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NOVEL MECHANISMS IN ZEBRAFISH OTOLITH NUCLEATION,
TETHERING, AND DYSFUNCTION

By

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A DISSERTATION

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ABSTRACT

Deflecting biomineralized crystals attached to mechanosensory hair cells are necessary for maintaining bodily balance. Zebrafish (*Danio rerio*) are useful organisms to study these biomineralized crystals called otoliths, as many required genes are homologous to human otoconial development. This study examines the underlying mechanisms of zebrafish otolith nucleation, tethering, and dysfunction. Here I show that 1) otolith nucleation is impaired in three zebrafish mutants with mutations in polyketide synthase, 2) *otolin-1b* is a putative component of the cuticular plate and involved with otolith growth and maintenance, and 3) *otolin-1a*, a component of the otolithic membrane, could be used to monitor risk for vestibular dysfunction during Benign Paroxysmal Positional Vertigo (BPPV) onset and following microgravity exposure. While previous studies have identified polyketide synthase as a mediator of biomineralization events in chordates and echinoderms, I demonstrate that it is evolutionarily conserved in zebrafish. The role and function of vertebrate Otolin-1 has been expanded by the characterization of hair-cell specific *otolin-1b*. Finally, I provide the groundwork for future studies on the genetic and environmental factors of vestibular dysfunction. Each one of these processes provides insight into novel mechanisms in otolith formation and maintenance.
DEDICATION

For my wife, Alexandra, without whom, none of this would have been written. Her steadfast love and support have been the foundation for my well-being and successes. To my mental health support team of Dr. Michael Kelley and Dr. Staci Rosche, you provided me with a sense of calm and enabled me with the tools to become the best version of me.
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personal life and professional performances. I will cherish the last eight years that I have spent under his fatherly guidance and mentorship.

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<td>Acetylated-tubulin</td>
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<tr>
<td>AM</td>
<td>Anterior macula</td>
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<tr>
<td>APB</td>
<td>Alkaline phosphatase detection buffer</td>
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<tr>
<td>ApoA</td>
<td>Apolipoprotein-A</td>
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<tr>
<td>AT</td>
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<td>BCIP</td>
<td>5-bromo-4-chloro-3’-indolyphosphate p-toluidine salt</td>
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<td>BPPV</td>
<td>Benign Paroxysmal Positional Vertigo</td>
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<td>BSA</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>Ethylenediamine tetra-acetic acid</td>
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<td>eNOS</td>
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<td>Green fluorescent protein</td>
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<td>Horseradish Peroxidase</td>
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<td><em>in situ</em> hybridization</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KS</td>
<td>Ketoacyl Synthetase</td>
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LNA  Locked nucleic acid
M    Molarity
MDR  Medium chain reductase
MFI  Mean fluorescence intensity
miR  microRNA
MMAPPR  Mutation Mapping Analysis Pipeline for Pooled RNA-seq
MO   Morpholino
mRNA messenger RNA
NBT  nitro-blue tetrazolium chloride
nco  no content
NDD  NAD(P)-dependent dehydrogenase
Oc90 Otoconin-90
OMP-1 Otolith Matrix Protein-1
OPBF Otolith Precursor Binding Factor
OPP  Otolith Precursor Particles
ORF  Open Reading Frame
Otog Otogelin
Otogl Otogelin-like
<table>
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<td>Otolin-1</td>
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<td>Phosphate Buffered Saline</td>
</tr>
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<td>PBS-T</td>
<td>Phosphate Buffered Saline with Triton</td>
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<td>Polymerase Chain Reaction</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PKS</td>
<td>Polyketide synthase</td>
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<tr>
<td>PM</td>
<td>Posterior macula</td>
</tr>
<tr>
<td>PP</td>
<td>Phosphopantheteine-binding</td>
</tr>
<tr>
<td>PTU</td>
<td>Phenylthiourea</td>
</tr>
<tr>
<td>RCCS</td>
<td>Rotary cell culture system</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNA sequencing</td>
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<tr>
<td>RPKM</td>
<td>Reads per kilobase per million mapped reads</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>S</td>
<td>Somite</td>
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<td>s.d.</td>
<td>Standard deviation</td>
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<td>sgRNA</td>
<td>Short guide RNA</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SL</td>
<td>Standard length</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
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<tr>
<td>Su(H)</td>
<td>Suppressor of hairless</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>Tecta</td>
<td>Alpha-tectorin</td>
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<tr>
<td>Tectb</td>
<td>Beta-tectorin</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>VOR</td>
<td>Vestibulo-ocular reflex</td>
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<tr>
<td>vns</td>
<td>vanished</td>
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<td>WT</td>
<td>Wild-type</td>
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CHAPTER 1

INTRODUCTION
Use of Zebrafish

Otoconia and otoliths act as a mass load that increase the sensitivity of mechanosensory hair cells to the effects of gravity and linear acceleration in mammals and fish, respectively. While the morphology of otoconia (“ear particles”) and otoliths (“ear stones”) differ, the initial formation of bio-crystals rely on many homologous protein components [1]. Zebrafish (Danio rerio) is a superb model organism for studying inner ear development and dysfunction, due to their external development and the optical transparency of the early embryo. While the last common ancestor of zebrafish and humans was extant 420 million years ago, the genome shows 70% sequence similarity to the human genome. They also develop quickly, with zebrafish larvae having fully functional ears by 96 hours post fertilization (hpf) [2]. Finally, zebrafish are genetically tractable, allowing the organism to be manipulated to over-express or under-express genes involved in inner ear development.

Zebrafish inner ear development is similar to other vertebrates and humans. The anatomical structures of the vestibular labyrinth are conserved in both structure and function. This includes the three semicircular canals and the two or more macular organs. The crista, or the mound of sensory epithelium, is at the base of each canal. The long kinocilia of the hair cells are within each crista, embedded in the membrane called the cupula [2]. While both human and zebrafish utricles serve the same function of balance detection, the saccule is responsible for auditory function in zebrafish but detects only balance in humans. Zebrafish do not possess a structure homologous to the mammalian cochlea, which is responsible for the human auditory function [3].
There are two types of microtubule-containing cilia within the developing inner ear of zebrafish: motile and immotile [4, 5]. During hair cell maturation, a single, immotile cilia called the kinocilia emerges next to actin-rich stereociliary bundles. Movement across the stereocilia causes depolarization of the hair cells, which relay their signal to the brain through the vestibular-ocular nerve. [6]. Any perturbation to the system can result in vestibular dysfunction, such as benign paroxysmal positional vertigo (BPPV). The surrounding epithelial cells within the inner ear have short, motile cilia that create fluid flow within the otocyst. While it was previously thought that this fluid flow is required for otolith biomineralization [5], morpholino-mediated knockdown of cilia formation or maturation does not affect otolith nucleation in zebrafish.

Zebradish Otolith Development

In mammalian inner ear development, Otoconin-90 (Oc90, the major protein component of otoconia) is necessary for otoconial seeding and nucleation [7, 8]. Oc90 can bind Otolin-1 (Otol1), which is produced by vestibular support cells, to establish a protein-rich matrix that serves as a scaffold for subsequent deposition of calcium carbonate [9, 10]. While it is not the major protein component in zebrafish otoliths, Oc90 plays an important role in otolith seeding and early development as oc90-morphants do not develop otoliths [1, 11, 12].

During early inner-ear development in zebrafish, the otic placode cavitates around 18 hpf, forming the fluid-filled otocyst (Figure 1.1A). At this time, a single kinocilia emerges from an immature tether cell. At the tip of the kinocilia, otolith precursor particles (OPPs) bind with the help of an otolith precursor binding factor (OPBF) and begin to nucleate into a nascent otolith. A previous study established a critical period for otolith nucleation using
a small molecule to inhibit otolith formation from 14-26 hpf [13]. Subsequent studies have demonstrated that otolith seeding and nucleation occurs at 18-18.5 hpf [1, 4, 14]. From 18.5-24 hpf, the beating of motile cilia within the otocyst moves OPPs towards the site of nucleation and they continue to aggregate near the kinocilia tip (Figure 1.1B). During this time point (~21 hpf) is the first instance that the otolithic membrane can be detected. Currently, there are four known components of the vertebrate otolithic membrane, including Otogelin-like (Otogl), Beta-tectorin (Tectb), Alpha-tectorin (Tecta), and Otogelin (Otog) [14-17]. In the absence of these components, otoliths form but fail to tether to the sensory macula. As the tether cell fully matures into a mechanosensory hair cell (as indicated by the presence of stereociliary bundles), the aggregated mass of OPPs biomineralizes and continues to incorporate calcium carbonate within the structure (Figure 1.1C).
Figure 1.1 Otolith formation during inner-ear development: Graphical abstract of zebrafish otolith nucleation and growth during the critical period of otolith formation (18-24 hpf).
Importance of inner ear hair cells

Hair cells are necessary and sufficient for otolith formation. When hair cells are ablated \((\text{atoh1b}^- \text{+ atoh1a-morphants})\) during inner ear development, otoliths fail to nucleate (Figure 1.2C) [18, 19]. When hair cell formation is delayed, \((\text{atoh1b-morphants})\), a single otolith forms at 26 hpf and becomes tethered by 32 hpf (Figure 1.2A-B) [18, 20]. When supernumerary hair cells are generated by attenuated Notch signaling \((\text{rbpja}^- \text{+ rbpjb- or Su(H)-morphants})\), extra ectopic otoliths form throughout the otocyst (Figure 1.2D) [21-23]. Interestingly, otic epithelial cells that would be otherwise fated for support cells differentiate into hair cells in these morphants [24].

Identifying the Otolith Precursor Binding Factor

The presence of an OPBF was proposed over two decades ago and has still proved to be elusive [25]. Recent studies suggest that one or more OPBFs are expressed by tether-cells and helps to mediate otolith nucleation by binding other otolith precursor particles [5, 14, 20]. An OPBF helps facilitate otolith seeding, which occurs when OPPs bind to the tip of tether-cell kinocilia during the critical period of otolith nucleation [13, 20]. In zebrafish, the mature mechanosensory hair cells can be identified by morphology (i.e. presence of stereocilia) at 21.5 hpf [6, 26]; however, otoliths nucleate near immature, tether cells, which can be identified by a single immotile kinocilia at 18.5 hpf [4, 20]. Neither cilia nor ciliary motility are necessary for otolith tethering to the underlying sensory epithelium [5, 20]. In the absence of tether cilia \((\text{ift88-morphants})\), an OPBF is still made as OPPs are able to nucleate and adhere to at the apical surfaces of hair cells (Figure 1.2E-F) [20].
Figure 1.2 Tether cells are necessary and sufficient for otolith formation: (A) In *atoh1b*-morphants, hair cell development is delayed and, as a result, the otolith does not form. (B) Only until the hair cells form later in development, does the otolith finally nucleate. (C) When hair cells are completely ablated, OPPs do not nucleate, leading to otolith agenesis. (D) In the presence of supernumerary hair cells, extra ectopic otoliths form within the inner ear. (E) In the absence of cilia, OPPs are still able to nucleate near the apical surfaces of hair cells and (F) eventually biomineralize into otoliths.
Zebrafish Otolith Composition

The otolith itself is comprised of more than 380 protein components that make up 0.1-1.0% of the total otolithic mass with the remaining 99% being calcium carbonate [27]. The largest protein component in zebrafish otoliths is otolith matrix protein-1 (OMP-1) [12, 28]. While OMP-1 is not required for otolith nucleation, OMP-1 morphants exhibit reduced otolith size [12]. OMP-1 is also involved with deposition of other otoconins. For example, in OMP-1-morphants, Otol1a is no longer localized in the posterior otolith (sagitta) and the anterior otolith (lapillus) [12]. Other major protein components include Starmaker, Sparc, and Apolipoprotein-A (ApoA). Starmaker is primarily involved with otolith morphology as morphant otoliths are unusually shaped and are composed of calcite [29]. The highly acidic Sparc protein is involved with otolith growth as morphants are characterized by smaller otoliths compared to WT [30]. The role of ApoA remains questionable, although it has been suggested that it can bind the product of Polyketide synthase (Pks1), which has been implicated in otolith nucleation in Japanese medaka (Oryzias latipes) [27, 31].

Otolith growth is diurnal, as layers of proteins are typically deposited during the day while alternate layers of calcium carbonate are primarily deposited at night [32]. Otolith growth takes place near the distal edge closest to the underlying sensory epithelium. Between these two structures lies a unique microenvironment that promotes otolith biomineralization and calcium carbonate deposition. The rest of the endolymph is composed of anti-calcifying agents that slowly degrade the otoliths [32]. As a result, the outer layer of the otolith is typically smooth, while the distal edge is ruffled.
Research Objectives

My first objective was to identify and characterize a set of zebrafish mutants that showed otolith agenesis. Preliminary analysis of the mutants identified diffusely expressed otoconins in the otocyst. My central hypothesis was that otolith agenesis in the mutants was due to an error in ion homeostasis within the endolymph of the inner ear. My rationale was that, while the otoconins within the inner ear have the capability to form otoliths, they are unable to do so due to extrinsic factors. After several failed attempts at sequencing likely candidates, our lab used two complementary approaches to identify the causative gene. The first approach used whole genome homology mapping to determine sites of high similarity in the zebrafish mutants that lack otoliths. The second approach was an RNA sequencing (RNA-seq) analysis during the critical period of otolith nucleation in order to determine aberrant expression of the causative gene.

My second objective was to identify the functional role of Otol1b during early zebrafish inner ear development. Our lab used morpholino-mediated knockdown of otol1b to demonstrate that otolith nucleation is markedly decreased in morphant embryos. This suggests that otol1b has a role in otolith nucleation and tethering similar to the tether-cell specific OPBF. Consequently, my hypothesis was that Otol1b is an OPBF that (1) acts with Oc90 to form a scaffold to support otolith nucleation and bio-mineralization and (2) mediates the attachment of the utricular and saccular otoliths to the sensory macula during early inner ear development in zebrafish. My rationale was that the function of Otolin-1 during otolith and otoconia early inner ear development are conserved across vertebrates. My approach was to generate a genetic knockout (KO) using CRISPR-Cas9 genome editing system and characterize its behavioral responses to auditory and vestibular assays.
My third objective was to establish genetic and environmental models of vestibular dysfunction and identify potential blood plasma markers for vertigo susceptibility. My central hypothesis was that prior to vestibular dysfunction, dislodgement of otoconia within the inner ear causes degradation of the otoconial membrane and, thus, elevates levels of otoconial membrane proteins, such as Oto11, in blood plasma. Our lab previously generated a tecta-null zebrafish that exhibited abnormal swimming behaviors that are reminiscent of vertigo. My rationale was that the tecta-null zebrafish can be used as a genetic model for BPPV. Additionally, zebrafish were exposed to microgravity-like conditions, which are known to exacerbate feelings of vertigo. My approach was to isolate blood plasma from susceptible fish and test for high levels of Oto11a and other otolithic membrane proteins.

Research Significance

Application of CRISPR/Cas9 Genome Editing System

My research has benefitted from the recent emergence of the CRISPR-Cas9 genome editing system [33]. Our lab has utilized this novel tool to generate genetic knockouts of several components of otoliths and the otolithic membrane including otog and tecta. By using genetic knockouts for comparisons, it will help alleviate any concerns of non-specific phenotypes that may arise from relying solely on targeted knockdown of genes using antisense morpholino oligonucleotides [34, 35]. However, it has been recently demonstrated that truncated mRNA from crispants can activate the overexpression of similar genes to compensate for the loss of function [36].
First Characterization of otoll Genetic Knockout In A Model Organism

Many genes required for mammalian otoconial development have homologs that are similarly necessary for zebrafish otolith development. This will serve as the first study describing a vertebrate otoll genetic knockout as an otoll-null mouse has not been described yet. A previous study demonstrated that a zebrafish otoll homolog, otolin-1a (otolla), is not required for or expressed during the critical period of otolith nucleation [12]. The role and function of its paralog, otolin-1b (otollb) has not been characterized yet.

Novel Fixation Method

Zebrafish otoliths are primarily composed of calcium carbonate, in the form of aragonite, which accounts for ~99% of the total otolithic mass [37, 38]. The center of the otolith yields a proteinaceous core that acts as a site for otolith nucleation and biomineralization. This matrix lays the foundation for further otolith growth, which is mediated by daily deposition of additional otoconins and calcium carbonate molecules [37]. While deposition of calcium carbonate is important for increasing the mass load atop mechanosensory hair cells, it has provided a challenge for antibody penetration. In order to address this issue, I have adapted CLARITY to elucidate otolithic protein expression using antibodies that could not be resolved using other fixation methods [39]. This fixation method using hydrogels has allowed us to keep the integrity of the protein matrix while efficiently removing calcium carbonate (using EDTA) in order to preserve and retrieve antigen sites.
Discovering Novel Mechanisms that lead to Otolith Agenesis

These studies are innovative in that they address whether key mechanisms appear to be evolutionarily-conserved during otolith nucleation in vivo. This study attempts to minimize the list of unknown factors that contribute to otolith nucleation by identifying and characterizing causative genes in zebrafish mutants that exhibit otolith agenesis. Known factors include a hair cell calcium sensor (otopetrin-1) and an ionocyte maturation factor (gcm2) [40, 41].

Currently, there are two known ENU-mutagenesis mutants including keinstein and no content along with several spontaneous mutants such as corkscrew and vanished. Insight from these mutants might elucidate novel mechanisms for otolith nucleation. Furthermore, it may provide the identity of the OPBF(s), which have yet to be identified.

Implications for the Prevention of Vestibular Dysfunction

The final goal of this study will attempt to address the etiology of BPPV and other factors that contribute to vertigo. While BPPV is treatable, the instance of acute vertigo leaves individuals susceptible to falls, fractures, breaks, and even death in some circumstances [42]. By establishing genetically- and environmentally-induced zebrafish models of vertigo, this study could lead to the development of precautionary measures such as potential drug targets to reduce vertigo incidence in humans.
CHAPTER 2

MATERIALS AND METHODS
Husbandry and maintenance

All zebrafish were maintained in a temperature-controlled (28.5°C) and light-controlled (14 hours on/10 hours off) room per standardized conditions.

*nco* strain (jj149) was generated by an ENU screen on the AB background and obtained from Zebrafish International Research Center (Eugene, OR, USA). *csr* was a spontaneous mutant generated in a *bre-KO2/ntl-GFP* line (AB background). *vns* was a spontaneous mutant generated in a AB/TL background. *Tg(pou4f3:GAP-GFP)* was used to mark mechanosensory hair cells [43]. Wild-Type (WT) strain TuAB was used to generate CRISPR/Cas9-mediated genetic knockouts of targeted genes.

All protocols were approved by Creighton University Animal Care and Use Committee.

Whole genome and RNA-sequencing

Mutant *nco* embryos and WT clutchmates were phenotyped and collected during the critical period of otolith nucleation (24 hpf) and the whole embryo lysates (n=50) were submitted for RNA sequencing. Analysis was completed using MMAPPR (Mutation Mapping Analysis Pipeline for Pooled RNA-seq) as previously described [22]. Whole genome sequencing of *csr* mutant embryos (identified by phenotype)(n=150) was performed and analyzed using MegaMapper as previously described [23]. Common SNPs were removed by the Single Nucleotide Polymorphism Database (dbSNPs). Reference sequences for both experiments were mapped to the Zv9 reference genome. All sequencing was conducted at the University of Nebraska Medical Center Genomics Core Facility.
Protein modeling

PHYRE2 was used to generate hypothetical protein models of relevant genes. Analysis was performed using Hidden Markov models under an intensive conditions [44]. Accession numbers used were: zebrafish Pks1 (XP_682975.3), medaka Pks1 (NP_001295960.1), human Otol1 (NP_001073909.1), zebrafish Otol1a (NP_001093211.1) and Otol1b (NP_001296385.1).

mRNA and plasmid DNA rescue

WT mRNA and pks1^{L905P} were synthesized using mMessage Machine from a full length clone provided by Dr. Hiroyuki Takeda (University of Tokyo), cleaned on an RNeasy column, and subsequently injected into single-cell csr and nco embryos. Naked plasmid of the medaka pks1 clone was injected into vns embryos. Overall penetrance of otolith formation was determined in all three mutants. Site-directed mutagenesis (Agilent) was used to generate the mutant clone containing the causative mutation in csr (pks1^{L905P} in Japanese medaka, pks1^{A911P} in zebrafish). Primers used for site-directed mutagenesis were:

\( pks1_{\text{L905P}}_{\text{Forward: 5'}}-\text{GATATGGCGTGATGTCCGGTGACAGGTTGAAGATC-3'} \)

\( pks1_{\text{L905P}}_{\text{Reverse: 5'}}-\text{ATCTTCAACCTGTCAACCGGACATCACGCCATATC-3'} \)

Pathway analysis

Pathway analysis of nco was performed using Ingenuity Pathway Analysis (IPA, QIAGEN Inc., [45]. The Ensembl Gene IDs were assigned to each gene and uploaded to IPA.
Cut-off for gene expression analysis was set at 0.75 RPKM. The calculated z-score indicates a pathway with genes exhibiting increased mRNA levels (positive) or decreased mRNA levels (negative). No change in mRNA levels results in a z-score of zero.

Genotyping

Primers below were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/ primer3plus.cgi) using the following accession numbers for *otol1b* (NM_001309456.1), *otol1a* (NM_001099741.1), and *tecta* (XM_009301924.3).

*otol1a* Forward: 5’- GCATCAATAGACAAGACCACACACAGTG-3’

*otol1a* Reverse: 5’-CCAATTGCTGTTGGACTCACAGTG-3’

*otol1b* Forward: 5’- TGCTCTCGTGTGTTTCGATG-3’

*otol1b* Reverse: 5’-ATGCTCTCATCTCTCCTCTCTTTC-3’

*tecta* Forward: 5’-CTGCTCTGAGGGCTGTCAG-3’

*tecta* Reverse: 5’-GCCTTCGTTCTCTTGGCAGTAC-3’

*csr, nco, and vns* samples were PCR-amplified and submitted for Sanger sequencing using the following primers:

*nco* Forward: 5’-GGGAGGATGCTTGTTGG-3’

*nco* Reverse: 5’-GTGGCCCAATAGGATCCA-3’

*csr* Forward: 5’-AAGACGCGGCACATGACTC-3’

*csr* Reverse: 5’-TTCAACAAACAGTGCTCCGG-3’
vns_Foward: 5’-GCCATCATTTGGAATTGGATG-3’

vns_Reverse: 5’-GGTGTTCCAGTCCCATGAGC-3’

Morpholinos

Morpholinos (MOs) were either injected into the embryo or the yolk at the one-cell stage. Concentrations for the morpholinos were 4ng for otol1b and 2ng for p53. Sequence information for morpholinos made by Gene Tools (https://www.gene-tools.com/) were the following:

**otol1b MO**: CACCTTTATTACCCATCTCACCTGT

**p53 MO**: GCGCCATTGCTTTGCAAGAATTG

CRISPR/Cas9

Crispants were generated and identified using previously established methods [46, 47]. Oligos were annealed using T4 DNA polymerase (NEB, M0203) and transcribed by MEGAscript SP6 Transcription Kit (ThermoFisher, AM1330). For single-cell injections, 2nl of sgRNA and Cas9 protein (PNA Bio, CP04) were injected. sgRNA sequences were designed using CHOPCHOP (http://chopchop.cbu.uib.no/) [48] for the following genes:

**otolin1b_sp6**: 5’-ATTAGGTGACACTATAGGCTTTCTTCTGATATGTGGTCTTAG AGCTAGAAATAGCAAG-3’

Antibodies

Affinity-purified rabbit polyclonal antibodies were generated to Otog (CGNRVDGPSASKG, 1:1000), Oc90 (CNTQSDTVDRKPTQSKPQ, 1:1000), Starmaker
(DSKESPDTDKPEGPDS, 1:1000), Tecta (NGDPIDDLRLPSGRQADS, 1:1000), Otol1b (KGNSGSKGDKEKGDQGN, 1:1000), Otol1a (NGTDGLPGSKGPKGDPGP, 1:1000) by conventional methods (GenScript, USA) and directly labelled before immunofluorescence. Other antibodies used were Keratan Sulfate (MZ15, 1:2000, DSHB), Hair Cell Specific-1 (HCS-1, 1:500, DSHB), and acetylated-tubulin (Ac-tubulin, 1:500, Sigma T6793). Secondary antibodies include: Goat anti-Mouse IgG2b (Invitrogen, A21140), Goat anti-Rabbit IgG (Invitrogen, A21071), Goat anti-Mouse IgG2a (Invitrogen, A21133) and Goat anti-GFP (Rockland, 600-101-215). Phalloidin (ThermoFisher, A12379) was used at a concentration of 1:500.

Immunofluorescence

Embryos and larvae were collected at different stages in early inner ear development, euthanized, fixed with hydrogel overnight and washed in CHAPS-based (1% by weight) CLARITY-clearing solution [39]. Embryos were decalcified with EDTA (120 mM in 0.1% PBS-Triton) before blocking (0.1% PBS-Triton with 3.33% sheep serum and 3.33% BSA), incubating in primary and secondary antibodies diluted in blocking buffer, mounting in 50% Glycerol-PBS solution, and imaging by confocal microscopy (Leica TCS SP8).

Image processing

Confocal microscopy images were processed using Leica LAS software. Hair cells for nco embryos and the mean fluorescent intensity (MFI) levels were quantified in Leica Application Suite X. Agarose gels and immunobLOTS were imaged and quantified in Image Lab software (Bio-Rad Laboratories). DNA sequences were visualized in SnapGene
software (from GSL Biotech, available at snapgene.com). Figures were generated using Microsoft PowerPoint and GraphPad Prism.

Alizarin

To assess complex calcium deposits in the otocyst [49], embryos were incubated in E3 Embryo Medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) containing 7 μM alizarin-3-methyliminodiacetic acid (Sigma A3882) diluted in DMSO and imaged at 5 days post fertilization (dpf).

Mitotracker staining

Mitotracker Red (ThermoFisher, M22425) was resuspended in DMSO (0.25 mM) and diluted to 200 nM in E3 Embryo Medium. nco and csr embryos were then incubated in the dark for 20 minutes before removing Mitotracker solution and replacing with fresh E3 Embryo Medium. Samples were allowed to stabilize in the dark for 30 minutes before imaging at 21 hpf. Then, embryos were phenotyped at 27 hpf.

Exogenous salt solutions

To test the effects of exogenous ions on otolith formation, embryos were kept in E3 Embryo Medium until early gastrulation. Embryos were washed, dechorionated, and transferred to 1X Basic Solution (58 mM NaCl, 0.4 mM MgSO₄ and 5 mM HEPES) supplemented with 0.7 mM potassium chloride, 0.6 mM calcium nitrate or 0.6 mM calcium chloride. Embryos were then transferred to fresh 1X Basic Solution with respective supplement for the remaining development. Embryos were scored by the presence or absence of otoliths at 27 hpf and then genotyped by high resolution melt (HRM) and analyzed in Rotor-Gene ScreenClust HRM Software (Qiagen, USA).

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In situ hybridization

The in situ hybridization protocol was adapted from Moens, et. al. [50] PTU-treated embryos were collected at 48 hpf, euthanized, fixed with 4% PFA in 1X PBS, dehydrated in 100% methanol, and kept at -20°C. Embryos were serially transferred from 100% methanol into 1X PBS-Tween (0.1%). Embryos were then treated with proteinase-K solution (10µg/mL) for 30 minutes before re-fixation and transferred back to 1X PBS-Tween. Embryos were pre-hybridized in hybridization mix at 70°C for 3 hours before adding the appropriate DIG-labeled probes (100-200µg/mL) for overnight incubation at 70°C. Samples were serially transferred from hybridization mix to 2X SSC buffer to 1X PBS-Tween. Embryos were pre-blocked at room temperature with Block-LNA (2% sheep serum with 0.2% BSA by weight in 1X PBS-Tween) before adding anti-DIG antibody (Roche 11093274910, 1:5000 in Block-LNA) overnight at 4°C. Samples were thoroughly washed in 1X PBS-Tween and transferred to APB solution (pH 9.5) before the staining reaction. Embryos were placed in staining solution (APB with NBT/BCIP (Roche 11681451001, 10µL/mL)) at 4°C until expression was observed. The staining reaction was stopped by adding 0.1M glycine (pH 2.5) for 10 minutes. Embryos were washed in 1X PBS before serially transferring to 80% glycerol/20% PBS solution before imaging.

Blood plasma collection

Our lab modified a blood collection technique previously described by Babaei et al. to collect protein from the blood plasma of zebrafish and to quantify protein levels by immunoblot [51]. β-actin was used as a sex-independent control [52]. Adult and juvenile fish were euthanized using ice baths before cutting off the tail immediately posterior to the
anal fin. Fish were propped upright in tubes using a sequin pin that punctured the fish posterior to the gill slit. Heparin (Sigma, H3393) was added to the bottom of the tube to prevent blood coagulation. Fish were originally spun at 100g to collect blood. Plasma was separated from the blood at 13,700g before freezing at -80°C where it was kept before processing for immunoblot.

Immunoblot

Blood plasma was thawed and added to 1X NuPAGE LDS Sample Buffer (Invitrogen), denatured at 70°C and loaded into a 12% polyacrylamide gel for protein separation by electrophoresis. Gels were transferred to nitrocellulose paper using iBlot Dry Blotting System (ThermoFisher). The protocol for immunoblotting was adapted from Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, USA). The buffering solution for the protocol was TBS-Tween (0.1%). Blots were blocked using WesternBreeze Blocker/Diluent (ThermoFisher, WB7050). Primary antibodies were diluted at 1:1000 and include: Oto1la, Tecta, and β-actin (Sigma A1978). Secondary antibodies were diluted to 1:2000 and include anti-rabbit IgG HRP (Rockland, 18-8816-33) and anti-mouse IgG HRP (18-8817-30). Immunoblots were chemically developed using ECL Prime Western Blotting System (GE Healthcare, RPN2232) and imaged using Image Lab software (Bio-Rad Laboratories).

Microgravity

To mimic microgravity-like conditions, adult zebrafish were placed in a custom Synthecon Rotary Culture Max (Figure 2.1) for five consecutive days (14 hours light on/10 hours light off) at 44 rpm. The apparatus was stopped twice daily to feed the fish with brine
shrimp. A flexible electroluminescent panel (Knema, LLC, EL2006) was uniformly wrapped around the culture chamber and then covered with a blackout blanket to suppress the vestibulo-ocular reflex. Following microgravity exposure, abnormal swimming behaviors were noted before euthanizing and processing the fish for blood plasma and whole-body cortisol collections.

For larval studies on the effects of microgravity, 3 dpf fish were placed in a 50mL culture chamber, filled with E3 Embryo Medium, attached to a Synthecon Rotary Cell Culture System (RCCS-4D) for two consecutive days at 16 rpm. Larvae were immediately tested for vestibular function (see Prepulse inhibition assay) by ZebraLab software (Viewpoint Behavior Technology) before euthanizing and fixing in hydrogel for subsequent experiments.
Figure 2.1 Apparatus to mimic microgravity-like conditions: (A) Synthecon Rotary Culture Max was customized to our specifications to accommodate adult zebrafish. Dimensions of culture chamber: 5.325” length by 2.745” diameter. (B) The culture chamber was uniformly lit and adjusted to a light cycle of 14h on/10h off. (C) In order to suppress the vestibulo-ocular reflex, the entire apparatus was covered with a blackout blanket.
Prepulse inhibition assay

A ZebraBox Revolution (Viewpoint Behavior Technology) was used to record the 
startle response of zebrafish larvae at 1000 frames per second. The prepulse stimulus was 
set at 660 Hz for 5 ms at 10 dB. The follow-up stimulus was delayed by 5 ms and was set 
at 440 Hz for 10 ms at 9 dB. Analysis was performed using ZebraLab software (Viewpoint 
Behavior Technology).

Cortisol assay

Cortisol was extracted from adult fish following the previously described protocol 
[53]. Fish carcasses were pulverized in deionized water using a tissue homogenizer. The 
homogenizer was rinsed with an additional deionized water and combined with the 
homogenized sample and placed on ice. Samples were transferred to a 15mL polypropylene 
conical tube and extracted twice with diethyl ether. For each extraction, samples were 
vortexed and centrifuged (2700 rpm for 3 minutes) to separate aqueous and ether layers. 
The aqueous layer was aspirated off and added to a new tube before allowing to air dry 
completely in a fume hood. Dried samples were reconstituted in PBS-gelatin (0.1%) and 
then frozen at -80°C before processing. Cortisol concentrations were analyzed using the 
Cortisol ELISA Kit (Cayman Chemical, 500360) following vendor’s protocol. Absorbance 
was read at 405nm (Bio-Tek, USA). Standard curves were generated and applied in 
Microsoft Excel.
Statistical analyses

Statistical significance was calculated using Fisher’s Exact Test, Student’s t-test, G-test for Independence, and Chi-Squared Distribution. Statistical analysis was performed in GraphPad Prism (San Diego, USA).
CHAPTER 3

ZEBRAFISH OTOLITH BIOMINERALIZATION REQUIRES POLYKETIDE SYNTHASE
Abstract

Deflecting biomineralized crystals attached to vestibular hair cells are necessary for maintaining balance. Zebrafish are useful organisms to study these biomineralized crystals called otoliths, as many required genes are homologous to human otoconial development. I sought to identify and characterize the causative gene in a trio of homozygous recessive mutants, nco and csr, and vns, which fail to develop otoliths during early ear development. I show that nco, csr, and vns have potentially deleterious mutations in pks1, a multi-modular protein that has been previously implicated in biomineralization events in chordates and echinoderms. I found that Oc90 expression within the otocyst is normal in nco and csr; therefore, it is not sufficient for otolith biomineralization in zebrafish. Similarly, normal localization of Otog, a protein required for otolith tethering in the otolithic membrane, is not sufficient for Oc90 attachment. Furthermore, eNOS signaling and Endothelin-1 signaling were the most up- and down-regulated pathways during otolith agenesis in nco, respectively. My results demonstrate distinct processes for otolith nucleation and biomineralization in vertebrates and will be a starting point for models that are independent of Oc90-mediated seeding. This study will serve as a basis for investigating the role of eNOS signaling and Endothelin-1 signaling during otolith formation.

Introduction

Otoconia and otoliths act as a mass load that increase the sensitivity of mechanosensory hair cells to the effects of gravity and linear acceleration in mammals and fish, respectively. While the morphology of otoconia and otoliths differ, the initial formation of bio-crystals rely on homologous proteins [1].
Zebrafish otoliths are primarily composed of calcium carbonate (CaCO₃), in the form of aragonite, which accounts for ~99% of the total otolithic mass with the remainder consisting of proteins called otoconins [37, 38]. Further analysis of teleost otoliths has identified more than 380 protein components [27]. Based on the level of protein expression or changes in the rate of otolith growth, the polymorph of calcium carbonate crystals can change [1, 54]. For example, knockdown of Starmaker results in otoliths made of calcite rather than aragonite [55]. There are three pairs of otoliths in zebrafish, which include the sagitae, lapilli, and asterisci. While the lapillus and sagitta nucleate early in zebrafish development, the asteriscus does not form until 11-12 days in development [56]. The center of the otoliths contains a proteinaceous core that acts as a site for otolith nucleation and biomineralization. This matrix lays the foundation for further otolith growth, which is mediated by daily deposition of additional otoconins and calcium carbonate molecules [37]. Otolith nucleation occurs when the OPPs bind to the tips of the immotile kinocilia of tether cells within the otic vesicle [4, 20]. Subsequent studies have demonstrated that the critical period of otolith seeding and nucleation starts at 18-18.5 hpf and ceases by 24 hpf [1, 4, 13, 14, 26].

In mammalian inner ear development, Oc90 (the major protein component of otoconia) is necessary for otoconial seeding and nucleation [7, 8, 57]. Oc90 can bind Otol1 to establish a protein-rich matrix that serves as a scaffold for subsequent deposition of calcium carbonate [9, 10]. Additionally, in vitro studies have suggested that Oc90 and Otol1 act synergistically to modulate otoconial crystal morphology [10]. While Oc90 is not the major protein component in zebrafish otoliths, it plays an important role in otolith seeding and early development as oc90-morphants do not develop otoliths [1, 11]. While
additional gene mutations have been identified that lead to otolith agenesis in zebrafish [40, 41, 58-61], the genes responsible for several zebrafish otolith mutants have been undetermined.

In this study, I sought to identify and characterize the causative gene in a trio of zebrafish mutants, nco, csr, and vns, which fail to develop otoliths during early inner ear development. I provide genetic evidence that the causative gene is polyketide synthase (pks1, currently wu:fc01d11), a candidate gene that was previously identified as a key factor of biomineralization in Japanese medaka (Oryzias latipes) and sea urchin (Hemicentrotus pulcherrimus) [31]. Furthermore, I suggest potential signaling pathways for pksl function during inner ear development in the zebrafish.
Figure 3.1 The *csr*, *nco*, and *vns* mutant phenotypes fail to form otoliths within the inner ear: (A-D) However, semicircular canal formation appears to be normal. (A’-D’)
All mutants fail to inflate their swim bladders, which is lethal. Imaged at 5 dpf.
Magnification 6.3X. (*) indicates swim bladder.
Results

csr and nco are genetically-linked

The most apparent phenotype of the homozygous recessive csr, nco, and vns mutants is that they fail to form otoliths (lapillus and sagitta) or any observable complex calcium deposits within the inner ear (Figure S1). Furthermore, the mutant larvae are homozygous lethal by 7 dpf as the swim bladder fails to inflate and they are unable to feed (Figure 3.1). As a result, it is not known whether asterisci formation is affected. While it is still unknown why the swim bladder fails to inflate when otoliths are absent, it is a common phenotype in other mutants with otolith agenesis [11, 40, 41, 58-61]. Due to this commonality within csr and nco, I sought to determine if these phenotypes would complement each other. The results of the complementation test showed that 29.25% of offspring failed to develop otoliths (n=106), supporting that nco and csr likely are allelic.

Exogenous ions influence otolith nucleation in csr embryos; not nco or vns embryos

As an aquatic species, the environment of zebrafish can be easily controlled and adapted to assess its impact on embryonic development. Previously, small molecules have been used to block otolith development by inhibiting otolith nucleation [13]. I hypothesized that there was an error in ion homeostasis that could be affected by exogenous solutions. In water treatments supplemented with calcium chloride (n=51), I found a significant decrease in csr penetrance of otolith agenesis in homozygous embryos ($\chi^2=19.27$, df=6, p=0.0037) compared to treatments supplemented with potassium chloride (n=46) or calcium nitrate (n=54). Additionally, I observed no significant change in nco mutant phenotype penetrance for water treatments supplemented with potassium chloride
(17.76%, n=107), calcium chloride (16.67%, n=120) or calcium nitrate (16.9%, n=112) (p=0.975, G-test for independence). Similarly, the penetrance of otolith formation in \textit{vns} was not affected by exogenous salts (data not shown).

Building on the hypothesis that there was an error in ion homeostasis, Mitotracker was used to mark mitochondria-rich cells (i.e. presumptive ionocytes) in \textit{csr} and \textit{nco} embryos. While \textit{nco} embryos appear normal, I observed that \textit{csr} embryos show a lack of Mitotracker localization at 21 hpf (Figure S2). Altogether, this suggests the nature of the \textit{nco} and \textit{csr} mutation, while likely allelic, are inherently different.

Potentially deleterious mutations identified in polyketide synthase for \textit{csr, nco,} and \textit{vns}

To positionally clone the gene responsible for \textit{nco} and \textit{csr}, I used complementary approaches for each strain. MMAPPR analysis of \textit{nco}-derived RNA sequencing (Figure 3.2 A) [61] and MegaMapper analysis of \textit{csr}-derived whole genome sequencing (Figure 3.2 B) [62] both identified a genomic region with high homology surrounding the \textit{pks1} locus. While several other genes were in that region, a previous study on otolith biomineralization in Japanese medaka made \textit{pks1} the likely gene candidate [31]. Potentially deleterious mutations were identified in \textit{pks1} for \textit{csr} (A911P) and \textit{nco} (L681*), which were both located within a conserved acyl transferase domain (Figure 3.2 C). Furthermore, a deleterious mutation in \textit{vns} (G239R) was serendipitously found to be linked to a neighboring gene during a separate study. The deleterious point mutation was identified by Sanger sequencing of the \textit{pks1} locus and confirmed by relatively high penetrance of otolith agenesis (95%).
Figure 3.2 Complementary approaches for causative gene discovery: MMAPP analysis of RNA sequencing data for neo (A) and whole genome homology mapping for csr (B) identified regions of high homology on the 24th chromosome near the pks1 locus (~33 Gb). (C) Deleterious mutations were identified in pks1 for neo and csr within the acyl transferase (AT) domain and vns within the polyketide synthase (PKS) domain. Sanger sequencing confirmed SNPs in csr, neo, and vns mutants. Other domains include Ketoacyl Synthetase (KS), Medium Chain Reductase (MDR), NAD(P)-dependent dehydrogenase (NDD), and Phosphopanthetheine-Binding (PP).
Japanese medaka pks1 mRNA or plasmid DNA rescues otolith biomineralization in csr, nco, and vns.

While the last common ancestor of Japanese medaka and zebrafish was estimated to be 150 million years ago [63], I sought to assess if the function of pks1 within the inner ear is conserved, based on high peptide sequence similarity (Figure S3). I injected Japanese medaka pks1 mRNA or DNA into single-cell embryos of csr, nco, and vns heterozygous in-crosses. Microinjection of Japanese medaka pks1 mRNA (300 ng/µL) rescued otolith biomineralization in both csr (p<0.0001, χ²<0.0001, n=93) and nco (p=0.0032, χ²=0.0022, n=84) mutants (Figure 3.3A,B). Additionally, microinjection of the Japanese medaka pks1 plasmid (20 ng/µL) rescued otolith biomineralization in vns (p<0.0001, χ²=0.0004, n=39).

Using site-directed mutagenesis, I introduced the non-synonymous mutation (A911P) in csr to the Japanese medaka mRNA construct (L905P). I repeated injections into single-cell embryos and failed to rescue otolith biomineralization in csr and nco. WT medaka pks1, but not pks1L905P, rescued otolith biomineralization in csr and nco embryos (Figure 3.3C).
Figure 3.3 WT pks1 nucleic acid rescues otolith formation in csr, nco, and vns: (A) Normal frequencies of mutant phenotypes in each uninjected strain. All four pairings follow homozygous recessive mode of inheritance. (B) Results of injected embryos show that Japanese medaka pks1 mRNA (300 pg) rescues both csr and nco mutants and pks1 DNA (20 pg) rescues vns mutants. (*, p<0.0001, paired t-test)(**, p<0.0032, paired t-test)(***, p=0.0001, paired t-test). Site-directed mutagenesis was used to introduce a conserved mutation in csr (A911P) into the Japanese medaka construct (L905P) (C) Injection of pks1L905P mRNA (300 pg) fails to rescue csr or nco mutant phenotypes.
Ingenuity pathway analysis of *nco* embryos

While *pks1* is thought to produce an otolith nucleation factor [31], its broader role during inner ear development is unknown. Ingenuity Pathway Analysis of *nco* mRNA expression at 24 hpf identified eNOS and Endothelin-1 signaling as the top up- and down-regulated pathways, respectively (Figure 3.4A). Among the down regulated genes was *rdh12l*, a gene adjacent to *pks1*, suggesting possible local control of transcription at that locus. *mir-92a*, the top down-regulated gene, has a predicted binding site in the 3’UTR of *rdh12l* (Figure S4) [64]. In addition, several genes listed in the top ten up- or down-regulated lists are also enriched in adult mechanosensory hair cells such as *il11b, fosab, fosb, fosl1a, socs3a, scg5*, and *dnaaf3* (Figure 3.4 B-C) [65]. Of these genes, *il11b* is up-regulated during neuromast hair cell regeneration [66]. Notably, *dnaaf3* causes primary ciliary dyskinesia and morpholino knockdown of *dnaaf3* causes abnormal otolith growth [67]. While its role in inner ear development is unknown, *scg5* is expressed within the anterior and posterior poles of the otic placode during the critical period of otolith nucleation [68].
Figure 3.4 Gene expression and pathway analysis of nco embryos: (A) Ingenuity Pathway Analysis shows the top up-regulated and down-regulated pathways, which are eNOS Signaling and Endothelin-1 Signaling, respectively. Positive z-score indicated increased mRNA levels. Negative z-score indicates decreased mRNA levels. No change in mRNA levels results in a z-score of zero. (B) Differential gene expression in the top up-regulated genes. (C) Differential gene expression in the top down-regulated genes. (**, expressed in adult zebrafish mechanosensory hair cells) [65].
Aberrant expression of proteins involved in otolith development in *csr* and *nco*

In mammalian inner ear development, Oc90 is necessary for otoconial seeding and nucleation [7, 8]. Similarly, the role of Oc90 is evolutionarily-conserved in zebrafish and has been previously thought to be necessary for otolith nucleation [11]. Using immunofluorescence (IF), I saw diffuse expression of Oc90 in *csr* and *nco* otocysts (Figure 3.5B-D), which demonstrated that Oc90 expression within the otocyst is not sufficient for otolith biomineralization in zebrafish. Similarly, normal localization of Otog, a protein required for otolith tethering in the otolithic membrane is not sufficient for Oc90 attachment. Additionally, other otoconins that are important for calcium deposition and growth were detected with diffuse expression within the otocyst such as Starmaker and Keratan Sulfate (Figure S5) [16, 29].
Figure 3.5 Aberrant expression of proteins involved in otolith development in *csr* and *nco*: (A) Schematic of anterior macula (AM) tethered to otolith at 27 hpf. (B) In WT, Otoconin-90 (Oc90) is expressed within the mineralized otolith, which is situated atop the otolithic membrane (Otogelin, or Otog), at 27 hpf. Scale bar=5 μm. (C-D) Oc90 has diffuse expression within the otocyst of *csr* and *nco*. In *csr* and *nco*, Otog is localized near the apical surface of hair cells. (E-F) Expression showing hair cells in WT and *nco* larvae at 5 dpf. Scale bar=25 μm. (G) Quantification of hair cell numbers in the posterior and anterior macula of WT and *nco* (n=4).
Polyketide synthase as an otolith precursor binding factor?

Otolith nucleation is thought to be mediated by a tether-cell specific otolith precursor binding factor (OPBF), which lays the foundation for the successive biomineralization of the otolith [14, 20, 25]. The presence of an OPBF was proposed more than two decades ago and its identification proves to be elusive [25]. Recent studies suggest that one or more OPBFs are expressed by tether-cells and help to mediate otolith nucleation by binding other OPPs [5, 14, 20].

I sought to assess if pks1 or its enzymatic product is a tether-cell specific nucleation factor. First, using publicly available RNA-seq data [65], I found that pks1 mRNA is enriched (7.46-fold increase) in adult mechanosensory hair cells compared to support cells within the zebrafish inner ear. Additionally, this data suggests pks1 mRNA to be transcriptionally regulated in support cells. Support cells predominantly express a 300bp region of the 5′UTR of the pks1 transcript while hair cells express the full open reading frame [65]. A search for transcriptional regulatory motifs in the 5′UTR of pks1 found a predicted binding site for TCF-3 [69], a transcription factor highly expressed in adult mechanosensory hair cells [65]. While the role of TCF-3 in the inner ear is unknown, it is expressed within the otic vesicle during the critical period of otolith nucleation [68].

Then, I demonstrated that the total number of hair cells remain unchanged during early development in nco, suggesting there are no differences in tether cell maturation and maintenance (Figure 3.5 E-G). However, in situ hybridization (ISH) data showed ubiquitous expression of pks1 in the otic vesicle of zebrafish [31]. While pks1 might be enriched in adult hair cells, early expression shows that it is ubiquitously expressed in the otic vesicle and, therefore, not the tether-cell specific OPBF.
Discussion

The homozygous recessive mutants csr, nco, and vns were chosen for this study because each lack the necessary factors such as an OPBF for otolith seeding and biomineralization. To determine the genes responsible for otolith agenesis in these mutants, I used two complementary approaches. The first approach was Whole Genome Sequencing of the csr mutant genome to identify regions of high homology. This indeed was difficult as the csr background strain was heavily inbred, resulting in multiple peaks of high homology. Since I demonstrated csr and nco are genetically-linked, I sought to further clarify the responsible locus using a second method (i.e. RNA-seq of the nco transcriptome) for comparison. This result pinpointed a region of high homology near the end of the 24th chromosome. While deciphering potentially deleterious mutations within that region, I focused on pks1 following evidence that it is responsible for otolith nucleation in Japanese medaka [31]. While these species are evolutionarily divergent, the shared phenotype between medaka and our mutants suggested that the role of pks1 is conserved. As a result, I chose to use medaka pks1 nucleic acid to rescue otolith formation in csr, nco and vns mutants. Similarities can also be drawn with other zebrafish mutants such as keinstein, which has diffused expression of Starmaker within the otocyst and exhibits similar circling swimming behaviors [70, 71]. Furthermore, keinstein may be another pks1 allele due to its predicted chromosomal location [72].

While WT medaka pks1 rescues otolith biomineralization in csr and nco, differential penetrance in response to exogenous ion treatments on otolith formation suggested the nature of each mutation is fundamentally different. This was confirmed by Sanger sequencing that nco has a premature stop codon while csr likely makes a defective protein
that may be stabilized by exogenous ions. This defective protein may be the explanation for the differences in Mitotracker localization in csr. Due to its surface stain expression, I hypothesize that Mitotracker was localized to mitochondria-rich ionocytes [73]. Ionocytes have previously been implicated in otolith formation as mutations in gcm2, which is responsible for ionocyte maturation, leads to otolith agenesis [40, 74]. I hypothesize that the endolymph in csr and nco mutants has the necessary components for otolith nucleation [37] but lack a trigger factor produced by pksl. The absence of pksl does not visibly appear to affect hair cell development that are required for otolith nucleation either [20]. It has been previously suggested that ApoA could potentially bind the product of polyketide synthase [27, 31]. Given the RNA-seq analysis of nco, I see no significant change in any apolipoprotein expression. Publicly-available in situ data does not support ApoA expression within the inner ear [68]. Additionally, IF of csr and nco embryos demonstrated that expression of a critical otoconial seeding protein, Oc90, within the otocyst is not sufficient for otolith biomineralization in the presence of the otolithic membrane.

One caveat is that the penetrance of otolith formation is influenced by the genetic background of zebrafish. When treated with the small molecule 31N3, WT embryos in the AB/EKW background fail to develop otoliths [13]. However, 31N3 fails to inhibit otolith formation in the TL and TU strains, suggesting that there are potential genetic modifiers that influence otolith nucleation in these backgrounds. While the csr mutation (A911P) leads to otolith agenesis in the AB background, homozygosity at the locus is compatible with proper development in the AB/TL background (data not shown). This suggests csr may be a hypomorphic allele and the AB background can overcome the loss of Pks1 function with enhanced ion flux. Ironically, the mutant phenotype was lost when csr was
outcrossed to the WIK background. It was only until *csr* was backcrossed to the AB background that the mutants were recovered. Altogether, I suggest that the AB background heavily influences the penetrance of otolith formation.

While *pks1* likely acts as an enzyme whose expression is enriched in adult mechanosensory hair cells [65], its product is required for otolith nucleation in zebrafish. However, the molecular function of *pks1* remains unknown. Using *nco* RNA-seq data, I performed an Ingenuity Pathway Analysis, which identified eNOS and Endothelin-1 signaling as the most up- and down-regulated pathways, respectively. eNOS signaling could be impacted by *pks1* metabolites such as iromycin, which has been shown to inhibit this pathway [75]. Both eNOS and Endothelin-1 have been implicated in inner ear development and function. Notably, it has been demonstrated that these pathways are inversely related in sensorineural hearing loss [76]. eNOS has been associated with noise-induced hearing loss and gentamycin ototoxicity [77]. Another example of this is Waardenburg syndrome Type IV, caused by mutations in endothelins, which cause abnormal pigmentation, sensorineural hearing loss, and Hirschsprung Disease related to neural crest cell abnormalities [78]. During early development, Endothelin-1 mRNA turns on during the critical period of otolith nucleation [68, 79] and is detected in the otic vesicle at 24 hpf [80]. Endothelin-1 and its receptor (*ednraa*) are both enriched in adult zebrafish inner ear support cells [65]. Additionally, Endothelin-1 has been identified as a potential modifier of osteoblast function to increase bone mineralization [81]. Furthermore, Endothelin-1 has been implicated with the FOS-family of genes (*fosab, fosp*, and *fos1a*) and *socs3a*, which are all differentially expressed in *nco* at 24 hpf. These genes are all part of a regulatory network during hypergravity-mediated bone formation [82]. Moreover, the
presence of osteoblast-associated proteins within teleost otoliths suggest a common mechanism between bone mineralization and otolith biomineralization [27]. Future studies will attempt to clarify the roles of Endothelin-1 and eNOS signaling pathways during biomineralization events.
CHAPTER 4

THE ROLE OF OTOLIN-1b DURING ZEBRAFISH INNER EAR DEVELOPMENT
Abstract

In zebrafish, calcium carbonate bio-crystals called otoliths attach to the underlying sensory macula by intimate tethering to the kinocilia of inner ear hair cells and by embedding themselves within a gelatinous otolithic membrane. To date, only Tecta and Otog have been demonstrated as putative components of the otolithic membrane. While Otolla has been proposed, it is not expressed during early development (prior to 35 hpf). Previous studies have suggested another Otolin-like component might mediate early attachment. Here, I addressed whether Otolla’s paralog, Otollb, has a role in early otolith attachment. Unexpectedly, I found that Otollb is hair-cell specific and a component of the cuticular plate, an F-actin rich meshwork of proteins at the apical surface of hair cells. Our results reveal a potential novel role for an Otolin-1 homolog during vertebrate inner ear development that is independent of otolith nucleation and attachment.

Introduction

Mammalian Otoll is an inner-ear specific glycoprotein that can form higher order multimers through additional post-translational modifications (such as hydroxylations and glycosylations) [9]. Previous studies have suggested two potential roles of Otoll. The first role involves Otoll as a scaffold protein for the deposition of calcium carbonate to form otoconia. *In vitro* studies have suggested that otoconia nucleation and biomineralization is mediated by the concerted expression of Otoll and Oc90 [10]. The localization of Otoll is tightly linked to Oc90 and OMP-1 as the absence of each protein leads to a reduction of Otoll in the otoconial matrix. [8, 12, 16]. The second role of Otoll is that it may be a component of fibrous membranes within the inner ear. *In vivo* studies have shown that it is
part of the otoconial and tectorial membranes [9, 83]. However, no knockout model has
been described yet to determine the functional role of Otol1.

Analogous to mammalian otoconial membrane, the otolithic membrane is a gelatinous,
acellular matrix that lies between the sensory macula and the otolith within the inner ear of
zebrafish [14, 41]. Following otolith seeding and nucleation, growing otoliths are
embedded in the otolithic membrane, which helps mediate otolith attachment to the
mechanosensory hair cells [1, 14]. Recent studies of the mammalian otoconial membrane
have identified several protein components including Otog, Otogl, Tecta, and Otol1 [9, 14,
15, 84]. Tecta, Otog, and Otol1a (Figure 5.1) have been proposed as putative components
of the otolithic membrane in zebrafish [14]. In *einstein (otog)* mutants, otoliths nucleate
but remain detached until 26-28 hpf when the otolithic aggregate frequently attaches to the
posterior macula [14]. Additionally, *rolling stone (tecta)* mutants display normal otolith
development until 72 hpf when the sagitta detaches from the sensory epithelium [14]. An
initial study of zebrafish Otol1 had focused on its morpholino-mediated knockdown of its
paralog, *otol1a*, which does not appear to be required for otolith nucleation and early
attachment during zebrafish inner ear development [12].

Similar in structure to mammalian Otol1, both zebrafish paralogs are predicted to be
secreted proteins as they contain functional globular c1q domains and several collagen-like
Gly-X-Y domains that can bind extracellular Oc90 (Figure 4.1) [16, 44]. It is predicted that
the c1q domain of Otol1a can form multimers [85]. While the full length of Otol1b has not
been cloned, predicted conservation of the c1q domain in the Otol1b open reading frame
suggests it can form multimers as well, which is necessary for scaffold formation [85, 86].
No Otolin-like protein has yet been identified to mediate attachment to vestibular hair cells
in zebrafish. Since the role of *otol1b* remains unknown, this study is significant in that it addresses whether Otol1-mediated functions are evolutionarily-conserved within the inner ear.
Figure 4.1 Structural similarity between Otol1 homologs: Predicted secondary structure models of (A) human Otol1 and the two zebrafish paralogs, (B) Otol1a and (C) Otol1b exhibit similar structures, which implies that they have conserved functional roles during development. [44]
Results

Temporal and Spatial Expression of \textit{otollb} during Inner Ear Development

I originally hypothesized that \textit{otollb} may be an unknown OPBF, which is expressed by the tether-cell, and helps to mediate otolith nucleation by binding other OPPs such as Oc90 [4, 14, 20]. Using publicly available RNA-seq data on adult zebrafish inner ear tissue [65], I found that \textit{otollb} expression was enriched in mechanosensory hair cells compared to non-sensory support cells (5.80 fold change). I confirmed the expression of \textit{otollb} by \textit{in situ} hybridization, showing punctate expression within the anterior and posterior macula of the inner ear at 48 hpf (Figure 4.2A). A notable feature of the OPBF is that it is expressed during the critical period of otolith nucleation and biomineralization. Using RT-PCR, our lab confirmed \textit{otollb} is expressed at the time of otolith seeding, approximately 18 hpf (Figure S6).

Localization of Otol1b during inner ear development

Teleost Otol1 was first isolated by Murayama \textit{et al.} in over a decade ago [87]. Expression of their Chum salmon anti-Otol1 antibody has demonstrated zebrafish Otol1 is highly concentrated in the lapillus and sagitta at 72 hpf [12]. This suggested that an Otolin-like protein is expressed in the otolithic membrane prior to 35 hpf, when Otol1a is thought to be expressed. The most obvious candidate is its paralog, Otol1b. Using an anti-Otol1b antibody, I unexpectedly observed concentrated expression of protein within the cuticular plate of hair cells along with diffuse expression in the lapillus and sagitta as early as 18 hpf (Figure 4.2B,C). By 48 hpf, Otol1b expression can be detected within the otolithic membrane of the utricle and saccule in WT embryos (Figure 4.2C). When looking at
expression in zebrafish mutants that have otolith agenesis and mutations in pks1 (csr and nco) [88], Otol1b is concentrated near the kinocilia tips in addition to expression within the cuticular plate (Figure S7).
Figure 4.2 Otol1b localization changes during inner ear development: (A) In situ hybridization of *otollb* showed that mRNA expression is limited to the anterior (AM) and posterior (PM) sensory macula within the inner ear. An Otol1b-specific antibody revealed that Otol1b is expressed in (B) both anterior and posterior otoliths at 21 hpf and (C) near the apical surface of mechanosensory hair cells at 27 hpf and (D) otolithic membrane at 48 hpf.
Generation of \textit{otollb} morphants and crispants

Using a morpholino designed against \textit{otollb}, I discovered aberrantly small and fragmented otoliths that were firmly attached at 27 hpf in morphants, suggesting that Otol1b is not involved with tethering to the hair cell (Figure 4.3). The otic phenotype precedes that of its paralog, \textit{otolla}, whose morphants appeared normal until after 35 hpf when the sagitta detached from the sensory macula [12]. While otoliths remain attached after 72 hpf, the morphants soon become necrotic at the concentrations tested, even while suppressing \textit{p53} activation [19]. My first attempt at generating a germline crispant (T81*) yielded a normal looking embryo with no noticeable otic phenotype in the F2 generation.
Figure 4.3 Morpholino knockdown of *otol1b*: (A) Frequency of otic phenotypes within controls (n=40) and *otol1b*-morphants (n=39). Images of otic vesicles for control with normal otolith development (B) and *otol1b* morphants with disrupted otolith formation (C-C’) at 27 hpf.
Discussion

The first studies of fish Otolin-1 focused on a peptide isolated from Chum Salmon (Oncorhynchus keta), which detected Otol1 expression within the sagitta, lapillus, and otolithic membrane of teleosts [28, 87]. Morpholino-mediated knockdown of zebrafish otolla indicated that it is not involved with otolith formation and early attachment to the sensory macula. This is supported by data showing zebrafish Otol1a is only detected in the otolithic membrane (Figure 5.1B). The expression patterns of mammalian Otol1 is conserved between both zebrafish paralogs as Otol1b is expressed in both the lapillus and sagitta during early development and both Otol1a and Otol1b are both detected in the otolithic membrane at 48 hpf.

The novelty of zebrafish Otol1b is that it is expressed in the cuticular plate. The cuticular plate is an F-actin rich region near the apical surface of the mechanosensory hair cells that provides a rigid foundation for the stereociliary bundles [89]. Pollock et al. suggests two models for cuticular plate development. The first model assumes that the cuticular plate forms prior to stereocilia development, while the other model proposes that the stereocilia grow into the developing cuticular plate. Our data suggests that the first model is correct, as Otol1b is expressed in the apical region of tether cells at 18 hpf, which precedes stereocilia development (~22 hpf). Otol1b is expressed in vestibular and auditory hair cells, which might suggest that balance and hearing are affected in its absence. Mutations in putative genes that are expressed in the cuticular plate have resulted in auditory and vestibular disorders. For example, mice with mutations in plastin 1 or LMO7 have progressive hearing loss [90, 91]. Additionally, it worthwhile to examine whether knockdown of otollb would affect attachment of the stereocilia or kinocilia to the hair cell.
It appears that there is gene compensation when a premature stop codon is introduced in *otollb* by Cas9 as truncated mRNA likely activates ancestrally-similar genes [36]. For example, Spark-like 1 can compensate for Oc90 during otoconial nucleation in *oc90*-null mice [92]. This might suggest that another gene, such as *otolla*, is over-expressed in the crispants to compensate for the lack of Otol1b. An alternative approach would be to excise the transcription start site so that the mutant allele does not activate these compensatory genes [93, 94]. The morphant phenotype shows markedly small otoliths that are still attached to the sensory macula, showing distinct differences between the zebrafish *otoll* homologs. However, the induction of non-specific phenotypes clouds the putative function of Otol1b. Regardless, novel localization in the cuticular plate expands on the role of *otoll* in inner ear development and function. Future studies will attempt to clarify the potential roles of the cuticular plate in auditory and vestibular function.
CHAPTER 5

OTOLIN-1a AS A BIOMARKER FOR VESTIBULAR DYSFUNCTION IN ZEBRAFISH
Abstract

Within the inner ear, dislodgment or degradation of calcium carbonate bio-crystals (otoconia) can result in an acute sensation of vertigo and loss of balance. This can leave an individual susceptible to falling, which is the leading cause of accidental death in the elderly. The most common form of this vestibular disorder among human adults is known as Benign Paroxysmal Positional Vertigo (BPPV). Although the exact mechanisms of BPPV are still unknown, elevated plasma levels of a key inner ear protein, Oto11, have been proposed to be an early clinical marker for BPPV susceptibility. I examined Oto11a (a homolog of mammalian Oto11) levels in blood plasma of zebrafish that are genetically predisposed to vestibular defects and/or susceptible to vertigo following exposure to environmental factors. I found that Oto11a is normally expressed within the otoconial membrane between vestibular hair cells and otoconia. It could not be detected within the blood plasma of WT or tecta-null zebrafish at four months of age. At 1.5 years old, tecta-null adults had elevated levels of plasma Oto11a compared to normal clutch-mates. This suggests an age-related increase in plasma Oto11a levels. Additionally, plasma Oto11a levels were elevated in age-matched ‘astrofish’ compared to normal gravity clutchmates. Lastly, in aged zebrafish and ‘astrofish’, tecta-null fish exhibited abnormal dorsal-light reflexes that exacerbates the incidence of vertigo. Our results suggest the otoconial membrane is degraded before the onset of vertigo, which causes an increase in Oto11a plasma levels. Altogether, Oto11 plasma levels could be used to monitor risk for vestibular dysfunction because of genetic and environmental factors.
Introduction

The deflection of bio-mineralized crystals attached to vestibular hair cells is necessary for maintaining bodily balance. Dislodgment or degradation of these crystals, called otoconia, can result in periodic episodes of dizziness and loss of balance that can lead to falls and bone fractures. The most common form of this balance disorder is known as Benign Paroxysmal Positional Vertigo (BPPV), which affects 20-40% of patients diagnosed with vertigo [95-97]. BPPV has been recognized as the most common vestibular disorder among human adults and the elderly with a lifetime prevalence of 2.4 percent [42, 95, 96]. While the molecular etiology of BPPV remains unknown, it is characterized by otoconial particles dissociating from the utricle and relocating to the semicircular canals (canalithiasis) or cupula (cupulolithiasis) forming an acute sensation of vertigo [42, 97]. Translocation of otoconial fragments expose mechanoreceptors on vestibular hair cells to atypical responses to gravity and linear acceleration [83]. As a result, this can lead to sudden feelings of disequilibrium. Although the body can compensate for these changes over time [42], acute vertigo can leave a person vulnerable to falls, which is the leading cause of accidental death in the elderly [97, 98].

Elevated plasma levels of a key otoconial protein, Otol1, has been proposed as an early clinical marker for BPPV susceptibility [99] While the exact mechanism that leads to increased serum Otol1 levels remains unknown, it is thought that Otol1 is capable of crossing the blood-inner ear labyrinth (IEL) barrier following potential otoconial degeneration [99]. Otol1 would be an ideal marker for otoconia health as it is only expressed in the mammalian inner ear [9]. It acts as the primary scaffold protein forming a hexagonal substructure of fibrils that allows for Oc90 attachment and subsequent calcium
carbonate deposition [83]. Loosening of these Otolin fibrils can lead to otoconial detachment, which could contribute to the etiology of BPPV [83]. This study will help to clarify the importance of Otol1 during disease onset and will be the foundation for future studies that can investigate preventative treatments for patients at an increased risk of otoconial degeneration and dislodgement. To accomplish these goals, I hypothesize that Otol1a (a zebrafish homolog of mammalian Otol1) blood plasma levels would be elevated in fish that were (1) genetically predisposed to vestibular defects (i.e. carry nonsynonymous mutations in the gene tecta) and/or (2) susceptible to vertigo following exposure to microgravity (termed ‘astrofish’).

Results and Discussion

Genetic model of BPPV: tecta-null zebrafish

In both zebrafish and mammals, several components of the otolithic membrane have been identified including otog, otogl, otolla, tecta, and tectb [9, 12, 14, 15, 17, 84]. In order to generate a genetic model of vestibular dysfunction, our lab focused on tecta, as mutations in humans can cause deafness and vestibular dysfunction [100]. Furthermore, it’s predicted that Nidogen domain of Tecta binds to the Otol1 collagenous Gly-X-Y domains [101]. This might suggest that without Tecta, Otol1 would be unable to correctly incorporate within the otolithic membrane.

In zebrafish, mutations in tecta show a wide range of phenotypes in the saccular otolith (sagitta), including malformations and otolith detachment from the sensory macula [14]. Even though tecta-null embryos exhibit normal growth and attachment of the utricular otolith (lapillus), adult tecta-null fish begin to show abnormal dorsal-light reflexes and
sporadic circular swimming behaviors at approximately 1.5 years of age [14]. Altogether, these suggest that the fish with this mutation are experiencing acute onsets of vertigo that are observed in the age-related onset of BPPV incidence.

**Age-related increase of plasma Otol1a in tecta-null zebrafish**

While mammalian Otol1 is exclusively expressed atop vestibular hair cells within the inner ear [9], zebrafish Otol1a is expressed within the otolithic membrane of both auditory (saccule) and vestibular (utricle) organs (Figure 5.1B). While an otoll-null mouse has not been described yet, morpholino-mediated knockdown of otoll in zebrafish exhibits defects in otolith tethering and circling swimming behaviors [12]. While I could not detect Otol1a within the blood of 4-month old fish (SL 3cm) (Figure 5.2C), we detected Otol1a in the blood of fish aged over 1.5 years (Figure 5.2A). This supports human data showing an age-related increase of Otol1 plasma levels in individuals [102]. When looking at my proposed zebrafish BPPV-model, I show increased levels of Otol1a in aged tecta-null fish compared to WT clutchmates (Figure 5.2B). Again, this supports human data showing that individuals with an increased risk for BPPV have increased levels of plasma Otol1 [99].
Figure 5.1 Localization of Otol1a in the inner ear: (A) Confocal image of the otolithic membrane at 27 hpf showing Otog and Tecta as putative components. (B) At 48 hpf, Otol1a is expressed between mechanosensory hair cells and otoliths located in the utricle (above) and the saccule (data not shown). Scale bar=10μm
Figure 5.2 Otol1a elevated in aged vertigo-susceptible and in microgravity-exposed zebrafish: (A) Representative immunoblot showing increased plasma Otol1a expression in tecta-null zebrafish at approximately 1.5 years of age. (B) Quantitation of immunoblot in A. (C) Representative immunoblot showing no detectable plasma Otol1a in WT fish at 4 months old (SL 3cm). (D) Representative image of plasma Otol1a levels in astrofish following 5 days of microgravity exposure. (E) Quantitation of C and D with bar indicating average (n=12).
Environmental model of vestibular dysfunction: Microgravity exposure

Following microgravity exposure, individuals are susceptible to several neuro-vestibular symptoms that can last from several minutes to more than a week. These symptoms include space-motion sickness, spatial disorientation, muscle fatigue, which can lead to falls, dizziness, and vertigo [103]. Microgravity can also disrupt the vestibulo-ocular reflex (VOR) which stabilizes vision by compensatory eye movements based on signals from the vestibular organs [103, 104]. Additionally, asymmetry in otolithic mass of the sagitta and lapillus can lead to interruptions in the perception of motion and spatial orientation [105]. These observations are not limited to humans. For example, it has been demonstrated that cichlids (*Oreochromis mossambicus*) are susceptible to the effects of “motion sickness” due to the organizational changes of otolithic protein matrices in response to gravity [106].

Microgravity exposure increases plasma levels of otolithic membrane proteins

Following five days of microgravity exposure in a Synthecon Rotary Culture Max, 4-month old astrofish (SL 3cm) had detectable and significantly higher Otol1a plasma levels compared to normal gravity clutch-mates. Additionally, another otolithic membrane protein, Tecta, was detected in the blood plasma (data not shown). Immediately following microgravity exposure, all astrofish were lethargic (n=12), and those that were genetically-predisposed to vertigo (*tecta*-null) had abnormal dorsal-light reflexes and circling swimming behaviors (n=4). These behavioral responses by *tecta*-null astrofish have not been observed in normal gravity *tecta*-null fish until they are older than approximately 1.5 years old. This suggests that microgravity exposure triggers early onset of vertigo in fish with a genetic susceptibility. These observations were likely not a result of stress responses
as we did not detect significant differences in cortisol levels of astrofish compared to normal gravity clutchmates (Figure S8) [107].

Microgravity exposure alters morphology of utricular hair cells

The typical morphology of inner ear hair cells can be characterized as columnar in form and span the entire otic epithelium [26]. Hair cells can be identified by a single kinocilium and stereociliary bundles on their apical surface. In astrofish, I observed severe changes in the morphology of hair cells in the adult utricle (Figure 5.3). Additionally, utricular hair cells underwent apical constriction, which was not observed in WT. This change in hair cell morphology (specifically, apical constriction) suggests that hair cell function is attenuated following microgravity exposure [108].
Figure 5.3 Abnormal morphology of hair cells following microgravity exposure: (A) WT control utricular hair cells at 4 months of age with normal morphology. (B) Utricular hair cells from astrofish at 4 months of age exhibit apical constriction. Scale bar=20µm.
Effects of microgravity on zebrafish larvae

A previous study on the effects of microgravity on inner ear development identified a critical period for normal vestibular function between 72 hpf and 120 hpf [104]. I sought to exploit this critical period to determine whether or not the otolithic membrane would be altered or degraded following microgravity exposure. In order to mimic the effects of microgravity, we placed zebrafish larvae in a Synthecon rotary cell culture system (RCCS) at 72 hpf and removed them at 120 hpf. When looking at the ratio of Oto1l1a to Pou4f3 expression (Figure 5.4A,B), I observed no significant changes in mean fluorescence intensity (MFI)(Figure 5.4C).

While there were no gross morphological differences within the inner ear, I wanted to test whether there were any significant changes in behavior of larvae exposed to microgravity. Using a prepulse inhibition assay to detect the startle response of individuals to external stimuli, the larvae experienced no significant change in their latency response times or distance travelled (Figure 5.5A,B) [109]. The response rates for both WT and microgravity-exposed larvae were comparable to previously recorded data with a similar stimulus frequency (Figure 5.5D)[109]. However, the displacement of WT larvae was increased compared to larvae exposed to microgravity suggesting a reduction in vestibular function (Figure 5.5C). Future studies will assess larval responses to vibrational stimuli at higher frequencies (i.e. more than the tested 440 Hz).
Figure 5.4 Effects of microgravity on the zebrafish larval inner ear: Representative images of WT (A) and microgravity-exposed (B) adult zebrafish utricles at 5 dpf showing no significant difference between the ratio Oto1a MFI over Pou4f3:mGFP MFI (n=4, p=0.2775, unpaired t-test). Scale bar=20µm.
Figure 5.5 Prepulse inhibition assay of microgravity-exposed larvae: There was no significant change in the (A) latency response ($n=33$, $p=0.1487$, unpaired t-test) or (B) distance travelled ($n=33$, $p=0.2286$, unpaired t-test) between groups. (C) Displacement of microgravity-exposed larvae was decreased compared to WT ($n=33$, $p=0.0552$, unpaired t-test). (D) Response rates for microgravity-exposed larvae was slightly decreased compared to normal gravity controls ($n=33$, $p=0.2905$, Fisher’s exact test).
Application and Significance

In this study, I characterize two novel models of vestibular dysfunction that coincide with elevated plasma levels of an otolithic membrane protein. These results support observations of elevated Otol1 plasma levels seen in the aging adults and persons susceptible to BPPV [99, 102]. The tecta-null zebrafish provides a testable avenue for potential therapeutic intervention to decrease the incidence of vertigo. However, I still do not know when vestibular impairment starts relative to Otol1a plasma levels. A follow-up to this study would be to compare Otol1a plasma levels with fish at various stages to determine if the degradation of the otolithic membrane precedes vestibular impairment to allow for intervention.

The utility of a blood plasma biomarker would make it possible to assess an individual’s ability to adapt and/or readapt to transitions in gravity. The predictive measure of a blood plasma biomarker could clarify an individual’s capacity for sensorimotor adaptation to microgravity exposure and countermeasures could be individually customized to reduce the incidence of vestibular dysfunction [110]. However, elevated Otol1a plasma levels in all astrofish may suggest that this protein might not be applicable as a diagnostic measure. Future studies will quantify vestibular function (e.g. measuring VOR) in relation to otolithic membrane protein plasma levels.

Altogether, this opens the possibility to use blood plasma levels of otoconial membrane components as prognostic biomarkers for predicting and, potentially, preventing the onset of vestibular dysfunction.
APPENDIX

SUPPLEMENTAL MATERIAL
Figure S1 No detectable calcium deposits in the csr otocyst: Brightfield images of 5 dpf WT (A) and csr mutant (C). Alizarin-staining of WT (B) and csr (D) shows no complex calcium deposits in csr during early development. Arrows indicate otocyst.
Figure S2 Spatial differences in mitochondrial membrane potentials: (A) While Mitotracker marks active mitochondria in WT, (B) csr embryos show a lack of Mitotracker expression during early development. Arrow indicates otic vesicle.
Figure S3 Japanese medaka and zebrafish Pks1 is highly conserved: Similar secondary structures of (A) zebrafish Pks1 and (B) Japanese medaka Pks1 imply that their roles are conserved even though they are separated by 150 million years of evolution. (C) Ensembl genomic data comparison of Japanese medaka pks1 and zebrafish pks1 show a high rate of homology.
Figure S4 miR-92a binding site in the 3’ UTR of *rdh12l*. TargetScanFish 6.2 of *rhdl2l* in zebrafish shows potential microRNA binding sites including *miR-92a*, which is the most down-regulated gene in *nco* embryos at 24 hpf.
Figure S5 Diffuse expression of otolith matrix proteins within the otocyst of csr embryos: Normal expression of otolith matrix proteins (A,C). Starmaker (B) and Keratan Sulfate Proteoglycans (D) show diffuse expression inside the otocyst of the csr mutant. Anterior to left. Scale bar=20μm
Figure S6 Expression of *otollb* during the critical period of otolith nucleation: RT-PCR results (on total embryonic RNA) showing *otollb* expression at 18 hpf.
Figure S7 Expression of Otol1b in mutants that exhibit otolith agenesis: (A) Normal expression of Otol1b is restricted to the cuticular plate located near the apical surface of mechanosensory hair cells at 27 hpf. (B) In nco, Otol1b expression appears normal whereas (C) Otol1b expression in csr is located near the tips of kinocilia. Scale bar=10µm.
**Figure S8 Stress cortisol assay shows no difference in stress response:** A comparison of cortisol levels between astrofish and normal gravity clutchmates ($p=0.2272$, unpaired t-test).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-type</th>
<th>Otoliths absent</th>
<th>Total (n)</th>
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<tr>
<td>csr</td>
<td>83.81%</td>
<td>16.19%</td>
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<tr>
<td>nco</td>
<td>80.70%</td>
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<td>csr x nco</td>
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<td>csr + L905P mRNA</td>
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Table S1 Frequency of WT and mutant phenotypes for un-injected and injected csr, nco, and vns embryos.
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<th>ORF RPKM</th>
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<td>Support cells SD</td>
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Table S2 Differential expression of pks1 in adult zebrafish hair and support cells. Fold change (Log2) values of pks1 expression shown for adult inner ear hair cells and support cells. SRA=Sequence Read Archive; RPKM=Reads Per Kilobase Million; ORF=Open Reading Frame.
REFERENCES


