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VIEWPOINT

Sensory transduction in auditory hair cells—PIEZOs can't touch this

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The molecular identity of the components of the hair cell mechanosensory transduction apparatus, which transforms information carried by sound into electrical signals, has been the focus of intensive research over the past 40 years. In recent years, pieces of this molecular puzzle have come to light, with a handful of components now firmly established (reviewed in Zheng and Holt, 2021; Qiu and Müller, 2022). The tip link, extending from the top of one hair cell stereocilium to the side of an adjacent taller neighbor is formed by CDH23 and PCDH15. At the upper end of the tip link, CDH23 interacts with USH1C (harmonin), USH1G (sans), and MYO7A to anchor the tip link and regulate tip-link tension, respectively (Fig. 1). At the lower end, PCDH15 binds to LHFPL5 and conveys tension to the channel complex which includes TMIE (transmembrane inner ear protein), the pore-forming subunits TMC1 and/or TMC2, and CIB2. All of these molecular components satisfy essential criteria that must be met to claim a rightful place in nature's most exquisite mechanosensor (Qiu and Müller, 2022), which is capable of detecting movements as small as ~1 Å (Denk and Webb, 1992).

In a recent manuscript, Lee et al. (2024) propose a direct role for PIEZO proteins in hair cell sensory transduction. PIEZOs 1 and 2 form bona fide ion channels and are involved in the sense of touch and other forms of mechanotransduction throughout the body (Wu et al., 2017a). Thus, at first glance, the notion that PIEZO proteins might contribute to mechanotransduction in sensory hair cells does not seem far-fetched. However, as we discuss below, PIEZOs have not satisfied essential criteria to be considered components of the hair cell transduction complex, while TMCs 1 and 2 and other transduction apparatus components do meet these criteria.

Gene expression

First, any gene contributing to auditory transduction must be expressed in sensory hair cells at the right time during development and into adulthood. Rodent auditory hair cells become mechanosensitive during the first postnatal week (Waguespack et al., 2007; Lelli et al., 2009) and auditory function commences during the second postnatal week. Quantitative PCR data demonstrate *Tmc2* gene expression transiently during the first postnatal week followed by *Tmc1* expression, which rises and is maintained into adulthood (Kurima et al., 2002; Kawashima et al., 2011), preceding the developmental onset of hair cell transduction and auditory function, respectively. *Tmc1* and *Tmc2* mRNA have also been detected in hair cells using single-cell RNA sequencing (Elkon et al., 2015; Cai et al., 2015; Scheffer et al., 2015; Kolla et al., 2020). *Piezo1* was not detected in the same gene expression databases. *Piezo2* mRNA expression was detected at low levels but peaks early in the first postnatal week and then declines to near zero by the end of the first postnatal week (Scheffer et al., 2015). Wu et al. (2017b) used in situ hybridization with antisense probes directed against *Piezo1* and *Piezo2*. *Piezo1* expression was not detected in the inner ear, while *Piezo2* expression was evident in hair cells. The Lee et al. (2024) group used fluorescent in situ hybridization (FISH) and suggested that a few faint puncta are evidence that both *Piezo1* and *Piezo2* are expressed in auditory hair cells. Confirmation of the specificity of the probes would boost confidence in this result. While gene expression for transduction molecules may be low, it is detectable with modern methods. Currently, there is no compelling evidence for *Piezo1* expression in auditory hair cells. *Piezo2* does seem to be expressed transiently during the first postnatal week.

Protein localization

Second, protein components of the mechanosensory transduction complex must be localized correctly in the hair bundle, specifically, at the tips of shorter row stereocilia. Using iontophoretic application of transduction blockers or calcium imaging in hair cell stereocilia several groups have demonstrated localization of sensory transduction channels to the tips of hair cell stereocilia (Jaramillo and Hudspeth, 1991; Denk et al, 1995; Lumpkin and Hudspeth, 1995). With higher resolution, Beurg et al. (2009) convincingly showed channel localization at the

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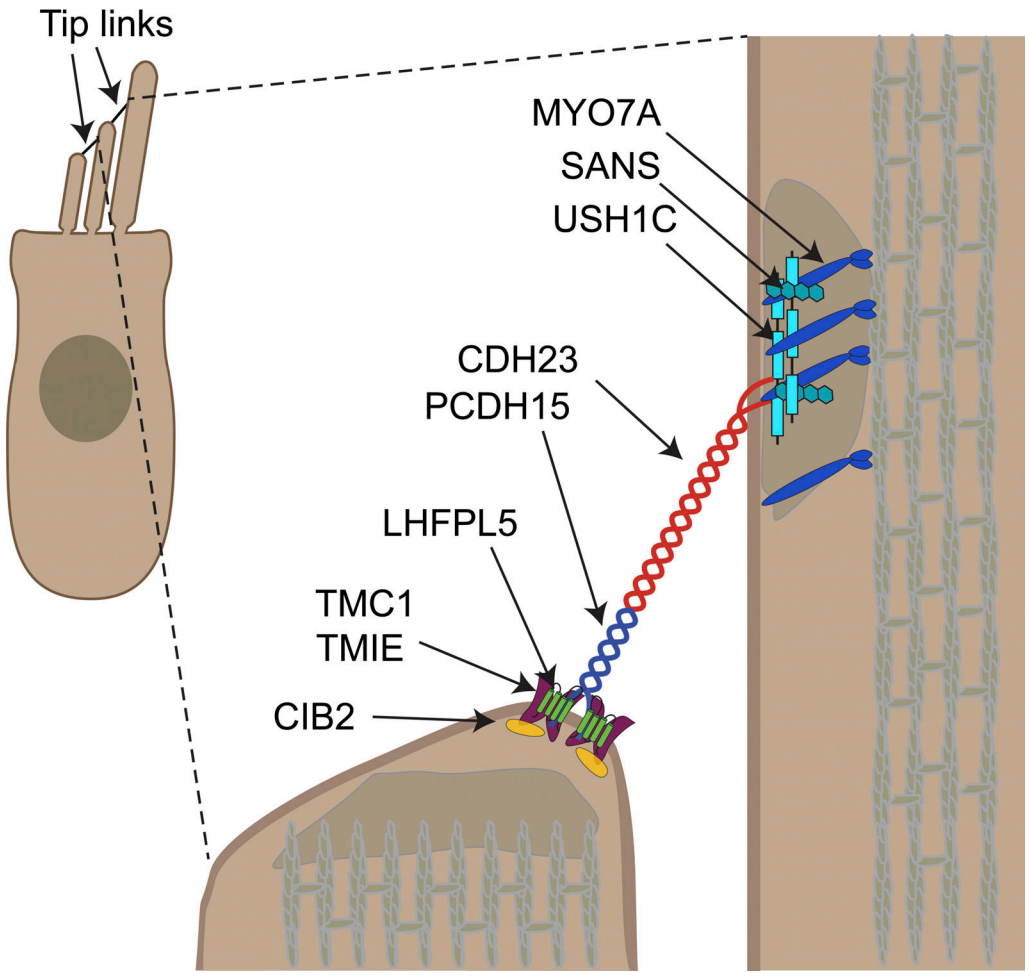

Figure 1. **Schematic diagram of an auditory hair cell (inset) with an expanded view of a single pair of stereocilia (right).** Reprinted from Qiu and Müller (2022). Essential and well-established components of the sensory transduction apparatus are labelled, including myosin 7a (MYO7A), harmonin (USH1C), sans (USH1G), cadherin 23 (CDH23), protocadherin 15 (PCDH15), lipoma HMGIC fusion partner-like 5 (LHFPL5), transmembrane inner ear protein (TMIE), transmembrane channel-like 1 (TMC1), and calcium and integrin binding protein 2 (CIB2).

tips of the second and third rows of stereocilia, at the lower end of tip links. TMC localization is consistent with these patterns. Using viral delivery of TMC coding sequences in frame with coding sequences for fluorescent proteins or a 3xFlag tag, TMCs 1 and 2 were localized to the tips of hair bundles (Kawashima et al., 2011; Askew et al., 2015). Kurima et al. (2015) used a similar approach, but rather than viral delivery, they generated transgenic mice with TMC1 fused to mCherry and TMC2 fused to GFP and observed native fluorescence at the tips of second and third row stereocilia, consistent with the Beurg et al. (2009) data. Two groups have shown anti-TMC1 antibody localization at hair bundle tips and, importantly, a lack of staining in TMC1 knockout controls (Beurg et al., 2015; Giese et al., 2017). Lastly, the Müller group generated transgenic mice with TMC1 fused to an HA tag and TMC2 fused to a MYC tag and reported robust punctate localization at stereocilia tips in two publications (Liang et al., 2021; Qiu et al., 2023). Thus, using four different localization strategies, six different groups have reported localization of TMC proteins in the right place at the right time, greatly boosting confidence in this conclusion. Similarly, several laboratories have localized TMIE and CIB2 to the tips of stereocilia in hair cells (Riazuddin et al., 2012; Wang et al., 2017; Giese et al., 2017; Michel et al., 2017; Liang et al., 2021; Cunningham et al., 2020; Zhao et al., 2014).

Localization data for PIEZO proteins in hair bundles are limited. Wu et al. (2017b) reported no expression of *Piezo1* mRNA and no PIEZO1 protein localization in hair bundles. They did find PIEZO2 protein in hair cells but not in hair bundles. The staining was localized to the apical surface of the hair cell, near the convex side of the hair bundle base (Wu et al., 2017b). Importantly, the staining was absent in *Piezo2* knockout controls. Lee et al. (2024) generated knock-in mice with PIEZO1 fused to GFP and PIEZO2 fused to tdTomato. Faint puncta were distributed somewhat randomly throughout the hair bundle but were not concentrated at hair bundle tips, the site of sensory transduction. Control data using the same localization and imaging approach in *Piezo* knockout mice or wild-type mice that lacked the fusion constructs were not presented but, if available, could boost confidence in the Lee et al. (2024) suggestion that PIEZOs are present in hair bundles. Although detection of faint fluorescence

from just a few transduction molecules at stereocilia tips is challenging, the current lack of compelling data indicates this criterion remains unfulfilled for PIEZO proteins.

Genetic deletion

A third criterion is loss of sensory transduction with genetic deletion of the candidate proteins. For TMCs, eight publications report consistent results demonstrating that deletion of *Tmc1* and *Tmc2* results in complete loss of sensory transduction in auditory and vestibular hair cells throughout the inner ear at all developmental stages tested (Kawashima et al., 2011; Pan et al., 2013, 2018; Kim et al., 2013; Beurg et al., 2014; Askew et al., 2015; Nist-Lund et al., 2019; Cunningham et al., 2020). Similar results have been reported for zebrafish, where mutations in *tmcs* disrupt sensory transduction in auditory, vestibular, and lateral line hair cells (Chou et al., 2017; Erickson et al., 2017; Smith et al., 2020). The same also holds true for *Tmie* and *Cib2*, where several groups have reported that mutations disrupt sensory transduction in mice and zebrafish (Zhao et al., 2014; Cunningham et al., 2020; Pacentine and Nicolson, 2019; Gleason et al., 2009; Wang et al., 2017, 2023; Michel et al., 2017; Giese et al., 2017). There are no publications reporting contrary results. Thus, there is little doubt about the conclusion that expression of either TMC1 or TMC2, as well as TMIE and CIB2, are necessary for sensory transduction in vertebrate hair cells.

Conditional deletion of floxed *Piezo1*, *Piezo2*, or both in mice expressing an inner ear–specific *Pax2-Cre* yielded no deficit in hair cell sensory transduction (Wu et al., 2017b). Interestingly, *Piezo2* expression was found to be associated with anomalous mechanotransduction currents evoked by mechanical stimulation of the hair-cell cell-body (Beurg et al., 2016) in a similar location to where Wu et al. (2017b) detected PIEZO2 protein. The current was observed only during the first postnatal week, coincident with the transient expression of *Piezo2* during this time frame. Conditional deletion of *Piezo2* abolished the anomalous current, solidly linking *Piezo2* with this current and confirming *Pax2-Cre* effectively excised *Piezo2* (Beurg and Fettiplace, 2017). Conditional knockout of *Piezo2* was necessary because of the lethality of the full knockout due to effects on lung function. However, recordings of transduction currents from *Piezo2* full knockouts were possible immediately after birth and resulted in abolition of the anomalous current but no effect on conventional hair cell transduction currents (Beurg and Fettiplace 2017). This laid to rest any concern regarding incomplete efficacy of *Pax2-Cre* in the *Piezo2* conditional knockout. Lee et al. (2024) generated mice conditionally deficient in *Piezo1* and *Piezo2* but did not present transduction currents recorded from those mice. Thus, there is no evidence suggesting that deletion of either *Piezo1*, *Piezo2*, or both results in a deficit in hair cell sensory transduction.

Point mutations

Fourth, while genetic deletions linked to loss of function can establish the necessity of a gene or protein for a biological process, perhaps a stronger criterion is a change in function that results from more subtle genetic manipulations. For TMC1, the point mutation p.M412K, termed *Beethoven* (Vreugde et al., 2002), was found by three different groups to reduce calcium selectivity (Pan et al., 2013; Beurg et al., 2015; Corns et al., 2016), strongly linking TMC1 with pore properties of hair cell transduction. Beurg et al. (2019), (2021) went on to examine six additional mouse lines, each bearing unique point mutations in TMC1 and reported those substitutions also changed permeation properties.

The strongest evidence linking TMC1 with the pore of the hair cell sensory transduction channel was generated using a chemical-genetic approach (Pan et al., 2018). 17 amino acids within transmembrane domains 4–8 were selected for cysteine substitution. The TMC coding sequence was packaged into AAV vectors, which were introduced into the inner ears of *Tmc1/Tmc2* double knockout mice. 16 of the constructs yielded robust transduction currents. Application of cysteine modification reagents reduced current amplitudes for five constructs. 11 constructs led to reduced calcium selectivity. Importantly, the effect of cysteine modification reagents was inhibited by transduction channel closure or by pre-incubation with established open channel blockers, which confirmed the cysteine substitutions were within the pore region of the channel. Three research groups, six publications, and 20 different point mutations have yielded data showing reduced current amplitude, reduced calcium selectivity or both, strongly supporting TMC1 as a pore-forming subunit of the hair cell transduction channel. Notably, a point mutation in TMIE has also been shown to affect conductance and ion selectivity of hair cell transduction channels (Cunningham et al., 2020), which further supports the argument that both TMC1 and TMIE are essential components of the ion channel complex.

There are no reports of single point mutations in either PIEZO1 or PIEZO2 that alter the pore properties of hair cell transduction. Lee et al. (2024) did report PIEZO constructs that included four mutations in the C-terminal domain of PIEZO1 or 2 which when overexpressed in hair cells disrupted transduction current amplitudes. They argue that the reduced currents provide evidence that the mutant constructs co-assemble with endogenous PIEZOs in the native transduction complex in mouse hair cells. However, they did not examine whether overexpression of the exogenous mutant PIEZO proteins caused developmental consequences, altered the localization or function of TMC1 or TMC2, or affected hair cells in other non-specific ways.

Auditory function in mice and humans

Genetic disruption of *Tmc1* causes profound deafness in mice (Kurima et al., 2002; Vreugde et al., 2002; Marcotti et al., 2006) and double knockout of *Tmc1* and *Tmc2* causes complete loss of auditory and vestibular function (Kawashima et al., 2011). *Tmc2* deletion alone does not cause auditory dysfunction, but does yield mild vestibular dysfunction, suggesting a role for *Tmc2* in semicircular canal function (Kawashima et al., 2011; Ratzen et al., 2024). 10 unique mouse lines with *Tmc1* mutations have been reported which also have complete loss of auditory function (Vreugde et al., 2002; Marcotti et al., 2006; Kawashima et al., 2011; Manji et al., 2012; Beurg et al., 2019, 2021; Marcovich et al., 2022). In addition, restoration of *Tmc1*

Table 1. **Essential criteria for components of the hair cell sensory transduction channel complex are satisfied for TMC proteins (+) but not for PIEZO proteins (–)**

Criteria	TMC1	TMC2	PIEZO1	PIEZO2
Gene expression in neonatal hair cells	+	+	–	+
Gene expression in adult hair cells	+	–	–	–
Protein localization at hair bundle tips	+	+	–	–
Loss of sensory transduction in KOs	+	+	–	–
Point mutations alter channel properties	+	+	?	?
Loss of auditory function in mice	+	–	–	–
Loss of auditory function in humans	+	–	–	–

expression via gene replacement therapy for recessive mutations (Askew et al., 2015; Nist-Lund et al., 2019; Wu et al., 2021; Marcovich et al., 2022) or genome editing for dominant mutations (Gao et al., 2018; Gyorgy et al., 2019; Yeh et al., 2020; Wu et al., 2021) restores auditory function in deaf *Tmc1* mutant mice.

Conditional deletion of *Piezo1* in the inner ear does not cause auditory dysfunction (Wu et al., 2017b). Wu et al. (2017b) do report mild elevation (10–20 dB) of auditory brainstem response (ABR) thresholds at three of seven frequencies tested in mice with conditional inner ear deletion of *Piezo2*. However, this result was not investigated by Lee et al. (2024) who instead opted to generate transgenic mice overexpressing a mutant form of *Piezo1* or *Piezo2*. The *Piezo* mutants included an AAAA substitution, previously reported as nonfunctional (Zhao et al., 2016). When overexpressed in the inner ear via a constitutively active CAG promoter, Lee et al. (2024) reported elevated ABR thresholds in response to broadband click stimulation. However, the elevation of ABR thresholds progressed overtime from 4 to 12 wk of age and paralleled degeneration of sensory hair cells in the transgenic mice, raising the possibility that the loss of auditory function was a consequence of a non-specific degenerative effect of overexpression of the mutant constructs. The level of overexpression was not quantified despite the inclusion of a GFP tag on the C-terminus of the mutant constructs. Potential consequences of these *Piezo* mutants, such as developmental effects, on the expression of TMC1, TMIE, and CIB2 were not examined.

In humans, over 100 genetic mutations in *TMC1* have been identified that cause hereditary hearing loss (reviewed in Jung and Müller [2023]; genome variation database: http://dgv.tcag.ca/dgv/app/home). Both dominant and recessive mutations have been identified. Recessive loss of function mutations typically cause profound, congenital deafness, while dominant *TMC1* mutations tend to cause progressive hearing loss. In addition, numerous genetic mutations have been identified in each of the other components of the hair cell transduction apparatus, *MYO7A*, *USH1C*, *CDH23*, *PCDH15*, *LHFPL5*, *TMIE*, and *CIB2*, all of which cause deafness in mice and humans (reviewed in Jung and Müller [2023]; genome variation database: http://dgv.tcag.ca/dgv/app/home). However, while humans with homozygous loss-of-function mutations in *PIEZO1* and *PIEZO2* have been identified with a range of hereditary disorders, hearing loss is not associated with any of these genetic mutations, suggesting that PIEZOs are not essential for human hearing.

Summary and conclusions

PIEZO proteins have been extensively studied in other systems and play critical roles in the sense of touch and other forms of mechanotransduction, yet there is little evidence suggesting PIEZOs play an essential role in the auditory system. Thus far, neither PIEZO1 nor PIEZO2 have met basic criteria required for other bona fide components of the hair cell mechanosensory transduction apparatus (Table 1). As outlined above, there is now abundant and rigorous evidence supporting the molecular identity of 8 to 10 different protein components of the hair cell mechanosensory transduction apparatus. While the identity of these components remained a mystery for decades, recent advances, fueled by genetic studies in humans and functional investigations in mouse and zebrafish models, have propelled the field forward. These studies have led to the identification of core-components of the sensory transduction apparatus in auditory hair cells, including molecular components of the tip–link complex (PCDH15, CDH23, LHFPL5) and of the sensory transduction channel (TMC1/2, TMIE, CIB2; Fig. 1). The genetic studies are well supported by functional studies and biochemical studies, which have demonstrated that the proteins of the transduction apparatus directly bind to each other (reviewed in Qiu and Müller [2022]; Holt et al. [2021]).

Remarkably, TMC-dependent ion channel complexes are also expressed in invertebrates. The structures of the native ion channel complexes, purified from *Caenorhabditis elegans*, were solved by cryo-electron microscopy (Jeong et al., 2022; Clark et al., 2024). The native channels consist of TMCs, TMIE, and a CIB2 homolog, CALM-1, which assemble into an ion channel complex with striking twofold symmetry. A similar twofold symmetry is predicted for the sensory transduction complex in mammalian hair cells (Pan et al., 2018; Ballesteros et al., 2018). Collectively, these findings provide strong evidence that this ion channel complex is an evolutionary invention that predates the emergence of mammals but is ideally suited for mechanosensory transduction enabling the sense of hearing.

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