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The actin cytoskeleton in hair bundle development and hearing loss

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Abstract

Inner ear hair cells assemble mechanosensitive hair bundles on their apical surface that transduce sounds and accelerations. Each hair bundle is comprised of ~ 100 individual stereocilia that are arranged into rows of increasing height and width; their specific and precise architecture being necessary for mechanoelectrical transduction (MET). The actin cytoskeleton is fundamental to establishing this architecture, not only by forming the structural scaffold shaping each stereocilium, but also by composing rootlets and the cuticular plate that together provide a stable foundation supporting each stereocilium. In concert with the actin cytoskeleton, a large assortment of actin-binding proteins (ABPs) function to cross-link actin filaments into specific topologies, as well as control actin filament growth, severing, and capping. These processes are individually critical for sensory transduction and are all disrupted in hereditary forms of human hearing loss. In this review, we provide an overview of actin-based structures in the hair bundle and the molecules contributing to their assembly and functional properties. We also highlight recent advances in mechanisms driving stereocilia elongation and how these processes are tuned by MET.

Keywords

Actin; Actin binding protein; Stereocilia; Hair bundle; Deafness; Hearing loss

1. Introduction

The inner ear houses the auditory and vestibular end organs, which are responsible for the sensation of hearing and balance, respectively. Although these organs vary considerably in architectural complexity, from the 3D intricacy of the cochlea to the more planar maculae of the vestibular system, they all share a common transduction machinery necessary for sensory function. At the heart of sensory transduction are hair cells, which convert mechanical displacements into perturbations of hair cell receptor potential. To transduce mechanical

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stimuli originating from sound or accelerations, each individual hair cell assembles a complex sensory bundle consisting of approximately 100 stereocilia that elongates from its apical pole (Schwander et al., 2010). Within each hair bundle, stereocilia are precisely arranged into rows of graded heights and widths (Fig. 1A) and are interconnected by an array of extracellular links that merge them into a mechanically cohesive unit (Richardson and Petit 2019). Tip-link filaments, formed of proto-cadherin-15 (PCDH15) and cadherin-23 (CDH23), bridge between the tips of shorter stereocilia (row 2 + 3) to the shaft of the nearest taller stereocilia neighbor (Kazmierczak et al., 2007). Displacement of the hair bundle towards the tallest row tensions tip links and gates mechanoelectrical (MET) channels located on these shorter stereocilia rows (Qiu and Müller, 2022). The overall architecture of the hair bundle is essential for the MET apparatus to be optimally stimulated by sound and accelerations. Highlighting the exquisite precision of this system, this architecture ensures detection of sub-nanometer hair bundle displacements at the threshold of hearing.

Unlike their name implies, stereocilia are not true cilia assembled from microtubules. Instead, they are actin-based organelles that develop from microvillus-like precursors using a complex program of elongation and thickening (Barr-Gillespie, 2015). The actin cytoskeleton is central to this process, forming the structural core within each stereocilium defining its size, shape and mechanical properties (Fig. 1B). Stereocilia vary tremendously throughout the mammalian inner ear, ranging from a few microns long in the cochlea to over 100 μm long in the vestibular organs. Stereocilia further vary in size and length depending on their position within each sensory organ; this variation is particularly evident in the cochlea where stereocilia lengths scale along the tonotopic axis. Stereocilia lengths are further tuned within each hair bundle to establish the graded heights of the staircase architecture. This spectrum of stereocilia shapes and sizes are reproduced during development to within tight tolerances, revealing the presence of a precise and tunable cytoskeletal assembly template. How this template is specified at a molecular level remains an enduring question. A substantial number of proteins necessary for establishing stereocilia architecture have now been identified, providing the component parts-list for this mechanism. Perhaps unsurprisingly, this parts-list is dominated by actin and actin-associated proteins, confirming their indispensable role in stereocilia assembly and sensory transduction (see Table 1 and Fig. 2). In this review, we explore the fundamental properties of the actin cytoskeleton, and how these proteins are harnessed to enable the development, plasticity and long-term structural integrity of stereocilia; processes that are indispensable for sensory function.

2. Actin as the fundamental building block of stereocilia

Actin is the most abundantly expressed protein in eukaryotic cells and is the key building block of the filamentous actin cytoskeleton. The mammalian genome encodes six actin genes: four muscle (*ACTA1, ACTC1, ACTA2, ACTG2*) and two cytoplasmic actins (*ACTB, ACTG1*) (Vandekerckhove and Weber, 1978). The actin isoforms are highly conserved with an overall 95–99% sequence identity and share a similar capacity to reversibly polymerize into filaments (Kashina, 2020). Each actin monomer (42 kDa, 375 residues) consists of four distinct subdomains that together bind to a single adenine nucleotide (ATP, ADP-Pi or ADP) and has intrinsic ATPase activity (Fig. 1D). In the presence of mono and divalent

cations, globular actin monomers (G-actin) above the critical concentration (C_c = ~ 0.1 μM for Mg-ATP-actin) spontaneously polymerize into filamentous actin (F-actin) polymers. This process of salt-induced actin polymerization is well characterized and initiates with actin monomers undergoing an energetically unfavorable nucleation to form a trimeric "seed" proto-filament, which can then be extended by the incorporation of additional Mg-ATP-actin monomers. Actin filaments form an apparent right-handed helix with two chains (see Fig. 1E). Each turn of the helix has approximately 13 actin monomers with an overall helical pitch of 36 nm and a helical rise of 2.76 nm per monomer (Pollard, 2016). F-actin exhibits structural polarity with Mg-ATP-actin monomers preferentially incorporated at the fast-growing barbed ends, so called for their appearance under TEM when decorated with the proteolytic S1 fragment of muscle myosin. As G-actin monomers are incorporated into F-actin, their ATPase activity acts as a built-in timer to hydrolyze ATP (the rate of hydrolysis of Mg-ATP-actin is 0.3 s^{-1}) and measure the age of the monomer within the filament (Blanchoin and Pollard, 2002). Mg-ADP-actin monomers preferentially dissociate from the filament pointed end. The polymerization reaction continues until equilibrium, where the rate of pointed end monomer removal is equal to the rate of barbed end monomer addition (*i.e.*, the barbed end association rate constant multiplied by the free actin monomer concentration). When both rates are balanced at equilibrium, actin filaments maintain a steady state length as monomers are continually added, and removed, in a process called treadmilling (Wegner 1976). The critical concentration (C_c) for this reversible polymerization is defined as the concentration of free actin monomer that co-exists with actin polymer at equilibrium (C_c = ~ 0.1 μM for Mg-ATP-actin). At [G-actin] concentrations more than C_c, the rate of barbed end actin monomer addition exceeds dissociation from the pointed end, and the filament can elongate. Treadmilling occurs *in vitro*, but also has been observed in cellular structures such as lamellipodia, filopodia and microvilli, confirming the importance of this basic biochemical phenomenon in cells (Carlier and Shekhar 2017).

To prevent spontaneous and uncontrolled actin polymerization within the cytosol, where [G-actin] >> C_c, actin filament polymerization is tightly controlled by actin-binding proteins (ABPs). ABPs influence almost every aspect of the polymerization cycle, from sequestering actin monomers, promoting actin seed nucleation, capping and stabilizing filament barbed or pointed ends, to severing existing filaments (Pollard, 2016). Actin severing proteins promote actin depolymerization, thereby controlling actin filament length and recycling actin monomers. Severing proteins can also promote actin polymerization, by uncovering new barbed ends available for elongation (Ono, 2007). Precise cellular control of these activities allows the assembly of actin filaments to be spatially coordinated within the cell. An additional class of ABPs can cross-link actin filaments into macromolecular networks, and these are particularly important for building larger cellular architectures such as the cytokinetic ring of dividing cells, contractile actin stress fibers, lamellipodia as well as stereocilia, a large actin structure of elaborate complexity. A large and constantly growing catalog of mutations have been identified in genes encoding ABPs that cause hereditary forms of human hearing loss (see Table 1), and many of these pathogenic alleles interfere with the structure and function of stereocilia.

As actin filaments polymerize and assemble into larger macro-molecular structures, filament mechanics become foundational to the emergent properties of the polymer network. A key

mechanical property of biological polymers, such as actin filaments, is their persistence length, which is a measure of bending stiffness and the energetic cost of deformation. Polymers much shorter than the persistence length can be modelled as rigid, whereas polymers much longer than the persistence length are flexible and are described by random-walk statistical models. Individual actin filaments have a persistence length of ~18 μm, meaning that they are flexible on the cellular scale (Gittes et al., 1993). The bending stiffness of actin filaments and networks can be dynamically tuned, and one important mechanism is through the availability of cations in solution. Two divalent cation-binding sites have been identified in the actin monomer, with one controlling "polymerization" and the other "stiffness" (Kang et al., 2012). Binding of divalent cations to the "stiffness" site located in proximity to the actin D-loop increases actin filament torsional stiffness and persistence length (Hocky et al., 2016; Kang et al., 2012). Actin filament cross-linking by ABPs also massively increases persistence length and allows the assembly of more complex viscoelastic networks (between a viscous fluid and elastic solid) that enable cells to generate and respond to mechanical forces. Depending on the physical properties of crosslinking ABPs, including their dissociation constant for actin binding (K_D) and inter-filament cross-link spacing, ABPs can generate diverse network topologies with tunable mechanical properties (Claessens et al., 2006). Stereocilia are an extraordinary example of how individual actin filaments can be organized and cross-linked by ABPs to create a large, ordered cellular structure (Fig. 2).

Further diversity in actin filament properties originates from the expression of specific actin isoforms, which can co-polymerize into filaments and create subunit heterogeneity (Bergeron et al., 2010). Only beta (ACTB) and gamma (ACTG1) cytoplasmic actin isoforms are detected in sensory hair cells (Andrade, 2015; Furness et al., 2005; Perrin and Ervasti, 2010; Slepecky and Savage, 1994). These ACTB and ACTG1 isoforms are 99% identical, differing by only four amino acid substitutions in the first ten N-terminal residues. Despite the overall amino acid identity between ACTB and ACTG1 isoforms, biochemical assays using purified proteins reveal these two actin isomers have significantly different polymerization kinetics, with ACTG1 having notably slower nucleation and filament elongation kinetics as compared with ACTB (Bergeron et al., 2010). There is extensive evidence supporting distinct functions for ACTB and ACTG1 in cells, with differences in sub-cellular localization, post-translational modifications and isoform specific interactions with ABPs (Kashina, 2020). The cumulative effects of different actin isoform activity are emphasized in mutant animal models. Knockout *Actb*(−/−) mice are embryonically lethal at E8.5 (Bunnell et al., 2011; Shawlot et al., 1998), whereas *Actg1*(−/−) mice are viable, albeit with increased perinatal lethality (Belyantseva et al., 2009). Remarkably, *Actg1*(−/−) mice have a progressive hearing loss phenotype that results from degeneration of the stereocilia core, arguing that ACTB1 cannot compensate for the loss of ACTG1. Conversely, conditional deletion of *Actb1* specifically from hair cells also leads to postnatal degeneration of the stereocilia core and hearing loss, highlighting the need for both ACTB and ACTG1 in hair cells (Perrin et al., 2010).

Further insight into the function of actin isoforms comes from the identification of pathogenic *ACTB* and *ACTG1* alleles that co-segregate with hereditary hearing loss in humans. Dominantly inherited mutations in *ACTB* and *ACTG1* have been

respectively reported in Baraitser-Winter cerebrofrontofacial syndromes BRWS1 and BRWS2, pleiotropic diseases characterized by cranio-facial abnormalities, defective CNS development, coloboma and sensorineural hearing loss, amongst other phenotypes (Cuvertino et al., 2017; Yates et al., 2017). Mutations in *ACTG1* separately cause a phenotypically milder autosomal dominant, non-syndromic hereditary hearing loss DFNA20/26, characterized by a post-lingual progressive sensorineural hearing loss (Morell et al., 2000; Zhu et al., 2003). A number of pathogenic variants have been reported since the first confirmation of a causative deafness-causing mutation (p.T89I) in *ACTG1* (Zhu et al., 2003). Missense *ACTG1* variants do not cluster in specific hot spots but are distributed throughout the actin monomer. Deciphering the biochemical effects of ACTB and ACTG1 mutations has proven challenging, as mutant actins studied in, or purified from, recombinant expression systems are contaminated with wild-type actin to varying degrees. To address this, one approach has been to introduce mutations into the endogenous wild-type actin gene in yeast. Using this technique, DFNA20/26 mutations have been found to have varying effects upon increased nucleotide exchange rate and actin monomer conformational flexibility (Bryan and Rubenstein, 2009; Morín et al., 2009). DFNA20/26 mutations in ACTG1 can interfere with F-actin binding to ABPs, such as the severing protein cofilin, and the actin nucleator Arp2/3 complex (Bryan and Rubenstein, 2009; Kruth and Rubenstein, 2012) and have been reported to prevent polymerization into filaments (Miyajima et al., 2020). Newer approaches using actins tagged with cleavable profilin / beta-thymosin-4 fusions allow isoforms and mutants to be easily purified away from contaminating endogenous actin and will greatly accelerate structural and biochemical analyses (Ceron et al., 2022; Hatano et al., 2018). Although mouse models carrying *Actg1* variants have not been reported to date, it is clear from the broad spectrum of molecular phenotypes identified *in vitro* that DFNA20/26 hearing loss likely has multiple etiologies.

3. Actin filaments form the para-crystalline stereocilia core

The stereocilia core acts as a scaffold to shape and support the overlaying plasma membrane and provides the necessary rigidity for the hair bundle to be coherently stimulated by sounds and accelerations (Tilney et al., 1980). Pioneering ultra-structural studies using transmission electron microscopy (TEM) showed that stereocilia consist of thousands of parallel actin filaments, that are so precisely arranged, they exhibit para-crystalline order along the stereocilia long axis (DeRosier et al., 1980; Tilney et al., 1980). Individual actin filaments span the entire length of the stereocilia core and are all orientated unidirectionally with their fast-growing barbed-ends towards the tip (Flock and Cheung, 1977; Tilney et al., 1980). As a result of this exquisite structural order the stereocilia core can behave as a “light pipe”, which has inadvertently confounded the interpretation of fluorescence microscopy images (Egelman, 1981; Kohllöffel, 1977). Hair cells express cytoplasmic actins ACTB and ACTG1, and both proteins are distributed throughout the stereocilia actin core (Andrade, 2015; Belyantseva et al., 2009; Furness et al., 2005; Höfer et al., 1997; Patrinostro et al., 2018; Perrin et al., 2010), with some indication that ACTG1 might be more concentrated at the stereocilia tips (Patrinostro et al., 2018). These studies indicate that the bulk of stereocilia actin filaments might be heterogenous co-polymers of ACTB and ACTG1. Significant differences in actin isoform distribution emerge following mechanical damage to

the actin core. Noise trauma to the cochlea causes breaks (or “gaps”) in the stereocilia actin core, and ACTG1 specifically accumulates in these damaged regions, potentially to repair the core (Belyantseva et al., 2009). In *Actg1(−/−)* knockout mice, ACTB is recruited to these breaks instead of ACTG1, but this is unable to prevent overall stereocilia degeneration (Belyantseva et al., 2009; Perrin et al., 2010). The relative expression of ACTG1 relative to ACTB decreases in geriatric mice at 22 months of age, suggesting that attenuation of this repair process might contribute to age-related hearing loss (Andrade, 2015).

Stereocilia lengths typically exceed the persistence length of naked actin filaments, and thus assembly of each stereocilium depends upon ABPs to cross-link filaments and increase their flexural rigidity. Mass-spectrometry of hair bundles have catalogued a set of major cross-linking proteins, including fascin-2 (FSCN2), espin (ESPN), plastin-1 (PLS1) and Xin actin binding repeat containing 2 (XIRP2) (Krey et al., 2016; Krey et al., 2015; Shin et al., 2010). Together, these proteins contribute to assembling the mature stereocilia core with a 10–13 nm inter-filament spacing (Song et al., 2020; Tilney et al., 1980). The structural and mechanical properties of cross-linked actin filaments are determined by the biophysical and biochemical properties of actin crosslinkers, including cross-linker size, flexibility, dissociation constant (K_D) and binding kinetics (k_{on} and k_{off}) (Bathe et al., 2008; Claessens et al., 2006). In this section we will discuss the properties of these key actin cross-linkers and how they contribute to the mature stereocilia structure.

ESPN is the most critical actin cross-linking for hair bundle formation, evidenced by the severe stereocilia phenotype in the *jerker* mouse that prevents normal stereocilia widening and leads to rapid hair bundle degeneration and deafness (Rzadzinska et al., 2005; Sekerková et al., 2011; Sjöström and Anniko, 1992; Zheng et al., 2000). Mutations in *ESPN* cause autosomal recessive human hearing loss, DFNB36 (Naz et al., 2004). The mouse *Espn* gene encodes multiple ESPN protein isoforms (ranging from ~ 25 kDa to 110 kDa) that all share a common, calcium insensitive actin-filament binding module in their COOH-terminus (Bartles et al., 1998; Sekerková et al., 2004). Actin filaments crosslinked by ESPN form unipolar parallel bundles with ~ 12 nm inter-filament spacing, with ESPN molecules sparsely bound every 20–50 actin monomers (Kitajiri et al., 2010; Purdy et al., 2007). ESPN isoforms are detected along the length of stereocilia, consistent with their function building the para-crystalline core (Salles et al., 2009; Zheng et al., 2000) All ESPN isoforms additionally contain a WH2 domain that can bind to actin monomers but does not stimulate Arp2/3 actin nucleation activity (Loomis et al., 2006). Longer ESPN isoforms (ESPN-1, ESPN-2B, ESPN-3A) bind to profilin (Sekerková et al., 2004), and the longest isoforms ESPN-1 and ESPN-2 contain an additional actin filament binding site (Chen et al., 1999) that is autoinhibited by the ankyrin repeat domain in ESPN-1 (Zheng et al., 2014), and also have anti-capping activity (Zheng et al., 2022). The localization of specific ESPN isoforms has not been determined, except for ESPN-1, which concentrates at the tips of all stereocilia rows in a similar pattern to MYO3A (Ebrahim et al., 2016; Zheng et al., 2014). Interestingly, the auto-inhibition of ESPN-1 is relieved by binding to the tail domain of either MYO3A or MYO3B, providing an important connection to the stereocilia length regulation machinery (Liu et al., 2016). Consistent with this inhibition, *Espn-1* mutant hair cells in the vestibular organs have over-elongated stereocilia and lack the graded staircase architecture (Ebrahim et al., 2016). Stereocilia lengths are curiously unaffected in cochlear

hair cells of *Espn-1* mutant mice, showing that there are additional components to this mechanism (Ebrahim et al., 2016).

Plastin-1 (PLS1, also known as I-fimbrin) is another major stereocilia ABP; it crosslinks actin filaments into parallel bundles with ~ 12 nm inter-filament spacing (Bretscher, 1981; Volkmann et al., 2001). PLS1 has two actin binding domains (ABD) with each ABD containing two calponin homology domains, in addition to a regulatory domain with EF-hand motifs that confer Ca^{2+} sensitive actin binding (Namba et al., 1992). Mutations in *PLS1* are associated with autosomal dominant hearing loss (Diaz-Horta et al., 2019; Morgan et al., 2019) and *Pls1(−/−)* mouse models similarly exhibit a progressive hearing loss (Krey et al., 2016; Taylor et al., 2015). PLS1 is an abundant crosslinker detected in stereocilia and is localized along the entire stereocilia shaft in both auditory and vestibular hair cells (Krey et al., 2016; Shin et al., 2013; Taylor et al., 2015; Tilney et al., 1989). The stereocilia of *Pls1(−/−)* knockout hair cells are moderately shorter and thinner than wild-type hair cell stereocilia (Krey et al., 2016). Actin filaments within *Pls1(−/−)* stereocilia are packed hexagonally in the presence of FSCN2 and ESPN, rather than in a random liquid order normally observed in wild-type stereocilia with PLS1 (Krey et al., 2016). Because the binding sites for FSCN2 and ESPN are not distributed at equal 60° intervals along the actin filament helix it is energetically expensive to deform actin filaments to fit into a hexagonal lattice, and this limits the size of actin filament bundles due to internal strain (Claessens et al., 2008). PLS1 thus allows stereocilia cores to widen by biasing actin filaments towards liquid packing, releasing the internal strain within the actin paracrystal that would otherwise be limiting (Krey et al., 2016).

The third major crosslinker of the stereocilia paracrystal is fascin 2 (FSCN2), which is a paralog of FSCN1 and FSCN3 (Lin-Jones and Burnside, 2007). Fascin proteins have four β-trefoil domains with the two major actin-binding sites located in β-trefoil 1 and 3 (Jansen et al., 2011). By homology to FSCN1, FSCN2 is expected to form densely packed, parallel actin bundles with ~ 8 nm inter-filament spacing (Aramaki et al., 2016; Jansen et al., 2011; Sedeh et al., 2010). FSCN1 assembles stiff actin bundles with a high persistence length of ~ 170 μm at a 2:1 actin to fascin stoichiometry (Takatsuki et al., 2014), and is able to promote the formation of filopodia in heterologous cell line models (Vignjevic et al., 2006). FSCN2 is the only fascin gene expressed by mammalian hair cells and its expression increases as stereocilia undergo differential elongation, suggesting a role in the initial assembly of the hair bundle (Shin et al., 2010). FSCN2 is localized along the stereocilia paracrystal and is additionally concentrated at the stereocilia tips of mouse (Perrin et al., 2013; Shin et al., 2010). Hair cells in the zebrafish otocyst express the ortholog fascin 2b that is similarly localized along the length of the stereocilia paracrystal (Chou et al., 2011). Consistent with a role in regulating the stereocilia paracrystal, over-expression of FSCN2 increases stereocilia width and length, and is able to displace endogenous PLS1 and ESPN (Chou et al., 2011; Roy and Perrin, 2018). Furthermore, a missense substitution in *Fscn2* (p.R109H) causes postnatal shortening of stereocilia and results in progressive hearing loss (Perrin et al., 2013; Shin et al., 2010). Zebrafish fascin 2b and mouse FSCN2 both have moderate affinities for actin filaments with K_D estimates ranging from 0.4 to 5 μM, respectively (Perrin et al., 2013; Chou et al., 2011). Interestingly, the p.R109H mutation does not significantly affect FSCN2 binding to actin filaments but does potently inhibit actin filament cross-linking activity

(Perrin et al., 2013). These data suggest that filament bundling by FSCN2 is critical to stabilizing actin filaments in the paracrystal and prevents their depolymerization in postnatal stereocilia (Perrin et al., 2013). Despite FSCN2 extensively cross-linking actin filaments *in vitro*, fluorescence recovery after photobleaching (FRAP) experiments in hair cells reveal a significant mobile fraction of fascin 2b / FSCN2 with half-lives of ~8 min in mouse and ~1 min in zebrafish (Roy and Perrin 2018; Hwang et al., 2015) These rates of exchange are several orders of magnitude faster than turnover of actin monomers in the stereocilia core (Hwang et al., 2015) This dynamic exchange of fascin cross-linkers in the paracrystal may facilitate the localized exchange of actin monomers and filaments that are required for stereocilia remodeling, repair and ongoing proteostasis (Hwang et al., 2015; Roy and Perrin, 2018). Interestingly, fascin paralogs are sensitive to post-translational modifications and phosphorylation of fascin 2b on serine 38 reduces its actin binding affinity and localization to filopodia and stereocilia (Chou et al., 2011; Ono et al., 1997). It remains to be determined if a balance of kinase and phosphatase activities modulate FSCN2 *in vivo* to promote actin remodeling in precise stereocilia subdomains.

Xin-actin-binding-repeat-containing protein 2 (XIRP2) is another major class of actin crosslinker localized to the stereocilia paracrystal. XIRP2 binds and crosslink actin filaments via its 28 Xin domains (Pacholsky et al., 2004). Two major isoform classes are encoded from the *Xirp2* gene and detected in the inner ear. Two long isoforms have been identified, containing the N-terminal Xin repeats and a C-terminal LIM (Lin11, Isl-1, and Mec-3) domain. A short isoform, also known as XEPLIN, splices out the large exon 7 containing Xin repeats and encodes only the C-terminal LIM domain (Francis et al., 2015; Scheffer et al., 2015). XIRP2 isoforms display different localizations in hair cells, with the short isoform accumulating primarily in stereocilia, whilst the longer isoforms are detected in the cuticular plate, peri–cuticular necklace and stereocilia (Francis et al., 2015; Scheffer et al., 2015). A CRISPR induced mutation in *Xirp2* causes a progressive high frequency hearing loss in mouse that becomes evident 6 weeks after birth (Francis et al., 2015). Consistent with XIRP2 localization in stereocilia and a late onset hearing loss phenotype, hair cells in *Xirp2* mutants initially develop a normal hair bundle with functioning MET currents, but start to exhibit stereocilia degeneration by P7, with the shorter rows being particularly severely affected (Francis et al., 2015; Scheffer et al., 2015). The degeneration process is associated with structural disorganization and loss of the normal, periodic spacing of actin filaments (Scheffer et al., 2015). These data argue that XIRP2 is either required for the normal development of the stereocilia paracrystal or is involved in its maintenance. In support of the latter interpretation, a preliminary report has found that XIRP2 is recruited to sites of paracrystal damage following acoustic trauma, similar to ACTG1, and that this is dependent upon the C-terminal LIM domain (Wagner et al., 2021). LIM domain containing proteins are specifically recruited to actin filaments under tension (Sun et al., 2020), and XIRP2 may similarly function to recognize damaged actin filaments and potentially catalyze their repair.

4. Tapers, rootlets and the cuticular plate

Analogous to a tall building requiring deep structural foundations for stability, stereocilia project actin-based rootlets down into the hair cell body where they are anchored within

the cuticular plate, which is an actin filament meshwork at the apical pole of the hair cell. The assembly and sculpting of these regions are critical for MET and dependent upon the unique properties of actin filaments. As stereocilia approach their contact point with the hair cell, stereocilia diameters narrow to form a taper region, which allows stereocilia to mechanically pivot as they are deflected (Flock et al., 1977). In order to taper the stereocilia diameter, the paracrystal reduces from several hundreds of parallel actin filaments, to fewer than 50 (Song et al., 2020; Tilney et al., 1980). Actin filaments in the taper region remain parallel with filaments in the main paracrystal and their pointed ends terminate in close juxtaposition to the stereocilia membrane (Song et al., 2020). Within this tapered region, the osmiophilic rootlet is visualized as an intensely stained rod using transmission electron microscopy (TEM) (Fig. 1C). The rootlet extends bidirectionally, with the upper rootlet typically penetrating between a third to a half of the stereocilia length as measured from the cuticular plate insertion site (Furness et al., 2008). The lower rootlet core penetrates into the cuticular plate with its length scaling with the size of its corresponding stereocilium, such that the row 1 stereocilia have the longest lower rootlets (Furness et al., 2008; Kitajiri et al., 2010).

Rootlets are formed from actin filaments that have the same polarity as the stereocilia paracrystal, with barbed ends orientated towards the tips, but are spaced significantly closer together at an 8 – 9 nm inter-filament distance (Itoh and Nakashima, 1980; Song et al., 2020). Using tannic acid negative staining, longitudinal TEM images show a distinct banding pattern in the rootlet filaments with a periodicity of ~36 nm, which is identical to actin's half-pitch helical distance (Itoh, 1982). The organization of actin filaments in such a tight register suggests that significantly different crosslinking proteins are utilized within the rootlet. Similar to stereocilia, both ACTB and ACTG1 isoforms are detected in rootlets (Furness et al., 2008, 2005), however, their distinct functions in rootlets, if any, have not been explored. As the lower rootlets penetrate into the cuticular plate, radial fibers extend from the rootlets and anchor into the surrounding cuticular plate (Furness et al., 2008; Itoh and Nakashima, 1980; Itoh, 1982; Slepecky and Chamberlain, 1982). These filaments are not decorated by the muscle myosin S1-fragment in TEM micrographs, indicating they are unlikely to be actin filaments (Slepecky and Chamberlain, 1982). The cuticular plate itself is comprised of randomly orientated actin filaments, containing both ACTB and ACTG1, that are cross-linked to form a gel at the apical pole of the hair cell (DeRosier and Tilney, 1989; Furness et al., 2008; Slepecky and Chamberlain, 1982). The rootlet and cuticular plate are independently complex cellular structures, and we refer the reader to more in-depth treatment (Pacentine et al., 2020; Pollock and McDermott, 2015).

The molecules required to assemble stereocilia rootlets have only been recently identified. Central amongst these is TRIOBP (TRIO and F-actin binding protein) that is mutated in DFNB28 hereditary human hearing loss (Riazuddin et al., 2006; Seipel et al., 2001; Shahin et al., 2006). The *Triobp* gene in mouse encodes three splice isoforms, TRIOBP-1, TRIOBP-4 and TRIOBP-5 (Kitajiri et al., 2010). TRIOBP-5 encodes the full-length protein, whilst the shorter TRIOBP-4 and TRIOBP-1 isoforms are formed from its N-terminal and C-terminal exons, respectively (Kitajiri et al., 2010). Within hair cells, TRIOBP-4 protein is primarily concentrated within the upper rootlet in the stereocilia core, whereas TRIOBP-5 is restricted to the lower rootlet within the cuticular plate (Katsuno et al., 2019; Kitajiri

et al., 2010). The simultaneous loss of TRIOBP-4 and TRIOBP-5 in a mutant mouse model completely blocks the development of rootlets (Kitajiri et al., 2010). In a more selective ablation of just TRIOBP-5, hair cell rootlets are dysmorphic (Katsuno et al., 2019). Supporting the role of TRIOBP catalyzing stereocilia rootlet formation, purified TRIOBP-4 binds actin filaments *in vitro* via its R1 motif and uniquely cross-links filaments into bundles that are significantly denser than cross-linked by ESPN (Bao et al., 2013). The inter-filament spacing in TRIOBP-4 bundles is 8.2 ± 1.4 nm (Kitajiri et al., 2010), similar to the diameter of the actin filament itself, implying that actin filaments are so tightly packed there are likely steric constraints on proteins cross-linking from within the bundle. TRIOBP may therefore wrap around the filaments to bundle them, although this mechanism awaits biochemical confirmation (Kitajiri et al., 2010).

In addition to revealing a key structural assembly pathway for rootlets, *Triobp −4 / −5* null mice allowed the physiological function of rootlets to be experimentally tested for the first time. Rootlets do not form in *Triobp −4 / −5* null mice and stereocilia bending stiffness is reduced several-fold as a result, leaving the hair bundle susceptible to mechanical overstimulation (Kitajiri et al., 2010). Stereocilia bending stiffness is similarly reduced in *Triobp – 5* null animals with dysmorphic rootlets, confirming their key function in establishing the rigidity of hair bundles (Babahosseini et al., 2022). Intriguingly, genome-wide association studies (GWAS) have uncovered a statistically significant association between single-nucleotide polymorphisms (SNPs) in *TRIOBP* and presbycusis risk, teasing that a change in actin bundling and rootlet stiffness may clinically impact resilience to acoustic trauma (Hoffmann et al., 2016; Trpchevska et al., 2022). Consistent with this, a common effect of acoustic trauma is to physically damage stereocilia rootlets (Liberman, 1987; Tilney et al., 1982).

Additional proteins cooperate with TRIOBP to help cross-link actin filaments to correctly form stereocilia rootlets. The ankyrin repeat protein ANKRD24 forms a ring at the stereocilia insertion point on the hair cell surface, and bridges between the extracellular membrane and TRIOBP-5 in the rootlet (Krey et al., 2022). The association of ANKRD24 with TRIOBP-5 continues along the entire length of the lower rootlet, forming a boundary that separates the rootlet from the surrounding cuticular plate (Krey et al., 2022). In *Ankrd24* knock out hair cells, TRIOBP-5 is not efficiently recruited to the rootlets and this correlates with rootlets being abnormally patterned in the cuticular plate, as well as being significantly thicker (Krey et al., 2022). Consistent with the TRIOBP-5 / ANKRD24 complex being required for normal rootlet development, *Ankrd24* knock out hair cells are more susceptible to noise trauma and mechanical overstimulation (Krey et al., 2022). Pejvakin (PJVK), a member of the gasdermin protein family, also co-localizes with the lower rootlet structure and can interact with TRIOBP-1 *in vitro* (Kazmierczak et al., 2017). Whilst endogenous TRIOBP-1 is not detected in the stereocilia rootlet (Babahosseini et al., 2022), it contains the common C-terminal exons of the longer TRIOBP-5 isoform, and thus PJVK likely interacts with TRIOBP-5 in the lower rootlet *in vivo*. Despite this specific association with the major rootlet actin cross-linker TRIOBP, mutant *Pjvk* mice do not display any gross rootlet deformities, although they are deaf and exhibit degeneration of shorter stereocilia with active MET currents (Kazmierczak et al., 2017). The correct targeting of TRIOBP-5 is further dependent upon LIM domain protein 7 (LMO7), which is concentrated uniformly

throughout the hair cell cuticular plate (Du et al., 2019). *Lmo7* knock-out mice have reduced recruitment of TRIOBP-5 to rootlets, and rootlets are both shorter and disorganized, which ultimately results in hearing loss (Du et al., 2019).

Proteins resident at the stereocilia taper region are intimately connected with rootlet formation. Taperin (TPRN) concentrates to the tapered zone of the stereocilia as it contacts the hair cell cuticular plate (Rehman et al., 2010), and super-resolution fluorescence imaging further localizes it to the center of the stereocilia core, within the rootlet proper (Zhao et al., 2016). *Tprn* knockout mice have hearing loss, recapitulating DFNB79 human deafness, and exhibit abnormally curved lower rootlets that have a hollow core within them (Chen et al., 2016; Men et al., 2019). The changes to the lower rootlet likely reflect altered cross-linking of actin filaments in this region. A series of protein interactions are required for TPRN's critical localization at the base of stereocilia. TPRN forms a complex with chloride intracellular channel protein 5 (CLIC5), myosin 6 (MYO6) and protein tyrosine phosphatase receptor Q (PTPRQ) that provides an anchor to the plasma membrane and actin filaments (Salles et al., 2014). RIPOR2 (also known as FAM65B) is also involved in correctly localizing TPRN. RIPOR2 localizes to the base of stereocilia, where it forms a membrane-associated ring-like structure at the taper (Diaz-Horta et al., 2014; Zhao et al., 2016). Although TPRN does not directly bind to RIPOR2, TPRN is mis-localized along the stereocilia shaft in *Ripor2* knock-out hair cells, which exhibit stereocilia degeneration and hearing loss (Zhao et al., 2016). Similar to RIPOR2, glutaredoxin domain-containing cysteine-rich protein 2 (GRXCR2) also concentrates at the base of stereocilia where it interacts directly with CLIC5 and TPRN (Liu et al., 2018; Li et al., 2021). TPRN is either absent, or mis-localized along the stereocilia shaft of *Grxcr2* knockout mice which have dysmorphic stereocilia and develop hearing loss (Avenarius et al., 2018; Liu et al., 2018). Whilst the rootlet morphology in *Gxrcr2* and *Ripor2* mutant mice has not been reported, due to the mislocalization of TPRN in these models, we speculate that rootlets will similarly be disrupted. The restriction of TPRN to the stereocilia base thus appears to be an important theme in regulating the stereocilia actin cytoskeleton. TPRN shares a 34% similarity with phostensin (Rehman et al., 2010), a known pointed-end actin capping protein (Lai et al., 2009), and additionally drives cytoskeletal remodeling when overexpressed in COS-7 cells (Liu et al., 2018), suggesting that it can directly regulate the actin cytoskeleton.

5. Mechanisms driving stereocilia growth

Nascent stereocilia emerge from the lawn of microvilli that are present on the apical surface of a presumptive hair cell. Microvilli have a core of ~ 30 parallel bundled actin filaments but are significantly shorter and thinner than stereocilia. The transformation of these microvilli-like precursors into the mature stereocilia paracrystalline core is one of the marvels of inner ear biology and requires extraordinary remodeling of the actin cytoskeleton. Nascent stereocilia undergo four phases of elongation and thickening to form the mature staircase architecture with graded stereocilia heights (Tilney et al., 1988; Krey et al., 2020; Kaltenbach et al., 1994; Krey et al., 2023). During these growth phases, actin monomers are concentrated and polymerized into the paracrystalline core at the distal stereocilia tips (Drummond et al., 2015; Narayanan et al., 2015; Schneider et al., 2002). Whilst stereocilia originate from microvilli-like precursors, there are major differences in the stability of their

respective core actin filaments. The microvillar core continually treadmills, a process where new actin monomers are added to the microvillar tip, and simultaneously removed from the base at an equal rate, to maintain a steady-state length (Loomis et al., 2003). In contrast, stereocilia do not appear to treadmill at an appreciable rate, and their core actin filaments are remarkably stable (Narayanan et al., 2015; Drummond et al., 2015; Zhang et al., 2012); however, stereocilia actin exchange is observed in zebrafish (Hwang et al., 2015). Fine control of actin polymerization at the stereocilia tip is thus likely responsible for setting the ultimate length of each stereocilium. How stereocilia heights are set remains a major question.

At the heart of the stereocilia growth mechanism is the unconventional molecular motor myosin 15 (MYO15A), which traffics to the stereocilia tip compartment where actin polymerization occurs (Belyantseva et al., 2003). Mutations in *MYO15A* cause autosomal recessive hearing loss DFNB3 (Friedman et al., 1995; Wang et al., 1998). A missense substitution in the mouse ortholog of *Myo15a* causes hearing loss in the *shaker 2* mutant mouse by preventing stereocilia elongation, leaving the resulting hair bundles too short to be effectively stimulated (Probst et al., 1998). Multiple isoforms of MYO15A are expressed by hair cells, however it is the short isoform (MYO15A-2, aka MYO15A-S) that is expressed by embryonic hair cells as stereocilia initially start to elongate (Fang et al., 2015). In agreement with this, EGFP-tagged MYO15A-2 is also sufficient to rescue stereocilia growth in *shaker 2* hair cells cultured *in vitro* (Belyantseva et al., 2005). A longer isoform (MYO15A-1, aka MYO15A-L) is expressed by postnatal hair cells as early at P4.5, but is dispensable for the initial phase of stereocilia elongation (Fang et al., 2015; Krey et al., 2020). A third isoform (MYO15A-3) has been identified in inner ear mRNA, and a splice-site mutation uniquely affecting this isoform has been reported in a DFNB3 pedigree (Abu Rayyan et al., 2020; Ranum et al., 2019; Rehman et al., 2016). These findings indicate that MYO15A-3 may also be required for hearing, but its function remains unknown.

How does MYO15A-2 drive stereocilia elongation? One key function is delivering the molecular components required for elongation to the growing stereocilia tip. The ATPase motor domain of MYO15A-2 is kinetically optimized for long-range trafficking along actin filaments, and as a barbed end directed motor can move along stereocilia actin filaments towards the tips (Jiang et al. 2021; Bird et al., 2014; Belyantseva et al., 2003). Live imaging of zebrafish hair cells reveals there is a continual turnover of myosin 15a (equivalent to mouse MYO15A-2) into the tip compartment, with a half-life of 12 h measured using FRAP (Hwang et al., 2015). MYO15A interacts with the elongation complex (EC) consisting of four proteins: whirlin (WHRN), epidermal growth factor receptor pathway substrate 8 (EPS8), G-protein signaling modulator 2 (GPSM2) and G-protein subunit alpha$_{i3}$ (GNAI3). Each EC protein is normally concentrated at the tips of the tallest row 1 stereocilia and are all mislocalized in *Myo15a(sh2/sh2)* hair cells, demonstrating their dependence upon MYO15A for targeting (Belyantseva et al., 2005; Delprat et al., 2005; Manor et al., 2011; Mauriac et al., 2017; Mburu et al., 2003; Tadenev et al., 2019; Tarchini et al., 2016; Zampini et al., 2011). Furthermore, knock-out mouse models of individual EC proteins all phenocopy the short stereocilia phenotype observed in *Myo15a(sh2/sh2)* hair cells (Manor et al., 2011; Mauriac et al., 2017; Mburu et al., 2003; Tarchini et al., 2016). Detailed measurement of protein distributions reveals that MYO15A-2 and EPS8 localize first to the elongating hair

bundle during embryogenesis, and later recruit the WHRN-GPSM2-GNAI3 module as row 1 undergoes postnatal differential elongation (Tadenev et al., 2019; Krey et al., 2023). This data supports a model where MYO15A-2 delivers the EC to stereocilia tips, and where the EC proteins subsequently stimulate growth of the paracrystalline actin core in addition to specifying row identity.

How the EC complex drive stereocilia growth is unclear; none of the EC proteins are known to directly stimulate actin polymerization. EPS8 is the most compelling candidate in this regard and can influence actin polymerization in heterologous cell lines through activating the Rac GTPase (Offenhäuser et al., 2004). To date there is limited evidence for Rac GTPases being present in stereocilia, leaving this mechanism unsubstantiated (Grimsley-Myers et al., 2009). EPS8 has also been demonstrated to have “leaky” capping activity that can allow the regulated addition of actin monomers to the filament barbed end (Disanza et al., 2004). If leaky capping activity of EPS8 is key for stereocilia elongation, then the loss of EPS8 in a mouse model might be expected to cause stereocilia over-elongation, rather than the short stereocilia phenotype observed experimentally (Manor et al., 2011). Another proposed actin regulatory mechanism is through WHRN binding and regulating the bundling activity of ESPN1 (Wang et al., 2012). Whilst the EC proteins are unquestionably required for stereocilia growth, how they mechanistically contribute to stereocilia elongation and actin polymerization is still a major unanswered question.

New insight into the stereocilia growth mechanism comes from the discovery that MYO15A can directly stimulate actin polymerization, in addition to its known role trafficking within stereocilia. Using a reconstituted system of purified proteins, the actin-binding motor domain of MYO15A potently nucleates actin seeds *in vitro*, bypassing the rate limiting step of actin polymerization (Gong et al., 2022). The MYO15A motor domain nucleates by bridging across two actin monomers, whilst simultaneously preserving structural plasticity within the D-loop at the interprotomer contact (Gong et al., 2022). A missense substitution (p.D1647G) in the MYO15A motor domain, that causes hearing loss in mouse (Bowl et al., 2017), locks the actin protomer’s D-loop into a single conformation and prevents it from flexing (Gong et al., 2022). A preliminary report shows that the p.D1647G substitution inhibits MYO15A’s ability to catalyze actin nucleation *in vitro*, arguing that the change in D-loop flexibility is a key regulatory effect of MYO15A upon actin at the stereocilia tip (Moreland et al., 2021). The potential involvement for actin nucleation in stereocilia growth is counterintuitive, as actin filament barbed ends already exist at the stereocilia tips (Flock and Cheung, 1977; McGrath et al., 2021). One possibility is that MYO15A nucleates short actin polymers that could anneal to existing stereocilia barbed ends to extend the actin core (Gong et al., 2022). Actin filament extension by polymer addition has been demonstrated *in vitro* (Murphy et al., 1988) and *in vivo* (Okreglak and Drubin, 2010), but whether this occurs in stereocilia remains speculative.

Given the activities of MYO15A and the EC proteins upon actin polymerization, how might these be integrated together at the stereocilia tip? Fascinating additional insight into this question comes from the discovery that MYO15A and EC proteins undergo liquid–liquid phase separation (LLPS) and self-assemble into biomolecular condensates (Lin et al., 2021; Shi et al., 2022). LLPS is driven by multi-valent interactions between constituent proteins

and underpins the formation of membrane-less cytosolic, nuclear and synaptic particles that are involved in a range of cellular processes (Wang et al., 2021). Purified WHRN protein undergoes LLPS at high protein concentrations *in vitro* (> 5 μM) and recruits EPS8 and the tail MyTH4 domain of MYO15A as client proteins into the resulting spherical biomolecular condensates (Lin et al., 2021). GPSM2 and GNAI3 catalyze the formation of MYO15A / WHRN / EPS8 biomolecular condensates and lowers the protein concentration required for LLPS to ~ 1 μM *in vitro* (Shi et al., 2022), providing a molecular mechanism for how GPSM2 and GNAI3 stabilize the MYO15A + EC complex on the tallest row 1 stereocilia (Tadenev et al., 2019). Though the demonstration of LLPS *in vitro* is somewhat obscure, there is evidence for this phenomenon occurring in stereocilia. The MYO15A + EC complex spatially coincides with the tip density, which is an osmiophilic protein-dense structure visualized by TEM at the stereocilia tips (Furness and Hackney, 1985; Rzadzinska et al., 2004). The tip-density is severely diminished, or absent, in *Myo15a* and *Whrn* mouse mutants *in vivo* (Mogensen et al., 2007; Rzadzinska et al., 2004), and the introduction of hearing loss mutations into purified MYO15A or GPSM2 inhibits LLPS *in vitro* (Lin et al., 2021; Shi et al., 2022). Although further investigation is now needed to confirm if LLPS truly occurs *in vivo*, these data point to the biomolecular condensation of MYO15A + EC proteins as a potential feature of their combined activity at the stereocilia tips. MYO15A / EC biomolecular condensates can cross-link actin filaments to form large bundles *in vitro* (Lin et al., 2021; Shi et al., 2022) and we speculate that other emergent activities are waiting to be discovered.

A parallel system to regulate elongation of the stereocilia actin core has been identified that uses a separate myosin molecular motor. The myosin-3 paralogs (MYO3A and MYO3B) are expressed by cochlear hair cells in mouse as early as E16.5 and accumulate at the distal tips of all stereocilia rows (Lelli et al., 2016; Merritt et al., 2012; Salles et al., 2009; Schneider et al., 2006). MYO3A and MYO3B are both barbed-end directed motors that are kinetically tuned for trafficking along actin filaments, consistent with them moving towards and accumulating at the tips of filopodia and stereocilia (Dosé et al., 2007; Komaba et al., 2003; Les Erickson et al., 2003; Merritt et al., 2012; Salles et al., 2009). Mutant knock-out *Myo3a(−/−)*, or *Myo3b(−/−)* mice exhibit subtle, to no hearing loss (Ebrahim et al., 2016; Lelli et al., 2016), whereas double knock-out *Myo3a(−/−):Myo3b(−/−)* mice have severe hearing loss and suffer from a high rate of embryonic lethality (Ebrahim et al., 2016; Lelli et al., 2016). This data indicates that *Myo3a* and *Myo3b* share some degree of functional redundancy and can compensate for each other. Interestingly, knock-in *Myo3a* hypomorphic alleles cause a more severe hearing loss than *Myo3a* knock-outs (Li et al., 2018; Walsh et al., 2011) and are potentially better models of DFNB30 human deafness caused by mutations of *MYO3A* (Walsh et al., 2002).

The hair bundles of *Myo3a(−/−):Myo3b(−/−)* mice are highly dysmorphic with all stereocilia rows having a similar length, so that the graded staircase architecture is lost (Lelli et al., 2016). Thus, stereocilia rows over-elongate in the absence of both MYO3A and MYO3B, but not when only one paralog is absent (Lelli et al., 2016). How do the myosin-3 paralogs influence the size of the stereocilia actin paracrystal? MYO3A and MYO3B both independently bind isoform 1 of espin (ESPN-1) and espin-like (ESPNL) and traffic these proteins to the distal tips of filopodia in heterologous cells, where they

co-operate to stimulate actin polymerization and filopodia growth (Ebrahim et al., 2016; Merritt et al., 2012; Salles et al., 2009). ESPN-1 accumulates at the tips of all stereocilia rows, whereas ESPNL is restricted to the shorter stereocilia rows 2 + 3 with active MET currents (Ebrahim et al., 2016; Lelli et al., 2016; Salles et al., 2009). Curiously, both ESPN and ESPNL are still localized to the stereocilia tips of *Myo3a(−/−):Myo3b(−/−)* double knock-out hair cells, indicating there may yet be additional, hair cell specific mechanisms that target ESPN-1 and ESPNL to the stereocilia tip compartment (Lelli et al., 2016). Supporting the role of ESPN-1 at the stereocilia tip regulating growth of the actin paracrystal, shorter row stereocilia in *Espn-1* knock out hair cells over-elongate, eliminating the staircase architecture as in *Myo3a(−/−):Myo3b(−/−)* hair cells (Ebrahim et al., 2016; Lelli et al., 2016). Both MYO3A and MYO3B can relieve ESPN-1 autoinhibition, consistent with myosin-3 paralogs activating ESPN-1 at the tip (Liu et al., 2016). The over-elongation phenotype is only seen in the extrastriolar region of *Espn-1* KO hair cells of the utricle and saccule, and not within cochlea hair cells (Ebrahim et al., 2016). *Espnl* knockout hair cells do not exhibit stereocilia over-elongation, and instead display a specific loss of row 3 stereocilia in the cochlea (Ebrahim et al., 2016). These differing phenotypes of *Espn-1* and *Espnl* knockout mice demonstrate that MYO3A and MYO3B likely influence stereocilia architecture by engaging different effector proteins in a hair cell specific manner. Additional proteins that bind the myosin 3 paralogs include MORN4 and coronin 1A (CORO1A), though their potential involvement in regulating stereocilia size remains to be explored (Lelli et al., 2016; Li et al., 2019; Mecklenburg et al., 2015).

6. Tuning of stereocilia architecture by mechanoelectrical transduction (MET)

Initial evidence for MET influencing stereocilia architecture used a conditional knockout of the tip-link associated protein Sans (USH1G) to postnatally disrupt tip-links and block MET (Caberlotto et al., 2011a,b). This approach circumvented the otherwise severe hair bundle phenotype caused by the loss of tip-links earlier in development (Alagramam et al., 2011; Caberlotto et al., 2011a,b; Kazmierczak et al., 2007). Following the delayed loss of tip-links in postnatal *Sans* knock-out hair cells, the stereocilia tips of row 2 + 3 stereocilia lost their normal prolate shape and were shorter in height, implying a significant remodeling of the actin cytoskeleton (Caberlotto et al., 2011b). Using an *in vitro* model of the mouse cochlea cultured at P4, chelating extracellular Ca^{2+} with BAPTA reveals that row 2 + 3 stereocilia begin shrinking within 1 h of tip-link breakage, whilst row 1 is unaffected (Vélez-Ortega et al., 2017). What is the cellular signaling that leads to this rapid change of the actin cytoskeleton, specifically in the shorter mechanosensitive rows? The loss of tip-links decouples shorter stereocilia from both transduction-associated mechanical forces in addition to MET currents. To separate these two possible stimuli, acute pharmacological inhibition of MET currents using benzamil or tubocurarine, that preserve tip-links intact, also induce shortening of row 2 and 3 stereocilia specifically (Fig. 3A)(Vélez-Ortega et al., 2017). Remarkably, row 2 and 3 stereocilia regrow after MET blocker washout, arguing that these stereocilia are not only highly plastic, but also tune their size in response to MET (Vélez-Ortega et al., 2017). Targeted intracellular perfusion of BAPTA into hair cells *in vitro* triggers a shortening and thinning of row 2 + 3 stereocilia, further arguing that it is Ca^{2+}

influx, as part of the MET current, that is a key signal for remodeling the actin cytoskeleton (Vélez-Ortega et al., 2017). Genetic approaches to determine the contribution of MET *in vivo* have revealed different contributions to hair bundle architecture (Kawashima et al., 2011; Beurg et al., 2018; Krey et al., 2020; Krey et al., 2023). Stereocilia have abnormal widths and heights in mutant *Tmie* or *Tmc1:Tmc2* double KO (dKO) hair cells that retain tip links but lack MET currents (Kawashima et al., 2011; Beurg et al., 2018; Krey et al., 2020; Krey et al., 2023). Several changes are evident in *Tmie* and *Tmc1:Tmc2* dKO hair cells compared to controls; row 1 stereocilia are thinner and shorter, and row 2 stereocilia are thinner but do not shorten (Krey et al., 2020; Krey et al., 2023). These different effects of pharmacological (Vélez-Ortega et al., 2017) *versus* genetic (Krey et al., 2020) perturbation of MET upon stereocilia architecture are potentially due to timing of MET inhibition. Pharmacological inhibition was performed at P4 onwards *in vitro* (Vélez-Ortega et al., 2017), whereas in the case of *Tmie* and *Tmc1:Tmc2* dKO mutant hair cells (Krey et al., 2020), MET is already absent by P4 and likely significantly earlier (Kawashima et al., 2011; Cunningham et al., 2020). Taken together, this exciting body of work experimentally supports the longstanding postulate that MET and Ca^{2+} influx is critical to establishing the size of stereocilia and the overall architecture of the hair bundle (Tilney and Tilney, 1988).

How is Ca^{2+} sensed in the shorter stereocilia rows, and what effector molecules drive plasticity of the stereocilia actin cytoskeleton? The prolific nature of Ca^{2+} as an intracellular messenger has the potential to couple to the actin cytoskeleton through multiple independent pathways. The loss of Ca^{2+} influx causes a general redistribution of MYO15A and EC proteins within the hair bundle. In mutant *Tmie* and *Tmc1:Tmc2* dKO hair cells, or in wild-type hair cells treated with pharmacological MET blockers, EPS8, GNAI3, WHRN and MYO15A-2 (MYO15A-S) are less concentrated on the tallest row 1 and begin accumulating at row 2 + 3 stereocilia (Krey et al., 2020; Krey et al., 2023). As MYO15A is required to traffic these molecules in stereocilia, one question is how Ca^{2+} could alter the row targeting of MYO15A-2? The MYO15A motor domain can bind the EF-hand protein calmodulin (*e.g.*, CALM1) as a light-chain and this may allow for Ca^{2+} regulated motility (Bird et al., 2014). If Ca^{2+} were to inhibit MYO15A-2 this would be a mechanism to limit trafficking of the EC into row 2 + 3 in wild-type hair cells with active MET channels (Krey et al., 2020).

Another candidate Ca^{2+} sensor well positioned to sample MET associated Ca^{2+} influx to stereocilia is calcium and integrin binding protein 2 (CIB2). CIB2 binds Ca^{2+} via three functional EF-hand motifs and, along with its paralog CIB3, is an integral component of the MET channel complex on the shorter stereocilia rows (Giese et al., 2017; Liang et al., 2021). Mutations in *CIB2* cause DFNB48 human hearing loss and mutant mouse models reveal dysregulation of row 2 and 3 stereocilia architecture, consistent with phenotypes from genetic / pharmacological disruption of MET (Giese et al., 2017; Michel et al., 2017; Riazuddin et al., 2012; Wang et al., 2017). In addition to being a component of the MET complex, CIB2 can interact with WHRN, providing a potential mechanism to couple Ca^{2+} sensing with the activity of the elongation complex (EC) (Riazuddin et al., 2012). Calmodulin, a prototypical Ca^{2+} sensor, is also abundant in stereocilia, including being concentrated at the stereocilia tips where can integrate into the MET complex (Furness et al., 2002; Jeong et al., 2022; Walker et al., 1993). Calmodulin can bind to several actin regulatory proteins (Izadi et al., 2018), including the actin nucleator cordon-blue (COBL)

that is needed to build microvilli in the intestinal brush border (Grega-Larson et al., 2015), in addition to binding as a regulatory light chain to the various myosin motors in stereocilia (Heissler and Sellers, 2014). Ca^{2+} may additionally regulate ABPs that are directly involved in forming the stereocilia paracrystal. Plastin-1 (PLS1) has two EF hand domains that confer Ca^{2+} sensitivity to its ABD. Actin cross-linking by PLS1 is inhibited by elevated Ca^{2+} (Schwebach et al., 2017), and this could result in localized changes in actin filament structure allowing different complements of ABPs / myosin motors to bind and engage the filament. These potential sources of Ca^{2+} sensitive signaling remain to be explored in hair cells.

Following the loss of MET currents, in addition to shortening of the actin paracrystal there is evidence for a change in actin filament structure at the tips of row 2 stereocilia. Uncapped actin filament barbed ends, detected by the incorporation of fluorescently labeled actin monomers (*i.e.*, a barbed end assay), are normally detected at the tips of all stereocilia rows but are particularly enriched at the tips of row 2 stereocilia (McGrath et al., 2021). In *Tmie(−/−)* cochleae that lack MET currents, or in wild-type cochleae treated with the MET channel blocker tubocurarine (Fig. 3B), free actin barbed ends no longer accumulate at the tips of row 2 stereocilia (McGrath et al., 2021). These data indicate that stereocilia shortening in the absence of MET is correlated with a loss of actin barbed ends that would be potentially available to extend the core. Actin severing proteins that can generate barbed ends, including WDR1 (also known as AIP1), DSTN (also known as ADF) and cofilin-1 (CFL1) are required to maintain the lengths of the shorter stereocilia rows, hinting at their involvement in the MET regulation pathway (McGrath et al., 2021; Narayanan et al., 2015). DSTN and CFL1 are concentrated at the tips of row 2 stereocilia, and this localization is lost in *Tmie(−/−)* knockout mice, confirming their dependence upon MET currents for normal activity (McGrath et al., 2021). The availability of actin barbed ends at the tips of row 2 stereocilia is also significantly reduced in mutant *Dstn(−/−):Cfl1(fl/+)* mice that have one functional allele of *Cfl1*, arguing that both actin severing proteins are critical for generating barbed ends in response to MET (McGrath et al., 2021). Actin severing proteins may cooperate with other factors such as Molecules Interacting with CasL (MICAL) that can oxidize actin filaments and potentiate their cleavage by CFL1 (Grintsevich et al., 2016; Hung et al., 2010). Whether MICAL is localized in stereocilia is yet to be determined, however a recent study identified MICAL as a cargo protein of *Sisyphus (Myo10)*, the MYO15A ortholog in *D.melanogastor*, teasing that it may also be trafficked to the stereocilia tips by MYO15A (Rich et al., 2021).

In parallel with severing proteins, the availability of barbed ends is influenced by the presence of multiple capping proteins that stabilize filaments and prevent polymerization and depolymerization. A number of cappers concentrate at the tips of the shorter mechanosensitive rows, including heterodimeric capping protein CAPZB isoform 2 (CAPZB2), twinfilin-2 (TWF2), EPS8L2, and to a lesser extent EPS8 (Avenarius et al., 2017; Furness et al., 2013; Peng et al., 2009; Rzadzinska et al., 2009). Conditional deletion of *Capzb* specifically from hair cells causes severe hearing loss in mouse, and although mutant hair bundles appear normal at P1, stereocilia shorten and thin by P21 (Avenarius et al., 2017). TWF2 interacts directly with CAPZB to potentiate capping activity (Johnston et al., 2018) and is also concentrated on row 2 stereocilia (Peng et al., 2009; Rzadzinska et

al., 2009). Overexpression of TWF2 in hair cells by biolistic transfection shortens stereocilia lengths in rows 2 and 3, consistent with increased capping activity (Peng et al., 2009). EPS8L2 is a member of the EPS8 family of proteins and shares the same C-terminal domain that binds to actin filaments and has barbed end capping activity (Disanza et al., 2004). Knockout *Eps8l2* hair cells exhibit a slowly progressing hearing loss that is caused by the thinning, over-elongation and degeneration of the shorter stereocilia rows (Furness et al., 2013). Thus, in parallel with actin severing proteins generating new barbed ends at the tips of shorter rows, there is an opposing mechanism to cap and stabilize barbed ends. The normal tip localizations of CAPZB2, TWF2 and EPS8L2 on row 2 stereocilia are all disrupted in the hair bundles of *Tmie(−/−)* mutant mice, further demonstrating that transduction, and likely Ca^{2+} influx, is central to controlling their activity (Krey et al., 2020, 2023).

Recent reports have described an additional ABP complex that localizes to the shorter stereocilia rows 2 and 3 with MET currents. Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2 (BAIAP2L2) contains an inverse-BAR domain that supports formation of membrane curvature, in addition to a WASP-homology domain 2 (WH2) domain that can bind to actin filaments (Pykäläinen et al., 2011). Hair cells in *Baiap2l2* KO cochleae have reduced MET current amplitudes by P11 and stereocilia rows 2 and 3 degenerate resulting in progressive hearing loss (Carlton et al., 2021; Yan et al., 2022). BAIAP2L2 forms a tri-partite complex with the tail domain of MYO15A and EPS8, and its localization to stereocilia tips is lost in either *Myo15a(sh2/sh2)* or *Eps8(−/−)* hair cells, showing the necessity of this complex *in vivo* (Carlton et al., 2021; Halford et al., 2022). Even though the largest MYO15A-1 isoform co-localizes on the shorter stereocilia row tip with BAIAP2L2, MYO15A-1 is dispensable for the normal distribution of BAIAP2L2, implying the involvement of either MYO15A-2 or MYO15A-3 (Halford et al., 2022). BAIAP2L2 is absent from the hair bundles of *Tmie* KO and *Tmc1:Tmc2* dKO transduction mutants, or in hair cells loaded with intracellular BAPTA-AM, strongly arguing that influx of Ca^{2+} is necessary for its recruitment to stereocilia tips (Halford et al., 2022). Consistent with this, BAIAP2L2 interacts with CIB2 *in vitro*, and is mislocalized in *Cib2(−/−)* hair cells (Yan et al., 2022). BAIAP2L2 may therefore be closely associated with the MET channel via CIB2 and retained by the influx of Ca^{2+} ions through the mechanotransduction pore. How BAIAP2L2 then influences the stereocilia actin cytoskeleton, potentially through its WH2 domain, remains to be explored.

7. Conclusions + outlook

The ability of hair cells to build a mechano-sensitive bundle using the actin cytoskeleton is a magnificent feat of biology fundamental for the sense of hearing and balance. From a large body of work encompassing genetics, cell biology and biochemistry, a plethora of ABPs orchestrating this complex process have been identified, with mutations in many of these proteins causing hereditary forms of human hearing loss. A major question is how these molecules are all biochemically integrated and coordinated within the hair bundle. For example, how are factors driving elongation of stereocilia actin filaments balanced with mechanisms promoting shortening? Or more specifically, how are the activities of MYO15A, EC proteins, MYO3A / B, along with actin severing and barbed end capping

proteins kept in equilibrium to precisely control actin filament growth and stereocilia length? Influx of Ca^{2+} through the MET channel has emerged as a key physiological signal coordinating these processes, creating a feedback mechanism that adaptively changes bundle architecture in response to MET. Significant unresolved questions remain, including how the absolute length and width of stereocilia is specified, and how the stereocilia cytoskeleton responds to oxidative stress, mechanical noise trauma and aging.

Exploring these processes will require uncovering the full spectrum of ABP activities in hair cells. It will also necessitate, in our opinion, *in situ* high-resolution structural mapping of actin filaments within the stereocilia core. Actin filaments are structurally heterogenous and state-of-the-art technical refinements to cryo-EM and image processing are providing unprecedented resolution (approaching 2 Å) to identify the conformational landscape of individual protomers (Chou and Pollard, 2019; Galkin et al., 2015; Oosterheert et al., 2022; Reynolds et al., 2022). Actin filaments are themselves mechanosensitive and externally applied forces can induce localized conformational changes that regulate the binding and activity of specific ABPs (Jégou and Romet-Lemonne, 2021). Changes to actin's nucleotide-bound state (ADP *vs* ADP•Pi) also mediate filament bending and deformation, arguing that actin filaments can actively alter their mechanical properties (Reynolds et al., 2022). We speculate that similar structural plasticity is present within the stereocilia paracrystal, and that localized differences in actin filament conformation underlie key aspects of stereocilia biology. As an example, the LIM domain motif recognizes a stretched conformation of actin that allows it to bind selectively to tensed filaments (Hoffmann et al., 2014; Sun et al., 2020; Winkelman et al., 2020). Within the cuticular plate and stereocilia, several LIM domain containing ABPs are present, including XIRP2 and LMO7 (Du et al., 2019; Scheffer et al., 2015). Actin filament mechanosensation may explain why XIRP2 is recruited to sites of stereocilia core damage (Francis et al., 2015; Scheffer et al., 2015), especially following noise trauma where stereocilia core actin filaments are known to be mechanically disrupted (Wagner et al., 2021). Evidence for conformational changes at the level of individual actin protomers is currently lacking; however, visualization of the stereocilia paracrystal by electron cryo-tomography is rapidly progressing, and advances in direct electron detectors combined with image motion correction are expected to ultimately yield atomistic detail of stereocilia actin filaments (Elferich et al., 2021; Metlagel et al., 2019; Song et al., 2020). The integration of actin filament nanoscale architecture alongside exhaustive cataloging of ABP activities will help decipher how stereocilia dimensions are established, how they respond to MET and displacement forces, and potentially reveal therapeutic opportunities for stimulating repair of these critical mechanosensory organelles.

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Data availability

No data was used for the research described in the article.

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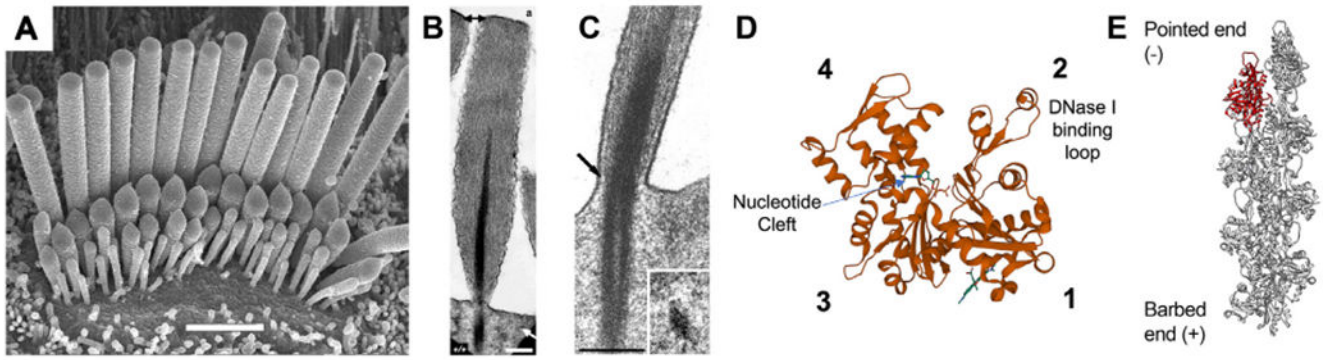

Fig. 1.

The actin cytoskeleton in hair cell stereocilia. **(A)** Scanning electron microscopy (SEM) of a cochlear hair cell, demonstrating the graded stereocilia sizes that contribute to the hair bundle architecture. Row 1 stereocilia are the tallest, with the shorter rows 2 + 3 having active MET channels gated by bundle deflection. Scale bar is 2 μm. **(B)** Transmission electron microscopy (TEM) of a sectioned stereocilia revealing its highly ordered core of actin filaments. The rootlet structure is darkly stained and penetrates into the stereocilia and cuticular plate. Scale bar is 250 nm. **(C)** TEM image of the stereocilia taper region demonstrating how the rootlet deforms during stereocilia deflection. **(D)** Structural model of an actin monomer (PDB: 1J6Z) with subdomains 1–4 and the nucleotide binding cleft labeled. The DNase I binding loop (D-loop) forms part of the interface between adjacent protomers in a filament. **(E)** Structural model of an actin filament (PDB: 6BNO) with an individual monomer highlighted in red. Actin filaments have structural polarity with a fast-growing barbed end and a pointed end where depolymerization occurs. Images reproduced with permission from: (A) Beurg M, et al. (2006) Journal of Neuroscience 26 (43) 10,992–11,000, DOI: 10.1523/JNEUROSCI.2188-06.2006, Copyright © 2006, Society for Neuroscience. (B) Mogensen et al. (2007) Cell Motil Cytoskeleton 64 (7): 496–508, DOI: 10.1002/cm.20199. Copyright © 2007, Wiley-Liss, Inc. (C) Furness et al. (2008) Journal of Neuroscience 28 (25): 6342–53, DOI: 10.1523/JNEUROSCI.1154-08.2008, Copyright © 2008, Society for Neuroscience. Molecular structures were rendered in VMD and Chimera.

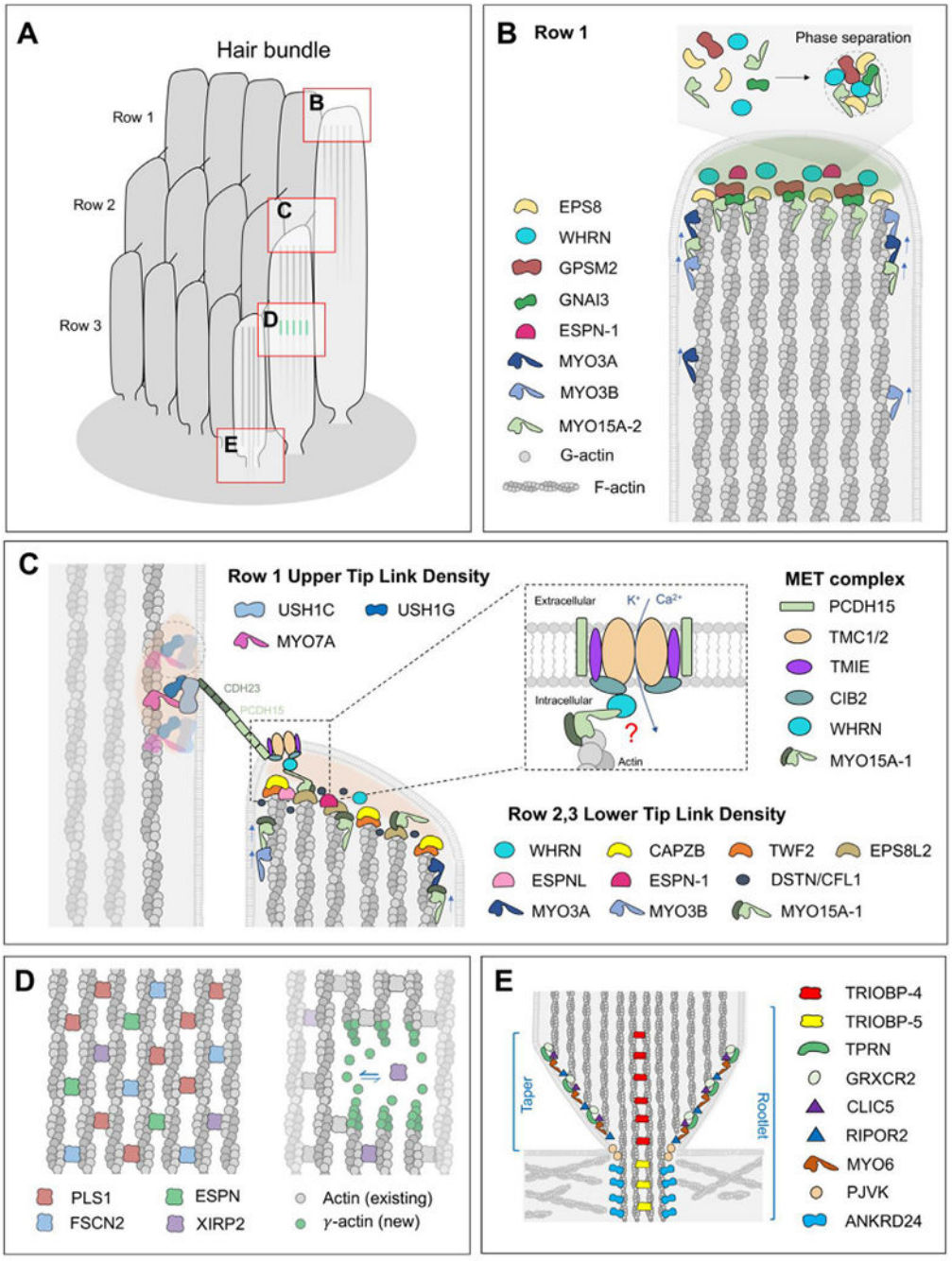

Fig. 2.

Distribution of actin-associated proteins in hair cell stereocilia. **(A)** Cartoon of a mammalian hair cell bundle with specific stereocilia zones expanded in panels B–E. Each stereocilium consists of thousands of parallel actin filaments that are cross-linked together and decorated by actin-associated proteins to form the structural core and provide signaling to control polymerization / depolymerization. **(B)** In the tallest row 1 stereocilia, MYO3A, MYO3B and MYO15A-2 traffic and deliver critical molecular cargoes to the tip. MYO3A / B transports ESPN-1 whilst MYO15A-2 delivers the elongation complex (EPS8, WHRN,

GPSM2, GNAI3) that is hypothesized to form a biomolecular condensate as part of the tip density. Actin polymerization is concentrated at the stereocilia tip, where actin filament barbed ends are polarized and available for turnover during stereocilia development and maturation. **(C)** Mechanoelectrical transduction (MET) channels, comprised of TMC1/2, TMIE and CIB2 (LHFPL5 is not shown) are localized to the lower tip link density (LTLD) at the tips of shorter row 2 and 3 stereocilia that are also enriched with actin filament barbed ends. The tip link protein PCDH15 inserts into the LTLD. At the other end of the tip link is the upper tip link density (UTLD) that associates with CDH23 via MYO7A, USH1C (Harmonin) and USH1G (Sans). Barbed ends of row 2 actin filaments are capped with a combination of TWF2, EPS8L2 and CAPZB. Actin severing proteins DSTN1/CFL1 can uncover actin filament barbed ends to promote actin polymerization, and this process is stimulated by MET. CIB2 may interact with actin filaments via WHRN and MYO15A-1, although this interaction remains to be demonstrated *in vivo*. **(D)** Actin-binding proteins (PLS1, FSCN2, ESPN, XIRP2) extensively cross-link actin filaments to increase stiffness of the stereocilia core (left panel). Following mechanical trauma, XIRP2 and new γ-actin (ACTG1) monomers are incorporated to repair the stereocilia core. **(E)** TRIOBP-4 and TRIOBP-5 cross-link actin filaments to form a dense rootlet that stabilizes stereocilia in the actin meshwork of the cuticular plate. Additional proteins, including TPRN, GRXCR2, RIPOR2, CLIC5 and MYO6 localize to the taper region, where actin filament pointed ends terminate and the stereocilia diameter reduces.

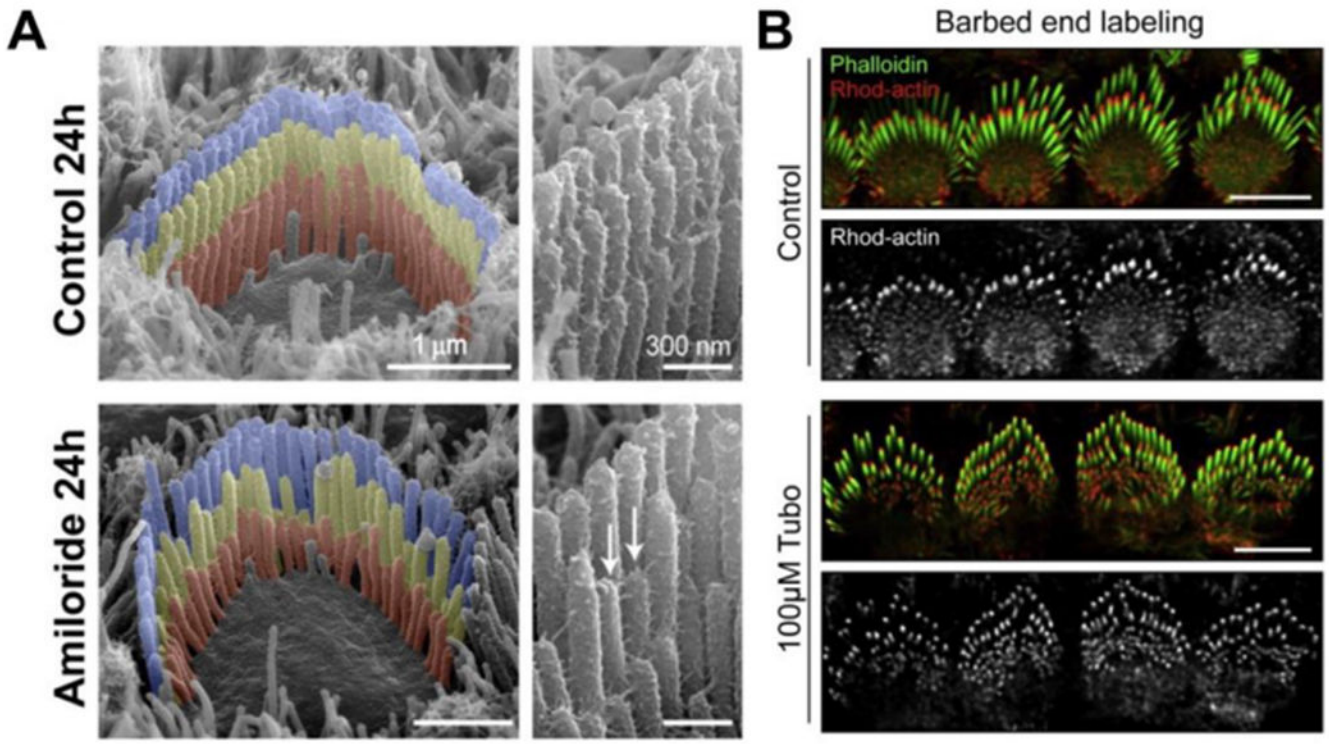

Fig. 3.

MET currents adaptively shape the stereocilia cytoskeleton. **(A)** Scanning electron microscopy (SEM) of hair bundles treated with a pharmacological inhibitor of the MET channel (amiloride), leaving tip-links intact. After 24 h of treatment, row 2 and 3 stereocilia with active MET channels have abnormal architecture, indicating a significant remodeling of the actin cytoskeleton. **(B)** Fluorescence confocal imaging of permeabilized hair cells incubated with rhodamine-labeled actin monomers to reveal the presence of barbed ends available to support polymerization. Free barbed ends are normally concentrated at the tips of row 2, but this is reduced following pharmacological blockade of MET currents using tubocurarine. Scale bars are 5 µm. Images reproduced with permission from: (A) Vélez-Ortega et al. (2017) eLife 6:e24661, DOI:10.7554/eLife.24661. (B) McGrath et al. (2021) Current Biology 31 (6): 1141–1153, DOI: 10.1016/j.cub.2020.12.006.

Table 1

Selected actin and actin-associated proteins in stereocilia.

Protein	Gene	Human deafness
Beta cytoplasmic actin	*ACTB*	
Gamma cytoplasmic actin	*ACTG1*	DFNA20/26
Espin	*ESPN*	DFNB36
Fascin 2	*FSCN2*	
Plastin-1	*PLS1*	
Xin-actin binding repeat containing 2	*XIRP2*	
Whirlin	*WHRN*	DFNB31
Epidermal growth factor receptor pathway substrate 8	*EPS8*	DFNB36
EPS8-like 2	*EPS8L2*	
G-protein signaling modulator 2	*GPSM2*	DFNB82
G-protein subunit alpha i3	*GNAI3*	
Unconventional myosin 3	*MYO3A, MYO3B*	DFNB30
Unconventional myosin 6	*MYO6*	DFNA22, DFNB37
Unconventional myosin 7	*MYO7A*	DFNA11DFNB2USH1B
Unconventional myosin 15	*MYO15A*	DFNB3
Harmonin	*USH1C*	DFNB18, USH1C
Sans	*USH1G*	USH1G
Calcium + integrin binding family member 2	*CIB2*	DFNB48
BAI1-associated protein 2-like 2	*BAIAP2L2*	
Twinfilin-2	*TWF2*	
Actin capping protein β2	*CAPZB2*	
Destrin (actin depolymerization factor)	*DSTN*	
Cofilin 1	*CFL1*	
TRIO and F-actin binding protein	*TRIOBP*	DFNB28
Ankyrin repeat domain 24	*ANKRD24*	
Pejvakin	*PJVK*	DFNB59
Taperin	*TPRN*	DFNB79