TMC1 and TMC2 Localize at the Site of Mechanotransduction in Mammalian Inner Ear Hair Cell Stereocilia

Highlights

- TMC1 and TMC2 localize at the site of hair cell mechanotransduction
- Expression of TMC2 in cochlea is limited to developing stereocilia bundles
- TMC1 is an essential component of the mechanotransduction channel complex

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In Brief

The molecular makeup of hair cell mechanoelectrical transduction (MET) complex remains elusive. Kurima et al. show that fluorophore-tagged TMC1/2 localize to MET sites at stereocilia tips and that their expression in TMC1/2-null mice rescues MET. These findings support the model that TMC1/2 are part of the MET complex.
SUMMARY

Mechanosensitive ion channels at stereocilia tips mediate mechanoelectrochemical transduction (MET) in inner ear sensory hair cells. Transmembrane channel-like 1 and 2 (TMC1 and TMC2) are essential for MET and are hypothesized to be components of the MET complex, but evidence for their predicted spatiotemporal localization in stereocilia is lacking. Here, we determine the stereocilia localization of the TMC proteins in mice expressing TMC1-mCherry and TMC2-AcGFP. Functionality of the tagged proteins was verified by transgenic rescue of MET currents and hearing in Tmc1<sup>-/-</sup>;Tmc2<sup>-/-</sup> mice. TMC1-mCherry and TMC2-AcGFP localize along the length of immature stereocilia. However, as hair cells develop, the two proteins localize predominantly to stereocilia tips. Both TMCs are absent from the tips of the tallest stereocilia, where MET activity is not detectable. This distribution was confirmed for the endogenous proteins by immunofluorescence. These data are consistent with TMC1 and TMC2 being components of the stereocilia MET channel complex.

INTRODUCTION

Mechanoelectrochemical transduction (MET), whereby mechanical stimuli are converted to electrical signals, is an integral property of inner ear hair cells, accomplished by their mechanosensory organelle, the stereocilia hair bundle. Each bundle comprises dozens of actin-based protrusions with graded lengths, organized in a staircase array. Tip links, extracellular protein filaments composed of cadherin-23 (CDH23) and protocadherin-15 (PCDH15), connect pairs of adjacent stereocilia near their tips in the direction of optimal mechanosensitivity of the bundle (Kazmierczak et al., 2007; Sakaguchi et al., 2009). At each end of the tip link are densely packed macromolecular complexes underlying the stereocilia membrane, known as tip-link insertion plaques. The upper insertion plaque is presumed to contain a cluster of motor proteins (Grati and Kachar, 2011) that maintains a resting tension on the tip link (Schwander et al., 2010). The lower tip-link insertion site is thought to be the site for the MET channel complex (Beurg et al., 2009). Stereocilia-mediated MET occurs as a consequence of stereocilia bundle deflection toward the tallest row of stereocilia, which exerts tension on tip links, opening MET channels and causing depolarization of the hair cell. The developmental acquisition of MET, which has been spatiotemporally characterized in rat (Waguespack et al., 2007) and mouse (Lelli et al., 2009) in the organ of Corti between postnatal day (P) 1 and P2 and fully mature MET being reached by P8.

Aside from the tip-link proteins, the molecular composition of the MET apparatus remains elusive (Fuchs, 2015; Gillespie et al., 2005). The tetraspanin TMHS/LHFPL5 (Beurg et al., 2015; Xiong et al., 2012) and a protein with two transmembrane domains, TMIE (Zhao et al., 2014), have recently been identified as MET channel accessory components. However, the precise spatiotemporal localization of these proteins and the identities of the pore-forming units and other essential auxiliary elements are yet to be determined. Two members of the transmembrane-channel-like (TMC) family, TMC1 and TMC2, are candidates to be part of the MET complex based on several lines of evidence: (1) Tmc1 and Tmc2 mRNA are selectively expressed in developing hair cells at the onset of acquisition of mechanosensitivity (Kawashima et al., 2011); (2) mutations of TMC1 cause deafness in humans and mice (Kurima et al., 2002; Vreugde et al., 2002); (3) in the absence of both functional TMC1 and TMC2, hair cells...
of the mouse auditory and vestibular system lack mechanosensory responses to forward deflection of the hair bundle (Beurg et al., 2014; Kawashima et al., 2011); (4) TMC1- and TMC2-deficient hair cells fail to take up FM1-43 or gentamicin (Kawashima et al., 2011), which enter wild-type hair cells via the MET channel (Gale et al., 2001; Marcotti et al., 2009); (5) transient exogenous expression of either TMC1 or TMC2 restored mechanosensitivity in hair cells from double homozygous null mice (Tmcl1Δ/Δ;Tmcl2Δ/Δ), demonstrating that these proteins are necessary for generating MET currents (Kawashima et al., 2011); (6) fish and mouse orthologs of the cytoplasmic domain of PCDH15 were recently shown to interact with both TMC1 and TMC2 (Maeda et al., 2014); and (7) a more-recent study shows interactions between PCDH15 as well as the MET protein LHFPL5 with TMC1 and TMC2 (Beurg et al., 2015).

Whereas these data collectively suggest that TMC1 and TMC2 are crucial for the development and gating of MET currents (Kawashima et al., 2015), to ascertain whether they are indeed components of the MET complex, it is necessary to verify their localization at the site of the MET channel complex, at the lower tip-link insertion sites on shorter stereocilia, from the early postnatal period onward (Beurg et al., 2009). Previous studies reported targeting of fluorophore-tagged TMC1 and TMC2 to the apex of all stereocilia when exogenously expressed using biolistic gene-gun mediated transfection of hair cells cultured in vitro (Kawashima et al., 2011), and Beurg et al. (2015) localized TMC1 to stereocilia and kinocilia using antibody labeling. However, a definitive conclusion on the precise spatiotemporal localization of the proteins during postnatal development was precluded by the transient nature of the protein expression, the likelihood of overexpression due to the non-native promoter used, and potential for cell damage by the gene-gun method. We addressed this in the current study by generating and characterizing transgenic mice that express TMC1-mCherry and TMC2-AcGFP under their native promoters. We first verified that the fluorophore-tagged TMC proteins mimic the function of the native TMC proteins by confirming rescue of hearing and vestibular deficits, as well as hair cell MET, in Tmc1Δ/Δ;Tmc2Δ/Δ mice expressing TMC1-mCherry and TMC2-AcGFP under their native promoters. We next investigated the effects on whole-cell MET currents in the transgenic mice that are further validated by immunofluorescence localization of the native proteins using specific anti-TMC1 and -TMC2 antibodies. Finally, we also examined the relationship of TMC1 and TMC2 to the recently described non-canonical, reverse-polarity mechanosensitive currents elicited after disruption of conventional MET (Barr-Gillespie and Nicolson, 2013; Kim et al., 2013; Marcotti et al., 2014).

RESULTS

Generation of TMC1 and TMC2 BAC Transgenic Mice

To visualize TMC proteins in hair cells, we generated transgenic mice expressing TMC1 or TMC2 fused to fluorophore tags at their C termini. The transgenes were derived from bacterial artificial chromosomes (BACs) encoding mouse genomic Tmc1 or Tmc2 to mimic endogenous expression. The native stop codons were replaced with cDNA encoding mCherry or AcGFP, respectively (Figure 1A). We obtained four founder lines segregating Tmcl1-mCherry and four founder lines segregating Tmcl2-AcGFP. Using qPCR, we estimated that the transgene copy numbers for Tmcl1-mCherry lines 1, 2, 3, and 4 were 3, 7, 7, and 18, respectively; transgene copy numbers for Tmcl2-AcGFP lines 1, 2, 3, and 4 were 2, 3, 3, and 5, respectively. To determine whether the TMC1-mCherry and TMC2-AcGFP were functional and could substitute for the function of native TMC1 and TMC2 in vivo, each transgenic founder line was crossed with the previously described null Tmc1Δ/Δ, Tmc2Δ/Δ, and Tmc1Δ/Δ;Tmc2Δ/Δ lines (Kawashima et al., 2011). All transgenic mice were fertile on the Tmc1Δ/Δ;Tmc2Δ/Δ background and transmitted the transgenes to offspring. In Tmcl1-mCherry line 3, however, transmission of the transgene through males was restricted to female offspring, indicating X chromosome integration of the transgene in this line.

Functional Evaluation of TMC1-mCherry and TMC2-AcGFP

Fluorophore tags may alter the configuration of proteins to which they are appended. To evaluate the functionality of TMC1-mCherry, we recorded auditory brainstem response (ABR) threshold measurements from transgenic mice from founder lines 1, 2, or 4 segregating Tmcl1-mCherry on a Tmcl1Δ/Δ background. Tmcl1Δ/Δ mice are profoundly deaf, but we determined that hearing in these mice was fully rescued when Tmcl1-mCherry was present (Figure 1B), indicating that the mCherry-tagged TMC1 can replace the native protein without detectable changes in auditory function. No difference was observed in the ABR thresholds among the different Tmc1-mCherry founder lines despite differences in transgene copy number, suggesting that the targeting and function of TMC1 are not significantly affected by gene dosage. Only partial rescue of auditory function was observed in females from line 3 (Figure 1B), where the Tmcl1-mCherry transgene segregated in a pattern consistent with X-linked inheritance (Lyon, 1961). The presence of Tmc2-AcGFP did not rescue hearing in Tmc1Δ/Δ mice (Figure 1B), consistent with previous reports on the inability of TMC2 to compensate for lack of TMC1 in the mature auditory system (Kawashima et al., 2011). Finally, the abnormal motor vestibular phenotypes, including head bobbing and arching, present in Tmc1Δ/Δ; Tmc2Δ/Δ mice (Kawashima et al., 2011), were not detected when either Tmcl1-mCherry, Tmc2-AcGFP, or both transgenes were present (data not shown). This rescue of function indicates that the fluorophore tags did not significantly affect the function of TMC1 or TMC2 in the vestibular system and that either TMC1 or TMC2 are sufficient to restore function in the vestibular system.

We next investigated the effects on whole-cell MET currents when native TMC1 or TMC2 were replaced with fluorophore-tagged TMC1 or TMC2. As previously reported, inner hair cells (IHCs) and outer hair cells (OHCS) from Tmcl1Δ/Δ;Tmc2Δ/Δ mice have no detectable MET currents (Kawashima et al., 2011). We recorded whole-cell MET currents from IHCs and OHCS of Tmcl1Δ/Δ;Tmcl2Δ/Δ mice expressing either Tmcl1-mCherry (line 2), Tmcl2-AcGFP (line 3), or both transgenes, in developing (P3) and mature (P7) stereocilia (Figure 1C). At P3, we observed partial rescue of MET currents in both OHCS and IHCs in the
The data also support the hypothesis that, when it is expressed, TMC2 is able to partially compensate for TMC1 function (Kawashima et al., 2011), indicating an agreement between spatiotemporal expression patterns of the transgenes in IHCs and OHCs: in OHCs, the presence of Tmc1-mCherry fluorescence in the form of diffraction-limited puncta in stereocilia of auditory and vestibular hair cells (Figures 2A–2D). The presence of discrete puncta, rather than a uniform diffuse fluorescence along the stereocilia, indicates an enrichment of a few closely associated TMC1 molecules in small clusters within each punctum. In cochlear hair cells at P8 (Figures 2A and 2B), TMC1-mCherry puncta were observed at the tips of the shorter rows of stereocilia but were absent or rarely detected at the tips of the tallest stereocilia. A few puncta were observed along the length of stereocilia, suggesting that there may be a population of TMC molecules not located at the site of MET. We also noted that the TMC1-mCherry fluorescence signal was absent from the tips of a few stereocilia in the shorter rows (Figure 2A, arrows). This may be due to decay or bleaching of the fluorophores or local damage of the stereocilia. Another possibility is that some MET sites lack TMC1, at least transiently, and that the presence of TMC is not required to sustain stereocilia morphology. The pattern of TMC1-mCherry puncta appeared to be indistinguishable between TMC1-mCherry mice on either Tmc1<sup>D/D</sup>, Tmc2<sup>D/D</sup> (Figure 2A) or Tmc1<sup>D/D</sup>, Tmc2<sup>D/d</sup> backgrounds (Figure S1A), suggesting that the presence of endogenous protein does not affect or preclude the localization of mCherry-tagged TMC1 at stereocilia tips. This pattern of predominant distribution of TMC1-mCherry at the tips of IHC and OHC stereocilia did not change mice and that different transgene copy numbers appear to have no significant effect on MET function or ABR thresholds.

**Localization of TMC1**

In Tmc1-mCherry mice on a Tmc1<sup>D/D</sup> background, we observed mCherry fluorescence in the form of diffraction-limited puncta in stereocilia of auditory and vestibular hair cells (Figures 2A–2D). The presence of discrete puncta, rather than a uniform diffuse fluorescence along the stereocilia, indicates an enrichment of a few closely associated TMC1 molecules in small clusters within each punctum. In cochlear hair cells at P8 (Figures 2A and 2B), TMC1-mCherry puncta were observed at the tips of the shorter rows of stereocilia but were absent or rarely detected at the tips of the tallest stereocilia. A few puncta were observed along the length of stereocilia, suggesting that there may be a population of TMC molecules not located at the site of MET. We also noted that the TMC1-mCherry fluorescence signal was absent from the tips of a few stereocilia in the shorter rows (Figure 2A, arrows). This may be due to decay or bleaching of the fluorophores or local damage of the stereocilia. Another possibility is that some MET sites lack TMC1, at least transiently, and that the presence of TMC is not required to sustain stereocilia morphology. The pattern of TMC1-mCherry puncta appeared to be indistinguishable between TMC1-mCherry mice on either Tmc1<sup>D/D</sup>, Tmc2<sup>D/D</sup> (Figure 2A) or Tmc1<sup>D/D</sup>, Tmc2<sup>D/d</sup> backgrounds (Figure S1A), suggesting that the presence of endogenous protein does not affect or preclude the localization of mCherry-tagged TMC1 at stereocilia tips. This pattern of predominant distribution of TMC1-mCherry at the tips of IHC and OHC stereocilia did not change mice and that different transgene copy numbers appear to have no significant effect on MET function or ABR thresholds.

**Figure 1. Rescue of Hair Cell Function in Tmc1<sup>D/d</sup>, Tmc2<sup>D/d</sup> Mice Expressing TMC1-mCherry and TMC2-AcGFP**

(A) Bacterial artificial chromosome (BAC) transgenes. The stop codons of Tmc1 or Tmc2 were replaced with cDNA encoding mCherry or AcGFP, respectively.

(B) Mean (± SEM) maximal mechanotransduction currents recorded from hair cells excised from mice of the indicated genotypes. Transduction currents were evoked using saturating hair bundle deflections and were recorded at −84 mV using the whole-cell, tight-seal technique. Number of hair cells is indicated for each genotype excised from three (wild-type), one (Tmc1<sup>D/D</sup>, Tmc2<sup>D/D</sup>), two (Tmc1<sup>D/D</sup>, Tmc2<sup>D/d</sup>), four (Tmc1<sup>D/D</sup>, Tmc1-mCherry, Tmc2<sup>D/d</sup>-AcGFP), and five (Tmc1<sup>D/D</sup>, Tmc2<sup>D/d</sup>-AcGFP) mice.
significantly after exposure to 5 mM BAPTA for 15 min (Figure S2), which is known to disrupt the tip links and abolish MET (Assad et al., 1991). Similar to the observations in cochlear hair cells, TMC1-mCherry puncta localized predominantly at the tips of the shorter rows of stereocilia, counterstained in green with Alexa 488-phalloidin.

To confirm the TMC1-mCherry localization results, we generated antibodies to detect endogenous protein. The specificity of the antibodies for TMC1 was verified in COS7 cells transfected with constructs encoding TMC1 and TMC2 and in stereocilia from Tmc1<sup>D/D</sup> mice (Figure S3). To detect and localize native TMC1 in cochlear and vestibular stereocilia, we used immunostaining based on a protocol (Grati and Kachar, 2011) optimized to detect immunofluorescence signal from low-copy-number proteins within the crowded molecular environment of the tip-link insertion site (Kachar et al., 2000). We labeled both mouse and rat inner ear tissue to verify the localization of endogenous TMC proteins in an additional species. Consistent with the localization of TMC1-mCherry, TMC1 immunofluorescence was observed at the tips of the second and third row stereocilia of mouse OHCs, but not at the tips of the tallest row (Figures 2E and 2F). The same pattern of TMC1 immunofluorescence was also observed in rat IHCs (Figure 2G) and OHCs (Figure 2H). Once again, we observed that the immunofluorescence signal was absent in some shorter stereocilia (Figures 2E–2H). Whereas this may be because TMC1 can be absent in some stereocilia tips, we cannot exclude that their absence may have been due to epitope masking when labeling a few protein molecules within the densely packed (Kachar et al., 2000) lower-tip-link insertion site. TMC1
immunofluorescence puncta were also observed predominantly at the tips of vestibular hair cell stereocilia (Figure 2I).

**Distinction between mCherry Fluorescence and Autofluorescence**
It is noteworthy that some autofluorescence was detected in wild-type bundles not expressing TMC1-mCherry (Figure S1 B). This is likely due to the high gain setting of the system and long exposure times needed to detect the low-abundance TMC1-mCherry. Furthermore, the presence of endogenous autofluorescent molecules is exacerbated in stressed or dying tissue (Buschke et al., 2012). To reduce bleaching of TMC1-mCherry and TMC2-AcGFP and limit autofluorescence, we performed rapid dissection and fixation. The residual autofluorescence signals, which were clearly much lower in number compared to TMC1-mCherry signal (Figure S1A), were likely the result of debris deposited on the stereocilia surface during dissection of the tissue. The distinction between TMC1-mCherry signal and background autofluorescence was clearly evident when we examined organ of Corti tissue from Tmc1-mCherry line 3 mice. Consistent with X-linked inactivation of the Tmc1-mCherry transgene, we observed a mosaic expression of TMC1-mCherry fluorescence in OHCs (Figures 3 A and 3B) and IHCs (Figures 3C and 3D) of female mice from this line. A similar expression pattern was previously described for a Myo7a transgene inserted in the X chromosome (Prosser et al., 2008). Quantifying the number of fluorescence puncta within hair bundles of Tmc1-mCherry line 3 mice, we found that some bundles showed puncta predominantly at the tips of stereocilia of the second and shorter rows (Figures 3A and 3B). The average number of puncta

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**Figure 3. Mosaic Expression of TMC1-mCherry in Mouse Line 3**
(A) Confocal image of OHC stereocilia (green) from P10 mice, showing mosaic expression of TMC1-mCherry fluorescent puncta (red). Cells that are not expressing TMC1-mCherry are highlighted with asterisks.
(B) Corresponding red channel (TMC1-mCherry) shown in gray.
(C) Stereocilia (green) from IHCs, of which only one cell (asterisk) is expressing TMC1-mCherry (red).
(D) Corresponding red channel (TMC1-mCherry) shown in gray.
(E–G) Confocal images of P10 mouse cochlear hair cells exposed to 5 μM FM1-43 FX. IHCs and OHCs from male Tmc1-mCherry line 3 mouse (E), from female Tmc1-mCherry line 3 mouse (F), and from Tmc1<sup>Δ/Δ</sup>;Tmc2<sup>Δ/Δ</sup> mouse (G). The Tmc1-mCherry line 3 mice were on a Tmc1<sup>Δ/Δ</sup>;Tmc2<sup>Δ/Δ</sup> background.

*The scale bars represent (A–D) 5 μm and (E–G) 100 μm. See also Figure S1.*
Localization of TMC2

Localization of TMC2-AcGFP in Hair Cell Stereocilia

(A) Confocal images of IHC stereocilia (labeled in red with Alexa Fluor 568 phalloidin) from Tmc2-AcGFP transgenic mice at P4 (line 4), showing localization of TMC2-AcGFP (green).

(B and C) TMC2-AcGFP (green) localizes at the tips of vestibular hair cell stereocilia (red) at P7 (line 2).

(D) In addition to stereocilia tip labeling, some hair bundles at P7 also show abundant TMC2-AcGFP puncta along the length of stereocilia.

(E) The expression of TMC2-AcGFP (green) within stereocilia (red) was seen to vary from cell to cell in vestibular organs (line 4; P8).

(F and G) Immunofluorescence confocal images of rat IHC and OHC stereocilia (green) at P7, showing immunofluorescence of TMC2 (red) using anti-TMC2 antibody PB361.

(H) Immunofluorescence staining of TMC2 (red) in stereocilia (green) of rat vestibular hair cells at P3. The scale bars represent (A–D and F–H) 2 μm and (E) 5 μm. See also Figure S3.

Localization of TMC2

Similar to TMC1-mCherry, TMC2-AcGFP expressed in Tmc2-AcGFP transgenic mice on a Tmc2-D/D; Tmc1-D/D background was also observed in stereocilia as diffraction-limited puncta (Figures 4A–4E). Consistent with the reported spatiotemporal expression of Tmc2 mRNA (Kawashima et al., 2011), the expression of TMC2-AcGFP in auditory hair cells was transient and disappears as the hair cells mature. To assess the temporal expression during organ of Corti development, we focused our analyses to the mid-apical turn of the cochlea, i.e., 30%–50% from the apex of the cochlea. In IHCs at P4, TMC2-AcGFP fluorescence puncta were localized to the tips of the second and shorter rows of stereocilia and absent from the tips of the tallest row (Figure 4A). Some puncta were also randomly distributed along the stereocilia, distant from the site of MET (Figure 4A). Similar to TMC1-mCherry, we also noted that the TMC2-AcGFP fluorescence signal was absent from the tips of some stereocilia in the shorter rows (Figure 4A, arrows). Localization of TMC2-AcGFP puncta at the tips of stereocilia was also observed in vestibular hair cells (Figures 4B and 4C). However, when compared to cochlear hair cells, greater variations in both the expression level and pattern of distribution of TMC2-AcGFP puncta were observed between stereocilia bundles of different vestibular hair cells, with much larger numbers of puncta present along the length of stereocilia in some cells (Figures 4D and 4E). These differences may be correlated with variations in the type, developmental stage, or both of the different hair cells in the vestibular sensory epithelia or may underlie previously unrecognized functional variations between them. Alternatively, they may be the result of transgene overexpression. Immunofluorescence using antibodies specific for TMC2 (Figure S3) in rat inner ear tissue confirmed localization of TMC2 at the tips of stereocilia in IHCs, OHCs, and vestibular hair cells (Figures 4F–4H). The observation of stereocilia tip localization of TMC2 in rat cochlear hair cells as late as P15 (Figures 4F and 4G) may be due to differences in the time course of Tmc2 expression in rat hair cells compared with mouse hair cells.

Spatial and Temporal Colocalization of TMC1 and TMC2

We next investigated the temporal expression and extent of colocalization of TMC1-mCherry and TMC2-AcGFP during postnatal development in stereocilia of mice expressing both transgenes on a null Tmc1-D/D; Tmc2-D/D background (Figures 5 and...
The earliest that we could consistently detect TMC1-mCherry or TMC2-AcGFP in auditory hair cell stereocilia was at P2 (Figure 5A), when TMC2-AcGFP signal was quite evident. By P3, both TMC1-mCherry and TMC2-AcGFP fluorescent puncta were abundant and localized along the length of stereocilia as well as at the tips, sometimes overlapping (Figure 5A). At P7, TMC2-AcGFP puncta were less evident in OHC stereocilia (Figure S4), but both TMC1-mCherry and TMC2-AcGFP puncta were equally present at stereocilia tips of IHCs (Figure 5A). At P10, TMC2-AcGFP puncta also disappeared from IHC stereocilia, whereas TMC1-mCherry puncta persisted at stereocilia tips of both IHCs (Figure 5A) and OHCs (Figure S4).

To examine the extent of overlap of localization of TMC1 and TMC2, we analyzed confocal images of IHCs at P7, where both TMC1-mCherry and TMC2-AcGFP were equally abundant (Figures 5A and 5B). The TMC1-mCherry and TMC2-AcGFP puncta were first identified as local, high-intensity regions (maxima) in the image, using a custom written code in MATLAB. At each of the identified local maxima, the center of mass of image intensity under a disc-shaped mask roughly the size of the point spread function (five-pixel radius) was then computed to obtain the centroids of each punctum (Figure 5B). The distance between the computed centroid of each TMC1-mCherry punctum and its closest TMC2-AcGFP punctum was used as a measure of separation between puncta. A histogram of the frequency distribution of TMC1-mCherry puncta centroids according to their distance from the centroid of the corresponding closest neighboring TMC2-AcGFP punctum (histogram bin size = 30 nm; number of puncta = 709; number of hair cells = 12).

Similar results were observed when plotting the frequency of TMC2-AcGFP puncta as a function of the distance to their closest TMC1-mCherry puncta (Figure S5A). Many of the overlapping TMC1-mCherry and TMC2-AcGFP puncta were in close proximity, with approximately 25% of the fluorescent TMC1-mCherry puncta having a TMC2-AcGFP punctum that was within a 30-nm radius and thus potentially part of the same molecular aggregate or complex (number of puncta = 709; number of cells = 12).
puncta localize to the tips of the second row and shorter stereocilia (Figure 5B, arrows).

We next wanted to determine whether TMC1 and TMC2 compete with each other as interchangeable subunits of a heteromeric aggregate. To do this, we first calculated the intensities of each punctum by integrating the pixel intensity within a point spread function-sized disc (five-pixel radius; pixel size = 30 nm). We then plotted the fluorescence intensities of each TMC1-mCherry punctum as a function of the distance to its closest TMC2-AcGFP neighbor (Figure 5D). The average intensity of TMC1-mCherry puncta that had a TMC2-AcGFP punctum neighbor within 30 nm was 5.86 ± 2.1, in a.u. (number of puncta = 93; number of cells = 10). The average intensity of TMC1-mCherry puncta whose nearest TMC2-AcGFP neighbor was more than 100 nm away was 4.84 ± 1.7 (number of puncta = 331; number of cells = 10). Conversely, we also plotted the fluorescence intensities of the TMC2-AcGFP puncta as a function of the distance of its closest TMC1-mCherry neighbor (Figure S5B). The average intensity of TMC2-AcGFP puncta that had a TMC1-mCherry punctum neighbor within 30 nm was 8.1 ± 3.8 (number of puncta = 93; number of cells = 10). The average intensity of TMC2-AcGFP puncta when the distance to the closest TMC1-mCherry neighbor was more than 100 nm was 7.5 ± 3.9 (number of puncta = 399; number of cells = 10). These data collectively suggest that the amount of each protein was not reduced when both proteins co-existed within a 30 nm range and potentially as part of the same molecular cluster.

In the vestibular organs, we did not observe temporal expression differences between TMC1-mCherry and TMC2-AcGFP from P3 (earliest point we examined) to adulthood, and both TMC1-mCherry and TMC2-AcGFP showed a higher degree of cell-to-cell variability compared to cochlear hair cells. Whereas most stereocilia bundles expressed both proteins to some degree, some cells showed predominance of either TMC1-mCherry or TMC2-AcGFP (Figure 5E). When both were expressed in the same hair cell, we observed partial overlap of TMC1-mCherry and TMC2 fluorescence at stereocilia tips (Figure 5F). Of note, the fluorescence puncta sometimes appear to be slightly separated above the stereocilia tip. This apparent mismatch is the combined result of the differential size of the point spread function for the fluorophores and for the actin counterstained stereocilia (stereocilia were stained with phalloidin and thinner-beveled tip where the membrane can be slightly pulled away or tented by tension on the tip link (see Figure 5F). Of note, the fluorescence puncta sometimes appear to be slightly separated above the stereocilia tip. This apparent mismatch is the combined result of the differential size of the point spread function for the fluorophores and for the actin counterstained stereocilia (stereocilia were stained with phalloidin conjugated to Alexa Fluor 405, whose emission wavelength is much shorter than the emission wavelengths of mCherry or AcGFP nm) with the fact that the stereocilia often have an elongated and thinner-beveled tip where the membrane can be slightly pulled away or tented by tension on the tip link (see Figure 4E in Kachar et al., 2000). Together, these parameters give the false impression that the TMC fluorescence puncta are separated from the tip of the actin-counterstained stereocilia.

TMC proteins have been reported to interact with PCDH15 isoforms, and PCDH15 also interacts with CDH23 to form the kinociliary links between the tallest stereocilia and the kinocilium (Gillespie et al., 2005; Goodyear et al., 2010; Kazmierczak et al., 2007). Based on this, we looked for localization of TMC1-mCherry and TMC2-AcGFP along the tallest stereocilia in the bundle but did not detect any enrichment of either protein at this region.

Whereas the stereocilia tip localization of TMC1 and TMC2 support their having a role in mechanotransduction, we next investigated how TMC1-mCherry and TMC2-AcGFP fluorescence intensity compares between hair cell body and stereocilia to explore the possibility for additional roles of these proteins elsewhere in the cell. The level of fluorescence signal from the cell bodies of hair cells expressing TMC1-mCherry and TMC2-AcGFP on a Tmc1<sup>−/−</sup>;Tmc2<sup>−/−</sup> background (Figures S6A and S6B, asterisk) was difficult to differentiate from autofluorescence, which is also present in neighboring supporting cells that are not believed to express the tagged proteins (Figures S6A and S6B, double asterisks). When we compared fluorescence in the red channel between hair cells from Tmc1<sup>−/−</sup>mCherry mice and wild-type mice (Figures S6C–S6F), a distinct fluorescence signal was detected in the hair cell bodies (Figures S6C–S6F, asterisk) of the transgenics compared to wild-type controls under identical image acquisition conditions. This fluorescence likely corresponds to TMC1-mCherry in endocytic or ER-associated vesicles. No enhancement of fluorescence signal was detected in the hair cell basolateral membrane.

Reverse-Polarity Currents in Tmc1<sup>−/−</sup>;Tmc2<sup>−/−</sup> Mice

Recently, non-canonical, reverse-polarity MET currents evoked by deflecting the hair bundle in the direction away from the tallest stereocilia (inhibitory) were recorded from hair cells (Beurg et al., 2014; Kim et al., 2013; Marcotti et al., 2014). These reverse-polarity currents have been shown to occur after tip-link disruption by the calcium-chelating agent BAPTA (Kim et al., 2013; Marcotti et al., 2014). Whereas their physiological significance and underlying mechanisms are yet to be elucidated, it was suggested that these reverse-polarity currents may be caused by the relocation of the MET channel as a result of tip link disruption, which might allow for its activation by inhibitory stimuli (Barr-Gillespie and Nicolson, 2013). Because the TMC proteins are hypothesized to be associated with MET, we investigated whether these proteins are required for the reverse-polarity MET currents (Beurg et al., 2014; Kim et al., 2013; Marcotti et al., 2014).

We first confirmed the presence of the reverse-polarity MET currents in wild-type hair cells following treatment with 5 mM BAPTA (Figure 6A). We next examined our Tmc1<sup>−/−</sup>;Tmc2<sup>−/−</sup> double mutants that are verified null alleles (Kawashima et al., 2011) that do not exhibit conventional transduction when stimulated with a glass probe (Figure 1). We did not detect any conventional transduction upon delivery of a 40-Hz sine wave stimulus using fluid jet simulation. However, a 25%–50% increase in the sine wave stimulus amplitude, which in wild-type hair cells evoked maximum MET currents, resulted in appearance of the reverse-polarity currents (Figure 6B). Our data are consistent with recent reports that recorded reverse-polarity currents in Tmc1 and Tmc2 mutant mice (Beurg et al., 2014; Kim et al., 2013).

DISCUSSION

Whereas earlier studies suggested that TMC1 is involved in molecular trafficking or intracellular regulatory signaling for hair cell differentiation (Marcotti et al., 2006), more-recent studies have provided genetic and physiological evidence for a direct role
for TMC1 and TMC2 in hair cell MET (Kawashima et al., 2011; Kim et al., 2013; Pan et al., 2013), suggesting that these proteins are components of the MET complex. One of these reports (Pan et al., 2013) further suggests that TMC1 and TMC2 may be components of the pore-forming element, and the varying conductance properties of the channel may be the result of variations in the relative stoichiometry of these proteins in the channel complex. However, an important milestone in corroborating this hypothesis (Liedtke, 2014) is verifying the precise localization of these proteins at the site of MET (Beurg et al., 2009). The current study shows that TMC1 and TMC2 localize to the specific site of MET at stereocilia tips. We addressed this outstanding question by using two different approaches to detect the proteins. First, we generated transgenic mice expressing mCherry-tagged TMC1 and AcGFP-tagged TMC2. Second, we generated antibodies specific for native TMC1 and TMC2 that we used to label mouse and rat inner ear tissue.

Mice expressing both TMC1-mCherry and TMC2-AcGFP on a Tmc1<sup>+/−</sup>;Tmc2<sup>+/−</sup> background showed normal vestibular phenotypes, normal hearing thresholds, and normal whole-cell MET currents within the ranges reported for mice segregating different numbers of wild-type alleles of endogenous Tmc1 and Tmc2 (Pan et al., 2013). These observations demonstrate that the fluorophore-tagged proteins are functional, properly temporally expressed, and targeted to their site of function.

Our spatiotemporal characterization of TMC1 and TMC2 localization in cochlear hair cells provides insights into the intricate and complex nature of MET currents. In the cochlea at P1, neither TMC1-mCherry nor TMC2-AcGFP is detectable in the stereocilia. By P3, both are abundantly expressed along the length and at the tips of stereocilia. The overall expression of both proteins then declines until about P7, with localization now predominantly at the tips of stereocilia, and a much-smaller population of TMC puncta still present along the stereocilia. TMC2-AcGFP expression declines until it virtually disappears by P10, whereas TMC1-mCherry remains at stereocilia tips through to adulthood. The observed spatiotemporal refinement of TMC localization is reminiscent of the pruning of extracellular stereociliary links and, in particular, lateral links and ankle links, which are present in early postnatal hair bundles but progressively disappear, leaving only tip links and top connectors in mature hair cells (Goodyear et al., 2005; Waguespack et al., 2007). Furthermore, the developmentally correlated changes in TMC1-mCherry and TMC2-AcGFP localization are consistent with the reported spatiotemporal expression of Tmc1 and Tmc2 mRNA (Kawashima et al., 2011) as well as the acquisition of MET in mouse cochlear hair cells (Lelli et al., 2009). In cochlear hair cells, the TMC proteins were rarely detected at the tips of the tallest row of stereocilia, where functional MET channels are thought to be absent. In vestibular organs, both TMC1-mCherry and TMC2-AcGFP localize to stereocilia tips of mature hair cells showing a cell-to-cell variability in the degree of colocalization of these proteins. Some hair cells show more TMC1 whereas others show more TMC2. The localization pattern of the fluorophore-tagged TMC1 and TMC2 was confirmed in stereocilia from mice and rats by immunofluorescence detection of endogenous proteins using antibodies specific to TMC1 or TMC2.

We observed a variable number of TMC1-mCherry and TMC2-AcGFP puncta along the length of all mature hair cell stereocilia, which may be due to increased copy number of the proteins in the transgenic mice. However, another possibility is that hair cells retain a pool of TMC proteins along the stereocilia plasma membrane that are actively trafficking or have a yet-to-be-detected function. That we were able to measure reverse-polarity MET currents recently reported by several groups (Barr-Gillespie and Nicolson, 2013; Kim et al., 2013; Marcotti et al., 2014) in our double homozygous null mice (Tmc1<sup>+/−</sup>;Tmc2<sup>+/−</sup>) confirms that neither TMC1 nor TMC2 is required for these currents. It is worth noting that we do not detect enrichment of TMC1 or TMC2 along the taller stereocilia contacting the kinocilium where PCDH15 is concentrated, in either developing cochlear hair cells or in vestibular hair cells (Kazmierczak et al., 2007). This suggests that interaction of TMC1/2 with PCDH15 is limited to the site of MET at the tips of stereocilia and does not involve kinociliary

Figure 6. Reverse-Polarity Currents in WT and Tmc1<sup>+/−</sup>;Tmc2<sup>+/−</sup> Hair Cells

(A) Example of MET current (black trace) recorded from a WT OHC (from mid-apical turn of the cochlea; P7 mouse) in response to a sine wave stimulus (black) with a fluid jet device. Upon application of 5 mM BAPTA, the MET current of the same cell is abolished (red trace) and followed by the progressive appearance of the reverse-polarity currents.

(B) Representative MET current recordings from a Tmc1<sup>+/−</sup>;Tmc2<sup>+/−</sup> OHC. No conventional MET currents were detected during regular stimulation (black trace). A higher stimulus (red sine wave) evoked reverse-polarity currents (red trace). All recordings in (A) and (B) are averages of three to eight individual traces (n = 5 cells).
Inconsistent with our data, a recent attempt to localize TMC1 by immunofluorescence using an antigen retrieval method in wild-type mice (Beurg et al., 2015) showed immunoreactivity along the length of the stereocilia, at the tips of the tallest rows of stereocilia, and in the kinocilium—sites where no mechano-transduction is thought to occur.

There are currently no direct data on molecular-level interactions of TMC1 and TMC2. Distinct putative single-channel conductances recently reported from wild-type IHCs (Pan et al., 2013) led the authors to propose that variations in the stoichiometry of TMC1 and TMC2 might explain tonotopic gradients in the conductance properties of the MET channel. Our localization study provides evidence that TMC2 is only transiently present in stereocilia of cochlear hair cells and thus might not contribute to tonotopic gradients in channel properties (Pan et al., 2013) in mature hair bundles. However, different conductance levels can arise from differential abilities of TMC1 and TMC2, alone or together, to interact with other proteins that affect single-channel MET conductance (Beurg et al., 2014), simultaneous gating of multiple MET channels in the same complex on a single stereocilium, or different channel complexes on different stereocilia.

Our results also show that, when both proteins are expressed at about the same levels, as in IHC stereocilia at P7, only ~25% of TMC1-mCherry puncta have a TMC2-AcGFP punctum located within a 30-nm radius and therefore potentially within the same molecular complex or cluster. The remaining puncta were randomly dispersed along the stereocilia. It is possible that TMC1 and TMC2 proteins traffic to the stereocilia membrane as independent clusters or homo-oligomers and co-accumulate in the limited space at the stereocilia tips by interacting with other MET components such as PCDH15 (Maeda et al., 2014). This interpretation is supported by our observation that the fluorescence intensity signal emitted by either TMC1-mCherry or TMC2-AcGFP was on average the same regardless of whether the puncta were within interacting distance (<30 nm) or not (>100 nm). This observation may indicate that the presence of one TMC does not exclude the presence of the other at the same locus. It may also indicate that the quantities of TMC1 and TMC2 do not vary whether or not the other is present. One could interpret this to argue against a role for these proteins as pore-forming elements of the MET channel (Pan et al., 2013).

In our current study localizing TMC1 and TMC2 to the site of canonical MET in hair cell stereocilia strongly supports the hypothesis that they are members of the MET channel complex (schematic model in Figure 7). Whether they are in fact the MET channel or non-pore-forming subunits/accessory proteins modulating the kinetics of gating, channel properties, stability, or subcellular targeting of the actual channel is yet to be conclusively established. Association of TMC1 and TMC2 with other MET proteins to form a stable complex may explain TMC1 enrichment at stereocilia tips even after tip link disruption by BAPTA. Similar to TMC, PCDH15 remains at stereocilia tips following BAPTA treatment (Indzhykulian et al., 2013). Other proteins, such as PCDH15 and TMIE, which were recently shown to interact with TMC proteins (Beurg et al., 2015; Fuchs, 2015; Maeda et al., 2014), and TMHS/LHFPL5 identified as an MET channel accessory component (Xiong et al., 2012) may contribute to the spatiotemporal distribution of TMC1 and TMC2 in stereocilia.
EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice
We used bacterial artificial chromosomes (BACs) encoding mouse Tmc1 or Tmc2 genes. Details of the BACs and procedures for their modification, purification, and injection into mouse eggs to generate the transgenic mice, as well as backcrossing onto mice segregating targeted deletion alleles of Tmc1 and Tmc2, are fully described in the Supplemental Experimental Procedures. Founder mice and offspring carrying the BAC transgenes were identified by PCR analysis of genomic DNA isolated from tail biopsies. All animal protocols and procedures were approved by a NIH Animal Care and Use Committee.

Hair Cell Electrophysiology
Whole-cell, tight-seal technique was used to record mechanotransduction currents from P3–P8 mice by deflecting the hair bundles of IHCs and OHCs using a stiff glass probe. To measure reverse-polarity currents, hair bundles were mechanically stimulated with a fluid jet system. The position of the stimulating pipette was adjusted to elicit a maximal MET current. All experiments were performed at room temperature. Details are provided in Supplemental Experimental Procedures.

Detection of TMC1-mCherry and TMC2-AcGFP and Immunofluorescence Microscopy
Inner ear tissue from mice expressing TMC1-mCherry and/or TMC2-AcGFP was dissected and mounted between slide and cover slip and viewed in a Nikon inverted fluorescence microscope, outfitted with a spinning disk confocal scan head, 100× Apo TIRF 1.49 N.A. objective, and an EM-CCD camera. NIS-Elements imaging software was utilized for image acquisition and analysis.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.058.

AUTHOR CONTRIBUTIONS
K.K. conceived, designed, and generated transgenic mice and collected and analyzed data. S.E. collected and analyzed data. B.P. collected and analyzed conventional MET electrophysiology data. M.S. collected and analyzed electrophysiology data acquired with fluid jet stimulation. B.A.M. helped acquire confocal microscope images. P.S. helped with analyses and quantification of fluorescence data. R.C., H.N., T.F., and Y.K. helped collect data. B.Y.C. and K.M. helped generate transgenic mice. J.R.H. analyzed conventional MET data. A.J.G. conceived the overall study and designed transgenic mice. K.K., S.E., A.J.G., and B.K. wrote the paper. All authors provided comments and approved the final submission of the manuscript.

CONFLICTS OF INTEREST
K.K. and A.J.G. hold the following U.S. patents: 7,166,433 (Transductin-2 and Applications to Hereditary Deafness), 7,192,705 (Transductin-1 and Applications to Hereditary Deafness), and 7,659,115 (Nucleic Acid Encoding Human Transductin-1 Polypeptide).

ACKNOWLEDGMENTS
We thank Elizabeth Wilson, James McGeehee, and Patrick Diers for mouse management. Work was supported by NIH intramural research funds Z01-DC00002 (to B.K.), Z01-DC000060 (to A.J.G.), and Z01-DC000039. B.P. and J.R.H. were supported by NIH/NINDS RO1-DC013521.

Received: December 23, 2014
Revised: June 11, 2015
Accepted: July 28, 2015
Published: August 27, 2015

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