

**THE BETA SUBUNIT OF THE AP-1 COMPLEX IS
ESSENTIAL FOR HAIR CELL FUNCTION AND
BASOLATERAL TARGETING OF NKA**

By

Rachel Clemens Grisham

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Oregon Health and Science University

CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of
Rachel Clemens Grisham
has been approved

Dr. Teresa Nicolson, Ph.D.

Dr. Caroline Enns, Ph.D.

Dr. Peter Barr-Gillespie, Ph.D.

Dr. Anthony Barnes, Ph.D.

Dr. John Brigande, Ph.D.

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Abbreviations used

AC	Anterior crista
AP	Adaptor protein
<i>ap1b1</i>	<i>adaptor protein 1 beta 1 subunit</i>
Cav1.3	Ca ⁺ voltage-dependent channel, L-type, α 1D
<i>cdh23</i>	<i>cadherin 23</i>
D3	Cameleon Ca ⁺⁺ sensor
dpf	days post fertilization
HC	hair cell
hpf	hours post fertilization
LRO	lysosomal related organelle
MAGUK	Membrane-associated guanylate kinase
MC	Medial crista
MET	mechanoelectrical transduction
<i>myo6b</i>	<i>myosin 6b</i>
NKA	Na ⁺ /K ⁺ ATPase
PC	Posterior crista
<i>pcdh15</i>	<i>protocadherin 15</i>
PM	plasma membrane
SC	supporting cell
<i>tmc1</i> , <i>tmc2a</i> and <i>tmc2b</i>	<i>transmembrane channel-like genes</i>
<i>tmhs</i>	<i>tetraspan membrane hair cell stereocilia</i>
<i>tmhsa</i> and <i>tmhsb</i>	zebrafish paralogs of <i>tmhs</i>

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Abstract

Mechanosensation in hair cells is a fascinating and intricately regulated process. Not only are the components of the mechanotransduction complex themselves critical for faithful transduction of mechanical stimuli, but this process also depends on the trafficking of those components to their correct cellular location in hair cells. Correct sorting of proteins within hair cells is important for a number of processes including ion homeostasis. In Chapters 3 and 4 I explore the consequences of the zebrafish mutations in the *adaptor protein 1 beta 1* (*ap1b1*) gene the beta-subunit of the AP-1 clathrin dependent sorting complex on mechanotransduction and regulation of intracellular Na⁺ concentrations. I show that the basolateral protein, the Na⁺/K⁺ATPase pump, is missorted to apical compartments of hair cells in *ap1b1* mutants. I also demonstrate that *ap1b1* mutants fail to regulate intracellular Na⁺ levels, likely a consequence of reduced Na⁺/K⁺ATPase pump at the basolateral membrane. In Chapters 2 and 5, I investigate the roles of two putative members of the mechanotransduction complex on transduction of mechanical stimuli. In Chapter 2, I show that *tmhsa* expression in hair cells is necessary for auditory response behaviors in zebrafish. I also show that expression of *tmhsa* specifically in hair cells in the mutant background is sufficient to rescue the auditory and vestibular phenotypes. Additionally, I demonstrate that the GFP-Tmhsa transgenic construct localizes to the tips of stereocilia in zebrafish hair cells, a characteristic that is consistent with the protein being apart of the transduction complex. In Chapter 5, I provide evidence that zebrafish Tmc2a is involved in hair-cell mechanotransduction. I show that while exogenous expression of the cytoplasmic, N-terminal fragment of

Tmc2a reduces mechanosensitivity, exogenous expression of the cytoplasmic, C-terminal fragment of Tmc2a increases mechanosensitivity, suggesting that Tmc2a is involved in mechanotransduction, but that the N- and C-terminal domains play different roles in this process.

Chapter 1 – Introduction

ZEBRAFISH AS A MODEL FOR HEARING RESEARCH

Aspects of the vertebrate auditory and vestibular systems are conserved across species. Both sensory systems are mediated by the reception of vibrational stimuli through the specialized mechanosensory hair cell, which converts fluid motion caused by sound waves or head movements into electrical impulses (Hudspeth, 1989). These impulses are then transmitted through a chemical synapse to nerves that carry the information to the brain where it is processed and decoded.

There are several ways that zebrafish are an ideal model to study hearing and hair cell biology (Nicolson, 2005; Whitfield et al., 2002), especially when compared to the mouse model system. First is accessibility: in mice, hair cells are housed in the inner ear, which is located in the skull encased in bone. On the other hand, zebrafish hair cells exist in two main organs, the inner ear and the lateral line (Figure 1.1) (Ghysen & Dambly-Chaudière, 2007; Whitfield, 2005). The lateral line system is a network of nerves connected to endorgans called neuromasts, which lie on the surface of the animal and are exposed to the surrounding aqueous environment. A dozen or more mechanosensory hair cells are clustered into the neuromast endorgans and are easy to visualize as they are located on the surface of the animal between skin cells. Zebrafish hair cells of the larval inner ear are visible through the transparent skin layer.

Development of the sensory epithelia of auditory and vestibular organs in zebrafish takes place during the external embryonic development, while the same process occurs

in mice during development *in utero* (Ladher et al., 2010; Torres & Giráldez, 1998; Whitfield et al., 2002). These factors make access to study mouse hair cells challenging for *ex vivo* or *in situ* study, and nearly impossible for *in vivo* study. By contrast, hair cells in zebrafish are not only useful for *in vivo* analysis in an intact organism, they are also amenable to time-course study during development.

Neuromasts of the lateral line are also especially amenable to manipulation through pharmacology and to physiological assays, as well as testing with vital dyes. A common method of assaying mechanotransduction in hair cells in both zebrafish and mice is the lipophilic dye FM 1-43 (Dambly-Chaudière et al., 2003; Meyers et al., 2003; Santos et al., 2006). This dye is able to rapidly traverse the mechanotransduction channel in hair cells as the mechanotransduction channel has a high open probability. Once inside the hair cell, FM 1-43 fluoresces 300 times brighter upon intercalation into membranes (Meyers et al., 2003). The FM 1-43 dye is used in Chapters 2 and 3 as a simple assay for mechanotransduction.

FM 1-43 labeling is a quick but crude method for determining if hair cells are mechanotransducing and is not sensitive enough to pick up the more subtle effects of a mutation on transduction. Other methods, however, can be used to analyze the degree to which mechanotransduction is affected. The Nicolson lab measures mechanotransduction activity more accurately by looking at microphonics and Ca^{++} transients in response to bundle deflection (Kindt et al., 2012; Trapani et al., 2009). Bundle deflection opens the mechanotransduction channel allowing positively charged ions, mainly K^+ and Ca^{++} , to flow through the mechanotransduction channel into the hair cell. This general movement of ions across the membrane can be measured as microphonics and can be useful in determining the mechanotransduction capability of

the channel. The flow of K^+ and Ca^{++} ions into the cell results in a graded receptor potential that triggers the release of intracellular Ca^{++} stores. This increase in Ca^{++} can be measured with Ca^{++} sensitive genetically encoded fluorescent molecules such as R-GECO or G-GECO, red or green monochromatic sensors, or Cameleon, a FRET-based sensor (Kindt et al., 2012; Palmer et al., 2006). And due to the transparency of the larval zebrafish, relative changes in these fluorescent indicators can be measured in the lateral line or inner ear of a live and intact animal.

The auditory and vestibular systems become functional within a shorter time frame than other vertebrates. By 5 days post-fertilization (dpf), zebrafish larvae are freeswimming with their dorsal sides up, indicative of a functional vestibular system, and their acoustic startle response is robust (Mo et al., 2010; Zeddies & Fay, 2005). The auditory system of a mouse is not fully functional until 21 days after birth (Ehret, 1976). The rapid, external development of the zebrafish auditory system also facilitates the analysis of large numbers of individuals and allows for experiments to be repeated at a relatively higher rate compared to mice.

Like mice, zebrafish are a genetically tractable system. Generating transgenic constructs is fairly straightforward in zebrafish and is useful for assaying how expression of a gene globally, conditionally or in selected cell-types affects cell physiology or behavior (Lieschke & Currie, 2007). In this dissertation I describe constructs assembled into the Tol2 Gateway cloning system and is commonly used to generate zebrafish transgenics (Kwan et al., 2007). This method is often used to generate a construct where a gene of interest is fused to both a promoter, to control its pattern of expression, and a fluorescent molecule so that the protein can be located in the cell where it is expressed. There are several methods to introduce transgenes into the

zebrafish genome. Among them, the Tol2 Gateway system is useful for generating large numbers of animals carrying transgenes with various levels of expression. In fact, this system is so efficient that its use occasionally results in animals with more than one copy of transgene.

An additional benefit to using zebrafish as a model to study hair cells is that the molecular components that comprise a functional hair cell in zebrafish are analogous to those components necessary for hair cell function in mammalian systems (Nicolson, 2005; Nicolson et al., 1998; Whitfield et al., 2002). To date, nearly all of the genes uncovered in screens for zebrafish with hearing and balance deficits have human orthologs that when mutated are causative for deafness.

GENETIC SCREENING AND CLONING OF ZEBRAFISH MUTATIONS

Traditionally, zebrafish have been used in forward genetic screens. Although, tiling mutants, a high throughput method for identifying mutations, and gene targeting techniques are an emerging approach for reverse genetics as well (Moens et al., 2008). Forward genetic screening is a process by which a specific phenotype is assayed for first followed by identification of the causative genetic mutation. Compare with a reverse genetic approach where the genetic mutation is identified first and then the phenotype is determined. The power of forward genetic screens lies in the unbiased identification of novel molecular components in any given pathway (Amsterdam & Hopkins, 2006). Chemical mutagenesis of zebrafish is easily accomplished through the use of ENU (Nethyl-N-nitrosourea) (Solnica-Krezel et al., 1994) and in Chapters 2 and 3, we utilize mutants obtained through this process. ENU mutagenesis induces small, usually single

base pair, changes to the genome, although the mutations can occasionally be larger (Knapik, 2000). Offspring of ENU treated zebrafish can be screened for phenotypes, in this case for deficits in the vestibular and auditory reflexes.

Screening for auditory and vestibular deficits in zebrafish involves a simple, two-part assay. Once the larvae are at the free-swimming stage at 5 dpf, they should be swimming “upright” with their dorsal sides up. Animals with a vestibular deficit are easily identified as they are usually found lying on their sides at the bottom of the Petri dish, which results from combination of a vestibular deficit and an un-inflated swim bladder. With the animals lying on their sides, the vestibular deficit can be further confirmed by touching the tail of the zebrafish larva which should elicit an escape response. Larvae with vestibular deficits will often swim in a circle, which is why they are also called “circling” mutants. Mutants that fail to respond to a tail-touch are excluded as they also have touch sensitivity defects. To assay for auditory function the side of the Petri dish containing free-swimming larvae is lightly tapped with a pen or probe, which should elicit a startle response. Animals with a functional auditory system will swim away from the sound, while animals with compromised auditory function will not respond or exhibit a weak response to the tapping sound. In our hands we usually observe both auditory and vestibular phenotypes together; however, vestibular deficits can occur without an obvious auditory component (Einhorn et al., 2012; Nicolson et al., 1998).

Once a mutant has been identified by its phenotype, it can be outcrossed to lines that are rich in polymorphisms. By documenting the recombination rate between these polymorphisms and the mutation, the location of the genetic lesion that causes the phenotype can be narrowed down. After quantifying the number of recombination

events in over one thousand fish, a number fairly easy to obtain due to large clutch sizes, a critical interval in the genome is defined. The critical interval will be flanked by rare recombination events on the order of 1/1000 or 1/2000. The majority of the zebrafish genome is sequenced and annotated (Howe et al., 2013), so after a manageable interval has been defined, mRNAs from genes within that interval can be systematically cloned, sequenced and scanned for genetic anomalies. This is the approach we took to identify the mutations responsible for the *skylab* auditory-vestibular phenotype (Chapter 3).

The process described above is useful in identifying most mutations isolated through mutagenesis screens, however with some mutations there are challenges to using this approach. Though the zebrafish genome is almost completely sequenced, the genome still has gaps (Howe et al., 2013), which are sections where there is not enough data to connect sequenced contigs. This results in gaps in the genomic sequence. Additionally, the above described technique for mapping a mutation can be problematic when trying to locate lesions near telomeres or centromeres where the rates of recombination are higher and lower, respectively, than in other regions of a chromosome (Demarest et al., 2011). In situations like this, deep sequencing, a more thorough method of identifying genetic lesions, is useful (Schneeberger et al., 2009). Deep sequencing was used in collaboration with another lab to identify the *astronaut* mutation (Obholzer et al., 2012) (Chapter 2).

ZEBRAFISH LATERAL LINE AND INNER EAR ANATOMY

As previously noted, hair cells in zebrafish are grouped into patches inside the inner ear and also in neuromast endorgans. The zebrafish inner ear develops from an

ectodermal thickening into an otic vesicle around 18 hours post-fertilization (hpf) (Whitfield et al., 1996; Whitfield et al., 2002). Discrete patches of hair cells develop shortly thereafter, beginning with the utricular and posterior maculae and followed by the anterior, medial and posterior cristae (Figure 1.1). The anterior macula will ultimately become the utricle, with a primarily vestibular function, and the posterior macula will develop into the saccule, capable of sensing auditory stimuli. The three cristae will form the semicircular canals important for sensing head rotations in vertebrates (Hudspeth, 1989; Whitfield et al., 2002). The majority of inner ear hair cells analyzed in this dissertation are from the medial crista.

The lateral line organ, unique to aquatic organisms, allows the zebrafish to sense and respond to water movements, which is important for rheotaxis, sensation of water currents, and predator avoidance behaviors (Stewart et al., 2013; Suli et al., 2012). The sensory aspect of this organ is mediated by mechanosensory hair cells that reside in neuromast endorgans found on the surface of the animal on the head and along the trunk (Figure 1.1A). The neuroepithelium of the lateral line and inner ear is comprised primarily of hair cells, supporting cells and a few mantle cells (Figure 1.1D). Although little is known about supporting and mantle cells, they are thought to maintain a

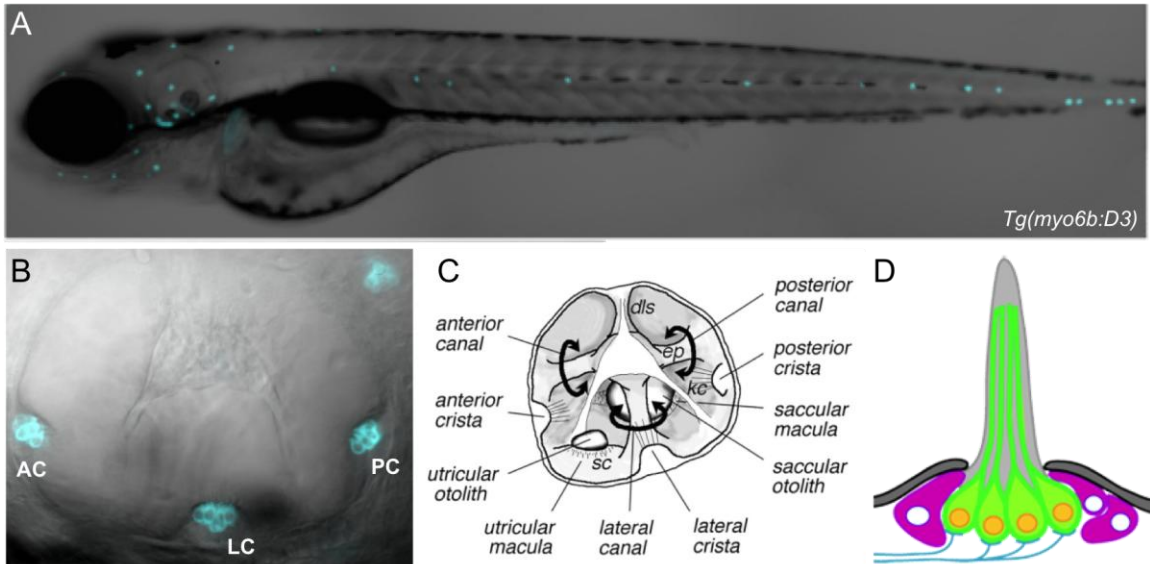


Figure 1.1: Zebrafish have hair cells in both the inner ear and lateral line organs.

A-B, A transgenic fish at 5 dpf expressing a fluorescent protein, the Ca^{++} sensor D3-Cameleon, specifically in hair cells of the lateral line and inner ear, respectively, using the *myosin 6b* (*myo6b*) promoter. Indicated are the anterior crista (AC), medial crista (MC) and posterior crista (PC). **C**, Diagram of the zebrafish inner ear at 4 dpf with labels indicating the locations of the cristae and maculae as well as the otoliths. Also indicated are the epithelial pillars (ep), the kinocilia of the cristae (kc) protruding into the semicircular canal, and the stereocilia (sc) of the maculae that appear to be in near direct contact with the otoliths, from Whitfield et al., 2002. **D**, A schematic displaying the organization of a neuromast with hair cells in green, support cells in magenta and innervating nerves in blue, adapted from Dambly-Chaudiere et al., 2003.

favorable environment for hair cells, for example by maintaining extracellular ion concentrations. Supporting cells can also give rise to new hair cells (Jones & Corwin, 1996; López-Schier & Hudspeth, 2006; Ma et al., 2008; Namdaran et al., 2012).

STRUCTURE AND MOLECULAR BIOLOGY OF THE HAIR CELL

Hair cells have both apico-basal polarity, where the apical and basolateral compartments are structurally and functionally distinct, and planar polarity, where the cell is asymmetrical and anterior and posterior sides are different. This configuration of apico-basal and planar polarity is essential to the reception and transmission of sensory signals as well as directional sensitivity. At the apical end of the hair cell are several rows of actin filled stereocilia, collectively referred to as the hair bundle. Many hair cells also have a true tubulin-filled cilia called the kinocilium (Schwander et al., 2010). At the basolateral end, hair cells make contact with afferent neurons through specialized ribbon synapses and also receive inputs from efferent synapses (LoGiudice & Matthews, 2009).

Stereocilia are arranged in a staircase fashion, whereby each successive row of stereocilia is taller than the last (Schwander et al., 2010). The planar polarity of hair cells is defined by this organelle. The staircase of stereocilia spans the width of the cell across the apical end, with the shortest row of stereocilia at one end and the tallest connected to the kinocilia at the other. These are universal features of auditory and vestibular hair cells across species.

In addition to the conserved structure of the hair cell and its associated cells, the molecular components that are responsible for transduction and transmission are also well conserved. Tip links connect the tips of shorter stereocilia to the side of taller neighboring stereocilia. These tip links are composed of two adhesion proteins, Protocadherin 15 (Pcdh15) and Cadherin 23 (Cdh23). PCDH15 resides at the lower end of the tip link while CDH23 is at the upper end (Kachar et al., 2000; Kazmierczak et al., 2007). The tip link mechanically gates the mechano-electrical transduction (MET)

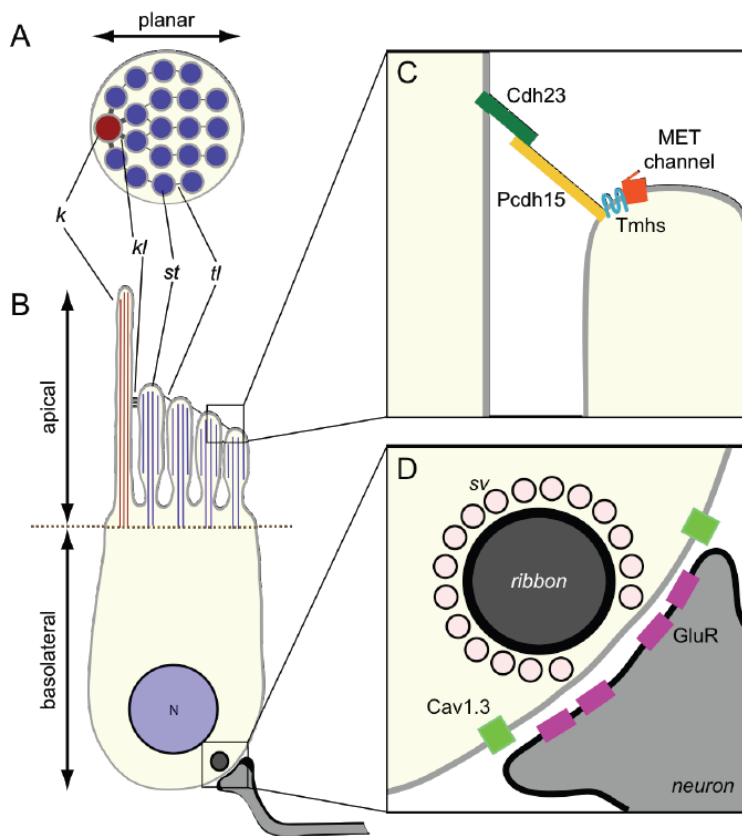


Figure 1.2: Key structures and proteins of the hair cell.

A, B, Top-down and side views of the hair cell. The tubulinfilled kinocilia (k) is indicated in burgundy. The actin filled stereocilia (st) are indicated in blue. Kinocilial links (kl) and tip links (tl) are also indicated. The planar polarity, determined by the location of the kinocilia, is indicated

in **A**. The apical and basolateral compartments of the hair cell are indicated in **B**. Also in **B**, boxes indicate areas of the hair cell highlighted in panels **C** and **D** in greater detail. **C**, A more detailed depiction of a tip link at the tips of stereocilia. The upper end of the tip link, consisting of two Cdh23 molecules, connects the side of the taller stereocilia to the lower end of the tip link, made up of two Pcdh15 molecules, which are integral to the membrane of the tip of the shorter stereocilia. Evidence suggests that Tmhs and the MET channel are at the lower end of the tip link and may somehow interact with Pcdh15. **D**, A more detailed diagram of the ribbon synapse showing the electron dense ribbon body surrounded by synaptic vesicles (sv). Also shown are presynaptic voltage-gated calcium channels, Cav1.3, and postsynaptic Glutamate receptors (GluR).

channel, which is thought to reside at the tips of the stereocilia near the base of the tip link and likely attaches to Pcdh15 either directly or indirectly (Beurg et al., 2009). Kinocilial links connect the tallest stereocilia to the kinocilium and is composed of Pcdh15 and Cdh23 (Goodyear et al., 2010). In addition to tip links, stereocilia are connected by numerous ankle- and lateral-links. Ankle-links are located between the base of stereocilia and lateral links are located along the length between stereocilia (Hackney & Furness, 1995). The tip links, however, are necessary for the integrity of the bundle. Without either Cdh23 or Pcdh15, stereocilia lose their association with one another and “splay” (e.g., see Figures 2.4 and 3.2) (Di Palma et al., 2001; Kachar et al., 2000; Kazmierczak et al., 2007; Seiler et al., 2005; Washington et al., 2005; Wilson et al., 2001). The tip link, MET channel and other closely-associated proteins are collectively referred to as the MET complex. In general, bundle splaying can indicate that a component of the MET complex is disrupted.

At the basal end of the hair cell, the ribbon synapse organizes synaptic machinery necessary for transmission of sensory inputs to the afferent nerve. Ribeye is a major component of the ribbon synapse and is essential for the organization of other associated proteins (Schmitz, 2009). Ribeye in hair cells self assembles into a spherical or oblong structure called a ribbon at the pre-synapse. It is thought that the ribbon facilitates and coordinates the fusion of glutamate-filled synaptic vesicles, which are tethered to the ribbon (Lenzi et al., 1999). Ribeye is also thought to organize the voltage-gated calcium channel at the presynapse (Moser et al., 2006; Sheets et al., 2011).

MECHANOTRANSDUCTION

Mechanically sensitive ion channels convert mechanical stimuli into electrical signals in cells and are important for sensory processes such as touch and hearing (Martinac, 2004; Sukharev & Sachs, 2012). Several mechanosensitive channels have been identified in archaea, bacteria, *C. elegans*, *Drosophila*, and cell lines and have been shown to play roles as diverse as salt sensation, acid sensation and sensing turgor pressure (Coste et al., 2010; Hong & Driscoll, 1994; Sukharev et al., 1994; Walker et al., 2000). Auditory and vestibular hair cells specialize in converting vibrational stimuli into graded receptor potentials, which lead to synaptic release. They accomplish this through a mechanically gated transduction channel. Fluid motions in the ear deflect the actin filled stereocilia at the apical end of the hair cell (Hudspeth, 1989). When the bundle is deflected toward the taller stereocilia tension is applied across the tip links, which are thought to gate the MET channel. An increase in tension across the tip links increases the open-probability of the MET channel.

The MET channel of the auditory and vestibular hair cell is yet to be identified, but some of its properties have been defined. For example, the MET channel is relatively non-selective for positive ions, with a preference for Ca^{++} ions (Corey & Hudspeth, 1983). The channel is also thought to have a large pore diameter, as it permits the passage of larger molecules such as FM 1-43, a vital dye used in Chapters 2 and 3 (See Figures 2.3 and 3.2 for examples) (Meyers et al., 2003). In addition to the properties of the MET channel, only one to two individual channels are thought to reside at the tips of each stereocilia (Fettiplace, 2009). The low number of channel molecules has been a major barrier to identification of the MET channel through traditional biochemical

methods and has demanded a more creative approach. In Chapter 5, I describe such a method that used Pcdh15 as bait in a split-ubiquitin yeast-two-hybrid to pull out MET channel candidates and follow up experiments that indicate Transmembrane channel-like 2a (Tmc2a) as a potential component of the MET channel.

TARGETING AND TRAFFICKING OF MEMBRANE PROTEINS IN HAIR CELLS

How membrane proteins are sorted specifically in hair cells has not been extensively researched. For example, studies using FM 1-43 as a marker for membrane dynamics in mammalian hair cells provide evidence that there is an appreciable amount of trafficking between two pools of membrane bound compartments. One of these pools of compartments is at the mid-basal end of the cell while the other is at the apical end of the cell body just below the stereocilia. In outer hair cells, this apical pool of FM 1-43 labeled vesicles overlaps with Hensen's body, a calcium store organelle found in outer hair cells just below the cuticular plate (Griesinger et al., 2002; Mammano et al., 1999; Meyer et al., 2001). Structures like these are often found at the base of primary cilia and sometimes referred to as the "ciliary pocket." These structures are thought to be involved in cilia-associated trafficking, among other roles (Benmerah, 2013).

Additional studies have looked at the trafficking of specific proteins of interest, such as plasma membrane calcium ATPase (PMCA) and PCDH15. Hair cells mainly express PMCA1 and PMCA2, both of which are localized to the plasma membrane, but have splice variants that exclusively sort to either the apical or basolateral membranes (Dumont et al., 2001). Specifically for PMCA2, the PMCA2xb variant localizes to the basolateral compartment of hair cells while PMCA2w is apically targeted (Grati et al.,

2006; Hill et al., 2006). Later studies revealed what components of the PMCA2 splice variants controlled this polarized expression profile (Grati et al., 2006; Hill et al., 2006).

Although there is a known mechanism for sorting membrane proteins targeted to the basolateral surface of cells, the mechanism of apical targeting of proteins is still unknown. A few amino acid motifs have been identified that are necessary for the correct sorting of apically targeted membrane proteins in other polarized cell types (Chmelar & Nathanson, 2006; Dwyer et al., 2001; Emi et al., 2012; Youker et al., 2013). The cellular pathway taken by some proteins on their way to the apical membrane has also been painstakingly described. For example, some membrane proteins can sort directly from the Golgi while many others are observed to pass through a common endosome termed the “apical recycling endosome” (Weisz & Rodriguez-Boulau, 2009). Specifically in hair cells, however, it is not understood how components destined for the stereocilia and the MET complex are correctly targeted.

Targeting of MET complex components, which localize to discrete microdomains within the apical surface of the hair cell, probably utilizes additional mechanisms. Strong evidence suggests that tetraspan membrane hair cell stereocilia (TMHS), a component of the MET complex, and PCDH15 are both necessary for each to localize to the tips of stereocilia (Xiong et al., 2012). This supports the idea that MET complex assembly is necessary for trafficking and implies that the MET complex is trafficked at least as a partially assembled complex.

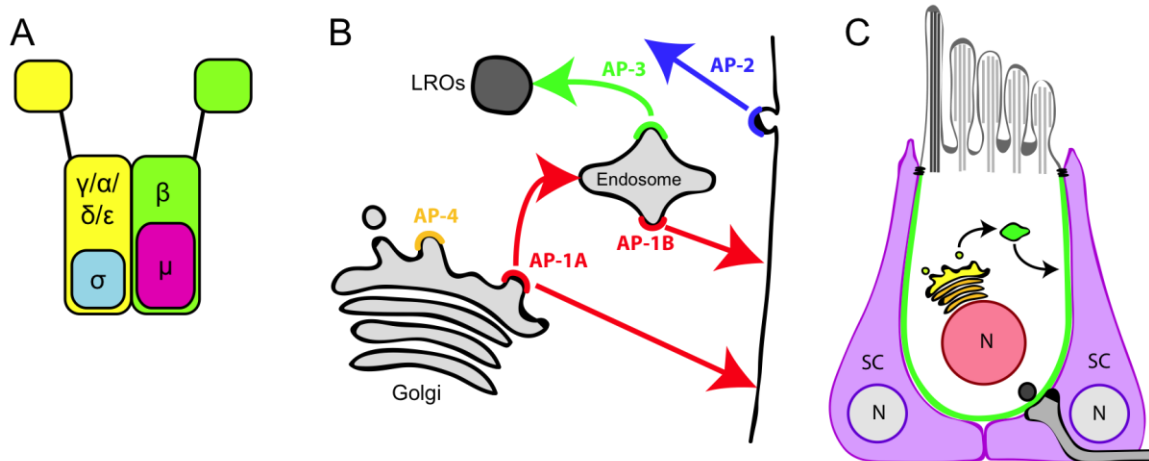


Figure 1.3: Adaptor proteins and their mechanisms of sorting.

A, A generalized schematic of an AP complex containing a large β subunit, a medium μ subunit, a small σ subunit and a variable large subunit γ, α, δ or ϵ belonging to AP complexes 1-4, respectively. **B**, A diagram of the sorting mechanisms of AP complexes 1-4. Indicated are a lysosomal related organelle (LRO) and the plasma membrane (PM). **C**, Diagram depicting likely sorting pathway of the AP-1 complex in hair cells sorting to the basolateral membrane, shown in green. Also indicated are the surrounding supporting cells (SC) and the nuclei (N) for each cell.

SORTING OF MEMBRANE PROTEINS

Movement of integral membrane proteins along the secretory pathway is a well characterized process. Critical components for trafficking membrane proteins include coat proteins such as COPI, COPII, and clathrin (Szul & Sztul, 2011). COPI and COPII are involved in shuttling membrane proteins from the endoplasmic reticulum to the Golgi network and back, respectively. Post-Golgi sorting of most membrane proteins is accomplished by a mechanism involving any of five adaptor protein (AP) complexes,

AP-1, -2, -3, -4 or -5 (Figure 1.3) (Hirst et al., 2011; Nakatsu & Ohno, 2003; Robinson, 2004). AP-1 and AP-2 are involved in clathrin mediated transport, AP-3 and AP-4 sort membrane proteins through clathrin independent means. In either case, the AP complexes interact with sorting motifs on cargos and then, in the case of AP-1, -2 and -3, bind to clathrin, providing selectivity in the initial step of transport.

AP complexes 1 through 4 are all heterotetrameric complexes made up of a small σ subunit, a medium μ subunit, a large β subunit and another variable large subunit, γ , α , δ or ϵ (Robinson & Bonifacino, 2001). The AP-1, -3 and -4 complexes are involved in sorting membrane proteins out of the trans-Golgi network. AP-1 sorts proteins to endosomal compartments or the plasma membrane (PM), AP-3 sorts proteins to lysosomal related organelles (LROs) and some evidence indicates that AP-4, like AP-1, is involved in basolateral targeting (Simmen et al., 2002). The AP-2 complex functions in the opposite direction, retrieving membrane proteins at the plasma membrane and sorts them to the early endosome (Rappoport, 2008; Traub & Apodaca, 2003). All of these subunits work independently to ensure membrane proteins reach their intended location.

Much work has been invested to understand the mechanisms by which proteins are sorted in polarized cells. The pathway of proteins destined for the basolateral membrane has been elegantly documented (Fölsch et al., 2009). As mentioned previously, AP-1 and AP-4 have been shown to be involved in the basolateral targeting of proteins (Nakatsu & Ohno, 2003). Sorting signals for proteins targeted to the basolateral membrane are well documented. The most studied are tyrosine-based (for example Yxx $\square$ and NPxY) and dileucine- based (specifically L[L/I]xxx[D/E]) signals, which are recognized by the μ and β subunits, respectively (Bonifacino & Traub, 2003; Heilker et al., 1996; Ohno et al.,

1998; Rapoport et al., 1998). Other sorting signals recognized by AP complexes for polarized sorting are continually being discovered, indicating many sorting signals exist adding a 16 level of mechanistic complexity (Deora et al., 2004; Dwyer et al., 2001; Mishra et al., 2012; Reales et al., 2011).

The AP-1 complex has been studied primarily in cell culture models. There are two possible medium subunits for the AP-1 complex, either μ 1A or μ 1B, which identify the AP-1A and AP-1B complex, respectively (Fölsch, 2005; Fölsch et al., 2003; Heldwein et al., 2004; Ma et al., 2009). In pig and dog kidney cell-lines, when the μ 1B subunit is mutated or missing AP-1B dependent cargoes that are normally restricted to the basolateral surface of polarized cells, instead sort to both apical and basolateral surfaces. Recent work has shown that AP-1A is also involved in basolateral targeting of membrane proteins in polarized cells (Gravotta et al., 2012). This suggests that both AP-1A and AP-1B play roles in polarized sorting of membrane proteins.

The trafficking routes taken by AP-1B dependent cargo to the basolateral membrane are also well described and can either occur via an endosome, particularly the recycling endosome where endocytosed membrane proteins may be processed and recycled back to the plasma membrane. AP-1B dependent cargoes can also occur through a pathway not involving the recycling endosome (Ang et al., 2004; Farr et al., 2009; Fölsch et al., 2009).

A couple studies analyzed the role of AP-1 in sorting of proteins specific to hair cells. Grati and colleagues looked at the influence of AP-1B on sorting of the basolaterally targeted PMCA2xb. They found that although mutation of a di-leucine motif in the cytoplasmic C-terminal end of PMCA2xb affected basolateral targeting in hair cells, consistent with an AP-1 dependent mechanism, PMCA2xb was not missorted in kidney

Table 1.1: Summary of known AP-1 subunit mutations in multicellular organisms

adapted from Ohno (2006) and updated.

Subunit	Organism	Mutation cause	Phenotype	Citation
σ 1A	<i>H. sapiens</i>	natural mutation	Mental retardation, enteropathy, deafness, peripheral neuropathy, ichthyosis and keratoderma (MEDNIK)	(Montpetit et al., 2008)
σ 1B	<i>H. sapiens</i>	natural mutation	Mental retardation, hydrocephaly and calcification of the basal ganglia.	(Saillour et al., 2007; Tarpey et al., 2006)
γ	<i>M. musculus</i>	gene targeting	embryonic lethal	(Zizioli et al., 1999)
μ 1A	<i>M. musculus</i>	gene targeting	embryonic lethal	(Meyer et al., 2000)
μ 1B	<i>M. musculus</i>	Unknown	chronic colitis	(Takahashi et al., 2011)
β 1	<i>D. rerio</i>	ENU mutagenesis	auditory and vestibular deficits, larval lethality after yolk absorption	(Clemens-Grisham et al., 2013)
σ 1	<i>D. rerio</i>	morpholino	reduced pigment, impaired spinal cord development, motor deficits, larval lethality	(Montpetit et al., 2008)
γ	<i>C. elegans</i>	dsRNAi	embryonic lethal	(Shim et al., 2000)
β 1	<i>C. elegans</i>	dsRNAi	embryonic lethal	(Shim et al., 2000)
μ 1 (<i>unc-101</i>)	<i>C. elegans</i>	natural mutant	50% larval lethality, uncoordinated movement, compromised sensory neuron function, defective development of primary cilia	(Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010; Lee et al., 1994; Margeta et al., 2009; Shim et al., 2000)
μ 1 (<i>amp-1</i>)	<i>C. elegans</i>	dsRNAi	lethal at larval stages	(Shim et al., 2000)
σ 1	<i>C. elegans</i>	dsRNAi	embryonic lethal	(Shim et al., 2000)
μ 1	<i>D. melanogaster</i>	EMS mutagenesis	reduced eye size, vein thickening, deficits in Notch signaling	(Benhra et al., 2011)

cells where the μ 1B subunit was mutated, rendering the AP-1B complex non-functional (Grati et al., 2006). This suggested that the AP-1B complex was not involved in basolateral targeting of this protein. This study could not exclude the possibility that PMCA2xb could be basolaterally targeted by AP-1A or any of the other AP complexes.

An additional study looking at PCDH15 and VLGR1 (very large G-protein coupled 17 receptor 1), a developmental component of stereocilia ankle-links, showed that AP-1 was required for exit of these proteins from perinuclear compartments in cell culture (Zallocchi et al., 2012). This finding indicates that AP-1 is involved in movement of stereocilia proteins out of the Golgi, albeit in a cell culture line.

The role of the AP-1 complex in an intact organism is less well studied (see Table 1.1 for summary). A morpholino knockdown study of the σ subunit highlighting the role of this gene in zebrafish (Montpetit et al., 2008). This group demonstrated that knocking down *ap1s1* caused skin malformation and severely impaired motility attributed to neurodevelopmental defects, mirroring some of the symptoms of MEDNIK (Mental retardation, enteropathy, deafness, peripheral neuropathy, ichthyosis and keratoderma) syndrome. The most extensive analysis of any AP-1 subunit in a multicellular organism comes from work on the *unc-101* mutant in which one of the two μ subunits is mutated. Initial analysis of this mutant revealed it had uncoordinated movement, defective dye filling of chemosensing cells and developmental defects. In *C. elegans* dye filling is a simple method to measure chemosensory activity in chemosensory cells (Tong & Bürglin, 2010), much like FM 1-43 labeling is a measure of mechanosensation. Subsequent work showed that this subunit was involved in sorting of membrane proteins in neurons, apical targeting of olfactory receptors and formation of the primary cilium in the chemosensing neurons (Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010; Lee et al., 1994; Margeta et al., 2009; Shim et al., 2000).

In this thesis I describe experiments that I and my colleagues performed in order to address outstanding questions on protein sorting in hair cells. In Chapters 3 and 4, I

investigate the effect of mutations to the $\beta 1$ subunit of the AP-1 complex (*ap1b1*) on protein sorting and hair cell function *in vivo*. We find that mechanotransduction is defective in *ap1b1* mutant hair cells, which easily explains the auditory and vestibular phenotypes observed in these animals. We also describe the missorting of the Na^+/K^+ ATPase (NKA), which is normally restricted to the basolateral surface of hair cells, to the apical surface and generally reduced amounts of NKA at the plasma membrane. We propose that the reduction of NKA at the membrane results in the observed build-up of intracellular sodium in hair cells.

In Chapters 2 and 5, we investigate the function of potential components of the MET complex, the transmembrane containing Tmhsa and Tmc2a, and their role in mechanotransduction in zebrafish hair cells. Tmhsa is known to localize to the tips of stereocilia, consistent with being a part of the MET complex. Tmhsa has also been shown to be important for mechanotransduction in mouse hair cells. Work I present in Chapter 2 mirrors both of these findings and suggests that Tmhsa performs a conserved mechanism in vertebrate hair cells. I also present evidence that the auditory and vestibular deficits exhibited by *tmhsa* mutants can be rescued by expressing GFP-Tmhsa specifically in hair cells. This suggests that the role of endogenous Tmhsa, which is potentially expressed in other cell types, is not critical to the auditory and vestibular systems.

And in Chapter 5, I present evidence indicating that Tmc2a plays a regulatory role on mechanotransduction on hair cells, specifically the cytoplasmic N- and C-terminal ends of Tmc2a. Interestingly we find that the N-terminus of Tmc2a reduces mechanotransduction in hair cells, while the C-terminus potentiates

mechanotransduction. This finding indicates a direct role of Tmc2a in mechanotransduction.

Chapter 2 – Tmhsa is necessary for hair cell morphology and function and localizes to the tips of stereocilia

AUTHORS

Rachel Clemens Grisham, Katie Kindt, Teresa Nicolson

ABSTRACT

TMHS is a known human deafness gene found in the DNFB67 locus that may play a role in hair cell mechanotransduction. In this study, we show that the highly conserved zebrafish ortholog, *tmhsa*, plays a similar role in zebrafish hair cells. Through analysis of the *astronaut* mutant, we observe that the *tmhsa* gene is essential for hair-cell physiology, specifically hair cells of the inner ear as we saw no defects in lateral-line hair-cells. This suggests that a different gene may play a similar role in hair cells of the lateral-line neuromasts. We also demonstrate that expression of *tmhsa* solely in hair cells is sufficient to rescue the zebrafish *tmhsa* mutant.

INTRODUCTION

Auditory and vestibular hair cells translate vibrations into graded electrical signals that allow animals to hear and maintain their balance. Sound vibrations are converted to fluid motions in the ear and are ultimately received at the apical end of hair cells where they deflect actin-filled stereocilia. This deflection opens a mechanically gated channel of unknown molecular identity, allowing K^+ and Ca^{++} ions to flow into and depolarize the hair cell (Hudspeth, 1989). Many of the molecular components of the mechanotransduction (MET) complex have been identified through the analysis of human mutations and forward genetic screens in mice and zebrafish. This type of analysis lead to the identification of the tip link proteins protocadherin 15 and cadherin 23, which have subsequently been shown to mechanically gate the MET channel (Ahmed et al., 2006; Kazmierczak et al., 2007; Seiler et al., 2005; Siemens et al., 2004; Söllner et al., 2004). In addition to these key molecular components, the MET complex is made up of several other proteins (Schwander et al., 2010), all of which play a significant role in transducing mechanical stimuli.

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human mutations and forward genetic screens in mice and zebrafish. This type of analysis lead to the identification of the tip link proteins protocadherin 15 and cadherin 23, which have subsequently been shown to mechanically gate the MET channel (Ahmed et al., 2006; Kazmierczak et al., 2007; Seiler et al., 2005; Siemens et al., 2004; Söllner et al., 2004). In addition to these key molecular components, the MET complex is made up of several other proteins (Schwander et al., 2010), all of which play a significant role in transducing mechanical stimuli.

Here, we describe the effect to hair cells of the newly identified of *tmhsa* mutation in the *astronaut* zebrafish mutant (Obholzer et al., 2012). Many of the observations we made confirm what have been described in the mouse studies, including localization of the Tmhsa protein to the tips of stereocilia. We also show that *tmhsa* expression solely in hair cells is sufficient to rescue the *astronaut* phenotype.

MATERIALS AND METHODS

Animal lines

Zebrafish were kept on a 12 hr light-dark cycle at 28°C. Cloning of the *astronaut* mutant line is described \cite{Obholzer:2012}. The *Tg(myo6b: β -actin-GFP)* and *Tg(myo6b:D3cpv)* cameleon lines have been previously described \cite{Kindt:2012}.

Cloning and molecular biology

To identify the relevant *tmhsa* transcript, a forward primer F1 was designed against the predicted first exon and reverse primers were designed against predicted exon 2 (R1 and R2), Genescan-predicted exon 3 (R3) and Ensembl-predicted exon 3 (R4), see Table 3.1 for primer sequences. PCR was performed on cDNAs with a SuperScriptIII (Invitrogen) kit from total RNA isolated from Tubingen 5 dpf wild-type (WT) larvae.

Vector construction and transgenic lines

Expression clones are based on the Tol2/Gateway zebrafish kit \cite{Kwan:2007}. After determining the correct *tmhsa* transcript, primers were designed to amplify the coding region of *tmhsa* and recombine into both middle entry (ME) and 3' entry (3E) clones (Table 1). The pME-*tmhsa* and p3E-*tmhsa* clones were then combined with a destination vector containing a green heart marker (395), the 5' entry vector containing the *myo6b* promoter \cite{Obholzer:2008}, and either p3E-GFP (366) or pME-GFP (no stop, 455) to generate the *myo6b:tmhsa-GFP* and *myo6b:GFP-tmhsa* constructs, respectively.

To generate transgenic lines, one-cell stage embryos were injected with 1 nl of the following solution: 30 ng/ul of expression clone, 20 ng/ul Tol2 transposase RNA and 10% Phenol Red (Sigma). Embryos were allowed to develop and at 2-5 dpf larvae were

analyzed for transient expression of the construct. Animals expressing the construct were grown to adult stages and crossed to Tubingen or Long Fin wild type lines to find animals with germline transgenesis. Three founder fish were isolated and two transgenic lines were maintained.

Table 3.1: Primer sequences used in this study

Primer name	Primer sequence
F1	GCAACTCGGGCAGTGTTTAT
R1	GTCTCTGGCAGCAGTTTGT
R2	TCTTCTCCTCCTGGAAGTCCT
R3	ACGGCGATGTCCTCTGTATA
R4	GTCATCTCCCAGCCTGACC
<i>tmhsa_ME_att1fwd</i>	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGCG AAAATGCTATCTGCC
<i>tmhsa_ME_att2rev</i>	GGGGACCACTTTGTACAAGAAAGCTGGGTGTGCTTCCT CTTCTTCTCCTC
<i>tmhsa_3E_att2fwd</i>	GGGGACAGCTTTCTTGTACAAAGTGGCGATGGCGAAAA TGCTATCTGCC
<i>tmhsa_3E_att3rev</i>	GGGGACAACCTTTGTATAATAAAGTTGGTCATGCTTCCTC TTTCTTCTC

Microscopy

Images were captured on a Zeiss LSM 700 upright confocal microscope using the Zen acquisition software (2009 release, Zeiss). Live larvae were mounted in 1% low melt agarose (Life Technologies) dissolved in E3 embryo medium and imaged with a 63x/0,95 water immersion lens. Images were subsequently processed with ImageJ software.

FM 1-43 labeling

For the FM 1-43 experiments, zebrafish larvae were incubated for 20 sec in E3 containing 3 uM N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino)styryl)Pyridinium Dibromide (FM 1-43, Life Technologies).

Calcium imaging and hair bundle stimulation

Calcium imaging and analysis was performed as described elsewhere \cite{Kindt:2012}.

RESULTS

The *astronaut* mutation was first identified in an ENU (N-ethyl-N-nitrosourea) mutagenesis screen for zebrafish mutants with hearing and balance impairments and was subsequently cloned using deep sequencing techniques (Nicolson et al., 1998; Obholzer et al., 2012). The *tmhsa* gene is found on chromosome 11 and its paralog, *tmhsb*, is located on chromosome 8. The *astronaut* nonsense mutation should cause a truncation early in Tmhsa and loss of the majority of the protein (Figure 2.1A).

The Tmhsa protein sequence is highly conserved through vertebrate species (Figure 2.1A). Most of the human mutations and one of the mouse mutations arise from missense mutations to residues that are conserved in all species analyzed here (Figure 2.1A) (Kalay et al., 2006; Shabbir et al., 2006). There is a potential dominant human mutation that substitutes a conserved Arg residue to a Leu, though it was observed in only one individual. Lastly, a human frame-shift mutation, which causes mis-splicing of the last exon, results in loss of these three residues and of the native stop site. The number of missense mutations found combined with its high level of identity across species, suggests that many specific residues are essential for protein function. These data also suggest the amino acids at the C-terminus are essential to TMHS protein function.

In order to identify key residues for protein function, we first analyzed the alignment of *tmhsa* genes across species (Fig 3.1A) Upon analysis of mouse, rat, cow and human sequences for Lhfpl5/Tmhs, we observed that the last three residues encoded in the 3rd exon in all sequences were two Glu residues followed by a nonpolar Val. We next

consulted two exon prediction programs, Genescan and Ensembl, for the zebrafish protein sequence. With both predictions, the zebrafish amino acid sequence had near perfect agreement with the sequences from other species, but unexpectedly we observed key differences particularly at the C-terminus. One difference we observed in the predicted zebrafish sequence was two additional amino acids between exons 1 and 2 in the Ensembl prediction, which did not match the alignments from other species. The next difference we noticed was that Genescan and Ensembl predicted completely different 3rd exons encoding the C-terminal residues for *tmhsa* (Figure 2.1A). Importantly, the exons predicted by Genescan would generate a protein with better alignment to the sequences from other vertebrates, including the three C-terminal amino acids, Glu-Glu-Ala (Figure 2.1A).

We hypothesized that only one of these predicted protein-products was correct. We therefore aimed to verify the Genescan and Ensembl predicted coding regions for zebrafish *tmhsa* by using RT-PCR (Figure 2.1B-C). I designed a single forward primer (F) and four reverse primers; two primers to exon 2 to serve as an internal control (R1, R2), one primer to the Genescan predicted exon 3 (R3), and one primer to the Ensembl predicted exon 3 (R4). We successfully amplified product using the primers spanning from exon 1 to exon 2 and to Genescan predicted exon 3, but we were unable to amplify product spanning from exon 1 and the Ensembl predicted exon 3 (Figure 2.1C). Subsequent sequencing revealed that the subtle difference in splicing between exon 1 and 2 agrees with the Genescan prediction and not the sequence predicted by Ensembl. These data suggest that the Genescan prediction represents the correct transcript for the *tmhsa* gene and that the transcript predicted by Ensembl is either not expressed at a high enough level to be detected by RT-PCR or is not ever transcribed. This result combined

with the observed human *TMHS* frame-shift mutation indicates the highly conserved C-terminus could be important for protein function.

The *tmhsa* mutant exhibits clear auditory and balance deficits arising from a specific effect on inner-ear hair-cells (Nicolson et al., 1998; Obholzer et al., 2012). A possible cause of this behavioral phenotype is a dysfunctional MET complex. To test whether MET channel activity is affected in *tmhsa* mutants, I tested FM 1-43 labeling of lateral line hair cells. The lipophilic dye FM 1-43 reliably labels hair cells with functional MET channels as this dye easily flows through open MET channels, which have a high open probability (Meyers et al., 2003). The FM 1-43 vital dye labels both mutant lateral-line hair-cells as well as wild-type (Figure 2.2A-B). In agreement with the FM data, microphonics (Nicolson et al., 1998) and Ca^{++} transients (Figure 2.2C) measured from mutant lateral-line hair-cells were not significantly reduced compared to wild type. As the hair cells that reside in the inner ear are responsible for mediating the vestibular and auditory reflexes, we next tested Ca^{++} responses of the cristae in the developing ear. In the inner ear, we recorded significantly reduced Ca^{++} responses in mutant hair cells compared to wild-type siblings (Figure 2.2D). Combined, these results indicate that the effect of the *tmhsa* mutation is most significant on hair cells of the inner ear and not those of the lateral line.

We hypothesized that the morphology of the hair bundles would be perturbed in the mutants as compromised MET channel function can lead to splayed bundles (Nicolson et al., 1998). We observed that the stereocilia of inner-ear hair-cells in *tmhsa* mutants were splayed while wild-type siblings had no bundle splaying (Figure 2.2E-F). This observation agrees with data from the mouse *Tmhs* mutant studies describing perturbations to bundle morphology and tip link formation (Longo-Guess et al., 2005;

Xiong et al., 2012). Mutations to components of the MET complex often compromise the integrity of the tip-links between individual stereocilia and bundles will fall apart or “splay.” As the *tmhsa* mutation also results in splayed bundles, this observation supports the idea that the Tmhsa protein is part of the MET complex.

Another indicator that Tmhs is a member of the MET complex is that immunolabeling experiments show the protein localizes to the tips of stereocilia in mice. We sought to determine if this was also true for zebrafish Tmhsa by cloning the *tmhsa* transcript, fusing it to GFP and expressing it specifically in hair cells using the *myo6b* promoter. We generated *myo6b:GFP-tmhsa* and *myo6b:tmhsa-GFP* constructs and observed that transient expression of the N-terminally tagged version (*myo6b:GFP-tmhsa*) expressed more efficiently than the C-terminally tagged version (*myo6b:tmhsa-GFP*) (data not shown). When expressed in hair cells transiently and stably, the *myo6b:GFP-tmhsa* construct labeled a number of intracellular compartments and faintly labeled the tips of stereocilia (Figure 2.3A-C). This observation combined with physiological and bundle morphology data suggests that as in mice, zebrafish Tmhsa may be a component of the MET complex.

In mice, *Tmhs* expression is not restricted to hair cells and is also expressed in select neurons (Longo-Guess et al., 2007). This suggests that expression of *Tmhs* in both cell types may be necessary for auditory and vestibular function. To determine if expression of *tmhsa* only in hair cells could rescue the *tmhsa* mutant phenotype, we crossed the F1 generation of *Tg(myo6b:GFP-tmhsa)* into the *tmhsa* mutant background. We then incrossed *tmhsa* mutant transgenics with non-transgenic *tmhsa* mutants to determine if expression of GFP-Tmhsa only in hair cells was sufficient to rescue the hearing and balance deficits in mutants. We observed that 25.7% of incrossed larvae not expressing

the transgene displayed the hearing and balance phenotype, an expected ratio for a recessive mutation. Of the incrossed larvae that were expressing the transgene, however, we were unable to observe a single instance of the expected *tmhsa* hearing and balance phenotype (Figure 2.3D). This strongly suggests that expression of GFP-Tmhsa only in hair cells is sufficient to rescue the hearing and balance deficits of *tmhsa* mutants and any potential expression in neurons is not essential to auditory and vestibular function.

DISCUSSION

In this study we showed that the *tmhsa* gene is necessary for the physiology of zebrafish hair-cells, specifically those of the inner-ear. We cloned zebrafish *tmhsa* and showed that the protein fused to GFP localizes to the tips of hair cell stereocilia as well as unidentified compartments within the hair cell. We also demonstrated that expression of GFP-Tmhsa specifically in hair cells is sufficient to rescue the *tmhsa* auditory and vestibular deficits.

In other systems, Tmhs plays a clear role in hair cell physiology (Xiong et al., 2012). However, it was unclear whether Tmhs expression in hair cells was sufficient for auditory and vestibular function or if downstream expression of this protein in neurons was also required. Here we show that *tmhsa* expression solely in hair cells is sufficient to rescue the *astronaut* phenotype. This clearly demonstrates that the importance of *tmhsa* expression to hearing and balance in zebrafish is most profound in the hair cell, and any potential effect of the *tmhsa* mutation in neurons is subtle.

Our findings also support other data illustrating the importance of Tmhs/Lhfpl5 in MET activity. It is interesting to note that the *tmhsa* mutation seems to profound consequences for mechanotransduction in hair cells of the inner ear while there are no obvious defects to mechanotransduction in neuromast hair cells (Figure 2.2). This suggests that the Tmhsa protein is either not necessary in neuromast hair-cells or that a different protein expressed in neuromast hair-cells performs a similar function with respect to mechanotransduction as Tmhsa in the inner ear. An attractive hypothesis is that the paralog of *tmhsa*, *tmhsb*, plays a similar role in neuromast hair-cells. During the

course of the evolution of zebrafish there was a genome-wide duplication, the zebrafish genome is rife with paralogs (Amores et al., 1998; Gates et al., 1999; Postlethwait et al., 1998). And as mentioned previously, there is a paralog for *tmhsa*, called *tmhsb*. Although this study did not evaluate the expression or function of *tmhsb*, it is possible this gene is expressed and has a similar function in neuromast hair cells. We have seen this type of segregated function with other zebrafish paralog genes before (Seiler et al., 2005) (See also Chapter 5).

With four transmembrane spanning domains and two extracellular loops, the topology of *Tmhsa* is analogous to tetraspanins and claudins. The tetraspanin family of proteins are increasingly appreciated for their roles in numerous processes including fusion, cell adhesion, cell migration and cell-cell signaling (Boucheix & Rubinstein, 2001; Hemler, 2005). The unifying feature of many tetraspanins is their ability to organize multimolecular complexes through lateral interactions with several different membrane proteins (Hemler, 2005). These complexes, often termed Tetraspanin Enriched Microdomains, form high density structures that are resistant to disruption by mild detergents. With this in mind, it is possible that *Tmhsa* is acting as an organizer for the MET complex, which would make its role in MET secondary to its role in organizing and maintaining the meticulously constructed MET complex. However, still other tetraspanins are known to facilitate channel activity (Payne, 2008; Tomita et al., 2005). And in mice, it has been suggested that *Tmhs* facilitates transduction in hair cells through binding to Protocadherin 15 and potentially mediates an interaction between Protocadherin and the elusive MET channel (Xiong et al., 2012). Splaying of stereocilia in zebrafish *tmhsa* mutants could be explained by a disruption in this process.

Another possibility is that Tmhsa plays a role in organization of actin in the stereocilia. Not only do we observe bundle splaying in hair cells, but the amount of actin in *tmhsa* bundles appears to be decreased (Figure 2.2). The organization of macromolecular complexes by tetraspanins has been shown to be somewhat dependent on the actin cytoskeleton (Berditchevski & Odintsova, 1999; Delagouillauie et al., 2004). It has been speculated that MET activity and the actin cytoskeleton are somehow linked (Caberlotto et al., 2011; Kikkawa et al., 2008; Mogensen et al., 2007). In fact, the Usher Syndrome gene, CLARIN-1, which has similar topology to Tmhsa, has been shown to initiate actin polymerization in cell culture models (Tian et al., 2009). It would be interesting to see if Tmhsa had a similar role in actin polymerization. It is possible, however, that any effect on actin density or organization is secondary to its role in MET (Figure 2.2). Overall, determining the connection between MET and actin organization needs further study.

This study also shows that the amino acid sequence of Tmhsa has a high level of identity with those sequences from its orthologs in other species (Figure 2.1). The level of conservation in this protein also indicates that domains essential to Tmhsa function and interaction with other proteins are well conserved across species. This idea is supported by observations that single amino acid changes in Tmhs/TMHS sequence have profound consequences on auditory and vestibular function.

Zebrafish are an ideal model to study protein localization *in vivo* particularly because of their transparency and ease of expressing fluorescently tagged proteins (Beis & Stainier, 2006; Slaughter & Li, 2010). Additionally, there are a number of tools available to study the physiology of hair cells in intact zebrafish with either florescent tools or with more traditional neurophysiology techniques (Baker et al., 2012; Kindt et al., 2012;

Trapani & Nicolson, 2010). The zebrafish *tmhsa* mutant therefore presents an opportunity to map out some of these functional domains and their potential roles in protein trafficking, hair cell physiology and bundle morphology.

The precise role of Tmhsa in MET activity and the MET complex is still unclear and evidence that Tmhsa plays a direct role in mechanical sensitivity is lacking. The zebrafish *tmhsa* mutant, however, could give researchers the opportunity to identify other lesser-known components of the MET complex and how they and Tmhsa work together to form this remarkable complex, which is specially designed to sense physical deflections.

FIGURES

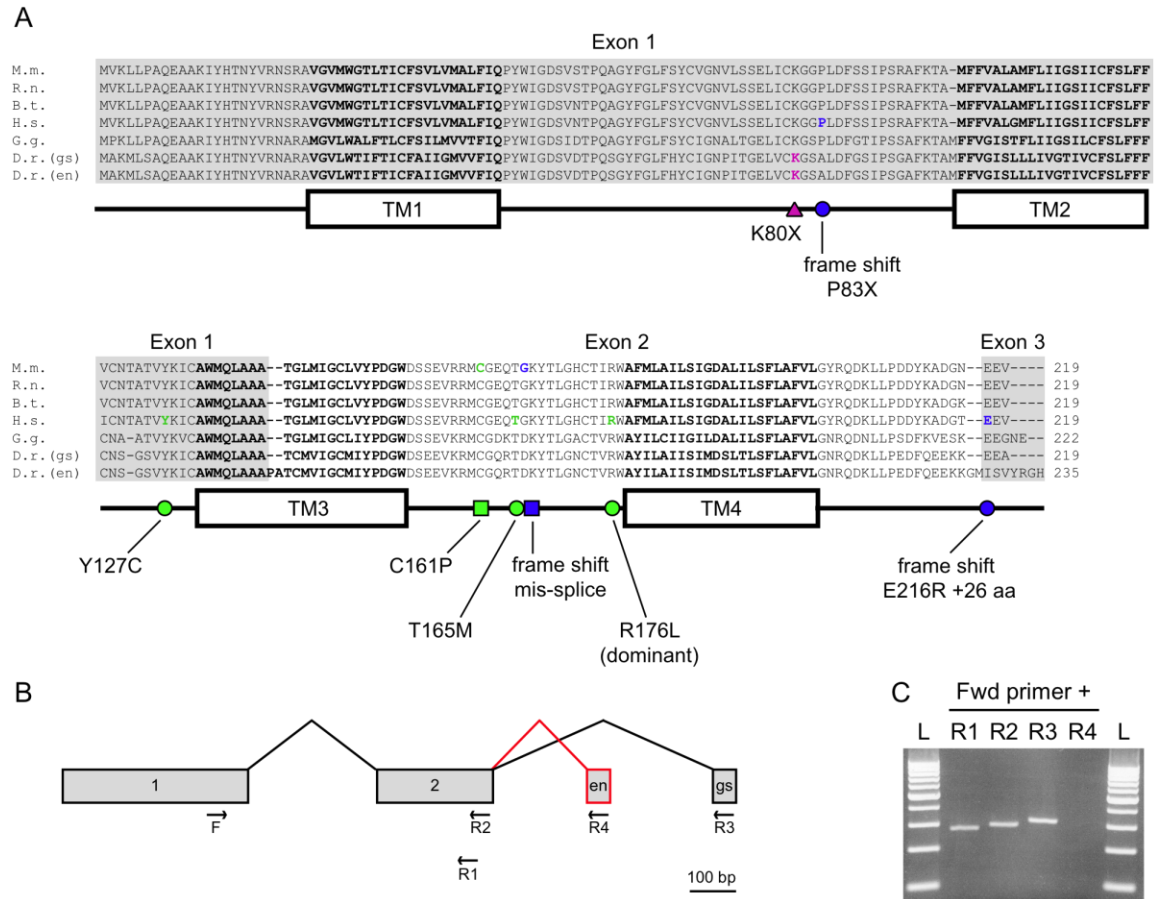


Figure 2.1: Alignment and cloning of zebrafish *tmhsa*

A, Alignment of mouse, rat, cow, human, chicken and zebrafish Genescan (gs) and Ensembl (en) predicted amino acid sequences. Transmembrane domain residues predicted by TMHMM 2.0 are in bold and are also shown below the sequence in a diagram of *tmhsa*. Exons 1 and 3 for all sequences are signified by grey boxes and exon 2 is between them. Published mutations are shown in the diagram of *tmhsa* below the alignment. Human mutations are indicated with circles, mouse mutations with squares and the zebrafish mutation as a triangle. Nonsense mutations are magenta, frame-shift

mutations are blue and missense mutations are green. **B**, A diagram of the Ensembl and Genescan predicted exons, with the forward (F) and reverse (R1-4) primers indicated below. **C**, Results from RT-PCR showing the expected sized products amplified by F (exon 1) with R1, R2 and R3 (exon 2 and Genescan exon 3), but not R4 (Ensembl exon 3). The ladder (L) is Generuler 100 bp.

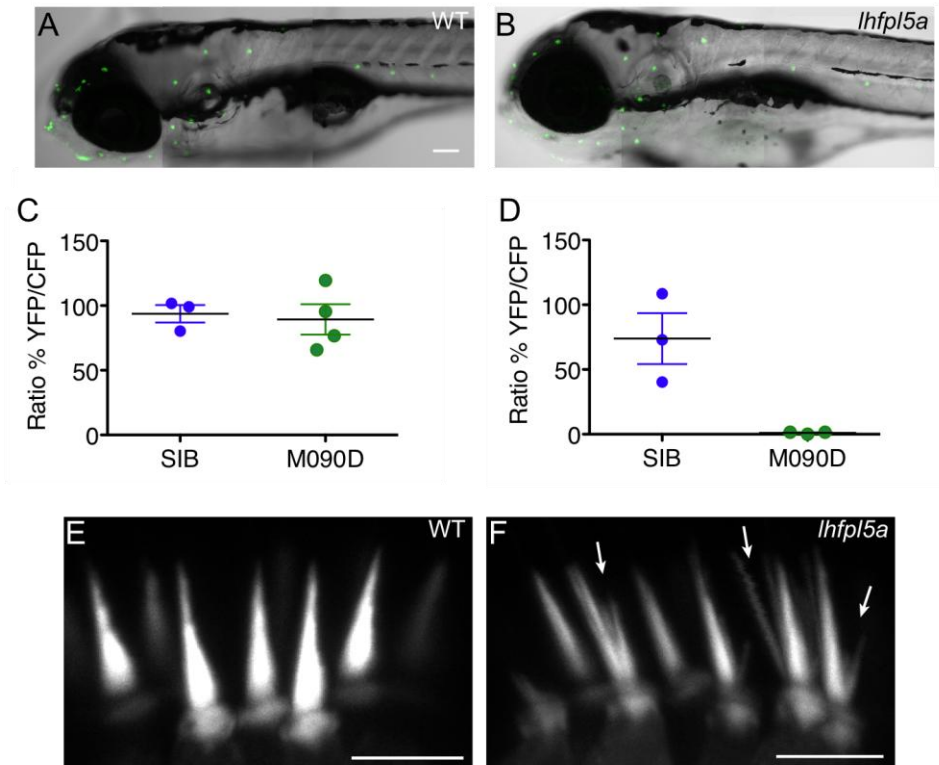
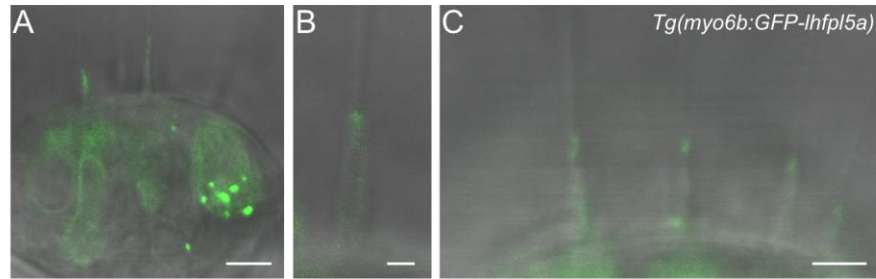


Figure 2.2: Inner ear hair cells of *tmhsa* mutants have physiology and morphology defects

A-B, FM 1-43 labeling of lateral line hair cells in a WT sibling and *tmhsa* mutant, respectively. **C**, Average Ca^{++} response of neuromast hair cells to a 2 sec, 10 Hz square wave waterjet stimulus, $n = 3$ fish. Ca^{++} transients from neuromast in *tmhsa* mutants are not significantly different from those of WT siblings, $p = 0.782$. **D**, Average Ca^{++} response of a patch of hair cells in the medial crista, $n = 3$ fish. Ca^{++} transients recorded from inner ear hair cells of *tmhsa* mutants are significantly different than WT siblings, $p < 0.01$. **E-F**, GFP-actin expressed specifically in hair cells to highlight bundle morphology in a WT sibling and *tmhsa* mutant, respectively. Scale bar in A, 100 μm , and in E and F, 5 μm .



D

<i>Tg(myo6b:GFP-lhfp15a)</i> +	# Mutant	Total N	% N mutant
No	9	35	25.7
Yes	0	39	0

Figure 2.3: GFP-Tmh5a localizes to the tips of stereocilia and rescues the *astronaut* phenotype.

A-C, Transgenic expression of GFP-*tmh5a* in hair cells of the inner ear in 5 dpf zebrafish. **A**, In hair cells, GFP-*tmh5a* can be seen localized to intracellular compartments. **B-C**, GFP-*tmh5a* also localizes to the tips of stereocilia. **D**, Table showing the number of circling mutants observed when the *GFP-tmh5a* transgene is or is not expressed. Percentage of observed larvae displaying the circling phenotype in either transgene expressers or non-expressers is shown at the far right column. Scale bar in A, 5 μ m, in B, 1 μ m, and in C, 2 μ m.

Chapter 3 – Mutations in *ap1b1* cause mechanotransduction deficits in hair cells and several other pathologies to mechanosensitive neuroepithelium

AUTHORS

Rachel Clemens Grisham, Katie Kindt, Karin Finger-Baier, Battina Schmid, Teresa Nicolson

ABSTRACT

The mechanosensory neuroepithelium of the inner ear is a tissue made up of a few important cell types, among them hair cells, supporting cells and mantle cells. All of these cell types perform unique and important roles to maintain functionality of this endorgan. Here we describe auditory/ vestibular mutants isolated from forward genetic screens in zebrafish with lesions in the *adaptor protein 1 beta subunit 1* (*ap1b1*) gene. In *ap1b1* mutants we observed that although the overall development of the inner ear and lateral-line organ appeared normal, the sensory epithelium showed progressive signs of degeneration. Mechanically-evoked calcium transients were reduced in mutant hair cells, indicating that mechanotransduction was also compromised. We also report several cellular defects to *ap1b1* mutant hair cells, including thinner stereociliary hair bundles, apical blebbing and a build up of intracellular vesicles and multivesicular bodies inside hair cells as well as interstitial edema in the neuroepithelium.

Interestingly, we observe slight perturbations in the development of the neuroepithelium as cells expressing proteins specific to hair cells can be found in the lower layer of supporting cells. These “out of place” hair cells also do not exhibit the characteristic morphology of a typical hair cell. All of this implicates *ap1b1* having diverse roles in hair cell function and development and maintenance of the neuroepithelium.

INTRODUCTION

The neuroepithelium of the larval zebrafish inner ear is divided into five patches: three cristae, one for each of the semicircular canals, and two maculae, each associated with an otolith. In addition to the ear, zebrafish also have small patches of neuroepithelium in neuromasts, which are part of the lateral-line system. All neuroepithelial patches are primarily composed of three cell types: hair cells, supporting cells and a few mantle cells (Behra et al., 2012; Driver & Kelley, 2009; Ghysen & Dambly-Chaudière, 2007). Hair cells respond to vibrational stimuli received from auditory or vestibular inputs and are embedded in a network of supporting cells. Supporting and mantle cells are also known to give rise to hair cells during development (Hernández et al., 2007). The number of hair cells in the neuroepithelium is tightly controlled and when this process is not regulated and supernumerary hair cells develop, auditory deficits can develop (Kanzaki et al., 2006). Although many transcriptional and signaling factors are known to regulate this process (Hawkins & Lovett, 2004), the cell biology of how these factors are trafficked and made functional within cells is not well explored.

In the present study, we isolated two alleles of *ap1b1* from two independent, largescale ENU (N-ethyl-N-nitrosourea) mutagenesis screens for auditory and vestibular zebrafish mutants (Granato et al., 1996) (Tübingen 2000 Screen Consortium). Cloning of the lesions in *ap1b1* revealed two early stop mutations. Here we investigate the effect of mutations in the zebrafish $\beta 1$ subunit of the AP-1 complex (*ap1b1*) on the development and function of the auditory/vestibular neuroepithelium *in vivo*.

The AP-1 complex is composed of four subunits: γ , $\beta 1$, $\sigma 1$ and either $\mu 1A$ or $\mu 1B$ (Farr et al., 2009; Fölsch, 2005; Fölsch et al., 2003; Gravotta et al., 2012; Heldwein et al., 2004; Ma et al., 2009). The two different μ -subunits distinguish the AP-1A from the AP-1B complex. As the $\beta 1$ subunit is common to both AP-1A and AP-1B complexes, for the purposes of this study we will refer to them both simply as the AP-1 complex. The AP-1 complex has been studied primarily in cell culture models, however the role of the AP-1 complex in an intact organism is less well understood.

In the present study, we quantify the vestibular and auditory deficits in *ap1b1* mutants and show that mechanotransduction is compromised in mutant hair cells. We also show some striking pathologies in hair cells, which likely precede the cell death we observe in the neuroepithelium.

In addition to dead and dying cells, we also report developmental defects in the neuroepithelium of *ap1b1* mutants. Specifically we show that occasionally cells expressing hair cell specific proteins do not adopt the characteristic morphology of a hair cell and are located below the normal plane of hair cells. In this chapter, we also explain in more detail the blebbing and spaces phenotypes we observe in the neuroepithelium. Together these results indicate that AP-1 is involved in many aspects of hair cell maintenance and development.

MATERIALS AND METHODS

Ethics statement: This study was performed with the approval of the Oregon Health & Science University Institutional Animal Care and Use Committee and in accordance with NIH guidelines.

Animal lines: Zebrafish were kept on a 12 hr light-dark cycle at 28°C. The mutant alleles of *skylab*, *tm246a* and *t20325* were isolated from two independent ENU mutagenesis screens (Granato et al., 1996) (Tubingen 2000 Screen Consortium) and maintained in either Tubingen or Top long fin wild-type (WT) backgrounds. The *Tg(myo6b:β-actin-GFP)*, *Tg(scm1:GFP)* and *Tg(myo6b:D3cpv)* cameleon lines have been previously described elsewhere (Behra et al., 2012; Kindt et al., 2012).

Behavioral assays: Vestibular-induced eye movements were recorded from 5 dpf larvae using techniques described in detail elsewhere (Mo et al., 2010; Obholzer et al., 2008). Data was analyzed in Matlab. The auditory escape response was quantified using methods described in (Einhorn et al., 2012). Statistical analysis was performed using Prism 5 (GraphPad).

Cloning and molecular biology: The *skylab* critical interval was determined by crossing WT WIK fish with Tubingen fish heterozygous for the mutation. Genetic mapping with F2 mutant larvae was performed through PCR amplification of SSLP markers. Genes within the critical interval were analyzed by PCR using the Advantage2 kit (Clontech) to amplify 400-600 bp fragments of the open reading frame from cDNAs generated by the SuperScriptIII kit (Invitrogen). These fragments were scanned for variations by comparing obtained sequence from mutants and siblings to sequences deposited in

Ensembl (http://uswest.ensembl.org/Danio_rerio/Info/Index). To pinpoint the *tm246a* lesion, primers were designed to amplify the genomic DNA around the splice acceptor site of exon 9 (forward primer, TCGTAAAAGCTGCAGACCCTA; reverse primer, TGATCAGACAGCTGGTGGAA). Before sequencing, all PCR products were purified using the QIAquick Gel Extraction kit (Qiagen).

Otoferlin immunolabeling: The rabbit anti-Otoferlin a was raised against the zebrafish protein and is described in detail elsewhere (Obholzer et al., 2008).

FM 1-43 labeling: For the FM 1-43 experiments, zebrafish larvae were incubated for 20 sec in E3 containing 3 μ M N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino)styryl)Pyridinium Dibromide (FM 1-43, Life Technologies). Fluorescence intensity measurements for FM-143 were processed, analyzed or quantified using ImageJ software from maximal z-projections for average intensity analysis.

In Situ Hybridization: Phenylthiourea (PTU) treated larvae were fixed overnight in 4% paraformaldehyde, washed in PBS + 0.1% Tween and stored in methanol at -20°C. The probe template used to detect *ap1b1* transcript corresponds to 1772-2114 bp of the coding region. The *in situs* were performed according to an established protocol (Thisse & Thisse, 2008). The GenBank accession number for *ap1b1* mRNA is NM_001128530.1.

Microscopy: DIC and *in situ* images were captured with a Leica DMLB widefield microscope equipped with an AxioCam MRm (for DIC) or an AxioCam MRc 5 (for *in situ*) camera (Zeiss) using AxioVision acquisition software (Release 4.5, Zeiss). All other images were captured on a Zeiss LSM 700 upright confocal microscope using the Zen acquisition software (2009 release, Zeiss). To view live larvae or the medial cristae of fixed larvae, animals were mounted in 1% low melt agarose (Life Technologies) dissolved in E3 embryo medium and imaged with a 63x/0,95 water immersion lens. To

view superficial neuromasts after immuno-labeling, larvae were mounted in Elvanol (0.1 M Tris pH 9.0, 10% polyvinyl alcohol, 88-89% hydrolyzed, 30% glycerol and 1% DABCO) and imaged with a 63x/1.4 oil lens.

Electron microscopy: Whole larvae (5 dpf; $n \geq 5$) were anesthetized with 0.02% MESAB and then fixed by immersion in 2.0% glutaraldehyde and 1.0% paraformaldehyde in normal solution (145 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.2) overnight to several days at 4°C. Specimens were fixed with 1.0% OsO₄ in H₂O for 10 min on ice, followed by fixation and contrast with 1.0% uranyl acetate for 1h on ice, and then dehydrated with several steps in ethanol and embedded in Epon. Ultrathin sections were stained with lead citrate and uranyl acetate.

Calcium imaging and hair bundle stimulation: Calcium imaging and analysis was performed as described elsewhere (Kindt et al., 2012). To deflect hair bundles, we used a fluid-jet composed of a pressure clamp HSPC-1 (ALA Scientific, New York) attached to a glass micropipette (tip diameter 25-35 μ m for 2 dpf, and 40-50 μ m for 3 and 5 dpf), positioned 100 μ m from a given neuromast. Deflections were sustained for the duration of the stimulus, and confirmed visually. A 25 mmHg fluid-jet stimulus was used to deflect hair bundles approximately 5-10°.

RESULTS

Positional cloning of *skylab* and expression of *ap1b1*

In two independent forward genetic screens for mutants that fail to respond normally to an acoustic tap, two recessive alleles of *skylab* were isolated, *tm246a* and *t20325* (Granato et al., 1996) (Tubingen 2000 Screen Consortium). Both mutants develop

normally, but do not inflate their swimbladders and die around 8-9 days postfertilization (dpf). To identify the mutations in *skylab*, we undertook a positional cloning approach. The *tm246a* lesion was finely mapped to a 330 kb critical interval on chromosome 5 using approximately 1900 homozygous larvae. This critical region contained no gaps and included all or part of 7 genes (Figure 3.1A). Amplification and sequencing of cDNAs from these 7 genes subsequently revealed that both alleles have early stop mutations in the *ap1b1* gene, which encodes the β -subunit of the AP-1 complex.

The *tm246a* mutation arises from a 23-base pair deletion near the splice acceptor site in intron 8 (Figure 3.1B and C). Although the core AG sequence of the acceptor site is intact, the deletion causes mis-splicing and results in an in-frame inclusion of four codons into the coding region between exons 8 and 9. The added fourth codon is a stop codon (Figure 3.1C and D). This mutation truncates the predicted Ap1b1 protein in the middle of the head domain, which is required for cargo binding and complex assembly, and completely removes the ear domain, which binds to clathrin (Dell'Angelica, 2001). The *t20325* allele contains a single c2340>t mutation changing a Glu residue in the ear domain to a stop in the open reading frame (Figure 3.1B and D). This mutation may disrupt clathrin binding and therefore formation of the clathrin lattice. The two early stop mutations uncovered in the *tm246a* and *t20325* alleles provide strong evidence that the phenotype observed in *skylab* mutant larvae is due to mutations in *ap1b1*.

To determine where *ap1b1* is expressed in developing zebrafish, we used *in situ* hybridization. In other species, the AP-1 complex is expressed in all cell types. We observed that *ap1b1* is expressed throughout the embryo at 24 and 48 hours postfertilization (hpf; Figure 3.1E-I), including in the developing ear at both stages. The

ubiquitous expression of *ap1b1* is consistent with published expression data of the AP-1 μ -subunits (Zizioli et al., 2010). These results also support a role for *ap1b1* in the sensory epithelium of the auditory and vestibular system.

***ap1b1* mutants exhibit auditory and vestibular defects**

Hair cells populate two sensory organs, the inner ear and lateral-line organ, in aquatic vertebrates such as fish and frogs. In the inner ear, hair cells are organized into several epithelial patches called cristae or maculae and mediate auditory and vestibular responses. In the larval lateral-line organ, hair cells form superficial clusters called neuromasts. Neuromasts are responsible for sensing water movements and are important for schooling and predator/prey behaviors. At the free-swimming stage of development, 4-5 dpf, the zebrafish inner ear is fully functional and larvae maintain an upright position while resting and exhibit a robust startle reflex to acoustic/vibrational stimuli. Qualitative characterization of the *tm246a* allele revealed that mutant larvae are only partially sensitive to an acoustic tap stimulus (Nicolson et al., 1998). We quantified the acoustic startle reflex at 5 dpf and observed that both mutant alleles have a significantly reduced startle reflex compared to their WT siblings in response to either a loud pure-tone stimulus (146 dB, 1000 Hz), or a multispectral tap stimulus (Figure 3.2A).

In accordance with having an auditory deficit, *ap1b1* mutants also swim in a circular pattern and fail to maintain an upright resting position, indicating that they also have balance defects. To quantify the deficit in vestibular function, we tested vestibular-induced eye movements in *tm246a* mutant and sibling larvae at 5 dpf (Mo et al., 2010). Upon head rotation, mutant larvae moved their eyes in response to visual cues (data not shown), suggesting that their vision is not disrupted. In contrast to WT siblings, when rotated in the dark, vestibular-induced eye movements were nearly undetectable in

tm246a mutant larvae (Figure 3.2B). A similar response was also observed in *t20325* mutants (data not shown). The average amplitude of the vestibular-induced eye movement in *tm246a* mutants was severely reduced compared to WT siblings (Figure 3.2C). Combined, these results demonstrate that *ap1b1* mutants have pronounced deficits in both auditory and vestibular function.

Mechanotransduction is disrupted in *ap1b1* mutant hair cells

Behavioral deficits in hearing and balance may be due to defects in either the peripheral or central components of the auditory/vestibular system. A previous study of *tm246a* larvae noted a reduction in FM 1-43 label of hair cells and reduced hair cell sensitivity to ototoxic drugs (Nicolson et al., 1998; Seiler & Nicolson, 1999). As both FM1-43 and ototoxic drugs are thought to permeate hair cells with functional transduction channels, FM 1-43 labeling and drug sensitivity are commonly used as indicators of hair cell function (Gale et al., 2001; Marcotti et al., 2005; Meyers et al., 2003; Steyger et al., 2003; Waguespack & Ricci, 2005). The behavioral deficits reported here and the previous studies with *tm246a* larvae suggest that mechanotransduction is only partially functional in *ap1b1* mutants. To determine the onset of hair-cell dysfunction, we measured the intensity of FM 1-43 label in mutant lateral-line hair cells at 3 dpf, when many hair cells are still maturing, and at 5 dpf, when the majority of the hair cells are mature. We observed a striking reduction in the amount of FM 1-43 label in *ap1b1* mutant hair cells compared to WT at 5 dpf (Figure 3.3A-C). The reduction was comparable in both mutant alleles and was seen as early as 3 dpf (Figure 3.3D). At 5 dpf, when the proportion of mature hair cells is greater than at 3 dpf, the difference between mutants and WT siblings in FM 1-43 label was more prominent. The pronounced reduction in FM 1-43

label over time suggests that both mutant alleles have the same effect on mechanotransduction.

As FM 1-43 labeling of lateral-line hair cells is robust only at later stages of development (Kindt et al., 2012), we sought to determine if mutations in *ap1b1* also had an effect on mechanotransduction at early stages, when hair cells first become mechanically sensitive. To assay early hair-cell activity, we used the transgenic line *Tg(myo6b:D3cpv)* that stably expresses D3-cameleon in hair cells and examined evoked calcium transients, which is a more sensitive method for measuring hair cell function (Kindt et al., 2012). D3-cameleon is a genetically encoded calcium indicator that uses fluorescence resonance energy transfer (FRET)-based technology to measure changes in intracellular calcium (Kindt et al., 2012; Palmer et al., 2006). For our experiments, we mechanically stimulated lateral-line hair cells with a fluid jet and recorded evoked calcium transients in individual hair cells at early stages of development (2 dpf), earlier than when FM 1-43 labeling is detectable (Kindt et al., 2012). We crossed the *Tg(myo6b:D3cpv)* line into the *t20325* background, and compared the responses in WT and mutant hair cells. In *t20325* fish, far fewer hair cells responded to the stimulus than in WT siblings at all stages examined (Figure 3.3E). Of the *t20325* mutant hair cells that responded to stimuli, the responses were comparable to those in WT at 2 dpf, but thereafter were significantly reduced compared to the WT siblings (Figure 3.3F and G). The decrease in hair cell activity that we observe in *ap1b1* mutants is consistent with the reduction of acoustically-evoked hindbrain calcium transients reported in an earlier study of this mutant (Nicolson et al., 1998). The calcium imaging experiments demonstrate that at all stages of development, the majority of hair cells fail to respond to mechanical stimuli. Over time, the calcium responses in mutant hair cells remain low

and fail to increase in size as seen in WT cells (Figure 3.3G). Together, the FM 1-43 and calcium imaging data indicate that mechanotransduction in *ap1b1* mutants is compromised at an early stage in hair cell development.

Aside from the lack of FM 1-43 labeling and reduced calcium transients, a common feature among zebrafish transduction mutants is splayed hair bundles (Nicolson et al., 1998). Splaying occurs when components of the transduction complex are missing or mutated, notably the tip link proteins Pcdh15 and Cdh23 (Seiler et al., 2005; Söllner et al., 2004). Because *ap1b1* mutants have reduced FM 1-43 labeling and calcium transients, we examined hair-bundle morphology by labeling actin filaments with fluorescently-tagged phalloidin. At 5dpf, the intensity of the label was reduced in *t20325* bundles (Figure 3.4A and C). Quantification of the amount of phalloidin labeling revealed a significant decrease in the amount of actin in mutant bundles when compared to WT bundles (WT: 61.5 A.U. $\pm$ 5.1; *t20325*: 48.0 A.U. $\pm$ 1.2, Mann-Whitney U-test: $p = 0.0006$). The *tm246a* mutant bundles, however, showed no significant change in the amount of actin (Figure 3.4B, 60.5 A.U. $\pm$ 5.1) compared to WT bundles. As suboptimal fixation can lead to mild splaying or morphological artifacts, we examined bundle morphology in live hair cells using a transgenic fish that expresses GFP tagged β -actin in hair cells, *Tg(m6b: β -actin-GFP)* (Kindt et al., 2012). In the *t20325* mutant background, we observed thinner hair bundles in the medial cristae of mutants (Figure 3.4D and E). The width of the hair bundle at its base was significantly decreased (WT: 1.4 μm $\pm$ 0.1; *t20325*: 1.2 μm $\pm$ 0.1; Mann-Whitney U-test: $p = 0.0002$). The height of mutant bundles appeared to be unaffected compared to WT bundles (WT: 4.7 μm $\pm$ 0.2, $n = 80$ bundles from 3 larvae; *t20325* mutant 4.6 μm $\pm$ 0.2, $n = 49$ bundles from 3 larvae; Mann-Whitney U-test: $p = 0.5885$). Overall these data suggest that maintenance of the bundle structure is affected

by the *t20325* mutation. The bundle defects are subtle, however, and are not likely to fully account for the strong reduction of FM1-43 labeling and calcium transients in *t20325* hair cells.

***ap1b1* mutants show degeneration of inner ear and lateral-line neuroepithelia**

In previous work, larvae carrying the *tm246a* allele were classified as a hair cell degeneration mutant due to the cellular defects in the inner ear neuroepithelia (Nicolson et al., 1998). To determine whether the degenerative phenotype was also present in the *t20325* mutant, we examined inner-ear and lateral-line neuroepithelia in *t20325* mutants at 5 dpf. At this stage we also observed comparable abnormalities in the *t20325* allele. As previously reported, we noticed floating cellular debris and apical blebs in the endolymphatic space, a fluid-filled cavity that envelops the apical surface of hair cell patches in the inner ear (Figure 3.5A-C). While we observed debris and apical blebs in every mutant ear in both alleles of *ap1b1* by 5 dpf (*tm246a*: n = 4, *t20325*: n = 6), we never observed this phenotype in WT siblings (WT *tm246a*: n = 5; WT *t20325*: n = 3). Further signs of cellular degeneration in *ap1b1* mutant hair cells included pyknotic nuclei that were also occasionally observed in the neuroepithelia of ear and neuromasts, but were rare in WT larvae (data not shown). This could be a direct result of the *ap1b1* mutations, but it is more likely that the various defects within the neuroepithelium of *ap1b1* mutants lead to the death of the hair cell.

In addition to blebs in the inner ear of *ap1b1* mutants, we also observed fluid-filled spaces between cells (interstitial edema), which were particularly apparent in neuromasts (Figure 3.5D-G). The sensory epithelium of neuromasts is made up of three main cell types, an upper layer of hair cells, a lower layer of supporting cells, and peripherally located mantle cells. All three of these cell types are important for

neuromast development and function (Ghysen & Dambly-Chaudière, 2007; Jones & Corwin, 1993; Tritsch et al., 2007). To determine the location of intercellular spaces between the three cell types, we utilized the *Tg(scm1:GFP)* transgenic line that labels only supporting cells due to the insertion of a GFP expression construct in the *tankyrase 1 binding protein 1* gene (Behra et al., 2009; Behra et al., 2012). In WT neuromasts at 5 dpf, we observed minimal intercellular spaces between GFP-positive supporting cells and non-GFP hair cells or mantle cells (Figure 3.5D). Additionally, we observed minimal intercellular spaces between adjacent supporting cells in WT (Figure 3.5E). Using the *Tg(scm1:GFP)* transgenic line in a *t20325* mutant background we observed, similar to WT neuromasts, minimal fluid-filled spaces between hair cells (Figure 3.5F). However, below the plane of hair cells, we observed abundant spaces between the GFP-positive supporting cells in *t20325* mutant neuromasts (Figure 3.5G). It should be noted that the interstitial edema had no effect on the general arrangement of cells within the neuromast.

To determine whether the phenotype of interstitial edema was progressive in nature, we examined neuromasts at earlier stages. In immature neuromasts at 2 dpf, fluid-filled spaces in mutant or WT neuromasts were not apparent (*tm246a*: WT = 3/18 neuromasts, mutant = 0/6 neuromasts; *t20325*: WT = 1/34 neuromasts, mutant = 0/14 neuromasts). Extracellular spaces were first detected in mutant neuromasts at 3 dpf (*tm246a*: WT = 0/8 neuromasts, mutant = 12/12 neuromasts; *t20325*: WT = 1/24 neuromasts, mutant = 7/9 neuromasts). These spaces remained more prevalent in mutant neuromasts and also appeared enlarged and more extensive at 5 dpf.

Given the signs of degeneration and the role of the AP1 complex in protein sorting, we examined the cellular morphology and membrane compartments within hair cells in

more detail using TEM (Figure 3.6). In sections of the anterior macula of the ear (5 dpf), interstitial edema was evident in mutant sensory epithelia, with many extracellular spaces present between cells (Figure 3.6A and B). This data agrees with what we observe by DIC (Figure 3.5) We also observed that the overall health of hair cells was compromised in *tm246a* mutants (Figure 3.6B). Occasionally, we observed hair cells largely devoid of cytoplasm in *tm246a* mutants, indicative of an abnormal physiological state (Figure 3.6B, asterisk). Another indication of an abnormal physiological state was the increase in multivesicular bodies in mutant hair cells compared to WT (Figure 3.6D and E, arrowheads, quantified in Figure 3.6F). In addition to an increased number of multivesicular bodies, the number and size of membranous compartments localized below the cuticular plate were greater in mutants compared to WT hair cells (Figure 3.6G and H, arrows, quantified in Figure 3.6I). Both enlarged vesicles and multivesicular bodies were not restricted to the cell body but could also be seen populating the blebs being extruded apically, adjacent to the bundle of stereocilia (Figure 3.6C). The interstitial edema and blebbing could arise from dysfunctional ionic homeostasis in hair cells caused by *ap1b1* mutations. These phenotypes may also be due to an inability to maintain membrane compartments of hair cells.

Hair cells found “out of place” in *ap1b1* mutants

The AP-1 complex in *Drosophila* has been shown to regulate the recycling of the Notch ligand, *Sanpodo*, out of the recycling endosome back to the surface of cells in the sensory organ precursor (Benhra et al., 2011). This loss of regulation of the cell-surface expression of *Sanpodo* in the developing sensory organ precursor resulted in stochastic effects on cell-fate decisions. This group observed that sensory organ precursors lacking functional AP-1 developed with some cell-types missing from the fully developed

sensory organ, while there were duplicates of other cell-types. These results indicated that AP-1 played a role in the development of the cell types that comprise the sensory organ by regulating the amount of Sanpodo expressed at the cell surface. We therefore hypothesized that the development of hair cells in the neuroepithelium of *ap1b1* mutants was disrupted.

To investigate whether development or specification of hair cells was disrupted in *ap1b1* mutants, we used the antibody against zebrafish Otoferlin to label cells that had adopted a hair cell fate and analyzed the location of these cells. We observed Otoferlin positive cells lacking the typical morphology of hair cells and found well below the plane of hair cells in *ap1b1* mutant neuromasts while we never observed either of these phenomena in wild-type neuromasts (Figure 3.7A and B). Quantification revealed that 7.8% of Otoferlin positive cells were “out of place” in *ap1b1* mutants (Figure 3.7C). We also observed “out of place” hair cells when we labeled with other hair cell specific antibodies Vglut3 and Rib b (not shown). These data support the hypothesis that AP-1 regulates cell surface expression of molecules implicated in development, such as Notch ligands, leading to the developmental defects in the neuroepithelium.

DISCUSSION

It was previously thought that disrupting the entire AP-1 complex was lethal and hence, many of the seminal studies on this complex have been done in cell culture. Here we present to date the first reported mutations to the β -subunit of the AP-1 complex, which should disrupt all AP-1 dependent functions, and analyze the genetic and cellular consequences of this mutation in zebrafish. Our findings indicate that lesions in *ap1b1* disrupt the function and integrity of hair cells in inner ear and lateral-line organs. Mutant hair cells show progressive signs of degeneration, including blebbing and interstitial edema, and an accumulation of vesicles and multivesicular bodies. In addition, mechanotransduction is compromised, evidenced by the significant reduction in both FM 1-43 label and mechanically-evoked calcium transients.

Auditory and vestibular functions are especially susceptible to *ap1b1* mutations

Despite the ubiquitous expression of *ap1b1*, *ap1b1* mutants display no other obvious behavioral phenotypes aside from auditory and vestibular deficits. The development and function of cell types other than hair cells appears to be unaffected in *ap1b1* mutants. This specific phenotype is unexpected considering the expression of *ap1b1* during development and the deleterious effects of lesions in the AP-1 complex in other species. For example, deletion of either the AP-1 γ or μ 1A subunit in mice is embryonic lethal, as is removal of both AP-1 μ subunits in *C. elegans* (Meyer et al., 2000; Shim et al., 2000). It is possible that the normal development of *ap1b1* mutants could be due to genetic redundancy, as paralogs are common in zebrafish. To date, however, we are unable to find a second copy of *ap1b1* in the latest assembly of the zebrafish genome (Zv9, July

2010 release). Alternatively, if *Ap1b1* is required in other cell types, maternal mRNA may sustain embryos through earlier stages of development. Supporting the idea that the maternal contribution of mRNA ameliorates the loss of the AP-1 complex during early development, transcripts for both AP-1 μ subunits have been detected at the 2-cell stage (Zizioli et al., 2010).

Further corroboration that AP-1 complexes are critical for the function of the auditory and vestibular system comes from studies of AP-1 mutations in humans. Mutations in *AP1S1*, one of the three $\sigma 1$ subunit genes in humans, results in MEDNIK (mental retardation, enteropathy, deafness, neuropathy, ichthyosis and keratoderma) syndrome (Montpetit et al., 2008). This rare, recessive disorder causes congenital hearing loss, although the pathological consequences of loss of *AP1S1* function in the inner ear are not known. Knockdown of *ap1s1* in zebrafish results in the disruption of the integrity of embryonic keratinocytes and spinal cord development, however, knockdown was lethal at larval stages, precluding the assessment of auditory/vestibular function (Montpetit et al., 2008). Nevertheless, the MEDNIK syndrome highlights the importance of AP-1 function in several epithelial and neuronal cell types.

***ap1b1* mutations disrupt normal development in the neuroepithelium**

AP-1 is known to be involved in sorting of integral membrane proteins to the plasma membrane. In *Drosophila*, AP-1 was shown to regulate the cell surface expression of the Notch ligand, *Sanpodo* (Benhra et al., 2011). This group demonstrated that when AP-1 was unable to regulate the amount of *Sanpodo* at the plasma membrane of the developing sensory organ, cell specification went awry resulting in too many of some cell types while too few of others. In much the same way, Notch signaling is also important for the development of prosensory patches in the auditory and vestibular

neuroepithelium and specification of hair cells in zebrafish and mammals (Haddon et al., 1998; Kiernan, 2013; Lanford et al., 1999; Rothschild et al., 2013; Slowik & Bermingham-McDonogh, 2013). The “out of place” hair cells we observe in *ap1b1* mutants echoes what we see in *Drosophila* sensory organ precursors lacking AP-1 function and suggests that there is a slight dysregulation of hair cell specification in *ap1b1* mutants. This dysregulation leads to the appearance of hair cells in neuroepithelium that do not have the typical morphology and are found below the plane of hair cells. Given what was seen in the Benhra et al. study, it would be interesting to monitor the development of supporting cells or mantle cells in *ap1b1* mutants, looking for any differences in the number and placement of these cells. This kind of analysis could also inform how these “out of place” hair cells develop. Regardless, it seems likely that AP-1 could play a role in regulating hair cell specification in neuroepithelium. As AP-1 regulates the Notch pathway in *Drosophila* sensory organ precursors, future experiments could explore the cell surface expression of Notch or any of its ligands, along with any other developmental players of cell specification within the neuroepithelium.

The neuroepithelium degenerates in *ap1b1* mutants

In addition to sorting proteins to the plasma membrane, the AP-1 complex is also important for maintaining the size and number of intracellular membrane compartments (Nonet et al., 1999; Zhang et al., 1998). Consistent with this role for AP-1, we noted changes within hair cells, including the accumulation of multivesicular bodies and enlarged vesicles. It is not clear, however, if the differences in membrane compartments in *ap1b1* mutants are due to a block in AP-1-mediated protein trafficking, or to secondary, degenerative changes within the hair cells. Indeed, dystrophic

conditions in axons can lead to an increase in the number of multivesicular bodies, suggesting that formation of new multivesicular bodies is driven by pathological conditions (Altick et al., 2009). Future work may address this distinction between direct and indirect consequences of AP-1 dysfunction.

As the lesions described here are the first reported mutations to the AP-1 β -subunit in a vertebrate animal model, the *ap1b1* mutant presents an opportunity for future studies to investigate how the β -subunit of AP-1 is involved in setting up and maintaining cell surface expression of proteins in the neuroepithelium. Importantly for hair cells, the AP53 1 complex plays an important role in basolateral targeting of membrane proteins (Fölsch et al., 2009). And polarized distribution of proteins is an important pathway for many processes including development, cellular function and homeostasis. With this in mind, the *ap1b1* mutant presents an opportunity to understand how basolaterally targeted proteins are affected and potentially how missorting of proteins results in the phenotypes observed.

FIGURES

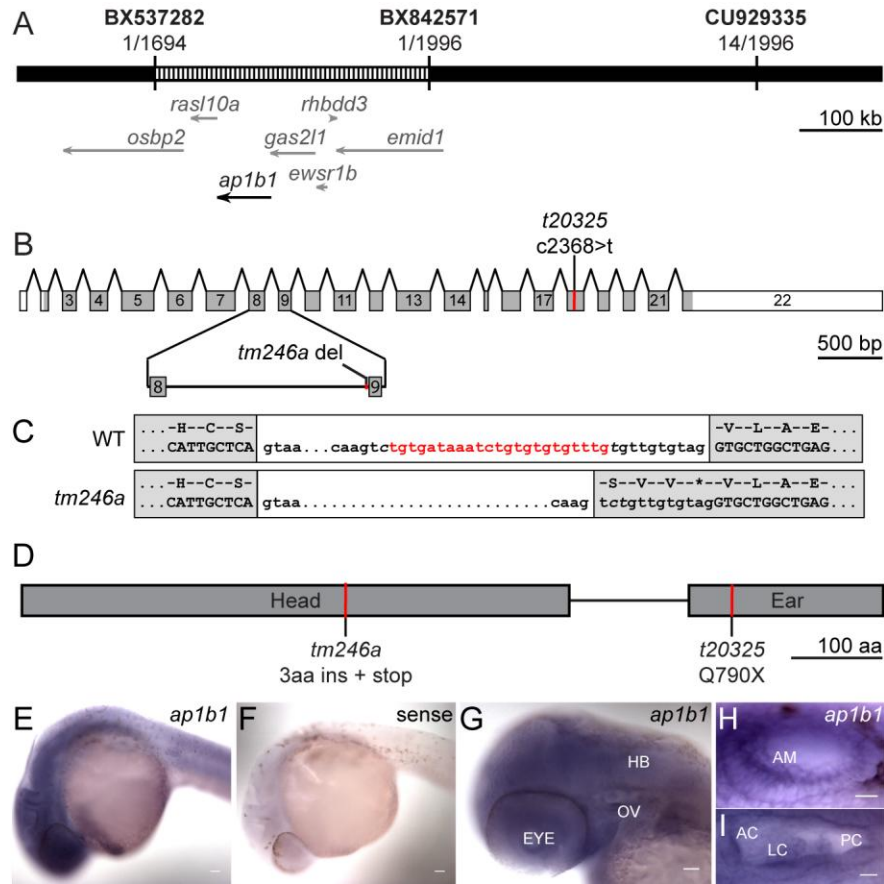


Figure 3.1: Positional cloning of *skylab* mutations and expression of *ap1b1*

A, A diagram of the 330 kb *skylab* critical interval (striped region) obtained through mapping of the *tm246a* allele. The critical interval encompasses the coding regions of five annotated genes as well as part of two other genes. **B**, An exon diagram of the *ap1b1* gene. The coding region is depicted in grey and the 5' and 3' UTRs are depicted in white. The locations of the *t20325* C-T transition and the *tm246a* 23 bp deletion between exons 8 and 9 are indicated. **C**, The nucleotides deleted from the splice acceptor site between exons 8 and 9 in the *tm246a* mutant are highlighted in red in the WT transcript. The resulting translations are shown above the WT and *tm246a*

transcripts. **D**, Diagram showing the location of *tm246a* and *t20325* mutations in the Ap1b1 protein. **E**, *ap1b1* is expressed ubiquitously at 24 hpf. **F**, Sense control for *ap1b1* *in situ* experiments. **G**, Expression of *ap1b1* persists in the head at 48 hpf. Scale bars in E-G, 5 μ m. **H, I**, Magnified images of the developing ear at 24 and 48 hpf, respectively. Scale bars in H and I, 10 μ m. HB, hindbrain; OV, otic vesicle; UM, utricular macula; AC, anterior crista; MC medial crista; PC, posterior crista.

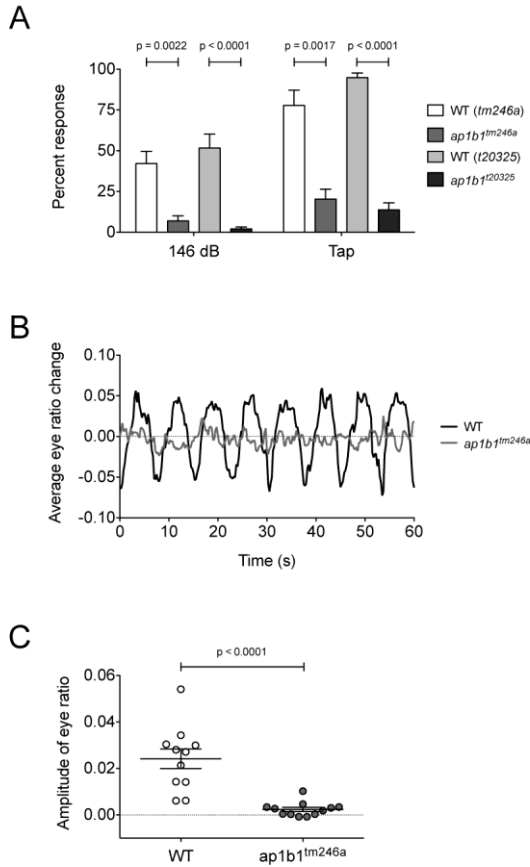


Figure 3.2: *ap1b1* mutants have deficits in auditory and vestibular behavioral responses

A, Graph showing the average startle response to either a 1000Hz stimulus at 146 dB or a tap stimulus of both mutants (*tm246a*: n = 9, *t20325*: n = 23) and their WT siblings (*tm246a*: n = 15, *t20325*: n = 19). **B**, Averaged traces of vestibular-induced eye movements from WT siblings (n = 11) and *tm246a* mutants (n = 12) over 60 sec. **C**, Average of peak amplitude of vestibular-induced eye movements at 0.25 Hz. Each dot represents one eye from an individual larva at 5 dpf (WT: n = 11, *tm246a*: n = 12). A Mann-Whitney U-test was used to compare differences between mutants and WT siblings.

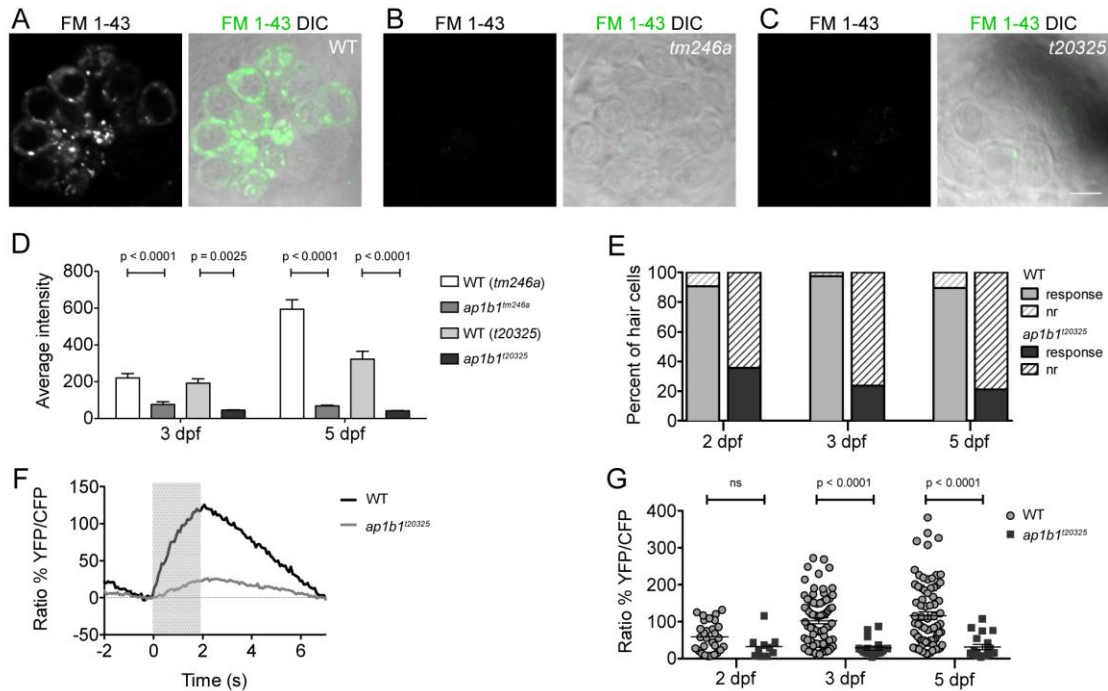


Figure 3.3: *ap1b1* mutants have deficits in hair cell mechanotransduction

A-C, FM 1-43 label of neuromast hair cells in WT, *tm246a* and *t20325* mutants at 5 dpf.

Scale bars, 5 μ m. **D**, Average intensity (A.U.) of FM 1-43 label in *tm246a* and *t20325*

mutants quantified at 3 dpf (*tm246a*: WT n = 18, mutant n = 5; *t20325*: WT n = 16,

mutant n = 8 neuromasts) and 5 dpf (*tm246a*: WT n = 9, mutant n = 10; *t20325*: WT n =

12, mutant n = 11 neuromasts) from at least 3 larvae along with WT, age-matched

siblings. **E**, The proportion of hair cells displaying calcium transients in response to a

water-jet stimulus (solid) compared to those that do not respond (nr = non-responders,

hatched lines). The percent of non-responding hair cells in the *t20325* mutants is greater

than the percent non-responders in WT at all stages of development assayed; Chi

Squared test, $p < 0.0001$. **F**, Trace representing the average calcium responses to a 2

sec water-jet stimulus from 5 dpf WT and *t20325* mutant larvae (n = 20 hair cells). The

grey box indicates the timing of the water-jet stimulus. **G**, Dot plot showing calcium

transients in WT and *t20325* larvae at 2, 3 and 5 dpf (non-responders were excluded).

Each point represents an individual hair cell. Error bars represent SEM. Statistical

analysis performed using a Mann-Whitney U-test.

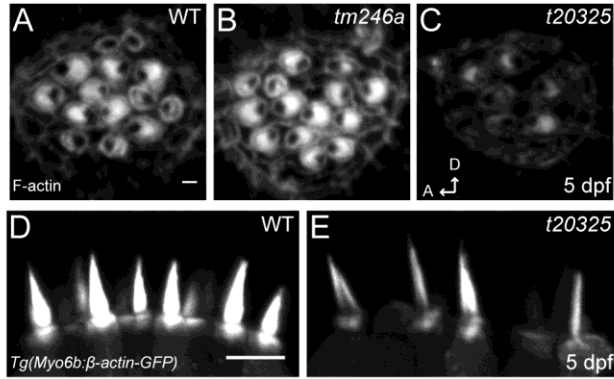


Figure 3.4: Stereociliary bundles of *ap1b1* mutant

A-C, Representative confocal images of neuromast hair bundles in WT, *tm246a* and *t20325* mutants at 5 dpf. Bundles were viewed from a top-down angle and actin was labeled with phalloidin-Alexa 488. This view shows the planar cell polarity of hair-bundles. Scale bar, 1 μ m. **D, E**, Side view of stereocilia from the medial cristae of 5 dpf WT and *t20325* mutants in the *Tg(myo6b:βactin-GFP)* background (z-projections, 2 μ m thick). Scale bar, 5 μ m.

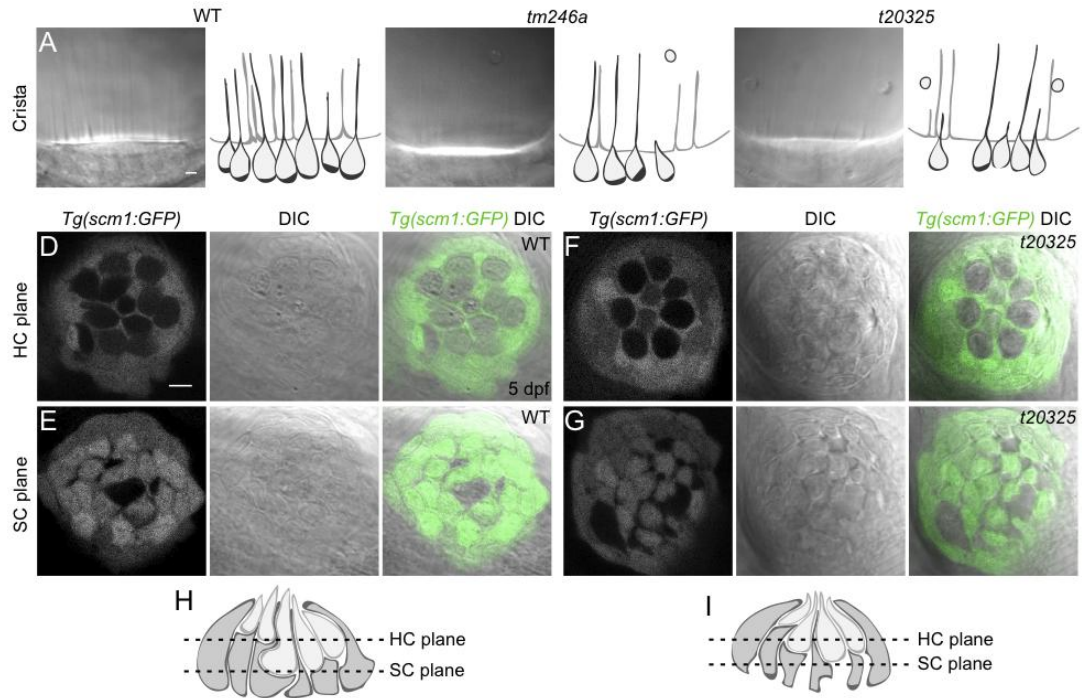


Figure 3.5: Loss of integrity of inner ear and lateral-line neuroepithelia in *ap1b1* mutants

A-C, DIC images with corresponding drawings of the neuroepithelium showing dorsal views of the posterior cristae of WT, *tm246a* and *t20325* mutants at 5 dpf. Arrows indicate blebs and an arrowhead indicates a space in the *t20325* mutant neuroepithelium. **D-G**, GFP expression in supporting cells of 5dpf WT and *t20325* mutant neuromasts in the *Tg(scm1:GFP)* background. **D**, GFP expression was excluded from hair cells. **E**, GFP expression in supporting cells below the plane of hair cells. **F**, *Tg(scm1:GFP)* expression in a *t20325* neuromast; hair cells looked similar to WT. **G**, In the supporting cell plane, an increase in the number of spaces between supporting cells in a *t20325* mutant neuromast was evident compared to WT supporting cells. Scale bar in all panels, 5 μ m.

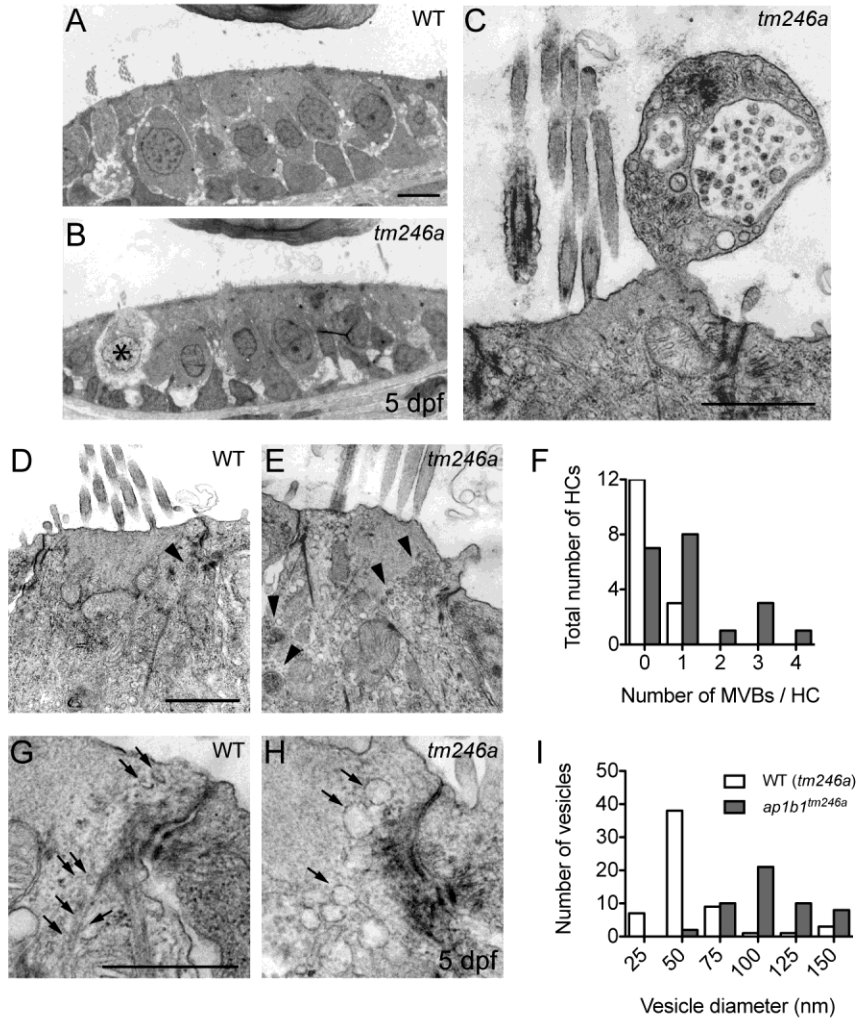


Figure 3.6: Decreased cell integrity and an increased number of intracellular membrane compartments in *ap1b1* mutant hair cells

A, B, Comparable sections of the utricular macula of WT and *tm246a* mutant at 5 dpf.

An asterisk indicates a *tm246a* mutant hair cell where the cytoplasm is breaking down, indicative of a dying cell. Scale bars, 3 μ m. **C,** Example of an apical bleb extruding from a mutant hair cell containing several vesicular compartments and a multivesicular body. Scale bar, 1 μ m. **D-E,** Comparable close-ups of WT and *tm246a* mutant hair cells just below the cuticular plate. Scale bar, 1 μ m. In *tm246a* mutant hair cells, more multivesicular bodies (arrowheads) were present compared to WT hair cells. **F,**

Quantification of the observed number of multivesicular bodies in WT sibling and *tm246a*

mutant hair cells. **G-H**, Sections of hair cells near the tight junctions showing an increased number of large vesicles in the mutants compared to WT. Vesicles are indicated with arrows. **I**, Observed sizes of vesicles in WT sibling and *tm246a* mutant hair cells. For quantification in **F** and **I**, WT: n = 15, *tm246a*: n = 20 hair cells.

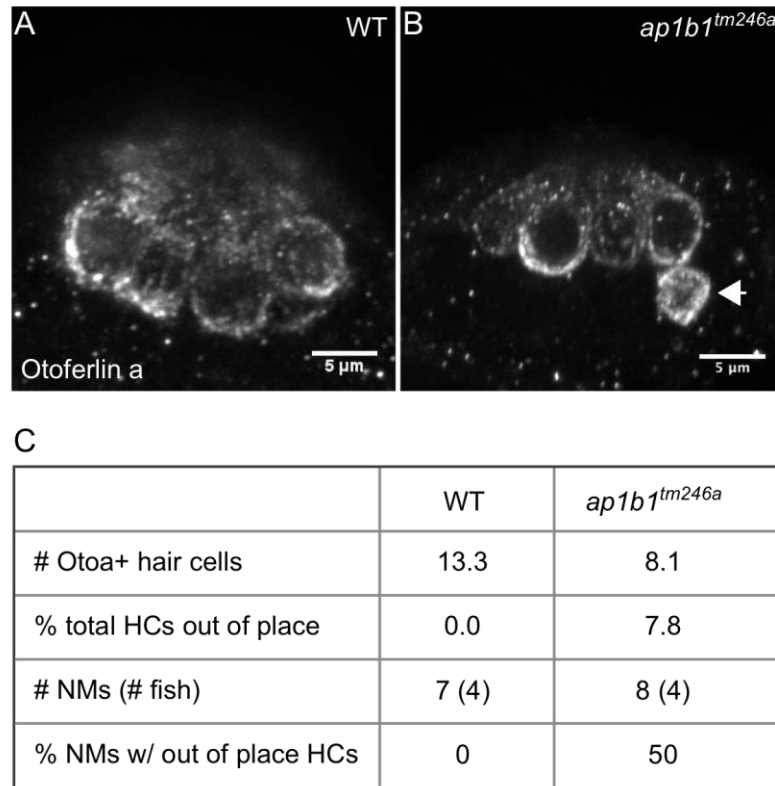


Figure 3.7 Cells expressing a hair cell specific protein found outside the expected location for hair cells.

A, B, Neuromast hair cells labeled with the Otoferlin a antibody in 5 dpf wild-type and *tm246a* mutant larvae. Arrow indicates “out of place” hair cell. Scale bars = 5 μ m. **C,** Table showing average number of hair cells and percent “out of place” hair cells observed in 5 dpf wild-type and *tm246a* mutant larvae. Table also indicates percent of neuromasts containing “out of place” hair cells.

Chapter 4 – Mutations in *ap1b1* cause mistargeting of the Na⁺/K⁺-ATPase pump in sensory hair cells

AUTHORS

Rachel Clemens Grisham, Teresa Nicolson

ABSTRACT

The hair cells of the inner ear are polarized epithelial cells with a specialized structure at the apical surface, the mechanosensitive hair bundle. Mechanotransduction occurs within the hair bundle, whereas synaptic transmission takes place at the basolateral membrane. The molecular basis of the development and maintenance of the apical and basal compartments in sensory hair cells is poorly understood. The Ap1b1 protein is a subunit of the adaptor complex AP-1, which has been shown to be involved in the post-Golgi sorting of membrane proteins, particularly to the basolateral compartment of cells. To gain insight into the cellular and molecular defects in *ap1b1* mutants, we examined the localization of several membrane proteins specifically targeted to the apical or basolateral compartment in hair cells. We report several sorting defects in *ap1b1* mutants. Importantly, we observed that the Na⁺/K⁺-ATPase pump (NKA) was less abundant in the basolateral membrane and was mislocalized to apical bundles in *ap1b1* mutant hair cells. Accordingly, intracellular Na⁺ levels were increased in *ap1b1* mutant

hair cells. Our results suggest that Ap1b1 is essential for maintaining integrity and ion homeostasis in hair cells.

INTRODUCTION

Clathrin-mediated transport requires Adaptor Proteins (APs) that interact with sorting motifs on membrane proteins, providing selectivity in the initial step of transport. Several distinct classes of AP complexes (AP-1, AP-2, AP-3, and AP-4) facilitate sorting along various trafficking routes (Fölsch et al., 2009). The Adaptor Protein 1 (AP-1) complex has been shown to mediate trafficking of membrane proteins to the plasma membrane from either the trans-Golgi network (TGN) or recycling endosomes. In polarized epithelial-cells, AP-1 is important for basolateral sorting of cargo proteins (Fölsch et al., 1999). Here we explore the consequence of the *ap1b1* mutation on the targeting of membrane proteins restricted to either the basolateral or apical compartments of hair cells.

Auditory and vestibular hair cells are polarized epithelial cells with a unique morphology essential for mechanosensation (Schwander et al., 2010). The hair bundle at the apical end of a hair cell is comprised of several rows of actin-filled stereocilia and a single primary cilium called the kinocilium. Upon deflection induced by sound or head movements, hair bundles transduce mechanical stimuli into graded receptor potentials. Within the basolateral compartment, hair cells transmit signals to afferent neurons, and in some cases receive signals from efferent neurons (Dambly-Chaudière et al., 2003; Metcalfe et al., 1985).

The correct regulation of membrane proteins at the cell surface is important for maintenance of the cell as well as development of organs in certain contexts (Bae et al., 2006; Benhra et al., 2011; Dwyer et al., 2001; Kaplan et al., 2010; Margeta et al., 2009). These proteins are critical for maintaining the electro-chemical gradients necessary for hair cell function (Anniko & Wróblewski, 1986; Boyer et al., 2001; Couloigner et al., 2006; Hibino & Kurachi, 2006; Kim & Marcus, 2011; Lang et al., 2007). How the hair cell orchestrates apical and basolateral trafficking of membrane proteins for its unique requirements has not been explored.

Evidence that the AP-1 complex may also be involved in apical targeting of membrane proteins has been presented in other systems (Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010). We show that the apically targeted Pcdh15 and Tmhs are missorted in hair cells, providing supporting evidence to the hypothesis that AP-1 is involved in apical targeting of membrane proteins.

In addition to the transduction and synaptic machinery, a number of channels and transporters are spatially restricted to the apical or basolateral ends of hair cells. Though AP-1 has been implicated in the sorting of basolateral membrane proteins, hair cell synapses appear to be largely intact in *ap1b1* mutants. In contrast, *zns-5* and the Na⁺/K⁺-ATPase pump, both proteins restricted to basolateral surfaces, are missorted to the apical surface in hair cells. Our results suggest that a lack of sorting by AP-1 leads to mislocalization of the NKA pump and is likely to account, in part, for the defects associated with *ap1b1* mutant hair cells.

MATERIALS AND METHODS

Ethics statement: This study was performed with the approval of the Oregon Health & Science University Institutional Animal Care and Use Committee and in accordance with NIH guidelines.

Animal lines: Zebrafish were kept on a 12 hr light-dark cycle at 28°C. The mutant alleles of *skylab*, *tm246a* and *t20325* were isolated from two independent ENU mutagenesis screens (Granato et al., 1996) (Tubingen 2000 Screen Consortium) and maintained in either Tubingen or Top long fin wild-type (WT) backgrounds.

Vital Dyes: For the FM 1-43 experiments, zebrafish larvae were incubated for 20 sec in E3 containing 3 uM N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino)styryl)Pyridinium Dibromide (FM 1-43, Life Technologies). For the Sodium Green experiments, zebrafish larvae were incubated for 20 min in E3 containing 10 uM tetra(tetramethylammonium) salt (Sodium Green, Molecular Probes) and 1% DMSO at room temperature in the dark. After either treatment, larvae were washed with E3 plus 0.02% 3-amino benzoic acid ethylester (MESAB, Western Chemical Inc.) for 1-2 min at room temperature.

Vector construction and expression: See Chapter 2 for description of *myo6b:GFP-tmhs* expression construct. Plasmid containing the expression construct was injected into eggs from *ap1b1* mutant incrosses at the one-cell stage with 20 pg of RNA encoding the Tol2 enzyme to facilitate genomic integration.

Immunostaining: The mouse IgG1 NKA, zns-5 and MAGUK (Membrane-associated guanylate kinase) antibodies were obtained from Developmental Studies Hybridoma Bank. Mouse anti-Pcdh15, rabbit anti-Cav1.3 and mouse IgG2a anti-Rib b antibodies

were raised against zebrafish proteins. Cav1.3 and Rib b antibodies are described in detail elsewhere (Obholzer et al., 2008; Sheets et al., 2011). For Pcdh15 antibody label, larvae were fixed in 4% paraformaldehyde solution containing 4% sucrose (Sigma) in PBS for 5 hrs or overnight at 4°C. Larvae were then washed for 5 min. three times in PBS containing 1% DMSO, 1% TritonX and 0.25% Tween-20 at room temperature followed by incubation in Pcdh15 antibody block (1x PBS, 1% BSA, .5% Fish skin gelatin (Sigma), .02% Sodium Azide, 1% DMSO, 1% TritonX and 0.25% Tween-20) for several hours. Primary Pcdh15 antibody serum was diluted 1:20 in antibody block solution containing 2% goat serum and 5 mM BAPTA and incubated overnight at 4°C.

For NKA labeling, zebrafish larvae were fixed in 4% paraformaldehyde in PBS for 4.5 hrs or overnight, washed several times in PBST before an overnight incubation in NKA primary at 4°C. NKA antibody was diluted (1:500) in 1% BSA, 0.5% fish skin gelatin, and 0.02% sodium azide in 1x PBS plus 2% goat serum. MAGUK, Cav1.3 and Otoferlin primary antibodies were diluted in 1% BSA, 0.5% Fish Skin Gelatin, and 0.02% Sodium Azide in 1x PBS plus 2% Goat Serum and used at the following concentrations: MAGUK (1:500), Cav1.3 and Otoferlin (1:1000) and Rib b (1:10,000).

After primary antibody incubation, larvae were washed in PBS with 0.01% Tween 6 times over 3 hrs and incubated in anti-mouse-Alexa 488, anti-mouse-Alexa 546, antimouse-Alexa 647 (Life Technologies) or anti-rabbit-DyLight 549 (Jackson ImmunoResearch) (1:1000) overnight. To detect MAGUK and Rib b in the same prep, we used anti-mouseIgG1-Alexa 568, anti-mouseIgG2A-Alexa 647 secondary antibodies (Life Technologies). When used, phalloidin conjugated to Alexa 488 (Life Technologies) was added along with secondary antibodies at 1:500.

Ouabain treatment: Ouabain (Sigma) from a 5 mM stock solution in water was diluted in E3 embryo medium to a 500 μ M working solution with FM 1-43 (Life Technologies) added at 3 μ M. Individual larvae were incubated in the working solution for 2 min at room temperature. Larvae were anesthetized with Mesab (Western Chemical Inc.) during the last minute, after which they were mounted in 1% low-melt agarose (Life Technologies) for image capture.

Microscopy: Images were captured on a Zeiss LSM 700 upright confocal microscope using the Zen acquisition software (2009 release, Zeiss). To view live larvae or the medial cristae of fixed larvae, animals were mounted in 1% low melt agarose (Life Technologies) dissolved in E3 embryo medium and imaged with a 63x/0,95 water immersion lens. To view superficial neuromasts after immuno-labeling, larvae were mounted in Elvanol (0.1 M Tris pH 9.0, 10% polyvinyl alcohol, 88-89% hydrolyzed, 30% glycerol and 1% DABCO) and imaged with a 63x/1,4 oil lens.

Image analysis: Fluorescence intensity measurements of maximal z-projections from FM-143 and Pcdh15 assays were processed, analyzed or quantified using ImageJ software.

Images were processed using ImageJ software. Maximal z-projections of confocal images were generated to quantify the amount of NKA in the medial crista. Integrated intensity of the z-projections was measured in MetaMorph software (Molecular Devices). To quantify colocalization of NKA and phalloidin labeled actin in the stereocilia, sections every 3 μ m were analyzed from confocal stacks of medial cristae. Regions encompassing stereocilia were defined using the phalloidin stain as a guide and included the base of the bundle just above the cuticular plate. Colocalization was

performed using MetaMorph; percent of overlap is defined as the percent of phalloidin positive pixels that overlap with NKA positive pixels.

Profiles of NKA fluorescence were plotted using ImageJ. At least three hair cells from medial cristae from two experiments were used for analysis. The regions to measure profiles were drawn across cells from single sections of a z series. The regions were 1.0 μm thick and wide enough to cross both membranes of the cell. The regions were placed equidistant from the cuticular plate and the nucleus. Membrane NKA was calculated as the average of grey values 1.0 μm wide about the peak of NKA labeling at the edge of the cell. Cytoplasmic NKA was quantified using a 1.0 μm wide centrally located area between these regions.

To quantify length and width of stereociliary bundles, maximal z-projections of individual bundles from confocal images were made and measurements were made in ImageJ using the line tool. To quantify Sodium Green fluorescence, a circular region 6 μm in diameter was drawn around the base of the cell with the circle tool at a plane in the center of the nucleus of each cell. The DIC channel was used to determine the center of the nucleus for each cell analyzed.

Statistical analysis for all assays was performed using Prism 5 (GraphPad).

RESULTS

Apically targeted proteins missorted in *ap1b1* mutants

Some evidence from work in *C. elegans* supports the idea that the AP-1 complex is involved in sorting of apical proteins (Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010; Margeta et al., 2009). Many proteins expressed in hair cells are specifically targeted

to the apical stereocilia, but of major importance are the proteins that are a part of the MET complex. The tip link protein Pcdh15 (Ahmed et al., 2006; Kazmierczak et al., 2007; Seiler et al., 2005) was recently shown in cell culture to be an AP-1 dependent cargo (Zallocchi et al., 2012). Another study also in mice showed that Tmhs was required for trafficking Pcdh15 to the tips of stereocilia and vice versa (Xiong et al., 2012). The same study also showed that in cell culture Tmhs was necessary for localization of Pcdh15 to the plasma membrane, raising the possibility that Tmhs is also an AP-1 dependent cargo. We therefore hypothesized that Tmhs and Pcdh15 proteins would be missorted in *ap1b1* mutants.

To analyze Pcdh15 sorting, we utilized an antibody against an N-terminal epitope of zebrafish Pcdh15. In wild-type hair-cells, the Pcdh15 antibody labels an area just below the stereocilia (Figure 4.1A). This sub-apically localized structure may represent a pool of Pcdh15 that is ready to be trafficked to the tips of stereocilia. In *ap1b1* mutants, we observed that this pool of Pcdh15 just below the stereocilia was missing or reduced (Figure 4.1B). Quantification revealed there was significantly less Pcdh15 antibody label in *ap1b1* mutant hair cells compared to wild type (Figure 4.1C).

Unfortunately, the conditions for this antibody have not been optimized for reliable labeling of tip links, so quantification of Pcdh15 in the tips of stereocilia from either mutants or wild types was not possible. We did, however, observe occasional labeling with the Pcdh15 antibody in the tips of stereocilia (not shown), suggesting that some amount of Pcdh15 is able to be incorporated into the tip links.

To analyze sorting of Tmh_{sa}, we transiently expressed the *myo6b:GFP-tmhsa* construct (described in Chapter 2), a potential component of the MET complex (Xiong:2012). When transiently expressed in wild-type hair-cells, GFP-Tmh_{sa} localizes to the tips of

stereocilia (not shown) and also in unidentified intracellular compartments of hair cells (Figure 4.2C). The localization of this construct to intracellular compartments is also seen when it is stably expressed in hair cells (Figure 2.3). At 3 dpf in *ap1b1* mutants, the amount of GFP-Tmhsa localized to the intracellular compartments did not appear much different than wild type (Figure 4.2A). Imaging a cell two days later at 5 dpf in the same crista of the same fish from Figure 4.2A, we observed the compartments where GFP-Tmhsa localized appeared much larger than similar compartments in wild type (Figure 4.2B). This suggests that GFP-Tmhsa may not be efficiently sorted out of these unidentified compartments in hair cells

Localization of ribbon-synapse membrane-proteins in *ap1b1* mutants

Since the AP-1 complex has been implicated in transport to the basolateral membrane, we hypothesized that the deficit in mechanotransduction and the cellular phenotypes in *ap1b1* mutants could be due to a gross defect in localization of proteins within the hair cell plasma membrane. To determine whether *ap1b1* is essential for trafficking of basolateral synaptic components, we examined a membrane protein targeted to the hair cell ribbon synapse. At the ribbon synapse, the L-type Ca^{++} channel Cav1.3 coordinates vesicle release by mediating Ca^{++} influx near the ribbon body (Brandt et al., 2003; Platzner et al., 2000; Sidi et al., 2004). In both mutant alleles, we observed that Cav1.3a label was indistinguishable from the WT pattern and colocalized tightly with Ribeye b, a major component of the ribbon synapse (Figure 4.3A-D). To investigate the morphology of pre- and postsynaptic components of the hair-cell synapses, we immunolabeled Ribeye b and Membrane-associated guanine kinase (MAGUK), a marker of the postsynaptic density. In both WT sibling and mutant synapses, MAGUK was tightly juxtaposed to the ribbon synapse (Figure 4.3E-H).

Interestingly, there appeared to be some expansion of MAGUK immunolabel in *ap1b1* mutants (Figure 4.3D). As reduced neurotransmission is a common defect in other transduction mutants (Trapani & Nicolson, 2011), signaling by hair cells to afferent neurons is likely to be reduced in *ap1b1* mutants. A reduction in synaptic activity may lead to compensatory responses in the afferent neurons, resulting in expansion of post-synaptic densities. Overall, however, we did not observe abnormally localized Cav1.3, Ribeye b or MAGUK, suggesting that AP-1 is not necessary for assembly of ribbon synapses in hair cells.

Basolateral membrane proteins NKA and zns-5 epitope is missorted in *ap1b1* mutants

As the AP-1 complex has been implicated in transport of membrane proteins to the basolateral membrane, we hypothesized that the deficit in mechanotransduction and the cellular phenotypes in *ap1b1* mutants are due in part to defective localization of proteins within the plasma membrane of hair cells. One of the proteins we analyzed was the zns-5 epitope. The zns-5 antibody recognizes an unknown protein that localizes to the basolateral membrane of hair cells and is also found to label a number of other cell type membranes including osteoblasts, adult fin and neurons (Asharani et al., 2012; Fleming et al., 2004; Johnson & Weston, 1995; Sims et al., 2009; Wills et al., 2008). In 5 dpf wildtype larvae, the zns-5 antibody indeed labels the basolateral membranes of hair cells and is excluded from the apical membrane surrounding the actin-filled stereocilia, indicated by phalloidin labeling (Figure 4.4A). In *ap1b1* mutants, however, this antibody labels the zns-5 epitope in both the basolateral and apical compartments (Figure 4.4B). These data suggest that the protein recognized by the zns-5 antibody is an AP-1-dependent cargo.

AP-1 cargo membrane proteins are sorted from the TGN or endosome to the plasma membrane through binding of the AP-1 μ or β -subunits to either tyrosine-based ($Yxx\Phi$) or di-leucine-based ($[D/E]xxxL[L/I]$) sorting signals, respectively (Bonifacino & Traub, 2003; Heilker et al., 1996; Ohno et al., 1998; Rapoport et al., 1998). Sorting motifs are usually present within the cytoplasmic C-terminal tails of cargo membrane proteins. Despite the absence of a canonical sorting signal, previous evidence from cell culture experiments suggests that the α -subunit of the basolateral pump NKA is sorted by AP-1 (Efendiev et al., 2008). We hypothesized that if NKA is an AP-1 dependent cargo, then the pump would be mislocalized in *ap1b1* mutant hair cells. To examine AP-1 dependent sorting of NKA, we used an antibody that recognizes a highly conserved epitope found on all NKA α -subunits (Lowery & Sive, 2005). In WT animals at 3 and 5 dpf, this antibody labels the basolateral membranes of hair cells of the medial crista (Figure 4.5A) and lateral-line neuromasts (data not shown). NKA label is also observed on fibers that innervate the hair cells and supporting cell membranes (Figure 4.5A-C), as well as other cell types such as ionocytes, neurons, and the pronephritic duct (data not shown). In the *tm246a* mutants, NKA was present in the basolateral membrane of hair cells (Figure 4.5B), but the overall intensity appeared greatly reduced compared to WT (Figure 4.5A). In the *t20325* mutant, the overall intensity of NKA label appeared somewhat reduced compared to WT (Figure 4.5C), but not nearly to the same degree as the *tm246a* mutant. Strikingly, in larvae carrying either *ap1b1* mutant alleles, we observed that NKA was mislocalized to apical hair bundles (Figure 4.5E-F). In contrast, we never observed immunoabeling of NKA in WT hair bundles (Figure 4.5D). Quantification of NKA colocalization with phalloidin-labeled bundles at 3 and 5 dpf showed that a significant amount of NKA was missorted to the hair bundle at both developmental stages (Figure

4.5M). These data indicate that without a functional AP-1 complex, NKA is sorted indiscriminately to both basal and apical compartments in hair cells.

To determine how efficiently NKA is targeted to the plasma membrane, we plotted the fluorescence profile of NKA across individual hair cells and quantified the amount of NKA at the plasma membrane (Figure 4.5G-L). Both mutant alleles showed reduced NKA expression at the hair cell plasma membrane (Figure 4.5N). Given that NKA is reduced at the plasma membrane, we attempted to address whether NKA had accumulated within the cell body of hair cells by quantifying intracellularly localized NKA using the central region of the fluorescence profiles (Figure 4.5O). In addition to inefficient sorting of NKA to the plasma membrane, the amount of NKA localized to the intracellular compartment was also significantly reduced in the *tm246a* mutant allele compared to WT siblings. And though not significant, there was a trend towards reduction in *t20325* mutants. The reduction of both plasma membrane and intracellular levels of NKA suggests that in both mutants, NKA is degraded. The fluorescent profiles (Figure 4.5J-K) indicated that, despite an overall decrease in NKA levels, mutant hair cells showed greater relative staining of NKA in the cytoplasm than WT hair cells. We therefore calculated the ratio of intracellular to plasma membrane NKA signal and found that indeed the ratio (CYTO/PM) was significantly increased in hair cells harboring either allele (Figure 4.5P). This result suggests that targeting of NKA to the plasma membrane is reduced in *ap1b1* mutant hair cells.

Ouabain treatment does not rescue hair cell mechanotransduction in *ap1b1* mutants

The NKA pump is missorted to the apical stereocilia in *ap1b1* mutants. NKA pumps Na⁺ ions out of and K⁺ ions into the cell. This pump works to maintain the relatively low intracellular Na⁺ and K⁺ ion concentrations with respect to the extracellular

concentration at the basolateral surface of hair cells. The concentration of the endolymph that surrounds the apical end of the hair cell is high in K^+ and low in Na^+ compared to other extracellular fluids (Anniko & Wróblewski, 1986; Russell & Sellick, 1976; Wangemann, 2006). As NKA at the apical end of the hair cell could be disrupting the concentration gradient of Na^+ and K^+ ions across the apical membrane, we hypothesized this ionic disruption would also upset the driving force for K^+ through the MET channel and explain the lack of mechanosensitivity in *ap1b1* mutant hair cells. To counteract the potentially disruptive effect of NKA at the apical end of hair cells, we incubated 5 dpf larvae from an *ap1b1* ID incross in E3 containing 500 μ M of ouabain and assayed MET function using the lipophilic vital dye, FM 1-43. As we were interested in how the apically mislocalized NKA affected MET function we wanted to block only the apically localized NKA while avoiding inhibition of NKA at the basolateral end. To minimize the possibility that the drug would penetrate the neuroepithelium and inhibit basolaterally localized NKA, ouabain was applied in the absence of DMSO. We observed that FM 1-43 label was not significantly different in ouabain-treated animals compared to vehicle control in either wild-type or mutant hair cells (Figure 4.6). This suggests that mislocalization of NKA to the apical end of hair cells does not alone account for the lack of MET activity in *ap1b1* mutant hair cells.

The amount of intracellular Na^+ is increased in *ap1b1* mutant hair cells

In nearly all cell-types, NKA is the primary pump for maintaining the relatively low level of Na^+ and high level of K^+ inside the cell. With the observation that NKA is less abundant in the hair cell plasma membrane of both *ap1b1* mutants, we hypothesized that this reduction would lead to increased Na^+ in hair cells. This idea is consistent with previous reports that inhibition of NKA in goldfish hair cells increases the concentration

of intracellular sodium (Mroz et al., 1993). In addition, it has been demonstrated that blebbing in hair cells can be triggered through an influx of Na^+ (Shi et al., 2005), and blebbing occurs in both *ap1b1* mutants (Figure 3.6 and data not shown). To assay relative amounts of intracellular Na^+ , we incubated intact 5 dpf larvae in the fluorescent Na^+ indicator Sodium Green. Compared to WT siblings, larvae carrying either mutant allele of *ap1b1* had significantly increased levels of Na^+ in individual hair cells (Figure 4.7A-C). Although the level of fluorescence varied among mutant hair cells within a neuromast, overall it was increased in *ap1b1* mutant hair cells compared to WT hair cells (Figure 4.7D). This increase in Sodium Green label suggests that *ap1b1* mutants are unable to maintain appropriate intracellular Na^+ levels. To determine whether an increase in Na^+ levels was specifically due the trafficking defects in *ap1b1* mutants, and not secondary to its transduction defects, we tested another transduction mutant carrying the *pcdh15*^{th263b} allele. At 5 dpf, *pcdh15* mutant hair cells did not show an increase in intracellular Na^+ (Figure 4.7D). This result suggests that Na^+ build up is a unique consequence of mutations to *ap1b1*, and not due to the loss of mechanotransduction.

DISCUSSION

The polarized distribution of membrane proteins in epithelial cells is essential for cellular function and is accomplished in part through the AP-1 complex (Shafaq-Zadah et al., 2012; Zhang et al., 2012). It is well accepted that the AP-1 complex plays an important role in basolateral targeting of membrane proteins (Fölsch et al., 2009). In support of this, we show that zebrafish AP-1 is involved in the sorting of NKA and *zns-5* to the basolateral compartment of hair cells. We also contribute preliminarily to the

growing evidence that AP-1 also plays a part in the pathway to sort proteins apically (Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010). Importantly, however, we find that the missorting of NKA in the *ap1b1* mutant has profound consequences on ion homeostasis in hair cells, particularly with respect to Na⁺. Based on our observations, we propose that these defects are caused primarily by the missorting of basolaterally targeted proteins including NKA. Incorrect targeting of NKA leads to the presence of this pump in the apical hair bundle of mutant hair cells. Loss of Ap1b1 function also results in a reduction of NKA at the basolateral membrane, leading to an increase in intracellular Na⁺. Collectively, these findings suggest that ion homeostasis and mechanotransduction are disrupted in hair cells when basolateral proteins such as NKA are missorted to the apical surface.

Sorting of apical proteins Pcdh15 and Tmhsa disrupted in *ap1b1* mutants

The AP-1 complex has been implicated in the apical targeting of some membrane proteins (Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010). We provide *in vivo* evidence that Pcdh15 is an AP-1 dependent cargo in cell culture lines (Zallocchi et al., 2012) (Figure 4.1). Interestingly, the defects with Pcdh15 antibody labeling we observe in *ap1b1* mutants are opposite of what we see with transiently expressed GFP-Tmhsa (Figure 4.2). While we see significantly reduced Pcdh15 in *ap1b1* mutant hair cells compared to wild type, we preliminarily see that GFP-Tmhsa accumulates in an intracellular compartment larger than similar compartments seen in wild type. This could be indicative of slightly different methods of trafficking for Pcdh15 and Tmhsa. This could also be an artifact of different methods of quantifying proteins in hair cells, one being antibody labeling of fixed tissues while the other is a transgene expressed and

visualized in live animals. Taken together, *ap1b1* appears to be important for the sorting of Pcdh15 and Tmhsa suggesting that both proteins are AP-1 dependent cargos.

***ap1b1* is required for proper localization of NKA in hair cells**

AP complexes selectively recognize cargo via intrinsic sorting signals, such as the dileucine and tyrosine motifs (Bonifacino & Traub, 2003; Heilker et al., 1996; Ohno et al., 1998; Rapoport et al., 1998). However, in the case of NKA, a canonical AP-1 sorting motif has not been identified. Instead, novel motifs within the α -subunit of NKA appear to be necessary for basolateral targeting in cell lines (Dunbar & Caplan, 2001; Efendiev et al., 2008; Muth et al., 1998). Additionally, unlike other well-known AP-1 dependent cargoes, the post-Golgi transport pathway taken by NKA to the plasma membrane does not involve recycling endosomes, but rather goes directly from the TGN to the plasma membrane (Farr et al., 2009). In our study, we observe that a fraction of NKA is sent to the apical surface in *ap1b1* mutant hair cells (Figure 4.5). As apical missorting of AP-1 dependent cargoes is a common outcome when AP-1 is disrupted (Mellman & Nelson, 2008; Shteyn et al., 2011), our observations support the hypothesis that the AP-1 complex is required for sorting of NKA to the basolateral membrane.

In accordance with our observations that AP-1 is required for basolateral sorting of NKA, there is also a striking reduction in the level of NKA within the basolateral membrane in both *apb1* mutant alleles. Intracellular NKA was significantly reduced in the *tm246a* allele, and there was a trend towards reduction in the *t20325* allele.

Additionally, immunolabeled Pcdh15 is also reduced in *ap1b1* mutant hair cells (Figure 4.1). In contrast to NKA and Pcdh15, we did not observe reductions in immunolabel of several components of the hair-cell synapse including the membrane α subunit of Cav1.3 (Figure 4.3). A reduction in NKA and Pcdh15 implies that the proteins may be trafficked

through a competing AP pathway, such as AP-3, which targets proteins to the lysosome, where NKA is likely degraded. Recent evidence in kidney cells demonstrates that AP-1 complexes are required for both stability and trafficking of the anion exchanger 1 (AE1) to the plasma membrane, suggesting that as observed with NKA in *ap1b1* mutants, mistargeted proteins are degraded as a consequence of missorting (Almomani et al., 2012).

Missorting of NKA causes a Na⁺ imbalance in mutant *ap1b1* hair cells

In hair cells, the activity of NKA is necessary to clear the build up of intracellular Na⁺ generated by other Na⁺-coupled transport activities (Mroz et al., 1993). Exchangers such as the Na⁺/H⁺ and Na⁺/Ca⁺⁺ pumps are thought to account for most of the Na⁺ flowing into hair cells. Consistent with a role for regulating intracellular Na⁺, our data suggest that decreases in the cell-surface expression of NKA lead to an increase in intracellular Na⁺ concentrations in mutant hair cells. Though overall Na⁺ levels are increased in mutant hair cells, these levels were also highly variable among individual cells. The variability suggests that Na⁺ build-up may be progressive. We propose that the failure of NKA to balance Na⁺ loading within hair cells leads to increased intracellular Na⁺. A rise in internal Na⁺ may disrupt several cellular processes, including Na⁺-coupled transport activity, and potentially the resting membrane potential. An increase in Na⁺ can also lead to overt signs of necrosis. A previous report demonstrated that excessive Na⁺ influx causes apical blebbing in cultured hair cells (Shi et al., 2005). Thus, elevated Na⁺ is likely to disrupt hair cell function and eventually lead to blebbing and cell death, which we observe in mutant hair cells of both *ap1b1* alleles.

Polarized epithelial cells have the unique challenge of maintaining two functionally distinct domains of the plasma membrane. In polarized, electrically active cells, the expression and sorting of ion channels and transporters to distinct compartments is critical for cell activity. Based on our data, we propose that the AP-1 complex is vital for the correct sorting of NKA and the maintenance of ion homeostasis in hair cells.

FIGURES

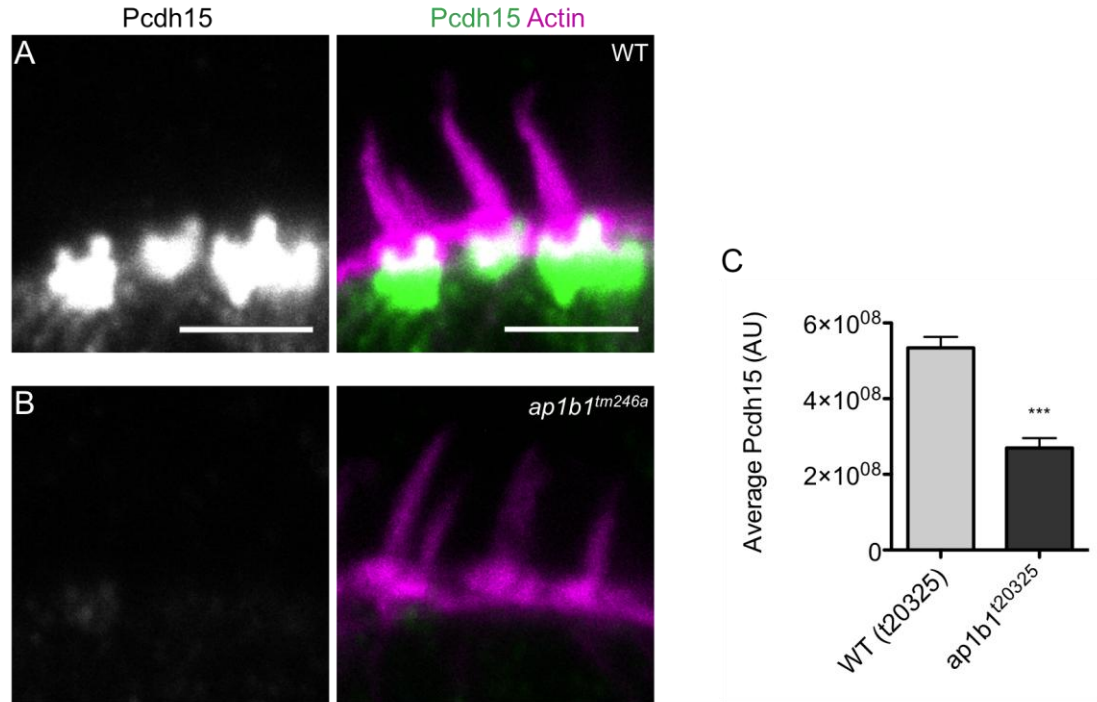


Figure 4.1: Pcdh15 is reduced in *tm246a* mutant hair cells compared to wild type.

A, B, Pcdh15 label of the apical end of wild-type and *tm246a* mutant inner ear hair cells of 5 dpf larvae. Scale bars = 2 μ m. **C,** Quantification of Pcdh15 label in wild-type and *tm246a* mutant medial cristae (n = 21 wild-type larvae, n = 18 mutant larvae).

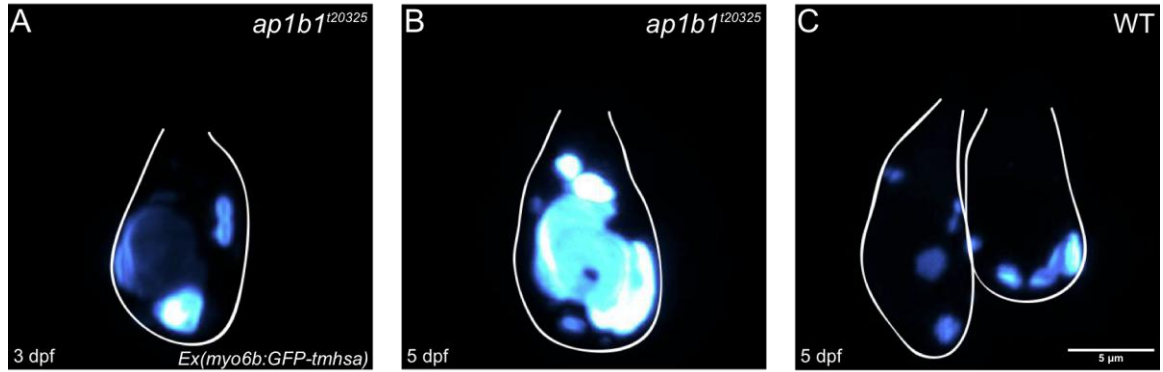


Figure 4.2: Build-up of transiently expressed GFP-*tmhsa* in *t20325* mutant hair cells.

A, B, GFP-*tmhsa* transiently expressing in larval hair cells from 3 and 5 dpf *t20325* mutant larvae, respectively. **C,** Transient expression of GFP-*tmhsa* in hair cells from a 5 dpf larva. Scale bar = 5 μm.

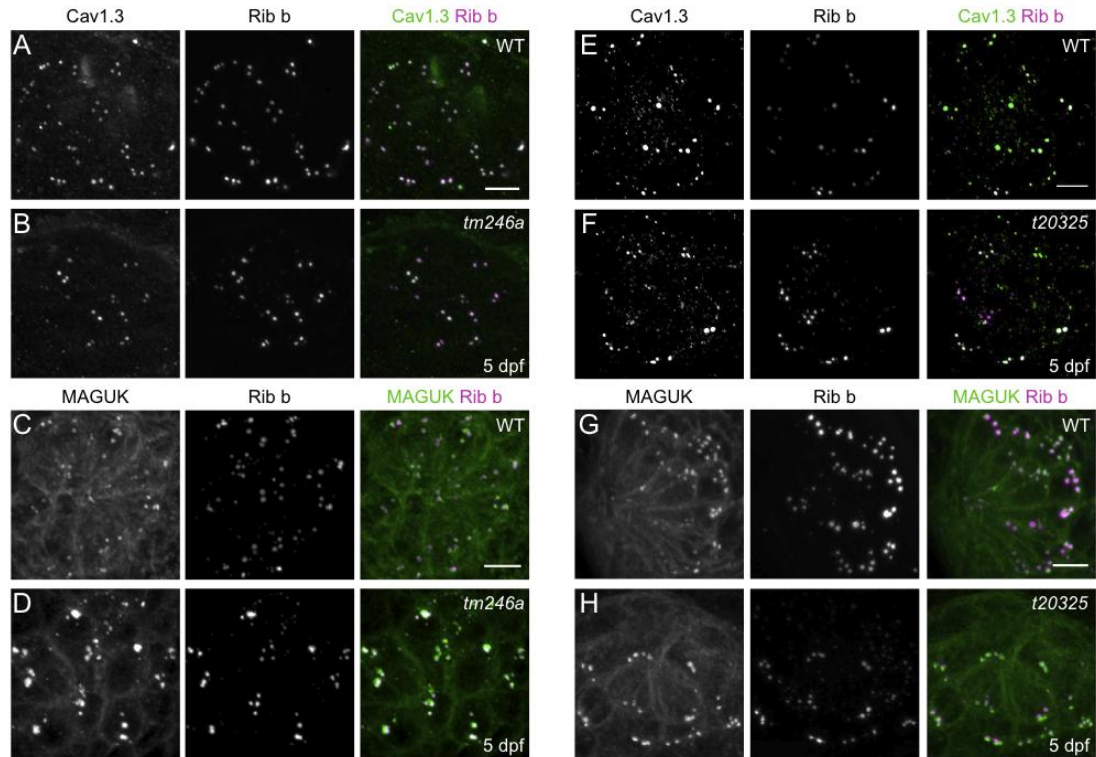


Figure 4.3: Components of the basolateral ribbon synapse are intact in *ap1b1* mutant hair cells

Representative confocal images of immunolabeled synaptic proteins in WT and mutant neuromasts (top down view, z-projections). A-B, E-F, Immunolabel of Cav1.3 and Rib b in neuromast hair cells of A, WT (*tm246a*), B, *tm246a* mutant E, WT (*t20325*) and F, *t20325* mutant. C-D, G-H, Images of presynaptic Rib b and postsynaptic MAGUK immunolabel in neuromast hair cells of C, WT (*tm246a*), D, *tm246a* mutant G, WT (*t20325*) and H, *t20325* mutant. All images were taken at 5dpf. Scale bars, 5 μ m.

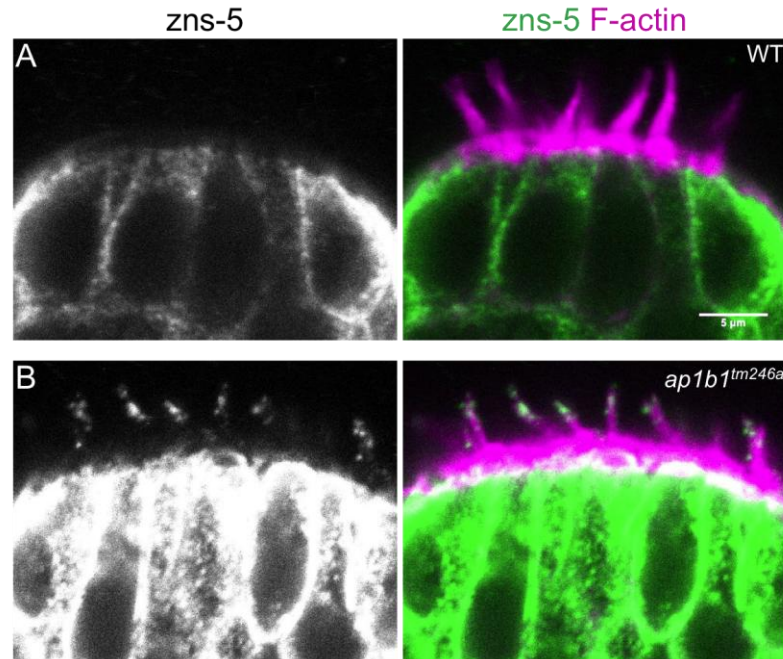
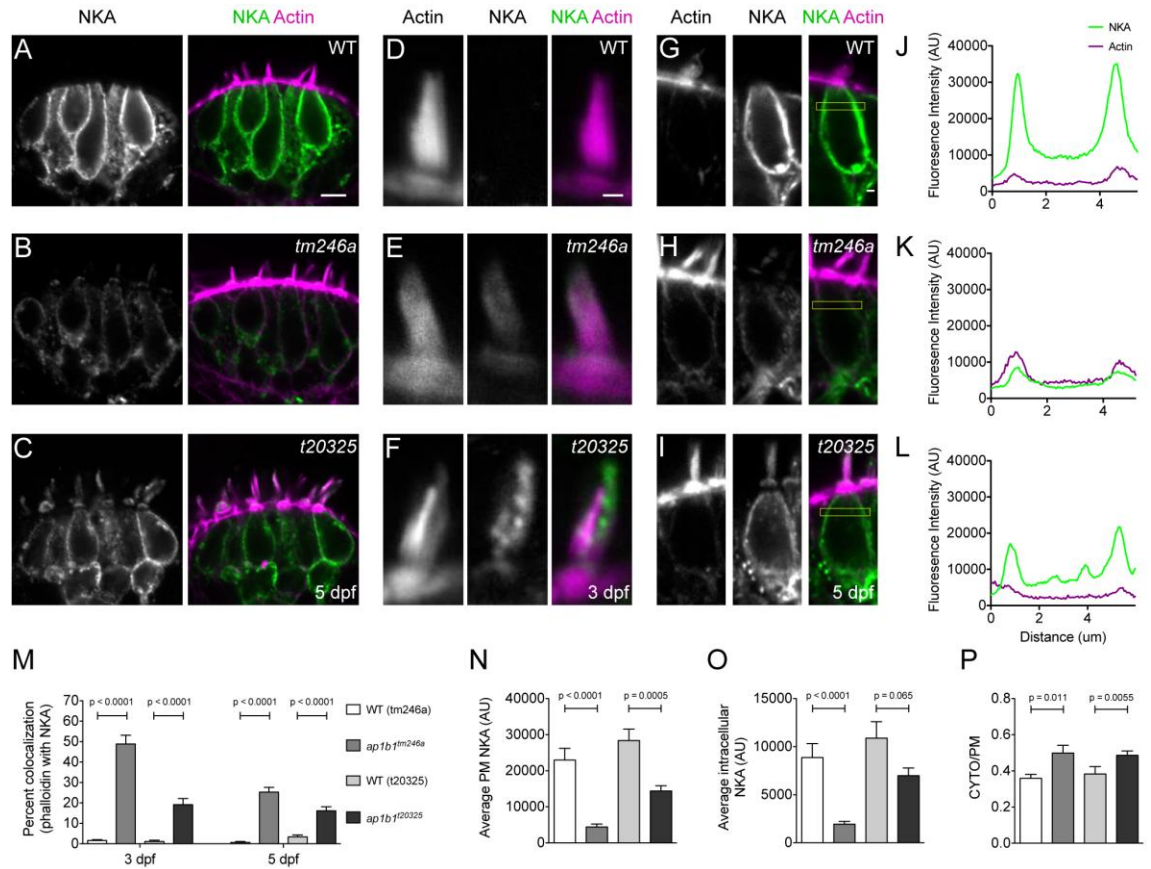


Figure 4.4: zns-5 epitope is missorted in *tm246a* mutant to stereocilia.

A, B, zns-5 antibody labeling in wild-type and *tm246a* mutant hair-cells, respectively.

Panels on the right depict zns-5 fluorescence alone. Panels on the left are a merge of zns-5 labeling, green, and phalloidin labeling, magenta.



44, mutant n = 62 bundles) from ≥ 4 larvae. **N**, Quantification of NKA fluorescence (A.U.) at the membrane at 5 dpf. **N**, Quantification of intracellular NKA fluorescence (A.U.). **O**, Quantification of intracellular NKA fluorescence (A.U.) at 5 dpf. **P**, The ratio of intracellular NKA (CYTO) to plasma membrane localized NKA (PM). For **N-P**, *tm246a*: WT n = 19, mutant n = 16; *t20325*: WT n = 18, mutant n = 21 hair cells. Error bars in **M-P** represent SEM and statistical difference determined with a Mann-Whitney U-test.

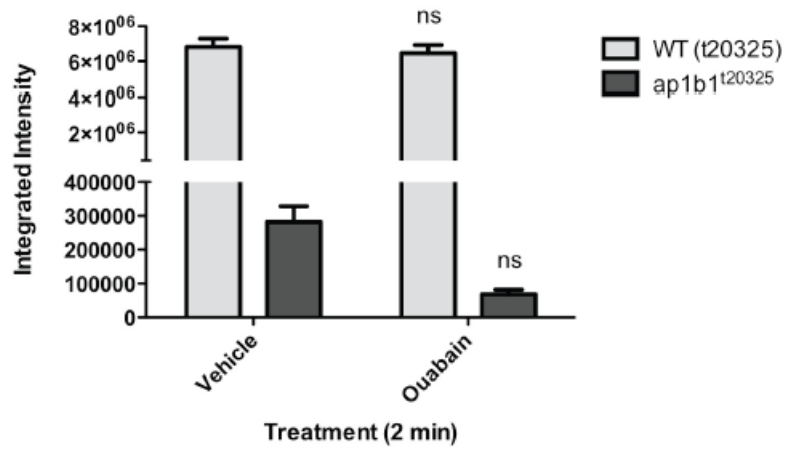


Figure 4.6: Ouabain treatment has no effect on FM 1-43 labeling of *t20325* mutant hair cells.

Vehicle (embryo medium; wild type $n = 8$, *t20325* $n = 9$) and ouabain (500 μM ; wild type $n = 9$, *t20325* $n = 4$) treatment of 5 dpf larvae. FM 1-43 fluorescence was unchanged in both wild type and mutant larvae when treated with ouabain compared to control. Error bars represent SEM and statistical difference determined with a Mann-Whitney U-test.

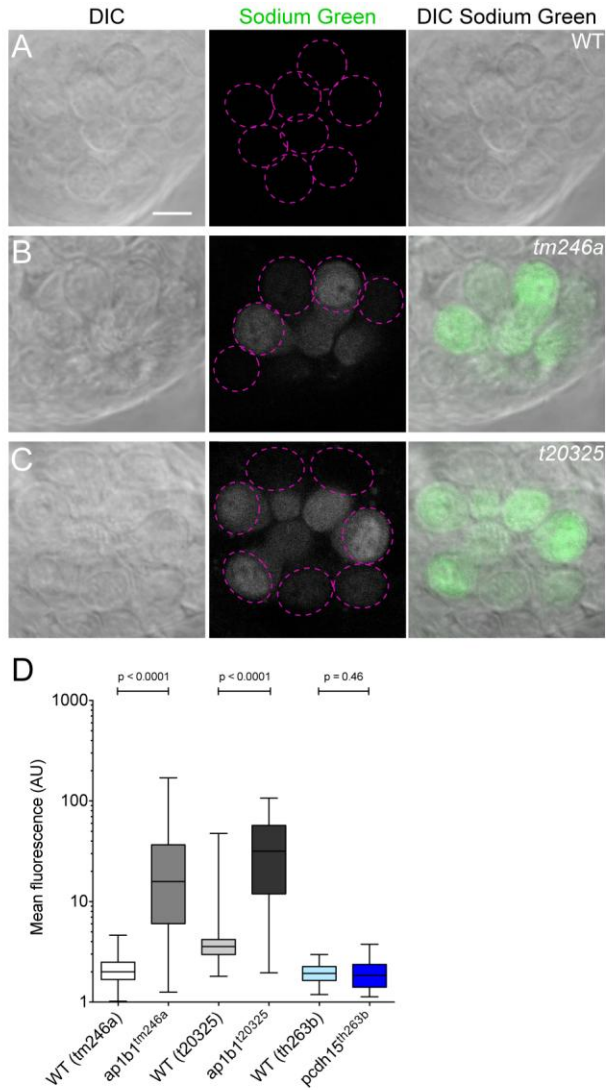


Figure 4.7: Increase of intracellular Na⁺ levels in mutant hair cells

A-C, Sodium Green label in WT, *tm246a* and *t20325* mutant hair cells. Dotted magenta circles outline hair cells that were used for quantification in that plane of view. Scale bar, 5 μ m. **D**, Quantification of Sodium Green label in *ap1b1* mutant, *pcdh15*^{th263b}, and corresponding WT hair cells. (*tm246a*: WT n = 124, mutant n = 90; *t20325*: WT n = 125, mutant n = 74; *th263b*: WT n = 100, mutant n = 84 hair cells). Error bars represent SEM. Statistical analysis performed with a Mann-Whitney U-test.

Chapter 5 – The N- and C-terminal domains of zebrafish

Tmc2a play opposing roles in MET activity in hair cells

AUTHORS

Rachel Clemens Grisham, Weike Mo, Katie Kindt, Tim Erickson, Teresa Nicolson

ABSTRACT

Hair cells convert sound vibrations to electrical impulses through the use of the mechanotransduction channel (MET). Though many properties of the MET channel have been defined, the molecular identity remains elusive. Mutations in Transmembrane channel-like 1 (TMC1) cause hearing loss in humans. This same gene in *C. elegans* was recently shown to have channel activity. Together, this makes TMC1 a strong candidate for the MET channel. In the present study, we explore the role of zebrafish Tmc2a in mechanotransduction and show that the N- and C-termini of Tmc2a oppositely affect mechanically evoked calcium responses in hair cells. This provides compelling evidence for Tmc2a playing an important role in MET channel function and gives insight to a regulatory role.

INTRODUCTION

Mechanotransduction is an ancient and fascinating process that has been documented in organisms from archea to mammals and is often conducted through the use of mechanically gated channels (Martinac, 2004; Sukharev & Sachs, 2012). Although the molecular identities of some mechanosensitive channels are known, the identity of the mechanoelectrical transduction (MET) channel in hair cells of the auditory and vestibular systems has eluded scientists for decades (Fettiplace, 2009). Despite the lack of a molecular identity for the MET channel, its localization and much about its properties have been described. The MET channel resides at the tips of stereocilia, which are actin-filled projections at the apical end of the hair cell arranged in a staircase manner where rows of stereocilia are taller the more proximal they are to kinocilia. Deflection of the stereocilia towards the tallest row places tension across the tip links, which connect the tops of the stereocilia. These tip links gate the MET channel and under tension cause the channel to open, allowing positively charged ions to flow into the cell. The tip links are made of Cadherin 23 (Cdh23) at the upper end and Protocadherin 15 (Pcdh15) at the lower end (Kachar et al., 2000; Kazmierczak et al., 2007). Recent work has shown that the MET channel is located at the lower end of the tip link, which suggests that it might directly or indirectly interact with Pcdh15 (Beurg et al., 2009). Armed with this information, our lab performed a screen for proteins that interact with zebrafish Pcdh15 and among several proteins, we found an interaction with Tmc2a (Transmembrane channel-like 2a).

TMC1 is the causative gene for deafness in humans at the dominant DFNA36 locus and the recessive DFNB7/11 locus (Davoudi-Dehaghani et al., 2013; Gao et al., 2013; Hildebrand et al., 2010; Kurima et al., 2002; Heer et al., 2011). Mutations to *Tmc1* in mice also cause both dominant and recessive forms of deafness (Bock & Steel, 1983; Manji et al., 2012; Steel & Bock, 1980; Vreugde et al., 2002). Mouse studies have demonstrated that *Tmc1* and *Tmc2* are both important for hair cell mechanotransduction (Kawashima et al., 2011). *Tmc1* is thought to have 6 transmembrane domains and was recently shown to have channel properties in *C. elegans* chemosensing neurons (Chatzigeorgiou et al., 2013; Labay et al., 2010). Together, these data make the *Tmc1* and *Tmc2* proteins strong candidates for the mechanotransduction channel.

In this study, we find zebrafish *tmc1*, *tmc2a* and *tmc2b* genes expressed in hair cells, but differentially in different hair cell populations in the ear and lateral line. We also attempt to block MET activity by exogenously expressing the C- and N-terminal fragments of *Tmc2a* specifically in hair cells and find that while the N-terminal fragment moderately reduces mechanically evoked Ca^{++} transients, exogenous expression of the C-terminal fragment facilitates mechanically evoked Ca^{++} transients.

METHODS

Animal lines: Zebrafish were kept on a 12 hr light-dark cycle at 28°C. The *Tg(myo6b:β-actin-GFP)* has been previously described (Kindt et al., 2012). The *Tg(myo6b:R-GECO)* line was created by combining the R-GECO open reading (Tewson et al., 2012) frame with the zebrafish *myo6b* promoter using the Tol2/Gateway zebrafish kit (Kwan et al., 2007) (See Vector Construction).

Cloning and molecular biology: Full length *tmc2a* transcript was cloned from cDNAs obtained with SuperScriptIII (Invitrogen) kit from total RNA isolated from Tubingen 5 dpf wild-type (WT) larvae. Subsequent N- and C-terminal fragments were cloned from the full length clone.

Vector construction and transgenic lines: Expression clones are based on the Tol2/Gateway zebrafish kit (Kwan et al., 2007). N- and C-terminal fragments of *tmc2a* were recombined into the middle entry (ME) donor vector. The pME-*tmc2a*, pME93 *tmc2aNterm* and pME-*tmc2aCterm* clones were then combined with a destination vector containing a red heart marker (395), the 5' entry vector containing the *myo6b* promoter (Obholzer et al., 2008), and the 3' entry vector p3E-GFP_{CAAX} to generate the *myo6b:tmc2aNterm-GFP_{CAAX}*, and *myo6b:tmc2aCterm-GFP_{CAAX}* constructs.

In Situ Hybridization: Phenylthiourea (PTU) treated larvae were fixed overnight in 4% paraformaldehyde, washed in PBS + 0.1% Tween and stored in methanol at -20°C. The *in situs* were performed by T. Erickson according to an established protocol (Thisse & Thisse, 2008).

Microscopy: Images were captured on a Zeiss LSM 700 upright confocal microscope using the Zen acquisition software (2009 release, Zeiss). Live larvae were mounted in 1% low melt agarose (Life Technologies) dissolved in E3 embryo medium and imaged with a 63x/0,95 water immersion lens. Images were subsequently processed with ImageJ software.

Calcium imaging and hair bundle stimulation: Calcium imaging and analysis was performed by K. Kindt as described elsewhere (Kindt et al., 2012).

RESULTS

Using a split-ubiquitin yeast-two-hybrid method (Snider et al., 2010; Thaminy et al., 2004), we aimed to identify zebrafish proteins that interacted with the zebrafish tip link protein Pcdh15 (data not shown). Assaying for protein interactions between membrane proteins is challenging with conventional yeast-two-hybrid methods because interacting proteins bring together transcription factors that must translocate to the nucleus. The split ubiquitin is designed so that interactions bring together two halves of a transcription factor, which can then be cleaved off from the interacting proteins and translocate to the nucleus. This method used two cDNA libraries generated from larval zebrafish ears to uncover an interaction between Pcdh15 cytoplasmic domain and the N-terminus of Tmc2a.

In zebrafish, *tmc2a* has a paralog, *tmc2b*. Paralogs are common in zebrafish and can have non-overlapping or non-identical expression patterns. The different expression patterns of the paralogs can result in different mutant phenotypes when one or the other paralog is mutated (Amores et al., 1998; Gates et al., 1999; Postlethwait et al., 1998). Interestingly, it is human *TMC1* that is implicated in hereditary hearing-loss, not *TMC2* (Davoudi-Dehaghani et al., 2013; Gao et al., 2013; Hildebrand et al., 2010; Kurima et al., 2002; Heer et al., 2011). A recent study, however, revealed that in mice, both *Tmc1* and *Tmc2* are necessary for hair cell mechanosensitivity (Kawashima et al., 2011). These data suggest that while *tmc1* may be necessary for hair cell function, *tmc2a* and perhaps *tmc2b* in fish could play a role in this process as well. We therefore set out to determine if *tmc1*, *tmc2a*, and *tmc2b* are expressed in hair cells. To accomplish this aim, we first performed RT-PCR on cDNAs obtained from isolated ear or neuromast endorgans. In both

populations, we observed expression of the hair cell specific transcripts *cadherin 23* (*cdh23*), *vesicular glutamate transporter 3* (*vglut3*) and the expression of all three *tmc* transcripts tested. However, while the expression of *cdh23* and *vglut3* differed little between inner ear or neuromast cDNAs, there were notable differences in the relative amounts of each *tmc* expressed (Figure 5.1A). We noticed that *tmc2a* was more highly expressed in the inner ear while its level of expression in neuromasts was fairly low. In contrast, *tmc1* and *tmc2b* were both more highly expressed in neuromasts compared to their expression in the inner ear.

To ascertain whether the *tmcs* were expressed in the hair cells of these organs specifically, we performed *in situ* hybridization at various ages and analyzed expression of each gene in zebrafish. We observed that *tmc2a* was expressed early in the anterior and posterior maculae of the developing ear at 1 dpf soon after the hair cells in these endorgans begin to develop (Figure 5.1B). Expression in the anterior macula persists through 2 to 5 dpf, during which *tmc2a* can also be found expressed in the anterior, medial and posterior cristae (Figure 5.1C-E). Further scrutinizing of the anterior macula at 5 dpf revealed that *tmc2a* was indeed expressed in hair cells and was not expressed in supporting cells, indicating that the *tmc2a* gene is important for a hair cell specific process (Figure 5.1E).

By 4 dpf, *tmc1* is expressed in the three cristae of the inner ear, most strongly in the medial crista (Figure 5.1F). We were unable to detect *tmc1* expression in the neuromasts of larval stages we assayed (data not shown). At 4 dpf, we also noted robust *in situ* labeling of *tmc2b* transcripts in lateral-line neuromasts. Together these data suggest that *tmc1*, *tmc2a*, and *tmc2b* are expressed at different levels in the various hair-cell containing endorgans, with *tmc2a* being highly expressed in the inner ear hair cells, *tmc2b*

expressing highest in neuromast hair cells and *tmc1* expressing in both ear and neuromast hair cells at a potentially higher level in neuromasts by adult stages.

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Both N- and C-terminal cytoplasmic domains of Tmc2a are predicted to be intracellular. We therefore considered the possibility that both termini are sites of protein-protein interaction that are important for protein function. We then performed a similar scan of the C-terminus of Tmc2a and found that it also possessed a predicted coiled coil adjacent to the last transmembrane domain (Figure 5.2B-C). Again using the COILS program we observed that in all species analyzed there were seven layers of interaction in this coiled-coil. This suggested that both the N- and C-terminal tails of Tmc2a could be involved in mediating interactions with other proteins. We therefore hypothesized that exogenous expression of both N- and C-terminal fragments would disrupt the endogenous association of Tmc2a and Pcdh15 and have deleterious effects on the function of the MET complex.

A common effect of a disrupted MET complex is splayed bundles (Di Palma et al., 2001; Kachar et al., 2000; Kazmierczak et al., 2007; Seiler et al., 2005; Washington et al., 2005; Wilson et al., 2001), we therefore assessed bundle morphology of inner-ear hair-

ells that expressed either N- or C-terminal fragments of Tmc2a fused to GFP_{CAAX}.

GFP_{CAAX} is a Green florescent protein containing a CAAX prenylation motif, which increases association with membranes (Hancock et al., 1991). We injected this construct into a zebrafish line that transgenically expresses β -actin fused to the red fluorophore mCherry. We found that expression of neither the N- nor C-termini had any effect on the integrity of the hair bundle, suggesting that *tmc2a* does not play a role in maintaining bundle morphology (Figure 5.3A and C).

We next sought to determine if exogenous expression of the N- and C- termini of *tmc2a* in hair cells affected mechanically evoked activity. To do this we recorded Ca⁺⁺ responses from neuromast hair-cells transiently expressing either fragment and compared these responses to hair cells not expressing the construct. To our surprise, the N- and C-terminal fragments had opposite effects on mechanically evoked Ca⁺⁺ signaling in hair cells. As expected, the N-terminal fragment reduced the mechanically-evoked Ca⁺⁺ response of hair cells that expressed the transgene (Figure 5.3D-E). This reduction of Ca⁺⁺ response was irrespective of the intensity of the stimulus (Figure 5.3E-E'). In opposition to what we expected, we found that expression of the C-terminal fragment of Tmc2a facilitated the mechanically-evoked Ca⁺⁺ transients of hair cells (Figure 5.3B). These results support the idea that the Tmc2a protein is involved in MET activity, but that N- and C-terminal ends of the protein may have opposing functions with regard to mechanosensitivity.

DISCUSSION

In this study, we analyzed the expression patterns of three genes *tmc1*, *tmc2a*, and *tmc2b*, and explored the function of *tmc2a* in hair cell mechanotransduction. We found that while *tmc1* and *tmc2b* are more highly expressed in neuromast hair cells, *tmc2a* is more highly expressed in hair cells of the inner ear (Figure 5.1). We also found that exogenous expression of the N-terminus of Tmc2a in hair cells causes the reduction in mechanically-evoked Ca^{++} transients, presumably through disruption of the interaction between endogenous Pcdh15 and Tmc2a proteins. We also report that exogenous expression of the C-terminus of Tmc2a increases mechanically-evoked Ca^{++} responses in hair cells.

tmc2a is highly homologous with *tmc1*. In *C. elegans*, *tmc-1* was recently found to have channel activity when ectopically expressed in the CHO (Chinese hamster ovary) cell line (Chatzigeorgiou et al., 2013). This finding with the *C. elegans tmc-1* protein coupled with our data showing zebrafish Tmc2a interacts with Pcdh15 provides compelling evidence for Tmc2a being a channel component of the MET complex. We also provide evidence that Tmc2a is physiologically relevant for MET complex function (Figure 5.3). The fact that expression of the N-terminal fragment of Tmc2a causes a reduction in mechanically-evoked Ca^{++} transients is consistent with our hypothesis that there is an endogenous interaction between Tmc2a and Pcdh15 in hair cells, which is necessary for transduction of mechanical stimuli. We also report that expression of the C-terminus of Tmc2a increases the response of hair cells to mechanically-evoked stimuli. This suggests that the C-terminus is involved in mechanical gating, but through a different mechanism than the N-terminus. It is likely that the cytoplasmic C-terminal domain of Tmc2a is

interacting with some member of the MET complex. One possibility is that the C-terminus of Tmc2a interacts with a regulatory protein for MET channel activity, and expression of this fragment disrupts that interaction inducing a “loss of control” over the level of MET channel activity. Another possibility is that the C-terminus is involved in recruiting the MET channel or another protein that facilitates MET activity and over-expression of the C-terminal domain of Tmc2a leads to greater channel activity. Further biochemical and physiological experiments are needed to explore these hypotheses.

In either case, it is difficult not to implicate the role of the coiled-coil domains in N- and C-termini as these domains are well known for their roles in protein-protein interaction (Mason & Arndt, 2004). In some contexts, coiled-coil domains mediate the assembly of channel oligomers (Molland et al., 2010; Tsuruda et al., 2006). A recent study demonstrated the coiled-coil domain of the voltage sensitive H⁺ channel can even be engineered to form channel monomers, dimers, trimers and tetramers (Fujiwara et al., 2013). Further study of the N- and C-termini of Tmc2a would expand our knowledge of how coiled-coil domains are used in channel activity and could potentially shed light on how the MET channel functions and is regulated.

FIGURES

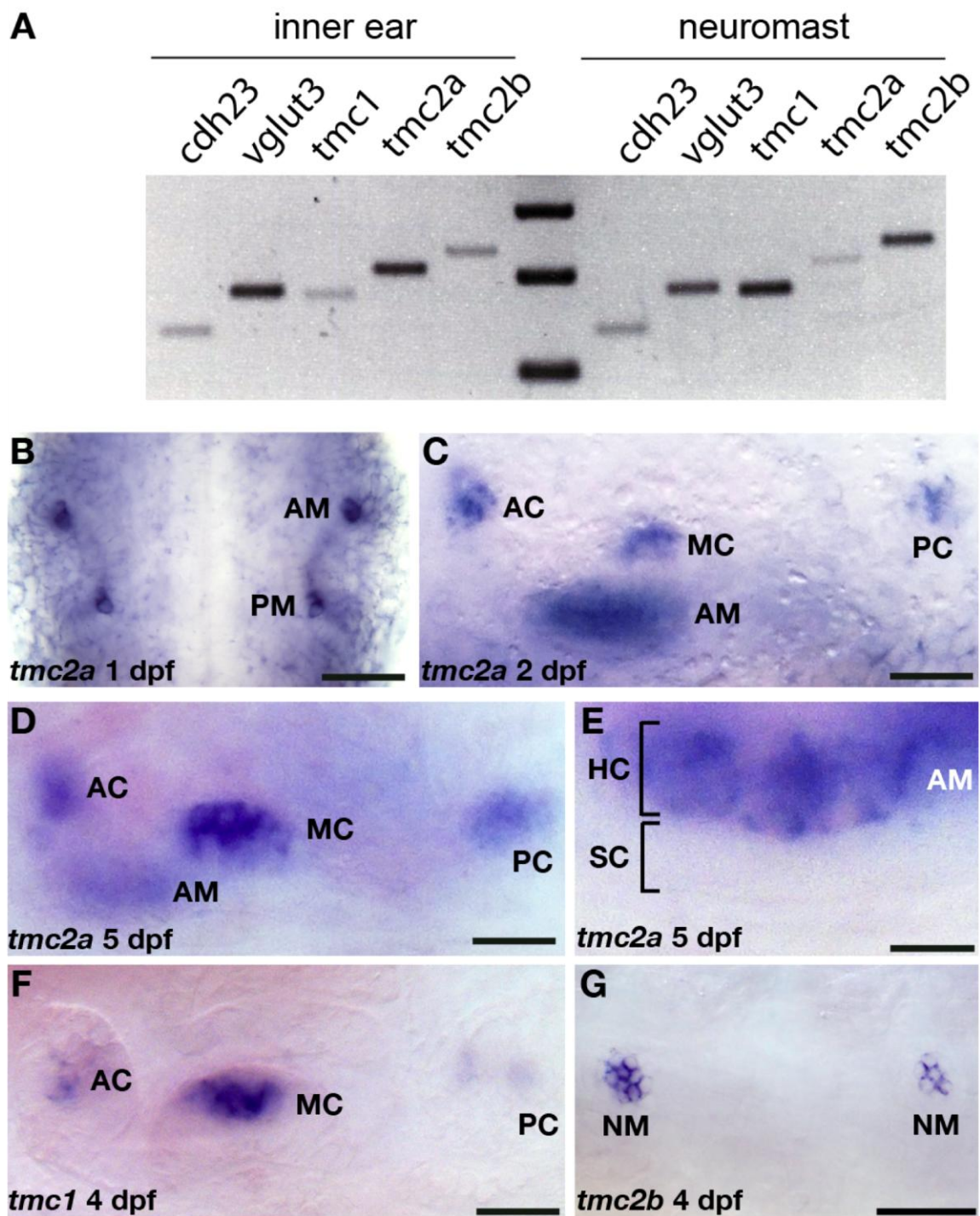


Figure 5.1: The *tmc1*, *tmc2a*, and *tmc2b* genes are differentially expressed in different hair-cell subpopulations.

A, Semi-quantitative RT-PCR of RNAs collected from either inner ear or neuromast endorgans showing expression of known hair-cell specific transcripts, *cadherin 23* (*cdh23*) and *vesicular glutamate transporter 3* (*vglut3*), as well as *tmc1*, *tmc2a*, and *tmc2b*. **B-E**, *In situ* hybridization showing expression of *tmc2a* in developing hair cell patches in the inner ear at 1, 2, and 5 dpf, respectively. **F**, *In situ* showing expression of *tmc1* in the inner ear hair cell patches at 4 dpf. **G**, *In situ* showing expression of *tmc2b* in hair cells in lateral-line neuromasts at 4 dpf. AM = utricular macula, PM = saccular macula, AC = anterior crista, MC = medial crista, PC = posterior crista HC = hair cell, SC = supporting cell, NM = neuromast.

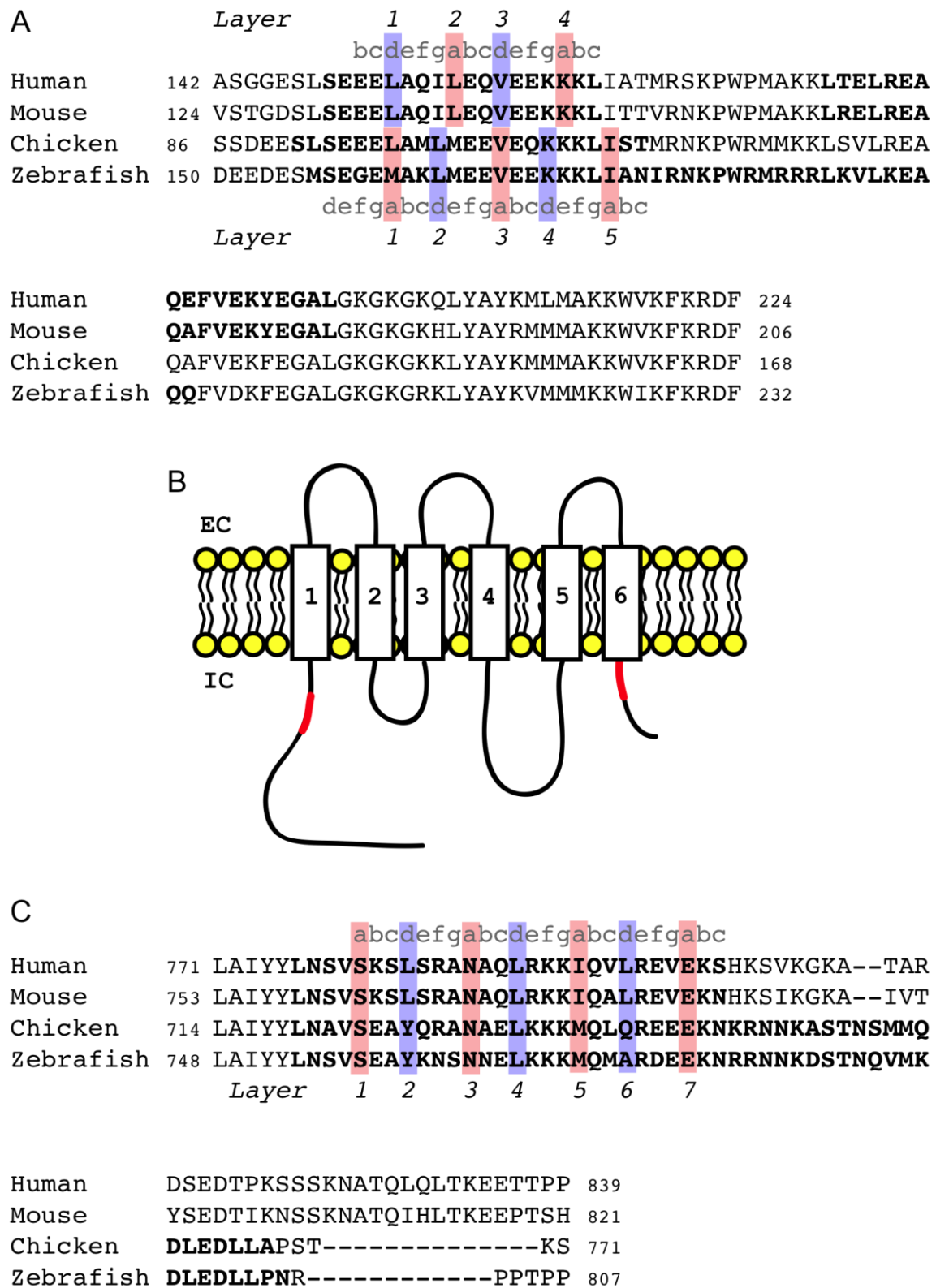


Figure 5.2: Putative coil-coiled regions of Tmc.

A, C, Sequence alignment for part of the N-terminal and C terminal tail, respectively, of Tmc2 from zebrafish with alignments to human, mouse and chicken. The “a” and “d” residues of the predicted coiled coil domain are highlighted in red and blue. The amino acid residues that scored ≥ 0.1 in the COILS program for each species specific sequence are in bold. **B,** Diagram of Tmc2a at plasma membrane with topology based on studies on mouse Tmc1 \cite{Labay:2010}. Coil-coiled regions in the N- and C-termini are highlighted in red.

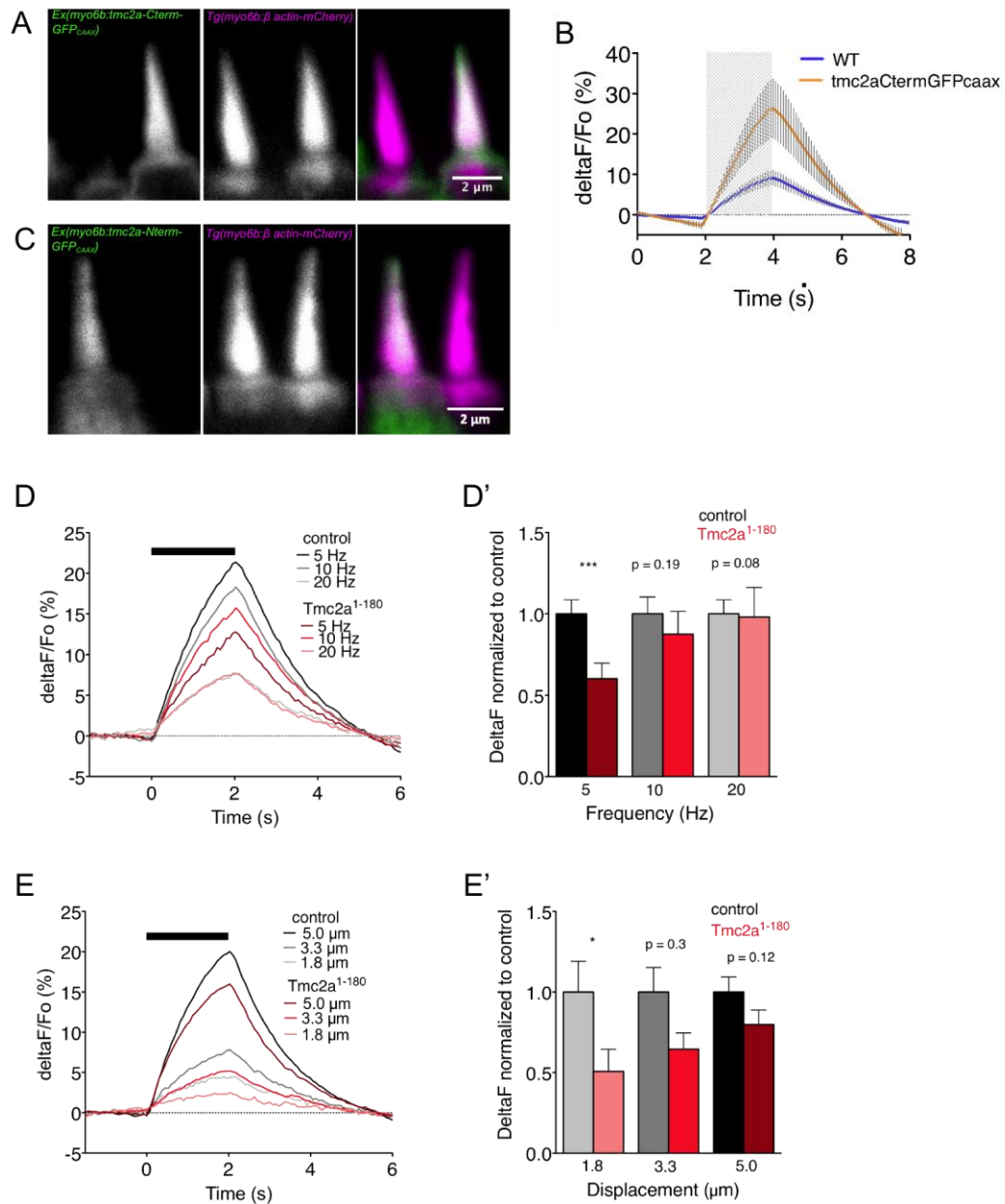


Figure 5.3: Tmc2a N-terminus reduces while the C-terminus increases the response of hair cells to mechanical stimuli.

A, Transient expression of the C-terminal fragment of Tmc2a fused to GFP_{CAXX} in a hair cell of the medial crista of 3 dpf larvae that also contains the *Tg(myo6b:β-actin-mCherry)* transgene. **B**, Trace representing the average calcium responses to a 2 sec, 10 Hz, 25

mmHg water-jet stimulus from lateral-line neuromasts of 3 dpf larvae (n = 23 cells WT, n = 17 cells Tmc2aCterm). Error bars are represented by dotted lines. **C**, Transient expression of N-terminal fragment of Tmc2a fused to GFP_{CAAX} in a hair cell of the medial crista of 3 dpf larvae that also transgenically expresses β -actin fused to mCherry in hair cells. **D**, Traces representing the average neuromast calcium responses to a 2 sec, 10 Hz water jet stimulus to a 1.8 mm (n = 50 WT, 33 Tmc2a¹⁻¹⁸⁰ cells), 3.3 mm (n = 50 WT, 43 Tmc2a¹⁻¹⁸⁰ cells) and 5.0 mm (n = 86 WT 84, Tmc2a¹⁻¹⁸⁰ cells), respectively, of 3 dpf larvae. **D'**, Same data as **D**, but quantified and normalized to WT control, * p > 0.05. **E**, Traces representing the average calcium responses to a 2 sec 5 Hz (n = 58 WT, 46 Tmc2a¹⁻¹⁸⁰ cells), 10 Hz (n = 62 WT, 37 Tmc2a¹⁻¹⁸⁰ cells) and 20 Hz (n = 57 WT 46, Tmc2a¹⁻¹⁸⁰ cells) water jet stimulus respectively, of 3 dpf larvae. **E'**, Same data as **E**, but quantified and normalized to WT control. *** p > 0.0001.

Chapter 6 – Discussion and future directions

GENERAL SUMMARY

Two main factors lead to dysfunction in hair cells: (1) defects in mechanotransduction, an apically regulated process; and (2) defects in synaptic transmission, a basolaterally regulated process. The data presented in this dissertation focus on two potential components of the mechanotransduction apparatus and another aspect that indirectly affects mechanotransduction. In Chapter 2, we report that genetic disruptions to the AP-1 complex result in a loss of hair-cell mechanosensitivity. In Chapter 3, we demonstrate that *tmhs* is necessary for hair cell mechanosensitivity. And in Chapter 4, we report on the opposing effects of exogenously expressing N- and C-terminal tails of Tmc2a on hair cell mechanosensitivity. Using a novel animal model, the goal of the research presented in this dissertation was to understand the functional importance of members of the MET complex and also the role of AP-1 in maintaining MET activity. This work builds on our understanding of how mechanotransduction in hair cells operates, an important component of functional hair cells.

CONCLUSIONS AND FUTURE DIRECTIONS

The AP-1 complex is necessary for hair cell mechanotransduction

Sorting and trafficking of proteins in hair cells is not well defined. Hair cells are polarized cell types containing distinct apical and basolateral membrane compartments with two distinct functions: mechanotransduction and synaptic transmission. Polarized

cells have the unique challenge of maintaining two functionally distinct domains (Drubin & Nelson, 1996; Nelson, 2003) and in the case of the hair cell the polarized expression of membrane proteins at the cell-surface must be regulated to ensure proper functioning. Moreover, the extracellular environment of the hair cell is very different at the apical and basolateral surfaces, further highlighting the importance the correct sorting particularly of channels and transporters in hair cells (Anniko & Wróblewski, 1986; Boyer et al., 2001; Couloigner et al., 2006; Hibino & Kurachi, 2006; Kim & Marcus, 2011; Lang et al., 2007). The data presented in Chapter 3 and 4 clearly demonstrate the necessity of AP-1-dependent protein sorting to establish and maintain functional MET in hair cells.

We show that two early-stop mutations in the *ap1b1* gene result in similar hearing and balance deficits, which are likely due to the loss of hair cell MET activity. We also show that the *ap1b1* mutations lead to missorting of the NKA as well as an epitope recognized by zns-5. Both of these proteins are normally restricted to the basolateral surfaces of hair cells, but in the *ap1b1* mutant these proteins sort to both basolateral and apical compartments of the cell. This missorting phenotype is also seen in polarized cells where either the $\mu 1A$ or $\mu 1B$ subunits of the AP-1 complex were mutated or missing (Fölsch et al., 1999; Gravotta et al., 2012). This confirms that the missorting of NKA is due to a dysfunctional AP-1 complex.

Interestingly, our data indicates that not all membrane proteins are missorted in *ap1b1* mutant hair cells. We analyzed several components of the afferent ribbon synapse and found that their localization was unaffected by mutations to *ap1b1*. These results indicate that the assembly and function of the ribbon synapse is unaffected by mutations to *ap1b1*.

Consistent with the involvement of AP-1 in vesicle formation and sorting membrane proteins, we also observe an accumulation of large membrane compartments and multivesicular bodies *ap1b1* mutant hair cells compared to wild type. The AP-1 complex is specialized for the sorting of membrane proteins from the Golgi apparatus and endosomal compartments to the early endosomes or the plasma membrane (Fölsch et al., 2009). Without a functional AP-1 complex, membrane proteins may be shuttled to the multivesicular body or lysosome through other compensatory sorting mechanisms, such as the AP-3 or ESCORT complexes. The absence of functional AP-1 complex may thus lead to the accumulation of membrane proteins normally targeted to the plasma membrane in intracellular endosomes and are subsequently degraded.

Several reports investigating AP-1 function in *C. elegans* suggest that the protein may regulate apical sorting processes. (Bae et al., 2006; Dwyer et al., 2001; Kaplan et al., 2010; Margeta et al., 2009). Preliminary data on Pcdh15 immunostaining and transient expression of GFP-Tmhsa in hair cells suggest that the *ap1b1* mutation can impact sorting of these membrane proteins. These experiments need to be repeated with the appropriate controls. As the optimal conditions for the Pcdh15 antibody are not yet determined, we were unable to confirm our immunolabeling with a *pcdh15* mutant for a negative control. And GFP-Tmhsa needs to be expressed transgenically in the *ap1b1* mutant to ensure all cells analyzed express similar amounts of the fusion protein.

All together, our results confirm what is observed in other systems when the AP-1 complex is missing or malfunctioning in polarized epithelial cells. Specifically, we see basolaterally targeted membrane proteins missorted to both the apical and basolateral plasma membrane in hair cells. We also have preliminary results indicating that sorting of apically targeted membrane proteins are also affected by mutations to *ap1b1*. Future

experiments with the *ap1b1* mutant should pursue these preliminary results as they could give valuable insight into how the *ap1b1* mutations more directly affect MET complex function. More broadly, however, the *ap1b1* mutant provides a unique opportunity to study the sorting of membrane proteins in hair cells in a model system that is especially suited to fluorescence imaging *in vivo*. Future studies could analyze sorting of proteins that are unique to hair cells and have polarized expression.

NKA localization to the basolateral compartment is necessary for hair cell function

In the presence of *ap1b1* mutations NKA missorts to apical membranes, and the amount of NKA is significantly reduced at the basolateral membrane. Given the importance of NKA in the balance of intracellular Na⁺ and K⁺ concentrations, we hypothesized that reductions in basolateral cell-surface expression of NKA resulted in a reduced capacity to maintain the appropriate intracellular Na⁺ and K⁺ concentrations. Indeed, we find that in *ap1b1* mutants the intracellular Na⁺ concentrations in the hair cell are significantly increased compared to wild type and this likely occurs over time. This ionic imbalance could lead to a disruption of the driving force for MET. A rise in internal Na⁺ may disrupt several cellular processes, including Na⁺ coupled transport activity, the electrochemical driving force for many ions including Na⁺, and therefore the resting membrane potential (Brodie et al., 1987; Mroz et al., 1993).

The observed ionic imbalance resulting from *ap1b1* mutants may lead to MET dysfunction in hair cells. However, the progressive disruption of membrane potential does not fit well with our data showing that MET activity in *ap1b1* mutants is immediately disrupted when the lateral line hair cells just become mechanosensitive at 2

dpf. This suggests that disruption of MET activity is due to some other consequence of *ap1b1* mutations.

One possible explanation for the *ap1b1* mutation-dependent MET disruption may be the loss of the driving force for K^+ and Ca^{2+} through the MET channel due to the missorting of NKA. The NKA pump plays an important role in maintaining intracellular concentrations of K^+ and Na^+ . The basolateral end of the hair cell is surrounded by a standard extracellular fluid, which is relatively high in Na^+ and low in K^+ . In contrast, the apical end of hair cells is surrounded by the endolymphatic fluid, which is high in K^+ and low in Na^+ , relative to intracellular concentrations (Anniko & Wróblewski, 1986; Russell & Sellick, 1976; Wangemann, 2006). This chemical gradient across the apical membrane is important for MET channel function and the driving force through the channel is derived from the gradient of K^+ across the membrane. At the basolateral surface, NKA maintains the relative concentrations of Na^+ and K^+ across the cell membrane, but in *ap1b1* mutants NKA is missorted to the apical compartment. This could disrupt the chemical gradient necessary for MET activity. This hypothesis does not, however, account for any perturbations to ionic concentrations in the endolymph or any other extracellular fluid that could have a disruptive effect on mechanotransduction.

However, when we blocked NKA activity with ouabain and assayed for MET function with FM 1-43, we saw no improvement to MET in lateral-line hair-cells of *ap1b1* mutants. This leads us to a different rationale for the malfunctioning of the MET complex. Although a chemical driving force across the MET channel is important for channel activity, another important component is the electrical driving force. At the basolateral end of the hair cell, the resting membrane potential is estimated to be around -60 mV, depending on the cell (Marcotti, 2012; Patuzzi, 2011). At the apical end of the

hair cell, the membrane potential can be anywhere between 0 mV and +90 mV. This difference in membrane potentials from the apical to basolateral ends of hair cells sets up the endocochlear potential and is thought to play a large role in the transduction of ions through the MET channel (Hibino et al., 2010; Wangemann, 2006). In the *ap1b1* mutant, we report an increase in intracellular Na⁺ compared to wild types. An increase in intracellular Na⁺ could cause an increase in the resting membrane potential at the basolateral end of the hair cell, which in turn would reduce the endocochlear potential. And when the endocochlear potential is reduced, the driving force for ions into hair cells through the MET channel would also be compromised, resulting in reduced mechanotransduction.

In conclusion, these results suggest that missorting of the NKA pump leads to misregulation of intracellular Na⁺ concentrations, which could have profound effects on the resting membrane potential of the hair cell. Future studies could look for potential disruptions to other ionic concentrations in hair cells. The results from these studies could indicate other pumps that may also be missorted in zebrafish hair cells.

***tmhsa* is both necessary and sufficient for hair cell function**

The precise function of Tmhs in the MET complex is still unknown. Recently, mouse *tmhs* was hypothesized to facilitate MET currents in hair cells (Xiong et al., 2012). The proposed mechanism for facilitation is similar to that of stargazin, another tetraspanin, which is a subunit of the AMPA receptor. We also show that Tmhsa is highly conserved between zebrafish, mice and humans suggesting its function in these species is also conserved. The functional conservation makes zebrafish a useful model to study the effects of *tmhsa* mutations on protein localization and hair cell function because of its

utility for studying fluorescently tagged proteins *in vivo* and hair cell physiology using Ca^{++} imaging techniques.

In Chapter 2, we demonstrate that transgenic GFP-Tmhs protein localizes to the tips of stereocilia, consistent with its suggested role in mechanotransduction (Xiong et al., 2012). We also show that expression of this transgenic construct specifically in hair cells is sufficient to rescue the hearing and balance phenotype in *astronaut* mutants. This confirms that the *astronaut* phenotype is due to a mutation in *tmhsa*, but it also demonstrates that expression of *tmhsa* only in hair cells and not the nervous system is sufficient for auditory and vestibular function in zebrafish.

The research by Xiong and colleagues provide evidence that mouse Tmhs is necessary to traffic the tip link protein Pcdh15 to the cell surface in culture (Xiong et al., 2012). How Tmhs mediates movement of Pcdh15 to the cell surface is still unknown. Sorting signals, like those recognized by AP-1, are commonly found on the cytoplasmic N- and C-terminal domains of membrane proteins (Bonifacino & Traub, 2003; Kelly & Owen, 2011). For this reason, it is interesting to consider the impact of the human TMHS mutation that exchanges the last three amino acids for 26 novel ones has on protein trafficking. Alternatively, this mutation could also affect protein stability or function. To determine the effect of this and other mutations, truncated versions of Tmhsa could identify potential motifs important for protein sorting and trafficking. With a similar approach and using additional methods to assay bundle morphology and mechanically evoked activity, we could map out regions of the protein that are also important in these pathways. Additional structure-function experiments like these could also determine the role of different regions of the protein and their importance to MET channel function.

Cytoplasmic N- and C- termini of *Tmc2a* affect hair cell MET

TMC1 is a deafness gene known to cause both dominant and recessive forms of deafness (Davoudi-Dehaghani et al., 2013; Gao et al., 2013; Hildebrand et al., 2010; Kurima et al., 2002; Heer et al., 2011). Its predicted topology has made it an interesting candidate for the MET channel (Kurima et al., 2003; Labay et al., 2010), the molecular identity of which is still unknown. In Chapter 5, we demonstrate that the *tmc1*, *tmc2a* and *tmc2b* genes are all expressed specifically in hair cells, but are each expressed in different populations of hair cells at differing expression levels and at different times. These patterns of gene expression could have implications on the sensitivity of hair cells to certain frequencies. Analysis of *Tmc1* and *Tmc2* in mice indicate that portions of the Organ of Corti are more sensitive to loss of one of these genes than the other, but in other sections an effect is only seen after loss of both genes (Kawashima et al., 2011). Hair cells of neuromasts may be tuned to relatively lower frequencies compared to hair cells in the inner ear. Furthermore, differences in frequency detection within the inner ear may exist between different patches of maculae and cristae. Future work should incorporate use of cutting-edge techniques, like TALEN (transcription activator-like effector nuclease) technology, to target and mutate each of these genes and test how they affect auditory and vestibular behaviors. TALENs are a new technology that is revolutionizing research in zebrafish as they can be targeted to introduce a double-stranded DNA break in the gene of interest (Cermak et al., 2011; Dahlem et al., 2012). This double-stranded break leads to non-homologous end joining, a mechanism of DNA repair which is error-prone and can result in small or large deletions or insertions.

We also show that exogenous expression of the cytoplasmic N- and C- terminal regions of *tmc2a* oppositely affect mechanosensitivity in neuromast hair cells. While the

N-terminal domain significantly reduces mechanically evoked Ca^{++} transients, the C-terminal tail significantly increases mechanically evoked Ca^{++} transients in neuromast hair cells. This implicates the N- and C- terminal regions in opposing aspects of the same pathway.

As the N-terminus of Tmc2a was identified in a screen for proteins that interact with Pcdh15, a key component of the MET complex, we hypothesize that exogenous expression of the N-terminal fragment of Tmc2a competes for the putative interaction between endogenous Tmc2a and Pcdh15. This would suggest that the interaction of Tmc2a with Pcdh15 is important for MET channel function in hair cells. Introduction of the N-terminal fragment of Tmc2a does not fully block mechanically evoked Ca^{++} . This could be due to the N-terminus not fully blocking the interaction between endogenous Tmc2a and Pcdh15, therefore residual MET activity remains. It could also indicate another component of the MET complex is maintaining its mechanosensitivity. Our lab recently found that kinocilial links between the kinocilia and stereocilia confer some mechanosensitivity (Kindt et al., 2012). In cells expressing the N-terminal fragment of Tmc2a, the residual mechanically-evoked Ca^{++} transients are potentially caused by responses originating in the kinocilia. These responses can have bidirectional sensitivity or “reversed” directional sensitivity, with respect to wild-type hair cells responding when the hair bundle is deflected toward the taller stereocilia. Careful examination of the directional sensitivities of hair cells expressing the N-terminal fragment could lead to insights into whether mechanosensitive elements originating from stereocilia or the kinocilia are disrupted in these cells.

Our original hypothesis was that exogenous expression of the C-terminus of Tmc2a would also reduce mechanical evoked activity in hair cells, as we assumed that

interrupting interactions of Tmc2a with other proteins at either the N- or C-terminus would have the same effect. However, we observe that the C-terminal region of Tmc2a alone increases mechanically evoked Ca^{++} activity, which is opposite of what we expected. This observation still places Tmc2a in the MET complex, however, it suggests that the C-terminus of Tmc2a may facilitate assembly of a larger MET complex, or disrupts an inhibitory interaction to hair cell mechanosensation.

A common feature between the cytoplasmic N- and C-terminal domains are predicted coiled-coil domains. Coiled-coil domains are known for mediating assembly of multimeric channel proteins and regulating channel activity (Fujiwara et al., 2013; Molland et al., 2010; Tsuruda et al., 2006). Analysis of mechanosensitivity in hair cells expressing deletion constructs lacking the coiled-coil regions in each of the N- and C-termini of Tmc2a would be a useful method to determine if these protein regions are important. This method could also be employed to identify any other protein regions within the two cytoplasmic domains that may play a role in mechanosensation.

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