INVITED REVIEW

The how and why of identifying the hair cell mechano-electrical transduction channel

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Abstract Identification of the auditory hair cell mechano-electrical transduction (hcMET) channel has been a major focus in the hearing research field since the 1980s when direct mechanical gating of a transduction channel was proposed (Corey and Hudspeth J Neurosci 3:962–976, 1983). To this day, the molecular identity of this channel remains controversial. However, many of the hcMET channel's properties have been characterized, including pore properties, calcium-dependent ion permeability, rectification, and single channel conductance. At this point, elucidating the molecular identity of the hcMET channel will provide new tools for understanding the mechanotransduction process. This review discusses the significance of identifying the hcMET channel, the difficulties associated with that task, as well as the establishment of clear criteria for this identification. Finally, we discuss potential candidate channels in light of these criteria.

Keywords Mechanotransduction · Mechano-electrical transduction · Hair cell · Hair bundle · Hearing · MET channel · Auditory

Introduction

Mechanosensation is a primordial sense. Because sensing ones environment is fundamental to an organism's survival,

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several sensory modalities have developed based on mechanosensation. Touch, proprioception, perception of osmotic stresses, and, of course, hearing are a few examples of such sensory modalities. As we understand it, all of those sensations employ directly mechanically gated transduction channels, the mechano-electrical transduction (MET) channels. Some of these MET channels have been identified, such as the osmotic stress detectors MscL [15, 82, 83, 101] and MscS [6] in *Escherichia coli* or the MEC-4 touch receptor in *Caenorhabditis elegans* [13, 39, 73]. The channels piezo [24, 25], TREK-1 [1], and TRAAK [72] have been implicated in mechanosensitivity of mammalian dorsal root ganglia. None of these channels have to operate at frequencies required for mammalian hearing [93], which can reach as high as 150 kHz in dolphins [46]. As for MET channels implicated in hearing, NOMPC (TRPN1) in *Drosophila melanogaster* [29, 30, 112, 116] is currently supported by the strongest evidence. However, even though NOMPC fulfills all criteria for a bona fide transduction channel involved in gentle touch sensation [112], its function in hearing is still a matter of debate [60]. Several MET channel candidates important for mammalian hearing have been proposed, but none meets all requirements, as will be discussed later in this review.

In recent decades, many proteins required for hearing and more specifically hair cell mechanotransduction have been identified; among those identified are the tip link proteins (protocadherin 15 PCDH15 and cadherin 23 CDH23) [54]. Tip links connect the shorter rows of stereocilia to their next taller neighbor and relay mechanical forces resulting from a sheering motion between those stereocilia to the hair cell MET (hcMET) channels. Other proteins identified include harmonin [5], myosin VIIa [41], myosin 3 [87], whirlin [109], and numerous actin-binding proteins (see review [81] and proteomics screen from chicken utricle [99]). The loss of these molecules results in compromised mechanotransduction and a deterioration or even complete loss of hearing. Yet the

molecular identity of the hcMET channel remains unknown, even after three decades of intensive search. Many consider the hcMET channel as the "holy grail" of the hearing field. But is this molecule more important than other components such as the tip link proteins or any of the other molecules known to underlie genetic human diseases resulting in hearing loss? We would argue that the significance of the final identification of the hcMET channel has grown more because of the immense amount of effort and degree of difficulty in resolving the question than due to its pure scientific impact. That said, identifying the channel is important, as it will make a plethora of new experiments possible. For example, it will allow us to determine how the channel is gated and where specifically the channel is located to more directly probe the functioning of numerous accessory proteins thus far identified as well as to provide insight into how MET channels of different sensory modalities are related to one another. Assuming that the hcMET channels did not develop de novo but from existing MET channels of, i.e., touch, we could investigate how (if) those channels changed during evolution to achieve ever higher frequency sensitivity.

The auditory field has been searching for the molecular identity of the hcMET channel for more than three decades, and although a number of candidates have been proposed, the channel's identity remains elusive. Assessment of channel candidacy has varied based on each investigator's approach; some perceive localization and molecular interactions as critical, whereas others regard the biophysical correlation to native properties as the linchpin for identification. Attesting to the difficulty of identifying the unequivocal experiment is the fact that there are currently three molecules where data is remarkably similar in support of their involvement in mechanotransduction, transmembrane channel-like (TMC [10, 53, 57, 69, 76]), LHFPL5 (formerly known as TMHS [67, 98, 111]), and transmembrane inner ear (TMIE [38, 78, 100, 113]). Mutation or knockout of two of these molecules (TMC [76], LHFPL5 [111]) results in changes in single channel conductance, a property typically considered intrinsic to the channel, yet it is unclear whether these molecules can form an ion channel by themselves or interact with the hcMET channel. Thus, even a property typically considered intrinsic to the channel protein, like single channel conductance, must be interpreted carefully. This illustrates the technical difficulty and molecular complexity of hair cell mechanotransduction that makes it difficult for a single piece of data to conclusively prove that a given molecule is the transduction channel (see [21] and [7, 59]). It is much more likely that a series of investigations using multiple approaches will be necessary to substantiate channel identity. The following discusses the potential criteria necessary to identify this channel, how these criteria could be weighted, and the reasons why these criteria have been so difficult to meet.

Why is the hcMET channel so hard to find?

The sensory auditory organelle, the hair bundle, consists of specialized microvilli called stereocilia, connected by tip links at their top and side links along their side [50, 81]. Under normal conditions, hcMET channels are opened when stereocilia are deflected towards the tallest row [84, 85] (Fig. 1) of stereocilia. The resulting sheering motion between stereocilia is transferred into a force at the top of the next shorter row, mediated by the tip link [54, 84, 85]. The location of tip links and Ca^{2+} imaging suggest that functional hcMET channels are located near stereocilia tops (in all but the tallest row), thereby placing it in close proximity to PCDH15 at the bottom end of the tip link [9]. Whether the channel is directly or indirectly
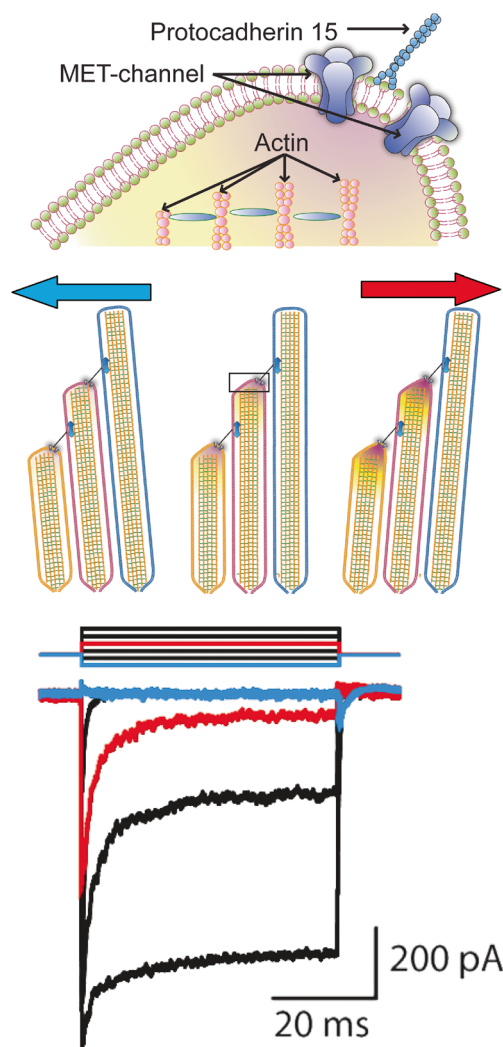
Fig. 1 MET current activation. The mammalian hcMET channels are thought to be located to the tip of stereocilia. Forces resulting from a deflection towards the shortest row (*blue arrow*) are thought to close hcMET channels while deflections towards the tallest row (*red arrow*) opens hcMET channels, allowing for the inflow of cations. Displacements of different amplitudes elicit currents of different sizes (*lower panel*). The tip link, comprised of protocadherin 15 and cadherin 23, is thought to be essential for the force relay to the hcMET channel

coupled to PCDH15 is unknown, making it difficult to design or interpret data that uses protein interactions assays. For example, can PCDH15 be used as bait to pull down the channel? This example exemplifies a common theme when it comes to hcMET channel identification, which is that there is so little data at the functional molecular level that critical assumptions need to be made at both the design and interpretation levels. The underlying assumptions required for data interpretation are discussed below along with each of the selected criteria for identification.

Further exacerbating channel identification, electrophysiological data indicates that there are as few as two functional channel proteins per stereocilium [8, 9, 26]. Consequently, there may only be about 200 active hcMET channel proteins in a given hair cell hair bundle. With approximately 16,000 hair cells per mammalian cochlea, this amounts to about 3.2 million proteins. Compared to the 640 billion copies of γ actin [99] and hundreds of billions of other proteins in the hair cell hair bundles alone, isolating the hcMET channel proteins is similar to finding a needle in a hay stack. If a large number of nonfunctional channels are present, the problem might not be quite as bad. However, if the channel's molecular components vary with frequency, as does the single channel conductance [8, 90], then the problem of identifying those varying components may become considerably more difficult. Thus, the limited data regarding the molecular interactions underlying mechanotransduction and the limited protein availability severely hamper attempts to identify the hcMET channel.

Is the hcMET channel unique to the ear?

Not knowing the molecular identity of the channel inspires much discussion as to how unique an entity this channel might be. Will it only be found in the ear? Does it have only one function? Is it inherently mechanosensitive? Properties of the hcMET channel suggest that it is specialized to operate at high frequencies [81] as compared to other mechanosensitive channels [15, 30, 39, 73, 83, 101, 112]. Other mechanosensory modalities operate at low frequency, at most a couple of hundred Hertz; the hcMET channel can detect signals over 100 kHz [46]. Are the kinetic differences between systems intrinsic to the MET channels or imparted by the microenvironment in which they reside? If the former is the case, then the hcMET channels likely represent a novel class of mechanosensitive channels; if the latter is the case, it is possible that a specialized environment surrounding a more conventional MET channel is responsible for the frequency sensitivities. Hereto, the answer has consequences towards channel identification.

If the microenvironment dictates the channel properties, then its removal from the native stereocilia environment may result in a functionally different hcMET channel, making identification much more difficult. If so, methods could be devised in which "hair cell-like" cells (where morphology, proteome, and functionality resemble auditory hair cells) are used as the template from which to investigate hcMET channel candidates. These cells would emulate the natural microenvironment more closely, perhaps providing as yet undescribed factors to allow functional expression. Previous attempts to generate hair cell-like cells resulted in very low yields, rendering them presently nonpractical [44, 63, 75]. Alternatively, cell lines such as COS-7 [97] could be used. Compounding this problem is the possibility that the channel is a heteromeric complex of different proteins so that expressing a single subunit in a heterologous system will not produce native channel properties [27, 28]. Not knowing these answers makes designing experiments and interpreting results difficult. How much one values these questions and associated criteria will shape how one interprets data regarding potential channel identity.
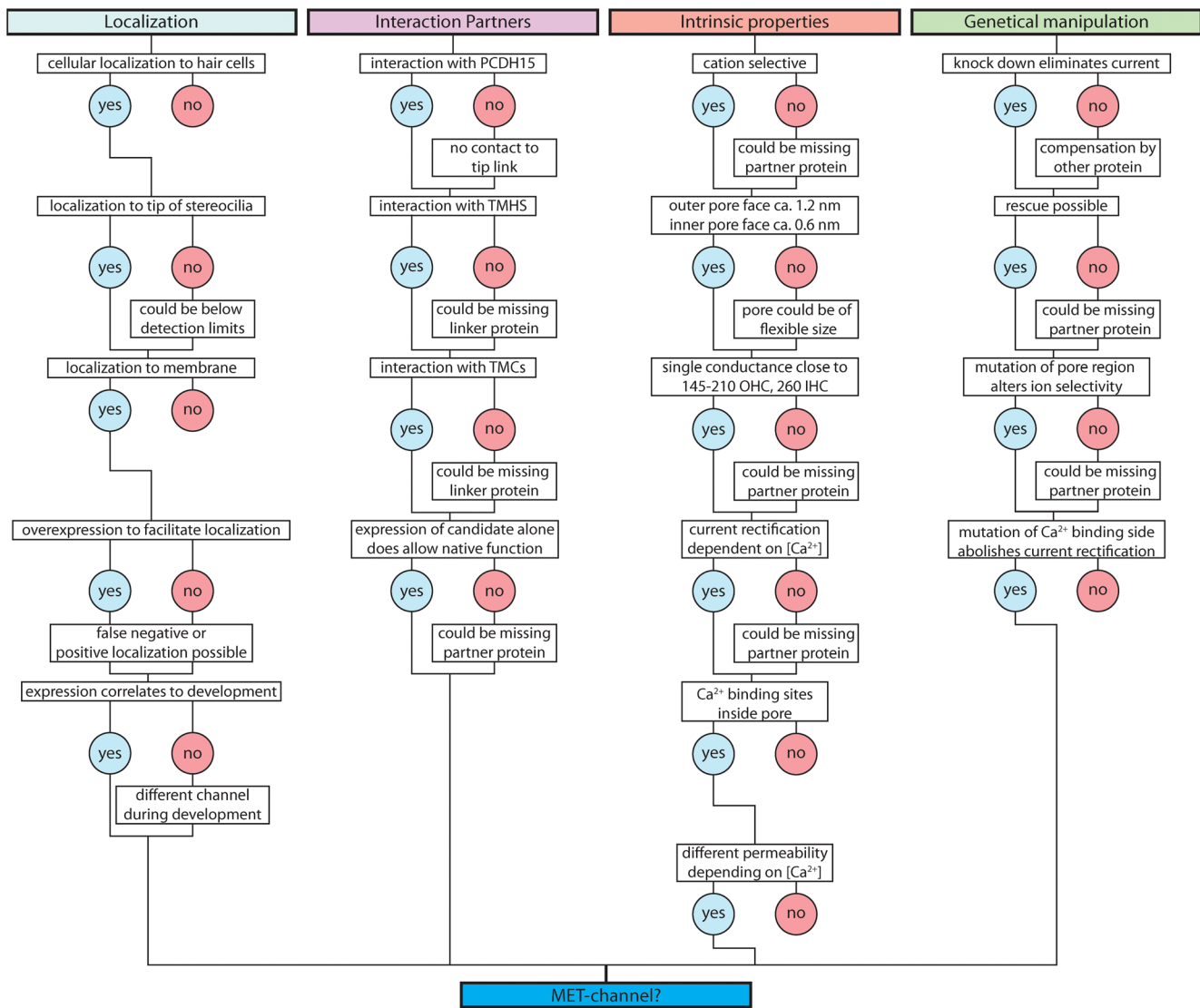
What does it take to validate a potential hcMET channel candidate?

A hcMET channel candidate must fulfill several criteria. In the following sections, we separate those criteria into four categories: channel localization, possible interaction partners, intrinsic channel properties, and genetic considerations (Fig. 2).

Localization criteria

The expectations as to channel localization for a given candidate would be the following: (i) expression in auditory hair cells but not necessarily exclusively, (ii) localization to the tips of stereocilia but not necessarily exclusively, (iii) localization to the membrane, and (iv) overlap between temporal expression of candidate and onset of mechanosensitivity. We will expand on those requirements and caveats in the following paragraphs.

In situ hybridization allows for cellular localization of a channel candidate's mRNA and is used to determine if the candidate protein can be expressed in hair cells. This does not, however, mean that the protein need only be in hair cells as it may serve multiple functions and so be found in a variety of cell types. An appropriately localized signal should also appear prior to the onset of mechanosensitivity. With only a small number of channel proteins present in a given hair cell, and possible low protein turnover, there could be only a very small amount of coding mRNA present. It is possible that the in situ technique would not be sensitive enough to detect this low signal leading to false-negative results. Alternatively, one could employ several PCR methods on isolated hair cells, which should allow for the detection of very low levels of mRNA. Also, RNA fluorescence in situ hybridization

Localization

cellular localization to hair cells — yes / no

localization to tip of stereocilia — yes / no → could be below detection limits

localization to membrane — yes / no

overexpression to facilitate localization — yes / no → false negative or positive localization possible

expression correlates to development — yes / no → different channel during development

Interaction Partners

interaction with PCDH15 — yes / no → no contact to tip link

interaction with TMHS — yes / no → could be missing linker protein

interaction with TMCs — yes / no → could be missing linker protein

expression of candidate alone does allow native function — yes / no → could be missing partner protein

Intrinsic properties

cation selective — yes / no → could be missing partner protein

outer pore face ca. 1.2 nm inner pore face ca. 0.6 nm — yes / no → pore could be of flexible size

single conductance close to 145-210 OHC, 260 IHC — yes / no → could be missing partner protein

current rectification dependent on [Ca²⁺] — yes / no → could be missing partner protein

Ca²⁺ binding sites inside pore — yes / no

different permeability depending on [Ca²⁺] — yes / no

Genetical manipulation

knock down eliminates current — yes / no → compensation by other protein

rescue possible — yes / no → could be missing partner protein

mutation of pore region alters ion selectivity — yes / no → could be missing partner protein

mutation of Ca²⁺ binding side abolishes current rectification — yes / no

MET-channel?

Fig. 2 Criteria flow chart for identifying the hcMET channel. These flow charts show different experimental criteria that a candidate must pass to fit our current understanding of the characteristics and physiology of the hcMET channel using immunohistochemistry, electrophysiology, pharmacology, and genetic techniques. However, there are instances where a candidate may fail the set criterion but still be the hcMET channel, as is explained in the boxes leading to the next criteria

methods are sensitive enough to detect single RNA copies in a given cell [61]. Some progress has been made in recent years to identify the transcriptome of cochlea hair cells [65] and the proteome of the hair bundles of chicken utricle hair cells [99]. This data is of high importance as it helps in establishing a functional network of proteins involved in hair cell function. However, we are still at the very beginning of solving this particular puzzle, although knowing its pieces is a good start. The leading MET channel candidate, the TMC proteins, shows an appropriate temporal and spatial distribution that supports their candidacy as hcMET channels [53]. Care must be taken though as a previous candidate TRPA1 [7, 21, 59] as well as several other transient receptor potential (TRP) channels show a similar temporal [3] or spatial [4, 51, 79, 86, 105, 106] expression pattern.

The next step after cellular localization of the channel candidate is the subcellular localization. Immunohistochemistry can be used to locate a candidate protein subcellularly. To be the hcMET channel, the candidate protein must be a transmembrane protein that localizes to stereocilia, specifically near to the stereocilia top. In addition, an antibody to localize a channel candidate must also be tested on hair cells that are missing the targeted protein (knockout animals for example) to avoid false-positive results (hair bundles are notoriously sticky and tend to bind antibodies nonspecifically). However, if the channel turns over regularly, the protein may be more distributed throughout the cell. As a result, the major immunohistochemical signal might not be at the location of functional relevance, similar to CDH23, a tip link protein, which is most highly expressed in the hair cell body and supporting

cells [115]. If hcMET channel protein turnover is limited, then there may only be two functional channels per stereocilium, likely resulting in a very low immunohistochemical signal. Finally, if the hcMET channel is part of a complex protein network and membrane bound, antigenicity might be compromised, leading to a false-negative result. Antigen retrieval technology is available to overcome these difficulties, so it is likely an answer will be obtained, but it is also likely that the road to this answer will not be straightforward.

Overexpression of a fluorescently tagged candidate protein is often used for localization. However, these data may be difficult to interpret as both tagging proteins and overexpressing proteins can lead to mislocalization [64, 66]. Additionally, expression of proteins in the bundle does not imply a functional relevance for mechano-electrical transduction. For example, other channels are associated with the stereocilia including ATP-gated channels [47], TRPML3 [35, 51], TRPV4 [51, 62], and TRPC3 and 6 [86] (for review, see [81]). Thus, localization data, though necessary for identifying the hcMET channel, must be interpreted carefully.

Besides the spatial expression pattern, temporal expression of a candidate protein should also match the onset of mechano-electrical transduction, which can be used to infer when hcMET channel protein is present and correctly localized [107]. However, a positive match of this onset and candidate expression can still be misleading (see TRPA1 developmental expression pattern [21]). Also, maturation might involve different channel isoforms being differentially expressed in time and location along the cochlea. This argument was used for the most recent candidates TMC1/2 [53, 76]. Hence, a match between the candidate's temporal expression pattern and the onset of mechano-electrical transduction does not necessarily validate the candidate, though in the end, the protein must be expressed prior to the channel being functionally mature.

All of the above assume that the investigated candidate is the hcMET channel. However, the candidate could also be a nonchannel protein necessary for correct channel localization or for channel transport and have no functional role for mechanosensitivity, yet provide positive results to each of the localization criteria listed.

Channel interaction partners

Some hcMET channel-interacting partners are predicted, based on the proposed molecular mechanisms involved in the mechano-electrical transduction process and also, as in the case of LHFPL5, by the unexpected finding of changes in hcMET single channel properties when mutated [111].

Since the existence of a directly mechanically gated transduction channel was proposed [23, 48, 49], the relay of forces to the channel has been a matter of debate. There is, to date, no conclusive evidence of direct coupling between the tip link and hcMET channel, so four mechanisms of force relay

between tip link and channel are possible. First, the PCDH15 portion of the tip link directly or indirectly (possibly involving additional proteins, like LHFPL5 [111]) connects to the hcMET channel, which is tethered to the cytoskeleton [81]. Second, the hcMET channel is directly or indirectly coupled to PCDH15 but does not have a connection to the cytoskeleton. Third, forces are relayed to the channel from the insertion point of PCDH15 via the membrane, with or without intermediate proteins, and the channel is tethered intracellularly to the cytoskeleton. Fourth, the channel is tethered neither to the cytoskeleton nor to PCDH15 but completely regulated by membrane lipid properties (Fig. 3; see also [81]).
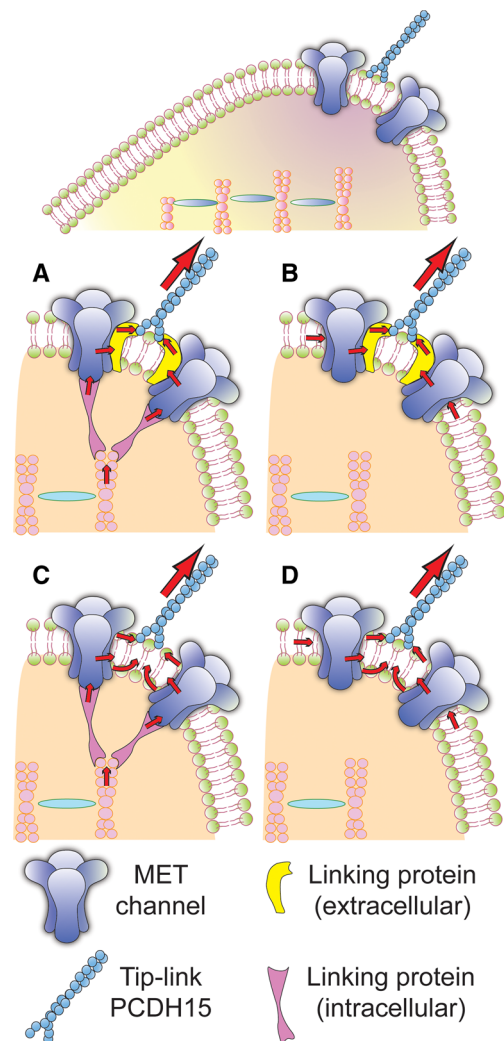
Fig. 3 Channel tethering. *A* Model showing protein-protein interactions both intra- and extracellularly. The tip link pulls directly on the channel, possibly through accessory proteins, which is itself attached to the actin cytoskeleton. *Red arrows* show the direction of force during bundle deflection. *B* Model showing extracellular tethering only. The tip link pulls directly on the channel, possibly through accessory proteins. *C* Model showing intracellular tethering only. The tip link pulls on the stereocilia membrane, and the channels are attached to the actin cytoskeleton. *D* Model showing no tethering. The tip link pulls on the stereocilia membrane only

Keeping these models in mind is important when interpreting results of experiments involving the expression of a hcMET channel candidate in vitro because expected interactions are quite different between models. For example, a candidate interacting with PCDH15 might support a tethering model and the conclusion that the candidate was indeed the hcMET channel. Unfortunately, other channels like HCN1 [88, 89] can interact with PCDH15, and so this conclusion needs to be tempered. Given that a tethered model is not proven, a negative result regarding the candidate interacting with PCDH15 is uninterpretable.

From a functional perspective, if the hcMET channel requires tethering, then its in vitro expression may require interaction partners in order to mimic native mechanosensitive responses. Eventually, the necessity of accessory proteins could be used to identify possible channel interaction partners but in the short term may lead to expression of a channel candidate that cannot be activated mechanically. For example, in the leading hcMET channel candidate, the mammalian TMC proteins neither localize to plasma membrane nor act as an ion channel in any expression system. The *C. elegans* homologue *tmc-1* functions as a Na^+-sensitive channel [20] but only possesses about 25 % amino acid homology to the mammalian TMCs [58]. What does this mean? If tethering is not required, hcMET channel in vitro expression and activation may be achievable; however, recent data suggest an additional problem. TREK1 can be gated by alterations of the lipid, showing that a mammalian MET channel can be activated/regulated by its lipid environment [17] (see review [2]). If this were true for the hcMET channel, then the expression system would also need to provide the appropriate lipid environment for channel activation.

Intrinsic channel properties

To validate a candidate as *the* hcMET channel, one needs to discriminate between intrinsic channel properties and properties that could be due to accessory partner proteins. To test the electrophysiological properties of a candidate channel, one must be able to express the protein in a heterologous system, locate it to the membrane, and then open it. As previously discussed, this is a nontrivial undertaking. For example, whether TMC proteins are mechanosensitive or act as ion channels, with properties similar to the native hcMET channel, remains to be determined due to the difficulties in localization and activation in heterologous systems (see above for potential reasons).

In addition, ion selectivity, single channel conductance, and even force sensing may not necessarily be intrinsic or exclusive properties of the hcMET channel. Accessory proteins interacting with different parts of the channel may impart these properties. For example, the channel's ion selectivity could be altered by proteins interacting with its vestibule, as is the case in the MEC complex [18] or recently suggested for the hcMET channel [10]. An accessory protein that alters the structure of the channel could impact the pore diameter or the residues facing the pore lumen, which is one possible explanation for the phenotype seen in LHFPL5 knockout [111] or the TMC1 *Beethoven* mutant mice [10, 76]. Thus, even with properties traditionally considered intrinsic to the channel, care must be taken when interpreting data in order to account for the potential role of extrinsic factors.

Pore properties

A channel's pore is arguably its most defining part; it allows ions to flow and thus for fundamental channel function. The outer and inner faces of the hcMET channel pore are asymmetric. The outer face minimum diameter is estimated at ~1.25 nm and the inner face at ~0.6 nm [31, 77], a difference considered the result of Ca^{2+} binding inside the membrane electrical field partially occluding the pore [77]. These estimates are based on pharmacological data that tested the pore size with molecules of different size, charge, and form. A channel candidate could be tested the same way. However, this approach has similar issues to single channel conductance measurements in terms of tonotopic variations and whether the properties are truly intrinsic, again making this measurement less than ideal as a sole criterion for identifying the hcMET channel. Nonetheless, this approach is useful because it uses macroscopic currents that may be easier to elicit in a heterologous system compared to the single channel measurements.

There is a growing body of literature to demonstrate that hcMET channels' single channel properties vary with frequency location [8, 92] ranging between 145 and 210 pS for OHCs and being approximately 260 pS for IHCs throughout the organ of Corti (in 20 μM external Ca^{2+}) [10–12]. The underlying mechanism for this tonotopic arrangement remains unknown, though splice variants or posttranslational modifications of a single protein could be responsible [96]. The hcMET channel may also be heteromeric with different subunit distributions giving rise to the differences in single channel conductance. If true, it is unlikely that homomeric expression of a single subunit will recapitulate native properties. If the hcMET channel is comprised of different subunits, the loss of one subunit does not necessarily render the channel complex nonfunctional as remaining subunits might compensate (compare to [53, 76, 111]). Given that we do not know the function of any particular subunit, whether it be to impart mechanosensitivity, to regulate trafficking and insertion, or control pore properties, it is complicated to predict outcomes from expression systems. Nonetheless, these experiments are critical, though likely frustrating, for the complete reconstruction of the hair cell MET complex. While heteromeric composition of the hcMET channel increases the difficulty of

identifying channel components, it can be used as a tool to probe for channel constituents, similar to the discovery of different subunits in cyclic nucleotide-gated ion channels [14, 32, 52, 108, 114]. Here, the mismatch between native channel properties and those found in expression systems was critical for the identification of accessory subunits [36, 43].

The native hcMET channel has a nonspecific inwardly rectified cation conductance, where rectification is based on the ability of external Ca^{2+} to limit current flow [22, 74, 77, 91]. The hcMET channel not only exhibits a 10× higher permeability for Ca^{2+} over Na^+ [68, 91] but also shows a mole fraction effect which alters ion selectivity and permeability based on external Ca^{2+} concentration (compare to [37, 45]). It is generally accepted that Ca^{2+} alters the permeation properties at all frequencies and regulates both conductance and rectification [33, 90, 93]. Thus, investigating candidate channels for their Ca^{2+} permeation could be quite telling. Furthermore, as Ca^{2+} binding sites are well documented in other channel classes (see [19, 34, 40, 103]), genetically modifying the Ca^{2+} binding site of a candidate and measuring changes in permeation in the native channel would be definitive in identifying the hcMET channel.

Genetics

If there is only one essential channel protein, its loss would have a significant impact on hearing performance and one might expect it to have been discovered by now in mutational screens investigating hearing loss. That no such protein is known suggests two possibilities: (a) loss of the hcMET channel function is a lethal mutation, indicating that the channel is essential for more than just hearing, and (b) there is redundancy; in this case, another protein could compensate for the loss of a given channel component. Tonotopic variations in hcMET channel properties suggest that no single protein comprises the channel. Therefore, redundancy could compensate for a knockout of a channel protein, though some subtle phenotype might be expected [73]. For example, it was recently demonstrated that TMC proteins compensate for one another and only loss of both TMC1 and 2 results in a loss of mechano-electrical transduction and thus hearing [53, 76]. Therefore, hearing loss as a screen for identifying the hcMET channel may not be sufficiently stringent to identify these elusive molecules.

Genetic manipulation allows for a detailed investigation of channel candidates where the most common manipulation is the knockdown or knockout of the protein of interest. A loss of mechano-electrical transduction in these experiments suggests that the gene product is part of the MET cascade and, possibly, though not necessarily, the hcMET channel. For example, the candidate protein may be important for hcMET channel trafficking or force relay. Also, the results of a

knockdown (with siRNA for example) and those of a knockout can be quite different. Although siRNA knockdown of TRPA1 in cultured organ of Corti showed a loss of mechanosensitivity [21], the knockout of TRPA1 in mice resulted in no hearing loss [7, 16]. Thus, specificity of the genetic manipulation as well as possible compensation by other proteins need to be carefully considered when drawing conclusions from these data types.

Discussion of current and previous channel candidates

Over the past 30 years, several hcMET channel candidates have arisen. In the following paragraphs, we revisit some of these candidates and discuss why they were candidates, what led to their exclusion, and if revisiting those channels could be worthwhile.

ENaC

Epithelial sodium channels (ENaC) are implicated in mechanosensation of *C. elegans* and had been discussed as a possible candidate for the mammalian auditory hcMET channel. Interest in ENaCs as a hcMET channel candidate was due to the amiloride sensitivity of the proteins and their role as a mechanotransduction channel in *C. elegans* [73]. An antibody raised against the purified ENaC channel of the bovine renal papilla [104] was used in an immunogold labeling of hair cells, which showed labeling close to the tip of stereocilia but below the tip link insertion point and thus closer to the shaft links [42]. However, subsequent studies showed that the amiloride sensitivity of the hcMET channel is about 100-fold higher than that of ENaCs, and the single channel conductance was about 10-fold higher for the auditory hcMET channel (~150–250 pS for the hcMET channel and ~10–15 pS for ENaCs). Further electrophysiological examinations also revealed that ENaCs have a higher Na^+ selectivity (PNa/PK= 5–100) and a lower Ca^{2+} selectivity (PCa/PK<0.4) than the hcMET channel [55]. Although the α-ENaC subunit and another member of the DEG/ENaC family, ASICS, have been shown to be expressed in chick cochlea, the knockdown of those genes in postnatal mice did not reveal a loss of mechanosensitivity [80, 94, 95, 110]. As a result, the ENaC family was abandoned as possible candidates for the mammalian auditory hcMET channel. As discussed earlier, none of the listed experiments completely disqualified ENaC, but the sum of evidence makes it rather unlikely.

TRPA1

The TRP family of ion channels was and remains a focus of interest as some of its members showed expression patterns in

the cochlea that matched requirements for the hcMET channel [21, 51, 102]. The most prominent of those TRPs was TRPA1, whose developmental expression pattern matched that of the onset of mechanosensitivity [21]. Also, the localization of TRPA1 to the tips of stereocilia fulfilled one of the criteria for being the hcMET channel. The immunohistochemical data was supported by electrophysiological data from a siRNA knockdown in E18-cultured vestibular hair cells that showed an almost complete loss of hcMET current. Also, the high conductance, Ca^{2+} permeability, and nonspecificity of the TRP pore made it an interesting candidate, though the outward channel rectification never matched that of the native channel. This combined data made TRPA1 a strong hcMET channel candidate. However, subsequent pharmacological and electrophysiological investigation of TRPA1 showed that it was different from the auditory hcMET channel. TRPA1 was 10–20 times less sensitive to amiloride block than the hcMET channel and ~100 times more sensitive to Gd^{3+} [71]. TRPA1 was dropped as a candidate after the TRPA1 knockout mouse, missing essential parts of the protein, showed normal hearing and unchanged hcMET currents [7, 59]. The problem with this, though, was the nonconditional nature of the knockout. As discussed before, the hcMET channel complex might be able to compensate for the systemic loss of TRPA1 while it could not in the siRNA knockdown situation. Although TRPA1 was quickly discarded as a hcMET channel candidate, it is still possible that it is part of the channel complex.

TMC1/2

TMC1 and TMC2 are the latest addition to the list of auditory hcMET channel candidates. Loss of TMCs results in profound hearing loss [53, 57]. TMCs 1 and 2 are the genes involved in previously described dominant and recessive nonsyndromic hearing loss, associated with the DFNA36 and DFNB7/B11 loci. How mutating these genes causes hearing loss remained unknown until recent findings suggest that TMC1 and 2 are important for a functional hvMET channel complex [53, 56, 76]. However, it is currently a matter of debate as to whether these proteins constitute (at least in part) the hcMET channel proper or are involved in trafficking/regulating the channel in some other way. While hair cells of single knockout mutants of either TMC1 or 2 retained MET currents, currents were lost in double knockouts [76]. Measurements of single channel conductance of either TMC1 or 2 knockouts were similar to native hcMET channels. Also, in situ hybridization data showed an expression of TMC1/2 in the hair cells of the organ of Corti and utricle. Immunohistochemical data of cultured utricle hair cells that overexpress a TMC2::AcGFP construct showed a labeling not only at the tip of stereocilia but also throughout the rest of the hair bundle [53]. Probably, the strongest data derived from investigating a point mutation of TMC1 (*Beethoven*) in a TMC2 null background showed an

effect on the Ca^{2+}-dependent block of MET current in 1.3 mM external Ca^{2+}. While mutant mice possessing one allele of TMC1 in a TMC2 null background would show a reduction of ~40 % MET current, compared between 50 μM and 1.3 mM external Ca^{2+}, the TMC1-Bth mutant mice would only show 30 % reduction [76]. This difference as well as an apparent reduction in single channel current (from 12.4pA for one copy of TMC1 to 8.4 in the TMC1-Bth mutant, both on a TMC2 null background) led the authors to argue that TMC1 forms part of the pore complex of the hcMET channel [76]. Most recently, an interaction between TMCs and PCDH15 [69] was identified. The interaction of TMCs and PCDH15 seems to be based on a common sequence at the C-terminus of different PCDH15 isoforms. Loss of that common sequence abolishes interaction. Overexpression of TMCs in zebrafish hair cells reduced mechanically evoked Ca^{2+} inflow into the cells, indicating that alterations of the ratio between TMCs and PCDH15 alter mechanotransduction. Thus, although TMCs have not been convincingly shown to localize at the tip of hair bundles, this possible interaction with PCDH15 infers that they will localize to the right spot. Therefore, localization and timing of expression as well as genetic and functional assays support an argument for TMCs as components of the hcMET channel.

What issues remain to be resolved? TMCs have not been shown to localize to plasma membrane, a requirement for any MET channel. Localization at the stereocilia tips of endogenous proteins remains to be elucidated (but see above regarding molecular interactions). Mammalian TMCs have not been demonstrated to be ion channels or to be mechanically sensitive, clearly important attributes of the hcMET channel (but as described herein, arguments also exist as to why these data may be difficult to generate). Although it has been shown that *C. elegans* TMC1 forms Na^+-sensitive ion channels [20], the experiments have not been repeated for the mammalian TMCs and there is only about 25 % sequence homology between mammalian and *C. elegans* [58]. Recent data showing that a hcMET current with reversed mechanosensitivity persists in TMC1 and 2 double knockouts further clouds the issue [56]. The pharmacology of this anomalous MET current in TMC1 and 2 knockouts was comparable to that of the control hcMET channel [56], prompting authors to suggest that TMCs are not forming the channel proper but might be involved in trafficking, localization, or functionality of the hcMET channel [56]. They suggest a similar role for the TMCs, as was suggested for LHFPL5 (formerly known as TMHS) [111], and argue against TMCs being integral to the hcMET channel pore. The basic argument being, if MET currents persist in the double knockout, then the knocked out proteins cannot be the channel proper but likely serve some other capacity needed to localize and position the actual hcMET channel to its native location. However, an independent study suggests that the anomalous MET currents were two orders of magnitude less sensitive to a

block by the permeable aminoglycoside blockers and that the blocking effect was not released at large negative holding potentials [70], suggesting they were impermeable. Thus, these authors argue that the anomalous current represents a novel channel that is not identical with the normal, tip link-dependent hcMET channel [70], but a new channel unmasked by loss of normal mechanotransduction (either with the knockout animals or also with treatments that break tip links). Hereto, care must be taken when interpreting dose response curves with aminoglycosides as the efficacy is dependent on channel open time which might be very different under conditions where the anomalous currents are produced [90]. Most recent evidence revisits both the pharmacology and the single channel data, concluding that TMCs may provide an accessory subunit to the channel that confers both single channel properties and pharmacological sensitivities but is not in itself the pore-forming subunit [10]. Clarifying the role and molecular distinction of the anomalous current from the native MET current is important for interpreting all existing data. It is clear that additional work is required to determine whether TMCs are pore-forming mechanosensitive ion channels in hair cells or whether they play some other critical role in the mechanotransduction process. It is also clear that regardless of molecular role, the TMCs are an important component of the mechanotransduction machinery.

LHFPL5 (formerly known as TMHS)

One hcMET channel candidate, LHFPL5, is present at the top of stereocilia, exhibits a large reduction in MET currents when knocked out, and shows a decreased single channel conductance and a slowing of activation and adaptation MET current kinetics [111]. Mutation in LHFPL5 also underlies the human deafness locus DFNB67 and the deafness in hurry-scurry mice [67, 98]. The hair bundles of LHFPL5 null mutants showed mild differences to control hair bundles, and the developmental expression pattern of LHFPL5 correlated to the maturation of MET currents [67, 111]. LHFPL5 interacts with PCDH15 (immunoprecipitation), one component of the tip link, suggesting a linker role for LHFPL5 between the force-relaying tip link and the hcMET channel [111]. Coexpression of LHFPL5 and PCDH15 leads to a localization of PCDH15 to the membrane, while PCDH15 stayed cytosolic when expressed without LHFPL5. This also indicated a possible reason for the deafness phenotypes of hurry-scurry mice, as PCDH15 mislocalization would affect tip link formation. The phenotype could be rescued by postnatal expression of the wild-type protein in cultured cochlea explants. The loss of LHFPL5 also led to a significant decrease of MET currents in whole cell voltage-clamp recordings of mechanically stimulated hair cells, as well as a slowing down of activation kinetics [111]. In summary, the effect on PCDH15 localization, the immunoprecipitation data, the effect on MET currents, and the effect on hair bundle morphology led the authors to hypothesize that LHFPL5 is a part of the hcMET channel complex, although not the pore-forming component of the channel itself, perhaps functioning as a linker of PCDH15 and the hcMET channel.

Conclusion

Finding a channel candidate is difficult, and proving that it is the hcMET channel is even more difficult. So much remains unknown about the mechano-electrical transduction process that interpretation of data regarding channel identity often depends on the perspective of the investigator. Additionally, we do not know if there is just one unique hcMET channel protein or multiple heteromeric forms. Criteria for identification typically include the following: localization to the membrane, localization to the tip of stereocilia, developmental expression comparable to the onset of mechano-electrical transduction, specific ion selectivity and permeability, differences in ion selectivity relative to the Ca^{2+} concentration of the surrounding solutions, ability to express the candidate in a heterologous system while maintaining mechanosensitivity, and possible interaction with tip link proteins or possible accessory proteins like LHFPL5. Each criterion has advantages and disadvantages. So far, no candidate has fulfilled all criteria. As discussed though, a candidate might not fulfill all criteria and still be a part of the channel complex. Additionally, only a few criteria are decisive, while the majority only hint at confirming or disproving a candidate. Identifying the hcMET channel is a decade-old challenge that tests the limits of technology and rigor. A great deal has been learned about hair cell mechanotransduction during this search. Perhaps in this case, the journey is as relevant as the destination.

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