FASCIN 2B IS A COMPONENT OF ZEBRAFISH STEREOCILIA AND A REGULATOR OF THEIR DIMENSIONS

By

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ABSTRACT

Title: Fascin 2B is a Component of Zebrafish Stereocilia and a Regulator of Their Dimensions

SHIH-WEI CHOU

Hearing is among the most important senses because this sense enables verbal communication, the basis of human culture. Since 1 newborn in 1000 is born deaf this puts a heavy burden on the afflicted. Hearing is mediated by hair cells, which are specialist in mechanotransduction, the conversion of mechanical stimuli into electrical responses. The organelles that carryout mechanotransduction are the stereocilia, actin-filled organelles that flex at their bases.

Hair bundles, highly organized groups of stereocilia, are commonly damage by excessive noise or genetic lesions resulting in hair cell death and hearing loss. Unfortunately, humans and other mammals do not regenerate hair cells; therefore, understanding the molecular mechanisms that build and maintain the integrity of the hair bundle, not only will help us elucidate the genetic factors involve in inherited deafness and age-related hearing loss, but also may open therapeutic opportunities for hearing restoration.

During the course of my thesis research, I identified a new class of actin cross-linking protein, fascin 2b, that localizes specifically to stereocilia. Using zebrafish transgenesis, I determined that fascin 2b is a modulator of stereociliary length and elucidated the mechanism of its specific localization. Additionally, using several types of newly invented genome editing technologies, I created several novel zebrafish gene knockouts, which are hypothesized to play a role in actin cross-linking or mechanotransduction.
My studies advance what is known about the mechanism of actin filament assembly in stereocilia and therefore hearing.
INTRODUCTION
Hair cell, hearing, and balance

Hearing, one of the most important sensory systems, is mediated by the highly specialized mechanosensory hair cells in the inner ear. It is reported by the National Institute on Deafness and other Communication Disorders (NIDCD) that about 17 percent (36 million) of American adults experience some degree of hearing loss. Hearing disabilities include those caused by inherited genetic mutations, such as dominant nonsyndromic deafness DFNA, recessive nonsyndromic deafness DFNB, Usher syndrome—which is described as carrying vision loss, vestibular dysfunction and congenital deafness. Noise-damaged or chemical-induced hair cell death can also result in acute or gradual hearing loss. As Beethoven poignantly wrote in his communication book after he lost his hearing¹, "... Oh how happy I should be if my hearing were completely restored, then I would hurry to you...". It emphasizes that deafness affects not only the ability to appreciate music but also importantly the ability to communicate with others and to feel involved in the community. Unfortunately, our society still does not quite know how to handle hearing disorders, as it was reported from the Disability Statistics Compendium in 2013 that the unemployment rate of deaf people is 40 - 60 %. Therefore, how hearing happens in the ear and how can it be repaired if broken is a substantial question in biology and for modern society.

The human ear is composed of the outer ear, the middle ear, and the inner ear (Figure 1.1 A). Sounds collected by the pinna pass through the external ear canal, and reach the middle ear where the compression of the air vibrates the tympanic membrane, also known as the eardrum, and the three ossicles attached to it. Then, this vibration is transmitted to the fluid-filled cochlea in the inner ear. The cochlea is a spiral-shaped cavity that consists of the Organ of Corti, where the sensory epithelium is distributed. In the Organ of Corti, vibration

¹
of the basilar membrane makes the hair cells sitting on it move back and forth relative to the tectorial membrane above the cells (Figure 1.1 B). These displacements result in the depolarization of the hair cells, and leads to the release of neurotransmitter to the contacting afferent fibers. Hence, hair cells, as mechanoreceptors, convert mechanical stimuli into electrical signals that are passed onto axon terminals and finally to the brain. Mechanotransduction is carried out by hair cells for hearing, balance, and the detection of water movement in some aquatic vertebrates; hair cells are found in the vestibular system and semicircular canals of the inner ear and also in the lateral line neuromasts of fishes and amphibians.

Figure 1.1

Figure 1.1. Hearing is mediated by mechanosensitive hair cells in the inner ear. (A) Structure of a human ear. Sound waves are collected by the external ear canal, and then vibrate the eardrum and ossicles in the middle ear. The inner ear contains sensory organs, the cochlea and the vestibular labyrinth, for hearing and balance, respectively. The stapes in the middle ear aids in transmitting vibrations to the oval window of the cochlea. (B) Schematic
of a cross section of the cochlea reveals that one row of inner hair cells and three rows of outer hair cells reside between the basilar and the tectorial membranes in the Organ of Corti. Figure adapted from Frolenkov et al., 2004².

Hair cells are specialized epithelial cells that have a hair bundle comprised of stereocilia, actin filament based structures, which protrude from the apical surface with gradually increasing lengths (Figure 1.2 A). Sound waves enter the inner ear and deflect the stereocilia towards the taller rows, opening up the transduction channels on the tips of each stereocilium and generating the receptor potential³. Located in the inner ear, hair cells are surrounded by supporting cells (Figure 1.2 A, B). Supporting cells maintain the integrity of the sensory cells by playing a role in ion homeostasis.
Figure 1.2. The sensory epithelium is composed of hair cells and supporting cells. (A) A schematic diagram shows a hair cell surrounded by two supporting cells. The hair cell has a prominent hair bundle, stereocilia and kinocilium, at its apical surface, and is innervated by afferent and efferent neurons at the basal region of the cell. Notch signaling specifies the cell fate of the hair cell. Adapted from Kandel, Schwartz & Jessell. Originally published in Principles of Neural Science, 4th Edition⁴. (B) An electron micrograph of a longitudinal section of the apical surface of the hair cell reveals rows of stereocilia (S) and the rootlets (R), which insert into the cuticular plate (CP) resting above a bed of mitochondria (M). Short microvilli (MV) are also present at the apical surface of the supporting cell (SC). Figure adapted from Tilney et al., 1989⁵.
Mechanotransduction in the hair cell

It has been demonstrated that the deflection of the hair bundle toward the tallest row of stereocilia opens the mechanotransduction channels located at the tips of the protrusions permitting entry of cations into the cell\(^6\), depolarizing the cell (Figure 1.3 A). If the deflection of the hair bundle occurs in the opposite direction, it closes the mechanotransduction channels. In hair cells, mechanotransduction is defined as the conversion of mechanical forces to electrical signals, and is thought to be conducted by tip links connecting the neighboring stereocilia, the gating spring molecules associate to the transduction channel, and the pore-forming ion channel located at the tip of the hair bundles\(^7\)-\(^10\). The widely accepted model now is the gating spring model proposed by Corey and Hudspeth in 1980s\(^9\), based on their observations in hair cells of bullfrogs: (1) the activation of the transduction is in a linear relationship with the distance of the hair bundle displacement\(^6,9\); (2) this reaction responds to the stimulation, is fast, and has a rapid relaxation\(^6\); (3) there is a short delay of current generation after the rise of signals of stimulation can be seen\(^13\). Thus, it is hypothesized that sliding the shorter stereocilium towards the taller one tightens the tension placed on the tip link, which connects directly to the gating spring, an elastic element that pulls on the transduction channel. Thus the force applied to the stereocilia is transmitted to the mechanically gated channel and opens the ion channel (Figure 1.3 A). The characteristics of the mechanotransduction mediated by the hair bundle have been described physiologically and morphologically in the past 40 years, but molecular identities of the tip links have just begun to be found in the last 15 years, and identities of the elastic element and the transduction channel remain controversial\(^14\). Hair cell
mechanotransduction has two types of adaptation, fast and slow, which are defined as a decreased current while stimulating with constant force over time, and the further stimulation recaptures the current\textsuperscript{15,16}. Adaptation is implicated in shifting resting potentials. Therefore, this unique quality is important for refining the operating range of hair bundles, preserving high signal-to-noise sensitivity, and preventing damage of hair cells from overstimulation\textsuperscript{17,18}. Studies in non-mammalian cells showed that the fast adaptation happens over a period of less than one millisecond. This process is thought to be because of an intrinsic property of the channel protein itself in responding to calcium ions, or a function of the hypothetical gating spring molecule\textsuperscript{15,19,20}. The slow adaption on the other hand takes place in tens of milliseconds. This process is thought to be mediated by the movement of myosins along the actin filaments, which relaxes the tension on the tip link\textsuperscript{21-23}. Hair bundles are situated in a fluid-filled environment known as endolymph in the mammalian inner ear. The endolymph environment is high in potassium and low in calcium. When the mechanotransduction channel opens, a current is generated mainly by the influx of Ca\textsuperscript{2+} and K\textsuperscript{+} through the non-selective cation channel at the tips of the stereocilia\textsuperscript{24}. Depolarization triggers the influx of Ca\textsuperscript{2+} at the basolateral surface of the hair cell, thus inducing the release of neurotransmitter (Figure 1.3 B). Subsequently, the mechanical stimuli are converted to electrical signals that propagate along the afferent fibers to the central nervous system to elicit sensation.
Figure 1.3
Figure 1.3. Mechanotransduction through hair cell stereocilia. (A) A schematic diagram shows that the opening of the mechanotransduction channel is in response to the deflection of the hair bundle in the positive direction, towards the tallest stereocilium. This permits the entry of positively charged ions. (B) Once the mechanotransduction channels are open, influx of Ca\(^{2+}\) and K\(^+\) at the tips of the stereocilia depolarizes the hair cell. Calcium-induced neurotransmitter release takes place at the basal-lateral region of the cell that is contacted by afferent neurons. Micrograph of hair cell captured by A.J. Hudspeth.

Actin and the cytoskeletal composition of the apical surface of the hair cell

Actin serves as a major structural component of all cells’ cytoskeletons, including microfilaments, thin filaments, and part of the contractile apparatus in myofibers. In animal cells, actin filaments are important for constructing the cell shapes and are responsible for actively adjusting the cell shape to the environment. Actin filaments also play a critical role in generating focal adhesions and guiding directional cell motilities. Intracellular motile processes such as the vesicle movement, phagocytosis, and cytokinesis are also mediated by the dynamics of actin filaments\(^25\).

A small group of actin monomers form a nucleation core that acts as a starting point for further assembly, and ATP-bound actin monomers are added onto the existing filament during actin polymerization. Filamentous actin, F-actin, is formed when monomers of G-actin, a globular protein, align themselves head-to-tail to generate a filament. Two parallel protofilaments wind around each other and form a right-handed helix with a twist repeating every 37 nm (Figure 1.4 A). To supply diverse cellular functions, actin filaments are actively arranged and remodeled into different structures in a motile cell\(^25\): (1) At the leading edge of
the cell, branched and cross-linked actin filaments, known as lamellipodia, form beneath the cell membrane. This filamentous network contributes to cell movements. (2) Bundles of parallel actin filaments are assembled to form a finger-like protrusion, the filopodium, at the front of the cell. This thick rod of actin bundles is involved in directional migration of the cell. (3) Antiparallel actin filaments are cross-linked to build contractile fibers. (4) At the trailing edge of the cell, branched and unbranched actin filaments are assembled to create a think layer of actin network that supports the cell structure.

F-actin can also be de-polymerized. During de-polymerization, hydrolysis of ATP occurs on the actin subunits followed by the release of ADP-bound actin monomers at which time the filament shortens. In a very organized manner F-actin can sometimes treadmill. Actin treadmilling is characterized by the addition of actin monomers at the plus end of an actin filament and the removal of actin monomers at the minus end to maintain a steady-state length of the filament (Figure 1.4 B). The dynamics of actin filaments is not only important for regular cellular functions but also contributes to disease progression when it is mis-regulated. For example, actin filaments are involved in motility of invading pathogens\textsuperscript{26}, cancer metastasis and invasion\textsuperscript{27}, and neurodegenerative disease\textsuperscript{28}.

In hair cells, actin filaments are concentrated in stereocilia, rootlets, cuticular plates, and circumferential belts\textsuperscript{29,30} (Figure 1.2 A). Myosin subfragment 1 (S1) labeling demonstrated that all actin filaments in the stereocilium run in the same direction in which the minus ends point towards the cell body\textsuperscript{31} (Figure 1.4 C). In these studies, transmission electron microscopy (TEM) further illustrates the highly organized framework of actin filaments in each stereocilium of hair cells in lizard and chicken\textsuperscript{32,33}. Longitudinal sections of the stereocilium reveal a configuration of hundreds of parallel actin filaments tightly bundled
by cross-linkers that run perpendicular to the longitudinal axis of the stereocilium. Bridges between actin filaments are seen as stripes uniformly spaced every ~12.5 nm (Figure 1.4 H). Cross sections of a stereocilium show a hexagonal packing of actin filaments as well as a festooned pattern when the slice is thinner than 37.5 nm, a full turn of the F-actin helix (Figure 1.4 E, F). The uniform festooned pattern seen from stereocilia of all lengths in a hair cell is thought to be a result of the well-aligned crossover points in parallel actin filaments.

Electron micrographs of bent stereocilia, which were bent either at the bases or in the middles of the protrusions have given insight into how cross-linkers behave during deflections. When a mechanical force displaces the stereocilium, actin filaments bend, but neither stretch nor compress, they simply slide against each other. Moreover, the cross-bridges remain parallel to the apical surface of the cell (Figure 1.4 G, H).

Most of the actin filaments end at the taper region where the base of the stereocilium joins with the cell body, and only those in the center extend below the cell surface into the cuticular plate to form the rootlet (Figure 1.4 D). There are as many as 3000 actin filaments in a stereocilium of hair cells in lizards, but only 18-29 in the rootlet, and therefore stereocilia taper off at the base. This is an important feature of the stereocilium that is different from the microvillus that is also composed of parallel actin filaments. The purpose of the rootlets is to anchor the stereocilia during deflections. The rootlets anchor into a unique hair cell organelle called the cuticular plate. The cuticular plate is an actin-based cytoskeletal structure found in the apical surface of the hair cell. In contrast to stereocilia, the cuticular plate is composed of a meshwork of actin filaments (not parallel actin bundles) that sits near the apical surface of the hair cell just beneath the stereocilia. However, the actual function of the cuticular plate is largely undiscovered. At the perimeter of the cuticular plate
is a circumferential belt formed by parallel filaments with alternating polarity in chicken hair cells\textsuperscript{36}. Function of the circumferential belt is not clear, but it has been proposed to either provide the stiffness to the apical cell surface or transmit distortion to stereocilia when sounds come into the ear as if it works as a contractile ring that squeezes the apical surface of the hair cell\textsuperscript{36}.

There are numerous lines of evidence to show that actin is important in stereocilia. Actin filaments in stereocilia are composed of both isoforms of cytoplasmic actin, $\beta$-actin and $\gamma$-actin\textsuperscript{37}. Mutations in $\gamma$-actin, $ACTG$, are responsible for dominant nonsyndromic deafness, DFNA20/26\textsuperscript{38}. Lacking of either $\beta$- or $\gamma$-actin specifically in hair cells results in progressive hearing loss in mice\textsuperscript{39,40}. Initially, both knockouts exhibit regular hearing with normal morphology of stereocilia; however, over time in each animal, stereocilia degrade, coinciding with gradual hearing loss within weeks after birth. This demonstrates that either actin isoform is sufficient for building stereocilia during development and hearing at early stages. Yet, distinct pathologies demonstrated in $\beta$- and $\gamma$-actin knockout mice imply that both isoforms have non-redundant functions, which differentially influence the maintenance of stereocilia\textsuperscript{40}. These two actin isoforms share high amino acid sequence identity, such that only four amino acids are different; however, some variance has been identified in expression patterns, kinetic properties, and interacting partners\textsuperscript{41-43}. Therefore, actin is of vital importance in the hair bundle.
Figure 1.4

Actin filaments

Minus (pointed) end

Facing T zone

Plus (barbed) end

Facing leading edge

Transport for recycling

Actin filaments

Microtubules
**Figure 1.4. Actin organization in the stereocilium.** (A) Arrangement of actin monomers in the actin filament. Two protofilaments wind around each other to form a right-hand helix, which is 8 nm in diameter and has a full twist repeated every 37 nm (between the white bars). Helical structure of actin filaments as observed by electron microscopy. Figure is adapted from McGough et al., 1997.\(^4^4\) (B) Graphic of actin treadmilling, a dynamic process describing the addition of new actin monomers at the plus end of the actin filament and the depolymerization from the minus end. Figure adapted from Lowery and Van Vactor, 2009.\(^4^5\) (C) An electron micrograph of a longitudinal section, cut through the basal end of a stereocilium labeled with myosin S1 reveals that all F-actin is oriented with the pointed ends of the actin monomers, towards the cell body. TEM images adapted from Tilney et al., 1980.\(^3^4\) (D) Transverse section across the hair cell apical region, just basal to the stereocilia, demonstrates that fewer but thicker strands of actin bundles form rootlets that insert into the cuticular plate (lower half of the figure). Black circles outline the positions of rootlets. Upper portion of the figure is cut through the base of several stereocilia just above the cuticular plate. (E) EM of a thin transverse section cut at an oblique angle to the long axes of stereocilia demonstrates the hexagonal packing of actin filaments in stereocilia represented by black lines marked on the figure. (F) The same EM as (E), but black lines emphasize the festoon-pattern, which is a property of the stereocilium due to the pattern of cross-linkers that are all in register with the actin filaments. (D-H) TEM images adapted from Tilney et al., 1983.\(^3^3\) (G) EM of thin section through the apical portion of a hair cell shows bending stereocilia. (H) Enlarged view of the boxed area in (G) depicts that cross-linkers in the stereocilium retain spacing at 12.5 nm intervals and run parallel to the surface of the hair cell.
**Actin-associated proteins in stereocilia**

In order for actin to be effective in any cell type, the F-actin must be regulated by actin-associated proteins. Proteomic studies revealed the presence of several classes of actin-binding proteins in stereocilia, and many associated genes were found to be linked to hereditary hearing loss\(^4^6\). One of the categories is protein that regulates actin polymerization. Seeding of actin monomers to form dimers and tetramers for stable polymerization is usually a kinetically unfavorable event but can be achieved by providing actins at concentrations much higher than the critical concentration or can be facilitated by adding actin-binding proteins that catalyze nucleation such as formin, Ena/VASP, or Arp2/3 complex. Formins mainly function to nucleate actins, and also act as a leaky capping protein at the barbed end of actin filaments to allow elongation\(^4^7\). Mutations in a formin, diaphanous 1 (DIAPH1), were found to co-segregate with DFNA1 hearing loss, but the precise mechanism of pathogenesis is still uncertain\(^4^8\). Furthermore, we do not know how initiation of actin polymerization is carried out in stereocilia nor do we know the exact proteins that may carry out this process.

In general, another important class of actin-associated protein involved in shaping and maintaining actin filaments are cap binding proteins. Cap binding proteins may either stabilize the actin filaments or increase the rate of disassembly. Barbed end capping proteins, gelsolin and twinfillin 2 were shown to localize to the tips of stereocilia in hair cells\(^4^9,^5^0\). The Ca\(^{2+}\)-dependent F-actin severing function of gelsolin is important for actin dynamics in stereocilia because outer hair cells of mouse cochlea with mutant gelsolins develop long thread-like stereocilia\(^4^9,^5^1\). In addition, reduction of stereociliary length was a result of
overexpressing Twf2 in the inner ear hair cells. These findings together demonstrate the importance of actin-capping proteins in stereocilia formation and maintenance.

Another class of actin-associated proteins that have been demonstrated to be critical for hair bundle formation is the actin cross-linker. Cross-linkers are proteins that connect neighboring actin filaments and facilitate their organization into stiff bundle protrusions or gel-like mesh-networks. Actin cross-linking proteins generally have at least two actin-binding domains or they behave as dimers during binding. The size of a cross-linking protein and the distance between the actin-binding domains determines packing density of a bundle.

Espin is a deafness gene, DFNB36, and when the ortholog is mutated in mice, shorter stereocilia are the result. Espin was first isolated from Sertoli cell junctional plaques, and was shown to bind and cross-link actin filaments into parallel actin bundles. Isoforms of espin encode different amino acids at the N-terminus, which mediate their interactions with distinctive protein partners. Both isoforms of espins are localized to the stereocilia of hair cells but not the rootlet. Mouse hair cells deficient in espin develop hair bundles with thinner and shorter stereocilia, but different locations and types of hair cells in the cochlea and the vestibular system vary in the severity of phenotypes. These studies indicate a function of espin in assembling and stabilizing actin filament bundles in stereocilia.

Plastin is a class of actin cross-linking proteins that helps in forming parallel actin bundles in microvilli. Both the I- and T-isoforms were detected in stereocilia of hair cells in the inner ear, but each exhibits a distinct temporal expression pattern; which the I-plastin is found in developing and mature hair bundles but T-plastin shows a transient expression in immature hair bundles. However, there is not yet any hair bundle deficiency reported to be a result of plastin mutation. TRIOBP, which localizes to the rootlet of stereocilia, is able
to pack actin filaments into organized bundles in mice\textsuperscript{64} and the orthologous protein when defective is the cause of DFNB28 in human\textsuperscript{65}. Although there are a number of actin-bundling proteins identified in stereocilia, there are likely to be many more types of cross-linkers that participate in formation of stereocilia, which have over 3000 filaments precisely organized for mechanotransduction.

The other category of molecules that could regulate the formation of actin filaments includes unconventional myosins that walk along actin filaments and in most cases transport cargo. Stereocilia-localizing motor proteins, myosin Ic, IIIa, VI, VIIa, and XVa are found associated with human deafness DFNA48, DFNB30, DFNB37, DFNB2, and DFNB3, respectively\textsuperscript{2,66}. Mouse models deficient in these unconventional myosins develop hair bundles with various types of morphological abnormalities. For example, shaker 2 mice with mutations in the myosin XVa gene exhibit irregularly short stereocilia\textsuperscript{67}. In addition, whirlin proteins, usually found at the tips of stereocilia, are mis-localized in hair bundles that are deficient in myosin XVa\textsuperscript{68,69}. This suggests that hair cells rely on motor protein such as myosin XVa to actively transport cargos to the tips of stereocilia for maintaining the structure or function of the hair bundle. These observations implicate an important role of myosin motor proteins in regulating the lengths of stereocilia in mature hair cells, yet to identify the temporal expression patterns of myosins should give us some insights into how myosins influence actin polymerization during stereocilia development.

**Hair cell differentiation**

Hair cells and supporting cells come from the same group of progenitor cells arising at the thickening epithelium of the otic vesicle. Epithelial cells in the otic vesicle develop into
three different cell types: hair cells, supporting cells, and statoacoustic ganglion cells\textsuperscript{70}. The Notch-Delta signaling pathway and proneural gene expression drives the differentiation of hair cells and supporting cells. First, presumptive hair cells express high levels of Notch ligands; in the neighboring cells, this triggers high levels of Notch, the receptor protein. Lateral inhibition prevents formation of excessive numbers of hair cells: only a cell that expresses high levels of Delta, a Notch ligand, from the beginning of the process will become a sensory cell (\textbf{Figure 1.2 A}). The surrounding cells become supporting cells\textsuperscript{71,72}.

Expression of the proneural gene, atonal in vertebrates, is also essential for defining the presumptive hair cells. Manipulating the expression of \textit{Math1}, a murine atonal homolog, is sufficient to alter the total number of cochlear hair cells\textsuperscript{73,74}. Hair cell regeneration has only been reported in lower vertebrate such as fish, amphibians, and birds, but not in mammals\textsuperscript{75,76}. In species that do regenerate hair cells, it is hypothesized that after losing a hair cell, supporting cells may undergo two distinct processes to reproduce new hair cells. In one process, the supporting cell directly transdifferentiates into a hair cell. In a second process, asymmetric division of a support cell results in one newly formed hair cell and a new supporting cell.

The third lineage derived from the thickening epithelial cells in the otic vesicle is the statoacoustic ganglion (SAg) neurons. Epithelial cells in the anteroventral region of the otic vesicle delaminate from lamina at around 22 hours post fertilization (hpf), and undergo an epithelial to mesenchymal transition to become neuroblasts at which point they leave the otic vesicle\textsuperscript{70}. Matured SAg neurons interact with sensory cells in the ear through their axons which project onto the hair cells\textsuperscript{77}.
Hair bundle development

The hair cell obtains its name from its striking staircase-shaped stereociliary bundle that emanates from the apical surface of the hair cell to protrude into the lumen of the otic vesicle. Despite the confusing nomenclature, the stereocilium is a modified microvillus composed of actin filaments. The stereocilia are taller and thicker than the microvilli, and have a prominent taper at their base (Figure 1.2 B and 1.4 C). These differences between stereocilia and microvilli may partially be regulated by the effect of different actin-associated proteins on the rate of actin monomer renewal between protrusions.

Hair bundle development has been extensively characterized in the chicken (Figure 1.5). At embryonic day 8 (E8), randomly distributed microvilli arise at the apical surfaces of the hair cells. The kinocilium, a true cilium, forms at the center of the apical surface at E9. The kinocilium then moves aside to the peripheral edge of the hair cell. Once this asymmetry is realized, microvilli closer to the kinocilium are believed to elongate and then increase in length and width between E13 to E17. As stereocilia lengthen at the plus end, several core strands of actin filaments at the minus end extend below the apical surface and form a much more condensed packed actin filament structure named the rootlet. A tapering towards the base of each stereocilium is formed by the systematic, successive ending of actin filaments, from outer to inner relative to the center of the actin core. The rootlets are embedded in the cuticular plate, a dense actin meshwork (Figure 1.2 B). These two structures develop simultaneously during the development of the hair bundle. The rootlets are thought to function as an anchor point for stereocilia to enable it to pivot on the cuticular plate in response to stimuli. At the end of development, the mature hair bundle is shaped like a staircase with the tallest row of stereocilia sitting beside the kinocilium. In contrast to
chicken hair cells, kinocilia degenerate and disappear in mature hair cells in the cochlea, but not the vestibular system, of mammals\(^2\). Therefore, the kinocilium is considered to be involved in setting up the organization of the hair bundle initially, but does not play a direct role in mechanotransduction for hearing in mammals. However, most lower vertebrates, such as chicken, frog, and fish, retain their kinocilia throughout the lifetime of the hair cells. The general feature of the hair bundle, the staircase-shaped hair bundle, is apparent in hair cells of human, mouse, chicken, lizard, and fish; however, each might differ in the height, the width and the numbers of rows of stereocilia. Even in a given sensory organ, the morphology of hair cell stereocilia changes gradually across the whole sensory epithelium\(^79,80\). The rigidity of actin-filaments impacts the stiffness of stereocilia, and this is one of the important features to stereocilia that determines its deflection capability; therefore, these morphological variations could impact the mechanosensitivity of these different hair cells, and it is thought to be one of the models enabling fine frequency discrimination in ears.

![Figure 1.5](image)

**Figure 1.5**
Figure 1.5. Hair bundle development is a systematic process. A schematic diagram demonstrates the progression, from left to the right, of hair bundle formation. A single kinocilium erupts from the center of the apical surface of the hair cell. Polarization of the hair cell is first observable when the kinocilium localizes asymmetrically. After the growth of stereocilia, rootlets, and the cuticular plate a mature hair cell bears hair bundles with graded heights and extended rootlets, with an expanded cuticular plate near its apical region.

The extracellular linkages between neighboring stereocilia are important for maintaining the integrity of the hair bundle (Figure 1.6)\textsuperscript{81,82}. At the tapper regions of the stereocilia, a protein complex forms zipper-like connections called the ankle links. Mutations in genes that contribute to the ankle link cause deafness in the human and mouse\textsuperscript{83,84}. Above the ankle link is the shaft connector, which is composed of a member of the receptor-like protein tyrosine phosphatase family called PTPRQ. Deficiency of Ptprq in mice abolished the shaft connector, caused splayed hair bundles in postnatal stages, and resulted in a progressive loss of cochlear hair cells\textsuperscript{85}. Joining the upper regions of the stereocilia are the horizontal top connectors, which are also known as lateral links in mice. This lateral links perhaps provide constrains that bond rows of stereocilia together to assist the stereocilia to move as a whole\textsuperscript{81,86}. The tip links are composed of cadherin 23 and protocadherin 15, the former attaches on the side of the taller stereocilia and the latter locates at the tip of the shorter stereocilia\textsuperscript{87-90}. Displacement of stereocilia applies force on the tip link and tension on this link is thought to either directly pull the gate on the channel protein or change the membrane configuration; and thus, opens the mechanotransduction channel. Cadherin 23 and protocadherin 15 are transmembrane proteins that contain 27 and 11 repeats of cadherin domains, respectively. Each protein forms a homodimer in a calcium-dependent manor.
Extracellular domains on the most amino-terminus of each protein, EC1 and EC2, interact and form an antiparallel heterodimer with an extended “hand-shake” structure that is 5 nm wide and 13 nm long\(^1\). Hair cells lacking either cadherins or carrying mutations in cadherins that interfere with the interaction interfaces between cadherin 23 and protocadherin 15 will lead to tip-link breakages as well as hearing deficiency\(^2,3\). However, on the other hand, tip links broken by loud sound stimulations or chemical treatments are restored and resume normal mechanotransduction in 24 hours\(^4\).
Figure 1.6. Stereociliary links. Electron micrographs show links that contact stereocilia. From the bottom to the top, neighboring stereocilia are connected with ankle links, shaft connectors, horizontal top connectors, and tip links. The kinocilium and the tallest row of stereocilia are also bridged by filamentous linkers named the kinocilium links. Figure is adapted from Nayak et al., 2007.
**Zebras as a model for hearing and human deafness**

The zebrafish (*Danio rerio*) model system has proven to be an invaluable tool for the investigation of hearing and hair cell development. *Ex utero* egg development, transparent embryogenesis, and rapid maturation allows for efficient manipulation and observation of developmental processes *in vivo*. In addition, the zebrafish genome has been sequenced, annotated, and is available on several on-line databases. There are several methods that have been used to determine a gene’s function within zebrafish, such as knocking down the expression of a target gene through the injection of sequence specific morpholinos or small interfering RNA (siRNA). Other methods include isolating zebrafish mutants after random mutations are induced by ethyl-nitrosourea (ENU), viral insertion, or gamma rays. More recently, several novel whole genome-editing tools, such as Zinc Finger Nucleases (ZFN), Transcription Activator-like Effector Nucleases (TALEN), and the CRISPR-Cas9 system, make it possible to create gene-specific knockouts or carry out site-directed mutagenesis zebrafish. In addition to these methods, function of a gene could also be studied by microinjecting zebrafish embryos with cDNA or RNA of the gene of interest, which prompts gain-of-function or dominant negative effects at the cellular level. In this thesis, we used many of these genetics approaches to investigate molecular regulatory mechanisms of ear development in zebrafish.

Zebrafish is a vertebrate organism possessing organs that are similar to those of humans. Despite the lack of outer and middle ear structures, zebrafish have an analogous system using otolith for hearing, and several human syndromic and nonsyndromic deafness genes have a zebrafish counterpart. Protein sequence homology among species indicates that human and zebrafish may share a conserved pathway for ear development. However,
it is worth mentioning that fish underwent an additional whole genome duplication that did not occur in land vertebrates\textsuperscript{101}. This whole genome duplication event caused zebrafish to often have two copies of a gene, giving them the ability to retain, subdivide, or lose the function of the ancestor gene\textsuperscript{101}. If subdivision of a gene has occurred, the phenotype generated by a single gene mutation in zebrafish may be less severe than a mutation in the orthologous gene in mammals.

**Zebras fish inner ear development**

Anatomic descriptions of fish ears had been described, but it was not until 1930 that von Frisch and colleagues began to experimentally prove that fish are capable of perceiving sounds\textsuperscript{102}. By emitting a sound before or at the same time of food feeding, they showed that fish could respond to sounds and be trained for a sound-triggered feeding reward behavior without the assistance of visitional input, as the eyes were removed in their experiments. Sound detection is mediated by the sensory epithelia and their overlaying otoliths in the inner ear of fish. Otoliths are composed of proteins and dense calcium carbonate crystals, which are denser than rest of the fish’s body. Thus, when sound vibrations reach the fish, the otolith will vibrate at a different velocity than the sensory epithelium beneath it, and this relative motion causes the bending of hair bundles, which initiates the depolarization of hair cells\textsuperscript{103}. In some fishes, vibration of the ear can also be achieved indirectly by movements of the swim bladder and the Weberian ossicles\textsuperscript{103}. It is thought that these acoustic couplings enhance the hearing ability in fish and broaden the hearing bandwidth. The majority of fish can hear between 100 to 3000 Hz, while zebrafish are most sensitive at 800-1000 Hz\textsuperscript{104}. In extreme cases fish can hear up to 180kHz, as in the genus *Alosa*\textsuperscript{105}.
Inner ear development in zebrafish is a complex process that requires the coalescence of many different molecular cascades to form an organ capable of sensing mechanical stimuli. These cascades includes growth factors, transcription factors, myosins, members of the retinoic acid pathway, members of the notch-delta signaling pathway, and the cadherin family of proteins\textsuperscript{100}. The inner ear of zebrafish develops from the thickening epithelium, later known as the otic placode, which resides in the ventral side of the embryo at the shield stage. At the early somite stage, the transcription factor pax2/5/8 family specifically labels the otic preplacode region, and these genes are required for inducing the formation of the otic placode in zebrafish\textsuperscript{106}. Additionally, \textit{pax2.1} regulates the expression of \textit{deltaA} which mediates the specification of hair cells\textsuperscript{107}. Hereafter, the placodal ectoderm develops into an otic placode; this is induced by tissue interaction of the hindbrain and the mesoderm\textsuperscript{108}. To form the otic vesicle in the zebrafish, the placode beneath the surface ectoderm undergoes cavitation and forms a vesicle with a narrow lumen that appears in the placode at around the 18-somite stage of development. While the lumen is extended, the otic vesicle keeps growing in size without a large amount of cell proliferation\textsuperscript{109}. Later, epithelial thickening starts again ventrally and progresses to the dorsal roof within the vesicle; expression of neurogenic genes of the Notch-ligand family marks these regions as the future sensory patches\textsuperscript{110,111}. The zebrafish adult inner ear has six sensory patches that contain hair cells to perform mechanotransduction (Figure 1.7). These sensory patches include three maculae (saccule, utricle, and lagena) and three cristae (anterior, lateral, and posterior). Maculae hair cells are responsible for the vestibular and hearing function while hair cells in cristae are used for detecting angular accelerations.
The first sensory epithelium to develop is the anterior maculae\textsuperscript{100}. Hair cells could be identified based on their specialized bundle structure as early as 24 hours post-fertilization (hpf), which reach functional maturity at 27 hpf\textsuperscript{412,113}. The first pair of hair cells that emerge from the maculae are tether cells. Tether cells are atypical premature hair cells, and they originate from ciliated cells with elongated cilia at 21.5 hpf\textsuperscript{112}. The motility of kinocilia of the tether cell is essential for capturing the seeding materials as calcium carbonates that give rise to otoliths\textsuperscript{114}. To prevent otolith seeding at places other than future maculae, seeding materials are continuously agitated by beating cilia that are distributed around the whole otic vesicle\textsuperscript{112}. Tether cells eventually develop into mature hair cells and share common characteristics with other mature hair cells after passing the initial critical period of otolith formation, which is between 18-24 (hpf)\textsuperscript{112} in zebrafish. Otolith size expands rapidly within this critical period. Unlike humans and rodents, otoliths of fish continue to grow as the fish increase in body size. Adult zebrafish have three distinct otoliths that differ in size, shape, and location\textsuperscript{115} (Figure 1.7). Sagitta, the saccular otolith, located over the posterior maculae, is large and round at embryonic stages, but becomes thin and elongated in adulthood. The ovoid-shaped lapillus, the utricular otolith, is positioned above the anterior maculae in the otic vesicle. Asteriscus, associated with the lagena macula, does not develop until 9-11 days post fertilization (dpf). Observation of monolith (mnl) mutant zebrafish revealed that a loss of the anterior (utricular) otolith disrupted vestibular function but not audition\textsuperscript{116}. After the initial formation of maculae, development of semicircular canals begins with the growth of finger-like protrusions of epithelia inward toward the lumen of the ear at the rostral, caudal, ventral, and lateral sides of the otic vesicle. The lateral protrusions further divide and form three bulges. Each bulge fuses with the other epithelial protrusion at the end according to
their position. Rearrangement of these epithelia results in the formation of the canals and their accompanying sensory patches known as cristae\textsuperscript{117}.

In summary, zebrafish inner ear development starts at an early embryonic stage. The first hair cell in the inner ear can be identified at around 24 hpf. After differentiated neurons have made contact with the sensory hair cells, and the swim bladder has fully developed, larvae fish show swimming behavior at 96 hpf. Muscular reflex induced by auditory, vestibular, or lateral line stimulation can then be observed around 96 hpf \textsuperscript{100,118}. The number of hair cells continues to increase until 10 months of age in zebrafish; however, having more hair cells does not hugely enhance on their hearing ability\textsuperscript{104}. After 10 months, cell death and proliferation in the sensory epithelium can be detected by the TUNEL assay and the BrdU labeling, respectively\textsuperscript{104}. This suggests that unlike adult ears of mammals, which do not show an ability to regenerate hair cells after over-stimulation- or chemically- induced hair cell death\textsuperscript{119}, the adult fish has a way to manage the total amount of hair cells through a regenerative process, even at the older ages.
Figure 1.7. Structure of the developing zebrafish inner ear. (A) Schematic of zebrafish ear illustrates the positions of each sensory patch in a 4-dpf wild-type zebrafish; dls, dorsalateral septum; ep, epithelial pillars; kc, kinocilia of crista hair cell; sc, stereociliary bundles. (B) DIC (Differential Interference Contrast) microscopic image of the zebrafish inner ear, with the focus set on the utricular otolith. (C) Dorsal view of a 5-dpf zebrafish larva, two inner ears are separated from the notochord (nc). Five sensory patches found inside the otic vesicle with the stereocilia labeled with phalloidin: ac, anterior crista; lc,
lateral crista; pc, posterior crista; mu, somatic muscle; sm, saccular macula; and um, utricular macula. (D) Lateral view of developing zebrafish inner ears from 3, 5, 10, 15, 17, 20 dpf, and adult. aa, rostral ampulla; ac, rostral canal; cc, common crus; D, dorsal; ha, horizontal ampulla; hc; horizontal canal; la, lagena; P, posterior; pc, posterior canal; s, saccule; and u, utricle. Figure adapted from Whitfield et al., 2002. 

The lateral line system of zebrafish

In addition to the ear, hair cells are found on the body surface of zebrafish in an organ system called the lateral line system (Figure 1.8 A). The lateral line serves as a mechanosensory system to detect water movement and is involved in a variety of behaviors, such as schooling, sexual courtship, predator avoidance, and prey detection. Neuromasts, which are composed of a group of sensory hair cells and supporting cells (Figure 1.8 B), are present on the head region and along the trunk to the tail, and form the anterior lateral line (ALL) and the posterior lateral line (PLL), respectively. The distribution of lateral line neuromasts provides easy accessibility to manipulate hair cells or to observe morphological changes in the developing zebrafish.

The PLL originates from a thickening placode that sits caudal to the otic placode. Although the molecules involved in induction of the lateral line placode are still unclear, PLL placode formation begins at approximately 19 hpf. The anterior portion of the PLL placode gives rise to the PLL ganglion that later innervates the deposited neuromasts. The posterior portion of the PLL placode becomes the primordium, which starts to migrate caudally along the horizontal myoseptum at 20 hpf and deposits a cluster of 20 to 30 cells, the proneuromast, every 5 to 7 somites. The PLL primordium is segmented along the anterior-posterior axis.
A subset of cells at the trailing edge of the PLL primordium is arranged into a rosette structure, termed the protoneuromast, while the leading edge remains unpatterned\textsuperscript{125}. The organization of the PLL primordium resembles an assembly line. The most mature protoneuromast rosettes are at the trailing edge, while the more recently formed protoneuromast rosettes from the progenitor pool are added closer to the leading edge\textsuperscript{126}. Deposition of the protoneuromast happens when the fourth rosette is generated behind the leading edge\textsuperscript{127}. After the PLL primordium completes migration at ~ 48 hpf, 5 to 6 primary neuromasts are formed along the trunk while 2 to 3 terminal proneuromasts are present at the tail\textsuperscript{128}. With the exception of the terminal neuromasts, the distance between two neighboring neuromasts remains consistent, regardless of the actual position on the body surface of zebrafish. The spacing mechanism governing proneuromast deposition is not due to the physical distance but to a temporal distance related to the speed of primordium migration\textsuperscript{128}.

Each proneuromast continues differentiation and reaches a mature state a few hours after deposition\textsuperscript{128}. A mature functional neuromast is defined as a group of hair cells with oppositely polarized hair bundles and well-formed synaptic contacts with innervated afferent and efferent neurons\textsuperscript{122} (Figure 1.8 B). In the zebrafish lateral line, the Notch-Delta signaling pathway mediates lateral inhibition and was shown to be involved in cell fate determination events at different developmental stages. First, Notch-Delta signaling allows separate fates of the PLL ganglion neurons and the migrating PLL primordium from the PLL placode. The zebrafish mutant, \textit{mind bomb} (\textit{mib}), which perturbs an ubiquitin ligase for Delta and Jagged, exhibits an increased number of neurons and the number of primordium cells are less abundant\textsuperscript{110,129}. Second, hair cell specification is also determined by Notch-Delta signaling. In the PLL, hair cells are derived from cells that express a high level of Delta\textsuperscript{130}. 
Neurons that innervate neuromasts are bipolar with axons that project to the targeting neuromasts and the hindbrain\textsuperscript{131}. Cell bodies of these neurons reside in the ganglia located between the eye and the ear for the ALL, and behind the ear for the PLL. The ganglion of the PLL develops from the same placode as the primodium. The migration of the primodium is suggested to be a main driving force that guides the path finding mechanisms of these neurons\textsuperscript{124,132}. Studies that observed the re-innervation of hair cells in the neuromast, during the regeneration period, after chemically-induced hair cell death, demonstrate that neurons are strict selectors which only form stable synaptic contacts with hair cells of the identical polarity\textsuperscript{133}. Counting of the numbers of dye-label neurons and neuromasts reveals several different patterns of innervation in neuromasts of various lateral lines. A single neuromast is at least innervated by two neurons for its contrasting polarity of hair cells, each neuron for groups of oppositely polarized hair cells. On the other hand, one neuron could also innervate many neuromasts, and this plasticity in organization is hypothesized to be related to the sensitivity, resolution, or the ability to adjust to environmental challenges of lateral lines\textsuperscript{134}. Lateral lines also receive inhibitory and excitatory input from the efferent system, which is proposed to play a role in preventing overstimulation or inducing sensitivity in hair cells\textsuperscript{135-137}. 
Figure 1.8. Zebrafish lateral line system. (A) Position of anterior and posterior lateral line in larval zebrafish. (B) Schematic of a neuromast. The neuromast is composed of hair cells, mantle cells, supporting cells, and neuronal endings. Afferent neurons synapse with hair cells. Each hair cell has stereocilia and a long kinocilium covered by a gelatinous structure, the
cupula. Afferent contact each hair cell. Inhibitory and excitatory efferent fibers form contacts with hair cell and afferent nerve endings, respectively.

Creating gene-specific knockout zebrafish by ZFN, TALEN, and CRISPR-Cas9 technologies

For decades, zebrafish had been a less attractive model organism for genetic studies because of the technical barrier of using embryonic-stem-cell-based gene targeting in zebrafish. RNA interference (RNAi) is an effective way to diminish the expression of genes at post-transcriptional or translational level, yet it has not been very successful in zebrafish embryos. Morpholinos, an anti-sense based knockdown strategy, have been widely used in zebrafish; however, decreased efficacy over time, the off-targeting silencing, and the toxicity-induced side effects can constrain it for studying gene function.

In 2008, zinc finger nucleases (ZFNs) were shown to induce site-directed mutations in zebrafish upon the injection of ZFN RNAs. ZFNs are engineered restriction enzymes that fuse the DNA-binding domain with a FokI endonuclease domain (Figure 1.9 A). Sequence-dependent DNA recognition and binding rely on the ability of an array of zinc finger (ZF), Cys2His2 domains. Upon DNA-ZF binding, two FokI endonucleases are brought in close proximity to allow for dimerization, which elicits a DNA double-strand break in situ. Non-homologous end joining (NHEJ) repairs DAN and tends to introduce insertions or deletion mutations at the point of DNA double-stand breaks. These are referred to indels. The efficiency of creating indel mutations by a given ZFN in vivo relies on sufficient DNA binding; however, the DNA-recognition specificity of a ZF is affected by its adjacent ZFs. Therefore, in this thesis, we use the context-dependent assembly (CoDA) method, which
selects the target site via constructing the three-fingers array from two two-finger modules that share a common middle unit (ZF2) and each has been pre-screened to have a high-affinity DNA targeting by a yeast-based assay97 (Figure 1.9 B). CoDA provides a user-friendly platform for assembling ZFNs and increases the success rates for ZFN-induced mutations in zebrafish.

To overcome the constrains CoDA, transcription activator-like (TAL) effector, a DNA-binding protein identified from plant pathogenic Xanthomonas, was used143,144. A naturally occurring TAL effector is composed of a tandem repeat, nuclear localization signal (NLS), and a transcription activation domain (AD), respectively ordered from the amino-to-carboxyl terminus (Figure 1.9 C). Proteins in this family are conserved but vary in the number of central repeat domains, which mediate DNA recognition via the repeat variable di-residue (RVD)145 that is about 33 - 35 amino acids in length. Each repeat unit binds a single DNA base pair independently with a simple but specific amino acid code (Figure 1.9 D); thus, by joining a series of repeat units together, the TAL effectors array is capable of targeting any given DNA sequence. After artificially assembling the desired TAL effector repeats with a FokI endonuclease, this TAL Effector Nuclease (TALEN) can direct a site-specific DNA cleavage after dimerization (Figure 1.9 E). Repair of ZFN- or TALEN-induced double-strand breaks have successfully generated mutations of genes at the genomic DNA level in yeast, plants, nematodes, zebrafish, mouse, and human cultured cell lines144,146,147. In general, compared to ZFN, TALEN that can design to recognize a longer target site sequence, 9 bp and 16 bp for a half site of ZFN and TALEN, respectively, make it to have less non-specific binding and a higher efficacy146.
Figure 1.9. Introducing DNA double-strand breaks by ZFNs and TALENs. (A) Schematic of the binding positions of the ZFNs, including DNA binding domains, the left and the right zinc-finger arrays, and cleavage domains, a dimer of FokI enzyme, on the targeted DNA sequence. Figure is originally published in Miller et al., 2007\(^{148}\). (B) ZFNs assembled with CoDA. Two different three-zinc-finger arrays and their corresponding 3-bp DNA-binding sites are depicted. By combining the F1 unit of the first array (left), the common F2 unit, and the F3 unit from the second array (right), a ZFN for the DNA target site
is created (bottom). Figure is adapted from Sander et al., 2011\textsuperscript{97}. (C-E) TALEN-mediated gene targeting. Figures are adapted from Boch et al., 2009\textsuperscript{143}, Miller et al., 2011\textsuperscript{144}, and Clark et al., 2011\textsuperscript{149}, respectively. (C) Graphical demonstration of a single TAL effector found in nature that has the repeat domain, the nuclear localization signal (NLS), and an acidic transcription activation domain (AD). Amino acid positions 12 and 13 in the first repeat are the repeat variable di-residues (RVD) responsible for DNA recognition of the repeat number 1. (D) Preference of base binding for four common RVDs. (E) Engineered TALENs with TAL effectors selected for desired target DNA sequences. Binding of two TALENs on the DNA brings \textit{FokI} endonucleases to dimerize and create the DNA double-strands break at the target site.

In addition to these DNA-mediated gene-targeting methods, a RNA-based DNA-binding method, CRISPR-Cas9 is also being utilized as a tool for creating site-specific mutations. Clustered regularly interspaced short palindromic repeats (CRISPRs) are used as an adaptive immune system in bacteria to destroy invasive DNA elements\textsuperscript{150-152}. After infection, fragments of viral DNAs will be inserted into the genomic loci close to the endogenous CRISPR array of the host. Then, when the host cell transcribes the whole CRISPR array region, it produce precursor CRISPR RNA (pre-crRNA) that can pair in complementary to the invading viral targets. The CRISPR type II system requires an additional trans-activating crRNA (tracrRNA) which base pairs with the pre-crRNA, and this RNA duplex will guide the CRISPR-associated proteins, Cas9, to a specific DNA region to induce a double-strand DNA cleavage (Figure 1.10 A). Engineered single guide RNA (sgRNA), which has a structural domain resembling the critical instructive motif of
crRNA:tracrRNA complex, successfully transforms the CRISPR-Cas9 system to be an efficient and easy-to-build genome editing tool in human cultured cells, mouse, and zebrafish\textsuperscript{99,153,154} (Figure 1.10 B). The minimum selection criterion for a target site is to have a protospacer adjacent motif (PAM) sequence (NGG) that is positioned at the 3’-end of the desired target region in genomic DNA\textsuperscript{155}. The success rate of targeted indel mutations induced by engineered CRISPR/Cas9 is comparable to that of TALENs\textsuperscript{156}. Yet, because the length of the target sequence needed for CRISPR is shorter than TALENs, a higher off-target rate of CRISPR/Cas9 can be a concern. The CRISPR/Cas9 system has been shown to induce a biallelic conversion at the somatic level\textsuperscript{157}. In addition, this system can also generate precise DNA sequence mutations into the zebrafish genome when used with single-stranded oligodeoxynucleotides\textsuperscript{158}. These modifications in the genomic DNA sequences can be heritably transmitted through the zebrafish germline. Together, these novel whole genome-editing tools, ZFN, TALEN, and CRISPR/Cas9, have made it possible to create gene-specific knockout zebrafish as an alternative to using reverse genetic approaches to study the importance of a gene in zebrafish. In my thesis, I used all three targeting technologies based on which ever is the best to answer the question at hand.
Figure 1.10. CRISPR/Cas systems. (A) Schematic demonstration of a naturally occurring crRNA:tracrRNA:Cas9 complex. The complementary strand of the DNA target site sits adjacent to the PAM sequence, which is paired with crRNA. Structure of the crRNA:tracrRNA duplex guides the docking of Cas9 and the cleavage of the DNA. (B)
Engineered CRISPR/Cas9 system used in vivo. The function of dual RNA (crRNA:tracrRNA) is replaced by a structurally similar sgRNA. 5’-end of the sgRNA is the variable region designed based on the target site of interest. Figures are adapted from Hwang et al., 2013.

Objectives of the study

Hearing is mediated by structurally and functionally unique cells, the hair cells. There were two overarching goals of my thesis. One, identify structural proteins of the hair bundle, their functions, and mechanisms of their regulation. More specifically, I sought to identify a new actin-bundling protein and to determine its functional significance in generating the unique morphology of the stereocilia. My second goal was to identify and target a new class of hair cell mechanotransduction channel candidate in zebrafish.
-Chapter 2-

Identification of Fascin 2b as a Constituent of Stereocilia and Understanding its Function Using Zebrafish Transgenesis and Gene-specific Knockouts
ABSTRACT

In vertebrates, the senses of hearing and equilibrium are enabled by hair cells in the inner ear. Hair cells are specialized epithelial cells that are mechanosensitive. Mechanosensation depends on the precise regulation of the morphology of the hair bundle protruding from the apical surface of the hair cell. Hair bundles are composed of actin-based stereocilia that are graded in length, and this delicate shape relies on an intricate network of actin-binding proteins. However, the mechanisms that govern the morphology of the hair bundle remain largely undefined.

To identify proteins that organize the cytoskeleton of stereocilia, we scrutinized the hair-cell transcriptome of zebrafish. One promising candidate encodes fascin 2b, a filamentous actin-bundling protein found in retinal photoreceptors. In this study, we verified fascin 2b as being present in zebrafish inner ear hair cells using reverse transcription polymerase chain reactions (RT-PCR) and in situ RNA hybridization. Immunofluorescence studies in both adult and larval zebrafish revealed that fascin 2b protein preferentially localizes to the stereocilia of hair bundles. Overexpression of wild-type (WT) fascin 2b and fascin 2b phosphomimetic mutants in transgenic zebrafish suggests that the phosphorylation state of fascin 2b regulates the recruitment of this protein to stereocilia. Finally, stable transgenic zebrafish embryos that express GFP-WT fascin 2b in hair cell stereocilia possess longer hair bundles when compared to their non-transgenic siblings, indicating that fascin 2b regulates the length of stereocilia.

Furthermore, to understand whether fascin 2b contributes to the formation of stereocilia, we performed time-lapse studies with doubly transgenic embryos, which express GFP-WT fascin 2b and β-actin-mCherry in hair cells. β-actin-mCherry labels stereocilia and cuticular
plates in hair cells allowing for observation of stereocilia formation. In the doubly transgenic zebrafish, the developing stereocilia contain fascin 2b at all time points observed, suggesting that fascin 2b participates in the formation of stereocilia because it is present during the inception of these protrusions.

**INTRODUCTION**

**Actin cross-linking protein, fascin, and its roles in cytoskeletal structures**

Fascin was first purified from sea urchin oocytes and coelomocytes \(^{159,160}\), and its name refers to its ability to form tight bundles with F-actin. Over the past 30 years, fascin homologs in fly, frog, zebrafish, mouse, and human have been discovered and characterized \(^{161-164}\). There are three isoforms of fascin in mammals: fascin 1, fascin 2, and fascin 3. Each of these has a distinct expression profile. Fascin 1 is largely found in the brain, ovary, and testis \(^{163-165}\). Fascin 2 is expressed in the inner and outer segments of retina photoreceptor cells \(^{166}\). Fascin 3 is located in the testis \(^{167}\). In contrast to vertebrates, invertebrates have just one fascin gene \(^{168}\).

Out of the large variety of actin-binding proteins, fascin 1 is notable for being highly expressed in neurons, glial cells, skeletal, smooth muscle cells, mature dendritic cells, and certain types of epithelial tumor cells. Within the cell, fascin localizes to cortical cell protrusions, including filopodia, spikes, lamellipodial ribs, oocyte’s microvilli, and dendrites of dendritic cells. Fascin 1 works by bundling actin filaments parallel to each other to form a tightly packed array; thus, the major function of fascin in these sites is to induce the formation of and provide stiffness to these highly organized actin filament structures \(^{169}\). Transfection of fascin in epithelial cells is sufficient to induce the formation of filopodia or
increase cell migration activity in certain cells\textsuperscript{170}. Fascin expressed in microspikes and ruffles is required for organization of the cytoskeleton at the leading edge as well as mediating signaling involved in cell-cell adhesion\textsuperscript{171}. Additionally, a high level of fascin expression is found within metastatic cancers\textsuperscript{172}. Metastatic tumor cell migration is suppressed when the binding of fascin to actin is blocked by migrastatin analogues; therefore, fascin has been considered to be a new therapeutic target for cancer treatment\textsuperscript{173}.

Human fascin 1 is a globular monomeric protein that has a molecular mass of ~55 kDa\textsuperscript{174}. X-ray crystallography studies revealed that fascin 1 belongs to the β-trefoil group of proteins\textsuperscript{175,176} (Figure 2.1 A). All fascins are predicted to have four highly conserved repeat fascin domains that are unique and not similar to other known actin binding proteins, such as villin. As an actin cross-linking protein, it is hypothesized that fascin contains at least two actin-binding regions, where each binds to neighboring actin filaments, permitting them to be bundled. However, no single fascin domain encompasses a complete actin-binding domain. One actin-binding region is thought to be located in the most conserved region of all the fascins (Figure 2.1 B). This region resides between amino acid residues 11 and 50, an area containing sequences similar to an actin-binding site on myristoylated alanine-rich C-kinase substrate (MARCKS)\textsuperscript{165}. The phosphorylation state of the serine 39 residue within this region has demonstrated an important regulatory function by affecting fascin’s actin binding ability\textsuperscript{177-179}. Initially, another actin binding region was predicted in the C-terminus of fascin through a rough proteolysis assay\textsuperscript{178}. Preincubation of human fascin 1 with an anti-fascin immunoglobulin that specifically targets the C-terminus of fascin 1 blocked the actin binding of fascin 1 \textit{in vitro}. In experiments that addressed the function of the interaction in cultured cells, introducing the anti-fascin immunoglobulin into cells also partially inhibited cell
migration\textsuperscript{180}. Point mutations that selectively altered amino residues in β-trefoil domain 3 severely affected the F-actin-bundling activity of fascin\textsuperscript{181}. Collectively, these data demonstrate that there are two major actin-binding sites of fascin within β-trefoil domain 1 and 3, which together create a groove for interacting with actin due to its pseudo-2-fold symmetry (Figure 2.1 A). Further evidence supporting these regions as critical for actin binding is found in mutant fascins within \textit{Drosophila}. Fly fascin with mutations at amino residues 277 and 492 disrupted the formation of actin bundles in cells and bristles\textsuperscript{168}.

High and low speed centrifugation experiments have yielded information on fascins’ capacities to directly bind and bundle actin filaments, respectively. These \textit{in vitro} tests indicate that the binding of fascin 1 and fascin 2 to filamentous actin reaches saturation at a stoichiometry of 1:4.76 and 1:3, respectively\textsuperscript{174,182}. The location of the binding site for fascin 1 on F-actin was suggested to be close to the vicinity of the tropomyosin binding site on F-actin as was demonstrated by competition experiments\textsuperscript{183,184}. Other actin-binding proteins, such as caldesmon and skeletal muscle tropomyosin, were shown to compete actin binding sites with fascin 1 and inhibit fascin 1 actin-binding and -bundling activities when all are co-expressed in the \textit{in vitro} experimental setting\textsuperscript{185}. However, the structural interface between the F-actin and fascin in a cellular context remains largely unknown. We believe that the hair bundle, with a highly organized F-actin core, provides a great model to investigate how the interaction between F-actin and its cross-linkers are regulated under physiological conditions.
Figure 2.1

**Figure 2.1.** Proteins in the fascin family are greatly conserved. (A) Surface of fascin 1 structure displayed in two orientations. The blue-to-red scale represents high-to-low residue...
conservation calculated from aligning fascin 1 from 12 species from human to zebrafish. The small diagrams show colored β-trefoil domains and their corresponding positions in each view. Figure adapted from Jansen et al., 2011. (B) For all fascin homologs, amino acid residues in fascin domain 1 (green) that resemble the MARCK domain are highly conserved. (C) Fascin proteins found in different species share a high degree of amino acid similarity at the protein level.

MATERIALS AND METHODS

Zebrafish strain and embryo maintenance

Wild-type Tübingen (Tü) strain zebrafish were raised in fish-system water at 28 °C by standard procedures and kept according to Case Western Reserve University Institutional Animal Care and Use Committee standards. The embryonic zebrafish were staged according to Kimmel et al., 1995.

Verification of the expression of fascin2a and 2b in zebrafish

To confirm mRNA presence in zebrafish hair cells, we performed polymerase chain reaction (PCR) for fascin 2a (NM_001123516.1) product and fascin 2b (NM_200770.2) product using zebrafish hair cell cDNA as a template with the interexonic primer sets ZF F2A 5’ EX2, ZF F2A 3’ EX5, ZF F2B 5’ EX4, and ZF F2B 3’ EX5.1, respectively (Table 2.1). PCR conditions were: 94°C, 1 min; 94°C, 30 sec; 64°C (fascin 2a) or 68°C (fascin 2b), 1 min; 72°C, 1 min; 35 cycles (steps 2-4), and a final extension for 1 min at 72°C. These PCR primers and parameters were designed to amplify a segment of the fascin 2a and fascin
2b cDNA, but not the genomic locus. All PCR amplicons were confirmed to be either *fascin 2a* cDNA or *fascin 2b* cDNA by Sanger sequencing.

**RNA in situ hybridization**

To generate the template plasmids, pCRII::fascin 2a and pCRII::fascin 2b, for the generation of digoxigenin (DIG)-labeled RNA probes for *in situ* hybridizations, PCR was used to amplify the fascin 2a and fascin 2b cDNAs with the primer pairs ZF fascin 2a 5’ start and ZF fascin 2a 3’ and ZF fascin 2b 5’ start and ZF fascin 2b 3’, respectively (Table 2.1). The products of these reactions were separately subcloned into pCRII-TOPO (Invitrogen). The plasmids, pCRII::fascin 2a and pCRII::fascin 2b, were used to generate antisense and sense probes for fascin 2a and fascin 2b mRNA, respectively. Whole-mount *in situ* hybridizations were conducted on 4-days post fertilization (dpf) wild type zebrafish larvae treated with 1-phenyl-2-thiourea to reduce pigmentation. Paraformaldehyde-fixed samples were incubated with digoxigenin-UTP (DIG)-labeled probes. Hybridizations were detected with alkaline-phosphatase-conjugated anti-DIG antibody, and visualized with 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as the formation of insoluble blue precipitate. Frozen sections of 16 µm prepared from labeled embryos were immobilized in optimum cutting temperature (OCT) compound (Sakura Finetek), and sectioned using a cryostat (Leica), according to the procedure described in Söllner et al., 2004.187

**Fluorescent labeling of zebrafish**
To label larvae with anti-fascin 2 serum and fluorophore-coupled phalloidin, 4-dpf larvae were fixed (Cytoskelfix; Cytoskeleton) for six minutes at −20°C. Then embryos were rinsed in 1× PBS four times for 5 minutes at RT and permeabilized in 1.5-3% Triton in 1× PBS overnight at RT. Following permeabilization, embryos were incubated with the anti-fascin 2 serum diluted 1:600 at 4°C overnight. Secondary antibody (Alexa Fluor 488 goat anti-rabbit) was used at a 1:200 dilution. F-actin was labeled with Alexa Fluor 546 phalloidin and anti-actin serum in figure 2.2 and 2.6, respectively. All samples were mounted with Vectashield (Vectorlabs) and stored at 4°C in the dark before imaging.

**Absolute quantitative real-time PCR**

Primers were designed to recognize fascin 2a cDNA (zf F2A 5'E4.1 and zf F2A 3'EX5), fascin 2b cDNA (zf F2B 5'E3.1 and zf F2B 3'E4.1), and beta-2 microglobulin cDNA (zf b2m qPCR F and zf b2m qPCR R), a product of a housekeeping gene. The primers were used in absolute quantitative real-time PCR studies and to produce plasmids to generate standard curves for these experiments (Table 2.1). To generate the plasmids, PCR amplifications were performed using hair-cell cDNA as template. The product of each reaction was subcloned separately into the pCR8/GW/TOPO vector ( Invitrogen). The identities of the PCR products were confirmed by the Sanger sequencing method. Standard curves were generated, using defined quantities of these plasmids, to determine the number of copies of each cDNA in question in the pool of hair-cell cDNAs. The concentrations of the purified DNA plasmids were measured by spectrophotometry (NanoDrop; Thermo Scientific). Plasmids containing fascin 2a cDNA, fascin 2b cDNA, or beta-2 microglobulin cDNA were serially diluted to obtain three standard series ranging from 1 x 107 copies per µl to 1 x 102 copies per µl.
PCR amplifications conducted in absolute quantitative real-time PCR experiments were performed in triplicate using hair-cell cDNA or the plasmid standards (SYBR Green PCR Master Mix; Applied Biosystems). Each reaction contained 12.5 µl of SYBR Green PCR Master Mix, 1 µl each of forward and reverse primer (20 µM), 2 µl of hair-cell cDNA, and 8.5 µl of nuclease-free water. For non-template controls, nuclease-free water was substituted for cDNA. The reactions were performed (7300 Real-Time PCR System; Applied Biosystems) with the following PCR parameters: 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 15 s at 55°C, and 40 s at 72°C. Fluorescence signals were collected during the elongation phase. A dissociation cycle was used as the final cycle of the reaction: 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C.

Three standard curves were each plotted using the natural logs of the number of copies per µl on the abscissa and the threshold cycle (Ct) values on the ordinate (7300 Real-Time PCR System; Applied Biosystems). With the linear trendlines determined as $y = -3.1777x + 35.909$, $y = -3.5936x + 38.787$, and $y = -3.5684x + 39.012$, the primer efficiencies were calculated to be 106%, 89.7%, and 90.7% for fascin 2a, fascin 2b, and beta-2 microglobulin cDNA amplicons, respectively. The dissociation curves of the non-template controls showed flat profiles (data not shown). The remaining aspects of the absolute quantitative real-time PCR protocol were carried out according to Leong and colleagues.

**Construction of expression vectors for GFP-fusion proteins**

Creation of the vector pMT/PV3b/EGFP/WT- fascin 2b for the expression of GFP-WT fascin 2b in hair cells involved multiple steps. To create pMT/SV/PV, a multiple cloning site was generated by annealing two oligonucleotides, 5’ MCS-pBSISK+ and 3’ MCS-
pBSII SK+. All oligonucleotides are listed in Table 2.1. The product was then ligated (T4 ligase; Promega) into pBluescript II SK(+) (Stratagene), which had been digested with SpeI and SacII. The resulting construct was digested with NotI and AflIII to insert a polyadenylation addition sequence, which had been excised from pEGFP-1 (Clontech) using NotI and AflIII. The multiple cloning site with the polyadenylation addition sequence was removed with SpeI and BglII; this digested product was then ligated with the pminiTol2/MCS vector\(^{190}\), which had been digested with the same enzymes, to create pMT/SV. The zebrafish parvalbumin 3b promoter, which drives expression in hair cells, was amplified from the Ppv3b-4 vector\(^{191}\) by a PCR reaction (Pfu polymerase; Stratagene) that introduced BamHI sites onto the product termini using primers Bam Pv3b 1 and 3′_no_G_Pv3b. The promoter was subcloned into pCRII-TOPO (Invitrogen), and the resulting plasmid was digested with BamHI. The fragment containing the promoter was then ligated into BamHI-digested pMT/SV, resulting in pMT/SV/PV. This was digested with Agel and Xmal, and a DNA fragment containing the GFP cDNA in frame with fascin 2b cDNA from pGFP:DrF2B\(^{192}\), also digested with Agel and Xmal, was inserted to yield pMT/PV3b/EGFP/WT-fascin 2b. In all constructs made, the parvalbumin 3b promoter is abbreviated as PV3b.

To generate the GFP-S38D fascin 2b expression construct, the S38D fascin 2b cDNA was amplified by PCR from pGST:DrF2B S39D\(^{192}\) using the primers F2B XhoI 5′ F2FD and F2B XmaI 3′ L2FD (Table 2.1). The PCR product was digested with XhoI and XmaI and ligated into XhoI- and XmaI-digested pMT/PV3b/EGFP/WT-fascin 2b vector. To generate pMT/PV3b/EGFP/S38A- fascin 2b and S38E- fascin 2b, appropriate substitutions were introduced by mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit;
Stratagene) using primer pairs, a51g_g52a_c53g, a51g_g52a_c53g.1, a51g_g52c, and a51g_g52c.1 listed in Table 2.1.

For dominant negative fascin 2b DNA constructs, the DNA fragments containing the first and the last two fascin domains were amplified from pMT/PV3b/EGFP/WT-fascin 2b by PCR with the primer sets XhoI 5’F2B F2FD, Xmal 3’ F2B F2FD, XhoI 5’F2B L2FD, and Xmal 3’F2B L2FD (Table 2.1). Sequence-confirmed amplicons were subcloned with Xmal and XhoI to replace the full length fascin 2b cDNA, and create pMT/PV3b/EGFP/ fascin 2b F2FD and pMT/PV3b/EGFP/ fascin 2b L2FD.

Table 2.1. Primers used for molecular biology experiments in Chapter 2.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence 5' -&gt; 3'</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZF F2A 5’ EX2</td>
<td>CAGGACGATGAGACGGACATGG</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>ZF F2A 3’ EX5</td>
<td>AGACCACTGCTGGAGACGTACC</td>
<td></td>
</tr>
<tr>
<td>ZF 2B 5’ EX4</td>
<td>CGAGGACGAGCAGCTGATTCTGA</td>
<td></td>
</tr>
<tr>
<td>ZF 2B 3’ EX5.1</td>
<td>GTATTTCCAGAGGGAAGAGC</td>
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</tr>
<tr>
<td>ZF fascin 2a 5’ start</td>
<td>ATGTCTACAAACCGAATAAGCGCA</td>
<td>RNA <em>in-situ</em> hybridization</td>
</tr>
<tr>
<td>ZF fascin 2a 3’ end</td>
<td>GTGCTCCCAACAAGGATGAGGCA</td>
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</tr>
<tr>
<td>ZF fascin 2b 5’ start</td>
<td>ATGCCCTCAATGCGACACAAAGC</td>
<td>Absolute real-time PCR</td>
</tr>
<tr>
<td>ZF fascin 2b 3’ end</td>
<td>TCGATTTCCAGAGGGAAGAGC</td>
<td></td>
</tr>
<tr>
<td>zf F2A 5’EX4.1</td>
<td>TCGTCCCAATCCTGGTCTGCG</td>
<td></td>
</tr>
<tr>
<td>zf F2A 3’EX5</td>
<td>AGACCACTGCTGGAGACGTACC</td>
<td></td>
</tr>
<tr>
<td>zf F2B 5’E3.1</td>
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<td></td>
</tr>
<tr>
<td>zf F2B 3’E4.1</td>
<td>CATAAACGAGCGACTGCGTC</td>
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<tr>
<td>zf b2m qPCR F</td>
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</tr>
<tr>
<td>zf b2m qPCR R</td>
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<td>CTAGTTTGGATCCTTATAAAGTTAAAGC</td>
<td>pMT/SV/PV</td>
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<tr>
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<td>3’_no_G_Pv3b</td>
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</table>

Table 2.1. Primers used for molecular biology experiments in Chapter 2.
Zebrfish transgenesis

Fish transposon, Tol2, is a non-viral cargo for delivering foreign DNA fragments into the Danio rerio genome\(^{190}\). To increase the efficiency of the minitol vector system, we co-injected the vector with Tol2 transposase RNA. To make Tol2 transposase RNA, we linearized the pT3TS/Tol2 with *Xba*I, and transcribed it *in vitro* with the mMESSAGE mMACHINE kit (Ambion). The quality and the size of the RNA were confirmed by RNA bioanalyzer. Within each experimental group we co-injected 100 pg of vector DNA and 25 pg of Tol2 RNA into one-cell stage zebrafish embryos. All novel transgenics made and used in this chapter is listed in Table 2.2.

### Table 2.2. Novel transgenic zebrafish used in Chapter 2.

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Transgenic zebrafish name, type</th>
<th>Primary subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMT/PV3b/EGFP/WT-</td>
<td><em>Tg(pvalb3b: EGFP-fscn)</em></td>
<td>Stereocilia, GFP-fascin 2b</td>
</tr>
</tbody>
</table>

The transgenic zebrafish used in this chapter include:

- **a51g_g52c**: GTGAACGCTTCAGCTCCAGCCCTCAAGAA\_GAAGCAG\_CGTCAC
- **a51g_g52c.1**: AGGGAAGCAGCTCCAGCTCCAGCCCTCAAGAA\_GAAGCAG
- **a51g_g52a_c53g**: AGGTGAACGCTTCAGCTCCAGCCCTCAAGAA\_GAAGCAG
- **a51g_g52a_c53g.1**: CAGATCTGCTTCTTCTTGAGCTCTGGAGCTG
- **pMT/PV3b/EGFP/S38A**: pMT/PV3b/EGFP/S38A- fascin 2b
- **XhoI 5’F2B F2FD**: AACTCGAGCAGCATGCCCTCCAATGGCACCAAAG
- **pMT/PV3b/EGFP/S38E**: pMT/PV3b/EGFP/S38E- fascin 2b F2FD
- **XmaI 3’ F2B F2FD**: CTCCCCGGTCATTGCGGATGGCTTTCCTCAAG
- **pMT/PV3b/EGFP/fascin 2b F2FD**: CTCCCCGGGTCTATGAGCTCTTTCTCAAG
- **XhoI 5’F2B L2FD**: AACTCGAGCAGAGGAAGCCATCCCAATGGCACCAAAG
- **pMT/PV3b/EGFP/fascin 2b L2FD**: CTCCCCGGTCAGATGAGCTCTTTCTCAAG
- **XmaI 3’F2B L2FD**: CTCCCGGGTCACTATGAGCTCTTTCTCAAG
- **pMT/PV3b/EGFP/fascin 2b L2FD**: CTCCCGGGGTCACTATGAGCTCTTTCTCAAG

*The table shows the transgenic zebrafish used in Chapter 2, including their plasmid DNA, transgenic zebrafish name, type, and primary subcellular localization.*
<table>
<thead>
<tr>
<th>fascin 2b</th>
<th>2b), stable</th>
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<tr>
<td>pMT/PV3b/EGFP/S38A-2b</td>
<td>Tg(pvalb3b: EGFP-fscn)</td>
</tr>
<tr>
<td>fascin 2b</td>
<td>2b_S38A), stable</td>
</tr>
<tr>
<td>pMT/PV3b/EGFP/S38E-2b</td>
<td>Tg(pvalb3b: EGFP-fscn)</td>
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<tr>
<td>fascin 2b</td>
<td>2b_S38E), stable</td>
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<tr>
<td>pMT/PV3b/EGFP/S38D-2b</td>
<td>Tg(pvalb3b: EGFP-fscn)</td>
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<td>fascin 2b</td>
<td>2b_S38D), somatic</td>
</tr>
<tr>
<td>pMT/PV3b/β-actin/mCherry</td>
<td>Tg(pvalb3b: actb-mCherry)193, stable</td>
</tr>
<tr>
<td>Ppv3b-4</td>
<td>Ppv3b-4191, stable</td>
</tr>
</tbody>
</table>

**Confocal imaging**

All images were taken with a confocal microscope (TCS SP2; Leica Microsystems Inc.) using a 40 X/1.4 NA oil-immersion objective.

**Measurement of stereocilium length**

A method similar to Peng et al. was used to measure the lengths of phalloidin-labeled hair bundles50. Briefly, to measure the maximum lengths of hair bundles, all images were acquired using a confocal laser-scanning microscope (Leica) equipped with a 40 × objective lens and visualized using the manufacturer’s software. For each anterior macula studied, a stack of images captured in the z-plane was collected and compiled into image sequences using Volocity software (Improvision). The maximum length of each phalloidin-labeled hair bundle was measured in the xy-plane. Student’s t-tests were performed, and the results were plotted with GraphPad Prism.
**Preparation of fascin 2b-filamentous actin complexes for electron microscopy**

MBP-fascin 2b fusion protein was expressed in the *E. coli* strain BL21 and purified using amylose resin\(^{192}\). For actin-fascin 2b complex formation, rabbit skeletal muscle actin (Cytoskeleton) was incubated in G-buffer (5 mM Tris [pH 8.0], 0.2 mM CaCl\(_2\), 0.5 mM DTT, 0.2 mM ATP) on ice for 1 h at 2.5 mg/ml and then centrifuged at room temperature for 20 min at 14,000 \(\times g\); the supernatant was collected. The MBP-fascin 2b fusion protein stock was centrifuged at 100,000 \(\times g\) for 1 h at 22°C; the supernatant was collected and its protein concentration determined. 5 \(\mu\)M MBP-fascin 2b was combined with 2.4 \(\mu\)M G-actin in G-buffer. Actin polymerization was stimulated by adding filamentous buffer (500 mM KCl, 20 mM MgCl\(_2\), 10 mM ATP), at a volume 1/10\(^{th}\) of the final reaction volume, to the G-buffer that contained the proteins.

**Negative staining and transmission electron microscopy**

Five \(\mu\)l of MBP-fascin 2b-filamentous actin complexes were placed on a 400-mesh glow discharge/carbon-coated copper grid. After 1 min, the grid was washed with water and then stained with 1% uranyl acetate in water. After 2 min, excess fluid was removed from the grid. Samples were viewed with a transmission electron microscope (FEI Tecnai F30; FEI).

**Fascin 2b knockout fish**

Zebrafish embryos injected with *fscn2b* TALEN RNAs were a gift from Dr. C. Moens. Founder fish that transmit the mutant allele to the offspring were identified using high-resolution melting analysis (HRMA). Genomic DNA from embryos obtained from
potential founder fish were extracted using the HotSHOT method, in which we first incubated fish samples in a basic solution (pH~ 12) at 95°C for 30 min, and then added a neutralizing solution (pH~ 5) after the samples had cooled down to room temperature. Samples were then vortexed and spun down. The resulting supernatant containing genomic DNA was then used for subsequent analysis. To detect mutations by HRMA, 5’ HRM primer and 3’ HRM primer, were used to amplify a 102 bp amplicon containing the full region of the fascin 2b TALEN target site. The thermo-settings for HRMA include: preincubation at 95°C for 5 min, 35 cycles of amplification (95°C, 10s; 68°C, 15s; 72°C, 10s), and the melting curve test (95°C, 30s; 65°C, 1 min) (LightCycler 480, Roche). The genotypes of fascin 2b TALEN knockouts, both adult fish or embryos used for further experiments, were determined by Sanger sequencing with the primer pair, fscn 2b TALEN 5’seq and fscn 2b TALEN 3’seq2. Sequences of the primers used for TALEN experiments are listed in Table 2.3.

Table 2.3. Primers used for identifying fscn2b TALEN knockouts.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5' -&gt; 3’</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>5’ HRM primer</td>
<td>CTCTCTGGGCTTGTGGGTTGT</td>
<td>102</td>
</tr>
<tr>
<td>3’ HRM primer</td>
<td>GCCGTTAGGCTAGCGATTCTC</td>
<td></td>
</tr>
<tr>
<td>fscn 2b TALEN 5’seq</td>
<td>ACTCAGCGGAAAGGCGCAAGGAC</td>
<td>437</td>
</tr>
<tr>
<td>fscn 2b TALEN 3’seq2</td>
<td>CTTTGTCAAGGCGCACGAGCG</td>
<td></td>
</tr>
</tbody>
</table>

FM1-43 dye staining in neuromasts

Free swimming zebrafish larvae at 5 dpf were placed in a 12-well culture plate and immersed in membrane probe solution containing FM1-43 (n-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)-styryl)pyridinium dibromide (Invitrogen), for 1 min, and then rinse three times in regular fish water.
Electrophysiological recordings from zebrafish neuromasts

Larvae at 5 – 7 dpf were anaesthetized with Tricaine (MS-222, Sigma-Aldrich) in a fish external solution containing (in mM): NaCl (120), KCl (2), HEPES (10), CaCl₂ (2), and NaH₂PO₄ (0.7) at pH ~7.3. For recording from the lateral line neuromasts, fish were tied down, on their sides, with strands of dental floss in the recording chamber. Heart beat and blood flow were visually monitored to access viability of the fish throughout the recording period. An upright Olympus BX51WI microscope was used to guide the manipulations and recordings were made with a PC-505B amplifier (Warner Instruments, Hamden, CT) and a PCI-6221 digitizer (National Instruments, Austin, TX). Images were captured with a Grasshopper3 CMOS camera (Point Grey, Richmond, BC, Canada) using the software provided by the manufacturer. Recordings were all made from neuromasts of the posterior lateral line. Stimulation of neuromast hair cells were made by deflection of the cupula using a fluid jet controlled by a HSPC-1 High Speed Pressure Clamp (ALA Scientific Instruments, Farmingdale, NY). The fluid jet pipette loaded with the fish external solution was ~50 µm from the neuromast. Borosilicate glass electrodes with a resistance of 5-6 MΩ were filled with fish external solution and placed proximal to the stereocilia. At room temperature, 50-Hz sinusoidal stimuli were administered and the microphonic potentials were recorded using jClamp (Scisoft) in a current-clamp mode, amplified by 20 (SIM983, Stanford research, Sunnyvale, CA), and low-pass filtered at 200 Hz. All records represent an average of at least 500 responses.
FRAP acquisition and analysis

Embryonic or larval stage zebrafish were anesthetized in 650 µM Tricaine (MS-222, Sigma-Aldrich) in fish water and then mounted on glass-bottom dishes (MatTek) with 0.1 % (wt/vol) low-melting-point agarose (Promega). The immobilized embryos were then covered with fish water containing Tricaine to prevent desiccation and twitching during time-lapse imaging. FRAP experiments were performed using laser scanning confocal microscopes (TCS SP2 or SP5; Leica Microsystems Inc.) with 40 × or 63 × / 1.4 NA oil-immersion lens. Regions of interest in the hair bundle (ROI 1, 100 nm wide) were selected to be photobleached with the full power of 488 nm laser beam. To quantify the fluorescence recovery, the averaged intensity of the bleached zone (region of interest 1, ROI 1), a non-bleached hair bundle (ROI 2) as a control, and a region that did not have high levels of fluorescence (ROI 3) for the background measurements at each time point were measured. The intensity of ROI 1 at any given time point was first corrected by subtracting the background signals (ROI 3, equation 1) and corrected for a global fluorescence loss during imaging (ROI 2, $I_{\text{precell}}/I_{\text{infcell}}$, equation 2). $I_{\text{precell}}$ is the fluorescence intensity of ROI 2 before photobleaching\textsuperscript{196}. $I_{\text{infcell}}$ stands for postbleached ROI 2 at the equilibrium time point—when the intensity of ROI 1 reached the plateau value. Finally, we normalized the fluorescence intensity at the equilibium ($I_{b,\text{corr}}(\text{inf})$) to 100 % (equation 3)\textsuperscript{197}. To determine recovery half times, the data points ($I_{b,\text{corr,normAxelrod}}(t)$) were fitted to a one-phase exponential equation (equation 4). $Y = Y_0 + (\text{plateau} - Y_0)\left(1-\exp \left(-Kx\right)\right)$, where $Y_0$ is the Y value when x (time) is zero, \textit{plateau} is the Y value at infinity, and $K$ is the rate constant. Recovery half times ($t_{1/2}$) were calculated as $\ln(2)/K$ (GraphPad Prism).
RESULTS

*fascin 2b is expressed in hair cells of the zebrafish otocyst*

To identify proteins that may be important for stereocilia development, maintenance, or function, we searched the zebrafish-hair cell transcriptome for the expression of genes encoding proteins that bundle strands of filamentous actin. One gene that we found in the dataset that was considered to be a strong candidate for this function was *fascin 2b*, an actin-bundling protein that belongs to the highly conserved fascin subfamily (Figure 2.1 C). To verify the presence of *fascin 2b* mRNA, and determine if *fascin 2a* mRNA is present, in hair cells, we performed reverse transcription-polymerase chain reactions (RT-PCR) using RNA isolated exclusively from adult zebrafish hair cells. The primers for these reactions recognized different exons of the fascin 2b gene and were used with PCR parameters designed to amplify a segment of the fascin 2b cDNA, but not the genomic locus (Table. 2.1). The amplification experiments readily produced *fascin 2b* cognate products from the hair cell cDNA (Figure 2.2 F), implying a high level of *fascin 2b* mRNA is present in the hair cells of adult zebrafish maculae. Since the zebrafish genome underwent a whole genome duplication event during evolution, this resulted in two fascin 2 genes, *fascin 2a* and *fascin 2b*. To determine the primary isoform expressed in hair cells, we performed absolute quantitatively real-time PCR and demonstrated that in hair cells *fascin 2b* is expressed at a level 42 times greater than *fascin 2a* (Figure 2.3).

In order to distinguish the mRNA expression patterns of these two fascin 2 genes in whole animals, we conducted *in situ* hybridization experiments on 4-dpf zebrafish. Using probes specifically targeted to the individual fascin 2 genes’ mRNAs, we found that the transcript of *fascin 2b* (Figure 2.2 A, C), but not *fascin 2a* (Figure 2.2 G, H), to be the
predominant isoform of fascin-2 that is expressed in the zebrafish inner ear at the embryonic stage. Furthermore, imaging of cyro-sectioned zebrafish ears, from whole-mount embryos, exposed to the anti-sense probe (Figure 2.2 D) revealed that fascin 2b expression was relatively specific to hair cells. On the other hand, the probe specific for fascin 2a mRNA localized within the eye (arrow in Figure 2.2 G) and a structure deeper in the head, not the inner ear (Figure 2.2 G). For comparison, negative control experiments using DIG-labeled sense RNA probes for fascin 2b mRNA (Figure 2.2 B, E) and fascin 2a mRNA (Figure 2.2 I, J) were also performed but did not strongly label.
Figure 2.2. Localization patterns of fascin 2 mRNAs and proteins in zebrafish. (A–E, G–J) RNA in situ hybridizations were performed on larvae at four-days postfertilization (4
Fascin 2b mRNA is detected in the otocysts (A, arrowhead; C) and the eye (A, arrow). Fascin 2a mRNA is expressed in the eye (G, arrow), but not the otocyst (H). Fascin 2b sense RNA (B, E) and fascin 2a sense RNA (I, J), each labeled, were used as controls. A cryosectioned ear labeled with fascin 2b antisense probe is displayed (D). AM, PC, and asterisk indicate the anterior macula, the posterior crista, and the lumen of the otocyst, respectively (C, D, E, H, J). (F) RT-PCR analysis shows expression of fascin 2b in zebrafish hair cells. Agarose gel confirms the expected product size of the fascin 2b amplicon, left lane (+ hair-cell cDNA); no product is observed without cDNA template, right lane (no template control). (K-O) Labeling using fascin 2 antiserum reveals strong fluorescent signals (green) in hair bundles of an anterior macula (K, O) and a posterior crista (L) from larvae at 4 dpf and of an adult lagena (M, N). In red, fluorophore-coupled phalloidin labels the filamentous actin of stereocilia and cuticular plates (K–O). Higher magnification of M is displayed (N). Scale bar is 2 µm. Soma is indicated by asterisk (K). (O) An enlarged view of a hair bundle from an anterior macula is shown with regions of interest (ROI) selected for the stereocilia (orange line) and the cuticular plate (blue line). (P) Fluorescence intensity profiles of stereocilia, using the orange-line ROI from O, show that the fascin 2b- (green) and phalloidin-associated (red) signals are overlapping. (Q) Intensity profiles of the cuticular plate, using the blue-line ROI from O, demonstrate no significant labeling with fascin 2 antiserum (green). Intensity scales are linear, but the units are arbitrary (P, Q). X-axes (P, Q) represent the lengths of the respective orange and blue lines (O).
Fascin 2b protein localizes to stereocilia

Proteins in the fascin family are found localized to actin-rich structures with great specificity. We investigated our hypothesis that fascin 2b may localize to stereocilia and assist in assembly or stabilization of the hair bundle. Using an antibody against fascin 2, we detected fascin 2 proteins in the maculae and cristae of the inner ear at 4 dpf (Figure 2.2 K, L) as well as in adult zebrafish lagenae (Figure 2.2 M, N). The anti-fascin 2 serum (a gift from Dr. Beth Burnside) was generated by injecting Xenopus fascin 2 together with zebrafish fascin 2a and fascin 2b into rabbits; thus, the antiserum does not distinguish between the two fascin 2 isoforms. However, both the absolute quantitative RT-PCR and in situ hybridization experiments suggest that fascin 2b is the principal gene expressed in the ear. Fluorescence intensity plots (Figure 2.2 O, P, Q) show that fascin 2b is evenly distributed among the stereocilia and that its localization strongly correlated with phalloidin labeling in the hair bundle (Figure 2.2 P). This pattern contrasted drastically with that of the cuticular plate (Figure 2.2 Q) where no significant fascin 2b staining was observed in the region of the stereociliary rootlets or anywhere in the cuticular plate.
Figure 2.3

A

Ct

fascin 2a mRNA      fascin 2b mRNA      beta-2 microglobulin mRNA

B

Ct

30.99 ± 0.11

fascin 2a mRNA

27.30 ± 0.15

fascin 2b mRNA

C

Number of copies per µl

fascin 2a mRNA

fascin 2b mRNA

78
Figure 2.3. Detection of the levels of fascin 2 transcripts in hair cells using absolute quantitative real-time PCR. (A) Graph shows levels of transcripts for fascin 2a, fascin 2b, and beta-2 microglobulin as measured using absolute quantitative real-time PCR. Each column represents a threshold cycle (Ct) value measured using primers directed towards fascin 2a cDNA, fascin 2b cDNA, or beta-2 microglobulin cDNA, with adult hair-cell cDNA used as a template. (B) The average Ct value for fascin 2a cDNA and fascin 2b cDNA is 30.99 ± 0.11 (mean ± SEM) and 27.30 ± 0.15, respectively. (C) The number of copies of each fascin 2 cDNA per µl of total hair-cell cDNA is shown. mRNA levels in the hair cell are proportional to calculated cDNA copy numbers. The concentration of fascin 2b cDNA (1612 copies per µl of hair-cell cDNA) is approximately 44 times greater than that of fascin 2a cDNA (37 copies per µl of hair-cell cDNA).

Phosphorylation of fascin 2b on residue serine 38 modulates this proteins localization in hair cells

Phosphorylation of fascin 1 at serine 39 inhibits this protein’s capacity to bind and bundle actin. This alteration influences the formation of actin-rich structures, such as microspikes and filopodia that mediate cell migrations\textsuperscript{177-179}. Cells that express a S39A fascin1 mutant, which possesses a constitutively active actin-binding site, form elongated filopodia; in contrast, the fascin 1 phosphomimetic, S39E, did not enable the formation of filopodia\textsuperscript{171}. In support of the notion that phosphorylation governs the activity of fascin proteins, previous study showed that recombinant protein, the zebrafish fascin 2b phosphomimetic S38D mutant, has lower actin-binding and -bundling activities than the wild-type form \textit{in vitro}\textsuperscript{192}. 
To test our hypothesis that the phosphorylation state of fascin 2b modulates this proteins localization to stereocilia, we used zebrafish transgenesis. Here, we created stable transgenic zebrafish that express wild-type GFP-fascin 2b, Tg(pvalb3b: EGFP-fscn 2b), non-phosphorylatable phosphomutant, GFP-fascin2b S38A, Tg(pvalb3b: EGFP-fscn 2b S38A), or a phosphomimetic form of fascin 2b, GFP-fascin2b S38E, Tg(pvalb3b: EGFP-fscn 2b S38E), in hair cells (Figure 2.4 A) and measured the amount of fusion protein in hair bundles and somata for each protein. The bundle-to-soma florescence intensity ratios (I_{bundle}/ I_{soma}) of 4-dpf zebrafish were 27.9 ± 2.44 (n = 28), 18.1 ± 2.40 (n = 26), 0.63 ± 0.04 (n = 27), and 0.05 ± 0.007 (n = 31) for wild-type fascin 2b, GFP-S38A, GFP-S38E and GFP, respectively (Figure 2.4 B), establishing that phosphorylation diminishes the steady-state level of stereocilia-localized fascin 2b. This phosphoregulatory mechanism persists in mature hair cells, demonstrating that this is an intrinsic property of fascin 2b regardless of developmental stage of the hair cell (Figure 2.4 B, 7 dpf).
Figure 2.4. Stable transgenesis with phosphomutant fusion proteins demonstrates that
phosphorylation of serine 38 of fascin 2b modulates fascin 2b localization in hair cells.

(A) Confocal images (from left to right) were taken of live transgenic zebrafish (4 dpf) that express GFP-fascin 2b, GFP-S38A fascin 2b, GFP-S38E fascin 2b, or GFP, respectively. GFP-associated fluorescence is predominately in stereocilia (yellow bracket) in transgenic zebrafish that express GFP-fascin 2b or GFP-S38A fascin 2b; in contrast, the majority of GFP-S38E fascin 2b and GFP localized to somata (S), with minimal presence in stereocilia.

(B) The ratio of the mean hair-bundle fluorescence intensity (I\text{Bundle}) versus the mean somata fluorescence intensity (I\text{Soma}) was plotted for each fusion protein. Scale bar is 5 µm.

**Fascin 2b and filamentous actin together form highly ordered bundles *in vitro*.

A salient property of actin-bundling proteins that localize to stereocilia is that they organize strands of filamentous actin into parallel bundles\textsuperscript{56,57,199}. By a low-speed centrifugation assay, recombinant fascin 2b protein can cause actin bundling *in vitro*\textsuperscript{192}, but it is not known if this bundling forms loose, orthogonal networks, like those that can be formed by filamin\textsuperscript{200}, or regular, closely packed, parallel actin bundles as in stereocilia. To examine actin bundling by fascin 2b, this protein was expressed with an N-terminal maltose-binding protein (MBP) tag (MBP-fascin 2b) and then purified from bacterial lysates. MBP alone does not bind or bundle actin. Actin from isolated rabbit skeletal muscle was incubated with recombinant fascin 2b in filamentous buffer and the structure of the resultant actin filaments were negatively stained and viewed by transmission electron microscopy. The ratio of recombinant fascin 2b fusion protein to monomeric actin was 2.1 to 1. The electron micrographs showed the formation of regular, closely packed actin bundles with filaments bearing a centerline arrangement, characteristic of parallel actin bundles (*Figure 2.5*). This
indicates that fascin 2b causes the formation of highly ordered bundles of filamentous actin rather than loose networks.

Figure 2.5

**Figure 2.5. Physical characterization of the interaction between fascin 2b and filamentous actin.** (A, B) Negative staining and electron microscopy reveal actin bundling by recombinant fascin 2b fusion protein. Micrographs display filamentous actin in the absence of MBP-fascin 2b (A) and in the presence of MBP-fascin 2b (B); bundled filaments are observed in B. Scale bars are 100 nm.

Long filopodia are generated when fascin 2b is expressed in COS-7 cells, and the effect is augmented by coexpression with espin

Some actin-associated proteins that participate in stereociliary development and maintenance have the capacity to induce the formation of long filopodia or long microvilli in cultured cells. To test whether fascin 2b acts similarly, in COS-7 cells, we expressed
wild-type fascin 2b fused to green fluorescent protein (GFP-WT fascin 2b). The COS-7 cell line has been used for in vitro expression to reveal the fundamental attributes of proteins, including those proteins that are required for hearing. In COS-7 cells in which GFP alone was introduced (number of cells, \( n_{\text{cells}} = 102 \)), fluorescent filopodia were not observed under our culturing conditions (Figure 2.6 C, G, K). We expressed GFP-WT fascin 2b fusion protein in COS-7 cells and observed the formation of long filopodia (mean length ± standard error of the mean (SEM) = 6.04 ± 0.21 \( \mu \)m; number of filopodia, \( n_{\text{filopodia}} = 130 \); number of cells for which filopodial lengths were measured, \( n_{\text{cells}} = 26 \)) (Figure 2.6 A, E, I) with even distribution of GFP along the length of each protrusion. 71% of the transfected cells (\( n = 236 \)) displayed long filopodia, but the remainder did not show this type of protrusion. For comparison, we expressed another actin-bundling protein, espin fused to GFP (GFP-espin), in COS-7 cells and observed filopodia (5.97 ± 0.11 \( \mu \)m; \( n_{\text{filopodia}} = 400 \); \( n_{\text{cells}} = 80 \)) (Figure 2.6 B, F, J) with lengths similar to those of cells that expressed GFP-WT fascin 2b (Figure 2.6 M). 100% of the espin-expressing cells (\( n = 300 \)) formed long filopodia. To determine how the presence of both proteins influences filopodia, we coexpressed GFP-WT fascin 2b and espin in COS-7 cells to simulate conditions in stereocilia and found the mean filopodial length to be longer (8.43 ± 0.17 \( \mu \)m; \( n_{\text{filopodia}} = 330 \); \( n_{\text{cells}} = 66 \)) (Figure 2.6 D, H, L) than the mean filopodial lengths of cells that expressed either protein alone. The mean filopodial length was approximately 2 \( \mu \)m greater than those of cells that expressed either GFP-WT fascin 2b (\( P<0.0001 \), Student’s \( t \) test) or GFP-espin (\( P<0.0001 \)) (Figure 2.6 M), indicating synergism between these actin-bundling proteins. 100% of cells (\( n = 300 \)) that coexpressed these proteins exhibited long filopodia. To confirm that these protrusions were filopodia and therefore composed of actin, we labeled COS-7 cells that expressed both GFP-
WT fascin 2b (Figure 2.6 N) and mCherry-espin (Figure 2.6 O) with fluorophore- coupled phalloidin (Figure 2.6 P). In all cases, the protrusions were shown to be filled with actin by homogeneous phalloidin labeling (Figure 2.6 P, Q). These studies demonstrate that long filopodia are generated when fascin 2b is expressed in cultured cells, and this effect is enhanced by coexpression with espin. Thus, fascin 2b and espin, separately or together, can modulate the lengths of filopodia and can also induce their formation.

Figure 2.6. Roles of fascin 2b proteins in the formation of long filopodia. Schematics of proteins expressed in COS-7 cells and corresponding confocal micrographs are displayed:
GFP-WT fascin 2b (A, E, I), GFP-espin (B, F, J), GFP (C, G, K), GFP-WT fascin 2b and espin (D, H, L). Confocal images are of representative live cells. Filopodia are observable in cells expressing GFP-WT fascin 2b (E, I) and GFP-espin (F, J). None are apparent in cells in which only GFP was introduced, as exhibited by this representative cell (G, K). Cells that express both GFP-WT fascin 2b and espin have longer cytoplasmic protrusions (H, L, M) than cells that express GFP-WT fascin 2b or GFP-espin separately (E, F). The mean lengths ± SEM of the cellular protrusions are shown for cells expressing GFP-espin ($n_{filopodia} = 400$), GFP-WT fascin 2b ($n_{filopodia} = 130$), or GFP-WT fascin 2b together with espin ($n_{filopodia} = 330$), and they indicate synergism between fascin 2b and espin (M). Asterisk indicates $P<0.0001$ for Student’s $t$ test. To visualize actin-based filopodia, cells were labeled with Alexa 633 phalloidin (blue) (P, Q). Coexpression of GFP-WT fascin 2b (N) and mCherry-espin (O) shows that both proteins colocalize (white) (Q) to phalloidin-labeled filopodia (P) in a fixed COS-7 cell. Scale bars of confocal images are 5 µm. Figure courtesy of P. Hwang.

**Lengthening stereocilia by overexpression of GFP-WT-fascin 2b in the hair cell**

To probe how the actin cross-linker, fascin 2b, contributes to the growth of stereocilia, if it has a synergistic effect with other actin cross-linkers as happened in filopodia (Figure 2.6), we measured the stereociliary length in hair bundles of somatic transgenic zebrafish that overexpress GFP-fascin 2b. Embryos injected with transgene constructs were fixed at 4 dpf, and F-actin was visualized with fluorophore-coupled phalloidin (Figure 2.7 A, A’). In injected zebrafish, transient over-expression of GFP-fascin 2b in hair cells had two different patterns of localization, in bundle and in soma. We suspected this is a dose-dependent effect in which a lower level of fusion protein has a stereocilia-exclusive localization pattern.
(Figure 2.7 A’), similar to those seen by antibody labeling (Figure 2.7 K), but a higher level of expression leads to saturation of GFP-fascin 2b in the bundle, which overflows to the soma (Figure 2.7 A). The lengths of each of the hair bundles were determined by measuring the signals from the phalloidin allowing for an accurate comparison between transgenic and non-transgenic hair cells. In the anterior macula, the averaged length of the hair bundle of hair cells not expressing GFP-fascin 2b was 2.36 ± 0.02 µm (n = 480 hair cells) while the average bundle length in hair cells with GFP-fascin 2b expression was 2.85 ± 0.06 µm (n = 132 hair cells). Therefore, bundle lengths of cells expressing GFP-fascin 2b were longer, than those that did not, by greater than ~0.49 µm (P = 0.0001, ** in Figure 2.6 C). To demonstrate that this was indeed an effect cause by fascin 2b and not GFP, we also measured the lengths of bundles of hair cells that expressed GFP alone (Figure 2.7 B), which showed no significant difference in bundle lengths between hair cells with (2.41 ± 0.05 µm, n = 148 hair cells) and without (2.38 ± 0.08 µm, n = 45 hair cells) EGFP (P = 0.8653, * in Figure 2.7 C) in the same animal. This result agreed with our observations in which COS-7 cells transfected with GFP-fascin 2b possessed lengthened filopodia; while, cells transfected with a construct that produces GFP alone lacked filopodia altogether204. Together, these results imply that adding extra actin cross-linkers, such as fascin 2b, increases the lengths of stereocilia.
Figure 2.7. The effect of GFP-WT fascin 2b expression on stereociliary length. (A, A’, B)

Representative confocal images of transgenic hair cells in the anterior maculae of zebrafish at 4 dpf are displayed. (A, A’) Hair cells expressing GFP-WT fascin 2b and (B) GFP are labeled with fluorophore-coupled phalloidin (red). The fusion proteins and GFP appear green. When high levels of GFP-WT fascin 2b are expressed in hair cells (number of cells, n = 192), the fusion protein localizes to the stereocilia and the somata (A’), but when low levels are...
expressed, it localizes specifically to the hair bundles (A). (B) In contrast, cells expressing GFP ($n = 71$) generally exhibit greatly reduced levels of GFP fluorescence in their hair bundles. Scale bars are 5 µm. (C) A graph represents that cells that express GFP-WT fascin 2b ($(+)$ GFP-WT fascin 2b, $n = 132$) have an increased mean hair-bundle length when compared to that of non-transgenic hair cells ($(-)$ GFP WT-fascin 2b) in transgenic animals that mosaically express the transgene. The means of the bundle lengths of cells that lack expression of the fluorescent proteins ($(-)$ GFP WT-fascin 2b, $n = 480$; $(-)$ GFP, $n = 45$) and that of those that express GFP ($(+)$ GFP, $n = 148$) are similar, indicating that there is no substantial experimental variation of the controls within and between the two groups. The means of the bundle lengths ± SEM are plotted. Single and double asterisks indicate $P = 0.0001$ and $P = 0.8653$, respectively, for Student’s $t$ tests.

**Fascin 2b participates in the development of stereocilia.**

Stereocilia formation can be described in four stages: 1) induction of short microvilli at the apical surface of the hair cell, 2) differential elongation of each row of stereocilia, 3) expansion of stereocilia widths, and 4) rootlet growth and extension below the cuticular plate (Figure 1.6). To determine the stage at which fascin 2b first participates in stereocilia development, we monitored the growth of stereocilia in doubly transgenic zebrafish that express both β-actin-mCherry and GFP-fascin 2b in hair cells. In all time-lapse images taken under a confocal microscope, no hair bundle, as identified by β-actin-mCherry expression, was seen without the co-localization of GFP-fascin 2b (Figure 2.8 C). This result demonstrates a synchronization of stereocilia formation and fascin 2b incorporation. Together these results suggest that fascin 2b is actively involved in the induction of F-actin
protrusions at the beginning stage of stereocilia formation, elongation of stereocilia, and maintenance of the heights of stereocilia at later stages.
Figure 2.8
Figure 2.8. GFP-fascin 2b localization in transgenic hair cells faithfully mimics the localization pattern of the endogenous protein. (A) Confocal micrographs of the apical region of a single macular hair cell in a zebrafish larva fixed at 4 dpf. Actin in the stereocilia (arrow) and the cuticular plate (arrow head) was labeled with anti-actin serum (red). Immunolabeling with anti-fascin 2 serum (green) showed that fascin 2b protein localizes to the stereocilia but not to the cuticular plate. (B) A hair bundle from the lateral crista of a doubly transgenic zebrafish at 4 dpf. This cell expresses β-actin-mCherry (red), which labels stereocilia (arrow) and the cuticular plate (arrowhead). GFP-fascin 2b (green) localizes to stereocilia and not to the cuticular plate—faithfully emulating the endogenous localization of fascin 2b. Scale bars are 1 µm. These animals have normal swimming and startle behaviors, indicating that hair cells function normally (data not shown). (C) Time-lapse confocal imaging used to monitor the growth of a hair bundle from the crista of a doubly transgenic zebrafish expressing both β-actin-mCherry (red) and GFP-fascin 2b (green) in hair cells. Fascin 2b is actively incorporated in stereocilia during elongation of the hair bundle. Scale bar is 2 µm

All four fascin domains of fascin 2b are required for its subcellular localization to stereocilia.

Every fascin protein is predicted to have four fascin domains that each folds into a β-trefoil structure (Figure 2.9 A, B). Instead of a single fascin domain, the pseudo 2-fold symmetry of the whole protein was thought to create two actin-binding sites. We therefore constructed two GFP-fascin 2b vectors that each contains only two fascin domains, under the control of the parvalbumin 3b (PV3b) promoter that drives gene expression specifically in
hair cells, to investigate the effect of overexpressing a dominant-negative form of fascin 2b. One vector contains the first two fascin domains (pMT/PV3b/EGFP/fascin 2b F2FD) (Fig 2.9 C), and the other only has the last two fascin domains (pMT/PV3b/EGFP/fascin 2b L2FD) (Figure 2.9 D). When viewed under a confocal microscope, neither of these two fusion proteins localized to the hair bundle in somatic transgenic zebrafish (Figure 2.9 C, D). Also, we did not observe any major morphological changes in stereocilia of these transgenic animals. Our data, agreed with the mutational analysis done on fascin 1, which all four domains together bind F-actin, is true of fascin 2b. However, because these fish expressed endogenous wild-type fascin 2b in addition to the proposed dominant-negative version of the protein, we could not conclude any functional significance of fascin 2b in this experiment. This could be because these truncations may not fold correctly. Therefore, we went on to generate fascin 2b knockout zebrafish to conduct loss-of-function experiments.
Figure 2.9. All four fascin domains are required for proper F-actin binding by fascin 2b. (A) X-ray crystal structure of fascin 1 shown by ribbon representation, image adapted from Chen et al., 2010\(^{173}\). (B) Zebrafish fascin 2b has five exons, which encode four fascin domains. Schematic diagram shows the relative positions of the fascin domains juxtaposed to the corresponding segment of full-length cDNA. (C, D) Schematic diagram of potential
dominant-negative constructs used in our study, with each possessing two fascin domains fused to a GFP reporter. Maximum projection of confocal images of hair cells somatically expressing GFP-fascin 2b F2FD (C) and GFP- fascin 2b L2FD (D) in 4-dpf zebrafish maculae. Maximum projection is a volume rendering method that projects a stack images taken with increments at the z-axis using the maximum signal intensity at each pixel. Localization of fusion proteins (green) is present in the soma but not the stereocilia that are counter-labeled by phalloidin (arrow) in red. This finding suggests that all four fascin domains together manage this protein’s actin-binding and -bundling activity \textit{in vivo}. Scale bars are 5 µm.

\textit{fascin 2b} knockout zebrafish exhibit hair bundles with normal morphology and mechanotransduction

Because transfection of fascin 2b induced filopodia formation in COS-7 cells\textsuperscript{204}, this suggests that fascin 2b may also be important for stereocilia genesis; therefore, fascin 2b knockout zebrafish were created to test this hypothesis. We used a transcription activator-like effector nuclease (TALEN) to create deletions in the exon 1 of the fascin 2b gene (Figure 2.10 A and B). Identified genotypes resulted in frameshift mutations that were predicted to make truncated proteins (detailed method described in the materials and methods). Fascin 2b knockout zebrafish have otic vesicles of regular size in addition to exhibiting both a normal startle response and swim behavior to wild type fish. Protein expression of fascin 2b was not detected in hair cells of the fascin 2b null zebrafish (Figure 2.10 D), the gene symbol of \textit{fascin 2b} is \textit{fscn 2b}. According to the F-actin binding and bundling activity of fascin 2b seen \textit{in vitro} (Figure 2.5), we expected several possible phenotypes for fascin 2b knockouts (Fig
model): (1) no stereocilia formation, if bundling by fascin 2b is important for initiating
the assembly of F-actin at the apical surface of hair cells; (2) stereocilia become shorter or
equal in length, if cross-linking by fascin 2b is crucial for elongation or maintenance of F-
actin; (3) thinner stereocilia, if the number of fascin 2b proteins is proportional to the number
of actin filaments that contributes to the width of each stereocilium. However, we observed
no major differences in hair cell numbers or overall lengths of hair bundles between wild
type and fascin 2b null zebrafish (Figure 2.10 C).
Figure 2.10. Homozygous *fscn 2b* null embryos generated by TALEN-mediated gene deletion no longer have fascin 2b protein in hair bundles. (A) A schematic diagram of the
full-length \textit{fscn 2b} gene, gray boxes represent exons, and black lines represent introns. The TALEN target site (blue line) at the gene locus is within the first exon of \textit{fscn 2b}. (B) Sequencing results of TALEN-induced DNA mutations in the \textit{fscn 2b} locus in the F1 generation of our knockout zebrafish. (C) Hair bundles of cristae were visualized by Alexa-546-conjugated phalloidin in 5-dpf zebrafish. No significant difference in hair bundle morphology and hair cell numbers were detected among each genotype. Asterisks label the soma of the hair cells, and the white brackets indicate the area of hair bundles. (D) At 5 dpf, heterozygous (left) and homozygous (right) fascin 2b TALEN knockout zebrafish were immunolabeled with fascin 2 antiserum (green). Homozygous fascin 2b knockout zebrafish are deprived of fascin 2b protein in hair cells of the inner ear macula. Yellow lines outline a single hair cell. No detectable green signal associated with hair bundles could be observed in \textit{fscn 2b}^{-/-} hair cells (right). Scale bars are 10 µm.
Figure 2.11. Potential phenotypes of hair cells in *fscn 2b* knockout zebrafish. In wild-type zebrafish, a normal hair cell has rows of stereocilia that are graded in height, and a single kinocilium located next to the tallest stereocilia. *fscn2b* knockout may result in no hair bundle, stereocilia with equal lengths, shorter stereocilia, thinner stereocilia, or floppy stereocilia.

Other technologies may help to identify a phenotype in fascin 2b null zebrafish. Due to the size of zebrafish hair bundles, we were not able to resolve an individual stereocilium with regular confocal microscopy, but we think this may be possible with super-resolution microscopy, such as stimulated emission depletion (STED) or structured illumination (SIM), which give spatial resolution between 50-100 nm, such resolution would be adequate to resolve individual stereocilia. We are also currently working on assaying the fine structure of stereocilia in *fscn 2b* with transmission electron microscopy (TEM), but have faced some
technical difficulties preserving the integrity of stereocilia during fixation. Alternatively, without further experiments, we cannot completely rule out the possibility that no phenotype exists in the fascin 2b knockout zebrafish due to possible compensatory upregulation of other actin cross-linking proteins in hair cells that find their way to the stereocilia, such as fascin 1 and fascin 3. However, we did not detect *fascin 1* expression in hair cells of wild-type zebrafish by gene-specific RT-PCR. Effective antibodies against zebrafish proteins are still needed to determine the level of fascin 1 and fascin 3 in the *fascin 2b* null knockout.

It remains possible that strands of F-actin within the stereocilia of knockout zebrafish may not be tightly bundled because of the loss of the most abundant cross-linker, fascin 2b; therefore, it could lead to floppier stereocilia, which, while not morphologically different, are no longer stiff enough for effective mechanotransduction (Figure 2.11). Hence, we tested if loss of fascin 2b hindered mechanotransduction mediated by stereocilia. We first conducted the FM1-43 dye-loading assay, which provides a gross visual assessment of hair-cell mechanotransduction because FM1-43 enters and accumulates in hair cells through functional mechanotransduction channels. Our result showed uptake of FM1-43 in hair cells of homozygous fascin 2b knockout zebrafish (Table 2.4). To further identify possible defects in mechanotransduction ability of hair bundles we quantified mechanotransduction by conducting electrophysiology experiments that record microphonic potentials from neuromast hair cells of the *fascin 2b* knockout zebrafish (Figure 2.12 A, B). In neuromasts, the average microphonic potentials (mean ± SEM) in wild type, heterozygous, and homozygous fascin 2b knockout zebrafish were 9.80 ± 1.33 µV (n = 4), 8.07 ± 0.61 µV (n = 7), and 9.32 ± 0.76 µV (n = 5), respectively. No differences between any two groups were statistically significant via Student’s T-test (Figure 2.12 C, Table 2.4). In addition, voltage
changes per bundle in all groups tested were very similar, as it was $0.59 \pm 0.098 \, \mu V$ for wild-type cells, $0.52 \pm 0.079 \, \mu V$ for heterozygous mutants, and $0.57 \pm 0.073 \, \mu V$ for homozygous mutants. Therefore, loss of fascin 2b does not affect the mechanotransduction in hair cells in neuromasts. In the future, to determine the importance of fascin 2b in hair cells mediating hearing; we will measure microphonic potentials and mechanotransduction currents of hair cells from the inner ear of $fascin \, 2b$ knockout zebrafish.
Figure 2.12

**A** puff pipette

**B**

**C**

- **wild type**
- **fscn 2b +/-**
- **fscn 2b -/-**
Figure 2.12. Microphonic recordings from neuromast hair cells. (A) A schematic diagram demonstrates the setup of our microphonic recording experiment. Hair cell mechanotransduction is stimulated by the deflection of the cupula, which contain stereocilia, and the kinocilia (red). Frequency of movement of the cupula is regulated by a water jet delivered by the puff pipette. Corresponding voltage changes are recorded from the electrode, which is placed close to the hair bundle. (B) DIC micrographs show the proper positioning of the pipettes and the neuromast that is under examination from a top-down view. Left panel, the puff pipette (*) is placed ~50 µm away from the neuromast and is in focus with the middle sections of kinocilia (arrow head). Right panel, the recording pipette (**) is placed just above the apical surface of hair cells and next to stereocilia. Scale bar is 20 µm. (C) Representative traces of the microphonic potentials recorded from wild-type zebrafish and heterozygous and homozygous fascin 2b knockout animals. The bottom trace demonstrates the stimulus delivered through the puff pipette. Microphonic potentials recorded from fscn 2b -/- zebrafish remain similar to the wild type.

Table 2.4. Summary of the phenotypes of fascin 2b knockout zebrafish.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hair cell number (mean ± SD)</th>
<th>Stereocilia morphology</th>
<th>Startle reflex</th>
<th>FM1-43 dye loading</th>
<th>Microphonic potentials (µV) mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>22.3 ± 2.5</td>
<td>normal</td>
<td>yes</td>
<td>yes</td>
<td>9.8 ± 1.33</td>
</tr>
<tr>
<td>fscn 2b +/-</td>
<td>20.9 ± 2.4</td>
<td>normal</td>
<td>yes</td>
<td>yes</td>
<td>8.07 ± 0.61</td>
</tr>
<tr>
<td>fscn 2b -/-</td>
<td>20 ± 3</td>
<td>normal</td>
<td>yes</td>
<td>yes</td>
<td>9.32 ± 0.76</td>
</tr>
</tbody>
</table>

Fast and non-biased movement of fascin 2b in zebrafish stereocilia

For many F-actin-based cellular structures, such as neuronal growth cones and filopodia, actin assembly, actin disassembly, and the dynamical activities of other actin-
associated proteins are crucial for their normal function\textsuperscript{206,207}. Despite their high structural similarity to filopodia, the permanency of stereocilia throughout the lifetime of the hair cell has driven efforts to understand the protein dynamics within the stereocilia. Studies of cultured mouse hair cells transfected with GFP-actin showed that the actin of stereocillia “treadmill” from the tip to base on an hourly timescale\textsuperscript{208}. On the other hand, isotope labeling experiments suggested that most protein, up to > 90%, in the shafts of stereocilia is stationary for up to a few weeks, and the majority of protein renewal occurs at tips\textsuperscript{209}. In this study, we ask how fascin 2b protein behaves in stereocilia of a live zebrafish. Hence, we generated the stable transgenic zebrafish Tg(pvalb3b: EGFP-fscn 2b), which has GFP-fascin 2b protein localized exclusively to stereocilia but no other subcellular domains (Figure 2.8 B). And we performed fluorescent recovery after photobleaching (FRAP) with this transgenic zebrafish. We demonstrated a fast fascin 2b exchange rate in mature stereocilia of $t_{1/2}$ of $76.3 \pm 8.55$ s ($n = 11$) (Figure 2.13 A-D) and this movement is unbiased (Figure 2.13 E, F). We bleached the top half (Figure 2.13 E) or the bottom half (Figure 2.13 F) of the stereocilia, and watched the fluorescent signals recover from the bottom up or top down, respectively. These results demonstrate that fascin 2b can move unbiased in stereocilia. Interestingly, the exchange rate of fascin 2b recovery in zebrafish stereocilia is slower than that had been observed for fascin 1 in filopodia in cultured cells\textsuperscript{210}. Perhaps the number of actin filaments, one of the fundamental differences between stereocilium and filopodia, of which the former contains more of, contributes to the mobility of actin-associated proteins.
Figure 2.13. Stereociliary fascin 2b exchanges rapidly and without bias. (A) A schematic of the hair bundle with GFP-fascin 2b. (B) A lateral crista hair bundle before (Pre) and after a 100-nm-wide photobleach, which targeted the mid-region (yellow bracket). (C) FRAP profiles from B. Within 315 s, the mean intensity in the post-bleached region matches the mean intensity along the length of the stereocilia (dashed line), indicating complete GFP-fascin 2b exchange. (D) Recovery plot, from C, fit to a one-phase exponential function. The exchange rate of this particular hair bundle is determined to be $t_{1/2} = 42.6$ s. (E, F) Color-coded scale of recovery, after upper half (E) or lower half (F) of bundles were bleached (dashed line), demonstrates that GFP-fascin 2b migrates towards the stereociliary tips or bases, respectively. Blue- to-red color scale represents low-to-high GFP signal, respectively. Scale bars are 1 µm. Figure courtesy of P. Hwang.
DISCUSSION

Function of actin cross-linking proteins in regulating F-actin in stereocilia

Our data reveal the expression of fascin 2b in the inner ear hair cells of zebrafish, and the localization of fascin 2b to be exclusive to the stereocilia (Figure 2.2). With electron microscopy (EM), we show that recombinant MBP-fascin 2b binds to F-actin and organizes it into highly ordered bundles rather than loose networks (Figure 2.5). Using a supernatant-depletion assay, we determined the dissociation constant of filamentous actin and MBP-fascin 2b to be $K_d \approx 0.37 \, \mu M^{204}$. A proteomic-based study revealed the expression of multiple actin cross-linking proteins in stereocilia of chicken vestibular hair cells and that the level of expression of each protein varies$^{205}$. The most abundant actin cross-linker is fascin 2 followed by plastin1 and espin. Interestingly, the actin affinity we determined for fascin 2b is similar to that of espin$^5^7$ ($K_d \approx 0.22 \, \mu M$). By co-transfection of fascin 2b and espin in cultured cells, we showed a synergistic function on lengthening the filopodia$^{204}$.

For future investigations, we propose a two-pronged approach to address how two different actin cross-linkers act on F-actin simultaneously in stereocilia. One, discern the bundling capacity of each actin cross-linker alone or together. Using EM, we can measure the thickness of and number of actin filaments actin within bundles created by incubation of F-actin with different combinations of actin cross-linking proteins. In addition, EM tomography could be utilized to reveal the functional binding site of the cross-linkers on F-actin and patterns of bundling within the cross-linked F-actin. Two, reveal whether each actin cross-linker occupies different compartments of the stereocilia. We propose to generate transgenic zebrafish expressing fluorescent-tagged actin cross-linker proteins that can be visualized after diaminobenzidine (DAB) photooxidation in EM$^{211}$. Under EM, samples taken in views of
longitudinal and transverse sections of the stereocilia can reveal localization of protein-DAB precipitates within a single stereocilium. This would help us to elucidate if any actin cross-linkers have a preferable affinity to actin filaments at the core or the rim of the stereocilium.

**Overexpression of fascin 2b lengthens actin-based protrusions**

In cultured cells, we demonstrated that expression of fascin 2b induced the formation of long filopodia (**Figure 2.6**), and extra-long filopodia were observed when fascin 2b was co-expressed with espin, another actin-bundling protein. This synergism is also found in hair cell stereocilia. In our transgenic animals, hair bundles with GFP-fascin 2b fusion protein are significantly longer than bundles from their non-transgenic littermates or GFP-expressing zebrafish (**Figure 2.7**). This result suggests a role for fascin 2b in the elongation of stereocilia during hair bundle genesis. These interesting findings indicate that multiple actin cross-linkers, or at least fascin 2b and espin together, stimulate lengthening of actin-based protrusions *in vivo*. On the other hand, in our real-time imaging performed in the doubly transgenic zebrafish, we concluded that incorporation of fascin 2b proteins in stereocilia coincides with the growth of the hair bundle (**Figure 2.8 C**). The drawback of this experiment is that the parvalbumin 3b promoter we used is a hair-cell specific promoter that turns on early during the formation of the hair cell; therefore, it could be argued that this experiment does not truly represent the timing of expression of fascin 2b. With this caveat in mind, we believe that fascin 2b is incorporated into developing stereocilia and plays a positive role in organizing F-actin. Moreover, perhaps this earlier entry of fascin 2b explains the lengthening effect on stereocilia in the transgenic animals when compared to the wild-
type zebrafish. In order to fully understand the role of fascin 2b in the developing hair bundle it may be necessary to identify and clone its endogenous promoter.

**A two-hit model in stereocilia maintenance**

To test if fascin 2b is necessary for building stereocilia, we created fascin 2b null knockouts (Figure 2.10 A, B). Depletion of fascin 2b in the knockout zebrafish did not abolish the formation of, or alter the morphology of, stereocilia (Figure 2.10 C). Mice with mutant fascin 2 (R109H) form stereocilia but they degenerate with aging when in a strain with a mutation in cadherin 23; this leads to progressive hearing loss\(^{212}\). In addition, conditional knockouts of either β- or γ- actin genes in hair cells of mice interfere with the stereocilia morphology in aging hair cells\(^{40}\). Hair bundles form normally at the beginning in either β- actin or γ- actin single-gene-knockout animals, but stereocilia degenerate progressively and animals develop deafness gradually\(^{40}\). Whether mutation of fascin 2b in fish causes progressive hair cell damage, it is not clear. It is a challenge to study progressive hearing loss in fish because zebrafish regenerate their hair cells.

On the other hand, there are multiple actin cross-linkers in stereocilia with different level of abundance; therefore, we propose that the lack of a strong stereocilia defect in fascin 2b knockout zebrafish could be explained by the two-hit model. The idea of a two-hit model was first introduced to explain cancer progression by Dr. Knudson in the 1971\(^{213}\). By comparing numerous case studies of inherited and non-inherited forms of retinoblastoma, Dr. Knudson observed that inherited cases of retinoblastoma tend to arise later than inherited forms. He hypothesized this is because it takes time to accumulate mutations in both copies of a tumor suppressor gene. To loosely apply this concept to inner ear biology, we propose
that actin bundling proteins are so necessary for hair bundle development that in order to cause damage to a hair bundle at least two genes must be non-functional. A mouse line that has GFP-knock-in at the fascin 2 locus that disrupts its protein production was not reported to have an auditory phenotype\textsuperscript{214}. In other words, functions of different actin cross-linking proteins may be similar to each other, and the shared activities help to support the formation of stereocilia. Hence, creating double knockout zebrafish targeting different actin cross-linking proteins would allow us to test our two-hit model of stereocilia development and deafness.

**Fascin 2b movement within stereocilia supports the active modulation of actin filaments during hair bundle deflection**

The fascin 2b exchange rate ($t_{1/2}$ of 76.3 ± 8.55 s) represents a golden mean\textsuperscript{215}, enabling two opposing functions: stable cross-linking at fast timescales and malleable cross-linking at slower timescales. On the one hand, during deflections of stereocilia fascin 2b proteins serve as stationary cross-links. This is because the fascin 2b exchange rate is much slower than the rate of stereocilia deflections (subsecond timescale). On the other hand, fascin 2b mobility should enable actin exchange. Previous studies had revealed discontinuous gaps within the F-actin strands of stereocilia\textsuperscript{39}. Here, we proposed that exchange of fascin 2b is required for locally regulating actin dynamics as fascin 2b cross-links dissociate to permit localized F-actin disassembly and re-cross-link during re-polymerization to enable reformation of the paracrystal. Since fascin 2b exchanges without bias (Figure 2.13 E and F), this suggests that fascin 2b plays a non-instructive role in the F-actin disassembly-re-polymerization process.
-Chapter 3-

Understanding Hair Bundle Development with Zebrafish Transgenesis
ABSTRACT

Development of the hair bundle is a complicated process. The timeline of development has been determined in chickens and mice, but the time course of bundle development needs to be established in zebrafish. Three decades ago, an elegant experiment that examined the structure of chicken hair bundles using electron microscopy was performed by Tilney and colleagues\textsuperscript{216}, and it demonstrated several morphologically distinct stages during the development of hair bundles. Hair bundle development starts from the growth of a single kinocilium, followed by the differential elongation of stereocilia, the extension of rootlets, and simultaneously the formation of the cuticular plate. Mutations in many genes can result in a malformed hair bundle or result in a bundle with defects that occur over time. These morphological defects usually leads to hearing loss. Through our genetic approach, we will identify more actin-associated proteins, such as fascin 2b in chapter 2, in stereocilia; thus to understand the significance of each gene in each step of hair bundle development, we aimed to visualize the localization of relevant structural proteins within developing hair bundles in live animals in real time. We first sought to characterize the growth of stereocilia and the kinocilium under normal conditions. For these studies, we took advantage of the optical transparency of zebrafish larvae and transgenesis with a hair-cell-specific promoter, parvalbumin 3b, to express fluorescently-tagged fusion proteins in hair cells to monitor behaviors of target proteins during hair bundle development.

To view the development of the hair bundle under a confocal microscope in real time, we first created stable transgenic lines that express cytoskeletal proteins. One set of lines, β-actin-mCherry or GFP-tubulin α 1 were used for visualizing whole stereocilia or kinocilia, respectively, in the maculae, cristaed, and neuromasts. The rate of F-actin and microtubule
assembly in stereocilia and the kinocilium was measured with time-lapse imaging of fusion proteins in the hair bundle. In addition, to probe the timing for differential elongation of stereocilia, we established a unique transgenic zebrafish line expressing actin motor protein GFP-MYO15a, which is known to localize at the tip of stereocilia in mature mouse hair cells, to track their localization pattern during hair bundle development. Finally, to understand the possible timing of mechanotransduction apparatus assembly during hair bundle development, we cloned zebrafish *lhfpl5a* and *lhfpl5b*, homologs of a human deafness gene lipoma HMGIC fusion partner-like 5 (*LHFPL5*) suggested to be part of the mechanotransduction complex in hair cells, and generated transgenic zebrafish with GFP-Lhfpl5b labeling at the tip of stereocilia. In conjunction with confocal laser scanning microscopy, these transgenic strains will not only allow us to monitor the sequence of hair bundle development but also serve as a new tool for detecting morphological phenotypes in novel knockouts.

**INTRODUCTION**

**Variations of hair bundle structure**

Hair cells are specialized epithelial cells that form hair bundles for mechanosensitivity, but to be able to initiate the process of hearing, the hair bundle must have the correct morphology and be placed in the proper location. Structures of hair bundles from various species have been investigated by electron microscopy\(^{32,216,217}\). One of the pioneering works of the hair bundle focused on the lizard ear. It showed that basilar papilla of the lizard ear has approximately 150 hair cells, and the maximum height of the hair bundles vary from 7 to 31 \(\mu\)m from the basal to the apical region of the papilla\(^{34}\). In contrast, the 7- to 10-day-old chicken basilar papilla contains about 500 hair cells, and are grouped
into tall and short hair cells from the superior and the inferior margin of the papilla, respectively\textsuperscript{216}. The hair cells of the papilla are remarkably well organized, as with other auditory organs, so the number, distribution, lengths, and widths of the stereocilia together create an inner ear with remarkable sensitivity. From the distal end of the papilla, a hair cell may have 50 stereocilia with the tallest one being 5.5 µm in length and 0.12 µm in width. There are increasing numbers and widths, but decreasing lengths, of stereocilia from the distal to proximal end of the papilla\textsuperscript{216}. In the cochlea of mammals, the basal turn of the sensory epithelium, responsible for detecting high frequency stimuli, has stereocilia with shorter lengths than those that are located more apically\textsuperscript{218}. Also, in the cochlea of mammals, there are two types of hair cells, inner and outer hair cells. Inner hair cells organize their stereocilia in a flat U-shape, and outer hair cells arrange their three distinct rows of stereocilia into a V/W-shape with the kinocilium at the midpoint between left and right symmetrical halves\textsuperscript{219}. One of the factors that influence frequency tuning in different species are the lengths and widths of stereocilia, so the molecular mechanisms that govern their shapes are vitally important to determine; moreover, the mechanisms that result in interspecies hair bundle morphological differences are even less understood.

\textbf{Planar cell polarity in the inner ear}

Like other epithelial cells, hair cells grow with distinct identity of apical-basal polarity, in which hair bundles protrude from the apical surface and the spiral ganglion neurons form synaptic contacts at the basal lateral membrane of each hair cell in mammals. In addition, planar cell polarity (PCP) is involved in setting up three tiers of asymmetrical patterns in the inner ear\textsuperscript{220}. First, staircase-shaped hair bundles define the positive direction
of the bundle deflection that activates mechanotransduction in individual hair cells (Figure 3.1 A-C). Second, the orientations of neighboring hair cells are highly coordinated (Figure 3.1 D). Third, the orientations of polarized bundles in the auditory epithelium are non-random and are either uniform across the base to the apex of the cochlear duct in mammals (Figure 3.1 D) or follow a global trend (Figure 3.1 E) in zebrafish ear.

The process of building uniformly polarized hair bundles is linked to the core PCP pathway. PCP proteins, such as Van Gogh-like, Frizzled, and Dishevelled, were found asymmetrically localized in hair cells. Often mutant mice that reduce or change the expression patterns of the PCP proteins in the ear have no significant defects in the shape, the number, and the distribution of stereocilia, but their hair bundles are disoriented. PCP proteins also govern placement of the kinocilium. In many cases of PCP-mutant mice, kinocilium movement persists but with miscellaneous final positions. Although we know PCP proteins play a significant role, the order in which the kinocilium and the tissue planar polarity develops still needs to be elucidated.
Figure 3.1. Development of hair bundles and the innervation of hair cells. (A) SEM image of a mature hair cell from the mammalian Organ of Corti demonstrates three rows of stereocilia on the apical surface and the organization in a staircase shape. Figure adapted from Frolokov et al., 2004². (B) Hair cells in a single neuromast have two opposite polarities. Zebrafish labeled with fluorophore-conjugated phalloidin (green) shows the bundle orientation in neuromast hair cells. Position of the kinocilium is a hollow region (arrow), which is depraved of F-actin on the apical surface of the hair cells. Scale bar is 1 µm. (C) Afferent fibers selectively innervate hair cells with the same polarity. A and P represent the anterior and posterior axis of zebrafish. (D) SEM image of the mouse organ of Corti at
the middle turn. All three rows of OHCs and IHCs have stereocilia with a uniform orientation. Figure adapted from Montcouquiol et al., 2003\textsuperscript{226}. (E) Phalloidin labeling of hair cells in the anterior macula of zebrafish. Stereocilia and cell boundaries are stained but not the kinocilia, thus leave a black dot at the apical surface of each hair cell (G). A and L represents the anterior and lateral axis of the fish, respectively. (G’) An arrow can be drawn from stereocilia to the kinocilium to represent the polarity of the hair bundle. (F) Hair cells polarities of (E) depict as arrows. Scale bar is 10 μm. Figure adapted from Inoue et al., 2013\textsuperscript{227}.

**MATERIALS AND METHODS**

**Generation of transgenic zebrafish**

Zebrafish β-actin cDNA was amplified with primer pairs Xmal\_beta\_actin and PacI\_beta\_actin. The product was inserted into pMT/SV/PV3b/mCherry (West and McDermott, 2011) to create pMT/PV3b/β-actin/mCherry. To construct pMT/PV3b/EGFP/tuba1, the full-length tubulin α-1 cDNA, which was amplified from zebrafish hair cell cDNA with primer pair XhoI\_G\_5’ tubal and XmaI\_3’ tuba1, was ligated to XhoI and XmaI digested pMT/PV3b/EGFP/WT-fascin 2b. The vector for overexpressing GFP-MYO15A in hair cells was created by subcloning mouse Myo15a cDNA amplified from pEGFP-C2-myo15a\textsuperscript{67} with primer pair Myo15a inf F and Myo15a inf R to Xho I and Xma I digested pMT/PV3b/GFP/WT-fascin2b vector (In-Fusion HD cloning kit; Clontech). For expression vectors encoding TMHS-fusion proteins, GFP proteins cDNA was fused to the cDNA encoding the N-terminus of Lhfpl5b and the C-terminus of Lhfpl5a. Transcripts of lhfpl5b were amplified with lhfpl5b infu F and lhfpl5b infu R primers from zebrafish whole fish.
cDNA and *lhfpl5a* transcripts were amplified from zebrafish hair cell cDNA with *lhfpl5a* C-term *infu* F and *lhfpl5a* C-term *infu* R primers. In-Fusion cloning was then used to create pMT/PV3b/GFP/*lhfpl5b* and pMT/PV3b/*lhfpl5a*/GFP. Primers used for constructing expression vectors are listed in Table 3.1, and transgenic animals created are listed in Table 3.2. DNA constructs were microinjected into one-cell-staged zebrafish embryos and fluorescent signals were screened in ears of larval zebrafish between 3 – 6 dpf with a Leica MZFLIII fluorescence stereomicroscope.

Table 3.1. Primer pairs used for creating expression vectors used in microinjection.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequences 5' (\rightarrow) 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>XmaI_beta_actin</td>
<td>CCGGGATGGATGATGAAAATTGCGGCACT</td>
</tr>
<tr>
<td>PacI_beta_actin</td>
<td>TTAATTAAGAAGCATTTGCGGTGGACGATG</td>
</tr>
<tr>
<td>XhoI_G_5’ tuba1</td>
<td>AACTCGAGGATGCGGTAGTGAGATCTCTCTATCC</td>
</tr>
<tr>
<td>XmaI_3’ tuba1</td>
<td>AACCCGGGTGGACAGAAACACAGC</td>
</tr>
<tr>
<td>Myo15a inf F</td>
<td>GGACTCAGATCCTGAGATTCATGCACCTCATAAGCAACCT</td>
</tr>
<tr>
<td>Myo15a inf R</td>
<td>TAAGGATCCACCCGGCGATCGACTGACACGAGGATCT</td>
</tr>
<tr>
<td>lhfpl5b inf F</td>
<td>GGACTCAGATCCTGAGCTCTGAGATCTCTAAACATGAGCAAAGG</td>
</tr>
<tr>
<td>lhfpl5b inf R</td>
<td>TAAGGATCCACCCGGATCGTCTGCAGTGNAGTTTTC</td>
</tr>
<tr>
<td>lhfpl5a C-term infu F</td>
<td>CAGTTTGAATATCCGGAGACCAACTGAAAAAGCGGTTC</td>
</tr>
<tr>
<td>lhfpl5a C-term infu R</td>
<td>TTGCTACATTGTTAAAACGCGGATCATGCGTTTTC</td>
</tr>
</tbody>
</table>

Table 3.2. Transgenic animals used in Chapter 3.

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Transgenic zebrafish</th>
<th>Primary Subcellular localization pattern, protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMT/PV3b/β-actin/mCherry</td>
<td><em>Tg(pvalb3b: actb-mCherry)</em></td>
<td>Stereocilia and cuticular plate, β-actin-mCherry</td>
</tr>
<tr>
<td>pMT/PV3b/EGFP/tuba1</td>
<td><em>Tg (pvalb3b: EGFP)</em></td>
<td>Kinocilium and microtubules,</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Antigen</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>pMT/PV3b/EGFP/Mgm1</td>
<td>Somatic</td>
<td>GFP-tubulin α1</td>
</tr>
<tr>
<td>pMT/PV3b/EGFP/myo15a</td>
<td>Tips of stereocilia,</td>
<td>GFP-MYO15A</td>
</tr>
<tr>
<td>pMT/PV3b/EGFP/lhfpl5b</td>
<td>Tips of stereocilia,</td>
<td>GFP-Lhfpl5b</td>
</tr>
<tr>
<td>pMT/PV3b/lhfpl5b/mGFP</td>
<td>Somatic</td>
<td>Lhfpl5a-GFP</td>
</tr>
</tbody>
</table>

**Immunolabeling of the hair bundle in zebrafish larvae**

Larval zebrafish from *Tg(pvalb3b: actb-mCherry)* were fixed with 4% PFA, permeabilized with TritonX-100, and stained with selected antibodies. Alexa Fluor 488 phalloidin is used to label F-actin. For labeling microtubules, monoclonal antiserum against acetylated-tubulin and AlexaFluor 405 goat anti-rabbit serum were used and the primary and the secondary antibodies, respectively. Processed zebrafish samples were mounted with Vectashield prior and during imaging.

**Confocal imaging**

All images were taken with a confocal microscope (TCS SP2 or SP8; Leica Microsystems Inc.) using a 40× or 63× / 1.4 NA oil-immersion objective.
RESULTS

Elongation of stereocilia in zebrafish hair cells is 33 times faster than chicken

Since the exact time line of the development of stereocilia is unknown in zebrafish, we want to determine the spatiotemporal relationships of the development of this structure so we created transgenic zebrafish Tg (pvalb3b: actb-mCherry) (Figure 3.2 A, B). This strain of transgenic zebrafish expresses β-actin-mCherry in the stereocilia and the cuticular plate of hair cells (Figure 3.2 C-E) and enables the visualization of the formation, elongation, and the pattern development of these actin-rich structures (Figure 3.2 F-F‴) in real-time. Fusion protein, β-actin-mCherry, labels actin-filament-based but not microtubule-based structures with high specificity (Figure 3.2 G). Live imaging by confocal microscopy showed a single hair cell with stereocilia at 58 hours post fertilization (hpf) (Figure 3.2 H). Four hours later, the stereocilia from the same cell elongated (Figure 3.2 I), and continued to grow for another 6 hours (Figure 3.2 J).

We also observed that the cuticular plate grew in thickness in the same cell we imaged. Overexpression of β-actin-mCherry in hair cells does not change the length of the hair bundles as mature stereocilia with β-actin-mCherry have similar lengths when compared to non-transgenic hair cells. Interestingly, our preliminary data reveals two different rates of stereocilia lengthening in zebrafish embryos with similar ages. First, a rapid initial growth of stereocilia (Figure 3.2 K, bundle 3) was found, during which the length of stereocilia increased about 2 µm in three hours. Second, a slow growth of stereocilia (Figure 3.2 K, bundle 1) was seen, during which stereocilia elongate about 0.2 µm in 14 hours. However, we do need more data to validate whether these two different growth rates of stereocilia, fast and slow, respectively, can represent a fast promotion of actin filament formation and a
steady state elongation of actin filaments after the stereocilia reach a certain length. Yet, the fastest rate of stereocilia elongation we observed in the cristae of our transgenic zebrafish is 33 times faster than that was reported from the chicken basilar papilla\textsuperscript{78}.

\textbf{Figure 3.2}
Figure 3.2. Using transgenic zebrafish to monitor the development of stereocilia in hair cells. (A) A schematic diagram of the embryonic zebrafish with the otic vesicle in pink, sensory hair cells of cristae (green), maculae (blue), and neuromast organs (red dots) of the lateral line. (B) At 7dpf, a live transgenic Tg (pvalb3b: actb-mCherry) animal expresses β-actin-mCherry (bright red) specifically in hair cells of neuromasts and maculae. (C-J) Confocal images of Tg (pvalb3b: actb-mCherry) zebrafish at 4 dpf, unless otherwise indicated. β-actin-mCherry fusion proteins localize in the stereocilia, the cuticular plate, and the circumferential ring of hair cells in cristae (C), neuromast (D), and maculae in live animals (E). At the apical surface of the hair cells, the kinocilium resides in the foniculus (arrow head in D). (F and G) Fixed samples demonstrate that the expression of β-actin-mCherry is highly colocalized with F-actin (green) but not microtubules (cyan). Neuromast hair cells viewed from the top with β-actin-mCherry signals in red (F and F’’) and phalloidin-stained actin filaments in green (F’ and F’’). (F’’) Polarity of hair cells within the neuromast depicted by arrows point to the position of the kinocilium. (G) Immunolabeled microtubules (blue) in hair cells validate the specificity of the fusion protein to actin in this transgenic zebrafish. (H-J) Time-lapse recording from Tg (pvalb3b: actb-mCherry) zebrafish displays the growth of stereocilia (bracket) in 10 hours. A single hair cell from a lateral crista is displayed. (K) Length of the stereocilia of three representative hair cells imaged over 23 hours. Asterisks indicate the soma of the hair cells. Scale bars are 5 µm.

The kinocilium keeps lengthening while the stereocilia start to elongate

Mechanosensitivity of the hair cell is initiated with deflection of the hair bundle in a positive direction, from the stereocilia to the kinocilium; therefore, the position of the
kinocilium is critical for determining the polarity during stereocilia development, which then defines the direction of functional bundle displacement. In some cases, the kinocilium remains present throughout the lifetime of the hair cell, such as hair cells in the vestibular system of mammals although whether this contributes to a different mechanism for mechanotransduction remains controversial\textsuperscript{228-230}. Mouse cochlea lacking Kif3a, a protein that encodes the microtubule motor subunit, developed a shortened cochlear duct, and lost the v-shaped organization of the stereocilia in addition to the absence of kinocilia in the hair cells\textsuperscript{231}. This study suggests a possible instructive role of the kinocilium in setting up the planar cell polarity for positioning the stereocilia. To further understand the patterning of the kinocilium during hair bundle development, we thought it would be useful to create a transgenic zebrafish that labels the kinocilia of hair cells for monitoring the initial migration of the kinocilium and its relative position to developing stereocilia. Transgenic zebrafish, \textit{Tg (pvalb3b: EGFP-tuba1)}, expressing EGFP-tagged tubulin \(\alpha\)1 along the kinocilium in hair cells (\textbf{Figure 3.3 A-D}) were generated.

To study the lateral migration of the kinocilium relative to the timing of stereocilia elongation, we obtained transgenic zebrafish that express both \(\beta\)-actin-mCherry and EGFP-tubulin \(\alpha\)1 in hair cells. Unfortunately, the massive amount of EGFP-tubulin \(\alpha\)1 in the soma of hair cells, particularly at the apical surface, hinders imaging of the base of the kinocilium for defining their position (\textbf{Figure 3.3 C}). Although, it is possible to view the position of the kinocilium from a top-down view of hair cells in the neuromast, it then becomes impractical to measure the growth of stereocilia because they are only 1.5 \(\mu\)m in length when mature\textsuperscript{232}. However, this stable transgenic zebrafish with the expression of \textit{GFP-tuba1} in hair cells can
still provide us with an opportunity to monitor the growth of the kinocilium during hair cell development.

We also attempted to simultaneously view the kinocilia and stereocilia to determine their relationship during development. It is known that stereocilia start to elongate after the lateral migration of the kinocilium, but it is not clear whether the length of the kinocilium also provides an initial sign to the nascent stereocilia to stimulate their elongation. We imaged a neuromast of a doubly transgenic zebrafish expressing both β-actin-mCherry and EGFP-tubulin α1 in hair cells with confocal microscopy. In most of the neuromasts we imaged at 4 dpf, all the visible kinocilia in the same neuromast were equal in their height (Figure 3.3 D). Four hair bundles were observed at 72 hpf (Figure 3.3 E), five hours later, four additional hair bundles formed (Figure 3.3 F). The new hair bundles had an obvious staircase-shaped array of stereocilia, and newly formed kinocilia (Figure 3.3 G, kinocilia number 5-8). However, these kinocilia had not yet reached their mature length as seen in the originally observed bundles (Figure 3.3 G, kinocilia number 1-4). Therefore, we showed that though the position of kinocilium may set up polarity for stereocilia at the apical surface of the hair cell, the kinocilium is continuously elongating with the growth of stereocilia.
Figure 3.3. Transgenic labeling of tubulin α 1 in the kinocilium of hair cells of Tg (pvalb3b: EGFP-tubα1) zebrafish. (A, B) Schematic diagram shows the EGFP-tubulin α1 fusion protein and its localization in the kinocilium of the hair bundle. (C) Fluorescent signals from EGFP-tubulin α1 (green) are seen in the kinocilium (arrow) as well as the soma.
but not the nucleus (asterisk) of transgenic zebrafish at 4 dpf. Confocal images are overlaid with the corresponding bright field image to reveal the relative position of the full-length kinocilia (bracket). (D) Maximum projection of a confocal image series from a neuromast of the transgenic fish at 4 dpf. (E and F) A neuromast of a doubly transgenic zebrafish, which expresses β-actin-mCherry (red) and EGFP-tubulin α1 (green) shown in a 3D view from a series of confocal images. Four kinocilia (number 1-4) were observed at 72 hpf (G), and four additional kinocilia (number 5-8) grew in a period of five hours (F). (G) Schematic diagram shows the apical region of the neuromast in (F) and the newly present stereocilia (red) and kinocilia (green). Scale bars are 5 µm.

**Myosin XVa populates the tips of the stereocilia**

Nonsyndromic hearing loss and deafness, DFNB3, is genetically linked to mutations in human MYO15A. Shaker2 mice, which produce a truncated version of myosin XVa, are deaf with shorter stereocilia that lack tip-links. Immunolabeling experiments revealed that the localization of MYO15A at the tips of stereocilia starts at around E18.5, which coincides with differential elongation of stereocilia in mice. Also, the amount of myosin XVa accumulated at the tip of stereocilia is in direct proportion to the heights of stereocilia. Phenotypes found in Myo15a sh2 mice and the spatial and temporal expression pattern of Myo15a in hair cells make myosin XVa an ideal candidate to be a molecular regulator that is responsible for generating or maintaining the stereocilia graded in height. Thus, we proposed that using zebrafish transgenesis to express fluorescently-tagged myosin XVa in hair cells would provide us with a unique tool to monitor stereocilia staircase formation.
In our study, somatic expression of GFP-MYO15A was found localized at the tips of stereocilia of zebrafish hair cells. This tip-exclusive localization pattern was seen for both short and long stereocilia in hair cells of cristae, maculae, and neuromasts (Figure 3.4 A-D) and the level of fluorescent intensity of GFP-MYO15A increases with stereociliary length as was reported in other mammalian hair cells. Interestingly, however, excessive amounts of myosin XVa did not lead to an additional elongation of mature stereocilia (Figure 3.4 E), indicating that myosin XVa is necessary but not sufficient for lengthening.
Figure 3.4. Tip-exclusive localization pattern of GFP-MYO15A in stereocilia. (A) GFP-tagged myosin XVα. (B) Schematic diagram of a hair bundle of a doubly transgenic hair cell with β-actin-mCherry (red) in stereocilia and GFP-MYO15A (green) populating the tips of stereocilia in a length-dependent manner, in which the taller rows of stereocilia have more fusion protein molecules. (C-E) Confocal images taken from hair cells expressing both β-actin-mCherry (red) and GFP-MYO15A (green). GFP-MYO15A localizes to the tips of stereocilia of hair cells in the (C) cristae and (D) neuromasts. (E) An extraordinarily high level of GFP-MYO15A expression (arrow) did not alter the morphology of the hair bundle. Scale bars are 1 µm.

**TMHS concentrates at the tips of stereocilia**

The onset of hearing has been shown to correlate with the formation of tip-links by imaging and electrophysiological recording. By imaging of Ca^{2+}, the location of the mechanotransduction channels was mapped to the tips of all stereocilia, which do not populate the tallest row of each bundle. Studies aiming to identify the molecules involved in mechanotransduction had identified cadherins and protocadherins as components of the tip link structure, transmembrane channel-like (TMC) protein superfamily for potential channels, and myosins for component transportation or regulating membrane tension. Moreover, mutations in the integral membrane protein Tetraspan Membrane protein of Hair cell Stereocilia (TMHS) were identified to be the cause of deafness in hurry-scurry (hscy) mice and non-syndromic hearing loss, DFNB67, in humans. In cochlear and vestibular hair cells of *Tmhs^{−/−}* mice, disruption of the colocalization pattern of TMHS and PCDH15 at the tips of stereocilia suggests a function of TMHS in efficient transportation of PCDH15.
In addition to the reduced number of tip links seen in $Tmhs^{−/−}$ mice, mechanotransduction currents observed in TMHS-deficient outer hair cells were also attenuated with a loss of fast adaptation$^{240}$. Changes in the properties of mechanotransduction indicate a role of TMHS as a regulatory subunit for the pore forming channels in hair cells resembling the discovery of the transmembrane AMPA receptor regulatory proteins (TARPs)$^{241}$. Here, we set out to define the localization of TMHS in zebrafish hair cells.

In zebrafish, there are two paralogs of TMHS with substantial similarity in sequence identity, $lhfpl\,5a$ (ENSDARG00000045023) and $lhfpl\,5b$ (ENSDARG00000056458) found in the zebrafish database in Ensembl (Zv9) (Figure 3.5 A). Expression of both transcripts was verified by RT-PCR from maculae of zebrafish. Zebrafish microinjected with GFP-Lhfpl5a cDNA reveal a predominant localization in the tips but not the shafts of stereocilia (Figure 3.5 C). Expression of the paralogous protein GFP-Lhfpl5b shows a similar tip-exclusive pattern in the stereocilia (Figure 3.5 E), and this is also consistent with what has been observed in mouse hair cells by immunolabeling using anti-TMHS serum$^{240}$. In addition to being targeted to the tips of stereocilia, both transiently/somatically expressed GFP-Lhfpl5a (Figure 3.5 B, C) and stably expressed GFP-Lhfpl5b (Figure 3.5 D, E) have the fusion proteins diffusely spread out in the soma of the hair cell. This is likely due to excessive expression of fusion protein, which has saturated all of the available TMHS binding sites in the tips of stereocilia.

Nevertheless, in this study, we conclude that the tip-specific localization pattern is conserved between fish and rodents. Interestingly, transgenic hair cells in the maculae had a strong expression of GFP-Lhfpl5 a/b in the soma but no fluorescent signals could be detected in the stereocilia (data not shown). This could be due to technical reasons. The optical clarity
might be improved by making thin slices of tissue for imaging. Or, this result could imply that there is a versatile composition of the mechanotransduction apparatus between sensory epithelia mediating different tasks. Next, we would like to investigate whether the products of these two paralogs, *lhfpl5a* and *lhfpl5b*, form a heterodimer and how each one interacts with other known tip-localized proteins in stereocilia through testing the protein-protein interaction in live zebrafish.

Figure 3.5 TMHS localized to the tip of stereocilia. (A) Alignment of protein sequences of human TMHS and the zebrafish orthologs. (B-E) Confocal images of live zebrafish at 5 dpf (B and C) or 11 dpf (D and E). The white brackets (B and D) and the expression of β-actin-mCherry in red (C and E) outline the stereocilia of hair cells. Expression of GFP-Lhfpl 5a
(cyan signals in B and C) and GFP- Lhfpl 5b (green signals in D and E) are found concentrated at the tips of the stereocilia. Aggregations of the fusion proteins are seen in the somata (asterisks). Scale bars are 1 µm.

**DISCUSSION**

**Intricate networking of F-actin in stereocilia**

Using transgenic zebrafish, Tg (*pvalb3b: actb-mCherry*), we measured the rate of stereocilia elongation in developing zebrafish hair cells (Figure 3.2 H-K), and revealed that it is 33 times faster than that what has been observed in embryonic chicken hair cells\(^{242,243}\). In both systems, the final lengths of stereocilia reached within the period of observation is very similar, thus the difference in elongation rates is not a result of structure requirement. There might be other molecular factors, exclusive to zebrafish hair cells, that facilitate the rate of actin polymerization. Thus, the novel transgenic zebrafish, Tg (*pvalb3b: actb-mCherry*), with mCherry-labeled stereocilia, allows us to monitor the hair bundle structure in real time and can be used as a tool to pinpoint the actin-regulatory proteins needed for hair bundle development, maintenance, and regeneration.

A next step using this transgenic line could be to investigate the stability of F-actin in mature stereocilia. F-actin continuously undergoes assembly and disassembly in filopodia; however, how F-actin behaves in a long-lived protrusion structure such as the stereocilium, remains controversial. Some studies proposed a treadmill-like movement of actin in stereocilia\(^ {208}\), while others believe that mature stereocilia are static, with only very little protein turnover\(^ {209}\). Applying FRAP experiments to stereocilia expressing β-actin-mCherry should shed light on the dynamics of F-actin in stereocilia.
**Microtubules in hair cells**

Our data showed that the kinocilium does not reach its final length before the surrounding stereocilia start to elongate, and both cytoskeletal structures continue to lengthen simultaneously. In the future, to understand how the PCP sets up in individual hair cells, we would like to use this new transgenic zebrafish, *Tg (pvalb3b: EGFP-tuba1)*, to study the relative expression patterns between the kinocilium and other PCP proteins during the hair bundle development. Although removing the kinocilium from a mature hair cell of the bullfrog does not affect mechanotransduction stimulated by bundle deflection in the positive direction\(^{244}\), the kinocilium was proposed to possess functional mechanotransduction that is independent from the tip links of immature hair cells\(^{232}\). In the future, it would be interesting to understand the possible cross-talk between the kinocilium and stereocilia during development by: 1) determining the rate of microtubule assembly in the kinocilia of the hair cells and how it contrasts to other cell types; 2) comparing the elongation rate of the kinocilium with and without stereocilia formation; and 3) analyzing the function of the mechanosensitivity mediated by the kinocilium in nascent hair cells and whether entrance of Ca\(^{2+}\) promotes actin polymerization in stereocilia.

**Stereociliary length and myosin XVa**

In our study, we showed that in hair cells expressing GFP-MYO15A, the fusion protein decorates the stereocilia only at the tips (Figure 3.4 C and D). In addition, the amount of myosin XVa localization to the tips is proportional to the lengths of stereocilia in both mouse and zebrafish hair cells; this indicates that this protein play a similar role in zebrafish as it dose in mice and that functions are conserved across species. Thus, our
transgenic hair cells expressing GFP-MYO15A recapitulate the endogenous localization of myosin XVa. In the future, performing live imaging on β-actin-mCherry transgenic fish to monitor the rate of stereocilia elongation with or without massive expression of myosin XVa expression might give us some insight whether or not myosin XVa can directly induce and facilitate the rate of actin assembly in stereocilia. In addition, myosin XVa is a plus-end-directed motor protein in filopodia\textsuperscript{68}; therefore, it may transport some essential components for mechanotransduction to the tip. So far, whirlin has been determined to be a cargo protein of myosin XVa, and the mutation of whirlin also results in deafness in mice. In the future, identifying additional cargo proteins of myosin XVa would expand our knowledge of proteins required for either stereocilia elongation or mechanotransduction. Also, with our transgenic zebrafish, we could examine the mobility of myosin XVa in stereocilia by FRAP. In addition, this unique reagent could be used to trace potential co-migration with other known tip proteins in real time.

**Determining the organization of the mechanotransduction apparatus with zebrafish transgenesis**

In this study, we showed that both Tmhs isoforms densely cluster at the tips of stereocilia of live zebrafish (Figure 3.5 B-E) and this pattern resembles the same pattern observed in mice\textsuperscript{239,240}. We propose using zebrafish transgenesis to determine the relative spatial relationship of protein subunits of the mechanotransduction apparatus. Specifically, the potential physical interactions between TMHS and hypothetical channel proteins, such as Tmcs or Piezos, requires further investigation. Also, previous studies suggest that structural proteins within the stereocilia are relatively stable\textsuperscript{209}; however, chemically-broken tip links
could be restored in a day\textsuperscript{245}. It would be worthwhile examining the behavior of Tmhs proteins, a subunit of the mechanotransduction complex, upon and after breakage in live animals.

To probe the functional importance of \textit{tmhs} for mechanotransduction, we are generating zebrafish knockouts with truncated \textit{Lhfpl5a} and \textit{Lhfpl5b}. So far, we have successfully introduced indel mutations in the coding regions of \textit{lhfpl5a} and \textit{lhfpl5b} by TALENs. In the future, we will evaluate the electrophysiological properties of hair cells in individual knockout lines or double knockouts by patch clamp and measurement of microphonic potentials.
Chapter 4

Investigating Roles of the Piezo Protein Family in Hair Cell Mechanotransduction in Zebrasfish
ABSTRACT

Mechanotransduction, converting physical forces to electrical signals, plays important roles in physiology. The sensory cells that respond to mechanical stimuli, such as sound, touch, acceleration, and changes in blood pressure, are mechanotransducers with specialized cellular structures and mechanosensitive ion channels. Recently, Piezo proteins were proposed to be pore-forming ion channels involved in mechanotransduction. Mammalian cells transfected with reconstituted mouse Piezo proteins exhibit mechanically activated currents\(^{(246)}\). Here, we study if piezo proteins mediate mechanosensation in zebrafish by generating knockouts of piezo genes using TALENs. We have determined that there are 4 piezo genes (\textit{piezo}, \textit{piezo 1}, \textit{piezo 2a}, and \textit{piezo 2b}) in zebrafish and we have targeted them all for mutation. Our data demonstrates that zebrafish with a mutation in piezo 2b lack a response to gentle touch in early developmental stages. This result supports a role of piezo 2b in mechanotransduction in living vertebrates.

INTRODUCTION

Complications of identifying channel proteins that operate mechanotransduction

In the past, many studies have aimed to identify the molecules that are responsible for mechanotransduction\(^{(247)}\); though this was met with some success in bacteria\(^{(248)}\) and invertebrates\(^{(249,250)}\), the findings in mammals have created more questions than answers\(^{(237,251-254)}\). A general property of a mechanotransduction ion channel is that upon stimulation, an ion channel protein opens rapidly with a short latency and allows mostly cations such as Na\(^+\), K\(^+\), and Ca\(^{2+}\) to flux in through the pore-forming region. Using genetic approaches, the first breakthrough for mechanotransduction in multicellular animals was the identification of the
DEG/ ENaC proteins as mechanotransduction channels that mediate gentle touch in *C. elegans*\(^{255}\). However, there is no conclusion as to whether the DEG/EnaC protein homologs in the fly and the mouse behave similarly.

There are numerous reasons, which make it challenging to identify mechanically-gated channels in vertebrates. First, sensory cells, which carry the mechanosensitive channels, are sparsely located and are often rare. Second, it is predicted that the number of channels needed for mechanotransduction for each individual sensory cell is low. Together, it is difficult to isolate a sufficient number of the target cells to purify an adequate amount of channel candidates for quantitative biochemical research. Third, it has been challenging to reconstitute a functional eukaryotic channel in cultured cells or synthetic lipid bilayers. Exogenously expressed channel candidates are not always able to conduct a mechanically activated current in cells. Although it might indicate that these candidates are not channels, it may also emphasize the heteromeric composition of the channel complex and the need of auxiliary proteins to fulfill the minimum functional unit for mechanotransduction. Fourth, loss-of-function experiments are excellent in distinguishing genes for a certain function, in general, but a broader functional influence of a single gene or redundantly-acting genes can lead to a lethal event or no observable phenotypes, respectively. Fifth, genetic screens usually look for the loss of mechanosensation in mutants, but this could underestimate the importance of some channel components that serve in a repressor function in wild-type conditions.

**Piezo proteins as a new channel protein family involved in mechanotransduction**

Cells employ numerous mechanosensations to interpret physiological environments
surrounding them; however, mechanotransduction channels that mediate hearing have not been cloned, and it is still not yet clear whether there is a similar mechanism among all types of mechanosensitive channels. Piezos, which are named after a Greek word ‘πιεση’ (piesi) meaning ‘pressure’, each encode a large-transmembrane protein predicted to have about 24 to 36 transmembrane domains and share no significant similarity to other known channel protein families (Figure 4.1 A). In mouse, expression profiles of piezo mRNAs are found strongly expressed in the lung, bladder, skin, and dorsal root ganglion (DRG), suggesting a role for piezos in mechanotransduction. Reducing the protein level of Piezo 1 in cultured mouse neuroblastoma cells (N2A) by siRNA suppressed the mechanically activated (MA) currents that are reliably observed in N2A cells. Overexpressing mouse Piezo 1 and Piezo 2 cloned from N2A cells and DRG neurons, respectively, are sufficient to induce MA currents in human embryonic kidney (HEK) 293T cells. Reconstituted lipid bilayers with Piezo1 was shown to conduct both sodium and potassium currents, and the induced MA currents are prevented by known channel blockers such as ruthenium red and gadolinium which usually block many cationic MA currents. Imaging with total internal reflection microscopy (TIRF) on GFP-MmPiezo 1 revealed that piezo proteins assemble into homotetramers, therefore form a huge channel with 120 to 160 transmembrane segments spanning the membrane. Mutations identified in PIEZO1 and PIEZO2 have been linked to human diseases such as dehydrated hereditary stomatocytosis and Marden-Walker syndrome, respectively (Figure 4.1 A). In most of the clinical cases, mutations result in altering the inactivation property of piezos rather than a complete loss of mechanotransduction. Characterizing the functions of Piezo 1 and Piezo 2 in mechanosensitive sensory cells has become an important area of research since the identification of the piezo protein family of
channels. The discovery of piezos opens up a new era in the mechanotransduction field, as they might be components of the channel that have been missing for so long in the hearing field. However, it is unfortunate that Piezo 1 and Piezo 2 null knockout mice die during the embryonic stage or at birth, respectively, before the onset of hearing; that is approximately P18 in mice\textsuperscript{263,264}. Instead of making conditional knockouts mice for specific tissue types, we propose to take advantage of the rapid development of zebrafish, and investigate the function of piezos in mechanosensation in fish, specifically focusing on hair cells that are electrophysiologically tractable as early as 3 and 5 dpf for inner ear and lateral line neuromasts, respectively.

Figure 4.1

**Figure 4.1.** Piezos are a family of large transmembrane proteins in both vertebrates and invertebrates. (A) Schematic diagram shows the predicted transmembrane topology of
human PIEZO 2. Amino acids associated with human diseases when mutated are mapped on the schematic, hPIEZO1 (blue) and hPIEZO2 (red) are indicated. Figure adapted from Volkers et al., 2014265. (B) Sequence alignments compare amino acid sequences of piezos from mouse, zebrafish, and fly.

Expression of piezo 2b but not piezo 1 nor piezo 2a mRNA has been detected in Rohon-Beard (RB) neurons and the trigeminal ganglions of zebrafish at 24 hpf by in situ hybridization266. RB neurons that innervate the skin at 1 dpf are important for touch sensation in zebrafish embryos267. Recently, the piezo 2b gene was knocked down in zebrafish by morpholinos266. In the study, RB neurons of piezo 2b morphants developed normally as the numbers and the morphology of its peripheral neurites remained significantly unchanged when compared to the wild type zebrafish. Yet, piezo 2b morphants showed a reduced response to the gentle touch stimulation. Thus, it suggests that Piezo 2b is the mechanotransducer responsible for the touch response in RB neurons266. However, Faucherre and colleagues only showed the absence of the correctly spliced mRNA of piezo 2b in embryos injected with piezo 2b-specific morpholino, they did not provide the rescue experiment for their morpholino knockdown test. In addition, effectiveness of morpholinos is restricted to early developmental stages, and usually becomes too diluted to have an effect at later developmental stages. Thus, we believe that by creating piezo channel knockout zebrafish, we could develop an understanding of the function of each piezo gene in mechanosensitive tissues of vertebrates, including in hair cell transduction.
MATERIALS AND METHODS

Assembly of TALEN-encoding expression vectors for inducing DNA mutations in *piezo*, *piezo 1*, and *piezo 2a* genes by TALENs in zebrafish

TALEN target sites were selected and corresponding TAL units were obtained from ZifiT (http://zifit.partners.org/ZiFiT/)\textsuperscript{268,269}. To assemble a full length TALEN for a single target site using FLASH assembly, we performed the following procedures according to Reyon et al., 2012\textsuperscript{270}. First, the biotinylated $\alpha$ unit was ligated with the extension unit 1 and attached onto a streptavidin-coated plate. Second, repetitive digestions and ligations allowed for the addition of two more extension units and a termination unit onto the existing $\alpha$ unit/extension unit 1 complex. Third, the final digestion was used to release the full-length TALE from the plate and produced cohesive ends for ligation with the expression vector. The final expression vectors containing the full-length TALENs (Table 4.1) were confirmed by sequencing before they were used in RNA *in vitro* transcription.

Table 4.1. A list of plasmid vectors used in the FLASH assembly for constructing expression vectors containing desired TALENs for targeting piezo genes. Unit numbers are based on the FLASH ID given in Reyon et al., 2009\textsuperscript{270}.

<table>
<thead>
<tr>
<th>Gene/ Target Site</th>
<th>$\alpha$ unit</th>
<th>Extension unit 1</th>
<th>Extension unit 2</th>
<th>Extension unit 3</th>
<th>Termination unit</th>
<th>Expression vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>piezo TALEN1 Left</td>
<td>374</td>
<td>123</td>
<td>121</td>
<td>112</td>
<td>284</td>
<td>JDS74</td>
</tr>
<tr>
<td>piezo TALEN1 Right</td>
<td>374</td>
<td>37</td>
<td>213</td>
<td>142</td>
<td>277</td>
<td>JDS71</td>
</tr>
<tr>
<td>piezo1 TALEN1 Left</td>
<td>373</td>
<td>231</td>
<td>144</td>
<td>158</td>
<td>322</td>
<td>JDS78</td>
</tr>
<tr>
<td>piezo1 TALEN1 Right</td>
<td>376</td>
<td>24</td>
<td>149</td>
<td>177</td>
<td>273</td>
<td>JDS74</td>
</tr>
<tr>
<td>piezo2a TALEN2</td>
<td>375</td>
<td>133</td>
<td>234</td>
<td>65</td>
<td>350</td>
<td>JDS78</td>
</tr>
</tbody>
</table>
Generation of ZFN-encoding plasmid for *piezo 2b* gene-specific knockout zebrafish

ZFN target site, *piezo 2b* ZFN 6-1, was selected in exon 4 of the *piezo 2b* gene (ENSDARG00000076722) by ZiFit. Methods in Foley et al., 2009\textsuperscript{271} and Sander et al., 2011\textsuperscript{97} were followed to generate the ZFN-expression vectors. First, DNA fragments encoding zinc finger arrays for the left and the right target sites of *piezo 2b* were synthetized by Integrated DNA Technologies (IDT) in the format of pIDTsmart-ZF (Table 4.2). Second, the synthetic DNA fragments were subcloned from pIDTsmart-ZF into ZFN-expression vectors, pMLM290 and pMLM292, with *XbaI* and *BamHI* restriction enzymes (New England Biolabs).

Table 4.2. DNA sequences encoded the left and the right *piezo 2b* ZF half sites.

<table>
<thead>
<tr>
<th>ZF half site</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>piezo2b</em> ZFN6-1 Left</td>
<td>GAAAAAAAATCTAAGACCCGGGAGGCACCCCTTCCAGTGTGCAATTTGCAATGCGGAAC&lt;br&gt;TTTCGCAAGGCTCTAACTTGACCGCTGACCTACTACCCGTACTACCCCGTGAAAAACC&lt;br&gt;GTTCAGTGTCCGATTGCAATTTTCTCCAGTGCAACCGACTACCCCGTGAAAAACC&lt;br&gt;TCTACGTACCCCACTTGGAAGCCATTCAATGCCGAATAATGCATGCGCAACT&lt;br&gt;TCAGTGACATGGGTAACCTGGGTCGCCTCACTAATACCCACCTGAGGGGATCCAG&lt;br&gt;AAGGA</td>
</tr>
<tr>
<td><em>piezo2b</em> ZFN6-1 Right</td>
<td>GAAAAAAAATCTAAGACCCGGGAGGCACCCCTTCCAGTGTGCAATTTGCAATGCGGAAC&lt;br&gt;TTTCGCAAGGCTCTAACTTGACCGCTGACCTACTACCCGTACTACCCCGTGAAAAACC&lt;br&gt;TCTACGTACCCCACTTGGAAGCCATTCAATGCCGAATAATGCATGCGCAACT&lt;br&gt;TCAGTGACATGGGTAACCTGGGTCGCCTCACTAATACCCACCTGAGGGGATCCAG&lt;br&gt;AAGGA</td>
</tr>
</tbody>
</table>
**RNA in-vitro transcription**

ZFNs and TALENs for injections were in vitro transcribed from expression vectors linearized with *PmeI* digestion (Ambion mMACHINE Ultra-T7 kit). Quality and quantity of RNAs were verified by RNA bioanalyzer (Agilent Technologies, Inc.)

**Zebrafish microinjection**

For each half site, 150 to 250 ng/µl of ZFN and TALEN RNAs were prepared on ice with phenol red and 1x Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES pH 7.6) and injected into one-cell stage zebrafish embryos.

**High-Resolution Melting Analysis (HRMA)**

To examine the targeting efficiency of each pair of ZFNs or TALENs, individual embryos injected with ZFN or TALEN RNAs were subjected to HRM analysis. General HRMA conditions are the same as had been described in the method section of chapter 2. Primers and optimal annealing temperature used for individual gene-specific target site are listed in Table 4.3.

**Genotyping**

For genotyping, genomic DNA obtained from ZFN or TALEN RNA-injected embryos, embryos of the founders, or a small sample of fish tail, were used as templates for PCR. A fragment of DNA containing the target site region was amplified with gene specific primers listed in Table 4.3. Purified PCR products were subcloned to pCR4-TOPO-TA
sequencing vector (Invitrogen). Indel mutations were identified by ClustalW alignment analysis with MacVector (MacVector, Inc).

Table 4.3. A list of primers used for HRMA and genotyping.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequences (5' -&gt; 3')</th>
<th>Annealing Temp (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>piezo TALEN1 HRM F1</td>
<td>TCTGGGGTGTGTGCAGGTTTGG</td>
<td>65</td>
<td>132</td>
</tr>
<tr>
<td>piezo TALEN1 HRM R1</td>
<td>GCAAGGAAGGAGCGCTGAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo TALEN1 5'seq</td>
<td>CTGGGACTATTTGTAGCCCTCC</td>
<td>65</td>
<td>377</td>
</tr>
<tr>
<td>piezo TALEN1 3'seq</td>
<td>CACCTTTGAGCAAGGTTGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo1 TALEN1 HRM F1</td>
<td>GGGAGGAAATTGAGTGAGGAAAGTTG</td>
<td>68</td>
<td>121</td>
</tr>
<tr>
<td>piezo1 TALEN1 HRM R1</td>
<td>GGTGTAAGGAGGAAACACGGTTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo1 TALEN1 5'seq2</td>
<td>AGCGCGAGGCTTTAAGCAATG</td>
<td>65</td>
<td>333</td>
</tr>
<tr>
<td>piezo1 TALEN1 3'seq2</td>
<td>GGTGTAAGGAGGAAACCGTTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo2a TALEN2 HRM F2</td>
<td>CTGTGGTGCTTTTCTCAAGCCGG</td>
<td>66</td>
<td>125</td>
</tr>
<tr>
<td>piezo2a TALEN2 HRM R2</td>
<td>GGGGACTGAACTTTTCAGGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo2a TALEN2 5'seq</td>
<td>CAGGCTGTCCACAAACTGTG</td>
<td>65</td>
<td>505</td>
</tr>
<tr>
<td>piezo2a TALEN2 3'seq</td>
<td>GCTCAGGTCTCTCATCATTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo2b ZFN6-1 HRM F1</td>
<td>GCAAGCTGGTGATGGCTGCTGCT</td>
<td>68</td>
<td>129</td>
</tr>
<tr>
<td>piezo2b ZFN6-1 HRM R1</td>
<td>CGCAGAAGCAGCAGCATCATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>piezo2b ZFN6-1 5'seq</td>
<td>GAGGAATGGAGTGAACGGACC</td>
<td>65</td>
<td>414</td>
</tr>
<tr>
<td>piezo2b ZFN6-1 3'seq2</td>
<td>GGATGCCATCTCAAACGGTGCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FM 1-43 dye loading**

Free swimming zebrafish larvae at 5 dpf were placed in a 12-well culture plate and immersed in staining solution containing FM1-43 (n-(3-triethylyammoniumpropyl)-4-(4-(dibutylamino)-styryl)pyridinium dibromide (Invitrogen) for 1 min, and rinse three times in regular fish water.

**Behavior tests**
At 1 dpf, zebrafish embryos with chorions, an acellular envelope surrounding the fertilized egg, were individually observed for the spontaneous contraction under an upright dissection microscope. To test for the touch response, chorions were manually removed and individual embryos were put into a well of 24-well culture plate. All wells were filled with standard fish water. A Dumont #5 forceps or a customized hair-loop was used to place a gentle touch on the surface of zebrafish larvae. To exam in the startle response, larvae that tested positive for touch response were transferred from the 24-well plate to a 100 mm x 15 mm polystyrene Petri dish, and the startle response were elicited by lightly tapping on the rim of the dish with forceps.\textsuperscript{272}

**RESULTS**

**Piezo 2b is required for gentle touch in zebrafish larvae.**

Two copies of *PIEZO* genes are each found in mouse and human, but there is only one ortholog in invertebrates.\textsuperscript{273} To understand the scenario in lower vertebrates, we searched for orthologs of mouse *Piezo 1* and *Piezo 2* in zebrafish via BLAST (tBLASTx) in Ensembl zebrafish database (ZV. 9). We identified four genomic loci that encode proteins that have a high similarity to mammalian piezos (Figure 4.1 B). To probe whether piezo proteins are involved in mechanotransduction in zebrafish, we generated piezo gene knockouts. We utilized the Fast Ligation-based Automatable Solid-phase High-throughput (FLASH) method to assemble TALENs for targeting (see the method section for the details). The efficacies of each pair of TALENs were determined by the High Resolution Melting Analysis (HRMA) (Figure 4.2). We have successfully crated zebrafish with mutations in genomic loci of *piezo*, *piezo 1*, and *piezo 2a* (Figure 4.3). These indel mutations are germline transmittable as we identified heterozygous mutants of all three genes in the F1 generation.
piezo TALEN1

piezo 1 TALEN 1

piezo 2a TALEN 2

Figure 4.2

Figure 4.2. HRMA reveals the induction of somatic mutations in piezo genes by TALENs. Graphs of melting curves generated from HRMA tests on 22 zebrafish embryos injected with TALEN RNA specific to piezo, piezo 1, and piezo 2a as well as two non-
injected control embryos. Each melting curve represents the profile of a single embryo. In
our analysis, melting curves obtained from two of the control wild-type fish are colored in
blue. Melting curves of samples that run similar to the wild type in HRMA also are colored
in blue. Samples that did not closely resemble the wild-type fish were further separated into
groups as showed in different colors (other than blue) of curve lines. Samples that most
significant deviate from the wild type are colored in green (arrows). Changes of melting
curve profiles reveal a successful induction of multiple genetic variations, in other words,
mutations, at the target loci.
Figure 4.3. Sequencing results of mutant genotypes identified from zebrafish that are heterozygous for piezo mutant alleles. Genomic DNA segments in the vicinity of the selected TALEN target sites from the fin of adult zebrafish were amplified, cloned, and sequenced. The wild-type sequence of TALEN target sites, both the left and the right, are highlighted in yellow. Deleted bases, dashed lines highlighted in grey, are apparent in mutant zebrafish sequences. Extra bases inserted to generate mutants are highlighted in blue. These results demonstrate that we effectively used TALENs to create three stable gene-specific knockout zebrafish lines targeting piezo, piezo 1, and piezo 2a.
Using ZFNs, we created zebrafish with several base-pair deletions in the coding region of piezo 2b (Figure 4.4). As embryos developed, we documented several general locomotor behaviors such as spontaneous muscle contraction, which appears between 17 to 27 hpf, touch-evoked coiling, touch-evoked escape, and, organized swimming\textsuperscript{274}. All 28 embryos tested, with 7 of them identified as wild types, 11 heterozygous, and 9 homozygous mutants, exhibit spontaneous tail contractions at 24 hpf as that has been characterized for normal wild type zebrafish\textsuperscript{274}. Between 24 to 48 hpf, piezo 2b\textsuperscript{−/−} larvae show no response to a gentle touch initiated from the head or the tail. Control wild type and heterozygous (piezo 2b\textsuperscript{+/−}) larvae both display normal touch responses. However, by 72 hpf, all larvae, regardless of their genotype, show an normal escape response elicited from touching their tails.

To examine whether piezo 2b plays a role in mechanotransduction in hair cells, we performed the acoustic-vibrational startle response test as well as the FM 1-43 dye uptake. Hair cells in the ear and the lateral line can be activated by acoustic or vibrational stimulation, and this action fires the Mauthner cells. Motor neurons activated by Mauthner cells then triggers a C-start bending and swimming behavior away from the source of stimulation\textsuperscript{272,275,276}. At 5 dpf, all groups of zebrafish larvae in the same clutch demonstrate normal startle responses and swimming behaviors. FM1-43 dye enters hair cells through mechanotransduction channels and this specific labeling would be inhibited by the addition of cation-channel blockers\textsuperscript{277}, thus the rapid FM1-43 dye uptake by hair cells is used to represent the presence of functional mechanotransduction. Any changes that affect mechanosensitive properties of the mechanotransduction channels or the channel composition itself should result in a loss of sufficient FM1-43-loading in hair cells. In our knockout animals, all hair cells in lateral line neuromasts of 5-dpf piezo 2b\textsuperscript{−/−} zebrafish
showed efficient FM1-43 dye loading. These results indicate that Piezo 2b plays an important role in touch sensation at early developmental stages but hair cell mechanotransduction is grossly normal in piezo 2b knockout zebrafish.

**Figure 4.4.** Generation of piezo 2b knockout zebrafish by ZFN. (A) Schematic diagram illustrating a portion of the 43 exons (grey boxes) and some of the introns (black lines) of zebrafish piezo 2b. ZFN recognition and binding sites were designed to target exon 4 of piezo 2b. ZFN DNA-binding domains for a sequence-specific triplet of nucleotides are individually colored with pink, green, and blue represent the fingers for the right ZFN and yellow, purple, and orange represent the fingers for the left ZFN. FokI nucleases (red) dimerize and create DNA double-strand breaks in the spacer region, in this case the six base pairs between the

**Figure 4.4.** Generation of piezo 2b knockout zebrafish by ZFN. (A) Schematic diagram illustrating a portion of the 43 exons (grey boxes) and some of the introns (black lines) of zebrafish piezo 2b. ZFN recognition and binding sites were designed to target exon 4 of piezo 2b. ZFN DNA-binding domains for a sequence-specific triplet of nucleotides are individually colored with pink, green, and blue represent the fingers for the right ZFN and yellow, purple, and orange represent the fingers for the left ZFN. FokI nucleases (red) dimerize and create DNA double-strand breaks in the spacer region, in this case the six base pairs between the
left and the right ZFN targeting sites. UTR stands for the untranslated region. (B) Sequencing results of mutations identified from zebrafish injected with *piezo 2b* ZFN mRNAs. The most common genotype observed are several base pair deletions within the spacer region, which is flanked by two ZFN binding sites (blue highlights).

**DISCUSSION**

**Touch zebrafish with Piezo 2b**

Our data shows that *piezo 2b*−/− larvae do not respond to touch between 1 – 2 dpf, which supports the finding in *piezo 2b* morphants that the mechanotransduction function of Piezo 2b is necessary for RB neurons in responding to touch in early developmental stages.\(^{266}\) Surprisingly, we also observed that *piezo 2b*−/− knockouts retrieved response to touch after 3 dpf. Degeneration of RB neurons, the normal course of zebrafish, starts from 2 dpf and only 22% of RBs survive till 4 dpf.\(^{278}\) During this period, DRGs form and start axogenesis and eventually replaced the receptive field covered by RBs.\(^{278,280}\) Thus, our data suggests that the mechanotransduction function of Piezo 2b is restricted to RB neurons, and there are mechanotransduction channels other than Piezo 2b could drive the touch sensation mediated by DRGs. Our knockout animals provide a reliable model to differentiate touch sensation mediated by RB neurons or DRGs. In the future, we would like to obtain an antibody for detecting endogenous levels of Piezo 2b proteins and to perform patch clamping to determine the electrophysiological aspects in *piezo 2b* deficient RB neurons.

On the other hand, not like larvae, we have not yet been able to obtain any adult fish, out of 40 fish screened, that are homozygous for the mutant allele but adult zebrafish with one mutant allele (*piezo 2b*+−) show no significant deficiencies in survival rates, swimming,
hearing, and mating. We suspect that either the touch responses mediated by RB neurons in early developmental stages contribute to the survival of zebrafish to adulthood or piezo 2b is involved in other types of mechanosensation in different tissue types later in development. Further experiments are needed to determine the expression profiles of piezo 2b from various tissues at multiple developmental ages to determine which tissues require this protein’s function.

A potential mechanotransduction function of piezo in hearing

Although many deafness genes have been cloned from patients with hearing deficiencies and various mutant proteins have indicated their role in hearing, so far, the pore-forming mechanotransduction channel is still undetermined. Recently, absence of the mechanotransduction channel current in hair cells of mice deficient in transmembrane channel-like (Tmc) 1 and 2 genes, indicates that the TMC1 and TMC2 proteins contain, at the least, a component of the channel\textsuperscript{237,253}. However, the presence of cation currents activated by deflecting the hair bundle in the reverse direction in Tmc1\textsuperscript{-/-}Tmc2\textsuperscript{-/-} double knockouts weakens the notion that TMC1 and TMC2 are the sole components of the channel\textsuperscript{254}. If TMC1 and TMC2 are not the pore-forming subunits, could this new class of mechanosensitive channel, PIEZO, be the key? Or do these two proteins interact to modulate the channel core? Our preliminary data demonstrates the expression of piezo 1 mRNA in hair cells in the otic vesicle of zebrafish larvae (data not shown). In the future, we will investigate functions of each of the piezo proteins in hair cells with the piezo-specific knockout zebrafish we created. These novel piezo knockouts would advance our understanding about the physiological relevance of piezo-mediated mechanotransductions in zebrafish.
-Chapter 5-

Discussion
Building of hair bundles is a process-dependent challenge to hair cells

Formation of functional stereocilia is a multi-step process, which requires integration of many different actin-associated proteins. In our real-time imaging studies (Chapter 3), we observed different stages of hair bundle development: the appearance of a kinocilium, the formation of stereocilia, and the elongation of stereocilia. In this thesis (Chapter 2), I identified fascin 2b, an actin-bundling protein, as a stereociliary protein that lengthens stereocilia when overexpressed. However, the lack of a strong stereociliary defect in fascin 2b knockout hair cells suggests that there are more actin-bundling proteins involved in the process of stereocilia formation. Moreover, I determined the developmental timeline of the hair bundle through real-time imaging on various transgenic zebrafish I created (Chapter 3). These fish will also be useful tools for visualization of cytoskeletal proteins, actin and microtubules, in hair cells that have subtle structural defects when we alter the expression of proteins that are required for hair bundle development.

Stereocilia and hearing

Currently, many researchers are seeking methods that can induce hair-cell regeneration in order to restore human hearing loss. Irregular morphology of hair bundles not only hinders mechanotransduction but also leads to hair cell death. Therefore, in the long-term, we believe that our work of identifying and understanding the molecules required for the process of the growth and the maintenance of stereocilia could contribute to therapies aimed at the reprogramming and production of functional hair cells for restoration of hearing loss.
Chapter 6

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