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☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.

For hair cell transduction current recording, a minimum of 6 hair cells (6-18) were recorded for each condition. In experimental groups to show reproducible and robust hearing, we used a minimum of 11 animals (from 11 to 30) for each of the frequency studied. For auditory startle evaluation, we used 9 injected animals to better measure the response and variations, and used 5 uninjected control animals due to their uniform unresponsiveness. We used a minimum of 4 animals in all control studies. Other experiments were done in biological triplicate, n=3 on different days. In previous studies we determined this sample size to be sufficient to ensure reproducibility.

2. Data exclusions

Describe any data exclusions.

For ABR measurements, a small fraction of frequencies for which thresholds were not apparent were excluded to ensure that only ABR thresholds scored accurately were used.

3. Replication

Describe whether the experimental findings were reliably reproduced.

All the experimental findings were replicated with the number of replicates, animals and variations shown by n, SD (in vitro assays), and SEM (in vivo experiments).

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

The animals of the same genotypes (Bth/+ or C3H) were randomly chosen to be in experimental or control groups according to experimental design.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

All data from in vivo experiments were collected and analyzed by at least two researchers who were both blinded to experiment.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Prism 6 from GraphPad was used for statistic analysis. Matlab were used for indel-identification and acoustic startle reflex data analyses. ImageJ (NIH image) was used for IHC and OHC counting. Adobe Photoshop CS3 was used to composite images showing the whole cochlea. Custom LabVIEW software controlling National Instruments 24-bit soundcards (6052E) generated all ABR/DPOAE stimuli and recorded all responses.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Immunofluorescence: rabbit anti-MYO7A (25-6790, Proteus BioSciences), chicken anti-GFP (ab13970, Abcam), goat anti-SOX2 (sc-17320, Santa Cruz Biotechnology), goat anti-chicken Alexa Fluor 488 (A-11039, Invitrogen), donkey anti-rabbit Alex488 (A21206) or Alex 594 (A21207), donkey anti goat Alex594 (A11058), and Alexa-488-phalloidin (A12379). All of them have been used in previously published studies on the adult mouse cochlea (Shu et al.,Hum Gene Ther. 2016 Sep;27(9):687-99; Huang et al., J Neurosci. 2013 Sep 18;33(38):15086-94; Kurima et al., 2015, Cell Reports 12, 1606–1617)

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HEK293T cells were originally purchased from ATCC (catlog CRL-3216).
Wild-type, Bth/+ and Bth/Bth fibroblasts were obtained from P5 pups.

b. Describe the method of cell line authentication used.

HEK293T (catlog CRL-3216) is a commercial cell line and authenticated by ATCC.
Primary fibroblasts from Bth mice were genotyped by the lab.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Mouse strains used in this study are:
C3HeB/FeJ (C3H),
heterozygous Tmc1 Bth/+ , in C3H background
homozygous Bth/Bth, in C3H background
Tmc1 Bth/Bth;Tmc2 Δ/Δ, in C3H background
Tmc1 Δ/Δ;Tmc2 Δ/Δ, in C3H background
Mice of either sex at P0-P6 were used for culture and injection. Acoustic tests were performed 1-2 month after the injection.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The research did not involve human research participants.

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Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.	For samples with GFP plasmid transfection, the fraction of cells with GFP expression was measured by flow cytometry 24 h after delivery. For samples treated with Cas9: FitC-Tmc1-mut3 RNP, or Cas9:CrRNA-Tmc1-mut3:atto-550-TracrRNA RNP, media was removed 6 h after delivery. The cells were trypsinized, washed three times with 500 µL PBS containing 20 U/mL heparin, and prepared for counting and flow cytometry.
6. Identify the instrument used for data collection.	Beckman Coulter Cytoflex
7. Describe the software used to collect and analyze the flow cytometry data.	CytExpert
8. Describe the abundance of the relevant cell populations within post-sort fractions.	The purity of cells was high as determined by microscopy.
9. Describe the gating strategy used.	Cells lacking the treatment of interest were used as negative controls for gating. For RNP delivery samples (FitC or Atto-550), control samples of fluorescent RNP only (without Lipofectamine 2000) were used for delivery to determine the efficiency of the washing procedure to remove extracellularly bound RNPs. The gating is the same with untreated samples.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☐