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Molecular Structure of the Hair Cell Mechanoelectrical Transduction Complex

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Cochlear hair cells employ mechanically gated ion channels located in stereocilia that open in response to sound wave–induced motion of the basilar membrane, converting mechanical stimulation to graded changes in hair cell membrane potential. Membrane potential changes in hair cells cause neurotransmitter release from hair cells that initiate electrical signals in the nerve terminals of afferent fibers from spiral ganglion neurons. These signals are then propagated within the central nervous system (CNS) to mediate the sensation of hearing. Recent studies show that the mechanoelectrical transduction (MET) machinery of hair cells is formed by an ensemble of proteins. Candidate components forming the MET channel have been identified, but none alone fulfills all criteria necessary to define them as pore-forming subunits of the MET channel. We will review here recent findings on the identification and function of proteins that are components of the MET machinery in hair cells and consider remaining open questions.

Mechanosensory hair cells of the mammalian cochlea are bestowed with unique structural specializations that allow them to convert sound wave–induced vibrations into electrical responses that are relayed to the central nervous system (CNS) with astonishing precision and fidelity. Each hair cell contains at its apical surface a bundle of 50–150 actin-rich stereocilia that are arranged in a staircase pattern with multiple rows of ascending heights (Fig. 1). Physical deflection of the hair bundle toward the tallest row of stereocilia leads to a depolarization of the hair cell (Gillespie and Muller 2009; Peng et al. 2011; Fettiplace and Kim 2014). Critical for mechanoelectrical transduction (MET) are the tip-link filaments that connect the stereocilia of a hair bundle along the axis of its mechanical sensitivity (Pickles et al. 1984; Assad et al. 1991; Basu et al. 2016). The tip link consists of two homodimers, one composed of two cadherin 23 (CDH23) molecules and one of two protocadherin 15 (PCDH15) molecules, which associate in *trans* at their respective amino termini to connect a shorter stereocilium to its next tallest neighbor (Siemens et al. 2004; Sollner et al. 2004; Ahmed et al. 2006; Kazmierczak et al. 2007). The tip link conveys tension force to the MET channel thus leading to channel opening and an inward flow of Ca^{2+} and K^{+} ions (Fig. 1) (Fettiplace and Kim 2014).

Hair cells in the mammalian cochlea are arranged in one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) that run along the length of the cochlear duct. Hair cells in the cochlea show a tonotopic organization where the highest frequency tones activate hair

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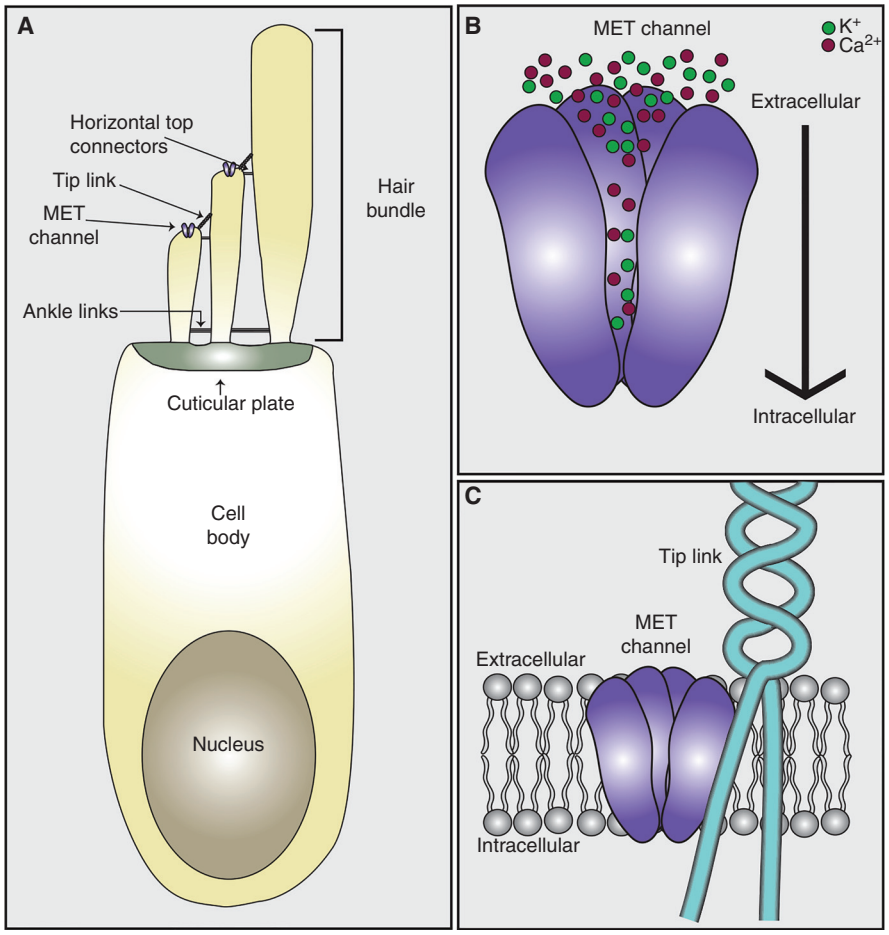

Figure 1. Cochlear hair cell anatomy. (*A*) Hair cells contain actin-rich stereocilia embedded in the cuticular plate at their apical surface. Stereocilia are interconnected via extracellular linkages including tip links, ankle links, and horizontal top connectors. The mechanoelectrical transduction (MET) channel that mediates auditory processing is located at the tips of shorter stereocilia at the lower end of tip links. (*B*) The MET channel pore is nonselective for cations with ion fluxes in hair cells composed predominantly of Ca^{2+} and K^+. Upon normal stimulation, ions flow from the extracellular space containing endolymph into the hair cell. (*C*) The lower end of the tip link, composed of protocadherin 15 (PCDH15), is inserted into the membrane at the tip of the stereocilia in close proximity to the MET channel pore. It is currently unknown whether the tip link binds directly to the channel pore, is connected via linker protein to the pore, or transmits force to the channel pore by impacting the local lipid environment.

cells at the base of the cochlea, the lowest tones hair cells at the apex, with a gradient in between. OHCs amplify input sound signals, whereas IHCs connect to afferent neurons and transmit sound information to the CNS (Dallos 2008; Schwander et al. 2010; Appler and Goodrich 2011; Fettiplace 2017). In rat cochlear hair cells, the conventional sensory MET channel that is critical for auditory perception is localized near the lower end of tip links in the two shorter rows of stereocilia (Beurg et al. 2009). Each hair cell has relatively few mechanotransduction channels, perhaps one to two per stereocilium (Ricci et al. 2003; Beurg et al. 2009), which show a tonotopic gradient in single-channel (unitary) conductance with increasing conductance from apex to base. This tonotopy in conductance is specific for OHCs and not IHCs (He et al. 2004;

Beurg et al. 2006; Jia et al. 2007). There is also a gradient in Ca^{2+} permeability (decrease from apex to base) in OHCs, but apparently not in IHCs (Beurg et al. 2006; Kim and Fettiplace 2013).

MOLECULES THAT ARE CRITICAL FOR MECHANOTRANSDUCTION

The search for the molecules that form the sensory MET channel in mammalian hair cells and regulate its function has been going on for decades. Many candidates have been examined, but almost all failed most of the criteria defining a bona fide MET channel. Some compelling candidates that have been ruled out are TRPN1/NOMPC (Sidi et al. 2003; Shin et al. 2005), TRPV4/VR-OAC (Liedtke et al. 2000), TRPA1 (Corey et al. 2004; Kwan et al. 2006), TRPV6, TRPM6, TRPM7 (Morgan et al. 2017), HCN1 (Ramakrishnan et al. 2009; Horwitz et al. 2010), TRPML3 (Grimm et al. 2007; Nagata et al. 2008; van Aken et al. 2008; Jors et al. 2010), PKD1 (Steigelman et al. 2011; Wu et al. 2016), PKD2 (Fettiplace 2009; Wu et al. 2016), TRPC3/TRPC6 (Quick et al. 2012; Wu et al. 2016), and TRPM1 (Gerka-Stuyt et al. 2013; Wu et al. 2016). However, recent studies based on the analysis of the function of genes linked to hearing loss have finally identified proteins that are integral components of the MET complex of hair cells and regulate its assembly and function. Some of these proteins are plausible candidates for the pore-forming subunits of the MET channel, whereas others affect MET via other mechanisms. We will review here current knowledge of the function of proteins that are intricately linked to MET in hair cells.

Criteria for a Mechanosensitive Channel

There are four established criteria for a candidate protein to be confirmed as a pore-forming subunit of a MET channel (Christensen and Corey 2007; Arnadottir and Chalfie 2010; Yan et al. 2013; Ranade et al. 2015). Until recently, no mammalian channels met all requirements. This changed with the discovery of the PIEZO1/2 proteins, which are bona fide mammalian MET channels with important functions in many cell types (Coste et al. 2010, 2012, 2015). Although PIEZO2 is expressed in hair cells, it is not essential for auditory function (Wu et al. 2017) and instead carries the "reverse-polarity current" or "anomalous mechanosensitive current" that can be observed in early developing hair cells, following damage to more mature hair cells, and in hair cells from mice with mutations in several genes associated with MET function (Stepanyan and Frolenkov 2009; Alagramam et al. 2011; Kim et al. 2013; Zhao et al. 2014; Beurg et al. 2016; Wu et al. 2017). The precise function of the reverse-polarity channel is not well defined and will not be considered here.

The four established criteria for a MET channel are as follows:

1. The protein must be expressed in the proper time and place. The MET response in murine cochlear hair cells begins around postnatal day 0 (P0) in the base, and then follows in the apex 2–3 days later (Waguespack et al. 2007; Lelli et al. 2009). Thus, candidate proteins must be expressed before or concurrent with the emergence of the MET response and maintained into adulthood. Furthermore, the MET channel is localized near the lower end of tip links in proximity to the tip-link component PCDH15 (Kazmierczak et al. 2007; Beurg et al. 2009). Thus, for any protein to be considered a candidate for the MET channel, it must be expressed by P0 and localize near the lower end of tip links.
2. The protein must be required for mechanosensitivity in hair cells. Disruption of the function of the protein must affect MET in hair cells. Importantly, knockdown and knockout data need critical evaluation. Knockdown using morpholinos or short hairpin RNAs (shRNAs) has the potential for off-target artifacts and has led to incorrect conclusions regarding the mechanotransduction channel in hair cells (Corey et al. 2004; Kwan et al. 2006). Knockouts address essential functions of a protein but genetic compensation by a homolog is possible.
3. Mutations in the protein cause changes to the properties of the mechanical response. Mutations of the protein should change the prop-

erties of the MET response. For example, mutations to critical residues in PIEZO proteins lead to changes in ion selectivity and conductance (Coste et al. 2015; Zhao et al. 2016). One needs to carefully examine the impact of all mutations and verify that the mutant protein is normally expressed and localized. Notably, trafficking/accessory/modifying functions can change properties of currents, and changes in ion selectivity and conductance are not always caused by changes to the pore. Accessory proteins (e.g., MinK modulates Kv7.1 [McCrossan and Abbott 2004]; STIM1 modulates CRAC1 [McNally et al. 2012]) and even the local lipid environment (e.g., phosphoinositol-4-5-bisphosphate [PIP_2] modulates MET channel properties [Borbiro et al. 2015; Effertz et al. 2017]) can impact these seemingly inherent pore properties.

4. Heterologous or ectopic expression leads to mechanical sensitivity in otherwise insensitive cells or lipid bilayers. This criterion is most critical in demonstrating that a protein is a pore-forming subunit of a mechanically sensitive ion channel. For example, PIEZO proteins confer mechanical properties upon otherwise mechanically insensitive cells, and reconstituted PIEZO proteins demonstrate channel activity in lipid bilayers (Coste et al. 2010, 2012). Recreating the correct stimulus that activates the hair cell MET channel may be challenging, as is properly expressing the pore-forming protein at the surface of cells without the full complement of trafficking partners, auxiliary subunits, and/or chaperone proteins.

In the following, we will critically evaluate the extent to which membrane proteins that have been linked to the MET channel in hair cells, namely, lipoma high mobility group IC fusion partner-like 5 (LHFPL5)/tetraspan membrane protein of hair cell stereocilia (TMHS), transmembrane inner ear (TMIE), and transmembrane channel-like proteins 1 and 2 (TMC1/2), fulfill these criteria before discussing the function of proteins without transmembrane domains (CIB2, TOMT) and lipids (PIP_2), which are also critical for MET.

Candidates for the Pore-Forming Subunit of the Mechanotransduction Channel

LHFPL5/TMHS

LHFPL5, also known as TMHS, is a protein with four predicted transmembrane domains. Mutations in LHFPL5 cause deafness in humans and mice (Longo-Guess et al. 2005; Kalay et al. 2006; Shabbir et al. 2006). LHFPL5 is a member of the tetraspan junctional complex superfamily, which includes the transmembrane α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) regulatory proteins (TARPs)—auxiliary channel subunits of glutamate receptors (Jackson and Nicoll 2011). LHFPL5 is part of a subfamily of six proteins collectively known as the LHFPL tetraspans (LHFPL1-6). LHFPL3 and LHFPL4 contribute to synaptic receptor targeting and clustering (Davenport et al. 2017; Yamasaki et al. 2017). Notably, both accessory proteins like TARPs/CACNG2/stargazin (Letts et al. 1998; Twomey et al. 2016) and pore-forming ion channel subunits like CRAC/ORAI1 (Feske et al. 2006; Prakriya et al. 2006; Vig et al. 2006) and LRRC8a/SWELL (Qiu et al. 2014; Syeda et al. 2016) have a four-transmembrane topology similar to LHFPL5 (Fig. 2).

Expressed at right place and time? *Lhfpl5* messenger RNA (mRNA) is expressed in the mouse cochlea concurrent with the emergence of MET currents, and expression continues through adulthood (Xiong et al. 2012; Scheffer et al. 2015). LHFPL5 protein is localized in hair cells throughout stereocilia during the first postnatal week, with some protein concentrated at the tip-link region (Xiong et al. 2012; Mahendrasingam et al. 2017). Exogenously expressed LHFPL5 (HA-LHFPL5) also localizes to the tips of stereocilia (Xiong et al. 2012). With immunogold electron microscopy (EM) (Mahendrasingam et al. 2017), LHFPL5 protein is detected throughout developing stereocilia, but is concentrated in mature hair cells near the lower end of tip links where the MET channel is located. Consistent with the localization data, LHFPL5 binds in vitro to the tip-link component PCDH15 and to TMIE but binding to TMC1/2 has so far not been demonstrated (Xiong et al. 2012; Zhao et al. 2014; Beurg

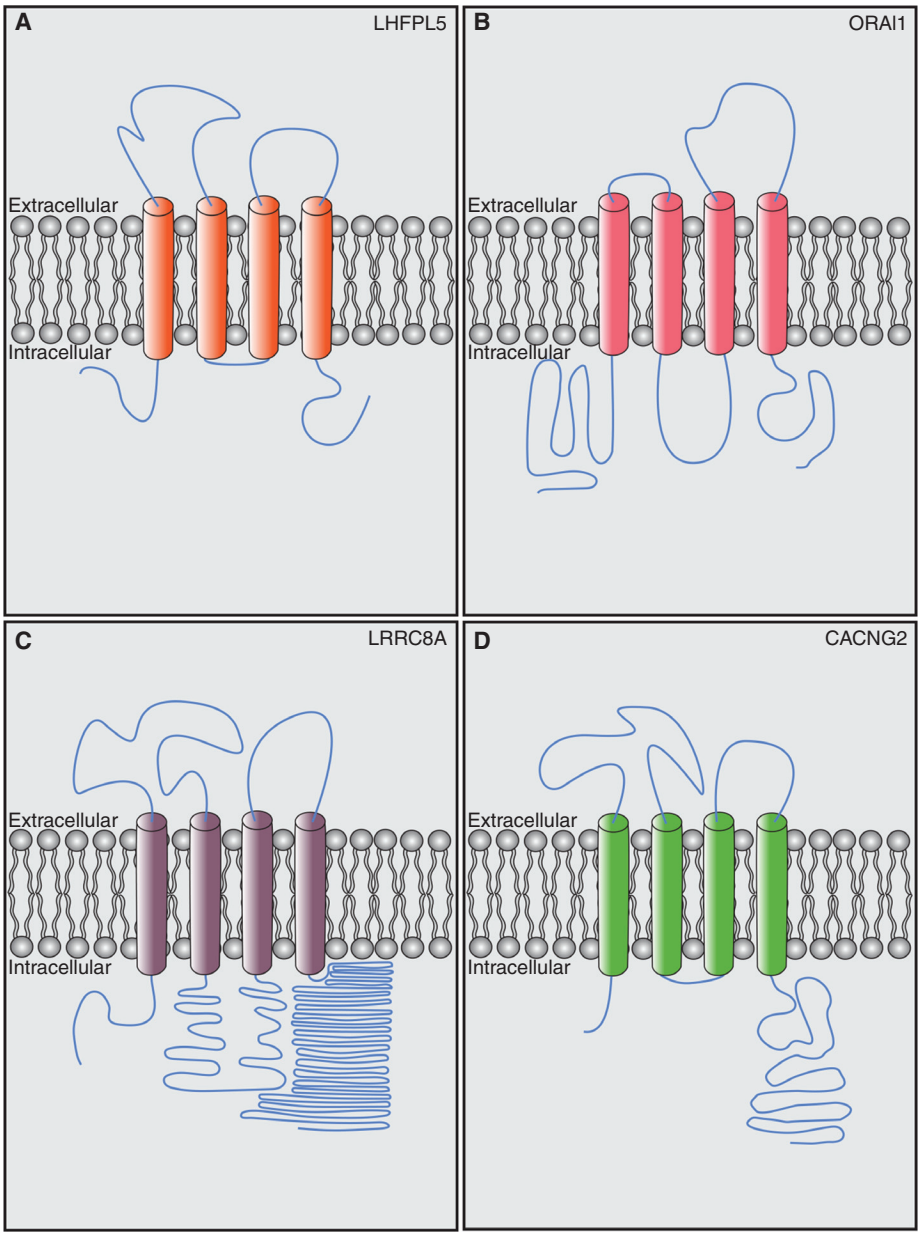

Figure 2. Proteins with predicted membrane topology similar to lipoma high mobility group IC fusion partner-like 5 (LHFPL5). (*A*) LHFPL5/tetraspan membrane protein of hair cell stereocilia (TMHS) contains four predicted transmembrane domains with cytoplasmic amino- and carboxy-terminal domains. (*B*) ORAI1 (Prakriya et al. 2006), the pore-forming domain of the calcium-release activated calcium channel (CRAC), has a similar topology to LHFPL5. (*C*) LRRC8A (Qiu et al. 2014), the pore-forming subunit of the volume-regulated anion channel (VRAC), contains four transmembrane domains with cytoplasmic amino- and carboxy-terminal domains. (*D*) CACNG2/Stargazin (Twomey et al. 2016) is an accessory subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) receptors with similar topology to LHFPL5. Lengths of domains are to relative scale. Diagrams are depicted as extracellular and intracellular at the plasma membrane, but for endoplasmic reticulum (ER) proteins, "extracellular" = ER lumen. (Figures generated based on predicted topologies from Uniprot.)

et al. 2015b). LHFPL5 expression is higher overall in hair cells located in the low-frequency apex of the cochlea relative to the high-frequency base, but there are similar numbers of LHFPL5 puncta per stereocilial tip along the cochlear spiral (Mahendrasingam et al. 2017).

Required for mechanosensitivity? Macroscopic MET currents are decreased by ~90% in LHFPL5-deficient hair cells relative to wild-type (Xiong et al. 2012). Reductions in macroscopic currents are in part caused by defects in the assembly of tip links because the number of tip links are significantly reduced in LHPFL5-deficient hair cells (Xiong et al. 2012). However, the unitary conductance of MET channels in stereocilia maintaining tip links is also decreased, channel activation is slowed, and fast adaptation is impaired (Xiong et al. 2012; Beurg et al. 2015b), suggesting that LHFPL5 is intimately linked to the channel pore. The fact that current is still detected in the absence of LHFPL5 could mean that LHFPL5 does not contribute to the channel pore and rather acts like a TARP for the MET channel (Xiong et al. 2012). However, other proteins, perhaps from the LHFPL family, could partially compensate for LHFPL5 loss.

The normal tonotopic gradient in the conductance of the transducer channel is drastically reduced in *Lhfpl5* mutants (Beurg et al. 2015b), suggesting that LHFPL5 is a component of the channel pore or regulates localization/function of a pore protein. Differences in LHFPL5 expression could underlie the tonotopic gradient in conductance that occurs along the cochlear spiral consistent with a possible tonotopic gradient in LHFPL5 levels (Mahendrasingam et al. 2017). Importantly, TMC1 is no longer present in the stereocilia of *Lhfpl5*$^{-/-}$ mutant mice, but LHFPL5 is still present in *Tmc1*$^{dn/dn}$*Tmc2*$^{-/-}$ mice (Beurg et al. 2015b). Thus, LHFPL5 may contribute to hair cell mechanosensitivity by regulating proper localization of TMC1. Adding weight to this hypothesis is the fact that the tonotopic gradient of MET conductance is similarly impacted in *Tmc1*$^{-/-}$ as in *Lhfpl5*$^{-/-}$ mice. The putative effect of LHFPL5 on TMC1 may be indirect, as coimmunoprecipitation experiments have so far not shown clear evidence for interaction in vitro (Beurg et al. 2015b). Curiously, TMC2 localization appears not to be impacted by loss of LHFPL5, suggesting that TMC1 has unique trafficking and localization properties relative to TMC2 (Beurg et al. 2015b).

Do mutations cause changes to mechanotransduction response? Mutations in *Lhfpl5* that do not impact localization to stereocilia have not been identified or tested. *The hurry-scurry* mutation in *Lhfpl5* (Longo-Guess et al. 2005; Xiong et al. 2012), which causes deafness and significant loss of MET currents, appears to affect trafficking of LHFPL5 in heterologous cells and presumably hair cells, which precludes its utility in providing insights into the role of LHFPL5 in the MET response. Other mutations linked to human deafness (Shabbir et al. 2006) do not affect binding to PCDH15 (Xiong et al. 2012), but their cell surface localization has yet to be tested.

Does heterologous or ectopic expression confer mechanosensitivity? Efficient cell-surface expression of LHFPL5 in heterologous cells is dependent upon coexpression with PCDH15 (Xiong et al. 2012). When LHFPL5 and PCDH15 are expressed in heterologous cells, mechanically evoked responses have not been successfully evoked, suggesting that LHFPL5 alone does not form a channel (Zhao et al. 2014). LHFPL5 is still present in the stereocilia of *Tmc1/2* dKO mice, which suggests that LHFPL5 alone is not sufficient for the MET response in the absence of TMC1/2 (Beurg et al. 2015b).

Summary. The current working hypothesis is that LHFPL5 acts like a TARP to allosterically regulate the properties of the pore-forming subunit of the MET channel and helps in the assembly of the channel complex by regulating the transport of PCDH15 and TMC1. However, it cannot be excluded that LHFPL5 contributes directly to the pore, possibly in combination with TMC1/2 and TMIE or with yet-to-be-identified proteins.

TMIE

TMIE causes deafness in humans, mice, and zebrafish (Mitchem et al. 2002; Naz et al. 2002; Chung et al. 2007; Gleason et al. 2009). TMIE

contains two predicted transmembrane domains with cytoplasmic amino and carboxyl termini, although it has a predicted signal sequence at the amino terminus that if cleaved would leave a single transmembrane domain with an extracellular amino terminus and intracellular carboxyl terminus (Fig. 3). It is unclear which form of the protein predominates in vivo.

Expressed at right place and time? *Tmie* mRNA is expressed in the mouse cochlea (Mitchem et al. 2002; Scheffer et al. 2015), and X-gal staining for LacZ expressed from the endogenous *Tmie* locus labels only hair cells in the cochlea (Zhao et al. 2014). Immunolocalization of TMIE with antibodies showed TMIE localized to stereocilia, with a concentration at the tips of shorter stereocilia, beginning at early postnatal stages (Zhao et al. 2014). Epitope-tagged TMIE, expressed in hair cells by injectoporation, is localized to stereocilia of hair cells as well (Zhao et al. 2014). Consistent with the localization data, TMIE interacts in vitro directly with one of three major PCDH15 isoforms (PCDH15-CD2) that are generated by alternative splicing. It binds to two additional major splice variants (PCDH15-CD1, PCDH15-CD3) when in a ternary complex with LHFPL5 (Zhao et al. 2014). The temporal dynamics of TMIE expression need to be further investigated, but it seems true that TMIE is expressed in the proper time and place to contribute to MET function.

Required for mechanosensitivity? In zebrafish, mutations in *Tmie* cause defects in microphonic recordings indicative of MET defects (Gleason et al. 2009). MET currents are completely abolished in *Tmie*-null mice (Zhao et al. 2014). The MET currents cannot be rescued by exogenous expression of LHFPL5 or TMC1/2 but can be rescued by acute expression of TMIE. The morphologies of stereocilia of $Tmie^{-/-}$ mice during the time periods when MET currents were assayed are relatively normal, with no apparent loss of tip links. Interestingly, localization of LHFPL5, PCDH15-CD2, and TMC2 proteins appear normal in $Tmie^{-/-}$ mice, suggesting that TMIE is not primarily responsible for the transport of the transduction complex, and further implicate an integral role for TMIE in the MET channel complex (Zhao et al. 2014).

Do mutations cause changes to mechanotransduction response? The *Tmie* mutation in spinner j (sr^j) mice is predicted to cause a premature truncation of TMIE within the carboxy-terminal cytoplasmic domain and causes profound deafness (Mitchem et al. 2002). However, it is not known whether the truncated protein is expressed and localized properly in hair cells. Several mutations in TMIE are associated with deafness in humans (Naz et al. 2002). These mutations are located in the carboxy-terminal cytoplasmic tail of TMIE and affect binding to PCDH15 (Zhao et al. 2014). Full-length TMIE can completely rescue MET currents in $Tmie^{-/-}$ hair cells, whereas some of the mutants linked to deafness localize to stereocilia but rescue transduction only partially (Zhao et al. 2014). Additional mutational analysis is necessary to further define the function of TMIE in hair cells.

Does heterologous or ectopic expression confer mechanosensitivity? Exogenous TMIE localizes to the surface of heterologous cells. However, mechanically activated currents could not be evoked with TMIE alone, or with TMIE in the presence of either LHFPL5, TMC1, TMC2, or PCDH15 (Zhao et al. 2014). Zhao et al. (2014) were able to measure PIEZO1-mediated currents in the same heterologous cells used to study MET channel components, suggesting that (1) the MET channel requires a different stimulus for activation, (2) one of the components is not localized properly (e.g., TMC1/2), or (3) the channel is not assembled properly because another factor is missing. Investigators employ stimuli including sheer stress, membrane stretch, suction stress, and mechanical indentation to stimulate mechanically sensitive channels (Ranade et al. 2015), but the most effective stimulus for the MET channel is currently unknown. Basu et al. (2016) showed that tension on the MET channel (via pulling directly on the tip link) is sufficient to open the MET channel in the absence of hair bundle deflection. This suggests that if the channel is assembled and expressed properly, an appropriate mechanical stimulus in heterologous cells should be able to open the channel.

Summary. TMIE localizes to the stereocilia, is essential for MET, and deafness-associated

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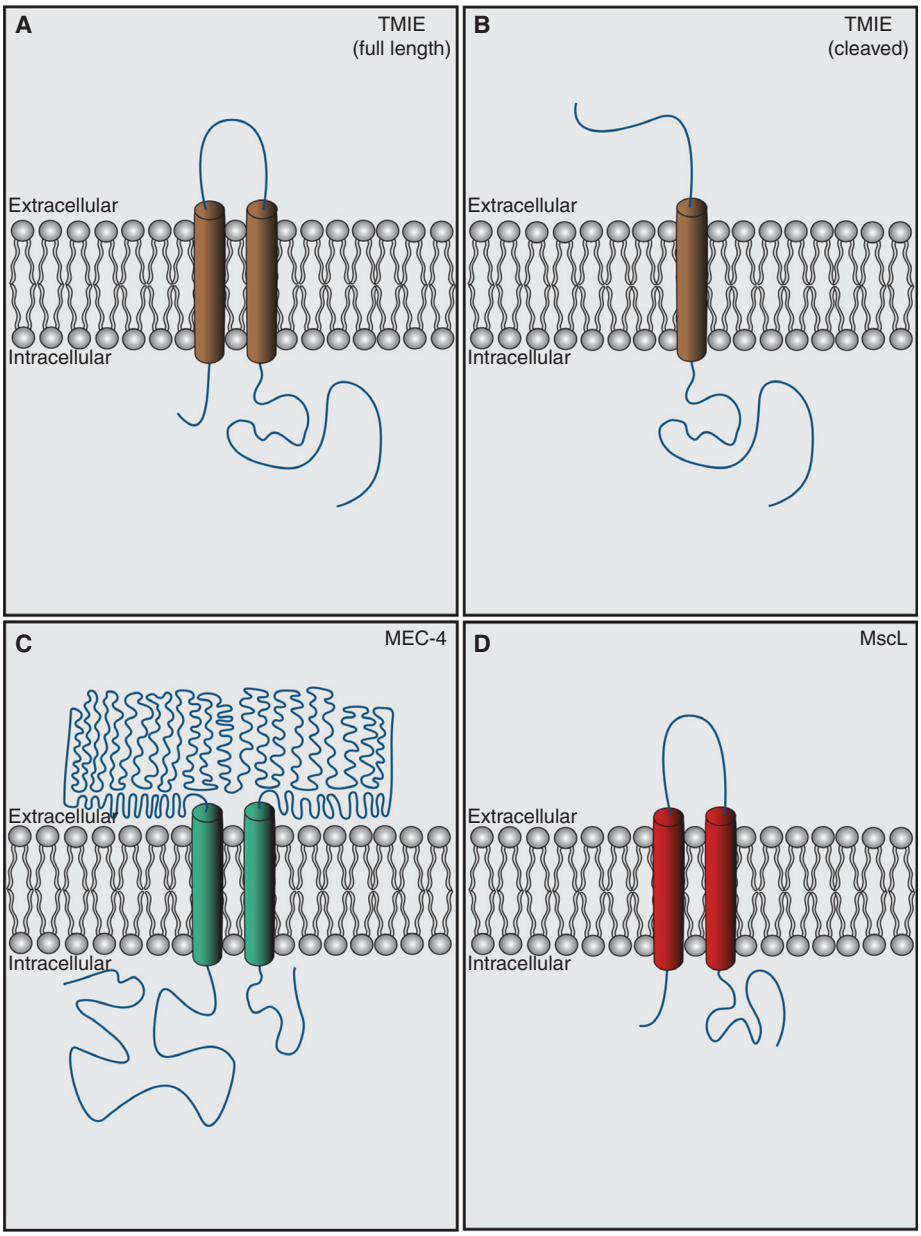

Figure 3. Proteins with predicted membrane topology similar to transmembrane inner ear (TMIE). (*A*) Full-length TMIE (Zhao et al. 2014) contains two transmembrane domains with intracellular amino and carboxyl termini. (*B*) After cleavage of a predicted amino-terminal signal sequence, TMIE contains a single transmembrane domain with an extracellular amino terminus and an intracellular carboxyl terminus. It is unknown which version of TMIE predominates in vivo, or whether both full-length and cleaved versions are present. (*C*) MEC-4 (Lai et al. 1996), the pore-forming subunit of the *Caenorhabditis elegans* light-touch mechanosensory channel, contains two transmembrane domains with cytoplasmic amino- and carboxy-terminal domains similar to full-length TMIE. (*D*) MscL (Chang et al. 1998), a mechanosensory channel expressed in prokaryotes has similar topology to full-length TMIE. Lengths of domains are to relative scale. Diagrams are depicted as extracellular and intracellular at the plasma membrane, but for endoplasmic reticulum (ER) proteins, "extracellular" = ER lumen. (Figures generated based on predicted topologies from Uniprot.)

mutations in TMIE can affect conductance through the channel. Localizations of other known MET complex components are not affected in *Tmie*$^{-/-}$ animals, yet MET currents are abolished. Interestingly, the predicted topology of TMIE is similar to known mechanosensitive channel subunits, including *Caenorhabditis elegans* MEC-4 (ENac/DEG channel pore subunit [Lai et al. 1996; O'Hagan et al. 2005]) and MscL (the bacterial mechanosensitive channel [Chang et al. 1998]), both of which are composed of two transmembrane domains and oligomerize to form a channel pore (Fig. 3). Alternatively, TMIE, which binds PCDH15 and LHFPL5 in vitro, could link the lower end of the tip link to the channel, or properly localize an as-yet-unknown channel pore protein to the lower end of the tip link.

TMC1/2

TMC1/2 are multipass transmembrane proteins. *TMC1* but not *TMC2* has been associated with dominant and recessive forms of deafness (Kurima et al. 2002; Vreugde et al. 2002; Kawashima et al. 2011, 2015). *TMC1/2* are members of a family of eight genes (*TMC1-8*) that are grouped into three smaller subfamilies based on sequence similarity (Keresztes et al. 2003). TMC orthologs have been reported in *Drosophila melanogaster* and *C. elegans* (Chatzigeorgiou et al. 2013; Zhang et al. 2015, 2016; Guo et al. 2016). TMC1/2 have cytoplasmic amino and carboxyl termini, six predicted transmembrane domains, and a large intracellular cytoplasmic loop between transmembrane domains 4 and 5. This topology is similar to the α subunit of Shaker K^+ channels and TRP channels (Yool and Schwarz 1991; Vannier et al. 1998; Long et al. 2005; Labay et al. 2010), but is also similar to transporter proteins with intracellular functions like ZnT-1 (Fig. 4) (Palmiter and Huang 2004). It is still unknown whether TMC proteins are ion channels. The large intracellular domain between TM4 and 5 of TMC1 is the site of the *dn* mutation in mice, and some alternative topologies suggest that this region has two additional transmembrane domains that could act as a reentrant loop for a potential pore (Labay et al. 2010; Fettiplace 2016).

TMC proteins have a variety of functions in different species and tissues but the relationship to MET is unclear for most TMCs. TMC in *C. elegans* was initially reported to be essential for salt sensation, but this was not confirmed (Chatzigeorgiou et al. 2013; Wang et al. 2016). Research by the Garcia Laboratory suggests a developmental function for *C. elegans* TMC1 with little indication for a role in sensory transduction (Zhang et al. 2015), and Wang and colleagues (2016) have demonstrated that TMC1 in worms is critical for alkaline sensation. Intriguingly, Yue et al. (2018) linked *C. elegans* TMC proteins to egg laying and showed that manipulating TMC expression could modulate resting membrane potential via a depolarizing background leak conductance. Furthermore, expression of mammalian TMC1/2 rescued defects in worms deficient for *C. elegans* TMC proteins (Yue et al. 2018). Additional disparate functions have been attributed to the TMC ortholog in *Drosophila*. Fruit flies with mutations in *tmc* have defects in larval locomotion that could be rescued by mammalian TMC1/2 (Guo et al. 2016), and impaired food texture discrimination facilitated by a putative mechanical stimulus (Zhang et al. 2016). However, mechanically sensitive currents could not be evoked in S2 cells expressing *Drosophila* TMC (Guo et al. 2016). In humans, mutations in TMC6/EVER1 and TMC8/EVER2 are linked to epidermodysplasia verruciformis (Ramoz et al. 2002) and TMC6/8 regulate cellular zinc distribution in concert with the zinc transporter ZnT-1 (Lazarczyk et al. 2008).

Expressed at right place and time? *Tmc1* and *Tmc2* mRNA are expressed in hair cells. Quantitative polymerase chain reaction (PCR) experiments show that *Tmc2* mRNA is expressed first, starting around the onset of MET sensitivity at birth and then maintained until P10, when it stops being expressed. *Tmc1* mRNA starts to be detected around P3, with levels subsequently increasing, and is maintained into adulthood (Kawashima et al. 2011).

Using immunolocalization, TMC1 protein was detected in stereocilia and kinocilia as early as P5 (Beurg et al. 2015b). Kurima and colleagues (2015) used bacterial artificial chromo-

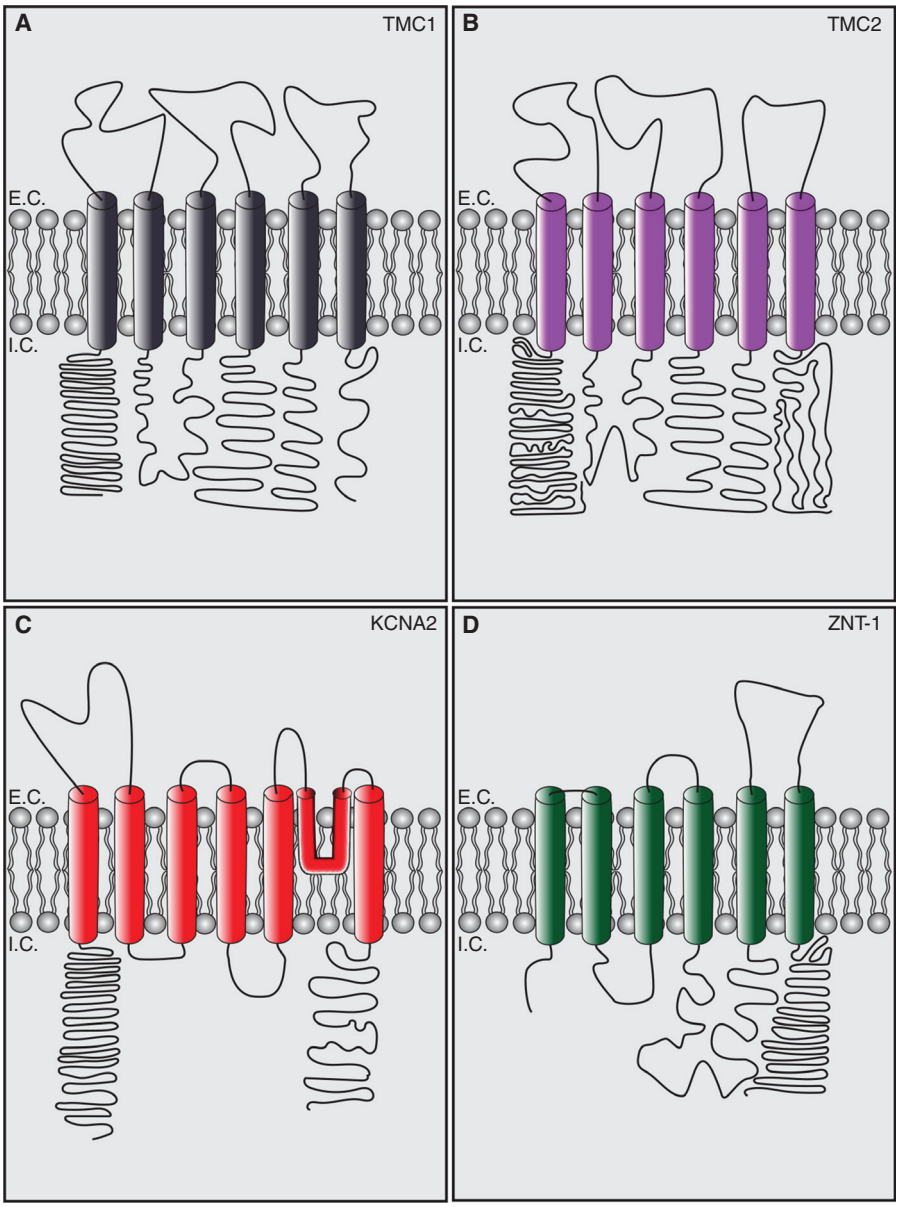

Figure 4. Proteins with predicted membrane topology similar to transmembrane channel-like proteins 1 and 2 (TMC1/2). (*A*) Full-length TMC1 (Labay et al. 2010) contains six predicted transmembrane domains with intracellular amino and carboxyl termini. (*B*) Full-length TMC2 (Kurima et al. 2003) contains six predicted transmembrane domains with intracellular amino and carboxyl termini. (*C*) KCNA1 (Long et al. 2005), the pore-forming subunit of the Shaker K^+ channel Kv1.2, contains six transmembrane domains, a membrane reentrant loop forming the pore between TM5 and TM6 and cytoplasmic amino- and carboxy-terminal domains. Some alternate topologies for TMC1/2 predict a possible pore reentrant loop between TM4 and TM5 (Labay et al. 2010). (*D*) ZnT-1 (Palmiter and Huang 2004), a zinc transporter that regulates transport of zinc between the endoplasmic reticulum (ER) and the cytoplasm, has a similar predicted topology to TMC1/2. Lengths of domains are to relative scale. Diagrams are depicted as extracellular and intracellular at the plasma membrane, but for ER proteins, "extracellular" = ER lumen. E.C., extracellular; I.C., intracellular. (Figures generated based on predicted topologies from Uniprot.)

some (BAC) transgenic techniques to generate TMC1-mCherry and TMC2-AcGFP mice. These mice showed expression of the transgenes with a similar temporal pattern as for TMC1 and TMC2 mRNAs. TMC1-mCherry and TMC2-AcGFP localized to tips of stereocilia and appeared to be at higher levels in shorter stereocilia. There was some colocalization of TMC1-mCherry and TMC2-AcGFP, although a significant number of puncta were not colocalized. TMC1-mCherry was also expressed in the cell body of hair cells and Kurima and colleagues (2015) used antibodies to demonstrate immunolabeling for TMC1 and TMC2 at the tips of stereocilia. Overexpressed epitope-tagged TMC1/2 also localizes to stereocilia (Kawashima et al. 2011; Zhao et al. 2014; Beurg et al. 2015b; Cunningham et al. 2017). Consistent with the localization data, TMC1/2 interact with PCDH15, but not LHFPL5 and TMIE when expressed in heterologous cells (Maeda et al. 2014; Beurg et al. 2015b). TMC1/2 in heterologous cells remain in the ER, suggesting that these interactions occur in an intracellular compartment.

Required for mechanosensitivity? Initially, it was reported that the deafness-causing mutations in *Tmc1* in mice (*Bth* and *dn*) affect maturation and survival of hair cells because the cells do not acquire mature potassium currents (Marcotti et al. 2006). Later studies demonstrated that MET current properties in these mutants were also affected, along with substantial residual current being carried by TMC2 transiently during the first few postnatal days. Double mutants of $Tmc1^{dn/dn}$;$Tmc2^{-/-}$ and $Tmc1^{-/-}$; $Tmc2^{-/-}$ (Kawashima et al. 2011) completely lack MET current without loss of tip links (Kawashima et al. 2011; Kim et al. 2013). By P9-P10, when TMC2 expression is down-regulated in the cochlea, MET currents in $Tmc1^{dn/dn}$ single mutants dramatically decrease and eventually are lost, suggesting that at later stages, TMC1 alone is essential for the MET current (Kim and Fettiplace 2013). TMC1 and TMC2, when overexpressed individually from viral vectors, can partially rescue MET function in $Tmc1^{-/-}$; $Tmc2^{-/-}$ hair cells (Kawashima et al. 2011; Askew et al. 2015). Overexpression of the amino terminus of zebrafish TMC2a can act as a dominant negative and decreases the MET response in wild-type zebrafish hair cells (Maeda et al. 2014). This manipulation altered the localization of PCDH15, which suggests that TMC proteins may play a role in linking the tip link to the channel if they do not compose the channel themselves.

Do mutations cause changes to mechanotransduction response? MET currents in hair cells expressing TMC1 or TMC2 alone have different properties from those expressing both TMC1 and TMC2 (Kim and Fettiplace 2013; Pan et al. 2013; Corns et al. 2017). TMC1-only expressing hair cells have lower unitary conductances and lower Ca^{2+} permeabilities, whereas TMC2-only expressing hair cells have higher unitary conductances and higher Ca^{2+} permeabilities. TMC2 also confers a reduced affinity of the MET channel to the permeant blocker dihydrostreptomycin, similar to the TMC1 *Bth* mutation (see below) (Corns et al. 2017). It has been proposed that TMC1 and TMC2 coassemble into channels thus leading to the tonotopic gradient in single-channel (unitary) conductance and Ca^{2+} permeability in wild-type hair cells (Kim and Fettiplace 2013). This is unlikely because TMC2 is not expressed later than P10, and TMC1 and TMC2 do not exhibit notable colocalization in hair cells (Kurima et al. 2015).

Additionally, fast adaptation properties are distinct in TMC2-expressing IHCs relative to TMC1-expressing IHCs (Pan et al. 2013), similar to that observed in $Lhfpl5^{-/-}$ animals (Xiong et al. 2012). If LHFPL5 is responsible for TMC1 (but not TMC2) localization to stereocilia and thus the residual current in *Lhfpl5-null* animals is carried by TMC2 (Beurg et al. 2015b), then perhaps the effects on adaptation in TMC2-only hair cells (*Tmc1*-null) and $Lhfpl5^{-/-}$ hair cells is the result of a loss of TMC1.

Mutations in *Tmc1* (*dn* and *Bth*) cause changes to the MET response (Kim and Fettiplace 2013; Pan et al. 2013; Beurg et al. 2015a; Corns et al. 2016). However, it is currently unclear whether the mutations affect protein localization. The *dn* mutation is caused by an in-frame deletion of amino acids 463–519, located in the long internal cytoplasmic loop (Kurima et al. 2002). The eventual complete loss of MET

current in the *dn* mutants after the down-regulation of TMC2 at P9–10 suggests that localization or expression of the mutant protein may be affected (Kim and Fettiplace 2013). The *Bth* mutation (M412K) adds a positive charge in a predicted extracellular loop between TM domain 3 and 4 (Vreugde et al. 2002) and alters properties of the MET current (Pan et al. 2013; Beurg et al. 2015a; Corns et al. 2016) with a decrease in unitary conductance and Ca^{2+} permeability of IHCs (Pan et al. 2013) and OHCs (Corns et al. 2016), although it was also reported that only Ca^{2+} permeability is affected (Beurg et al. 2015a). Pan et al. 2013 reported an increase in a Ca^{2+}-dependent block in IHCs from *Bth* mice, whereas Corns et al. 2016 reported a decreased sensitivity of the MET channel to block by the permeant blocker dihydrostreptomycin in *Bth* OHCs (Pan et al. 2013; Corns et al. 2016). These data hint at the possibility that TMC1/2 form the MET channel pore. However, these changes in ion selectivity could be caused by mutations in accessory proteins as has been reported for other ion channel complexes (e.g., MinK or Stim1 [Goldstein and Miller 1991; McNally et al. 2012]). Furthermore, changes to the local lipid environment (Effertz et al. 2017) lead to similar changes in conductance and Ca^{2+}-selectivity of the MET channel in hair cells and in effects of blockers as reported for TMC1 mutations. There are other mouse mutants of *Tmc1* that cause profound deafness (Manji et al. 2012)—*Baringo* (Y182C), *Nice* (Y449A), and *Stitch* (Y554L), but their effects on MET have not been studied.

Does heterologous or ectopic expression confer mechanosensitivity? Heterologously expressed TMC proteins localize to the ER, even in the presence of other MET-associated proteins (Labay et al. 2010; Sirianant et al. 2014; Zhao et al. 2014; Cunningham et al. 2017; Giese et al. 2017), and no reports show mechanosensitive currents in TMC-expressing cells, although many laboratories have tried (Labay et al. 2010; Kawashima et al. 2011; Zhao et al. 2014; Guo et al. 2016). Perhaps a chaperone or trafficking partner for TMC1/2 is missing in heterologous cells, because TMC1/2 go to stereocilia in hair cells (Kurima et al. 2015). However, other TMC proteins localize to the ER and have reported functions there (Lazarczyk et al. 2008; Sirianant et al. 2014).

Summary. TMC1/2 proteins are clearly components of the MET channel complex in hair cells but they have several possible specific roles: (1) TMC1/2 alone or together with other proteins (i.e., LHFPL5 or TMIE) form a channel pore; (2) TMC1/2 make up an external vestibule of the channel that regulates conductance and selectivity similar to the Slo Ca^{2+}-activated K^+ channel (Brelidze et al. 2003; Beurg et al. 2014); (3) TMC1/2 are auxiliary subunits responsible for proper localization of the channel pore; or (4) TMC1/2 link the tip link to the channel pore. Heterologous expression and structural studies will be crucial to distinguish between these possibilities.

Additional Molecules Critical for the Mechanosensitive Response in Cochlear Hair Cells

Additional molecules have been identified that affect MET in hair cells, although they are not similar in structure to ion channels, and thus unlikely to contribute to the pore of the MET channel. CDH23 and PCDH15 comprise the tip link and are critical for transmitting force onto the MET channel either directly or indirectly via linker proteins or the local lipid membrane (Siemens et al. 2004; Sollner et al. 2004; Ahmed et al. 2006; Kazmierczak et al. 2007). MYO7A, HARMONIN, and SANS are present at the upper end of the tip link and might regulate transduction via effects on the tip-link protein or by regulating hair bundle stiffness (Fig. 5) (Holt et al. 2002; Kros et al. 2002; Adato et al. 2005; Grillet et al. 2009; Michalski et al. 2009; Grati and Kachar 2011). Others such as WHIRLIN, MYOXVa, and CLRN1 have roles in hair bundle development and might affect MET secondarily (Fig. 5) (Delprat et al. 2005; Ahmed et al. 2006; Stepanyan and Frolenkov 2009; Geng et al. 2012; Xiong et al. 2012). A direct link to the transduction machinery is likely for CIB2 and TOMT, as well as for PIP_2, a phospholipid component of the stereociliary membrane (Fig. 5), and these molecules will be discussed here further.

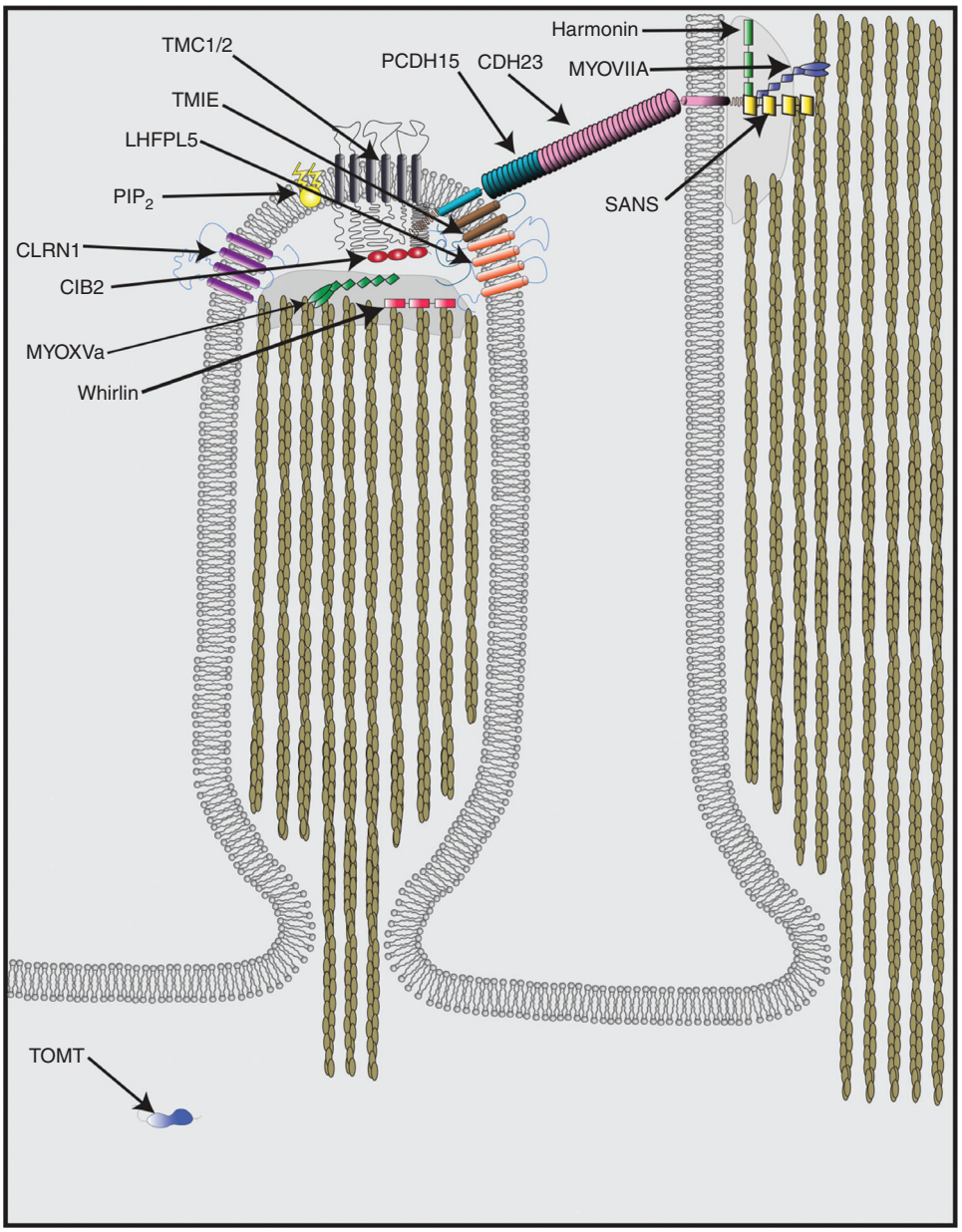

Figure 5. Molecules that contribute to mechanoelectrical transduction (MET) in cochlear hair cells. CDH23 and PCDH15 interact to form the tip link, which connects the tip of shorter row stereocilia to the side of taller row stereocilia. Harmonin, SANS, and MYOVIIA all localize to the upper tip-link density and interact in a complex with CDH23 to regulate MET and tip-link tension. LHFPL5, TMIE, and TMC1/2 all localize to the tips of stereocilia at the lower end of the tip link and are essential for MET function. PIP_2 and CIB2 localize to the tips of stereocilia and directly contribute to MET function. CLRN1, MYOXVa, and Whirlin localize to the tips of stereocilia and impact MET function, but these effects may be secondary to their effects on stereocilia morphology. TOMT localizes exclusively to the cell body but is essential for MET function by regulating TMC1/2 localization to stereocilia.

CIB2

Mutations in calcium and integrin-binding family member 2 (CIB2) cause deafness (Riazuddin et al. 2012). CIB2 is a member of a family of proteins (CIB1-4) containing EF hand domains (Gentry et al. 2005), which are thought to mediate intracellular Ca^{2+} signaling. CIB2 contains no transmembrane domains but contains three EF-hand domains, of which the second and third bind Ca^{2+} (Blazejczyk et al. 2009). CIB2 interacts in vitro with WHIRLIN, MYO7A, TMC1, and TMC2, but not LHFPL5 or TMIE (Riazuddin et al. 2012; Giese et al. 2017). Several deafness-associated mutations weaken the interactions with TMC1/2 (Giese et al. 2017).

CIB2 is expressed in the stereocilia of hair cells (Riazuddin et al. 2012; Giese et al. 2017; Michel et al. 2017; Wang et al. 2017) and both a null mutation and a point mutation that are linked to disease in humans (F91S) abolish MET (Giese et al. 2017; Michel et al. 2017; Wang et al. 2017). MYO7A, HARMONIN, PCDH15, TMC1, and TMC2 appear to localize properly in *Cib2* mutants (Giese et al. 2017), suggesting that CIB2 does not play a role in the transport of the MET complex. In heterologous cells, CIB2 is localized in intracellular compartments and is not sufficient to transport TMC1/2 to the cell surface (Giese et al. 2017).

Hair bundle morphology is altered in *Cib2* mutants, especially after the first postnatal week. However, the morphology was relatively normal during the time when MET was assayed, suggesting that the morphological defects are not causative for the MET defects (Giese et al. 2017; Michel et al. 2017). Although it is clear that CIB2 is essential for mechanotransduction, its precise role merits further study.

TOMT/LRTOMT2/COMT2

Mutations in transmembrane *O*-methyltransferase (*TOMT*, also known as *COMT2* or *LRTOMT* in humans) cause deafness in zebrafish, mice, and humans (Ahmed et al. 2008; Du et al. 2008; Erickson et al. 2017). The gene in humans (*LRTOMT*) is a fusion gene that contains two alternate reading frames, encoding the proteins LRTOMT1 and LRTOMT2 (Ahmed et al. 2008). LRTOMT2 is homologous to TOMT, which is a protein conserved from zebrafish to humans that is similar in sequence to catechol-*O*-methyltransferase (COMT). COMT regulates catecholamine levels in the brain and periphery (Mannisto and Kaakkola 1999) by enzymatic activity of its methyltransferase domain. TOMT has residual methyltransferase activity in vitro (Du et al. 2008). The human protein (LRTOMT2) contains a single transmembrane domain (Ahmed et al. 2008), and the zebrafish protein (TOMT) is predicted to have a single transmembrane domain (Erickson et al. 2017), but most analysis programs suggest that mouse TOMT has no transmembrane domain (Cunningham et al. 2017).

Tomt mRNA is expressed in hair cells (Ahmed et al. 2008; Du et al. 2008; Erickson et al. 2017) and epitope-tagged TOMT localizes exclusively to the cell body of hair cells. The pattern appears relatively diffuse throughout the cell body of zebrafish and mouse hair cells and may be enriched in the Golgi apparatus of zebrafish (Cunningham et al. 2017; Erickson et al. 2017). Immunolocalization with an antibody to TOMT showed expression in the cytoplasm of hair cells (Ahmed et al. 2008). In heterologous cells TOMT also localizes to the ER and can interact with PCDH15, TMIE, TMHS, and TMC1/2 (Cunningham et al. 2017; Erickson et al. 2017).

MET currents are abolished in zebrafish and mouse *Tomt* mutants (Cunningham et al. 2017; Erickson et al. 2017) and the defect can be rescued at least partially by the expression of TOMT mutants devoid of methyltransferase activity, suggesting that TOMT contributes to MET independent of its enzymatic function (Cunningham et al. 2017; Erickson et al. 2017). Hair bundle morphology (Cunningham et al. 2017; Erickson et al. 2017) was relatively normal during the period in which MET currents were measured, and tip-link numbers are normal in IHCs and OHCs from *Tomt* mutant mice (Cunningham et al. 2017). However, TMC1/2 are no longer localized properly to stereocilia in zebrafish or mice, suggesting a transport or assembly function for TOMT (Cunningham et al. 2017;

Erickson et al. 2017). In heterologous cells, TOMT alone is not sufficient to target TMC1/2 to the cell membrane (Cunningham et al. 2017; Erickson et al. 2017). Thus, TOMT likely works in concert with other unidentified proteins in protein transport. The function of TOMT is perhaps similar to that of MEC-6 and POML-1 in regulating proper surface localization of MEC-4, the mechanosensitive channel in *C. elegans* that is critical for gentle touch (Arnadottir and Chalfie 2010; Chen et al. 2016).

PIP_2

PIP_2 is a phospholipid component of cell membranes that can alter ion channel function (Hardie and Franze 2012; Zhang et al. 2013; Borbiro et al. 2015). PIP_2 has been localized to the stereocilia of frog (Hirono et al. 2004), mouse (Goodyear et al. 2008), and rat hair cells (Effertz et al. 2017). Depleting PIP_2 pharmacologically from hair bundles reduces adaptation in bullfrog saccular hair cells and decreases the MET current (Hirono et al. 2004). In hair cells from the rat cochlea, PIP_2 depletion leads to surprising changes in MET function, including loss of fast adaptation, decreases in MET single-channel conductance, changes in ion selectivity, slower activation of the channel, an increase in the open probability at rest, and a decrease in the Ca^{2+} pore block (Effertz et al. 2017). Considering the fact that PIP_2 is a component of the local cell membrane, these data reinforce the fact that seemingly inherent properties of the channel such as single-channel conductance and ion selectivity can be modulated by factors not inherent to the channel pore. Interestingly, PIP_2 modulation also affects PIEZO channel activity (Borbiro et al. 2015).

CONCLUSION

Recent years have seen significant progress in the identification of core components of the mechanotransduction machinery of hair cells with the identification of the proteins that form tip links and proteins that are intricately linked to the function of the MET channel (Fig. 5), either by contributing to the channel complex directly or by regulation of assembly and function of the MET complex. Several integral membrane proteins have been identified (LHFPL5, TMIE, TMC1/2) that are candidates to contribute to the pore of the MET channel or to regulate the function of pore-forming subunits. The specific function of these proteins still needs to be defined. Intriguingly, seemingly intrinsic properties of the channel pore (i.e., channel conductance, ion selectivity, and channel block) can be impacted by modifying the lipid environment (i.e., PIP_2) surrounding the channel. It is thus imperative to develop a heterologous system to study the mechanisms that regulate MET channel assembly and the function. A major stumbling block is the fact that TMC proteins in heterologous cells remain in the ER. Interestingly, some members of the TMC family even appear to be resident ER proteins (Lazarczyk et al. 2008; Sirianant et al. 2014). Perhaps the MET channel is similar to VRAC channels, which are heteromeric channel complexes composed of SWELL1/LRRC8A and up to four additional LRRC8 subunits (LRRC8B-E) that regulate cell volume (Qiu et al. 2014; Voss et al. 2014; Syeda et al. 2016). Possibly relevant is the fact that VRAC subunit composition can differ and different subunit combinations confer distinct variations of channel properties (Syeda et al. 2016). A heteromeric channel complex could explain tonotopic differences in the properties of the MET channel, a feature of the channel that may be critical for our ability to encode sound of different frequencies to provide our sense of hearing.

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