

Who needs tip links? Backwards transduction by hair cells

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The molecular identity of the channel that mediates mechanotransduction by hair cells remains uncertain, despite being biophysically characterized since the late 1970s (Corey and Hudspeth, 1979). Two recent reports from Jeffrey Holt, Gwenaelle Géléoc, Andrew Griffith, and their colleagues suggested that the hair cell's transduction channel is made up of members of the transmembrane channel (TMC)-like family of membrane proteins (Kawashima et al., 2011; Pan et al., 2013) (Fig. 1 A). They showed that the TMC paralogs TMC1 and TMC2 are selectively expressed in the inner ear, appearing at the onset of transduction, and that at least one of them must be expressed for normal mechanotransduction. In this issue of the *JGP*, Robert Fettiplace and colleagues (Kim et al.) challenge the Holt–Géléoc–Griffith model, suggesting instead that the TMCs do not make up the transduction channel but instead couple those channels to tip links, the mechanical elements that impart directional sensitivity to hair cells. Examining transduction in a *Tmc1;Tmc2* double mutant, they found persistence of a conductance with properties very similar to those of the transduction channel, except that it is activated by mechanical deflections of the opposite polarity. This surprising result raises the possibility that the transduction channel is a membrane protein distinct from TMC1 and TMC2 that only becomes functional as the transduction channel once other key molecules, like the TMCs and tip-link cadherins, are expressed. Nevertheless, a few reservations remain about this interpretation, and the conclusions are not as clear-cut as Kim et al. (2013) imply.

In wild-type hair cells, the transduction channel is located in the hair bundle, the mechanically sensitive structure decorating the apical surface of the cell. The bundle is composed of a single asymmetrically located kinocilium, an axonemal cilium, as well as dozens of actin-filled stereocilia arranged in rows of increasing height. External stimuli like sound deflect the bundle, which leads to the opening of transduction channels when the deflection is toward the taller stereocilia, referred to as a positive stimulus. Many biophysical features of the channel are known (Gillespie and Müller, 2009); for example, it is a nonselective cation channel (reversal potential near zero under normal ionic conditions),

with a modestly elevated calcium permeability (P_{Ca}/P_{Cs} of ~ 5). At 100–300 pS, its single-channel conductance is large; the large conductance compensates for the scarcity of channels, as only one to two active channels are present in each stereocilium. The tip link, a narrow (~ 8 -nm) and long (~ 150 -nm) extracellular filament, runs from each short stereocilium to its tallest neighbor, parallel to the bundle's plane of mirror symmetry; all tip links are located along the axis of mechanical sensitivity. Tip links must be present for gating of the transduction channel (Assad et al., 1991). Bundle deflection increases tip-link tension, which opens transduction channels either directly through protein–protein interactions or indirectly by stretching the membrane at the tip of stereocilia.

Many key molecules participating in mechanotransduction have been identified, mostly through genetics (Fig. 1). For example, the tip link is composed of a dimer of cadherin-23 (CDH23) molecules that interact end-to-end with a dimer of protocadherin-15 (PCDH15) molecules (Kazmierczak et al., 2007). The PCDH15 dimer is located at the base of the tip link, anchored in the tip membrane of the shorter stereocilium. Calcium imaging experiments demonstrated convincingly that channels are associated with the PCDH15 end of the tip link (Beurg et al., 2009), suggesting that PCDH15 interacts directly or indirectly with the transduction channel. Other transduction molecules identified through genetics (e.g., USH1C, USH1G, and MYO7A) apparently are located at the other end of the tip link, associated with CDH23 (Gillespie and Müller, 2009). One exception is tetraspanin protein lipoma HMGIC fusion partner-like 5 (LHFPL5/TMHS), which coimmunoprecipitates with PCDH15 in cell lines; knockout of *Tmhs* alters PCDH15 targeting and affects the conductance properties of the transduction channel in mouse outer hair cells (Xiong et al., 2012). However, TMHS is proposed to be an auxiliary component rather than a pore-forming subunit of the transduction channel (Xiong et al., 2012).

Assembly of the transduction apparatus during development is complex. In zebrafish, newly formed hair cells initially respond by opening transduction channels

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when hair bundles are stimulated in the negative direction, i.e., away from the kinocilium (Kindt et al., 2012). As development proceeds, the response polarity flips to its final state in which positive stimuli open channels; in the middle, the polarity can switch back and forth. Kindt et al. (2012) showed that kinocilia are necessary for this reverse-polarity response. In addition, the reverse-polarity response requires the tip-link proteins Pcdh15 and Cdh23 and shows sensitivity to known antagonists of hair cell transduction channels. Similarly, in early postnatal rat outer hair cells, transduction is activated by bundle stimuli of either normal or reverse polarity (Waguespack et al., 2007).

Despite the progress just cited in identifying transduction components and describing the development of the transduction complex, a major question for the field has remained unanswered: what is the molecular identity of the transduction channel itself? The TMC molecules are only the most recent of many that have been advanced. The TMC family has eight members, each with multiple (6–10) predicted transmembrane domains (Keresztes et al., 2003; Kurima et al., 2003); epitope tagging experiments suggest that TMC1 adopts a six-transmembrane domain structure (Labay et al., 2010). Although none of the mammalian TMCs have been shown to conduct ions, the TMC1 structure is certainly reminiscent of that of other ion channels.

Evidence for the TMCs being part of the transduction complex is strong; expression of the TMCs correlates with the onset of transduction in mouse cochlea and utricle, hair cells lack mechanotransduction in double knockouts of *Tmc1* and *Tmc2* (*Tmc1^{Δ/Δ};Tmc2^{Δ/Δ}*), and the Beethoven point mutant of *Tmc1* changes both the calcium permeability and the single-channel conductance of the transduction current (Kawashima et al., 2011; Pan et al., 2013). As argued elsewhere (Morgan and Barr-Gillespie, 2013), these data fall short of conclusive

proof that the TMCs form the conductance pathway of the transduction channel. Nevertheless, the TMCs certainly are critically important for hair cell mechanotransduction, regardless of whether they form the channel pore itself.

Using the *deafness* (*dn*) recessive allele of *Tmc1*, which causes an in-frame deletion, and a different knockout of *Tmc2* than the one used by the Holt–Géléoc–Griffith group, Kim and Fettiplace (2013) showed that the relative calcium permeability of transduction channels in *Tmc1* and *Tmc2* single mutants differed substantially, and that these differences correspond to apical-basal gradients of *Tmc1* expression (Kawashima et al., 2011) and the wild-type variance in calcium permeability in outer hair cells of the mouse cochlea (Kim and Fettiplace, 2013). These data are consistent with the idea that TMCs form the transduction channel pore, and that TMC1 is predominant at the basal (high frequency) end of the cochlea.

However, just as the idea that the TMCs are good candidates for the mechanotransduction channel has begun to sink in, Kim et al. (2013) have thrown a wrench into the works. They generated a double mutant (*Tmc1^{dn/dn};Tmc2^{Δ/Δ}*) of the alleles used in their previous study and characterized the transduction properties of its hair cells. In contrast to the results of the Holt–Géléoc–Griffith group (Pan et al., 2013), *Tmc1^{dn/dn};Tmc2^{Δ/Δ}* hair cells could be activated by mechanical displacements; using a fluid jet stimulator to deliver a sinusoidal stimulus, outer hair cells from the double mutant displayed mechanotransduction but required a substantial stimulus amplitude for activation. Remarkably, in P4 (postnatal day 4) to P8 hair cells, the conductance was only activated by reverse-polarity stimuli, i.e., stimuli that normally close channels in wild-type hair cells. Although it is not clear whether the reverse-polarity current studied by Kim et al. (2013) is the same as that appearing during

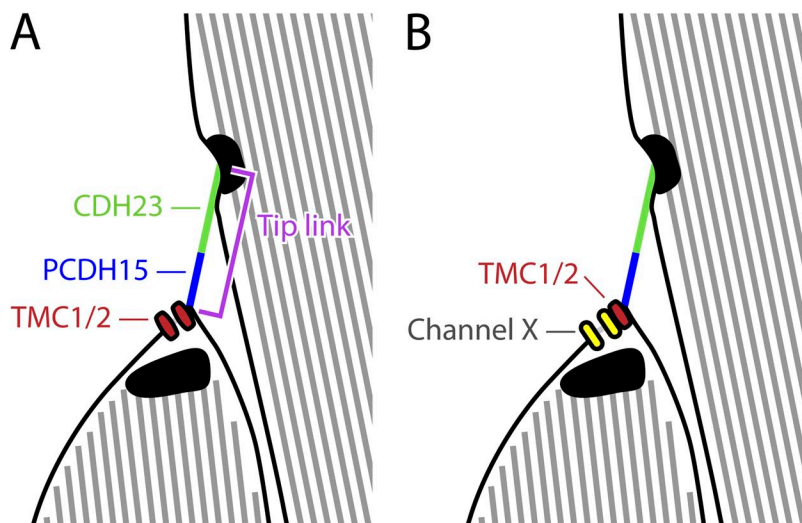


Figure 1. Molecular anatomy of the transduction apparatus. (A) Model favored by the Holt–Géléoc–Griffith group, with TMC proteins forming the transduction channel pore. In this model, the PCDH15 end of the tip link interacts directly with TMC proteins. (B) Model consistent with the data of Kim et al. (2013), with TMC proteins coupling the tip link to the transduction channel. The interaction of PCDH15 is still with TMCs; when either tip links or TMCs are disrupted, the channel is no longer anchored at stereocilia tips and could move to a new location where reverse-polarity activation is possible.

zebrafish development (Kindt et al., 2012), it is telling that both currents appear to be present only during early development.

The reverse-polarity current has similarities to transduction currents in wild-type hair cells: it has roughly the same total conductance per cell; it has a large single-channel conductance; it is blocked by Ca^{2+} , streptomycin, and FM1-43; and it has a similar $P_{\text{Ca}}/P_{\text{Cs}}$. There are major differences, however, between the two conductances: the reverse-polarity conductance requires large stimuli to even begin to evoke it, its onset is slower, it appears to inactivate instead of adapt, and it is not sensitive to tip-link breakage by calcium chelators.

This latter observation is crucial. Tip links are abolished by extracellular BAPTA treatment (Assad et al., 1991), and the insensitivity of the reverse-polarity current to BAPTA suggests that tip links are not involved in its gating. Although not fully explored, and activated mainly at positive voltages, similar reverse-polarity currents were reported in mouse hair cells expressing strong alleles of *Pcdh15* and *Cdh23* (Alagramam et al., 2011); these mutant hair cells lack normal tip links. Remarkably, BAPTA treatment of P0–P2 hair cells of wild-type mice also uncovered a reverse-polarity current, which developed over the span of ~ 5 min (Kim et al., 2013). Either a new conductance was unmasked over this time frame or the authentic transduction channel relocated so that it now could be activated by inhibitory stimuli.

The novelty of tip link-free mechanotransduction in hair cells raises important questions about the data. Only one other report using adult outer hair cells showed that receptor currents could occur after BAPTA treatment, although the currents were tonic, suggesting that channels were stuck open (Meyer et al., 1998). Importantly, the present study by Kim et al. (2013) did not demonstrate saturation of the reverse-polarity current. Although the inhibitor experiments largely rule out most artifacts, such as mechanically activated current leak around the recording electrode, the current must eventually saturate if it passes through a discrete channel. The size of the reverse-polarity current was also unusually variable. Finally, further mapping of stimulus polarity would have been useful; do hair cells respond to orthogonal stimuli?

Considering all of the data and potential caveats, we can think of three broad interpretations of the data:

Loss of TMC proteins in the double mutant converts the transduction channel so that it now can be activated by reverse-polarity stimuli. This is the interpretation favored by the authors. In this case, wild-type TMC proteins are important for coupling the tip link to the transduction channel but are not the channel itself (Fig. 1 B); in the absence of TMCs, channels relocate so that they are activated by stimuli of the opposite polarity and have an altered calcium permeability. As noted above, *Pcdh15* and *Cdh23* mouse mutants that lack tip links

display a similar phenotype. Although TMC proteins and the transduction channel are presumably present in these mutants, tip links are not, and so the channel may adopt a similar reverse-polarity state. In this scenario, the changes in calcium permeability seen in single or double *Tmc1* and *Tmc2* mutants would not be directly caused by changes in a pore formed by the TMCs, as suggested by Pan et al. (2013); rather, the data suggest that when TMCs bind the channel, they influence the pore's properties, similar to TMHS (Xiong et al., 2012).

A different channel is unmasked in the double mutant. Although the properties of the reverse-polarity current are indeed similar to those of the transduction channel, the criteria used (block by Ca^{2+} , streptomycin, and FM1-43) are not stringent, as other channels could have a similar inhibition profile. Many channels show stretch activation, e.g., activation by lateral membrane tension, and it is plausible that a distinct channel appears under the conditions favoring the reverse-polarity conductance. In this interpretation, the TMCs make up the native transduction pore. The observation that the “destruction” of the hair bundle has no effect on the reverse-polarity current suggests that a type of stretch activation, albeit with some form of directional sensitivity, may be in play.

The dn allele is not a null, and a TMC1 mutant channel remains. Critically, whether *Tmc1^{dn}* is a null allele has not been established; the mutation leads to an in-frame deletion of 57 amino acids in a large intracellular loop, and this deletion might not prevent protein expression. This interpretation suggests that the protein product of the *Tmc1^{dn}* allele cannot couple to tip links, but TMC1-mediated transduction currents nonetheless remain and produce the reverse-polarity conductance. The likelihood of this interpretation is reduced by the observation that in the *Tmc1^{dn/dn};Tmc2^{+/+}* single mutant, Fettiplace and colleagues (Kim and Fettiplace, 2013; Kim et al., 2013) see similar changes in calcium permeability in inner hair cells, as do the Holt–Géléoc–Griffith group with the *Tmc1^{Δ/Δ};Tmc2^{+/+}* mutant (Pan et al., 2013). The similarity in calcium permeability suggests that the two *Tmc1* alleles are equivalent. Direct comparison of hair cells from *dn/dn* and knockout mice using the same stimulus and conditions would be required to tease out any subtle differences between these two genotypes. Nevertheless, whether the *dn* allele of *Tmc1* is a null mutation and whether *Tmc1^{Δ/Δ};Tmc2^{Δ/Δ}* hair cells have a reverse-polarity conductance must be investigated in the near future.

Where do we go from here?

Full tests of any of these hypotheses should address two key questions: what masks the reverse-polarity

conductance in wild-type hair cells, and how is the conductance unidirectionally activated? The first and third hypotheses negate the masking issue by suggesting that the loss of wild-type TMCs uncouples the transduction channel (whether TMC1 or another channel) from the tip link. In contrast, the new-channel hypothesis explains what is seen in extreme conditions (complete loss of tip links) or at very early stages of development, where reversed and mixed polarity responses are present.

How the reverse-polarity response is activated directionally and opposite to normal transduction remains baffling. Kim et al. (2013) emphasize that the reverse-polarity current remains substantial even after badly damaging the hair bundle, damage that presumably includes splayed stereocilia that lack filamentous interconnections (Fig. 2). This observation suggests that rather than being gated by links between stereocilia, as are channels in wild-type hair cells, channels are activated by bending the stereocilia in parallel or by deflecting the kinocilium, which remains present in mouse outer hair cells before P10 (Sobkowitz et al., 1995).

If independent stimulation of stereocilia or the kinocilium is required for activation of the reverse-polarity current, bending forces are likely to be largest at the bases (Fig. 2). When the cilia are deflected, channels in these regions could be activated by interaction with other apical membrane proteins or by lateral membrane tension. In either case, the channels or the activation mechanism must be asymmetrically localized in the hair cell's apical membrane or cilia. Moreover, if the

reverse-polarity conductance is derived from the native transduction channel and channels are located at ciliary bases, then upon BAPTA treatment, channels must move from stereocilia tips to their bases in a few minutes. As membrane diffusion is unlikely to move channels this fast, active transport by minus end-directed myosin VI motors may mediate this redistribution. The hypothesis of a stretch-activated channel with similar pharmacological sensitivities as the transduction channel may not require transport to the asymmetric location, but the chelation of calcium is still required to unmask its activity. These hypotheses beg for experimental investigation.

Localization of the channels on one side of the kinocilium is an intriguing model (Fig. 2). Molecular clues suggest that hair cells are derived from an ancestor cell that used microtubule-based mechanotransduction (Bermingham et al., 1999; Senthilan et al., 2012), and asymmetric localization of proteins around the kinocilium is a possibility, as this structure demonstrably responds to the planar cell polarity signals that orient hair cells (Grimsley-Myers and Chen, 2013). Moreover, asymmetry in the relationship between the kinocilium and apical structures was originally used to explain directional sensitivity of hair bundles (Hillman, 1969). In this proposal, kinocilia coupled to stereocilia in an intact hair bundle “plunge” into the cell body when deflected in the positive direction, opening channels located at the kinocilium base. Although this model does not explain wild-type transduction (Hudspeth, 1982), it could apply

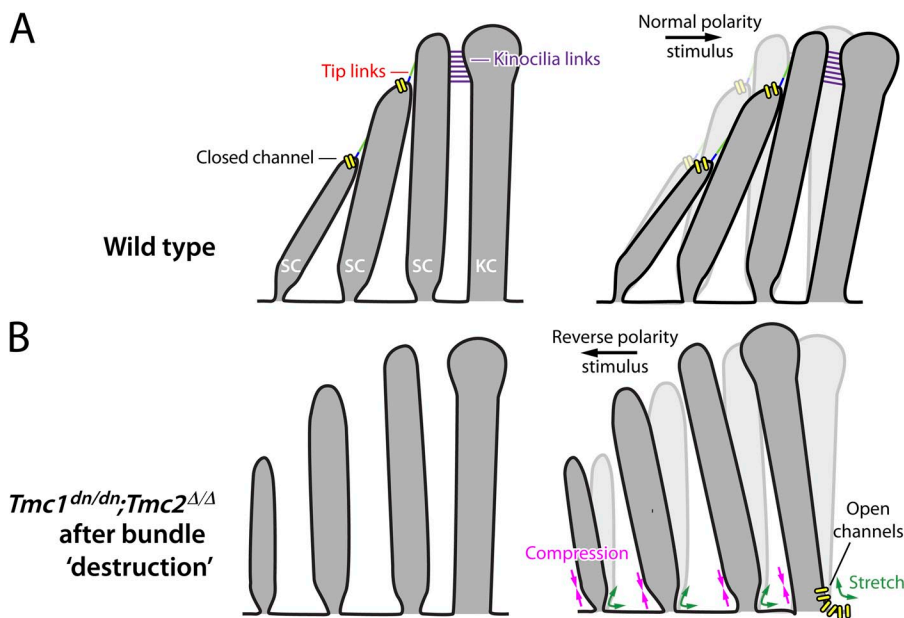


Figure 2. Stimulation of wild-type and double mutant hair bundles. (A) Wild-type hair bundle. Stimulation of the bundle in the positive direction (right; “Normal polarity stimulus”) puts tension on tip links, which tug open transduction channels. SC, stereocilium; KC, kinocilium. (B) *Tmc1^{dn/dn};Tmc2^{Δ/Δ}* hair bundle after stimulus-induced bundle “destruction.” Although not described in detail in Kim et al. (2013), we presume that means a bundle that has lost all connections between its cilia. Stimulation of the bundle in the negative direction with a fluid jet stimulator (right; “reverse polarity stimulus”) leads to stretching on the positive side of each stereocilium and the kinocilium and compression on the negative side. Here, channels are depicted as localizing only to the kinocilia base, although there is no direct evidence for this model. Channels could be located in the stereocilia bases as well. In addition, this diagram illustrates channel activation as occurring through membrane stretch, although it is plausible that compression could open channels as well.

to the case of *Tmc1*;*Tmc2* double mutants. Here, rotation of the kinocilium in the negative direction (reverse polarity) would stretch the apical membrane between the kinocilium and cell junctions, leading to activation of channels located there. Although we are unaware of any reports of asymmetry in the distribution of proteins adjacent to the kinocilium, this location is at least a plausible one that could explain many of the surprising data. Zebrafish mutants lacking kinocilia no longer respond to negative deflections (Kindt et al., 2012), so examination of transduction currents in triple mouse mutants lacking kinocilia (Jones et al., 2008) and without *Tmc1* and *Tmc2* expression could test this model. Another experimental test of the model would be to carry out calcium imaging near the base of kinocilia.

The results from Kim et al. (2013) are fascinating and, if confirmed, could assist in the definitive identification of the transduction channel. If the channel is composed of TMC subunits, it becomes more important than ever to identify the pore region and mutate amino acids within these proteins; moreover, whether or not TMC1 is the channel, it will be crucial to describe the nature of the protein expressed by the *dn Tmc1* allele. In contrast, if the channel is not a TMC protein, the present results suggest that the authentic channel nevertheless interacts with the TMCs, an observation that could assist in the channel's identification. Moreover, the data suggest that the TMCs interact directly or indirectly with PCDH15 at the tip-link base, which provides further hints as to the molecular makeup of the transduction apparatus. Whether the TMCs are the transduction channel or not, it is clear that they play a central role in organizing the mechanotransduction complex of hair cells.

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