Dual Perspectives

Dual Perspectives Companion Paper: Are TMCs the Mechanotransduction Channels of Vertebrate Hair Cells?, by David P. Corey and Jeffrey R. Holt

Molecular Identity of the Mechanotransduction Channel in Hair Cells: Not Quiet There Yet

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Hair cells in the mammalian cochlea are specialized mechanosensory cells that convert sound-induced vibrations into electrochemical signals. The molecular composition of the mechanotransduction channel underlying auditory perception has been difficult to define. The study of genes that are linked to inherited forms of deafness has recently provided tantalizing clues. Current findings indicate that the mechanotransduction channel in hair cells is a complex molecular machine. Four different proteins (TMHS/LHFPL5, TMIE, TMC1, and TMC2) have so far been linked to the transduction channel, but which proteins contribute to the channel pore still needs to be determined. Current evidence also suggests that the channel complex may contain additional, yet to be identified components.

Key words: **hair cell; LHFPL5; mechanotransduction; tip link; TMC1; TMC2; TMHS; TMIE**

Introduction

Hair cells in the mammalian cochlea are the receptor cells that convert sound-induced vibrations into mechanical signals. Hair cells carry their name because of their characteristic shape where a bundle of stereocilia (the "hair bundle") crowns the apical hair-cell surface (Fig. 1). The hair bundle is the mechanically sensitive organelle of hair cells. Within the bundle, the stereocilia are organized in rows of decreasing heights; they are connected by several types of extracellular filaments, including the tip links, which are thought to convey mechanical force onto the transduction channels (Fig. 1).

Electrophysiological recordings from hair cells provided the first evidence for the existence of an ion channel directly activated by mechanical force (Hudspeth and Corey, 1977). Subsequent studies demonstrated that the transduction channel in hair cells is a nonselective cation channel, admitting Ca^{2+} as well as monovalent ions (Corey and Hudspeth, 1979). This property has been explored to localize the channel in hair cells without knowledge of its molecular identity. High-speed Ca^{2+} imaging has shown that, upon mechanical stimulation of hair bundles in rodents, Ca^{2+} enters stereocilia near the lower end of tip links (Beurg et al., 2009), thus indicating that in mammals transduction channels are present only at one end of this filament (Fig. 1). However, the identification of the molecular components of the ion channel that carry these currents has been challenging. Channel identification has been hampered by the scarcity of hair cells and the low expression levels of the mechanotransduction channels in stereocilia with only one or two functional channels per tip link (Ricci et al., 2003; Beurg et al., 2009).

Breakthroughs in the discovery of components of the mechanotransduction machinery of hair cells have been fueled by genetic studies. Deafness is the most common form of sensory impairment in humans, and mutations in >80 genes have been linked to the disease (http://hereditaryhearingloss.org/). The study of these genes initially led to the identification of the proteins that form the tip link (Fig. 1) (Siemens et al., 2004; Söllner et al., 2004; Ahmed et al., 2006; Kazmierczak et al., 2007). Subsequently, four integral transmembrane proteins, tetraspan membrane protein in hair cell stereocilia (TMHS; official nomenclature LHFPL5), transmembrane inner ear expressed gene (TMIE), and transmembrane channel-like proteins 1 and 2 (TMC1 and TMC2) have been closely linked to the transduction process (Fig. 1) (Kawashima et al., 2011; Xiong et al., 2012; Kim and Fettiplace, 2013; Kim et al., 2013; Pan et al., 2013; Beurg et al., 2014; Zhao et al., 2014). TMC1 and TMC2 have been proposed to be pore-forming subunits of the mechanotransduction channel (Pan et al., 2013), although compelling evidence supporting this conclusion has not yet been provided. We will summarize here our current knowledge on the molecular identification and function of putative components of the mechanotransduction channel in hair cells.

TMC1 and TMC2: a link to deafness and mechanotransduction

TMC1 was initially identified through the positional cloning of the gene underlying the sensory deficits in families afflicted with

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specific forms of dominant and recessive deafness (Kurima et al., 2002). Mouse models carrying dominant and recessive mutations in *TMC1* have also been described (Kurima et al., 2002; Vreugde et al., 2002). Studies in mice demonstrated that TMC1 and its close homolog TMC2 are expressed in hair cells in the inner ear, albeit each with a different developmental time course. In vestibular hair cells, TMC1 and TMC2 are coexpressed from developmental stages into adulthood. By contrast, in cochlear hair cells, TMC1 and TMC2 are coexpressed for the first few days after birth, but only TMC1 expression is maintained into adulthood (Kawashima et al., 2011).

The most conclusive evidence for the subcellular localization of TMC1 and TMC2 within hair cells was provided by a recent elegant study. Using BAC-transgenic mice that express TMC1 and TMC2 tagged with fluorescence proteins, it was shown that TMC1 and TMC2 are each concentrated near the lower end of tip links (Kurima et al., 2015), where the transduction channel is localized (Beurg et al., 2009). This localization was confirmed with antibodies to the endogenous TMC1 and TMC2 proteins (Kurima et al., 2015). The localization pattern is consistent with the observation that TMC1/2 interacts in heterologous cells with PCDH15 (Maeda et al., 2014; Beurg et al., 2015b), which forms the lower part of tip link (Fig. 2) (Kazmierczak et al., 2007).

Analysis of the sequences of TMC1 and TMC2 prompted speculation that the two proteins might encode subunits of the mechanotransduction channel in hair cells. Computer algorithms predict that TMC1 contains six putative transmembrane domains, as well as two additional hydrophobic domains that are not predicted to span the membrane (Kurima et al., 2002). The membrane topography of TMC1 has been experimentally verified (Labay et al., 2010); and with respect to the number of transmembrane domains (but not in other ways), the proteins resemble voltage-gated potassium channels and members of the Trp ion-channel family (Yool and Schwarz, 1991; Vannier et al., 1998). As one caveat to these studies, it has been difficult to express significant amounts of TMC1 and TMC2 at the cell surface of heterologous cells. The majority of the protein is retained in the ER and transmembrane topography has been mapped with epitope tags using ER-retained TMC1 (Labay et al., 2010). The same caveat applies to the biochemical studies that have demonstrated interactions between PCDH15 and TMC1/2. Because only minor amounts of TMC1/2 reach the cell surface, interactions with PCDH15 likely occur in an intracellular compartment (Maeda et al., 2014; Beurg et al., 2015b).

Electrophysiological analysis of cochlear and vestibular hair cells from *Tmc1/2* double-mutant mice further solidified a link between the two proteins and the mechanotransduction process. Mechanically evoked transducer currents are abolished in cochlear and vestibular hair cells from mutant mice lacking both TMC1 and TMC2 (Kawashima et al., 2011; Pan et al., 2013). Transducer currents are also absent in cochlear hair cells from *Tmc1* mutant mice but only ~P10 when TMC2 is no longer expressed in the cochlea, indicative of functional redundancy between the two proteins (Kawashima et al., 2011; Pan et al., 2013). Transduction defects in hair cells lacking TMC1 and TMC2 can be rescued by overexpression of either TMC1 or TMC2 alone, which suggests that the two proteins can function independently of each other to support transduction (Kawashima et al., 2011; Pan et al., 2013; Askew et al., 2015). Interestingly, mutations in *Tmc1* but not *Tmc2* have been shown to cause deafness (Kurima et al., 2002; Vreugde et al., 2002; Santos et al., 2005; Tlili et al., 2008), which can likely be explained by the fact that TMC2 is expressed only transiently in developing cochlear hair cells whereas TMC1 expression is maintained into adulthood (Kawashima et al., 2011).

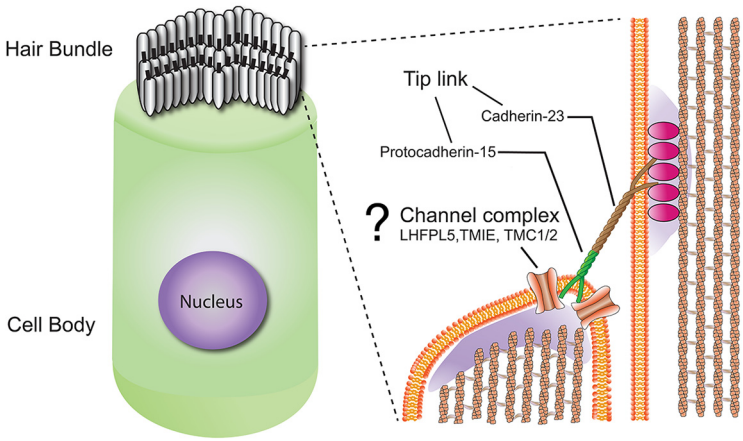

Figure 1. Hair bundles and tip links. Left, Diagram of a hair cell is shown highlighting the hair bundle and the tip-link filaments that connect the stereocilia in the direction of their mechanical sensitivity. Right, Molecules that form tip links and putative components of the mechanotransduction channel. Cadherin-23 interacts with protocadherin-15 to form the upper and lower parts of tip links. LHFPL5, TMIE, and TMC1/2 localize at the lower end of tip links near PCDH15 where transduction channels are located.

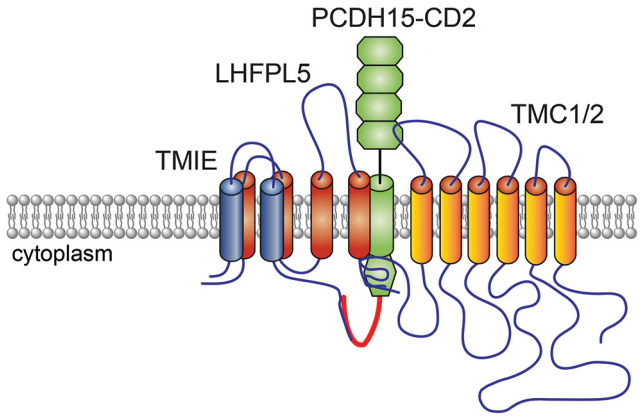

Figure 2. Model of the transduction channel complex of hair cells. TMC1/2, LHFPL5, and TMIE interact with PCDH15. TMIE specifically binds to the C terminus (red) unique to the PCDH15-CD2 isoform.

TMC1 and TMC2: pore-forming subunits of mechanotransduction channels?

Single-channel recordings in developing outer hair cells (OHCs) from rodents have shown that the conductance of the transducer channel increases from the apex to the base of the cochlea, whereas the Ca^{2+} permeability of the channel decreases (Beurg et al., 2006; Kim and Fettiplace, 2013). No such changes are observed in inner hair cells (IHCs) (Beurg et al., 2006; Kim and Fettiplace, 2013). Because developing cochlear hair cells coexpress TMC1 and TMC2, several investigators asked whether conductance properties or ion selectivity of the mechanotransduction channel was altered in hair cells from mice carrying mutations affecting the expression of either TMC1 or TMC2. Changes in channel conductance and/or ion selec-

tivity would further support a role for TMC1 and TMC2 as integral components of the transduction complex. Intriguingly, the tonotopic gradient in channel conductance is blunted in cochlear OHCs of mice lacking *Tmc1* but not *Tmc2* (Kim and Fettiplace, 2013; Beurg et al., 2014). Conversely, the Ca^{2+} selectivity of the transducer channel is affected in OHCs and IHCs lacking *Tmc2* but not *Tmc1* (Kim et al., 2013; Pan et al., 2013). These effects of *Tmc1/2* mutations on the transducer current are curious in that TMC1 affects conductance and TMC2 affects Ca^{2+} selectivity in subtle ways (Kim and Fettiplace, 2013; Kim et al., 2013; Pan et al., 2013; Beurg et al., 2014). The findings suggest that the two proteins are functionally, albeit not entirely, redundant, which is somewhat surprising given the observation that expression of TMC1 alone or TMC2 alone can rescue transduction in hair cells lacking both TMC1 and TMC2 (Kawashima et al., 2011). Perhaps further detailed analysis of transducer currents in rescue experiments will reveal subtle differences that so far have escaped detection. One study also reported that the reduction in Ca^{2+} permeability and channel conductance is more pronounced in hair cells from mutant mice termed *Beethoven* that express a TMC1 protein with a missense mutation linked to progressive deafness (Pan et al., 2013). Together, these findings suggest that TMC1 and TMC2 are integral components of the mechanotransduction machinery of hair cells.

Holt and colleagues also developed a new method for mechanical stimulation of individual stereocilia to measure single-channel events (Pan et al., 2013). Using this method, they reported a range of conductance for the mechanotransduction channel in IHCs. They concluded that differences in the stoichiometry of TMC1/2 may explain the variations in conductance they measured and that this is the underlying reason for the tonotopic changes in the conductance of the transducer channel along the cochlear tonotopic axis (Pan et al., 2013).

Despite these interesting findings, the extent to which TMC1 and TMC2 are pore-forming subunits of the transduction channel remains unclear. Adult cochlear hair cells only express *Tmc1* (Kawashima et al., 2011). Thus, variations in the stoichiometry of TMC1/2 cannot explain tonotopic variations in transducer currents of the adult cochlea. In addition, using fluorescent-protein-tagged TMC proteins expressed from BAC transgenes, Kachar and colleagues showed that, even in developing hair cells, TMC1 and TMC2 colocalize in only ~30% of the fluorescence puncta observed in stereocilia (Kurima et al., 2015). Some stereocilia express only TMC1 or TMC2 (Kurima et al., 2015). These data make it less likely that TMC1 and TMC2 associate in various stoichiometries to form channels with different conductance states important for tonotopic gradients. It seems more likely that with their new stimulation and recording protocol, Holt and colleagues did not only record single-channel events but a composite of several channels, thus leading to the false impression of several conductance states of individual channels consisting of different subunits.

In addition, although measurements from the *Beethoven* mice performed by Holt and colleagues (Pan et al., 2013) suggest that TMC1 may be a pore-forming subunit of the transduction channel, the precise phenotype of *Beethoven* mice is not clear. A second study by the Fettiplace laboratory reported that in *Beethoven* mice there is a subtle change in Ca^{2+} permeability of the transducer channel similar to what Holt reported, but the Fettiplace group did not observe the changes in channel conductance described in the study from the Holt laboratory (Beurg et al., 2015a). This is a significant and important difference between the two studies because changes in conductance would link the protein more closely to the pore of the channel than small changes in Ca^{2+} permeability alone. The reason for the difference in results is unclear but may be explained at least in part by the fact that the former study analyzed IHCs, whereas the latter study analyzed OHCs. In addition, the method used by Holt and colleagues to measure single-channel conductance involves deflection of an unidentified number of stereocilia within a hair bundle. The method has not been verified and thus might have given inaccurate results. Clearly, further studies are important to resolve this issue.

Notably, although changes in ion selectivity might be caused by mutations in pore residues of transduction channels, this is not universally true. For example, a point mutation in the hydrophobic domain of MinK leads to altered ion selectivity and a change in open channel block of a slowly activating potassium current in *Xenopus* oocytes (Goldstein and Miller, 1991). While these authors concluded from these data that MinK, a single pass transmembrane protein, is an ion channel, it was later shown that MinK associates with the potassium channel KvLQT1 (Kv7.1) without directly contributing to the pore (Barhanin et al., 1996; Sanguinetti et al., 1996). Another example is provided by the calcium release-activated calcium channel (CRAC1). STIM1 is not a pore-forming subunit of the CRAC1 channel and is localized in the ER. Nevertheless, the channel-associated STIM1 protein alters the ion selectivity of the CRAC1 channel (McNally et al., 2012). These findings demonstrate that accessory proteins can modulate the ion selectivity of an ion channel.

As an alternative, it has been proposed that TMC1/2 might form a vestibule of the transduction channel that shuttles ions toward the pore (Beurg et al., 2014; Marcotti et al., 2014). Mutations that cause variations in conductance could affect the charge of an external vestibule and ion flux toward the pore. A similar mechanism has been proposed as an explanation for the large conductance of the Slo Ca^{2+}-activated K^+ channel, which contains an internal vestibule lined with glutamate residues. Replacement of glutamate with neutral residues drastically reduces the channel conductance (Brelidze et al., 2003). In the mechanotransduction channel of hair cells, there may be such a vestibule exposed to the extracellular environment, which could explain the high permeability of the channel for larger ions (Farris et al., 2004; van Netten and Kros, 2007). Further experiments are necessary to distinguish between a role for TMC1 and TMC2 as accessory or pore-forming subunits of the mechanotransduction channel in hair cells.

The curious case of the "reverse-polarity" current

In mature healthy hair cells, only deflections of the hair bundle in the direction of the longest stereocilia leads to an increase in the open probability of mechanically gated ion channels (Corey and Hudspeth, 1983). Hair bundles of immature hair cells are less directionally sensitive and deflections both in the direction of the longest stereocilia and in the opposite direction lead to an increase in channel open probability (Waguespack et al., 2007; Kindt et al., 2012; Kim and Fettiplace, 2013; Marcotti et al., 2014). The currents that are evoked by deflection toward the smallest stereocilia have been dubbed "reverse-polarity" currents (Kim et al., 2013). Curiously, when tip-links are broken, normal polarity currents can no longer be evoked but robust reverse-polarity currents emerge (Alagramam et al., 2001; Kim et al., 2013; Beurg et al., 2014; Marcotti et al., 2014). Whereas normal-polarity currents can no longer be evoked in hair cells from *Tmc1/2* double mutant mice, these hair cells nevertheless show robust reverse-polarity currents similar to hair cells lacking tip links (Kim et al., 2013; Beurg et al., 2014). It is intriguing that the kinetics of ap-

pearance of the reverse-polarity current and disappearance of the normal-polarity current are inversely correlated (Kim et al., 2013; Beurg et al., 2016), suggesting that the channel carrying the forward current is converted into the channel carrying the reverse current. The two currents also show similar properties, including block by a high concentration of extracellular Ca^{2+} and by standard pharmacological channel inhibitors (Kim et al., 2013), although others have emphasized the differences in the two currents (Marcotti et al., 2014).

Recent findings suggest that the reverse-polarity current is carried by an ion channel localized to the apical surface of the cell body of hair cells with highest concentration near the longest stereocilia (Beurg et al., 2016). Interestingly, in *Tmc1* and *Tmc2* single mutants, fewer tip links are occupied with channels compared with *Tmc2*-deficient hair cells expressing mutant TMC1 (Pan et al., 2013). Thus, it has been proposed that TMC1/2 may not be the pore-forming subunits of the normal-polarity channel and instead function to transport channels into stereocilia and/or to stabilize interactions with tip links (Kim and Fettiplace, 2013; Kim et al., 2013; Beurg et al., 2014; Beurg et al., 2015b). In the absence of TMC1/2 or tip links, the pore-forming subunits may then relocalize to the apical cell surface. Notably, recent studies also show that the reverse-polarity current is more accurately described as a stretch-activated current that response to pulling forces applied to the apical membrane domain of the hair cell body (Beurg et al., 2016). Further studies are necessary to define the molecular identity of the ion channel carrying the reverse-polarity current and its relationship to the normal-polarity current.

Loose ends and open questions regarding TMC1 and TMC2

TMC1 and TMC2 are intriguing proteins that are very likely closely linked to the transduction channel in hair cells. One might argue that the analysis of the effects of additional mutations in *Tmc1* and *Tmc2* on the properties of mechanotransduction currents in hair cells might clarify their role in mechanotransduction. For example, cysteine mutations can be engineered into the protein, and the effect of cysteine-modifying compounds on mechanotransduction can be evaluated (Akabas et al., 1992; Karlin and Akabas, 1998). However, these studies will probably not distinguish between a role of TMC1 and TMC2 as pore-forming or accessory channel subunits that form a vestibule. For these experiments to be conclusive, it first needs to be shown that TMC1 and TMC2 actually can form an ion-conducting pore. This could perhaps be achieved by crystallography or cryoelectron microscopy but would be more convincingly established by showing that TMC1 and TMC2 form pores following their expression in heterologous cells and ultimately in lipid bilayers. Attempts have so far failed to demonstrate that heterologous expression of TMC1 and TMC2 can confer mechanical sensitivity on native cells (Kawashima et al., 2011; Zhao et al., 2014). This failure has been attributed in large part to the inability of TMC1 and TMC2 to be efficiently shuttled to the cell-surface or heterologous cells; most of the protein remains in the ER (Labay et al., 2010). The fact that TMC1 and TMC2 can be transported to the tips of stereocilia (Kurima et al., 2015) suggests that the proteins are capable of being inserted into the plasma membrane; heterologous cells might lack critical chaperones or transport components. A *Tmc* ortholog in worm has been reported to be necessary for salt sensation and to generate a sodium-sensitive channel when expressed in heterologous cells (Chatzigeorgiou et al., 2013). However, others have shown that the phenotype of nematodes with mutations in *Tmc1* is complex and pleiotropic, with perturbations of sexual and metabolic function without an obvious specific role in sensory transduction (Zhang et al., 2015). Thus, further studies are necessary to show whether TMC proteins encode ion channels and whether they can be activated by mechanical force.

The tetraspan protein LHFPL5 and mechanotransduction

Recent studies have taken advantage of a large collection of genetically modified mice obtained from ENU mutagenesis screens and of mice genetically engineered to carry specific mutations to identify mouse lines that are deaf and have defects in mechanotransduction. Using this strategy, Xiong et al. (2012) demonstrated that mutations in *Lhfpl5*, also known as *Tmhs*, cause deafness and lead to a nearly 90% reduction in mechanotransduction in cochlear hair cells. LHFPL5 is a protein with four transmembrane domains that directly binds to PCDH15 (Xiong et al., 2012). Consistent with the biochemical data, LHFPL5 localizes in hair cells near the lower end of tip links (Xiong et al., 2012). Subsequent studies demonstrated that LHFPL5 regulates the transport of PCDH15 into stereocilia, thereby controlling the efficiency of tip-link formation (Xiong et al., 2012). In addition, LHFPL5 directly affects the properties of the transduction channel. Single-channel recordings demonstrated that, in the absence of LHFPL5, the conductance of the transduction channel is reduced and adaptation is severely impaired (Xiong et al., 2012). These findings are consistent with the model that LHFPL5 is a subunit of the mechanotransduction channel. LHFPL5 alone cannot be an essential part of the pore, however, because substantial mechanically evoked currents can still be recorded in hair cells lacking LHFPL5 (Xiong et al., 2012). Interestingly, LHFPL5 shares certain features with the TARP subunits of AMPA receptor. Like LHFPL5, TARPs have four transmembrane domains and they regulate both the transport of the pore-forming subunits of AMPA receptors and channel conductance (Jackson and Nicoll, 2011). In analogy, LHFPL5 may act as a TARP-like protein for the transduction channel and allosterically modulate the properties of the pore-forming subunits (Xiong et al., 2012). However, it can at present not be excluded that LHFPL5 might also contribute to the pore of the transduction channel. An instructive example is provided by the *swell* channel that is regulated by osmotic changes (Qiu et al., 2014; Voss et al., 2014; Syeda et al., 2016). The *swell* channel is a heteromeric channel consisting of several proteins with four transmembrane domains where subunit composition affects the properties of the ion channel (Syeda et al., 2016). Interestingly, LHFPL5 is a member of a small gene family (Longo-Guess et al., 2005), but the function of other members of this family in hair cells has not yet been reported. Alternatively, LHFPL5 might affect membrane properties locally, which is a function attributed to some tetraspans (Yáñez-Mó et al., 2009).

Recently, an intriguing link was established between LHFPL5 and TMC1. Although TMC1 does not bind directly to LHFPL5, TMC1 is no longer localized to the stereocilia of *Lhflp5*-deficient hair cells (Beurg et al., 2015b). Thus, both the transport of PCDH15 (Xiong et al., 2012) and TMC1 (Beurg et al., 2015b) depends on LHFPL5, suggesting that the proteins act in a molecular complex. Both LHFPL5 and TMC1 bind to PCDH15, which might mediate formation of the ternary complex, but other proteins might also contribute. In mice lacking *Lhfpl5* and in mice lacking *Tmc1*, the tonotopic gradient in the conductance of the transducer channels is similarly blunted (Beurg et al., 2015b). Perhaps variations in levels of LHFPL5 and/or TMC1 along the

cochlear axis could explain tonotopic differences in the conductance properties of the transducer channel.

TMIE is essential for mechanotransduction by hair cells

In search for additional components of the mechanotransduction machinery of hair cells, yeast two-hybrid screens were performed with proteins linked to transduction, including LHFPL5, PCDH15, and TMC1. One of the proteins that was identified as a binding partner for LHFPL5 and PCDH15 but not TMC1 was TMIE (Zhao et al., 2014). TMIE contains two predicted transmembrane domains and is linked to deafness in both humans and mice (Mitchem et al., 2002; Naz et al., 2002). Studies in zebrafish have shown that *Tmie*-deficient hair cells show degenerative changes and lack microphonic potential in response to vibratory stimulation, indicating that transduction is affected (Gleason et al., 2009). In murine hair cells, TMIE is localized in the tip-link region and essential for normal mechanotransduction without affecting the assembly of tip links (Zhao et al., 2014). The transduction defect in the *Tmie*-deficient hair cells can be rescued by acute reexpression of TMIE at early postnatal ages, demonstrating that mutations in *Tmie* directly affect transduction and do not act by a more general developmental mechanism (Zhao et al., 2014).

The localization of TMIE in hair cells and binding to PCDH15 and LHFPL5 suggest that TMIE is an integral component of the transduction machinery. PCDH15 is expressed in three major alternative spliced variants (PCDH15-CD1, -CD2, -CD3). The three splice variants are identical in their extracellular and transmembrane domains as well as in a short membrane proximal cytoplasmic domain that mediates interactions with TMC1/2 and LHFPL5 (Fig. 2) (Ahmed et al., 2006; Xiong et al., 2012; Maeda et al., 2014; Beurg et al., 2015b). However, they have distinct cytoplasmic C termini (Ahmed et al., 2006). Genetic studies suggest that PCDH15-CD2 is the PCDH15 isoform critical for mechanotransduction once hair cells have reached maturity (Pepermans et al., 2014), and biochemical data show that TMIE binds to the unique C terminus of PCDH15-CD2 (Fig. 2) (Zhao et al., 2014). TMIE also interacts with all three PCDH15 isoforms in a ternary complex through its interaction with LHFPL5 (Zhao et al., 2014). A TMIE fragment that consists of the C-terminal half that mediates interactions with PCDH15 and LHFPL5 acts as a dominant negative inhibiting interactions of TMIE with PCDH15. When this dominant negative TMIE fragment is expressed in wild-type hair cells, mechanotransduction is reduced (Zhao et al., 2014). Transduction is similarly reduced by overexpressing the CD2 domain of PCDH15, a protein fragment that affects interactions between TMIE and PCDH15-CD2 (Zhao et al., 2014). These findings suggest that TMIE acts in a ternary complex with LHFPL5 and PCDH15 to regulate transduction.

The relationship of TMIE to TMC1/2 is currently unclear. TMIE does not bind to TMC1/2, and the localization of TMC1/2 in stereocilia does not appear to be affected in hair cells lacking TMIE (Zhao et al., 2014). Similarly, LHFPL5 is still present in the stereocilia of *Tmie* mutant hair cells and tip links form in the mutant mice (Zhao et al., 2014). Thus, the mechanism by which TMIE affects transduction remains to be established. It is astonishing that mechanotransduction is completely abolished in hair cells lacking TMIE, yet all known components of the transduction complex in addition to TMIE appear to be present in their stereocilia. Perhaps TMIE affects the expression and/or localization of a yet to be identified component of the transduction machinery. Alternatively, TMIE might contribute to the channel pore. Indeed, proteins with two transmembrane domains, such as ENac/DEG in *Caenorhabditis elegans*, oligomerize to form a channel pore (Delmas and Coste, 2013). However, it has so far not been demonstrated that TMIE has ion channel properties, either alone or together with other proteins, such as LHFPL5 and TMC1/2 (Zhao et al., 2014).

In conclusion, the study of genes that are linked to hearing loss has provided important insight into the molecular machinery that is critical for mechanotransduction by hair cells. The study of the affected genes has also revealed an unexpected complexity in the transduction machinery of hair cells. Available evidence suggests that LHFPL5, TMIE, and TMC1/2 bind to PCDH15 and are integral components of a protein complex that is present at the lower end of tip links, where the mechanotransduction channel of hair cells is localized. The data also suggest that TMC1 and LHFPL5 have a critical function in establishing variations in the properties of the transduction channel along the tonotopic axis of the cochlea. However, the extent to which LHFPL5, TMIE, and TMC1/2 contribute to the channel pore is unclear. A recent study established that Piezo1, a mechanically gated ion channel in mammals (Coste et al., 2010), contains a miniature pore-forming module, an extracellular ion-selective module, and an intrinsic mechanotransduction module (Zhao et al., 2016). It is unknown whether the mysterious mechanotransduction channel in cochlear hair cells contains these modules within one protein or within separate molecular components. Ultimately, this question can likely only be conclusively answered by reconstitution of the mechanically gated ion channel in a heterologous system, such as heterologous cells or lipid bilayers. These experiments are important to demonstrate that the candidate proteins can form a channel pore. Given the complexity in the composition of the transduction complex in hair cells, reconstitution will be a difficult but essential task. Additional experiments, such as the identification of the channel pore using mutagenesis and subsequent expression of mutant channels with altered pore properties in hair cells, will be necessary as ultimate proof that a particular ion channel forms the pore of the mechanotransduction channel important for hearing.

Response from Dual Perspective Companion Authors—David P. Corey and Jeffrey R. Holt

The Perspective by Wu and Müller does a good job summarizing the important findings regarding the role of TMC proteins, and also summarizes their recent work on TMHS/LHFPL5 and TMIE. As the two Perspectives cover much of the same material, it is not surprising that we agree on most of the key points. Disagreement about the specific roles of these proteins lies mostly in how we interpret and weigh the relative importance of some experiments.

One difference is in the importance of the reverse or anomalous current which is apparent when conventional transduction current is absent. The current has recently been shown to be activated by negative pressure on or near the apical hair-cell surface rather than by bundle deflection (Beurg et al., 2016). We agree with Wu and Müller that the anomalous current cannot be carried by TMC1 or TMC2, since it persists in TMC1/TMC2 knockouts. Although the pharmacological similarity of the two currents has lead some to suggest that the conventional and anomalous currents are carried by the same channels, we think the

(modest) pharmacological similarity—never a strong argument with low-affinity blockers— does not offer much guidance for protein identity.

Müller and Wu also take issue with the single-channel measurements of Pan et al. (2013), suggesting that multiple channels may have been activated. Certainly the same may also be true for the Beurg et al. (2015) measurements, which used a fluid jet directed at the entire bundle. In both cases the standard single-channel criterion of selecting the smallest unitary current was used to avoid such artifact. Notably, both studies report a similar broad range of single channel conductances in wild-type auditory hair cells.

Pan et al. showed that single-channel conductance in neonatal wild-type inner hair cells varies among cells, mostly in the range between cells with just TMC1 and those with just TMC2. They suggested that heteromultimerization could produce such variation. Since older outer hair cells do not express TMC2 (Kawashima et al., 2011; Kurima et al., 2015), we agree with Wu and Müller that heteromultimerization with TMC2 cannot explain tonotopic variation in mature cells. Nonetheless, the point remains valid that molecular differences between TMC1 and TMC2 may produce differences in single-channel properties when co-expressed at neonatal stages.

Furthermore, the mean of three single-channel measurements from *Bth* inner hair cells reported by Beurg et al. (2015) was nearly identical to the mean of 17 measurements reported by Pan et al. (2013) when corrected for a 1.4-fold difference in amplitude, the latter a consequence of different external calcium concentrations. Pan et al. (2013) recorded from 25 inner hair cells that expressed wild-type *Tmc1*, which revealed $p < 10^{-10}$ significance relative to *Tmc1-Bth* cells, and did not have the complication of concurrent TMC2 expression that Beurg et al. (2015) faced.

Finally, Müller and Wu feel strongly that nothing can be proven until a TMC protein in a heterologous cell or lipid bilayer is shown to create a mechanosensitive channel. We feel that this will be extremely difficult, given all the other proteins known to be essential for hair cell transduction. In the meantime, we suggest that site-directed mutagenesis, if rationally guided by sequence, may help confirm the location of TMC residues that line the channel pore.

Most importantly, we all agree that—after years of slow going—there has recently been remarkable progress in molecular identification of proteins in the hair-cell mechanotransduction complex. The next few years will be exciting indeed, as these proteins are assembled into an integrated model.

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