adult deaf from the Biobank, with audiograms documenting their phenotype, and the hearing loss diagnosed before the age of 60. Following in-depth bioinformatic analysis, pathogenic variants are being evaluated to determine genotype-phenotype-ethnicity correlations and diagnoses are transferred and validated at the medical genetics clinics to provide personalized genetic counseling based on the identified variant. Novel variants are being functionally characterized in our laboratory using in-vitro cell culture assays and by generating mutant knock-in mouse models using CRISPR/Cas9 technology. Among our cohort, the most frequently mutated genes were GJB2, TMC1, MYO15A, MYO7A, SLC26A4 and MYO6 (in this order) and rarer genes, including SYNE4. Additionally, we identified a novel frameshift variant (p.Asn1110Valfs43Ter) in ATP11A, a phospholipid flippase gene, in a Jewish Israeli family. Adeno-associated virus gene therapy has and is being used to rescue hearing in several mouse models for deafness, with a perspective to treat human deafness. Implementing precision medicine in the field of hearing loss and translating it into the clinic will provide patients with improved risk-based prevention, medical management and rehabilitation by otolaryngologists and audiologists.
Hearing Loss-Causative GJB2 Mutations can be Studied in an hiPSC-Derived Model System

Brett M. Colbert, Pei-Ciao Tang, Christian del Castillo, Derek M. Dykxhoorn, Xue Zhong Liu*

University of Miami Miller School of Medicine

Hearing loss (HL) is the most common sensory disorder. There are 120+ genes that have been implicated in genetic HL. The most common is Gap Junction Beta 2 (GJB2). It codes for the gap junction protein Connexin26 (Cx26), expressed by the supporting cells of the inner ear. Cx26 is vital for nutrient and signaling molecule distribution in the inner ear. A common GJB2 variant is c.109G>A which introduces a missense mutation and a single amino acid change (p.V37I), leading to mild-to-moderate hearing loss. As human cochlear tissue is hard to obtain, previous in vitro studies to characterize GJB2 variants have been carried out in non-hearing relevant expression systems, such as HeLa cells. These systems have greatly aided our understanding of Cx26, however do not recapitulate the expression from the endogenous loci. To better account for these factors, we have used an established model of Cx26-expressing otic supporting-like cells (Cx26-OE) differentiated from human induced pluripotent stem cells (hiPSCs). We reprogrammed an hiPSC line from a GJB2 c.109G>A+/+ individual using Sendai virus reprogramming. qPCR and immunocytochemistry (ICC) for pluripotency markers Oct4, NANOG, Tra1-80, and Sox2, and directed trilineage differentiation with ICC for markers of each germ layer were used to assess pluripotency. The c.109G>A+/+ line was then corrected with CRISPR to generate an isogenic control line. The c.109G>A+/+ and GJB2WT/WT were shown to express equivalent levels of GJB2 transcripts by qPCR (n=3, p=0.39) and Cx26 by Western blotting compared to control IPSC-derived otic progenitor cells. Cx26 was seen to localize to the plasma membrane by ICC. FRAP showed equivalent levels of fluorescent recovery after photobleaching. Organoids expressing SOX2, MYO7a, and Cx26 were derived from the mutant and control line. Variants in GJB2 are the most common cause of genetic hearing loss. There are several proposed mechanisms of dysfunction, but a precise understanding of these factors in human tissue is still elusive, due to the scarcity of human cochlear tissue. We have shown that iPSC-derived otic supporting-like cells endogenously express Cx26 and functional assays can be performed on these cells. Inner ear organoids also express hair cells and Cx26 expressing supporting cells. Adequate characterization of these hiPSC-derived, Cx26-expressing otic supporting cell models will allow future studies into the mechanism of dysfunction for various GJB2 variants, affording a better understanding of Cx26 functionality in HL and providing a platform for therapeutic development.
HAP1 regulates the surface expression of KCNQ4

Jung Ah Kim, Kyung Seok Oh, Young Ik Koh, Heon Yung Gee

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In the mammalian cochlea, K⁺ recycling and homeostasis is important for the auditory function and voltage-gated channel subfamily Q member 4 (KCNQ4) plays a pivotal role in the K⁺ recycling. Mutations in KCNQ4 have been reported to cause non-syndromic sensorineural deafness type 2 (DFNA2), an autosomal dominant form of progressive hearing loss. KCNQ4 comprises six transmembrane domains with a long cytoplasmic C-terminal tail. This C-terminal tail is known to involve in interaction with number of proteins. For example, calmodulin (CaM) decreases KCNQ4-mediated currents by binding to the C-terminal tail of KCNQ4. In this study, we hypothesized that there will be additional proteins which interact with the KCNQ4 C-terminus, thus regulating KCNQ4 activity.

To identify interactors of KCNQ4, we performed yeast two-hybrid (Y2H) screening with murine adult inner ear cDNA library as a prey and KCNQ4 C-terminal tail as a bait. The Y2H revealed HAP1 and MMP14 as novel interactors in addition to CaM, a known interactor. We confirmed this novel interaction by co-immunoprecipitation in HEK 293 cells. GST pull-down assay with purified interaction domain of HAP1 and KCNQ4 C-terminal tail corroborates the direct interaction of KCNQ4 and HAP1. In order to investigate the functional effect of the interaction, we performed electrophysiology and found that HAP1 overexpression decreased KCNQ4-mediated currents, whereas MMP14 did not modify KCNQ4-mediated currents. Since HAP1 was previously reported to involve in vesicular trafficking and to interact with membrane proteins including GABAA receptor, InsP3R1, EGFR, and IP3 receptor, we performed surface biotinylation assay to examine whether HAP1 affects the membrane trafficking of KCNQ4. Consequently, we identified that the surface KCNQ4 expression was downregulated by HAP1 overexpression. To validate if the decrease of surface KCNQ4 under HAP1 overexpression results from intercellular endocytosis defects, we conducted endocytic assay in HEK 293 cells. Endocytic Assay confirmed when HAP1 co-expressed with KCNQ4, endocytosed KCNQ4 level remained unchanged while KCNQ4 gradually endocytosed by time-dependent manner in the absence of HAP1. We generated HAP1 knockout cell lines by utilizing CRISPR/Cas9 system HAP1 KO cell lines showed elevated membrane expression of KCNQ4 and increased KCNQ4 currents. Furthermore, we discovered that the HAP1 is expressed in the mouse outer hair cells and co-localized with KCNQ4. These findings suggest that HAP1 regulates KCNQ4 through its interaction with KCNQ4 C-terminal tail in the inner ear.
Genetics of Age-Related Hearing Loss in the Lebanese Population: A First Step in this Fertile Landscape

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Age-related hearing loss (ARHL) defined as progressive bilateral sensorineural loss, is the most common sensory deficit in humans. Approximately 25% of people over 45 years, and half of those in their 7th decade, exhibit severe ARHL that causes social isolation and depression. The major obstacle hampering the development of new treatments for ARHL is our poor understanding of its underlying mechanisms.

This study aims to identify the pathophysiological processes and molecular pathways leading to ARHL in the Lebanese population, known to be a mosaic of various religious factions where social, religious, cultural, political, and economic factors favor consanguineous marriages. The availability of next-generation sequencing (NGS) technologies and the access to large and informative families in Lebanon will facilitate the discovery of new ARHL genes.

Six large families with ARHL were recruited. DNA sequencing of one proband from each was achieved through the OtoSCOPE, a NGS platform targeting >150 genes linked to deafness. Causative variants were identified in two families: a new frameshift variant, c.1192dup, p.(Thr398AsnfsTer24) in MYO6 and a known missense variant p.(Arg1388His) in TECTA, while uncertainty remains in the other families. Indeed, a variant of unknown significance (VUS): p.(Met143Val) was identified in MYO6 in one family while three known heterozygous pathogenic variants in genes involved in autosomal recessive deafness were identified in one affected patient in the 4th family. On another hand, three different heterozygous VUS in genes involved in autosomal dominant deafness were found in the proband of the 5th family. The last family remains genetically undiagnosed. Segregation studies are ongoing in the families with candidate VUS while whole exome sequencing was adopted for the last one.

The present work confirms the complexity of the genetics of hearing loss and paves the way for a better understanding of the genetic etiologies of ARHL in Lebanon.
Tailored WES Data Analysis and Reanalysis in the Lebanese Population and Lessons Learned

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The recent implementation of next generation sequencing technologies significantly improved the molecular biology field. Indeed, it accelerated the linkage of diseases to their causative mutations while also allowing the identification of incidental findings that are of a medical value despite being unrelated to the patient’s condition. Besides, it sheds the light on private pathogenic variants frequent in consanguineous populations.

In this study, 500 Lebanese patients, presenting a wide spectrum of genetic disorders were referred to us for molecular diagnosis. Whole Exome Sequencing (WES) was performed. Incidental findings in 73 genes were evaluated as per the ACMG guidelines, in addition to the private Lebanese mutations listed in the CTGA database1. This allowed us to identify the causative mutations in 50% of the cases, in line with other international studies. To improve the diagnostic yield, WES data, generated during the first 2 years of this study, was reanalyzed for all patients who were left genetically undiagnosed. Reanalysis, based on updated bioinformatics tools and novel gene discoveries, enabled us to increase the diagnostics yield to 57%. An association between the rate of positive diagnosis and the disease group was noted. Indeed, the highest diagnosis success rate corresponds to the group of congenital hearing and visual disorders (100%), followed by neuromuscular disorders (85%), metabolic and mitochondrial disorders (84.2%), bone diseases and leukodystrophy (75%), epilepsy (66.7%) and neurodevelopmental disorders (30.4%). In parallel, dominant actionable variants were found in 6% of our cohort where genes associated with dominant cardiac diseases were the most frequently mutated (in 2% of our cohort). Genetic predisposition to cancer was observed in 1% of the cases while 2.5% carry a recessive disease allele.

In conclusion, the present work pinpoints the contribution of WES to an efficient genetic diagnosis and better clinical management. Lessons learned from WES reanalysis will also be shared.

1 Bizzari S et al., Genes (Basel). 2021 Sep 27;12(10):1518. doi: 10.3390/genes12101518
Exome Sequencing Leads to the Identification of a Rare Case of a Dominant Non-syndromic Hearing Disorder in a German Family

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Hearing impairment is the most common sensorineural disorder in humans. Approximately 1 - 3 out of thousand newborns suffer from severe hearing loss or deafness at birth or in the first years of life. Based on the form of the physiological defect, hearing disorders classified into conductive hearing loss, sensorineural hearing loss, or a combination of both. Hearing disorders caused by environmental factors or viral infections, strong sources of noise, ototoxic substances and genetic causes. About ~ 60% of all prelingual, hearing disorders are genetic. A total of 187 gene locations have been described to date, for which 143 genes have so far been identified, 44 genes at least still unknown. Autosomal non-syndromic hearing loss (ANSHL) is a genetically heterogeneous sensorineural disorder, with prelingual hearing loss and absence of other clinical manifestations. Based on the clinical diagnosis it is not possible to recognize in which genes mutations are present. The aim of this study is to identify the pathogenic gene in a non-consanguineous German family over three generations, with seven affected members. Mutation analysis were performed, in two affected family members using direct sequencing of the genes GJB2 and GJB6, including deletion analysis. Followed by whole exome sequencing, with the “INVIEW HUMAN EXOME” platform, array Agilent Genomics SureSelectXT All Exon V5. In the gene locus, DFNB1 no alteration was detected. A further targeted analysis of other genes was not possible, therefore complete exome sequencing took place. A heterozygous pathogenic variation was only detected in the gene TMC1, c.1249G> A, Gly417Arg, confirmed by Sanger sequencing. The inheritance pattern of the mutation in the family indicates a dominant non-syndromic hearing impairment. So far, mutations have been described in the TMC1 (MIM 606706) gene which, on the one hand, leads to recessive (DFNB7/B11; MIM 600974) or dominant (DFNA36; MIM 606705) inherited hearing impairment.
Expanding KITLG to Include Autosomal Recessive Inheritance, Oculocutaneous Albinism and Waardenburg/Albinism-Deafness Syndromes

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Pathogenic variants in KITLG, a key player in melanocyte proliferation and pigment production, are well-established causes of autosomal dominant non-syndromic hearing loss, Waardenburg syndrome type 2, familial progressive hyperpigmentation and familial progressive hyper- and hypopigmentation. Untangling the all-encompassing possibilities with respect to inheritance patterns is critical work to aid a rapid and accurate molecular genetic diagnosis. This work expands understanding of KITLG to include a recessive disorder characterized as a hypomelanosis spectrum with or without hearing impairment.

Through multiple sequencing approaches and extensive networking, we characterize the largest case series with biallelic KITLG variants comprised of six unrelated individuals. We greatly expand the hypomelanosis spectrum to include distal hypopigmentation, partial depigmentation resembling Tietz albinism-deafness syndrome and complete depigmentation reminiscent of oculocutaneous albinism. Sensorineural hearing impairment was identified congenitally or in the neonatal period and exhibited a unilateral (n=1), asymmetrical (n=2), or bilateral (n=2) hearing impairment in individuals from whom this information was available. We speculate variants with loss-of-function cause oculocutaneous albinism while those with residual function cause Waardenburg syndrome type 2 or albinism-deafness syndrome.

This work broadens the understanding of the mode of inheritance of Waardenburg syndrome type 2 to include autosomal recessive transmission. We emphasize the importance of genetic testing in diagnosing patients with unilateral or asymmetrical hearing impairment. This work defines KITLG as a new molecular cause of autosomal recessive albinism-deafness syndrome and oculocutaneous albinism.
Etiology of Auditory Neuropathy Spectrum Disorder in a Large Pediatric Cohort

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Auditory Neuropathy Spectrum Disorder (ANSD) is characterized by abnormal auditory nerve response (absent/abnormal ABR) and normal outer hair cell function (presence of cochlear microphonic or present otoacoustic emissions). It is clinically heterogeneous and accounts for nearly 10% of children with congenital hearing loss. Risk factors include prematurity, perinatal hyperbilirubinemia, and genetic syndromes. We report on the findings on etiology from a retrospective review of records on 266 individuals with a confirmed diagnosis of ANSD, and subsequent prospective evaluation of children with ANSD. From 24 probands ascertained, 7 family trios had genomic sequencing as a pilot study to elucidate the molecular etiology for ANSD.

In the retrospective study we identified cochlear nerve hypoplasia/aplasia by temporal bone imaging in 17% of the cases. Hyperbilirubinemia was more common in children with ANSD born at <37 weeks gestation compared to term infants, and rarely reported in those with cochlear nerve hypoplasia. Interestingly, 15% of children with cochlear nerve hypoplasia had an associated chromosomal or syndromic diagnosis including trisomy 21, Beckwith Wiedemann syndrome, and VACTERL association. In the ANSD cohort, a familial case of recessive pathogenic OTOF variants, and two children with Brown–Vialetto–Van Laere (BVVL) syndrome have been identified. A systematic referral for genetic evaluation in a prospective cohort has resulted in the identification of additional cases with chromosome aneuploidy, copy number variants, and pathogenic variants in SOS2, ARID1B, COL2A1, COL11A2, CABP2, and HARS2 genes, not all of which were on a hearing loss gene panel. These diagnoses were made due to additional findings, prompting a genetic referral. A pilot study for genomic testing on children with ANSD who had negative results for a hearing loss gene panel is in progress. Based on our experience we strongly recommend a clinical genetic and diagnostic evaluation to identify a genetic etiology in children with ANSD.
Investigations of *In Silico* Splice Prediction Tools in the Context of *SLC26A4*-Related Hearing Loss

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Variants in *SLC26A4* are a common cause of autosomal recessive hearing loss (HL) associated with an enlarged vestibular aqueduct (DFNB4/non-syndromic enlarged vestibular aqueduct) and Pendred syndrome (hearing loss and thyroid goiter). In ~15-30% of cases, biallelic variants *SLC26A4* are not identified, suggesting the presence of an unidentified second pathogenic variant. We hypothesized that in some cases, the second variant is a splice-altering synonymous change.

To identify potential splice-altering synonymous variants, we analyzed rare (MAF < 0.00007) synonymous *SLC26A4* variants in gnomAD (n = 162) with Human Splicing Finder (HSF). HSF predicts that 55% (n = 89) of these variants alter splicing. We performed *in vitro* splicing assays on a subset (n = 18) of predicted splice-altering variants and a subset (n = 4) of predicted nonsplice-altering variants using a pre-constructed pET01 Exontrap vector (MoBiTec) encoding a 5’ and 3’ exon separated by a multiple cloning site.

We find that HSF is a sensitive, but not specific method of assessing splicing. 17% (3/18) of synonymous variants predicted to alter splicing *in silico* alter splicing *in vitro*. When grouped by mechanism of splice alteration, 0% (0 of 5) of variants predicted to alter splicing via cryptic splice (CS) effects, 17% (1 of 6) of variants predicted to alter splicing via exonic splice enhancer and silencer (ESE/ESS) effects, and 29% (2 of 7) of variants predicted to alter splicing via both CS & ESE/ESS effects altered splicing *in vitro*. 100% (4 of 4) of the *in silico* predicted non-splice-altering variants were non-splice-altering *in vitro*.

In conclusion, while it is important to assess synonymous variants for splicing effects and HSF is a valid screening tool, an *in silico* prediction of a splice-altering effect could be verified *in vitro* in only 17% of cases. This result indicates the need to identify factors impacting the accuracy of HSF predictions.
Nonsyndromic Hearing Loss Mimics: Beyond Usher and Pendred Syndrome

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Syndromic hearing loss (SHL) accounts for approximately 30% of genetic diagnoses for pediatric hearing loss (HL) and has important implications for patients and families. Current clinical algorithms for genetic testing do not include all HL phenotypes, such as unilateral and asymmetric HL. Some recent studies suggest that a larger number of SHL genes than was previously appreciated can mimic a nonsyndromic HL phenotype. This raises the possibility of underdiagnosis of SHL. Our goal was to identify and study cases of nonsyndromic HL mimics with genetic diagnoses of SHL in our pediatric patient population via retrospective chart review. Demographics, medical and family history, audiological data, and genetic testing were assessed for patients at Boston Children’s Hospital with SHL who were initially evaluated for nonsyndromic HL. While many SHL patients who presented with apparently isolated HL had Usher or Pendred syndrome, 13 patients with isolated HL received SHL diagnoses that are typically associated with additional features in early childhood. Pathogenic variants were identified in nine genes (MITF, PAX3, SOX10, FGFR3, SIX1, EYA1, LARS2, KMT2C, and TFAP2A). In some cases, subtle syndromic features were appreciated after genetic diagnosis. Ten (77%) of these 13 patients had unilateral or asymmetric HL. Of the 13 diagnoses, 6 had Waardenburg syndrome, 2 subjects had Muenke syndrome or branchiootorenal syndrome, and 1 subject had Kleefstra syndrome type 2 or Perrault syndrome type 4. One patient had isolated unilateral HL and a dual diagnosis of branchiooculofacial syndrome and mosaic chromosome 5p15.33p13.3 gain. In the majority of these cases, a genetic diagnosis of a nonsyndromic hearing loss mimic was associated with asymmetric or unilateral hearing loss. Increased access to comprehensive genetic testing for patients with any hearing loss phenotype will improve SHL diagnosis and facilitate early referral to appropriate specialists, tailored intervention, and improved prognostic and recurrence information for families.
Defining the Genetic Landscape of OTOA-related Hearing Loss

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Pathogenic variants in OTOA are responsible for autosomal recessive non-syndromic hearing loss at the DFNB22 locus. Copy number variants (CNVs) are the most frequently reported variant in OTOA and reflect non-allelic recombination due to a nearby highly homologous pseudogene (OTOAP1). However, the mutational spectrum of DFNB22 is diverse, and in addition to CNVs that result in deletions and gene-pseudogene conversions, also includes single nucleotide variants (SNVs) and small indels. Here, we present the largest patient cohort exploring the mutational landscape of OTOA. We used targeted genomic enrichment and massively parallel sequencing to screen all known deafness-associated genes in a large ethnically diverse cohort with hearing loss. All variants were discussed in the context of clinical and family history data. Manual review of highly homologous regions was performed using Integrative Genomics Viewer (IGV). A probable or definitive genetic diagnosis was identified in 2548 of 5939 probands with hearing loss (43%). Variants in OTOA accounted for 3% of all positive cases (74 probands). A whole gene deletion was the single most frequently detected pathogenic variant, and was detected in 36 probands (48.6%), representing 31.7% of causative alleles (47/148). In addition to the whole gene deletion, we identified a broader mutational spectrum totaling 68 unique variants. Eight CNVs were detected including 4 deletions, 3 conversions, and 1 duplication. Among the 60 identified SNVs, 14 were loss-of-function variants (3 stop-gained, 3 frame-shift, and 8 splice variants), 3 were indels and 43 were missense variants. Within our cohort, both rare and recurrent CNVs, as well as SNVs which require manual review of sequence data for detection, represent an important etiology of DFNB22. Our findings emphasize the importance of sufficient coverage of OTOA and OTOAP1 for detection of CNVs, as well as manual review of this region to resolve genetic diagnoses.
Genotype-Phenotype Correlation for STRC-Related Hearing Loss

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STRC gene variants are the most common cause of autosomal recessive mild-to-moderate hearing loss. Unique to STRC-related hearing loss (HL) is the fact that copy number variants (CNVs) are the most commonly identified genomic change. The high prevalence of STRC-associated CNVs reflects the genomic landscape of this region, which includes a large duplication of four contiguous genes, CKMT1B, STRC, CATSPER2 and PPIP5KI. The highly homologous pseudogene, STRCP1, gives rise to gene-to-pseudogene conversions and continuous deletions of STRC and the neighboring CATSPER2 gene. Here, we present a comprehensive genotypic and phenotypic analysis of a large multiethnic cohort of patients with STRC-related HL.

We used targeted genomic enrichment and massively parallel sequencing to screen all known deafness-associated genes in a large cohort of patients with HL. Of 5756 patients tested, a genetic cause of HL was identified for 2461 individuals (43%), 310 (12.6%) of which had positive STRC diagnoses. Nearly 94% (291) of probands with STRC-related HL carried at least one CNV, including 142 gene-to-pseudogene conversions, 350 STRC-CATSPER2 deletions, and only 2 partial STRC deletions. 65.8% of the total diagnoses of STRC-related HL were due to CNV/CNV, 28% were due to CNV/SNV, and 6% were due to SNV/SNV. Contrary to previous studies, STRC whole gene (no involvement of CATSPER2) or partial gene deletions are ultra-rare, as we only identified two cases. To investigate genotype-phenotype correlation, we reviewed clinical and genetic data from our cohort and published literature (119 probands). One hundred ninety audiograms were analyzed in the context of genotypic data. We confirm that STRC-related HL is consistently associated with mild-to-moderate HL. We found no significant differences in the severity of hearing loss between genotypes type (missense/missense, missense/Loss-of-Function (LoF), and LoF/LoF).

These findings will contribute to our on-going goal of defining the genetic and phenotypic landscapes of this common type of autosomal recessive non-syndromic HL and define its natural history.

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Identification and Evaluation of Autosomal Recessive Non-Syndromic Hearing Impairment Genes in Rwanda

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The incidence of hereditary hearing impairment (HI) is higher in developing countries compared to developed countries and more than 120 independent genes have been identified as responsible for almost 50% of profound HI. Non-syndromic HI is the most common form accounting for 70% of cases of which 80% are autosomal recessive. Reported mutations in GJB2, GJB6, and GJA genes are the most common cause of HI but studies among Cameroonian and South African participants did not identify a significant association, hence the need for further genetic exploration of other responsible genes in African population. In Rwanda, more than 50% of HI among children was attributed to hereditary causes but no genetic evidence has been established. This study aims at identifying genes responsible for autosomal recessive non-syndromic hearing impairment (ARNSHI) among Rwandan population. We will recruit 100 affected families and 100 control individuals without HI or family history. One proband per affected family will be recruited together with one unaffected individual, both parents, and at least one affected relative for blood sampling. Standard audiomtry including air and bone conduction will be performed, and clinical history will be assessed to exclude exposure to ototoxic drugs or infection including prenatal exposure. For each family, we will exome-sequence samples of the affected members and use Sanger sequencing to follow up variants segregating in their parents and non-affected sibling, and controls. At the end of the study, we expect to establish a dataset of genes that cause ARNSHI in Rwanda with possible novel ARNSHI genes that have not yet been discovered in the African population. This will help to advance the science of HI and establish appropriate medical care including proper genetic counseling necessary for affected individuals and families as well as plan for prevention and control measures against genetic HI.
**GJB2 is a Major Cause of Non-Syndromic Hearing Impairment in Senegal**

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The prevalence of *GJB2*-related non-syndromic hearing impairment (NSHI) varies widely (0-50%) with reported population specific mutations. In sub-Saharan Africa, except for Ghana, the prevalence of *GJB2*-related NSHI is close to zero. To investigate the contribution of *GJB2* pathogenic variants and *GJB6*-D13S1830 deletion in autosomal recessive non-syndromic hearing-impaired families, we recruited a total of 129 affected and 143 unaffected individuals from 44 multiplex families segregating autosomal recessive non-syndromic HI, 9 simplex hearing-impaired families of suspected genetic origin, and 148 controls individuals without personal or family history of HI. The DNA samples were screened for *GJB2* coding region variants and *GJB6*-D13S1830 deletion. Mean age at the medical diagnosis of the affected individuals was 2.93 ± 2.53 years [range: 1-15 years]. Consanguinity was present in 40 out of 53 families (75.47%). In total variant in *GJB2* explained HI in 34.1% (n=15/44) of multiplex families. A bi-allelic pathogenic variant, *GJB2*: c.94C>T; p.(Arg32Cys) accounted for 25% (n=11/44 families) of familial cases of which 80% (n=12/15) were consanguineous. Interestingly, the previous reported “Ghanaian” founder variant, *GJB2*: c.427C>T; p.(Arg143Trp), accounted for 4.5% (n=2/44 families) of the families investigated. Among normal controls, carrier frequency of *GJB2*: c.94C>T and *GJB2*: c.427 was estimated at 1.35% (2/148) and 2.70% (4/148) respectively. No *GJB6*-D3S1830 deletion was identified in any of the HI patients. This is the first report of genetic investigation of HI in Senegal, and suggests that *GJB2*: c.94C>T; p.(Arg32Cys) and *GJB2*: c.427C>T; p.(Arg143Trp) should be tested in clinical practice for congenital non syndromic hearing impairment in Senegal.

**Keywords:** Hearing impairment; *GJB2*; *GJB2*: c.94C>T; p.(Arg32Cys) ; Senegal; Africa.
Session 2

Molecular Basis of
Mechanotransduction Machinery
Mechanical Gating of the Auditory Transduction Channel TMC1 Involves the Fourth and Sixth Transmembrane Helices

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The transmembrane channel-like (TMC) 1 and 2 proteins play a central role in auditory and vestibular transduction, forming ion channels that convert sound and head movement into electrical signals. TMC1 occurs as a dimer, and a structure has been suggested based on related channels. A pore in each subunit of the dimer likely involves transmembrane domains TM4-7, with specific residues identified that affect permeation. However, the molecular mechanism of gating—how tip-link tension causes the pore to open—remains unknown. Other proteins, including PCDH15, LHFPL5, TMIE and CIB2, appear to be part of the transduction complex but it is not clear which protein senses tension. Here, using structural models predicted by AlphaFold2 and related applications as a guide to mutagenesis, we probed the effects of twelve mutations on mechanical gating of the transduction currents in native hair cells. We used whole-cell electrophysiological recording from cochlear explants of TMC1/2-null mice in which we expressed TMC1 mutants by AAV injection into neonatal inner ears. These revealed that mutations of a critical set of residues in the pore-lining helices TM4 and TM6 modified gating, reducing the force sensitivity or shifting the open probability of the channels, or both. For some of the mutants, these changes were accompanied by a change in single-channel conductance. Our observations are in line with a model in which TMC1—and by homology TMC2—are the force-sensing subunits of the complex, and in which at least the TM4 and TM6 helices are involved in the mechanical gating of the channel.
MYO15A-CETN2 Phase Separation Underlies Formation of the Stereocilia Tip Density

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Stereocilia are actin-rich mechanosensory organelles that convert fluid motion into electrical impulses in the inner ear. A key protein in stereocilia development is the molecular motor MYO15A; mutations of which cause hereditary human hearing loss, DFNB3. MYO15A localizes to stereocilia tips where it delivers several proteins, referred to as the elongation complex (EC), all of which are required for stereocilia growth. The presence of MYO15A and the EC coincide with the tip density, a protein dense region that is hypothesized to regulate actin polymerization at the stereocilia tip. Formation of the tip density is dependent upon MYO15A. Here, we show that MYO15A in conjunction with a newly identified EC protein, CETN2, phase separates to form a biomolecular condensate that may contribute to formation of the tip density.

MYO15A-2 is well-documented in heterologous cells to accumulate at the tips of actin- based filopodia, before undergoing retrograde flow back to the cell body. Whilst imaging filopodia retrograde flow, we discovered that puncta of MYO15A-2 were ejected into the cytoplasm, where they accumulated. Membrane-dye experiments revealed that these puncta were not membrane bound, consistent with MYO15A forming a biomolecular condensate. To characterize these structures, we used FRAP and found that they maintained a small mobile fraction representative of a solid-like condensate. Active concentration of MYO15A-2 at the filopodia tip was critical for phase separation to occur. Mutation of the MYO15A-2 3\textsuperscript{rd} IQ domain, to preclude CETN2 binding, did not affect trafficking to filopodia tips. However, loss of CETN2 binding to MYO15A-2 disrupted the condensation reaction from occurring at filopodial tips, resulting in dissociation of the puncta during retrograde flow. We speculate that a MYO15A-CETN2 biomolecular condensate contributes to the formation of the stereocilia tip density, and that CETN2 is necessary for driving this reaction.

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Regulation of Resting Tension in the Mechanotransducer of Mammalian Auditory Hair Cells via MET-Dependent Stereocilia Remodeling

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In order to detect soft sounds, auditory hair cells tension their mechano-electrical transduction (MET) machinery at rest. A classical model developed for non-mammalian hair cells postulates that myosin motors at the upper end of the tip links climb along the actin cores of stereocilia, applying constant upward tension. However, some reports suggest that proteins associated with the upper end of the tip link may have limited mobility, at least in mammalian auditory hair cells. Therefore, we explored an alternative mechanism based on recent findings that transducing stereocilia in the hair bundle retract or elongate when resting current through the MET channels is decreased or re-established, correspondingly. We argued that MET-dependent retraction of stereocilia may either: i) not affect resting MET current if the upper end of the tip link is freely moved by myosin motors or, ii) increase resting MET current if it is somehow locked to the stereocilia actin core. We recorded MET currents in young postnatal cochlear outer and inner hair cells using fluid-jet stereocilia deflections while initiating MET-dependent stereocilia retraction in four different ways: i) blocking MET channels, ii) closing MET channels with negative hair bundle deflection, iii) preventing Ca2+ entry into stereocilia with cell depolarization, iv) using intracellular Ca2+ chelators. In all these experiments, presumable stereocilia retraction caused an increase in resting MET currents which persisted for several seconds. Drugs disrupting actin polymerization inhibited recovery of these after-stimulation MET current “overshoots”. Confocal imaging revealed seconds-long Ca2+ influxes during these overshoots in some but not all transducing stereocilia, consistent with reported non-uniformity of MET-dependent stereocilia remodeling. Thus, we conclude that auditory hair bundles can self-adjust resting tension of their mechanotransducers through MET-dependent stereocilia remodeling, also suggesting that the upper end of the tip link is likely to be locked to the stereocilia actin core.

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CETN2 Promotes Ca2+ - Dependent Assembly of Myosin 15 Biomolecular Condensates

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Stereocilia transduce sound and are packed with a para-crystalline array of actin filaments that determine their size and shape. Myosin 15 (MYO15A) traffics molecules critical for the growth and maintenance of the stereocilia actin core. Mutations in MYO15A cause human hereditary hearing loss, DFNB3. The motor domain of MYO15A consists of an ATPase with three IQ domains that bind to light chain proteins that together are necessary for force production and motility. Identification and characterization of the endogenous light chain complement is critical to understanding how MYO15A operates within stereocilia. Here, we identify the centrosome – associated protein, centrin-2 (CETN2) as a bone fide light chain protein that binds MYO15A.

To identify light chains binding to MYO15A, we performed co-IP experiments using pituitary AtT20 cells expressing an EGFP-tagged MYO15A-3IQ-FLAG construct. LC-MS/MS analysis of co-immunoprecipitated proteins identified CETN2 as a candidate light chain. We confirmed CETN2 binding to MYO15A using expressed proteins purified from Sf9 insect cells, and further mapped the CETN2 binding site to the third IQ domain (IQ3) of MYO15A using truncation analysis. CETN2 is an EF-hand containing protein that can polymerize upon binding calcium. We found that CETN2 similarly drives calcium-dependent assembly of MYO15A into high molecular weight structures. Visualization of these structures using confocal fluorescence microscopy revealed that MYO15A-CETN2 forms a biomolecular condensate in the presence of Ca2+. Critically, MYO15A does not form biomolecular condensates in the absence of CETN2. Using orthogonal light scattering we found that condensate formation was a multi-step reaction, indicating the presence of at least two MYO15A-CETN2 intermediate species. We speculate these structures are critical for MYO15A to traffic along stereocilia and regulate actin polymerization within the stereocilia tip density. Our data further suggests that CETN2 is a candidate gene for studies of human hereditary hearing loss.

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Single-cell RNA Sequencing Reveals Age-associated Patterns of Hair Bundle Gene Expression

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The hair bundle of cochlear hair cells is responsible for mechanosensation of the sound pressure wave. Several genes associated with human hearing loss, including ACTG1, STRC, and TMC1, encode proteins that localize to the hair bundle, with mutations in these genes associated with changes in bundle morphology. However, the temporal pattern of expression of these hair bundle genes beyond the onset of hearing has not been thoroughly investigated. We sought to examine their pattern of expression from the onset of hearing to middle age.

Outer hair cells (OHCs) were isolated from cochlear tissue from C3H/FeJ mice at the onset of hearing (P15), maturity (P70), and middle age (P228) using a manual micropipetting approach for single-cell RNA sequencing. Review of published literature was used for manual curation of a set of hair cell genes whose products localize to the hair bundle. Genes were evaluated for differential expression using Wilcoxon rank-sum testing, with meaningful change defined as \( p < 0.05 \) after Bonferroni correction for multiple hypothesis. The pattern of gene expression was classified as up- or down-regulation from baseline expression at P15.

High-quality sequencing data were obtained from 180 OHCs. Expression analyses showed that 5 genes (Actg1, Anxa5, Cib2, Homer2, and Triobp) out of 71 genes (7%) were differentially expressed while the rest exhibited stable expression across timepoints. Actg1, Homer2, and Triobp were downregulated from P15 to P70 then maintained stable expression till P228. In contrast, Cib2 exhibited late downregulation from P70 to P228. Finally, Anxa5 showed progressive downregulation across all timepoints.

The expression of hair cell genes whose products localize to the hair bundle changes beyond the onset of hearing in mice. Our dataset is valuable in understanding normal age-associated changes in expression profiles of hair bundle proteins.
Session 3
Molecular Biology of Cochlear Development
Inhibitory G Proteins Have Multiple Roles During Hair Cell Polarized Morphogenesis

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Previous studies suggest that the inhibitory G alpha (GNAI) proteins are critical for hearing and have multiple roles with distinct regulatory partners during the polarized morphogenesis of hair cells (HCs). This knowledge remains fragmentary, however, because to date functional analyses relied largely on the use of Pertussis toxin that globally downregulates functionally redundant GNAI1, GNAI2, GNAI3 and GNAO proteins, but may also induce unrelated defects. We thus embarked on a comprehensive study to definitively determine the role(s) of individual GNAI proteins. We obtained or generated single \textit{Gnai1}\textsuperscript{ko}, \textit{Gnai2}\textsuperscript{ko}, \textit{Gnai3}\textsuperscript{ko} and \textit{Gnao}\textsuperscript{ko} constitutive mouse mutants, then derived viable double mutants: \textit{Gnai1}\textsuperscript{ko}; \textit{Gnai2}\textsuperscript{ko} and \textit{Gnai1}\textsuperscript{ko}; \textit{Gnai3}\textsuperscript{ko}. Because combined loss of \textit{Gnai2}; \textit{Gnai3} is embryonic lethal, we also generated a \textit{Gnai3}\textsuperscript{Flox} allele and conditional \textit{FoxG1-Cre}; \textit{Gnai2}\textsuperscript{ko}; \textit{Gnai3}\textsuperscript{Flox} mutants.

We find that only GNAI3 is essential for the organization and elongation of stereocilia, as well as for auditory function. However, both GNAI2 and GNAI3 were similarly polarized at the HC apex with their binding partner GPSM2, whereas GNAI1 and GNAO were not detected or polarized. As reported previously (Beer-Hammer et al., 2018), we confirmed that GNAI2 can mitigate the loss of GNAI3 during hair bundle morphogenesis. Interestingly, functional rescue by GNAI2 was incomplete because GNAI2 progressively failed to fully occupy the subcellular compartments where GNAI3 was missing. Strikingly, our \textit{FoxG1-Cre}; \textit{Gnai2}\textsuperscript{ko}; \textit{Gnai3}\textsuperscript{Flox} mutants also recapitulated for the first time two distinct types of defects so far only observed with Pertussis toxin: 1) a delay or failure of the basal body to migrate off-center in prospective HCs, and 2) a graded reversal of OHC orientation. We thus conclude that besides regulating hair bundle morphogenesis with GPSM2, GNAI proteins have two other earlier, physiological roles. These results complement our identification of the GPCR GPR156 as the GNAI regulator reversing HC orientation (Kindt et al., 2021).
The Role of Nrxn3 in Synapse Formation in Lateral-Line Hair Cells

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Hair cells rely on a specialized ribbon synapse to reliably encode sensory stimuli. Previous studies have identified molecules at the mature ribbon synapses which are required for structure or function, but what molecules help initiate hair cell to afferent contacts during development to promote synapse formation is less clear. My work investigates the role of the presynaptic adhesion molecule Nrxn3, a gene shown to be enriched in hair cells. My research uses the zebrafish lateral line to examine the role of Nrxn3 in hair cell synapse formation. To study synapse formation, I examined zebrafish mutants with lesions in the long, α-form of Nrxn3. I used immunohistochemistry to stain for presynaptic ribbons and postsynaptic densities in mature hair cells. This staining revealed dramatically fewer complete synapses and more unpaired pre- and post-synapses in nrxn3-α mutants. I also examined synapses in developing hair cells to understand if Nrxn3 is important for synapse formation or maintenance. This analysis revealed that even during development synapse formation is disrupted in nrxn3-α mutants compared to controls. These findings indicate that Nrxn3-α is essential for synapse formation in lateral-line hair cells. Currently, I am using CRISPR-Cas-9 to create zebrafish mutants to identify the postsynaptic partner of Nrxn3. Additionally, I plan to use behavioral assays and functional imaging to understand how the loss of synapses in nrxn3-α mutants impacts behavior and synapse function. Overall, my work has identified a novel player required for synapse formation in hair cells.
Feature Characteristics and Signaling Mechanisms Involved in SGN Neurite Guidance

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The cochlea exploits an intricate tonotopic organization of afferent innervation to effectively process complex auditory stimuli. To create this precisely organized pattern, neurites from spiral ganglion neurons (SGNs) navigate a complex milieu of cells, extracellular matrix, and biochemical gradients to reach their peripheral and central targets in the organ of Corti and cochlear nuclei, respectively. This process of a neurite growing through its environment is called pathfinding. In pathfinding, the tip of the neurite, the growth cone, senses, turns, and grows toward a target in response to biochemical and biophysical cues. To study this in vitro, we use photopolymerization and various engineering techniques to investigate the biophysical and biochemical factors that direct SGN neurite growth and probe the pathways SGNs use to pathfind in response to these cues. Here, we demonstrate that topographical feature geometry (amplitude and angle) determines neurite turning efficiency. Specifically, we show that increasing feature amplitude promotes neurite turning to increasing angle turn challenges in a dose dependent manner. Using this multiangled channel patterned substrate, we show that both inositol triphosphate (IP3) signaling and ryanodine sensitive receptors (RyR) are needed for SGNs to turn in response to these channels. In related work, we see that IP3 signaling and RyR are utilized by SGNs to align their neurite growth in response to chemo-repulsive features, while only IP3 is implicated in aligning growth to chemopermissive features. IP3 signaling appears to be required for SGNs to turn in response all cues, while RyRs are only required for SGNs to turn in response to repulsive cues. Overall, this research informs the key, basic biological process of how an SGN neurite senses and turns in response to substrate cues. Thus, informing the signaling and engineering required to guide SGN neurite growth towards desired targets, such as cochlear implants.
**Initial Characterization of a Itga8-Pcdh15 Signalosome During Inner Ear Development**

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An organism’s perception of its surrounding environment depends on sensory function. Neurosensory cells from the inner ear are involved in key biological processes associated with hearing and balance. To be able to achieve their function these cells depend on a complex array of membrane receptors, ion channels and signaling molecules that are concentrated at extremely sophisticated structures positioned at the apical (hair cell bundle and kinocilium) and basal (ribbon synapses) poles. Disruption of this network due, for example, to mutations, results in morphological and functional abnormalities and forms the bases of many human genetic disorders. The aim of this study is to assess the role of Pcdh15-Itga8 (Protocadherin-15-Integrin alpha8) complex during hair cell (HC) development and the downstream signaling pathway activated by it. Our published work and the work from others demonstrated that absence of Itga8 and/or Pcdh15 results in HC bundle abnormalities, and alterations in Rhoa activity. Moreover, we found a physical interaction between Itga8 and Pcdh15 in hair cells.

During inner ear development, Itga8-Pcdh15 complex regulates downstream signaling cascades that results in hair cell cytoskeletal rearrangements. Thus, Itga8 dysfunction may contribute to the hearing deficits observed in Pcdh15 mutants.

Kinome analysis using Itga8 knockout epithelial cells demonstrated several key signaling molecules were affected. Among them, we focused on ezrin-radixin-moesin (ERM) and Yap. Lack of Itga8 resulted in a decrease in ERM activity and an increase in Yap activity. This was demonstrated in vitro and in vivo in an Itga8 hair cell specific conditional knockout mouse. Moreover, these animals developed progressive hearing loss.

Collectively, these studies establish a functional link between Usher syndrome and an integrin-downstream signaling cascade, in which disruption of the complex results in cytoskeletal disarrangements that, in turn, alter ERM’s and Yap’s activities, two key factors necessary for organ of Corti development.
Dual Embryonic Origin of Intermediate Cells of the Stria Vascularis

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Intermediate cells of the stria vascularis are neural crest derived melanocytes. They are essential for the establishment of the endocochlear potential in the inner ear, which allows mechanosensory hair cells to transduce sound into nerve impulses. Despite their importance for normal hearing, how these cells develop and migrate to their position in the lateral wall of the cochlea has not been studied. We find that as early as E10.5 some Schwann cell precursors in the VIIIth ganglion begin to express melanocyte specific markers while neural crest derived melanoblasts migrate into the otic vesicle. Intermediate cells of both melanoblast and Schwann cell precursor origin ingress into the lateral wall of the cochlea starting at around E15.5 following a basal to apical gradient during embryonic development, and continue to proliferate postnatally.
Semaphorin3D and Neuroblast Delamination in the Chicken Otic Vesicle

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During inner ear development, the movement and fate of progenitor cells must be carefully regulated for the developing organism to hear properly. One critical step of inner ear development, neuroblast delamination, is the migration of neuronal progenitor cells from the developing otic epithelium and towards the hindbrain to create the audiovestibular nerve. The purpose of this research is to investigate the expression and regulatory role of the Semaphorin3D (SEMA3D) gene during neuroblast delamination in the chicken otic vesicle. SEMA3D is a secreted protein that directs axon movement through receptor-dependent attraction or repulsion of neurons and is known to be expressed later in chicken inner ear development. We hypothesize that SEMA3D is promoting neuroblast delamination in the chicken otic vesicle by forcing neuroblasts to migrate from the otic epithelium. Using a retrovirus-mediated RNA knockdown system to decrease SEMA3D expression in embryonic-day three chicken otic vesicles, subsequent changes in neuroblast development are assessed using quantitative PCR (qPCR) and immunofluorescence. Preliminary results indicate that SEMA3D knockdown causes a reduction in expression of neuronal markers like Isl1 and Tubb3, suggesting decreased neuroblast delamination in cultured otic vesicles. Current work is underway to determine whether neuroblasts delaminate when SEMA3D is knocked down and which receptor for SEMA3D is potentially used in this process. Understanding the molecular mechanisms underlying neuroblast delamination during chicken inner ear development will add to our knowledge of this developmental process and of the role of the SEMA3D gene itself.
Session 4

Transcriptome Profiling

of the Auditory System
Single-Cell Analysis of the Zebrafish Inner Ear

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A major cause of human deafness is permanent loss of the mechanosensory hair cells of the inner ear. Although adult zebrafish can regenerate hair cells following injury, mammals do not. The degree to which the inner ear of fish and mammals share common cell types remains unresolved. To further characterize the zebrafish inner ear as a model for hair cell regeneration, we performed single-cell RNA and ATAC sequencing of the zebrafish inner ear at multiple stages to catalog the diversity of hair, support, and progenitor cells and their homology to mammalian counterparts. We identify bipotent progenitors for hair and support cells that appear to be unique for the zebrafish inner ear versus the mechanosensory lateral line. We identify several types of support cells, including distinct types in the macula versus cristae. In the macula, we identify two types of hair cells that share gene expression with mammalian type I versus II cells. In situ hybridization reveals that these hair cell subtypes occupy distinct spatial domains within the two major macular organs, the utricle and saccule, consistent with the reported distinct electrophysiological properties of hair cells within these domains. These findings suggest that primitive type I and II hair cells likely arose in the last common ancestor of fish and mammals, with subsequent modifications along the mammalian lineage. Similarities of inner ear cell type composition between fish and mammals support zebrafish as a relevant model for understanding inner ear-specific hair cell regeneration.
Transcriptome Profiling and Novel Isoform of the Cochlear Slc26a4-Expressing Cells.

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Mutations in SLC26A4 are known to cause a majority of Pendred syndrome cases (PDS, OMIM#274600) and non-syndromic enlarged vestibular aqueducts (NSEVA/DFNB4, OMIM#600791). The genotype-phenotype correlation varies depending on ethnicity, degree of hearing loss, and number of mutated SLC26A4 alleles. However, little is known about how variation is accounted for at the isoform level. The recently developed single-cell RNA-sequencing (scRNA-seq) technique has expanded our knowledge of gene expression profiles and isoform variety. In this study, we used scRNA-seq to identify the cochlear stria vascularis (SV) cells including Slc26a4-expressing cells based on gene expression profile. We identified various transcript isoforms of Slc26a4 in the cochlear Slc26a4-expressing cells to better understand genotype-phenotype correlation based on isoform level, specifically whether novel isoforms affect hearing phenotype based on mutation loci. To determine if the novel isoforms are transcribed, we measured the mRNA expression of the cochlear lateral wall (LW) by reverse transcriptase (RT)-PCR. The sequence of the novel isoform was confirmed by sequencing using the 3500 Genetic Analyzer. To validate whether the novel isoforms are translated, we measured the protein levels of the pendrin by western blotting and immunostaining. We found that the novel isoform is transcribed as mRNA and is translated as protein. These findings shed light on the molecular mechanism of PDS-NSEVA and expand our isoform-guided understanding of pathogenetic variants.
The Role of Unfolded Protein Response in Noise-Induced Hearing Loss.

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In modern society, we are at risk of noise-induced hearing loss (NIHL) as we are increasingly exposed to a variety of external noises. Noise-induced hearing loss can be temporary (TTS) or permanent (PTS), depending on the intensity and duration of exposure. The mechanism by which hearing is restored after temporary hearing loss has not yet been elucidated. We investigate the time-dependent transcriptomic changes after transient threshold shift (TTS) and permanent threshold shift (PTS) noises stimulation in cochlea. As a result of DEGs analysis, most of the transcriptomes showed the same expression pattern for TTS and PTS noise stimuli, and showed the greatest change one day after exposure to noise. As such, most transcriptomes are expressed in the same pattern, but there is a difference in the recovery of hearing, so we focused on transcriptomes showing the same expression pattern for TTS and PTS. Based on gene ontology analysis, we found that genes involved in the unfolded protein response (UPR) pathway were upregulated in the cochlea after TTS and PTS noise stimulation. As a result of western blot analysis of the cochlea, genes related to the PERK and IRE1a pathways were significantly induced one day after noise exposure among the three UPR branches. After 2 weeks, when hearing was restored, TTS and PTS showed differences in UPR activation. Activated PERK and IRE1a in TTS returned to baseline levels after 2wks, whereas in PTS, the increased PERK branch was still activated. To further elucidate the role of PERK branch in hearing recovery, we investigated the effects PERK inhibitor, GSK2656157 on TTS. Interestingly, GSK2656157 treatment inhibited the hearing recovery in TTS, suggesting that UPR is required for hearing recovery in TTS. Our findings indicate that UPR is required to restore hearing after temporary noise-induced hearing loss, and that modulation of the UPR can be utilized to prevent NIHL.
Time Lapse Transcriptomic Analysis of Human Otic Organoids Define Cell Types and Developmental Lineages of the Inner Ear

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The human inner ear contains specialized sensory hair cells, neurons, and many different supporting cells that provide critical mechanical, ionic, and nutritive structure. Because each cell type plays an important role in the proper development and function of the inner ear, any model used to study this system should replicate as much cell diversity and maturity as possible. While animal models provide notable insights into gene programs critical to the vertebrate inner ear, they often fall short of replicating key mechanisms, both known and unknown, that are unique to humans. In our lab, through directed differentiation and self-organization, we convert human induced pluripotent stem cells into 3D cell aggregates containing sensory, neural, and support cells of the inner ear. Using these “otic” organoids, we investigated the developmental cues and cell diversity that emerges during the organoid differentiation and maturation process. To accomplish this, we performed single-cell RNA sequencing at ten time points throughout the first 36 days of induction. We then determined the pathway of on-target cells from starting undifferentiated pluripotent stem cells through sequential hallmarks of inner ear development to culminate in mature sensory, neural, and supporting cells. We then classified the time course of emergence of a diverse set of on- and off-target cell types. We validated the modulation of gene programs via RNA expression associated with exogenously delivered FGF, BMP, and WNT small molecule activators and inhibitors. Importantly, we observed previously underappreciated endogenous gene expression pathways may be influencing the efficacy of on-target differentiation, and propose modifications to differentiation protocols that may increase otic differentiation efficacy. Preliminary experiments already confirm that our database, collectively known as the Inner ear Organoid Developmental Atlas (IODA), is a powerful tool to make hypothesis-driven investigations into developmental pathways and may yield a new generation of highly efficient and robust differentiation protocols.
Sessions 6 and 7
Pathological Mechanisms
Underlying Deafness
Maturation and Development of Ribbon Synapses is Affected in a New Pex1 Mouse Model for Zellweger Spectrum Disorder

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Zellweger spectrum disorders (ZSDs) are a group of rare genetic multisystem disorders characterized by defect in peroxisome synthesis, assembly, and function. ZSDs are associated with neurosensory hearing loss, retinopathy, multiple organ dysfunction and psychomotor impairment. Mutations in PEX1 are the most common cause of ZSDs, representing 70% of all cases (Reuber et al. 1997). In more severe cases, patients died before 1 year of age. Limited research has focused on the impact of peroxisomal disorders on auditory function, hampering the development of treatments for ZSDs patients. Peroxisomes are mainly involved in lipid synthesis and oxidative stress balance. As hair cells (HCs) are particularly sensitive to metabolic changes, we hypothesize that mutations in PEX1 cause hearing loss by affecting HC functions along the cochlea.

We created a conditional knockout (cKO) mouse by breeding a novel floxed Pex1 mouse with HC specific CRE expressing mice: Gfi1-CRE (all HCs) and VGluT3-CRE (Inner HCs). We show that Pex1Gfi1 is associated with a decrease in auditory brainstem responses (ABRs) wave-1 amplitude in response to click and pure tones. This decrease in wave-1 amplitude is not associated with ABRs or distortion product otoacoustic emissions threshold change. This result suggests that inner HCs (IHCs) are more sensitive to neonatal Pex1 excision than outer HCs. Due to the mosaic nature of Gfi1-CRE, we decided to use another CRE-expressing mouse to delete Pex1 specifically in IHCs. Using Pex1VGluT3 mice, we show that Pex1 excision in IHCs leads to hearing loss and is also associated with significant decrease in wave-1 amplitude. We also observed a significant decrease in ribbon synapse number and volume in Pex1VGluT3 mice. This result is correlated with an alteration of oxidative stress level.

These results suggest a critical function of Pex1 in development and maturation of IHCs-Spiral Ganglion Neurons synapses as well as hearing function.
Early-onset Hearing Loss in a Mouse Model Lacking the Neuronal AP3B Complex

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AP complexes play a crucial role in the transport of proteins between organelles. The heterotetrameric AP3 complex is either ubiquitously expressed (AP3A) or neuronal-specific (AP3B) depending on which subunits are present. Evidence that the AP3 complex is important for hearing comes from the mocha mouse mutant (Ap3d null allele), which exhibits deafness and vestibular dysfunction. However, the Ap3d subunit is ubiquitously expressed being present in both AP3A and AP3B complexes. The neuronal AP3B complex is reported to be important for the generation of synaptic vesicles and the sorting of cargo proteins to nerve terminals. In order to investigate the requirement of the AP3B complex for hearing we have generated an Ap3m2 loss-of-function mutant, as this subunit is only present in neurons. Using CRISPR/Cas9 we have generated an Ap3m2 mutant in which there is a frameshifting single nucleotide duplication causing an early truncation in the encoded protein that lacks the critical cargo-protein recognition domain.

We find that homozygous mutants exhibit an early-onset hearing loss phenotype, with moderately elevated ABR thresholds. However, these do not worsen by six-months of age, when animals begin to develop spontaneous seizures.

Further analysis of the ABR waveforms suggest a large synaptic component to the phenotype, with young animals exhibiting severely reduced Wave I amplitudes. Moreover, DPOAE and ultrastructural studies (SEM) indicate no OHC dysfunction, while histological analyses reveal normal spiral ganglion neuron density. However, differences in vesicular distributions are evident in the mutants.

In addition to the hearing phenotype, homozygous mutants show an anxiety-like reaction to novel objects and environments.

Our results demonstrate a role for the neuronal AP3B complex in hearing and neurobehaviour, and identify AP3M2 as a gene that should be considered when assessing patients with idiopathic hearing loss. Further studies are required to fully elaborate upon the requirement of the AP3B complex for hearing.
Fate Mapping Reveals Heterogeneity In Cochlear Macrophages In Steady-State And After Acoustic Trauma

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Cochlear injury results in activation of resident macrophages and recruitment of monocytes from circulation, which may differentiate into macrophages. The precise roles of resident and recruited macrophages in hearing loss are unclear. We have reported that macrophages promote the survival of spiral ganglion neurons (SGNs) via fractalkine (CX3CL1-CX3CR1) signaling after cochlear injury. However, it remains unclear if CX3CR1-expressing resident and recruited macrophages are distinct and differentially promote SGN survival. Here, we used a robust fate mapping technique wherein CX3CR1-expressing resident and recruited macrophages are endogenously labeled with different fluorescent reporters to define heterogeneity in cochlear macrophages in terms of origin, spatial distribution, morphology, fate, phenotype and function after acoustic trauma. Tamoxifen inducible CX3CR1YFP−CreER/YFP−CreER mouse line was crossed with Rosa-Isl-tdTomato (R26RFP) reporter mouse line. The progeny CX3CR1YFP−CreER/wt:R26RFP were injected with tamoxifen and euthanized at various time points post injection to determine Cre recombination efficiency. A cohort of tamoxifen-injected CX3CR1YFP−CreER/wt:R26RFP mice were allowed to recover for 60 days (“wash out”) followed by noise exposure. Tissue was analyzed by flow cytometry and confocal imaging. By 60 days post tamoxifen administration, CX3CR1-expressing cochlear resident macrophages (98 ± 1.7% recombination efficiency) and blood circulating CX3CR1 lineage (2.5 ± 1.1% recombination efficiency) displayed distinct YFP+ RFP+ and YFP+ RFP− phenotype. Examination of resident macrophages for one year indicate that their turnover rate is considerably slower than circulating monocytes/macrophages (1-3 days). Acoustic trauma in “washed out” mice show the presence of proliferating resident and monocyte-derived macrophages of both pro- and anti-inflammatory phenotypes in the spiral ganglion and laminaris compared to sham exposed mice containing only naïve macrophages. Morphometric analysis indicate that morphology is not a good indicator to distinguish CX3CR1-expressing resident and recruited macrophages. These data establish the use of genetic fate mapping to distinguish CX3CR1-expressing resident macrophages from recruited macrophages in both normal and injured cochlea.
Exploring Structure, Activity, and Regulation in Deafness-Associated Variants of Diaphanous 1 (DIAPH1)

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DFNA1 is one of the earliest mapped deafness loci and is associated with autosomal dominant progressive hearing loss. The gene that is affected in the DFNA1 mutations encodes Diaphanous 1 (DIAPH1), a member of the formin family of cytoskeletal regulators. Like other formins, DIAPH1 interacts directly with the actin and microtubule cytoskeletons and is regulated by an auto-inhibitory interaction between its N- and C-termini. Little is known about the mechanisms underlying DFNA1 and more generally about the role of formins in the inner ear. There are at least five distinct mutant alleles of DIAPH1 that are linked two deafness two missense mutations in the N-terminal region of the protein (in or adjacent to the regulatory DID domain) and three in the C-terminal regulatory DAD domain (adjacent to the actin-binding Formin Homology 2 domain). Several variants are classified as non-syndromic, while others are associated with the platelet disorder macrothrombocytopenia. We are biochemically testing hypotheses about the etiology of hearing loss associated DFNA1 mutations. All mutants so far are well-folded in vitro, and the mutated C-terminal regions maintain their ability to stimulate actin polymerization. The C-terminal mutants show varied affinities for the regulatory DID domain; the Caa mutation¹ has an affinity for the DID domain similar to WT, while the 1213X² and Δgo³ show almost no inhibition. The N-terminal mutant I521S⁴ forms a well-folded dimer despite the fact that this mutation is in the predicted dimerization interface.⁵ We are extending these studies to include microtubule interactions and actin end binding.

Genotype Phenotype Correlations of 37 DFNB9 Patients with Auditory Neuropathy and 17 New OTOF Pathogenic Variants

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Auditory neuropathy represents 5-10% of child’s hearing loss. Pathogenic bi-allelic variations of OTOF result in autosomal recessive deafness DFNB9. We retrospectively studied the genotype-phenotype correlations of 37 cases from 30 families with pathogenic bi-allelic OTOF variations. Seventeen new pathogenic variants were identified. All patients had isolated auditory neuropathy. Hearing loss was pre-lingual in 78% of cases and profound in 70%. Hearing loss was progressive in 30%, fluctuating in 30% and temperature-sensitive in 22%. The diagnosis of auditory neuropathy was mainly based on the discordance of electrophysiological tests with acoustic otoemissions present (78%) and brainstem auditory evoked responses absent or desynchronized (81%). All patients with homozygous or compound heterozygous "loss of function" variants had congenital bilateral profound hearing loss, patients compound heterozygous for a "loss of function" variant and a missense variant had variable presentations. Those with two missense variants had a mild to severe hearing loss, which could be of secondary onset. 54% received cochlear implant rehabilitation, 16% of which were bilateral. Our study confirms a successful hearing rehabilitation with cochlear implants, with open word perception increasing from 0% before surgery to 80% at 8 years after implantation. However, cochlear implantation cannot be considered as a treatment (disturbance in noise, social difficulties and professional integration...). Gene therapy trials in mutant OTOF -/- adult mice have shown prolonged hearing rehabilitation, making it possible to consider a short-term therapeutic trial for this isolated congenital form of deafness (RHU AUDINNOVE 2019). This phenotype-genotype study is an essential prerequisite for the future therapeutic trial.
MyD88 Regulates Expression of TLR4 and TRPV1 at the Plasma Membrane

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Aminoglycosides remain clinically necessary to treat bacterial infections that elicit severe systemic inflammation. Unfortunately, parenteral administration of these drugs can cause permanent hearing loss following entry into mammalian sensory hair cells via non-selective cation channels, such as TMC1, TRPA1 and TRPV1. Bacteriogenic-induced activation of TLR4 (via lipopolysaccharides, LPS) upregulates and sensitizes TRPV1 channels increasing cellular uptake of aminoglycosides and thereby exacerbating hearing loss (Jiang et al., 2019). LPS binding of TLR4 activates an intracellular signaling cascade via the adaptor protein, MyD88, while the loss of TLR4, TRPV1 or MyD88 activity ameliorates severe inflammation and drug-induced hearing loss. (Jiang et al., 2019; unpublished data). We hypothesize that MyD88 facilitates internalization of, and/or a conformational change, in plasmalemmal TRPV1 to increase cellular uptake of aminoglycosides.

The TRPV1 agonist, capsaicin, enhances, while the TRPV1 antagonist capsazepine attenuates, cellular uptake of GTTR, with or without MyD88 or TLR4 knockdown, suggesting that plasmalemmal TRPV1 is functional independent of MyD88 or TLR4 expression. However, after MyD88 knockdown, cells (with or without LPS treatment) had reduced uptake of GTTR compared to their control cells. MyD88 knockdown increased TRPV1 expression that is further elevated by LPS exposure suggesting that MyD88 is required for TRPV1 internalization and degradation. Also, increased MyD88 and TRPV1 protein expression after LPS treatment is attenuated after TLR4 knockdown. In PLA experiments, MyD88-TRPV1 and TLR4-TRPV1 interactions are increased after LPS treatment. Knockdown of MyD88 attenuated LPS-induced degradation of TLR4 expression observed in MyD88* cells in immunoblots. This suggests that LPS-increased internalization of TLR4 is dependent on MyD88, as previously demonstrated.

Our data suggest that MyD88 traffics a TLR4-TRPV1 complex away from the membrane, while knockdown of MyD88 prevents this internalization - ‘trapping’ these proteins at the plasma membrane. These results implicate that MyD88 activity is required for inflammation-exacerbated aminoglycoside-induced cytotoxicity and hearing loss.

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Reorganization of Afferent Cochlear Synapses on Residual Inner Hair Cells in Mice with Partial Loss of Hair Cells

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While each inner hair cell (IHC) is innervated by multiple spiral ganglion neurons (SGNs), each SGN contacts just a single IHC. While not the initial pattern, this is a consequence of synaptic remodeling and synapse elimination late in cochlear development. In a series of experiments intended originally for a different purpose, diphtheria toxin (DT) injected into postnatal day 5 DTR mice was used to eliminate HCs. We found, serendipitously, that some of the mice had only a partial loss of HCs. We quantified synapses on surviving IHCs to ask whether a reduced number of IHCs would result in a reduction in synapse elimination during postnatal synaptic remodeling, or induce reorganization of synaptic innervations during maturation and, consequently, an increased number of synapses on the surviving IHCs. ~6 month-old DTR mice in a CBA/CaJ background were examined and compared to ~4 month-old wild-type CBA/CaJ mice. ABR thresholds were elevated in the DTR mice. ABR amplitudes were also significantly reduced in these mice compared to the wild-type control mice. Consistent with the ABR results, these mice showed diffuse both IHC and OHC loss over the entire cochlear length, including apical region. Synapse counts, defined as colocalized CtBP2 and PSD95 – or CtBP2 and GluA2 – immunofluorescent puncta, on surviving IHCs, increased by 25.8%, 28.7% and 37.4% at 8, 16 and 32 kHz cochlear regions, respectively, relative to the control mice. Synapse counts on the IHCs in clusters are similar to those on isolated IHCs. We suggest that after partial loss of hair cells in the DTR mouse model, SGNs do not necessarily lose their peripheral axons or degenerate but can shift their synapses to residual IHCs in a large-scale reorganization of synapses. Because the phenomenon was noticed in adult female mice, determining the age and sex difference at which this occurs requires further investigation.
Quinoxaline Derivatives Protect from Excitotoxicity in Zebrafish

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More than 300 million people worldwide suffer from noise-induced hearing loss (NIHL). The effects of noise are rapid, with hair cell degeneration and/or neuronal excitotoxicity, being observed immediately after the exposure. Unfortunately, despite the enormous impact of NIHL, its molecular mechanism is poorly understood, not to mention any therapeutic interventions that may help to prevent or alleviate this condition. Published work supports the therapeutic potential of quinoxaline derivatives to treat ototoxin- and noise-induced hearing loss by NF-kB pathway inhibition. Quinoxaline and its derivatives have an excellent malleable structure for chemical modifications, and can cross the blood brain barrier, which make them attractive as putative ototherapeutic compounds. Moreover, quinoxalines have been approved by the FDA for their use in the pharmaceutical and food industries, which would expedite their developmental phase for use in the auditory system as repurposed compounds.

We employed a zebrafish model for excitotoxicity to screen a library of 68 quinoxaline derivatives. The number of hair cells and ribbon synapses, as well as the neuronal innervation of neuromast hair cells were assessed in the treated animals. NF-kB pathway was studied in the top ten identified otoprotective compounds.

The results presented here show that quinoxaline derivatives can confer protection against excitotoxicity-induced neuromast damage. Treatment with the top ten candidates resulted in preservation of the hair cell number, and ribbon synapses. Moreover, there was a significant reduction of NF-kB pathway activation, determine in a zebrafish NF-kB reporter fish line.

Overall, this work provides initial evidence of the potential therapeutic effect of quinoxaline derivatives to protect against NIHL. Future studies are aimed at the characterization of the top ten compounds in a mouse model for NIHL.
The Role of ATF Signaling in Response to PTS-inducing Noise

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The ATF signaling pathway has known roles in regulating stress response and cell survival within a variety of tissue types. In a recently published paper from our laboratory, we identified the ATF signaling pathway as uniquely upregulated in the type 1A spiral ganglion neurons (SGNs) following traumatic noise exposure. Here we explored the spatiotemporal expression of the ATF transcription factors and some of their canonical target genes in the spiral ganglion, organ of Corti, and lateral wall in response to PTS-inducing noise.

Male and female B6CBAF1/J mice were divided into sham-exposed and noise-exposed groups at 10 weeks of age. Noise trauma was induced with an 8-16kHz octave band of noise at 105 dB SPL administered for 2 hours, and cochleae from both groups were collected 6 hours, 24 hours, and 7 days following exposure. Using fluorescent in situ hybridization, changes in both localization and level of expression of Atf3, Atf4, Gadd45a, and Ddit3 were assessed and quantitatively compared.

Consistent with our single cell RNA-seq analysis of the spiral ganglion neurons, Atf3, Atf4, Gadd45a, and Ddit3 exhibited a slight increase in expression within the SGNs beginning 6 hours after noise, with robust upregulation occurring in the type 1A SGNs at the 24-hour time point before returning to baseline expression at 7 days. No changes in expression were seen within the sensory cells or supporting cells of the organ of Corti. Transient upregulation of all four Atf targets was observed within the spiral limbus and lateral wall 6 hours following noise.

Using transcriptomics and in situ hybridization, we have identified the ATF signaling pathway as a potentially significant contributor in the stress response against traumatic noise. These results underscore the importance of identifying key signaling regulators of cell survival for the development of targeted therapeutics to prevent and treat noise-induced hearing loss.
A Novel Mutation in the FerA Domain of Otoferlin Causes Age-Related Hearing Impairment

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The protein otoferlin is essential for hearing. Mutations in OTOF, the gene encoding otoferlin, lead to autosomal recessive profound prelingual deafness, DFNB9. The protein consists of six to seven C2 domains and a phospholipid binding FerA domain, the function of which is unclear to date. Here, we analyzed a mouse line with a mutation in the FerA domain. Our new mouse line Otof-p.KL>M has a three base pair deletion which leads to a replacement of the lysine 824 and leucine 825 by a methionine.

By performing ABR measurements, we could observe that homozygous Otof-p.KL>M mice exhibit an age-related hearing impairment. One and six month old Otof-p.KL>M mice showed no difference in their ABR responses compared to wild type mice, while at the age of 12 months, mutant mice revealed significantly higher ABR thresholds. At very low and high frequencies, the majority of mice exhibited no apparent auditory responses for stimuli up to 100 dB sound pressure level (SPL). DPOAE measurements were unaltered in homozygous Otof-p.KL>M mice compared to the wild type.

Immunohistochemical analyses of excised whole mount organ of Corti tissue revealed no loss of outer hair cells in Otof-p.KL>M mice compared to the wild type controls at any age. In contrast, in Otof-p.KL>M mice the number of inner hair cells decreased at the apical turn as well as at the basal turn of the organ of corti. The degeneration of inner hair cells started as early as at 1 month of age.

Taken together, our findings give us first insights into the importance of the otoferlin FerA during for auditory function, and in particular in the survival of inner hair cells.
Macrophage Activation in Normal Aging Cochlea: Evidence in C57BL/6 and CBA/CaJ Mice

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Macrophage function extends beyond innate immunity to include in homeostasis, clearance of cellular debris, tissue repair and remodeling. In this regard, the activity of resident or recruited macrophages is involved in age-related neurodegenerative diseases. Noble et al (2019), using immunofluorescence reported site-specific age-related changes in human cochlear macrophage morphology. In this study, macrophage density, morphology, and activation as a function of cochlear site were compared using confocal and electron microscopy in aging mice. CX3CR1YFP-CreER/wt:R26RFP (2-12 moa) mice on a C57BL/6 background to differentiate resident vs. recruited macrophages and CBA/CaJ (3-24 moa) mice were used. Both resident and recruited macrophages were present in all sites (OC, SGN, LW, SV) of the aging cochlea whereas young cochlea contained only resident macrophages. In the aging cochlea OC, both macrophage subtypes were found to be engulfing dying hair cells. Ultrastructurally, thin macrophages in the spiral laminaris, oriented parallel with nerve fibers, included few cytoplasmic elements and long filopodia at either end. Macrophages in Rosenthal’s canal were similar to those of the spiral laminaris or amoeboid in shape with short filopodia. In contrast SV macrophages in the aging cochlea localized to intrastrial space in close proximity to the vascular network. Confocal microscopy showed that macrophages were amoeboid-shaped with cytoplasmic dark, nonfluorescent areas and vacuoles. These regions via TEM were shown to contain phagocytosed debris including lipid droplets and compacted melanin granules. Our aging mouse data corroborates published aging human data. Combined confocal and electron microscopy establishes site-specific morphologic and cytoplasmic content diversity of macrophages in the aging mouse cochlea. These findings imply that macrophage function could be specialized by cochlear location. Future work will determine the diversity in macrophages at a single cell ‘omics’ level and study the precise role of these cells in the aging cochlea via depletion & repopulation of macrophages.
Characterization of Prestin Knock-In Mouse Models Carrying Deafness-Associated Missense Variants

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SLC26A5, also known as prestin, mediates electromotility of cochlear outer hair cells (OHCs). Although prestin is known to be essential for mammalian hearing, only a limited number of deafness associated SLC26A5 variants have been reported, including p.A100T (c.298G>A) and p.P119S (c.355C>T). Our previous in vitro examination revealed that these missense changes negatively affected prestin function. In this study, we investigated these prestin variants in vivo to understand their pathological roles in hearing loss.

We generated A100T- and P119S-prestin knock-in (KI) mouse models in the FVB/NJ background using a standard CRISPR/Cas9 technique at Northwestern University’s Transgenic and Targeted Mutagenesis Laboratory. After several rounds of backcrossing to wild-type (WT) mice, we measured distortion product otoacoustic emissions (DPOAEs) of littermates from heterozygous breeding pairs to evaluate their hearing at f2 frequencies ranging from 2 to 47 kHz. Auditory brainstem responses (ABRs) were also acquired. Both A100T- and P119S-prestin homozygous mice exhibited significantly elevated ABR and DPOAE thresholds across frequency by ~5 weeks of age, while their heterozygous littermates did not. Electrophysiological measurements on OHCs isolated from the KI animals recapitulated the impaired electromotility observed in our previous in vitro study for both A100T and P119S homozygotes. Immunofluorescence with a custom prestin antibody (Zheng et al., 2005) confirmed lateral membrane localization of the two variants. Interestingly, in A100T homozygous mice, OHC loss was limited only to the basal region of the cochleae while in P119S homozygous mice it was more widespread, indicating distinct pathogenic effects of the two variants.

Our results demonstrate that both A100T and P119S missense variants are indeed disease-causing alleles of SLC26A5. Further examination is needed to elucidate how these prestin variants differentially affect OHC health to gain insights into possibilities for mitigation.

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Immediate Effects of Noise Exposure to the Actin Structures Supporting Stereocilia Bundles in Mammalian Auditory Hair Cells

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During sound stimulation, each stereocilium of the auditory hair cell bundle pivots around its base, where actin core of stereocilium becomes denser and forms a rootlet inserting the stereocilium into the cuticular plate. It is believed that actin-based cuticular plate provides a stable mechanical support for stereocilia deflections, while rootles are responsible for pivotal flexibility and life-long resilience of stereocilia to mechanical stimuli. Not surprisingly, classical studies identified damage to the stereocilia bundles and rootlet displacements as hallmarks of the permanent noise-induced hearing loss (NIHL) (Liberman, 1987). Yet, despite decades of NIHL studies, it is still unknown whether these ultrastructural changes in stereocilia rootlets occur immediately after acoustic trauma or resulted from secondary reorganization of the cytoskeleton.

Previous data from our lab showed that fluid-jet overstimulation of the stereocilia bundles in-vitro may cause rotation of the rootlets within the cuticular plate minutes after overstimulation. To determine whether a similar phenomenon occurs in-vivo, we exposed adult CD-1 mice to broadband noise (120 dB SPL for 1 hour) that produces permanent NIHL. Then, unexposed control and noise-treated organs of Corti were examined with super-resolution confocal microscopy and serial sectioning with focused ion-beam (FIB) and backscatter scanning electron microscopy (FIB-SEM). Although Alexa Fluor 488 phalloidin did not label rootlets in most of the samples, rootlet insertions were clearly seen as prominent label-free holes in the cuticular plates. We quantified the distances between these insertions and, so far, did not find significant changes after noise exposure. However, preliminary FIB-SEM data revealed noise-induced changes in the actin density at the bottom of cuticular plates. In addition, noise damaged hair cells exhibited cytocauds – abnormal F-actin bundles extending from the cuticular plate into the cell body. We hypothesize that noise-induced depolymerization of cuticular plate actin may facilitate rootlet rotation and cause formation of cytocauds.

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Actin-based stereocilia assemble into rows of precisely graded heights, and the preservation of this architecture is essential for mechanoelectrical transduction (MET). Mutations in MYO15A, encoding the molecular motor myosin 15, disrupt the hair bundle and cause human hereditary hearing loss, DFNB3. Alternative splicing creates multiple MYO15A isoforms with different functions in the hair bundle. A long isoform (MYO15A-1, aka MYO15A-L) postnatally maintains the size of shorter row stereocilia with active MET. A short isoform (MYO15A-2, aka MYO15A-S) controls stereocilia growth during developmental trafficking of the elongation complex (EC), WHRN, EPS8, GNAI3, GPSM2, in addition to potentially stimulating actin polymerization directly. We recently identified a new isoform (MYO15A-3) with an alternative transcription start site encoding a novel 50-residue N-terminal domain. Here, we investigate the expression of MYO15A-3 in cochlear hair cells and examine if it traffics the EC, like MYO15A-2.

We measured expression of *Myo15a* mRNA by qPCR through key stages of hair bundle development and maturation. *Myo15a*-3 expression was upregulated during postnatal development in C57B6/J cochleae, increasing significantly by P60. To test our hypothesis that MYO15A-3 can traffic the EC *in vitro*, we examined the colocalization of EGFP-MYO15A-3 and EPS8 in HeLa cell filopodia. The relative intensity of endogenous EPS8 labeling at filopodia tips was significantly increased in cells expressing EGFP-MYO15A-3 or EGFP-MYO15A-2. As a negative control, EGFP-MYO10 did not enhance EPS8 accumulation at filopodia tips. To test if EPS8 is trafficked by MYO15A-3 *in vivo*, we generated an isoform specific *Myo15a*-Δ3 null allele in mouse using CRISPR-Cas9. EPS8 antibody labeling was absent from the stereocilia tips of mutant *Myo15a*(Δ3/Δ3) hair cells compared to control *Myo15a*(+/Δ3) samples that had robust labeling at P30. Our results suggest that MYO15A-3 traffics EPS8 in adult stereocilia, and that these proteins may be involved in maintaining stereocilia architecture.

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GluA3 Subunits are Required for the Appropriate Assembly of AMPA Receptors at Cochlear Afferent Synapses and for Presynaptic Modiolar-Pillar Features

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Hearing depends on the activation of AMPA-type glutamate receptors (AMPARs) on the post-synaptic terminals of auditory nerve fibers. Noise-induced cochlear synaptopathy is caused by excitotoxic overactivation of those receptors; antagonizing the calcium-permeable subset of AMPARs (CP-AMPARs) pharmacologically can prevent this synaptopathy. Cochlear AMPARs are tetrameric heteromers comprised of the pore-forming subunits GluA2, 3, and 4 where absence of GluA2 results in a CP-AMPAR channel with increased permeability to Ca2+ and Na+. AMPAR tetramers form as dimers of dimers, with the GluA2/3 dimer being energetically favored and prominent. In the absence of GluA3, GluA2/4 would be the only heterodimer. We used global Gria3KO mice to ask what happens to cochlear ribbon synapses in the absence of subunit GluA3. Male WT and KO mice were compared at 5 weeks of age following normal rearing in an animal facility with ambient noise. ABR thresholds and wave-I amplitudes of Gria3KO mice were similar to male WT, as were the numbers of paired and unpaired synaptic puncta. However, synaptic molecular anatomy and ultrastructure were altered. Analysis of confocal images showed that ribbon synapses of Gria3KO mice had smaller AMPAR arrays that contained less GluA2 and more GluA4 relative to WT. Changes to the GluA4:GluA2 ratio in Gria3KO were greater for synapses on the pillar side of the inner hair cell (IHC). Ultrastructurally, the IHC modiolar-pillar differences in presynaptic ribbon size, ribbon shape, and vesicle size seen in WT were diminished or reversed in Gria3KO. Our results show that loss of GluA3 may increase the number of GluA2-lacking CP-AMPARs at cochlear ribbon synapses. Additionally, changes to the presynaptic ribbon suggest transsynaptic developmental effects. We hypothesize these molecular-anatomical alterations to AMPAR subunits result in synapses with increased vulnerability to AMPAR-mediated excitotoxicity that may lead to synapse loss and hearing loss as the mice age.
Session 8
Hair Cell Damage, Repair, and Regeneration
The Effect of a Viral-Induced Inflammation Model on Cochlear Uptake of Aminoglycosides

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The effect of virogenic inflammation on drug induced hearing loss is unknown. This study tests whether a TLR7 agonist (gardiquimod) induces COVID-19-like inflammation and modulates cochlear and serum levels of aminoglycosides. We also tested different gardiquimod doses to compare their efficacy in inducing inflammatory responses. We also tested different doses of gardiquimod to determine if increasing doses increase cochlear uptake of gentamicin, an ototoxic aminoglycoside, and a specific dose that does not elevate serum levels of gentamicin.

C57BL/6 mice received DPBS (control) or gardiquimod (1-20 mg/kg) through subcutaneous injection (N≥4 per group). Three and 24 hours later, blood and cochlear tissues were collected to obtain cytokine expression levels via qRT-PCR or Luminex multiplex ELISA assays, including: IFNα, IFNβ, IFNγ, MCP1, MIP1α, NFκB, TNFα, IP10, IL1α, IL1β, IL2, IL6, IL10, IL12α, IL12β. To determine the effect of gardiquimod-induced inflammation on cochlear and serum concentrations of gentamicin, C57BL/6 mice received DPBS (control) or gardiquimod (up to 20 mg/kg; N=6 per group), and 24 hours later, mice received gentamicin for 1 hour (i.p.). Blood and cochlear samples were then collected to evaluate gentamicin levels using an ELISA kit.

Gardiquimod induced dose-dependent inflammatory responses, with increased serum and cochlear levels of cytokines associated with clinical COVID-19 infections, e.g., IP10, MCP1, MIP1α, IL6, TNFα, compared to DPBS groups. Inflammatory responses at 24 hours were reduced compared to 3 hours after treatment. Gardiquimod dose-dependently increased cochlear levels of gentamicin, without modulating serum levels of gentamicin.

Our data show that gardiquimod induced a robust inflammatory response that mimics viral infection-induced inflammation in humans. Furthermore, a wide range of gardiquimod doses increased cochlear, but not serum, levels of gentamicin. Thus, activated TLR7 signaling could increase the risk of ototoxin-induced hearing loss.
Session 9
Deafness: from Gene to Therapeutics
Genetic Etiology of Hereditary Hearing Loss in the Gulf Cooperation Council Countries

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Hearing loss (HL) is the most prevalent sensory disorder worldwide, with approximately 1 affected in 500 newborns. The last 30 years have seen exponential growth in identifying genes and variants responsible for hereditary hearing loss (HL) worldwide. This has led to a significant increase in understanding of hearing and deafness molecular mechanisms, which has improved diagnoses for populations with hereditary HL. However, many communities worldwide, especially in the Gulf Cooperation Council countries (GCC), have a high prevalence of consanguineous marriages. Congenital monogenic conditions, such as recessive HL, are more common in these populations. Many studies have shown that high consanguinity, endogamy and first cousin marriages were observed in the six countries of GCC. An extant literature review found that genes and variants were responsible for HL in 138 family cases within which 89 recessive DNA pathogenic variants were revealed. In addition, 21 genes responsible for non-syndromic hearing loss (NSHL) and 17 genes responsible for syndromic hearing loss (SHL) have been reported in cases from the GCC region. Specifically, this data indicates that in the Gulf Cooperation Council, 72% of HL forms are non-syndromic and 28% are syndromic. Individuals with NSHL had 66% of variants in four genes (GJB2, OTOF, TMC1 and CDH23), with most variants occurring in the GJB2 gene (37.5%). In comparison, the Usher syndrome has been more common among SHLs, as observed in 41% of the syndromic GCC cases.

In conclusion, this study revealed that one factor contributing to the diagnosis of HL in the GCC was the utilization of next-generation sequencing (NGS)-based diagnostic techniques and genetic testing, with approximately 58% of those variants being identified using this technology.

Key word: Hearing loss, syndromic, non-syndromic, genes, NGS.
AAV-mediated Clarin-2 Gene Therapy prevents Progressive Hearing Loss in Mice

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We still know little about the key pathways to post-natal progressive hearing/balance impairments. Clarin-1 is a tetraspan-like glycoprotein involved in post-lingual HL and variable vestibular and vision deficits in humans. Clarin-2 is also essential for hearing in zebrafish, mice and humans. Our findings support key evolutionarily conserved inner ear role(s) of these clarins. Here, we further characterize the function of clarin-2 in HCs, and evaluate gene therapy in clarin-2 knockout mice.

We used the Clrn2clarinet deficient mouse. Confocal imaging was used to monitor hair bundle markers and synaptic substructures, and SEM for bundle architecture. ABRs, DPOAEs, plus MET and capacitance measurements assessed hearing sensitivity, hair cell MET and synaptic exocytosis. Vestibular-evoked potentials (VsEPs) and vestibulo-ocular responses (VORs), and balance behavioral assays documented vestibular function. For gene supplementation therapy, mice were injected at P0-P1 through the RWM using AAV9-PHP.B-Clrn2.

In addition to contributing to MET and bundle integrity, clarin-2 is required for auditory IHC synapses. Capacitance measurements in clarin-2 knockouts reveal defective calcium currents and decreased IHC exocytosis. VsEPs amplitudes are decreased, remaining stable up to P90. However, follow-ups show normal bundle structure and function, and no overt balance deficit. Importantly, all clarin-2-mediated deficits are prevented by AAV9-Clrn2 gene supplementation. Re-expression of clarin-2 prevented MET current decrease and loss of the ‘transducing’ short row stereocilia. Furthermore, ABRs, DPOAEs, MET, SEM and capacitance recordings confirm restoration of hearing and normal transduction currents, hair bundles, calcium currents and IHC synaptic exocytosis.

Our study reveals an unrecognized role of clarin-2 in IHC synaptic function. In spite of decreased VsEPs from P30, no overt balance deficit was observed regardless of age. Unlike in Clrn1−/− knockout mice, the post-natal progressive HL in Clrn2clarinet can be durably prevented. Our findings support the applicability of gene supplementation for progressive HL, a disorder with a post-natal intervention window.
Metformin, a Candidate Treatment for Noise-Induced Hearing loss, Exhibits Sex-Dependent Efficacy

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Our laboratory recently profiled cell type-specific transcriptional changes in the mouse cochlea following permanent threshold shift (PTS)-inducing noise exposure. By intersecting the list of dysregulated genes with the DrugCentral database, we identified candidate therapeutics to prevent noise-induced hearing loss (NIHL). The top-ranking candidate was the FDA-approved antidiabetic drug, metformin. To date, there exists no comprehensive evaluation of the effects of metformin treatment on protection from PTS-inducing noise in animals of both biological sexes. Here, we examine the physiological and histological outcomes of PTS-inducing noise exposure in mice of both sexes, with and without metformin treatment.

We utilized intact male, intact female, and ovariectomized (OVX) female B6CBAF1/J mice in this study (bilateral ovariectomy performed at 8-weeks of age). Baseline auditory brainstem response (ABR) thresholds were established at 9-weeks of age. Following baseline testing, mice were administered metformin (2 mg/kg/day) or a vehicle (saline) in their drinking water for the remaining duration of the study. At 10-weeks of age, mice were exposed to a PTS-inducing noise (102.5 or 105 dB SPL, 8-16 kHz, 2h). Auditory thresholds were determined via the ABR 24-hours (compound threshold shift or CTS) and 1-week post-exposure (PTS). Following the 1-week ABR, the mice were euthanized, and cochlear tissue was collected for histological analysis.

Our data demonstrate that metformin treatment reduces the CTS and PTS in male mice but not in intact female mice. Furthermore, metformin treatment ameliorates outer hair cell loss in male mice. This study is the first, to our knowledge, that uses physiological and histological approaches to evaluate the FDA-approved drug, metformin, as a candidate treatment for NIHL in mice of both sexes. Results from the OVX female mice will be reported in the meeting.
Rescue of Auditory Function in a Mouse Model of Human Recessive Deafness with a Single Administration of AAV Gene Therapy

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Patients with mutations in the TMPRSS3 gene suffer from recessive deafness as the result of deficits in hair cells and auditory ganglion neurons for which cochlear implantation (CI) is the only treatment option. However, in some patients poor CI outcomes are seen potentially due to the involvement of the spiral ganglion. To develop sustained biological treatment for these patients, we generated a knock-in (KI) mouse model with a frequent human TMPRSS3 mutation (c.916G->A). The Tmprss3 mice displayed late onset progressive hearing loss similar to human DFNB8 patients. We used AAV2 to carry a human TMPRSS3 gene to inject into adult (13 month of age) KI mice inner ears. We showed that AAV2-TMPRSS3 delivery resulted in the re-expression of TMPRSS3 gene in the auditory hair cells and the modiolus region. AAV2-TMPRSS3 injection rescued auditory function in the KI mouse model to a level of the wildtype mice of the same age, with hearing being maintained with a single administration. Inner ear study showed both hair cells and spiral ganglions were rescued as the result of AAV2-TMPRSS3 delivery in the KI mouse. This proof of principle study demonstrates that gene therapy could be successfully implemented at a late-stage in life, using a mouse model of human genetic deafness. It further indicates that a single administration of AAV gene therapy could achieve noticeable restoration of hearing and long-term therapeutic effect. Our study strongly supports the development of AAV2-TMPRSS3 gene delivery as a standalone therapy or in combination with CI to treat DFNB8 patients.
Targeted Genome Editing in Adult Cochlea Hair Cells Rescues Non-syndromic Progressive Hearing Loss in the MicroRNA-96\textsuperscript{Dmdo/+} Mouse Model

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MicroRNA MiR-96 is involved in the differentiation of the auditory hair cells and is required for hearing. Heterozygous point mutations in the seed region of MiR-96 result in dominant late onset progressive hearing loss in humans, which offers an opportunity for therapeutic intervention in adult patients. Genome editing has been proven to be efficient to treat genetic hearing loss in neonatal mice. It is yet to be determined if it’s sufficient to target MiR-96 in adult inner ear to rescue hearing. We performed study using editing to disrupt a MiR96 dominant mutation Diminuendo (Dmdo) in adult mice with progressive hearing loss. Due to the small size of MiR96 and limited gRNA sequence availability, we screened gRNAs for 5 CRISPR nucleases and the gRNAs for KKH-saCas9 and SpCas9 yielded the highest editing efficiency. Considering that KKH-Cas9-gRNA can be inserted into one AAV whereas the SpCas9/gRNA will require two AAVs, we packaged the KKH-saCas9-gRNA-Dmdo into AAV2, which targets inner and outer hair cells efficiently. AAV2-KKH-Cas9-gRNA was injected into adult (6-wks) MiR-96\textsuperscript{Dmdo/+} cochlea through the round window membrane with semi-circular.

Hearing test showed in the injected ears, significantly lower ABR and DPOAE. Thresholds were detected across most of the frequencies. Six months after the injection, the hearing rescue effect was even greater than one month post injection, due to the continuous deterioration of hearing in the uninjected ears whereas hearing was robustly maintained in the injected inner ears. This is the first time that gene editing is used to target a MicroRNA mutation to robustly rescue hearing in adult mice in a sustained manner. As mouse Dmdo mutation is identical to the human mutation, as well as a 100% identity between mouse and human MiR96 sequences, our approach including the gRNA design lays the foundation to develop treatment for human MiR96 hearing loss.
Dual-AAV Gene Therapy for Usher Syndrome Type 1F Deafness and Blindness

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Usher syndrome type 1F, a recessive deafness and blindness, is caused by mutations in the *PCDH15* gene, which encodes the tip-link protein PCDH15. Gene addition therapy could be an attractive treatment, however the PCDH15 coding sequence of ~5.8 kb is too large to fit into a single AAV capsid. We used a dual-AAV strategy to circumvent the size limitation and to treat the deafness in an Usher 1F mouse model.

We engineered two vectors that each encode half of PCDH15 carrying an N-terminal HA tag. *Pcdh15* conditional knockout mice were injected with dual AAVs through the round window membrane at P1. Histological analyses and hearing tests were performed at P30. Hair cells in treated mice displayed HA immunoreactivity at the tips of stereocilia, and robust rescue of hair bundle morphology as observed by actin labeling and SEM. Rescue of mechanotransduction was confirmed with FM1-43 loading at P30. We tested hearing at P30 with ABR recording: while uninjected *Pcdh15* conditional knockout mice were deaf, mice treated with dual AAVs demonstrated good hearing rescue at low and middle frequencies. To test vestibular function we used constitutive knockout mice and treated them with dual AAVs encoding PCDH15. Uninjected control *Pcdh15* null mice showed a severe vestibular phenotype: head bobbing, circling behavior, hyperactivity, swimming difficulties and inability to stay on a rotarod, but treated mice performed at wild-type levels.

To assess the potential of dual AAVs in human retina, we used retinal organoids made from human iPSCs in culture. SEM showed that a majority of photoreceptors developed inner segments, while some formed outer segments and connecting cilia. We observed nascent calycal processes at the apical ends of inner segments. Five weeks after dual-AAV delivery, retinal organoids showed anti-HA labeling in photoreceptors, which was localized on the surface of the inner segments and at the inner/outer-segment junction where calyceal processes develop.

Rescue of Hearing by Adenine Base Editing in a Humanized Mouse Model of Usher Syndrome Type 1F

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Mutations in the tip-link protein protocadherin-15 (PCDH15) cause Usher syndrome 1F, characterized by congenital deafness, lack of balance and degenerative blindness. Within the Ashkenazi Jewish population, many Usher 1F cases result from the R245X mutation—a single C>T transition which converts the R245 codon to a stop codon.

Working towards a treatment for Usher 1F, we explored the potential for adenine base editors (ABEs) to revert the C>T mutation by converting A>G on the complementary strand. We first created a humanized mouse model in which 60 bases of the mouse gene in this region were replaced with the human sequence bearing the R245X mutation. With this model, editors and guide RNAs could be tested in mouse but optimized for human sequence. Mice homozygous for the R245X allele were deaf and exhibited profound balance deficits, while heterozygous mice were unaffected.

We first tested different ABEs and gRNAs in HEK293 cells carrying the R245X mutation, and assessed efficacy with sequencing. We then packaged the best ABEs, divided into two parts, into dual AAV9-PHP.B vectors. These were injected into cochleas at P1, and recombination of the ABE protein within hair cells was mediated by intein joining. In homozygous Pcdh15-R245X null mice, we observed base editing but not hearing rescue, most likely because cochlear hair bundles were disorganized from birth. We then tested rescue in mice bearing one R245X allele and one conditional knockout allele, with Cre expression delayed to allow normal bundle development. In these, we observed moderate hearing rescue after dual-vector delivery of the split-intein ABE. This study introduces a relevant Usher 1F model for studying the common R245X mutation, and demonstrates the ability of an adenine base editor to correct the mutation within the cochlea.
Exome Sequencing Provides Effective Genetic Diagnosis for Unilateral and Asymmetric Pediatric Hearing Loss

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Genetic testing is the current standard of care for evaluation of bilateral symmetric pediatric sensorineural hearing loss (SNHL). Current algorithms generally do not recommend genetic evaluation as an initial test for asymmetric and unilateral SNHL because prior studies showed low diagnostic rates. However, these studies did not focus on children, routinely include trios, use exome sequencing (ES), or have a large sample size. Our goal was to investigate the efficacy of ES in pediatric patients with unilateral and asymmetric SNHL compared to bilateral symmetric SNHL.

ES was performed from buccal-derived DNA for pediatric patients with confirmed SNHL without a known genetic or environmental etiology and their biological relatives (typically trios) as part of a large cohort study. ES mapping and variant calling (including copy number variants) was performed with the DRAGEN pipeline. Primary variant analysis focused on 366 known and candidate deafness-causing genes.

ES was performed for 218 probands and 333 relatives (551 participants). The cohort was clinically heterogeneous, with various lateralities, severities, configurations, and onsets of SNHL. A genetic cause of SNHL was identified for 31.2% of probands (n=68) with causative variants in 37 genes. The overall genetic diagnostic rate was 40.7% for bilateral, 23.1% for asymmetric, and 18.0% for unilateral, with syndromic diagnoses made in 20.8%, 33.3%, and 54.5% of cases in each group, respectively. There were more variants of unknown significance in the asymmetric and unilateral groups (66.7% and 70.6%, respectively), compared to bilateral symmetric SNHL (32.3%).

We identified a genetic cause of SNHL in a significant percentage of pediatric patients with asymmetric and unilateral SNHL. Syndromic SNHL was more common in these cases. Increased access to genetic testing for patients with all SNHL phenotypes will provide valuable prognostic information and facilitate timely access to appropriate habilitation and therapies.
Session 10
Precision and Translation
Medicine for Deafness
Comprehensive Newborn Hearing Screening through SEQaBOO
(SEQuencing a Baby for an Optimal Outcome)

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Early genetic screening in congenitally deaf and hard of hearing (DHH) newborns or in non-penetrant (at birth) DHH infants is an integral component of comprehensive newborn hearing screening (NBHS). Compelling data indicate the union of genetic testing and physiologic screening improves clinical outcomes and quality of life for DHH individuals. SEQaBOO (SEQuencing a Baby for an Optimal Outcome) is a platform for assessing DHH newborns and evaluating parental attitudes concerning genomic testing. Parents of newborns referred following a positive NBHS or at confirmatory diagnostic audiometry may enroll for SEQaBOO (comprehensive genomic sequencing including ACMG v3.0 secondary findings, plus annual surveys) or annual surveys only. Sequencing data and interpretation are possible prior to diagnostic audiometry and can influence follow-up care.

Comprehensive NBHS through SEQaBOO includes genome sequencing and variant interpretation of DHH genes, cCMV analysis, and genome wide CNV analysis in addition to state mandated physiologic screening. This platform has identified chromosomal aneuploidy (n=1), absence of heterozygosity (n=1) and chromosomal structural rearrangements (n=4). Over 70% of families approached have enrolled with >50% choosing genome sequencing. Among SEQaBOO babies ultimately diagnosed as DHH, a genetic etiology of \( \text{GJB2} \) and \( \text{SLC26A4} \) variants was reported (n=6). Genetic diagnoses were inconclusive (n=13) with most having only one pathogenic/likely pathogenic variant in a recessive gene or a VUS in a gene associated with dominant hearing loss. Three ACMG secondary findings were disclosed (\( \text{COL3A1} \), \( \text{NF2} \) and \( \text{PKP2} \)) and followed up with primary care physicians.

Annual surveys aim to gather data on family medical history, health information and evolving attitudes on genomic testing. Feedback is positive with 61.5% of parents acknowledging the benefits of receiving genome sequencing results on themselves, 67.6% acknowledging benefits to both themselves and their child and 62.6% acknowledging that the genetic sequencing results have helped them understand the cause(s) of their child’s DHH.
CRISPR-Cas9 Mediated Targeting of the Dominant Coch p.A449T Pathogenic Variant in a Patient-derived Skin Fibroblast Cell Line and in a New Humanized Mouse Model

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Dominant gain-of-function mutations in COCH cause DFNA9, a progressive Mendelian sensorineural hearing loss (HL) and vestibular disorder. Of interest, a recent analysis of common and rare genetic variation associated with adult hearing loss (medRxiv doi.org/10.1101/2021.09.27.21264091) revealed a genome-wide significant single-variant association of large effect size in COCH (p.C542F, OR=81.4). Individuals with the p.A449T variant, also located in the vWFA domain, display an early-onset hearing loss, typically in the first to second decades of life, compared with individuals with variants in the more upstream LCCL domain. Specific disruption of p.A449T using CRISPR-Cas9 strategies, could keep the normal allele intact and might rescue hearing and balance. We generated a fibroblast cell line using a skin biopsy from an individual with the p.A449T mutation. Through transfection with a plasmid carrying SaCas9-KKH and COCH guide-RNA expression plasmid, we successfully created indels disrupting only the p.A449T mutated allele with an editing efficiency of 43%.

We generated a humanized mouse model in CBA/CaJ harboring the p.A449T mutation and flanking human sequence. Auditory Brainstem Responses (ABRs) as well as Distortion Product Otoacoustic Emissions (DPOAE) analyses of 3-4 months-old mice have shown significantly elevated thresholds at all tested frequencies for both CochA449T/+ (heterozygous) and CochA449T/A449T (homozygous) mice, as compared to their Coch+/+ (wild-type) littermates. Vestibular sensory evoked potentials (VsEP) were measured and thresholds were significantly elevated for heterozygous and homozygous mice compared to wild-type littermate controls. Having the appropriate animal model, we are currently poised to perform in vivo studies, including round window micro-injection of an AAV encoding CRISPR-Cas9 and guide RNA, to assess phenotypic rescue. If successful, this strategy is expected to inform gene therapy in other autosomal dominant HL disorders, the majority of which are due to missense pathogenic variants rather than truncation or loss of gene function.
Mutation Agnostic RNA Interference with Concomitant Engineered Gene Replacement Rescues Hearing in a Mature Murine Model of DFNA36

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Hearing loss is the most common sensory deficit, of which genetic etiologies are a frequent cause. Dominant and recessive mutations in TMC1, a gene encoding the pore-forming subunit of the hair cell mechano-transduction channel, cause DFNA36 and DFNB7/11 respectively, which combined account for approximately 2.3% of genetic hearing loss. Previous work has established the efficacy of mutation-targeted RNA interference (RNAi) in the treatment of murine models of autosomal dominant nonsyndromic hearing loss, including DFNA36. However, the clinical application of such approaches is limited by the infeasibility of development and validation of novel constructs for each deafness-causing variant. To address this problem, we developed a mutation agnostic approach that consists of RNAi suppression of both mutant and wild-type alleles with concomitant delivery of an engineered, RNAi-resistant wild-type allele. This approach was investigated in the treatment of a murine model of DFNA36 and achieved robust hair cell, auditory brainstem response, and cochlear amplification preservation. These data suggest that this strategy may be broadly applicable to autosomal dominant nonsyndromic hearing loss.
Session 11
Emerging Themes
and Methods
Auditory Central Gain and Neural Synchrony in Aging Mice and Humans

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The auditory nerve (AN) degrades with age, leading to decreased afferent input, poorer neural synchrony, and auditory processing deficits. Decreased neural synchrony may underlie the difficulties in speech understanding that begin presenting in middle age. The auditory midbrain compensates for a loss of peripheral AN activity via amplification (increased central gain), but synchrony may not recover. We hypothesized that aging would be associated with decreased afferent input, increased central gain, and decreased neural synchrony, and that these effects would be increasingly prevalent with advancing age.

AN and midbrain function were assessed via electrophysiologic responses (CAP and ABR). Measurements of amplitude and neural synchrony in the AN and midbrain were compared across younger, middle-aged, and older groups of mice (CBA/CaJ) and human participants.

In both mice and humans, results were consistent with increased central gain, with larger age-related amplitude reduction observed at the level of the AN than at the midbrain. In contrast, measures of neural synchrony were significantly lower in both the auditory nerves and midbrains of older mice and humans, compared to younger groups. Early evidence suggests that changes in neural synchrony and central gain begin manifesting in middle age, but analysis is ongoing.

These translational findings demonstrate that age-related peripheral neural degeneration contributes to central gain, largely preserving midbrain potentials, but central gain fails to recover losses in neural synchrony. Persistent deficits in neural synchrony may contribute to auditory processing deficits observed in older mice and humans. Furthermore, we can use this translational model to further investigate the specific sites of pathology and underlying mechanisms, including decreased inhibitory control, that contribute to functional deficits in the aging auditory system.
A Comparative Hearing Model to see if Age Related Hearing Loss has a Metabolic Basis

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A leading cause of age-related hearing loss is hypothesised to be a decrease in metabolism. Metabolic decline is a conserved feature in many different systems as animals get older, however there is a lack of research measuring metabolism in an auditory organ over a lifespan. Using Müller's organ of the Dessert Locust (Schistocerca gregaria), we quantified metabolism as the ear ages. We also measured sound-evoked and baseline activity directly from the auditory nerve in vivo and from individual auditory neurons ex vivo as a function of age.

To test the hypothesis that metabolic decline is responsible for age-related hearing loss, we slowed metabolism with a decrease in rearing temperature and then measured their auditory function against locusts reared in standard (desert) temperature. Finally, we quantified expression of key metabolic genes within the locust ear (RNA Seq.) as a function of age and compare against mammalian ear transcriptomic datasets.
Complex Phenotypes; Complex Genetics; Complex Approaches
Combination of Hearing Loss and Retinitis Pigmentosa is Not Always an Usher Syndrome

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Usher syndrome (USH) is a genetic disorder, with autosomal recessive inheritance, characterized by retinitis pigmentosa (RP), congenital sensorineural hearing loss and in some cases, vestibular dysfunction. It is the most common cause of deafblindness in humans. There are some cases that, despite presenting RP associated with deafness, cannot be classified as USH. Here we report the phenotypic and genotypic characterization of an individual and her family with RP and hearing loss, initially diagnosed with Usher syndrome.

A clinical evaluation was carried out in the affected individual, and her family. WES was performed to the propositus.

A total of 5 individuals were studied. The propositus, initially diagnosed with Usher syndrome type I, 3 siblings with isolated RP and a healthy cousin. The studied individuals were clinically and genetically characterized. The propositus presented congenital profound sensorineural hearing loss and RP starting at 17 years old. From the variant analysis were identified two, one homozygous pathogenic variant in GJB2 gene, p.S199F and homozygous likely pathogenic variant in EYS gene, c.6816_6817del.

Clinical and genetic differential diagnoses is important when hearing loss and RP are both present in a patient. Although not always possible, a careful assessment of the medical and family history is important. WES analysis is essential in the diagnostic definition in these cases.
Successful development of therapeutics for hearing and balance disorders depends on detailed knowledge of the transcriptional regulatory networks involved in hair cell (HC) development and function. Our laboratory previously identified a striking over-representation of the RFX transcription factors (TFs) binding motif in the promoters of HC expressed genes, suggestive of an important role for these ciliogenic TFs in HC development. The RFX TFs are divided into groups based on their functional domains, where group 1 consists of RFX1, RFX2, and RFX3. We subsequently showed that conditional deletion of Rfx1 and Rfx3 together (Rfx1/3) from HCs results in an abrupt loss of outer hair cells shortly after the onset of hearing, profound hearing loss, and a mild, late onset vestibular phenotype. However, the Rfx1/3 conditional knock out (cKO) mutants did not exhibit kinocilia or planar cell polarity defects.

With significant homology in functional domains, similar role in ciliogenesis, and expression in cochlear and vestibular HCs, we hypothesize that RFX2 compensates for the loss of RFX1/3 in inner ear HCs.

Using an Rfx2 knock out mouse model (Rfx2Gt), we established that Rfx2 is expressed in cochlear HCs from postnatal day (P)1-P7 and vestibular HCs from P1 - adulthood. However, Auditory Brainstem Response (ABR) and Vestibular Sensory Evoked Potential (VsEP) thresholds of Rfx2Gt mutant mice were not significantly elevated compared to control littermates. To assess the compensatory role of RFX2 in the vestibular system, we created a triple conditional knockout mouse, Rfx1/2/3 cKO, and measured VsEPs and assessed vestibular stereociliary morphology. Loss of Rfx1/2/3 together causes significantly elevated VsEP thresholds as early as 1-month-old and abnormal kinocilia at 6-months-old. RFX2 alone does not have an overt functional role in the auditory or vestibular system but has a compensatory role for RFX1/3, revealing the essential role of group 1 RFX TFs in vestibular function.
ATP1A3 Gene is Responsible for Isolated and Syndromic Auditory Neuropathy (CAPOS Syndrome)

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CAPOS syndrome combines progressive hearing loss (auditory neuropathy type, (AN)), optic atrophy, hypotonia, and cerebellar ataxia. The disorder is described as appearing in childhood, with acute episodes of febrile neurological deterioration resembling encephalitis. We conducted a cohort study of 39 families (43 patients) with isolated (73%) or syndromic (27%) AN without cochlear nerve malformation. Their DNA was analyzed by Next Generation Sequencing using a panel of 216 genes involved in isolated or syndromic deafness. Four unrelated patients had the same heterozygous pathogenic variant of the ATP1A3 gene, c.2452G>A, p.(Glu818Lys), already reported as responsible for CAPOS syndrome (OMIM-601338). The diagnosis of the hearing loss was made in post-lingual period from 5 to 12 years old. The deafness progressively worsened with very low word recognition (10%) despite a classical hearing aid. A single or bilateral cochlear implantation allowed recovering a word recognition score close to 100% (up to 12 years post-implant). Two patients have never had any of the febrile episodes classically described. Optic nerve damage was not present in two patients, one of whom was 16 years old. The ataxia described in the CAPOS syndrome is attributed to cerebellar damage but the implication of a vestibular deficit was present in 2/3 of the patients tested. We have identified the ATP1A3 p.(Glu818Lys) variant in patients with isolated neuropathy with or without inaugural febrile episodes. Balance disorders could involve peripheral vestibular damage. Cohort studies should confirm efficacy in auditory perception in these patients.
ACTG1: a Spectrum ranging from Non-Syndromic Hearing Impairment to Polymalformative Fetal Presentations

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Pathogenic variants of \textit{ACTG1} have been reported for two distinct phenotypes: Autosomal dominant isolated deafness DFNA20/26 and Baraitser-Winter syndrome 2, that associates intellectual deficiency, ocular malformations, dysmorphism, epilepsy and cerebral malformations. Surprisingly, hearing impairment is seldom associated to Baraitser-Winter syndrome 2. There is a high prevalence of DFNA20/26 patients identified through gene panel sequencing presenting with isolated sensorineural hearing impairment of dominant transmission. DFNA20/26 usually presents as non-syndromic, progressive, postlingual, hearing impairment with an onset between the first and third decade. The objective is to better characterize the phenotypes associated with \textit{ACTG1} variants.

This is a retrospective study on a French cohort of 35 patients and two fetuses.

Most of the patients have a typical presentation of DFNA20/26. Three patients present with developmental delay and a recognizable dysmorphism with flat face and arched eyebrows. Four patients present with auditory neuropathy. In the two fetal cases we found corpus callosum and cerebral anomalies, associated to cardiac and skeletal malformations for one of them.

Conclusion: \textit{ACTG1}-associated phenotype is broader than currently described. We have identified extra-auditory symptoms and a recognizable dysmorphism in a number of patients.
Highlighted Abstracts
De Novo Mutations are a Common Cause of Genetic Hearing Loss

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De novo mutations (DNMs) are a well-recognized cause of genetic disorders. The contribution of DNMs to hearing loss (HL) is poorly characterized. We hypothesized that DNMs are an underappreciated etiology of HL, and leveraged our cohort of individuals with HL to assess their contribution.

Targeted genomic enrichment (TGE) and massively parallel sequencing (MPS) were used for genetic testing of all exons and flanking intronic sequences of known deafness-associated genes. Segregation analysis was performed in all families in which a potentially causative variant was detected in the proband. Familial testing results and systematic review of previous DNM reports in the Deafness Variation Database (DVD), PubMed and ClinVar were reviewed to characterize the rate, distribution, and spectrum of DNM.

DNMs were detected in 10% (24 of 238) of probands for whom testing of both parents was completed. DNMs were detected in 15 deafness-associated genes, including 9 novel variants in ACTG1 (n = 2), AIFM1, ATP2B2, CDH23, GATA3, MITF, MYO6, and NR2F1. DNMs in MITF were most common (21% of all DNMs), followed by GATA3 (13%), STRC (13%), and ACTG1 (8%). Review of novel and previously reported DNMs revealed a gene-specific de novo mutational spectrum and variability in the contribution of DNM to pathogenic variation.

DNMs are a common cause of genetic HL, and must be considered in all cases of sporadic HL. Gene-specific variability in the contribution of DNM to genetic HL necessitates the development of gene-specific criteria for interpretation of variants with de novo origin.
The Role of Ezrin and Radixin Proteins in “Blooming” of Nascent Stereocilia in Auditory Hair Cells

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Stereociliary development occurs in multiple steps. These have been well-characterised by Tilney et.al. in chick and Kaltenbach et.al. in golden hamster. Based on these studies, stereocilia formation starts an initial bloom of actin microvilli. These then elongate, staircase pattern is formed, and excess microvilli resorbed to give rise to a mature stereociliary bundle. In this study, using Scanning electron microscopy and super-resolution microscopy, I have characterised hair bundle development in the mouse organ of Corti from embryonic day (E) 14.5 to Postnatal (P) 12. These stages provide a comprehensive account of hair bundle initiation at E15.5 in IHCs, establishment of staircase patterning by P2 in Inner hair cells (IHCs) and Outer hair cells (OHCs), and resorption of extra microvilli by P11 in OHCs. To understand the initiation of the stereocilia, we examined the molecular mechanisms that may promote “microviliogenesis”. Using light microscopy, we examined the expression of ezrin and radixin. We find that they are expressed in the apical plasma membrane from E15.5 onwards with radixin and ezrin coexpressing in microvilli-like immature stereociliary bundle of IHCs. ERM (Ezrin, Radixin and Moesin) proteins have been found to link plasma membrane to actin filaments. We have perturbed ERM proteins using small molecule inhibitors in organotypic cultures of mouse organ of Corti and chick basilar papilla. We have used electron microscopy and super-resolution microscopy to observe the effects of these inhibitors. Following ezrin inhibitor treatment, we observed that there was a stage dependant perturbation in planar cell polarity of hair bundles. In contrast, radixin inhibitor treated explants showed specific morphological changes such as reduction in number and fusion of immature stereocilia at early embryonic stages of mouse and chick. Our findings suggest specific roles of the ERM proteins Ezrin and Radixin in initiation of immature stereocilia.
Reversal of Hearing Loss in Spns2 Mutant Mice

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Spinster homolog 2, Spns2, is a sphingosine-1-phosphate (S1P) transporter, and Spns2tm1a mutant mice were previously described by our group (Chen et al., 2014) showing a rapidly progressive hearing loss associated with a decline in endocochlear potential (EP). As EP appears to develop normally at first in mutants, we considered ways of restoring it to normal levels after the onset of hearing loss. In this study, we asked if progressive hearing loss can be reversed after it has developed as a proof-of-concept.

We used a genetic approach to initiate expression of the Spns2 gene, using tamoxifen injection to activate Flp recombinase which recognises FRT sites in the Spns2tm1a allele, leading to restoration of Spns2 gene activity. Tamoxifen was injected at 4 different ages (Postnatal day (P)14, P17, P21 and P28) and ABRs were recorded at intervals before and after injection up to 8 weeks old when the EP was also measured.

By comparing pre and post tamoxifen ABR thresholds in the same mouse, we observed that ABR thresholds are recovered at 6, 12 and 18 kHz in the injected mice at P14 and P17. Some improvement was found at 12kHz with injection as late as P28. EP levels at 8 weeks old were generally higher in mutants injected at younger ages than in those injected at P21 or P28, and lower 12 kHz ABR thresholds correlated with higher EP levels. Hair cell degeneration was observed in mice injected at P28, but not in mice injected at P14.

Overall, our results show that hearing loss due to the Spns2 mutation can be reversed. This study provides a proof of concept that certain forms of hearing loss can be reversed after the loss has occurred, which is important support for the development of new treatments for humans.
Regeneration of Hair Cells in the Mature Mouse Cochlea Following Reprogramming with Atoh1, Gfi1, and Pou4f3

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Hair cells in the mature mammalian cochlea do not regenerate and any hair cell loss is permanent. Neonatal cochlear cells respond to the ectopic expression of the hair transcription factor Atoh1 by differentiating into hair cells. In the mature cochlea, however, Atoh1 alone does not induce the conversion of supporting cells into hair cells. Moreover, in neonatal mice as well as non-mammalian species, the regenerative ability of supporting cells depends on the removal of regulator signaling from HCs. It is possible that mature hair cells regulate mature supporting cells, and that the loss of the hair cells can improve the response to reprogramming factors.

We targeted the ROSA locus to conditionally express Gfi1, Atoh1, and Pou4f3 (Rosa-GAP). When combined with Lfng-CreER, Rosa-GAP expresses the transcription factors in supporting cells. We ablated hair cells in Lfng-CreER:Rosa26-GAP mice by using the Pou4f3DTR mouse. For acute damage, reprogramming and hair cell killing were induced simultaneously at 3 weeks of age. For chronic damage, hair cell killing was induced at 3 weeks of age followed by reprogramming at 6 weeks of age.

Following both acute and chronic HC damage, regeneration was observed throughout the length of the cochlea with inner and outer HCs identifiable by location. Examination of regenerated hair cell-like cells following acute damage revealed neural connections and phalloidin labeled disorganized stereocilia bundles that were also detected via SEM. Further work is ongoing to characterize regenerated hair cells and investigate the expression profile of these cells.

Currently, the best therapeutics for hearing loss are hearing aids and cochlear implants. While these enable users to re-gain some hearing, they provide incomplete recovery. Understanding the response of supporting cells to hair cell death and reprogramming provides insight into the molecular pathways regulating supporting cell identity.
The Tetraspan LHFPL5 Establishes Force Sensitivity of the Mechano-transduction Channel of Cochlear Hair Cells

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The mechanoelectrical transduction (MET) channel of cochlear hair cells is located in hair cells near tip links, the fine extracellular filaments that are thought to gate the MET channel. TMC1/2, TMIE, LHFPL5 and CIB2/3 are essential components of the MET channel complex. TMC1/2 and TMIE bind to each other to form a functional ion channel complex and mutations in each of these proteins affect channel pore properties. CIB2/3 binds to TMC1/2 and serves as an auxiliary subunit that regulates MET channel localization and function. The function of LHFPL5 in the MET complex has remained elusive. Here we show that LHFPL5 is critical to regulates the response for the MET channel to mechanical force, including the regulation of optimal channel activation and maximal force sensitivity. Using structure-based predictions and mutational analysis, we have identified domains critical for the function of LHFPL5 in regulating MET channel activity. Our studies thus provide insights into the gating mechanisms of the MET channel in hair cells and define a crucial role for LHFPL5 in establishing the channels force-sensitivity.
Effects of Jag1-Mediated Notch Activation in Inner Ear Patterning: Insights from the Nodder Mouse Model for Alagille Syndrome.

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Proper auditory function requires a precisely ordered mosaic pattern of mechanosensitive inner hair cells (IHCs), outer hair cells (OHCs) and subtypes of associated supporting cells (SCs) within the Organ of Corti. This pattern is established by a series of cell fate decisions mediated, in part, by Notch signalling, starting from early pro-sensory domain induction followed by inhibitory cues specifying HCs versus SCs.

Defects in Notch signalling lead to patterning defects that include extra IHCs and fewer OHCs, and subsequent hearing deficits, as exemplified by mice with mutations in the Notch ligand Jagged1 (Jag1), and patients with JAG1-disorder Alagille syndrome, a multisystem disorder that includes hearing loss. However, research into the role of Jag1 in cochlear development has been 1) limited to conditional and heterozygous loss of function models due to late embryonic lethality of Jag1 germline mutants and 2) incomplete, due to a predominant focus on late embryonic HC phenotypes, while Jag1, like Notch target genes, is expressed prior to sensory fate specification and remains expressed in SCs postnatally.

In this study, we aim to identify the roles of Jag1-mediated Notch activation in inner ear development by using immunohistochemistry and RNAscope to characterize changes in cochlear development in Nodder (Jag1Ndr/Ndr) mice, which are viable in the homozygous condition and recapitulate Alagille syndrome. Nodder mice display profound hearing loss and patterning defects that include an increased number of IHCs and associated SCs, fewer OHCs and associated SCs and atypical OHC-like cells located in the IHC- compartment that share morphological and molecular characteristics with OHCs.

Our data highlights the importance of Jag1 in several aspects of cochlear development and we are in the process of generating single cell sequencing data of our Nodder mice to provide mechanistic insights in Jag1-mediated pattern formation and to identify potential regenerative strategies to combat hearing deficits.
Exploring the Pathological Mechanisms of miR-96 Mutations in the Inner Ear

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The microRNA miR-96 is important for hearing, as it acts as a transcriptional regulator in the inner ear and coordinates hair cell maturation. Point mutations in the seed region of miR-96 cause dominant progressive hearing loss in humans and mice. Here, we present two mouse mutants carrying two point mutations identified as underlying progressive hearing loss in humans (Mir96s403 and Mir96s1334). This study aims to determine the underlying pathological mechanisms in the inner ear.

Auditory brainstem response (ABR) measurements, scanning electron microscopy (SEM) and immunolabelling of pre- and post-synaptic components were used to determine the onset of hearing impairment, the hair bundles' morphology, and look for synaptic defects, respectively. We performed RNAseq of the organ of Corti and RT-qPCR to determine how the different mutations affect the gene expression profile. We are currently using several approaches such as gene set enrichment analysis (GSEA) to construct the miR-96 regulatory network.

Our results indicate that the two mutations lead to different physiological, structural and transcriptional phenotypes. Homozygotes of both mouse lines exhibit profound hearing loss, but only Mir96s1334 heterozygous mice have a mild progressive hearing loss. Structural analyses showed hair cell degeneration and misshapen hair bundles in both mutants, with Mir96s1334 mice being more severely affected. Moreover, Mir96s403 homozygotes show a reduction in the number of inner hair cell synapses. The structural phenotype of Mir96s1334 mice is more severe than that of Mir96s403 mice, consistent with the audiological features displayed by humans carrying those mutations. However, the lack of hearing impairment in Mir96s403 heterozygotes in contrast to the human findings might indicate that mutant miR-96 is acquiring different targets in mice and humans. Identifying the critical pathways underlying hearing impairment will ultimately allow the pharmacological modulation of the miR-96 regulatory network for the prevention or delay of hearing loss.
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