independent observers. Wave 1 amplitude was defined as the difference between the average of the 1-ms pre-stimulus baseline and the wave 1 peak (P1), after additional high-pass filtering to remove low-frequency baseline shifts.

For DPOAE measurements, the cubic distortion product was measured in response to primaries *f*1 and *f*2. The primary tones were set so that the frequency ratio (*f*2/*f*1) was 1.2 and so that the *f*2 level was 10 dB below the *f*1 level. For each *f*2/*f*1 primary pair, primaries were swept in 5-dB steps from 20 dB SPL to 80 dB SPL (for *f*2). At each level, the amplitude of the DPOAE at 2*f*1–*f*2 was extracted from the averaged spectra, along with the noise floor. Threshold was computed by interpolation as the *f*2 level required to produce a DPOAE at 5 dB SPL.

Acoustic startle reflex. Mice were placed into a small, acoustically transparent cage resting atop a piezoelectric force plate in a sound attenuated booth. Acoustic stimuli and amplified force plate signals were encoded by a digital signal processor (Tucker-Davis Technologies, RX6) using LabView scripts (National Instruments). Mice were placed in silence for 2 min and 60 dB broadband white noise for 5 min to acclimate to the test environment before real measurements. Broadband white noise was presented at a background level of 60 dB SPL throughout the experiment and a 16-kHz tone was presented at randomized intervals from an overhead speaker (80 dB to 120 dB SPL, 20 ms duration with 0 ms onset and offset ramps). Ten repetitions were recorded for each of the intensities per test subject. Startle response amplitude was measured as the root mean square (RMS) voltage of the force plate signal shortly after sound presentation.

Immunohistochemistry and histology. Injected and non-injected cochleae were removed after animals were killed by CO_2 inhalation. Temporal bones were fixed in 4% paraformaldehyde at 4 °C overnight, then decalcified in 120 mM EDTA for at least 1 week. The cochleae were dissected in pieces from the decalcified tissue for whole-mount immunofluorescence. Tissues were infiltrated with 0.3% Triton X-100 and blocked with 8% donkey serum for 1 h before applying the first antibody. Rabbit anti-MYO7A (1:500 ; #25-6790, Proteus BioSciences), chicken anti-GFP (1:750; ab13970, Abcam) and goat anti-SOX2 (1:350; sc-17320, Santa Cruz Biotechnology) were used at room temperature overnight. The second antibody was incubated for 1 h after three rinses with PBS rinses. All Alexafluor secondary antibodies were from Invitrogen: donkey anti-rabbit Alex488 (A21206) or Alex 594 (A21207), donkey anti goat Alex594 (A11058) or Alexa-488-phalloidin (A12379) and goat anti-chicken Alex488 (A-11039) were used at a 1:500 dilution. Specimens were mounted in ProLong Gold Antifade Mountant medium (P36930, Life Technologies). Confocal images were taken with a Leica TCS SP5 microscope using a 20× or 63× glycerin-immersion lens, with or without digital zoom. For IHC and OHC counting, we acquired *z*-stacks by maximum intensity projections of *z*-stacks for each segment by imageJ (NIH image), and composite images showing the whole cochlea were constructed in Adobe Photoshop CS3 to show the whole turn of cochlea. A frequency map was constructed for each case by measuring the spiral extent of all the dissected cochlear pieces and converting cochlear location to frequency using a plug-in of ImageJ (https://www.masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-histology-resources/imagej-plugin-for-cochlear-frequency-mapping-in-whole-mounts). MYO7A-positive IHCs and OHC were counted in the cochlear regions that respond to different sound frequencies, and any segments containing dissection-related damage were omitted from further analysis.

Hair cell transduction current recording. Wild-type or $Tmc1^{Bth/\Delta}Tmc2^{\Delta/\Delta}$ littermates were injected with 0.9 µl Cas9–Tmc1-mut3–Lipofectamine 2000 or Cas9–GFP sgRNA–Lipofectamine 2000. Wild-type C57B/L6 mice were injected with 0.9 µl Cas9–Tmc1-wt3–Lipofectamine 2000 at P0–P1 via cochleostomy. Cochleae were removed at P5–P6 and cultured in MEM(1×) + GlutaMAX-I medium with 1% FBS at 37 °C, 5% CO_2 for up to 15 days. For recording, the organs of Corti were bathed in standard artificial perilymph containing 137 mM NaCl, 0.7 mM NaH_2PO_4, 5.8 mM KCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 10 mM HEPES, and 5.6 mM D-glucose. Vitamins (1:50) and amino acids (1:100) were added to the solution from concentrates (Invitrogen, ThermoFisher Scientific), and NaOH was used to adjust the final pH to 7.4 (~310 mOsm/kg). Recording pipettes (2–4 MΩ) were pulled from R6 capillary glass (King Precision Glass) and filled with intracellular solution containing 135 mM CsCl, 5 mM HEPES, 5 mM EGTA, 2.5 mM $MgCl_2$, 2.5 mM Na_2-ATP, and 0.1 mM $CaCl_2$; CsOH was used to adjust the final pH to 7.4 (~285 mOsm/kg). Whole-cell, tight-seal, voltage-clamp recordings were conducted at −84 mV at room temperature (22–24 °C) with an Axopatch 200B amplifier (Molecular Devices). Hair bundles were deflected with a stiff glass probe fabricated from capillary glass with a fire polisher (MF-200, World Precision Instruments) to create a rounded probe tip of ~3–5 µm in diameter. Probes were mounted on a PICMA Chip piezo actuator (Physik Instrument) and driven by an LVPZT amplifier (E-500.00, Physik Instrumente). Sensory-transduction currents were recorded from uninjected and Cas9–sgRNA-treated hair cells. The data were filtered at 10 kHz with a low-pass Bessel filter and digitized at >20 kHz with a 16-bit acquisition board (Digidata 1440A, Molecular Devices) and pClamp 10 software (Molecular Devices).

Inner ear tissue dissection for HTS. $Tmc1^{Bth/+}$ mice were injected with Cas9–sgRNA at P1 as described above. All dissection instruments were thoroughly cleaned with 70% ethanol and DRNAase Free (D6002, ARgos), then autoclaved before dissection. Mice were euthanized at P5. Temporal bones were removed and immersed in clean PBS pH 7.4 (10010001, ThermoFisher) individually. Different forceps were used for each ear. The organ of Corti, spiral ganglion, and spiral ligament from the injected and non-injected ear, and tail tissue were all removed under microscope from each mouse.

Hair cells isolation for HTS. $Tmc1^{Bth/+}$ mice were injected with Cas9–Tmc1-mut3–Lipofectamine 2000 at P1 and euthanized at P5. Cochleae were dissected and immersed in 1 µM FM 1-43FX (PA1-915, ThermoFisher) dissolved in HBSS (ThermoFisher) for 10 s at room temperature in the dark. Cochleae were rinsed three times with HBSS and placed in 100 µl Cell Recovery Solution (354253, Discovery Labware) for 10 min at 37 °C, then transferred to 100 µl TrypleE Express Enzyme (12604013, ThermoFisher). Sensory epithelia were extracted by forceps. After incubation for 10 min at 37 °C, the tissues were pipetted up and down 30 times. FM 1-43-positive cells were isolated using a 1-µl pipette under a microscope (Axiovert 200M, Carl Zeiss), then subjected to whole-genome amplification by MALBAC Single Cell WGA Kit (YK001A, Yikon Genomics).

Statistical analysis. Statistical analyses were performed by two-way ANOVA with Bonferroni corrections for multiple comparisons for ABRs, DPOAEs, and acoustic startle response; and by Student's *t*-test for hair cell transduction currents using Prism 6.0 (GraphPad). No statistical methods were used to predetermine sample size. A total of 106 $Tmc1^{Bth/+}$ or C3H mice (P0–2) of either sex were used for injections. The mice were randomly assigned to the different experimental groups. The final 25% of the experiments were performed in a double-blinded manner.

Code availability. Labview software for cochlear function testing is available here: http://www.masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-engineering-resources. Matlab scripts used to quantify the acoustic startle response are available from the corresponding authors on request. Indel identification scripts are provided in the Supplementary Information.

Data availability. High-throughput sequencing data have been deposited in the NCBI Sequence Read Archive database under accession code SRP103108. All other data are available from the corresponding authors on reasonable request.

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