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Building and repairing the stereocilia cytoskeleton in mammalian auditory hair cells

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Abstract

Despite all recent achievements in identification of the molecules that are essential for the structure and mechanosensory function of stereocilia bundles in the auditory hair cells of mammalian species, we still have only a rudimentary understanding of the mechanisms of stereocilia formation, maintenance, and repair. Important molecular differences distinguishing mammalian auditory hair cells from hair cells of other types and species have been recently revealed. In addition, we are beginning to solve the puzzle of the apparent life-long stability of the stereocilia bundles in these cells. New data link the stability of the cytoskeleton in the mammalian auditory stereocilia with the normal activity of mechanotransduction channels. These data suggest new ideas on how a terminally-differentiated non-regenerating hair cell in the mammalian cochlea may repair and tune its stereocilia bundle throughout the life span of the organism.

Keywords

Inner ear hair cells; actin; mechanotransduction; deafness; noise

1. Introduction

The hair cells in the inner ear detect sound-induced vibrations or head movements via the deflections of their sensory organelles, the hair bundles. The hair bundle exhibits a complex architecture that continues to challenge our knowledge in cell biology. It is still unclear how a cell could build this precisely-organized structure and make it almost identical in neighboring hair cells. At the apical pole of mammalian auditory hair cells, dozens of microvillus-like projections, known as stereocilia, are arranged in three rows of increasing height, with heights and thicknesses being remarkably uniform within each row (Fig. 1A, 1C). Stereocilia are also interconnected by thin extracellular protein filaments (Fig. 1B, 1D),

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some of which (*i.e.* tip links) participate directly in mechano-electrical transduction, the major function of the hair bundle (Fig. 1E, 1F). In this review, we describe known molecules that play a role in the development and maintenance of the hair cell bundle cytoskeleton. Despite an overall similarity between the structure and function of the stereocilia bundles in hair cells of many vertebrate species, growing evidence indicates important distinctions in their underlying molecular machineries. Therefore, we will focus on the stereocilia bundles of mammalian auditory hair cells.

In many mammalian species, formation of the auditory hair cell bundles occurs during embryonic development. However, in small rodents like rats, hamster, or mice, development of stereocilia bundles starts in embryonic ages and continues until 2–3 weeks after birth, which makes them convenient experimental models. Therefore, several groups studied hair bundle development in rodents (Kaltenbach et al., 1994; Roth et al., 1992; Zine et al., 1996) comparing it with the findings of seminal papers describing the stereocilia bundle development in the chick cochlea (Tilney et al., 1992a; Tilney et al., 1986). In both chick and rodents, the hair bundle develops from a cluster of microvilli on the apical surface of hair cells. These microvilli-like projections, called stereocilia, grow into rows of different heights while they elongate and thicken to form the adult hair bundle. Simultaneously, supernumerary stereocilia are reabsorbed into the cuticular plate — a stiff structure in the apical region of the hair cell body supported by an actin mesh network — leaving a smooth cell surface area around the bundle. Although the general pattern is similar in chick and rodent auditory hair cells, independent phases of stereocilia bundle development are less noticeable in mammals, indicating potential differences in the timing of underlying molecular signals (for more details, see (Barr-Gillespie, 2015; Frolenkov et al., 2004).

2. Building the stereocilia paracrystalline actin core

The stereocilia cytoskeleton consists of highly-organized parallel actin filaments crosslinked into a mechanically rigid structure (DeRosier et al., 1980; Tilney et al., 1980). Hair cells express both β - and γ -actin isoforms. In chick and guinea pig cochlear hair cells, β -actin expression seems to be restricted to the hair bundle, while γ -actin is expressed in the hair bundle, the cuticular plate, and the lateral wall (Furness et al., 2005; Hofer et al., 1997). In adult cochlear hair cells from mouse and guinea pig, β - and γ -actin seem to be uniformly distributed along the entire stereocilia cytoskeleton (Perrin et al., 2010b), except at the stereocilia tips where γ -actin expression is significantly higher than β -actin, particularly in the outer hair cells (OHCs) (Furness et al., 2005; Patrinostro et al., 2018). There are discrepancies, however, in the distribution of actin isoforms within the stereocilium cross-section. In chick cochlear hair cells, electron microscopy of ultrathin sections simultaneously labeled with immunogold against β - and γ -actin showed no preferential expression of either isoform across the stereocilium (Hofer et al., 1997). However, a similar approach showed preferential localization of β -actin to the periphery of the stereocilium in guinea pig cochlear OHCs (Furness et al., 2005). A subsequent study using immunofluorescent labeling of adult mouse cochlear hair cells indicated that γ -actin, and not β -actin, was the isoform concentrated at the periphery of stereocilia in the inner hair cells (IHCs) (Belyantseva et al., 2009). However, it was later shown that the use of the secondary antibodies led to immunolabeling artifacts, probably due to the limited access of

the secondary antibodies to the core of the stereocilium (Perrin et al., 2010b). Even though β - and γ -actin are expressed by two separate genes, both isoforms are 99% identical in protein sequence since they differ only by four amino acids in the N-terminal region, and they are 100% conserved between birds and mammals (reviewed in (Perrin et al., 2010a)). In the absence of the either β - or γ -actin isoforms, auditory hair cell stereocilia develop normally but exhibit premature stereocilia degeneration (Belyantseva et al., 2009; Patrino et al., 2018; Perrin et al., 2010b). In humans, mutations in β -actin are typically associated with syndromic types of hearing loss, while mutations in γ -actin cause either syndromic or non-syndromic progressive hearing loss (Liu et al., 2008; Morin et al., 2009; Procaccio et al., 2006; Rendtorff et al., 2006; Riviere et al., 2012; van Wijk et al., 2003; Zhu et al., 2003). Therefore, it is reasonable to speculate that β -actin has a major structural role in stereocilia, while γ -actin also participates in stereocilia repair. This repair role of γ -actin is supported by the breaks (or “gaps”) in the stereocilia actin core observed in mice lacking γ -actin, and by the appearance of γ -actin filled gaps in stereocilia cores after *in-vivo* damage of the cochlea by intense acoustic noise (Belyantseva et al., 2009).

The actin filaments in the stereocilia core are polarized, with barbed/plus ends (*i.e.* the preferred site of actin polymerization) toward the stereocilia tips, and pointed/minus ends toward the stereocilia bases (Flock et al., 1977; Tilney et al., 1980). These filaments are interconnected in the stereocilia shaft by multiple actin crosslinkers from the espin, fascin, and plastin/fimbrin families (Shin et al., 2010; Taylor et al., 2015; Zheng et al., 2000) (Fig. 2A). Crosslinkers in these families act as monomers, have multiple actin-filament-binding domains, and lead to interfilament spacings between 8 to 14 nm. Espins are encoded by a single gene that, through multiple transcriptional start sites and splice variants, produces several espin (ESPN) isoforms: 1, 2A, 2B, 3A, 3B and 4 (Sekerova et al., 2004); all of which can crosslink actin filaments via their shared C-terminal actin-bundling domain (Bartles et al., 1998) and lead to actin bundles with filaments separated by ~12 nm (Kitajiri et al., 2010; Purdy et al., 2007). Fascins 1, 2 and 3 are encoded by three independent genes, with fascin-1 (FSCN1) and fascin-2 (FSCN2) proteins having the highest degree of similarity within the family (~73% similarity based on standard protein BLAST analysis between mouse fascin-1 [NP_032010](#), fascin-2 [NP_766390](#), and fascin3 [NP_062515](#)). Fascin-1 has four β -trefoil domains in tandem — two of which are the major actin-binding domains — and leads to an interfilament spacing of ~6–8 nm (Jansen et al., 2011; Van Audenhove et al., 2016; Winkelman et al., 2016). Plastins 1, 2 and 3, also known as I-plastin (fimbrin), L-plastin (lymphocyte cytosolic protein 1) and T-plastin, respectively, are encoded by three separate genes. Plastins have two actin-binding domains in tandem (de Arruda et al., 1990; Lin et al., 1994) and lead to the crosslinking of actin filaments with a separation of ~9–12 nm (Van Audenhove et al., 2016; Volkmann et al., 2001). Quantitative mass spectrometry of adult mouse vestibular hair bundles showed that plastin-1 (PLS1) is the most abundant cross-linker in the bundle, almost twice more abundant than fascin-2 and espin (Krey et al., 2016). Surprisingly, espin constituted only about 15% of the bundle crosslinkers (Krey et al., 2016), even though its deficiency results in the dramatic disorganization of stereocilia (Sekerova et al., 2011; Zheng et al., 2000).

In chick hair bundles, however, fascin-2 is the predominant crosslinker, with expression levels nearly 8- to 10-fold higher than plastin-1 and plastin-3 (PLS3), the second most

abundant crosslinkers in vestibular and cochlear hair cells, respectively (Avenarius et al., 2014; Shin et al., 2013). While actin filaments in hair bundles from the chick cochlea are packed into a uniform hexagonal paracrystalline arrangement (Tilney et al., 1983), actin packing in hair bundles from the lizard cochlea and the mouse utricle are more “liquid” (Krey et al., 2016; Tilney et al., 1980). It is very likely that the simultaneous expression of plastin (which bundles F-actin with larger interfilament spacing) together with espin/fascins (that favor tighter F-actin packing) results in an intermediate “liquid” arrangement of actin filaments (Krey et al., 2016). On the other hand, the preferential expression of fascins/espin over plastin in chick hair bundles allows for a highly ordered hexagonal arrangement. This hypothesis has been supported by the observation that mice lacking plastin-1 exhibit stereocilia with a uniform hexagonal packing of actin filaments (Krey et al., 2016).

In contrast to vestibular hair cell stereocilia, quantification of actin crosslinkers in mouse auditory hair cell stereocilia has not been performed. However, given the “liquid” packing of actin filaments in the mouse OHC stereocilia and patches of hexagonal packing in the IHC stereocilia (Krey et al., 2016; Mogensen et al., 2007), one may suggest that the expression patterns of F-actin crosslinkers in these two types of hair cells are quite different.

The physiological significance of the differences in crosslinker composition in different hair cell types and/or species, and hence in actin filament packing, is yet unclear. It has been argued that the hexagonal packing may limit the growth of a stereocilium in thickness while the “liquid” packing may favor the assembling of a thicker stereocilium (Krey et al., 2016). However, hexagonal packing is prominent in the thick IHC stereocilia but absent in the thin OHC stereocilia of wild type mice (Krey et al., 2016). More is known about the requirements of different crosslinkers for stereocilia development. Espin is expressed early in development, presumably initiates the formation of the stereocilia actin core, and its deficiency results in profound deafness associated with stereocilia disorganization (Sekerikova et al., 2011; Zheng et al., 2000). Mice lacking functional plastin-1 exhibit progressive hearing loss and largely normal stereocilia but with reduced length and width, indicating that this crosslinker participates in stereocilia core expansion during late stages of development (Krey et al., 2016; Taylor et al., 2015). Fascin-2 seems to be expressed later, is present in adult stereocilia, and may be required for their maintenance rather than initial formation (Perrin et al., 2013; Shin et al., 2010). Other types of actin-crosslinkers found in hair cell stereocilia are filamin B (FLNB), actinin 1 (ACTN1), actinin 4 (ACTN4), and XIRP2. Filamin B and α -actinins need to dimerize to be able to crosslink two actin filaments, and they typically lead to greater inter-filament spacings (*e.g.*, ~32 nm in the case of α -actinins (Winkelman et al., 2016)). Therefore, the main function of filamin B and α -actinins in stereocilia may not be the formation of the actin paracrystalline structure, but rather interaction between the actin cytoskeleton and other structures. Filamin dimers crosslink actin filaments via their actin-binding domains and can simultaneously interact with other intracellular or membrane proteins — such as transcription factors, signaling molecules, ion channels, integrins, or other cytoskeletal proteins — via their immunoglobulin-like domains (reviewed in (Razinia et al., 2012)). α -actinins are part of the spectrin superfamily. They contain one actin-binding domain in the N-terminus and a central rod region consisting of four spectrin domains that allows them to interact with phospholipids or transmembrane receptors in the plasma membrane (reviewed in (Sjoblom

et al., 2008)). α -actinins crosslink actin filaments by forming anti-parallel homodimers (Ylanne et al., 2001). Despite potential problems in the quantification of stereocilia proteins with low molecular mass (Krey et al., 2016), proteomics studies from the same group revealed potentially important differences between auditory and vestibular hair cells in chicks. In auditory stereocilia, filamin B seems to be present at high levels (similar to fascin-2), while actinin 1 and actinin 4 are expressed at low levels (Avenarius et al., 2014). In vestibular stereocilia, however, actinin 1, actinin 4 and filamin B are all detected at low levels (similar to fascin-1) (Shin et al., 2013). The XIRP2 gene leads to several protein isoforms that can be grouped into “long” or “short” isoforms depending on whether they express the large exon 7 which contains all Xin actin-binding domains (Francis et al., 2015; Scheffer et al., 2015). Thus, “short” XIRP2 may not be able to crosslink actin. While both isoforms are expressed in inner ear hair cells from chick and mouse, short XIRP2 is enriched in the stereocilia while long XIRP2 mainly localizes at the cuticular plate and pericuticular region (Francis et al., 2015; Scheffer et al., 2015). Moreover, in rat cochlear hair cells XIRP2 was shown to localize at the periphery of the stereocilia actin core (Francis et al., 2015). Thus, it is plausible that, instead of crosslinking actin filaments, XIRP2 may be involved in connections between the actin cytoskeleton and the plasma membrane. Interestingly, mice deficient in both the long and short isoforms of XIRP2 have a disorganized actin cytoskeleton in cochlear and utricle hair cells, evident as early as postnatal day 7 (Scheffer et al., 2015), indicating that XIRP2 is necessary for the proper formation of the stereocilia actin filaments during the early development of the hair bundle. It remains to be elucidated whether the paracrystalline arrangement of the stereocilia actin cytoskeleton remains intact in the absence of filamin B, actinin 1 or actinin 4.

3. Building the hair bundle staircase

The tips of stereocilia are not only the sites of actin polymerization (Schneider et al., 2002) but also the sites of mechano-electrical transduction (MET). In mammalian auditory hair cells, MET channels are located at the tips of the middle and shorter row stereocilia but not on the first, tallest row of stereocilia (Beurg et al., 2009) (Fig. 1E, 1F). It is yet to be re-evaluated whether this localization of the MET channels only at the lower end of the tip link is also valid for hair cells of non-mammalian species, since older reports indicated localization of MET channels at both sides of the tip link (Denk et al., 1995). There are several proteins that localize to the tips of the “transducing” stereocilia in the shorter rows of the mammalian hair cell bundles and that are known to be essential for the formation of the MET apparatus in these cells: TMC1, TMC2, TMHS/LHFPL5, TMIE and CIB2 (for a review, see Fettiplace, 2017; Qiu et al., 2018)). In addition to these MET-related proteins, several other proteins that localize at the tips of stereocilia are: myosin XVA (MYO15A), myosin IIIA (MYO3A), myosin IIIB (MYO3B), whirlin (WHRN), EPS8, EPS8L2, espin 1 (ESPN-1), ESPNL, twinfilin 2 (TWF2), CAPZA1, CAPZB-2, GPSM2 and GNAI3 (Avenarius et al., 2017; Belyantseva et al., 2003a; Belyantseva et al., 2005; Delprat et al., 2005; Fang et al., 2015; Furness et al., 2013; Manor et al., 2011; Mauriac et al., 2017; Peng et al., 2009; Salles et al., 2009; Tarchini et al., 2016). Proteins that exhibit different expression levels between stereocilia rows are attractive candidates for the regulation and/or formation of the characteristic staircase architecture of the hair bundle. In the mouse

cochlear hair cells, some proteins are clearly enriched at the tips of the tallest (non-transducing) stereocilia: EPS8, short isoform of myosin XVA, GPSM2 and GNAI3, while others show preferential expression at the tips of the transducing stereocilia: EPS8L2, twinfilin 2, ESPNL and long isoform of myosin XVA (Fig. 2B).

Among the molecules mentioned above, myosin XVA seems to be one of the “master keys” initiating the proper elongation of stereocilia within a hair bundle. *Shaker-2* mice exhibit profound deafness and severe vestibular defects due to a mutation in the motor domain of myosin XVA. In these mice, stereocilia are abnormally short in IHCs, OHCs, and vestibular hair cells (Belyantseva et al., 2003a; Beyer et al., 2000; Probst et al., 1998). Despite their abnormally small heights, a staircase arrangement is still observed in the *shaker-2* OHC stereocilia but barely noticeable (if present at all) in the *shaker-2* IHC bundles (Stepanyan et al., 2009). This observation highlights important, yet largely ignored in the literature, differences in the molecular mechanisms of stereocilia bundle formation in different hair cell types. Alternative splicing leads to two distinct isoforms of myosin XVA (Belyantseva et al., 2003a), but only one of them — the “short” isoform — seems to be involved in the elongation of stereocilia during the development of the hair bundle (Fang et al., 2015). Myosin XVA delivers to the tips of stereocilia other proteins required for the proper elongation of actin filaments, such as the scaffolding protein whirlin (Belyantseva et al., 2005; Delprat et al., 2005) and the actin-binding protein EPS8 (Manor et al., 2011).

Whirlin has three PDZ domains and a C-terminal proline-rich region (Mburu et al., 2003), which are involved in protein-protein interactions. *Whirler* mice have a 592 bp deletion in the whirlin gene that leads to a loss of ~200 amino acids, a frameshift and a premature stop codon in whirlin (Mburu et al., 2003). The stereocilia of IHCs and OHCs in *whirler* mice are abnormally short and resemble those found in the *shaker-2* mouse (Belyantseva et al., 2003b; Holme et al., 2002b; Mustapha et al., 2007). Thus, whirlin may be required for the proper delivery of other proteins that are crucial for the elongation of stereocilia. The wild type whirlin gene leads to a full “long” isoform, and a “short” truncated isoform that lacks two of the PDZ domains. While whirlin expression has been shown to remain at the tips of stereocilia even in adult IHC and OHC from mouse and rat (Delprat et al., 2005), it was reported that the short isoform of whirlin is only highly expressed during the initial development of the auditory hair cell bundles (Kikkawa et al., 2005). More importantly, this temporary expression of the short isoform starts at different time points (3 to 6 days apart) for each row within the hair bundle: it is first detected in the tallest row of stereocilia, later in the middle row, and last in the shortest row (Kikkawa et al., 2005). This expression pattern leads to differential expression levels of short whirlin between stereocilia rows and, therefore, could be a key player in the establishment of the staircase architecture of the bundle. However, the expression patterns of short whirlin do not seem to match perfectly the periods of stereocilia elongation. For instance, short whirlin expression disappears in IHCs several days before the elongation of the tallest row of stereocilia is complete (Peng et al., 2009). In contrast to these findings, it was also shown that short whirlin is only expressed at the tips of the tallest row stereocilia in both IHCs and OHCs, but this study was limited to the second postnatal week of the bundle development (Ebrahim et al., 2016a).

EPS8 has an N-terminal phospho-tyrosine binding (PTB) domain, a central Src homology 3 (SH3) domain, a C-terminal actin-binding effector domain, and exhibits plus-end actin-capping activity (Disanza et al., 2004; Offenhauser et al., 2004). EPS8 is enriched at the tips of stereocilia from the tallest row in cochlear hair cells and EPS8 knock-out mice exhibit abnormally short IHC and OHC stereocilia (resembling the phenotypes of the *shaker* -2 and *whirler* mice) (Manor et al., 2011; Zampini et al., 2011). EPS8 is completely absent from the tips of stereocilia in *shaker* -2 mice, found at lower levels at the tips of stereocilia in *whirler* mice, and able to localize at the tips of stereocilia even when lacking its capping activity, which strongly suggests that myosin XVA (partially via its cargo whirlin) is responsible for delivering EPS8 to the barbed ends of actin filaments within stereocilia (Manor et al., 2011). EPS8L2 is an actin-binding protein similar to EPS8 (Offenhauser et al., 2004) that is expressed at the tips of shorter row stereocilia in IHCs and OHCs (Furness et al., 2013), and it is presumed to have plus-end actin-capping activity. Interestingly, the tallest row of stereocilia on IHCs in EPS8L2 knock-out mice are significantly shorter, suggesting that EPS8L2 can regulate the elongation of the tallest stereocilia despite its absence or very low expression in that row (Furness et al., 2013). EPS8L2 is still detected at the tips of stereocilia in *shaker* -2 mice, indicating that it could reach the stereocilia tips even in the absence of functional myosin XVA (Furness et al., 2013).

Twinfilin 2 is a plus-end actin capping protein localized to the tips of transducing shorter row stereocilia in IHCs and OHCs, but not to the stereocilia of the tallest row (Avenarius et al., 2017; Peng et al., 2009). The onset of expression of twinfilin 2 correlates with the end of the elongation phase in the IHC middle row stereocilia, while its overexpression results in a significant reduction of stereocilia length (Peng et al., 2009). Therefore, it has been proposed that twinfilin 2 expression dictates the maximum height of transducing stereocilia (Peng et al., 2009). Another unconventional deafness-related myosin, myosin VIIA, may interact with twinfilin 2 and deliver it or stabilize it at the tips of the shorter row stereocilia in the hair bundle (Rzadzinska et al., 2009). *Shaker-1* mice carry mutations in the myosin VIIA gene that lead to premature stop codons or are deleterious for its motor function and, as a result, exhibit IHC and OHC with abnormally long and disorganized stereocilia (Gibson et al., 1995; Mburu et al., 1997; Prosser et al., 2008). Therefore, an interesting hypothesis was proposed that the staircase architecture of the stereocilia bundle is determined by the interplay between myosin XVA/whirlin complexes promoting stereocilia growth at the tallest row and myosin VII/twinfilin 2 complexes inhibiting stereocilia growth at the shorter rows (Rzadzinska et al., 2009). In addition, twinfilin 2 may not be the only molecule inhibiting stereocilia growth, since it interacts with CAPZB2, another plus-end actin-capping protein that localizes near the tips of stereocilia (Avenarius et al., 2017). Knock-out mice for CAPZB exhibit stereocilia height abnormalities in cochlear hair bundles followed by stereocilia degeneration (Avenarius et al., 2017).

Several other molecules have been reported to be essential for the formation of the staircase architecture of the stereocilia bundle in mammalian auditory hair cells. The signaling molecule GPSM2 and its binding partner GNAI3 are both expressed at the tips of the tallest row stereocilia in IHCs and OHCs (Mauriac et al., 2017; Tarchini et al., 2016). Mice deficient in either GPSM2 or GNAI3 have abnormally short stereocilia bundles in IHCs and OHCs, that appear very similar to the hair bundles of *shaker-2*, *whirler*, and EPS8 knock-out

mice (Mauriac et al., 2017; Tarchini et al., 2016). Gelsolin (GSN) is an actin capping and severing protein, temporarily expressed in OHCs but not in IHCs during the first two postnatal weeks of hair bundle development in mice (Mburu et al., 2010). In gelsolin knock-out mice, hair cell bundles in OHCs develop normally but stereocilia continue to elongate past the second postnatal week exclusively in the cochlear apex, even though gelsolin expression in wild type OHCs seems to be uniform throughout the entire cochlear length (Mburu et al., 2010). Cochlear hair bundles of mice deficient in myosin IIIA or myosin IIIB develop normally. However, in the absence of both myosin IIIA and IIIB, the staircase of the hair bundles in IHC and OHC is severely impaired and stereocilia are significantly overgrown (Lelli et al., 2016). These results indicate that myosin IIIA and IIIB have compensatory functions during the development of the cochlear hair bundles. The exact mechanism of how all these molecules participate in the hair bundle formation is yet to be elucidated.

Defects in the stereocilia actin crosslinkers can also lead to impaired stereocilia elongation. The expression levels of espin gradually increase in cochlear hair bundles during the elongation phase of stereocilia. Moreover, this increase in espin is greater toward the apex of the cochlea — where stereocilia are longer — than the base of the cochlea (Sekerikova et al., 2006). The *jerker* mouse, which has a frameshift mutation in the espin gene, lacks espin expression in hair cells and displays shorter stereocilia in IHCs and OHCs during the early stages of stereocilia elongation, particularly at the apex of the cochlea (Rzadzinska et al., 2005; Sekerikova et al., 2011; Zheng et al., 2000). By the second postnatal week, *jerker* mice have extensive stereocilia degeneration in both IHCs and OHCs (Sekerikova et al., 2011). Mice carrying a single copy of the *jerker* mutation express low levels of espin and slightly shorter stereocilia in cochlear hair cells during the first postnatal week of development, but adult cochlear bundles with an apparently normal staircase architecture (Sekerikova et al., 2011; Zheng et al., 2000). Therefore, the reported differences in stereocilia heights in heterozygous *jerker* mice are probably due to a developmental delay in stereocilia elongation. Espin 1 is the longest isoform of the espin gene and it is targeted to the tips of cochlear stereocilia by myosin III and other unidentified myosin motors (Lelli et al., 2016; Salles et al., 2009). Isoform-specific knock-out mice lacking espin 1 have normal hearing and cochlear hair bundle morphologies (except for delayed retraction of the supernumerary stereocilia during the early development of the hair bundle), but exhibit abnormally long and thin stereocilia in vestibular hair bundles (Ebrahim et al., 2016b). Thus, one or several of the shorter isoforms of espin — and not espin 1 — are the isoforms impacting the elongation of stereocilia in the cochlea of the *jerker* mutant mice. It is worth mentioning that all espin isoforms cause a dramatic elongation of microvilli when expressed in LLC-PK1-CL4 cells (Loomis et al., 2003).

XIRP2 knock-out mice also have shorter stereocilia in IHCs and OHCs as measured via confocal or electron microscopy images, but the cochlear hair bundles still exhibit a marked staircase arrangement (Scheffer et al., 2015). Stereocilia from XIRP2 knock-out mice start degenerating during the second postnatal week (Scheffer et al., 2015).

The espin-like (ESPNL) protein is expressed by a gene paralog to espin and it localizes at the tips of transducing stereocilia (Ebrahim et al., 2016b). Unlike espin, ESPNL appears to

have only one actin-binding domain and thus is unable to crosslink actin filaments (Ebrahim et al., 2016b). ESPNL knock-out mice exhibit high-frequency hearing loss, and OHCs in the base of the cochlea show missing stereocilia in the shortest row of the bundle (Ebrahim et al., 2016b).

RIPOR2 (also known as Fam65b) oligomers form a ring-like structure around the stereocilia cytoskeleton at the taper region of mouse cochlear hair cells and RIPOR2-deficient mice have cochlear bundles with disorganized stereocilia, abnormal bundle morphology (shape and staircase architecture) and abnormally long stereocilia (Zhao et al., 2016).

4. Regulating the thickness of the stereocilium

Several mutant mice with defects in the stereocilia actin crosslinkers exhibit stereocilia width abnormalities. *Jerker* mutant mice exhibit IHC and OHC bundles with abnormally thin stereocilia, while heterozygous *jerker* mice show tapered thinning along the stereocilia shaft (Sekerova et al., 2011; Zheng et al., 2000). Plastin 1 knock-out mice exhibit progressive hearing loss and thin stereocilia in IHCs and OHCs, in particular at the stereocilia tips, which is similar to the phenotype of *jerker* heterozygous mice (Krey et al., 2016; Taylor et al., 2015). Interestingly, in cochlear hair cells of plastin 1 knock-out mice, the thinning of the stereocilia actin cores may occur despite slightly larger interfilament distances and, therefore, it may be associated with the decreased number of actin filaments (Taylor et al., 2015). In contrast, vestibular stereocilia of plastin 1-deficient mice exhibit both a lower number of actin filaments and smaller interfilament distances (Krey et al., 2016). Mice with a mutation in fascin 2 as well as fascin 2 knock-out mice both exhibit progressive hearing loss and progressive degeneration of cochlear hair bundles (Liu et al., 2018; Shin et al., 2010). However, detailed characterizations of the changes in stereocilia morphology or the stereocilia cytoskeleton ultrastructure in the absence of fascin 2 are still missing. XIRP2 knock out mice have abnormally thin stereocilia in IHCs but slightly thicker stereocilia in OHCs (Scheffer et al., 2015).

Myosin III and XVA may be involved not only in delivering proteins required for stereocilia elongation but also regulating stereocilia thickness. *Shaker-2* mice exhibit apparently thick stereocilia most likely due to the lack of the myosin XVA cargo, whirlin. It has been reported that *whirler* mice exhibit thicker stereocilia in both IHCs and OHCs. However, actin filament counts in transmission electron micrographs showed the same number of actin filaments per stereocilium in mutant and wild type mice, indicating that whirlin may play role in the regulation of the interfilament spacing within the actin core of a stereocilium (Mogensen et al., 2007), perhaps via the delivery of specific actin crosslinkers (Wang et al., 2012). Mice lacking only the long isoform of myosin XVA have transducing stereocilia in IHCs that become abnormally thin (initially at the stereocilia tips) and eventually degenerate (Fang et al., 2015). The lack of the long isoform of myosin XVA also leads to stereocilia degeneration in OHCs, but without marked changes in stereocilia thickness. In myosin IIIA knock-out mice, cochlear hair bundles have normal morphology but accelerated stereocilia degeneration and progressive hearing loss (Lelli et al., 2016; Walsh et al., 2011). Myosin IIIB knock-out mice show no phenotype at all (Lelli et al., 2016). However, mice lacking myosin IIIB and having only one copy of the myosin IIIA gene exhibit occasional, yet

marked, thinning at the tips of IHC stereocilia (Lelli et al., 2016). This indicates that myosin IIIA and IIIB may have a role in determining the thickness of stereocilia but they may compensate for each other.

In addition to its actin-capping activity, EPS8 has been shown to regulate actin bundling via its binding to the SH3 domain-containing protein BAIAP2 (also known as IRSp53) (Disanza et al., 2006; Hertzog et al., 2010). It is yet unknown whether EPS8 has an impact on the crosslinking of actin filaments in the hair cell stereocilia. Nevertheless, EPS8 knock-out mice exhibit cochlear bundles remarkably similar to those from the *shaker-2* and *whirler* mice, with thicker stereocilia in the shorter rows of IHCs (Zampini et al., 2011). In contrast, EPS8L2 knock-out mice have abnormally thin shorter rows transducing stereocilia in IHCs (Furness et al., 2013). Given that EPS8L2 does not bind BAIAP2, it may not be involved in regulating actin bundling activity like EPS8. Therefore, the mechanisms by which EPS8L2 can regulate stereocilia thickness remain to be explored. Mice deficient for the actin-capping protein CAPZB also exhibit abnormally thin stereocilia (Avenarius et al., 2017).

5. Stereocilia cytoskeleton maintenance and repair

Birds, fish, amphibians, and reptiles can regenerate their hair cells. Mammalian auditory hair cells have lost this ability and, therefore, they are expected to survive for several years or even decades. As discussed below, several groups have reported that the actin core within stereocilia is rather stable and it does not undergo active protein turnover. Consequently, mammalian auditory hair cells must maintain their stereocilia bundles for years or decades without relying on active cytoskeleton turnover or cell regeneration. This implies the existence of largely unknown mechanisms of stereocilia repair in these cells.

a. Turnover of the stereocilia cytoskeleton

Initial reports on the incorporation of exogenous β -actin into the stereocilia of young postnatal rats hypothesized that the stereocilia actin core is maintained through the continuous treadmill of actin (Rzadzinska et al., 2004; Schneider et al., 2002). A similar relatively fast actin remodeling was demonstrated in zebrafish stereocilia but without evidence of treadmilling (Hwang et al., 2015). However, several independent groups have now established that, in adult and young mammalian and non-mammalian hair cells, active actin remodeling occurs only in a small ($\sim 0.5 \mu\text{m}$) region at the tips of stereocilia but not along their shafts (Drummond et al., 2015; Narayanan et al., 2015; Zhang et al., 2012) (Fig. 3A). In mammalian hair cells, the tip region of actin remodeling ($\sim 0.5 \mu\text{m}$) was observed in the relatively long vestibular stereocilia or the first and second row stereocilia in IHCs. The size of this region of actin remodeling in the shortest rows of IHC and OHC stereocilia is uncertain since the total height of these stereocilia could be as small as $\sim 0.5 \mu\text{m}$. In contrast to F-actin, the actin crosslinker fascin 2, however, shows continuous turnover in cochlear and vestibular hair bundles, indicating that the stereocilia cytoskeleton has both static and dynamic components (Roy et al., 2018).

b. Mechanotransduction current and cytoskeleton stability

Mechanotransduction in cochlear hair cells appears and matures in parallel with the hair bundle development (Geleoc et al., 2003; Lelli et al., 2009; Waguespack et al., 2007). Therefore, it is tempting to speculate, following Tilney et al. (1992b), that the MET current somehow drives the formation of the remarkable staircase morphology of the hair cell bundle. This idea has been recently revived after demonstration of stereocilia abnormalities in the non-mechanotransducing hair cells with developmentally delayed disruption of USH1G (also known as SANS, a scaffold protein likely responsible for tip link maintenance) or cadherin 23 (CDH23, a known tip link component) (Caberlotto et al., 2011a; Caberlotto et al., 2011b). Despite suggestive similarity of stereocilia changes, these *in vivo* models cannot convincingly separate the effects of MET disruption from the potential direct effects on stereocilia structure, since both USH1G and CDH23 are known to be essential for normal hair bundle architecture (Holme et al., 2002a; Kikkawa et al., 2003). More direct evidence came from experiments blocking the resting MET current *in vitro* (Velez-Ortega et al., 2017). At the resting bundle position, the tip links are slightly tensioned to guarantee MET responses to the smallest bundle deflections, which leads to a small but constitutive inward current (Corey et al., 1979). Elimination of this resting MET current in the mammalian auditory hair cells, either by blockage of the MET channels or by breakage of the tip links, both lead to the selective shortening of stereocilia in the shorter rows of a hair bundle, *i.e.* the stereocilia that harbor MET channels, without affecting the tallest non-transducing stereocilia bundles (Velez-Ortega et al., 2017). Furthermore, these effects could be recapitulated by manipulations of intra- and extra-cellular Ca^{2+} , suggesting that it is the Ca^{2+} influx through the MET channels that controls the stability of the stereocilia cytoskeleton (Fig. 3B). This Ca^{2+} -dependent control is dynamic since stereocilia are able to regrow after the washout of the MET channel blockers, the regeneration of the tip links, or the recovery of the intracellular Ca^{2+} buffering (Velez-Ortega et al., 2017). Upon retrospective examination of previously published images, we noticed that mice with deficits in the components of the MET machinery TMC1, TMC2, TMHS/LHFPL5, TMIE and CIB2 can develop nearly normal cochlear hair bundles up to postnatal days 4 or 5 (Giese et al., 2017; Kawashima et al., 2011; Xiong et al., 2012; Zhao et al., 2014). However, after this point, they all start exhibiting changes in the morphology of the stereocilia and/or the staircase architecture of the hair bundle that resemble the changes after pharmacological (Velez-Ortega et al., 2017) or genetic (Caberlotto et al., 2011a; Caberlotto et al., 2011b) deactivation of the MET channels. Thus, it is unlikely that the activity of MET channels controls the developmental formation of the staircase architecture of the hair bundle. However, this activity is very likely to be crucial for the apparent stability of the stereocilia cytoskeleton throughout the life span of the organism.

c. Repair of the stereocilia bundle

Current literature on the potential treatments of acquired hearing loss is largely ignorant to the intrinsic mechanisms of stereocilia repair that may exist in the mammalian cochlear hair cells. Yet, damage to the stereocilia bundles has been known as a hallmark of permanent noise-induced hearing loss for several decades (Liberman, 1987). New data on the stability of the stereocilia cytoskeleton provide a potential self-repair mechanism that may exist in stereocilia. In an undamaged cochlear hair cell, constitutive Ca^{2+} influx through the MET

channels stabilizes the actin core of stereocilia (Velez-Ortega et al., 2017). However, damage to the tip links during noise exposure (Husbands et al., 1999; Kurian et al., 2003; Pickles et al., 1987), partial block of the MET channels by ototoxic antibiotics (Ricci, 2002; Rusch et al., 1994), or even other cochlear insults such as the ones that affect the endocochlear potential (Asakuma et al., 1980), may inhibit this influx through the MET channels and initiate instability of stereocilia cytoskeleton. This instability may help repair not only the actin core of stereocilia but also the other components of the mechanosensory apparatus that are linked to the cytoskeleton. It is very interesting to see whether further studies would confirm or refute the existence of this, as of today, largely speculative mechanism. Interestingly, the region of actin turnover at the tips of the transducing second row stereocilia in the mouse IHCs is generally larger and more variable compared to that in the non-transducing tallest row stereocilia (Narayanan et al., 2015), and resembles the variations that can be seen in the Ca^{2+} influx through MET channels at the tips of individual stereocilia in these cells (Beurg et al., 2009).

6. Calcium sensitive elements in the stereocilia cytoskeleton

Since the stability to the stereocilia cytoskeleton depends on the influx of Ca^{2+} through the MET channels (Velez-Ortega et al., 2017), the Ca^{2+} -sensitive components of the stereocilia cytoskeleton may be the first candidates for the regulation of the stability and/or rearrangement of the stereocilia actin paracrystalline core. First, Ca^{2+} influx through the MET channels may have a variable effect on actin isoforms themselves. Indeed, when bound to Ca^{2+} , γ -actin exhibits slower polymerization and depolymerization kinetics than β -actin (Bergeron et al., 2010). In contrast to espins, that have no recognizable binding site for Ca^{2+} and whose actin bundling activity is not affected by Ca^{2+} *in vitro* (Sekerova et al., 2003), some F-actin crosslinkers from the plastin and α -actinin families are sensitive to changes in Ca^{2+} concentration. Plastins have two N-terminal Ca^{2+} -binding EF-hand domains (de Arruda et al., 1990). While plastin-1 and plastin-2 activities seem to be inhibited by Ca^{2+} , plastin-3 activity appears to be insensitive to changes in free Ca^{2+} concentrations (Giganti et al., 2005; Lin et al., 1994; Namba et al., 1992). α -actinins have a C-terminal Ca^{2+} -binding EF hand domain, and Ca^{2+} has been shown to regulate the actin-binding activity of actinin 1 and actinin 4 (Burridge et al., 1981; Tang et al., 2001). Members of the gelsolin family enhance actin dynamics upon an increase in intracellular Ca^{2+} and are also present in hair cell stereocilia (Kinosian et al., 1998; Mburu et al., 2010; Olt et al., 2014; Revenu et al., 2007).

CIB2 is a Ca^{2+} -binding protein that is part of the MET machinery in hair cells. Mice deficient in CIB2 exhibit overgrown transducing stereocilia (Giese et al., 2017), perhaps due to the lack of sequestering of free Ca^{2+} by CIB2 at the strategic location of the stereocilia elongation machinery at the very tip of a stereocilium. Calcium-binding protein 1 (CABP1) is a Ca^{2+} -binding protein that stabilizes the actin cytoskeleton in dendritic spines but, in response to a Ca^{2+} influx, allows the remodeling of the actin cytoskeleton and thus regulates activity-dependent synaptic plasticity (Mikhaylova et al., 2018). CABP1 is highly expressed in the spiral ganglion (Yang et al., 2016) where it plays a role in the excitability and synchrony of auditory nerve fibers (Yang et al., 2018), but it is also present in IHCs and OHCs (Yang et al., 2016) where its function and subcellular localization remain unknown.

In addition to potential regulation by structural proteins like CIB2 or other immobile Ca^{2+} -binding proteins, the concentration of free Ca^{2+} in the stereocilium is known to be tightly regulated by plasma membrane calcium ATPases (PMCA) (Crouch et al., 1995; Ficarella et al., 2007; Yamoah et al., 1998) — with the specific type 2a isoform (PMCA2a) being the only PMCA in OHC bundles and the predominant PMCA in IHC bundles (Dumont et al., 2001). The intrastereocilium concentration of free Ca^{2+} is also regulated by mobile (diffusible) buffers such as calmodulin, parvalbumin- β and calbindin-D28k (Beurg et al., 2010; Furness et al., 2002; Hackney et al., 2005). Therefore, all these proteins may be also involved in maintaining stereocilia stability and it is not surprising that mutations in PMCA2a result in profound disorganization of stereocilia bundles (Spiden et al., 2008). However, in contrast to Ca^{2+} -binding cytoskeletal proteins such as CIB2 or the long isoform of myosin XVA, whose expression is more pronounced in the transducing shorter row stereocilia (Fang et al., 2015; Giese et al., 2017), neither PMCA2a nor mobile Ca^{2+} -buffers exhibit any preferred localization between stereocilia rows within a hair bundle (Chen et al., 2012; Hackney et al., 2005). Thus, available data so far indicate modulatory rather than direct roles of PMCA2 and mobile Ca^{2+} buffers in the MET-dependent remodeling of stereocilia.

7. Conclusions

The initial development of the cochlear hair cell stereocilia seems to be regulated by temporarily and spatially restricted signals, resulting in the formation of characteristic stereocilia shapes and staircase architectures of the bundle. However, some of these signals vary between different hair cell types (*e.g.*, IHCs vs. OHCs), different cochlear regions, or specific rows within the hair bundle (*e.g.*, transducing vs. non-transducing stereocilia). Soon after the initial formation of the cochlear hair cells bundles, the stability of the stereocilia actin cytoskeleton becomes dependent on the constant influx of Ca^{2+} through the MET channels that are partially open at rest. Mature cochlear hair bundles have an exceptionally stable cytoskeleton that undergoes only limited actin turnover at the tips. Most likely, this stability is still regulated by the Ca^{2+} influx through the MET channels. Therefore, any cochlear insult that affects the resting MET current and, hence, the levels of free Ca^{2+} in the stereocilia could affect the balance between actin remodeling at the tips and the very stable cytoskeleton within the stereocilia shaft, converting a “stable” stereocilium into a “repairing” one. This mechanism may explain how our cochlear hair cells are able to maintain the precise shape of their stereocilia throughout the life span.

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Abbreviations

BAIAP2

BAI1 associated protein 2 (also known as IRSp53, insulin receptor substrate p53)

CABP1	calcium-binding protein 1
CAPZA1	capping actin protein of muscle Z-line subunit alpha 1
CAPZB	capping actin protein of muscle Z-line subunit beta
CIB2	calcium and integrin binding family member 2
EPS8	epidermal growth factor receptor pathway substrate 8
EPS8L2	EPS8-like protein 2
ESPNL	espin-like protein
GNAI3	G protein subunit alpha i3
GPSM2	G protein signaling modulator 2
IHC	inner hair cell
LHFPL5	LHFPL tetraspan subfamily member 5 (also known as TMHS)
MET	mechano-electrical transduction
OHC	outer hair cell
PDZ	PSD95 Dlg1 and ZO-1 domain
PTB	phospho-tyrosine binding domain
RIPOR2	RHO family interacting cell polarization regulator 2 (also known as Fam65b)
SH3	Src homology 3 domain
TMC1	transmembrane channel-like protein 1
TMC2	transmembrane channel-like protein 2
TMIE	transmembrane inner ear
TWF2	twincillin 2
USH1G	USH1 protein network component sans
XIRP2	Xin actin-binding repeat-containing protein 2

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Highlights

- Differentially expressed proteins regulate the hair bundle staircase architecture
- The stability of the stereocilia actin core depends on intrastereocilia Ca^{2+} levels
- Changes in intrastereocilia Ca^{2+} levels may trigger signals for stereocilia repair
- Ca^{2+} -sensitive proteins within stereocilia could be potential therapeutic targets

Fig. 1. Mouse cochlear hair cell bundles.

(A, C) Inner hair cell (IHC) at postnatal day 10 (A) and outer hair cell (OHC) at postnatal day 11 (B) showing the uniform stereocilia heights and diameters within each row of the hair bundle. (B, D) High-magnification views of the regions highlighted in panels A and C, showing the links that interconnect stereocilia in IHC (B) and OHC (D). Arrows point to the “tip links” that connect the top of a stereocilium from a shorter row to the side of a stereocilium from the next taller row, in the axis of mechanotransduction. (E, F) Diagram illustrating stereocilia from three different rows, interconnected by tip links, before (E) and during (F) bundle deflection. Notice that deflection of the bundle in the positive direction (*i.e.* toward the tallest row) leads to the gating of the non-selective cation MET channels at the tips of the shorter “transducing” stereocilia.

Fig. 2. Schematic representation of the paracrystalline actin core and proteins with differential trafficking within mammalian auditory hair cell bundles.

(**A**) Parallel actin fillaments are crosslinked into a rigid paracrystalline structure by members of the plastin, fascin, and espin families. Relative expression levels between members of these three families of crosslinkers were set to match the levels found in mouse vestibular hair cell bundles via quantitative mass spectrometry (Krey et al., 2016). (**B**) Tips of two neighboring stereocilia (from two different rows within the bundle) that are connected via a tip link, showing the proteins that are selectively enriched at the tips of transducing (left) versus non-transducing (right) stereocilia.

Fig. 3. Hypothesized activity-dependent remodeling of the stereocilia actin core.

(A) The constant influx of Ca^{2+} ions via the resting MET current stabilizes the stereocilia actin core, and active actin remodeling (green) is limited to the very tips of stereocilia. (B) When the MET channels are closed, due to tip link breakage or to the presence of MET channel blockers, the stability of the actin core is lost and the actin cytoskeleton within the stereocilia shaft undergoes active remodeling as well.