THE FUNCTION AND GENETIC INTERACTIONS OF ZEBRAFISH *atoh1* AND *sox2:* GENES INVOLVED IN HAIR CELL DEVELOPMENT AND REGENERATION

A Dissertation

by

BONNY BUTLER MILLIMAKI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Genetics

The Function and Genetic Interactions of Zebrafish *atoh1* and *sox2*: Genes Involved in Hair Cell Development and Regeneration.

THE FUNCTION AND GENETIC INTERACTIONS OF ZEBRAFISH *atoh1* AND *sox2*: GENES INVOLVED IN HAIR CELL DEVELOPMENT AND REGENERATION

A Dissertation

by

BONNY BUTLER MILLIMAKI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Bruce B. Riley
Committee Members,	Arne C. Lekven
	Brian Perkins
	Sumana Datta
Intercollegiate Faculty Chair,	Craig Coates

August 2010

Major Subject: Genetics

ABSTRACT

The Function and Genetic Interactions of Zebrafish *atoh1* and *sox2*: Genes Involved in Hair Cell Development and Regeneration. (August 2010)

Bonny Butler Millimaki, B.S., Middle Tennessee State University

Chair of Advisory Committee: Dr. Bruce B. Riley

The sensory cells of the inner ear, hair cells, provide vertebrates with the ability to detect auditory stimuli and balance. In mammals, cochlear hair cells, those responsible for hearing, do not regenerate. Zebrafish hair cells do regenerate. Gaining an understanding of the role and regulation of the genes involved in the formation and regeneration of these cells may provide information important for the development of genetic therapies.

We show that zebrafish *atoh1* acts as the proneural gene responsible for defining the equivalence group from which hair cells form. Expression of *atoh1* is dependent upon Fgf and Pax. Atoh1 induces expression of *delta,* resulting in activation of Notch and subsequent lateral inhibition. Another factor known to be important for hair cell formation in mice is Sox2. In zebrafish, *sox2* expression is downstream of Atoh1, Notch and Fgf. Zebrafish Sox2 is not required for hair cell formation, but rather Sox2 is important for hair cell maintenance.

In zebrafish, otic hair cell regeneration has not yet been characterized. We show that, following laser ablation, hair cells regenerate by way of transdifferentiation. We

further show that this regeneration requires Sox2 activity. These data suggest that Sox2 acts to maintain support cell plasticity. This role is likely conserved because Sox2 is also important for stem cell plasticity in mammals. This new understanding of the role and regulation of both Atoh1 and Sox2 provides essential information that can be used to further efforts to provide genetic therapies for hair cell regeneration in mammals.

DEDICATION

I dedicate this work to my husband, Ryan, for his unwavering love and support through this adventure, to our most successful genetics experiment, our daughter Evelyn, for she inspires me every day, and to my heavenly father for giving me such wonderful creations to explore and the gifts with which to do it.

Then Jesus said, “He who has ears, let him hear” Mark 4:9

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Bruce Riley, and my committee members, Dr. Datta, Dr. Perkins, Dr. Lekven, and Dr. Panin, for their guidance and support throughout the course of this research and for being great examples.

This work was supported by the National Institutes of Health, NIDCD grant R01-DC03806. We thank Bruce Appel and Jim Fadool for supplying heat shock dnSu(H) and NICD lines.

Thank you to all members of the zebrafish labs for your support and assistance. I especially thank the members of the Riley lab. I thank Dr. SuJin Kwak and Dr. Bryan Phillips for their guidance and assistance as I began my research. I thank Dr. Hye Joo Kwan for showing me how to balance home and work life. I thank Shruti Vemeraju, Mahesh Padanad, Reha Dahson, and BeWei Gho for their friendship and support throughout the years. I thank Neha Bhatt for her editing eye and wonderful pictures which were used to create my introductory figures. I especially thank Elly Mae Sweet for all her dedicated work and insights. You have been with me from the beginning and helped me to make it to the end. I thank your for your work on both of our papers and the many scientific discussions that have led me to new experiments and discoveries.

I want to thank all of my family. I thank my mother, father, step-mother, step-father and siblings who have lovingly supported, both emotionally and financially, my decision to be a career student. I am grateful for my graduate student family, Megan Reynolds and Sarah Black, who have stood by me in good times and bad. I am also

thankful for my church family whose prayers and support have guided me to the end. I thank my family of other mommies from LLL, especially Rowena and Amanda, without whom I would never had made it to this point as a mom or scientist. I thank my BVM family for caring for my angel with such care so that I can work without worry. Lastly, I thank my husband and daughter for their love, support and inspiration. We made it!!!

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

The vertebrate inner ear is a highly developed structure responsible for both hearing and balance. Within the ear are a number of sensory patches made up of hair cells that are responsible for auditory and vestibular transduction, and associated support cells. In mammals, hair cell loss within the auditory organ, the cochlea, is permanent. In lower vertebrates, including zebrafish, hair cell death is followed by a period of regeneration of hair cells from the surrounding support cells (reviewed by Brignull et al., 2009). To understand how mammalian hair cell regeneration may be initiated by way of gene therapy we must first gain an understanding of the genes required for initial hair cell development. In mouse, both *Atoh1* and *Sox2* are required for hair cell formation; however, much debate remains about the role of each transcription factor. Because *Atoh1* is a homologue of the *Drosphila* pronerual gene *atonal, Atoh1* may act as the proneural gene responsible for defining the equivalence group from which both hair cells and support cells form. *Sox2* is initially expressed within both hair cells and support cells but is subsequently lost from hair cells and maintained in support cells.

_____

This dissertation follows the style and format of *Development*.

Furthermore, *Sox2* plays a role in the maintenance of stem cell plasticity (Masui et al., 2007). Taken together one can hypothesize a role for *Sox2* in hair cell regeneration. Alternatively, Sox2 may regulate hair cell differentation alone. Studying both of these transcription factors in zebrafish can clarify their role in hair cell differentiation and regeneration.

INNER EAR MORPHOLOGY

The vertebrate inner ear is a labyrinth of interconnected chambers specially suited for detecting sound and acceleration, gravity and movement. The structure of the vertebrate inner ear includes semicircular canals and macular organs each associated with sensory cells (reviewed in Haddon and Lewis, 1996; Torres and Giraldez, 1998; Riley and Phillips, 2003). The sensory cells of the inner ear, hair cells, project ciliary bundles into the lumen of the ear. All the chambers of the inner ear are filled with endolymph, a specialized fluid filled with ions that are specially suited for hair cell function. Mechanical deflection of the hair bundles, as a result of sound waves or motion, opens ion channels and results in hair cell stimulation. Support cells surround the hair cells, with hair cells sitting apically relative to support cells (Fig. 1D). Neurons of the statoacoustic (VIIIth) ganglion (SAG), located between the ventromedial wall of the ear and the ventral portion of the neural tube, synapse along the basolateral membrane of the hair cells and project back to the hindbrain.

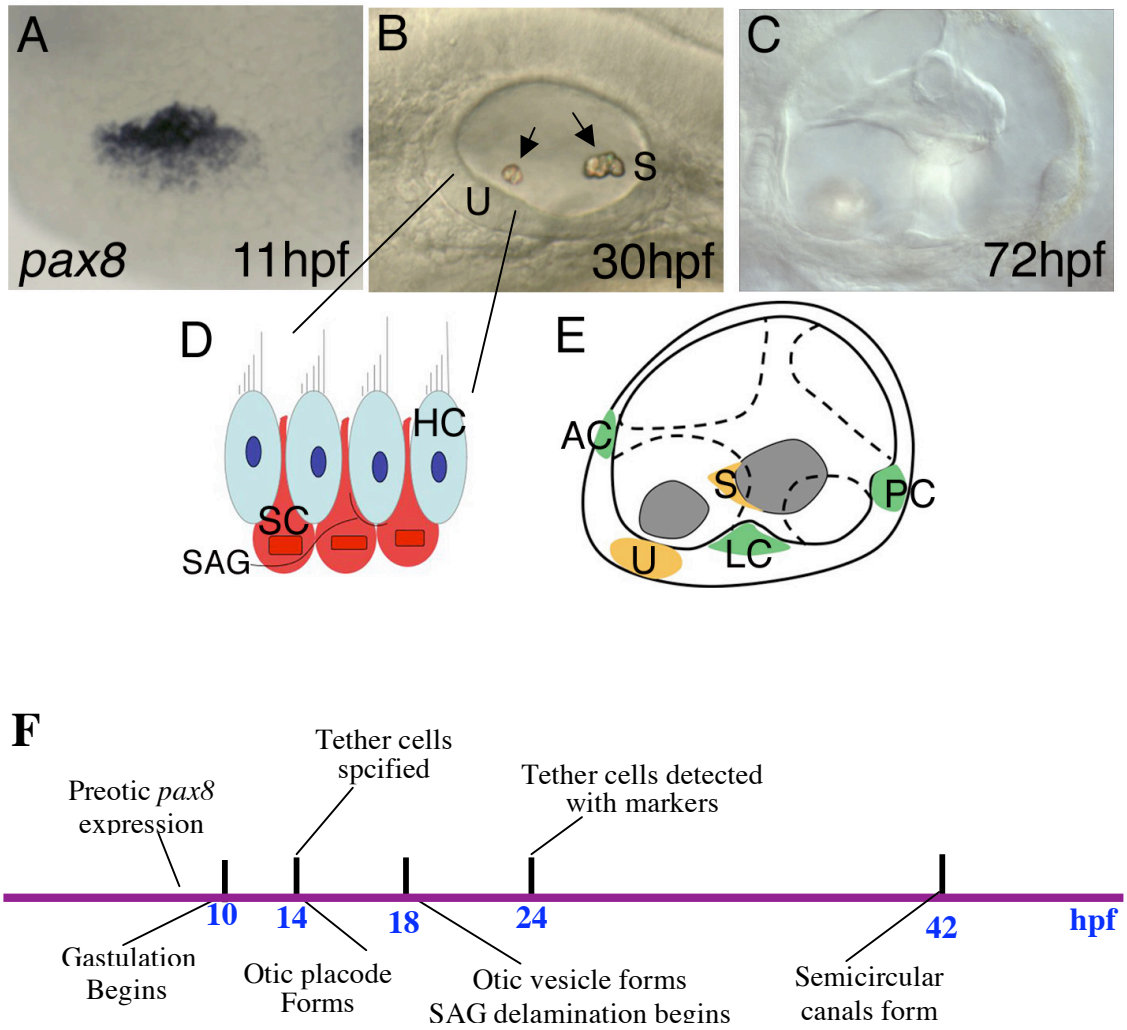


Figure 1: Ear Development and Morphology.
By 9 hours post fertilization (hpf) the cells of the preotic placode can be distinguished by expression of *pax8*, shown here at 11 hpf (A). (B) From the preotic placode forms an otic vesicle, seen in lateral view. Within the otic vesicle are two maculae, the utricle (U) and the saccule (S). Each macula is made up of an otilith (arrows) and a sensory epithlium with hair cells (HC) and support cells (SC) as depicted in D. Signals from the mechanosensory hair cells are transmitted to the brain by way of innervating SAG, statoacoustic ganglion, neurons. (C) The ear continues to develop into a structure of interconnected fluid filled chambers, semicircular canals, each associated with sensory epithelia, cristae. E is a cartoon representation of C with labeled utricle (U), saccule (S), anterior chritae (AC), posterior christae (PC), and lateral christae (LC). All pictures and cartoons represent a lateral view. F shows a time line of ear development.

All vertebrates have at least two maculae, the utricle (in the anterior) and saccule (in the posterior). A macula is a bed of sensory epithelia and associated extracellular accretions of calcium carbonate crystals. In fish these crystals coalesce into a singular structure known as an otolith (Fig. 1B), whereas in mammals crystals form "snow drift" called otoconia embedded in an organic matrix. Because the otolith is attached to hair cells, the otolith can amplify sound and movement. When the otolith moves, it deflects the associated hair cells and results in excitation of innervating neurons that signal to the central nervous system. In higher vertebrates the maculae are utilized only for the detection of vestibular (acceleration and gravitational) cues; however, fish and frogs also use the saccule for hearing (Popper and Fay, 1993).

Where most fish can hear between 10 and 1000 Hz, zebrafish can detect sound waves up to 4000 Hz (Nicolson, 2005). Osterophysine fishes, including zebrafish, are considered "hearing specialists" because they have bones (weberian ossicles) that connect the saccule to the swim bladder. Sound waves cause vibration of the swim bladder that is then transmitted to the saccule via the weberian ossicles (Popper and Fay, 1993). Higher vertebrates utilize a highly derived end organ that specializes in hearing, the cochlea, that also contains a sensory epithelium that includes hair cells and support cells. Although the organs utilized for hearing are somewhat different, all vertebrates use hair cells to transduce cues of sound, movement, and gravity.

Fish and amphibians also form a series of sensory epithelia along the length of the body called the lateral line. This organ detects low-frequency stimuli, such as pressure waves and laminar flow. The ability to detect the movement of water is vital

for a number of behaviors including schooling and preditor avoidance (Popper and Fay 1997).

All vertebrate ears also contain three semicircular canals that are important for sensation of angular acceleration. These interconnected tubes form through projections of epithelia that fuse in the center of the ear (Whitfield et al., 2002). At the closed end of each semicircular canal resides a mound of sensory epithelia known as crista (Fig. 1C,E). The hair cells of the cristae each have a single tubulin filled kinocilium that embeds into the gelatinous membrane (cupula) that spans the canal. Movement of the animal's head causes capula movement and subsequent hair cell deflection resulting in detection of head position and angular acceleration by the brain (Nicolson, 2005).

Amniotes form the nonsensory structures of the inner ear followed by the corresponding sensory epithelium. In fish, in contrast, the sensory cells of the inner ear form before morphogenesis of their associated non-sensory structures (Haddon and Lewis, 1996; Whitfield et al., 2002). There are 7 sensory patches formed within the fish ear. First to form are those within the utricle, in the anterioventral region of the ear, and the saccule, in the posteriomedial portion of the ear. By 60 hours post fertilizationa (hpf) the sensory cells associated with the semicircular canals begin to differentiate. Lastly the lagenar macula and macula neglecta begin to form after embryonic day 10.

INNER EAR DEVELOPMENT

The ear forms from the otic placode, an ectodermal thickening on either side of the head adjacent to the hindbrain. The otic placode becomes morphologically visible by 14 hpf, hours post fertilization, but can be detected through expression of specific factors much earlier. Many genes such as *dlx, eya* and *six4* are expressed throughout the precursors of multiple cranial placodes, including the otic placode, during early development. The first gene specific to the otic placode, *pax8*, can be detected in zebrafish by 9 hpf (Fig. 2B). Expression of *pax8* is found in preotic cells by late gastrulation in zebrafish, mouse and *Xenopus* (Pfeffer et al., 1998; Heller and Brandi, 1999).

Induction of the otic placode involves a number of factors. The sources of inductive signaling include the adjacent hindbrain and underlying mesoderm. Fgfs, fibroblast growth factors, are expressed in both inductive tissues. In mouse, loss of FGF3, expressed in the hindbrain, along with FGF10, expressed within the underlying mesenchyme, ablates otic development (Wright and Mansour, 2003). In zebrafish, Fgf3 and Fgf8 are both expressed within the hindbrain segment rhombomere 4. Loss of both Fgf3 and Fgf8 results in a complete loss of otic tissue and *pax8* expression (Pillips et al., 2001). Missexpression of Fgf also results in formation of ectopic otic vesicles (Phillips et al., 2004).

Although Fgf is important for otic induction, it does not act alone. Transcription factors confer cells the competence to respond to inductive signals (Fig. 2A). Foxi1 is a

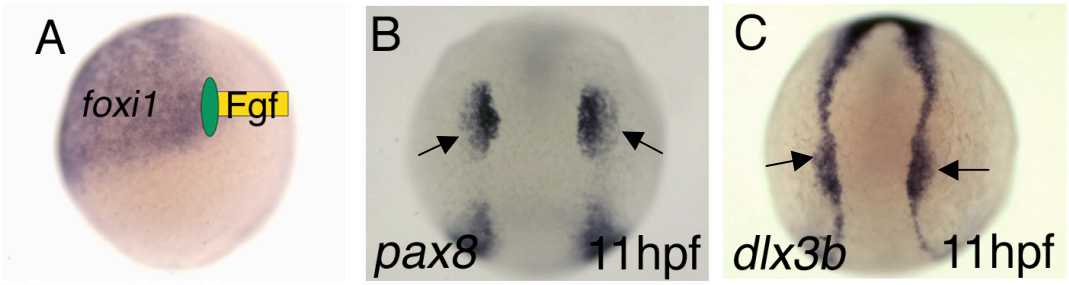


Figure 2: Otic Induction.
A is a cartoon representing a lateral view of preotic induction, shown at 8 hpf. The inner ear forms in the ectoderm where Fgf signals from the hindbrain abut *foxi1* expression. These cells, green oval, are induced to expresses *pax8* within the preotic placode, as seen in a dorsal view in B (arrow). (C) Other signals, such as *dlx3,* mark the entire preplacodal ectoderm and may include areas of upregulation within the preotic placode (arrows).

forkhead class transcription factor initially expressed in the anteroventral quadrant of late blastula stage zebrafish embryos (Solomon et al., 2003). By late gastrulation, expression of *foxi1* is lost ventrally and upregulated in two patches at the lateral edges of its initial domain, encompassing the preotic domain of *pax8.* Loss of Foxi1 results in a loss of *pax8* and severely impairs subsequent otic development (Solomon et al., 2003; Hans et al., 2004).

Other factors that play a critical role in preotic development, such as *dlx3b* and *dlx4b*, are initially expressed within the preplacodal domain (Fig. 2C), and are later restricted to individual placodes. Loss of zebrafish *dlx3b* results in formation of a small otic vesicle (Solomon and Fritz, 2002). Loss of both *dlx3b* and *dlx4b*, however, allows otic initiation, as indicated by *pax8* expression; however, these cells later lose otic fates.

In mouse and chick, the otic placode invaginates, pinching off and sinking into the mesenchyme under the surface ectoderm, to form an otic vesicle. In zebrafish, the otic vesicle forms through a process known as cavitation. During cavitation, the nuclei of the otic placode move to the placode surface. Cells lose cell-cell adhesion centrally in order to make a thin slit-like lumen (Haddon and Lewis, 1996). The otic cells now grow larger and divide to form an otic vesicle.

Once the otic vesicle forms, various genes are expressed within specific regions of the vesicle. Some factors, such as *nkx5.1* and *pax5,* are specific to the anterior domain (Pfeffer et al., 1998; Adamska et al., 2000). *pax2a,* initially expressed throughout the entire otic vesicle, is later restricted to the ventromedial portion of the vesicle, the area where the sensory epithelium will form.

Cells at the anterior and posterior poles of the otic vesicle are specified as the sensory epithelium. Expression of specific factors of sensory cell development begins by 14 hpf (Haddon et al., 1998). The first formed hair cells are known as tether cells because they tether otolith material together, assisting in formation of otoliths (Riley et al., 1997). The kinocilia of these precocious hair cells can be detected by 19 hpf. The neurons responsible for sending signals from the hair cells to the central nervous system are those of the statoacoustic ganglion. These cells form from cells within the otic vesicle near the utricular macula and, around 18 hpf, begin to delaminate, primarily from the anteroventral surface of the otic vesicle. These cells congregate in a region medial to the ear where they differentiate and send projections both to the hindbrain and to the hair cells (reviewed in Riley and Phillips 2003). The ear eventually forms a complex structure of interconnected chambers through coordinated folding of the otic epithelium, cell division and apoptosis.

MECHANOTRANSDUCTION BY HAIR CELLS

Hair cells provide both auditory and vestibular cues to the brain via mechanotransduction. This means of transforming a mechanical force into an electrical signal is utilized by many different systems including touch receptors, *Drosophila* chordotonal organs, and hair cells of the ear and lateral line. Hair-cell transduction is both very sensitive and able to detect a wide range of intensities. The mechanically sensitive portion of the hair cell resides within cilia located on the apical surface. Actin-

filled stereocilia are arranged on the cell surface in order of ascending height. In immature and vestibular hair cells there is an additional axonemal kinocilium adjacent to the tallest stereocilium that anchors the hair cell to the extracellular matrix (reviewed by Fettiplace and Hackney, 2006; Vollrath et al., 2007; Gillespie and Muller 2009).

Hair cells respond to either sound or movement through syncronous lateral deflection of the stereocilia. If deflected toward the stereocilia height gradient, channels will be open to allow an influx of cations (with a strong preference for Ca^{2+}). The influx of cations depolarizes the cell which activates neurotransmitter release at the base of the hair cell. This signal is received by the inervating statoaucustic ganglion neuron and transmitted to the central nervous system where it is processed as sound or motion (reviewed by Fettiplace and Hackney, 2006; Vollrath et al., 2007; Gillespie and Muller 2009).

Many studies over the past thirty years have focused on how deflection of stereocilia results in open cation channels. Opening of channels directly via mechanical force has been described to occur through a "gated spring" (Corey and Hudspeth, 1983). Electron microscopy shows that fine extracellular filaments connect each stereocilium tip to the adjacent taller stereocilium (Pickles et al., 1984). Loss of these tip-links results in a loss of mechanotransduction indicating a role for tip-links in hair cell activity (Assad et al., 1991). Tip-links are composed of a helical structure made of two strands that separate at the upper end of the connection just prior to adherence to the neighboring stereocillium (Kachar et al 2000). Recent studies suggest that the channel responsible for allowing ions into the hair cell is located at the base of the tip-link on the shorter

member of the stereocilia pair (Beurg et al., 2008). How the tip-link relates to the gating of transduction channels remains unclear.

LATERAL INHIBITION

The sensory epithelium of the inner ear comprises two cell types, hair cells and support cells, both of which develop from a common progenitor pool or equivalence group. To determine which cells from within the equivalence group will develop into hair cells and which will become support cells, the ear utilizes a process known as lateral inhibition via Delta-Notch signaling (Fig. 3). Initially, all cells within the equivalence group express a low level of the tethered ligand Delta (Dl). Dl ligands bind to and activate the Notch receptor on adjacent cells. During this early phase, the uniform level of Dl maintains cells in an uncommitted state allowing for continued cell division. For reasons that are not yet clear, a subset of the cells within equivalence group up-regulate expression of Delta. Elevated Notch activity in neighbors results in loss of hair cell specific gene expression and up-regulation of support cell specific factors. Hair cells maintain low or no Notch activity. It is through this process that a pattern of hair cells surrounded by support cells forms within the sensory epithelium (reviewed by Pi and Chien, 2007).

Evidence for this model includes analysis of zebrafish *mindbomb* (*mib*) mutants. Mib is an E3 ubiquitin ligase required for Delta-Notch signaling (Itoh et al., 2003). In

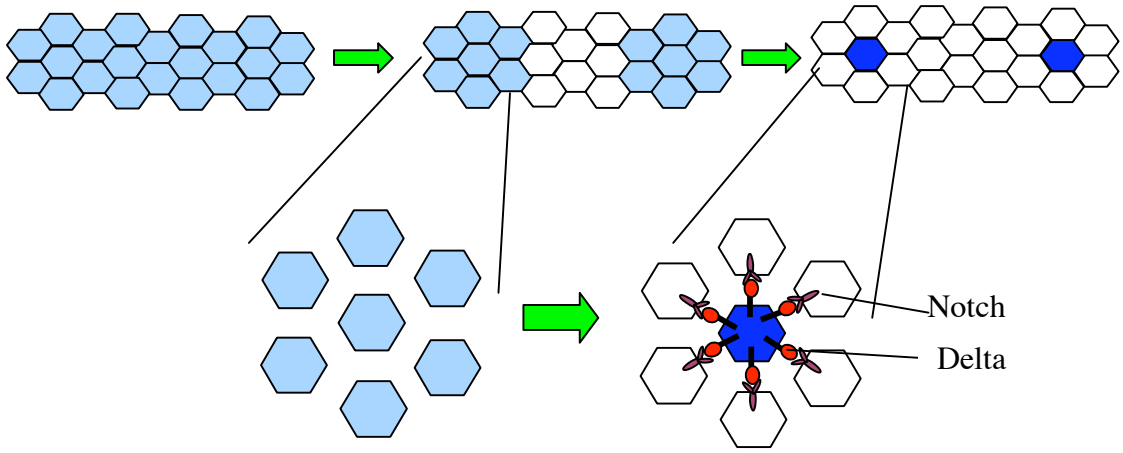


Figure 3: Lateral Inhibition.

Initially all the cells within the large prosensory patch express a low level of the proneural gene (light blue). Next the large prosensory patch is restricted into two patches. Within the patch one cell will upregulate proneural gene expression (dark blue). This will result in upregulation of Delta (Dl) (red). Dl will signal through Notch within the adjacent cells and result in loss of proneural gene exression. The cells that maintain proneural gene expression will develop into neurons or hair cells and the cells that lose proneural expression will develop into glial or support cells.

these mutants all Notch signaling fails, causing all cells to maintain hair cell fate and blocking support cell formation (Haddon et al., 1998). In mutants with reduced DeltaA activity, an increase in hair cell number is also observed (Riley et al., 1999). In mouse, two

Notch ligands, Delta-like 1 (DLK1) and JAGGED2 (JAG2), regulate the balance of hair cells and support cells. Loss of either of these ligands results in an increase in hair cell number in a manner consistent with the lateral inhibition model (Lanford et al., 1999, Kiernan et al., 2005a, Brooker et al., 2006).

PRONEURAL GENES

Neural development requires the activity of proneural genes. Proneural genes encode basic helix-loop-helix (bHLH) transcription factors whose expression marks the presence of an equivalence group. An equivalence group is a group of cells that each have equal potential to take a given developmental path. In sensory epithelia, it is believed that proneural gene expression marks the equivalence group from which hair cells and support cells form, though the identity of this gene has been debated (see below).

In *Drosophila*, *atonal* related genes are proneural genes that act to specify chordotonal sense organs as well as the photoreceptor cells of the eye (Jarman 1993). We can use the specification of the R8 cell in the eye as a model of how proneural genes

act. First, expression of *atonal* (*ato*) is initially detected in a broad pattern long before specification of the R8 cell (Jarman et al., 1993; Jarman et al., 1995). Delta-Notch signaling restricts the size of the equivalence group, and with it the domain of *ato* expression, by breaking the equivalence group into groups of *ato* expressing cells separated by non-expressing cells (Baker et al., 1996; Baker and Yu, 1997). Next, lateral inhibition instructs cells as to their fate within this group with some cells maintaining *ato* and becoming photoreceptors. In *ato* mutants, *ato* transcription is not restricted. This is because Ato normally activates *dl* expression, therefore without Ato activity there is no *dl* expression and no restriciton (Jarman et al., 1995; Baker and Yu, 1997). However, without Ato activity, R cell differentiation cannot begin. Notch mutants likewise fail to restrict *ato* expression due to failed lateral inhibition, but in this case all cells become photoreceptors (Baker et al., 1996).

To function, proneural genes heterodimerize with other bHLH transcription factors such as *daughterless*. This heterodimer binds to a specific DNA sequence known as an E-box and results in the expression of downstream targets such as *dl* (Murre et al., 1998; Cabrera and Alonso, 1991). There appear to be two distinct phases of Ato activity. During the initial (proneural) phase, low-level Notch activity stimulates *ato* expression (Baker and Yu 1997). During the second (fate specification) phase, elevated Notch activity blocks *ato* expression by way of the canonical Notch pathway consistent with lateral inhibition. This is in stark contrast to terminal differentiation factors, such as NeuroD, which are also bHLH factors but are not directly regulated by Notch.

By understanding the role *ato* plays in photoreceptor fate specification, we are able to gain an understanding of the general behavior of proneural. By convention, four criteria define proneural function. First, expression precedes sensory cell fate specification. Second, the gene is responsive to lateral inhibition via Delta-Notch signaling. Third, function is required for production of the equivalence group from which the sensory structure is produced. Fourth, missexpression of proneural function is sufficient to produce ectopic sensory cells. We will apply these criteria to inner ear studies to resolve a controversy regarding the identity of the proneural gene associated with the development of the sensory epithelia.

atoh1 GENES

A homologue of the *Drosophila* gene *ato, Atoh1,* is expressed within the developing sensory epithelium of the ear. Mouse *Atoh1* is a close homologue to Drosophila *ato*, with 82% amino acid similarity in the bHLH domain and 100% conservation of the domain required for DNA recognition (Ben-Arie et al., 1996; Chien et al., 1996). In fact, *Drosophila ato* can rescue *Atoh*1 knock out mice and *Atoh1* can rescue *ato* mutant flies (Wang et al., 2002).

The time at which *Atoh1* expression begins within the mouse ear is somewhat controversial. In situ data suggest that *Atoh1* expression does not begin within sensory cells until they become post-mitotic, implying that it cannot play a role in specifying the

equivalence group (Chen et al., 2002). In contrast, one study reports that *Atoh1* mRNA can be detected via RT-PCR 12 hours prior to that of in situ signals, suggesting that it may, in fact, mark the equivalence group from which the hair cells form (Matei et al., 2005). Overexpression of *Atoh1* in rat organ cultures results in an overproduction of cochlear hair cells (Zheng and Gao 2000). In mouse, embryos deficient in *Atoh1* fail to form hair cells or support cells (Bermingham et al., 1999). Even so, expression of some early upstream regulators of the sensory epithelium, Jag1 and Sox2, persists within some of the cells that do form (Woods et al., 2004). This has led some to suggest that *Atoh1* is not required as a proneural gene for specification of the equivalence group, but rather to promote hair cell formation (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005).

In zebrafish, two *atoh1* genes, *atoh1a* and *atoh1b*, are expressed within the developing sensory epithelium (Adolf et al., 2004). In order to elucidate the role of *atoh1* in sensory cell development, we must first determine if *atoh1* fulfills the criteria of proneural genes as discussed above. Furthermore, it is not yet understood how *atoh1* expression is regulated. We will examine the role and regulation of *atoh1* in Chapter II.

Sox2

SOX2 is an SRY-related (sex-determining region Y)HMG (high mobility group) box transcription factor and a universal marker of stem cells and neural progenitors within the developing central nervous system (Gubbay et al., 1990; reviewd in Shi et al.,

2008). Humans suffering from a *Sox2* mutation exhibit sensorineural hearing loss, in addition to sever eye malformations (Hagstrom et al., 2005). Studies in mouse show that loss of *Sox2* results in a failure to form the sensory epithelium and thus a loss of *Atoh1* expression (Kiernan et al., 2005b). This has lead some to speculate that *Sox2* is the proneural gene that establishes the prosensory equivalence group (Kiernan et al., 2005b; Dabdoub et al., 2009). In mouse, *Sox2* is initially expressed throughout the ventral half of the developing otic vesicle, long before sensory primordia formation, and subsequently expressed within the primordia of both hair cells and support cells. Later, expression is lost from hair cells and maintained within support cells alone (Kiernan et al., 2005b; Hume et al., 2007; Neves et al., 2007). This is inconsistent with a proneural gene; however, because loss of *Sox2* blocks formation of the sensory epithelium, the role of Sox2 in hair cell and support cell development and function remains to be fully resolved.

Sox2, along with *Sox1* and *Sox3*, is a member of the SoxB1 group of transcription factors known to maintain neural precursors in a progenitor state (Graham et al., 2003; Wright et al., 1993). Inhibition of *Sox2* results in precocious neural differentiation while constitutive expression of *Sox2* inhibits neural differentiation with cells maintaining progenitor characteristics (Avilion et al., 2003; Graham et al., 2003). One mechanism by which SOXB1 proteins are thought to act is through inhibition of bHLH-mediated neural differentiation (Bylund et al., 2003). Missexpression studies suggest that *Sox2* and *Atoh1* are mutually antagonistic in mouse choclear cell fate specification (Dabdoub et al., 2009). Knowing that support cells maintain plasticity and can develop into hair

cells following hair cell death and that *Sox2* is maintained in support cells (reviewed in Brignul et al., 2009; Oesterle et al., 2008), one can speculate that *Sox2* helps support cell maintain a capacity for regeneration. *Sox2* has also been implicated in neural stem cell maintenance within the adult brain because a reduction of *Sox2* expression results in a reduction in the proliferation of neural precursors (Ferri et al., 2004; Episkopou 2005). Since *sox2* expression begins after the specification of the sensory epithelium in zebrafish, we will use zebrafish to study Sox2 function and ask if it plays a role in support cell plasticity or maintenance or both.

Regulation of *Sox2* within the ear has not yet been studied thoroughly. In mouse, *Sox2* expression is required for *Atoh1* expression, and loss of ATOH1 does not affect *Sox2* expression (Kiernan et al., 2005b). In zebrafish, *atoh1* expression precedes that of *sox2*, suggesting a different regulatory mechanism. We will discuss the role and regulation of zebrafish *Sox2* in Chapter III.

OTHER SIGNALS IMPORTANT FOR HAIR CELL FORMATION

Signals from the hindbrain adjacent to the otic vesicle play a prominent role in otic placode induction. Additionally, these factors continue to affect patterning within the ear at later stages. Candidates for these hindbrain signals include members of the Fgf family of secreted ligands. In zebrafish, *Xenopus*, chick and mouse, Fgf3 is maintained within the hindbrain following otic placode induction (Mahmood et al., 1995; Mahmood

et al., 1996; Mckay et al., 1996; Lombardo et al., 1998; Phillips et al., 2001). Studies of mutations in the gene coding for the transcription factor *valentino* (*val*) in zebrafish gives indirect evidence that Fgf3 is important for hair cell formation. Embryos mutant for *val* form a hindbrain with expranded *fgf3* expression throughout the segments of the hindbrain adjacent to the ear. This results in an expansion of anterior specific otic markers and the production of ectopic hair cells within nearly all the otic tissue adjacent to the hindbrain. Furthermore, loss of either Fgf3 or Fgf8 results in ears that produce fewer than normal hair cells (Kwak et al., 2002). Although it is evident that Fgf is important for hair cell formation, the mechanism by which this hindbrain signal affects hair cell differentiation has not yet been identified.

One family of genes known to mediate Fgf signaling is the Pax2/5/8 family of transcription factors. Pax2 and Pax8 work together to maintain the otic placode (Hans et al., 2004, Mackereth et al., 2005). Expression of *pax8* precedes that of *pax2* genes and is lost from otic cells soon after formation of the otic vesicle. Disruption of Pax8 results in a reduction in the size of the otic placode and a significant reduction in hair cell number (Mackereth et al., 2005). The expression of *pax2a* and *pax2b* begins throughout the preotic cells but becomes restricted to the medial wall as the otic vesicle forms. Upon specification, tether cells, along with some other hair cells, upregulate expression of *pax2a* and *pax2b* (Riley et al., 1999). Although these two genes have similar expression patterns, their roles in hair cell formation appear to differ. Loss of Pax2a results in less robust upregulation of *dla* (*deltaA*) within hair cells. This weakened lateral inhibiton results in an overproduction of hair cells. Conversely, Pax2b depleted embryos produce

fewer than normal hair cells. Taken together one can suggest a role for Pax2b in hair cell specification and Pax2a in lateral inhibition. Although *pax2a* mutants initially produce more hair cells than normal, these mutants later exhibit a loss of utricular hair cells due to apoptosis (Kwak et al., 2006). Pax2a is required for the expression of another Pax gene, *pax5* (Kwak et al., 2006). Pax5 is initially expressed in the anterior end of the otic vesicle and is later localized to the utriclar macula (Kwak et al., 2006; Pfeffer et al., 1998). Hair cells initially form normally in embryos deficient for Pax5; however, a number of these cells subsequently undergo apoptosis and are extruded from the ear (Kwak et al., 2006). It is evident that *pax* genes play a role in lateral inhibition and hair cell maintenance but their role in hair cell differentiation has yet to be clarified.

HAIR CELL MAINTENANCE

In addition to Pax5, whose role in hair cell maintenance has been studied in zebrafish, many factors required for hair cell survival have been identified in mouse. One such protein is the transcription factor *Brn3c*. Expression of *Brn3c* begins within the postmitotic precursors of both auditory and vestibular hair cells, and is activated by high levels of Atoh1. Upon loss of *Brn3c,* murine ears produce hair cells but some of these cells fail to express markers of mature hair cells, appear disorganized and eventually die (Xiang et al., 1998). Loss of another factor expressed after *Brn3c*, *Barh11*, results in degeneration of all of the hair cells in the cochlea and consequently

causes hearing loss in mice (Li et al., 2002). *Gfi1* is another noted hair cell survival factor that is regulated by *Atoh1* and *Brn3c*. *Gfi1* knockout mice exhibit apoptosis within cochlear hair cells (Wallis et al., 2003; Hertzano et al., 2004). All of these hair cell survival factors are expressed within the hair cells themselves and act in a cell autonomous fashion.

Support cells also play an important role in hair cell maintenance. Zebrafish mutants for *mib* exhibit a vast overproduction of hair cells at the expense of support cells due to a loss of Delta-Notch mediated lateral inhibition. In these mutants, hair cell production continues to increase until 48 hpf at which time hair cells begin to die. By 60 hpf most ears are entirely devoid of hair cells (Haddon et al., 1998). It is thought that the hair cells begin to die in *mib* mutants because support cells provide something required for hair cell viability. Support cells could provide some necessary trophic factor or, alternatively, support cells may be required for proper formation of the epithelial structure surrounding hair cells.

HAIR CELL REGENERATION

The leading cause of human deafness is loss of sensory cells (Seidman et al., 2002). In fact, age related hearing loss affects more than 40% of individuals 75 years of age and older (Seidman et al., 2002). In mammals, cochlear hair cells do not regenerate leaving prosthetics, hearing aids and cochlear implants as the only treatment for hearing

loss in humans (Chardin and Romand 1995). In non-mammalian vertebrates, such as birds, frogs and fish, sensory cell death is not permanent because it is followed by hair cell regeneration.

Hair cell regeneration can occur either through transdifferentiation of support cells directly into hair cells or through asymmetric cell division of support cells fiving rise to a new support cell and a new hair cell (reviewed in Kwan et al., 2009). Neonatal mouse support cells can undergo transdifferentiation (Kelley et al., 1995). There is evidence in chick that both transdifferentiation and asymmetric cell division occur sequentially and may be regulated by different genetic pathways (Bhave et al., 1995; Duncan et al., 2006; Cafaro et al., 2007; Stone and Cotanche, 2007). The regulation of hair cell regeneration has been the focus of many recent studies. Studies in chick show that transdifferentiation correlates with *Atoh1* upregulation (Cafaro et al., 2007). Forced expression of *Atoh1* in the support cells of prenatal rodents causes differentiation into hair cells (Shou et al., 2003; Izumikawa et al., 2005).

Cochlear support cells are thought to be a highly specialized cell type compared to support cells in other sensory epithelia (Corwin and Oberhltzer, 1997). Support cells are held in a differentiated state through expression of genes downstream of Notch signaling including members of the *Hes* family (Kwan et al., 2009). This is evident because disruption of Notch in damaged cochlear tissue results in the production of extra hair cells within the mature auditory epithelium signifying re-entry of support cells into the cell cycle to produce new hair cells (Hori et al., 2007). One model of hair cell regeneration suggests that hair cell death results in a loss of Delta-Notch signaling

mediated by hair cells. Loss of sustained Notch signaling within a support cell is hypothesized to downregulate support cell specific factors and upregulate hair cell specific factors such as *Atoh1*. It is not yet understood why cochlear hair cells of adult mammals have lost the capacity for regeneration. One hypothesis suggests that high levels of the cyclin dependant kinase $P27^{Kip1}$ within support cells of the cochlea prevent re-entry into the cell cycle. Mammlian vestibular support cells, which are involved in hair cell regeneration, express $P27^{Kip1}$ at a much lower level (Chen and Segil, 1999). Regeneration of hair cells within the otic sensory epithelium of zebrafish has not yet been studied. Studies of the zebrafish lateral line indicate that regeneration may occur through both transdifferentiation and asymmetric cell division, depending upon the context (Hernandez et al., 2007: Ma et al., 2008**).** Damage to only mature hair cells results in a rapid regeneration without cell division where more extensive damage is repaired through long-term recovery involving increased cell division (Hernandez et al., 2007). The genes required for regeneration within the lateral line have not yet been identified; however, expression of the transcription factor Sox2, noted for its involvement in stem cell pluripotency, is consistent with a role in regeneration (Hernandez et al., 2007). We will address a role for Sox2 in otic hair cell regeneration in Chapter III.

DISSERTATION OBJECTIVES

The objectives of this dissertation are to address the role of both Atoh1 and Sox2 in hair cell development and regeneration, and to examine the regulation of *atoh1* and *sox2*, using zebrafish as a model system.

Atoh1 is a homologue of the *Drosophila* proneural gene *atonal*. Even though loss of ATOH1 in mouse results in a loss of hair cell formation, for various reasons some investigators have concluded that ATOH1 does not function as a proneural gene but rather as a late hair cell specification factor. To study the role of Atoh1 in zebrafish I performed loss of function studies using antisense oligonucleotides. Chapter II shows that Zebrafish *atoh1* operates by a mechanism fully consistant with classical proneural genes. Without Atoh1 no hair cells form while overexpression of *atoh1* is sufficient to result in the production of ectopic hair cells. Furthermore, *atoh1* is responsive to lateral inhibition via Delta-Notch signaling. We were further able to show that Fgf and Pax are both upstream activators of *atoh1*.

The transcription factor Sox2 is required for sensory formation in mouse; as such it is also upstream of Atoh1. In Chapter III I show that, in zebrafish, *atoh1* expression precedes that of *sox2* and that Sox2 activity is not required for hair cell formation. Rather, loss of Sox2 results in death of hair cells. In zebrafish, as in other organisms, *sox2* expression begins throughout the sensory epithelium and is restricted to the support cells upon hair cell maturation. I was able to show that hair cell regeneration occurs via transdifferentiation in zebrafish, and that this requires Sox2 activity. Furthermore,

Atoh1, Notch and Fgf are all upstream activators of *sox2*. Together these studies identify two transcription factors important for hair cell development, maintenance and regeneration and elucidate some of the regulators of these factors.

CHAPTER II

ZEBRAFISH *atoh1* GENES: CLASSIC PRONEURAL ACTIVITY IN THE INNER EAR AND REGULATION BY Fgf and Notch.*

OVERVIEW

This was a collaborative effort with my colleague E.M. Sweet. I contributed to every figure in this work and was responsible for the majority of figures on pages 34, 39, 42, 46 and 54.

INTRODUCTION

Sensory epithelia of the vertebrate inner ear consist of two cell types, hair cells and support cells. Both are produced from a prosensory equivalence group initially marked by expression of *Atoh1*, a homolog of the *Drosophila* proneural gene *atonal* (*ato*) (Bermingham et al., 1999). As the equivalence group develops, a few cells

---

*Reprinted with permission from "Zebrafish *atoh1* genes: classic proneural activity in the inner ear and regulation by Fgf and Notch."; by **Millimaki, B.B.†, Sweet, E.M.†, Dhason, M.S. and Riley, B.B.**, 2007, *Development* **134**, 295-305.

upregulate *atoh1* expression and complete differentiation as hair cells. The rest lose expression of *atoh1* and become support cells. As the principal regulator of hair cell differentiation, *Atoh1* has received great attention in recent years in both basic and applied research (Shailam et al., 1999; Lanford et al., 2000; Zheng and Gao, 2000; Itoh and Chitnis, 2001; Chen et al., 2002; Wang et al., 2002; Woods et al., 2004; Fritzsch et al., 2005; Izumikawa et al., 2005; Kelley, 2006). However, despite extensive analysis of *Atoh1*, a number of fundamental issues still need to be resolved. Most notably, there are conflicting reports as to the precise role(s) of *Atoh1* in otic development. Although *Atoh1* is maintained only in hair cells, it may function earlier to specify the equivalence group itself – a definitive proneural function. Accordingly, disruption of mouse *Atoh1* (*Math1*) ablates all hair cells and support cells in the cochlea (Woods et al., 2004). However, the persistence of cells expressing some early markers of sensory epithelia has been interpreted to mean that mouse *Atoh1* is not required for specifying the equivalence group per se, but instead only promotes the final stages of hair cell development (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005). Additionally, a key aspect of prosensory development does not require *Atoh1*: Prospective sensory cells begin to express $p27^{kip1}$ and exit the cell cycle prior to expression of *Atoh1*, and this process still occurs in *Atoh1* mutants. On the other hand, $p27^{kip1}$ expression and cell cycle withdrawal could be regulated independently from equivalence group specification. Indeed, sensory epithelia still form in $p27^{kip1}$ mutants despite the failure of cells to properly exit the cell cycle (Chen and Segil, 1999). This leaves open the

question of when the equivalence group forms and whether *Atoh1* acts early or late in the process.

Work on *Drosophila ato* provides a useful paradigm for testing vertebrate *Atoh1* function (Fig. 4). *ato* is initially expressed in a broad pattern (the equivalence group) well before cell fate specification (Jarman et al., 1995). The equivalence group then restricts its own size through activation of Delta-Notch (Dl-N) signaling (Baker et al., 1996; Baker and Yu, 1997). In this process, N-dependent downregulation of *ato* breaks the equivalence group into discrete "intermediate groups" of *ato*-expressing cells separated by non-expressing cells that are excluded from the sensory structure. Subsequently, the balance of *ato* and N activity selects between alternate fates within intermediate groups (lateral inhibition). Because *ato* is required for *Dl* expression, *ato* mutants fail to activate N-mediated restriction of *ato*, resulting in retention of a broad field of *ato*-expressing cells that are otherwise blocked from further development (Jarman et al., 1995; Baker and Yu, 1997). Similarly, *N* mutants also fail to restrict *ato* expression, but in this case all cells differentiate as sensory cells (Baker et al., 1996). Paradoxically, during the prosensory phase of development elevating N activity by expressing N intracellular domain (NICD) enhances *ato* expression (Baker and Yu, 1997). This involves a poorly characterized branch of the N pathway not requiring Su(H) (Ligoxygakis et al., 1998). During subsequent phases of development, NICD activates the canonical N pathway and abolishes *ato* expression. This work provides clear predictions for how vertebrate *Atoh1* might function assuming it acts as a classic proneural gene. In contrast, terminal differentiation factors like NeuroD are insensitive

to N activity and are not required for cell fate specification (Chitnis and Kintner, 1996; Reviewed by Brunet and Ghysen, 1999; Hassan and Bellen, 2000).

A crucial determinant of proneural gene function is the regulatory context in which it operates (Niwa et al, 2004). Activation of *ato* requires combinatorial signaling and specific regional identity genes like *eyeless* (*Pax6*), which also modify the sensory fate specified by *ato* (Niwa et al., 2004). The factors that induce *Atoh1* in the ear and cooperate in its function are largely unknown. *Sox2* is expressed broadly in the early otic vesicle in mouse and is required for induction of *Atoh1* several days later (Kiernan et al., 2005b). The lag in *Atoh1* expression suggests that in mouse Sox2 works combinatorially with other factors to initiate prosensory development. A number of signaling molecules have also been implicated in sensory epithelium development (Pirvola et al., 2002; Stevens et al., 2003; Daudet and Lewis, 2005; Brooker et al., 2006; Kiernan et al., 2006; Pujades et al., 2006), but their relationships to *Atoh1* expression remain unknown. Identifying the upstream activators of *Atoh1* is essential for understanding the regulatory network leading to formation and maintenance of hair cells.

Here we investigate the role of zebrafish *atoh1* genes, *atoh1a* and *atoh1b*, in hair cell development. Gene knockdown shows these genes play essential roles during successive stages of hair cell development, beginning in the preotic placode. Interactions with the Delta-Notch pathway strongly support a classic proneural role for

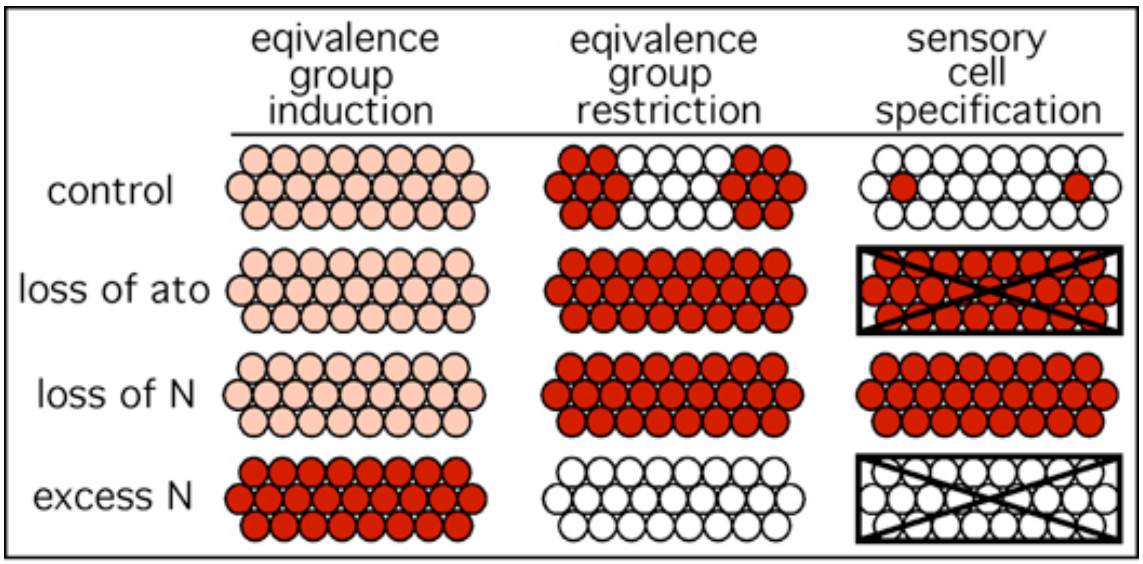


Figure 4. *Drosophila ato* as a Paradigm for Proneural Regulation and Function. Red circles represent *ato*-expressing cells. Stage-dependent refinement of the expression pattern is altered in distinctive ways by perturbing *ato* or *N* function. In addition, loss of *ato* or excess *N* blocks specification of sensory cells (crossed-out fields of cells).

atoh1. We also show that Fgf and members of the Pax2-5-8 family of transcription factors are required for induction or maintenance of *atoh1* expression. These data reveal a complex gene network in which *atoh1* genes play vital roles at multiple stages of sensory epithelium development.

MATERIALS AND METHODS

Strains and developmental conditions

The wild-type strain was derived from the AB line (Eugene, OR). The *mib*^{ta52b} and *noi*^{tu29a} mutations are likely null alleles (Lun and Brand, 1998; Itoh et al., 2003). *b380* is a deletion of *dlx3b* and *dlx4b* and mutants are easily identified after 1l hpf by lack of somitic segmentation (Fritz et al, 1996). The *hsp70-dnSu(H)* line was developed by Latimer et al. (2005), and the *hsp70-Gal4* and *UAS-NICD* lines were developed by Scheer and Campos-Ortega (1999). About 25% of embryos were affected by dnSu(H) and NICD in these lines, respectively. Embryos were developed in fish water containing methylene blue at 28.5° and staged according to standard protocols (Kimmel et al., 1995). At least 30 embryos were observed for each time-point except where noted.

In situ hybridization

In situ hybridization was performed at 67°C as described (Jowett and Yan, 1996; Phillips et al., 2001).

Immunofluoresence

Antibody staining was performed as described by Riley et al., (1999). Primary antibodies: Pax2 (Covance, diluted 1:100), acetylated tubulin (Sigma T-6793, diluted 1:100). Secondary antibodies: Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes A-11010, diluted 1:50) or Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes A-11001, diluted 1:50).

Misexpression

The *atoh1a* plasmid was obtained from Reinhard Köster. To misexpress *atoh1a* under the control of the CMV promoter, 30-90 pg of plasmid was injected into 1-cell embryos. For RNA misexpression, wild-type mRNA was synthesized in vitro using mMessage mMachine kit (Ambion). A total of 60-80pg of mRNA was injected into 1-cell embryos, or was coinjected with *atoh1a*/*atoh1b* double MO.

Morpholinos

Morpholinos were obtained from Gene Tools, Inc. For most experiments, 5 ng of morpholino was injected into 1-cell embryos. Morpholinos for *dlx3b*, *dlx4b*, *fgf3, foxi1, pax2b* and *pax8* were described previously (Solomon and Fritz, 2002; Mackereth et al. 2005). Additional morpholino sequences are as follows: *atoh1b*-MO 5' TCATTGCTTGTGTAGAAATGCATA T 3'; *atoh1a*-MO1 5' TCTGTTGGTTTGTGCTTTTGGGAGG 3'; *atoh1a*-MO2 5'AAAGTTTGTGGCTAT

GGATACAGGG 3'; *atoh1a*-MO3 5' ATCCATTCTGTTGGTTTGTGCTTT T 3'. *atoh1a*-MO3 was used for most experiments. The phenotypes caused by injection of *atoh1a* and/or *atoh1b*-MOs affected 90-100% of embryos, except where noted.

SU5402 inhibitor treatment

SU5402 was dissolved in DMSO to prepare a 40 mM stock solution. Embryos were treated in their chorions with 50 μM SU5402 (10-14 hpf), 80 μM (12-18 hpf), or 100 μM (18-24 hpf). Controls were incubated in an equal concentration of DMSO as that of treated embryos. To terminate treatment, embryos were washed several times and either allowed to develop further or fixed and processed immediately.

RESULTS

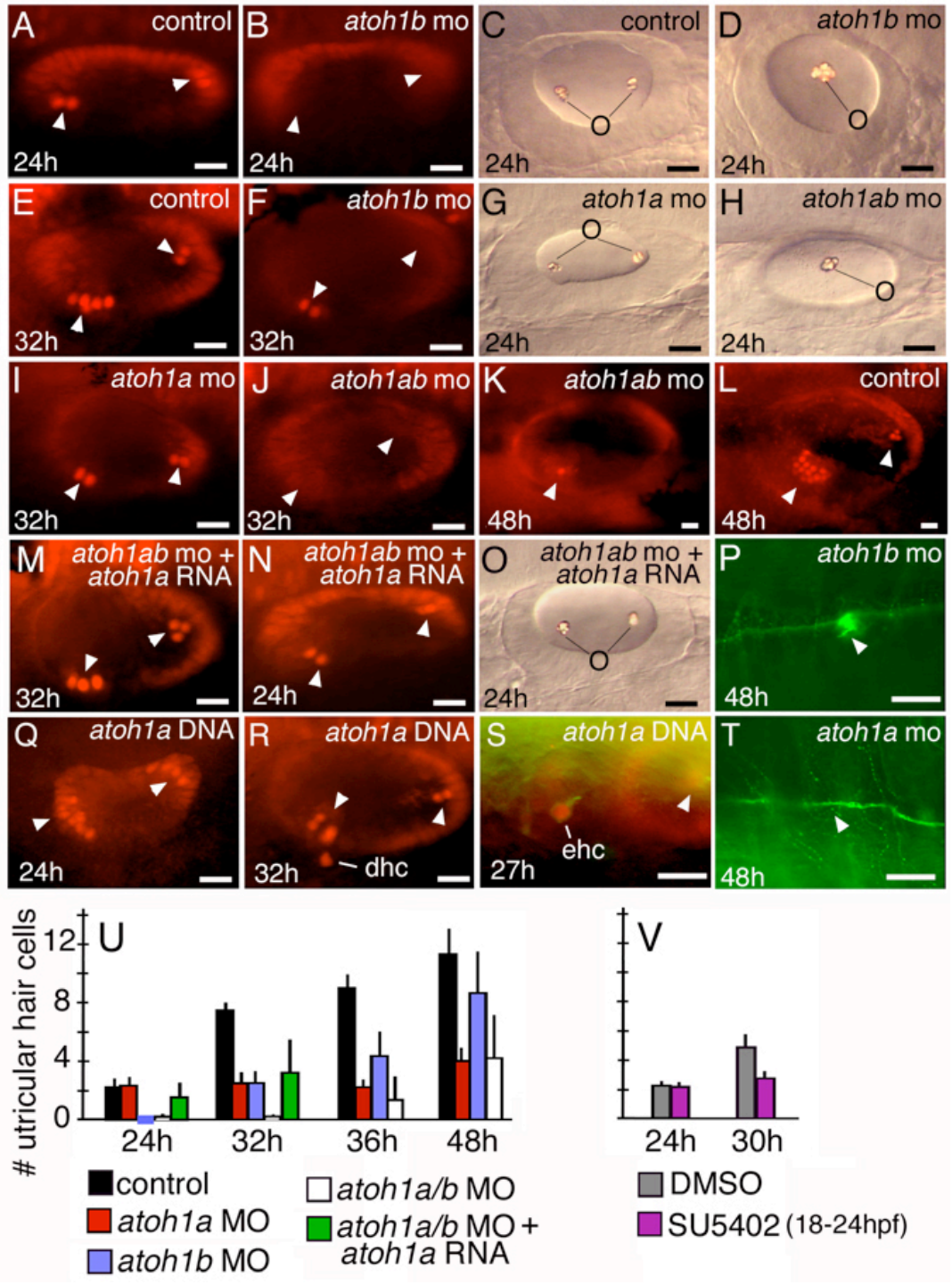
Requirement of *atoh1* genes for hair cell development

It was shown previously that zebrafish *atoh1a* (formerly *zath1*) is expressed in hair cells in the inner ear and lateral line (Itoh and Chitnis, 2001; Whitfield et al., 2002). We designed three different morpholino oligomers (MOs) to block translation of *atoh1a*, all of which affected hair cell development. While two of these MOs caused varying degrees of non-specific cell death in the neural tube, the third was effective at a dose that had no discernable toxicity and was therefore used for the remainder of this study.

Injection of *atoh1a*-MO strongly impairs formation of hair cells in the inner ear (Fig. 5U). Tether cells, an early-forming hair cell required for otolith localization (Riley et al., 1997), are not affected in *atoh1a* morphants and otoliths form normally (Fig. 5G). Tether cells, named for their precocious kinocilia, initially form in pairs at both ends of the nascent otic vesicle and later adopt the morphology of fully developed hair cells by 22 hpf. Normally, later-forming hair cells begin to accumulate soon after 24 hpf. However, later-forming hair cells are profoundly impaired in all *atoh1a* morphants as additional hair cells are not evident until 48 hpf (Fig. 5I,U, and data not shown).

Adolf et al. (2004) recently described a second zebrafish *atonal* homolog, *atoh1b*, that we hypothesized might also play a role in hair cell development. In contrast to *atoh1a*-MO, injection of *atoh1b*-MO ablates tether cells in both the utricle and saccule (Fig. 5B) in all specimens. Later-forming hair cells are still produced, albeit more slowly than normal (Fig. 5F,U). A single otolith is produced but initially forms as an untethered mass due to the absence of tether cells (Fig. 5D). Otoliths eventually bind to utricular hair cell cilia after 30 hpf (not shown). Coinjection of *atoh1a*-MO and *atoh1b*-MO ablates all hair cells in the inner ear in > 90% of specimens (Fig. 5J,U). This was confirmed using phalloidin to mark stereocilia and antiactetylated tubulin staining of kinocilia (not shown). A single untethered otolith is produced (Fig. 5H) reflecting loss of tether cells. Hair cells do begin to form by 48 hpf in *atoh1ab* double morphants (Fig. 5K,U), probably reflecting diminishing capacity of the MOs to knock down *atoh1*

Figure 5. Requirement for *atoh1* in Hair Cells in the Ear and Lateral Line. All panels show dorsolateral views with anterior to the left and dorsal up. (A, B, E, F, I-N, Q-S) Pax2 antibody staining of otic hair cells (arrowheads) at the indicated times in control embryos (A, E, L), *atoh1a* morphant (I), *atoh1b* morphants (B, F), *atoh1ab* double morphants (J, K), *atoh1ab* double morphant coinjected with *atoh1a* mRNA (M, N), and embryos injected with *atoh1a*-plasmid (Q-S). *atoh1a*-plasmid stimulates production of supernumerary hair cells at 24 hpf (Q), but these are not maintained at 32 hpf (R), and instead displaced hair cells (dhc) appear ventrally within subjacent mesenchyme, leaving gaps in the hair cell layer. An ectopic hair cell (ehc) is revealed anterior to the otic vesicle by co-staining with Pax2a (red) and acetylated-tubulin (green) (S). (C, D, G, H, O) Otoliths (o) produced in control (C), *atoh1a* morphant (G), *atoh1b* morphant (D) *atoh1ab* double morphant (H) and *atoh1ab* double morphant coinjected with *atoh1a* RNA (O). (P,T) Acetylated-tubulin staining of the lateral line and neuromasts (arrowheads) in *atoh1b* morphant (P) and *atoh1a* morphant (T) at 48 hpf. (U, V) The mean (± standard deviation) of Pax2-postive hair cells present in the utricle at the indicated times and under the indicated conditions. Sample sizes ranged from 15-35 embryos per time point. Scale bar, 15 μm.


A control
24h
B atoh1b mo
24h
C control
O
24h
D atoh1b mo
O
24h
E control
32h
F atoh1b mo
32h
G atoh1a mo
O
24h
H atoh1ab mo
O
24h
I atoh1a mo
32h
J atoh1ab mo
32h
K atoh1ab mo
48h
L control
48h
M atoh1ab mo + atoh1a RNA
32h
N atoh1ab mo + atoh1a RNA
24h
O atoh1ab mo + atoh1a RNA
O
24h
P atoh1b mo
48h
Q atoh1a DNA
24h
R atoh1a DNA
dhc
32h
S atoh1a DNA
ehc
27h
T atoh1a mo
48h
utricular hair cells
12
8
4
0
U
24h
32h
36h
48h
control
atoh1a MO
atoh1b MO
atoh1a/b MO
atoh1a/b MO + atoh1a RNA
V
24h
30h
DMSO
SU5402 (18-24hpf)

function at later stages. Thus, *atoh1* function is essential for hair cell formation in zebrafish as in mouse. Moreover, the data support a model in which *atoh1b* preferentially regulates development of tether cells while *atoh1a* regulates later forming hair cells.

Neuromasts of the lateral line are also ablated by knocking down *atoh1a* (Fig. 5T). However, knocking down *atoh1b* has no effect on neuromasts (Fig. 5P). These data are consistent with findings that neuromasts express and require *atoh1a* but not *atoh1b* (Itoh and Chitnis, 2001; Sarrazin et al., 2006; and our unpublished observations).

Misexpression of *atoh1a*

To test whether the effects of *atoh1*-MOs on hair cell development could be rescued, *atoh1ab* double morphants were coinjected with 80 pg of *atoh1a* mRNA. More than half of these coinjected embryos produce tether cells, tethered otoliths, and later-forming hair cells (Fig. 5M-O), indicating substantial rescue from the effects of the MOs. These data show that loss of hair cells in *atoh1*-morphants is a specific consequence of disrupting *atoh1* function.

Injecting 80 pg of *atoh1a* mRNA (with or without MOs) did not lead to formation of excess or ectopic hair cells. This is in contrast to mouse in which misexpression of *atoh1* promotes formation of ectopic hair cells in tissues immediately surrounding endogenous sensory epithelia (Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). Because injected mRNA may not be stable enough to strongly affect later stages of otic development, we injected zebrafish embryos with plasmid

DNA to misexpress *atoh1a* under the control of the powerful and ubiquitously expressed CMV promoter. Injection of 90 pg of *atoh1a* plasmid caused axial truncation in up to 30% of embryos whereas injection of 30 pg or 60 pg did not alter overall embryonic morphology (not shown). Embryos injected with 60 pg or 90 pg of *atoh1a* plasmid often showed expanded sensory patches at 24 hpf (Fig. 5Q). By 30 hpf, however, many supernumerary hair cells are lost while isolated Pax2-positive cells appear sporadically in the subjacent mesenchyme (Fig. 5R). The latter are likely to be dying hair cells as suggested by general elevation of acridine orange staining (not shown). We showed in another study that dying hair cells are often extruded from the otic vesicle to the underlying mesenchyme (Kwak et al., 2006). This also occurs in *mind bomb* (*mib*) mutants, which form supernumerary hair cells that are later extruded as they undergo apoptosis (Haddon et al., 1999). It is possible that excess hair cells die because forced expression of *atoh1a* bypasses vital processes required for hair cell maintenance. We also cannot exclude the possibility of non-specific toxicity associated with concentrated plasmid-injection. In addition to changes in the otic vesicle, about 1/3 of embryos injected with *atoh1a* plasmid also formed ectopic Pax2a-positive cells in the surface ectoderm just anterior or posterior to the otic vesicle. Double labeling with acetylated tubulin antibody confirms that some of these cells are hair cells (Fig. 5S). Although ectopic hair cells formed at the level of the lateral line, *pax2a* expression indicates these are not lateral line neuromasts. These data show that in zebrafish as in mouse, *atoh1* misexpression can induce excess and ectopic hair cells, but only in regions close to the endogenous hair cell domains. This is consistent with findings that bHLH proteins work

combinatorially with other transcription factors, such as Hox and Pax proteins, whose regional expression establishes restricted zones of competence (Niwa et al., 2004; reviewed by Westerman et al., 2003).

Expression of *atoh1a* and *atoh1b* during normal development

Otic expression of *atoh1a* begins at 14 hpf in two domains in the otic placode, marking the primordia of the utricular and saccular sensory epithelia (Fig. 6A). As hair cells begin to differentiate, *atoh1a* expression upregulates in the hair cell layer but weak expression is also detected in the basal cell layer. The latter may represent nascent hair cells in the earliest stages of differentiation (Fig. 6C). Expression continues in the sensory maculae through at least 48 hpf. Expression is also seen in the sensory cristae by 48 hpf (not shown).

Expression of *atoh1b* begins much earlier, marking the medial edge of the preotic placode by 10.5 hpf (Fig. 10A,B). This pattern resolves into two discrete patches by 14 hpf, encompassing the future sensory epithelia (Fig. 6D). At this stage, expression of *atoh1b* overlaps with *atoh1a*, but *atoh1b* is expressed at a higher level (compare Fig. 6A,D). By 22 hpf, *atoh1b* expression diminishes and marks only a subset of the *atoh1a* domain (Fig. 6E,F). These differences in temporal expression are consistent with the notion that *atoh1b* acts early in otic development while *atoh1a* predominates during later development of sensory epithelia.

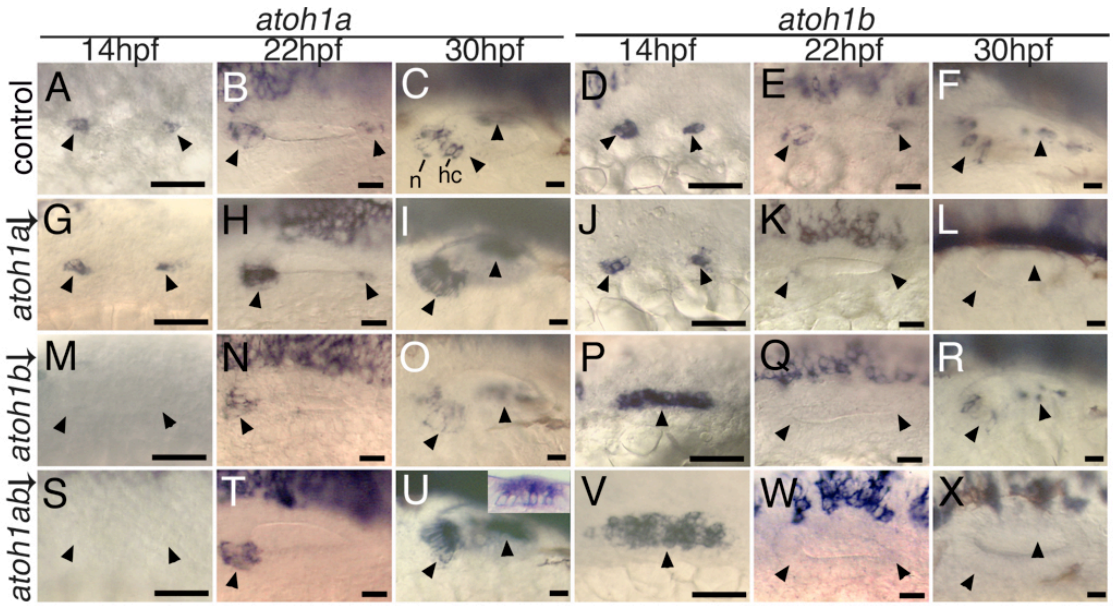


Figure 6. Atoh1-Dependent and Independent Expression of *atoh1* Genes. Dorsolateral views (anterior to left) showing expression of *atoh1a* (A-C, G-I, M-O, S-U) and *atoh1b* (D-F, J-L, P-R, V-X) in control (A-F) *atoh1a* morphant (G-L), *atoh1b* morphant (M-R) and *atoh1ab* double morphant (S-X) embryos at the indicated times. Expression of *atoh1a* at 32 hpf in mature hair cells (hc) and putative nascent hair cells (n) is indicated in (C). Arrowheads indicate observed or expected domains of otic expression. Inset in U shows a parasagittal section through the anterior *atoh1a* expression domain. Scale bar, 15 μm.

Auto- and crossregulation of *atoh1* gene expression

Because proneural genes often regulate their own expression, we examined expression of *atoh1a* and *atoh1b* in embryos knocked down for either or both functions. In *atoh1b* morphants, preplacodal expression of *atoh1b* is not altered (not shown). However, *atoh1b* expression fails to become restricted to two sensory primordia in the otic placode at 14 hpf (compare Fig. 6D,P). Expression of *atoh1b* ceases by 16 hpf in *atoh1b* morphants (Fig. 6Q and data not shown), indicating that *atoh1b* is required to maintain its own transcription. Interestingly, macular expression of *atoh1b* returns after 24 hpf (Fig. 6R).

atoh1a is not expressed in *atoh1b* morphants until around 20 hpf and is limited to the utricular (anterior) macula (Fig. 6M,N). By 30 hpf, *atoh1b* morphants show *atoh1a* expression in both utricular and saccular maculae, although the level of expression is lower than normal (Fig. 6O). These data show that *atoh1a* requires *atoh1b* for expression in the otic placode but not in the otic vesicle after 20 hpf. Once activated, *atoh1a* could be responsible for reactivation of *atoh1b* expression after 24 hpf (Fig. 6R).

In *atoh1a* morphants, *atoh1a* and *atoh1b* are expressed normally through 20 hpf (Fig. 6G,J, and data not shown). By 22 hpf, *atoh1a* morphants begin to express *atoh1a* at higher than normal levels (Fig. 6H,I). Conversely, *atoh1b* expression is nearly extinguished by 22 hpf and cannot be detected after 24 hpf (Fig. 6K,L). These data show that *atoh1a* is necessary to maintain *atoh1b* expression after 22 hpf and that *atoh1a* limits its own expression.

In *atoh1ab* double morphants, *atoh1b* is expressed in an expanded domain at 14 hpf but is not maintained in the ear after 16 hpf (Fig. 6V-X and data not shown). Expression of *atoh1a* cannot be detected until 22 hpf, after which it is expressed at higher than normal levels (Fig. 6S-U). Sections show that the epithelium has only a single layer of columnar cells that express high levels of *atoh1a* (Fig. 6U inset).

Taken together, these data show that *atoh1b* acts early to establish and refine the sensory equivalence group and to induce early expression of *atoh1a*, while *atoh1a* is required later to maintain expression of *atoh1b* and to limit its own expression. The requirement for *atoh1b* to restrict its own expression domain at such an early stage is consistent with the possibility that it acts as a classic proneural gene (Fig. 4). The data also confirm that *atoh1b* is required for differentiation of tether cells whereas *atoh1a* is required for later forming hair cells.

Involvement of *atoh1* genes in Delta-Notch signaling

Proneural genes often limit their own expression, or domain of expression by transcriptional activation of Delta (Dl), which in turn stimulates Notch (N) and thereby inhibits subsequent proneural gene expression in neighboring cells (Baker and Yu, 1997; Parks et al., 1997). In support, knocking down *atoh1b* strongly inhibits expression of *dlA* and *dlD* in the ear at 14 hpf (Fig 7C,D, and data not shown). Similarly, knocking down *atoh1a* diminishes *dlA* and *dlD* expression at 22hpf (Fig 7A,B, and data not shown). Thus, *atoh1* genes are required for normal activation of *delta* gene expression.

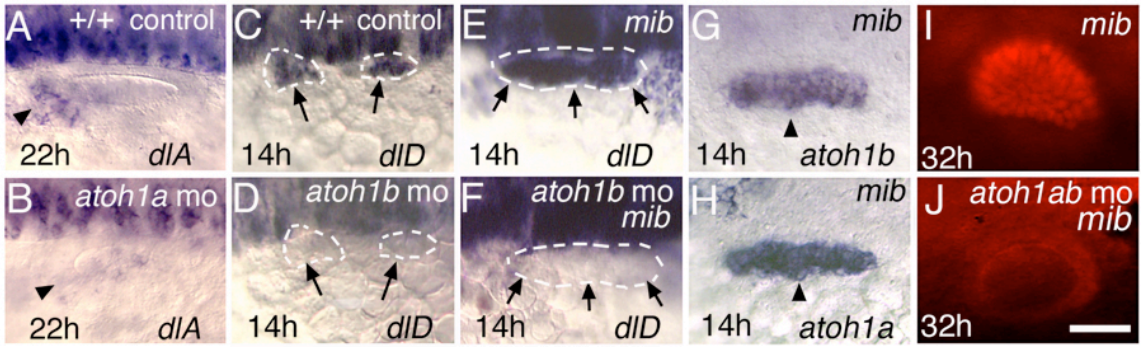


Figure 7. Interactions Between *atoh1* and the Delta-Notch Pathway. (A, B) Expression of *dlA* at 22 hpf in a control embryo (A) and *atoh1a* morphant (B). (C-F) Expression of *dlD* at 14 hpf in a control embryo (C), *atoh1b* morphant (D), *mib* mutant (E), and *mib* mutant-*atoh1b* morphant (F). (G, H) *mib* mutants show expanded otic domains of *atoh1b* (G) and *atoh1a* (H) at 14 hpf. (I, J) Pax2 antibody staining at 32 hpf reveals supernumerary hair cells in a *mib* mutant (I) but no hair cells in a *mib* mutant coinjected with *atoh1a*-MO and *atoh1b*-MO (J). Arrowheads and arrows indicate otic regions. All images are dorsolateral views with anterior to the left. Scale bar, 30 μm (A, E, I-P) or 15 μm (B-D, F-H).

To further investigate the role of Dl-N feedback, we examined *atoh1* function in *mind bomb* (*mib*) mutants. The *mib* gene encodes an E3 ubiquitin ligase essential for Dl-N signaling (Itoh et al., 2003). *mib* mutants produce an enlarged domain of both *atoh1a* and *atoh1b* at 14 hpf, mimicking the failure to restrict expression seen in *atoh1b* morphants (Fig. 7G,H). Because both *atoh1* genes remain fully active in *mib* mutants, *delta* gene expression is also greatly expanded and all cells in the equivalence group complete differentiation as hair cells (Haddon et al., 1999; Riley et al., 1999; Fig 7E,I). However, injection of *atoh1a*-MO and *atoh1b*-MO into *mib* mutants fully suppresses these latter defects, blocking *delta* gene expression and ablating all hair cells in all specimens (Fig. 7F,J). These data further support a role for *atoh1* genes as upstream activators of Dl-N signaling that normally acts to limit and refine *atoh1* expression and function.

To test the temporal requirements for the canonical N pathway, we used a transgenic line to express a dominant-negative form of Su(H) (Supressor of Hairless) (dnSu(H)) under the control of *hsp70* promoter (Wettstein et al., 1997; Shoji et al., 1998; Latimer et al., 2005). Su(H) is a required factor for Notch activity. This promoter induces high-level transcription within 15 minutes following heat shock, providing a pulse of protein accumulation lasting several hours (Scheer et al., 2002). Heat shock induction of dnSu(H) at 8 hpf does not alter *atoh1b* expression or hair cell development (not shown). However, heat shock at 10 hpf causes the initially broad domain of *atoh1b* to be maintained through at least13.5 hpf, about 2 hours longer than normal (Fig. 8B). By 14.5 hpf, expression becomes restricted to two discrete domains that are larger than

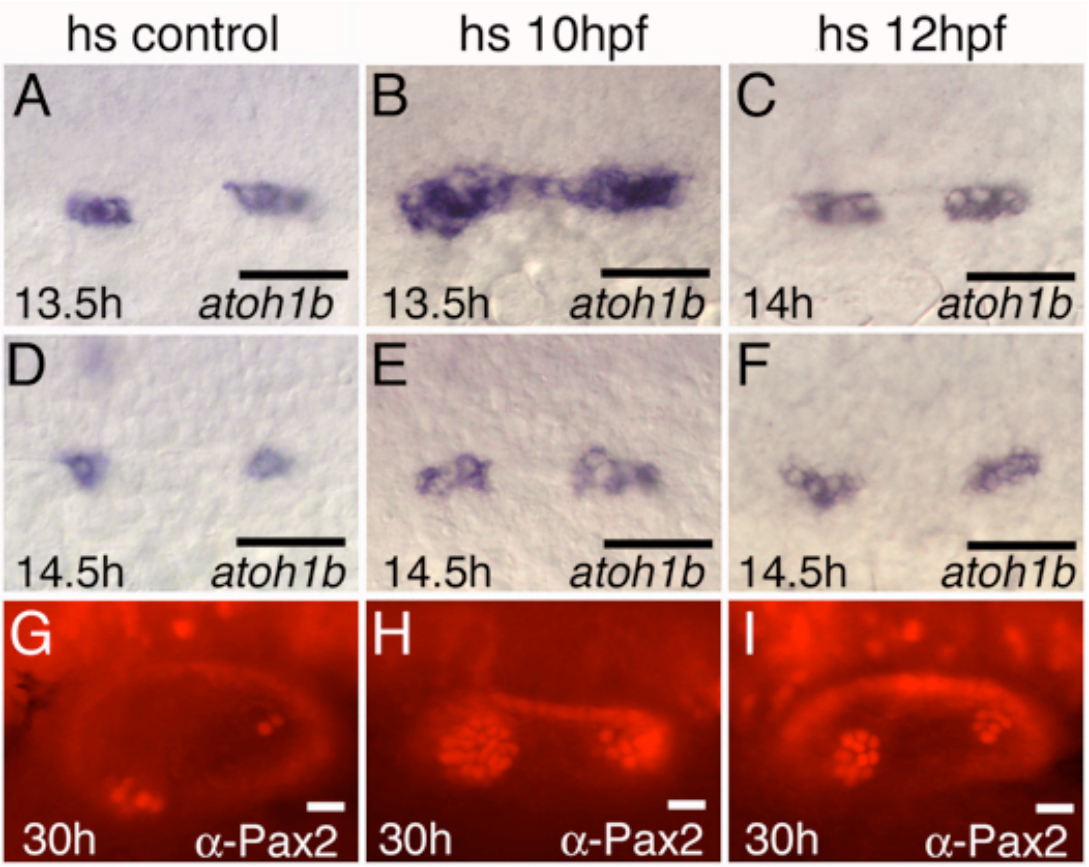


Figure 8. Heat Shock-Induction of dnSu(H). Expression of *atoh1b* at 13.5 or 14 hpf (A-C), *atoh1b* at 14.5 hpf (D-F) and Pax2 at 30 hpf (G-I) as seen in control embryos heat shocked at 10 hpf (A, D, G) or *hsp70-dnSu(H)* transgenic embryos heat shocked at 10 hpf (B, E, H) or 12 h pf (C, F, I). Images show lateral views with anterior to the left. Scale bar, 15 μm.

normal (Fig. 8E). This domain restriction presumably reflects resumption of Dl-N signaling as the pulse of dnSu(H) subsides. However, the enlarged domains show no further reduction after 14.5 hpf and go on to form supernumerary hair cells (Fig. 8H). Heat shock at 12 hpf (after equivalence-group restriction has already begun) also results in maintenance of two large domains and production of excess hair cells (Fig. 8C,F,I). Heat shock at 14 hpf has little effect on *atoh1b* expression or hair cell formation (not shown). These data show that equivalence group restriction can still occur after 13.5 hpf but then *atoh1b* expression stabilizes by 14.5 hpf regardless of domain-size, defining an interval during which cell fates are specified.

To test how N gain-of-function affects *atoh1* gene expression (as in Fig. 7), we used a heat shock-inducible Gal4-UAS system to drive expression of N intracellular domain (NICD) (Scheer and Campos-Ortega, 1999). In this system, heat shock induces sustained NICD expression for at least 17 hours (Scheer et al., 2002). Heat shock induction of NICD at 9 hpf or 10 hpf does not prevent induction of *atoh1b* in the preotic placode (Fig. 9B). However, *atoh1b* expression is lost by 12 hpf (Fig. 9D). In addition, *atoh1a* is never activated and no hair cells are produced (not shown). Heat shock induction of NICD at 18 hpf also rapidly extinguishes *atoh1* expression and blocks hair cell formation (not shown). We also examined the effects of NICD in *atoh1b* morphants, which usually have no functional equivalence group until 20 hpf when *atoh1a* is first expressed. In *atoh1b* morphants, activation of NICD at 18 hpf induces *atoh1a* by 19 hpf, one hour earlier than without NICD (Fig. 9E-G). Expression then subsides by 20 hpf and no hair cells are produced (Fig. 9H, and data not shown). Thus,

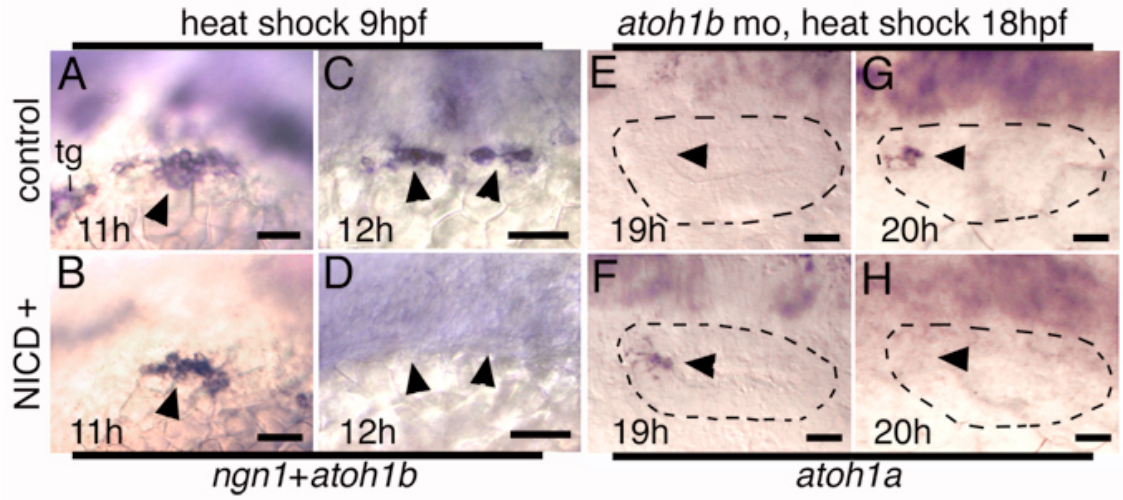


Figure 9. Heat shock induction of NICD. (A-D) Expression of *atoh1b* and *ngn1* at 11 hpf (A, B) and 12 hpf (C, D) in control embryos (A, C) or NICD-positive embryos (B, D) heat shocked at 9 hpf. Loss of *ngn1* expression, which is non-overlapping with *atoh1b*, confirms effective NICD-induction. (E-H) Expression of *atoh1a* at 19 hpf (E, F) and 20 hpf (G, H) in *atoh1b* morphants without NICD (E, G) or with NICD (F, H) heat shocked at 18 hpf. Otic vesicles are outlined. Arrowheads mark otic expression domains. tg, trigeminal ganglion. All are lateral views with anterior to the left. scale bar, 15 μm.

NICD initially stimulates, or at least does not block, upregulation of *atoh1* genes as the equivalence group forms but then rapidly extinguishes *atoh1* expression at all later stages.

In summary, the relationship between *atoh1* function and the Dl-N pathway is consistent with all predictions of the fly *ato* paradigm (Fig. 4). Moreover, *atoh1*-dependent restriction of the equivalence group precedes fate specification by several hours. These findings strongly support a classic proneural mechanism of action for zebrafish *atoh1* genes.

Regulation of *atoh1b* in preotic cells

Expression of *pax8* is the earliest known marker of otic placode induction (Pfeffer et al., 1998). *atoh1b* is expressed in a subset of *pax8*-expressing cells in the preotic placode (Fig. 10A,B), raising the possibility that *pax8* is required for early activation of *atoh1b*. Knocking down *pax8* reduces the size of the preotic domain of *atoh1b* (Fig. 10F), but the level of expression appears normal. We next asked whether factors that act upstream of or parallel to *pax8* might also regulate *atoh1b*. Induction of *pax8* requires Foxi1 autonomously within the preplacodal ectoderm, as well as stimulation by Fgf3 and Fgf8 secreted from adjacent hindbrain tissue (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al. 2003; Solomon et al., 2003; Hans et al., 2004). Knocking down *foxi1* causes severe reduction of *atoh1b* expression (Fig. 10G). To test the role of Fgf, embryos were treated with the Fgf signaling inhibitor SU5402. Induction of *atoh1b* is blocked in embryos treated from 10-14 hpf (not shown).

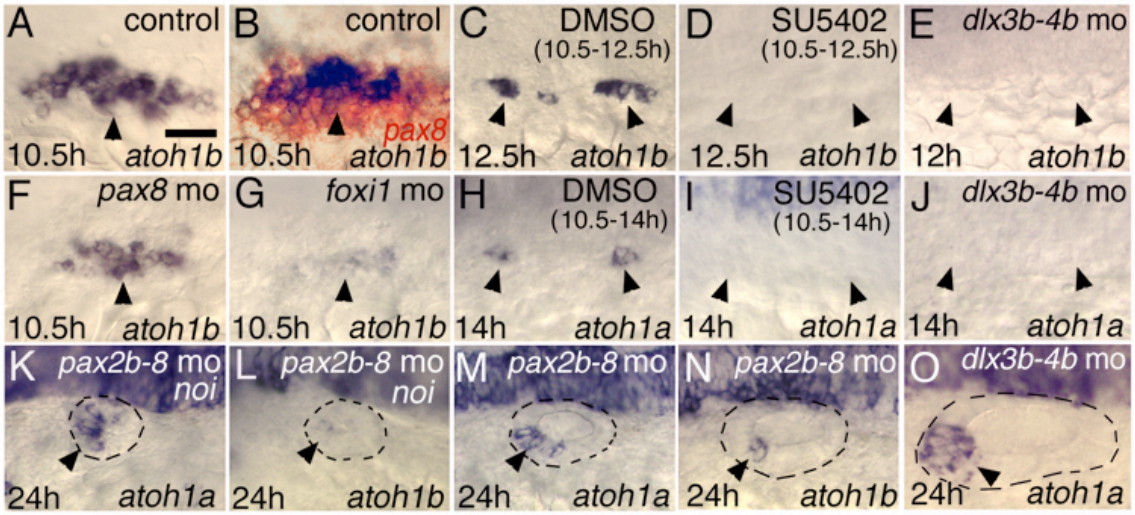


Figure 10. Inducers of Early *atoh1* Expression. (A, B, F, G) Expression of *atoh1b* at 10.5 hpf in a control embryo (A, B), *pax8* morphant (F) and *foxi1* morphant (G). The specimen in (B) was double stained to reveal *pax8* expression (red). (C, D) Expression of *atoh1b* at 12.5 hpf in embryos treated from 10.5-12.5 hpf with DMSO alone (C) or SU5402 in DMSO (D). (H, I) Expression of *atoh1a* at 14 hpf in embryos treated from 10.5-14 hpf with DMSO alone (H) or SU5402 in DMSO (I). (E, J, O) *dlx3b-dlx4b* morphants showing expression of *atoh1b* at 12 hpf (E) or *atoh1a* at 14 hpf (J) or 24h hpf (O). (K-N) Expression at 24 hpf of *atoh1a* (K,M) and *atoh1b* (L,N) in *noi* mutants injected with *pax2b-pax8*-MO (K,L), and in wild-type embryos injected with *pax2b-pax8*-MO (M,N). All are dorsolateral views with anterior to the left. Arrowheads indicate observed or expected domains of otic expression. Scale bar, 30 μm (A, B, F, G, K-O) or 10 μm (C-E, H-J).

When SU5402 is added beginning at 10.5 hpf, after the onset of *atoh1b* expression, expression of *atoh1b* is lost in all specimens by 12.5 hpf (Fig. 10D). Expression of *atoh1a* is also blocked (Fig. 10I), consistent with a requirement for *atoh1b* in *atoh1a* induction. Embryos coinjected with *fgf3*-MO and *fgf8*-MO also do not express *atoh1* genes (not shown). Thus, Foxi1 and Fgf signaling are required to initiate and maintain expression of *atoh1b* in the preotic placode, and Pax8 is needed to produce a normal sized domain.

Distal-less genes *dlx3b* and *dlx4b* also regulate early otic development but in a distinct pathway acting parallel to *foxi1-fgf-pax8*. Loss of *dlx3b* and *dlx4b* does not block induction of *pax8* but subsequent steps in otic development fail (Solomon and Fritz, 2002; Liu et al., 2003; Hans et al., 2004). Accordingly, neither *atoh1a* nor *atoh1b* are expressed in *dlx3b-dlx4b* morphants during placodal development (Fig. 10E,J). Similarly, *b380* mutants, which are deleted for *dlx3b* and *dlx4b* (Fritz et al., 1996) also fail to express *atoh1* genes in the otic placode (not shown). Later in development, *dlx3b-dlx4b* morphants produce small otic vesicles containing only anterior (utricular) sensory patches. Tether cells do not form, consistent with loss of early *atoh1b*, but later hair cells begin to form after 24 hpf (not shown) in association with belated expression of *atoh1a* (Fig. 10O). Dlx proteins could act directly on *atoh1b* transcription or indirectly by regulating competence to respond properly to Fgf after initial otic induction, as suggested by recent studies (Hans et al., 2004; Solomon et al., 2004).

Pax2 and Pax8 proteins maintain *atoh1b*

Pax8 normally cooperates with closely related proteins Pax2a and Pax2b to maintain the otic placode (Hans et al., 2004; Mackereth et al., 2005). Knockdown of *pax8* and *pax2b* in embryos homozygous for a null mutation in *pax2a* (*noi* mutants, Lun and Brand, 1998) causes progressive loss of otic tissue and no vesicles are produced. Accordingly such embryos do not express *atoh1a* or *atoh1b* in the otic region (not shown). Reducing the MO concentration by half allows the majority of *pax2a-pax2b-pax8*-deficient embryos to produce small otic vesicles. In 100% of these specimens, *atoh1a* is expressed at a high level in a nearly normal number of cells at the anterior end of the otic vesicle whereas *atoh1b* expression is barely detectable in any specimen (Fig. 10K,L). Partial knockdown of *pax8* and *pax2b* in wild-type embryos results in a moderately diminished otic vesicle expressing normal levels of both *atoh1a* and *atoh1b* (Fig. 10M,N), although *atoh1b* is typically expressed in only one or two cells. These data show that full expression of *atoh1b* requires Pax8 and Pax2 functions. In contrast, *atoh1a* expression is not strictly dependent on Pax2/8 function.

Continuing requirements for Fgf

As the otic vesicle forms, *fgf3* and *fgf8* begin to be expressed in domains encompassing the sensory epithelia (Leger and Brand, 2002). To test whether Fgf signaling regulates *atoh1* expression after placode formation, embryos were treated with SU5402 for various intervals at successively later stages of development. Treatment from 12-18 hpf did not affect *atoh1b* but reduced expression of *atoh1a* (Fig. 11A-D).

When embryos were treated at 18 hpf for one, two, four or six-hour intervals, expression of both *atoh1a* and *atoh1b* were strongly reduced but not eliminated (Fig. 11E-H). We hypothesized that the period of SU5402-insensitivity of *atoh1b* from 12 to 18 hpf reflects maintenance of *atoh1b* by autoregulation. Furthermore, since *atoh1a* and *atoh1b* help maintain each other at later stages, cross-regulation could account for residual expression seen in SU5402-treated embryos. In support, *atoh1b* morphants fail to express either *atoh1a* or *atoh1b* when treated with SU5402 from 18-22 hpf (Fig. 11J). We next tested the effects of SU5402 on hair cell formation. In embryos treated from 18-24 hpf, tether cells were

produced normally (not shown). This was not unexpected because tether cells are already present in the otic vesicle at 18 hpf and hence their specification cannot be blocked by this treatment. However, production of later forming hair cells was strongly impaired during the 6-hour period following removal of the inhibitor (Fig. 11L, Fig. 5V). Presumably the severe reduction in *atoh1* expression seen at 24 hpf delays resumption of macular development. These data show that *atoh1* expression and hair cell development require ongoing Fgf signaling. This marks the first identification of a signaling molecule required to both induce and maintain *atoh1* expression in the vertebrate inner ear.

***atoh1*-dependent and -independent expression of macular genes**

We next tested whether *atoh1* function affects *fgf* or *pax* gene expression. Otic expression of *fgf3* and *fgf8* is normal in *atoh1ab* double morphants (Fig. 12A-D).

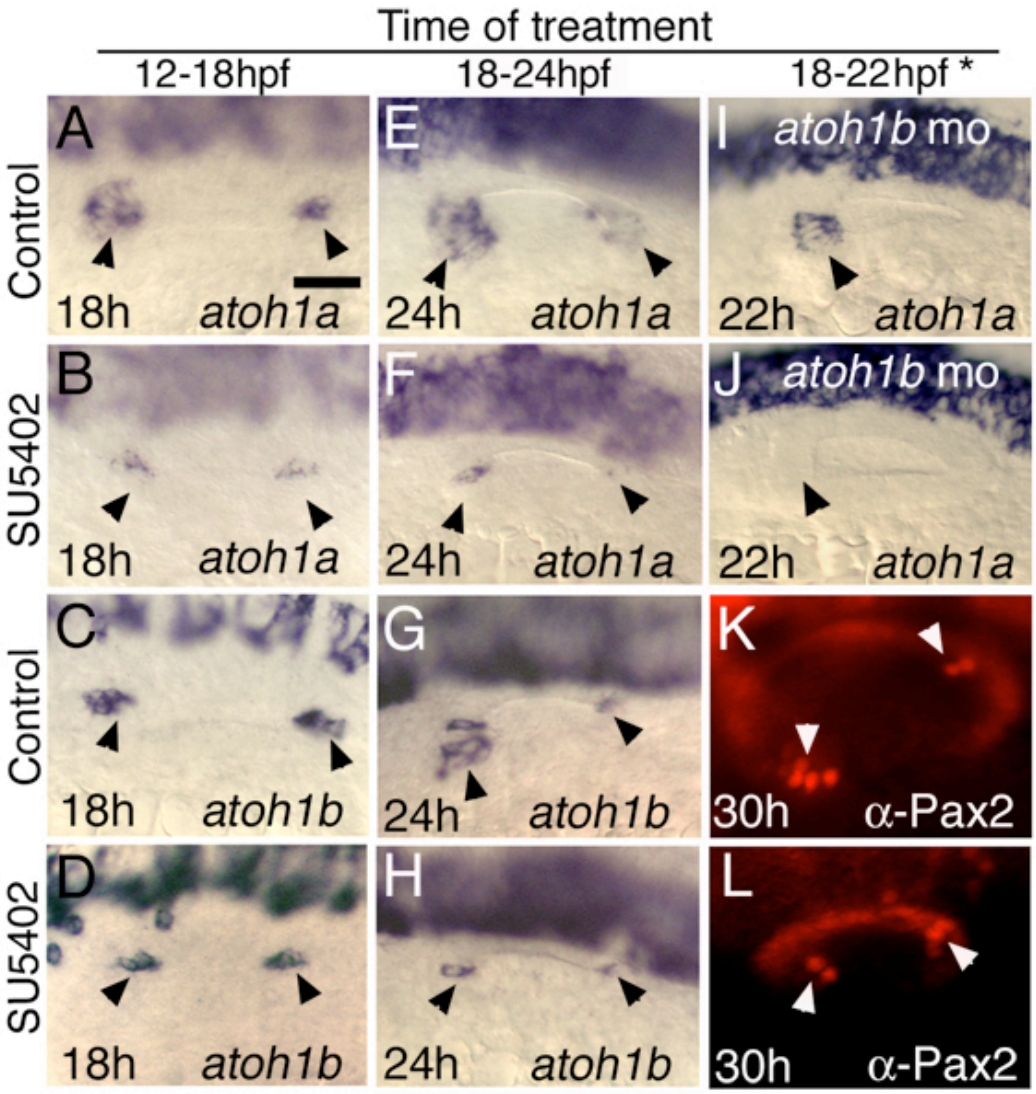


Figure 11. Stage-Dependent Requirements For Fgf. Embryos were treated with DMSO (control) or DMSO plus SU5402 for the indicated time intervals. (A-H) Expression of *atoh1a* in control and SU5402 treated embryos at 18hpf (A, B) and 24hpf (E, F), and expression of *atoh1b* in control and SU5402 treated embryos at 18hpf (C, D) and 24hpf (G, H). (I, J) Expression of *atoh1a* at 22 hpf in *atoh1b* morphants treated with DMSO (I) and DMSO and SU5402 (J). (K, L, *treatment from 18-24 hpf) Pax2 staining of hair cells at 30hpf in embryos treated with DMSO (K) or DMSO and SU5402 (L). All images are dorsolateral views with anterior to the left. Black arrowheads indicate otic expression. White arrowheads indicate sensory epithelia. Scale bar, 30 μm.

Likewise, expression of *pax5* in the utricle, which is regulated by Fgf signaling (Kwak et al., 2002; Kwak et al., 2006), is also unaltered in *atoh1ab* double morphants (Fig. 12F).In contrast, knockdown of both *atoh1a* and *atoh1b* strongly reduces the level of *pax2b* expression (Fig. 12H). *pax5* and *pax2b* are both required for normal development and maintenance of hair cells (Whitfield et al., 2002; and our unpublished observations) but only the latter is affected by *atoh1* function. Thus, expression of *fgf* genes and some downstream targets (*pax5*, *atoh1a*) continue in the macular region despite disruption of *atoh1* function and the absence of a sensory epithelium.

DISCUSSION

Our data support a model in which *atoh1a* and *atoh1b* act in a complex network leading to the establishment of a sensory equivalence group and subsequent differentiation of hair cells (Fig. 13). There are two distinct phases of *atoh1* function. In the first phase, *atoh1b* establishes a single prosensory domain during preplacodal development and subsequently activates Delta-Notch feedback to split the domain into separate utricular and saccular primordia in the nascent otic placode by 12 hpf. Lateral inhibition and specification of tether cells occurs by 14 hpf, when Atoh1b also activates expression of *atoh1a*. In the second phase, beginning soon after formation of the otic vesicle, *atoh1a* expression predominates in the maculae and maintains *atoh1b* in a subset

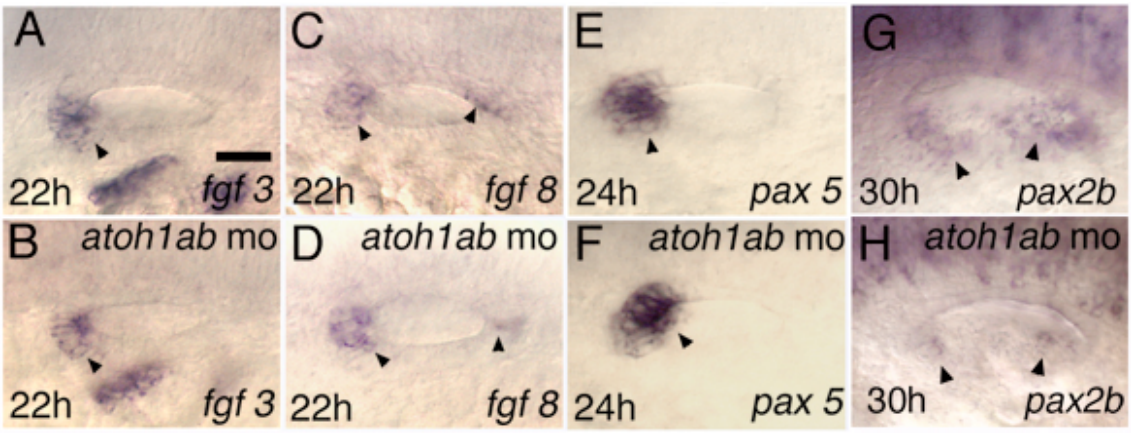


Figure 12. Expression of Macular Genes. Expression of *fgf3* (A, B) and *fgf8* at 22 hpf (C, D), *pax5* at 24 hpf (E, F) and *pax2b* at 30 hpf (G, H) in control embryos (A, C. E. G) and atoh1ab double morphants (B, D, F, H). All panels show dorsolateral views with anterior to the left and dorsal up. Arrowheads indicate expression in sensory epithelia. Scale bar, 30 μm.

of cells. Moreover, *atoh1a* is primarily responsible for specifying later forming hair cells and activating Delta-Notch mediated lateral inhibition. Fgf signaling is an essential upstream activator of *atoh1* expression during both phases, though *atoh1b* becomes independent of Fgf after 12 hpf. This could reflect the onset of *atoh1b*-autoregulation. A similar transition occurs with *Drosophila ato*, which becomes autoregulatory as it initiates domain-restriction and lateral inhibition (Sun et al., 1998). Fgf may facilitate *atoh1b*'s transition to autoregulation, similar to the role of EGFR and MAP Kinase activity in promoting autoregulation of *Drosophila ato* during sensory organ development (zur Lage et al., 2004). Unlike *atoh1b*, maintenance of *atoh1a* remains heavily dependent on Fgf but is not dependent on *atoh1* function after 20 hpf. Indeed, *atoh1ab* morphants maintain higher than normal expression of *atoh1a*.
This is probably because *fgf* genes continue to be expressed (Fig. 12) and promote *atoh1a* expression in the absence of N-mediated feedback inhibition.

The overlapping yet distinct functions of zebrafish *atoh1* genes likely reflects evolutionary "subfunctionalization" (Force et al., 1999). Following a genome duplication thought to have occurred early in the teleosts lineage, duplicate copies of genes often diverge in regulation to subdivide the ancestral function. Only *atoh1b* is required for development of tether cells, which are analogous to primary neurons. Because such precocious cell types are typical of anamniote embryos, this probably reflects an ancestral *atoh1* function. *atoh1a* has apparently lost regulatory elements required to respond to the *fgf-foxi1-pax* and *dlx* pathways involved in *atoh1b* induction early in development. However only *atoh1a* is essential for later hair cells, which

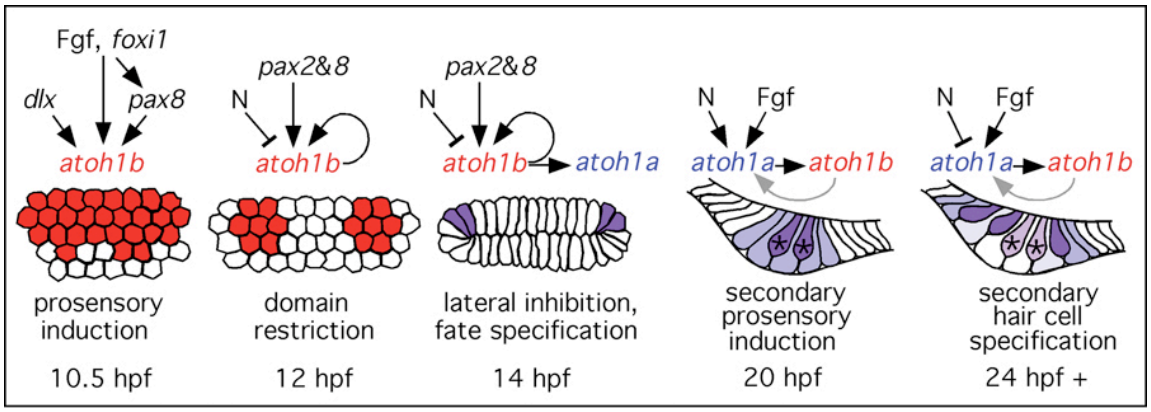


Figure 13. Summary of *atoh1* Regulation and Function. *fgf-foxi1-pax8* and *dlx* pathways induce expression of *atoh1b* (red) in medial preotic cells, specifying the prosensory equivalence group. By 12 hpf, the domain is restricted into two intermediate groups by Dl-N activity, which is activated by *atoh1b* function. Tether cells are specified around 14 hpf as *atoh1a* is activated (blue, coexpression with *atoh1b*, purple). At 20 hpf, N and Fgf activate a wider domain of *atoh1a* associated with later forming hair cells. Tether cells (asterisks) terminally differentiate. *atoh1a* is required to maintain or activate *atoh1b* in differentiating cells, and *atoh1b* helps maintain high levels of *atoh1a*. At 24 hpf and thereafter, later forming hair cells begin to differentiate and coexpress *atoh1* genes and N activity limits *atoh1* expression. Mature tether cells and hair cells downregulate *atoh1* expression.

continue to form well beyond embryonic development. This too is probably an ancestral *atoh1* function. Sensory epithelia continue to expand throughout life in teleosts, suggesting ongoing recruitment of new cells into the equivalence group. Fgf-dependent induction of *atoh1a* in adjacent cells might account for such recruitment, a function similar to the role of EGFR and *ato* in recruiting new sensory organ precursors in the *Drosophila* chordotonal organs (zur Lage et al., 1997). The two Atoh1 proteins probably retain similar DNA-binding properties, however, as misexpression of *atoh1a* can restore tether cell formation in *atoh1ab* double morphants (Fig. 5M-O).

Zebrafish *atoh1* genes have proneural function

There have been differing opinions as to whether vertebrate *Atoh1* genes act as classic proneural genes or only as terminal differentiation factors (Reviewed by Kelley, 2006). Specific comparisons between zebrafish *atoh1* genes and *Drosophila ato* (Fig. 4) reveal striking parallels. More generally, various authors have used four criteria to define proneural function (Brunet and Ghysen, 1999; Hassan and Bellen, 2000; Westerman et al., 2003) that can be applied to zebrafish *atoh1* genes. First, proneural genes are expressed prior to sensory fate specification. *atoh1b* is induced broadly in the preotic placode at 10.5 hpf whereas specification of tether cells (stabilization of *atoh1* expression) does not occur until 14 hpf. Second, proneural genes are subject to lateral inhibition (and the related process of domain-restriction) via N-mediated repression. Zebrafish *atoh1* genes, once induced, are readily repressed by N activity. Moreover, both *atoh1* genes facilitate their own repression by autonomously activating *delta*

expression. Third, proneural function is necessary for producing the equivalence group for the entire sensory structure. *atoh1ab* morphants produce only a simple epithelium lacking hair cells; and while support cell markers are not known in zebrafish, it is important to note that the epithelium continues to express *atoh1a*. Since loss of *atoh1* expression marks the first step in support cell specification, these cannot be support cells. Fourth, proneural function is sufficient to induce ectopic sensory development. Misexpression of *atoh1a* induces ectopic hair cells, though only in limited regions near the otic vesicle or endogenous sensory epithelia, as has been shown for *Atoh1* in mammals (Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). Competence to respond appropriately to Atoh1 may require a unique combination of additional factors. The zone of competence could be influenced by *pax2-5-8* genes, which are coregulated with *atoh1* genes by Fgf signaling. Other signaling pathways have also been implicated in this process. Misexpressing components of the Notch or Wnt pathways in chick can also induce ectopic sensory patches, but only in restricted regions near endogenous sensory patches (Steven et al., 2003; Daudet and Lewis, 2005). Combinatorial signaling and restricted zones of competence also influence the functions of proneural genes in *Drosophila* (Westerman et al., 2003; Niwa et al., 2004). Thus, while many additional details need to be resolved, zebrafish *atoh1* genes meet all four criteria used to define proneural function.

Conserved mechanisms?

While mammals show no early phase of specification analogous to tether cell development, and sensory epithelia develop only during a limited stage of embryogenesis, some aspects of sensory development have been conserved. The clearest example is the role of N signaling. *Dll1* and *Jag2* encode N ligands that regulate the balance of hair cells and support cells in the mouse cochlea. Loss of *Jag2* causes a modest increase in hair cells (Lanford et al., 1999; Kiernan et al., 2005a), as does anti-sense knockdown of *N1* in cochlear cultures (Zine et al., 2000). Loss of *Dll1* causes a larger increase in hair cells (Brooker et al., 2006), and disrupting both *Dll1* and *Jag2* causes a dramatic increase in hair cells and a modest decrease in support cells (Kiernan et al., 2005a). The number of support cells is greater than expected because support cells continue to divide longer than normal, partially offsetting earlier deficiencies. Although no phenotype comparable to zebrafish *mib* has been described in mouse, the mouse data nevertheless support the lateral inhibition model well. Residual support cell development likely reflects the activity of another N ligand, Jag1. *Jag1* is initially expressed throughout the prospective sensory region and later becomes restricted to support cells during differentiation. It has been proposed that Jag1 signaling between support cells augments lateral inhibitory signals from hair cells (Eddison et al., 2000). Indeed, partial loss of *Jag1* also leads to excess hair cell production (Zine et al., 2000; Kiernan et al, 2001). However, conditional knockouts of *Jag1* ablate much, though not all, of the sensory epithelia (Brooker et al., 2006; Kiernan et al., 2006). This supports a model in which Jag1's function changes with time, initially promoting the early

inductive phase of N signaling and later augmenting lateral inhibition. While the mechanistic basis for the shift from inductive to repressive N signaling remains unknown, similar transitions occur in the regulation of *Drosophila ato* and zebrafish *atoh1a* (Baker and Yu, 1997; Fig. 9F,H). It is not known whether mouse also shows N-dependent restriction of the initial equivalence group.

Fgf signaling may also play a conserved role in mammals. A number of Fgfs are expressed in the otic vesicle and developing sensory epithelia in mouse, but in most cases their role in hair cell formation is obscured by severe morphogenetic defects caused by specific gene knockouts. However, hypomorphic alleles of *Fgfr1* severely reduce hair cell production in the cochlea without blocking morphogenesis (Pirvola et al., 2002). Furthermore, Pirvola et al. (2002) have proposed that Fgfs produced by inner hair cells in the Organ of Corti stimulate differentiation of later forming outer hair cells through activation of Fgfr1.

A potential difference between mouse and zebrafish is the question of whether mouse *Atoh1* has proneural activity (reviewed by Kelley, 2006). This is especially evident when considering the mammalian cochlea, which is a highly derived structure that differs in important ways from the more primitive maculae and cristae. However, as summarized below, available data are complex and can be considered inconclusive. *Atoh1* is necessary for hair cell differentiation and is sufficient for inducing ectopic hair cells (Bermingham et al., 1999; Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). *Atoh1* is also subject to autoregulation (Helms et al., 2000), which in other species facilitates pattern refinement during lateral inhibition. Unfortunately a direct

link between lateral inhibition and *Atoh1* has not been shown in mouse. *Atoh1* is initially expressed in a broad domain that spans the full depth of the epithelium, approximately 4-5 cells thick (Bermingham et al., 1999; Lanford et al., 2000; Chen et al., 2002; Woods et al., 2004), but expression is not uniform and some cells appear to express little or no *Atoh1*. These data do not distinguish whether there is an earlier stage of low uniform *Atoh1* expression followed by rapid upregulation and pattern-refinement or, alternatively, whether *Atoh1* marks only differentiating hair cells after fate-specification. Several groups have concluded that mouse *Atoh1* lacks proneural activity based in part on the observation that sensory regions in *Atoh1* knockout mice contain a single layer of cells that morphologically resemble support cells (Bermingham et al., 1999). However, these cells express no definitive markers of mature support cells (Woods et al., 2005). Early non-restricted expression of *Jag1* occurs normally, but later expression normally associated with support cells is lost. Thus support cell differentiation is disrupted, though it is not clear whether the defect lies in specification or maintenance. Another early marker of the sensory epithelium, $p27^{kip1}$, normally precedes *Atoh1* in expression and continues to be expressed in the prosensory region in *Atoh1* mutants (Chen et al., 2002). This has been interpreted to mean that cells of the equivalence group are specified but fail to differentiate. However, $p27^{kip1}$ plays no role in fate-specification and there are no independent indicators of when the equivalence group forms in mouse. While expression of $p27^{kip1}$ is regulated partly by the same inductive signals that specify the equivalence group (Kiernan et al., 2006), upregulation of fate-specifying gene(s) need not follow precisely the same timecourse. Moreover,

even if *Atoh1* were necessary for prosensory induction, loss of *Atoh1* would not be expected to block any of the initial transcriptional responses to inductive signals. Thus expression of *p27^{kip1}* and *Atoh1* in the absence of *Atoh1* function (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005) could simply reflect ongoing parallel responses to common upstream activators in cells that are otherwise blocked at an early stage. Similarly, we have shown that several early markers of sensory epithelia in zebrafish (*atoh1a*, *pax5*) are coregulated by Fgfs and continue to be expressed in *atoh1ab* morphants (Fig. 12). A similar situation has been documented in *Drosophila ato* mutants, which produce no photoreceptors in the eye but continue to coexpress genes normally preceding formation of the prosensory equivalence group, including *ato* and the N target gene *hairy* (Jarman et al., 1995). In summary, gene expression and genetic studies in mouse do not necessarily contradict the notion that *Atoh1* might have proneural activity, but key supportive data are also lacking. Resolving this issue will require assessment of precisely when fate specification occurs relative to expression of *Atoh1* and *p27^{kip1}*, how these genes are coregulated, and the epistatic relationships between the various upstream factors including Sox2, Jag1 and Fgf.

CHAPTER III

Sox2 IS REQUIRED FOR MAINTENANCE AND REGENERATION, BUT NOT INITIAL DEVELOPMENT, OF HAIR CELLS IN THE ZEBRAFISH INNER EAR.*

OVERVIEW

This work was completed with help from the second author, E.M. Sweet. I performed some aspect of every experiment discussed within this work. E.M. Sweet was essential to the completion of Figures on pages 77 and 92.

INTRODUCTION

The capacity for maintenance and regeneration are fundamental properties of many mature tissues and organ systems. Regeneration often involves reactivation of developmental regulatory factors that coordinate growth and differentiation of pluripotent progenitor cells or stem cells. In the inner ear, sensory epithelia comprise interspersed patterns of sensory hair cells and support cells that in most vertebrates are

---

*Reprinted with permission from "Sox2 is required for maintenance and regeneration, but not initial development, of hair cells in the zebrafish inner ear."; by **Millimaki, B.B., Sweet, E.M. and Riley, B.B.**, 2010, *Dev Biol* **338**, 262-269. * Elsevier.

capable of self-renewal (Corwin and Oberholtzer, 1997; Ozeki et al., 2007; Edge and Chen, 2008). Hair cells are highly susceptible to a number of environmental insults that can trigger apoptosis. Lost hair cells can be regenerated from support cells through either of two processes: Support cells may directly transdifferentiate into hair cells or, alternatively, undergo asymmetric division to yield a hair cell and another support cell (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Adler and Raphael, 1996). Unfortunately, the capacity for regeneration has been lost in the mammalian cochlea (Ozeki et al., 2007; Corwin and Oberholtzer, 1997; Edge and Chen, 2008), accounting for progressive irreversible hearing loss in humans as we age. To some extent this may be due to elevated expression levels of the mitotic inhibitors p27(Kip1) and Ink4d in support cells (Chen and Segil, 1999; Lowenheim et al., 1999; Chen et al., 2003), thereby preventing regeneration through asymmetric cell division. However, it is not clear why cochlear support cells cannot undergo transdifferentiation.

A candidate for a regulator of maintenance and regeneration of hair cells is *Sox2*. *Sox2* encodes a transcription factor well known for its role in maintaining pluripotent stem cell populations, as well as differentiation during early development. For example, *Sox2* is required to maintain pluripotency in mouse embryonic stem cells (Avilion et al., 2003; Masui et al., 2007) whereas misexpression of *Sox2* facilitates conversion of adult differentiated cell types into pluripotent stem cells (Takahahsi and Yamanaka, 2006; Yu et al., 2007). *Sox2* is also one of the first regulators of early specification of neurectoderm during vertebrate gastrulation (Kishi et al., 2000; Graham et al., 2003). How *Sox2* orchestrates the mutually exclusive activities of maintaining pluripotency vs.

stimulating differentiation is not fully understood. In sensory epithelia of the inner ear, *Sox2* is initially expressed in progenitors of both hair cells and support cells (Kiernan et al., 2005; Hume et al., 2007; Neves et al., 2007). It is eventually lost from hair cells after differentiation but is maintained in support cells. The role of *Sox2* in support cells is unknown. In mouse, disruption of *Sox2* blocks initial formation of the entire sensory epithelium, thereby obscuring its subsequent role in support cells, as well as its possible involvement in hair cell maintenance (Kiernan et al., 2005).

We have investigated the role of *sox2* in zebrafish, taking advantage of the fact that it is not required for establishment of the sensory epithelium during early otic development. We find that knockdown of *sox2* does not prevent the emergence of hair cells and support cells but does lead to subsequent sporadic cell death of hair cells, and possibly support cells as well. We further show that, in wild-type embryos, regeneration of hair cells following laser-ablation involves transdifferentiation of support cells but not cell division, and that knockdown of *sox2* totally blocks the regeneration process. These findings suggest that *sox2* is required to maintain support cells in a pluripotent state or, alternatively, *sox2* facilitates a discrete aspect of support cell differentiation that provides the facultative ability to transdifferentiate under appropriate conditions. The data further indicate that *sox2* is required for survival of at least some hair cells, either directly by regulating early stages of hair cell differentiation or indirectly by regulating essential non-autonomous functions of support cells.

MATERIAL AND METHODS

Strains and analysis of gene expression

The wild-type strain was derived from the AB line (Eugene, OR). *hsp70:Gal4, UAS:NICD* and *brn3c:gfp* lines were previously described (Scheer and Campos-Ortega, 1999; Xiao et al., 2005). In situ hybridization was performed at 67°C as described (Millimaki et al., 2007). Where indicated in the text, statistical significance was assessed using t-tests.

Misexpression

To generate heat shock vectors for misexpression, full length cDNAs of *fgf8, atoh1a,* or *sox2* (Pujic et al., 2006) were ligated to *hsp70* heat shock promoter (Shoji et al., 1998) with flanking *I-SceI* meganuclease sites (Thermes, 2002; Rembold et al., 2006). Recombinant plasmid (10-40 pg/nl) was coinjected with *I-SceI* meganuclease (NEB, 0.5 U/μl) into 1-cell stage embryos. Stable transgenic lines *Tg(hsp70:fgf8a)*^{x17}, *Tg(hsp70:atoh1a)*^{x20} and *Tg(hsp70:sox2)*^{x21} were generated by raising injected embryos to adulthood and screening by in situ hybridization for overexpression of the transgene or PCR for germline transmission.

Morpholinos

Translation-blocking morpholino oligomers (MOs) were obtained from Gene Tools, Inc. Embryos were injected at the one-cell stage with MOs as follows: 5 ng *sox2*-MO, 5'- AACCGATTTTCTGAAAGTCTACCC-3' (Pujic et al., 2006); 2.5 ng

atoh1a-MO, 5'-ATCCATTCTGTTGGTTTGTGCTTTT-3'; 7.5 ng *atoh1b*-MO, 5'-TCATTGCTTGTGTAGAAATGCATAT-3' (Millimaki et al., 2007). In all knockdown experiments, embryos were coinjected with 7.5 ng of *p53*-MO (Robu et al., 2007) to inhibit non-specific cell death sometimes caused by off-target effects of MOs. Under the conditions used here, co-injection of *atoh1a*-MO, *atoh1b*-MO and *p53*-MO (2.5, 7.5 and 7.5 ng, respectively) resulted in complete absence of hair cells through at least 48 hpf in more than 90% of morphants. Efficacy of *sox2*-MO was confirmed by showing that staining with Sox2 polyclonal antibody (Millipore, 1:100 dilution) was undetectable in the otic vesicles of *sox2*-morphants at 36 hpf, and staining in the brain was strongly reduced (data not shown). Uninjected embryos of comparable stage and genetic background were used as controls for knockdown experiments.

SU5402 and DAPT inhibitor treatment

SU5402 was dissolved in DMSO to prepare a 20 mM stock solution. DAPT was dissolved in DMSO to prepare a 10mM stock solution and was diluted 100x for incubations. Embryos were treated in their chorions with 110 μM SU5402 and/or 100 μM DAPT beginning at 26 hpf, and then fixed at 30 hpf to examine changes in *sox2* expression.

Cell transplantation and laser-ablation

Ablations were performed using a MicroPoint laser system with either a 40x or 100x objective. Multi-cell ablations required sequential targeting of individual cells.

For lineage-tracing experiments, donor embryos were injected with lineage tracer (lysine fixable rhodamine 10,000 MW dextran, mixed 1:4 with biotinylated dextran in 0.2 M KCl) at the one-cell stage. Labeled donor cells were transplanted to unlabeled host embryos at the blastula stage. After allowing chimeras to develop to the indicated stages, hair cells in close proximity to lineage-labeled support cells were laser ablated. During ablations, we frequently observed temporary photo-bleaching of GFP in non-targeted hair cells. GFP fluorescence typically recovered within two hours. Laser irradiation also caused varying degrees of photo-bleaching of rhodamine-dextran in nearby support cells. Although rhodamine-fluorescence was still readily detectable several hours later, fluorescence often continued to diminish with time as lineage label accumulated in vesicles and appeared to be secreted into the lumen of the otic vesicle. In some cases rhodamine fluorescence could no longer be detected by 24 hours post-ablation. In such cases, staining for biotinylated dextran usually permitted detection of lineage-labeled cells. In other experiments, embryos were examined for evidence of regeneration 17 hours post-ablation, prior to complete loss of rhodamine fluorescence. Loss of lineage-label was never observed in non-laser irradiated embryos.

BrdU incorporation

BrdU pulse labeling was performed as described by Gray et al. (2001). Dechorionated embryos were incubated in fish water containing 10 mM BrdU and 10% DMSO for 30 min at 33°C. Embryos were rinsed and incubated twice in fish water for 15 min at 33°C. Embryos were then fixed in MEMFA (see in situ hybridization), briefly

rinsed, and incubated in 2N HCl for 1 h at 37°C. Embryos were washed and stained with anti-BrdU (Beckton-Dickinson, 1:250).

Cell death assay

For acridine orange staining, dechorionated embryos were incubated in 7 ml of 1μg/ml acridine orange solution in fish water for 30 minutes. Embryos were then washed with fish water 3 times, 10 minutes each wash. Analysis was completed immediately.

RESULTS

Expression of *sox2*

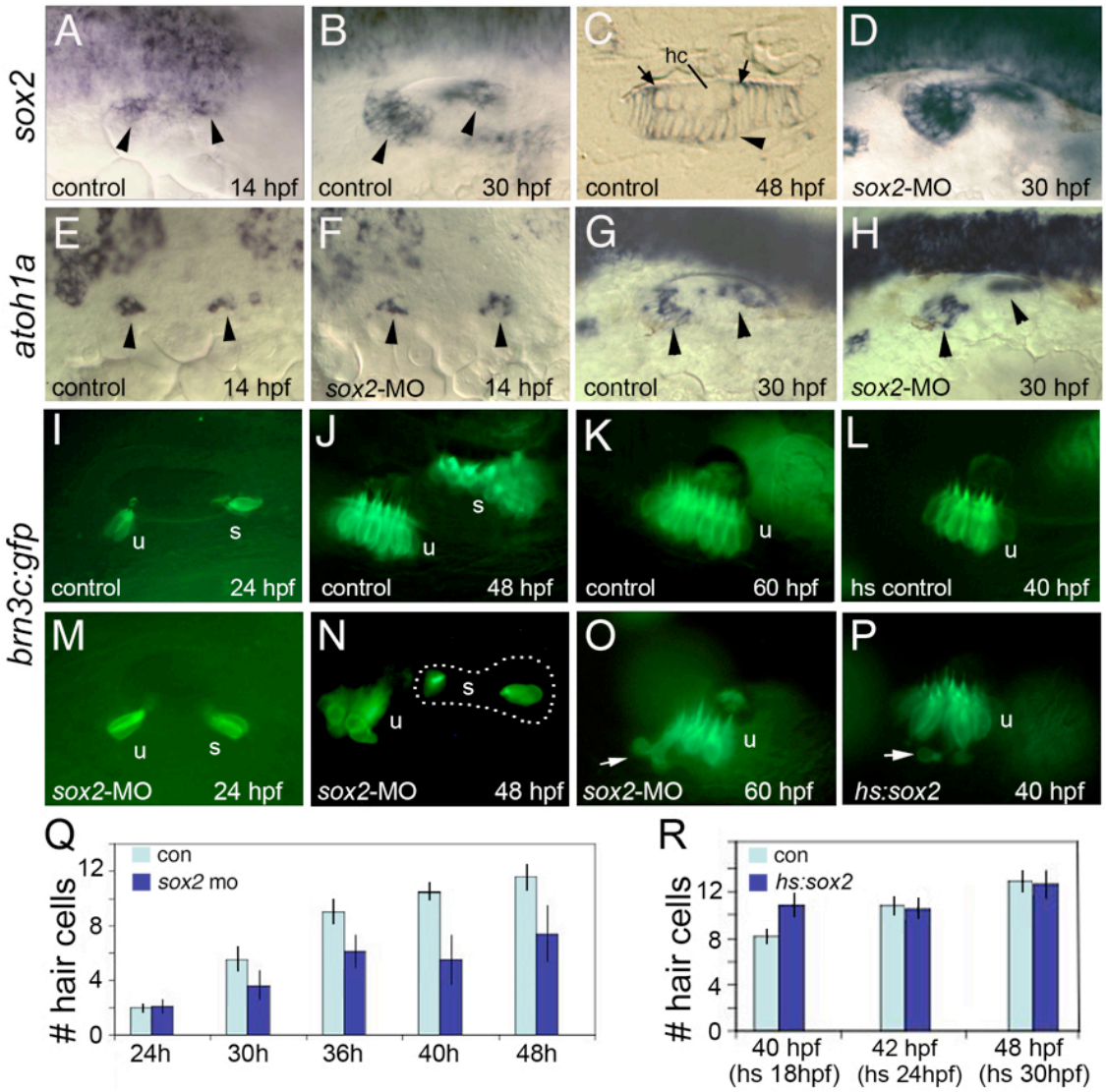
Otic expression of *sox2* begins at around 14 hpf in the nascent otic placode (Fig. 14A). This is 4 hours after the onset of *atoh1b*, the main gene responsible for specifying the prosensory equivalence group (Millimaki et al., 2007). Expression of *sox2* is contiguous along the medial edge of the otic placode with elevated expression in two domains marking the future utricular and saccular maculae. Expression is eventually restricted to the macular domains, which increase in size as the maculae expand within the otic vesicle (Fig. 14B). Sectioning reveals that nascent hair cells at the periphery of the maculae still express *sox2* but expression is lost as hair cells mature (Fig. 14C). Support cells maintain *sox2* expression, as has been seen in mouse and chick (Fig. 14C)

(Hume et al., 2007; Neves et al., 2007). By 48 hpf, primordia of the cristae also begin to express *sox2* (data not shown). Otic expression of *sox2* continues through at least 72 hpf, the latest stage examined (data not shown).

Effects of knocking down *sox2*

We next assessed the consequences of knocking down *sox2*. Injection of translation-blocking morpholino oligomer (MO) to knockdown *sox2* in zebrafish did not block early expression of *atoh1a* or *atoh1b* in the otic placode (Fig. 14F and data not shown). At later stages, the macular domains of *atoh1a* expression were nearly normal or slightly reduced in size (Fig. 14H). The macular domain of *sox2* expression appeared relatively normal in *sox2* morphants, though the level of transcript was higher than normal (Fig. 14D). To determine whether knockdown of *sox2* perturbs hair cell formation, we injected *sox2*-MO into transgenic embryos expressing *brn3c:gfp*, a marker of differentiated hair cells (Xiao et al., 2005). Tether cells, the first hair cells to differentiate during otic development (Riley et al., 1997), formed on time and appeared normal in *sox2*-depleted embryos (*sox2* morphants) (Fig. 14M). At later stages, additional hair cells continued to form but accumulated significantly more slowly than normal ($p < 0.0001$) (Fig. 14N, Q). Additionally, the saccule of *sox2* morphants usually showed a notable gap between newly forming hair cells (anterior) and the initial tether cells (posterior) (Fig. 14N). Finally, hair cells appeared disorganized in *sox2* morphants,

Figure 14. Sox2 is Not Required for Hair Cell Development. (A-C) *sox2* expression in control embryos at 14 hpf (A), 30 hpf (B) and in a cross section of the utricular macula at 48 hpf (C). *sox2* expression is lost from mature hair cells (hc) but is still detected in recently formed hair cells (arrows) and all surrounding support cells (arrowhead). (D) *sox2* expression at 30 hpf in a *sox2* morphant. (E-H) Expression of *atoh1a* in control embryos (E, G) and *sox2* morphants (F, H) at the indicated times. Arrowheads mark macular expression domains. (I-P) *brn3c:gfp* expression in control embryos at 24 hpf (I), 48 hpf (J) and 60 hpf (K); expression in a control embryo heat shocked at 24 hpf and photographed at 40 hpf (L); expression in *sox2* morphants at 24 hpf (M), 48 hpf (N) and 60 hpf (O); and expression in a *hs:sox2* transgenic embryo heat shocked at 24 hpf and photographed at 40 hpf (P). Positions of the utricular (u) and saccular (s) maculae are indicated. Note the absence of hair cells in the middle of the saccular macula in the *sox2* morphant (N). Arrows in (O, P) show hair cells being extruded from the utricular macula. All images show lateral views with anterior to the left and dorsal to the top. (Q) A time course showing the mean number of utricular hair cells in control embryos (con) and *sox2* morphants (*sox2* mo). Sox2 morphants exhibited a normal number of hair cells at 24 hpf ($p = 0.88$) but showed significantly fewer hair cells at later time points ($p < 0.0001$ for each time point). (R) Number of utricular hair cells in control embryos and *hs:sox2*/+ embryos subjected to heat shock at 18, 24 or 30 hpf, and counted at 40, 42 or 48 hpf, respectively. Transgenic embryos heat shocked at 18 hpf produced significantly more hair cells than normal ($p < 0.0004$), whereas the number of hair cells was not altered by heat shocking at 24 or 30 hpf ($p = 0.78$ or 0.73, respectively). Error bars in (Q, R) represent standard deviations, with $n \geq 15$ for each time point.


sox2
atoh1a
brn3c:gfp
A
control 14 hpf
B
control 30 hpf
C
hc
control 48 hpf
D
sox2-MO 30 hpf
E
control 14 hpf
F
sox2-MO 14 hpf
G
control 30 hpf
H
sox2-MO 30 hpf
I
u
s
control 24 hpf
J
s
u
control 48 hpf
K
u
control 60 hpf
L
u
hs control 40 hpf
M
u
s
sox2-MO 24 hpf
N
s
u
sox2-MO 48 hpf
O
u
sox2-MO 60 hpf
P
u
hs:sox2 40 hpf
Q
con
sox2 mo
hair cells
0
4
8
12
24h
30h
36h
40h
48h
R
con
hs:sox2
hair cells
0
4
8
12
40 hpf
(hs 18hpf)
42 hpf
(hs 24hpf)
48 hpf
(hs 30hpf)

and some hair cells appeared to be extruded into the underlying mesenchyme (Fig. 14O). Such displacement has been previously associated with loss of cells undergoing apoptosis (Kwak et al., 2006). Thus, hair cell production is not blocked in *sox2* morphants, but nevertheless occurs slowly and shows signs of irregular patterning. Such deficiencies could indicate faulty hair cell maturation or an increase in hair cell death or both.

To test whether *sox2*-deficiency causes increased cell death, we stained *sox2* morphants and control embryos with the vital dye acridine orange (AO) at 48 hpf. In *sox2* morphants, AO- positive cells were observed in the otic vesicle in 31 of 33 specimens examined and, on average, 2.6 positive cells were seen per ear (Fig. 15B). The majority (66%) of AO-positive cells were seen within the developing maculae of *sox2* morphants and marked both the apical and basal layers of the sensory epithelium, indicating the presence of dying hair cells and possibly support cells as well (Fig. 15C, D, F). In control embryos, only 20 of the 33 specimens exhibited AO- positive cells with an average of only 1 positive cell per ear examined. Moreover, only a single control specimen showed any AO-positive cells within the maculae (Fig 15A, E), a far lower incidence than was seen in *sox2* morphants ($p < 0.0001$). Thus, cell death is normally quite rare in sensory epithelia but is common in *sox2* morphants, confirming that *sox2* directly or indirectly influences hair cell survival.

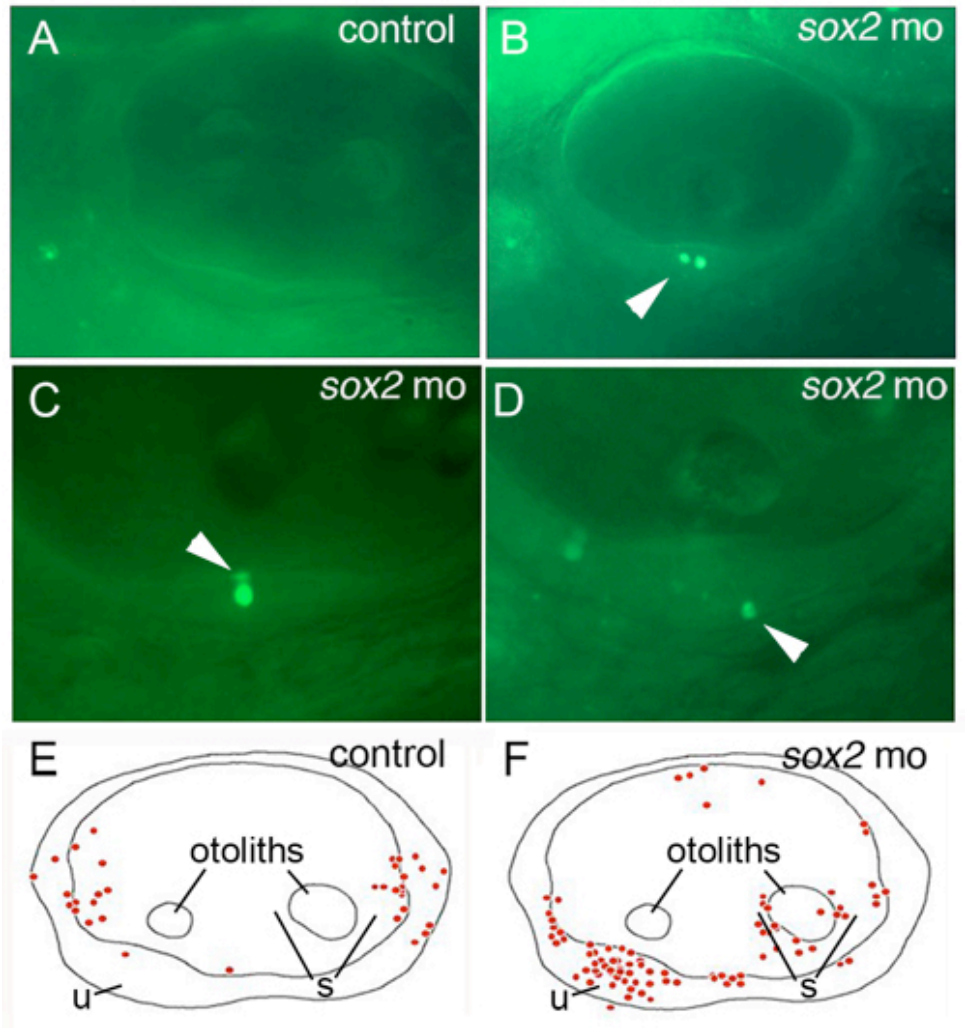


Figure 15. Loss of Sox2 Results in mMacular Death. (A-D) AO-labeling of dying cells in a control embryo (A) and *sox2* morphants (B-D). Morphants often contained multiple dying cells within sensory epithelia (B), and were observed in apical (C) or basal (D) regions of the maculae (arrowheads). (E, F) Schematic maps depicting the distribution of all AO-positive cells seen in otic vesicles of 33 control embryos (E) or 33 *sox2* morphants (F) at 48 hpf. Positions of the utricular macula (u), saccular macula (s) and otoliths are indicated. No AO-positive cells were detected in the lateral wall of the otic vesicle. All images show lateral views with anterior to the left and dorsal to the top.

Effects of *sox2* misexpression

Injection of *sox2* mRNA caused severe patterning defects throughout the embryo, confounding interpretation of its effects in the inner ear (data not shown). We therefore generated a transgenic line to misexpress *sox2* under the control of the heat shock-inducible promoter *hsp70* (Shoji et al., 1998). Activation of *hs:sox2* at 18hpf caused a 20-30% increase in the number of hair cells produced by 40 hpf (Fig. 14R). The resulting maculae appeared somewhat disorganized and occasionally (≤ 10% of embryos) exhibited hair cells being ejected from the macula (Fig. 14P). In contrast, activation of *hs:sox2* at 24 hpf or later had no discernable effect (Fig. 14R). At no time did activation of *hs:sox2* result in production of ectopic hair cells beyond the endogenous macular domains, indicating that, unlike *atoh1a/b* (Millimaki et al., 2007), *sox2* is not sufficient to establish a prosensory equivalence group.

Co-misexpression of Sox2 and Atoh1a

Misexpression studies in mouse suggest that Sox2 and Atoh1 are mutually antagonistic with respect to cell fate specification in the cochlea (Dabdoub et al., 2008). We therefore tested whether *hs:sox2* could block the ability of *hs:atoh1a* to stimulate hair cell production. Activation of *hs:atoh1a* at 24 hpf resulted in production of excess and ectopic hair cells throughout the ventromedial wall of the otic vesicle by 33-34 hpf (Fig. 16J). Co-activation of *hs:atoh1a* and *hs:sox2* also led to formation of ectopic hair cells (Fig. 16L), similar to activation of *hs:atoh1a* alone. Thus, misexpression of *sox2* does not antagonize *atoh1a* function sufficiently to block hair cell differentiation in

zebrafish. However, the pattern of ectopic hair cells was less orderly following co-activation of *hs:sox2* and *hs:atoh1a* (note the absence of straight rows of hair cells in Fig. 16B), suggesting that excess Sox2 weakly impairs the ability of Atoh1a to pattern the macula.

Regulation of *sox2* by Atoh1, Fgf and Notch

To better understand the role of sox2 in macular development, we examined its functional relationship to other genes known to regulate early steps in the process, Atoh1a/b, Notch, and Fgf (Millimaki et al., 2007). In *atoh1a/b* double morphants, which lack hair cells and support cells, *sox2* expression was not detectable until 20 hpf, a delay of six hours (Fig. 16E and data not shown). At 30 hpf, *atoh1a/b* double morphants continue to express *sox2* in two macular domains, though both domains are smaller than normal (compare Figs. 16F and 1B). These data show that Atoh1a/b activity is required for initiation of *sox2* expression at the correct time. To block Fgf signaling we incubated embryos with the pharmacological inhibitor SU5402. This does not block *sox2* expression but reduces its level of expression (compare Figs. 16G and 1B). To block Notch signaling embryos were treated with DAPT, which blocks proteolytic processing necessary to activate Notch. This also reduced the level of *sox2* expression (Fig. 16H). Treatment with both SU5402 and DAPT nearly eliminated *sox2* expression (Fig. 16I), suggesting that these signals act in parallel to regulate *sox2*.

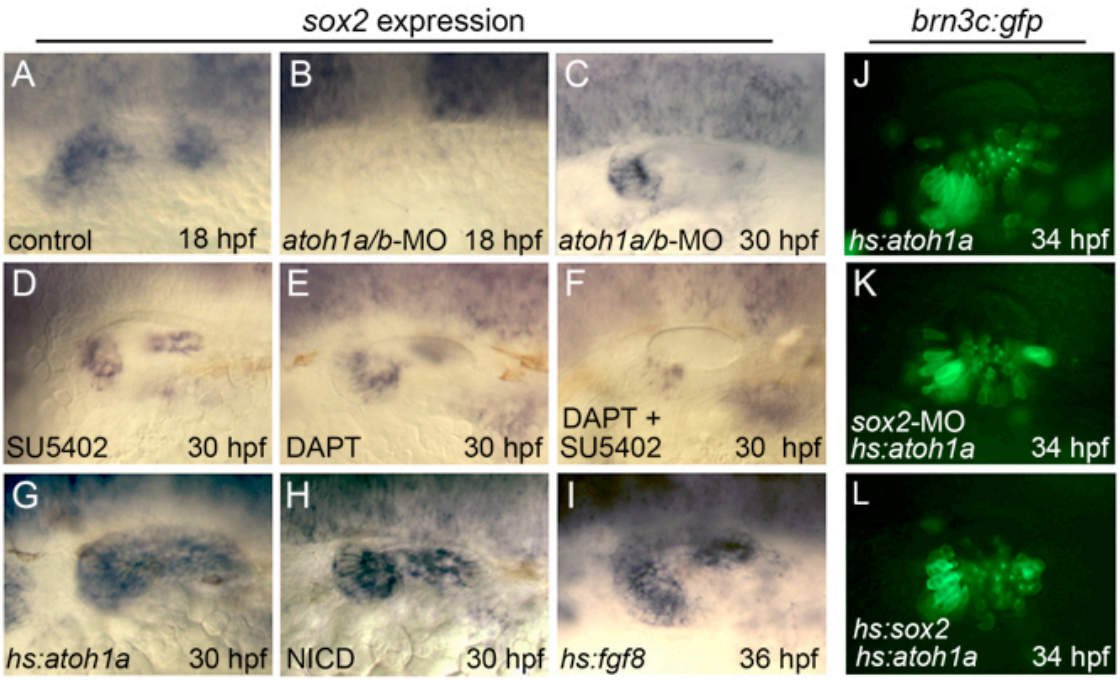


Figure 16. Relationship Between Sox2 and Upstream Regulators of Hair Cell Development. (A-I) *sox2* expression in a control embryo (A), *atoh1a/b* morphants (B, C), wild-type embryos exposed to SU5402 (D), DAPT (E), or both DAPT and SU5402 (F) beginning at 26 hpf, a *hs:atoh1a*/+ embryo heat shocked at 24 hpf (G), a *hs:gal4*/+;*UAS-NICD*/+ embryo heat shocked at 24 hpf (H), and a *hs:fgf8*/+ embryo heat shocked at 30 hpf (I). *sox2* expression is shown at 30 hpf, except (A, B, 18 hpf) and (I, 36 hpf). Expression in control embryos does not change appreciably between 30 and 36 hpf. (J-L) Expression of *brn3c:gfp* in *hs:atoh1a*/+ transgenic embryos (J, K) and a *hs:atoh1a*/+;*hs:sox2*/+ double transgenic embryo (L) heat shocked at 24 hpf and photographed at 34 hpf. The specimen in (K) was also injected with *sox2*-MO. All images show lateral views with anterior to the left and dorsal to the top.

To further test their roles in *sox2* regulation, we used heat shock lines to misexpress Atoh1a, Fgf8 or an activated intracellular domain of Notch (NICD) (Scheer and Campos-Ortega, 1999). Activation of *hs:atoh1a* at 24 hpf led to a dramatic expansion of the *sox2* domain to cover the entire ventromedial wall of the otic vesicle by 30 hpf (Fig. 16J). This correlated with production of ectopic hair cells in the same domain several hours later (Fig. 16A). However, expansion of the domain of *sox2* expression is not required for ectopic hair cell production, since activation of *hs:atoh1* in *sox2* morphants also led to overproduction of hair cells (Fig. 16C). Heat shock activation of NICD led to nearly as great an expansion in *sox2* expression (Fig. 16H). Activation of *hs:fgf8* caused a modest expansion of the macular domains of *sox2*, as well as a low level of ectopic expression in intervening tissue (compare Figs. 16L and 1B). Under the conditions used here, neither NICD nor Fgf8 were sufficient to stimulate ectopic hair cell formation. Thus, Atoh1a, Notch and Fgf activity are all able to activate ectopic expression of *sox2*, but this response is neither necessary nor sufficient for ectopic hair cell production.

Analysis of hair cell regeneration and the role of *sox2*

Regeneration of hair cells in the inner ear has not been previously examined in zebrafish embryos. To do so, we used a laser to ablate GFP-positive hair cells in *brn3c:gfp*/+ embryos and established a timeline for hair cell regeneration. We initially targeted only hair cells at the macular center to distinguish subsequent regeneration from normal developmental accumulation of hair cells along the periphery. When ablation

was initiated at 48 hpf, the resulting gap in the macula was still easily discernable 12 hours later (Fig. 17A). By 24 hours post-ablation most gaps had been largely filled with new hair cells (Fig. 17B). Thus, substantial hair cell regeneration takes place between 12 and 24 hours post-ablation. Next, to assess the capacity for wholesale regeneration, we ablated all visible hair cells in the utricular macula at 30 hpf, taking care to examine embryos at 34 hpf to confirm that all hair cells had been killed. We then counted the number of hair cells present at 38 hpf (before there is discernable regeneration) and again at 50 hpf (after regeneration has occurred). In unablated controls, the number of hair cells increased by an average of 3.6 ± 0.9, representing normal hair cell production as the macula grows (Fig. 17E, F). In ablated ears, 6.4 ± 0.5 hair cells were produced in this time, representing both normal and regenerative hair cell production (Fig. 17E, F). We infer that the difference between control and laser-irradiated groups (2.8 hair cells/16 hours, $p < 0.005$) represents the number of hair cells produced through regeneration.

We next examined whether regeneration involves transdifferentiation or asymmetric cell division. To examine whether regeneration involves cell division, hair cells were ablated in the center of the utricular macula at 48 hpf, embryos were allowed to recover for 3, 6, 10 or 20 hours post-ablation, and then BrdU was added for a 3 hour pulse-label. We examined a total of 46 embryos, with at least 8 specimens per time point. Although BrdU-positive cells were detected in many regions of the embryo at each time point, no BrdU incorporation was detected in the macula in any specimen (Fig 17G-I). This indicates that regeneration seen within 24 hours post-ablation does not

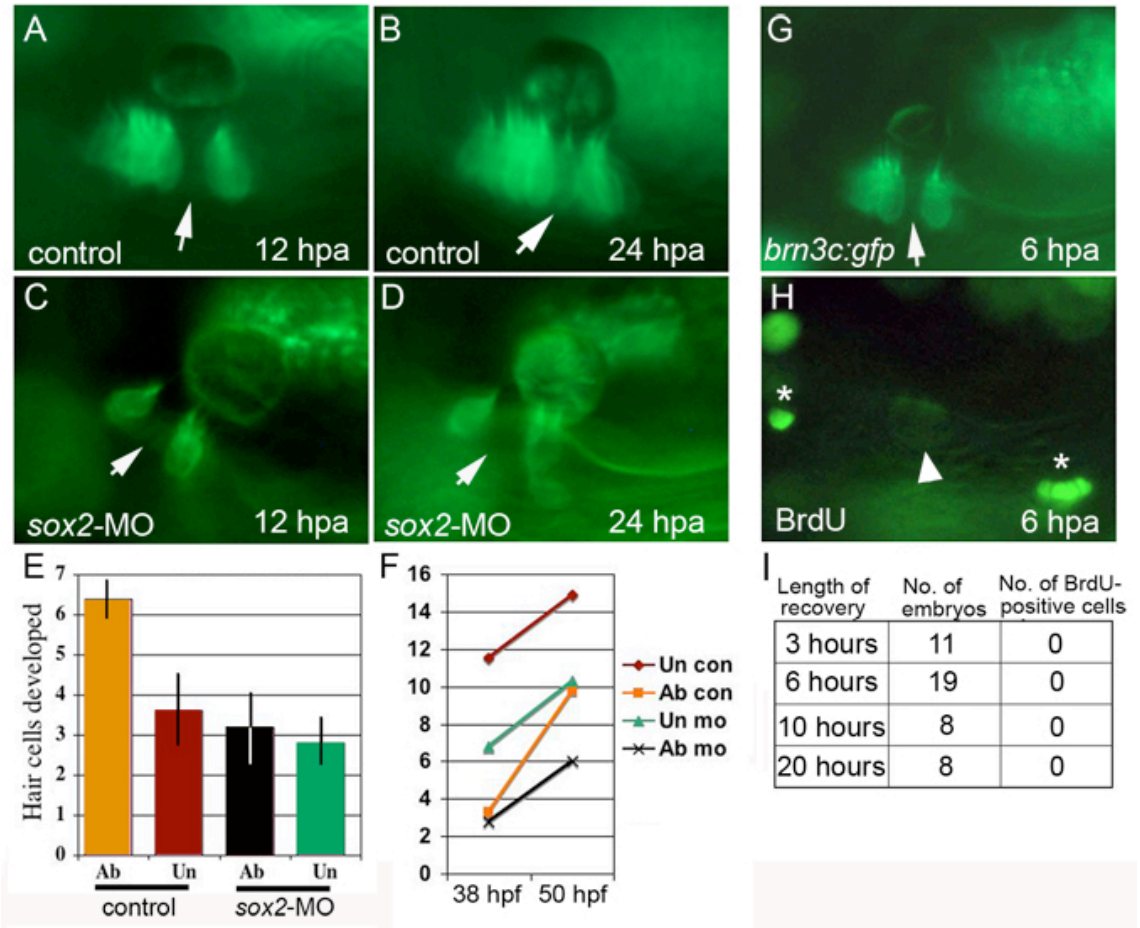


Figure 17. Hair Cell Regeneration Requires *sox2* But Does Not Involve Cell Division. (A-D) *brn3c:gfp* following ablation in a control embryo (A, B) and a *sox2* morphant (C, D). Hair cells were ablated at 48 hpf, and ablated regions (arrows) were still evident at 12 hours post-ablation (hpa) (A, C) and 24 hpa (B, D). By 24 hpa, the gap filled in with newly formed hair cells in the control (B) but not in the *sox2* morphant (D). (E, F) The number of hair cells produced following wholesale ablation of utricular hair cells. Ablation was conducted at 30 hpf, embryos were allowed to recover, and hair cells were counted at 38 hpf and again at 50 hpf. Typically 2 hair cells were produced during the recovery period. The number of hair cells produced between 38 and 50 hpf (E), and the total number of hair cells (F) are indicated for ablated (ab) and unablated (un) control embryos and *sox2*-morphants. Each time point shows the mean ± standard error of 3 or 4 experiments, with sample sizes of 19 to 23 embryos. (G-I) BrdU incorporation at various times following ablation initiated at 48 hpf. After 3, 6, 10 or 20 hours of recovery, embryos were incubated with BrdU for 3 hours and then fixed for processing. A specimen just before fixation at 6 hours post ablation (G) shows that the hair cell gap is still evident (arrow). After processing with anti-BrdU (H), dim GFP fluorescence is still detectable (arrowhead) and shows that no brightly labeled BrdU-positive cells (asterisks) are evident within the macula.

involve asymmetric cell division. To test whether regeneration involves transdifferentiation, we performed a lineage analysis in laser-irradiated *brn3c:gfp* embryos. Rhodamine-labeled cells were transplanted into unlabeled host embryos at the mid-blastula stage, and host embryos were screened at 36 hpf (n = 310 embryos) or 48 hpf (n = 280 embryos) toidentify rare cases in which lineage-label was detected in support cells but few or no hair cells (Fig. 17A-C). Of 590 embryos (1180 ears) screened, 38 showed appropriate labeling patterns. In these specimens, hair cells near the lineage-labeled support cells were laser-ablated. Because laser-targeting sometimes causes photo-bleaching without killing hair cells, specimens were examined again 3 hours post-ablation to confirm that targeted hair cells had indeed been killed (Fig. 18D-F). By 17-24 hours post-ablation, 16 out of 38 specimens showed rhodamine-positive hair cells, with a corresponding disappearance of rhodamine-positive support cells (Fig. 18G-I). The remaining 22 specimens gave inconclusive results due to variable loss of lineage label (see Materials and Methods). These data show that support cells can transdifferentiate into hair cells within 17-24 hours post-ablation, thereby facilitating regeneration in zebrafish embryos.

We next examined whether hair cell regeneration occurs in *sox2* morphants. Ablation of hair cells in the macular center in *sox2*-morphants at 48 hpf produced gaps that remained unfilled at 72 hpf, 24 hours after ablation (Fig. 17 C, D, n = 9). Similar results were obtained following wholesale ablation: In *sox2*-morphants in which all hair

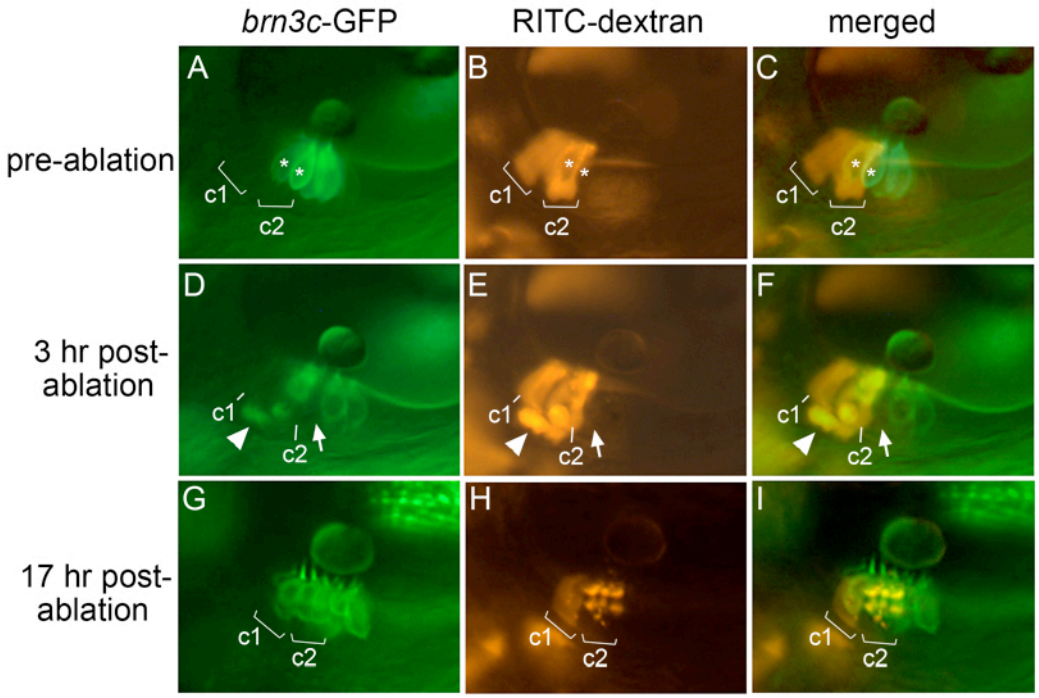


Figure 18. Regeneration Occurs Through Transdifferentiation. (A-C) Lineage-labeled embryo at 48 hpf, just before laser-ablation, showing *brn3c:gfp* labeled hair cells in the utricular macula (A), two clusters (c1 and c2) of lineage-labeled cells (B) and an overlay showing both labels (C). Most lineage-labeled cells are support cells. Asterisks mark hair cells that were subsequently targeted for ablation. (D-F) The same specimen 3 hours post-ablation. A notable gap in the hair cell layer (arrow) marks the position previously occupied by one of the targeted hair cells. Accumulation of lineage-label plus GFP beneath the macula appears to show a fragmenting apoptotic hair cell being ejected from the macula (arrowhead). Labeled support cells are still evident in clusters c1 and c2. (G-I) The same specimen 17 hours post-ablation. Support cells in cluster c1 are still evident, though fluorescence intensity has decreased as described in Materials and Methods. In contrast, lineage-label is no longer visible in the support cell layer in cluster c2. Instead, lineage-labeled cells now occupy the hair cell layer and express *brn3c:gfp*. Much of the lineage label is concentrated in vesicles, as is typical at this stage following laser irradiation (see Materials and Methods). All images show lateral views with anterior to the left and dorsal to the top.

cells were ablated at 30 hpf, an average of 3.2+/- 0.9 hair cells were produced between 38 hpf and 50 hpf. In unablated *sox2*-morphants an average of 2.8 +/- 0.6 hair cells were produced (Fig. 17E, F). Because there was no difference in the number of hair cells produced in ablated and unablated embryos ($p = 0.75$), we infer that no regeneration occurred by 50 hpf. Together these data suggest that *sox2* is required for hair cell regeneration in zebrafish embryos.

DISCUSSION

We have shown a requirement for *sox2* in maintenance and regeneration of hair cells in the zebrafish inner ear. It is possible that both functions are co-regulated in support cells or, alternatively, they could reflect independent functions in hair cells and support cells, respectively. Although *sox2* is not required for overt hair cell formation, the sporadic cell death seen later could reflect faulty regulation of early hair cell differentiation. Alternatively, the requirement for hair cell survival could indicate that *sox2* regulates an essential non-autonomous function in support cells. Analysis of *mib* mutants in zebrafish suggests that support cells are required for hair cell survival. In this background, the entire sensory equivalence group differentiates precociously as hair cells, all of which subsequently die by 36 hpf (Haddon et al., 1998). Deficiencies in support cell functions are clearly subtler in *sox2* morphants, and hair cell death occurs only sporadically over a protracted period. Additionally, it is possible that support cells

themselves die in *sox2* morphants, though this is difficult to resolve without reliable support cell-specific markers.

The requirement for *sox2* in regeneration clearly points to an essential function in support cells. We find that support cells directly transdifferentiate into hair cells following laser ablation in zebrafish, as has been observed in neonatal mice (Kelley et al., 1995). Maintenance of *sox2* expression might allow support cells to retain developmental plasticity even as they differentiate enough to execute their essential functions. Alternatively, *sox2* might regulate a discrete aspect of support cell differentiation that enables them to respond to macular damage by transdifferentiation into hair cells. The mechanism governing transdifferentiation is not well understood, but studies in chick suggest that *Atoh1* is involved (Cafaro et al., 2007). In this case, downregulation of *sox2* might be required for upregulation of *Atoh1*. It is also known that *Atoh1*-null cells can sometimes become hair cells when surrounded by wild-type cells, indicating the existence of an alternate hair cell pathway (Du et al., 2007). The status of *sox2* in this pathway is unknown. It will be interesting to explore whether the loss of regenerative processes in the mammalian cochlea involve changes in *Sox2* regulation. Support cells in mouse might lack the ability to reduce expression of *Sox2* enough to allow *Atoh1* activation. Alternatively, expression levels may be too low to maintain pluripotency. Cochlear support cells are highly specialized and differentiated, which could indicate a more stable commitment to these specific fates (Corwin and Oberholtzer, 1997). Expression of *sox2* in the lateral line in zebrafish is also consistent

with a role in regeneration, though this can apparently occur by transdifferentiation or asymmetric cell division (Woods et al., 2004; Hernandez et al., 2007; Ma et al., 2008).

The role of *sox2* in patterning of the inner ear and sensory epithelium shows some interesting parallels between zebrafish and mouse, though there are clearly also some important differences. We have shown that zebrafish *sox2* expression begins within the maculae downstream of *atoh1a/b*, and knockdown of *sox2* does not block *atoh1a/b* expression. In contrast, mouse *Sox2* is initially expressed throughout the ventral half of the otic vesicle well before formation of the sensory primordia (Kiernan et al., 2005). Moreover, *Sox2* mutant mice produce no sensory cells and fail to express *Atoh1*. These observations have led to the suggestion that mouse *Sox2* acts as a proneural gene to establish the prosensory equivalence group (Kiernan et al., 2005; Dabdoub et al., 2009). As a potential correlate, we detected a 20-30% increase in hair cell production following activation of *hs:sox2* at18 hpf. This corresponds to a brief period in zebrafish when Notch activity stimulates *atoh1a* expression (Millimaki et al., 2007), suggesting that the pulse of *sox2* misexpression may help mediate this effect. However, in contrast to *Atoh1* (Woods et al., 2004; Millimaki et al., 2007), misexpression of *Sox2* is not sufficient to activate formation of ectopic sensory epithelia in mouse or zebrafish, arguing against a simple prosensory role. An alternative explanation for the early requirement in mouse is that *Sox2* initially acts as a regional specifier for the floor of the otic vesicle without which all ventral fates are lost. This would explain why the prosensory inductive signal Jag1 is not expressed in *Sox2* mutants (Kiernan et al., 2005).

In a second phase of *Sox2* function, zebrafish and mouse appear much more alike in their expression and regulation of *Sox2*. In both species, *Sox2* is induced by Notch activity, and possibly Fgf signaling as well (Pirvola et al., 2002; Brooker et al., 2006; Kiernan et al., 2006; Hayashi et al., 2008) (Fig. 16). Interestingly, early expression of *Atoh1* is co-induced by these same signals (Pirvola et al., 2002; Woods et al., 2004; Brooker et al., 2006; Kiernan et al., 2006; Millimaki et al., 2007; Hayashi et al., 2008). Subsequent mutual antagonism between *Atoh1* and *Sox2* (Dabdoub et al., 2009) could then reinforce cell fate diversification mediated by Notch-dependent lateral inhibition (Haddon et al., 1998; Riley et al., 1999; Brooker et al., 2006; Kiernan et al., 2006; Millimaki et al., 2007). Perturbing the balance of these activities might explain why in our studies misexpression of *sox2* led to more chaotic arrangements of hair cells. However, unlike misexpression experiments in mouse (Dabdoub et al., 2009), we did not see a reduction in hair cell production following misexpression of *sox2*, arguing that Sox2 does not directly antagonize Atoh1 activity. It is possible that variation in the relative abundance or perdurance of misexpressed proteins influences how cells respond in different settings (Boer et al., 2007; Kopp et al., 2008).

CHAPTER IV

SUMMARY AND DISCUSSION

The sensory cells of the inner ear provide organisms with the ability to hear and detect gravity. Loss of these cells can be detrimental to an individual. Mammalian cochlear hair cells, necessary for the detection of sound, cannot regenerate. In zebrafish, hair cells can and do regenerate. Understanding the role and regulation of the genes involved in the formation and regeneration of these cells in zebrafish may provide information important for the development genetic therapies in humans. We showed that zebrafish *atoh1* is the proneural gene required to induce the equivalence group from which hair cells form. Although this is contrary to what has been previously suggested in mouse, the data in mouse does not refute the possibility that *Atoh1* acts as a proneural gene in mouse as well. We further showed that expression of *atoh1* requires Fgf and Pax and is restricted by Notch activity. One factor we identified as acting downstream of Atoh1 is *sox2*. This work indicated that Sox2 is required for hair cell survival and regeneration, but is not necessary for hair cell specification. This role is likely conserved, as Sox2 is important for stem cell plasticity. Taken together, this study reveals the role of both Atoh1 and Sox2 in hair cell development and regeneration and their regulation by Fgf, Notch and one another. This new understanding of the role and regulation of both Atoh1 and Sox2 provides essential information that can be used to further efforts to provide genetic therapies for hair cell regeneration in mammals.

atoh1 IS A PRONEURAL GENE

Much debate has surrounded the role Atoh1 plays in hair cell development. As a homologue of *Drosophila Atonal*, Atoh1 is a basic helix-loop-helix (bHLH) transcription factor and likely fufills a proneural role. We showed that, in zebrafish, the function of *atoh1* closely resembles that of *Drosophila ato*, suggesting that it is the proneural gene responsible for establishing the equivalence group from which hair cells form. We further showed that *atoh1* genes in zebrafish fulfill the criteria established for defining a proneural gene (Brunet and Ghsen, 1999; Hassan and Bellen, 2000; Westerman et al., 2003). First, the expression of a proneural gene precedes sensory fate specification. The expression of *atoh1b* is first detected broadly within the preotic placode by 10.5 hpf. The specification of the first hair cells, tether cells, does not occur until 14 hpf. Second, proneural genes respond to lateral inhibition and domain restriction via Dl-Notch signaling. We showed that, as with other proneural genes, Atoh1 is an upstream activator of *dl* expression. Additionally, *atoh1* genes are repressed by Notch activity. This is consistent with the role of proneural genes by which *atoh1* activates *dl* expression, which then inhibits *atoh1* in neighboring cells via Notch activation and lateral inhibition. We further showed that loss of Notch activity results in a larger domain of *atoh1* expression due to failed Notch mediated domain restriction. Third, a proneural gene is required for production of the entire equivalence group. Loss of both Atoh1a and Atoh1b through morpholino mediated targeted gene knockdown results in formation of an epithelium devoid of hair cells and support cells. All cells within this

epithelium express *atoh1,* normally lost from support cells, suggesting that the cells within the epithelium of *atoh1*mo respond to inductive signals but are unable to undergo differentiation into hair cells or support cells. Fourth, a proneural gene is sufficient to induce ectopic sensory epithelia production. Misexpression of *atoh1a* induces production of extranumerary hair cells.

Because *atoh1* fufills all the criteria to be difined as a proneural gene we can assume that induction of *atoh1* may act as a genetic therepy for hair cell loss. However, misexpression of *atoh1* only produces extra hair cells in regions in or near the ear, suggesting a requirement for a combination of signals. *Drosophila* proneural gene activity is also influenced by combinatorial signals and zones of restricted competence (Westerman et al., 2003; Niwa et al., 2004). Misexpression of *atoh1* alone may not be sufficient to treat hair cell loss. Identification of these addtional signals will further our understanding of how hair cells develop and what genes may be required for hair cell regeneration.

REGULATION OF Atoh1

The preotic placode forms in the region where Foxi1 expression abuts hindbrain expression of Fgf. The formation of the preotic placode is evident by induction of *pax8*, the first preotic marker (Pfeffer et al., 1998). Because *atoh1b* expression is first detected within a subset of *pax8* expressing cells, we reasoned that *pax8,* or the factors

responsible for *pax8* induction, may act upstream of *atoh1b*. Pax8 is required to produce a normal sized preotic domain and, as such, is required for a normal sized *atoh1b* domain. Loss of *pax8* does not affect the level of *atoh1b* expression within the domain of cells that continue to express *atoh1b* in the absence of *pax8*. In contrast, Foxi1 and Fgf are both required for normal initiation of *atoh1* expression. Loss of Foxi1 results in a severe reduction of *atoh1b* expression. Chemical inhibition of Fgf signaling prior to or just after the onset of *atoh1b* expression results in the loss of both *atoh1b* and *atoh1a*.

We also investigated a requirement for Fgf in maintaining *atoh1* expression following induction of the otic placode. In addition to Fgf in the hindbrain, Fgf is also expressed within the domains of the otic vesicle that encompass the sensory epithelia (Leger and Brand, 2002). We predicted that Fgf is required for Atoh1 maintenance. We showed that normal *atoh1* expression and subsequent hair cell development require continuing Fgf signaling. This is the first factor identified to be required for both induction and maintenance of *atoh1* in the vertebrate ear (Fig. 19).

Sox2 REGULATION

Another transcription factor expressed within the sensory epithelium is *sox2*. In mouse, *Sox2* is expressed very early and is required for formation of the sensory epithelia and *Atoh1* expression (Kiernan, et al., 2005b). In zebrafish, *sox2* expression begins later in the developing sensory primordia, hours after that of *atoh1b,* and is not

required for *atoh1* expression. As such we suspected that Atoh1 induces *sox2* expression. We showed that loss of Atoh1 results in a delay in *sox2* initiation. Futhermore, activation of *hs:atoh1a* results in strong upregulation of *sox2* expression throughout the ventromedial wall of the ear. This is likely an indirect affect because there is a delay between onset of *atoh1a* expression and *sox2* upregulation. One factor known to act downstream of Atoh1 is Notch. Loss of Notch results in reduction of *sox2* expression. Expression of the activated form of Notch leads to a vast expansion of *sox2* expression into the medial wall of the ear. These data suggest that after Atoh1 induces Notch activity, in neighboring cells, through upregulation of *delta* expression, Notch turns on *sox2* expression. This is consistent with the finding that *sox2* expression is maintained within support cells, the same cells in which Notch activity remains strong following hair cell specification.

Induction and continued expression of *aoth1* involve ongoing Fgf activity. Recent experiments show that overexpression of *atoh1a* also results in an upregulation of *fgf8* expression (Sweet and Riley, personal communtication). We showed that loss of Fgf, via chemical inhibition, reduces the level of *sox2* expression. We suspected that, since Atoh1 activity results in activation of both Fgf and Notch that they may act reduntantly to induce *sox2* expression. Loss of both Fgf and Notch results in a near ablation of *sox2* expression. Taken together these data suggest that both Fgf and Notch mediate Atoh1 to induce *sox2* expression in addition to activating *sox2* expression on their own.

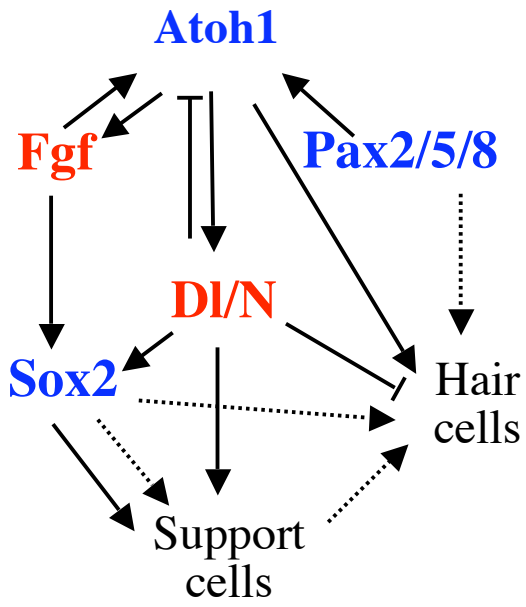


Figure 19: The Signals Involved in Hair Cell and Support Cell Differentiation and Maintanance. The specification of hair cells and support cells involves both transcription factors (blue) and signaling molecules (red). Fgf and Pax2/5/8 proteins activate *atoh1* expression within the preotic placode. Atoh1 acts as the proneural gene and, as such, is responsible for the formation of both hair cells and support cells. The cells that maintain *atoh1* expression will become hair cells, those that lose *atoh1* expression through Delta-Notch (Dl/N) mediated lateral inhibition will develop into support cells. Atoh1 activates *sox2* expression through upregulation of both *fgf* and *delta*. Sox2 acts to maintain support cell plasticity. Loss of Sox2 results in hair cell death. This could point to a role in support cell maintenance (dashed arrow). Support cells are known to be involved in hair cell survival (dashed arrow), as are Pax2/5/8. Alternatively, Sox2 may mediate hair cell survivial directly (dashed arrow).

Sox2 IS REQUIRED FOR HAIR CELL MAINTENANCE

In zebrafish, unlike mouse, Sox2 is not necessary for hair cell formation. Hair cells develop normally in *sox2* morphants until after 30 hpf, at which time hair cells begin to die. Because *sox2* expression begins within the hair cell primordia, subsequent cell death may reflect a flaw in regulation of early hair cell development. Alternatively, hair cell death may reflect a non-autonomous support cell function. Expression of *sox2* is maintained within support cells and may be required for proper support cell activity. *mib* mutants that have lost lateral inhibition form a sensory epithelium full of hair cells and void of support cells. These hair cells eventually die. This suggests a role of support cells in hair cell maintenance (Haddon et al., 1998). Alternatively, the cell death observed in *sox2* morphants may include that of both hair cells and support cells. Support cell death could result in subsequent hair cell death.

Sox2 IS REQUIRED FOR HAIR CELL REGENERATION

Expression of *sox2* is maintained within support cells of mouse, chick and zebrafish (Hume et al., 2007; Neves et al., 2007) (Fig. 14C). Little is known about the function of support cells; however, we do know that they are important for hair cell regeneration. As such, we speculated that *sox2* may be important for support cell

activity in hair cell regeneration. Because hair cell regeneration has not previously been studied within the zebrafish ear, we first characterized the process. We showed that hair cell regeneration following laser ablation occurs via transdifferentiation and require Sox2.

There are a number of ways that Sox2 could aid in hair cell regeneration. Expression of Sox2 may allow support cells to maintain pluripotency while being in a differentiated state. Alternatively, Sox2 may be required during support cell development to give them the ability to respond to macular damage and transdifferentiate. Little is known about how transdifferentiation occurs. In chick it correlates with *ATOH1* upregulation (Cafaro et al., 2007). Cells may first require downregulation of *SOX2* for *ATOH1* upregulation. Hair cells of the mammalian cochlea cannot regenerate. It is possible that loss of regenerative ability reflects an inability to respond to macular damage and transdifferentate. This may reflect some change in *Sox2* regulation. Support cells may be unable to reduce *Sox2* so that *Atoh1* may be upregulated. An alternative to this model stems from the suggestion that cochlear support cells may be more committed to their fate than those of maculae, as they are highly specialized. This may reflect a lower level of expression for some pluripotent factors including *Sox2* (Corwin and Oberholtzer, 1997). Thus, the expression of *Sox2* within cochlear support cells may be too low to maintain pluripotency.

Many studies currently focus on the signal that indicates to support cells that macular damage has occurred. The most likely candidate is Notch. Hair cells continue to present Notch ligands on their cell surface once mature. This allows support cells to

continue to receive activated Notch and maintain expression of support cell specific factors including *sox2* while ensuring that hair cell specific factors, such as *atoh1,* remain off. Upon hair cell death, support cells lose some of their Notch activity. This could in turn lead to a downregulation of *sox2* expression and upregulation of *atoh1* expression in what was the support cell. This would allow the support cell to transdifferentiate into a hair cell. This is just one model by which tight regulation of *atoh1* and *sox2* could lead to hair cell regeneration.

CONSERVED MECHANISM?

There are clear differences between mammalian hair cell development and that of zebrafish. In mammals there are no cells analogous to tether cells. Additionally, mammalian hair cell development stops after embryogenesis. Mammals form a specialized structure for hearing, the cochlea, with different types of hair cells and support cells. Even so, there is great similarity between zebrafish and mammals in the genes involved in hair cell specification.

We showed that zebrafish Fgfs are required for induction and are important for continued expression of *atoh1*. Additionally, we show that Fgf acts upstream of *sox2*. A number of FGFs are available within the mammalian otic vesicle and developing sensory epithelium. Loss of FGF in mouse causes such severe morphological defects that the role of Fgf in hair cell development is not discernable in FGF loss of function mutants.

Hypomorphic alleles of FGFR1 do not block morphogenesis but rather severely reduce hair cell production (Privola et al., 2002). Chemical inhibition of FGF results in a severe reduction in the number of both hair cells and support cells formed. This treatment also ablates *Atoh1* expression (Hayashi et al., 2008). It follows then that Fgf induces *atoh1* expression, which results in macular formation in mouse as well as zebrafish. Studies also indicate, as in zebrafish, FGF may activate *Sox2* expression in mouse (Hayashi et al., 2008).

Another similarity between mouse and zebrafish is the role of Notch signaling. In mouse, two Notch ligands, Dll1 and JAG2, regulate the balance between hair cell and support cell specification within the cochlea. Loss of either of these ligands results in the production of greater than normal numbers of hair cells (Lanford et al., 1999; Kiernan et al., 2005a; Zine et al., 2000; Brooker et al., 2006). In embryos lacking both ligands, many extra hair cells form (Kiernan et al., 2005a). More support cells form than expected in these embryos, possibly because support cell division continues longer than in control embryos, masking earlier lateral inhibition defects. Another Notch ligand, JAG1, is expressed throughout the sensory primordia and later is restricted to support cells. A partial loss of JAG1 function gives rise to extra hair cell production, (Keirnan et al., 2001; Zine et al., 2000). A conditional knockout of JAG1, in contrast, ablates much of the sensory epithelia and results in a loss of *Sox2* expression (Brooker et al., 1006; Kiernan et al., 2006). This supports a model by which JAG1 function, like Notch in *Drosophila* and zebrafish, changes from an early induction phase to a later lateral

inhibition phase (Baker and Yu, 1997). Additionally, this shows that in mouse, as in zebrafish, Notch acts to induce *sox2* expression.

One difference between mouse and zebrafish hair cell development is that, where mouse only has one ATOH1, in zebrafish there are two Atoh1 proteins. This has allowed us to identify two distinct phases of Atoh1 activity, each governed by a specific Atoh1. During the first phase, Atoh1b establishes a large prosensory domain within the preplacodal domain. Atoh1b activity results in Dl-Notch mediated domain restriction that separates the large prosensory domain into two smaller patches, the utricular and saccular primodia. Next, Atoh1b activity results in tether cell specification and *atoh1a* induction. The second phase begins by 14 hpf and is governed by Atoh1a activity. Atoh1a turns on Notch-mediated lateral inhibition, maintains *atoh1b* expression and specifies later forming hair cells. This shows that the two Atoh1 proteins take on distinct roles linked to their time of expression.

The specific functions of the two zebrafish *atoh1* genes are likely the product of evolutionary subfunctionalization (Force et al., 1999). Early in the teleost lineage whole genome duplication occurred. Many gene pairs divide the role of the ancestral gene and have distinct regulation. In the specification of the sensory epithelia within the ear, Atoh1b is required only for formation of the tether cells. Atoh1a is not required for tether cell formation as its expression begins after their specification. *atoh1a* is apparently unable to respond to the factors upstream of *atoh1b*. Once *atoh1b* activates expression of *atoh1a,* it functions to specify later forming hair cells. Formation of both hair cell types likely represents the ancestral functions of *atoh1*. The different functions

of *atoh1a* and *atoh1b* are probably dictated by their differential expression. The DNA binding domains of the two proteins are likely similar because early misexpression of *atoh1a* results in the formation of a greater than normal number of tether cells.

The role of mouse ATOH1 likely corresponds to the combined functions of zebrafish Atoh1a and Atoh1b. In mouse, as in zebrafish, ATOH1 is both necessary and sufficient for hair cell specification (Bermingham et al., 1999; Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). ATOH1 knockout mice form a cochlea with a single layer of cells within the sensory domain that all maintain expression of factors found within normal support cells, including $p27^{kip1}$ (Chen et al., 2002). This has led some to suggest that a sensory primordia does form in the absence of ATOH1, indicating that ATOH1 acts as a late specification factor and not as a proneural gene (Bermingham et al., 1999). However, expression of $p27^{Kip1}$ within the cochlea, preceeds that of *Atoh1*. As such, the factors that induce $p27^{Kip1}$ are not affected by a loss of ATOH1. Additionally, ATOH1 null cochlear cells also express *Atoh1,* not normally found within differentiated wild-type support cells (Fritzsch et al., 2005). Together this indicates that the cells that do form within the ATOH1 null cochlea may not be sensory primordia but rather a group of cells with a confused identity responding to inductive signals and express early markers of both hair cell and support cell markers. Although it is not yet conclusive, the data seen in mouse do not preclude the possibility that ATOH1 acts as a proneural gene.

Because SOX2 mutant mice form no sensory epithelia and lack *Atoh1* expression, some have suggested that SOX2 is likely the proneural gene responsible for

specifying the equivalence group from which hair cells form (Kiernan et al., 2005b; Dabdoub et al., 2009). Misexpression of *Sox2,* unlike that of *Atoh1*, does not result in ectopic sensory cells in mouse or in zebrafish, a necessary function for a proneural factor (Woods et al., 2004; Dabdoub et al., 2009). It is more likely that, in mouse, rather than acting as a proneural gene, SOX2 initially acts a regional specifier for the floor of the otic vesicle. As such, without SOX2 activity, all ventral fates, including that of the sensory epithelia, are lost. Zebrafish Sox2 does not play this role, as its expression begins within the equivalence group long after that of *atoh1*. Following this early role in mouse, *Sox2* expression is restricted to the sensory primordia and is eventually maintained only in support cells, as in zebrafish. This suggests that SOX2 may play a similar role in hair cell survival and regeneration in mouse and zebrafish.

CORRELATION OF FINDINGS WITH CANCER RESEARCH

Most, if not all, adult human tissues contain a small number of stem cells (reviewed in Blanpain and Fuchs, 2009). When these stem cells evade the tight genetic controls placed on their cell cycles they can become cancerous (Kashyap et al., 2009). One factor expressed in stem cells is *sox2*. In fact viral misexpression of Sox2, in concert with a number of other factors, can induce reprogramming of somatic cells into stem cells (Pei, 2009). The tight regulation of the genetic factors within stem cell populations includes regulation of *sox2,* overexpression of which may lead to cancer

formation. It has been shown that Sox2 expression is more frequently seen in tumor cells than in surrounding tissues (Ben-Porath et al., 2008). An example of a specific cancer in which Sox2 expression is found is that of colorectal polyps. Sox2 expression can be detected in this subset of colorectal cancers but not in the surrounding colon tissue (Park et al., 2008). It has been suggested that ectopic Sox2 expression may be associated with abnormal differentiation of colorectal cancer cells (Tani et al., 2007).

Colorectal tumor samples have a deficit of goblet cells, normally found throughout colon tissue (reviewed in Leow et al., 2005). In mouse, differentiation of goblet cells requires expression of Atoh1. Examination of colorectal tumor samples revealed a reduction in Atoh1 expression. Misexpression of Atoh1 within the tumor cell line results in significant inhibition of proliferation (reviewed in Leow et al., 2005). Taken together this data suggest that loss of Atoh1 expression results in a failure of colon cells to differentiate as goblet cells allowing the cells to form tumors.

We have identified both Fgf and Notch as factors that act upstream of Atoh1 and Sox2. Both Notch and Fgf have been shown to be involved in oncogenesis (Dvorak et al., 2006; Yao and Mishra, 2009). Much of the work regarding colorectal cancer has focused on the role of Wnt signaling. Recent studies have shown that Wnt acts along with Notch to maintain progenitor cell populations within the intestine, misregulation of which can lead to cancer formation (reviewed in De Lau et al., 2007; Radtke et al., 2006). A number of stem cell derived cancers exhibit overexpression or constitutive activity of Notch, including colorectal cancer (Yao and Mishra, 2009; Qiao and Wong,

2009). Because of this, Notch has become the target of many new cancer therapies (Qiao and Wong, 2009).

We have shown that Notch activity induces *sox2* expression and *atoh1* repression within the zebrafish ear. Studies in colorectal cancers exhibit both an overexpression of *sox2* and a loss of *atoh1*. Our data corrolate with the idea that misregulation of *sox2* and *atoh1* may be due to Notch overactivity. By understanding the role and regulation of both *atoh1* and *sox2* in the zebrafish ear we may further the understanding of their interactions within cancer formation.

REFERENCES

Adamska, M., Leger, S., Brand, M., Hadrys, T., Braun, T. and Bober, E. (2000). Inner ear and lateral line expression of a zebrafish Nkx5-1 gene and its downregulation in the ears fo FGF8 mutant, ace. *Mech. Dev*. **97**, 161-165.

Adler, H.J. and Raphael, Y., (1996). New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. *Neruosci. Lett*. **205**, 17-20.

Adolf, B., Bellipanni, G., Huber, V. and Bally-Cuif, L. (2004). *atoh1.2* and *beta3.1* are two new bHLH-encoding genes expressed in selective precursor cells of the zebrafish anterior hindbrain. *Gene Expr. Patterns* **5**, 35-41.

Assad, J.A., Shepherd, G.M.G. and Corey, D.P. (1991). Tip-link integrity and mechanical transduction in vertebrate hair cells. *Neuron* **7,** 985-994.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L. and Vivian, N., Lovell-Badge, R., (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.***17**, 126-140.

Baker, N. E., Yu, S. and Han, D. (1996). Evolution of proneural *atonal* expression during distinct regulatory phases in the developing *Drosophila* eye. *Curr. Biol.* **6**, 1290-1301.

Baker, N. E. and Yu, S. Y. (1997). Proneural function of neurogenic genes in the developing *Drosophila* eye. *Curr. Biol.* **7**, 122-132.

Ben-Arie, N., McCall, A.E., Berkman, S., Eichele, G., Bellen, H.J., and Zoghbi, H.J., (1996). Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. *Hum. Mol. Genet.* **5**, 1207-1216.

Ben-Porath, I., Thomson, M.W., Carey, V.J. Ge, R., Bell G.W., Regev, A. and Weinberg, R.A. (2008). An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat. Genet.* **40**, 499-507.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). *Math1*: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-1841.

Beurg, M., Nam, J.H., Crawford, A. and Fettiplace, R. (2008). The actions of calcium on hair bundle mechanics in mammalian cochlear hair cells. *Biophys. J.* **94,** 2639-2653.

Bhave, S.A., Stone, J.S., Rubel, E.W. and Coltrera, M.D. (1995). Cell cycle progression in gentamicin-damaged avian cochleas. *J. Neurosci.* **15**, 4618-4628.

Blanpain C. and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat. Rev. Mol. Cell Biol.* **10**, 207-217.

Boer, B., Kopp, J., Mallanna, S., Desler, M., Chakravathy, H., Wilder, P.J., Bernadt, C. and Rizzino, A. (2007). Elevating the levels of Sox2 in embryonal carcinoma cells and embryonic stem cells inhibits the expression of Sox2:Oct-3/4 target genes. *Nucl. Acids Res.* **35**, 1773-1786.

Brignull, H.R., Raible, D.W. and Stone, J.S. (2009). Feathers and fins: Non-mammalian models for hair cell regeneration. *Brain Res.* **1277**, 12-23.

Brooker, R., Hozumi, K. and Lewis, J. (2006). Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. *Development* **133**, 1277-1285.

Brunet, J.-F. and Ghysen, A. (1999). Deconstructing cell determination: proneural genes and neuronal identity. *BioEssays* **21**, 313-318.

Bylund, M., Andersson, E., Novitch, B.G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.

Cabrera, C.V. and Alonso, M.C. (1991). Transcriptional activation by heterodimers of the aschaete-scute and daughterless gene products of Drosophila. *EMBO J.* **10**, 2965-2973.

Cafaro, J., Lee, G.S. and Stone, J.S. (2007). *Atoh1* expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Dev. Dyn.* **236**, 156-170.

Chardin S. and Romand R. (1995). Regeneration and mammalian auditory hair cells. *Science* **267**, 707-711.

Chen, P. and Segil, N. (1999). p27kip1 links cell proliferation to morphogenesis in the developing organ of Corti. *Development* **126**, 1581-1590.

Chen, P., Johnson, J. E., Zoghbi, H. Y. and Segil, N. (2002). The role of *Math1* in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* **129**, 2495-2505.

Chen, P., Zindy, f., Abdala, c., Liu, F., Roussel, M.F. and Segil, N. (2003) Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. *Nat. Cell. Biol.* **5**, 422-426.

Chien, C.T., Hsiao, C.D., Jan, L.Y., and Jan, Y.N., (1996) Neuronal type information encoded in the basic-helix-loop-helix domain of proneural genes. *Proc. Natl. Acad. Sci. USA* **12**, 13239-12344.

Chitnis, A. and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embryos. *Development* **122**, 2295-2301.

Corey, D.P. and Hudspeth, A.J. (1983). Kinetics of the receptor current in bullfrog saccular hair cells. *J. Neurosci.* **3**, 962-976.

Corwin, J.T. and Cotanche, D.A. (1988). Regeneration of sensory hair cells after acoustic trauma. *Science* **240**, 1772-1774.

Corwin, J.T. and Oberholtzer, J.C. (1997). Fish n' chicks: model recipes for hair-cell regeneration? *Neuron* **19**, 951-954.

Dabdoub, A., Puligilla, C., Jones, J.M., Fritzsch, B., Cheah, K.S., Pevny, L.H. and Kelley, M.W. (2009). Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc. Natl. Acad. Sci. USA* **105,** 18396-18401.

Daudet, N. and Lewis, J. (2005). Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* **132**, 541-551.

De Lau, W., Barker, N. and Clevers, H. (2007). WNT signaling in the normal intestine and colorectal cancer. *Front. Biosci.* **12**. 471-491.

Du, X., Jensen, P., Goldowitz, D. and Hamre, K.M. (2007), Wild-type cells rescue genotypically *Math1*-null hair cells in the inner ears of chimeric mice. *Dev. Biol.* **305**, 430-438.

Duncan, L.J., Mangiardi, D.A., Matsui, J.I., Anderson, J.K., McLaughlin-Williamson, K. and Cotanche, D.A. (2006). Differential expression of unconventional myosins in apoptotic and regenerating chick hair cells confirms two regeneration mechanisms. *J. Comp. Neurol.* **499**, 691-701.

Dvorak, P., Dvorakova, D. and Hampl, A. (2006). Fibroblast gwth factor signaling in embryonic and cancer stem cells. *FEBS Lett.* **580,** 2869-2874.

Eddison, M., Le Roux, I. and Lewis, J. (2000). Notch signaling in the development of the inner ear: lessons from *Drosophila*. *Proc. Natl. Acad. Sci. USA* **97**, 11692-11699.

Edge, A. and Chen, Z-Y. (2008). Hair cell regeneration. *Curr. Opin. Neurobiol.* **18**, 377-382.

Episkopou, V. (2005). SOX2 functions in adult neural stem cells. *Trends Neurosci.* **28**, 219-221.

Ferri, A.L., Cavallaro, M., Braida, D., Di Cristofano, A., Canta, A., Vezzani, A., Ottolenghi, S., Pandolfi, P.P., Sala, M., DeBiasi, S. and Nicolis, S.K. (2004). Sox2 deficiencey causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development.* **131**, 3805-3819.

Fettiplace, R. and Hakeny (2006). Active hair bundle movements in auditory hair cells. *J. Physiol.* **576,** 29-36.

Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan., Y.-L. and Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**, 1531-1545.

Fritz, A., Rozkowski, M., Walker, C., and Westerfield, M. (1996). Identification of selected gamma-ray induced deficiencies in zebrafish using multiplex polymerase chain reaction. *Genesis* **144**, 1735-1745.

Fritzsch, B., Matei, V. A., Nichols, D. H., Bermingham, N., Jones, K., Beisel, K. W. Wang, V. Y. (2005). *Atoh1* null mice show directed afferent fiber growth to undifferentiated ear sensory epithelia followed by incomplete fiber retention. *Dev. Dyn.* **233**, 570-583.

Gillespie, P.G. and Muller, U. (2009). Mechanotransduction by hair cells: models, molecules, and mechanisms. *Cell* **139,** 33-44.

Graham, V., Khurdyakov, J., Ellis, P. and Pevny, L. (2003). Sox2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.

Gubbay, J., Collingnon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P. and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346**, 245-250.

Haddon, C. and Lewis, J. (1996). Early ear development in the embryo of the zebrafish, *Danio rerio*. *J. Comp. Neurol.* **365**, 113-128.

Haddon, C., Jiang, Y.J,, Smithers, L. and Lewis, J. (1998) Delta-Notch signaling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the *mind bomb* mutant. *Development* **125**, 4637-4644.

Haddon, C., Mowbray, C., Whitfield, T., Hones, D., Gschmeissner, S. and Lewis, J. (1999). Hair cells without supporting cells: further studies in the ear of the zebrafish *mind bomb* mutant. *J. Neurocytol.* **28**, 837-850.

Hagstrum, S.A., Pauer, G.J., Reid, J., Sipson, E., Crowe, S., Maumenee, I.H. and Traboulsi, E.I. (2005). SOX2 mutation causes anophthalmia, hearing loss, and brain anomalies. *Am. J. Med. Genet. A.* **138A**, 95-98.

Hans, S., Liu, D. and Westerfield, M. (2004). Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors. *Development* **131**, 5091-5102.

Hassan, B. A. and Bellen, H. J. (2000). Doing the MATH: is the mouse a good model for fly development? *Genes Dev.* **14**, 1852-1865.

Hayashi, T., Ray, C.A. and Bermingham-McDonogh, O. (2008). *Fgf20* is required for sensory epithelial specification in the developing cochlea. J. *Neurosci.* **28**, 5991-5998.

Heller, N. and Brandi, A.W. (1999). *Xenopus* Pax 2/5/8 orthologues: novel insights into *PaxI* gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. *Dev. Genet.* **24**, 208-219.

Helms, A. W., Abney, A. L., Ben-Arei, N., Zoghbi, H. Y. and Johnson, J. E. (2000). Autoregulation and multiple enhancers control *Math1* expression in the developing nervous system. *Development* **127**, 1185-1196.

Hernandez, P.P., Olivari, F.A., Sarrazin, A.F., Sandoval, P.C. and Allende, M.L. (2007) Regeneration in zebrafish lateral line neuromasts: expression of the progenitor cell marker Sox2 and proliferation-dependent and –independent mechanisms of hair cell renewal. *Dev. Neurobiol.* **67**, 637-654.

Hertzano, R., Montcouquiol, M., Rashi-Elkeles, S., Elkon, R., Yucel, R., Frankel, W.N., Rechavi, G., Moroy, T., Friedman, T.B., Kelley, M.W. and Avraham, K.B. (2004). Transcription profiling of inner ears from Pou4f3(ddl/ddl) identifies Gfi1 as a target fo the Pou4f3 deafness gene. *Hum. Mol. Genet.* **13**, 2143-2153.

Hori, R., Nakagawa, T., Sakamoto, T., Matsuoka, Y., Takebayashi, S. and Ito, J. (2007). Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea. *Neuroreport* **18,** 1922-1914.

Hume, C.R., Bratt, D.L. and Oesterle, E.C. (2007). Expression of LHX3 and SOX2 during mouse inner ear development. *Gene. Expr. Patterns* **7**, 798-807.

Itoh, M. and Chitnis, A. B. (2001). Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. *Mech. Dev.***102**, 263-266.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L., Weissman, A. M., Lewis, J., Chandrasekharappa, S. C. and Chitnis, A. B. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Izumikawa, M., Minoda, R., Kawamoto, K., Abrashkin, K. A., Swiderski, D. L., Dolan, D. F., Brough, D. E. and Raphael, Y. (2005). Auditory hair cell replacement and hearing improvement by *Atoh1* gene therapy in deaf mammals. *Nat. Med.* **3**, 271-276.

Jarman, A.P., Grau, Y., Jan, L.Y. and Jan, Y.N. (1993). Atonal is a proneural gene that directs chordotonal organ formation in the Drosophila periperal nervous system. *Cell* **73**, 1307-1321.

Jarman, A. P., Sun, Y., Jan, L. Y. and Jan, Y. N. (1995). Role of the proneural gene, *atonal,* in formation of *Drosophila* chordotonal organs and photoreceptors. *Development* **121**, 2019-2030.

Jowett, T. and Yan, Y. L. (1996). Double fluorescent *in situ* hybridization to zebrafish embryos. *Trends Genet.* **12**, 387-389.

Kachar, B., Parakkal, M., Kurc, M., Zhao, Y. and Gillespie, P.G. (2000). High-resolution structure of hair-cell tip links. *Proc. Natl. Acad. Sci. USA* **97,** 13336-13341.

Kashyap, V., Rezende, N.C., Scotland, K.B., Shaffer, S.M., Persson, J.L., Gudas,

L.J. and Mongan, N.P. (2009). Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the Nanog, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. *Stem cells Dev*. **18**, 1093-1108.

Kelley, M.W., Talreja, D.R. and Corwin, J.T. (1995). Replacement of hair cells after laser microbeam irradiation in cultured organs of Corti from embryonic and neonatal mice. *J. Neurosci*. **15**, 3013-3026.

Kelley, M. W. (2006). Regulation of cell fate in the sensory epithelia of the inner ear. *Nat. Rev. Neurosci*. **7**, 837-849.

Kiernan, A. E., Ahituv, N., Fuchs, H., Balling, R., Avraham, K. B., Steel, K. P. and de Angelis, H. M. (2001). The Notch ligand Jagged1 is required for inner ear sensory development. *Proc. Natl. Acad. Sci. USA* **98**, 3873-3878.

Kiernan, A. E., Cordes, R., Kopan, R., Glosser, A. and Gridley, T. (2005a). The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. *Development* **132**, 4353-4362.

Kiernan, A. E., Pelling, A. L., Leung, K. K. H., Tang, A. S. P., Bell, D. M., Tease, C., Lovell-Badge, R., Steel, P. K. and Cheah, K. S. E. (2005b). *Sox2* is required for sensory organ development in the mammalian inner ear. *Nature* **434**, 1031-1035.

Kiernan, A. E., Xu, J. and Gridley, T. (2006). The Notch ligand Jag1 is required for sensory progenitor development in the mammalian inner ear. *PloS Genet*. **2**, 27-38.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Kishi, M., Mizuseki, K., Sasai, N., Yamazaki, H., Shiota, K., Nakanishi, S. and Sasai, Y. (2000). Requirement of Sox2-mediated signaling for differentiation of early *Xenopus* neurectoderm. *Development* **127**, 791-800.

Kopp, J.L., Ormsbee, B. D., Desler, M. and Rizzino, A. (2008) Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. *Stem Cells* **26,** 903-911.

Kwak, S.-J., Phillips, B. T., Heck, R. and Riley, B. B. (2002). An expanded domain of *fgf3* expression in the hindbrain of zebrafish *valentino* mutants results in mis-patterning of the otic vesicle. *Development* **129**, 5279-5287.

Kwak, S.J., Vemaraju, S., Moorman, S. J., Zeddies, D., Popper, A. N. and Riley, B. B. (2006). Zebrafish *pax5* regulates development of the utricular macula and vestibular function. *Dev. Dynam.* **235**, 3026-3038.

Kwan, T., White, P.M. and Segil, N. (2009). Development and regeneration of the inner ear. *Ann. N.Y. Acad. Sci.* **1170**, 28-33.

Lanford, P. J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T. and Kelley, M. W. (1999). Notch signaling pathway mediates hair cell development in mammalian cochlea. *Nature Genetics* **21**, 289-292.

Lanford, P. J., Shailam, R., Norton, C. R., Gridley, T. and Kelley, M. W. (2000). Expression of Math1 and HES5 in the cochlea of wildtype and Jag2 mutant mice. *JARO* **1**, 161-171.

Latimer, A. J., Shin, J. and Appel., B. (2005). *her9* promotes floor plate development in zebrafish. *Dev. Dyn.* **232**, 1098-1104.

Leger, S. and Brand, M. (2002). Fgf8 and Fgf3 are required for zebrafish ear placode induction, maintenance and inner ear patterning. *Mech. Dev.* **119**, 91-108.

Leow, C.C., Polakis, P. and Gao, W.Q. (2005). A role for Hath1, a bHLH transcription factor, in colon adenocarcinoma. *Ann. N.Y. Acad. Sci.* **1059**, 174-183.

Ligoxygakis, P. Yu, S.-Y., Delidakis, C. and Baker, N. E. (1998). A subset of *Notch* functions during *Drosophila* eye development require *Su(H)* and the *E9spl)* gene complex. *Development* **125**, 2893-2900.

Li, S., Price, S.M., Cahill, H., Ryugo, D.K., Shen, M.M. and Xiang, M. (2002). Hearing loss caused by progressive degeneration of cochlear hair cells in mice deficient for the Barh11 homeobox gene. *Development* **129**, 3523-3532.

Liu, D., Chu, H., Maves, L., Yan, Y.-L., Morcos, P. A., Postlethwait, J. H. and Westerfield, M. (2003). Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. *Development* **130**, 2213-2224.

Lombardo, A., Isaacs, H.V. and Slack, J.M.W. (1998). Expression and functions of Fgf3 in Xenopus development. *Int. J. Dev. Biol.* **42**, 1101-1107.

Lowenheim, H., Furness, d.N., Kil, J., Zinn, C., Gultig, K., Fero, M.L., Frost, D., Gummer, A.W., Roberts, J.M., Rubel, E.W., Hackney, C.M. and Zenner, H.P. (1999). Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of Corti. *Proc. Natl. Acad. Sci. USA* **96**, 4084-4088.

Lun, K. and Brand, M. (1998) A series of *no isthmus* (*noi*) alleles of the zebrafish *pax2.1* gene reveals multiple signaling events in development of the midbrain-hindbrain boundary. *Development* **125**, 3049-3062.

Ma, E.Y., Rubel, E.W. and Raible, D.W. (2008) Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. *J. Neurosci.* **28**, 2261-2273.

Mackereth, M. D., Kwak, S.-J., Fritz, A. and Riley, B. B. (2005). Zebrafish *pax8* is required for otic placode induction and plays a redundant role with *Pax2* genes in the maintenance of the otic placode. *Development* **132**, 371-382.

Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C. and Mason, I. (1995). Multiple roles for Fgf3 during cranial neural development in the chicken. *Development* **121**, 1399-1410.

Mahmood. R., Mason, I.J. and Morrisskay, G.M. (1996). Expression of Fgf3 in relation to hindbrain segmentation, otic pit position and pharyngeal arch morphology in normal and retinoic acid exposed mouse embryos. *Anat. & Embryol.* **194**, 13-22.

Maroon, H., Walshe, J., Mahmood, R., Keifer, P., Dickson, C. and Mason, I. (2002). Fgf3 and Fgf8 are required together for formation of the otic placode and vesicle. *Development* **129**, 2099-2108.

Masui, S., Nakatake, Y., toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., Ko, M.S. and Niwa, H. (2007) Pluripotency governed by *Sox2* via regulation of *Oct3/4* expression in mouse embryonic stem cells. *Nat. Cell Biol.* **9**, 625-625.

Matei, V., Pauley, S., Kaing, S., Rowitch, D., Beisel, K.W., Morris, K., Feng, F., Jones, K., Lee, J. and Fritzsch, B. (2005). Smaller inner ear sensory epithelia in Neurog1 null mice are related to earlier hair cell cycle exit. *Dev. Dyn.* **234**, 633-650.

McKay, I.J., Lewis, J. and Lumsden, A. (1996). The role of Fgf3 in early inner ear development: an analysis in normal kreisler mutant mice. *Dev. Biol.* **174**, 370-378.

Millimaki, B.B., Sweet, E.M., Dhason, M.S. and Riley, B.B. (2007). Zebrafish *atoh1* genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. *Development* **134**, 295-305.

Neves, J., Kamaid, A., Alsina, B. and Giraldez, F. (2007) Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick. *J. Comp. Neurol.* **503**, 487-500.

Nicolson, T. (2005). The gentics of hearing and balance in zebrafish. *Annu. Rev. Genet.* **39**, 9-22.

Niwa, N., Hiromi, Y. and Okabe, M. (2004). A conserved developmental program for sensory organ formation in *Drosophila melanogaster*. *Nat. Genet.* **36**, 293-297.

Osterle, E.C., Campbell, S., Taylor, R.R., Forge, A. and Hume, C.R. (2008). Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *Assoc. Res. Otolaryngol.* **9**, 65-68.

Ozeki, H., Oshima, K., Senn, P., Kurihara, H. and Kaga, K. (2007). Development and regeneration of hair cells. *Acta. Orolaryngol. Suppl.* **559**, 38-44.

Park, E.T., Gum, J.R., Kakar, S., Kwon, S.W., Deng, G. and Kim, Y.S. (2008). Aberrant expression of SOX2 upregulates MUC5AC gastric foveolar mucin in mucinous cancers of the colorectum and related lesions. *Int. J. Cancer.* **122**, 1253-1260.

Parks, A. L., Huppert, S. S. and Muskavitch, M. A. (1997). The dynamics of neurogenic signalling underlying bristle development in *Drosophila melanogaster*. *Mech. Dev.* **63**, 61-74.

Pei, D. (2009). Regulation of pluripotency and reprogramming by transcription factors. *J. Biol. Chem.* **284**, 3365-3369.

Pfeffer, P. L., Gerster, T., Lun, K., Brand, M. and Busslinger, M. (1998). Characterization of three novel members of the zebrafish *Pax2/5/8* family: dependency of *Pax5* and *Pax8* expression on the *Pax2.1* (*noi*) function. *Development* **125**, 3063-3074.

Phillips, B. T., Bolding, K. and Riley, B. B. (2001). Zebrafish *fgf3* and *fgf8* encode redundant functions required for otic placode induction. *Dev. Biol.* **235**, 351-365.

Phillips, B.T., Storch, E.M., Lekven, A.C. and Riley, B.B. (2004). A direct role for Fgf but not Wnt in otic placode induction. *Development* **131**, 923-932.

Pi, H. and Chien, C.T. (2007) Getting the edge: neural precursor selection. *J. Biolmed. Sci.* **14**, 467-473.

Pickles, J.O., Comis, S.d. and Osborne, M.P. (1984). Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction. *Hear. Res.* **15**, 103-112.

Pirvola, U., Ylikoski, J., Trokovic, R., Hebert, J. M., McConnell, S. K. and Partanen, J. (2002). FGFR1 is required for the development of the auditory sensory epithelium. *Neuron* **35**, 671-680.

Popper, A.N. and Fay, R.R. (1993). Sound detection and processing by fish: critical review and major research questions. *Brain Behav. Evol.* **41**, 14-38.

Popper, A.N. and Fay, R.R. (1997). Evolution of the ear and hearing: issues and questions. *Brain Behav. Evol.* **50**, 213-221.

Pujades, C., Kamaid, A., Alsina, B., Giraldez., F. (2006). BMP-signaling regulates the generation of hair-cells. *Dev. Biol.* **292**, 55-67.

Pujic, Z., Omori, Y., Tsujikawa, M., Thisse, B., Thisse, C. and Malicki, J. (2006) Reverse genetic analysis of neurogenesis in the zebrafish retina. *Dev. Biol.* **293**, 330-347.

Qiao, L. and Wong, B.C. (2009) Role of Notch signaling in colorectal cancer. *Carcinogenesis.* **30**, 1979-1986.

Radtke, F., Clevers, H. and Riccio, O. (2006). From gut homeostasis to cancer. *Curr. Mol. Med.* **6**. 275-285.

Riley, B. B., Zhu, C., Janetopoulos, C. and Aufderheide, K. J. (1997). A critical period of ear development controlled by distinct populations of ciliated cells in the zebrafish. *Dev. Biol.* **191**, 191-201.

Riley, B. B., Chiang, M.-Y., Farmer, L. and Heck, R. (1999). The *deltaA* gene of zebrafish mediates lateral inhibition of hair cells in the inner ear and is regulated by *pax2.1*. *Development* **126**, 5669-5678.

Riley, B.B. and Phillips, B.T. (2003). Ringing in the new ear: resolution of cell interactions in otic development. *Dev. Biol* **261**, 289-312.

Robu, M.E., Larson, J.K., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., and Ekker, S.C. (2007) p53 activation by knockdown technologies. *PLoS Genetics* **3**, e78.

Ryals B.M. and Rubel E.W. (1988) Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* **240**, 1774-1776.

Sarrazin, A. F., Villablanca, E. J., Nuñez, V. A., Sandoval, P. C., Ghysen, A. and Allende, M. L. (2006). Proneural gene requirement for hair cell differentiation in the zebrafish lateral line. *Dev. Biol.* **295**, 534-545.

Scheer, N. and Campos-Ortega, J. A. (1999). Use of the Gal4-UAS technique for targeted gene expression the zebrafish. *Mech. Dev.* **80**, 153-158.

Scheer, N., Riedl, I., Warren, J. T., Kuwada, J. Y. and Campos-Ortega, J. A. (2002). A quantitative analysis of the kinetics of Gal4 activator and effector gene expression in zebrafish. *Mech. Dev.* **112**, 9-14.

Seidman, M.D., Ahmad, N. and Bai, U. (2002). Molecular mechanisms of age-related hearing loss. *Ageing Res. Rev.* **1:** 331-343.

Shailam, R., Lanford, P.J., Dolinsky, C. M., Norton, C. R., Gridley, T. and Kelley, M. W. (1999). Expression of proneural and neurogenic genes in the embryonic mammalian vestibular system. *J. Neurocytol.* **28**, 809-819.

Shi, Y., Sun, G., Zhao, C. and Stewart, R. (2008). Neural stem cell self-renewal. *Crit. Rev. Oncol. Hematol.* **65**, 43-53.

Shoji, W., Yee, C. S. and Kuwada, J. Y. (1998). Zebrafish Semaphorin Z1a collapses growth cones and alters their pathway *in vivo*. *Development* **125**, 1275-1283.

Shou, J., Zheng, J.L., and Gao, W.Q. (2003). Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of Hath1. *Mol. Cell Neurosci.* **23**, 169-179.

Solomon, K, and Fritz, A. (2002). Concerted action of two *dlx* paralogs in sensory placode formation. *Development* **129**, 3127-3136.

Solomon, K. S., Kudoh, T., Dawid, I. and Fritz, A. (2003). Zebrafish *foxi1* mediates otic placode formation and jaw development. *Development* **130**, 929-940.

Solomon, K. S., Kwak, S. J. and Fritz, A. (2004). Genetic interactions underlying otic placode induction and formation. *Dev. Dynam.* **230**, 419-433.

Stevens, C. B., Davies, A. L., Battista, S., Lewis, J. H. and Fekete, D. M. (2003). Forced activation of Wnt signaling alters morphogenesis and sensory organ identity in the chicken inner ear. *Dev. Biol.* **261**, 149-164.

Stone, J.S. and Cotanche, D.A. (2007). Hair cell regeneration in the avian auditory epithelium. *Int. J. Dev. Biol.* **51**, 633-647.

Sun, Y., Jan, L. Y. and Jan, Y. N. (1998). Transcriptional regulation of *atonal* during development of the *Drosophila* peripheral nervous system. *Development* **125**, 3731-3740.

Takahahsi K. and Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.

Tani, Y., Akiyama, Y., Fukamachi, H., Yanagihara, K. and Yuasa, Y. (2007). Transcription factor SOX2 up=regulates stomach-specific pepsinogen A gene expression. *J. Cancer Res. Clin. Oncol.* **133**, 263-369.

Thermes V. (2002) *I-SceI* meganuclease mediates highly efficient transgenesis in fish. *Mech. Dev.* **118**, 91-98.

Torres, M. and Giraldez, F. (1998) The development of the vertebrate inner ear. *Mech. Dev.* **71**, 5-21.

Vollrath, M.A., Kwan, K.Y., and Corey, D.P. (2007). The micromachinery of mechanotransduction in hair cells. *Annu. Rev. Neurosci.* **30,** 339-365.

Wallis, D., Hamblen, M., Zhou, Y., Venken, K.J., Schumacher, A., Grimes, H.L., Zoghbi, H.Y., Orkin, S.H., Bellen, H.J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development* **130**, 221-232.

Wang, V. Y., Hassan, B. A., Bellen, H. J. and Zoghbi, H. Y. (2002). Drosophila *atonal* fully rescues the phenotype of *Math1* null mice: new functions evolve in new cellular contexts. *Curr. Biol.* **12**, 1611-1616.

Westerman, B. A., Murre, C. and Oudejans, C. B. (2003). The cellular Pax-Hox-helix connection. *Biochim Biophys Acta.* **1629**, 1-7.

Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The *Xenopus* homolog of *Drosophila Suppressor of Hairless* mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.

Whitfield T. T., Riley, B. B., Chiang, M.-Y. and Phillips, B. (2002). Development of the zebrafish inner ear. *Dev. Dynam.* **223**, 427-458.

Wright, E.M., Snopek, B. and Koopman, P. (1993). Seven new members of the Sox gene family expressed during mouse development. *Nucleic Acids Res.* **21**, 744.

Wright, T.J. and Mansour, S.L. (2003). Fgf3 and Fgf10 are required for mouse otic placode induction. *Development* **130**, 3379-3390.

Woods, C., Montacouquiol, M. and Kelley, M.W. (2004). Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nat. Neurosci.* **7**, 1310-1318.

Woods, C., Montcouquiol, M. and Kelley, M. W. (2005). Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nat. Neurosci.* **7**, 1310-1318.

Xiang, M., Gao, W.Q., Hasson, T. and Shin, J.J. (1998). Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. *Development* **125**, 3935-3946.

Xiao, T., Roeser, R., Staub, W. and Baier, H. (2005). A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development* **132**, 2995-2967.

Yao, Z. and Mishra, L. (2009). Cancer stem cells and hepatocellular carcinoma. *Cancer Bio. Ther.* **8**, 1692-1698.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Sterwart, R., Slukvin, I.I. and Thomson, J.A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920.

Zheng, J. L. and Gao, W. Q. (2000). Overexpression of *Math1* induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* **3**, 580-586.

Zine, A., Van De Water, T.R. and de Ribaupierre, F. (2000). Nothc signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* **127**, 3373-3383.

zur Lage, P. I., Jan, Y. N. and Jarman, A. P. (1997). Requirements for EGF receptor signaling in neural recruitment during formation of *Drosophila* chordotonal sense organ clusters. *Curr. Biol.* **7**, 116-175.

zur Lage, P. I., Powell L. M., Prentice, R. R. A., McLaughlin, P. and Jarman, A. P. (2004). EGF receptor signaling triggers recruitment of *Drosophila* sense organ precursors by stimulating proneural gene autoregulation. *Dev. Cell* **7**, 687-696.

VITA

Name: Bonny Butler Millimaki

Address: 3258 TAMU College Station TX, 77843-3258

Email Address: bonnyb@tamu.edu

Education: Ph.D. Texas A&M University, 2010

B.S., Middle Tennessee State University, 2003

Publications: **Millimaki, B.B**., Sweet, E.M. and Riley, B.B. (2010). Sox2 is required for maintenance and regeneration, but not initial development, of hair cells in the zebrafish inner ear. *Dev. Biol.* **338**, 262-9.

Petko, J.A., **Millimaki, B.B**., Canfield, V.A., Riley, B.B. and Levenson R**.** (2008). Otoc1: a novel otoconin-90 ortholog required for otolith mineralization in zebrafish. *Dev. Neurobiol.* **68,** 209-22.

Millimaki, B.B., Sweet, E.M., Dhason, M.S. and Riley, B.B. (2007). Zebrafish atoh1 genes: classic proneural activity in the inner ear and regulation by FgF and Notch. *Development* **134**, 295-305.

Honors NSF GK12 Fellow (2007-2008).

First prize in Genetics Research Symposium, oral competition (2007). "*atoh1* Genes Play a Classic Proneural Role in Hair Cell Specification."

Finalist in poster presentation at the International Zebrafish Conference, Madison WI (2006). *"atoh1* Genes Play a Classic Proneural Role in Hair Cell Specification and are Induced by Fgf and Pax."