ER stress inhibitor attenuates hearing loss and hair cell death in Cdh23\textsuperscript{erl/erl} mutant mice

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Hearing loss is one of the most common sensory impairments in humans. Mouse mutant models helped us to better understand the mechanisms of hearing loss. Recently, we have discovered that the erlong (erl) mutation of the cadherin23 (Cdh23) gene leads to hearing loss due to hair cell apoptosis. In this study, we aimed to reveal the molecular pathways upstream to apoptosis in hair cells to exploit more effective therapeutics than an anti-apoptosis strategy. Our results suggest that endoplasmic reticulum (ER) stress is the earliest molecular event leading to the apoptosis of hair cells and hearing loss in erl mice. We also report that the ER stress inhibitor, Salubrinal (Sal), could delay the progression of hearing loss and preserve hair cells. Our results provide evidence that therapies targeting signaling pathways in ER stress development prevent hair cell apoptosis at an early stage and lead to better outcomes than those targeting downstream factors, such as tip-link degeneration and apoptosis.

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The cadherin 23 (CDH23) protein is localized in the upper part of the tip link in hair cells, where it functions as a key component.\textsuperscript{1–3} In humans, Cdh23 mutations cause nonsyndromic autosomal recessive deafness (DFNB12) and Usher syndrome type 1D, USH1D, characterized by deafness associated with retinitis pigmentosa and vestibular dysfunction.\textsuperscript{4–6} In mice, Cdh23 mutations lead to hearing loss with or without vestibular dysfunction.\textsuperscript{7–9} Recently, we identified a novel point mutation (T208C) of Cdh23 and named this mutation erlong (erl).\textsuperscript{10} The Cdh23\textsuperscript{erl/erl} mice (erl mice) proved to be animal models of DFNB12. Previously, we showed that hair cell apoptosis is one of the pathological mechanisms leading to hearing loss in this mutant.\textsuperscript{10} Here, we aimed to reveal that ER stress signaling is the upstream pathway leading to hair cell apoptosis in mice with the erl mutation, and we sought to find potential therapeutics.

The perturbation of endoplasmic reticulum (ER) homeostasis leads to the accumulation of unfolded or misfolded proteins in the ER lumen, resulting in ER stress. The unfolded protein response (UPR) is subsequently triggered to alleviate this stress and to restore ER homeostasis, promoting cell adaptation and survival. Conversely, if the stress is prolonged, or if the adaptive response fails, the apoptosis pathway will be initiated.\textsuperscript{11–13} The UPR is mediated through three ER transmembrane receptors: protein kinase RNA-like ER kinase (PERK); activating transcription factor-6 (ATF6); and inositol-requiring enzyme 1 (IRE1).\textsuperscript{11–14} Upstream of this network, the ER chaperone immunoglobulin-binding protein (BiP) and the pro-apoptotic factor CHOP in the mutant.10 Here, we aimed to reveal that ER stress signaling is the upstream pathway leading to hair cell apoptosis in mice with the erl mutation, and we sought to find potential therapeutics.

CDH23 partly failed to reach the top of hair bundles and were co-localized with BiP in the subapical regions of OHCs in erl mice. We measured the CDH23 distribution in...
cochlear outer hair cells (OHCs) in erl mice and B6 mice. At P4, the CDH23 protein was specifically localized at the top of the OHCs in B6 mice, as revealed by confocal 3D images, indicating the localization of the tip links in hair bundle stereocilia (Figure 1a). In contrast, the CDH23 protein localized from the stereocilia to the nuclei of the OHCs in erl mice. Portions of the CDH23 proteins failed to reach the top of the hair bundles and remained in the OHC cytoplasm. Immunostaining showed that the ER chaperone BiP was more readily detected in almost the same region and co-localized with cytoplasmic CDH23 in the erl mouse OHCs at P6 (Figure 1b), whereas no specific BiP signaling was detectable in the cytoplasm of the B6 mouse OHCs. Line charts confirmed the higher expression of BiP and its co-localization with CDH23 in the erl OHC cytoplasm (Figure 1c) when compared with the fluorescence intensity of CDH23 and BiP.

The PERK arm of UPR was activated in the erl mouse cochlea. The ER stress marker BiP was upregulated in the erl mouse inner ears, as compared with those of B6 mice. In the measurements of mRNA isolated from the erl mouse cochleae, BiP was upregulated at P12 but downregulated at P30 (Figure 2a). Immunostaining showed not only a greater expression of BiP in OHCs but also that BiP was expressed at higher levels in spiral ganglion (SG) cells and in stria vascularis (StV) in the erl cochleae at P6 and P12 (Figure 2b and c). Consistent with the confocal three-dimensional (3D) images, the BiP signals were mainly localized in the OHC cytoplasm and formed a hat-like pattern...
revealed that the BiP signals were expressed in the OHC area of the
Cdh23erl/erl genotypes of mice sharing the same genetic background (showed higher intensities in the StV, SG and especially OHCs of the erl Chop by crossing disruption in the Chop mice containing the erl stress-induced apoptosis in erl erl in Chop Disruption of the erl
a bare CHOP signal was present in B6 mouse cochleae. The same age showed that CHOP was highly detected in the P12 and P30 (Figure 4a). Immunostaining performed at the expression in the erl (Figure 4). At the mRNA level, Chop maintained a higher erl CHOP was also expressed at higher levels in the erl cochleae (Figure 3). Localization in the perinuclear regions of the OHCs in the erl
expression in the eukaryotic initiation factor (eIF2α
Figure 2
Downstream of the PERK arm, the pro-apoptotic factor Chop was also expressed at higher levels in the erl cochleae (Figure 4). At the mRNA level, Chop maintained a higher expression in the erl cochleae compared with B6 cochleae at P12 and P30 (Figure 4a). Immunostaining performed at the same age showed that CHOP was highly detected in the OHCs, SG and StV of erl mice and was mainly detected in the perinuclear regions of OHCs (Figure 4b and c). However, a bare CHOP signal was present in B6 mouse cochleae.

Disruption of the Chop gene protected hearing and OHCs in erl mice. To further confirm the involvement of Chop in ER stress-induced apoptosis in erl mice, we used double-mutant mice containing the erl mutation in the Cdh23 gene and a disruption in the Chop gene. The inbred mice were obtained by crossing Chop+/− mice with Cdh23+/+erl mice. Nine genotypes of mice sharing the same genetic background (C57BL/6) were generated: Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; Cdh23+/+Chop+/−; and Cdh23+/+Chop+/−. Supplementary Figure 1 shows the genotyping of the double-mutant Cdh23+/+Chop−/− mice. We measured the hearing and cytocochleograms of the Cdh23+/+Chop−/−; erl (Cdh23+/+erl Chop−/−); and Chop−/− (Cdh23+/+Chop−/−) mice. At 10 weeks, the auditory-evoked brainstem response (ABR) thresholds in the Cdh23+/+Chop−/− double-mutant mice were significantly better than those in the erl mice (Figure 5a). The surface preparation performed at the same age revealed less loss of OHC in the Cdh23+/+erlChop−/− double-mutant mice in comparison with Cdh23+/+erl mouse. In the erl mice, several spots of OHC loss were exhibited in the basal and middle turns, and a few OHC losses were shown in the apical turns of the cochlea. However, in the Cdh23+/+erlChop−/− double-mutant mice, no OHC loss was found in the entire length of the cochlea (Figure 5b). The quantitative study showed that the mean percentage of OHC loss in the Cdh23+/+erlChop−/− mice was significantly lower than that in the erl mice (Figure 5c).

Sal prevented hearing loss in erl mice. After receiving corresponding treatments, the erl mice in the test, vehicle and control groups underwent ABR and distortion product otoacoustic emission (DPOAE) testing at the same ages. The ABR thresholds in the test group were significantly lower than those in the vehicle and control groups in response to 16 (Figure 6a and b), 8 and 32 kHz tone-burst and click stimuli (Supplementary Figure 2). No significant difference was found between the vehicle and control groups. The otoprotective effect was further confirmed by the higher DPOAE amplitudes. At 8 weeks, the DPOAE amplitudes in the test group were significantly greater at high frequencies (Supplementary Figure 3a). At 12 weeks, the test group showed much higher DPOAE amplitudes at almost all frequencies (Figure 6c). At 16 weeks, mice in all three groups showed lower DPOAE amplitudes, whereas the test group’s amplitudes remained higher at low frequencies (Supplementary Figure 3b).

Sal protected against OHC death in erl mice. A cochlear surface preparation was performed on erl mice in the test,
Figure 3  The eIF2α phosphorylation in the B6 and erl cochleae at P30. The immunofluorescence labeling of phospho-eIF2α showed strong cytoplasmic and perinuclear localization in the cochleae of the erl mice. The line intensity analysis along the arrow shown in the panels to the left revealed that the p-eIF2α signals were expressed in the OHC and stria vascularis (StV) areas and were strongly detected around the nuclei in the erl cochleae (n = 3 mice per group). Scale bars, 50 μm (upper); 20 μm (lower).

Figure 4  CHOP expression in the B6 and erl mouse cochleae. (a) The Chop mRNA levels in the cochleae of the B6 and erl mice at P12 and P30. The Chop mRNA level was significantly higher at P12 and P30 in the erl cochlea than in the B6 cochlea. The error bars represent S.E.M. (*P < 0.05, n = 3 mice, t-test). (b,c) CHOP maintained greater intensities in stria vascularis (StV), spiral ganglion (SG), especially in the perinuclear region between nucleus and hair bundles of the OHCs in the erl mice at P12 and P30 (n = 3 mice per group). Scale bars, 50 μm (left); 20 μm (right).
vehicle and control groups at 12 weeks. OHC impairment was obvious in the vehicle and control groups, while OHC loss was very minimal in the test group (Figure 7a). In the vehicle and control groups, several substantial contiguous spots of OHC loss were observed in the basal and middle turns, and some OHC loss was seen in the apex turns. In contrast, the mice in the test group showed only small amounts of OHC loss in the basal turn and rare OHC loss in the middle turn. The mean percentage of OHC loss in the test group was significantly lower than that in the vehicle and control groups (Figure 7b). No significant difference was found between the vehicle and control groups.

A scanning electron microscope was used to analyze OHC morphology and subcellular structures at 12 weeks (Figure 7c). The images showed that untreated erl mice exhibited almost total OHC loss and no detectable subcellular structure of hair bundles in the basal turn. In contrast, the Sal-treated mice showed small amounts of OHC loss and approximate normal arrangements of hair bundles.

Sal suppressed ER stress-induced apoptosis in erl mice. ER stress and apoptosis-related genes and proteins were downregulated after the Sal treatments. The mRNA expression levels of Bip, Chop, caspase-3 and caspase-12 genes were tested at P30 and 12 weeks. The results showed that Bip and Chop decreased in the Sal group versus the vehicle group at P30, and caspase-3 was downregulated at 12 weeks (Figures 8a and b). However, no significant difference was found in the caspase-12 gene expression (Supplementary Figure 4). BIP and cleaved caspase-3 protein extracted from the...
erl mouse cochleae in the vehicle and test groups were measured by Western blot at P30. The results showed that BiP and cleaved caspase-3 were much less abundant in the Sal-treated cochleae than in the vehicle-treated cochleae (Figures 8c). Furthermore, CHOP was immunolabeled in the vehicle and test groups at P30. The results showed that the CHOP signals in the OHCs, SG and StV in DMSO-treated cochleae were distinctly weakened by Sal treatment, and the most significant difference in CHOP expression was seen in the perinuclear regions of the OHCs (Figures 8d).

Discussion
As a novel mutation of the Cdh23 gene, the erl mutant mice exhibited the postnatal onset of hearing loss starting at P27, which progressed to total deafness at ~P100. This is considered to be an animal model for human DFNB12.10
Given their time window from initial hearing-loss initiation to total deafness, these mutant mice are ideal tools for testing new otoprotective drugs. We previously revealed that erl mice treated with erythropoietin and Z-VAD-FMK could be significantly protected against OHC death and rescued from hearing loss through the blocking of the apoptosis pathway in erl mice.\textsuperscript{10,16} In the current study, the upstream apoptosis response in the erl cochleae was discovered to be relevant to ER stress and the UPR.

Under the quality-control surveillance of the ER, only correctly folded proteins can be transferred by the Golgi complex and sent to their final destinations, while unfolded or misfolded proteins are retained in the ER and ultimately degraded by the ubiquitin-dependent proteolytic pathway or autophagy.\textsuperscript{17,18} The accumulation and aggregation of unfolded or misfolded proteins in the ER lumen increase ER loading and disrupt ER homeostasis, ultimately leading to ER stress.\textsuperscript{19,20} Under mild-to-moderate stimulation, ER stress is an adaptive and restorative response that leads to cell adaptation. In contrast, if the stress is beyond the ER’s adaptive capacity, the protective signaling will switch to pro-apoptotic responses. ER stress has been linked to the pathogenesis of neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease, as well as to the pathogenesis of cell death, such as renal tubule lesions in diabetes.\textsuperscript{21–23} In zebrafish mutants, the defective CDH23 proteins in hair cells failed to be transferred and contributed to the formation of Usher protein complexes, therefore leading to ER stress.\textsuperscript{15} Taken together, these results offered us new insight into the pathological mechanisms of and potential therapies for sensorineural deafness. Using an animal model for mammals, we questioned whether similar protein-folding defects and the activation of ER stress happened in mice with the Cdh23 mutation.

We provided evidence that part of the ERL-CDH23 protein exhibited abnormal localization in the OHC (from the stereocilia to the nucleus, Figure 1a), failing to reach the top of hair bundles and thus leading to reduced migration to the tip links.

**Figure 8**  
Sal downregulated ER stress and apoptosis-related genes and proteins. (a) BiP and Chop mRNA were downregulated in the Sal-treated mice at P30. However, no significant difference was found in the Caspase-3 mRNA levels (\(^*\) \(P < 0.01\), \(*\) \(P < 0.05\), NS, not significant, \(n = 3\), t-test). (b) At 12 weeks, the Caspase-3 mRNA levels were remarkably downregulated in the Sal-treated mice compared with DMSO-treated mice; however, no significant differences were found in the BiP and Chop mRNA levels \((**\ P < 0.01\), *\(P < 0.05\), NS, not significant, \(n = 3\), t-test). (c) The BiP and cleaved caspase-3 protein levels in the cochlea from the Sal-treated mice were much lower than those from the DMSO-treated mice at P30. Significant differences were found between the DMSO and Sal-treated mice in their quantities of BiP and cleaved caspase-3. The error bars represent S.E.M. \(**\ P < 0.01\), *\(P < 0.05\), NS, not significant, \(n = 3\), t-test). (d) CHOP signals were more strongly detected in the OHCs, SG and StV of the DMSO-treated mice, showing high concentration in the perinuclear region in between nucleus and hair bundles of OHCs but attenuated by Sal (\(n = 3\) mice per group). Scale bars, 50 \(\mu m\) (left); 20 \(\mu m\) (right).
Moreover, the ERL-CDH23 protein in the subapical region of the OHC was co-localized with ER chaperone BiP (Figures 1b and c). The BiP protein was found to be highly expressed in OHCs, SG and STV in the erl cochlea at P6 and P12, and the BiP mRNA was upregulated at P12 but downregulated at P30 (Figure 2). Considering the UPR pathway, these results suggest that BiP was dissociated with the ER transmembrane receptor and then combined with defective CDH23 at the early stage of this response. Downstream of this pathway, eIF2α phosphorylation was detected in the erl cochlea (Figure 3), and the Chop mRNA and protein were upregulated at P12 and P30 (Figure 4). These results indicate that the PERK arm of the UPR was activated. CHOP was a key mediator when the ER stress-induced UPR signaling progressed to apoptosis.

The results of the auditory test, surface preparation and OHC counting in our double-mutant Cdh23<sup><small>−/−</small></sup> Chop<sup><small>−/−</small></sup> mice further confirm that the deletion of the Chop gene could partly protect hearing and preserve the OHC in mice with the Cdh23<sup><small>−/−</small></sup> mutation (Figure 5). Taking these data together with the previous study on the induction of apoptosis-related genes in the <i>erl</i> mouse cochlea, it was suggested that apoptosis is an event downstream of the induction of the UPR, with the effector PERK signaling pathway being a contributor. Thus, we selected the PERK arm of the UPR as a therapeutic target.

It is interesting to note that CHOP does not present nuclear localization in OHCs, as expected for a transcription factor (Figures 4 and 8). However, CHOP does not have a nuclear localization signal. Its interaction with other stress-induced transcription factors mediates its nuclear localization. It is therefore possible that in OHCs, CHOP is excluded from the nucleus due to the absence of interacting transcription factor partners. It was also reported that cells with cytoplasmic and nuclear localized CHOP gave distinct gene expression profiles. It was shown that that cytoplasmic CHOP inhibited the migration, while nuclear CHOP caused a G1 cell cycle arrest. In this <i>erl</i> model, particularly in OHCs, CHOP is localized in the perinuclear regions in between the nuclei and hair bundles of the OHCs. An OHC develops a polarized bundle of stereocilia that is important for mechanotransduction. Many hair bundle proteins migrate toward the hair bundle; thus, we speculate that the cytoplasmic CHOP may regulate the expression of protein migration-related genes. Further study is warranted on CHOP’s role(s) aside from inducing apoptosis on OHC degeneration and functionality.

Sal is a small molecular compound (480 Da) that prevents cell death from ER stress-induced apoptosis by selectively inhibiting the dephosphorylation of eIF2α. Studies show that Sal resists ER stress-induced cell death, improves cell survival and delays disease processes. Our hearing tests confirmed our hypothesis that Sal could protect hearing by inhibiting ER stress-induced hair cell apoptosis in the <i>erl</i> mice. In a previous work, we reported that progressive hearing loss started at P27 in <i>erl</i> mice. In this study, as early as 4 weeks postnatal, the 32-kHz-stimulus-evoked ABR thresholds in the test group were 10 dB better than in the control and vehicle groups (Supplementary Figure 1b). At all subsequent time points, the Sal-treated mice showed better ABR thresholds under all stimuli. Referring to the correspondence between high and low frequencies and to the basal-apical gradient of the basilar membrane, the auditory tests indicate that the OHC death specifically started in the basal turns and then spread through the entire cochlea, which could be remedied by Sal treatment. Our previous studies targeting anti-apoptosis treatments also showed otoprotective effects, but Sal showed an extended duration (up to 16 weeks). In our histological examination, we observed substantial contiguous and isolated OHC losses in the vehicle and control groups; in contrast, the Sal-treated mice showed only small amounts of OHC loss (Figure 7). This result provides a good correlation between our anatomical observations and functional tests.

After the Sal treatment, the upregulated <i>Bip</i> and <i>Chop</i> mRNA were decreased at P30, and the <i>caspase-3</i> was decreased at 12 weeks (Figure 8a), indicating that <i>Bip</i> and <i>Chop</i> were affected by Sal treatment at an early age and that the <i>caspase-3</i> mRNA was influenced later. However, the cleaved caspase-3 proteins (detected by western blot) were found to be decreased by Sal at P30. Our cleaved caspase-3 antibody (Asp175) was able to recognize the large fragment (17 kDa) of activated caspase-3 resulting from the cleavage adjacent to Asp175, but it was incapable of detecting the full-length caspase-3 protein. These results suggest that the caspase-3 cleavage was blocked at P30 by Sal treatment. The <i>caspase-12</i> mRNA remained unchanged at P30 and 12 weeks (Supplementary Figure 4), indicating that <i>caspase-12</i> might not be involved in the feedback of the Sal treatment. The BiP proteins (detected by Western blot) and CHOP proteins (detected by immunostaining) were also downregulated after Sal treatment (Figure 8). These results, together with the previous data, suggest that the defective CDH23 accumulated in the ER and then increased the ER loading and destroyed ER homeostasis, leading to the dissociation of the BiP from PERK. The downstream upregulated CHOP led to caspase activation and apoptosis. If the adaptive UPR reduces the protein load in the ER, then the restoration of protein synthesis will promote cell survival. Otherwise, if protein synthesis overrides the restoration of proteostasis, cell death will be triggered. Sal inhibits the dephosphorylation of eIF2α, which inhibits protein synthesis when it is phosphorylated by PERK. This increased phosphorylation of eIF2α limits protein synthesis (including that of CDH23 and CHOP), relieves ER loading, and promotes cell survival. Potential mechanisms of the pro-apoptotic function of uncontrolled protein synthesis during ER stress have recently been described.

As an animal model of DFNB12, the <i>erl</i> mice with Sal treatment began at an early age (starting from P7) and showed significant hearing protection for a long duration (up to 16 weeks). By considering the signaling pathway of ER stress-induced apoptosis, we suggest that therapies targeting this specific signaling pathway could prevent hair cell loss at an early stage and achieve more effective and sustained results. USH1D patients who carry different mutations of the same gene (<i>Cdh23</i>) as DFNB12 exhibit congenital deafness and postnatal-onset blindness. Thus, we assume that early treatment with Sal might prevent ophthalmic dysfunction in Usher syndrome, thus highlighting Sal’s application as a...
therapeutic agent in the treatment of animal models for Usher syndrome.

Conclusion

In summary, this study is the first to consider ER stress-induced hair cell apoptosis as having a key role in the hearing loss and hair cell death of mice with the Cdh23 mutation. Protein synthesis and related protein-folding processes are early molecular events in ER stress and apoptosis. Thus, protein-folding errors triggered by genetic mutations should be considered the earliest therapeutic target. Many Food and Drug Admission (FDA)-approved anti-ER stress drugs are presently available on the market and thus can be repurposed for otoprotection.

Materials and Methods

Experimental design. This study aimed at assessing ER stress-induced hair cell apoptosis in the mechanism of hearing loss and hair cell death in Cdh23ermutant mice. The measurements of ER stress indicators were performed between Cdh23ermutant mice, control B6 mice, as well as the Chop gene knockout (C57BL/6 background) mice. The ER stress inhibitor Salubrinal (Sal) was systematically given in Cdh23ermutant mice by intraperitoneal injection. The hearing, OHC reservation and ER stress indicators were tested between Sal-treated, vehicle-treated and untreated Cdh23ermice.

Mice. All of the procedures involving mice were approved by the Animal Research Committee of Case Western Reserve University School of Medicine (protocol R01DC009246). All of the mice were experimented upon in the same environment Committee of Case Western Reserve University School of Medicine (protocol R01DC009246). All of the mice were experimented upon in the same environment.

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Western blotting. The cochleae were lysed using ice-cold RIPA buffer. Equal amounts of protein were subjected to SDS-PAGE and transferred onto the PVDF (Bio-Rad) membrane. Afterward, the PVDF membrane was blocked for 1 h in 5% ECL prime blocking agent and then incubated overnight at 4 °C with 1:1000 diluted primary antibodies: anti-BiP (Cell Signaling 3177, Danvers, MA, USA), anti-caspase-3 (Cell Signaling 9661) and anti-Ij-actin (Santa Cruz sc-130657). After washing with TBST, the membrane was incubated in secondary antibodies (1:5000, goat anti-rabbit IgG, Santa Cruz sc-2054). Protein bands were visualized using the chemiluminescence-emanating ChemiDoc MP Imaging System (Bio-Rad).

Immunofluorescence staining. The inner ears were fixed in 4% parformaldehyde (PFA) overnight and then decalcified in 10% ethylenediaminetraacetic acid (EDTA). After being dehydrated in sucrose and embedded in tissue OCT freeze medium at −20 °C, 6 μm continuous sections were cut. These sections were permeabilized in 0.5% Triton X-100 for 30 min, blocked in 5% BSA for 2 h at room temperature, and then incubated overnight at 4 °C with 1:200 diluted primary antibodies: anti-CHD23 (Santa Cruz sc-26338), anti-BiP (Cell Signaling 3177), anti-CHOP (Santa Cruz sc-757), anti-elf2a (Cell Signaling 9722) and anti-phospho-elf2a (Cell Signaling 3597). Secondary antibodies with 1:400 dilution (donkey anti-rabbit IgG, Invitrogen A31573; rabbit anti-goat IgG, Invitrogen A10178) were added for 1 h; the sections were then counter-stained with DAPI. The negative controls (with no primary antibodies added) showed no staining.

ABR and DPOAE testing. A computer-aided evoked potential system (Intelligent Hearing Systems, the Smart-EP software 3.30, Miami, FL, USA) was used as previously published. Briefly, the mice were anesthetized, and their body temperature was maintained at 37 °C. Sub-dermal needle electrodes were used. The recording electrode was inserted at the vertex of the skull; the ground electrode was inserted in the apex of the nose; and the reference electrodes were fixed near each ear. Click and 8, 16 and 32 kHz tone bursts were channeled through an inserted earphone. The ABR threshold was identified as the lowest stimulus level at which clear and repeatable waveforms were recognized (Figure 2a). The DPOAE measurement was conducted for pure tones at frequencies ranging from 4.4 to 20.3 kHz by using the Intelligent Hearing System (Smart EP 3.30 Software). Frequencies were acquired with the F2:F1 ratio of 1:22 and with primary stimulus of 65/65 dB SPL.

Surface preparation and hair cell counting. As we previously described, the temporal bones were fixed in 4% PFA, and the basilar membrane was then carefully dissected and cut into three separate fragments: apical, middle and basal turn. The fragments were permeabilized in 2% Triton X-100, stained for F-actin with phalloidin (Invitrogen), and observed with a fluorescence microscope (Leica DM4500 B). Hair cells were counted as being present if cell bodies and the V-shaped hair bundles were intact.

Confocal 3D imaging. To determine CHD23 localization in OHCs, whole-mount immunostaining of inner ear basilar membranes from 4-day-old mice was conducted using primary antibody anti-CHD23 (Santa Cruz; sc-26338), secondary rabbit anti-goat IgG, Invitrogen A10178, Alexa 488 (1:1000 dilution), then counter-stained with DAPI. Confocal images were captured using a Leica (Germany) SP8 confocal microscope with a ×631.4 NA oil objective and a 488 nm laser-line. Imaging stacks acquired with voxel-size 512 × 512 × 0.054 μm3 and 1024 × 1024 × 0.027 μm3, respectively depending on the sizes of OHCs. All deconvolutions were processed using Leica Application Suite X (LAS X, LASX) software.

Scanning electron microscope. The mice were fixed with 2.5% glutaraldehyde, and then the whole cochleae were dissected. The bony capsule, spiral ligament, and Reissner's membrane were carefully removed to expose the
whole organ of Corti. Afterwards, the specimens were fixed in 1% osmium tetroxide (OsO4) for 1 h three times and in 1% thiocarbohydrazide (TCH) for 1 h twice (the OTOTO technique). The specimens were dehydrated in a gradient ethanol series, critical-point dried using CO2, and finally coated in palladium. Then, the samples were viewed through a high-resolution scanning electron microscope (FEI Helios NanoLab 650, Germany).

Statistical analysis. The data are presented as mean ± S.E.M. The analysis was performed using the SPSS 18.0 software. The data were statistically analyzed using one-way ANOVA and Student’s t-tests. P-values < 0.05 were considered significant.

Conflict of Interest. The authors declare no conflict of interest.

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