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Human deafness-associated variants alter the dynamics of key molecules in hair cell stereocilia F-actin cores

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Abstract

Stereocilia protrude up to 100 μm from the apical surface of vertebrate inner ear hair cells and are packed with cross-linked filamentous actin (F-actin). They function as mechanical switches to convert sound vibration into electrochemical neuronal signals transmitted to the brain. Several genes encode molecular components of stereocilia including actin monomers, actin regulatory and bundling proteins, motor proteins and the proteins of the mechanotransduction complex. A stereocilium F-actin core is a dynamic system, which is continuously being remodeled while

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Declarations

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maintaining an outwardly stable architecture under the regulation of F-actin barbed-end cappers, severing proteins and crosslinkers. The F-actin cores of stereocilia also provide a pathway for motor proteins to transport cargos including components of tip-link densities, scaffolding proteins and actin regulatory proteins. Deficiencies and mutations of stereocilia components that disturb this “dynamic equilibrium” in stereocilia can induce morphological changes and disrupt mechanotransduction causing sensorineural hearing loss, best studied in mouse and zebrafish models. Currently, at least 23 genes, associated with human syndromic and nonsyndromic hearing loss, encode proteins involved in the development and maintenance of stereocilia F-actin cores. However, it is challenging to predict how variants associated with sensorineural hearing loss segregating in families affect protein function. Here, we review the functions of several molecular components of stereocilia F-actin cores and provide new data from our experimental approach to directly evaluate the pathogenicity and functional impact of reported and novel variants of *DIAPH1* in autosomal-dominant DFNA1 hearing loss using single-molecule fluorescence microscopy.

Introduction

Hearing loss is a common neurosensory deficit of 1.33 per 1000 newborn babies (Fortnum et al. 2001; Korver et al. 2017; Watkin and Baldwin 2012). More than 50% of congenital hearing loss is caused by a heritable deficiency, most often a single gene defect. Prenatal infections, especially cytomegalovirus, and other factors including premature birth, injuries at birth and/or drugs are also contributors to newborn hearing loss (Chien et al. 2016; Korver et al. 2017; Marazita et al. 1993). Management of congenital sensorineural hearing loss requires long-term interventions using hearing aids, cochlear implants or brain-stem implants in some cases to develop sound-based speech (Alderson-Day and Fernyhough 2015; Nikolopoulos and Vlastarakos 2010; Wong et al. 2019). Sign languages are available when these devices are ineffective or not chosen. However, it can be difficult to achieve normal speech recognition and language development even with the most advanced hearing aids and cochlear implants (Ruben 2018; Tomblin et al. 2014). More than 30% of congenital deafness as well as noise induced hearing loss are related to damage of stereocilia, mechanosensory organelles that protrude from the apical surface of inner ear sensory hair cells. Regeneration or repair of damaged auditory hair cells, associated supporting cells and the entire mosaic architecture of the sensory epithelium is a major goal of hearing researchers in management of sensorineural hearing loss (Chien et al. 2016; Duan et al. 2004).

This review focuses on the actin cytoskeleton of cochlear hair cell stereocilia that function as mechanical switches to convert sound vibrations into electrochemical signals conveyed to the brain (Fig. 1). In all vertebrates, on the apical surface of each hair cell, stereocilia are organized in rows of increasing height, positioned close to each other and interconnected by extracellular filamentous links. In human hair cells, tightly packed F-actin bundles of each stereocilium within the longest row extend from the apical surface to a length of 4–7 μm for inner hair cells and 3–6 μm for outer hair cells (Wright 1984). Each tip of a stereocilium in the shorter row is connected to the side of an adjacent longer row stereocilium by a filamentous tip-link. Deflection of stereocilia toward the longest row applies tension to

tip-links and opens mechanotransduction channels located at the tips of the shorter-row stereocilia (Gillespie and Muller 2009). Stereocilia also emanate from the apical surface of vestibular hair cells, and their ranks are less prominent than for auditory hair bundles. The lengths of vestibular hair cell stereocilia bundles can be much longer allowing them to detect linear and rotational accelerations of the head.

The actin cytoskeleton of stereocilia is both a durable and a dynamic structure, being continuously remodeled while maintaining an overall architecture. For the lifespan of mammals, the F-actin architecture of stereocilia remains largely unchanged, while actin monomers are constantly turning over, mainly at the distal ends (Narayanan et al. 2015). However, the F-actin crosslinkers, espin and plastin, are refreshed along the entire length of stereocilia (Miyoshi et al. 2021; Roy and Perrin 2018). We posit that a comprehensive understanding of the mechanisms governing lifelong homeostasis of these stereocilia macromolecules will not only elucidate the pathophysiology of human syndromic and non-syndromic sensorineural hearing loss but eventually will provide the underpinnings of necessary knowledge for formulating therapeutic strategies to protect and, perhaps someday, restore hearing.

Currently, mutations in more than 130 genes have been identified as causes of human syndromic and non-syndromic sensorineural hearing loss (<https://hereditaryhearingloss.org/>). At least 23 of these genes encode proteins associated with the development and maintenance of stereocilia F-actin cores including actin monomers, actin regulatory proteins, bundling proteins, motor proteins and the mechanotransduction protein complex (Table 1). However, it is challenging to predict how each protein molecule contributes to homeostasis in stereocilia F-actin cores and how mutations identified in families segregating sensorineural hearing loss affect protein function. We speculate that in vivo functional analyses of protein molecules will be crucial given the recent exhaustive search capabilities available with whole exome and genome sequencing. These sequencing technologies can provide a long list of candidate causal variants some of which will no doubt prove to be just rare benign polymorphisms. An efficient pipeline to screen the functions of variant protein molecules is required to conclude that a variant is associated with hearing loss. Here, we review the genes encoding protein molecules that establish and maintain the F-actin cores of stereocilia. We then highlight how single-molecule fluorescence microscopy-based functional analysis can be employed to evaluate novel candidate variants of *DIAPH1*, a gene known to be associated with DFNA1 human deafness (Lynch et al. 1997).

Structure and dynamics of actin in living cells

Actin genes and products

Six paralogous actin genes are present in a mammalian genome: three α -actin genes (α -skeletal muscle, α -cardiac muscle and α -smooth muscle), one β -actin gene (β -cytoplasmic) and two γ -actin genes (γ -cytoplasmic and γ -smooth muscle) (Drummond et al. 2012; Vedula et al. 2017). In human, these six genes are named *ACTA1*, *ACTC1*, *ACTA2*, *ACTB*, *ACTG1* and *ACTG2*, respectively (Vedula and Kashina 2018). Actin is a 42-kDa globular protein that can form a polymer (F-actin) measuring approximately 7 nm in diameter

with a double-helix repeat every 37 nm (13 actin monomers) (Fig. 2, a and b). An actin filament has a “barbed end” and a “pointed end”. Actin monomers are more likely to polymerize to barbed ends. Actin is one of the most highly conserved proteins in eukaryotic cells, whose amino acid sequence differs by no more than 20% even between algae and human (Hanukoglu et al. 1983). The amino acid sequence is also highly similar between paralogs, and about 90% of the sequence is identical between the six mammalian actin genes (Khaitlina 2001). Different actin genes can replace the function of another in some organs, such as in the heart (Kumar et al. 1997). However, the unique phenotypes of knockout mice of the actin paralogs clearly indicate that each actin gene has a distinct biological function. Lack of *Actb* in mice causes early embryonic lethality (Tondeleir et al. 2014), while lack of *Acta1* or *Actc1* results in perinatal lethality accompanied by muscle weakness and myofibril disorganization, respectively (Crawford et al. 2002; Kumar et al. 1997). Mice lacking *Acta2* or *Actg1* are viable, but *Acta2*-knockout mice show defects in vascular contractility and blood pressure regulation (Schildmeyer et al. 2000), while *Actg1*-knockout mice suffer from growth abnormalities and progressive hearing loss (Belyantseva et al. 2009). Mutations of *ACTG1* also cause autosomal-dominant, nonsyndromic hearing loss, DFNA20/26 (Morell et al. 2000; van Wijk et al. 2003; Zhu et al. 2003).

An actin monomer has four subdomains, 1–4, with a large cleft dividing subdomains 1 and 2 from subdomains 3 and 4 (Fig. 2a). A nucleotide, either ATP or ADP, coupled with a divalent cation, typically Mg^{2+} , is held in the cleft by hydrogen and ion bonds to the surrounding amino acid residues (Kabsch et al. 1990; Schutt et al. 1993; Sheterline et al. 1995). Nucleotides control de novo nucleation and elongation of existing F-actin in addition to stabilizing the tertiary structures of actin monomers (Asakura 1961; Dominguez 2009). For example, ATP-actin is more likely to polymerize than ADP-actin both at the barbed and pointed ends. The critical concentration at which actin monomers are in equilibrium with F-actin is approximately 20-fold lower for ATP-actin, 0.1 μM , compared with that of ADP-actin at 1.8 μM (Pollard 1986). The nucleotide also controls the depolymerization of F-actin. After polymerization, ATP-actin rapidly hydrolyzes their ATP into ADP and phosphate with a half-life of approximately 2 seconds (s) and then slowly releases the phosphate with a half-life of approximately 350 s to become ADP-actin, which is more likely to dissociate from both ends of F-actin than ATP-actin (Pollard 2016; Pollard et al. 2008). Binding of other divalent cations, such as Ca^{2+} , also alters the speeds of actin polymerization and ATP hydrolysis and affects the critical concentration (Scipion et al. 2018; Strzelecka-Golaszewska et al. 1978). The ratio of Ca^{2+} -actin to Mg^{2+} -actin can be higher in a Ca^{2+} -rich condition such as in stereocilia, and the polymerization properties may be different in such environment since ATP hydrolysis of Ca^{2+} -actin is six times slower than with Mg^{2+} -actin (Bergeron et al. 2010; Blanchoin and Pollard 2002). Among the amino acid residues surrounding the nucleotide and the divalent cation, at least two missense mutations in *ACTG1*, p.K213R and p.M305T, are reported to cause DFNA20/26 due to loss-of-function (Merino et al. 2018; Miyajima et al. 2020; Park et al. 2013; Yuan et al. 2016).

Actin dynamics at the leading edge of living cells

Lamellipodia of motile cells provide useful models to understand the “dynamic equilibrium” of an actin cytoskeleton (Fig. 2c). The veil-like lamellipodium continuously protrudes and retracts resembling waves coming to the seashore. Additionally, F-actin bundle-containing filopodia protrude from the edges of lamellipodia. The quasi-two-dimensional actin mesh in lamellipodia is continuously remodeled to drive the “waves” while retracting toward the center of cells (retrograde flow). Cytoplasm in lamellipodia contains as high as 50–200 μM of ATP-actin. The critical concentration of ATP-actin at which ATP-actin monomers are in equilibrium with F-actin is only 0.1 μM indicating that lamellipodia without regulatory mechanisms would be packed with F-actin and eventually the cycle of polymerization and depolymerization would stall (Lodish et al. 2000; Pollard 1986).

Polymerization and depolymerization of actin monomers in a live cell are controlled by many actin regulatory proteins. Elongation of existing actin filaments is suppressed by barbed-end cappers, such as CapZ (an α/β heterodimer formed by CAPZA1 or CAPZA2, and CAPZB) (Pollard and Cooper 1984). Depolymerization of existing filaments is enhanced by severing proteins, such as cofilin and ADF (Bamburg et al. 1980; Poukkula et al. 2011). AIP1 preferentially promotes disassembly of ADF/cofilin-decorated actin filaments (Okada et al. 2006). In addition, polymerization activities of actin monomers are modulated mainly by two ubiquitous proteins of contrasting functions, thymosin- β 4 and profilin. Thymosin- β 4, a 43 amino acid peptide in human, can sequester a large pool of actin monomers and suppress nucleation and binding to existing filaments (Pollard 2016; Safer et al. 1991). Profilin, a 16 kDa protein, also suppresses nucleation although binding to the barbed ends of existing actin filaments is not inhibited (Pollard and Cooper 1984; Vinson et al. 1998). Furthermore, profilin competes with thymosin- β 4 and accelerates barbed-end elongation by catalyzing the exchange of actin-bound ADP to ATP (Mockrin and Korn 1980; Pantaloni and Carlier 1993). Inhibition of nucleation by these two proteins may be crucial to allow nucleation in a controlled manner, such as from the Arp2/3 complex in lamellipodia. Typical cytoplasm contains a high concentration of profilin-ATP-actin (50–100 μM in most eukaryotic cells), which substantially exceeds the critical concentration of ATP-actin and suggests that barbed-end cappers and severing proteins play crucial roles to balance polymerization and depolymerization in living cells (Pollard 2016; Pollard et al. 2000).

Single-molecule fluorescence microscopy has helped to elucidate actin cytoskeletal dynamics in lamellipodia, distinguishing and resolving single fluorescent molecules from the background signal and revealing actin and regulatory protein behavior in live cells. Thin lamellipodia of *Xenopus* XTC cells are useful to determine dissociation rates and trajectories of actin and actin regulatory proteins (Watanabe and Mitchison 2002). We reported that CapZ dissociates from barbed ends of lamellipodial actin with a half-life of 1–2 s, while for in vitro biochemistry experiments CapZ remains on barbed ends for more than a few hours (Miyoshi et al. 2006; Pollard and Cooper 1984). Fast dissociation was also observed for other proteins that can bind to barbed ends, such as EPS8, gelsolin (GSN) and VASP (Miyoshi et al. 2006). These results indicate that a common “uncapping” mechanism removes these proteins from barbed ends. Severing proteins may

depolymerize barbed ends more aggressively than other parts of F-actin to cause uncapping because the dissociation rate of CapZ decreased significantly after removal of cytoplasm by permeabilization, stabilization of F-actin using Jasplakinolide and inhibition of cofilin by LIM-kinase overexpression (Miyoshi et al. 2006). These findings are consistent with the localization of severing proteins at the tips of stereocilia in mice, where barbed ends accumulate (McGrath et al. 2020).

Dynamics of stereocilia components

Microvilli are transient structures on the cell apical surface and usually appear and disappear within a few minutes (Gorelik et al. 2003; Ida et al. 2017; Meenderink et al. 2019).

Compared with actin protrusions of microvilli, stereocilia have a unique characteristic that their overall architecture is largely unchanged for the lifetime of a mammal (Fig. 2d) (Jia et al. 2009). Actin dynamics is also different between microvilli and stereocilia. F-actin in microvilli treadmills from the barbed ends at the tips toward the pointed ends near the apical surface (Meenderink et al. 2019), while we now know that stereocilia replenish actin monomers mainly at the barbed ends near the tips without treadmilling the F-actin core (Drummond et al. 2015; Narayanan et al. 2015; Perrin and Ervasti 2010; Rzadzinska et al. 2004; Zhang et al. 2012). One unresolved question is the timing and molecular events responsible for cessation of F-actin treadmilling in a subset of microvilli on the apical surface of nascent hair cells during their transition to stereocilia. Espin, a major F-actin bundling protein in stereocilia, likely contributes to this transition since expression of espin in cultured cells can suddenly stop the treadmilling in filopodia (Loomis et al. 2003; Zheng et al. 2015).

Several of the actin regulatory proteins that function in lamellipodia also participate in stereocilia actin dynamics. CapZ is localized at the tips of shorter-row stereocilia and required for the regulation of their length (Avenarius et al. 2017). The severing proteins, cofilin-1 (CFL1) and ADF (DSTN), also localize at stereocilia tips suggesting an aggressive depolymerization dynamics near their barbed ends, as reported for the fast turnover of barbed-end capper CapZ in lamellipodia (McGrath et al. 2020; Miyoshi et al. 2006). One difference from the lamellipodial actin meshwork is that F-actin in stereocilia is tightly packed by the crosslinkers, such as plastin-1, espin and fascin-2 (Krey et al. 2016; Sekerkova et al. 2011). Unexpectedly, these crosslinkers can move freely along the entire length of stereocilia perhaps to allow for the paracrystal-like packing of F-actin (Miyoshi et al. 2021; Roy and Perrin 2018). The molecular mechanisms that maintain the entire architecture of stereocilia are not fully understood, especially those phenomena that balance polymerization and depolymerization of the F-actin cores and replace damaged actin monomers in the F-actin stereocilia cores (Belyantseva et al, 2009). Elucidating the dynamics of protein molecules that govern the homeostasis of stereocilia macromolecules will provide a basis to formulate therapeutic strategies for protecting hearing and perhaps reversing sensorineural hearing loss.

Hereditary hearing loss due to variants of actin and actin-associated proteins in stereocilia

ACTB and ACTG1

The *ACTB* and *ACTG1* genes encode the almost identical β -cytoplasmic actin and γ -cytoplasmic actin. Both actins are found in stereocilia (Hofer et al. 1997) and differ by only four residues among the first nine amino acids at their N-termini, MDDDAALVY (*ACTB*, NP_001092.1) and MEEEAALVI (*ACTG1*, NP_001092.1), in mammals and birds (Perrin and Ervasti 2010). At least during development of the inner ear, these two actin genes can substitute functionally for one another as indicated by normal appearing morphology of early postnatal *Actb*-null or *Actg1*-null stereocilia (Perrin et al. 2010). Gain-of-function variants of *ACTB* and *ACTG1* are associated with Baraitser–Winter cerebrofrontofacial (BWCFF) syndrome (OMIM #243,310) characterized by craniofacial features (widely spaced eyes, bulbous nose with broad nasal tip and prominent nasal bridge, congenital non-myopathic ptosis, prominent metopic ridge and highly arched eyebrows) and intellectual disability (Baraitser and Winter 1988). The prevalence of sensorineural hearing loss in cases of BWCFF syndrome ranges from 30 to 80% (Riviere et al. 2012; Verloes et al. 2015; Yates et al. 2017). The function gained by *ACTB* and *ACTG1* variants in the BWCFF syndrome appears to be upregulated polymerization of F-actin. Lymphoblasts expressing either of two *ACTB* variants discovered in BWCFF patients, p.S155F and p.R196H, accumulated F-actin in cytoplasm and increased the number of filopodia-like protrusions (Riviere et al. 2012). Another BWCFF variant of *ACTB*, p.E117K, assembles into unusually stable F-actin by a combination of increased polymerization speed and a resistance to depolymerization (Johnston et al. 2013). However, gain-of-function *ACTB* variants do not always result in BWCFF syndrome. Two studies report dystonia-deafness syndrome caused by the gain-of-function variant p.R183W (Procaccio et al. 2006; Skogseid et al. 2018).

Different phenotypes associated with loss-of-function variants of the genes encoding *ACTB* or *ACTG1* proteins indicate that these two actin paralogues cannot completely substitute for a loss of the other despite their nearly identical amino acid sequences. Two elegant studies suggest that both the amino acid sequences and the genomic context including non-coding regions are important for the functions of these two genes. In these studies, genetically-engineered mice using CRISPR/Cas9 and TALEN technologies express *ACTG1* and *ACTB* from the endogenous *Actb* and *Actg1* loci, respectively. Mice expressing *ACTG1* protein under the control of the endogenous *Actb* promoter and mice expressing actin almost identical to *ACTB* under the control of the *Actg1* promoter showed a normal phenotype in overall morphology and appearance of all major organs examined (Vedula and Kashina 2018). Expanding on these observations, a contemporaneous study using mice expressing *ACTG1* protein from the endogenous *Actb* locus, revealed a progressive elevation of the hearing threshold beginning in the high frequencies and degeneration of outer hair cell stereocilia by six months of age in homozygous mice (Patrinostro et al. 2018). Taken together, the interchangeability of the *Actb* and *Actg1* protein coding sequences in the limited number of organs examined indicates that the nucleotide sequence of noncoding regions, such as the promoters and other regulatory elements, the untranslated regions (UTRs) and also the mRNAs translation dynamic differences may be important determinants

of unique ACTB and ACTG1 functions (Patrinostro et al. 2018; Vedula and Kashina 2018). It is also apparent that in some cell types, the few amino acid sequence difference between ACTB and ACTG1 is likely responsible for their distinct functions, since these two actins are not able to substitute for one another in the long-term maintenance of stereocilia (Patrinostro et al. 2018), as concluded also from the analysis of the progressive hearing loss phenotype in *Actg1*-knockout mice (Belyantseva et al. 2009).

A microdeletion of 7p22.1 including the *ACTB* gene and a missense mutation of *ACTB* result in a pleiotropic phenotype including malformations of the heart and the renal tract, a distinctive facial feature of interrupted wavy eyebrows, dense eyelashes, a wide nose and mouth and a prominent chin as well as intellectual disability and a developmental delay (Cuvertino et al. 2017; Shimojima et al. 2016). In contrast, dominant missense mutations damaging *ACTG1* function predominantly cause DFNA20/26 sensorineural hearing loss (Miyajima et al. 2020; Morell et al. 2000; van Wijk et al. 2003; Zhu et al. 2003). The onset of sensorineural hearing loss in cases of DFNA20/26 ranges widely from first decade to sixth decade (Yuan et al. 2016). Mouse models reveal that ACTG1 likely contributes to repair processes within damaged stereocilia. Mice subjected to intense sound incorporate ACTG1 into gaps within stereocilia F-actin cores (Belyantseva et al., 2009). Mice engineered to express reduced levels of ACTG1 show elevation of ABR thresholds although less so than the severe consequences to hearing loss of *Actg1*-null mice (Perrin et al. 2010).

Barbed-end cappers and severing proteins associated with human deafness

Currently, mutations in two genes encoding barbed-end cappers, *EPS8* and *EPS8L2*, underlie human deafness genetically mapped to the *DFNB102* and *DFNB106* loci, respectively (Behloul et al. 2014; Furness et al. 2013). Epidermal growth factor receptor pathway substrate 8, EPS8, was initially identified as a substrate of EGFR and is involved in the transduction of signals between a small GTPase (Ras) regulating cell proliferation and another small GTPase (Rac) to cause actin remodeling and membrane ruffling in fibroblasts (Fazioli et al. 1993; Scita et al. 1999). EPS8 also binds to the barbed ends of F-actin and can directly inhibit actin elongation (Croce et al. 2004). EPS8L2 (EPS8-like protein 2) is one of three EPS8-related proteins and is predicted to remodel actin cytoskeleton in response to EGF signaling (Tocchetti et al. 2003). Although it is unclear how EPS8 and EPS8L2 contribute to the actin dynamics in stereocilia, experiments using mice suggest that these two proteins bind to the barbed ends of F-actin near the tips of stereocilia and are involved in maintaining the staircase arrangement of stereocilia. EPS8 mainly localizes at the tips of tallest row stereocilia while Eps8L2 distributes to the tips of the mechanosensitive shorter-row stereocilia (Furness et al. 2013; Manor et al. 2011). Consistent with this finding, mutant proteins discovered from patients, EPS8 (p.N30*) and EPS8L2 (p.S339Afs*15), likely disrupt the F-actin-binding domain at the C-termini (Behloul et al. 2014; Furness et al. 2013). Different distributions of these proteins are likely to be a result of different interacting partners. EPS8 is reported to be a cargo of MYO15 (Hadi et al. 2020; Manor et al. 2011). It is uncertain if EPS8L2 is also a cargo of a myosin or passively diffuses to the tips of stereocilia (Manor et al. 2011).

Knockouts of other F-actin barbed-end cappers and severing proteins are also associated with sensorineural hearing loss in mouse. CapZ is an abundant barbed-end capper in stereocilia. A conditional knockout of *CapZB* results in shortened or lost stereocilia in mice (Avenarius et al. 2017). Stereocilia contain the severing proteins, cofilin-1 (CFL1) and ADF (DSTN), and also AIP1 (WDR1), an F-actin-binding protein enhancing severing (McGrath et al. 2020; Narayanan et al. 2015). Loss-of-function in ADF and AIP1 causes hearing loss and shortened stereocilia in rows 2 and 3 of the staircase in mice (Narayanan et al. 2015). Stereocilia also contain twinfilin-2 (TWF2), a protein consisting of two ADF homology domains (Paavilainen et al. 2007). However, the exact function of twinfilins in actin dynamics is still unresolved as to whether it binds to ADP-actin, is involved in filament severing, is a barbed-end capper or is an uncapper of barbed ends (Hakala et al. 2021; Poukkula et al. 2011). In addition to other barbed-end cappers and severing proteins, it is likely that TWF2 is involved in length regulation of stereocilia after being transported to tips by MYO7A (Peng et al. 2009; Rzadzinska et al. 2009). Interestingly, knockout of CapZ, ADF and AIP1 results in shortened stereocilia and yet these actin regulatory proteins are thought to suppress elongation or promote depolymerization of existing F-actin.

Actin crosslinkers

Actin filaments in a stereocilium are bundled together by the crosslinkers espin (ESPN), espin-like protein (ESP NL), plastin-1 (PLS1), plastin-3 (PLS3), fascin-1 (FSCN1), fascin-2 (FSCN2), GRXCR1, GRXCR2, Xin-related protein 2 (XIRP2) and TRIOBP (Kitajiri et al. 2010; Krey et al. 2016; Odeh et al. 2010; Scheffer et al. 2015; Shin et al. 2010; Zheng et al. 2000). Among the genes encoding these actin crosslinkers, pathogenic recessive variants of *GRXCR1*, *TRI-OBP*, *ESPN* and *GRXCR2* are associated with hearing loss in human at the *DFNB25*, *DFNB28*, *DFNB36* and *DFNB101* loci, respectively (Imtiaz et al. 2014; Kitajiri et al. 2010; Naz et al. 2004; Schraders et al. 2010). One case study reported that three missense mutations in *PLS1* cause autosomal-dominant hearing loss (Morgan et al. 2019). Experimental demonstration using a functional assay for pathogenicity of these substitutions would add strength to this conclusion.

Most of the actin crosslinkers, such as espin (ESPN), plastin1 (PLS1) and fascin-2 (FSCN2), are found along the entire length of stereocilia F-actin cores and contribute to the paracrystal-like packing of actin filaments (Krey et al. 2016; Sekerkova et al. 2011). The Jerker mouse has a mutation in the *Espin* gene and develops thin, short stereocilia compared to the wild-type (Sekerkova et al. 2011). Two studies reported that stereocilia lacking PLS1 show hexagonal packing of F-actin, which is different from heterogenous F-actin packing in wild-type stereocilia (Krey et al. 2016; Metlagel et al. 2019). However, these two reports describe discrepant phenotypes of *Pls1*-null stereocilia perhaps due to different methodologies. Krey and co-authors reported a decrease in the diameters of *Pls1*-null stereocilia. In this study, utricular stereocilia were embedded in agarose melted at 42 °C, detached from cell bodies after cooling and then fixed in paraformaldehyde. Metlagel et al. performed CryoET using cryofixed vestibular stereocilia and reported an increase in the diameters of *Pls1*-null stereocilia. Therefore, morphological change of *Pls1*-null stereocilia is an unresolved question although *Pls1*-null and wild-type stereocilia may well behave differently during the treatments used in these studies. Mice expressing

mutant FSCN2 (p.R102H) develop stereocilia with heterogeneous packing, but the distance between filaments is larger than those in wild-type stereocilia (Krey et al. 2016). The F-actin packing is disorganized when XIRP2 is lacking (Scheffer et al. 2015). Absence of functional GRXCR1 in pirouette mice results in thin and slightly shortened stereocilia. GRXCR1 localizes along the entire length of auditory and vestibular stereocilia (Odeh et al. 2010) and based on data from zebrafish, may regulate the assembly of harmonin-sans Usher protein complex (Blanco-Sanchez et al., 2018).

In contrast to other crosslinkers, TRIOBP is unique in its localization to stereocilia rootlets and actin-based cytoskeletal structures in supporting cells of the organ of Corti (Kitajiri et al. 2010). The *TRIOBP/Triobp* genes in human and mouse encode three major isoform classes, TRIOBP-1, TRIOBP-4 and TRIOBP-5. Mice lacking both the TRIOBP-4 and TRIOBP-5 are congenitally profoundly deaf although initially stereocilia appear morphologically normal. Subsequently, stereocilia rootlets fail to develop and hair cells in these mice degenerate by P16. In contrast, stereocilia rootlets in mice lacking only TRIOBP-5 are thin and dysmorphic. Data from a recent study indicate that TRIOBP-5 is critical to form thick, stiff rootlets (Katsuno et al. 2019). Mice lacking TRIOBP-5 but expressing TRIOBP-4 develop less rigid stereocilia vulnerable to external force. The reinforcement of rootlets is likely provided by the ability of TRIOBP-5 to homopolymerize by its C-terminal coiled-coil domains since lack of these domains leads to frequent rootlet breakage and malformations. Loss of TRIOBP-5 also causes an expansion of TRIOBP-4 bundling within the actin core, which extends up to stereocilia tips, and these mice show progressive hearing loss (Katsuno et al. 2019) as do humans with pathogenic variants of *TRIOBP* that are limited to exons unique to *TRIOBP-5* mRNA (Pollak et al. 2017; Wesdorp et al. 2017).

Variants of myosins and cadherins associated with human deafness

Myosins move on F-actin using the energy of ATP hydrolysis. Myosins are classified into “conventional” myosins (also known as myosin II) originally found in muscles and “unconventional” myosins that were discovered in many other tissues (Cheney and Mooseker 1992). Two genes encoding conventional, non-muscle myosins, *MYH9* and *MYH14*, are associated with autosomal-dominant hearing loss DFNA17 and DFNA4A, respectively, although their exact functions in the inner ear are still debated (Donaudy et al. 2004; Lalwani et al. 2000). Among unconventional myosins, variants of *MYO3A*, *MYO6*, *MYO7A* and *MYO15A* are linked to autosomal recessive hearing loss, DFNB30, DFNB37, DFNB2 and DFNB3, respectively (Ahmed et al. 2003a; Liu et al. 1997; Walsh et al. 2002; Wang et al. 1998; Weil et al. 1997). Variants of *MYO6* and *MYO7A* are also associated with autosomal-dominant hearing loss, DFNA22 and DFNA11, respectively (Liu et al. 1997; Melchionda et al. 2001; Weil et al. 1997). Furthermore, mutations in *MYO7A* cause the most severe Type 1 form of Usher syndrome characterized by congenital, bilateral, profound sensorineural hearing loss, vestibular areflexia and adolescent-onset retinitis pigmentosa (Koenekoop et al. 1993). A few studies report that missense mutations in the kinase domain or motor domain of *MYO3A* can cause autosomal-dominant hearing loss (Dantas et al. 2018; Doll et al. 2020; Grati et al. 2016). Cargo transport in stereocilia is a major function of unconventional myosins (Friedman et al. 2020).

The amino acid sequence of the motor domains of unconventional and conventional myosins is well conserved, although their kinetic properties are adapted for unique functions (Bird et al. 2014). In contrast, the tail domains of myosins are divergent and have evolved several functions including auto-regulating the activity of its motor domain and delivering a variety of cargo including proteins and lipids (Li and Zhang 2020; Li et al. 2008; Yao et al. 2015). Among the unconventional myosins currently associated with hearing loss, MYO6, MYO7A and MYO15A have well-folded tail domains, while the tail domain of MYO3A is predicted to be almost unfolded (Li and Zhang 2020). The tail domain of MYO6 is characterized by two consecutively arranged regions for cargo binding, an N-terminal helical cargo-binding domain (HCBD) and a C-terminal cargo-binding domain (CBD), which is likely to interact with a protein tyrosine phosphatase receptor type Q (PTPRQ), a membrane protein crucial to tether the stereocilia membrane to F-actin (Goodyear et al. 2003; Sakaguchi et al. 2008). MYO6 is the only unconventional myosin that moves toward the pointed end of F-actin at the base of stereocilia and is thought to transport PTPRQ toward the base of stereocilia on the apical surface of hair cells (Avraham et al. 1997). The loss of MYO6 in the deaf Snell's waltzer mouse (*sv*) results in fused stereocilia and degeneration of the organ of Corti (Avraham et al. 1995).

Cadherin 23 (CDH23) and protocadherin 15 (PCDH15) are two proteins comprising stereocilia tip-links that gate the mechanotransduction channel and are encoded by the *CDH23* and *PCDH15* genes, respectively. Recessive variants of *CDH23* and *PCDH15* are associated either with type 1 Usher syndrome 1D and 1F or non-syndromic deafness DFNB12 or DFNB23, respectively, (Ahmed et al. 2003b; Bork et al. 2001). MYO7A is concentrated at the upper tip-link density (Grati and Kachar 2011) near the intracellular domain of cadherin 23 by binding to the CEN region of USH1G (SANS) using the N-terminal MyTH4-FERM (NMF) and to the PDZ3 domain of USH1C (Harmonin) using the C-terminal MyTH4-FERM domain (CMF) (Li et al. 2016; Wu et al. 2011). The tail of MYO15A also has tandem MyTH4-FERM domains structurally homologous to that of MYO7A although there is a ~ 370-residue insertion within the F1 domain of NMF (Liang et al. 1999). The short isoform of MYO15A that lacks the long N-terminal domain elongates stereocilia to a predetermined length (Belyantseva et al., 2003) and forms an elongation complex at the tips of stereocilia by transporting whirlin, a PDZ-domain-containing scaffold protein, using the PDZ ligand of MYO15A (Belyantseva et al. 2005). The short isoform of MYO15A is also reported to transport the barbed-end capper EPS8 to stereocilia tips and controls the lengths and probably the diameters of stereocilia (Hadi et al. 2020; Manor et al. 2011). The long isoform of MYO15A is localized at the lower tip-link density near PCDH15 and is essential for maintaining the length of the mechanotransducing stereocilia (Fang et al., 2015).

The MYO3A tail domain is mostly disordered but has a conserved THDI (tail homology domain I), which interacts with the espin isoform 1 (Espin1) and boosts elongation of stereocilia (Salles et al. 2009). Through the ARB1 and ARB2 motifs in the THDI domain, MYO3A interacts with two Espin1 molecules via the Ankyrin (ANK) repeats (Liu et al. 2016). Double-knockout mice of MYO3A and MYO3B result in an abnormal arrangement of stereocilia bundles with a deficient staircase architecture (Lelli et al. 2016).

Scaffolding proteins

Scaffolding proteins provide linkages between F-actin of the cell cortex and membrane proteins (Bretscher et al. 2002). Among the genes encoding scaffold proteins in stereocilia, harmonin (USH1C), radixin (RDX), whirlin (WHRN) and PDZD7 are associated with autosomal recessive hearing loss DFNB18, DFNB24, DFNB31 and DFNB57, respectively (Ahmed et al. 2002; Booth et al. 2015; Khan et al. 2007; Mburu et al. 2003; Ouyang et al. 2002). More debilitating variants of *USH1C* and *USH1G* encoding harmonin (USH1C) and SANS (USH1G) also cause Usher syndrome type 1 (Bitner-Glindzicz et al. 2000; Weil et al. 2003), while variants of whirlin are associated with Usher syndrome type 2D (Ebermann et al. 2007). RDX is a member of the ERM family of three proteins (ezrin, radixin and moesin) characterized by an N-terminal FERM (Four-point one, ezrin, radixin, moesin) domain and a C-terminal F-actin-binding domain (Bretscher et al. 2002). The FERM domain of RDX is reported to interact with several membrane proteins including CD43, CD44 and ICAM-1/2/3, but the partners of RDX in hair cells are yet to be determined (Shaw 2001; Tsukita et al. 1994; Yonemura et al. 1998). *Rdx*-null mice show degeneration of cochlear stereocilia at approximately P14 and subsequent hearing loss although there is no obvious malformation of stereocilia at P1 (Kitajiri et al. 2004). In *Rdx*-null mice, in vestibular hair cells but not cochlear hair cells, a radixin deficiency appears to be partially substituted for by ezrin, another member of the ERM family (Kitajiri et al. 2004). Whirlin (WHRN) is a PDZ-domain-containing scaffold protein, which is transported via an interaction with the PDZ ligand of MYO15A (Belyantseva et al. 2005). *Whrn* mutant mice (*whirler*) are deaf and have short stereocilia arrested in an immature state (Mogensen et al. 2007). PDZD7 has three PDZ domains, a similar domain composition to harmonin and whirlin, and is involved in scaffolding the ankle-link complex (Du et al. 2020; Grati et al. 2012). Monoallelic deleterious mutations of *PDZD7* and *GPR98* were reported as a digenic case of Usher syndrome type 2 (Ebermann et al. 2010).

Harmonin (USH1C) and SANS (USH1G), encoded by the Usher syndrome genes *USH1C* and *USH1G*, are transported to stereocilia upper tip-link densities by binding to the MyTH4-FREM domain of MYO7A (Grati and Kachar 2011). These proteins are necessary for tensioning tip-links that gate the mechanotransduction channels near the tips of stereocilia (Kros et al. 2002; Li et al. 2016; Wu et al. 2011). Among the three splice isoforms of harmonin (harmonin-a, -b and -c), cochlear hair cells express the longest isoform, harmonin-b, which has three PDZ domains (Verpy et al. 2000). Harmonin-b interacts with CDH23 both in retina and in the upper tip-link density using one or two PDZ domains (PDZ1 and PDZ2 in retina; PDZ2 in stereocilia) (Siemens et al. 2002). The PDZ3 domain interacts with the MyTH4-FERM domain of MYO7A (Yu et al. 2017). Mice expressing harmonin-b with a missense mutation in the PDZ2 domain are deaf with mislocalized CDH23 and disrupted morphology of stereocilia (Grillet et al. 2009). Harmonin-b also binds directly to F-actin using sequence between its PDZ2 and PDZ3 domains (Michalski et al. 2009). SANS is another critical component of the upper tip-link complex with the C-terminal SAM-PBM domains interacting with the PDZ1 domain of harmonin-b (Yu et al. 2017). *Ush1g*^{-/-} mice show hearing loss due to the lack of mechano-electrical transduction currents (Caberlotto et al. 2011).

DIAPH1 and DFNA1 deafness

Dominant variants of *DIAPH1* are associated with DFNA1 hearing loss (Lynch et al. 1997). The pathology associated with variants of *DFNA1* is unique from other F-actin regulatory proteins. DIAPH1 is a formin homology protein with a processive F-actin elongation activity (Fig. 3a). The average speed of movement can reach 2.0 $\mu\text{m/s}$, which corresponds to polymerization of ~ 700 actin subunit per second (Higashida et al. 2004). With such aggressive actin elongation activity, ectopic expression of DIAPH1 forms a large amount of F-actin in cultured cells (Watanabe et al. 1997). The actin elongation activity of FH1 and FH2 domains is suppressed by an autoinhibitory interaction between DID and DAD domains of DIAPH1, which is released by the binding of Rho, a small GTPase, to the DIAPH1 GBD domain (Watanabe et al. 1997). The function of DIAPH1 appears to be unnecessary to the development of stereocilia although abnormal activation of DIAPH1 is pathogenic (Ueyama et al. 2016). Loss of DIAPH1 function is associated with syndromic microcephaly, blindness and early-onset seizures in human but not with hearing loss (Al-Maawali et al. 2016; Ercan-Sencicek et al. 2015). Neither hearing loss nor abnormal morphology of inner ear was observed in mice lacking a functional *Diaph1* gene (Ueyama et al. 2016). However, a constitutively active mutation of DIAPH1 that has a truncated DAD domain (p.R1213X) does cause hearing loss both in human and mouse as a result of stereocilia degeneration (Ueyama et al. 2016). Variants of DIAPH1, such as p.A265S, p.A1210Sfs*31, p.A1210GfsX3130 and p.E1192_Q1220del30, located in the DID and DAD domains have been identified in families segregating hearing loss, suggesting a relationship between a gain-of-function of DIAPH1 and hearing loss (Kim et al. 2019; Neuhaus et al. 2017; Westbury et al. 2018). In mice, ectopically expressed GFP-DIAPH1 (p.R1213X), a constitutively active form, is observed at stereocilia tips and in the apical surface of hair cells as well as in Deiters' cells and pillar cells (Ninoyu et al. 2020). Although the DFNA1 inner ear pathology associated with variants of *DIAPH1* is still not entirely understood, we hypothesize that abnormally formed F-actin, monomer depletion or both due to upregulated actin polymerization may disturb the normal, routine maintenance of stereocilia F-actin cores.

Single-molecule fluorescence microscopy for functional analysis of DIAPH1 variants

Here, we introduce our experimental approach to use single-molecule fluorescence microscopy in cultured cells for functional analyses of novel DIAPH1 variants (Figs. 3 and 4). In *Xenopus* XTC cells expressing GFP-DIAPH1, single-molecule fluorescence microscopy can visualize processive actin elongation activities of DIAPH1 molecules as directional movements of fluorescent puncta (Higashida et al. 2004). Using this technique, we evaluated the actin elongation activity of a novel p.R1213X variant found in two Japanese families segregating hearing loss (Fig. 3b and c). This variant was predicted to show abnormal, constitutively active actin elongation activity due to the truncation at the C-terminus of the autoinhibitory DAD domain. We prepared XTC cells expressing GFP-DIAPH1 (p.R1213X) and evaluated the frequency of directionally moving molecules (Fig. 3d, Movie S1, indicated by circles and trajectories in time-lapse images) (Ueyama et al. 2016). We also prepared XTC cells expressing wild-type GFP-DIAPH1 for a negative

control and GFP-DIAPH1 (p.M1199D) for a positive control, which is a mutant with no DID-DAD interaction (Fig. 3e, Movie S1, indicated by circles and trajectories in time-lapse images) (Lammers et al. 2005). Directionally moving molecules were frequently observed in cells expressing GFP-DIAPH1 (p.R1213X) and GFP-DIAPH1 (p.M1199D) compared with cells expressing wild-type GFP-DIAPH1. These data indicate the disrupted DID–DAD autoinhibitory interaction of these two variants. We also performed semi-quantitative comparison using the density of moving molecules normalized to the expression levels of GFP-DIAPH1 (Ueyama et al. 2016). DIAPH1 (p.R1213X) was considered to be moderately active since the density of activated DIAPH1 (p.R1213X) molecules was significantly higher than wild-type DIAPH1 and also lower than DIAPH1 (p.M1199D) (Fig. 3f). From the phenotype of transgenic mice expressing DIAPH1 (p.R1213X), which show degeneration of stereocilia after approximately 10 weeks of age, we concluded that abnormal actin elongation activity of DIAPH1 is associated with DFNA1 deafness (Ueyama et al. 2016).

Single-molecule fluorescence microscopy was also used as a tool to screen for the pathogenicity of novel variants of DIAPH1 (Fig. 4). Two missense mutations, p.A265S and p.N1140S, were identified in a Korean family and a Japanese family, respectively (Fig. 4, a and b). The p.A265S variant was predicted to be pathogenic since GFP-DIAPH1 (p.A265S) expressed in XTC cells showed frequent directional movements (Fig. 4c, Movie S2, indicated by circles and trajectories in time-lapse images) similar to p.R1213X (Fig. 3d) (Kim et al. 2019). These data suggest that the p.A265S variant in the DID domain disrupts the autoinhibitory DID–DAD interaction. In contrast, the p.N1140S variant appears to be a benign polymorphism since GFP-DIAPH1 (p.N1140S) expressed in XTC cells showed only a few directional movements (Fig. 4c, Movie S2, indicated by circles and trajectories in time-lapse images), which is a phenotype indistinguishable from XTC cells expressing wild-type GFP-DIAPH1 (Fig. 3e). The p.N1140S substitution in the FH2 domain was unlikely to disrupt processive actin elongation activity of DIAPH1 since GFP-DIAPH1 (p.N1140S) could travel long distances similarly to wild-type GFP-DIAPH1. Subsequently, the p.N1140S variant was found in a healthy control in the same pedigree (Fig. 4b, a female with asterisk), and another patient in this pedigree with hearing loss did not have this variant (Fig. 4b, a male double asterisks) supporting the assumption that this variant of *DIAPH1* is a benign polymorphism. Semi-quantitative comparison also suggests that p.A265S is moderately active while the autoinhibition of p.N1140S is intact (Fig. 4d). Compared with conventional, macroscopic biochemistry experiments, our single-molecule microscopy-based approach can directly visualize the function of molecules, such as the autoinhibitory DID–DAD interaction of DIAPH1 and the actin elongation activity of FH1–FH2 domains. As we demonstrated in this section, direct analysis of a protein function could represent an efficient methodology to screen variants found in patients with hearing loss.

Conclusion

In the analyses of multiplex pedigrees segregating hearing loss, recent advances in whole exome or genome sequencing permit exhaustive searches for candidate variants. However, it remains even more challenging in affected isolated cases of deafness to determine if a protein variant or a noncoding variant is truly pathogenic or a rare benign polymorphism. Thus, individuals with rare inherited disorders may be left without fully solved molecular

genetic diagnoses. Methodologies that can directly evaluate the function of protein variants, such as single-molecule fluorescence microscopy, may be another valuable tool in the armamentarium to experimentally evaluate putative pathogenic variants of hearing loss.

Actin and its regulatory proteins form a dynamic system that is continuously remodeled in living cells. Stereocilia are also dynamic structures that, while maintaining the overall shape due to a relative stability of the F-actin core, possess an ongoing replenishment of many of their constituents, such as actin monomers and crosslinkers, through their constant binding and dissociation. Variants in human and mouse actin and partner proteins can disrupt this “dynamic equilibrium” in stereocilia resulting in abnormal morphological changes, damaged mechanotransduction and often sensorineural hearing loss caused by the death or dysfunction of hair cells. Single-molecule fluorescence microscopy helps to elucidate the molecular mechanisms of hearing loss as we detected abnormal actin elongation activity of DIAPH1 variants. Insight from a molecular diagnosis and understanding the underlying wild-type and defective functions are important for making headway towards therapeutics to protect and restore hearing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
DFNA	Locus for dominantly inherited nonsyndromic deafness
DFNB	Locus for recessively inherited nonsyndromic deafness
GFP	Green fluorescent protein
USH	Usher syndrome

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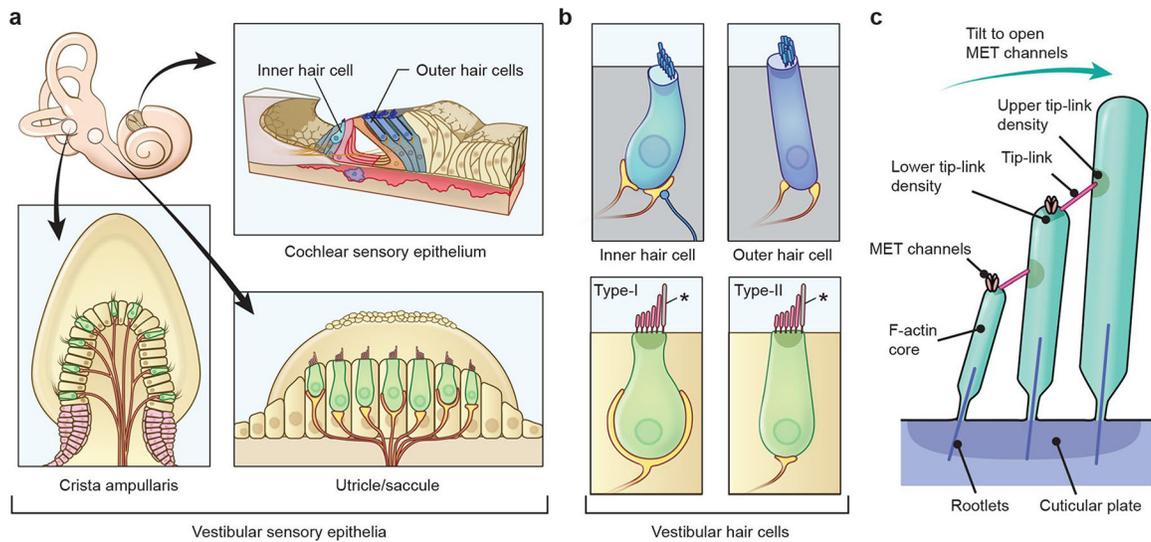


Fig. 1.

Sensory epithelia of the inner ear, hair cells and their stereocilia in mammals. **a** In the inner ear, there are three distinct types of sensory epithelia. The cochlear sensory epithelium detects and attenuates sound vibration using one row of inner hair cells and three rows of outer hair cells, which are a part of the organ of Corti. Vestibular sensory epithelia of utricle and saccule detect horizontal and vertical accelerations. The three cristae ampullaris detect angular accelerations of the head. **b** Alignment of stereocilia on the apical surface of hair cells. Each outer hair cell has three rows of V-shaped stereocilia while stereocilia of inner hair cell are organized in a straighter line. Vestibular hair cell stereocilia (type I and type II) are long and also align in a staircase manner although the alignment is not as organized as inner and outer hair cells. Asterisks indicate kinocilia, which are composed of microtubules. **c** Architecture of stereocilia F-actin cores and tip-links. Stereocilia shafts consist of F-actin, which are bundled more tightly near their bases to form rootlets and anchor stereocilia in the cuticular plate. Tip-links are tethered to F-actin cores via protein complexes in upper and lower tip-link densities. Tension caused by unidirectional deflection of all stereocilia within a bundle opens the mechanotransduction channels (MET channels) near the tip-links

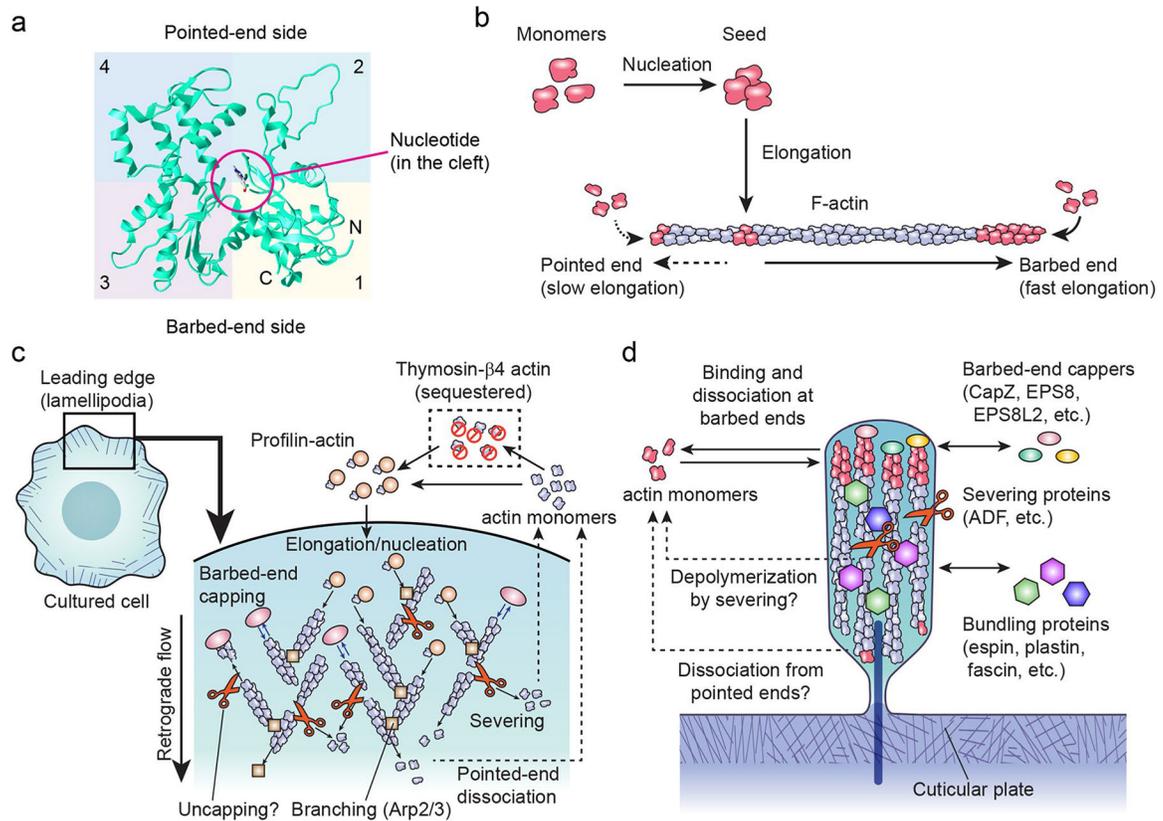


Fig. 2. Structure and dynamics of actin monomers and filaments (F-actin). **a** Tertiary structure of an ACTB actin monomer (PDB number: 3j82) (Hanc et al. 2015). A large cleft divides subdomains 1 and 2 from subdomains 3 and 4. ATP or ADP and a cation are held in a pocket deep in the cleft (ADP shown). **b** Assembly of F-actin from actin monomers in vitro. Formation of F-actin seeds from monomers is a slow nucleation step. F-actin has two ends, a barbed end that can rapidly elongate and a pointed end that can elongate but more slowly. **c** Actin dynamics in the lamellipodia, a thin veil-like structure at the leading edge of cells. Quasi-two-dimensional actin mesh is dynamically remodeled and tracked toward the center of cells by the retrograde flow. Lamellipodia can be used to study the dynamics of actin monomers, barbed-end cappers, Arp2/3 complex and severing proteins, many of which play crucial roles in stereocilia. Exchange of ATP and ADP in actin monomers is not shown to simplify the diagram. **d** Dynamics of stereocilia components. Proteins, such as actin, barbed-end cappers and bundling proteins, turn over through processes involving binding and dissociation that are hypothesized to be mechanisms to maintain the entire architecture of stereocilia but are not well understood, especially those that balance polymerization and depolymerization of the F-actin and replace damaged components in stereocilia F-actin cores

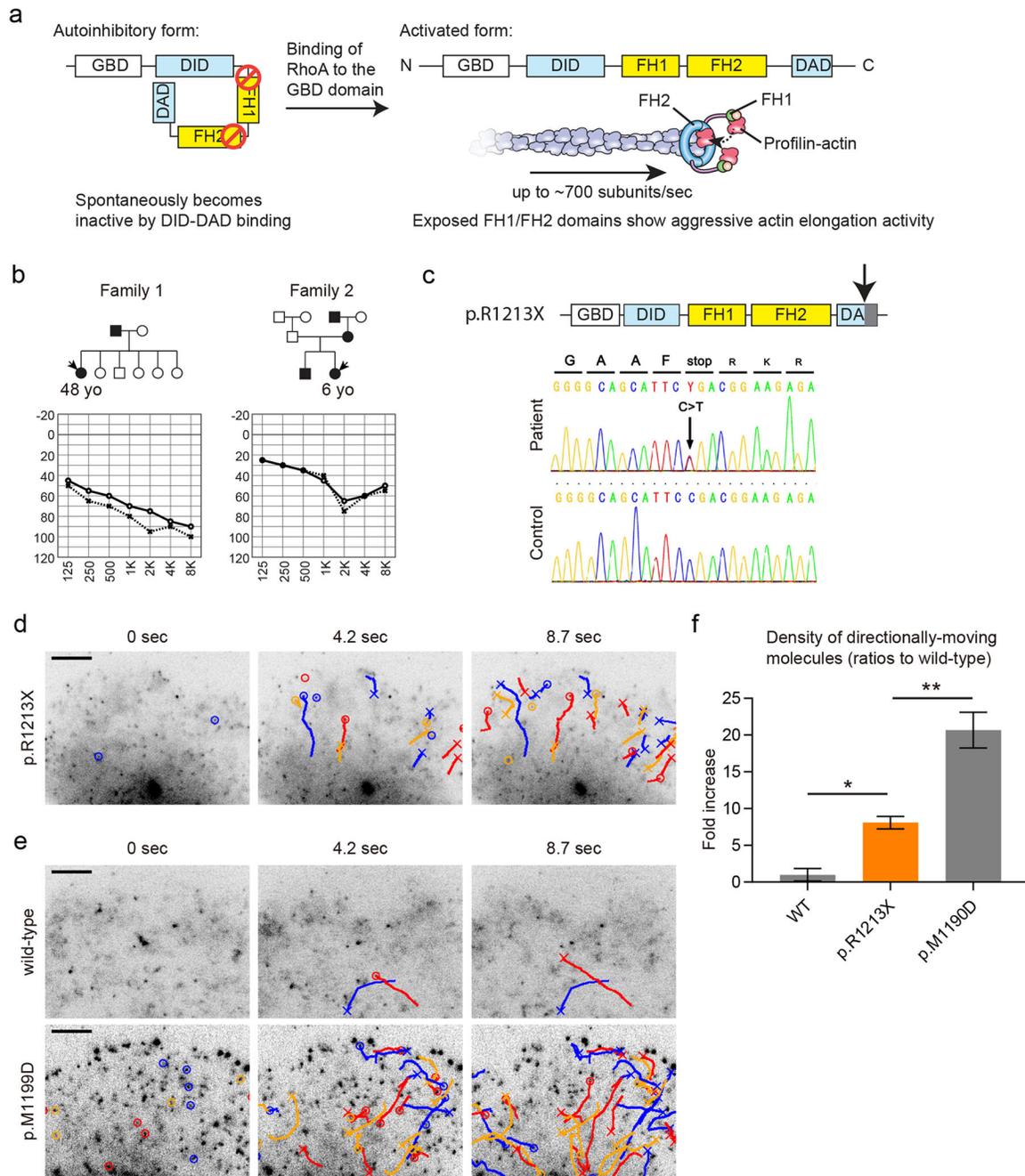
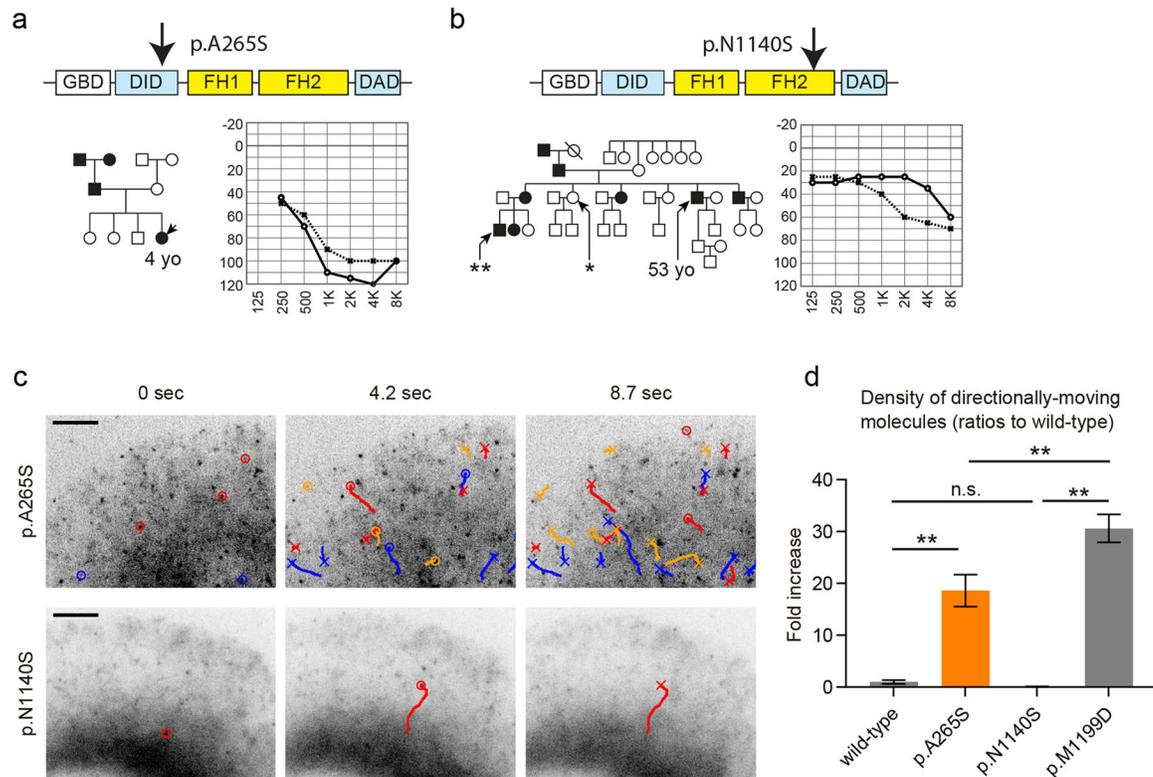


Fig. 3. Functional analysis of the DIAPH1 (p.R1213X) variant using single-molecule fluorescence microscopy. **a** Autoinhibitory mechanism regulating DIAPH1 actin elongation activity. The DID and DAD domains spontaneously interact with each other. Binding of RhoA to the GBD domain of DIAPH1 releases inhibition and allows for aggressive actin elongation activity by the FH1-FH2 domains of DIAPH1. The FH1 domain recruits profilin-actin while the FH2 domain holds onto the barbed end of F-actin to cause processive and high-speed actin elongation, which reaches an average speed of ~ 700 actin subunits/sec. **b** Two Japanese families segregating hearing loss analyzed to identify the *DIAPH1* (p.R1213X)

variant. Audiograms of probands are also shown. **c** Diagrams and Sanger sequencing results of *DIAPH1* (p.R1213X) variant found in families shown in **(b)**. The p.R1213X variant truncates the C-terminus of the DAD domain (downward pointing arrow). The DNA sequence is from the proband in Family 1. **d** Detection of abnormal, constitutive activation of DIAPH1 (p.R1213X) using single-molecule fluorescence microscopy. Time-lapse images of *Xenopus* XTC cells expressing GFP-DIAPH1 (p.R1213X) are shown. GFP-DIAPH1 (p.R1213X) molecules show frequent directional movements driven by actin elongation activity of FH1-FH2 domains, which were abnormally exposed by the disrupted DID-DAD interaction. Molecules moving directionally for more than two frames are indicated by circles and trajectories. Crosses indicate disappearances. Bar, 5 μ m. **e** Time-lapse images of negative and positive controls, wild-type GFP-DIAPH1 and GFP-DIAPH1 (p.M1199D). Only a few molecules show directional movements in XTC cells expressing wild-type GFP-DIAPH1. Moving molecules were frequently observed in XTC cells expressing GFP-DIAPH1 (p.M1199D), which is a variant lacking the DID-DAD autoinhibitory interaction (Lammers et al. 2005). Bar, 5 μ m. **f** Semi-quantitative comparison of directionally moving molecules showing moderate activation of DIAPH1 (p.R1213X). The densities of moving molecules were determined and normalized to the expression levels of GFP-DIAPH1 as described in our previous study (Ueyama et al. 2016). Directional movements of DIAPH1 (p.R1213X) were significantly more frequent than wild-type DIAPH1 and less frequent than DIAPH1 (p.M1199D). One-way ANOVA showed $p < 0.0001$. Post hoc Tukey's multiple comparison is indicated by asterisk ($p < 0.05$) and double asterisks ($p < 0.01$). Number of replicates: $n = 5$ for wild-type, $n = 6$ for p.R1213X and p.M1199D. Error bars, SEMs

**Fig. 4.**

Pathogenicity screening of DIAPH1 variants using single-molecule fluorescence microscopy. **a** A Korean family segregating hearing loss. The p.A265S variant was found in this family and considered to be pathogenic using single-molecule fluorescence microscopy analyses in (c). An audiogram of the proband is shown. **b** A Japanese family segregating hearing loss. The p.N1140S variant was found in the proband of this family and predicted to be a benign polymorphism based upon our single-molecule fluorescence microscopy assay in (c). The p.N1140S variant was subsequently found in a female with normal hearing (asterisk), and another male with hearing loss in this family did not have the p.N1140S variant (double asterisks). An audiogram of the proband is shown. **c** Functional analyses of the p.A265S and p.N1140S variants using single-molecule fluorescence microscopy and *Xenopus* XTC cells expressing GFP-fused variants. GFP-DIAPH1 (p.A265S) showed frequent directional movements similar to p.R1213X indicating the pathogenicity of p.A265S variant. In contrast, the p.N1140S variant is likely a benign polymorphism since GFP-DIAPH1 (p.N1140S) did not show frequent directional movements. The movement distances were also similar to wild-type DIAPH1 despite the amino-acid substitution in the FH2 domain. Indications of markers are similar to Fig. 3d. Bar, 5 μ m. **d** Semi-quantitative comparison of directionally moving molecules showing the constitutive activation of DIAPH1 (p.A265S) and the intact autoinhibition of DIAPH1 (p.N1140S). The densities of moving molecules were normalized by the expression levels as described in our previous study (Ueyama et al. 2016). Directional movements of DIAPH1 (p.A265S) were significantly more frequent than wild-type DIAPH1 and less frequent than DIAPH1 (p.M1199D) while DIAPH1 (p.N1140S) did not show an increase of moving molecules.

New XTC cells were prepared to obtain data for the wild-type and p.M1199D in this graph. One-way ANOVA showed $p < 0.0001$. Post-hoc Tukey's multiple comparison is indicated by double asterisks ($p < 0.01$). Number of replicates: $n = 5$ for p.N1140S, $n = 6$ for wild-type, p.A265S and p.M1199D. Error bars, SEMs

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Table 1
Genes encoding actin and actin-associated proteins in stereocilia necessary for human hearing

Genes	Locus	Encoded protein	Known function(s) in stereocilia
<i>DIAPH1</i>	<i>DFNA1</i>	Protein diaphanous homolog 1	Mediates processive actin elongation activity (constitutively active <i>DIAPH1</i> is pathogenic)
<i>MYO7A</i>	<i>DFNA11, DFNB2, USH1B</i>	Unconventional myosin 7A	Transports and assembles upper tip-link complex, tip-link tensing
<i>ACTG1</i>	<i>DFNA20/26*</i>	Actin, cytoplasmic 2 (γ -cytoplasmic actin)	Assembles F-actin cores, repairs gaps in filaments
<i>MYO6</i>	<i>DFNA22, DFNB37</i>	Unconventional myosin 6	Transports PTPRQ and associated proteins to stereocilia bases (toward <i>pointed</i> ends of F-actin)
<i>PTPRQ</i>	<i>DFNA73, DFNB84</i>	Phosphatidylinositol phosphatase (PTPRQ)	Forms ankle-links
<i>PLS1</i>	<i>DFNA**</i>	Plastin-1	Crosslinks F-actin in stereocilia cores
<i>MYO15A</i>	<i>DFNB3</i>	Unconventional myosin 15A	Transports the components of elongation complex to stereocilia tips, such as <i>WHRN</i> and <i>EPS8</i>
<i>CDH23</i>	<i>DFNB12, USH1D</i>	Cadherin 23	Composes tip-links with Protocadherin-15
<i>USH1C</i>	<i>DFNB18, USH1C</i>	Harmonin	Scaffolds upper tip-link complex
<i>PCDH15</i>	<i>DFNB23, USH1F</i>	Protocadherin-15	Composes tip-links with Cadherin 23
<i>RDX</i>	<i>DFNB24</i>	Radixin	Links between plasma membrane and F-actin cores
<i>GRXCR1</i>	<i>DFNB25</i>	Glutaredoxin domain-containing cysteine-rich protein 1 (GRXCR1)	Crosslinks F-actin in stereocilia cores
<i>TRIOBP</i>	<i>DFNB28</i>	TRIO and F-actin-binding protein (TRIOBP)	Crosslinks F-actin and forms rootlets (isoforms 4 and 5)
<i>MYO3A</i>	<i>DFNB30, DFNA**</i>	Unconventional myosin 3A	Recruits ESPN isoform 1 (involved in arrangement of stereocilia)
<i>WHRN</i>	<i>DFNB31, USH2D</i>	Whirlin	Forms an elongation complex at the tips with <i>EPS8</i> , involved in forming the staircase of stereocilia
<i>ESPN</i>	<i>DFNB36</i>	Espin	Crosslinks F-actin in stereocilia cores
<i>PDZD7</i>	<i>DFNB57</i>	PDZD7	Scaffolds ankle-link complex
<i>TPRN</i>	<i>DFNB79</i>	Taperin	Protein at the stereocilia taper
<i>GRXCR2</i>	<i>DFNB101</i>	Glutaredoxin domain-containing cysteine-rich protein 2 (GRXCR2)	Crosslinks F-actin in stereocilia cores
<i>EPS8</i>	<i>DFNB102</i>	Epidermal growth factor receptor kinase substrate 8 (EPS8)	Barbed-end capper, involved in forming the staircase of stereocilia
<i>EPS8L2</i>	<i>DFNB106</i>	Epidermal growth factor receptor kinase substrate 8-like protein 2 (EPS8L2)	Barbed-end capper, regulating stereocilia staircase architecture
<i>USH1G</i>	<i>USH1G</i>	Usher syndrome type-1G protein (SANS)	Scaffolds upper tip-link complex
<i>ACTB</i>	N/A*	Actin, cytoplasmic 1 (β -cytoplasmic actin)	Assembles F-actin cores

* Gain-of-function is associated with Baraitser-Winter cerebrotendofacial (BWCFF) syndrome accompanying sensorineural hearing loss in 30–80% of patients (Riviere et al. 2012; Verloes et al. 2015; Yates et al. 2017)

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