METHODS

Primary cell culture. Wild-type, *Tmc1*$^{Bth/+}$ and *Tmc1*$^{Bth/Bth}$ fibroblasts were obtained from P5 pups (see below). Mice were euthanized and cleaned with 70% ethanol. Underarm skin fragments (1–2 cm^2) were excised and submerged in cold HBSS (ThermoFisher). Subcutaneous fat was removed by forceps. Skin fragments were cut into ~1-mm^2 pieces with a 25G 5/8″ syringe (1180125058, Covidien). Tissues were digested with 0.5 mg/ml Liberase DL (Sigma 5401160001) at 37 °C for 1 h with occasional pipetting up and down to break cell clumps. Warm culture medium (1:1 DMEM:F12 medium (ThermoFisher) with 15% fetal bovine serum (FBS) (ThermoFisher) and 100 U/ml penicillin+streptomycin (ThermoFisher) was added to stop the enzyme digestion. The solution was filtered with a 70-μm cell strainer (Falcon) and centrifuged at 200*g* for 5 min. The pellet was resuspended in culture medium and transferred to a 25-ml culture flask, then incubated at 37 °C with 5% CO_2 and 3% O_2. Fibroblasts were cultured for about 2–3 days to reach ~90% confluence, then passaged in 100-ml flasks in DMEM plus GlutaMax (ThermoFisher) supplemented with 10% (v/v) FBS at 37 °C with 5% CO_2.

Delivery of proteins complexed with cationic lipids into mouse fibroblasts. Cultured fibroblast cells were plated in 24-well format (500 μl well volume) in Dulbecco's modified Eagle's medium plus GlutaMAX (DMEM, Life Technologies) with 10% FBS (no antibiotics) at a cell density sufficient to reach ~80% confluence at the time of usage. Purified sgRNA was incubated with Cas9 protein for 5 min before complexing with cationic lipid[15,26]. Delivery of Cas9–sgRNA was performed by combining 100 nM RNP complex with 3 μl cationic lipid in 50 μl OPTIMEM medium (Life Technologies) according to the manufacturer's protocol for DNA plasmid transfection. The above mixture containing cationic lipid and RNP was then added to cells. All complexing steps were performed at room temperature. Cells were harvested and genomic DNA was extracted for sequencing ~96 h after treatment.

GUIDE-seq and data analysis. Mouse fibroblasts were transfected using 1,000 ng Cas9 plasmid (pCas9), 300 ng sgRNA plasmid (pTmc1-mut3 sgRNA), and 50 pmol GUIDE-seq double-stranded oligodeoxynucleotides (dsODN) using a LONZA 4D-Nucleofector. Transfection programs were optimized following the manufacturer's instructions (CA158 and CA189, P2 Primary Cell 4D-Nucleofector X Kit). pmaxGFP Control Vector (400 ng; LONZA) was added to the nucleofection solution to assess nucleofection efficiency in primary cells. The medium was replaced ~16 h after nucleofection and cells were collected for genomic DNA extraction after ~96 h. For GUIDE-seq off-target DNA cleavage analysis, pCas9, pTmc1-mut3 sgRNA, pmaxGFP, and dsODN were nucleofected into *Tmc1*$^{Bth/+}$ heterozygous mouse primary fibroblasts. A sample nucleofected with dsODN only served as a negative control. About 400 ng genomic DNA for each sample was sheared acoustically using a Covaris m220 sonicator to an average length of 500 bp in 130 μl TE buffer. Each sample was sequenced on an Illumina Miseq following previously described protocols[23]. Reads were consolidated first by their Illumina indexes and then by the 8-nt molecular index that defines a single pre-PCR template fragment. The consolidated reads were mapped to the mouse reference genome (GRCm38) using BWA-MEM. Off-target sites were identified by first mapping the start position of the amplified sequences using a 10-bp sliding window, then retrieving the reference sequence around the site. Given the size of some of the deletions, the number of base pairs used as the flanking sequence was increased to 100 bp. The retrieved sequences were aligned to the Cas9 target sequence using a Smith–Waterman local-alignment algorithm.

High-throughput DNA sequencing of genomic DNA samples. Treated cells or tissues were collected after four days and genomic DNA was isolated using the Agencourt DNAdvance Genomic DNA Isolation Kit (Beckman Coulter) according to the manufacturer's instructions. On-target and off-target genomic regions of interest were amplified by PCR with flanking HTS primer pairs (listed in Supplementary Sequences). PCR amplification was carried out with Phusion high-fidelity DNA polymerase (ThermoFisher) according to the manufacturer's instructions using ~100 ng genomic DNA as a template. PCR cycle numbers were chosen to ensure the reaction was stopped during the log-linear range of amplification. PCR products were purified using RapidTips (Diffinity Genomics). Purified DNA was amplified by PCR with primers containing sequencing adaptors. The products were purified by gel electrophoresis and quantified using the Quant-iT^{TM} PicoGreen dsDNA Assay Kit (ThermoFisher) and KAPA Library Quantification Kit-Illumina (KAPA Biosystems). Samples were sequenced on an Illumina MiSeq as previously described[27].

Sequencing reads were demultiplexed using MiSeq Reporter (Illumina), and individual FASTQ files were analysed with a custom Matlab script (Supplementary Note). Each read was pairwise aligned to the appropriate reference sequence using the Smith–Waterman algorithm. Base calls with a *Q*-score below 31 were excluded from calculating editing frequencies. Sequencing reads were scanned for exact matches to two 10-bp sequences that flank both sides of a window in which indels might occur. If no exact matches were located, the read was excluded from analysis. If the length of this indel window exactly matched the reference sequence, the read was classified as not containing an indel. If the indel window was one or more bases longer or shorter than the reference sequence, then the sequencing read was classified as an insertion or deletion, respectively.

General *in vivo* experiments. All *in vivo* experiments were carried out in accordance with NIH guidelines for the care and use of laboratory animals and were approved by the Massachusetts Eye & Ear Infirmary IACUC committee. Isogenic heterozygous *Tmc1*$^{Bth/+}$ mice maintained on a C3HeB/FeJ (C3H) background were obtained as a gift from A. Griffith[21], and inbred with wild-type C3H mice obtained from Jackson Laboratory. Crossbred homozygous C3H-*Tmc1*$^{Bth/Bth}$ mice were caged with C3H mice to generate heterozygous *Tmc1*$^{Bth/+}$ mice. All mice were genotyped by Transnetyx. For mechanotransduction experiments, two genotypes of *Tmc* mutant mice: *Tmc1*$^{Bth/Bth}$*Tmc2*$^{\Delta/\Delta}$ and *Tmc1*$^{\Delta/\Delta}$*Tmc2*$^{\Delta/\Delta}$[16], were bred to generate *Tmc1*$^{Bth/\Delta}$*Tmc2*$^{\Delta/\Delta}$ mice.

Microinjection into the inner ear of neonatal mice. 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. At least five mice were injected in each group. All surgical procedures were done in a clean, dedicated space. Instruments were thoroughly cleaned with 70% ethanol and autoclaved before surgery. Fresh Cas9 and sgRNA were mixed before injection at a final concentration of 25 μM. One microlitre Lipofectamine 2000 was mixed with 1 μl Cas9–sgRNA RNP and incubated for 20 min at room temperature. Mice were anaesthetized by hyperthermia on ice. Cochleostomy was performed by preauricular incision to expose the cochlear bulla. Anatomical landmarks included the stapedial artery and tympanic ring, which were identified before injection. Glass micropipettes (4878, WPI) were pulled with a micropipette puller (PP83, Narishige) to a final outer diameter of ~10 μm. Needles held by a Nanolitre 2000 micromanipulator (WPI) were used to manually deliver the Cas9–sgRNA–lipid complexes into the scala media, which allows access to inner ear cells. The injection sites were the base, middle, and apex–middle turn of the cochlea. The volume for each injection was 0.3 μl with a total volume of 0.9 μl per cochlea. The release rate was 69 nl/min, controlled by a MICRO4 microinjection controller (WPI).

Microinjection into adult inner ear by canalostomy. Three 6-week-old Atoh1–GFP mice[28] were injected with Cas9–GFP sgRNA–lipid complex, with the same concentration and volume for each component as used in injection into neonatal inner ear. Mice were anaesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). The right post-auricular region was exposed by shaving and disinfected with 10% povidone iodine. For canalostomy, a 10-mm postauricular incision was made under the operating microscope, and the right pinna and the sternocleidomastoid muscle were extracted to expose the posterior semicircular canal (PSCC), located in the margin of the temporal bone. We used a Bonn microprobe (Fine Science Tools) to drill a small hole on the PSCC, then left it open for a few minutes until no obvious perilymph leakage was observed. The tip of the polyimide tube (inner diameter 0.0039 inches, outer diameter 0.0049 inches, Microlumen) was inserted into the PSCC towards the ampulla. The hole was sealed with tissue adhesive (3M Vetbond), and a lack of fluid leakage indicated the tightness of the sealing. The tubing was cut after injection, with approximately 5 mm of tubing left connected to the PSCC and sealed with tissue adhesive. The volume for each injection was 1 μl per cochlea. The release rate was 169 nl/min, controlled by MICRO4 microinjection controller (WPI). The skin was closed with 5-0 nylon suture (Ethicon Inc.). The total surgery time was approximately 20 min, including a 6-min injection period.

Acoustic testing. ABR and DPOAE were recorded as described previously[29] at 32 °C in a soundproof chamber. Mice of either sex were anaesthetized with xylazine (10 mg/kg, intraperitoneally (i.p.)) and ketamine (100 mg/kg, i.p.). Acoustic stimuli were delivered through a custom acoustic assembly consisting of two miniature dynamic electrostatic earphones (CDMG15008-03A, CUI) to generate primary tones and a miniature microphone (FG-23329-PO7, Knowles) to record ear-canal sound pressure near the eardrum. Custom LabVIEW software controlling National Instruments 24-bit soundcards (6052E) generated all ABR and DPOAE stimuli and recorded all responses.

For ABR measurements, needle electrodes were inserted at the vertex and ventral edge of the pinna, with a ground reference near the tail. ABR potentials were evoked with 5-ms tone pips (0.5-ms rise–fall with a cos2 onset, delivered at 35/s). The response was amplified 10,000-fold, filtered (100 Hz–3 kHz passband), digitized, and averaged (1,024 responses) at each SPL. The sound level was raised in 5 dB steps from 30 dB below threshold up to 90 dB SPL at frequencies from 5.66-45.24 kHz (in half-octave steps). Following visual inspection of stacked waveforms, "threshold" was defined as the lowest sound pressure level (SPL) at which any wave could be detected. In general, thresholds were defined by three