The Pennsylvania State University

The Graduate School

College of Medicine

MOLECULAR DETERMINATION OF NOVEL GENES AND PATHWAYS REQUIRED FOR VESTIBULAR MORPHOGENESIS IN ZEBRAFISH

A Dissertation in

Genetics

by

Jessica Ann Petko

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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

August 2008
The dissertation of Jessica Petko was reviewed and approved* by the following:

Robert Levenson  
Professor of Pharmacology  
Dissertation Advisor  
Chair of Committee

Keith C. Cheng  
Professor of Pathology

Laura Carrel  
Assistant Professor of Biochemistry and Molecular Biology

Victor A. Canfield  
Assistant Professor of Pharmacology

Sarah K Bronson  
Associate Professor of Cellular and Molecular Physiology  
Co-Chair, Intercollege Graduate Program in Genetics

*Signatures are on file in the Graduate School
The vestibular system of the vertebrate inner ear functions to detect gravity and motion in order to maintain balance. Functionally there are two divisions of the vestibule: the semicircular canals which detect angular acceleration and the otolith organs which perceive linear movements. Each division, although structurally distinct, detects movement and gravity through the deflection of mechanosensory hair cells.

The semicircular canals are three fluid filled tubes arranged in different orthogonal planes. Angular rotation of the head creates uneven flow of fluid through the canals and over the sensory hair cells that lie at each terminal, which can be translated to the brain via the eighth cranial nerve. The otolith organs, including the utricle and the saccule, are each composed of a patch of sensory hair cells associated with an ear stone composed of calcium carbonate. During linear acceleration, the stone, known as the otoconial matrix in mammals and the otolith in fish, serves as an inertial force for deflecting and stimulating the sensory hair cells below.

Combinatorial expression of many genes expressed within the hindbrain and the developing otic vesicle induce morphogenesis of inner ear structures involved in auditory and vestibular function. Dysfunction in these genes has been associated with deafness, balance disorders, and various craniofacial abnormalities. Although some genes that influence ear development have been elucidated, there are likely many more that have remained undiscovered. The goal of my research is to use zebrafish as a tool for the
identification and characterization of genes that participate in vestibular development. I have discovered several genes that are expressed in the zebrafish otic vesicle during embryogenesis, and I have shown that these genes are essential for the formation of vestibular structures.

The first genes that I analyzed were the two zebrafish orthologs of Pten, a well studied tumor suppressor gene that is involved in many human cancers. This gene is known to control cell growth and proliferation through its negative regulation of the Akt pathway. The zebrafish orthologs, *ptena* and *ptenb* are expressed during embryonic development, and their gene products are able to regulate the levels of phosphorylated Akt in embryogenesis. The two genes show distinct expression patterns, and predictably, knockdown of the individual genes also produce distinct phenotypes. *ptena*, but not *ptenb*, is expressed in the otic vesicle. Knockdown of *ptena* produces otolith defects at 24 hpf (hours post fertilization) and semicircular canal defects at 48 and 72 hpf. Knockdown of *ptenb* does not affect inner ear development. These results suggest that the function of one ancestral Pten may have been divided between two orthologs after the genome duplication in teleost fish. I also conclude that control of cell growth and survival may play a role in the formation of these vestibular structures.

I have identified and investigated a novel zebrafish ortholog of a mammalian gene involved in otolith development. The mammalian otolith organs contains thousands of minute biomineralized particles called otoconia, whereas the inner ear of teleost fish
contains three large ear stones called otoliths that serve a similar function. Otoconia and otoliths are composed of calcium carbonate crystals condensed on a core protein lattice. Otoconin-90 (Oc90) is the major matrix protein of mammalian and avian otoconia, while Otolith Matrix Protein-1 (Omp-1) is the most abundant matrix protein found in the otoliths of teleost fish. This difference in major matrix protein composition has been hypothesized to account for the morphological differences observed between mammalian otoconia and zebrafish otoliths. Therefore, it was unexpected that orthologs for these matrix proteins would be found other species. I have identified a novel gene, \textit{otoc1}, which encodes the zebrafish ortholog of Oc90. Expression of \textit{otoc1} is detected in the ear between 15 hpf and 72 hpf, and is restricted primarily to the macula. During embryogenesis, expression of \textit{otoc1} mRNA precedes the appearance of \textit{omp-1} transcripts. Knockdown of \textit{otoc1} mRNA translation with antisense morpholinos produces a variety of aberrant otolith phenotypes. My results suggest that Oc90 orthologs may serve to nucleate calcium carbonate mineralization of zebrafish otoliths, and that this protein is not strictly involved in determining mammalian otoconial morphology.

Previous experiments have shown that a zebrafish ortholog of Neuronal Calcium Sensor-1 (Ncs-1) is required for the formation of semicircular canal hubs in zebrafish otogenesis (Blasiole et al. 2005). In order to gain further insight into the pathways involved in this Ncs-1 dependent process, I studied the role of Ncs-1 interacting proteins (NIPs) in vestibular development. A yeast-2-hybrid screen was performed to identify novel NIPs. Several of these newly identified and other previously known interactors were analyzed as candidates for otogenic genes. I determined that many zebrafish NIP orthologs are
expressed in the developing semicircular canal structure. Morpholino knockdown of three of these genes, \textit{arf1}, \textit{pi4kβ} and \textit{dan} has demonstrated that these genes are indeed important for vestibular morphogenesis. Combinatorial knockdowns have also been used to show that \textit{arf1}, \textit{pi4kβ}, and \textit{ncs-1a} functionally interact. These functional interactions and direct physical associations suggest that these genes are involved in a unified pathway during inner ear development.
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<table>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium Ions</td>
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<td>CO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
<td>Carbonate Ions</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>Distance Matrix</td>
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<tr>
<td>dpf</td>
<td>Days Post Fertilization</td>
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<td>EST</td>
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<td>hpf</td>
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<td>Millimolar</td>
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<tr>
<td>MO</td>
<td>Morpholino Oligonucleotide</td>
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<td>MP</td>
<td>Maximum Parsimony</td>
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<tr>
<td>mRNA</td>
<td>Messanger Ribonucleic Acid</td>
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<td>N</td>
<td>Number</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PLA2</td>
<td>Secretory Phospholipase A2</td>
</tr>
<tr>
<td>PLA2L</td>
<td>Secretory Phospholipase A2 Like</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>RACE</td>
<td>Rapid Amplification of cDNA Ends</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecylsulphate</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast-Two-Hybrid</td>
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I would like to offer my sincerest gratitude to all of those who have contributed to the completion of my graduate work. First, I would like to thank Dr. Robert Levenson, my thesis advisor and mentor, for his unending support and encouragement and for providing an environment in which I could work and think independently. I am grateful to the members of my thesis committee, Drs. Victor Canfield, Keith Cheng, and Laura Carrel, for their advice and support throughout my graduate school career. I especially thank Dr. Victor Canfield for his technical advice and support.

Secondly, I would like to thank the past and present members of the Levenson Lab who have made the lab an enjoyable place to work. Each and every member of the lab has contributed to my education through their support, knowledge, and friendship.

I extend gratitude to my collaborators, including Drs. Isolde and Reudiger Thalmann, Drs. Bernard and Christine Thisse, Dr. Inna Hughes, Dr. Bruce Riley, and Bonny Millimaki, for their assistance and advice. I am also indebted to the faculty and staff of the Pharmacology Department and the Genetics Program. In particular, I thank Marie Duvall and Kathy Shuey for their guidance and friendship. I also appreciate Dr. Sarah Bronson for giving me the opportunity to help out with the genetics symposium and meet my idol, Nancy Hopkins.
Finally, I want to acknowledge my friends and family members. I thank my parents and my husband for their continued love and support throughout my schooling. I am truly lucky to have a mother who has encouraged me to receive the favor that I deserve and to do all that is in my heart.
Chapter 1

Literature Review
1.1 Structure and Function of the Vertebrate Vestibular System

1.1.1 Overview of the Inner Ear

Although hearing and balance are functionally different senses, information about sound, motion, and gravity are processed by similar mechanisms within the vertebrate inner ear. These stimuli are all detected by the same type of specialized mechanosensory cells with the assistance of various extracellular structures. The functional diversity between auditory and vestibular systems is explained by the structural variation within the individual compartments of the inner ear. While the auditory system has evolved in structural complexity, the anatomy and function of the vertebrate vestibular apparatus has remained conserved. All vertebrates sense angular movement of the head through the semicircular canal system, and they sense linear movements and gravity through the otolith organs.

1.1.2 Mechanosensory Hair Cells

The sensory epithelia of the vestibular and auditory organs are composed of the mechanosensory hair cells and the accessory supporting cells (Fig. 1.1, A). Sound, motion, and gravity are perceived by the mechanical stimulation of the sensory hair cells. Hair cells are named for the array of stereocilia extending from the apical end of the cell into the endolymphatic interior of the membranous labyrinth (Purves et al. 2008). These microvilli vary in size and are arranged in a graduated manner from shortest to tallest.
Kinocilia, the tallest cilia, utilizes microtubules to maintain its rigid structure, while the remainder of the stereocilia is packed with actin (Flock and Cheung 1977; Sobkowicz et al. 1995). The apical end of the hair cell converts mechanical energy to receptor currents (Wever 1971; Gillespie and Walker 2001). Depolarization of the hair cell membrane allows for the release of neurotransmitters from the basal end of the cell onto the peripheral end of cranial nerve VIII (Gillespie and Walker 2001). The molecular basis of the hair bundles response to stimuli resides in the tip links, elastic filaments that connect the tip of each stereocilia to its neighboring cilia (Gillespie and Walker 2001). When the stereocilia are deflected towards the kinocilia, tension in the tip links allows for the opening of mechanically sensitive nonselective cation channels (Fig. 1.1, C), depolarization of the cell (Fig. 1.1, D), and increased transmitter release (Hudspeth and Corey 1977; Gillespie and Walker 2001). Deflection of the stereocilia away from the kinocilium (Fig. 1.1, B and D) decreases tip link tension which prevents the opening of transduction channels and generates a hyperpolarized state (decreases transmitter release). Hair cells are normally coupled to extracellular structures such as the gelatinous cupula in the semicircular canals, and the otolithic structures of the saccule and utricle (Purves et al. 2008). These structures assist in the deflection of hair cells during movement of the head.

1.1.3 Otolith Organs

Gravity and linear acceleration are perceived through the otolith organs located in the saccular and utricular compartments of the vestibular system (Fig. 1.2, A). The saccule
and the utricle each contain a single cluster of sensory hair cells and supporting cells called a macula. These sensory regions are associated with an extracellular mass of calcium carbonate. Mammalian, avian, reptilian and amphibian otolithic masses are composed of thousands of tiny polyhedral particles called otoconia that are embedded in a gelatinous membrane (Fig. 1.2, B). In contrast, teleost fish have large, singular otoliths that are smooth in appearance. Both otoliths and otoconia are composed of calcium carbonate (CaCO$_3$) crystals condensed on an extracellular protein matrix; however, the CaCO$_3$ crystal polymorph utilized and the protein composition of the organic matrix vary between species (Pote and Ross 1991). The inertial properties of this mass load during periods of linear motion allow deflection and activation of the hair cells of the maculae.

1.1.4 Semicircular Canals

The semicircular canals consist of three fluid-filled tubes which are oriented in perpendicular planes (Fig. 1.2, A) (Purves et al. 2008). At the terminal end of each canal lies a cluster of sensory hair cells termed cristae. The cupula, an extracellular gelatinous membrane, covers the hair cells and deflects the stereocilia in response to fluid flow (Fig 1.2, C and D). Angular movement of the head causes a disproportional flow of
endolymphatic fluid through each canal and a differential activation of each cristae. These inequalities are translated from the hair cells to the brain via the eighth cranial nerve.

1.2 Zebrafish as a Developmental Model

1.2.1 General Model System Information

Zebrafish, *Danio rerio*, are fresh water fish that originate from streams in the Ganges River basin of East India and Burma. In the late 1970’s, Dr. George Streisinger introduced the scientific community to the power of the zebrafish as a developmental model organism. Zebrafish are attractive for the study of vertebrate development as they are small enough to maintain in large numbers as adults, and yet their embryos are large enough for classical embryonic manipulations. Unlike mammals and some other fish species, in zebrafish fertilization occurs externally allowing simple physical, molecular, and/or genetic manipulations and developmental observation from the very beginning of embryogenesis. The zebrafish embryo remains transparent from zygote formation through embryonic stages. Chemical inhibition of pigmentation after 24 hpf (hours post fertilization) by phenythiourea (PTU) can extend the transparency of the fish through early larval stage (Karlsson et al. 2001). The transparent nature of the embryo and larva allow for observation and comparison of organogenesis between normal and genetically altered states. Embryo transparency has also contributed to the success of using whole
mount *in situ* hybridization for determining gene expression during early development (Thisse and Thisse 1998).

Online resources for zebrafish have expanded significantly since the turn of the century. Sequencing of the zebrafish genome began in 2001 at the Sanger Institute in Cambridge, UK. The project incorporated two strategies: the traditional mapping and sequencing of BAC libraries and whole genome shotgun sequencing. As of January 12, 2008 the project is estimated to be 76% complete (Sanger 2008). The genome sequences are annotated and released semi-annually to Ensembl (www.ensembl.org) for use by the scientific community. There is also an extensive collection of Expressed Sequence Tags (ESTs) that have been generated, sequenced, and annotated by the Washington University Zebrafish Genome Resource (WUZGR) group. There are many sets of ESTs that were generated from different periods of development and/or different tissue types. These sequences are searchable on both GenBank (www.ncbi.nlm.nih.gov/) and Ensembl and are available for purchase.

Several procedures and technologies have been developed to alter gene expression or protein function in zebrafish. The most popular technique for reverse genetics in

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**Figure 1.2**: Anatomy of the Vestibular System

(A) An anatomical representation of the inner ear. (B) Cross section of the utriculæ macula showing hair bundles projecting into the extracellular otoconial membrane. (C) The position of the cupula without (left) and during (right) angular acceleration. Adapted from *Neuroscience* (Purves et al. 2008).
zebrafish is knockdown of gene expression using morpholinos. Morpholinos (MOs) are antisense oligonucleotides that bind complementary sequences in mRNA (Stirchak et al. 1989). This binding results in a steric blockage of the translation initiation complex or pre-mRNA splicing machinery, which, in turn, leads to decreased translation products or altered splice products, respectively (Fig. 1.3). In addition, morpholinos can bind and block the activity of miRNAs and ribozymes. Morpholinos are favorable over other RNAi techniques because their mode of action does not require RNAse H or RISC mediated mRNA degradation (Summerton 2007).

Morpholinos are different from natural nucleic acids in that the ribose or deoxyribose sugar moieties are replaced with a morpholine ring (Fig. 1.4) and the anionic phosphates are substituted with non-ionic phosphodiamidate linkages. These modifications allow suitable positioning of the DNA bases to bind its complementary site on mRNA via Watson Crick base pairing (Kang et al. 1992). Also, the morpholino oligo is not recognized by cellular enzymes or nucleases making them stable and unable to trigger an innate immune response (Hudziak et al. 1996). Morpholinos are delivered via microinjection into the yolk of a newly fertilized zebrafish zygote (Nasevicius and Ekker 2000). Gene knockdown is effective for 48-72 hours post fertilization (hpf) at which time the morpholino becomes too dilute due to cellular division. Therefore, morpholinos are most useful for determining gene function during early development from embryogenesis to the early phases of larval development. Electroporation techniques have been utilized by some researchers to introduce morpholinos to particular tissues later in development or adulthood (Thummel et al. 2006).
Figure 1.3: Mechanisms of morpholino functionality.

(A) Normal translation. The ribosome (purple) bound to the mRNA (blue) is translating the sequence into a peptide (orange). (B) Translation blockage by a morpholino. A morpholino (green) that binds the mRNA at the translation initiation codon or the 5’ untranslated region will block the ribosome from translating a peptide. (C) Splice-blocking morpholino. A morpholino targeting a splice donor or acceptor site in pre-mRNA will block the activity of the splicing machinery at that site. Splice site morpholinos may result in mature mRNA that is missing an exon, including an unspliced intron, or another abnormal splicing event due to a cryptic splice site.

Figure 1.4: Chemical structure of nucleic acids vs morpholinos.

(A) Structure of a ribose ring. (B) Structure of a deoxyribose ring. (C) Structure of a morpholine ring. (Summerton 2007)
1.2.2 Zebrafish as a Model for Inner Ear Development

1.2.2.1 Evolutionary Conservation of Inner Ear Anatomy

The inner ear of all vertebrates is composed of two functional divisions, the vestibular and auditory systems. However, structurally and evolutionarily the inner ear can be divided into the pars superior and the pars inferior. The term pars superior refers to the more anterior and dorsal structures, namely the semicircular canals and the utricle. The pars inferior is more ventral and posterior and is comprised of the saccule, lagena, and macula neglecta in fish; the sacule, lagena, basilar papilla and amphibian papilla in amphibians; the saccule, cochlea, and lagena in birds; and the saccule and cochlea in mammals. Schematics of the inner ear for each of these species are shown in Fig. 1.5 (Riley and Phillips 2003). The pars superior has changed very little in structure and function throughout evolution; however, the pars inferior has evolved a more complex structure and become adapted for sound detection in a variety of habitats.

In bony fish the structures of the inner ear are primarily cartilaginous and incorporate bone to a small degree (Carey and Amin 2006). In teleost fish, such as zebrafish, auditory sensitivity has been associated with all of the sensory regions in the pars inferior: the saccule, the lagena, and the macula neglecta. The zebrafish is a member of the Ostaryophysans, a group of fish that couple the saccule to the swim bladder through a set of bones called the webberian ossicles (Bang et al. 2001). This system transfers sound induced vibration of the swim bladder to the inner ear to enhance hearing sensitivity.
The amphibian pars inferior contains the sacule, lagena, basilar papilla and amphibian papilla (Bang et al. 2001). The lagenar pouch is reduced as compared to those of bony fish, and the saccule takes on a more anterior position. Although the saccule plays roles in motion and gravity sensation, it has also demonstrated the ability to detect sound stimuli (Lewis and Narins 1998). The papillae are the principle acoustic detectors in amphibians (Wever 1985; Elephandt 1996). Interestingly, the basilar papilla is often tuned to a component of the animals mating call, whereas the amphibian papilla is a “general purpose acoustic sensor” (Lewis and Narins 1998).

The primary auditory endorgan in birds and mammals is the cochlea. The cochea is believed to have arisen from extension of the basilar papilla observed in amphibians (Carey and Amin 2006). The saccule plays a strictly vestibular role in birds and mammals. The lagena is still present at the end of the cochlea in birds and is of unknown function (Riley and Phillips 2003). In contrast, mammals do not have a lagena and the cochlea is much longer and more coiled than in birds.

The studies I report in this thesis concentrate on the development of the semicircular canals and the otoliths of the saccule and utricle. The semicircular canals and the utricule strictly play vestibular roles, but the saccule is involved in sound detection in zebrafish. This is important, because the identification of genes involved in saccular function can be attributed to auditory and vestibular function. Studies of this organ may lead to further
**Figure 1.5:** Anatomical structure of the inner ear.

Representation of adult inner ear structures of zebrafish, *Xenopus*, chick, and mouse. Each diagram is a lateral view with the anterior to the left. Auditory regions are shaded in blue. Abbreviations: ap, amphibian papilla; bp, basilar papilla; c, cochlea; l, lagena; s, saccule; u, utricle. This figure was adapted from Riley and Phillips (2003).

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**Figure 1.6:** Development of the larval zebrafish ear.

The development of the zebrafish otic vesicle from placode to semicircular canal duct formation. All diagrams are lateral views with anterior to the left.
functional characterization of genes known to be involved in deafness and vestibular
dysfunction in humans.

1.2.2 Structural development of the Zebrafish Inner Ear.

An overview of zebrafish inner ear development from placode formation to the
completion of semicircular canal formation at 72 hpf is shown in Fig. 1.6.

Otic Vesicle Formation

Development of the inner ear begins in the mid-somite stage of zebrafish embryogenesis
with the formation of the otic placode, an ectodermal thickening visible on either side of
the hindbrain by 15 hpf (Haddon and Lewis 1996). The zebrafish otic vesicle is formed
by cavitation of the otic placode into a hollow ball of epithelium. To begin this process,
the placode thickens into a solid ovoid ball just below the surface of the embryo. The
initial cells in this complex have already become polarized as indicated by a
concentration of actin in the center of the mass (Haddon and Lewis 1996). Formation of
the lumen is accomplished by the movement of the cell nuclei to the periphery of the
thickened placode, and a loss of cell junctions at the placode center (Haddon and Lewis
1996). The lumen first appears as a slit at about 18 hpf, and expands maintaining its
simple ovoid shape until 24 hpf. This process differs in amniotes such as birds and
mammals in which the otic vesicle develops from a folding of ectodermal tissues and the
pinching off a vesicle (Haddon and Lewis 1996). Interestingly the formation of the otic
vesicle resembles the formation of the neural tube in these species. While fish develop the otic vesicle and the neural keel through a hollowing process, amniotes form the otic vesicle and the neural tube through a folding and pinching process (Haddon and Lewis 1996). The otic vesicle will derive all the structures of the vestibular labyrinth and the neurons of the statoacoustic ganglion (the VIIIth cranial nerve).

**Statoacoustic Ganglion Development**

Neurons of the statoacoustic ganglion are derived from neuoblasts which delaminate from the ventral side of the otic vesicle and migrate to an area just ventral of the vesicle. Delamination begins at about 22 hpf and over the next few hours, the number of cells that delaminate and migrate increases with the peak period being between 22 and 30 hpf (Haddon and Lewis 1996). This delamination process, which generates a few hundred neuronal precursors, slows and can no longer be observed after 42 hpf. As the cells delaminate, they collect in the developing ganglion. Between 36 and 48 hpf the ganglion becomes a separate compact mass oriented ventromedially to the otic vesicle that extends beneath the anterior and the posteriomedial sensory regions. Differentiation of ganglion neuroblasts to mature neurons begins between 24 and 30 hpf. By 3 dpf many neurons have developed and their dendrites contact the hair cells within the sensory regions.
Otolith and Macula Formation

Otoliths are extracellular structures made of calcium carbonate and proteins that associate with the sensory patches of the utricle and saccule. Otolith development in zebrafish requires two steps: the seeding phase and the growth phase (Riley et al. 1997). After formation of the otic vesicle, the apical side of the otic epithelium develops a high density of cilia which protrude into the lumen (Riley et al. 1997). As the lumen expands, two classes of cilia become evident (Fig. 1.6). The more dominant type of cilia corresponds to motile cilia that beat rapidly to circulate endolymphatic fluid. The other class of cilia corresponds to kinocilia of precociously forming hair cells called tether cells. A pair of tether cells forms at each primordial macula. While the motile cilia shrink from ~4um at 19 hpf to their disappearance at about 24 hpf, the tether cell kinocilia grow during this period from ~5um at 19 hpf to ~8um by 21 hpf. The formation and degeneration of each particular type of cilia correlates to the otolith seeding process. During this first part of otolith development, numerous small particles (<0.6 um) develop and float freely throughout the endolymph. As the motile cilia circulate these otolith precursors throughout the vesicle, they begin to accrete on the lengthening kinocilia of the tether cells (Fig 1.6). This process is known as otolith seeding. By 24 hpf, otolith precursor particles are no longer observed in the endolymph, and the motile cilia disappear.

The second phase of otolith development is a slow growth phase that occurs after 24 hpf (Riley et al. 1997). This growth involves daily deposition of calcium carbonate to the otolith that continues for the lifetime of the fish (Borelli et al. 2003). As the slow growth
phase begins, the development of sensory hair cells in the macula is already underway, and the size of the macula expands proportionally to match the size of the otolith (Haddon and Lewis 1996). By 48 hpf, there are 10-20 hair cells in each macula. From 72 hpf onward, hair cells are produced at a steady rate until adulthood when each macula contains a total of roughly 13,000 hair cells. (Platt 1993)

Development of the inner ear during later larval stages involves the subdivision of the otocyst into the utricle, saccule, and lagena (Haddon and Lewis 1996). Although the utricular and saccular otoliths and macula form by 24 hpf, the division of these macula in separate compartments occurs days later. The utricle is the earliest derived otolith organ in development and encompasses the anterior portion of the otic vesicle, including the anterior macula. This structure is evident as a separate compartment after 3 dpf when the semicircular canals become isolated from the otolith organs. From 8-12 dpf, a single outpocketing containing the saccular macula is generated (Bever and Fekete 2002). This pouch will elongate and become a separate compartment by 15 dpf. A thickening of epithelium that is contiguous with the saccular macula will begin to differentiate at 15 dpf to form the lagenar macula. The lagenar otolith can develop any time between the formation of the lagenar macula and 17 dpf (Riley and Moorman 2000). A fourth macula, the macula neglecta will develop between 17 and 20 dpf (Platt 1977). This macula, which lacks an otolith, is located near the utriculosaccular canal. The hair cells of the macula neglecta, like the lagenar macula, may be derived from cells surrounding the saccular macula (Bever and Fekete 2002).
**Semicircular Canal and Cristae Development**

The development of the semicircular canals begins between 42 and 48 hpf ([Fig. 1.6](#)) with the projection of three finger-like protrusions from the otic epithelium into the lumen: one from the middle of the lateral wall (the lateral protrusion), one from the middle of the anterior wall (the anterior protrusion) and one from the middle of the posterior wall (the posterior protrusion) (Haddon and Lewis 1996). Each protrusion consists of an epithelial covering and an acellular core made of extracellular matrix proteins. At about 60 hpf, the lateral protrusion bifurcates into an anterior and posterior branch that will meet and fuse with the anterior and posterior protrusions, respectively. Within a few hours, another protrusion arises from the lateral part of the ventral wall (the ventral protrusion) to meet fusion plate of the lateral hub by 72 hpf. During the same time period, a thin dorsolateral septum extends from the lateral part of the dorsal wall to the lateral protrusion. The toroidal space surrounding the newly formed cross shaped pillars constitutes the three semicircular canals. After fusion of the epithelial pillars, invading fibroblasts replace the ECM core of the hubs. Although the three canals have formed by 72 hpf, they must grow to acquire their distinctive adult form which can be seen by 17 dpf (Bever and Fekete 2002).

Hair cells become visible with the aid of phalloid staining in two (the anterior and posterior cristae) of the three developing crista at 60 hpf, although packing of the epithelium at 48 hpf marks the region where they will form (Haddon and Lewis 1996).
The mature hair cells of the third crista appears later at 72 hpf in the middle of the lateral wall. At 5 dpf, the each of the crista contains about 20 hair cells.

1.2.2.3 Genetic determination the Vestibular System

Otic Induction

It has long been known that signals from the hindbrain are responsible for the early induction of otic tissue in many species. The best known brain-derived factors required for otic induction are members of the fibroblast growth factor of proteins. Fgf3 was first proposed as an otic inducer because its expression persists in the hindbrain primordium from the end of gastrulation through placode induction (Wilkinson et al. 1988). Application of antibodies that block the function of Fgf3 to chick explants results in a blockage in the formation of the otic vesicle (Represa et al. 1991). Targeted disruption of Fgf3 expression in mice severely disturbs patterning of the otic vesicle, but induction of the placode remains normal (Mansour et al. 1993). These studies implicated that Fgf3 merely plays a role in placode to vesicle transformation and not in otic induction. However, further investigation of Fgf3 function in Xenopus, chick, and zebrafish suggests that this protein is required for otic induction and that functional redundancy compensates for its loss in the previous studies. Misexpression of Fgf3 in Xenopus and chick embryos induces ectopic otic placode development, demonstrating that Fgf3 expression is sufficient for placode induction (Lombardo et al. 1998; Vendrell et al. 2000).
Knockdown of fgf3 or fgf8 alone causes small malformed otic vesicles in zebrafish (Phillips et al. 2001; Leger and Brand 2002; Maroon et al. 2002), while co-injection of morpholinos targeting fgf8 and fgf3 ablates placode formation. The zebrafish fgf8 mutant, acerebellar, phenocopies the fgf8 morphant, and injection of a morpholino targeted to fgf3 into acerebellar mutants results in complete blockage of inner ear development (Whitfield et al. 1996; Reifers et al. 1998). These results indicate functional redundancy in the ability of Fgf3 and Fgf8 to induce otic development. Similar studies demonstrated that Fgf3 and Fgf10 show functional redundancy in mouse otic inducion, and that Fgf19 may work in concert with Fgf3 to achieve otic induction in chick embryos (Riley and Phillips 2003).

Alternate sources of otic induction have also been identified. In zebrafish, Pbx2 and Pbx4, essential binding partners of HOX proteins, are required for hindbrain specification (Waskiewicz et al. 2002). Disruption of these genes inhibits rhombomere differentiation and the entire hindbrain takes on a rhombomere 1 (r1) identity. Despite the absence of localized r4 expression of fgf3 and fgf8 in these morphants, low levels of broad fgf8 expression throughout r1 allows the formation of a small otic vesicle that develops in its proper location. Fgf8 alone is sufficient for otic induction, however proper localization of otic vesicle formation in these morphants indicates that signals from the mesendoderm may be involved in identifying the site induction. Further support for this theory comes from disruption of the zebrafish one-eyed pinhead protein, an important cofactor for the Nodal dosalizing signal (Gritsman et al. 1999). Blockage of Nodal signaling prevents the formation of the mesendoderm and causes a delay in otic induction, although expression
of \textit{fgf8} or \textit{fgf3} in the hindbrain is unchanged (Mendonsa and Riley 1999; Phillips et al. 2001). This evidence strengthens support for a role in the mesendoderm in defining the site of otic induction.

The earliest otic-specific marker of otic induction in vertebrates is \textit{Foxi1}, a transcription factor which provides the otic precursors the ability to respond to the Fgf induction signals by activating \textit{Pax8} expression (Solomon et al. 2003). \textit{Pax8} is a paired box transcription factor which can be detected in pre-otic cells during the latter half of gastrulation (Pfeffer et al. 1998). \textit{Pax8} responds to Fgf signals by activating transcription of several otic-specific transcription factors such as \textit{Pax2a}, \textit{Sox9a}, and \textit{Sox9b} (Liu et al. 2003). Expression of these genes signifies a commitment to an otic fate and provides necessary machinery to respond to otic induction signals and to regulate placode formation. A summary of this induction cascade is shown in \textbf{Fig. 1.7}.

\textit{Formation and patterning of the otic vesicle}

Signaling from tissues surrounding the ear including the notochord, the hindbrain, and the floor plate of the neural tube have an inductive role on formation and patterning of the otic vesicle (Brigande et al. 2000a; Brigande et al. 2000b; Fekete and Wu 2002). At midsomitogenesis, the expression of several genes including \textit{Pax2a}, \textit{Dlx3b}, and \textit{Eya1} is uniformly detected throughout the otic placode (Krauss et al. 1991; Akimenko et al. 1994; Sahly et al. 1999). The only known genes with distinct expression patterns at this stage are the \textit{Delta} genes which are expressed in the anterior and posterior domains of the vesicle (Haddon et al. 1998). \textit{Hox} genes from each hind brain rhombomere impart an
Cells of the future otic placode are able to respond to Fgf signals, emanating from the hindbrain, owing to the competence factor Foxi1 (green) and induce Pax8 (red arrows) during an early phase of development (85% epiboly). Pax8 is among the first factors expressed and its activity is required for the expression of Sox9a that maintains (blue arrows) expression of a second pair of competence factors, Dlx3b and Dlx4b, in the preotic domain. Together with Fgf signals and Pax8, Dlx3b-4b is required for the proper initiation of Pax2a in a second, later phase of development (three-somite stage). Once expressed, Pax2a functions with Pax8 in an overlapping manner. Pax2a maintains its own expression and establishes a positive feedback loop through Sox9a and Dlx3b that maintains expression of these factors even after the placode has formed and otic Pax8 expression has stopped. Later in development (as indicated by parentheses), expression of Sox9b probably also helps maintain this pathway. (Hans et al. 2004)
anterior-posterior (A-P) axis to the placode (Prince et al. 1998). Evidence suggests that Hedgehog signaling from ventral midline structures acts to induce posterior otic identity (Hammond et al. 2003).

As the placode begins to invaginate or hollow, an asymmetry in gene expression becomes apparent (Fekete and Wu 2002). Regional expression in the otocyst can be induced and/or maintained by external signals from the surrounding tissues. It is hypothesized that this regional gene expression in discrete developmental compartments that is necessary for organizing the ear as a whole (Brigande et al. 2000a; Brigande et al. 2000b; Fekete and Wu 2002)). This compartment-boundary model (Fig. 1.8) argues that the boundaries between compartments can serve as sites of focal cell-cell interactions to mediate local patterning and cell fate specification in the ear (Fekete 1996). Fig. 1.8 shows a representation of the compartment-boundary model. Several genes, many of which are transcription factors and homeobox genes, show restricted expression in the otocyst (Fig. 1.8, B). Fate mapping data from chick and mouse suggest that the dorsal portion of the otic vesicle is destined to become the pars superior, while the ventral portion will become the pars inferior (Fig. 1.8, C) (Li et al. 1978; Brigande et al. 2000a; Brigande et al. 2000b).

After formation and patterning of the otic vesicle occurs, differentiation begins. This includes the generation of different cell types including mechanosensory hair cells, supporting cells, and non-sensory epithelium. It is thought that sensory versus non-sensory regions are genetically predetermined in the otic vesicle. In the sensory region,
**Figure 1.8**: The compartment boundary model.

(A) Model of the compartmentalized otocyst viewed from an anteromedial perspective. (B) Several genes expressed in different parts of the otocyst are indicated, along with the compartments that they most likely encompass. (C) Predicted fate map for the early labyrinth showing where the cells from each compartment of the early endocyst are likely to reside after morphogenesis. The ear is viewed from an anteromedial perspective. Taken from Fekete and Wu (2002).
however, an alternating pattern of hair cells and supporting cells is generated through a process called lateral inhibition (Haddon et al. 1998; Lanford et al. 1999). Emerging hair cells upregulate Delta expression which, in turn, causes neighboring cells to increase Notch activity. Increases in Notch activity inhibits a sensory fate and induces support cell formation. Support cells are thought to perform a stem-cell like function for hair cell regeneration, and may also be essential for the maintainance of hair cells (Fekete et al. 1998; Baird et al. 2000; Stone and Rubel 2000). An additional role in support cells in otolith biogenesis has been suggested (Haddon et al. 1999).

**Genes Required for otoconia or otolith development**

The development and maintenance of ear stones is dependent upon several events: the correct induction and formation of the otocyst, specification and differentiation of the sensory macula and supporting cells, establishing the correct ionic environment, production and export of otolith or otoconial matrix proteins and gelatinous membrane, assembly of a protein core from free floating matrix proteins, and a local increase in Ca\(^{2+}\) and carbonate (CO\(_3^{2-}\)) concentrations for initiation of inorganic crystal formation (Hughes et al. 2006).

Development and patterning of the otic cyst was discussed in the previous section. It is not surprising that defects in this process would lead to malformed otoconia, as several different parts of the otocyst contribute to their development. For example targeted
deletion of the transcription factor Otx1 in mice leads to a complete absence of otoliths although small relatively normal sensory patches develop in the macular region (Morsli et al. 1999). Similarly, Tbx1 mouse mutants do not form otoconia over their single macula (Vitelli et al. 2003). Mutations in the fgf3 (valentino) or the fgf8 (acerebellar) gene in zebrafish lead to poorly adhered otoliths or the appearance of only one otolith, respectively (Kwak et al. 2002; Leger and Brand 2002). The foxi1 zebrafish mutant hearsay often forms a single otic vesicle with a single otolith (Solomon et al. 2003). Foxi1 is upstream of several factors required for otic morphogenesis, including Prendrin, an anion transporter required for ion balance in the endolymph.

Alterations in the size and patterning of the macular region contribute to the size of the otoconial or otolith complex. As I will discuss later, these regions are responsible for secreting otolith or otoconial components. A mutation in eya1 in the dog eared zebrafish mutants leads to widespread apoptosis in the otic vesicle and a reduction in the utricular and saccular macula (Whitfield et al. 1996; Kozlowski et al. 2005). As a result the otoliths are severely undersized. Similarly, the sox10 zebrafish mutant colourless has tiny otoliths that correspond to a reduced size in the macular organs (Dutton et al. 2001). This transcription factor is believed to play a role in specifying macular fate.

After macular specification, a strict pattern of alternating hair cells and supporting cells must be established by Notch lateral inhibition (Riley et al. 1999). Defects in this pathway lead to lack of supporting cells and an overrepresentation of hair cells. A deficiency of deltaA, a member of the Notch pathway, in zebrafish leads to malformed
and elongated otoliths that are likely due to an increase in the number of tethers making contact with the otolith (Riley et al. 1999). Similarly, the zebrafish mindbomb mutant fails to develop supporting cells and, in turn, fail to enlarge otoliths by 60 hpf. The otoliths in these mutants are poorly adherent and have increased mineralization (Haddon et al. 1999).

Endolymphatic fluid, the extracellular liquid that fills the internal lumen of the vestibular apparatus, is composed of a unique ionic milieu. Low levels of calcium and sodium and high concentrations of potassium are maintained by cells throughout the inner ear including cells of the nonsensory epithelium of the utricle (Fermin et al. 1990; Hsu 1991; Ichimiya et al. 1994), the striata vascularis (Kusakari et al. 1978; Kambayashi et al. 1982), the fibrocytes of the spiral ligament (Spicer and Schulte 1991), and the endolymphatic sac and duct (Salt et al. 1989; Thalmann and Thalmann 1999). A mutation in the mouse Tbx10 locus (dancer mutant) allows for ectopic overexpression of this gene and results in malformation of the endolymphatic sac and duct (Bush et al. 2004). Dancer mutants have abnormally high concentrations of phosphate in their otoconia (Anniko et al. 1988).

Specific loss of ion regulators has also been shown to disrupt otoconial or otolith morphology. Mice lacking plasma membrane calcium ATPase 2 (Pmca2) show an intact macula and gelatinous membrane, but no otoconia (Kozel et al. 1998). Pmca2 may be involved in maintaining localized calcium concentrations during otoconial formation. Pendrin is an anion transporter that specifically transports chloride and iodide (Everett et
Pendrin null mice exhibit loss of otoconia or develop giant otoconia, however, other defects such as stereociliary degeneration have been observed (Everett et al. 2001). Morpholino knockdown of two Na,K ATPase subunits, proteins hypothesized to play a major role in endolymph homeostasis, leads to complete absence or significant size reduction of otoliths (Blasiole et al. 2006).

Tight junctions in the otic epithelium and interactions with the basement membrane are required to create a tight seal and maintain the ionic environment within the vestibule and auditory organ (Hughes et al. 2006). Claudinj, a component of tight junctions, has also been shown to be important for zebrafish otolith development. claudinj mutants have severely reduced otoliths, and otolith core material can be seen floating about the vesicle (Hardison et al. 2005). Hardison et al. propose that loss of this protein results in a defective endolymph barrier for specific ions required for otolith core matrix aggregation (2005).

Otolith matrix proteins are proteins that make up the organic lattice for otolith mineralization. Each species has a major otolith matrix protein and several minor ones. The major matrix proteins are discussed in further detail in section 1.4. The major matrix protein in mice and birds is Otoconin 90 (Oc90) (Wang et al. 1998; Verpy et al. 1999). Oc90 null mice develop giant otoconia that lack much of the organic core, suggesting that this protein plays a role in recruiting other matrix proteins (Zhao et al. 2007). Several minor mammalian and avian otolith matrix proteins have been identified. The developing sensory macula produces Osteopontin (Takemura et al. 1994) and Calbindin
D28K (Baird et al. 1997; Balsamo et al. 2000) which are ubiquitous calcium binding proteins. Other minor constituents are Feutin (a proposed inhibitor of calcium phosphate based calcification), Myosin regulatory light polypeptide 9 (which has 2 EF-hand domains that bind calcium), SC-1 (protein that mediates cell/ECM interactions), and Laminin α3 (a component of the basal lamina involved in attaching cells to the ECM via interactions with integrins) (Thalmann et al. 2006). Three otolith matrix proteins have been discovered thus far in zebrafish: Starmaker, Otolith matrix protein-1 (Omp-1), and Otolin. Starmaker is the homolog of a dental sialophosphoprotein that is involved in tooth mineralization in mammals (Sollner et al. 2003). Knockdown of starmaker in zebrafish leads to a delay in otolith formation as well as a loss of the proteinaceous components of the developing crystal. At high doses, starmaker morphants displayed inorganic CaCO₃ with altered crystal structure. Omp-1 is the major matrix protein found in zebrafish otoliths (Murayama et al. 2005). Knockdown of this gene does not affect otolith seeding; however, the growth rates are severely reduced. Otolin has several collagen-like repeats and is thought to function in the organization of the inorganic and organic components of otolith biomineralization. In addition, Otolin participates in anchoring the otolith to the maculae (Murayama et al. 2005). otolin morphants have fragile otoliths that decalcify spontaneously and that lose adherence to the sensory maculae.

Several other genes are involved in otolith tethering in zebrafish. The mutants monolith, einstein, and menhir result in an inability of free floating otolith core particles to seed (Riley and Grunwald 1996; Whitfield et al. 1996; Riley and Moorman 2000). The
particles coalesce in the lumen and later attach to the hair cells of either macula. Interestingly *monolith* mutants lack supporting cells in the macula which suggests that these cells secrete factors important for the seeding process. *gp96* morphant fish mimic these adherence mutants (Sumanas et al. 2003). GP96 is a heat shock protein and molecular chaperone which is involved in processing of integrins and Toll-like receptors. In contrast, the *rolling stones* mutant has proper adhesion of otolith precursors during seeding, but after mineralization occurs, the otoliths detach and are free to move about the ear (Whitfield et al. 1996).

After the adherence of otolith core particles, rapid deposition of CaCO₃ occurs. Availability of Ca²⁺ and CO₃²⁻ ions is crucial for this process. The levels of CO₃²⁻ are mediated by carbonic anhydrase which is abundant in the ear (Lim et al. 1983; Shiao et al. 2005). Inhibitors of this protein result in lack of otoconia in mouse and chick (Kido et al. 1991). Several mouse mutants have primary defects in otoconial mineralization. The *head tilt* mutation has been identified to be carried in *NADPH-oxidase 3* (*Nox3*). The Nox3 protein creates reactive oxygen species (ROS) (Banfi et al. 2004). The role of ROS in otoconial formation has not been established; however, ROS generation is coupled to alterations in mitochondrial and endoplasmic reticulum Ca²⁺ regulation (Ermak and Davies 2002). Similar phenotypes to *head tilt* are seen in *tilted* mutants that have a defect in the gene Otopetrin 1 (*Otop1*) (Ornitz et al. 1998; Hurle et al. 2003; Besson et al. 2005). *Otop1* controls purinergic mediated Ca²⁺ homeostasis by the depletion of endoplasmic reticulum Ca²⁺ stores, specific inhibition of the purinergic receptor P2Y, and regulation of the influx of extracellular Ca²⁺ (Hughes et al. 2007). Knockdown of *otop1* in zebrafish
phenocopies the mouse mutants as otoliths do not develop at the proper time point (Hughes et al. 2004).

*Genes required for semicircular canal duct and crista development*

The two main components of the semicircular canal system are the ducts of non-sensory epithelium, and the associated sensory patches, the cristae. The developing cristae are molecularly defined well before the differentiation of their associated hair cells and include expression of markers such as *Bone morphogenetic protein (Bmp)* genes and *Msx* genes, which are orthologs of the *Drosophila* muscle segment homeobox genes (Ekker et al. 1992a; Oh et al. 1996; Wu and Oh 1996; Chang et al. 1999; Gerlach et al. 2000). The lateral crista is somewhat unique in that its development requires the expression of *Otx*-1, the vertebrate ortholog of *Drosophila* orthodenticle that is induced by Sonic hedgehog signaling from the notochord (Morsli et al. 1999). Bmps and their signaling mediators are the most well studied of these cristae protein markers as the regulation of this signaling is important for the proper expression of *Msxl* and other transcription factors that pattern the inner ear (Ekker et al. 1992a).

Members of the Bone morphogenetic protein family of TGF-β proteins are expressed in the presumptive cristae in all vertebrate model systems. By treating the chick inner ear with exogenous antagonists of Bmp signaling such as Noggin, defects in the crista were induced (Chang et al. 1999; Gerlach et al. 2000). Interestingly this treatment also leads to defects in duct formation, indicating that Bmp signaling from sensory regions is
responsible for both sensory and non-sensory epithelial changes that occur during inner ear development. In addition the endogenous Bmp antagonist Dan (differential screening-selected gene aberrant in neuroblastoma) has been shown to be expressed in the dorsomedial region of developing ear of the chick, an area that gives rise to the endolymphatic duct and sac (Yamanishi et al. 2007). Knockdown of Dan using siRNA causes severe reduction of the endolymphatic duct and sac and moderate deformities in the semicircular canal ducts. Additionally, knockdown of Dan results in altered expression of the transcription factor Nkx5.1, a known marker for the prospective semicircular canals and vestibule. A similar role for Bmp4 signaling in zebrafish has recently been established. The zebrafish mutant, gallery, displays an upregulation of bmp4 mRNA and in turn develop no protrusions or ectopic protrusions (Omata et al. 2007). These results were phenocopied by implanting a bead soaked with Bmp4 into the ear of wild type embryos. In addition, the gallery phenotype could be partially rescued by treatment with Noggin.

Little is known about the exact mechanism for outgrowth and fusion of non-sensory epithelium in amphibian and fish species; however, extracellular matrix proteins have been suggested to be involved in this process. Hyaluronic Acid (HA) or hyaluronin is a linear glycosaminoglycan that is a critical component of the extracellular matrix of supporting tissues such as bone and cartilage. HA also plays a direct role in the morphogenesis of the semicircular canal ducts in Xenopus and zebrafish. In Xenopus, HA accounts for at least 60% of the glycosaminoglycans in the acellular core of the epithelial protrusions that grow and fuse to form canal hubs (Haddon and Lewis 1991).
Injection of Hyaluronidase, an enzyme that degrades HA, directly into the epithelial protrusions in *Xenopus* causes the projections to collapse. Localized production of HA by the epithelial cells at the growing tips of the protrusions seems to provide the driving force in propelling the epithelium forward. In zebrafish, HA also seems to be involved in protrusion of epithelium during semicircular canal formation. *jekyll* is a zebrafish mutant that displays disruption of semicircular canals and pharangeal cartilage (Neuhauss et al. 1996). While protrusions form, they remain rudimentary and fail to elongate to the point of fusion. The protein disrupted in *jekyll*, Ugdh, is an enzyme required for the synthesis of proteoglycans including HA (Walsh and Stainier 2001). Expression of *dfna5*, a gene that has been shown to be involved in hearing loss in humans, is required for the expression of *ugdh* in zebrafish (Busch-Nentwich et al. 2004). Knockdown of *dfna5* leads to similar phenotypes as those seen in the *jekyll* mutant. Knockdown of either *dfna5* or *ugdh* resulted in a reduction of HA levels as measured by immunostaining with biotinylated HA binding protein and an anti-biotin antibody (Walsh and Stainier 2001; Busch-Nentwich et al. 2004).

Several genes have been identified that are involved in the formation of mammalian semicircular canal ducts. Again, factors from the cristae have been implicated in generation of non-sensory ear components. For instance, *Fgf* expression in the cristae induces a canal genesis zone by upregulating *Bmp2* (Chang et al. 2002). Distal-less related transcription factor 5 (*Dlx5*) null mice have dysmorphogenesis of the vestibule and absence of semicircular canals, a result that may explained by the decreased expression of *Bmp4* in these mutants (Merlo et al. 2002). *Nkx5.1*, a transcription factor
whose expression was disrupted in Dan (a Bmp antagonist) knockdowns in chick embryos, is also required for mammalian semicircular canal development (Hadrys et al. 1998).

Other genes involved in the formation of mouse and chick semicircular canal ducts regulate proliferation and fusion plate formation. Hmx2, a homeobox transcription factor, governs specification and commitment of epithelial cells in the pars superior to undergo the proliferative growth and fusion process (Wang et al. 2001b). Nuclear receptor Nor-1 is important for regulation of the proliferative growth (Ponnio et al. 2002). Netrin1, a protein that functions in axon guidance and cell migration in the CNS, has also been shown to participate in the detachment of fusion plate epithelium from the basement membrane (Salminen et al. 2000). It does so by stimulating proliferation of the mesenchyme which pushes the epithelial walls together to form a fusion plate. While Bmp signaling molecules have been implicated in zebrafish semicircular canal development, little is known about the role of proliferation in the generation of epithelial protrusions and fusion in this model. Fusion plate formation in chick requires an elevation in cell death at the point of contact, in order to merge the tissues (Lang et al. 2000). Zebrafish fusion plates are 5-10 times smaller than seen in chick and no upregulation of apoptosis is observed during fusion (Waterman and Bell 1984).
1.3 Phosphatase and Tensin Homolog

Pten (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor gene that is mutated in a number of human cancers including glioblastoma, advanced prostate cancer, breast cancer, endometrial carcinoma, and melanoma (Li and Sun 1997; Li et al. 1997; Steck et al. 1997; Podsypanina et al. 1999; Birck et al. 2000; Zhou et al. 2002). Mutations in the Pten gene are also responsible for the tumor predisposition syndromes Cowden disease (Liaw et al. 1997), Bannayan-Zonana (Marsh et al. 1997), and Lhermitte-Duclose disease (Zhou et al. 2003). Pten has been shown to have protein and lipid phosphatase activity \textit{in vitro}, although only the lipid phosphatase activity has been demonstrated \textit{in vivo} (Maehama and Dixon 1998). Pten acts as a negative regulator of the PI3-Kinase/Akt pathway via its ability to dephosphorylate phosphatidylinositol-3,4,5-triphosphate (PIP$_3$). The PI3-Kinase/Akt pathway is involved in regulating cell growth, motility, and survival (Thompson and Thompson 2004). Mutations or deletions of Pten lead to overactivation of the PI3-Kinase/Akt pathway which contributes to tumorigenesis (Wu et al. 1998; Stahl et al. 2003).

Pten also plays an essential role in early animal development, although the precise function of Pten in embryogenesis remains enigmatic. Homozygous knockouts of Pten in mice have been found to cause embryonic lethality, although the mutant phenotypes differed between various studies (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999). Since Pten is normally expressed ubiquitously during early murine development, embryonic lethality in Pten null mice has been interpreted to result
from abnormal development of multiple organs and tissues (Stiles et al. 2004). Tissue-specific knockout of *Pten* in mice suggests a possible role for Pten in the regulation of cell size and growth (Kishimoto et al. 2003). Pten has also been shown to be essential for *Drosophila* development as *dPten* null mutants die at early larval stages (Goberdhan et al. 1999; Huang et al. 1999; Gao et al. 2000). Pten appears to regulate the size of cells and organs as well as cell survival in *Drosophila* development. These properties of Pten have been attributed to its negative regulation of the PI3-Kinase/Akt pathway (Goberdhan et al. 1999).

### 1.4 Otoconins

The otolith organs of the vestibular system detect gravity and linear movements of the head. This stimulus is sensed through biomineralized particles coupled to the cilia of mechanosensory hair cells within the maculae. The vestibular function of these structures is conserved among all vertebrates; however, the saccular and lagenar macula are also utilized for hearing in fish and amphibian species. Despite the functional conservation of the otolith organs, there are species-specific differences in their structural morphology. The ear stones of mammalian, avian, and amphibian vestibular systems are composed of thousands of tiny polyhedral particles called otoconia that are embedded in a gelatinous membrane. In contrast, teleost fish have three large otoliths that are smooth and ovoid in appearance.
Otoliths and otoconia are both composed of calcium carbonate (CaCO₃) crystals that have mineralized on an organic lattice of extracellular matrix proteins. There are three crystal polymorphs of calcium carbonate: calcite, aragonite, and vaterite. The calcite polymorph is found in the otoconia of mammals, birds, and amphibians (utricular otoconia) (Pote and Ross 1991). The otoconia of the amphibian saccule and the otoliths of teleost fish are of the aragonitic polymorph. It has been proposed that the polymorph of CaCO₃ found in otoliths or otoconia is dependent on the matrix comprising the extracellular core (Pote and Ross 1991). The organic substances within the matrix include acidic proteins, glycosaminoglycans, and proteoglycans and are often called otolith matrix proteins. Known otolith matrix proteins are summarized in Table 1.1. Previously, such proteins were termed otoconins; however, “otoconin” has come to represent otolith matrix proteins that share homology with phospholipase A2 (PLA2). Two otolith matrix proteins have been discovered that have such homology.

Otoconin-90 (Oc90) constitutes at least 90% of the total protein found in calcitic CaCO₃ otoconia of mammals and birds (Wang et al. 1998; Verpy et al. 1999). Oc90 has two domains that share sequence homology with secretory phospholipase A2 (PLA2), but these domains do not posses enzymatic activity as several key residues have been altered. Otoconin-22 (Oc22) is the primary matrix protein of aragonitic CaCO₃ otoconia found in the saccule of amphibians (Pote et al. 1993). Oc22 shares only one domain of homology with PLA2. The primary matrix protein of amphibian utricular otoconia is unknown. The PLA2-like otoconins are extremely acidic, contain several potential glycosylation sites, retain the ability to bind calcium, and provide a rigid structure that can serve as a
scaffold for CaCO₃ deposition (Pote et al. 1993; Wang et al. 1998; Thalmann et al. 2001).

It is thought that these properties are important for sequestering Ca²⁺ to the macular region as the levels of these cations are relatively low in the endolymphatic fluid.

<table>
<thead>
<tr>
<th>Matrix Protein</th>
<th>Species</th>
<th>Known Homology/Domains/ Modifications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin D28K</td>
<td>Human</td>
<td>Secretion Motif, Calcium binding domain</td>
<td>(Usami et al. 1995; Balsamo et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetuin</td>
<td>Mouse</td>
<td>Secretion Motif, Glycosylated, Calcium binding domain</td>
<td>(Zhao et al. 2007)</td>
</tr>
<tr>
<td>Oc90</td>
<td>Human</td>
<td>Secretion Motif, Phospholipase A2 – like, Glycosylated</td>
<td>(Wang et al. 1998; Verpy et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oc22</td>
<td>Frog</td>
<td>Secretion Motif, Phospholipase A2 – like, Glycosylated</td>
<td>(Pote et al. 1993)</td>
</tr>
<tr>
<td>Omp</td>
<td>Zebrafish</td>
<td>Secretion Motif, Melanotransferrin-like</td>
<td>(Murayama et al. 2005)</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Rat</td>
<td>Secretion Motif, Phosphorylated, Glycosylated, Calcium binding domain</td>
<td>(Takemura et al. 1994)</td>
</tr>
<tr>
<td>Otolin</td>
<td>Zebrafish</td>
<td>Secretion Motif, Collagenous (type VIII and X collagen family)</td>
<td>(Murayama et al. 2005; Zhao et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starmaker</td>
<td>Zebrafish</td>
<td>Secretion Motif, Dental sialophosphoprotein</td>
<td>(Sollner et al. 2003)</td>
</tr>
</tbody>
</table>
Oc90 is expressed during the development of the mouse inner ear in non-sensory cells and extracellular locations (Verpy et al. 1999). At E10.5, Oc90 proteins can be detected by immunostaining in the epithelium of the dorsal wall of the otic vesicle. At E14.5, Oc90 is made by the non-sensory epithelia of the semicircular canal and the walls of the utriculo-saccular complex. Interestingly no sensory regions express Oc90. Extracellular Oc90 localizes to the core of the developing otoconia, and the cupula overlying the semicircular canal cristae. In *Xenopus*, embryonic Oc22 expression is similar to that of mouse Oc90 in that the protein is detected in the non-sensory epithelium of the developing utricle and saccule (Yaoi et al. 2004). In contrast to Oc90, however, Oc22 showed expression in the supporting cells of the cristae, but not in the non-sensory semicircular canal hubs.

Gene targeting has recently been used to confirm that Oc90 is required for mammalian otoconia formation (Zhao et al. 2007). Oc90 null mice fail to form the otoconial organic matrix, indicating that expression of Oc90 is necessary for the recruitment of other otoconins and organic components into the matrix core. Without formation of the organic matrix, the inorganic crystallites have the propensity to grow uninhibitedly as seen with inorganic calcite crystals in nature. In the same study, Zhao et al. identified the mouse ortholog of the zebrafish otolith matrix protein, Otolin. Notably, Otolin was absent from the Oc90 null otoliths, whereas Fetuin and Osteopontin were present. These results suggest that Oc90 recruits Otolin to the developing otoconia.
1.5 Neuronal Calcium Sensor-1

Calcium fluxes have been associated with many cellular processes. Transduction of these signals requires the action of various calcium sensing proteins. Ncs-1 is a member of a large family of EF hand containing calcium binding proteins, which are multifunctional regulators of many cellular processes including membrane trafficking, cell survival, and ion channels and receptor signaling.

While calcium sensing proteins are highly conserved throughout evolution, neuronal calcium sensor 1 (Ncs-1) is the oldest member of neuronal calcium sensor family of proteins with orthologs in yeast, C. elegans, Drosophila, zebrafish, Xenopus, rodents, and humans (Pongs et al. 1993; De Castro et al. 1995; Olafsson et al. 1995; Hendricks et al. 1999; Bourne et al. 2001). Ncs-1 was originally identified in Drosophila and subsequently named frequenin, because an overexpressing mutant displayed an enhanced synaptic facilitation (Pongs et al. 1993). In addition, a similar role for Ncs-1 has been identified in mammals to promote exocytosis from dense core vesicles in neurons and neuroendocrine cells (Rajebhosale et al. 2003). Not only does Ncs-1 promote exocytosis in neurons, but it also regulates intracellular calcium levels by influencing the activity of L-type Ca$^{2+}$ channels (Rousset et al. 2003), non-L type Ca$^{2+}$ channels (Weiss et al. 2000), N-type Ca$^{2+}$ channels (Wang et al. 2001a; Rousset et al. 2003), P/Q type Ca$^{2+}$ channels (Weiss and Burgoyne 2001; Tsujimoto et al. 2002; Rousset et al. 2003), Ca2+ permeable Trp channels (Hui et al. 2006), and the IP3 receptor (Schlecker et al. 2006). Ncs-1 has also been shown to interact with and inhibit the desensitization of the D2
dopamine receptor (Kabbani et al. 2002). Since the discovery of these roles of Ncs-1 in neurons, it is not surprising that Ncs-1 has been implicated in contributing to learning and memory in *C. elegans* (Gomez et al. 2001) and to disorders such as schizophrenia and bipolar disorder in humans (Koh et al. 2003).

Recently, Ncs-1 has been shown to play a role in cell survival. Nakamura et al. established that expression of Ncs-1 could be upregulated in response to stressors, neuronal damage, and in response to glial cell line derived neurotrophic factor (2006). Upregulation of Ncs-1 was protective against cell death in an Akt dependent manner (Nakamura et al. 2006).

Despite the role of Ncs-1 in promoting neurotransmission, the gene is found to be functional in many non-neuronal tissues during development and adulthood in several species (Weisz et al. 2000; Bourne et al. 2001; Guild et al. 2001; Guo et al. 2002; Mora et al. 2002; Blasiole et al. 2005; Gromada et al. 2005). For example, Ncs-1 overexpression enhances glucose mediated exocytosis of insulin from pancreatic β cells (Gromada et al. 2005) and FceRI dependent exocytosis of factors from mast cells of the immune system (Kapp-Barnea et al. 2003). The zebrafish ortholog, *ncs-1a*, is expressed in non-neuronal epithelium within the otic vesicle, ventral hematopoetic mesoderm, posterior somites, and pronephric tubules (Fig. 1.9) (Blasiole et al. 2005). Little is known about the role of Ncs-1 in these and other non-sensory tissues.
The most insight into the neuronal and non-neuronal cellular biology of Ncs-1 has come from studies of its influence on exocytosis. Ncs-1 has been shown to interact directly with phosphatidylinositol-4 kinase III beta (Pi4kβ) and ADP-ribosylation factor 1 (Arf1) by co-immunoprecipitation (Kapp-Barnea et al. 2003), GST (glutathione S-transferase) pulldown (Haynes et al. 2005), and fluorescence protein complementation (Haynes et al. 2007). Arf1, a small GTPase, is a key component in the formation of a number of vesicular coat complexes that traffic cargo to both the plasma membrane and the endosomal systems (Donaldson and Honda 2005). Arf1 also recruits Pi4kβ to the golgi complex and activates its kinase function (Godi et al. 1999). Activation of Pi4kβ leads to an increase in phosphatidyl-inositol 4,5-bisphosphate (Pl(4,5)P2), a lipid species that is important for golgi to plasma membrane trafficking (Hama et al. 1999; Walch-Solimena and Novick 1999; Godi et al. 2004). Ncs-1 also activates Pi4kβ and may contribute to its recruitment to the trans-golgi network (TGN) (Burgoyne and Weiss 2001; Zhao et al. 2001; Taverna et al. 2002; Rajebhosale et al. 2003). Interestingly, the interaction of Arf1 and Ncs-1 abolishes the ability of either protein to activate Pi4kβ (Haynes et al. 2005).

In terms of inner ear expression, neuronal expression of Ncs-1 has been detected in mice (Sage et al. 2000), but non-neuronal expression was observed in zebrafish (Blasiole et al. 2005). In mice, Ncs-1 expression first appears in the differentiating statoacoustic ganglion at E11 (Sage et al. 2000). Subsequently, Ncs-1 appears in differentiating neurons of the vestibular afferent processes (E14) and then in the cochlear afferent processes (E16). In the adult ear, additional expression of Ncs-1 is observed in vestibular and cochlear efferent fibers. No expression is detected in the hair cells of the sensory
Figure 1.9: Expression of ncs-1a mRNA during zebrafish embryogenesis.

Panels (D–F), (H), and (J,K) show lateral views with anterior to the left. (A,B) Early somitogenesis (ES), dorsal view. (C) Mid-somitogenesis (MS), dorsal view. (D) Mid-somitogenesis, lateral view. (E) 24 hpf, lateral view. (F) 24 hpf, lateral view of otic vesicle. (G) 36 hpf, dorsal view. (H) 48 hpf, lateral view. (I) 48 hpf, dorsal view. (J) 48 hpf, lateral view of ear. Arrowheads indicate expression of ncs-1a in outgrowing projections of semicircular canals. (K) 3 dpf, lateral view of ear. Arrowheads indicate expression of ncs-1a in mature pillars of semicircular canals. (L) 4.5 dpf, dorsal view.

aac, anterior; adaxial cells; am, anterior macula; ba, branchial arches; d, diencephalon; dt, dorsal telencephalon; hb, hindbrain nuclei; ht, hypothalamus; icm, intermediate cell mass; mb, midbrain; olf, olfactory vesicle; ov, otic vesicle; pp, pharyngeal pouches; ps, posterior somites; pt, pronephric tubules; vahb, ventral anterior hindbrain; vhm, ventral hematopoietic mesoderm. Taken from Blasiole et al. 2005.
epithelium, or in non-sensory otic structures in mice. In contrast, a zebrafish ortholog, \textit{ncs-1a}, can be detected in several non-neuronal parts of the ear during development (Fig. 1.9) (Blasiole et al. 2005). Expression is detected as early as 24 hpf in the anteroventral portion of the otic vesicle in the tissue corresponding to the developing uricular macula (Fig. 1.9, F). At 48 hpf expression remains strong in the utricular macula (Fig. 1.9, J). \textit{ncs-1a} transcripts also become apparent in the epithelial protrusions of the developing semicircular canals, and this expression persisted through the fusion of canal hubs at 72 hpf (Fig. 1.9, J-K). After 72 hpf, \textit{ncs-1a} expression diminishes in the ear. Morpholino knockdown of \textit{ncs-1a} causes a number of defects including inhibition of epithelial protrusion formation in the otic vesicle at 48 hpf (Fig. 1.10, I). By 5 dpf, \textit{ncs-1a} morphants do display epithelial pillars; however they are disorganized and in some cases, unfused (Fig. 1.11). The defects seen in semicircular canal morphogenesis upon knockdown of \textit{ncs-1a} confirm that the expression of \textit{ncs-1a} within the inner ear has a functional significance in this organ.

1.6 Rationale and Hypothesis

Vestibular disorders are common, and 42% of the American population will experience dizziness at least once in their lifetime (National Institute of Deafness and other Communication Disorders (NIDCD)). A variety of factors contribute to balance dysfunction including congenital disorders, exposure to ototoxic medication, infection, and trauma.
Figure 1.10: Expression of ncs-1a gene is essential for semicircular canal formation.

Effect of ncs-1a MO on zebrafish development. All panels are lateral views, anterior to the left. Morphants were injected with 1.5 ng of ncs-1a MO at the one-cell stage. (A) 27 hpf wild type embryo. (B) 27 hpf morphant. (C) 68 hpf wild type embryo. Arrow indicates presence of erythrocytes. (D) 68 hpf morphant. Arrow indicates presence of erythrocytes. (E) Otic vesicle (OV) of 27 hpf wild type embryo. (F) OV of 27 hpf morphant. (G) OV of 68 hpf wild type embryo. (H) Same as panel (G); epithelial pillars of semicircular canals are outlined. Red arrows indicate otoliths. (I) OV of 68 hpf morphant. Scale bars: (A–D) = 250 μm; (E–I) = 50 μm. Taken from Blasiole et al. 2005.
**Figure 1.11**: Aberrant semicircular canal formation in 5 dpf ncs-1a morphants.

(A–E) Lateral view of OVs at 5 dpf, anterior to the left. (A) WT embryo. (B) Morphant OV. Arrowhead indicates abnormal mass of epithelial tissue. (C) Morphant OV lacking lateral semicircular canal pillar (arrowhead). (D) Morphant OV. Arrowhead points to abnormal tissue mass in posterior canal. (E) Morphant OV. Arrowhead indicates posterior pillar that has failed to fuse with other pillars. Scale bar: 50 µm. Figure taken from Blasiole et al. 2005.
The goal of my research is to identify new genes involved in vestibular development which will lead to further understanding of vestibular function and dysfunction. I hypothesize that by studying the function of genes whose expression patterns are spatiotemporal regulated during the morphogenesis of vestibular structures I will be able to elucidate the roles of these genes in the developmental process.

I use zebrafish as a developmental model system. The structure and development of the vestibular system are highly conserved among all vertebrates, and many genes have been shown to be involved in the development of the vestibule in both mammalian and fish systems. The ability to knock down expression of virtually any gene using antisense morpholinos has allowed the dissection of genetic pathways involved in many developmental processes. In my studies, I have identified several genes that participate in vestibular morphogenesis. In particular, I show that zebrafish orthologs of Pten and Oc90 are involved in otolith development and that several Ncs-1 interacting proteins are involved in development of semicircular canal ducts. Because the genetic and structural development of the vestibular apparatus has been conserved throughout evolution, elucidating the molecular processes that regulate zebrafish ear development will provide insight into mechanisms underlying ear development in humans.
**Chapter 2**

*ptena and ptenb Genes Play Distinct Roles in Zebrafish Embryogenesis*

The work presented in this chapter was published in Developmental Dynamics (Croushore et al 2005). Robert Levenson was the principle investigator for this project. Victor Canfield identified the zebrafish orthologs of the human pten gene. Brian Blasiole and Ryan Riddle cloned ptena and ptenb and discovered alternative splicing for both genes. Christine and Bernard Thisse performed mRNA expression analysis of ptena and ptenb by whole mount insitu hybridization. I constructed the amino acid alignment of pten orthologs, performed RT-pcr analysis of ptena and ptenb at various developmental stages, performed in vitro translation experiments, used morpholino to knockdown each gene, and performed western blotting to compare levels of phosphorylated Akt to the total level of Akt. Gavin Robertson and Keith Cheng contributed knowledge and suggestions.
2.1 Introduction

Phosphatase and Tensin homolog is a tumor suppressor gene that exerts its function by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP₃) which in turn inhibits the Akt pathway. The PI3K/Akt pathway regulating cell growth, motility, and survival, therefore improper regulation of this pathway can lead to uncontrolled growth and tumorigenesis (Wu et al. 1998; Stahl et al. 2003). Pten has been shown to be mutated in a number of cancer types (Li and Sun 1997; Li et al. 1997; Steck et al. 1997; Podsypanina et al. 1999; Birck et al. 2000; Zhou et al. 2002) and in patients with the tumor predisposition syndromes Cowden disease (Liaw et al. 1997), Bannayan-Zonana (Marsh et al. 1997), and Lhermitte-Duclose disease (Zhou et al. 2003).

Pten plays an essential role in early animal development. Homozygous knockouts of Pten in mice have been found to cause embryonic lethality due to abnormal development of multiple organs and tissues, and dPten null mutant drosophila die in early larval stages (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999) Developmental studies have shown that Pten regulates cell size, growth, and survival in an Akt dependent manner (Kishimoto et al. 2003).

To analyze the role of Pten in vertebrate development, I took advantage of the powerful reverse genetic tools available in zebrafish. Antisense morpholino oligonucleotide (MO) gene knockdown provides a means to produce hypomorphs for any gene in the zebrafish
genome (Nasevicius and Ekker 2000). Graded knockdowns with MOs in zebrafish make it possible to study the developmental function of essential genes \textit{ex utero}, which is extremely difficult in mice. For example, homozygous \textit{looptail} (\textit{Ltap}) mutants (Kibar et al. 2001) or knockouts of \textit{Vegf} (Ferrara et al. 1996) in mice result in embryonic lethality. Alternatively, MO knockdown of the \textit{Ltap} ortholog \textit{strabismus/Van Gogh} (Park and Moon 2002) or \textit{vegf} (Nasevicius et al. 2000) in zebrafish provided further insight into the developmental function of these genes.

I have identified two paralogous \textit{Pten} genes in zebrafish, \textit{ptena} and \textit{ptenb}. These genes exhibit distinct but overlapping expression patterns early in embryogenesis. By 48 hpf, both genes show similar expression in the CNS, branchial arches, pectoral fin, and eye. \textit{ptena} and \textit{ptenb} appear to function as PIP3 lipid phosphatases based on their ability to decrease phosphorylation of Akt. Knockdown of \textit{ptena} caused irregularities in notochord and head shape, vasculogenesis, and ear development. In contrast, knockdown of \textit{ptenb} caused hooked tails, domed heads, and reduced yolk extensions. Since morpholino knockdown of \textit{ptena} and \textit{ptenb} mRNA translation in zebrafish produced unique phenotypes, it is likely that these two highly related genes play distinct roles in the developing zebrafish embryo.
2.2 Experimental Procedures

2.2.1 Identification and Characterization of Zebrafish *pten* Genes

A BLAST search of the GenBank EST database revealed numerous zebrafish cDNAs with a high degree of similarity to mammalian Pten. Assembly of overlapping sequences (ESTs fm55g11, fv35h02, faa38e03) yielded a full-length composite sequence corresponding to *ptena*. Two additional non-overlapping ESTs (fy71d09, fl55h02) encoded the 5' and 3' ends of a distinct *pten* gene (*ptenb*). PCR primer pairs were used to amplify the full-length *ptena* and *ptenb* cDNAs; *ptena* forward primer: 5' - GCTGTCAT GGCAATGAC - 3', *ptena* reverse primer: 5' - TCAGACTTTTTGTAATCTGTGCG - 3', *ptenb* forward primer: 5' - GACTCCTGTCACAGCCATGGCTGCG - 3', *ptenb* reverse primer: 5' - CTTCCCATAAAAATTTCAAC - 3'. BLAST searches of the zebrafish genome assembly (Version 4) available from the Zebrafish Sequencing Group (www.ensembl.org/) were used to assign linkage groups.

2.2.2 mRNA Expression Analysis

For RT-PCR, zebrafish embryos at various stages of development and ~ 6 month old adults were collected and homogenized in TRIzol Reagent (Invitrogen; Carlsbad, CA). Total RNA was extracted according to the method of Chomczynski and Sacchi (1987), and developmental stage-specific RNA (0.5 μg) was used as template to generate single stranded cDNA using the SuperScript First Strand Synthesis kit (Invitrogen). PCR was carried out with REDTaq DNA polymerase (Sigma; St. Louis, MO) using a RoboCycler
Gradient Temperature Cycler (Stratagene; La Jolla, CA) and primers that flank the alternatively spliced exons of *ptena* (forward primer 5’-CCAGCCAGCGCAGGTATGT GTA-3’ and reverse primer 5’-GCGGCTGAGGAAACTCGAAGATC-3’) and *ptenb* (forward primer 5’-GCTACCT-TCTGAGGAATAAGCTGG-3’ and reverse primer 5’-CTTGATGTCCCCCACACACAGGC-3’). PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The PCR products from each primer pair were verified by DNA sequencing.

Whole-mount *in situ* hybridization analysis was performed as described by Thisse et al. (1999; http://zfin.org/cgi-bin/webdriver?Mlval=aa-pubview2.apg&OID=ZDB-PUB-010810-1). The following antisense probes were utilized to characterize *pten* expression: *ptena* (GenBank accession no. AY398669, nucleotides 1-1371); *ptenb* (GenBank accession no. AY398670, nucleotides 1-1302). Additional antisense RNA probes include *dfna5* (Busch-Nentwich et al. 2004), *myoD* (Weinberg et al. 1996), *otx1* (from E. Weinberg), *ncs-1a* (Blasiole et al. 2005) and *starmaker* (Sollner et al. 2003), *VE-cadherin* (*cdh5*) (Larson et al. 2004).

### 2.2.3 Antisense Morpholino Knockdowns

Antisense morpholino oligonucleotides (MOs) (Gene Tools LLC; Philomath, OR) were designed to target the 5’UTR of each zebrafish *pten* gene. *ptena*-MO1 (5’-CCTCGCTCACCCT-TGACTGTGTATG-3’); *ptena*-MO2 (5’-CAGTTTTATTCGGTTTATTTGTCAG-3’); *ptenb*-MO1(5’-CTTTCGGACGGTC-
GGTCGTCTTTA-3’; ptenb-MO2 (5’-GGCTGTGACAGGAG-TCTTTAGGGTT-3’).

The MOs were resuspended in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) and microinjected into the yolk of single cell embryos.

The specificity of the pten MOs was tested using an in vitro translation assay as previously described (Blasiole et al., 2005). All experiments were performed with the short splice forms of the pten genes. ptena (GenBank accession no. AY398668) and ptenb (GenBank accession no. AY398671) cDNAs were subcloned into pBluescript II KS+ (Stratagene). Capped ptena and ptenb mRNAs were synthesized using the mMESSAGE mMACHINE (Ambion) transcription kit, and 0.5 μg of mRNA was used to program the synthesis of [³⁵S]-labeled proteins using an in vitro rabbit reticulocyte lysate translation kit (Ambion) under conditions described by the manufacturer. The translation of mRNA was tested in the presence of 4 μg of antisense MOs (ptena-MO1 and ptenb-MO1). An antisense MO targeted against the zebrafish Na,K-ATPase α₁a.1 gene (atp1a1a.1) (5’-GCCTTCTCTCCTGCCCCATTTTGCTG-3) (Shu et al. 2003) was used as a non-specific control. The entire reaction mixture (20 μl) of each in vitro translation assay was separated by SDS-PAGE. The gels were dried, exposed to X-ray film, and the relative intensity of the bands quantitated by laser densitometry (Molecular Dynamics; Sunnyvale, CA) and analyzed using the Quantity One software package (PDI, Inc.; Huntington Station, NY).
2.2.4 Phosphorylated Akt Assay

Pten activity was assayed in wild type and morphant embryos as follows. Wild type embryos, *ptena* morphants (injected with 6 ng *ptena*-MO1), and *ptenb* morphants (injected with 6 ng *ptenb*-MO1) were collected at 48 hpf and homogenized in lysis buffer (Blasiole et al. 2005). Thirty embryos were collected in each group. Lysates were incubated at 4°C for one hour, then centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants were normalized for total protein content, fractionated by SDS-PAGE (50 μg protein/lane), then transferred to a nitrocellulose filter. Immunoblots were probed with a rabbit anti-human Akt antibody (1:1000 dilution; Cell Signalling; Beverly, MA). Blots were stripped and reprobed with a rabbit anti-human phospho-Akt (Ser 473) antibody (1:1000 dilution, Cell Signalling). Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Immunoreactivity was visualized by enhanced chemiluminescence (ECL) using an ECL Plus kit (Amersham Pharmacia; Piscataway, NJ). Immunoblots were quantitated by laser densitometry (Molecular Dynamics) and analyzed using the Quantity One software package (PDI, Inc.). Statistical analyses of the data were performed using an unpaired one-tailed Student’s *t*-test.

2.3 Results

2.3.1 Identification and Characterization of Zebrafish *pten* Genes

Zebrafish *pten* genes were identified by BLAST searches of the zebrafish EST database.
These searches revealed two distinct classes of cDNAs, one class encoding *ptena* and the other *ptenb*. Primers were designed to amplify full-length *pten* ORFs using RT-PCR. Sequencing of the initial amplification products revealed the presence of two splice variants of the *ptena* and *ptenb* genes. The short splice variants were found to contain the same intron/exon organization as the mammalian *Pten* gene (Fig. 2.1). The long splice variant of both *ptena* and *ptenb* contained a 69 base pair (bp) insertion corresponding to the boundary of the 6th and 7th exons of the mammalian *Pten* gene (GenBank accession no. AH005966). The inserted 69 bp exon was found in zebrafish genomic DNA, and in the RT-PCR products amplified from embryonic and adult zebrafish mRNA. The additional exon found in the zebrafish *ptena* and *ptenb* genes is also present in the *pten* genes of Fugu, Tetraodon, and medaka (not shown), but not in chicken, human, or other tetrapod *Pten* genes.

The complete ORFs for the *ptena* long and short splice variants encode polypeptides of 454 and 431 amino acids. The complete ORFs for the *ptenb* splice variants encode polypeptides 422 and 399 amino acids in length. An amino acid sequence alignment of the zebrafish Ptena, Ptenb, and human Pten polypeptides is shown in Fig. 2.1. Zebrafish Ptena and Ptenb polypeptides exhibit 88% and 86% identity, respectively, with human Pten. The phosphatase motif (amino acids 123-130 of human Pten) shows complete identity between the human and zebrafish Pten polypeptides. The region of greatest divergence, including the alternatively spliced region of Ptena and Ptenb and a 32 amino acid insertion found in Ptena, occurs within the C2 domain, a region proposed to have a potential role in membrane localization (Lee et al. 1999).
**Figure 2.1**: Comparison of human Pten and zebrafish Pten polypeptides.

Human (hPTEN) and zebrafish (zfPTENA and zfPTENb) polypeptides were aligned using CLUSTALW. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the right of each line. Arrowheads indicate the location of introns and are flanked by the corresponding exon numbers. The asterisk indicates the extra exon found in the deduced zebrafish amino acid sequence. The region encompassing the C2 domain is underlined (Croushore et al. 2005).
BLAST searches of the zebrafish genome assembly (Version 4) available from the Zebrafish Sequencing Group indicate that the zebrafish \textit{ptena} gene is located on linkage group (LG) 17 (Zv4\_scaffold1476.4), and the \textit{ptenb} gene maps to LG 12 (GenBank accession nos. AL731788.8 and BX548001.4). These linkage groups have significant synteny with human chromosome 10, the location of the human Pten gene (Woods et al. 2000). These results suggest that the zebrafish \textit{ptena} and \textit{ptenb} genes most likely arose as a result of the genome-wide duplication that occurred in teleost fish (Amores et al. 1998; Postlethwait et al. 1998).

\subsection*{2.3.2 Expression of \textit{ptena} and \textit{ptenb} Genes in Zebrafish Embryos}

I used an RT-PCR approach to analyze stage-specific expression of the zebrafish \textit{pten} splice variants. Primers specific for \textit{ptena} were found to amplify both the long and short \textit{ptena} splice variants from all stages of embryonic development tested (6 hpf-72 hpf) as well as the adult (Fig. 2.2, A). Similarly, long and short splice variants of \textit{ptenb} were also detected at equivalent developmental stages (Fig. 2.3, A). It should be noted that the RT-PCR approach used was not quantitative, and therefore the relative abundance of reaction products may not accurately reflect \textit{ptena} and \textit{ptenb} mRNA levels \textit{in vivo}.

Whole-mount \textit{in situ} hybridization was used to analyze the expression patterns of \textit{ptena} and \textit{ptenb} during zebrafish embryogenesis. As shown in Fig. 2.2, B-C, \textit{ptena} mRNA was broadly expressed throughout the embryo at early and mid-somitogenesis. At 24 hpf, transcripts of \textit{ptena} were more abundant in the CNS, axial vasculature, retina, branchial
Figure 2.2: Expression of zebrafish \textit{ptena} mRNA during embryogenesis.

Expression of \textit{ptena} was analyzed by RT-PCR and whole-mount \textit{in situ} hybridization. (A) Expression of \textit{ptena} splice variants during zebrafish development determined by RT-PCR. Bands of 305 bp and 236 bp represent fragments of the long and short splice variants, respectively. (B-K) Expression of \textit{ptena} determined by whole-mount \textit{in situ} hybridization. (B) Early somitogenesis, lateral view. (C) Mid-somitogenesis, lateral view. (D) 24 hpf, lateral view. (E) 24 hpf, lateral view of head. (F) 24 hpf, lateral view of tail. (G) 36 hpf, lateral view of otic vesicle. (H) 24 hpf, transverse section of trunk. (I) 48 hpf, lateral view. (J) 48 hpf, transverse section of trunk. (K) 5 dpf, lateral view. A, aorta wall; AV, axial vasculature; BA, branchial arches; END, endoderm; FP, floor plate; LLP, lateral line primordium; NC, notochord; OV, otic vesicle; PF, pectoral fin; R, retina; SC, spinal cord; TEL, telencephalon (Croushore et al. 2005).
arches and pectoral fin bud (Fig 2.2, D-F). In the CNS, ptena was expressed predominantly in the telencephalon and the spinal cord (with the exception of the floor plate). Additional expression of ptena in the wall of the aorta and in the lateral line primordium was observed in transverse sections of 24 hpf embryos (Fig. 2.2, H). Between 24 hpf and 48 hpf, low levels of ptena expression were detected in the otic vesicle (Fig. 2.2, E and G). From 48 hpf through 5 dpf, robust ptena expression was detected in the eye, CNS, branchial arches, endoderm, and pectoral fin buds (Fig. 2.2, I-K).

From early somitogenesis through 24 hpf, low levels of ptenb mRNA were detected throughout the embryo with higher levels present in the caudal somites (Fig. 2.3, B-H). At the 10-somite stage, expression of ptenb was restricted to the posterior and lateral compartments of the somites, which are destined to become fast muscle fibers (Fig. 2.3, D-E). At the 18-somite stage, ptenb expression became restricted to the ventral portion of the somites, while transcripts of ptenb were detectable in neurons of the spinal cord and the cranial ganglia, particularly those in the trigeminal placode (Fig. 2.3, F-H). At 24 hpf, ptenb expression was observed in the spinal cord, the brain, and in the ventral portion of the somites (Fig. 2.3, I-J). In brain, ptenb transcripts were particularly abundant in nuclei of the diencephalon, telencephalon, and tegmentum (Fig. 2.3, J). Between 48 hpf and 5 dpf, ptenb transcripts were most abundant in the central nervous system, branchial arches, pectoral fin, and eye (Fig. 2.3, L-M). In contrast to ptena, there was no apparent enrichment of ptenb transcripts in the 36 hpf otic vesicle (Fig. 2.3, K).
Figure 2.3: Expression of zebrafish *ptenb* mRNA during embryogenesis.

Expression of *ptenb* was analyzed by RT-PCR and whole-mount *in situ* hybridization. (A) Expression of *ptenb* splice variants during zebrafish development determined by RT-PCR. Bands of 298 bp and 229 bp represent fragments of the long and short splice variants, respectively. (B-K) Expression of *ptenb* determined by whole-mount *in situ* hybridization. (B) Early somitogenesis, lateral view. (C-H) Mid-somitogenesis. (C) 10-somite stage, lateral view. (D) 10-somite stage, dorsal view of somites obtained with differential interference contrast microscopy. (E) 10-somite stage, transverse section. (F) 18-somite stage, lateral view. (G) 18-somite stage, dorsal view of tail. (H) 18-somite stage, dorsal view of head. (I) 24 hpf, lateral view. (J) 24 hpf, lateral view of head. (K) 36 hpf, lateral view of otic vesicle. (L) 48 hpf, lateral view. (M) 5 dpf, lateral view. AC, adaxial cells; BA, branchial arches; CG, cranial ganglia; D, diencephalon; EPI, epiphysis; E, eye; NC, notochord; OV, otic vesicle; PF, pectoral fin; S, somite; SC, spinal cord; TEL, telencephalon; TEG, tegmentum (Croushore et al. 2005).
2.3.3 Antisense Morpholino Knockdown of ptena and ptenb Expression

I have utilized antisense morpholinos (MOs) to analyze the role of the ptena and ptenb genes in zebrafish development. Two independent non-overlapping antisense MOs were designed to target the 5’UTR of each pten gene. One of each pair of MOs was used in an in vitro translation assay (Blasiole et al., 2005) to confirm the specificity of the MOs for their respective target sequences. The ptena MO (ptena-MO1) decreased the translation of ptena mRNA by 83% compared to ptena mRNA translated in the absence of MO (Fig. 2.4, A). Translation of ptena mRNA was not inhibited by a ptenb MO (ptenb-MO1) or an MO targeted to the zebrafish Na,K-ATPase α1a.1 subunit (atp1a1a.1-MO). Compared to in vitro translation of ptenb mRNA in the absence of MO, translation of ptenb mRNA was reduced 90% by ptenb-MO1, but was not decreased in the presence of either ptena-MO1 or the non-specific atp1a1a.1-MO (Fig. 2.4, B). These results strongly suggest that the ptena and ptenb MOs specifically block translation of their target mRNA sequences. Although the morpholinos are specific for their particular orthologs, it should be noted that these morpholinos will not be specific for the short or long splice variant of the target transcript.

I examined the effect of ptena-MO1 and ptenb-MO1 on zebrafish embryogenesis. At 15 hpf, high doses of ptena-MO1 (4 ng/embryo) produced extensive tissue disorganization, necrosis, and death of all injected embryos (n=273; data not shown). At a dose of 1 ng of ptena-MO1, >90% of injected embryos survived to 4 dpf (n=250). At 24 hpf, a variety of
**Figure 2.4:** Specificity of *pten* antisense morpholinos.

*ptena* and *ptenb* mRNAs were separately translated *in vitro* in the presence or absence of *ptena* or *ptenb* MOs (*ptena*-MO1 or *ptenb*-MO1), respectively. An MO targeted to the Na,K-ATPase α1a.1 subunit (*atp1a1a.1*-MO) was used as a non-specific control. (A) *In vitro* translation of *ptena*. (B) *In vitro* translation of *ptenb*. [35S]-labeled proteins were separated on an SDS-containing 10% polyacrylamide gel. The gel was dried and exposed to X-ray film. The ~60 kDa bands corresponding to Ptena and Ptenb polypeptides are shown at the bottom of each panel. Quantitation of bands by laser densitometry is shown at the top of each panel. Bars indicate the levels of [35S]-labeled Ptena or Ptenb in the presence of MOs compared to levels of [35S]-labeled Ptena or Ptenb in the absence of MOs (Croushore et al. 2005).

**Table 2.1:** Occurrence of *ptena* and *ptenb* morphant phenotypes at 48 hpf.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Wild type</th>
<th><em>ptena</em> morphants (<em>ptena</em>-MO1)</th>
<th><em>ptenb</em> morphants (<em>ptenb</em>-MO1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal Otoliths</td>
<td>0</td>
<td>59</td>
<td>5</td>
</tr>
<tr>
<td>Domed Head</td>
<td>0</td>
<td>72</td>
<td>83</td>
</tr>
<tr>
<td>Hooked Tail</td>
<td>0</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>Wavy Notochord</td>
<td>0</td>
<td>73</td>
<td>19</td>
</tr>
<tr>
<td>Lacking Axial Blood flow</td>
<td>0</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Lacking Intersegmental Blood Flow</td>
<td>7</td>
<td>83</td>
<td>28</td>
</tr>
<tr>
<td>Reduced Yolk Extension</td>
<td>0</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Total Number of Fish Assayed</td>
<td>100</td>
<td>100</td>
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</table>
developmental defects were observed in *ptena* morphants (Figs. 2.5, 2.6, and Table 2.1).

The most conspicuous morphological defects observed were smaller eyes, curved tails, and a shortened body axis. As outlined in Table 2.1, microscopic examination of *ptena* morphants (n=100) revealed defects in notocord shape (73%), ear morphology (59%), head shape (72%), and intersegmental blood flow (83%). By 4 dpf, the heads of *ptena* morphants failed to properly straighten, and a high percentage (>60%) of morphants displayed cardiac edema (Fig. 2.4, P).

I examined the effect of *ptena*-MO1 and *ptenb*-MO1 on zebrafish embryogenesis. At 15 hpf, high doses of *ptena*-MO1 (4 ng/embryo) produced extensive tissue disorganization, necrosis, and death of all injected embryos (n=273; data not shown). At a dose of 1 ng of *ptena*-MO1, >90% of injected embryos survived to 4 dpf (n=250). At 24 hpf, a variety of developmental defects were observed in *ptena* morphants (Figs. 2.5, 2.6, and Table 2.1). The most conspicuous morphological defects observed were smaller eyes, curved tails, and a shortened body axis. As outlined in Table 2.1, microscopic examination of *ptena* morphants (n=100) revealed defects in notocord shape (73%), ear morphology (59%), head shape (72%), and intersegmental blood flow (83%). By 4 dpf, the heads of *ptena* morphants failed to properly straighten, and a high percentage (>60%) of morphants displayed cardiac edema (Fig. 2.4, P).

A prominent defect in *ptena* morphants (73%) was the appearance of an irregularly shaped (wavy) notocord (Fig. 2.5, F). To analyze whether the notochord defect might be caused by alterations in the surrounding tissue, I examined the expression of *myoD* in the
adjacent somites. As shown in Fig 2.5, G-J, expression of myoD in wild type embryos and ptena morphants appeared very similar, suggesting that the defect in notochord morphology was not due to disorganization of the surrounding somites. At a dose of 2 ng of ptena-MO1, >75% of injected embryos survived through 4 dpf (n=250). I observed a lack of intersegmental blood flow in 83% of these ptena morphants. Analysis of ptena morphants with a VE-cadherin riboprobe, a marker of vascular endothelial tissue (Larson et al. 2004) revealed that the intersegmental vessels were irregularly formed and did not follow the well-defined chevron shape characteristic of intersegmental vessels seen in wild type embryos (Fig. 2.5, K-N). In addition, some of the intersegmental vessels in the morphants did not appear to completely traverse the trunk or form connections with the dorsal longitudinal anastomotic vessel (Fig. 2.5, N). These results suggest that ptena may play a role in vascular development.

Knockdown of the ptena gene produced a noticeable effect on ear development in zebrafish embryos. At 24 hpf, wild type otic vesicles contained two characteristic rounded otoliths (Fig. 2.6, A). In contrast, ptena morphants exhibited globular-shaped otoliths (Fig. 2.6, B), and/or a single otolith (Fig. 2.6, C-D). At 48 hpf, 59% (n=100) of ptena morphants contained only one otolith (Fig. 2.6, F). By 4 dpf, ptena morphants containing a single otolith failed to generate a second otolith (100%; n = 59). In addition, 22% of ptena morphants containing one otolith also exhibited a defect in semicircular canal formation (Fig. 2.6, H and J). Aberrant semicircular canal morphologies included failure of the epithelial pillars to fuse (Fig 2.6, H), as well as the presence of disorganized epithelial cell masses in the ear (Fig. 2.6, J). Using whole-mount in situ hybridization, I
analyzed expression of a number of ear markers involved in ear patterning (\textit{pax2a}, \textit{otx1}), otolith formation (\textit{starmaker}), and semicircular canal development (\textit{dfna5}, \textit{ncs-1a}). I did not observe differences in mRNA expression between \textit{ptena} morphants and wild type controls for any of the markers analyzed (\textbf{Fig. 2.6, K-T}), suggesting that the inner ear defects caused by \textit{ptena}-MO1 are not due to abnormal patterning of the otic vesicle or disrupted expression of genes involved in otolith formation or semicircular canal development.

To confirm the role of \textit{ptena} in zebrafish development, I analyzed the effects of a second non-overlapping morpholino (\textit{ptena}-MO2; 1.5 ng) on zebrafish embryogenesis.

At 24 hpf, \textit{ptena}-MO2 phenocopied the otolith and notochord defects in 45\% and 46\% of injected embryos (n=58), respectively. At 48 hpf, defects in intersegmental blood flow and head shape were observed in 47\% and 35\% (n=43) of injected embryos, respectively.
Figure 2.6: Effect of ptena-MO1 morpholino on zebrafish inner ear development.

All panels are lateral views of the otic vesicle with anterior to the left. (A) 24 hpf, wild type embryo. (B-D) 24 hpf, ptena morphants. (E) 48 hpf, wild type embryo. (F) 48 hpf, ptena morphant. (G) 4 dpf, wild type embryo. (H-J) 4 dpf, ptena morphants. (K-T) 48 hpf, in situ hybridization using ear-specific markers. (K) pax2a, wild type embryo. (L) pax2a, ptena morphant. (M) otx1, wild type embryo. (N) otx1, ptena morphant. (O) starmaker, wild type embryo. (P) starmaker, ptena morphant. (Q) dfna5, wild type embryo. (R) dfna5, ptena morphant. (S) ncs-1a, wild type embryo. (T) ncs1-a, ptena morphant. Scale bars: A-J = 25 µm. Arrowhead indicates site where epithelial pillars fail to fuse. Arrows point to abnormal tissue masses (Croushore et al. 2005).
None of the *ptena*-MO2 morphants exhibited defects in semicircular canal formation by 4dpf. These results are consistent with the view that in zebrafish, *ptena* plays an essential role in a variety of developmental processes including head and notochord formation as well as ear and vascular system development.

I next analyzed the effect of knocking down translation of *ptenb* mRNA in zebrafish embryos using the *ptenb* MOs. High doses of *ptenb*-MO1 (8 ng) or *ptenb*-MO2 (8 ng) severely affected embryogenesis, such that all tissues were extremely disorganized, the body axis was shortened, and all embryos died within 36 hpf (n=157; data not shown). At a dose of 4 ng of *ptenb*-MO1, 72% of *ptenb* morphants were viable at 48 hpf. At this stage, the principal defects I observed in *ptenb* morphants (n=100) were an upward hooked tail that occurred in 73% of the embryos, a domed head that occurred in 83% of the embryos, and a reduction in the yolk extension that occurred in 90% of the embryos (Fig. 2.7 and Table 2.1). Compared to *ptena* morphants, <20% exhibited defects in the shape of the notochord, and only 28% lacked intersegmental blood flow. By 24 hpf, >95% of *ptenb* morphants developed normal otoliths. Microinjection of *ptenb*-MO2 at a dose of 6 ng phenocopied the curved tail defect only. Taken together, these results suggest that *ptena* and *ptenb* play distinct roles in zebrafish development.
Figure 2.7: Effect of \( ptenb \)-MO1 morpholino on zebrafish development.

All panels are lateral views of 48 hpf embryos with anterior to the left. (A) Wild type embryo. (B) \( ptenb \) morphant. (C) Tail of wild type embryo. (D) Tail of \( ptenb \) morphant. Scale bars = 250 µm (Croushore et al. 2005).
Figure 2.8: Lipid phosphatase activity of zebrafish pten genes.

Lysates were prepared from wild type embryos as well as *ptena* and *ptenb* morphants at 48 hpf (30 embryos/group). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter. Blots were probed with an anti-Akt antibody, stripped, and reprobed with an anti-phospho-Akt (pAkt) antibody. (A) Western blot. (B) Quantitation of bands by laser densitometry. The bars for *ptena* (n=6 separate experiments) and *ptenb* (n=4 separate experiments) represent the relative ratio of pAkt to total Akt (tAkt) compared to wild type embryos. The error bars represent the standard error of the mean. The asterisk indicates a statistically significant increase in the ratio of pAkt to tAkt (p<.05) as calculated by an unpaired Students t-test (Croushore et al. 2005).
2.3.4 Biological Activity of Ptena and Ptenb

PTEN lipid phosphatase activity has previously been shown to negatively regulate phosphorylation of Akt by virtue of its ability to dephosphorylate PIP₃ (Maehama and Dixon 1998). Because knockdown of ptena and ptenb expression gave distinct developmental phenotypes, I asked whether the PIP₃ lipid phosphatase activity of Pten was conserved in the Ptena and Ptenb enzymes of zebrafish. To do this, I compared the level of activated (phosphorylated) Akt (pAkt) in lysates prepared from wild type zebrafish and ptena and ptenb morphant embryos. Expression of Ptena and Ptenb was knocked down using the ptena-MO1 and ptenb-MO1 morpholinos, and lysates were prepared from wild type and morphant embryos at 48 hpf. Western blot analysis was then used determine the relative percentage of total Akt (tAkt) that was phosphorylated (pAKT) in whole fish lysates. As shown in Fig. 2.8, MO knockdown of Ptena expression produced a significant increase in the relative amount of pAkt compared to uninjected control embryos. A similar effect on pAkt levels was also observed in lysates prepared from ptenb morphants (Fig. 2.8). These results indicate that zebrafish Ptena and Ptenb both exhibit PIP₃ lipid phosphatase activity and function to negatively regulate the PI3-Kinase/Akt pathway.
2.4 Discussion

Studies of mouse knockouts and *Drosophila* mutants indicate that the Pten tumor suppressor gene plays an essential role in early development. However, since homozygous null mutations in mice cause embryonic lethality, it has been difficult to gain a better understanding of the precise developmental function of Pten in vertebrates. Here I have studied Pten function using zebrafish as a model vertebrate developmental organism. Zebrafish offer many advantages for studying complex developmental processes. In particular, MO-based antisense knockdowns offer the possibility of creating hypomorphs of specific genes. Thus intermediate phenotypes may become manifest without the embryonic lethality associated with null mutations in species such as mouse. In zebrafish, I identified two paralogous *pten* genes, *ptena* and *ptenb*, that exhibit unique but overlapping expression patterns through development. Each gene encodes an enzyme that appears to negatively regulate Akt phosphorylation and is required for embryogenesis. However, my observation that knockdowns of *ptena* and *ptenb* expression produce distinct morphant phenotypes suggests that these two *pten* genes have different functional roles in zebrafish development.

Gene mapping data show that the zebrafish *ptena* and *ptenb* genes map to LGs 17 and 12, respectively, while the human Pten gene has been localized to chromosome 10 (Li et al. 1997). Both LG17 and LG 12 contain multiple orthologs of human genes located on chromosome 10 (Woods et al. 2000). The fact that these linkage groups share significant synteny with human chromosome 10 is consistent with the view that these are duplicate
chromosome segments. My studies indicate that both Ptena and Ptenb negatively regulate the PI3-Kinase/Akt pathway via their lipid phosphatase activity, and that each enzyme is required to support embryogenesis. However, it is of interest that knockdown of each pten gene produces a distinct morphant phenotype. This result suggests that the two genes are not able to compensate for one another in early embryogenesis. The duplication-degeneration-complementation model has been proposed to account for the retention of duplicated genes in the genome (Force et al. 1999). This model proposes that retention of duplicated genes in the genome is accompanied by changes in localization or function such that the duplicates together retain the original functions of the single ancestral gene. It will now be of considerable interest to determine whether the two zebrafish pten genes together perform the function of the single mammalian Pten gene, or whether either of these duplicates has evolved new functional properties. This question can be addressed using morpholino-based knockdown coupled with mRNA rescue approaches that are now possible in zebrafish.

Mammals contain a single functional Pten gene, and to date no splice variants of this gene have been identified. Both pten genes in zebrafish exhibit alternative splicing, with short and long transcripts of ptena and ptenb expressed throughout embryogenesis and in adult fish. The presence of the alternatively spliced exon in both fish genes indicates that it predates the duplication in the teleost lineage. However, available data are insufficient to determine whether this exon was present in an ancestral vertebrate and lost in the tetrapod lineage, or whether it arose in the teleost lineage. The long splice variant of each zebrafish Pten polypeptide contains a 23 amino acid insertion within one of the three
loops that form the putative membrane association region of the *pten* C2 domain (Lee et al. 1999). The location of the insertion suggests the possibility that the zebrafish *pten* splice variants may exhibit differences in membrane targeting, binding, or function.

The expression patterns of the zebrafish *pten* genes during embryonic development closely resemble those of the orthologous mammalian genes. In zebrafish, *ptena* and *ptenb* are expressed ubiquitously during early somitogenesis. At the 18-somite stage, *ptenb* shows strong expression in the cranial ganglia and brain nuclei. As development proceeds, *ptena* and *ptenb* genes are predominantly expressed in the central nervous system. In early mouse development, *Pten* expression is not spatially restricted at E7 (Luukko et al. 1999), whereas by E15, *Pten* transcripts are abundant in the central and peripheral nervous system (especially in the spinal cord and peripheral nerve ganglia). During embryogenesis, the mouse and human *Pten* genes are expressed in the vasculature, lung, kidney, thymus, thyroid gland, skin, and gastrointestinal tract (Luukko et al. 1999; Gimm et al. 2000). In zebrafish, I detected expression of *pten* genes in the axial vasculature (*ptena*), endoderm (*ptena*), and branchial arches (*ptena* and *ptenb*). Although expression of *pten* genes was detected in ear (*ptena*), somites (*ptenb*) and eye (*ptena* and *ptenb*) of zebrafish embryos, it is not yet clear whether *Pten* genes are expressed in these tissues during mouse or human embryogenesis.

Knockdown of *ptena* mRNA translation resulted in a number of morphological phenotypes including defects in the formation of intersegmental blood vessels. Previous studies have demonstrated that Vegf (vascular endothelial growth factor) signals through
the Akt pathway to regulate vasculogenesis and angiogenesis in zebrafish (Chan et al. 2002). My data indicates that knockdown of *ptena* causes an increase in the levels of pAkt, and that elevated pAkt levels may lead to abnormal vasculogenesis in zebrafish. My results thus provide the first indication that *ptena* may play a role in vascular development. *ptena* morphants also exhibited defects in notochord morphology. It is possible that the defect in notochord shape may arise early in development when *ptena* is ubiquitously expressed. It is of further interest that knockdown of *ptena* also produced a defect in otolith formation. Rather than the two rounded otoliths characteristic of 48 hpf wild type embryos, *ptena* morphants had abnormal numbers and/or morphology of otoliths. To my knowledge, a role for Pten in ear development has not been previously reported. It will clearly be of interest to determine whether *ptena* regulation of the Akt pathway may serve to mediate aspects of inner ear morphogenesis.

Studies of mouse *Pten* knockouts and *Drosophila Pten* mutants have shown that during development, Pten plays a role in regulating cell and organ size as well as cell proliferation (Goberdhan et al. 1999; Huang et al. 1999; Gao et al. 2000; Kishimoto et al. 2003). I did not observe gross increase in organ size in either *ptena* or *ptenb* morphants. While it is possible that *pten* knockdown may have caused changes in cell size, detailed histological analysis will be required to resolve this issue. I did observe that both *ptena* and *ptenb* morphants had heads that were abnormally shaped compared to wild type embryos. This domed appearance could possibly reflect an increase in cell number or cell size within the brain. Results from neuron-specific knockouts of *Pten* in mice showed that altering *Pten* expression caused an increase in neuron size leading to an
overall increase in brain size (Kishimoto et al. 2003). It will be of interest to determine if similar changes in cell size contribute to the domed head phenotype observed in pten morphant zebrafish.

In summary, I have identified two pten genes in zebrafish that have overlapping expression patterns. Using MO knockdown I showed that ptena and ptenb play distinct roles in embryonic development of zebrafish, while both have the ability to antagonize the PI3-Kinase/Akt pathway. It will be important to determine whether the developmental roles of ptena and ptenb are solely dependent on the PI3-Kinase/Akt pathway. In addition, it will be interesting to see whether the additional exon in the long splice variants of zebrafish pten genes affects their function.
Chapter 3

Otoc1: a Novel Otoconin-90 Ortholog Required for Otolith Mineralization in Zebrafish

The work presented in this chapter was published in Developmental Neurobiology (Petko et al 2008). Robert Levenson was the principle investigator for this work. Bonnie Millimaki and Bruce Riley contributed proof of intact hair cells in otoc1 morphants by performing morpholino knockdowns in a transgenic zebrafish line and by antibody staining for tubulin otoc1 morphants. Victor Canfield performed phylogenetic analysis of otoc1. I performed all other analyses presented in this chapter.
3.1 Introduction

The vestibular system of the vertebrate inner ear conveys information about gravity and motion to the brain. Otolith organs are specialized components of the vestibular system that detect gravity and linear movements of the head through biomineralized particles coupled to the cilia of mechanosensory hair cells. Although the function of these vestibular structures is conserved among vertebrates, there are species-specific differences in their structural morphology. The biomineral particles of mammalian, avian, and amphibian vestibular systems are composed of thousands of tiny polyhedral particles called otoconia that are embedded in a gelatinous membrane. In contrast, teleost fish have three large otoliths that are smooth and ovoid in appearance.

Otoliths and otoconia are composed of calcium carbonate (CaCO$_3$) crystals condensed around an extracellular matrix core composed of glycoproteins and proteoglycans (Ross and Peacor 1975; Lim 1980; Pote and Ross 1991). It has been proposed that the polymorph of CaCO$_3$ found in otoliths or otoconia is dependent on the matrix proteins comprising the extracellular core (Pote and Ross 1991). Otoconin-90 (Oc90) is the predominant matrix protein found in calcitic CaCO$_3$ otoconia of mammals and birds (Wang et al. 1998; Verpy et al. 1999), whereas Otoconin-22 (Oc22) is the primary matrix protein of aragonitic CaCO$_3$ otoconia found in the saccule of amphibians (Pote et al. 1993). Oc90 and Oc22 share sequence homology with secretory phospholipase A2 (PLA2). Oc90 contains two PLA2-like (PLA2L) domains, while Oc22 has one.
Although these PLA2L domains do not possess enzymatic activity, they are extremely acidic, contain several potential glycosylation sites, retain the ability to bind calcium, and provide a rigid structure that can serve as a scaffold for CaCO₃ deposition (Pote et al. 1993; Wang et al. 1998; Thalmann et al. 2001). Gene targeting has recently been used to examine the role of Oc90 in mammalian otoconia formation (Zhao et al. 2007). Oc90 null mice fail to form the otoconial organic matrix, indicating that expression of Oc90 is necessary for the recruitment of other otoconins and organic components into the matrix core. Without formation of the organic matrix, the inorganic crystallites are prone to form large, disaggregated structures.

In teleost fish, otoliths grow diurnally and form daily rings within their aragonitic CaCO₃ microstructure (Panella 1971). Each ring is composed of a zone in which CaCO₃ predominates and a discontinuous zone in which the organic matrix predominates (Degens et al. 1969). Three major otolith matrix proteins have been identified in teleost fish, Omp-1 (otolith matrix protein-1), Otolin-1, and Starmaker. Omp-1 is a mellanotransferin-like protein that is required for otolith growth and matrix formation (Murayama et al. 2000; Murayama et al. 2005). Omp-1, like Oc90, is glycosylated, rich in cysteine residues, capable of binding calcium, and required for the recruitment of other proteins into the otolith organic matrix. Otolin-1, on the other hand, is required for the correct anchoring of otoliths on the sensory maculae and provides a collagenous scaffold that appears to stabilize the otolith matrix (Murayama et al. 2002; Murayama et al. 2005). Starmaker is an ortholog of mammalian dentin sialophosphoprotein (DSPP), a protein that in humans is involved in the biomineralization of teeth (Sollner et al. 2003). In
zebrafish, Starmaker appears to play an important role in controlling the morphology of
the developing otolith. To date, however, there have been no otolith matrix proteins
described in fish that posses PLA2L domains or that share sequence homology with
either Oc90 or Oc22.

Here I have identified a novel gene, *otoc1*, which encodes the zebrafish ortholog of
mammalian Oc90. Additional Oc90 orthologs were also identified in medaka and
*Xenopus*. Each of these otoconins shares sequence conservation among their PLA2L
domains. Zebrafish *otoc1* is expressed in the ear from early somitogenesis through 72
hpf and becomes increasingly restricted to the macular region as development progresses.
Expression of *otoc1* was also detected in epiphysis, optic stalk, midbrain, diencephalon,
flexural organ, and spinal cord. Knockdown of *otoc1* mRNA translation with antisense
morpholinos produced a variety of abnormal otolith phenotypes ranging from reduced
size to complete absence. Co-injection of sub-effective doses of *otoc1* and *omp-1*
morpholinos produced a similar range of otolith defects. My data indicate that Otoc1 is
required for early events in otolith biomineralization and may be necessary for
recruitment of other proteins into the organic matrix.
3.2 Experimental Procedures

3.2.1 Cloning of zebrafish *otoc1* gene

I carried out a Blast search of the zebrafish EST database and identified two ESTs [GenBank accession nos. BM182076 (fv53d08) and BM861236 (fy47e07)] that showed significant amino acid sequence similarity to the PLA2L domains of murine Oc90, and termed this novel gene *otoc1*. Since the two ESTs together failed to encompass the complete *otoc1* open reading frame (ORF), I used RACE (rapid amplification of cDNA ends) to generate the remaining *otoc1* 5’ mRNA sequence. RACE-ready cDNA was prepared from 24 hpf zebrafish embryos using the GeneRacer Kit (Invitrogen; Carlsbad, CA). PCR was performed using the GeneRacer 5’ primer: 5’– CGACTGGAGCAGGACACTGA – 3’, and *otoc1*-race primer: 5’ – CTGCAGTTTCTCTCTTTAGCTCTTGTGAACATCTCAAG – 3’. cDNAs were sequenced using an ABI 377 automated DNA sequencer. Zebrafish *otoc1* (GenBank accession no. AY826978) is a 3315-bp long cDNA containing a complete ORF that spans nucleotides 169-2988. BLAST searches of the zebrafish genome assembly (Version Zv6), available from the Zebrafish Sequencing Group (www.ensembl.org/), was used to assign *otoc1* to chromosome 2.

Human and chicken Oc90, as well as bullfrog Oc22, have previously been identified (NM_001080399, AAZ15113, and AB091830 respectively). Blast searches of available cDNA and genomic databases identified Oc90 orthologs in medaka (BJ010323, partial sequence) and *X. tropicalis* (BX738908, BX729352, BX708116, CX380335), while Oc22
orthologs were identified in *X. tropicalis* (CN085057, CN085058, CN076276, and CN076275) and *X. laevis* (BC078486).

### 3.2.2 Phylogenetic analysis

Amino acid sequences of 31 PLA2 domains from PLA2 families I, II, V, and X, snake venom components, and otoconins were aligned using the PILEUP program (Devereux et al. 1984). Due to differences in spacing between conserved cysteines, alignments were unambiguous only at the 50 positions corresponding to amino acids 44-77 and 113-128 of human PLA2G1B (NP_000919). Phylogenetic analysis was performed using the Phylip suite of programs (version 3.573c) described by Felsenstein (1981). Maximum parsimony trees were calculated using PROTPARS. Evolutionary distance trees were constructed by using the algorithm of Fitch and Margoliash (1967). Only the PLA2 domains of otoconins are shown in the resulting tree.

### 3.2.3 *otoc1* mRNA expression

Whole-mount *in situ* hybridization analysis was performed as described previously (Thisse and Thisse 1998). The *otoc1* antisense probe was generated from an *otoc1* EST [GeneBank accession no. BM182076 (fv53d08)]. Additional antisense RNA probes used in this study include *pax2a* (from I. Dawid), *otxl* (from E. Weinberg), *dlx3b* (Ekker et al. 1992a), and *msxC* (Ekker et al. 1992b).
For RT-PCR analysis, zebrafish embryos at various stages of development, as well as 6
month old adults, were collected and homogenized in TRIzol Reagent (Invitrogen). Total
RNA was extracted according to the method of Chomczynski and Sacchi (Chomczynski
and Sacchi 1987), and 0.5 μg of RNA was used as template to generate cDNA
(SuperScript First Strand Synthesis kit; Invitrogen). For all samples, PCR was performed
with REDTaq DNA polymerase (Sigma; St. Louis, MO), 1μg of cDNA, and primers
specific for \textit{otoc1} (5’-CCAGCCAGCGCAGGTATGTGTA-3’ and 5’-GCGGCTGAGGA
AACTCGAAGATC-3’) or \textit{omp-1} (5’-GCTACCTTCTGAGGAATAAGCTGG-3’ and
5’-CTTGATGTCCCCACACACAGGC-3’). PCR products were separated by
electrophoresis on a 1.5% agarose gel, and imaged with a Fluorochem 8900 imaging
system (Alpha Innotech; San Leandro CA). The identity of the PCR products from each
primer pair was verified by DNA sequencing.

3.2.4 Antisense morpholinos

Antisense morpholino oligonucleotides (MOs) (Gene Tools LLC; Philomath, OR) were
targeted against either the initiating methionine [\textit{otoc1}-ATG MO (5’-GAAAATAAGAT
ACAGCATCCTCATC-3’)], or the donor splice site of intron 4 [\textit{otoc1}-SS MO (5’-
TCCGCTTCATCTACCCGTCGAGCG-3’)] of \textit{otoc1} mRNA. I also used an antisense
morpholino targeted against the initiating methionine of \textit{omp-1} [\textit{omp}-MO (5’-CAAGA
TGTCCTCCTGGAAGATCCAT-3’)]. This MO was generously provided by Dr.
Ruediger Thalmann (Washington University, St. Louis). MOs were resuspended in 1x
Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM
HEPES, pH 7.6), and microinjected directly into the yolk sac of single cell embryos. The ability of the otoc1 morpholino to specifically block translation of otoc1 mRNA was tested using an in vitro translation assay as previously described (Blasiole et al. 2005; Blasiole et al. 2006).

3.2.5 Immunofluorescence analysis of tether cells

Tether cells were visualized by immunostaining with antibody to acetylated tubulin as described previously (Riley et al. 1999; Millimaki et al. 2007). The primary antibody was acetylated tubulin (Sigma T-6793, diluted 1:100), while the secondary antibody was Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes A-11001, diluted 1:50). Tether cell kinocilia were compared between control and morphant ears using confocal microscopy. Hair cell bodies were detected using a transgenic zebrafish line expressing membrane-targeted GFP under control of the brn3c promoter/enhancer (Xiao et al. 2005). This fish strain was kindly provided by Herwig Baier (UCSF).

3.3 Results

3.3.1 Identification of otoc1

I carried out a Blast search of the zebrafish EST database and identified two ESTs [GenBank accession nos. BM182076 (fv53d08) and BM861236 (fy47e07)] that showed significant amino acid sequence similarity to the PLA2L domains of murine Oc90. I named this previously unidentified gene otoc1. Although the two ESTs overlapped,
together they failed to encompass the complete *otoc1* open reading frame (ORF). I therefore used 5’ RACE to generate the full-length *otoc1* coding sequence. The complete cDNA and deduced amino acid sequence for zebrafish *otoc1* is shown in Fig. 3.1. The zebrafish *otoc1* gene (GenBank accession no. AY826978) consists of a 2820 base pair ORF encoding a polypeptide of 939 amino acids with a predicted molecular weight of 106 kDa. Blast searches of the zebrafish genome assembly available from the Zebrafish Sequencing Group (http://www.ensembl.org/) indicate that *otoc1* maps to chromosome 2.

Blast searches of available cDNA and genomic databases identified Oc90 orthologs in additional fish species, including medaka. Surprisingly, both Oc22 and Oc90 transcripts were identified in *Xenopus*. A comparison of the gene structure for representative Oc90 and Oc22 orthologs is presented in Fig. 3.2, A. The genes share the greatest degree of sequence and structural similarity within the two PLA2L domains (Fig. 3.2, A-C) and the N-terminal secretory peptide.

It has been proposed that the structural rigidity of the PLA2L domains within otoconin proteins facilitates the formation of the organic otolith matrix (Thalmann et al. 2001). This rigidity results from the disulfide bonds formed between cysteine residues. The number of cysteine residues within exons encoding the PLA2L domains of *otoc1* and each of its orthologs is shown in Fig. 3.2, A. Zebrafish *otoc1* contains two fewer cysteines within the first PLA2L domain compared to other Oc90 orthologs. As shown in Figs. 3.1 and Figs. 3.2, B and C, the position of the cysteine residues within otoconin PLA2L domains is highly conserved across species.
A comparison of the molecular weight, predicted pI, and number of potential \( N \)-linked glycosylation sites for Otoc1 and other Otoconins is presented in Table 3.1. The predicted pI for Oc90 orthologs ranges from a pH of 4.53 to 4.94, with zebrafish \( otoc1 \) being the most acidic. Amongst otoconin family members there is a high degree of variability in the number of potential \( N \)-linked glycosylation sites. For example, there are only two potential \( N \)-linked glycosylation sites within human Oc90, while zebrafish Otoc1 contains 15 potential \( N \)-linked glycosylation sites (Fig. 3.1). Only three of the potential \( N \)-linked glycosylation sites are conserved between otoconin family members (Fig. 3.2, B and C).

### 3.3.2 Phylogenetic analysis

To confirm the evolutionary relationship between zebrafish \( otoc1 \) and its mammalian, avian, and amphibian orthologs, phylogenetic analysis was conducted using maximum parsimony (MP) (Felsenstein 1981) and distance matrix (DM) (Fitch and Margoliash 1967) methods (Fig. 3.3) using 13 otoconin PLA2L domains and 18 additional PLA2 sequences. The PLA2L domains of Otoc1 and its Oc90 orthologs cluster together in 73% (DM) and 69% (MP) of trees generated by bootstrap analysis, and clustering by position within the polypeptide is even more strongly supported, with the first domain clustering in 96% (DM) and 79% (MP), and the second domain in 89% (DM) and 74% (MP) of all trees. Clustering of Oc22 orthologs is also strongly supported by 61% (DM) and 80%
**Figure 3.1:** Nucleotide and deduced amino acid sequence of zebrafish *otoc1*.

The predicted secretory signal sequence is boxed, and the two PLA2L domains are shaded in gray. Circles indicate potential glycosylation sites and asterisks designate the location of conserved cysteines. Nucleotides are numbered with 1 representing the A of the initiating methionine (Petko et al. 2008).
<table>
<thead>
<tr>
<th>Otoconin</th>
<th>Predicted MW</th>
<th>Predicted pI</th>
<th>Predicted N-linked Gly sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Oc90</td>
<td>51.73487</td>
<td>4.73</td>
<td>2</td>
</tr>
<tr>
<td>Mouse Oc90</td>
<td>52.44458</td>
<td>4.72</td>
<td>4</td>
</tr>
<tr>
<td>Chicken Oc90</td>
<td>57.72786</td>
<td>4.94</td>
<td>4</td>
</tr>
<tr>
<td>Xenopus Oc90</td>
<td>59.76153</td>
<td>4.73</td>
<td>5</td>
</tr>
<tr>
<td>Zebrafish Otoc1</td>
<td>105.97479</td>
<td>4.53</td>
<td>15</td>
</tr>
<tr>
<td>Xenopus Oc22</td>
<td>16.68744</td>
<td>6.69</td>
<td>2</td>
</tr>
<tr>
<td>Bullfrog Oc22</td>
<td>16.59812</td>
<td>6.29</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 3.2: Comparison of Otoconin orthologs.

(A) Exon/intron organization of human, chicken, Xenopus tropicalis, medaka, and zebrafish Oc90 orthologs are shown to the left. X.tropicalis Oc22, and human PLA2 (Type IIA) genes are on the right. Exons are represented by open boxes drawn to scale. Introns (lines connecting exons) are not drawn to scale. PLA2L domains are shaded in gray. Numbers in shaded boxes denote the number of cysteines. A full-length sequence for medaka otoc1 is not currently available. Question mark in the medaka gene represents a gap in the known sequence. (B). Amino acid alignment of first PLA2L domain of Oc90 orthologs from mouse (m), chicken (c), X. tropicalis (x), and zebrafish (z) and the single PLA2L domain from bullfrog (bf) and X. tropicalis Oc22. (C) Amino acid alignment of the second PLA2L domain of Oc90 orthologs from mouse, chicken, X. tropicalis, and zebrafish and the single PLA2L domain from bullfrog and X. tropicalis Oc22. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left. Asterisks indicate cysteine residues and numbers above sequence indicate potential conserved sites of N-linked glycosylation (Petko et al. 2008).

(MP) of trees. In contrast, clustering of Oc90 with Oc22 is poorly supported (33% by DM and only 4% by MP).

3.3.3 Expression of otoc1 mRNA

I used whole-mount in situ hybridization to examine otoc1 mRNA expression during zebrafish embryogenesis. The expression profiles of zebrafish otoc1 are shown in Fig. 3.4. Expression of the otoc1 gene was detected as early as 15 hpf in the otic vesicle, midbrain-hindbrain boundary, optic stalk, and spinal cord (Fig. 3.4, A). Expression of otoc1 in these tissues persisted at 24 hpf, although otoc1 transcripts in the spinal cord became restricted to the floor plate (Fig. 3.4, B-C). At 24 hpf, otoc1 transcripts were also detected in the region of the circumventricular organs and developing branchial arches. In the ear at 24 hpf, otoc1 expression was detected throughout the otic vesicle except for
Figure 3.3: Phylogenetic analysis of PLA2L Otoconins.

The tree was rooted using 18 additional PLA2 sequences, including the seven alignable human PLA2 sequences from groups I, II, V and X. Numbers to the left of each node indicate percent support from bootstrap analysis (Fitch/Margoliash above, maximum parsimony below). Consensus trees obtained from both methods agree, except that the grouping of all Otoconin domains was supported by only 4% of trees in the MP analysis. Alternative clusterings using the MP method were supported by no more than 15% of trees (Petko et al. 2008).
a ventral portion of the epithelium (Fig. 3.4, D). At 48 hpf, otoc1 mRNA was present in the diencephalon, flexural organ, circumventricular region, and the otic vesicle (Fig. 3.4, E-H). In the otic vesicle, expression of otoc1 was predominant in the macular regions, with lighter staining also observed in the epithelial protrusions of the developing semicircular canals (Fig. 3.4, H). A similar expression pattern of otoc1 mRNA was observed at 72 hpf, although staining was less intense throughout the embryo and absent from the diencephalon (Fig. 3.4, I-J). By 5 dpf, only very low levels of otoc1 mRNA were detected and were localized predominantly in the flexural organ (Fig. 3.4, K-L). Virtually no transcripts were detectable in the ear at 5 dpf.

I next used RT-PCR to compare the temporal expression of otoc1 and omp-1, the major matrix protein of teleost otoliths. As shown in Fig. 3.4, M, primers specific for otoc1 amplified a product in all developmental stages analyzed from 12 hpf through adult. Although the PCR I performed was not quantitative, otoc1 amplicons generated from 5 dpf and adult were consistently (n=4) less intense than amplicons generated from earlier developmental stages, suggesting that levels of otoc1 transcripts may decrease during the course of development. These results are consistent with my in situ hybridization data which showed decreasing levels of otoc1 mRNA staining as a function of developmental age. In contrast, omp-1 expression was first detected at 15 hpf and persisted throughout embryogenesis including 5 dpf embryos (Murayama et al. 2005). Lower levels of omp-1 PCR products were observed at 15 hpf and 18 hpf compared to larval stages. As was the case with otoc1, omp-1 expression levels were consistently lower in the adult (n=4) compared to embryonic stages. These results suggest that expression of otoc1 precedes
Figure 3.4: Expression of zebrafish *otoc1* mRNA during embryogenesis.

Expression of *otoc1* was analyzed by whole-mount *in situ* hybridization (A-L). (A) Early somitogenesis, lateral view, (B) 24 hpf, lateral view, (C) 24 hpf, dorsal view, (D) 24 hpf, lateral view of ear, (E) 48 hpf, lateral view of head, (F) 48 hpf, dorsal view of head, (G) 48 hpf, ventral view of head, (H) 48 hpf, lateral view of ear, (I) 72 hpf, lateral view of head, (J) 72 hpf, lateral view of ear, (K) 5 dpf, dorsal view of head, (L) 5 dpf, lateral view of ear. BA, branchial arches; CVO, circumventricular organs; D, diencephalon; EP, epithelial protrusions of developing semicircular canals; FO, flexural organ; FP, floor plate; M, macula; MB, midbrain; MHB, midbrain/hindbrain boundary; OP, otic placode; OS, optic stalk; OV, otic vesicle; PF, pectoral fin; SC, spinal cord; SCC, semicircular canal pillars. (M) Comparison of *otoc1* and *omp-1* mRNA expression during zebrafish development determined by RT-PCR (Petko et al. 2008).
that of *omp-1*, raising the possibility that Otoc1 may be required for initiating formation of the organic matrix of zebrafish otoliths.

3.3.4 Morpholino knockdown of *otoc1* mRNA translation

To gain an understanding of the function of *otoc1*, I used antisense morpholinos (MOs) to knock down *otoc1* mRNA translation in developing zebrafish embryos. Two non-overlapping MOs were generated that targeted either the initiating methionine (*otoc1*-ATG MO), or the donor splice site of intron 4 (*otoc1*-SS MO) of zebrafish *otoc1* mRNA. Using an in vitro translation assay (Blasiole et al. 2005; Blasiole et al. 2006), the *otoc1*-ATG MO was found to specifically block expression of *otoc1* mRNA in a dose-dependent fashion (Fig. 3.5).

*otoc1*-ATG MO and *otoc1*-SS MO were separately microinjected into one-cell stage embryos. Microinjection of the *otoc1*-ATG MO (at doses ranging from 1-3 ng) into zebrafish embryos produced noticeable effects on ear development, whereas embryos injected with Danieau buffer alone showed no defects. At 24 hpf, morphant embryos appeared to develop normally, although morphants exhibited a slightly diminished head size compared to wild type embryos (Fig. 3.6, A and B). Injection of the *otoc1*-ATG MO induced a number of aberrant otolith phenotypes (Fig. 3.6, F-J). These defects ranged in severity and included small otoliths (Fig. 3.6, F and I), extra or missing otoliths (Fig. 3.6, G-I), or complete absence of otoliths (Fig. 3.6, J). The percentage of
Figure 3.5: Specificity of *otoc1* antisense morpholino.

*otoc1* and *ptenb* mRNAs were separately translated *in vitro* in the presence or absence of *otoc1* MO. [35S]-labeled proteins were separated on an SDS-containing 10% polyacrylamide gel. The gel was dried and exposed to X-ray film. Arrows indicate the location of Otoc1 and Ptenb protein bands. Dosage of morpholino is indicated below each lane and the mRNA being translated is indicated above the lanes (Petko et al. 2008).
Morphants were injected with 2 ng of `otoc1-ATG` MO. (A) wild type embryo, (B) `otoc1` morphant, (C-D) `pax2a` staining of (C) wild type embryo, (D) `otoc1` morphant, (E) otic vesicle, wild type embryo, (F-J) otic vesicle, `otoc1` morphants, (K-L) `otx1` staining of (K) wild type otic vesicle. (L) `otoc1` morphant otic vesicle. (N-O) `dlx3b` staining of (N) wild type otic vesicle (O) `otoc1` morphant otic vesicle. (M-P) `msxC` staining at 60 hpf of (M) wild type otic vesicle (P) `otoc1` morphant otic vesicle). Arrows indicate small otoliths, black arrowheads indicate missing otoliths, while white arrowheads indicate extra otoliths. Scale bars: A-B, 250 μm; E-J, 50 μm (Petko et al. 2008).

It is possible that the otolith defects in `otoc1` morphants reflect a general developmental delay. To address this possibility, I used the expression of `pax2a`, `otx1`, `dlx3b`, and `msxC` to analyze development of a variety of organ systems. In zebrafish, `pax2a` is commonly used as a marker for normal embryonic development (Shu et al. 2003; Blasiole et al.)
2005), while *otx1, dlx3b*, and *msxC* were used as markers for correct patterning of the ear (Ekker et al. 1992a; Ekker et al. 1992b; Hammond et al. 2003). As shown in Fig. 3.6, the expression patterns of *pax2a, otx1, and dlx3b* in 24 hpf morphants, and *msxC* in 60 hpf morphants, closely resembled that of wild type embryos. These results suggest that embryos treated with *otoc1*-ATG MOs develop normally during early embryogenesis.

Tether cells are precocious hair cells that are essential for otolith seeding (Riley et al. 1997). To determine whether knockdown of *otoc1* expression caused defects in tether cell development, tether cells were visualized in GFP-expressing transgenic fish (Xiao et al. 2005), and by immunostaining with antibody to acetylated tubulin. Tether cell kinocilia and cell bodies were compared between control and morphant ears as shown in Fig. 3.8. Intact tether cell bodies (16/16 morphants) and tether cell kinocilia were observed in wild type and *otoc1* morphants (15/15 morphants). Taken together, these results suggest that the otolith defects observed in *otoc1* morphants are not due to the absence of tether cell bodies or kinocilia.

Morpholinos targeted against *omp-1* have previously been shown to produce morphant fish with small otoliths (Murayama et al. 2005). Amongst the otolith phenotypes produced by knockdown of *otoc1* were morphants with small otoliths, suggesting a possible functional interaction between *otoc1* and *omp-1*. To test this idea, I co-injected sub-effective doses of *otoc1*-ATG MO (0.5 ng) and *omp-1* MO (0.5 ng) and analyzed the effect on otholith development. At a concentration of 0.5 ng, neither the *otoc1* MO
Figure 3.7: Phenotypes of *otoc1* morphants.

Bar graph depicts percent of fish at 24 hpf that displayed normal otoliths, small otoliths, aberrant number of otoliths, and complete lack of otoliths when injected with various doses of *otoc1*-ATG MO. The number of fish assayed at each dose of MO is displayed above the bars (Petko et al. 2008).
Figure 3.8: Tether cells are present in \textit{otoc1} morphant ears.

All panels show a lateral view of embryos at 24 hpf with anterior to the left. \textbf{(A and D)} DIC images of (A) wild type otic vesicle (D) \textit{otoc1} morphant otic vesicle containing only one otolith. Fluorescent image of (B) wild type otic vesicle from GFP-expressing Brn3c transgenic fish (E) \textit{otoc1} morphant otic vesicle from GFP-expressing Brn3c transgenic fish. A pair of tether cells (arrows) is present at the anterior and posterior poles. Anti-acetylated tubulin immunofluorescence of tether cell kinocilia in (C) wild type otic vesicle) and (F) \textit{otoc1} morphant otic vesicle. Arrowheads indicate a pair of tether cell kinocilia at the anterior and posterior poles of the otic vesicle. Asterisks indicate position of missing otolith (Petko et al. 2008).
Figure 3.9: Phenotypes of embryos co-injected with sub-effective doses of *otoc1*-ATG and *omp-1* morpholinos.

Bar graph depicts percent of fish at 24 hpf that displayed normal otoliths, small otoliths, aberrant number of otoliths, and complete lack of otoliths when injected with 0.5 ng of either *otoc1*-ATG MO alone, *omp-1* MO alone, or 0.5 ng of *otoc1*-ATG MO plus 0.5 ng of *omp-1* MO. The number of fish assayed for each treatment is displayed above the bars (Petko et al. 2008).
(n=37) nor the *omp-1* MO (n=41) produced severe otolith development defects, although 5-10% of fish displayed minor defects such as reduced otolith size and appearance of extra otolith particles. However, co-injection of sub-effective doses of the two MOs produced an otolith defect in 39% of the embryos (n=46) with 6.5% exhibiting loss of both otoliths (Fig. 3.9). Co-injection of the two MOs thus appears to amplify the otolith defects observed when either *otoc1* or *omp-1* MOs is used alone. The functional interaction observed in this assay suggests that *otoc1* and *omp-1* may act synergistically in formation of the organic matrix early in otolith development.

### 3.4 Discussion

I have identified a novel gene, *otoc1*, which encodes a zebrafish otolith matrix protein. Otoc1 is orthologous to Oc90, the major matrix protein of mammalian otoconia. Orthologs of Oc90 are also present in the genomes of medaka and *Xenopus*. Although otolith matrix proteins containing PLA2L structural domains have not previously been described in teleost fish, Otoc1 contains two PLA2L domains and shares sequence homology with Oc90 orthologs primarily within these regions of the protein. Identification of *otoc1* in zebrafish is somewhat surprising, since it has generally been assumed that variations in the CaCO₃ polymorph characteristic of mammalian, amphibian, or fish ear stones are dependent on differences in the protein species comprising the organic matrix core. Previous studies have implicated Oc90 as the predominant matrix protein of calcitic otoconia, and Omp as the predominant protein of
aragonitic otoliths. The identification of zebrafish *otoc1* therefore suggests that Oc90 orthologs may be necessary, but not sufficient, for formation of calcitic otoconia.

Besides containing nonfunctional PLA2L domains, Otoc1 shares a number of other structural features with otoconin family members. Each of these core matrix proteins appears to be a glycoprotein, and each is characterized by having an acidic pI that presumably aids in calcium binding. Otoc1 and Oc90 orthologs also share a cysteine-rich composition that may allow for the formation of multiple disulfide bridges. The fact that there is a high degree of conservation in the number and location of cysteines within the PLA2L domains of otoconin family members suggests that the formation of disulfide bridges may be important in building the rigid backbone of the otolith protein matrix and may act to form a scaffold for accretion of CaCO$_3$ crystals.

Three major otolith matrix proteins, Starmaker (Sollner et al. 2003), Omp-1 (Murayama et al. 2000; Murayama et al. 2005), and Otolin-1 (Murayama et al. 2002; Murayama et al. 2005), have been described to date in teleost fish. In zebrafish, the functional role of these proteins in early otolith development has been deduced by using antisense morpholinos to knock down translation of the cognate mRNAs. Morpholino-mediated knockdown of *starmaker* expression causes a delay in otolith formation as well as a change in the crystal polymorph of otoliths from aragonite to calcite, suggesting that Starmaker function is required for maintaining proper otolith morphology (Sollner et al. 2004). Omp-1 appears to be the predominant otolith matrix protein of teleost fish (Murayama et al. 2000). In zebrafish, *omp-1* morphants initiate otolith development, but
the otoliths fail to reach normal size, suggesting that Omp-1 is required for proper otolith growth (Murayama et al. 2005). In contrast, knockdown of *otolin-1* mRNA expression produces a very different phenotype. Morphant otoliths lose adherence to the sensory maculae and fuse, suggesting that Otolin-1 is required for the correct anchoring of the otoliths on the sensory maculae (Murayama et al. 2005).

Otoc1 represents the fourth otolith matrix protein to be identified in teleost fish. Overall, the effect of knocking down *otoc1* mRNA expression on otolith development is more severe than the morphant phenotypes produced by knocking down *omp-1* expression. The predominant phenotype of *omp-1* morphants is small otoliths (Murayama et al. 2005). In contrast, *otoc1* morphants exhibit a variety of otolith defects including small otoliths, extra or missing otoliths, and in some cases complete absence of otoliths. These phenotypic differences suggest that Otoc1 may function upstream of Omp-1 during otolith development. Interestingly, my *in situ* hybridization analysis of *otoc1* mRNA expression revealed the presence of *otoc-1* mRNA transcripts as early as 15 hpf (15 hpf being the earliest stage analyzed), while previous studies reported *omp-1* mRNA expression beginning at 16 hpf (Murayama et al. 2005). By using RT-PCR, *otoc-1* mRNA expression was clearly detectable at 12 hpf, whereas *omp-1* amplicons were not visualized until 15 hpf. These results suggest that expression of *otoc1* mRNA precedes that of *omp-1*. The availability of antibodies against zebrafish Otoc1 will clearly be necessary in order to compare the temporal pattern of Otoc-1 protein expression with that of OMP-1. However, my results suggest the possibility that Otoc-1 may be required for the initial steps in formation of the organic matrix of zebrafish otoliths, and for the
recruitment of other otoconins into the matrix core. Evidence for a functional interaction between Otoc-1 and Omp-1 (i.e. otolith defects produced by co-injection of sub-effective doses of \textit{otoc1} and \textit{omp-1} MOs) is consistent with this hypothesis.

The biomineralization of ear stones in the inner ear is a highly regulated process that requires that organic and inorganic components be brought together in the proper temporospatial context. The initial step in this process involves formation of an organic matrix which serves as a scaffold upon which CaCO$_3$ crystals can seed and grow. Morphant phenotypes produced by injecting \textit{otoc1} MOs suggest that \textit{otoc1} expression is required for otolith development and may serve as a scaffold for recruitment of other otoconins into the organic matrix.

OC90 has been shown to nucleate the organic lattice found in mouse otoconia by recruiting other matrix proteins, including the murian orthologs of Otolin. The identification of \textit{otoc1} has led us to propose a new model of otolith development in zebrafish in which Otoc1 initiates otolith seeding while Omp-1 is required for otolith growth. In addition, Otoc1 may serve to recruit Omp-1 and Otolin-1.

This hypothesis can be tested several ways. Recruitment of one protein by another implies physical interaction. I expect that Otoc1 directly interacts with Omp-1 and Otolin-1. This could be confirmed by utilizing GST pull down or directed yeast-2-hybrid methods. Secondly, recruitment of Omp-1 and Otolin-1 by Otoc1 can be demonstrated
by comparing Otoc1, Omp-1, and Otolin-1 protein distribution in the inner ear of wild
type, \textit{otoc1} morphant embryos, \textit{omp-1} morphant embryos, and \textit{otolin-1} morphant
embryos using antibodies specific for each of these proteins. I expect that \textit{otoc-1}
morphants will have little to no Omp-1 and Otolin-1 localized to the existing morphant
otoliths or the macula of missing otoliths. Consequently, I anticipate that more Omp-1
and Otolin-1 would be floating free in the endolymph of \textit{otoc-1} morphants. In contrast, if
Otoc-1 is responsible for the recruitment of Omp-1 and Otolin-1 to the otolith matrix,
Otoc-1 distribution in the otolith seed should not be disturbed in \textit{omp-1} morphants or
\textit{otolin-1} morphants.

Mutants that display otolith phenotypes similar to that of \textit{otoc1} morphants, may also be
useful for studying the role that \textit{otoc1} plays in otolith development. monolith, Einstein,
and menhir are mutants that fail to properly seed otoliths from free floating core particles.
It will be interesting to determine whether \textit{otoc1} protein secretion, localization or
production is abberent in these mutants as compared to wildtype zebrafish.
Chapter 4

A Screen for Ncs-1 Interacting Proteins and Their Role in Semicircular Canal Development

The principle investigator for this work was Robert Levenson. The yeast-2 hybrid screen for the identification of Ncs-1 interacting proteins was performed by Nadine Kabbani. Identification of zebrafish orthologs for human proteins was performed by Victor Canfield and me. Brian Blasiole obtained EST clones for several NIP orthologs. I obtained clones for the remaining NIP orthologs by ordering and confirming the identity of ESTs or by RT-PCR from cDNA. I am responsible for all other procedures performed in this chapter.
4.1 Introduction

The vestibular system of the inner ear functions to sense movement and gravity and is essential to the survival of all vertebrate species. The semicircular canals are one subdivision of the vestibular system responsible for detecting rotational head movements. Direction and speed of rotation is measured by differential flow of fluid through the hollow canals and over patches of sensory hair cells called cristae. The structural and functional properties of semicircular canals and their associated cristae are highly conserved among vertebrate species. Although some genes involved in the formation of these structures have been identified, little is known about the mechanisms that drive vestibular morphogenesis. Uncovering these mechanisms may lead to further understanding of human balance disorders and craniofacial abnormalities that are often associated with vestibular malformation.

Zebrafish have been a useful model for identifying genes involved in the development of the inner ear from early induction of the otic placode to the formation of mature semicircular canals and otolith organs. They serve as an excellent model for studying inner ear development, as they are easy to genetically manipulate and have the same complex vestibular structure as all other vertebrates. The formation of the semicircular canals in zebrafish begins at 45 hpf with the development of three epithelial outpocketings: one anterior protrusion, one posterior protrusion, and one lateral protrusion (Kimmel et al. 1995). At 60 hpf, the lateral protrusion bifurcates into an
anterior branch and a posterior branch that will fuse with the anterior and posterior protrusion, respectively (Haddon and Lewis 1996). Shortly after the formation of the horizontal hub, a ventral protrusion develops at the bottom of the vesicle and fuses with the lateral protrusion. Finally, a thin septum arises from the dorsal wall forming a dorsolateral partition. At 72 hpf, the four hubs are completely formed and appear as a cross shaped structure that spans the lumen. The precise mechanism driving epithelial outgrowth and fusion is unknown; however, some genes involved in hyaluronic acid synthesis and Bmp signaling have been implicated.

In *Xenopus*, the outgrowth of epithelium is directed by localized secretion of the extracellular matrix protein, hyaluronic acid (HA), into the acellular space between the epithelium and the underlying mesenchyme (Haddon and Lewis 1991). The secretion of HA is suggested to propel the protrusion forward into the lumen. Other studies in zebrafish have suggested a similar mechanism of protrusion formation. *jekyll*, a zebrafish mutant with interrupted semicircular canals, has a defect in *ugdh* (UDP-glucose dehydrogenase), a gene whose protein product is an enzyme that is required for the synthesis of proteoglycans including HA (Neuhauss et al. 1996). Disruption of *dfna5* (autosomal dominant nonsyndromic sensorineural deafness gene 5) expression results in a loss of *ugdh* expression and phenocopies the *jekyll* mutation (Busch-Nentwich et al. 2004).

Another zebrafish mutant, *gallery*, displays only a lateral protrusion and an immature anterior protrusion (Omata et al. 2007). The defective gene in this mutant is yet
unidentified; however, the defects are predicted to be caused by the overexpression of Bone morphogenetic proteins (Bmps) in the presumptive cristae. By implanting a Bmp4-soaked bead into the tissue surrounding the otic vesicle in a wild type embryo, Omata et al. were able to phenocopy the gallery mutation (2007). These results confirm that Bmp4 constitutes a signal from a sensory region that controls the formation of the non-sensory canal ducts. Altering the protein levels of Bmp antagonists, such as Dan (differential screening-selected gene aberrant in neuroblastoma) and Noggin, within the chick and zebrafish inner ear also disrupts development of the semicircular canal ducts (Chang et al. 1999; Gerlach et al. 2000; Omata et al. 2007; Yamanishi et al. 2007). Taken together, these results suggest that precise regulation of Bmp signaling is required for the formation of the semicircular canal structure in vertebrates.

Recent work has identified two zebrafish orthologs of Neuronal calcium sensor-1 (Ncs-1) and demonstrated that one ortholog, Ncs-1a, is required for semicircular canal morphogenesis (Blasiole et al. 2005). Ncs-1 is a calcium sensing protein that was originally identified in Drosophila for its ability to regulate exocytosis of neurotransmitters (Pongs et al. 1993). Despite the role of Ncs-1 in promoting neurotransmission, the gene is found to be expressed in many non-neuronal tissues during development and adulthood. For example, the zebrafish ortholog ncs-1a is expressed in non-neuronal epithelium during the formation of semicircular canal hubs (Blasiole et al. 2005). Morpholino knockdown of ncs-1a inhibits epithelial protrusion formation in the otic vesicle at 48 hpf. By 5 dpf, ncs-1a morphants display disorganized and sometimes
unfused epithelial pillars. Little is known about the mechanism by which Ncs-1 acts in this and other non-sensory tissues.

The goal of this project was to gain further understanding of non-neuronal roles of Ncs-1 and, in particular, to identify a mechanism or pathway for Ncs-1 in semicircular canal development. I hypothesize that protein interactors of Ncs-1 will also be involved in this process and may provide insight into the mechanism of morphogenesis. I have employed a multistep approach in order to answer this question which includes the identification of NIPs, distinguishing valid candidates, testing functional significance of NIPs, and establishing a common functional pathway involving NIPs and Ncs-1a.

A list of human Ncs-1 interacting proteins (NIPs) was constructed using both a yeast-2 hybrid screen and a primary literature search. I identified Zebrafish orthologs of these previously known and newly discovered NIPs through BLAST searches of the current assembly of the zebrafish genome and available EST databases. To confirm the interaction of NIPs with Ncs-1 and to show that this interaction is conserved in fish, I performed GST pulldowns and co-immunoprecipitations using zebrafish NIP orthologs and Ncs-1a.

For a protein-protein interaction to play a significant role in cellular processes, it is crucial for the proteins to co-localize in particular cell types. I expect that NIPs involved in vestibular morphogenesis will also be expressed in similar regions of the ear as ncs-1a. I utilized whole mount in situ hybridization to identify which zebrafish NIP orthologs are
expressed in these regions. I chose three NIPs that were expressed in the inner ear to explore further. Next, I knocked down the expression of these NIPs individually to determine whether they are involved in the development of the semicircular canal ducts. I expected to see that a knockdown of the chosen NIP orthologs would lead to dysfunctional protrusion development at 48 hpf and disorganized pillar formation at 72 hpf, the phenotypes observed in NCS-1a morphants.

After determining which NIP orthologs are expressed and functioning in the ear during development, I wanted to establish whether these genes are found in a common pathway with Ncs-1a during semicircular canal formation. This can be achieved in several different ways. By co-injecting sub-effective doses of morpholinos targeted to two different genes in a common pathway, synergism can be observed by the presence of the ear phenotype. I looked for synergistic effects of various combinations of NIP and ncs-1a morpholinos.

4.2 Experimental Procedures

4.2.1 Yeast-two-hybrid screen

A Y2H screen was performed as previously described (Lin et al. 2005). Briefly, bait plasmids containing full-length Ncs-1 fused to the yeast GAL4 DNA binding domain were transformed into the MaV103 yeast strain via the standard lithium acetate method. Transformed yeast was grown at 30°C in YTA media under -Leu selection for 48 hours.
Upon reaching maximal confluence, the adult human brain cDNA library plasmids were introduced using the same lithium acetate method as described previously (Lin et al. 2005). The genes represented in the cDNA library are fused to the yeast GAL4 transcriptional activation domain. Co-transformed cells were from for 14-36 hours at 30°C in YTA media under -Leu/-Trp/-His/-Ade to ensure selection for both bait and pray plasmids. Cells were then plated on -Leu/-Trp synthetic dropout plates and incubated at 30°C for a total of 5 days before clone analysis. Yeast colonies that grew on the dropout plates were subjected to a β-galactosidase nitrocellulose filter test for confirmation of interactions. cDNA was subsequently extracted from the yeast colony, sequenced, and subjected to a Blast search against the human genome to determine the identity of the clone.

4.2.2 Ortholog identification and cloning

Zebrafish orthologs of ten human NIPs were identified using a Blast search of the human sequence against the zebrafish EST database or the zebrafish genome. Table 4.1 illustrates the methods of identification for the gene sequence and the methods of obtaining clones for further investigation. For RT-PCR, zebrafish embryos at 48 hpf collected and homogenized in TRIzol Reagent (Invitrogen; Carlsbad, CA). Total RNA was extracted as previously described (Chomczynski and Sacchi 1987), and 0.5 μg of the RNA was used as template to generate single stranded cDNA using the SuperScript First Strand Synthesis kit (Invitrogen). PCR was carried out with the primer indicated in
Table 4.1 and REDTaq DNA polymerase (Sigma; St. Louis, MO) using a RoboCycler Gradient Temperature Cycler (Stratagene; La Jolla, CA).

4.2.3 Whole-mount in situ hybridization

24 and 48 hpf zebrafish embryos were preserved in 4% PFA in PBS. Whole-mount in situ hybridization analysis was performed as described previously (Thisse and Thisse 1998). Riboprobes were synthesized from clones listed in Table 4.2 for the following genes: dan, hint2, ip3r, pink1, pi4kβ, slc25a25b, trpc1, trpc4a, trpc5, and vamp2.

4.2.4 Morpholino Knockdowns

Antisense morpholino oligonucleotides (MOs) (Gene Tools LLC; Philomath, OR) were targeted against either the initiating methionine or the 5’ untranslated region of pi4kβ, arf1, ncs-1, or dan mRNAs. The sequence for each morpholino is listed in Table 4.2 MOs were resuspended in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6), and microinjected directly into the yolk sac of single cell embryos. Morpholino combinations for co-injections were mixed to appropriate concentrations before injection.

<table>
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<tr>
<th>Morpholino</th>
<th>Sequence</th>
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<tr>
<td>ncs1a-MO</td>
<td>5’-TAGTTTGCTGTGGATTTGCCCATC-3’</td>
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<tr>
<td>arf1-ATG MO</td>
<td>5’-TAAAGAGGTTTGGCGAATATGTTTCC-3’</td>
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<tr>
<td>arf1-UTR MO</td>
<td>5’-CGCCTTGTGCACACAAAGTTCCAAG-3’</td>
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<tr>
<td>pi4kβ-ATG MO</td>
<td>5’-AAGCTCCAGCTCTGTATCACCACCATG-3’</td>
</tr>
<tr>
<td>pi4kβ-UTR MO</td>
<td>5’-CACTTCCAGGCCCTCAAAATAAACC-3’</td>
</tr>
<tr>
<td>dan-ATG MO</td>
<td>5’-CGCGCACAACACATCACCACATCCTC-3’</td>
</tr>
<tr>
<td>dan-UTR MO</td>
<td>5’-TCAGTGGATTAGCAGCTCGGTTGT-3’</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Sequence Source and Genebank Accession Number</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Dan</td>
<td>EST (BC066387)</td>
</tr>
<tr>
<td>Hint2</td>
<td>EST (CN504819)</td>
</tr>
<tr>
<td>Ip3r</td>
<td>EST (CK017406)</td>
</tr>
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<td>Pink1</td>
<td>Genome Sequence (ch6 contig, NW_001513662)</td>
</tr>
<tr>
<td>Pi4kβ</td>
<td>EST (BM095643)</td>
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<td>S1c25a25a</td>
<td>Genome Sequence (ch 8 contig, NW_0011518928)</td>
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<tr>
<td>S1c25a25b</td>
<td>Genome Sequence (ch5 contig, NW_001513403)</td>
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<td>Trpc1</td>
<td>EST (EE302043)</td>
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<td>Trpc4a</td>
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<td>Trpc4b</td>
<td>Genome Sequence</td>
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<tr>
<td>Trpc5</td>
<td>EST (AW343190)</td>
</tr>
<tr>
<td>Vamp2</td>
<td>EST (BC059626)</td>
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4.2.5 Protein interaction assays

Directed Yeast-two-hybrids. Zebrafish arf1, pi4kβ, and dan, and human Trpc1 were inserted into pACT2. Human Ncs-1 was subcloned in pAS2-1. Bait and prey constructs were co-transformed into MaV103, and positive clones were identified by growth on -Leu/-Trp selection plates followed by expression of β-galactosidase activity. For each protein–protein interaction screen, β-galactosidase assays were repeated twice. For all β-galactosidase assays performed in this study, we detected either a robust signal indicating positive interactions (in the positive control only) or an absence of signal indicating no interaction.

Glutathione S-transferase pull-down. The fusion protein, glutathione S-transferase (GST)-Ncs-1 (amino acids 1–190), was constructed in the expression vector pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ). GST-Ncs-1 fusion protein was induced in Escherichia coli strain BL21 (DE3) then purified with glutathioneseapharose (Amersham) according to manufacturer’s instructions. arf1, pi4kβ and dan genes were cloned into the pet30c expression vector. Induction methods for these constructs are identical to the GST-Ncs-1 fusion protein. GST pull-down assays were performed as previously described (Lin et al. 2001). Eluted proteins were separated by SDS-PAGE and transferred to a PVDF filter. The filter was probed with a horseradish peroxidase conjugated S-tag antibody (1:5000 dilution, Santa Cruz Biotechnology).

Co-immunoprecipitations. Immunoprecipitations were performed from total cell lysates as previously described (Karpa et al. 2000). FLAG-tagged Pi4kβ was transfected into
HEK293 cells, collected, and immunoprecipitated using the M2 anti-FLAG monoclonal antibody. Western analysis of immunoprecipitated complexes was performed by using polyclonal anti-frequenin antibody (1:5000 dilution, Rockland Immunochemicals). Proteins were visualized with a horseradish peroxidase conjugated rabbit anti-goat (1:10,000 dilution) secondary antibody (Jackson ImmunoResearch).

4.3 Results

4.3.1 Identification of human NIPs

Previous studies have shown Ncs-1 to regulate Pi4kβ, Arf1, and several ion channels (Weiss et al. 2000; Weiss and Burgoyne 2001; Zhao et al. 2001; Tsujimoto et al. 2002; Rousset et al. 2003; Haynes et al. 2005). We hypothesize that Ncs-1 interacts with a multitude of cellular proteins, some of which are involved in a common pathway in inner ear development. To identify novel components of Ncs-1 signaling pathways, full length human Ncs-1 was used as bait in a Y2H screen of an adult brain cDNA library. This screen produced a total of 131 positive clones that passed high stringency selection. Clones were sequenced and subjected to a Blast search against the human genome for identification. Fourteen novel and unique interactors were identified. Table 4.3 summarizes the interactors identified in our screen and in previous work from other laboratories.
4.3.2 Identification and cloning of zebrafish NIP orthologs

Ten NIPs were chosen for analysis in zebrafish based on their known functions. Ncs-1 is known to be involved in calcium detection and exocytosis, therefore, I chose to further characterize interactors with similar known functions. Arf1, Pi4kβ, and Vamp2 have shown to play roles in exocytosis and neurotransmission (Balch et al. 1992; Gerst 1999; Krauss and Haucke 2007). Trpc1, Trpc5, and Ip3r are channels involved in calcium ion signaling (Clapham et al. 2001; Mikoshiba 2007), and Slc25a25 is a mitochondrial carrier with calcium binding motifs (del Arco and Satrustegui 2004). Although three mitochondrial proteins were identified in the screen for Ncs-1 interactors, whether or not Ncs-1 localizes to the mitochondria has not been investigated. To establish whether a mitochondrial role for Ncs-1 is important for ear development, I chose to study the two other mitochondrial localized Ncs-1 interactors in addition to Slc25a25: Hint2 and Pink1. Finally, I chose to study Dan (differential screening-selected gene aberrant in neuroblastoma) a secreted Bmp antagonist that has previously been shown to play a role in semicircular canals and the associated duct in chick embryos (Yamanishi et al. 2007).

Using a Blast search of the zebrafish EST database or genome, we were able to find at least one ortholog for each of these NIPs. The amino acid sequence of human NIPs and their zebrafish orthologs are compared in Fig. 4.1. Note that there are no sequence alignments for Trpc5, Ip3r and Vamp2, as the ESTs did not cover the entire open reading frame, and I was unable to identify a genomic sequence for them. Also, Dan is referred to by its synonymous name, Nbl1, in its amino acid alignment (Fig. 4.1, F). Multiple
<table>
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<th>Gene Name</th>
<th>Description</th>
<th>Identification</th>
<th>Function</th>
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<tbody>
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<td>Hint2</td>
<td>histidine triad protein</td>
<td>Y2H</td>
<td>Mitochondrial adenosine monophosphate-lysine hydrolase</td>
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<td>Spuf</td>
<td>'secreted protein of unknown function'</td>
<td>Y2H</td>
<td>Unknown</td>
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<td>Map3k10</td>
<td>kinase in JNK pathway</td>
<td>Y2H</td>
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</tr>
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<td>homolog of yeast SPT6, Ty suppressor</td>
<td>Y2H</td>
<td>Nuclear local.</td>
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<td>CLIP-115 homolog</td>
<td>Y2H</td>
<td>Early endosome/TGN transport</td>
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<tr>
<td>Nbl1/Dan</td>
<td>differential screening-selected gene aberrant in neuroblastoma</td>
<td>Y2H</td>
<td>Extracellular BMP antagonist</td>
</tr>
<tr>
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<td>peroxisomal enoyl-CoA isomerase</td>
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<td>Znf518</td>
<td>zinc finger protein</td>
<td>Y2H</td>
<td>Transcription factor</td>
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<td>Pink1</td>
<td>PTEN-induced kinase</td>
<td>Y2H</td>
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Table 4.3: Human Ncs-1 interacting proteins.
G

h_PI4KB 1 22
zf_PI4KB 1

h_PI4KB 32 22
zf_PI4KB 61 22

h_PI4KB 91 22
zf_PI4KB 120 22

h_PI4KB 151 22
zf_PI4KB 171 22

h_PI4KB 211 22
zf_PI4KB 231 22

h_PI4KB 260 22
zf_PI4KB 291 22

h_PI4KB 305 22
zf_PI4KB 351 22

h_PI4KB 365 22
zf_PI4KB 411 22

h_PI4KB 425 22
zf_PI4KB 471 22

h_PI4KB 485 22
zf_PI4KB 531 22

h_PI4KB 545 22
zf_PI4KB 591 22

h_PI4KB 605 22
zf_PI4KB 651 22

h_PI4KB 665 22
zf_PI4KB 711 22

h_PI4KB 725 22
zf_PI4KB 771 22

h_PI4KB 785 22
zf_PI4KB 831 22

ITTKLIDGFQYLTNGIM
ITTKLIDGFQYLTNGIM
orthologs were found for Trpc4 and Slc25a25 which are most likely due to the
duplication of the zebrafish genome during evolution (Holland 1999a; Holland 1999b).
Many of the protein sequences were highly conserved between human and zebrafish
orthologs. The most divergent peptides were Pink1, the N-terminus of Hint1, the C-
terminus of Dan (Nbl1), and the C-terminous of TRPC4.

4.3.3 Expression analysis of zebrafish NIP mRNAs

I used whole-mount in situ hybridization to examine mRNA expression for 10 NIPs
during zebrafish embryogenesis. The expression profiles of zebrafish for these genes are
shown in Fig. 4.2. Many of the genes showed distinct expression patterns, however for
the purposes of this project I focused on inner ear expression. Three genes that encode
mitochondrial proteins were analyzed (hint, slc25a25b and pink1), and all three showed
expression in the otic vesicle at 24 hpf, in the semicircular canal protrusions and the
anterior macula at 50 hpf, and in the fully formed semicircular canal hubs at 72 hpf. In
addition, pi4kb and dan showed similar expression patterns in the otic vesicle.

Two genes were unique in that they were expressed in only two out of the three
developmental stages examined. arfl transcripts were identified in the 24 hpf otic vesicle
and the 50 hpf epithelial protrusions, but expression was diminished by 72 hpf and undetectable in the semicircular canal hubs. In contrast, \textit{trpc1} was undetectable in the otic vesicle until 50 hpf when transcripts could be observed in the semicircular protrusions. \textit{trpc1} expression was sustained in the fully formed semicircular canal hubs at 72 hpf. Three genes (\textit{trpc5}, \textit{vamp2}, and \textit{ip3r}) were not expressed in the otic vesicle at any of the time points observed.

These results suggest that some of these genes may be involved in pathways leading to semicircular canal development based on their interaction with Ncs-1 as determined by the Y2H screen and the expression of the orthologous genes in the developing zebrafish otic vesicle. The genes which are not expressed in the ear could still play a role in Ncs-1 mediated pathways that occur in tissues outside the ear.

### 4.3.4 Morpholino Knockdown of Individual NIPS

To determine whether the NIP orthologs that are expressed in the inner ear are involved in semicircular canal development, I have chosen three of these genes to knockdown with antisense MOs: \textit{pi4kβ}, \textit{arf1}, and \textit{dan}. Two non-overlapping MOs were created for each gene to target either the start codon or the 5’ untranslated region of each gene. Each MO was individually injected in a range of doses (1-8ng) at the one cell stage.
4.3.4.1 Morpholino Knockdown of arf-1

Microinjection of \textit{arf1-UTR} MO at a dose of 2ng caused a variety of phenotypes beginning at 50 hpf (Fig. 4.3). In terms of gross morphology, \textit{arf1} morphants displayed a shortened body axis, reduced eye size, and a curved tail (Fig. 4.3, B and C). These defects persisted through 72 hpf (Fig. 4.3, I and J). At 50 hpf, wild type embryos had large otic vesicles with three semicircular canal protrusions (Fig. 4.3, D). In contrast, a majority of \textit{arf1} morphants (>90%) had small otic vesicles and no semicircular canal protrusions (Fig. 4.3, E-G).

Wild type embryos had developed all four hubs of the semicircular canals by 72 hpf (Fig. 4.3, K); however, morphant otic vesicles remained smaller than wild type and were only beginning to form epithelial protrusions. Only a misshapen lateral protrusions and an immature anterior protrusion was observed in most embryos (Fig. 4.3, L-O), while in some embryos the protrusions were extremely large without obvious origin (Fig. 4.3, P). Another defect observed in the morphant embryo was the stunted development of the jaw. In wild type embryos, the jaw can be seen to extend on the ventral side of the
embryo past the anterior boundary of the eye (Fig. 4.3, H). The morphant jaw does not extend past the middle of the eye (Fig. 4.3, I and J). In confirmation of these phenotypes, microinjection of a second, nonoverlapping MO (arf1-ATG MO) at higher doses (4-6ng) phenocopied arf1-UTR MO.

4.3.4.2 Morpholino Knockdown of pi4kβ

Knockdown of pi4kβ resulted in phenotypes that were very similar to those caused by arf1 knockdown (Fig. 4.4). Gross morphological changes were not apparent until 50 hpf at which time a shortened body axis and slightly smaller eyes were seen in a majority of pi4kβ-ATG MO morphants (4ng) (Fig 4.4, B and C). A curled tail was prominent in about half of the pi4kβ morphants analyzed (Fig 4.4, C). pi4kβ morphant ears at 48 hpf were smaller than wild type and contained no epithelial protrusions (Fig 4.4, E).

At 72 hpf, pi4kβ morphants remained stunted compared to wild type embryos (Fig 4.4, G and H). Those embryos that had curled tails at 48 hpf, retained this morphology at 72 hpf (Fig. 4.4 H). Growth retardation in the eye and body axis persisted, but was less severe than observed in arf1 morphants. Ear defects in 72 hpf pi4kβ morphants were more variable than those seen in arf1 morphants at the same stage. All pi4kβ morphants did display protrusions; however, the level of their development and fusion differed between embryos (Fig 4.4, J-L). In contrast to arf1 morphants, many pi4kβ morphants did display a posterior protrusion and a ventral protrusion. The ventral protrusions appeared before the fusion of the lateral, dorsal, and the posterior protrusion. In the wild
type ear, the ventral protrusion does not appear until the three early projections have fused. Additionally jaw development in pi4kβ morphants appeared to be stunted in many of the embryos, just as in the arf1 morphants (Fig 4.4, F-H). The pi4kβ -UTR MO phenocopied the pi4kβ-ATG MO but required higher doses (4ng) to induce ear defects and 6ng to induce body axis truncation.

4.3.4.3 Morpholino Knockdown of dan

A MO targeting the start codon of dan mRNA induced severe developmental abnormalities (Fig. 4.5). At a dosage of 2ng, dan-ATG MO caused a shortened body axis with misshaped tail and severe necrosis throughout the brain by 30 hpf (Fig. 4.5, C and D). Otic vesicles were reduced in size and in some cases were missing otoliths (Fig. 4.5, F and G). The 24 hpf dan morphants had a reduction in the size of the ventral portion of the eye. At higher doses (4ng), truncation was more pronounced and embryos displayed severely reduced otic vesicles or no vesicles at all (not shown). Morphants that received a lower dose of 1ng did not display gross abnormalities or inner ear defects at this time (not shown). At 48 hpf, defects were apparent in the low dose dan morphants (1ng dan-ATG MO). Body axis truncation and a reduction in pigment in the ventral most portion of the eye were the most obvious irregularities (Fig. 4.5, I-K). These phenotypes showed a dose dependency and were more severe at higher doses. No semicircular canal protrusions were apparent in dan morphants even at the low dose, while wild type embryos displayed three normal protrusions (Fig. 4.5, L-P). At higher doses, additional or abnormal otoliths were observed in morphants (Fig. 4.5, O and P).
Figure 4.3: Expression of *arf1* is necessary for normal semicircular canal development. All panels show a lateral view of embryos with the anterior to the left. Morphants were injected with 2 ng of *arf1*-UTR MO. (A-G) 48 hpf embryos (A) wild type embryo, (B-C) *arf1* morphant, (D) otic vesicle, wild type embryo, (E-G) otic vesicle, *arf1* morphant, (H-L) 72 hpf embryos (H) wild type embryo, (I-J) *arf1* morphant embryo, (K) otic vesicle, wild type embryo, (L-P) otic vesicle, *arf1* morphants. AP, anterior protrusion; PP, posterior protrusion; LP, lateral protrusion; DH, dorsal hub; AH, anterior hub; PH, posterior hub; VH, ventral hub; DH, dorsal hub; ?, protrusion of unknown origin.
Figure 4.4: Expression of \( pi4k\beta \) is necessary for normal semicircular canal development. All panels show a lateral view of embryos with the anterior to the left. Morphants were injected with 4 ng of \( pi4k\beta\)-ATG MO. (A-G) 48 hpf embryos (A) wild type embryo, (B-C) \( pi4k\beta \) morphant, (D) otic vesicle, wild type embryo, (E) otic vesicle, \( pi4k\beta \) morphant, (F-L) 72 hpf embryos (F) wild type embryo, (G-H) \( pi4k\beta \) morphant embryo, (I) otic vesicle, wild type embryo, (J-L) otic vesicle, \( pi4k\beta \) morphants. AP, anterior protrusion; PP, posterior protrusion; LP, lateral protrusion; DH, dorsal hub; AH, anterior hub; PH, posterior hub; VH, ventral hub; DH, dorsal hub; FP, fusion plate; ?, protrusion of unknown origin.
Figure 4.5: Expression of dan is necessary for normal semicircular canal development.

All panels show lateral views of embryos with the anterior to the left. Morphants were injected with dan-ATG MO. (A-G) 24 hpf embryos (A) wild type embryo, (B) head, wild type embryo (C) dan morphant, 2ng, (D) head, dan morphant, 2ng (E) otic vesicle, wild type embryo, (F-G) otic vesicle, dan morphant, 2ng, (H-P) 48 hpf embryos, (H) wild type embryo, (I) dan morphant embryo, 1ng, (J) dan morphant embryo, 2ng, (K) dan morphant embryo, 4ng, (L) otic vesicle, wild type embryo, (M-N) otic vesicle, dan morphants, 1ng, (O-P) otic vesicle, dan morphants, 2ng (Q-X) 72 hpf embryos (F) wild type embryo, (R-S) dan morphant embryo, (T) otic vesicle, wild type embryo, (U-V) otic vesicle, dan morphants, 1ng, (W-X) otic vesicle, dan morphants, 2ng. AP, anterior protrusion; PP, posterior protrusion; LP, lateral protrusion; DH, dorsal hub; AH, anterior hub; PH, posterior hub; VH, ventral hub; DH, dorsal hub; FP, fusion plate.
The most severely affected *dan* morphant embryos died by 72 hpf. Morphants that survived to 72 hpf had shortened body axis or curved trunks (*Fig. 4.5, R and S*). The ventral portion of the morphant eyes remained underdeveloped and unpigmented. The ear of 72 hpf *dan* morphants showed a variety of defects. At a low dose, *dan* morphants showed normal development of the three initial protrusions, some of which fused properly yet displayed an enlarged and abnormal fusion plate (*Fig. 4.5, U and V*). A higher dose of 2ng increased the severity of inner ear defects by causing abnormalities in the epithelial protrusions or missing protrusions (*Fig. 4.5, W and X*). The jaw appeared to be stunted in *dan* morphants at 72 hpf, a phenotype similar to that seen in *arf1* and *pi4kβ* morphants (*Fig. 4.5, Q-S*). A morpholino targeted to the 5’ untranslated region of *dan* mRNA was unable to phenocopy the *dan*-ATG MO and produced morphants that displayed no changes from wild type at the highest dose tested (8ng).

These results indicate that *arf1, pi4kβ*, and *dan* each play a common role in semicircular canal development. Knockdown each of these three genes resulted in the absence of epithelial protrusions in the inner ear at 48 hpf and abnormalities in protrusions or fusion of the pillars at 72 hpf. Disruption of *arf1, pi4kβ*, and *dan* gene expression also resulted in the stunted development of the jaw in 72 hpf fish as compared to wild type.

**4.3.5 Functional interaction between ncs-1a and multiple NIPs**

Ncs-1, Arf1, and Pi4kβ have been shown to interact functionally in a mammalian cell system to control neuronal secretion (Haynes et al. 2005). To determine whether there is
a functional interaction between these genes in ear development we have chosen to co-
inject combinations of subeffective concentrations of ncs-1a MO (.5ng), arf1-UTR MO
(.5ng), and pi4kβ-ATG MO (2ng) and observe the effects on semicircular canal
development at 48 and 72 hpf. The results are depicted in Fig. 4.6. Microinjection of
the subeffective dose of each morpholino alone produced defects in < 7% of the embryos
analyzed at both 48 hpf and 72 hpf. However, co-injection of sub-effective doses of pairs
of morpholinos caused an absence of epithelial protrusions in 48 hpf otic vesicles in 53%
of ncs-1a/arf1 morphants (n=68), 40% of ncs-1a/pi4kβ morphants (n=65), and 44% of
arf1/pi4kβ morphants (n=61). Additionally, at 72 hpf abnormal semicircular canal hub
formation was observed in 58% of ncs-1a/arf1 morphants (n=67), 38% of ncs-1a/pi4kβ
morphants (n=65), and 49% of arf1/pi4kβ morphants (n=61) respectively. Co-injection of
the ncs1a, arf1, and pi4kβ MOs in pairs thus appears synergistically induce semicircular
canal defects as compared to injection of each morpholino alone. The functional
interaction observed in this assay combined with a direct physical interaction of these
proteins suggests that ncs-1a, arf1, and pi4kβ may act in a common pathway leading to
the formation of semicircular canal ducts.

4.3.6 Direct interaction of Ncs-1 and NIP orthologs

Two NIPS, Pi4kβ and Arf1, have orthologs in zebrafish are essential for semicircular
canal duct formation. These genes functionally interact with each other and ncs-1a. A
functional interaction does not guarantee that two proteins are involved in the same
pathway, as a parallel pathway could yield similar results. A direct interaction between
two proteins that functionally interact increases the likelihood that the two are functioning in a common pathway. To confirm direct physical interactions between zebrafish proteins, I utilized directed Y2H, GST-pull down, and co-immunoprecipitation approaches. In the directed Y2H, human Ncs-1 was fused to the GAL4 DNA binding domain, while zebrafish Arf1, Pi4kβ, and Dan and human Trpc1 (positive control) were fused to the GAL4 activation domain. Human Ncs-1 was utilized, because it had previously been cloned into the pAS2-1 vector, and it sequence homology with the zebrafish polypeptide is extremely high. While human Trpc1 tested positively for an interaction with Ncs-1, Arf1, Pi4kβ, and Dan did not (data not shown).

Since antibodies have not been made that detect zebrafish Pi4kβ, Arf1 or Dan, epitope tags were used in GST-pull downs (Ncs-1-GST, Pi4kβ-S-Tag, Arf1-S-Tag, Dan-S-Tag). For the pull downs, Ncs-1-GST was able to pull down Arf1-S-Tag (Fig. 4.7), but not Pi4kβ-S-Tag or Dan-S-Tag (data not shown). GST and glutathione sepharose beads alone were unable to pull down Ars1-S-Tag, Pi4kβ-S-Tag or Dan-S-Tag. Additionally, I was unable to confirm the Ncs-1/Pi4kβ interaction by immunoprecipitation from HEK293 cells that exogenously expressed zebrafish Pi4kβ-Flag (data not shown). Therefore, thus far, I have confirmed the interaction of Ncs-1 with Arf-1, but not with Pi4kβ or Dan.
4.4 Discussion

By studying the zebrafish orthologs of genes that interact with Ncs-1 in humans, I have identified three new genes that are essential for the formation of semicircular canal ducts. Two of these genes in humans, *Arf1* and *Pi4kβ*, are known to be involved in a secretory pathway with Ncs-1 (Haynes et al. 2005). The other gene product, Dan, is an antagonist of Bmp signaling and has been shown to be involved in semicircular canal development in chick embryos (Yamanishi et al. 2007). Knockdown of these genes causes a number of phenotypes, but most interestingly, the semicircular canals fail to properly form. At 48 hpf, *arf1*, *pi4kβ*, and *dan* morphants had no epithelial protrusions in their otic vesicles. At 72 hpf these morphants had developed protrusions that were abnormal in number, appearance, and/or fusion.

I have shown that *arf1*, *pi4kβ*, and *ncs-1a* functionally interact. A functional interaction implies that these genes are either involved in the same pathway or in a parallel pathway that is also contributing to semicircular canal development. Indeed, Arf1, Pi4kβ, and Ncs-1 have been shown to be involved in common trafficking pathway from the trans-golgi network to the plasma membrane and the exocytosis of immune system factors, insulin, and neurotransmitters. Mammalian Arf1 and Ncs-1 individually activate Pi4kβ, but a physical interaction between Arf1 and Ncs-1 abolishes this affect. Pi4kβ generates
Figure 4.6: Phenotypes of embryos co-injected with sub-effective doses of ncs-1a, arfl, and pi4kβ, morpholinos.

Embryos were microinjected with sub-effective doses of ncs-1a Mo, arfl-UTR MO and pi4kβ-ATG MO alone or in conjunction with one another. The bar graph depicts the percentage of fish at 48 hpf that displayed normal epithelial protrusions, abnormal epithelial protrusions, and missing epithelial protrusions for each treatment and the percentage of fish at 72 hpf that displayed normal semicircular canal hub formation and abnormal semicircular canal hub formation for each treatment. The number of fish assayed for each treatment is displayed above the bars.
This western blot depicts the interaction of Arf1 and Ncs-1 by GST-pulldown followed by SDS-page and western blotting. The first lane was loaded with 30µg of ARF1-S-tag protein alone in order to determine the size of this protein on a western blot. The second lane was loaded with the experimental condition. Ncs-1-GST was incubated along with ARF1- S-tag and glutathione sepharose beads. The beads were washed with PBS to remove unbound protein. Proteins were denatured and loaded in to the second lane. The third lane was loaded with a mock-GST pulldown. Ncs-1-GST was incubated along with ARF1- S-tag and glutathione sepharose beads. The beads were washed with PBS to remove unbound protein. Proteins were denatured and loaded in to the second lane.
lipids that are important for targeting vesicles to the plasma membrane. Coincidentally, secreted molecules such as BMP antagonists must be secreted, implying that this pathway may be involved in trafficking Dan to the plasma membrane.

A direct physical interaction between these proteins would provide more evidence for the conservation of this common pathway in zebrafish. I was able to confirm the interaction between zebrafish Arf1 and Ncs-1 using a GST pulldown. Therefore, it is likely that these two proteins participate in a common pathway. It will be of interest to confirm a direct physical interaction between Ncs-1 and Pi4kβ or Dan. I have not yet been able to demonstrate a direct physical interaction between Ncs-1a and Pi4kβ using GST-pulldowns and IPs or between Ncs-1a and Dan using GST-pulldowns. Ncs-1 is a calcium binding protein that is myristoylated at its N-terminus, and these modifications lead to differential binding of proteins. Addition of calcium or utilization of a pure myristoylated Ncs-1 sample for co-IP or GST-pulldowns may be required to detect an interaction between Ncs-1 and Pi4kβ or Dan. Also, it remains to be discovered whether or not ncs-1a and dan functionally interact. Co-injection of subeffective doses of ncs-1a MO and dan MO should address this question. I hypothesize that they will functionally interact, and that coinjection of subeffective doses will produce semicircular canal defects.

Two mechanisms have been identified that lead to the proper development of semicircular canal ducts: secretion of hyaluronic acid into the epithelial protrusions and Bmp signaling (Haddon and Lewis 1991; Omata et al. 2007). Both of these processes
require the delivery of proteins to the plasma membrane. Hyaluronic acid is synthesized at the plasma membrane by hyaluronin synthase and is secreted during synthesis (Itano and Kimata 2002). The precursor of hyaluronic acid along with hyaluronin synthase must be delivered to the plasma membrane before synthesis can occur. Bmps and their antagonists are secreted signaling molecules. Trafficking of these molecules to the plasma membrane for secretion is essential for signaling. I hypothesize that Ncs-1a, Arf1, and Pi4kβ regulate the trafficking of hyaluronin synthases and Bmp signaling components to the plasma membrane for action or secretion.

More support for my model comes from the fact that *nsc-1a*, *arf1*, *pi4kβ*, and *dan* are all strongly expressed in the pharangeal arches, and knockdown of these genes results in jaw malformations. Several mutations affecting craniofacial development in zebrafish have also been associated with semicircular canal defects (Neuhauss et al. 1996). In this class of mutants the phenotypes are cause by failure of chondrogenic cells to differentiate (Bmp signaling is implicated) or abnormalities in extracellular matrix secretion (including HA). It is likely that that this link in phenotypes between craniofacial mutants and *nsc-1a*, *arf1*, *pi4kβ*, and *dan* morphants represents a similar mechanism.

If *nsc-1a*, *arf1*, *pi4kβ*, and *dan* are working in a common pathway in ear development morpholino knockdowns accompanied by mRNA rescue studies provide clue to the arrangement of the pathway components. By knocking down *nsc-1a*, *arf1*, *pi4kβ*, or *dan* and attempting to rescue the phenotype with mRNA encoding the other proteins, we can establish that the proteins are working in a common pathway and in a particular order.
Based on the role I have proposed for Ncs-1a in the secretion of Bmp antagonist Dan, I would expect that overexpression of Dan could rescue the semicircular canal defects seen in *ncs-1a*, *pi4kβ* and *arf1* morphants. Also, I predict that *pi4kβ* mRNA will be able to rescue the ear phenotype observed in *ncs-1a* and *arf1* morphants, but not those observed in *dan* morphants. Finally, since Arf1 and Ncs-1a are working toward a similar goal (the activation of Pi4kβ) in my model, I predict that these two genes will be unable to rescue *pi4kβ* or *dan* morphants.

In conclusion, we have taken a new approach to identifying genes involved in semicircular canal development in zebrafish. This approach has thus far yielded three new genes that participate in this process. It will be of great interest to clone more NIP orthologs, analyze the expression of these genes, and knockdown the ones that are expressed in the developing vestibule. Also, this approach can be used for other developmental processes for which few genes have been identified and a mechanism is unknown.
Chapter 5

Closing Discussion
5.1 *ptena* is involved in ear development

Pten is a lipid phosphatase that negatively regulates the Akt pathway. The tumor suppressing properties of Pten have been predominantly studied, while little is known about the role of Pten during development. Previous studies in null mutants have shown that Pten is essential for the survival of the embryo to term. Zebrafish have two orthologs of Pten. The expression of each ortholog is distinct yet overlapping, which suggests that the expression or function of one ancestral Pten may have been divided among the two orthologs in fish. In the case of functional redundancy, both *pten* orthologs would have a similar function in different tissues. It is also possible that various functions of the one ancestral Pten may have been split between the two orthologs. In either case, the existence of two orthologs provides a unique opportunity to study the function of Pten in one particular subset of tissues without disrupting the other subset.

I have shown that knockdown of *ptena* or *ptenb* generates distinct phenotypes. In particular, knockdown of *ptena* resulted in defects in the formation of otoliths, semicircular canals, intersegmental vasculature, and notochord, while *ptenb* did not cause any of these defects. The only overlapping phenotype I saw with both morpholinos was a domed head at 48 hpf.

Recently, Faucherre et al has shown that a mutation in either zebrafish *pten* gene alone has no effect on development (2007). Crossing the single mutants to create a double mutant resulted in embryonic lethality by 5 dpf (Faucherre et al. 2007). Morphological
defects in the double mutants did not arise until 48 hpf, when reduced body length, smaller eyes, enlarged head, heart edema, and lack of circulating blood cells was observed.

The differing results between morpholino knockdowns and mutants could be caused by a variety of factors. It is possible that maternal mRNA in the oocyte could account for the lack of phenotypes observed during ptena or ptena mutant development. Morpholinos would target both maternal and embryonic transcripts. Faucherre et al was unable to develop a zebrafish pten antibody to demonstrate loss of the pten protein in single or double mutants. I showed that the pten morpholinos I used were able to specifically knockdown the translation of their particular target in vitro. I also show that morpholino knockdown of either ptena or ptenb resulted in an increase in phosphorylated Akt in 48 hpf embryos. It would be of interest to determine whether changes in the levels of phosphorylated Akt are present in zebrafish pten mutants. It is also possible that the results I obtained using morpholinos targeting ptena and ptenb were the result of non-specific effects of the morpholino. However, this is unlikely, because two non-overlapping morpholinos for each gene were able to phenocopy each other. To eliminate this possibility I could co-inject a morpholino targeting P53 with the pten morpholinos as this approach has been shown to decrease the incidence of non-specific effects (Robu et al. 2007).

Knockdown of ptena resulted in vestibular abnormalities. At 24 hpf otoliths were misshapen, or there was one missing. In about \( \frac{1}{4} \) of embryos that displayed otolith
defects, semicircular canals did not properly fuse or formed abnormal epithelial masses. I expect that these defects may have been due to defects in proliferation. Since Pten negatively regulates the Akt survival and proliferation pathway, I would expect to see decreased levels of apoptosis and increased levels of proliferation in pten morphants embryos. Proliferation has been shown to be involved in semicircular canal development in mice. However, not much is known about the levels of proliferation at different stages of the developing zebrafish inner ear. It would be interesting to determine the levels of proliferation in the wild type and ptena morphant ears, to determine whether a difference in levels could account for the aberrant semicircular canal phenotype. Cell death is an important component of fusion plate formation in chick embryos, but little apoptosis is seen in the fusion plate of zebrafish. For this reason, I do not expect that the semicircular canal phenotypes observed in ptena morphants are related to decreases in apoptosis within the epithelial protrusions.

A low but persistent level of apoptosis is observed in all of the developing sensory regions in the zebrafish ear. This cell death is thought to be involved in the refinement of the hair cell/supporting cell pattern, elimination of inappropriately innervated hair cells, or for the generation of pathways for ingrowing nerve fibers. It is possible that knockdown of ptena decreases apoptosis and disrupts this correction process. It will be important to perform TUNNEL staining in order to determine whether the levels of apoptosis are changed in these sensory regions in ptena morphants. In addition, increased proliferation within the sensory regions could cause an expansion of these regions which may lead to aberrant patterning of the otic vesicle. It is imperative that
BrdU incorporation studies be completed to determine the basal level of proliferation in wild type embryos and the level of proliferation in morphants.

### 5.2 Otoc1 is a potential otolith matrix protein in zebrafish

I have identified a novel zebrafish ortholog of mammalian Oc90. The mammalian and avian otolith organs contain thousands of minute biomineralized particles called otoconia. The major component of the organic lattice in these otoconia is protein called Oc90 that shows two domains of similarity of phospholipase A2. Similarly the major matrix protein in amphibian uticular otoconia is Oc22, a protein with one domain of homology to phospholipase A2. In contrast, the inner ear of teleost fish contains three large ear stones called otoliths with Omp being the most abundant matrix protein. It has been hypothesized that the identity of the major matrix protein accounts for the morphological differences (quantity, shape, crystal structure) observed between mammalian otoconia and zebrafish otoliths. Therefore, it was unexpected that orthologs of Oc90 would be found in species other than birds and mammals. In contrast to this belief, I have identified a novel gene, *otoc1*, which encodes the zebrafish ortholog of Oc90. An amphibian ortholog of Oc90 was also discovered.

Expression of *otoc1* is detected in the zebrafish ear between 15 hpf and 72 hpf, and is restricted primarily to the macula. Morpholino knockdown of *otoc1* mRNA translation produces a variety of otolith defects. In combination, the sequence homology of Otoc1 to Oc90, the vestibular expression of *otoc1* mRNA, and the otolith abnormalities observed
in *otoc1* morphants suggests that Otoc1 is an otolith matrix protein in zebrafish. To further confirm this hypothesis it will be of great interest to show direct incorporation of Otoc1 into the otolith matrix. This can be accomplished two ways. The development of an Otoc1 antibody would allow for whole mount antibody staining or staining of sectioned otoliths. Since I am predicting that Otoc1 is involved in otolith seeding, I would expect to observe Otoc1 antibody staining of the central core of the otolith. In addition, removal of otoliths from embryos, decalcification of the structures, and 2-d gel electrophoresis followed by mass spectrometry of any proteins extracted from the otoliths would allow us to determine the identity of proteins found in the zebrafish otolith matrix, possibly including *otoc1*.

The discovery of Otoc1 as a likely matrix protein in zebrafish along with the discovery of other Oc90 orthologs in *Fugu* and *Xenopus* would suggest that this protein is not strictly involved in determining mammalian otoconial morphology. However, this idea cannot be ruled out, as the amino acid sequence outside of phospholipase A2 like domain is extremely divergent. One experiment that could be performed to confirm this hypothesis is expressing mouse Oc90 in *otoc1* morphant zebrafish embryos. This can be done by injecting mRNA of *Oc90* directly into the yolk of a newly fertilized zebrafish embryo. It has previously been demonstrated that loss of otopetrin in zebrafish leads to a loss of otoliths at 24 hpf; however, after 48 hpf otoliths began to develop that were of the calcitic crystal polymorph (Hughes et al. 2004). These calcitic otoliths were morphologically distinguishable from the normal aragonitic otoliths in zebrafish. Calcitic otoliths resemble salt crystals with large flat faces, while aragonitic otoliths are round and smooth.
in appearance. Therefore, if the injection mammalian Oc90 mRNA alone induces calcitic morphology, I should be able detect the morphological difference in the zebrafish otoliths.

Another experiment that I propose is to identify the expression pattern of the amphibian Oe90 ortholog. In Xenopus the utricular otoconia are of aragonitic morphology and the saccular otoconia are of calcitic morphology. It is already known that Oc22 is the major matrix protein found in utricular otoconia. It would be of interest to know whether the Oe90 ortholog is also expressed in the utricular otoconia or if it is restricted to the calcitic otoconia of the saccule. This data would provide further information on the ability of Oe90 orthologs to influence calcium carbonate polymorph in ear stone structures.

During embryogenesis, expression of otoc1 mRNA precedes the appearance of omp-1 transcripts. In addition, co-injection of morpholinos targeting otoc1 and omp-1 demonstrated that these genes exhibit a functional interaction. Muryama et al demonstrated that otolith growth was retarded in omp-1 morphants, but that seeding was unaffected (2005). My results suggest that otoc1 may serve to nucleate calcium carbonate mineralization of zebrafish otoliths and that omp-1 is responsible for growth after this nucleation. Oc90 in mice has been shown to recruit other proteins to the otolith matrix during embryogenesis (Zhao et al. 2007). It would be interesting to investigate whether the same is true for otoc1, and in particular, whether Otoc1 can recruit Omp-1 to the developing zebrafish otolith. By obtaining the Omp-1 antibody used by Muryama et
al., I should be able determine whether incorporation of Omp-1 into the otolith is hindered in \textit{otoc1} morphants.

During early embryogenesis in zebrafish, the otic vesicle represents the predominant expression site of the \textit{otoc1} gene. However, \textit{otoc1} transcripts were also detected in several peripheral tissues including floor plate, circumventricular organs, and the flexural organ. Interestingly, these anatomical sites of \textit{otoc1} expression coincide almost exactly with the expression of Reissner’s substance (Lichtenfeld et al. 1999; Lehmann and Naumann 2005). Reissner’s substance is a mixture of glycoproteins, including SCO-spondin and Reissner’s fibre-Gly1 (Herrera and Rodriguez 1990; Karoumi et al. 1990; Nualart et al. 1991; Gobron et al. 1996; Nualart et al. 1998; Didier et al. 2000; Lehmann et al. 2001; Meiniel 2001), that are secreted from the floor plate, flexural organ, and subcommissural organ (SCO) in zebrafish and other vertebrate species. Reissner’s substance is secreted by cells of the SCO into the ventricle where it aggregates into a threadlike structure called Reissner’s fibre which extends the entire length of the central canal of the spinal cord. Reissner’s substance is also produced by cells in the floor plate and flexural organ.

Analysis of the zebrafish mutants \textit{cyclops} and \textit{one-eyed pinhead} suggest that Reissner’s substance may play a role in axonal guidance and commisure formation (Lehmann and Naumann 2005). The predicted structural features of Otoc1 (rigid backbone and multiple sites for addition of N-linked sugars) suggest that Otoc1 may serve as a scaffold for deposition of the glycoprotein components of this fibrous complex. However, no
apparent morphological defects were detected in brain structures of otoc1 morphants, and in this study, axon guidance was not analyzed. It will be of considerable interest to learn whether the otoc1 gene product, in addition to its role in otolith development, is a component of Reissner’s substance. Although speculative, this hypothesis can be tested using Otoc1-specific antibodies to analyze Otoc1 protein distribution in wild type zebrafish and in the mutants cyclops and one-eyed pinhead that lack a floor plate and show altered deposition of Reissner’s substance (Lehmann and Naumann 2005). I expect that if Otoc1 is a component of Reissner’s substance, the protein will co-localize with Reissner’s fiber. In turn, elimination of the floor plate in cyclops and one-eyed pinhead mutants should ablate Otoc1 deposition in Reissner’s Fiber, as otoc1 mRNA expression is restricted to the floor plate at 24 hpf. Finally, if Otoc1 is a component of Reissner’s substance, I would expect axon guidance to be disrupted in otoc1 morphants. This can be investigated by fluorescently labeling neurons and observing the differences in axonal migration between wild type and otoc1 morphants.

5.3 Ncs-1a mediated pathway in semicircular canal development

Blasiole et al. (2005) has previously shown that ncs-1a, an ortholog of the mammalian calcium sensor Ncs-1 is expressed during embryonic development and is required for the formation of the zebrafish semicircular canals. I have hypothesized that by identifying and studying the interactors of a protein of interest, that I can gain insight into the pathways and mechanisms that this protein is involved in. The power of yeast-two-hybrid (Y2H) screens has been used to identify new interactors of G-protein coupled
receptors such as the D2 dopamine receptor and the Mu opioid receptor. The discovery of these physical protein interactions has lead to further functional characterization of the associated signaling pathways. Since little is known about the role of Ncs-1 in vestibular development, I chose to take a similar approach by studying the interactors of Ncs-1.

Human Ncs-1 interacting proteins (NIPS) were identified through aY2H screen performed by Nadine Kabbani. I also conducted a literature search to identify known interactors of Ncs-1. I have identified zebrafish orthologs for ten of these NIPs and performed whole mount in situ hybridization to determine their mRNA expression in the developing otocyst. Seven of these NIPs were expressed in the non-sensory epithelium of the developing semicircular canals, an expression pattern that overlaps with that of ncs-1a.

I chose three genes to knock down using morpholinos: pi4kβ, arf1, and dan. pi4kβ and arf1 were chosen on the basis that they are the most well characterized interactors of ncs-1a. Most of the known functions of Ncs-1 can be attributed to its control over activating Pi4kβ (Zhao et al. 2001). Arf-1 can also activate Pi4kβ, but in conjunction an Arf-1/Ncs-1 interaction antagonizes the ability of either protein to activate Pi4kβ (Godi et al. 1999; Haynes et al. 2005). Dan was chosen for further study, because previous experimentation in chick embryos has shown that this Bmp antagonist is involved in semicircular canal development (Yamanishi et al. 2007). Several Bmp signaling molecules are expressed in the developing zebrafish ear (Mowbray et al. 2001). Bmp4 signaling and regulation are essential for semicircular canal development (Omata et al. 2007). Knockdown of
pi4kβ, arf1, or dan alone results in a lack of epithelial protrusion formation at 48 hpf. By 72 hpf, some protrusions are evident but are abnormally shaped, unfused, and/or numbered. These results indicate that all three genes play a role in semicircular canal duct formation.

I have shown that Ncs-1a, Pi4kβ, Arf1, and Dan are all involved in semicircular canal development. However, further evidence is needed to demonstrate that these genes are acting in a common pathway. By showing a functional interaction, I can conclude that these genes act towards a common goal, the development of semicircular canals, either through a common or parallel pathway. By confirming a direct physical interaction between proteins with functional interactions, my hypothesis of a common pathway is much more likely than a parallel pathway. By co-injecting subeffective doses of ncs-1a MO and pi4kβ MO, ncs-1a MO and arf1 MO, and pi4kβ MO and arf1 MO, synergistic effects were observed for each pair of morpholinos. These results confirm a functional interaction between all three genes. Another method of demonstrating a functional interaction between is by performing complementation studies. This can be done early during zebrafish development by combining morpholino knockdown of one gene and mRNA rescue with another gene. If one gene is able to rescue the morphant phenotype of another gene, I would conclude that the proteins encoded by these genes act in a common pathway. In addition, the product of the rescuing gene acts downstream of the protein encoded by the morpholino target.
Although Ncs-1, Arf1, and Pi4kβ have all been shown to physically interact in mammalian systems, these interactions had not been tested for the zebrafish proteins. Sequence homology between human and zebrafish Ncs-1 proteins, Arf1 proteins, and Pi4kβ proteins is extremely high; therefore, it is likely that any physical interaction of these proteins in mammalian cells is also conserved in zebrafish. Using a GST-pulldown, I was able to confirm a physical interaction between zebrafish Arf1 and Ncs-1.

I have been unable so far to confirm the interaction of zebrafish Pi4kβ with Ncs-1 by GST-pulldown or co-immunoprecipitation from the lysates of transfected HEK-293 cells. While some groups have demonstrated a direct interaction of mammalian Pi4kβ and Ncs-1 via co-immunoprecipitation from COS-7 cell lysates and rat brain lysates (Zhao et al. 2001; Taverna et al. 2002), other researchers have had difficulties confirming this interaction by co-immunoprecipitation from adult and newborn rat brain lysates (Bartlett et al. 2000). It is recognized that co-immunoprecipitation may not be indicative of a direct physical interaction. Complexes of proteins can be isolated by immunoprecipitation, therefore, the physical interaction may be indirect. Indeed Arf1 is an interactor of both Pi4kβ and Ncs-1, therefore by immunoprecipitation of NCS-1, I should pull down Arf1 and any associated proteins, including Pi4kβ.

The current difficulties of co-immunoprecipitating Pi4kβ and NCS-1 may be indicative this interaction being calcium dependent. Calcium binding does change the conformation of NCS-1 and this change may be important for its interaction with other proteins. In addition NCS-1 is myristilated at its N-terminous. This lipid modification also
contributes to the structure of NCS-1 allowing selective interaction with other proteins. Interestingly, myristolation has been shown to be required for the Pi4kB/Ncs-1 interaction in Cos-7 cells. In my GST-pull down experiments, NCS-1-GST that is grown in bacteria is not myristolated, as bacteria lack the machinery to do so. By co-transforming N-myristol transferase and Ncs-1, bacteria will be able to produce myristolated Ncs-1 for future GST pulldown studies. Further experiments will need to be performed in order to confirm or disprove the Ncs-1/Pi4kβ physical interaction. Also, it will be of interest to determine whether zebrafish Ncs-1a and Dan demonstrate physical and/or functional interactions.

Another defect common among *pi4kβ*, *arf1*, *dan*, and *ncs-1a* morphants was an underdeveloped jaw. This is not surprising considering that these genes are highly expressed in the developing pharangeal arches. Interestingly, several mutations affecting craniofacial development in zebrafish have also been associated with semicircular canal defects (Neuhauss et al. 1996). These mutant phenotypes result from failure of chondrogenic cells to differentiate or from defects in genes encoding extracellular matrix proteins, including hyaluronic acid (HA). These processes are also important in inner ear development. HA secretion is required for semicircular canal development in both zebrafish and *Xenopus* (Haddon and Lewis 1991). Differentiation of mesenchymal cells into chondrogenic cells as well as semicircular canal development is thought to be dependent on BMP signaling (Chang et al. 2002; Omata et al. 2007). Based on the phenotypes and mechanism of dysfunction of craniofacial mutants in zebrafish, I hypothesize that *pi4kβ*, *arf1*, *dan*, and *ncs-1a* are important for HA synthesis or secretion.
of Bmp signaling molecules. Dan has already been shown to be a Bmp antagonist (Hsu et al. 1998); therefore, I propose that the phenotypes seen in dan morphants are due to aberrant Bmp signaling.

Pi4kβ, Arf1 and Ncs-1 are intimately related in trafficking from the trans golgi network to the plasma membrane. I propose a model in which Ncs-1 and Arf-1 positively regulate Pi4kβ activity which allows for trafficking of Dan to the plasma membrane for secretion (Fig. 5.1). By creating an antibody that recognizes zebrafish Dan, I should be able to use immunostaining of wild type or morphant embryos (with knockdown of pi4kβ, arf1, or ncs-1a) to look for secretion of the Dan protein. Also, I could look for markers of increased Bmp signaling such as increases in phosphorylated Smad proteins or altered expression of Smad target genes. It is also possible that this Ncs-1/Arf1/Pi4kβ trafficking pathway is indirectly involved in secretion of HA. It is known that hyaluronin synthase synthesizes HA at the plasma membrane, and that HA is exported from the cell as it is being synthesized. Therefore, an Ncs-1/Arf1/Pi4kβ mediated trafficking of HA precursors and/or hyaluronin synthases may be required for the delivery of these components to the plasma membrane (Fig. 5.1). To confirm this hypothesis, I would incubate pi4kβ, arf1, or ncs-1a morphants with a biotinylated HA-binding protein, followed by fluorescent immunostaining with an anti-biotin antibody to show a decrease in HA secretion.

Another interesting observation is that Ncs-1 is involved in protection of neuronal cells from stressors and damage through the Akt pathway (Nakamura et al. 2006). This may
suggest a potential link between the Akt pathway and semicircular canal development, as both Ncs-1 and Pten participate in this process (Fig. 5.1). Indeed proliferation within epithelial outpockets is required for semicircular canal development in mice. The role of proliferation in zebrafish semicircular canal development has not yet been determined. It will be important to study the normal proliferation rates in semicircular canal protrusions in zebrafish. To test whether the semicircular canal defects observed in ptena morphants and ncs-1a morphants are due to problems of proliferation, levels of proliferation in morphant embryos will need to be compared to the wild type levels. Since the elimination of Pten increases activity of Akt and the elimination of Ncs-1 decreases the activity of Akt in mice, I could coinject morpholinos directed to ncs-1a and ptena to test whether there is a functional interaction between the two genes. If there is a functional interaction, I expect that co-injection of the two morpholinos together will rescue the semicircular canal defects seen in the single morphants.
**Figure 5.1**: NIP model of intracellular trafficking.

NCS-1 and Arf1 alone activate Pi4kβ and plasma membrane trafficking. Ncs-1 and Arf1 together do not activate Pi4kβ or transport to the plasma membrane. Transport of pre-HA carbohydrates (D-glucuronic acid and D-N-acetylglucosamine: green fragments), HA synthase (black triangles), and Bmp signaling molecules (purple) are crucial for their rules in HA (green lightning) synthesis and extracellular signaling, respectively. This figure was adapted from College of Saint Benedict Website (http://employees.csbsju.edu/hjakubowski/classes/ch331/cho/ergolgi.jpeg).
5.4 Human Vestibular Dysfunction

A balance disorder is a disturbance that causes an individual to feel unsteady, giddy, woozy, or have a sensation of movement, spinning, or floating. Most commonly balance disorders are due to other congenital disorders, exposure to ototoxic medications, infection, or trauma. Benign paroxysmal positional vertigo (BPPV) is caused by dislodged otoconia from the utricular macula floating in the semicircular canals. At least half of BPPV cases are idiopathic, but studies of familial cases suggest a genetic predisposition (Gizzi et al. 1998). Meniere disease is described by episodic vertigo and hearing loss due to disruption of the fluid balance within the vestibule. Although the gene Cochlin has been shown to be mutated in a number of Meniere Disease patients, it does not account for all cases (Fransen and Van Camp 1999). Congenital disorders that are associated with deafness or craniofacial abnormalities are also many times associated with vestibular dysfunction and structural abnormality. For many of these disorders, the responsible gene has yet to be identified.

Vestibular dysfunction can negatively affect the quality of life for people of all ages. The National Institute of Deafness and other Communication Disorders (NIDCD) estimates that more than 40 percent of Americans will experience dizziness that is serious enough to go to a doctor. An understanding of the molecular mechanisms involved in the normal and abnormal development and function of the inner ear is an essential prerequisite for discovering the underlying causes of idiopathic balance disorders.
The studies described in this thesis illustrate essential roles for Pten, Otoc1, and Ncs-1 interacting proteins in vestibular morphogenesis in zebrafish. Since many genes have been shown to be conserved in mammalian and zebrafish otogenesis, it will be of interest to determine whether the genes I have identified are involved in mammalian development. It is hoped that by further studying the genes presented in this thesis, an underlying cause of vestibular function may be elucidated and become a therapeutic target.
REFERENCES


Vita
Jessica Petko

Academic Background

Sept. 1998 – May 2002    B.S. Major in Biology/ Minor in Psychology
California University of Pennsylvania
California, Pennsylvania

Aug. 2002 – Present    Ph.D. Candidate, Graduate Research Assistant
Intercollege Graduate Program in Genetics
Pennsylvania State University College of Medicine
Hershey, Pennsylvania

Honors, Awards, & Affiliations

April 2000- May 2002    Beta Beta Beta Biological Honors Society
Secretary (Sept. 2001-May 2002)

Nov. 2005- Nov. 2006    American Association of Anatomists

April 2006    First Place in Graduate Division
College Venture Challenge

Related Experience

Sept. 2007- Dec. 2007    Adjunct Faculty, Biology Department
Elizabethtown College
Elizabethtown, Pennsylvania

Publications


Abstracts