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MYOSIN-XVA IS KEY MOLECULE IN ESTABLISHING THE ARCHITECTURE OF MECHANOSENSORY STEREOCILIA BUNDLES OF THE INNER EAR HAIR CELLS

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MYOSIN-XVA IS KEY MOLECULE IN ESTABLISHING THE ARCHITECTURE OF MECHANOSENSORY STEREOCILIA BUNDLES OF THE INNER EAR HAIR CELLS

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in the College of Medicine at the University of Kentucky

By

Shadan Hadi

Lexington, Kentucky

Director: Dr. Gregory I. Frolenkov, Professor of Physiology

Lexington, Kentucky

2018

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ABSTRACT OF THESIS

MYOSIN-XVA IS KEY MOLECULE IN ESTABLISHING THE ARCHITECTURE OF MECHANOSENSORY STEREOCILIA BUNDLES OF THE INNER EAR HAIR CELLS

Development of hair cell stereocilia bundles involves three stages: elongation, thickening, and supernumerary stereocilia retraction. Although Myo-XVa is known to be essential for stereocilia elongation, its role in retraction/thickening remains unknown. We quantified stereocilia numbers/diameters in *shaker-2* mice ($Myo15^{sh2}$) that have deficiencies in "long" and "short" isoforms of myosin-XVa, and in mice lacking only the "long" myosin-XVa isoform ($Myo15^{\Delta N}$). Our data showed that myosin-XVa is largely not involved in the developmental retraction of supernumerary stereocilia. In normal development, the diameters of the first (tallest)/second row stereocilia within a bundle are equal and grow simultaneously. The diameter of the third row stereocilia increases together with that of taller stereocilia until P1-2 and then either decreases almost two-fold in inner hair cells (IHCs) or stays the same in outer hair cells (OHCs), resulting in a prominent diameter gradation in IHCs and less prominent in OHCs. *Sh2* mutation abolishes this gradation in IHCs/OHCs. Stereocilia of all rows grow in diameters nearly equally in $Myo15^{sh2/sh2}$ IHCs and OHCs. Conversely, *ΔN* mutation does not affect normal stereocilia diameter gradation until ~P8. Therefore, myosin-XVa "short" isoform is essential for developmental thinning of third row stereocilia, which causes diameter gradation within a hair bundle.

KEYWORDS: MYO-XVa, Auditory Hair Cells, Stereocilia Diameter Gradation, Postnatal Development, *Shaker-2*

Shadan Hadi

July 16th, 2018

MYOSIN-XVA IS KEY MOLECULE IN ESTABLISHING THE ARCHITECTURE OF MECHANOSENSORY STEREOCILIA BUNDLES OF THE INNER EAR HAIR CELLS

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July 16th, 2018

For my parents

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SECTION 1: BACKGROUND

1.1 The Anatomy and Physiology of the Ear

The mammalian ear is divided into three distinct parts that orchestrate the organism's ability to hear. The **outer ear**, the most external part, which receives sound wave stimuli; it is medially followed by the (3 cm) ear canal that serves as a bridge to transverse the sound waves to the **middle ear** and, therefore, resulting into the mechanical movement of the downstream tympanic membrane or the eardrum (Lehnhardt and Lehnhardt 2003).The tympanic membrane, which is approximately 85mm^2, is a structural mixture of collagen, epithelial, and connective tissues, in addition to neural axons and capillaries (Harrison 1988). The mechanical vibrations of the tympanic membrane by the sound stimulus causes the movement of three bones known as the auditory ossicles: the malleus, the incus, and the stapes; ultimately, the movement of these bones, mainly the stapes, transverses into the **inner ear** through an opening called the oval window, where the stapes is attached (Harrison 1988; Lehnhardt and Lehnhardt 2003). The three main ear parts are illustrated in Fig.1.1-1 below:

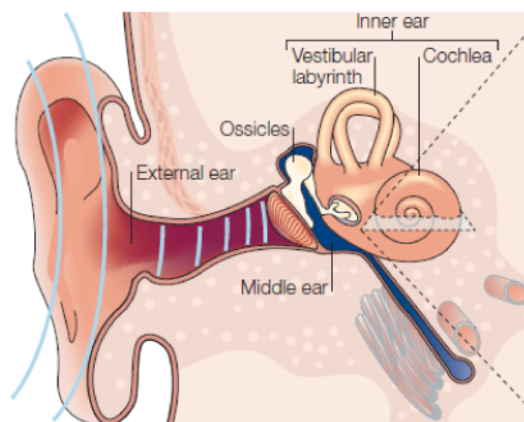


Figure 1.1-1 Anatomical overview of the mammalian ear
This figure is modified from (Frolenkov, Belyantseva et al. 2004).

The inner ear encompasses both the vestibular system, which allows us to maintain balance, and the cochlear system that is necessary for our auditory function (Fig.1.1-1). Both the vestibular and the cochlear organs are surrounded by a membrane that is encapsulated by a bony structure that well-fits into the temporal bones on both sides of the organisms's head (Lehnhardt and Lehnhardt 2003). The cochlear and the vestibular organs are exposed to a unique extracellular fluid with high amounts of potassium (K^+) and low amounts of sodium (Na^+), known as the endolymph (Kazmierczak, Harris et al. 2015) (Fig.1.1-2). The ionic composition of the endolymph is somewhat similar to that of the cytosol. In contrast, the other fluid-filled spaces in the inner ear contain the perilymph, which has high amounts of sodium (Na^+) and low amounts of potassium (K^+), just like the interstitial fluid in our body (Kazmierczak, Harris et al. 2015)(Fig1.1-2). The curled 35mm structure known as the cochlea encompasses three compartments: a middle compartment called the Scala media, which houses the hearing sensory organ (the organ of Corti is filled with endolymph, a top and bottom compartments known as the Scala vestibuli and Scala tympani, respectively, that are filled with perilymph (Harrison 1988), as shown in Fig1.1- 2 below:

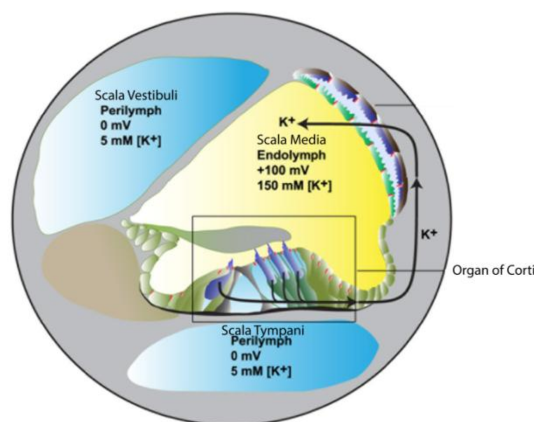


Figure 1.1-2: Fluids of the cochlea
This Figure is modified from (Harrison 1988; Kazmierczak, Harris et al. 2015).

Inside the cochlea, the organ of Corti tissue is comprised of the sensory auditory hair cells: one row of inner hair cells (IHCs) that detect the sound, and three rows of outer hair cells (OHCs) that amplify the sound (Fig.1.1-3) and (Fig.1.1-4). Additionally, other non-sensory cells, such as the supporting, Dieters, and pillar cells, provide mechanical support and stability for the hair cells (Harrison 1988).

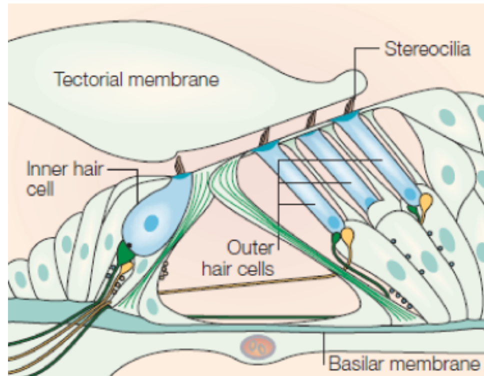


Figure 1.1-3: The sensory cells within the organ of Corti
This figure is modified from (Frolenkov, Belyantseva et al. 2004).

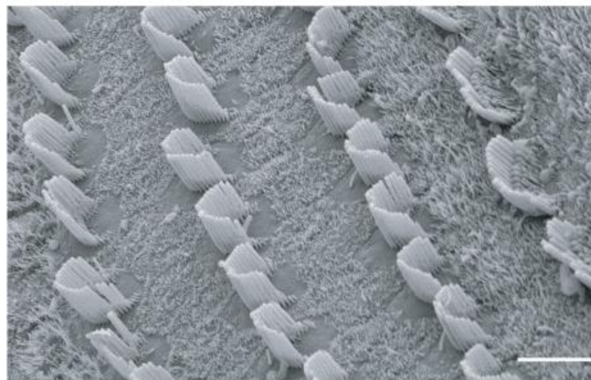


Figure 1.1-4: Scanning electron microscopy showing row arrangement of the hair cells
This figure is modified from (Schwander, Kachar et al. 2010).

The inner ear mechanosensory auditory IHCs and OHCs are characterized by their apical microvilli-like structures known as stereocilia (Fig.1.1-4). In mature mammalian hair cells, these stereocilia display three distinct rows that are graded in length forming a hallmark staircase hair bundle (Tilney and Saunders 1983). The length of rows/bundles

stereocilia is established in accordance with the frequency that is detected by these cells; for example, cells that detect high frequency sounds, (located at the base of the organ of Corti), have short stereocilia; in contrast, stereocilia in cells that detect low frequency sounds, (located at the apex), are long (Tilney and Saunders 1983). Mechanosensitive stereocilia are supported by parallel actin filaments that are connected by cross-bridges stabilizing the actin core (Tilney, Egelman et al. 1983). Running along the shaft of the stereocilia, these filaments turn into compact rootlets as they intercalate the cuticular plate at the tapered base (Fig.1.1-5). Around this tapered base, stereocilia are ultimately deflected by a mechanical stimulus (Tilney, Egelman et al. 1983), see (Fig.1.1-5).

The organ of Corti is confined between two acellular structures (Fig.1.1-3); on the top of the organ of Corti (covering the IHCs and OHCs) is the tectorial membrane, which physically contacts the first row of stereocilia in the OHC bundles (Fig.1.1-3). Conversely, at the bottom of the organ of Corti is the basilar membrane that specifically determines the location/hair cells that detect a certain sound frequency (Békésy 1960), see (Fig.1.1-3). As sound comes into the inner ear, its vibrations cause the movement of the tectorial membrane, the basilar membrane, as well as the endolymph; thus, this mechanical vibration causes the deflection of the mechanosensitive stereocilia in the positive direction (Fig1.1-5).

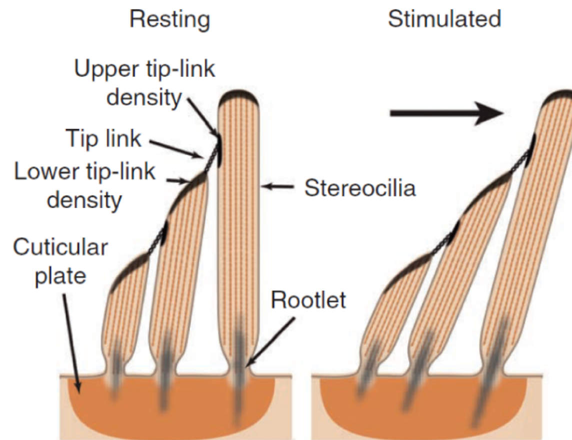


Figure 1.1-5: Stereocilia displacement during hair bundle stimulation
This figure is modified from (Peng, Salles et al. 2011).

Stereocilia displacement is produced by the stereocilia direct attachment with the vibrating tectorial membrane, such as in the OHCs, or through the vibration of the surrounding fluid, such as in the IHCs (Fig.1.1-3). Because of the large difference in electrical potential between the cytosol of the hair cells, (-70mV to -55mV) (Pujol, Nouvian et al. 2016) and the outside endolymph (+80mVto +100mV) (Pujol, Nouvian et al. 2016), see Fig1.1-2, deflection of the hair bundle in the positive direction allows the flow of positive ions into the cell (depolarization), through mechanosensitive channels that are located at the tips of transducing second and third row stereocilia (Beurg, Fettiplace et al. 2009), see Fig.1.1-5.Therefore, the mechanical signal of sound turns into electrical signal that is transmitted to the brain, predominantly from the inner hair cells, which are largely innervated by afferent nerve fibers (Harrison 1988). In contrast, the role of OHCs is different– they amplify the sound-induced vibrations in the organ of Corti in order for the IHCs to be able to detect them (Fettiplace and Hackney 2006).

1.2 Development of the Auditory Cells in the Inner Ear

The growth of stereocilia bundles into a sophisticated hallmark staircase-like structure is a relatively quick process, which primarily occurs within a maximum of 20-22 days, in both the mammalian and non-mammalian inner ear (Tilney, Tilney et al. 1986; Kaltenbach, Falzarano et al. 1994). With the interest of how the auditory cells form their unique architecture, Lewis Tilney and colleagues studied the embryonic development of hair cells using the chick organ of Corti (Fig.1.2-1). Similarly, James Kaltenbach and colleagues used hamsters to study the mammalian hair cell development to complete the understanding of critical developmental stages that are conserved, and therefore are fundamental for hearing (Tilney, Tilney et al. 1986; Kaltenbach, Falzarano et al. 1994).

Using chick embryos, Tilney demonstrated the organ of Corti maintains an average of 10,500 hair cell bundles that seem to be already developed by an embryonic age of 8 days (ED8) and are maintained throughout life (Tilney, Tilney et al. 1986). Tracking the development of bird inner and outer hair cells shows that most dramatic developmental events in the organ of Corti occur within the hair cells (Fig1.2-1).

According to Tilney et al., at an embryonic age of 9 days (ED9), development of the hair cell bundle in chicks starts when the previously grown kinocilium moves towards the outside of the developing bundle in order to determine the polarity of the future hair cell (Tilney, Tilney et al. 1986; Frolenkov, Belyantseva et al. 2004). At around (ED 10), the stereocilia on the cuticular plate are short (about half a micron long), and then they start to continuously elongate for the next 2-3 days as the staircase becomes prominent by ED11 (Fig1.2-1). All stereocilia in the entire chick auditory organ (from base to apex) grow to about 1.5 µm long by ED12, which would be the height of stereocilia in mature

hair cells at the base (Tilney, Tilney et al. 1986). When stereocilia height growth stops by around ED12, all stereocilia begin to increase in diameter up to ED17 (Tilney, Tilney et al. 1986). As the stereocilia thickening process end, stereocilia in the apical hair bundles start increasing in height again up to about 2.2-2.8 µm; therefore, hair cells at the base of the organ the organ of Corti mature before those in the apex, and the two are distinguished by their difference in stereocilia heights (Tilney, Tilney et al. 1986). The developmental stages described by Tilney are shown the Fig1.2-1 below:

Figure 1.2-1: Embryonic development of the hair bundle in birds
This figure is reproduced by (Frolenkov, Belyantseva et al. 2004) from (Tilney, Tilney et al. 1986) (modified)

Likewise, Kaltenbach observed analogous events during the postnatal development of stereocilia in the mammalian hair cell bundles (Fig.1.2-2). At postnatal day 0 (P0), hair bundles have similarly short and condensed stereocilia in addition to the kinocilium (Kaltenbach, Falzarano et al. 1994). During the time period between P2-6, stereocilia grow in length and width to form a "crescent" or a "W"-like staircase as in inner hair bundles or outer hair cells, respectively (Kaltenbach, Falzarano et al. 1994). Furthermore, during this period, stereocilia form their tip links at P4 (see Fig.1.1-5), and the extra stereocilia in the bundle start to retract (Kaltenbach, Falzarano et al. 1994). By P12-14, the cell's kinocilium also retracts, and P14 outer hair cell bundles become mature (although they have already stopped thickening and elongating by P4); on the other hand,

inner hair cells continue to grow to become mature at P16-18 (Kaltenbach, Falzarano et al. 1994). Just like in birds (Tilney, Tilney et al. 1986), mammalian hair cells at the base of the organ of Corti, ie. the ones that respond to high frequencies, mature before those at the apex (Kaltenbach, Falzarano et al. 1994). The described developmental events are shown in Fig.1.2-2 below:

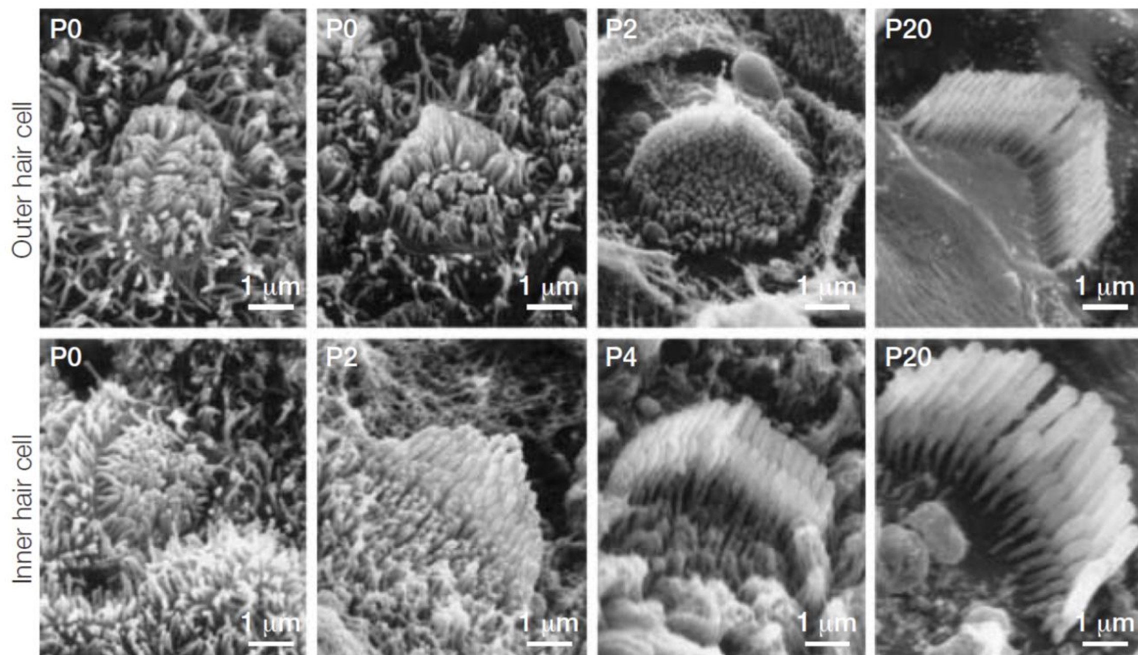


Figure 1.2-2: Electron microscopy of the hamster hair cell bundles during postnatal development. This figure is reproduced by (Frolenkov, Belyantseva et al. 2004) from (Kaltenbach, Falzarano et al. 1994) (modified).

1.3 Deafness Diseases in Humans

Following the most current statistics on deafness within the United States, the National Institute on Deafness and Other Communication Disorders (NIDCD) reports that 2-3 out of 1000 babies are born deaf (congenital hearing loss) (NIDCD 2016). Moreover, the Center for Disease Control and Prevention (CDC) states that "about 1 out of 2 cases of hearing loss in babies is due to genetic causes" (CDC 2015). The majority of deafness

cases are classified as “sensorineural” as they originate from the organ of Corti, specifically due to some form of physiological abnormality in the auditory hair cells or their neural connection with the brain (CDC 2015). Additionally, hearing loss can be progressive, which means the patient may hear at the beginning of his/her life and loses hearing over time, or congenitally profound, if the patient does not have the ability to hear from birth (CDC 2015). It is worth mentioning that, in contrast to birds and other non-mammalian species, mammalian auditory hair cells do not regenerate (Corwin and Cotanche 1988; Roberson and Rubel 1994). Therefore, they have to be maintained throughout life in order to maintain hearing.

1.4 Myosin-XVa in the Auditory Hair Cells and its Impact on Deafness

Cells may express diverse myosin proteins that are necessary for normal function; for example, in muscles, cells express the frequently known “conventional myosins” that interact with actin filaments allowing for muscle contraction. In contrast to myocytes, in the inner ear, auditory hair cells express “unconventional myosins”, which are involved in the molecular trafficking of cargo as the myosin protein “crawls” over actin filaments (Hasson 1997; Wang, Liang et al. 1998). In a hair cell bundle, as well as in the actin-rich cuticular plate at the apex of a hair cell, different myosin proteins are found along stereocilia actin filaments and have different localization patterns that are related to their function (Hasson 1997).

An unconventional myosin that we explore in our study is Myosin-XVa (Myo-XVa or Myo-15a). Myosin-XVa is expressed in the organ of Corti IHCs and OHCs as well as vestibular hair bundles, specifically at the tips of the stereocilia (Belyantseva, Boger et al. 2003), in addition to the kidney, the liver, and the pituitary and endocrine glands (Probst,

Fridell et al. 1998; Rehman, Bird et al. 2016). Myosin-XVa in humans, or Myosin-XV in mice, which we use as a model in our study, is produced by *Myo-XVA* gene on chromosome 17 in humans or *Myo-XV* on chromosome 11 in mice, respectively (Probst, Fridell et al. 1998; Rehman, Bird et al. 2016). *Myo-XVA* is 66 exon long and through alternative splicing during gene transcription, two isoforms of the Myo-XVa protein are made: an exceptionally big long isoform 1 and a short isoform 2 (Fig.1.4-1). Each of the Myo-XVa long/short isoforms have a motor domain allowing the Myo-XVa molecule to climb over actin, three IQ domains, and a tail domain that allows myosin to carry its molecular cargo (Fig.4-1) (Hasson 1997; Wang, Liang et al. 1998; Fang, Indzhykulian et al. 2015; Rehman, Bird et al. 2016). It is important to note that isoform 1 is named as "the long isoform" because of its bulky N-terminus (133 KDa), which is stated as " N-terminal extension" by (Fang, Indzhykulian et al. 2015), as illustrated in Fig,1.4-1 below:

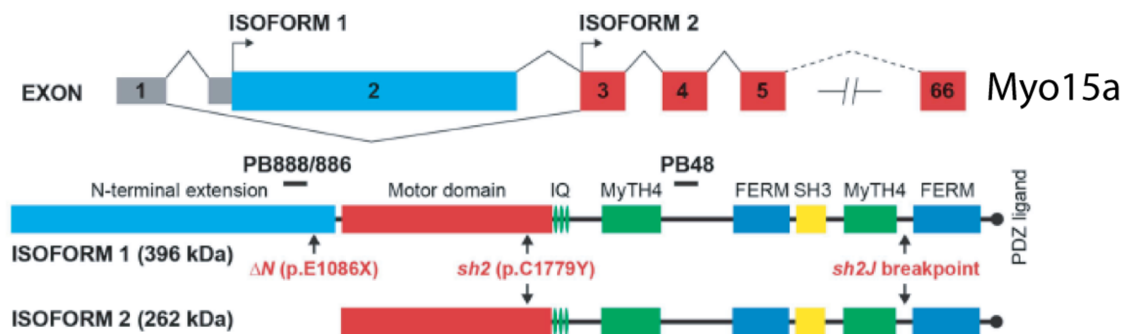


Figure 1.4-1: The structure of *Myo-XVa* and its protein isoforms
This figure is modified from (Fang, Indzhykulian et al. 2015)

Myo-XVa uses its motor domain to climb over actin filaments carrying necessary molecules for stereocilia elongation, such as whirlin (Belyantseva, Boger et al. 2005). Fig. 1.4-2 below illustrates actin filaments inside the stereocilia, which are crosslinked by

cross-linker proteins; note the localization of Myo-XVa and whirlin at the tips of stereocilia:

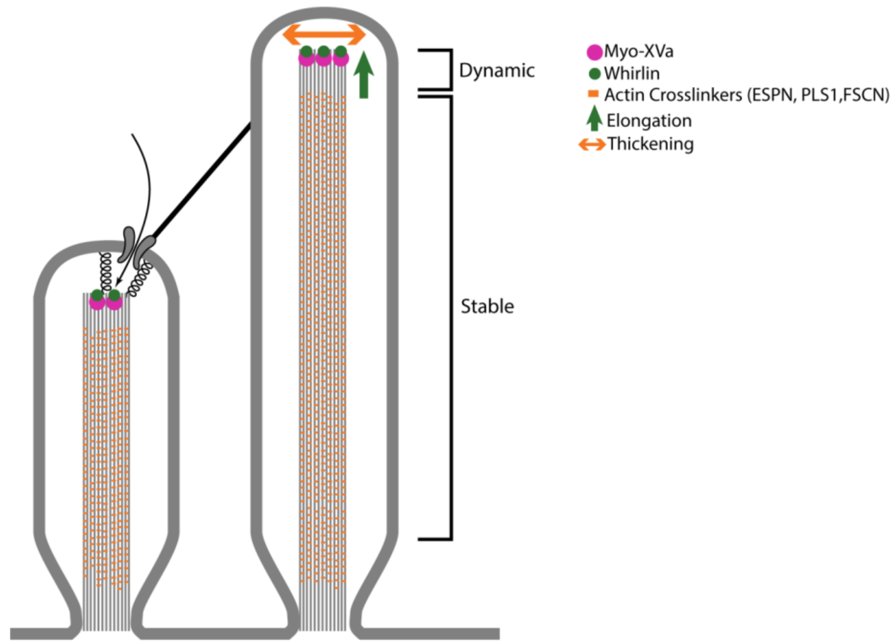


Figure 1.4-2: Some actin-binding proteins that are known to be necessary for the growth of stereocilia. This figure is redrawn from (Frolenkov, Belyantseva et al. 2004; Belyantseva, Boger et al. 2005; Krey, Krystofiak et al. 2016; McGrath, Roy et al. 2017)

Myo-XVa long and short isoform are expressed at various time points during hair bundle development (Fang, Indzhykulian et al. 2015). During early postnatal days, hair cell bundles express only the short isoform in order to develop the proper bundle architecture; however, by P6, protein expression is altered into the Myo-XVa long isoform, which is necessary for stereocilia maintenance in hair cells (Fang, Indzhykulian et al. 2015). Additionally, Myo-XVa long isoform have different expression patterns within the stereocilia hair bundles: it is expressed in all stereocilia rows in the OHCs and only second and third row stereocilia in IHCs (Fang, Indzhykulian et al. 2015), i.e. the ones that harbor mechanotransduction channels– “transducing” stereocilia (Beurg, Fettiplace et al. 2009).

Mutations in Myo-XVa long and short isoforms yield to different hair cell abnormalities with pronounced disruption in the stereocilia bundles; thus, leading to different deafness outcomes in both rodents and humans (Probst, Fridell et al. 1998; Fang, Indzhykulian et al. 2015; Rehman, Bird et al. 2016). For example, a point mutation in the motor domain of Myo-XVa, through amino acid replacement of Cysteine by Tyrosine in $Myo15^{sh2/sh2}$ (*Shaker-2*) mice (Probst, Fridell et al. 1998), Fig.1.4-1, affects both long and short isoforms; therefore, impairing the delivery of cargo to the tips of the stereocilia (Probst, Fridell et al. 1998; Fang, Indzhykulian et al. 2015). If Myo-XVa cannot climb over actin to carry "important" molecules, like whirlin, to the tips of the stereocilia, this can affect their normal development and elongation (Probst, Fridell et al. 1998; Belyantseva, Boger et al. 2005; Fang, Indzhykulian et al. 2015). Therefore, stereocilia bundles are short in $Myo15^{sh2/sh2}$ (*Shaker-2*) mice (Fig.1.4-3, A). These mice are disoriented, due to vestibular dysfunction, and profoundly deaf (Probst, Fridell et al. 1998).

On the other hand, loss of only the long isoform of *Myo15a* causes a different phenotype. Fang and co-authors introduced a nonsense mutation in the N- terminus of the long isoform that generates a stop codon and prevents the translation of the rest of the Myo-XVa long isoform; therefore, only the short isoform would be functional (Fang, Indzhykulian et al. 2015). According to Qing Fang et al., Myo-XVa isoform-specific knockout mutation does not affect the elongation of the stereocilia but causes progressive second and third row stereocilia degeneration in adult hair bundles, such as that seen in $myo15^{\Delta N/\Delta N}$ (ΔN) mice (Fig.1.4-3). Hair bundles from the two mouse strains (*shaker-2* and ΔN) are illustrated in Fig.1.4-3 below:

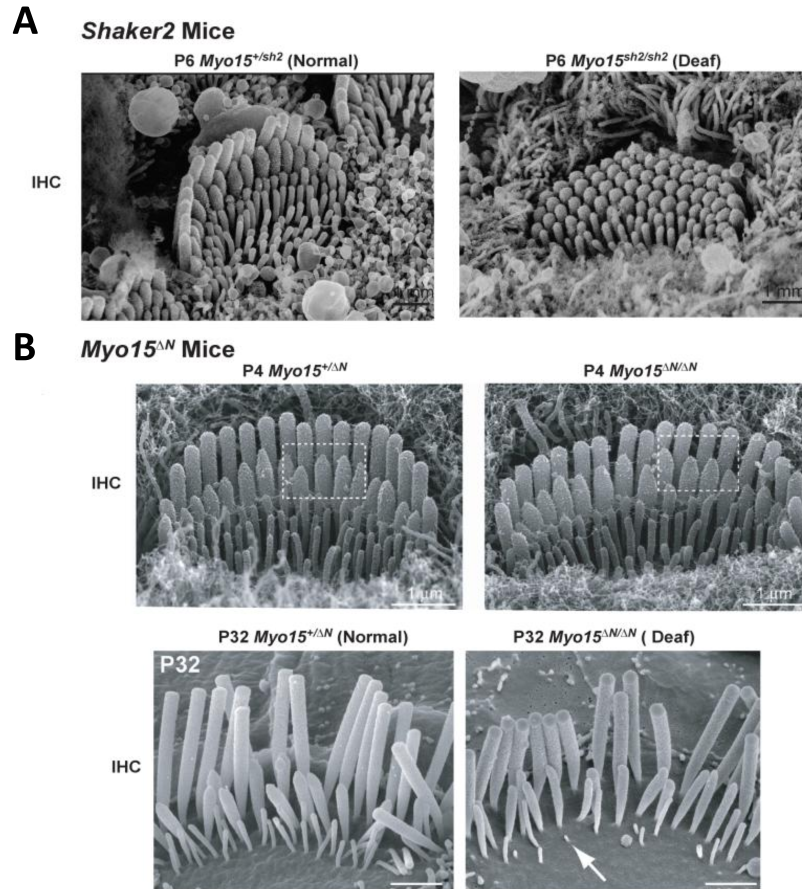


Figure 1.4-3: Morphological comparison of control heterozygous and homozygous hair cell bundles in different *Myo15* mouse strains. Panel A: *Myo15*^{sh2} inner hair cells; note short stereocilia in *Myo15*$^{sh2/sh2}$ bundles; images were acquired by Andrew Alexander in our lab. Panel B (top): *Myo15*$^{\Delta N}$ inner hair cells; note normal stereocilia in young *Myo15*$^{\Delta N/\Delta N}$ bundles; images are modified from (Fang, Indzhykulian et al. 2015). Panel B (bottom): *Myo15*$^{\Delta N}$ inner hair cells; note third row stereocilia degeneration in adult *Myo15*$^{\Delta N/\Delta N}$ bundles; images are modified from (Fang, Indzhykulian et al. 2015).

Both *Myo15*$^{sh2/sh2}$ (*Shaker-2*) mice and *Myo15*$^{\Delta N/\Delta N}$ (ΔN) mice are congenitally deaf.

Myo-XVa was the first unconventional myosin that was discovered to cause hearing loss, in the mutant *shaker-2* (*Myo15*$^{sh2/sh2}$) mice, and is responsible for DFNB3 “Non-syndromic Recessive Deafness 3” in humans (caused by mutation in MYO-XVA) (Probst, Fridell et al. 1998). The hallmark abnormally short stereocilia suggests that a mutation in Myo-XVa is linked to disruption in the normal development of stereocilia

elongation in the *shaker-2* hair bundles (Probst, Fridell et al. 1998). In fact, following studies have shown that whirler mice (*wi/wi*) which lack whirlin, a scaffolding protein that is carried by Myo-XVa to stereocilia tips (Belyantseva, Boger et al. 2005) (Fig.1.4-2), are also deaf and have short stereocilia (Belyantseva, Labay et al. 2003; Mogensen, Rzadzinska et al. 2007). Since Myo-XVa is needed for the normal elongation of stereocilia (Probst, Fridell et al. 1998; Belyantseva, Boger et al. 2003; Belyantseva, Boger et al. 2005), we were curious about whether it has a potential dual role in the thickening of stereocilia during development.

SECTION 2: INTRODUCTION

During the postnatal development of mammalian auditory hair cells, mechanosensory stereocilia arise from the numerous microvilli that originate from the surface of the cell (Kaltenbach, Falzarano et al. 1994). Through a series of consequent, but time-varied, developmental stages that are conserved in rodents and non-mammalian species, stereocilia grow in a region/row-specific graded-height manner forming the hallmark staircase (Tilney and Saunders 1983; Kaltenbach, Falzarano et al. 1994). In addition to stereocilia lengthening, the microvilli retract and the remaining stereocilia increase in thickness forming mature bundles (Tilney, Cotanche et al. 1992; Kaltenbach, Falzarano et al. 1994). To the best of our knowledge, although the molecules that may play a role in the programmed retraction of the microvilli are obscure, fortunately, the field has advanced to discover a number of proteins that are detrimental for stereocilia growth. For example, to attain the appropriate stereocilia length, whirlin, a scaffolding protein is carried by the tail domain of Myo-XVa to the tips of the stereocilia, where these two proteins are co-localized (Belyantseva, Boger et al. 2003; Mogensen, Rzadzinska et al. 2007). When the two of them arrive to the stereocilia tips, they initiate stereocilia elongation (Belyantseva, Boger et al. 2005), refer to (Fig.1.4-2). With their yet unknown direct impact on F-actin polymerization, whirlin and Myo-XVa interact with “actin bundling protein” eps8, at its N and C- termini respectively, creating a complex that is crucial for the elongation of stereocilia(Manor, Disanza et al. 2011). The scientific assumptions behind this process entail the hypothesis that whirlin stabilizes F-actin and eps8 prevents capping proteins from binding to the barbed (growing) end of F-actin (Manor, Disanza et al. 2011). Other actin binding proteins, such as twinflin-2 that is

localized to the tips of only transducding shorter row stereocilia, may hinder actin polymerization in the shorter rows of stereocilia, forming a characteristic staircase shape of the hair cell bundle (Peng, Belyantseva et al. 2009). In fact, modulating the expression of the proteins within Myo-XVa complex *in vitro* has been shown to alter the morphological definition of the staircase through length abnormalities, such as those seen in homozygous *shaker-2* and whirler stereocilia (Probst, Fridell et al. 1998; Mogensen, Rzadzinska et al. 2007; Manor, Disanza et al. 2011). Similarly, proteins like espin-1 which traffics along the F-actin by Myo-IIIa are also necessary for stereocilia elongation (Manor, Disanza et al. 2011). Conversely, molecules like eps8L2 may not contribute to stereocilia elongation per se but are responsible for maintaining the proper stereocilia height/diameter in mature postnatal bundles, possibly through environmentally driven dynamic adjustments in the actin core that is generally stable (Furness, Johnson et al. 2013)

Key actin bundling molecules, like espin, plastin-1, and fascin (Krey, Krystofiak et al. 2016), have been shown to be required for sterecoilia widening and increase in thickness during development (Sekerkova, Richter et al. 2011; Krey, Krystofiak et al. 2016), refer to (Fig.1.4.-2). For example, Plasin-1, a rich actin cross-linker, determines stereocilia diameters through preserving actin filaments from depolymerization and holding them at an appropriate spacing gap, about 10nm, to maintain the desired stereocilia thickness (Krey, Krystofiak et al. 2016). Likewise, actin-linking espin and glutaredoxin cysteine-rich 1 (Grxcr1) proteins allow for stereocilia thickening upon increasing the filaments within the actin core during development (Odeh, Hunker et al. 2010; Sekerkova, Richter et al. 2011). Early studies proposed that the thickness of stereocilia is determined by the

number of actin filaments and that the process of stereocilia widening/elongation are independent (Tilney, Tilney et al. 1986; Kaltenbach, Falzarano et al. 1994). However, past observations of short stereocilia in homozygous *shake-2 (Myo15*$^{sh2/sh2}$) and whirler (*wi/wi)* thick hair bundles drove our interest to investigate the hypothesis that this process may be concurrent. Since Myo-XVa is a "key" molecule affecting stereocilia growth, it may be responsible for determining not only stereocilia lengths but also their diameters. *Myo-XVa* produces two structurally similar isoforms of Myo-XVa, with the exception of a big N-terminus in the long isoform (Fang, Indzhykulian et al. 2015). Different mutations in Myo-XVa long/short isoforms lead to deafness that is associated with different bundle abnormalities ─ short stereocilia in *shaker-2* bundles that lack both isoforms and third row stereocilia degeneration in the long isofrom-specific knockout *Myo15*$^{\Delta N/\Delta N}$ mature bundles, confirming their role in elongation and maintenance, respectively (Fang, Indzhykulian et al. 2015). Therefore, to track the discrete functional role of Myo-XVa isoforms during development, we used Scanning Electron Microscopy (SEM) to image young and mature postnatal IHCs and OHCs in control (normal-hearing) heterozygous and deaf homozygous *Myo15*^{sh2} and *Myo15*$^{\Delta N}$ mice. We quantified the developmental changes in the stereocilia number and thickness. Our study indicates that Myo-XVa is not involved in the programmed retraction of the extra stereocilia during bundle maturation. Contrarily, we show that Myo-XVa, interestingly only its short isoform, indeed regulates stereocilia thickness and is responsible for the diameter gradation within the hair cell bundles.

SECTION 3: METHODS

3.1 Animals and Tissue Preparation

All animal procedures were approved by the University of Kentucky Animal Care and Use Committee (protocol# 903M2005).

We used *shaker-2* ($Myo15^{sh2}$) (Beyer, Odeh et al. 2000) and Myo-XVa isoform-specific knockout (C57BL6) mice ($Myo15^{\Delta N}$) (Fang, Indzhykulian et al. 2015), typically by breeding heterozygous/homozygous pairs that would generate $Myo15^{+/sh2}$ and $Myo15^{+/\Delta N}$ controls as well as $Myo15s^{sh2/sh2}$ and $Myo15^{\Delta N/\Delta N}$ mutants. Ages of interest were during postnatal days P0-20. For each postnatal age, 1-4 mice were used for each genotype from both mice strains. Mouse temporal bones were carefully dissected in cold Leibovitz (L-15) solution to extract the cochleae. Cochleae (age <P5) were micro-dissected and organs of Corti were fixed with 1ml of (3% Formaldehyde/Glutaraldehyde in 0.1M Sodium Cacodylate pH. 7.4) with 2mM Calcium Chloride $CaCl_2$(10 µm of 2M $CaCl_2$ is added to fixative prior to use). The tectorial membrane was removed prior to fixation. To prevent damage of the hair cells in the basal region of the organ of Corti that become very fragile in older animals, whole cochleae (age>P5) were perfused with fixative through the oval window and fixed overnight at 4°C. The organs of Corti were dissected in the following days. In the following days approximately 4ml of distilled water was added to dilute the fixative and prevent sample over-fixation. All organs of Corti were dehydrated with graded series of alcohol using 200 proof ethanol and critical point dried with liquid CO_2 using Leica EM CPD300 automated machine. Dried samples were carefully mounted over aluminum blots and sputter coated with platinum (5nm thickness) for later SEM imaging.

3.2 Sample Imaging

We used S-3400 Hitachi Scanning Electron Microscopy to image inner and outer hair bundles in the middle region of the organ of Corti. We imaged at least 6 neighboring IHCs and OHCs in control heterozygous and mutant homozygous mouse strains. To keep a reasonable compromise between a relatively large field of view with still good resolution, typically three hairs cells were included in one image. Images were taken at a scale of 2.5- 5µm and a working distance (WD) of 8-18µm. To obtain stereocilia count and diameter quantification, the angle of sample in relation to the EM beam did not matter as long as the stereocilia were clearly seen within the rows. Therefore, a front and top views of the hair bundle were sufficient for data analysis.

3.3 Data Analysis: Stereocilia Number and Diameter Measurements

Using ImageJ software (ver.1.51) each SEM image scale was converted to nm/pixel in order to obtain stereocilia diameter measurements in nanometers. For all postnatal ages (P0-P20), we manually counted total stereocilia/bundle for IHCs and OHCs from *shaker-2* ($Myo15^{sh2}$) and "Δ*N*" ($Myo15^{\Delta N}$) control and homozygous organ of Corti samples, see Fig.3.3-1, A. Then, we calculated the average stereocilia count per bundle of all IHCs/OHCs at the particular postnatal age and genotype. Control data from $Myo15^{+/sh2}$ and $Myo15^{+/\Delta N}$ mice combined together, since we did not see any significant differences. Data were presented as Means±SEM.

To quantify the diameter of the first, second, and third row stereocilia, using ImageJ, a straight line was drawn between the visibly defined edges of each stereocilium, see Fig. 3.3-1. The stereocilia diameters in each row were averaged in each cell and those

averages were further averaged to calculate the average diameter/standard error for all cells with same postnatal age and genotype. Control animals were combined together. All graphs were generated by OriginPro (ver.2018b). Average number of stereocilia per bundle and the average diameters of different rows for both *shaker-2* ($Myo15^{sh2}$) and ($Myo15^{\Delta N}$) IHCs and OHCs were plotted as a function of postnatal ages. Data were presented as Means±SEM. All statistical analysis was performed with two-sample-*t*-test; with Welch Correction. All figures were constructed using Adobe Illustrator CC (2017.1.0 Release).

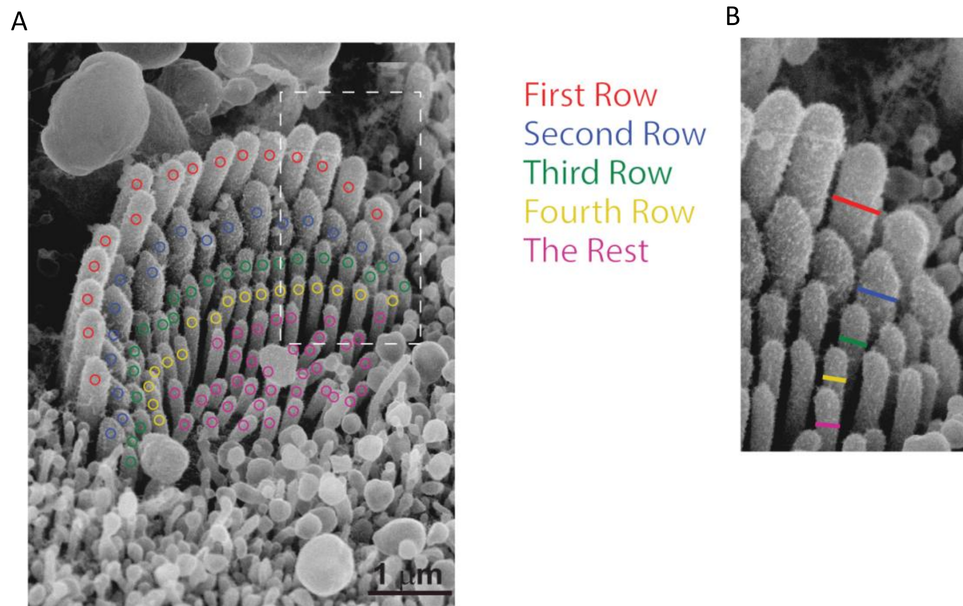
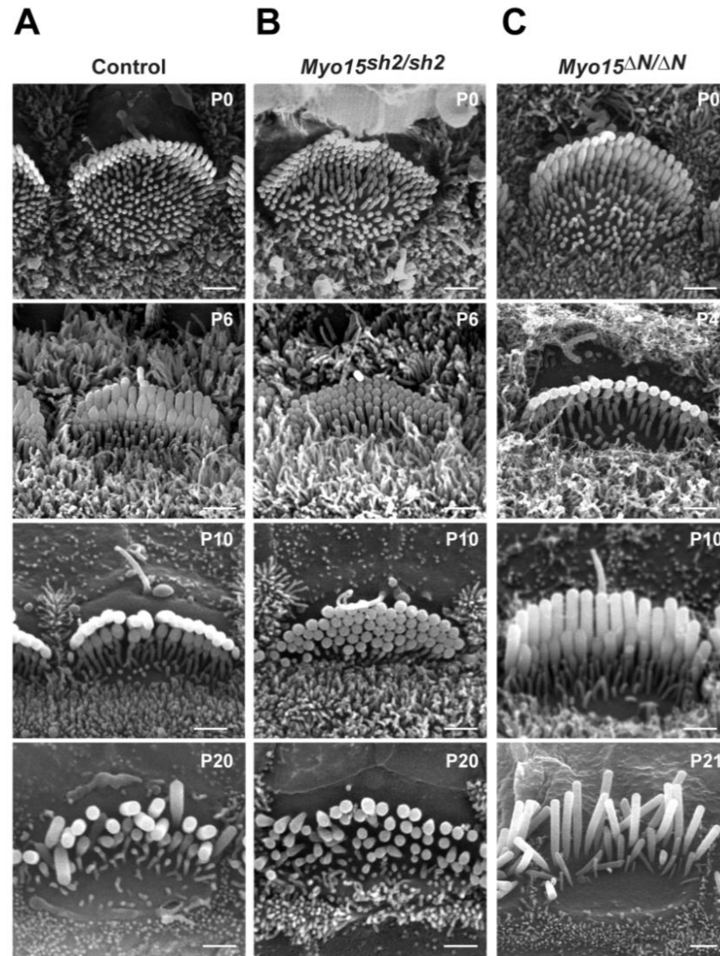


Figure 3.3-1 Quantification of stereocilia number and diameter in the hair bundles. Panel A: Total number of stereocilia in the IHC bundle. Panel B: Stereocilia diameter measurement (in nm) in different stereocilia rows in IHC bundle. Note color coded stereocilia rows within the bundle staircase. These two preliminary images were acquired by Andrew Alexander from our lab

SECTION 4: RESULTS

4.1 Myo-XVa does not regulate retraction of the extranumerary stereocilia in the auditory hair cells during their developmental maturation

A key developmental stage that we hypothesized to be regulated by Myo-XVa is the retraction of supernumerary stereocilia. Therefore, we tracked the changes of the total number of stereocilia per hair bundle in a presumably critical developmental time period during postnatal days (P0-P20). Our data indicate that starting from birth (P0), in all control and homozygous $Myo15^{sh2}$ and $myo15^{\Delta N}$ inner hair cells, nascent hair bundles have a large number of stereocilia, 200-280 per bundle. Later, this number decreases by 50%-60% through the retraction of the extra microvilli over the course of 6 days (P0-P6) (Fig. 4.1-1, D; see decrease in the number of stereocilia in the bundle in SEM images on Fig. 4.1-1, A, B, C). The total number of stereocilia remains constant and does not significantly change beyond postnatal days 6-8 throughout the adult age in both control and $Myo15^{sh2/sh2}$ mice. However, number of stereocilia per bundle continue to decrease in $Myo15^{\Delta N/\Delta N}$ mice due to selective 2nd and 3rd row degeneration in adult bundles, which confirms the previous observations by (Fang, Indzhykulian et al. 2015) (see blue line in Fig.4.1-1, D). Interestingly, there is a slightly smaller number of stereocilia in $Myo15^{\Delta N/\Delta N}$ bundles at P4-P6 compared to those in control and $Myo15^{sh2/sh2}$ mice. Yet, the overall programmed retraction of stereocilia is not affected in either $Myo15^{sh2/sh2}$ or $Myo15^{\Delta N/\Delta N}$ mice. Therefore, we conclude that both long and short isoforms of Myo-XVa do not regulate the resorption of extra stereocilia in the IHC bundles during postnatal development.



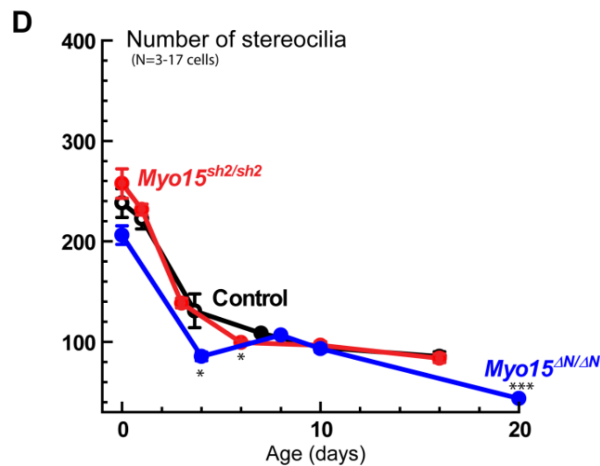
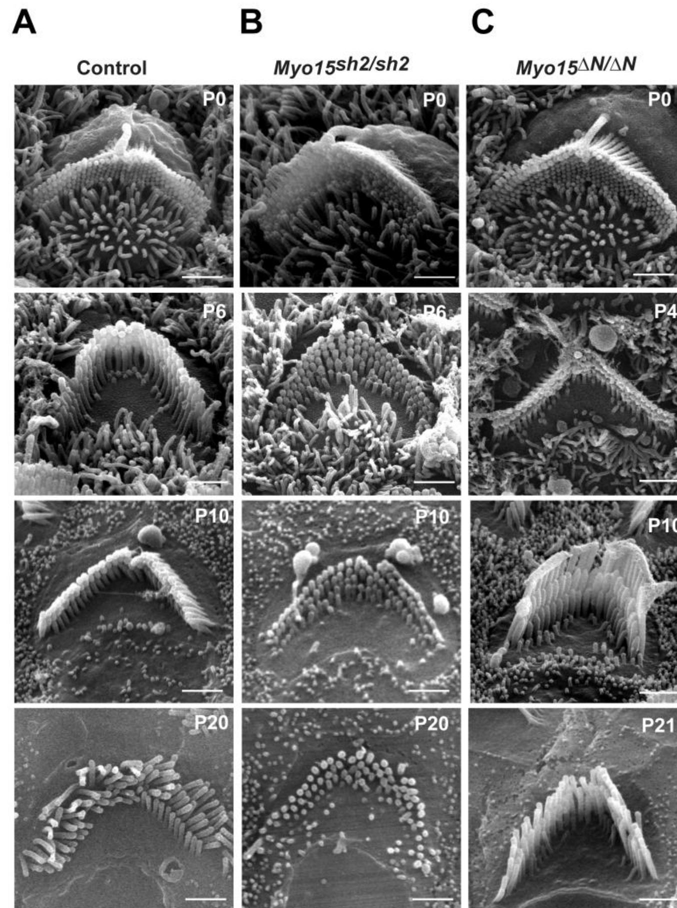


Figure 4.1-1: Myo-XVa does not affect the retraction of supernumerary stereocilia in IHCs. Panel A: SEM images of *Myo15*$^{+/sh2}$ and *Myo15*$^{+/\Delta N}$ control heterozygous IHC bundles (P0-20), B: *Myo15*$^{sh2/sh2}$ IHC bundles (P0-20), C: *Myo15*$^{\Delta N/\Delta N}$ IHC bundles (P0-P21), D: total number of stereocilia per bundle in control (black), *Myo15*$^{sh2/sh2}$ (red), and *Myo15*$^{\Delta N/\Delta N}$ (blue) (P0-P20) mice. Asterisk indicates significance (*P<0.05, **P<0.001, ***P<0.0001). Data are shown as Mean±SEM.

Similarly, quantification of total number of stereocilia per hair bundle in control, *Myo15*$^{sh2/sh2}$, and *Myo15*$^{\Delta N/\Delta N}$ OHCs shows a similar retraction of the extra stereocilia throughout P10. This reduction in stereocilia seems to be steeper in the OHC since they initially have a larger number of nascent stereocilia than that in IHCs. This is illustrated during P0-P10 in the IHCs (Fig.4.1-1, D) and OHCs (Fig.4.1-2, D); see also this evident stereocilia reduction on SEM images in (Fig.4.1-1,A,B,C) and (Fig.4.1-2, A,B,C). However, we did observe that *Myo15*$^{sh2/sh2}$ OHCs maintain a slightly larger number of stereocilia in the adult postnatal age (P18) compared to that of the control (P18) and *Myo15*$^{\Delta N/\Delta N}$ (P20). Overall, similar to the IHCs, neither short nor long isoforms of Myo-XVa disrupts the programmed retraction of the extra stereocilia in the OHC bundles. Therefore, we conclude that the programmed retraction of supernumerary microvilli within the auditory hair bundles is not regulated by Myo-XVa.



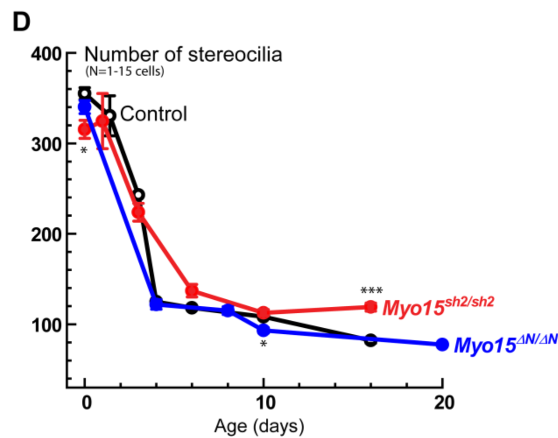


Figure 4.1-2: Myo-XVa does not affect the retraction of supernumerary stereocilia in OHCs. Panel A: SEM images of $Myo15^{+/sh2}$ and $Myo15^{+/\Delta N}$ control heterozygous OHC bundles (P0-20), B: $Myo15^{sh2/sh2}$ OHC bundles (P0-20), C: $Myo15^{\Delta N/\Delta N}$ OHC bundles (P0-P21), D: total number stereocilia per bundle in control (black), $Myo15^{sh2/sh2}$ (red), and $myo15^{\Delta N/\Delta N}$ (blue) (P0-P20) mice. Asterisk indicates significance (*P<0.05, **P<0.001, ***P<0.0001). Data are shown as Mean±SEM.

4.2 Myo-XVa is essential to form the differences in stereocilia diameters between the rows in the IHC bundles

Since Myo-XVa is essential for the elongation of stereocilia (Probst, Fridell et al. 1998), we were curious whether it is also needed to determine the thickness of stereocilia within the hair bundle. Therefore, we measured the diameters of stereocilia in three rows, (Fig.3.3-1, B), in all control, *Myo15*$^{sh2/sh2}$ and *Myo15*$^{\Delta N/\Delta N}$ IHC bundles during P0-P20 (Fig.4.2-1). Thickness measurements in control IHCs shows that, in order to achieve the proper wild type-like architecture, stereocilia of the first (tallest) and second row continuously increase in thickness reaching a maximum diameter of approximately 325-340nm by P18. Both, first and second row stereocilia show no significant difference in their diameter growth throughout postnatal development (see red and blue lines in Fig.4.2-1, D: control). In contrast to the taller rows, diameter of the third row stereocilia behaves differently. There is an initial increase in stereocilia thickness during the first postnatal days (P0-P1), which then remains steady up to P4, followed by a continuous reduction in stereocilia diameters (see green line in Fig.4.2-1, D: control). This major difference in stereocilia growth establishes the diameter gradation within the rows of the hair cell bundle (see also SEM images of P0-P20 control bundles in Fig.4.2-1, A). *Myo15*$^{sh2/sh2}$ hair bundles, that lack both Myo-XVa long and short isoforms, are characterized by absence of the staircase architecture due to disruption in stereocilia length gradation within the rows (Probst, Fridell et al. 1998). In this study, we discovered that stereocilia diameter gradation is also largely absent in *Myo15*$^{sh2/sh2}$ bundles. Our striking results come from observing third row stereocilia as they maintain the same thickness as first and second row, at least during the first 9 postnatal days (Fig.4.2-1, D:

Myo15$^{sh2/sh2}$). In addition, third row stereocilia in *Myo15*$^{sh2/sh2}$ bundles is significantly thicker than that in control bundles (Fig.4.2-1, D: *Myo15*$^{sh2/sh2}$; see green line for homozygous *shaker-2* and faint green line in background for control). Note the thick third row stereocilia in *Myo15*$^{sh2/sh2}$ SEM images (Fig.4.2-1, B). We believe that the absence of the Myo-XVa short isoform is responsible for the loss of stereocilia diameter gradation in the *Myo15*$^{sh2/sh2}$ bundles, because these abnormalities are not observed in the *Myo15*$^{\Delta N/\Delta N}$ IHC bundles that lack only Myo-XVa long isoform (SEM images in Fig.4.2-1, C). In *Myo15*$^{\Delta N/\Delta N}$ IHC, stereocilia increase in diameter in a similar fashion to that in control (Fig.4.2-1, D: *Myo15*$^{\Delta N/\Delta N}$). The normal process of stereocilia thinning in the third row is maintained and those stereocilia have significantly smaller diameters than the first row (See green vs. red line in Fig.4.2-1, D: *Myo15*$^{\Delta N/\Delta N}$). Unlike Myo-XVa short isoform, the long isoform is not essential for formation and maintaining the gradation of diameters within the IHC bundles.

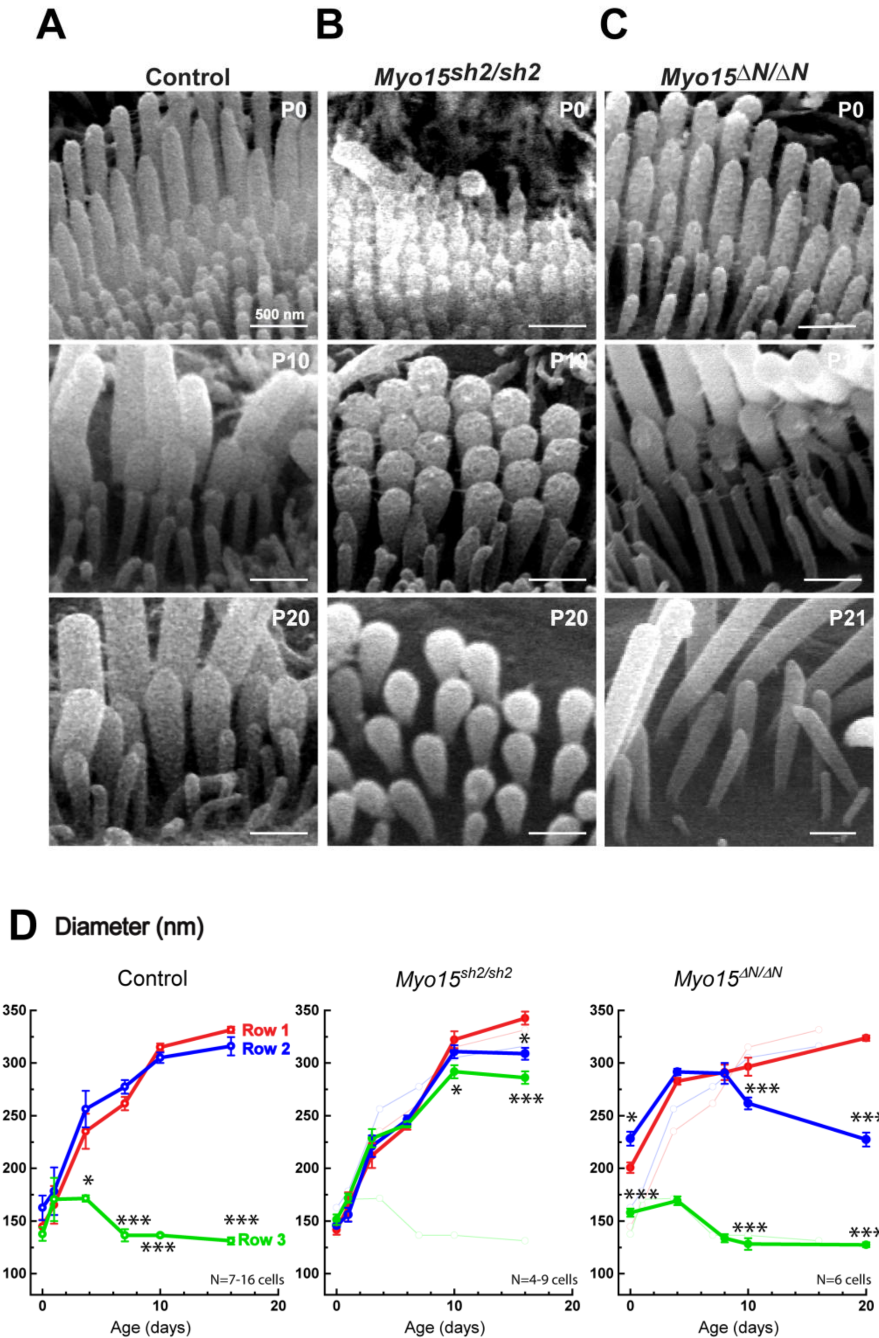


Figure 4.2-1: Myo-XVa regulates stereocilia thickness gradation in IHCs. Panel A: SEM images of *Myo15*$^{+/sh2}$ and *Myo15*$^{+/\Delta N}$ control heterozygous IHC bundles (P0-20), B: *Myo15*$^{sh2/sh2}$ IHC bundles (P0-20), C: *Myo15*$^{\Delta N/\Delta N}$ IHC bundles (P0-P21), D: quantification of stereocilia diameters (nm) in first (red), second (blue), and third (light green) stereocilia rows within the IHC staircase of (P0-P20) mice. Asterisk indicates significance (*P<0.05, **P<0.001, ***P<0.0001). All P-values were calculated relative to Row 1. Data are shown as Mean ± SEM.

Although there is a prominent disruption in the normal diameter gradation in *Myo15*$^{sh2/sh2}$ stereocilia bundles, we did observe some residual gradation that becomes significant during late postnatal period (P18). Yet this gradation was very different. In control bundles, first and second rows had similar stereocilia diameters, while the third row stereocilia were significantly thinner (refer to SEM images on Fig.4.2-1, A). In contrast, *Myo15*$^{sh2/sh2}$ IHCs had a small but progressive decrease of the diameters from the first to the third rows (refer to SEM images on Fig.4.2-1, B).

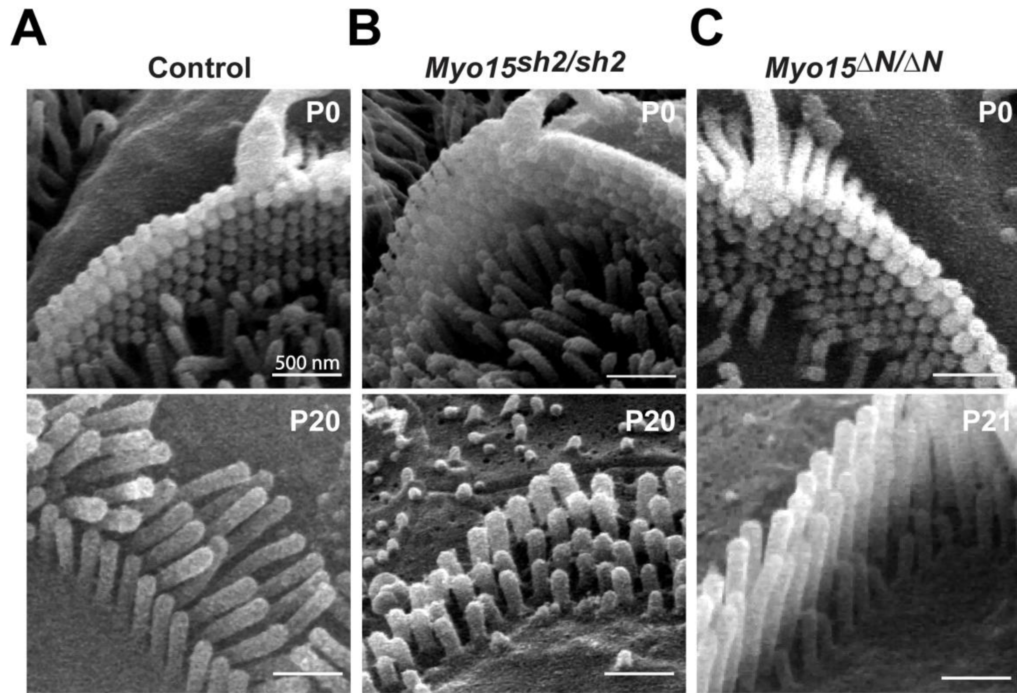
Additionally, we also found that, the second row stereocilia in *Myo15*$^{\Delta N/\Delta N}$ IHCs lacking long isoform of Myo-XVa become significantly thinner around P10 (see blue line in Fig.4.2-1, D: *Myo15*$^{\Delta N/\Delta N}$)- note the relative decrease in diameter ratio between first/second row stereocilia that becomes very prominent at P10-P20 in *Myo15*$^{\Delta N/\Delta N}$ SEM images (Fig.4.2-1, C) compared to that of control (Fig.4.2-1, A) and *Myo15*$^{sh2/sh2}$ hair cells (Fig.4.2-1, B). This is consistent with the previous report by (Fang, Indzhykulian et al. 2015), demonstrating thinning of the second row transducing stereocilia in the *Myo15*$^{\Delta N/\Delta N}$ IHCs that begins at P8 (Fang, Indzhykulian et al. 2015). Diameter quantification of second row stereocilia in *Myo15*$^{\Delta N/\Delta N}$ IHCs also revealed that at P0 these stereocilia are thicker than the stereocilia first row (note significant difference of first and second rows at P0 in Fig. 4.2-1, D: *Myo15*$^{\Delta N/\Delta N}$) and thicker than control. We speculate this can be due to the fact that *Myo15*$^{\Delta N/\Delta N}$ bundles have smaller number of nascent stereocilia than control and *Myo15*$^{sh2/sh2}$ IHCs (Fig.4.1-1, D).

4.3 Myo-XVa regulates the differences in stereocilia diameters in the OHC bundles

We hypothesized that, similar to the IHCs, Myo-XVa may also affect thickness gradation of stereocilia in the OHC bundles. We measured the diameters of stereocilia in all three

rows, (Fig.3.3-1, B), in control, *Myo15*$^{sh2/sh2}$, and *Myo15*$^{\Delta N/\Delta N}$ OHC's (P0-P20) (Fig. 4.3-1, D). In control bundles, stereocilia diameters in all rows increase throughout postnatal development and, thickness gradation becomes significantly prominent only late in development (Fig.4.3-1, D: Control). First and second row steroecilia were not significantly different in thickness until (P18), unlike third row which is significantly thinner than taller rows by P6 (Fig.4.3-1, D: control). As we expected, in *Myo15*$^{sh2/sh2}$ OHCs that lack both long/short Myo-XVa isoforms, the gradation of stereocilia diameters in different rows largely disappeared up to around P18, (SEM images in Fig.4.3-1, D *Myo15*$^{sh2/sh2}$)-note the non-significant change in diameter within the rows relative to row 1 in Fig.4.3-1: D, *Myo15*$^{sh2/sh2}$. Third row stereocilia is significantly thicker in *Myo15*$^{sh2/sh2}$ OHCs than in control OHCs and these stereocilia are almost as the thick as first and second row in *Myo15*$^{sh2/sh2}$ OHC - see SEM images in Fig.4.3-1, A, B at P20). Similar to IHCs, stereocilia diameter gradation is not affected in *Myo15*$^{\Delta N/\Delta N}$ OHC bundles that lack only Myo-XVa long isoform (Fig.4.3-1, D: *Myo15*$^{\Delta N/\Delta N}$). In these bundles, taller first and second row behave similar to control rows, and third row stereocilia is significantly thinner starting from P0, forming an evident gradation in diameter within the bundle. Thus, we conclude that the Myo-XVa short isoform, but not the long one, maintains thickness gradation within the rows in OHCs.

It is important to mention that, similar to IHCs, we did observe some subtle gradation of stereocilia diameters between different rows in *Myo15*$^{sh2/sh2}$ OHCs (Fig.4.3-1: D, *Myo15*$^{sh2/sh2}$), but it is very different from that in control.



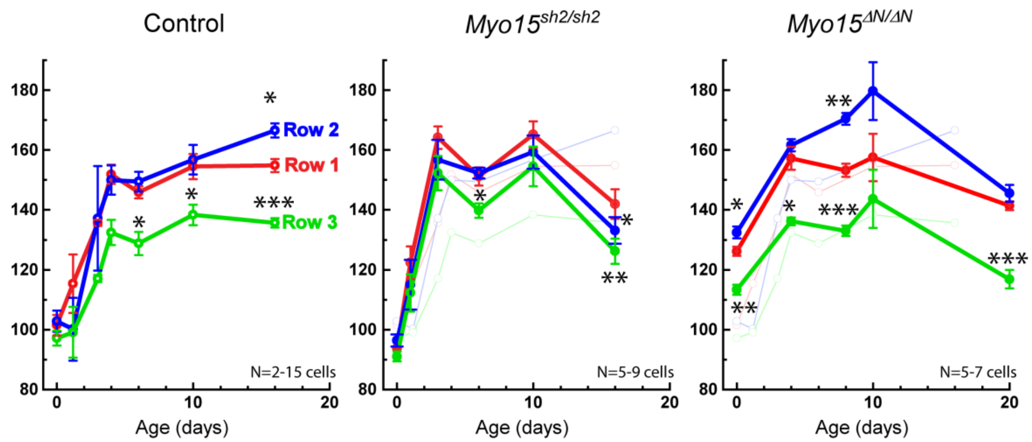


Figure 4.3-1: Myo-XVa regulates stereocilia thickness gradation in OHCs. Panel A: SEM images of *Myo15*$^{+/sh2}$ and *Myo15*$^{+/\Delta N}$ control heterozygous OHC bundles (P0-20), B: *Myo15*$^{sh2/sh2}$ OHC bundles (P0-20), C: *Myo15*$^{\Delta N/\Delta N}$ OHC bundles (P0-P21), D: quantification of stereocilia diameters (in nm) in first (red), second (blue), and third (light green) stereocilia rows within the OHC staircase of (P0-P20) mice. Asterisk indicates significance (*P<0.05, **P<0.001, ***P<0.0001). All P-values were calculated relative to Row 1. Data are shown as Mean±SEM.

SECTION 5: CONCLUSION

1) Myosin-XVa is not involved in the programmed retraction of supernumerary stereocilia during developmental maturation of the auditory hair cell stereocilia bundles.
2) “Short” isoform of myosin-XVa is essential for the programmed thinning of the third row stereocilia, which results in the formation of stereocilia diameter gradation in the stereocilia bundles of the auditory hair cells.

SECTION 6: DISCUSSION

6.1 Programmed resorption of supernumerary stereocilia is not affected by Myo-XVa deficiency

The retraction of supernumerary stereocilia in development is accompanied by wide-spread reduction in the number and heights of microvilli in all neighboring supporting cells (see SEM images on Fig. 4.1-1, A, B, C and Fig. 4.1-2, A, B, C). This observation suggests that auditory hair cells seem to have a conserved mechanism that favors systemic elimination of the supernumerary stereocilia within the organ of Corti and protecting “true” stereocilia. The mechanisms that determine the fates of stereocilia and microvili are indeed linked to the differences in their actin dynamics (Gorelik, Shevchuk et al. 2003; Drummond, Barzik et al. 2015; Narayanan, Chatterton et al. 2015). In contrast to the initial findings that postulated that stereocilia F-actin is dynamically “treadmilling” and the whole stereocillium core is replaced within 48 hours (Schneider, Belyantseva et al. 2002; Rzadzinska, Schneider et al. 2004), recent findings indicated that stereocilia are in fact very stable in normal physiological conditions (Zhang, Piazza et al. 2012; Drummond, Barzik et al. 2015; Narayanan, Chatterton et al. 2015). Mass spectrometry analysis of newly incorporated proteins at the EM level and the live cell imaging studies to trace transfected β-actin-GFP molecules within stereocilia have confirmed that the actin core is susceptible to slow and partial turnover only at the tips of the stereocilia (Zhang, Piazza et al. 2012; Drummond, Barzik et al. 2015), see Fig.1.4-2. Concurrent studies validated this observation and additionally suggested that while the actin core is continuously polymerizing at the tips of the stereocilia, other “actin-severing” proteins assist in “cutting” downstream filaments within the shaft to encourage

monomers removal through actin depolymerization and prevent stereocilia overgrowth (Narayanan, Chatterton et al. 2015).Velez-Ortega et al. have recently discovered that the stability of actin within the auditory stereocilia depends on the sustained influx of Ca^{2+} through partially open at rest mechano-electrical transduction (MET) channels. After blockage of this influx with chemical blockers of MET channels or tip link disruption, Velez-Ortega and colleagues observed significant retraction of second and third row transducing stereocilia in the auditory hair cells in rats and mice as well as reshaping of stereocilia tips (Velez-Ortega, Freeman et al. 2017). Thus, the staircase architecture of the auditory hair cell stereocilia bundles is stable in normal physiological conditions but can be easily disrupted through the disruption of the MET current.

In contrast to the stereocilia, short unspecialized microvilli at the surface of most epithelial cells are constantly forming and disassembling (Gorelik, Shevchuk et al. 2003). Cochlear epithelium seems to have a global program that reduces "unneeded" microvilli coat during development. According to our observations, this retraction is not restricted to the hair cells but extends also to the microvilli in the rim borders of neighboring supporting cells, as also observed by (Gorelik, Shevchuk et al. 2003)- see SEM images of IHC/OHC bundles starting from P10 and beyond in (Fig.4.1-1) and (Fig.4.1-2).

According to our data, this unnecessary supernumerary microvilli retraction is present in *Myo15*$^{sh2/sh2}$ and *Myo15*$^{\Delta N/\Delta N}$ bundles; therefore is independent of Myo-XVa (see Fig.4.1-1, D and Fig.4.1-2, D).

As mentioned earlier, inhibition of the resting MET current disturbs the stability of actin that is required to sustain lifelong stereocilia height and hair bundle architecture (Velez-Ortega, Freeman et al. 2017). In homozygous *Shaker-2*, mice that lack all isoforms of

Myo-XVa in their stereocilia, hair bundles are abnormally short and mutant animals are profoundly deaf. However, young postnatal auditory hair cells have prominent tip links and nearly normal MET current with the exception of loss of fast adaptation in IHCs (Stepanyan, Belyantseva et al. 2006; Stepanyan and Frolenkov 2009). For this reason, *Myo15*$^{sh2/sh2}$ stereocilia probably do not degenerate for quite a while in development (Stepanyan, Belyantseva et al. 2006). It is also important to note that Myo-XVa isoform-specific knockout *Myo15*$^{\Delta N/\Delta N}$ mice do show some decline in MET current and therefore selective degeneration of shorter row stereocilia that is already initiated by P11(Fang, Indzhykulian et al. 2015). Quantification of stereocilia in the adult Myo15$^{\Delta N/\Delta N}$ bundles indeed shows a significant decrease in the number of stereocilia (Fig.4.1-1), which confirms this degeneration and does explain the link between the necessity of hair cell machanotransduction and the stability of the actin core.

6.2 "Short" Isoform of Myo-XVa is essential for stereocilia thickness gradation within the auditory hair cell bundles

It has been established that myosin-XVa is essential for the programmed elongation of stereocilia in development by delivering its cargo, whirlin and other components of stereocilia elongation complex to the tips of the stereocilia (Belyantseva, Boger et al. 2005). However, the role of Myo-XVa in the control of other features of hair cell architecture, such as the diameter of stereocilia, has not been yet investigated. Our study not only demonstrated the effect of Myo-XVa dysfunction on the thickness of stereocilia in hair cells, but also provided a quantitative evidence of diameter regulation within the wildtype staircase bundle during postnatal development.

Building the actin core requires the assembly of thousands actin monomers that form the parallel actin filaments inside the stereocilia (Tilney, Egelman et al. 1983; Krey, Krystofiak et al. 2016). To maintain the structure this of parallel actin "reservoir", thousands of actin cross-linker proteins connect these filament to keep the stereocilia stable (Tilney, Egelman et al. 1983; Rzadzinska, Schneider et al. 2004; Drummond, Barzik et al. 2015; Krey, Krystofiak et al. 2016). In the mammalian auditory hair cells, those proteins are known to be different isoforms of: espin, plastin, and Fascin (Krey, Krystofiak et al. 2016), Fig.1.4-2. During development, the thickness of stereocilia is essentially determined by two key principles 1) the number of actin filaments inside the stereocilium 2) the number of actin associated cross-linker molecules (Tilney, Egelman et al. 1983). In fact, mice that lack plastin or plastin and fascin together are deaf and their stereocilia bundles have thinner diameters due to a decrease in their actin filaments and modifications in their packing (Krey, Krystofiak et al. 2016).

Previous immunolabeling experiments have shown that cross-linker proteins, espin, plastin, and fascin, are localized only to the shaft of the stereocilia (Krey, Krystofiak et al. 2016). These proteins are absent at the base of the stereocilia, which allows for flexibility during bundle deflection (Tilney, Egelman et al. 1983; Krey, Krystofiak et al. 2016). Interestingly, none of these proteins localizes to the tips of the stereocilia (Krey, Krystofiak et al. 2016). Yet, Myo-XVa is the first molecule to be detected at the tips (Belyantseva, Boger et al. 2003). According to our data, myosin-XVa is controlling the developmental thinning of third row stereocilia (Fig.4.2-1, D: Control). Therefore, it cannot simply bring some sort of "thickening" molecules to the stereocilia tips. More likely, Myo-XVa interacts with some yet unknown molecules that are expressed more

prominently at the tips of the third row stereocilia and control the number of actin filaments. One protein that we hypothesize to interact with Myo-XVa, specifically the "short" isoform, is "calcium and integrin-binding protein 2", CIB2, which has been shown to be fundamental for hair cell mechanotransduction and calcium signaling (Riazuddin, Belyantseva et al. 2012). Immunocytochemistry studies have demonstrated CIB2 localization at the tips of the third row stereocilia, and CIB2 mutant hair bundles have growth abnormalities such as increased height of the transducing stereocilia, occasional degeneration, and increased diameters of the IHC third row stereocilia (Giese, Tang et al. 2017).(**Note:** we also observe more prominent thickening of third row stereocilia in *Myo15*$^{sh2/sh2}$ IHCs (Fig.4.2-1, B, D: *Myo15*$^{sh2/sh2}$) and OHCs (Fig.4.3-1, B, D: *Myo15*$^{sh2/sh2}$). CIB2 also interacts with whirlin (Fig.6.2-1) (Giese, Tang et al. 2017) and the latter has been shown to regulate stereocilia heights (i.e. length of actin filaments) (Belyantseva, Boger et al. 2005).

Although in the absence of CIB2, whirlin is still carried by Myo-XVa to the tips of the stereocilia (Belyantseva, Boger et al. 2003; Giese, Tang et al. 2017), it is still possible that CIB2 cannot make it to the tips of the stereocilia without Myo-XVa. Since CIB2 labeling is also found along the actin filaments of the third row stereocilia (Giese, Tang et al. 2017), the alternative hypothesis is that it interacts directly with cross-linker proteins and serves as a "liaison" molecule between Myo-XVa and those cross-linkers. In conclusion, more studies are needed to confirm CIB2 co-localization with Myo-XVa at the tips of the stereocilia or even with cross-linker proteins in the shaft.

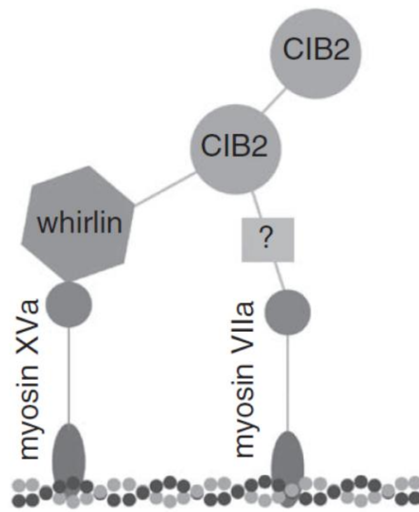


Figure 6.2-1: CIB2 interaction with Myo-XVa cargo whirlin. Figure is modified from (Geise et al., 2017)

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MANUSCRIPTS
Elkon R, Milon B, Morrison L, Shah M, Vijayakumar S, Racherla M, Leitch CC, Silipino L, **Hadi S**, Weiss-Gayet M, Barras E, Schmid CD, Ait-Lounis A, Barnes A, Song Y, Eisenman DJ, Eliyahu E, Frolenkov GI, Strome SE, Durand B, Zaghloul NA, Jones SM, Reith W, Hertzano R "RFX Transcription Factors are Essential for Hearing in Mice." *Nat Commun.* 2015 Oct 15;6:8549.

Hadi S, Alexander AJ, Velez-Ortega AC, Frolenkov GI "Short isoform of myosin-XVa is essential not only for staircase architecture but also for gradation of stereocilia diameters in the auditory hair cells" (In preparation).

ABSTRACTS/POSTER PRESENTATIONS
National Meetings
Hadi S, Alexander AJ, Velez-Ortega AC, Frolenkov GI (2018) "Myosin-XVa is Essential to Form the Gradation of Stereocilia Diameters Within the Hair Bundles of Inner but not Outer Hair Cells." *Association for Research in Otolaryngology MidWinter Meeting,* San Diego, CA, Abst. PS-125.

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Factors are Essential for Survival of the Terminally Differentiating Outer Hair Cells in Mice." *Association for Research in Otolaryngology MidWinter Meeting*, San Diego, CA, Abst. PS-292.

Regional Meetings

Hadi S, Alexander AJ, Velez-Ortega AC, Frolenkov GI (2017) "Myosin-15a is Essential for Gradation of Stereocilia Heights and Diameters in the Mechanosensory Bundle of the Auditory Hair Cells." *5th Annual Meeting of the Kentucky Chapter of the American Physiological Society*, Bowling Green, KY, Abst. 7. (Best poster award)

Local Meetings

Hadi S, Alexander AJ, Velez-Ortega AC, Frolenkov GI (2018) "Myosin-XVa is Essential to Form the Gradation of Stereocilia Diameters Within the Hair Bundles of Inner but not Outer Hair Cells."*University of Kentucky Department of Physiology Retreat,* Lexington, KY. (1st Prize- best poster award).

Hadi S, Alexander AJ, Velez-Ortega AC, Frolenkov GI (2017) "Myosin-15a is Essential not Only for Proper Elongation but Also for Thickening of the Mechanosensory Stereocilia in the Mammalian Auditory Hair Cells." *Neuroscience Clinical-Translational Research Symposium*, Lexington, KY.

Hadi S, Alexander AJ, Velez-Ortega AC, Frolenkov GI (2016) "Different Effects of Two Myosin 15a Isoforms on Stereocilia Development." *University of Kentucky Department of Physiology Retreat*, Lexington, KY.