Mechanosensory hair cells express two molecularly distinct mechanotransduction channels

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Auditory hair cells contain mechanotransduction channels that rapidly open in response to sound-induced vibrations. We report here that auditory hair cells contain two molecularly distinct mechanotransduction channels. One ion channel is activated by sound and is responsible for sensory transduction. This sensory transduction channel is expressed in hair cell stereocilia, and previous studies show that its activity is affected by mutations in the genes encoding the transmembrane proteins TMHS, TMIE, TMC1 and TMC2. We show here that the second ion channel is expressed at the apical surface of hair cells and that it contains the Piezo2 protein. The activity of the Piezo2-dependent channel is controlled by the intracellular Ca2+ concentration and can be recorded following disruption of the sensory transduction machinery or more generally by disruption of the sensory epithelium. We thus conclude that hair cells express two molecularly and functionally distinct mechanotransduction channels with different subcellular distributions.

Our senses of hearing, balance, proprioception and touch depend on the conversion of mechanical force into electrical signals. The molecules underlying mechanoelectrical transduction are not well defined, and the extent to which different mechanosensory phenomena depend on similar molecules to convert mechanical signals into electrical signals still needs to be defined. In the organ of Corti of the mammalian cochlea (Fig. 1a), hair cells are the mechanosensory cells for the perception of sound. Mechanotransduction channels in hair cells are localized in their hair bundles, which consist of stereocilia organized in rows of decreasing heights (Fig. 1b)1. In a healthy hair cell, deflection of the hair bundle toward the longest stereocilia leads to an increase in the open probability of the transduction channels, while deflections in the opposite direction decrease the channel open probability1. Mechanotransduction channels are gated by tip links, extracellular filaments that connect the stereocilia of a hair cell in the direction of the mechanical sensitivity of its hair bundle (Fig. 1b)1.

There is considerable uncertainty with regard to the molecular composition of the mechanotransduction channel in hair cells. Ca2+ enters stereocilia upon mechanical stimulation near the lower tip-link insertion site, thus defining the localization of the sensory-transduction channel2. TMHS (also called LHFPL5), TMIE, TMC1 and TMC2 are transmembrane proteins that are appropriately localized in stereocilia to be part of a transduction-channel complex3-5, but which of these proteins contribute to the channel pore is unclear. Functional studies in mice suggest that TMHS, a protein with four predicted transmembrane domains, is not a pore-forming subunit of the transduction channel alone but forms part of the channel complex4. TMIE contains two predicted transmembrane domains and is essential for mechanotransduction by hair cells, but its precise function within the transduction-channel complex remains to be established5. TMC1 and TMC2 contain at least six predicted transmembrane domains each6 and have been proposed to be components of the mechanotransduction channel7,8, possibly forming its pore8. However, it has so far not been possible to demonstrate that TMC1 and/or TMC2 encode ion channels.

During the developmental maturation of hair cells, their hair bundles are less directionally sensitive; transducer currents can initially be evoked by deflection of the hair bundle in the direction not only of the longest stereocilia but also in the opposite direction, toward the shortest stereocilia10-13. Similar reverse-polarity currents can be evoked in hair cells lacking tip links10,12,14 and in hair cells from mice carrying mutations in the genes encoding TMHS, TMIE, TMC1 and TMC2 (refs. 5,10,15). The appearance of reverse-polarity currents in damaged hair cells correlates with loss of normal-polarity currents, suggesting that the pore-forming subunits of the underlying ion channels may be shared. Consistent with this notion, ion channels carrying reverse-polarity currents show ion selectivity and responsiveness to pharmacological blockers similar but not identical to selectivity and responsiveness shown by ion channels for normal-polarity currents8,10,16. Based on these and other findings, it has been proposed that TMC1/2 might be accessory subunits of the transduction channel, regulating channel localization and/or forming an extracellular vestibule that controls ion flow toward the channel pore8,10,15-17.

Recent findings have identified Piezo1 and Piezo2 proteins as bona fide pore-forming subunits of mechanotransduction channels in mammals18,19. Piezo proteins have important functions in touch sensation and other mechanical processes, such as in sensing shear stress.

References:
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Received 29 July; accepted 27 October; published online 28 November 2016; doi:10.1038/nn.4449
in the vasculature and in regulating bladder function. However, the function of Piezo1 and Piezo2 in auditory hair cells has not been thoroughly explored. Here we show that Piezo2 is expressed in cochlear and vestibular hair cells. Studies in cochlear outer hair cells (OHCs) show that Piezo2 is not an essential component of the sensory-transduction channel in stereocilia. Instead, Piezo2 is localized

**Figure 1** Expression of Piezo1 and Piezo2 in the inner ear. (a) Diagram of the organ of Corti highlighting IHCs and OHCs. (b) Diagram of a cochlear hair cell showing the stereocilia and part of the cell body. Linkages between stereocilia are highlighted. (c) In situ hybridization on cochlear sections at P1 with probes for otoferlin, Piezo1 and Piezo2 (AS, antisense probe; S, sense control probe). Arrowheads highlight OHCs expressing Piezo2 mRNA. gV (dashed white line) indicates staining of trigeminal sensory ganglia. Scale bars, 200 µm in upper panel and 20 µm in lower panel. (d-i) td-Tomato fluorescence was visualized in cochlear whole mounts from Piezo2-GFP-IRES-Cre;AlgalDH/loxP mice at the indicated ages. (e,g,i) Arrows indicate td-Tomato expression in OHCs with occasional expression in IHCs. (h,i) Blood vessels also expressed td-Tomato. Scale bars, 20 µm in d–g and 100 µm in h and l. (j–o) Vestibular whole mounts at the indicated age revealed td-Tomato expression in hair cells (arrowheads) and in blood vessels. All experiments were repeated at least three times, and at least 10 images were collected for each independent experiment. Phalloidin is in blue in d, e, j, l and n. Scale bars, 100 µm.
apically within the hair cell body and required for the reverse-polarity currents that are observed during hair cell development, after tip-link breakage and in hair cells lacking TMC1/2. Piezo2 function is controlled by the intracellular Ca\(^{2+}\) concentration, suggesting that the two Ca\(^{2+}\)-permeable ion channels responsible for forward and reverse-polarity currents might engage in regulatory crosstalk. We thus conclude that hair cells contain two molecularly distinct mechanotransduction channels with different subcellular distribution and function. While Piezo2 is likely a pore-forming subunit of the reverse-polarity channel in hair cells, the proteins that form the pore carrying the normal-polarity sensory-transduction current still need to be determined. Overall, our findings show that mechanically gated ion channels important for different sensory modalities, such as perception of touch and sound, depend at least in part on different molecular components.

**RESULTS**

**Piezo2 is expressed in OHCs of the cochlea and in vestibular hair cells**

To determine the expression patterns of Piezo1 and Piezo2 in the inner ear, we carried out in situ hybridization in sagittal sections of the murine cochlea at postnatal day (P) 1 using antisense and control sense probes (Fig. 1c). As a positive control, we used a probe for Otof, which highlights both inner hair cells (IHCs) and OHCs at this age (Fig. 1c)\(^{1,2}\). We did not detect significant levels of Piezo1 mRNA in the inner ear. In contrast, Piezo2 expression was detected at low amounts in the cytoplasm of OHCs between the cell nucleus and the cuticular plate (Fig. 1c).

To confirm the expression pattern of Piezo2 in the inner ear, we crossed Piezo2-GFP-IRES-Cre mice expressing a Cre transgene from the endogenous Piezo2 genetic locus\(^{23}\) with the A9 mouse line, a Cre-dependent td-Tomato reporter\(^{24}\). In cochlear whole mounts at P7, td-Tomato expression was detected in OHCs throughout the length of the cochlear duct (Fig. 1d–g,i). We rarely observed Piezo2 expression in IHCs, but cannot rule out expression of low levels of Piezo2 that were hard to detect (Fig. 1e,g,i). We also observed td-Tomato expression in blood vessels. This was particularly obvious at P0, when expression levels of the fluorescence reporter in hair cells were very low (Fig. 1h). Analysis of the vestibule similarly revealed expression in hair cells and blood vessels (Fig. 1j–o).

Analysis of brain sections of mice obtained by crossing Piezo2-GFP-IRES-Cre mice with A9 mice revealed Piezo2 expression in blood vessels (Supplementary Fig. 1) but not in neurons throughout the auditory pathway, including neurons of the cochlear nucleus and superior olivary nucleus (Supplementary Fig. 1). Similar observations were made when we analyzed Piezo2 expression by in situ hybridization (data not shown).

**Hearing function in Piezo1/2-deficient mice**

To determine the function of Piezo proteins in the inner ear, we crossed Piezo2\(^{loxP/loxP}\) mice\(^{25}\) with Pax2-Cre mice, which express Cre in the presumptive otic ectoderm, thus leading to inactivation of Piezo2 throughout the inner ear\(^{25–28}\) (Pax2 is also expressed in midbrain neurons\(^{29}\), but we did not observe Piezo2 expression in midbrain neurons; Supplementary Fig. 1). While we did not observe Piezo1 expression in the inner ear, we also inactivated Piezo1 expression in combination with Piezo2 by crossing Piezo1\(^{loxP/loxP}\); Piezo2\(^{loxP/loxP}\) mice with Pax2-Cre mice. We will refer to the resulting mutant mice in this paper as Piezo2-cko and Piezo1/2-dcko mice. For some experiments, we inactivated Piezo1 alone by crossing Piezo1\(^{loxP/loxP}\) (ref. 30) mice with Pax2-Cre mice, and we will refer to the mutant mice as Piezo1-cko mice.

Measurement of the auditory brain stem response to broadband click-stimuli at P60 revealed a slight elevation (~15 dB) of auditory thresholds in Piezo2-cko and Piezo1/2-dcko mice relative to control wild-type and Pax2-Cre mice (Fig. 2a,b). Similar observations were made for pure tones between 8 and 20 kHz (Fig. 2c). Above 20 kHz, Pax2-Cre mice showed elevated thresholds compared to wild-type mice (Fig. 2c), suggesting that the genetic background of the Cre mouse line mildly affects auditory function at higher frequencies, preventing the analysis of Piezo1/2 function above 20 kHz.

We next recorded distortion-product otoacoustic emissions, which are mechanical distortions generated in the inner ear when two primary tones \(f_1\) and \(f_2\) are presented. OHCs amplify the distortions, \(2 f_1 - f_2\), which is consistent with the auditory brain stem response data. We thus conclude that OHC function is mildly affected in Piezo2-cko mice.

Piezo2-cko and Piezo1/2-dcko mice did not show obvious vestibular defects, which frequently manifest in mice by abnormal head posture...
and circling behavior. For quantification, we analyzed vestibular function with swim tests, scoring tail activity, hind leg activity, head position and body posture as described\(^3\). We did not observe any measurable defects between wild-type and mutant mice (Supplementary Fig. 2).

Given the mild auditory defects and lack of obvious vestibular defect, we did not evaluate vestibular function by electrophysiology.

**Piezo2 localization in hair cells**

To further define the function of Piezo1 and Piezo2 in hair cells and to confirm efficient ablation of Piezo1/2 proteins in the inner ear of mutant mice, we stained cochlear whole mounts with phalloidin to reveal their F-actin cytoskeleton and with antibodies to Piezo1 and Piezo2\(^2\),\(^3\),\(^3\). We used identical exposure times to collect images from wild-type and mutant animals. Consistent with our in situ hybridization data, we did not observe Piezo1 expression in the inner ear (data not shown). In contrast, cochlear whole-mount staining for Piezo2 revealed expression in OHCs at P5 (Fig. 3a–c). No expression was observed in Piezo2-cko mice, confirming the specificity of the staining signal (Fig. 3b). To analyze Piezo2 localization in hair cells more precisely, we collected optical sections from the tops of the stereocilia to the bottom of the hair-cell body (Fig. 3b,c). Piezo2 expression was detected near the apical surface of the cell body of hair cells where it appeared to be most concentrated close to the adherens junctions near the longest stereocilia (Fig. 3b–d). Piezo2 expression was not detected within the stereocilia, and very low to no expression was observed along the lateral membrane between hair cells and support cells (Fig. 3b). We collected higher magnification images and increased the exposure time dramatically but could not detect Piezo2 in stereocilia, while it was clearly detected near the apical surface of hair cells (Fig. 3c). We conclude that Piezo2 is probably not appropriately...
localized to be a component of the sensory-transduction channel that is present in hair cell stereocilia near tip links.²

Hair cell electromotility
The expression of Piezo2 in OHCs prompted us to investigate its role in amplification. Amplification by OHCs depends on length changes in the cell bodies of OHCs, termed electromotility. Electromotility is accompanied by a voltage-dependent gating-charge movement within the lateral membrane of OHCs, manifesting as a nonlinear capacitance.¹²–³⁵ Measurements of the nonlinear capacitance at P15 did not reveal significant difference between OHCs from wild-type and Piezo2-cko mice (Supplementary Fig. 3). We conclude that Piezo2 is likely not essential for normal amplification by OHCs.

Hair bundle morphology and mechanotransduction
We next asked whether Piezo2 might have a function in hair bundle development. We stained cochlear whole mounts at P80 with phalloidin but observed no obvious morphological defects in hair bundles or in the patterning of the sensory epithelium into three rows of OHCs and one row of IHCs (Fig. 4a,b). Similar observations were made at P5 (Fig. 3b). The findings were confirmed by scanning electron microscopy at P5 (Fig. 4c–l). Bundle polarity was maintained, stereocilia formed normal staircases and the stereocilia were connected by filamentosous linkages (Fig. 4m,n).

Although we did not detect Piezo2 expression in hair cell stereocilia, OHC function was mildly affected in Piezo2-cko mice (Fig. 2). We thus analyzed the extent to which mechanotransduction was affected in hair cells from Piezo1/2-dcko mice. We stimulated hair bundles of OHCs at P7 with a stiff glass probe (Fig. 5a) and recorded mechanotransduction currents in the whole-cell configuration. As reported,⁴,³⁶ OHCs from control mice had rapidly activating transducer currents, which subsequently adapted (Fig. 5a). Current displacement plots did not reveal an obvious difference in transducer current in hair cells from controls and Piezo2-1/2-dcko mice (Fig. 5b). The amplitude of saturated mechanotransduction currents in hair cells in the mid-apical part of the cochlea at maximal deflection with a stiff glass probe was at 498.47 ± 35.99 pA (mean ± s.e.m.) for control OHCs and 498.03 ± 54.19 pA (mean ± s.e.m.) for OHCs from mutants (Fig. 5b).

Severe defects in stiffness of hair bundles manifest as defects in mechanotransduction when hair bundles are stimulated with a fluid jet. We conducted fluid jet experiments at P7 but did not observe significant differences between OHCs from wild-type and mutant mice (Mann-Whitney test between control and Piezo2-cko groups: −10 V, P = 0.488; −8 V, P = 0.307; −6 V, P = 0.967; −4 V, P = 0.178; −2 V, P = 0.348; 0 V, P = 0.236; 2 V, P = 0.438; 4 V, P = 0.596; 6 V, P = 0.391; 8 V, P = 0.838; 10 V, P = 0.391; 12 V, P = 0.348; 14 V, P = 0.27; 16 V, P = 0.54; 18 V, P = 0.567; 20 V, P = 0.307; 22 V, P = 0.395; 24 V, P = 0.111; 26 V, P = 0.595; 28 V, P = 0.624; 30 V, P = 0.653; Supplementary Fig. 4).

Reverse-polarity currents in Piezo1 and Piezo2 single- and double-mutants
The reverse-polarity current originally reported for OHCs shows kinetic properties, with rapid onset followed by rapid adaptation, similar to those observed for Piezo2 (refs. 10,18). We thus tested the extent to which the reverse-polarity current was dependent on Piezo2. For this purpose, we used a fluid-jet stimulation system that has previously been used to deflect hair bundles in the normal-polarity and reverse-polarity directions.¹⁰ The experiments were carried out with 99 OHCs from 39 pups at P5. We recorded regular normal-polarity currents in hair cells from control, Piezo1-cko, Piezo2-cko and Piezo1/2-dcko mice (Fig. 5a–c; data not shown). Robust reverse-polarity currents were also recorded in OHCs from control and Piezo1-cko mice following the disruption of tip links with 5 mM BAPTA (Fig. 5c–f). However, no reverse-polarity currents could be evoked in the vast majority of OHCs from Piezo2-cko and Piezo1/2-dcko mice after tip-link disruption (Fig. 5c–f). The few hair cells that showed reverse-polarity currents might be a small population of OHCs that did not efficiently express the Cre used to inactivate Piezo1 and Piezo2. It is also possible that another channel is responsible for these rare reverse-polarity currents.

We next evaluated the extent to which Piezo2, expressed in heterologous cells and the reverse-polarity current in hair cells, was sensitive to dihydrostreptomycin (DHS). Previous studies have established that a 30% block of the reverse-polarity channel current can be achieved by 100 μM DHS in 1.5 mM extracellular Ca²⁺ (ref. 16). We showed that 100 μM DHS gives 40% and 25% blocks of Piezo2 expressed in human embryonic kidney (HEK) cells (Supplementary Fig. 5) at extracellular Ca²⁺ concentrations of 100 μM and 2.5 mM, respectively. We were unable to induce reverse-polarity currents in the majority of P5 IHCs both in wild-type mice following disruption of tip links and in TMC1/2 double mutants (Fig. 5g,h), which is consistent with the observation that we rarely observe Piezo2 expression in IHCs.

Effects of intracellular Ca²⁺ concentration on reverse-polarity currents
The appearance of reverse-polarity currents correlates with the disappearance of normal-polarity currents, which has led to the hypothesis that the two currents might depend on similar pore-forming ion-channel subunits.¹⁰,¹⁵,¹⁶,³⁷ Since our data suggest that different ion channels carry reverse-polarity and normal-polarity currents, we asked whether ion channels carrying normal-polarity currents suppress the activity of ion channels responsible for reverse-polarity currents. Importantly, transduction channels in OHCs are not completely closed at rest, and the intracellular Ca²⁺ concentration in OHCs is high.¹⁷,³⁸–⁴⁰ Channels close after tip-link breakage, thus preventing influx of Ca²⁺ through transduction channels.⁴¹ We therefore tested the extent to which the intracellular Ca²⁺ concentration might affect reverse-polarity currents. The reverse-polarity current was suppressed by elevating the intracellular Ca²⁺ concentration. This was exemplified by mechanotransduction currents in early postnatal mice (P0 basal OHCs; Fig. 6a), in which the hair cells displayed normal- and reverse-polarity currents when recordings were made with a patch pipette containing a solution that included the Ca²⁺ buffer EGTA (Fig. 6a). However, when recordings were made in the same preparation using an intracellular solution containing 1 mM Ca²⁺, reverse-polarity current was present immediately after rupturing the patch but quickly disappeared as Ca²⁺ was washed into the cytoplasm (Fig. 6b). There was no substantial reduction in the amplitude of the normal mechanotransduction current (Fig. 6b), indicating no general deterioration in the cell during perfusion with high Ca²⁺. Similar effects of Ca²⁺ wash-in were seen in five OHCs with 1 mM Ca²⁺ in the pipette solution; in these cells the mean reverse-polarity current was 600 ± 130 pA immediately after starting whole-cell recording and diminished after four minutes recording to 2 ± 0 pA. When 0.1 mM Ca²⁺ was present in the patch-pipette solution, the reverse-polarity current was unaffected after 14 min of recording. A similar Ca²⁺ block was obtained when the reverse-polarity current was elicited in Tmc1/Tmc2 double knockouts or after destruction of the tip links by exposing the hair bundle to BAPTA (data not shown).

The Ca²⁺ concentration needed to suppress the reverse-polarity current is high but not unrealistic, since we have previously estimated from Ca²⁺ reversal-potential measurements that Ca²⁺ in the stereocilia occurs in near-millimolar concentrations.³⁷ While the Ca²⁺ concentration is likely highest near the inner face of the mechanoelectrical transducer channel, the concentration further down the stereocilium
Figure 4  Hair bundle morphology in Piezo2-cko mice. (a,b) Analysis of the cochlea in P80 control and mutant animals by whole mount staining with phalloidin. There were no obvious defects in the patterning of the sensory epithelium or in the morphology of hair bundles of OHCs and IHCs. (c–n) Analysis of hair cells by scanning electron microscope at P5. Hair cells in the middle part (c,e,g,h,k,l) and base (d,f,i,j) of the cochlea were imaged. (m,n) Higher magnification view of stereocilia of IHCs showing linkages between them. The experiments were carried out three times for P80 animals and twice for s.e.m. At least 5 images were collected from each independent experiment. Scale bars: 10 µm in a,b; 5 µm in c-f; 1 µm in g-l; 100 nm in m,n. PZ2cko, Piezo2-cko mice.
and at the top of the cell will still be substantial as Ca\(^{2+}\) buffering in neurons is small\(^{12}\). Furthermore, the Goldman-Hodgkin-Katz equation used to infer the Ca\(^{2+}\) level\(^{17}\) applies to the bulk concentration just as the membrane potential refers to the potential difference between the bulk solutions and not to the membrane surface potential. During neonatal development, Ca\(^{2+}\) influx through the normal-polarity MT channels (which are two to three times more Ca\(^{2+}\)-permeable than reverse-polarity channels)\(^{16}\) therefore likely increases Ca\(^{2+}\) to a level sufficient to suppress the reverse-polarity current.

We next tested the extent to which the intracellular Ca\(^{2+}\) concentration affects Piezo2 function directly. We expressed Piezo2 in HEK293 cells and applied a series of force steps to the cell surface via a piezo-driven glass probe. Currents were recorded with whole-cell voltage clamp recordings with a pipette solution containing up to 10 mM Ca\(^{2+}\). There was no obvious effect of Ca\(^{2+}\) on the maximal response of Piezo2 to mechanical stimulation even after minutes of perfusion (Fig. 6c). These data suggest that Ca\(^{2+}\) affects Piezo2 indirectly, perhaps by regulating the activity of other proteins such as a kinase, which then regulates Piezo2 function or transport directly, or via Piezo2-associated proteins. This interpretation is consistent with the observation that it takes minutes for reverse-polarity currents to reach full amplitude following disruption of tip links\(^{10}\).

**Maturation of normal-polarity currents**

All recordings described above were carried out with hair cells from mice in which Piezo2 function had been inactivated by tissue-specific Cre-mediated recombination. Although our immunolocalization studies suggested that we efficiently inactivated Piezo2 function in Piezo2-cko mice (Fig. 3), we wanted to exclude that a small amount of Piezo2 protein might have persisted. We therefore analyzed transducer currents in hair cells from Piezo2-null mice. Since Piezo2-null mice die at birth\(^{43}\), we established cochlear cultures from mice at E18.5 and cultured them for several days. We stimulated OHCs from wild-type and mutant mice at similar positions in the medial part of the cochlea with a fluid jet, which allowed us to record normal-polarity and reverse-polarity currents. As reported earlier in rat\(^{43}\) and mice\(^{16,37,44}\), transducer currents in wild-type mice matured in vitro over several days (Fig. 7a). Similarly to previous reports using zebrafish and rodents\(^{10-13,37}\), we observed that currents in immature hair cells of wild-type mice at P0 revealed both normal- and reverse-polarity currents not having reached full amplitude (Fig. 7a). In wild-type mice, the amplitude of normal-polarity currents increased nearly eightfold after 1 d in culture and even further after 2 d (Fig. 7a), which is consistent with the progressive maturation of the transduction machinery even in vitro. In striking contrast, in OHCs from Piezo2-null mice, reverse-polarity currents were not detectable at P0 or after culturing hair cells for 1 or 2 d (Fig. 7a). However, normal-polarity currents were present, but they appeared to reach full amplitude on an accelerated time course. Normal-polarity currents were nearly fourfold larger in mutants at P0 compared to wild-type mice, about twofold larger after 1 d in culture but indistinguishable from those in wild-type mice after 2 d in culture. A similar delay in...
maturation was observed with acutely isolated hair cells from Piezo2-cko mice. In explants from Piezo2-cko mice, mechanotransduction currents at P0 were small and indistinguishable between wild-type and mutant mice, but over the next 3 d they matured slightly faster in hair cells from Piezo2-cko mice compared to wild-type cells (Fig. 7b).

**Induction of reverse-polarity currents by damage to the sensory epithelium**

Disruption of tip links and genetic mutations that affect components of the sensory-transduction machinery in stereocilia such as TMHS, TMIE and TMC1/2 unmask reverse-polarity currents in hair cells7,10,12,15. Reverse-polarity currents were not induced by careful dissection of the sensory epithelium of wild-type mice for electrophysiological recordings (Fig. 5c). Treatment of organ explants with chemicals that cause oxidative stress, such as H2O2, also did not lead to an activation of reverse-polarity currents (data not shown). However, when we mechanically disrupted the integrity of the sensory epithelium with a pipette, robust reverse-polarity currents were evoked in OHCs (Fig. 7c) near the stab wound (<10 cell-diameters from the wound) but not further away (>20 cell-diameters) where epithelial architecture was not affected (Fig. 7c). Notably, in hair cells near the stab wound, forward-polarity currents of normal amplitude and reverse-polarity currents could be evoked in parallel in the same cell (Fig. 7c). These findings suggest that reverse-polarity currents in OHCs can be evoked by mechanical damage to the sensory epithelium.

**DISCUSSION**

We provide here evidence that OHCs in the murine cochlea express two molecularly and functionally distinct mechanotransduction channels.

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**Figure 6** Effect of the intracellular Ca2+ concentration on reverse-polarity currents. (a) Normal and reverse-polarity currents recorded 30 s after patch rupture (top) and 4 min after attaining whole-cell recording. Internal solution containing 1 mM EGTA had no effect on either component. (b) Normal- and reverse-polarity currents 30 s after patch rupture (top) and 4 min after attaining whole-cell recording. Internal solution with 1 mM Ca2+ blocked reverse-polarity component but had no effect on normal MT currents. Similar effects of Ca2+ wash-in were seen in 5 OHCs with 1 mM Ca2+ in the pipette solution where the reverse-polarity current was 600 ± 130 pA immediately after attaining whole-cell recording and diminished after 4 min of recording to 2 ± 0 pA, but when EGTA was present in the patch-pipette solution, the reverse-polarity current was unaffected after 14 min of recording. (c) Piezo2-GFP was expressed in HEK cells and the cells were mechanically stimulated with a series of force steps to the cell surface via a piezo-driven glass probe. Currents were recorded with whole-cell voltage-clamp recordings with a pipette solution containing up to 10 mM Ca2+. Values are mean ± s.e.m.; paired t-test: \( P = 0.811, \ t_b = 0.247 \).

**Figure 7** Maturation of mechanotransduction in OHCs from Piezo2-null mice and effects of mechanical disruption of the sensory epithelium on channel activity. (a) Cochlear explants were prepared from control and Piezo2-null mice at P0 and were cultured for the indicated time (DIV, days in vitro). Mechanotransduction currents were evoked by sinusoidal deflection of hair bundles with a fluid jet using OHCs at a similar position in the medial part of the cochlea in controls and mutants. Left, typical recordings for an OHC from a wild-type and a Piezo2-null mouse. Right, quantitative analysis of the forward currents (top) and reverse-polarity currents (bottom) data from the experiment shown on the left. The numbers of hair cells used for recordings are indicated. Mann-Whitney test between WT and Piezo2-null mice; forward currents, * \( P = 0.013, U = 57; P0 + 1 \) DIV (forward currents), ** \( P = 0.004, U = 0; P0 + 2 \) DIV (forward currents), \( P = 0.762, U = 10; P0 \) reverse currents, *** \( P < 0.0001, U = 22.5; P0 + 1 \) DIV (reverse currents), \( P = 0.017, U = 2.5; P0 + 2 \) DIV (reverse currents), \( P = 0.476, U = 8 \). (b) Experiments similar to those in a on fresh cochlear explants from control and Piezo2-cko mice from P0 to P3. Values are mean ± s.e.m. Mann-Whitney test between WT and Piezo2-null mice: P0 forward currents, \( P = 0.057, U = 13; P0 + 2 \) DIV (forward currents), ** \( P = 0.005, U = 4; P0 \) (forward currents), \( P = 0.247, U = 8; P0 + 1 \) DIV (forward currents), *** \( P = 0.005, U = 9; P1 \) (reverse currents), \( P = 0.013, U = 5; P2 \) (reverse currents), * \( P = 0.03, U = 3; P3 \) (reverse currents), \( P = 1, U = 10 \). (c) Left: Top diagram shows experimental strategy for mechanically disrupting the sensory epithelium by insertion of a pipette; bottom, representative mechanotransduction currents that were evoked with a fluid jet after mechanical disruption of the sensory epithelium (P4). Right: quantitative analysis of the data. Values are mean ± s.e.m. Mann-Whitney test between OHCs at different distance from wound site: * \( P = 0.01, U = 0 \) for reverse-polarity currents and \( P = 0.257, U = 6.5 \) for normal-polarity currents. Cell numbers indicate the distance in cell diameters between recorded cells and wound site. (d) Model showing the localization of sensory-transduction channels and Piezo2-dependent channels in hair cells, and their cross-regulation by Ca2+.
One transduction channel is present in the stereocilia near tip links and is required for sensory-transduction. The second transduction channel contains Piezo2 and is concentrated at the apical surface of OHCs. The Piezo2-containing ion channel is observed in developing hair cells and can be unmasked at later developmental stages by breaking tip links or by damaging the sensory epithelium. Auditory function is mildly perturbed in the absence of Piezo2. As one possibility, the mild auditory defects in Piezo2-cko mice may indicate a role for Piezo2 in facilitating continuous repair in the sensory-transduction machinery, which might undergo turnover throughout life.

Hair cells of the mammalian cochlea are mechanosensory cells that convert sound-induced vibrations into electrochemical signals. The mechanically gated ion channel that is activated by sound has been studied for decades. This sensory-transduction channel is activated by deflection of the hair bundle in the direction of the longest stereocilia (normal polarity) and is present in stereocilia near the lower end of tip links. TMHS, TMIE, TMC1 and TMC2 are candidate subunits of the sensory-transduction channel. The observation that OHCs express a second mechanically activated ion channel at their apical surface is a surprise. We show that this ion channel contains Piezo2 and that it is activated in early developing hair cells by stimulation of the hair bundle in the direction opposite the normal direction of stimulation (reverse polarity). In wild-type hair cells, the sensory-transduction machinery matures postnatally over several days. Unexpectedly, our findings indicated that maturation was slightly accelerated in the absence of Piezo2. The mechanism by which Piezo2 affects the maturation of the sensory-transduction machinery is unknown, and the physiological relevance of this phenomenon needs further investigation.

We are intrigued by the observation that Ca\textsuperscript{2+} regulates the activity of the Piezo2-dependent ion channel. This observation perhaps explains why reverse-polarity currents are prominently detected in postnatal hair cells after but not before disruption of the sensory-transduction machinery. Sensory-transduction channels are not completely closed at rest, leading to influx of Ca\textsuperscript{2+} (ref. 38). The Ca\textsuperscript{2+} concentration in stereocilia in rodents has been estimated to be in the range of ~1 mM (ref. 17). Upon disruption of the transduction machinery, intracellular Ca\textsuperscript{2+} levels decline; this could then lead to the activation of the Piezo2-dependent ion channel (Fig. 7d). Consistent with this model, when we increase intracellular Ca\textsuperscript{2+} levels in hair cells following tip-link breakage, the activity of the Piezo2-dependent ion channel was reduced. When we expressed Piezo2 in heterologous cells, we could not alter its activity by changes in the intracellular Ca\textsuperscript{2+} concentration. This suggests that the effect of intracellular Ca\textsuperscript{2+} levels on Piezo2 function may be mediated by an intermediary protein (X) such as a kinase or phosphatase (Fig. 7d). Consistent with this model, reverse-polarity currents of full amplitude are observed in hair cells only several minutes after disruption of tip links. This could be in part attributed to slow changes in the intracellular Ca\textsuperscript{2+} concentration but might also signify intermediate steps in the regulation of Piezo2 function, for example by a Ca\textsuperscript{2+}-dependent kinase that phosphorylates Piezo2 directly or by a Piezo2-associated protein. Notably, Piezo2 accumulates at the apical surface of hair cells near the longest stereocilia, and its specific subcellular distribution might contribute to regulation by Ca\textsuperscript{2+}, for example by affecting insertion of Piezo2 into the membrane. Such Ca\textsuperscript{2+} dependence may not be easily recapitulated in a heterologous system.

Previous studies identified stretch-activated ion channels in the lateral membrane of hair cells. We consider it unlikely that Piezo2 is the ion channel responsible for the reported currents. We detected little to no Piezo2 in the lateral membranes of hair cells, and the properties of the reported channels are inconsistent with the properties of Piezo2. One study reported a stretch-activated channel that is a potassium channel, while a second study reported a channel that is permeable to cations and anions. A third study identified two types of stretch-activated ion channels, one with a voltage dependence inconsistent with the properties of Piezo2 and another that is likely a potassium channel.

The ion channel carrying the reverse-polarity current is activated by disruptions of the sensory-transduction machinery and by disruption of the sensory epithelium. The localization of Piezo2 at the apical surface of hair cells near junctional complexes between hair cells and support cells is intriguing. Perhaps Piezo2 is critical for sensing mechanical forces across the sensory epithelium that depend on adhesive contacts between hair cells and support cells. This might help to coordinate the maturation of the exquisitely organized sensory epithelium with the maturation of the sensory-transduction machinery.
It might also facilitate coordination or repair processes that are activated following pathological disruption of the sensory epithelium. In this regard, it is notable that Piezo1 and Piezo2 have been implicated in the mechanical injury response in cartilage.49 While speculative, Piezo2 might also serve as a backup to maintain residual drive for electromotility when hair bundles are damaged. As such, dual-channel specialization in OHCs might be viewed as a safeguard mechanism in situations of hair cell damage.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank S.-H. Woo and K. Nonomura (The Scripps Research Institute, La Jolla, California, USA) for providing genetically modified mice for Piezo2 and for comments on the manuscript. A. Patapoutian is an investigator of the Howard Hughes Medical Institute. L.S. was supported by National Institutes of Health grant. This research was funded by NIDCR grants DC005965, DC00704, DC014713 (U. Müller), DC01362 (Fettipace); NIDCR Deo22358 (A. Patapoutian); and the Dorris Neuroscience Center; the Skaggs Institute for Chemical Biology (U. Müller).

**AUTHOR CONTRIBUTIONS**

All authors contributed to experimental design. Z.W. and N.G. designed and performed most of the experiments. S.R. generated some of the genetically modified mice; C.C. and N.Z. participated in gene expression studies; B.Z. carried out electron microscopic studies; B.C. analyzed effects of pharmacological inhibitors on Piezo2 expressed in heterologous cells; S.H.-P. carried out auditory experiments and participated in data analysis and interpretation. U.M. and Z.W. wrote the manuscript with substantial input from the other authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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1. Gillespie, P.G. & Müller, U. Mechanotransduction by hair cells: models, molecules, and mechanisms. Cell 139, 33–44 (2009).
2. Beurg, M., Fettiplace, R., Nam, J.H. & Ricci, A.J. Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. Nat. Neurosci. 12, 553–558 (2009).
3. Kurima, K., TMC1 and TMC2 localize at the site of mechanotransduction in mammalian inner ear hair cell stereocilia. Cell Rep. 12, 1606–1617 (2015).
4. Xiong, W. et al. TMHS is an integral component of the mechanotransduction machinery of cochlear hair cells. Cell 151, 1283–1295 (2012).
5. Zhao, B. et al. TMIE is an essential component of the mechanotransduction machinery of cochlear hair cells. Neuron 84, 954–967 (2014).
6. Labay, V., Weichert, R.M., Makishima, T. & Griffith, A.J. Topology of transmembrane channel-like gene 1 protein. Biochemistry 49, 8952–8958 (2010).
7. Kawashima, Y. et al. Mechanotransduction in mouse inner ear hair cells requires Piezo2. J. Clin. Invest. 121, 4796–4809 (2011).
8. Kim, K.X. & Fettipace, R. Developmental changes in the cochlear hair cell mechanotransducer channel and their regulation by transmembrane channel-like proteins. J. Gen. Physiol. 141, 141–148 (2013).
9. Pan, B. et al. TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. Neuron 79, 504–515 (2013).
10. Kim, K.X. et al. The role of transmembrane channel-like proteins in the operation of hair cell mechanotransduction channels. J. Gen. Physiol. 142, 493–505 (2013).
11. Kindt, K.S., Finch, G. & Nicolson, T. Kinocilia mediate mechanosensitivity in developing zebrafish hair cells. Dev. Cell 23, 329–341 (2012).
12. Marzocchi, W. et al. Transduction without tip links in cochlear hair cells is mediated by ion channels with permeation properties distinct from those of the mechanoelectrical transducer channel. J. Neurosci. 34, 5505–5514 (2014).
13. Waguespack, J., Salles, F.T., Kacher, B. & Ricci, A.J. Stepwise morphological and functional maturation of mechanotransduction in rat outer hair cells. J. Neurosci. 27, 13890–13902 (2007).
14. Aalagramam, K. et al. Mutations in protocadherin 15 and cadherin 23 affect tip links and mechanotransduction in mammalian sensory hair cells. PLoS One 6, e19183 (2011).
HEK293 cells were directly transduced to express Piezo2-GFP18 using Lipofectamine 2000 (ThermoFisher Scientific). Transfections were performed with jClamp (SciSoftCo., New Haven, CT) and fitted to the first derivative of a two-state Boltzmann function with SigmaPlot (San Jose, CA)59:

\[
C_m = NLC + C_{\text{lin}} = Q_{\text{max}} \times (2e \nu \pi kT) \times \left(1 + e^{-\frac{ze(V_{m} - V_{p})}{kT}}\right)^{-2} + C_{\text{lin}}
\]

where \(Q_{\text{max}}\) is the maximum charge transferred, \(V_{m}\) is the peak of NLC, \(z\) is the number of elementary charge (e), \(k\) is Boltzmann's constant, and \(T\) is the absolute temperature. Membrane potential \(V_{m}\) was corrected for electrode access resistance (Rs).

Transfection and electrophysiology in cell lines. HEK293 cells were directly purchased from ATCC (Manassas, VA) and were authenticated by STR analysis with AuthentiFiler PCR Amplification Kit (ThermoFisher Scientific). Cells were tested for mycoplasma on a weekly basis with a mycoplasma kit (ThermoFisher Scientific). HEK293 cells were transiently transduced to express Piezo2-GFP18 using Lipofectamine 2000 (ThermoFisher Scientific). Twelve hours after transfection, cells were dissociated and plated onto coverslips precoated with poly-L-lysine and imaged. Cells were whole-cell patched to record mechanically evoked currents at −70 mV using an Axopatch 200B (Molecular Devices, CA). Mechanical stimulation of the cell surface was achieved by a glass probe driven by piezoelectric crystal microstage (E625 Amplifiers, Physik Instrument, Karlsruhe, Germany). The recording chamber was perfused with (in mM): 127 NaCl, 3 KCl, 2.5 CaCl2, 1 MgCl2, 10 glucose and 10 K–HEPES, pH 7.4. In some recordings, Ca2+ concentration was reduced to 0.20 mM. The patch pipette was filled with intracellular solution (133 mM CsCl, 1 mM MgCl2, 5 mM EGTA, 10 mM CaCl2, 4 mM Mg-ATP, 0.4 mM Na2-GTP and 10 mM H–HEPES, pH 7.3). Osmolarity was 295 mosm.

Data analysis and statistics. Data analysis was performed using Excel (Microsoft, Redmond, WA), OriginLab Northampton, MA) and MATLAB (MathWorks, Natick, MA). All data are mean ± s.e.m. or percentages. Data collection was randomized. Some electrophysiological recordings were performed blind to genotype; genotyping was performed by another person after recording (Figs. 5a–f and 7a,b). No statistical methods were used to predetermine sample sizes, but our sample size is similar to those reported in previous publications4,5,17,19,37. Data analysis was performed with statistical testing between different groups was analyzed with Fisher's exact tests (two groups) or χ2 tests (more than two groups). P < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001). Detailed statistics for all experiments are provided in the figure legends and in the Supplementary Methods Checklist.

Data availability statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.
50. Cahalan, S.M. et al. Piezo1 links mechanical forces to red blood cell volume. eLife 4, 2 (2015).

51. Woo, S.H. et al. Piezo2 is the principal mechanotransduction channel for proprioception. Nat. Neurosci. 18, 1756–1762 (2015).

52. Schwander, M. et al. A forward genetics screen in mice identifies recessive deafness traits and reveals that pejvakin is essential for outer hair cell function. J. Neurosci. 27, 2163–2175 (2007).

53. Grillet, N. et al. Mutations in LOXHD1, an evolutionarily conserved stereociliary protein, disrupt hair cell function in mice and cause progressive hearing loss in humans. Am. J. Hum. Genet. 85, 328–337 (2009).

54. Beurg, M., Tan, X. & Fettiplace, R. A prestin motor in chicken auditory hair cells: active force generation in a nonmammalian species. Neuron 79, 69–81 (2013).

55. Zhu, Y. et al. Active cochlear amplification is dependent on supporting cell gap junctions. Nat. Commun. 4, 1786 (2013).