Supplementary Information for

In vivo gene editing of outer hair cells prevents progressive hearing loss in a dominant-negative *KCNQ4* murine model

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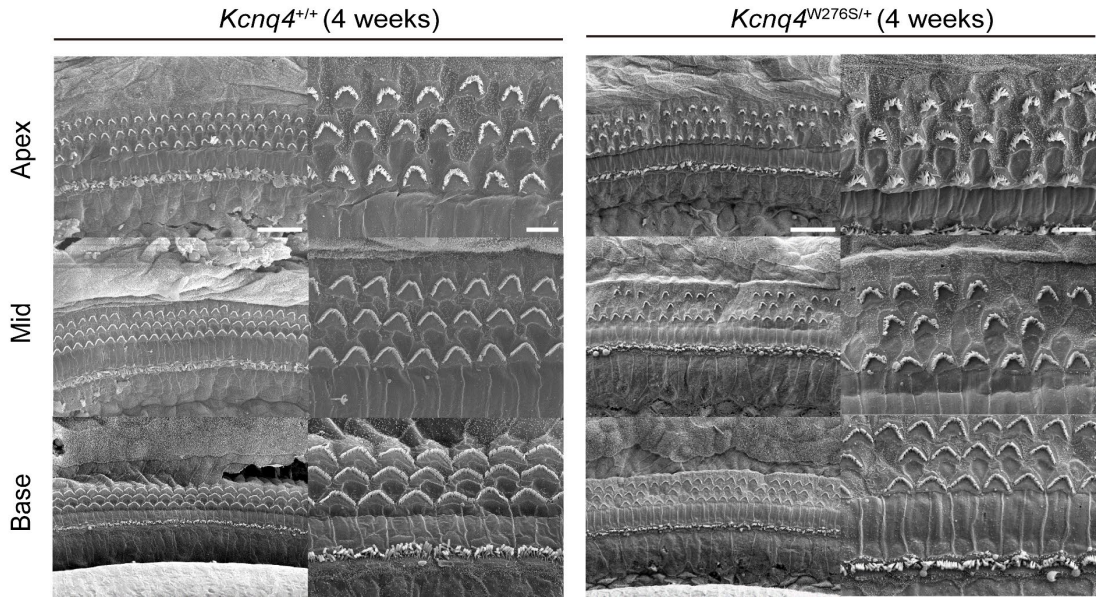
This PDF file includes:

Supplementary Figures

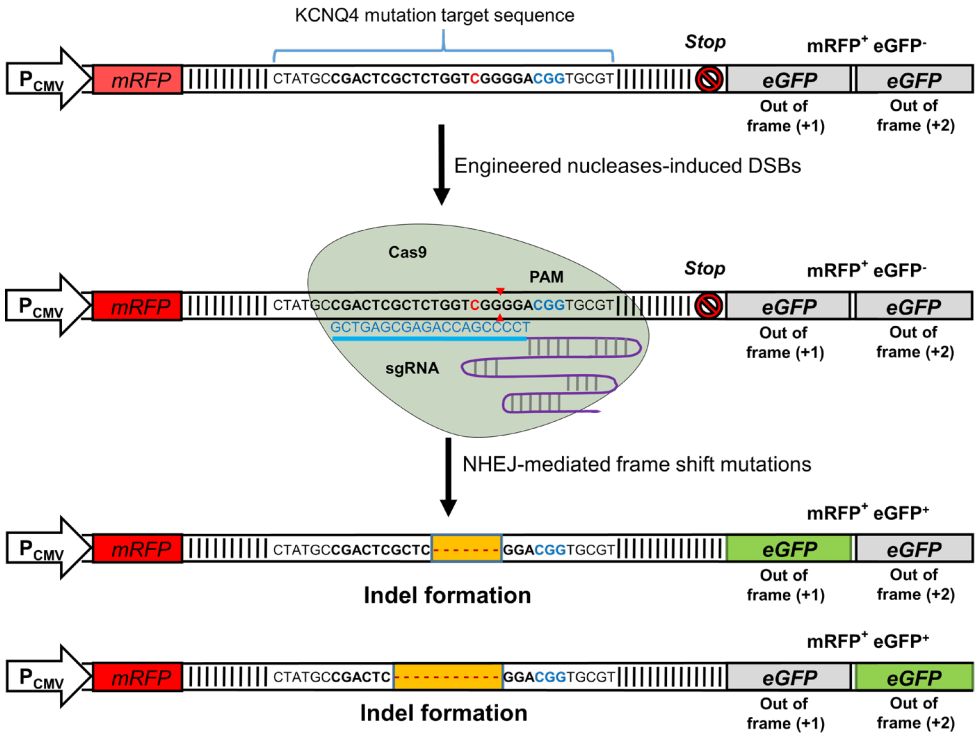


Supplementary Figure 1. Morphology of stereocilia in *Kcnq4*$^{W276S/+}$ mice.
Scanning electron microscopy images at the apex, mid, and base regions of the cochleae of 4-week-old wild-type and heterozygote mutant mice. Scale bar, 20 μm (low power) and 5 μm (high power).

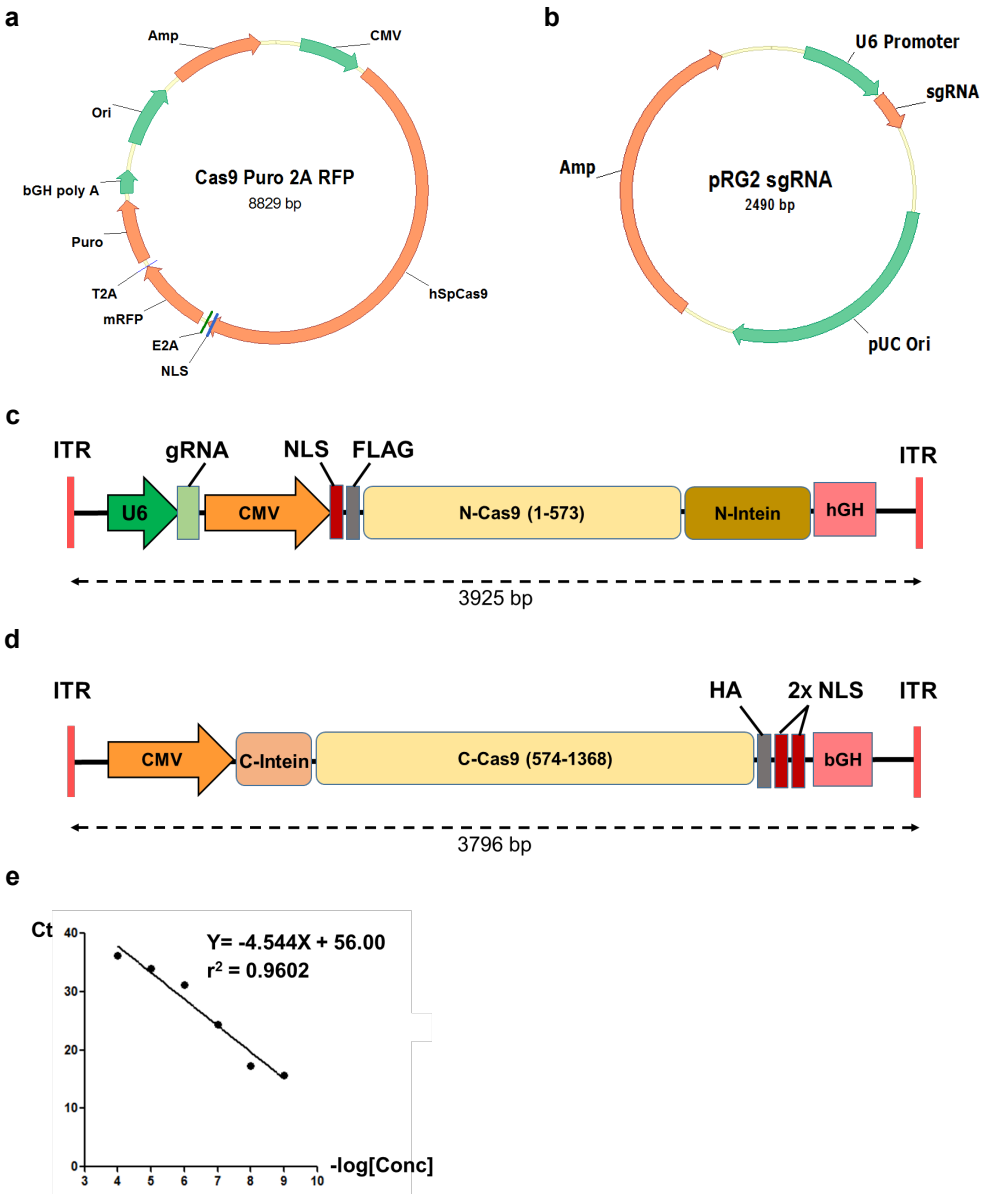
a

Target No.	Orientation	Cleavage position from mutation	Target sgRNA Sequence (5' to 3')	Target sgRNA Sequence with PAM (5' to 3')
T1	sense	2bp down stream	ATGCCGACTCGCTCTGGT**C**G	ATGCCGACTCGCTCTGGT**C**G**GGG**
T2	sense	3bp down stream	TATGCCGACTCGCTCTGGT**C**	TATGCCGACTCGCTCTGGT**C**GGG
T3	sense	3bp Upstream	CGACTCGCTCTGGT**C**GGGGA	CGACTCGCTCTGGT**C**GGGGA**CGG**
T4	sense	4bp down stream	CTATGCCGACTCGCTCTGGT	CTATGCCGACTCGCTCTGGT**CGG**

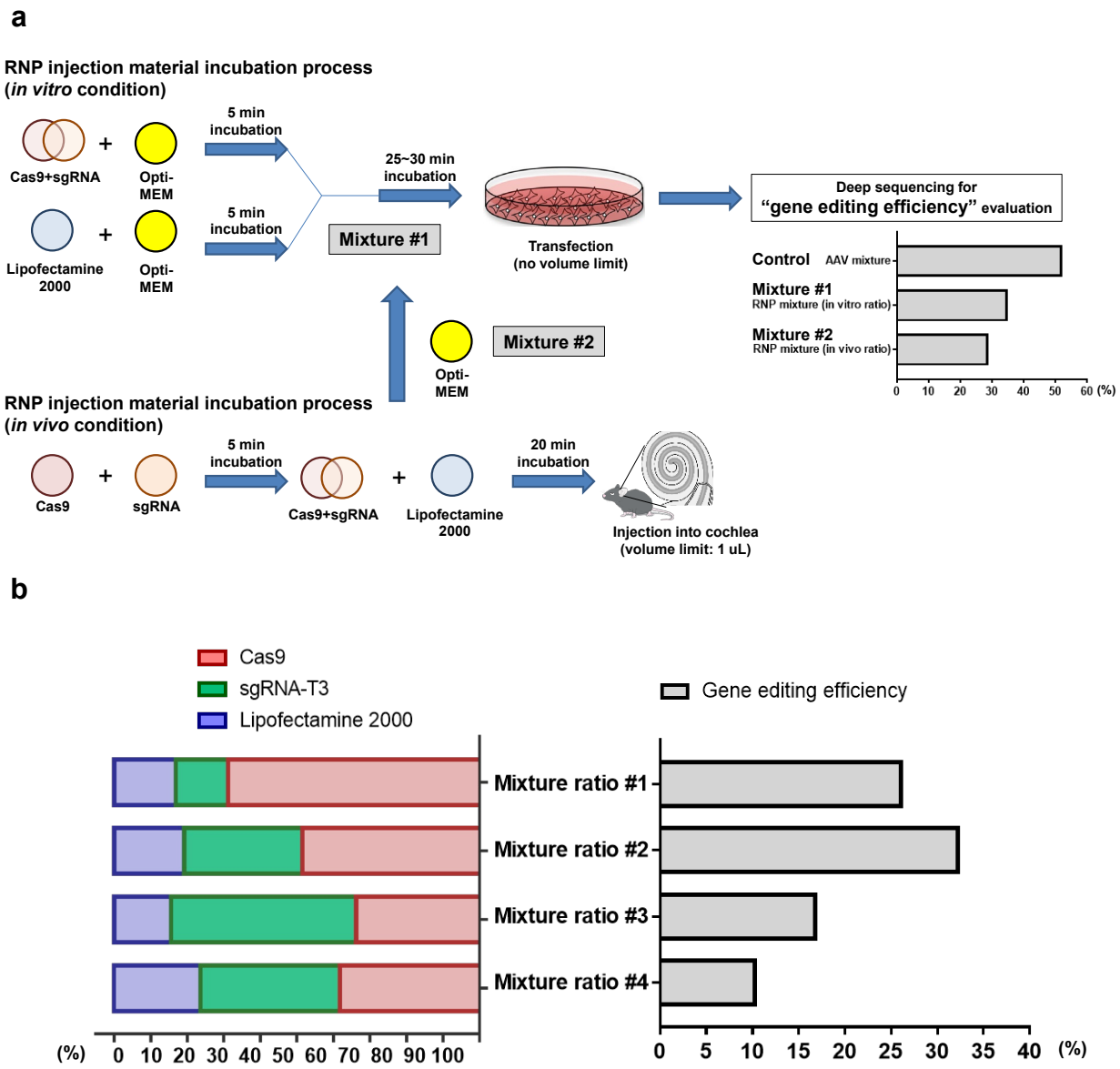
b



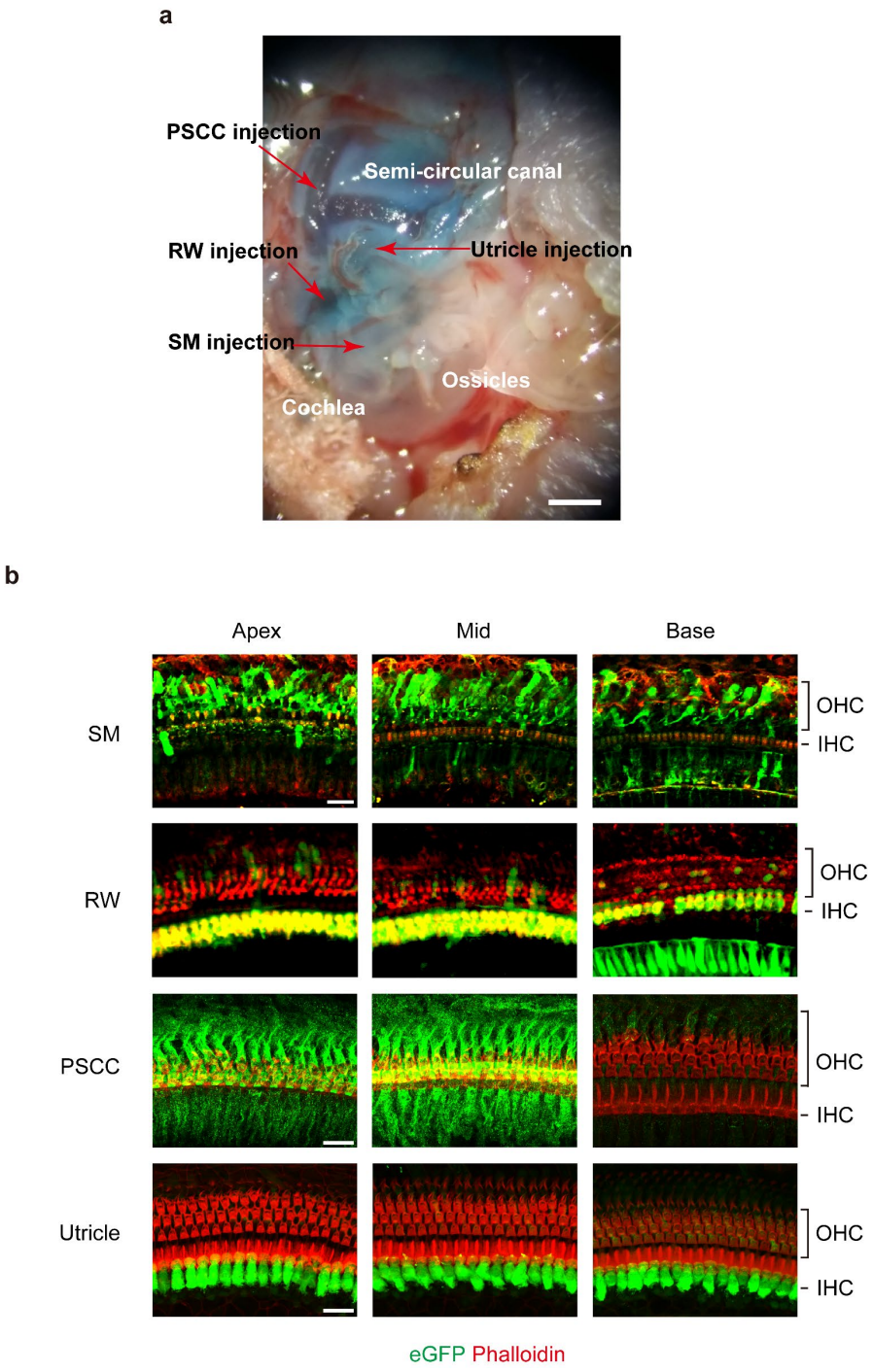
Supplementary Figure 2.| Candidate sgRNAs targeting the *Kcnq4* mutant allele.
(**a**) Profiles of four sgRNA candidates. PAM sequences are shown in bold. (**b**) Mechanism of the fluorescent reporter system in the sgRNA candidate selection process. The reporter plasmid constitutively expresses RFP and the target sequence encompassing the *Kcnq4* mutation site (c.830G>C) (35 bp in length; mutant nucleotide in red) by the CMV promoter (P_{CMV}), whereas *eGFP* is not expressed unless Cas9 is active because the *eGFP* sequence is out of frame. If a double-stranded break (DSB) is introduced into the target sequence by sgRNA (blue sequence) and Cas9 (light green), the DSB is repaired by error-prone nonhomologous end joining (NHEJ), which often results in an insertion/deletion (indel) mutation. This indel formation can cause frameshifts (orange bars with red dashes), leading to the expression of either *eGFP* gene visualized by green fluorescence. The predicted Cas9-gRNA cutting position is indicated with red bars after the arrowhead.



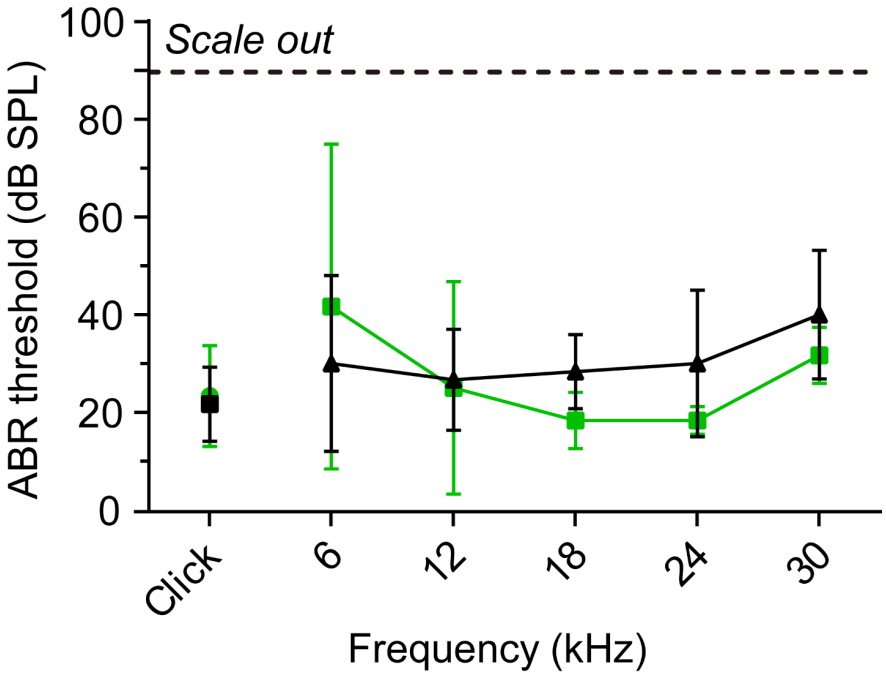
Supplementary Figure 3. Dual split AAV system for SpCas9 and sgRNA.
(**a**-**b**) Vector maps of Cas9-2A-mRFP-2A-Puro (**a**) and pRG2-sgRNA (**b**). hSpCas9 (human codon-optimized Cas9 nuclease derived from *Streptococcus pyogenes*) is expressed by the CMV promoter, and the sgRNA is transcribed by the U6 promoter (pU6). The ampicillin resistance gene (*Amp*) enables the selection of transformed bacterial cells. (**c-d**) Overview of the split-SpCas9 expression plasmids, namely U6-sgRNA- split–N-Cas9 N-intein (**c**) and C-intein-split–C-Cas9 (**d**). The U6 promoter was chosen (dark green) to express gRNA (light green). To ensure high expression, a strong synthetic mammalian promoter (CMV, orange) and a bovine growth hormone (bGH, red) polyadenylation site was used. Cas9 is shown in yellow, N-intein in dark brown, and C-Intein in light brown. FLAG and HA tags are shown in light and dark grey, respectively. All plasmid sequences used are in Supplementary Note 1. (**e**) Standard curve for the quantitation of split-SpCas9 encapsulated by Anc80L65 capsids. The viral titer was confirmed by quantitative RT-PCR. E2A, equine rhinitis A virus (ERAV) 2A; mRFP, monomeric red fluorescent protein; T2A, Thosea asigna virus 2A; *Puro*, puromycin resistance gene; ITR, inverted terminal repeat; NLS, nuclear localization signal.



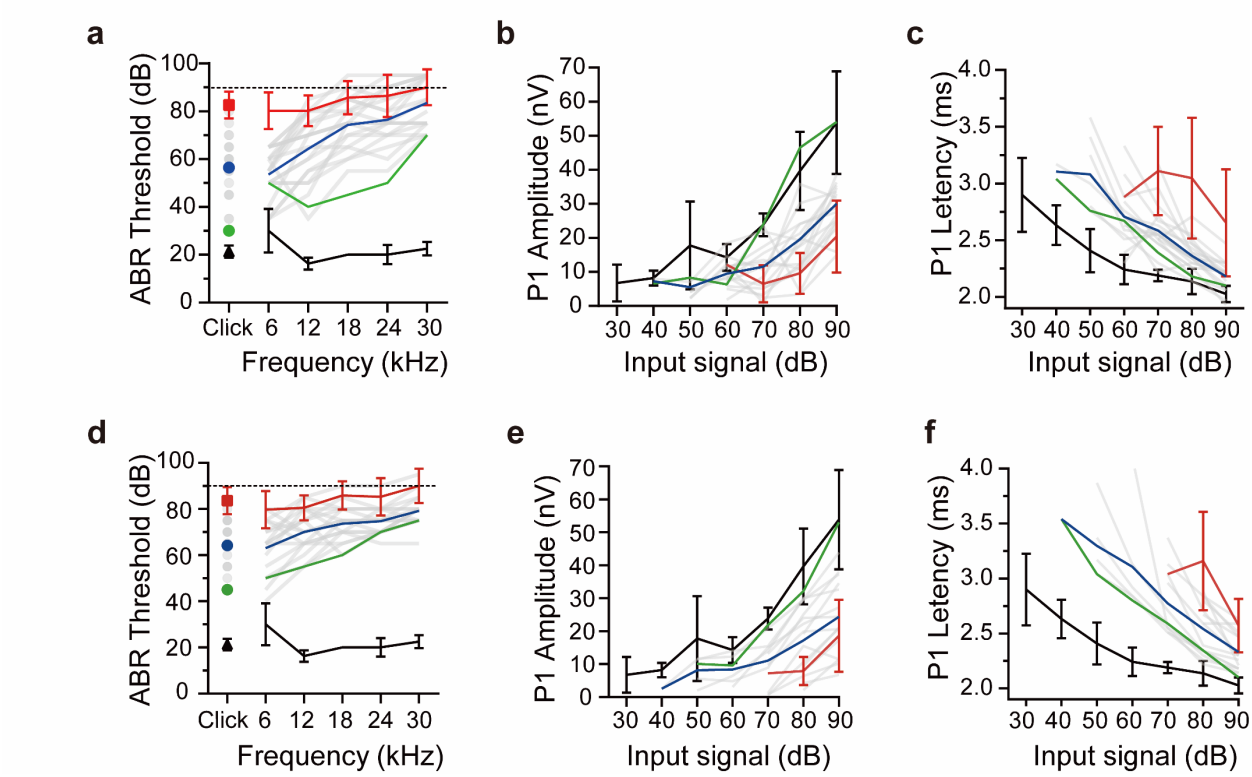
Supplementary Figure 4. Optimization of RNP vehicles.
(**a**) Schematic diagram of the validation of the Cas9 and sgRNA complex by lipofectamine. *In vivo* injection of the RNP mixture was performed using two stepwise incubations. *In vitro* and *in vivo* RNP mixtures were validated for the efficacy of gene editing by deep sequencing results with 28.56% indel sequence fragments. (**b**) *In vitro* optimization of the ratios of Cas9, sgRNA, and lipofectamine 2000 reagents. Various ratios of RNP mixtures were evaluated to identify the highest efficacy by increasing the concentrations of Cas9 and sgRNA and the volume of the lipofectamine reagent.



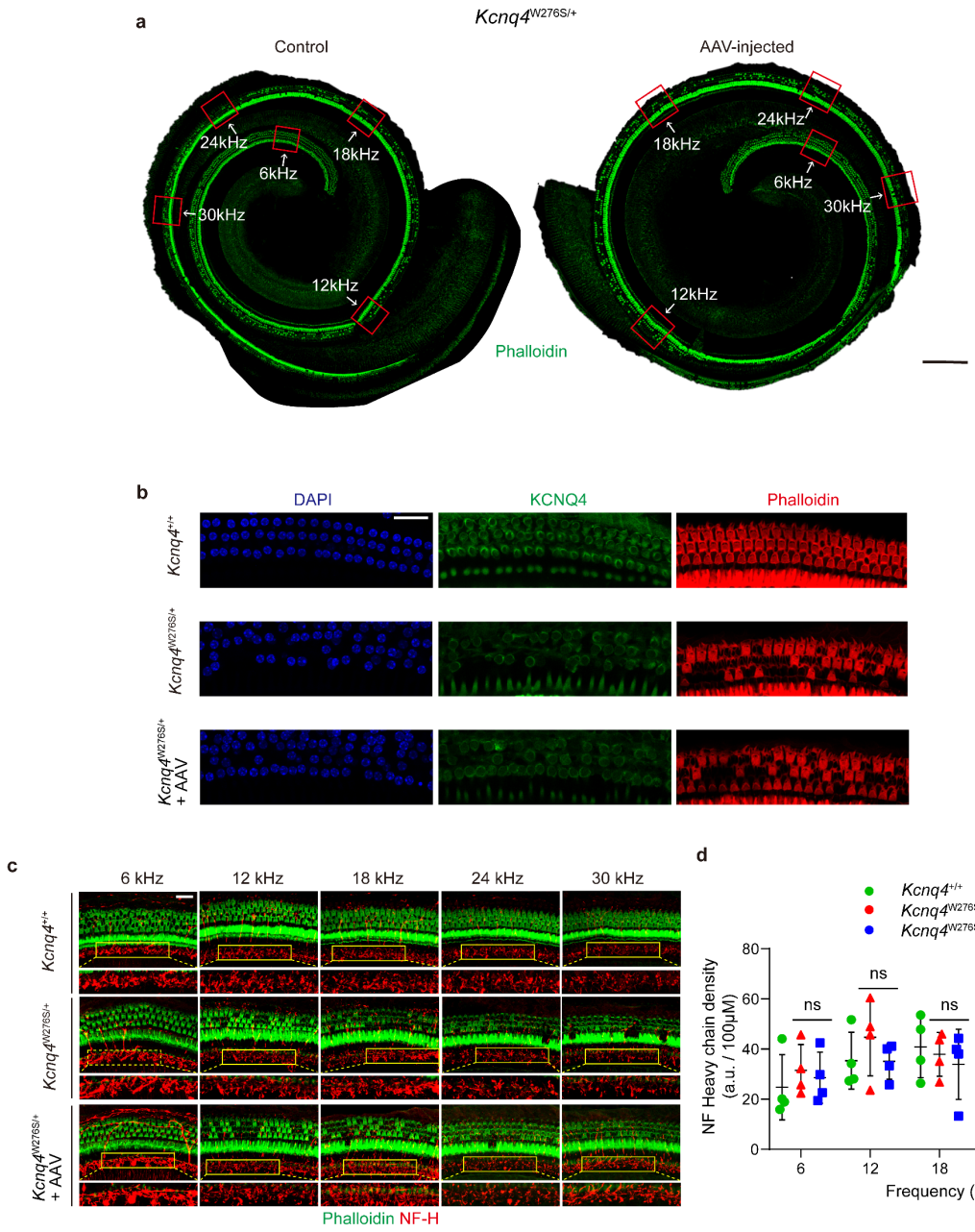
Supplementary Figure 5. Optimization of delivery routes for *in vivo* gene editing.
(**a**) Post-auricular incision image of the P1 pup representing four injection routes, i.e., posterior semi-circular canal (PSCC), round window (RW), scala media (SM); and utricle. Methylene blue was injected as a marker. Scale bar, 500 μm. (**b**) Representative images of the apex, mid, and base regions of the P10 pup injected with AAV2/Anc80-GFP at P1. AAV (AAV2/Anc80L65) delivery efficiency were validated according to the different injection methods. Green fluorescence indicates successful AAV delivery. Scale bar, 20 μm.



Supplementary Figure 6. Safety of RNP injection into the scala media.
ABR threshold values measured in 3-week-old wild-type mice after RNP injection at P1. There was no difference in ABR thresholds across all frequencies between the RNP-injected (green) and contralateral uninjected (black) cochleae ($p > 0.05$).

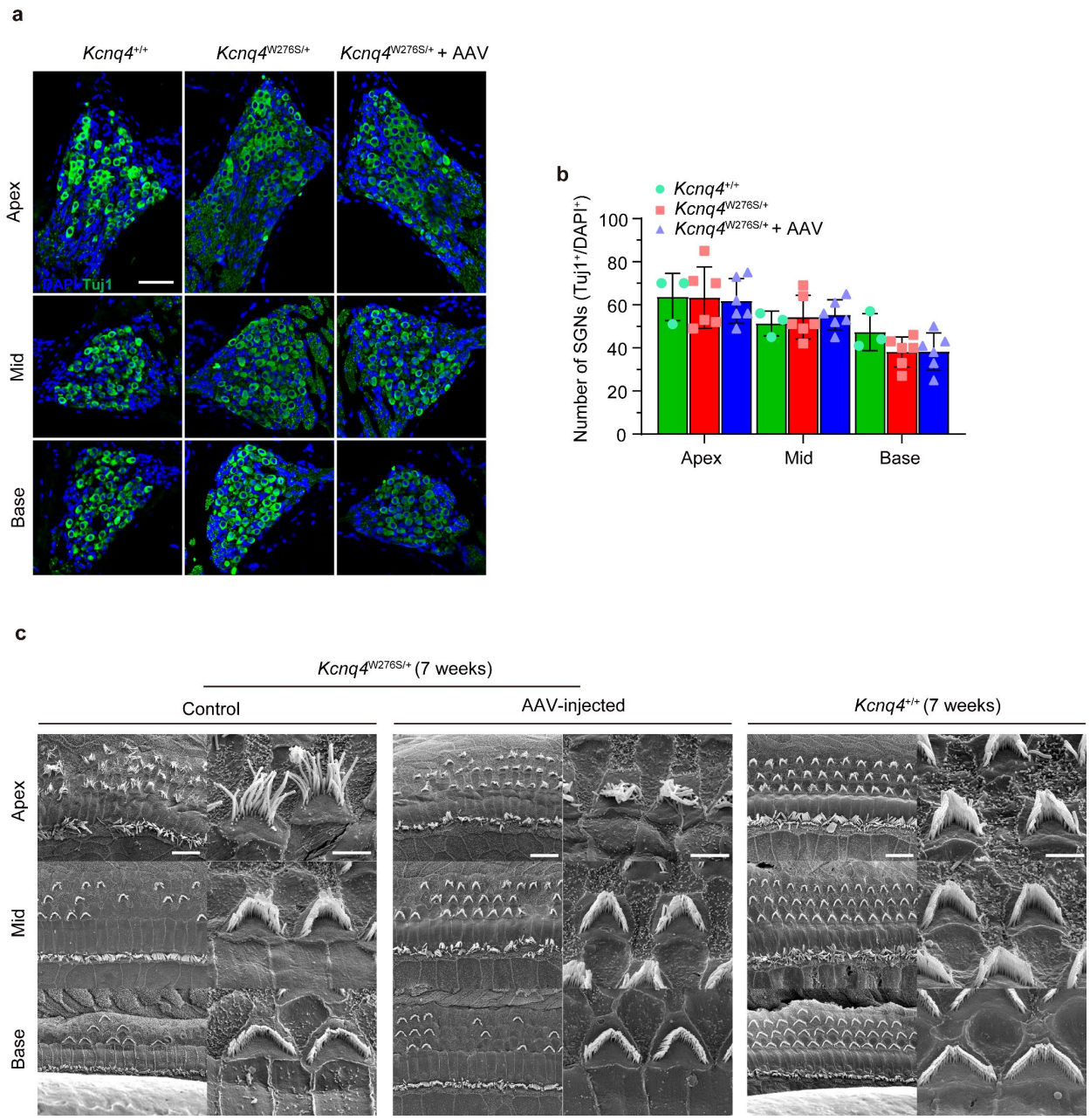


Supplementary Figure 7. Hearing restoration in *Kcnq4*$^{W276S/+}$ after *in vivo* gene editing.
(**a**) ABR thresholds across different sound frequencies in *Kcnq4*$^{W276S/+}$ AAV-injected mice at P49–P56 (gray lines, $n = 21$, selected cases from **Fig. 3c**). The best (green) and mean (blue) recovery traces are presented. Wild-type control is depicted in back color ($n = 4$). (**b-c**) Peak 1 amplitudes (**b**) and latencies (**c**) measured from 6 kHz ABR waveforms as shown in (**a**). (**d**) ABR thresholds across different sound frequencies in *Kcnq4*$^{W276S/+}$ AAV-injected mice at P49-P56 (gray lines, $n = 18$, selected cases from **Fig. 3e**). The best (green) and mean (blue) recovery traces are presented. Phenotypic rescue was observed in AAV-injected cochleae when compared to contralateral uninjected cochleae (red, n = 18) with statistical significances across all frequencies except for 30 kHz. However, phenotypic rescue degree did not reach the levels of wild-type control cochleae (black, n = 4). (**e-f**) Peak 1 amplitudes (**e**) and latencies (**f**) measured from 6 kHz ABR waveforms as shown in (**d**).



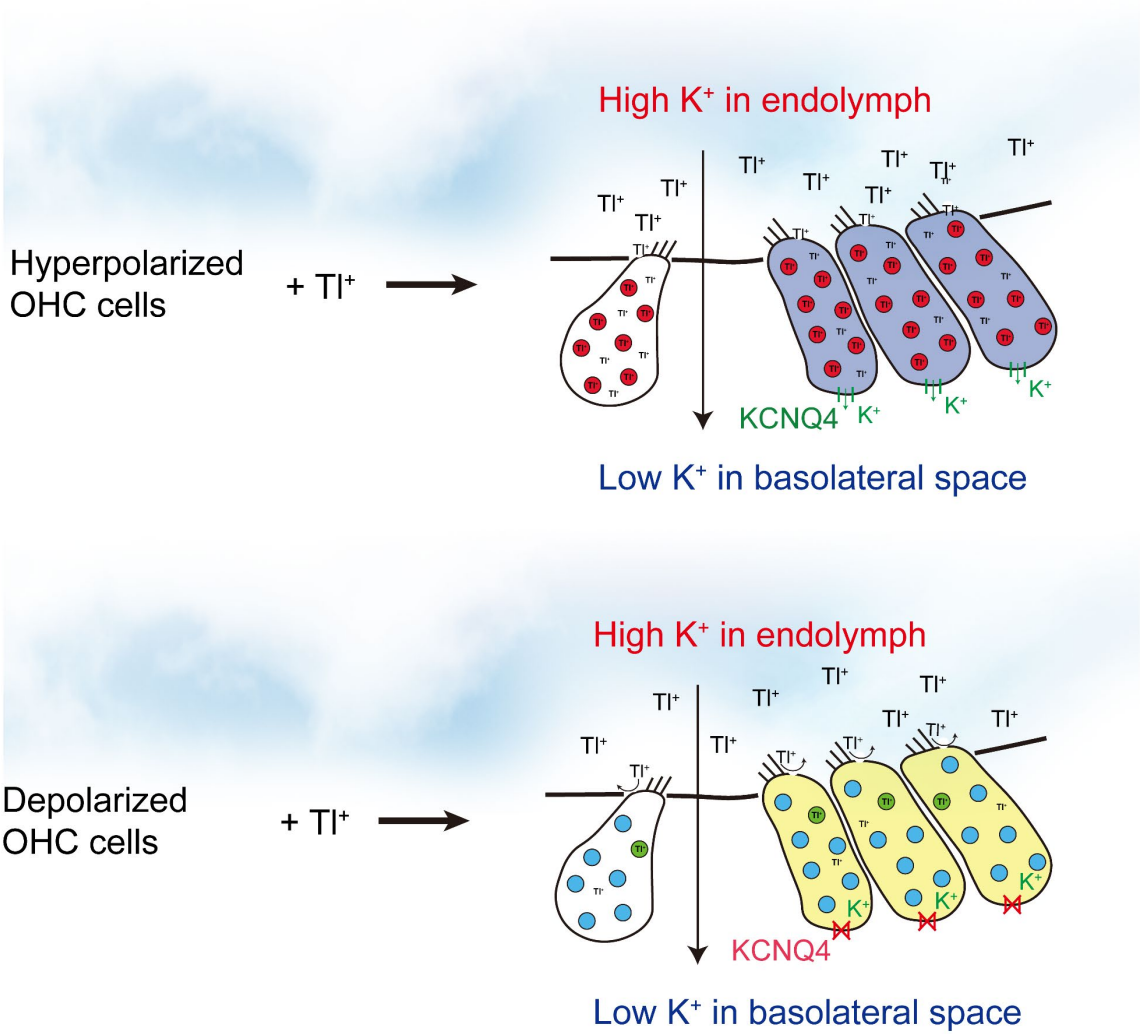
Supplementary Figure 8. Effect of *in vivo* gene editing on the hair cells and neurofilaments of *Kcnq4*$^{W276S/+}$ mice.

(**a**) Whole cochlear turns stained with phalloidin (green) from 7-week-old mutant mice uninjected or injected with AAV at P1 over five frequency regions (indicated by white arrows and red squares). Scale bar, 100 μm. (**b**) Cross-sectional images of wild-type, and uninjected mutant, and AAV-injected mutant cochlea (at the 12-kHz region) from 7-week-old mouse showing KCNQ4 expression. Scale bar, 20 μm. (**c**) Tonotopically mapped cochlear sections at 6, 12, 18, 24, and 30 kHz regions for wild type (top), uninjected mutant (middle), and AAV-injected mutant (bottom) mice. Areas inside the yellow rectangle are the 100-μm region used to quantify the fluorescence density of the inner spiral plexus (ISP). Scale bar, 20 μm. (**d**) Quantitation of fluorescence density. No statistical significance was observed in the mouse groups [wild type (black, $n = 3$), uninjected mutant (red, $n = 4$), and AAV injected mutant ($n = 4$, blue)] in all frequency regions. ns, not significant.



Supplementary Figure 9. Effect of *in vivo* gene editing on the neuronal survival and hair cell morphology in *Kcnq4*$^{W276S/+}$ mice.

(**a**) Cross-sectional images of the inner ear at the apex, mid, and base regions of cochlea of 7-week-old wild-type, uninjected mutant, and AAV-injected mutant mice showing ganglion survival. Tuj1 (green) positivity indicates alive spiral ganglion neuron (SGN). Scale bar, 50 μm. (**b**) Quantitation of the number of alive SGNs according to tonotopic regions in (a). No significant difference in the survival rates of SGNs among the wild-type (green, $n = 3$), uninjected mutant (red, $n = 6$), and AAV-injected mutant (blue, $n = 6$) mice was observed ($p > 0.05$). (**c**) SEM images at the apex, mid, and base from cochlea of 7-week-old uninjected mutant (control) and AAV-injected mutant mice. Scale bar, 20 μm (low power) and 5 μm (high power).



Supplementary Figure 10. Thallium (Tl^+) flux assay in the organ of Corti.
Graphical illustration of the Tl^+ flux assay performed in the organ of Corti of 7-week-old wild-type (top) and *Kcnq4* mutant (bottom) mice.

Supplementary Note 1.

The coding sequence of CMV-Cas9-Puro-2A-RFP

CMV enhancer colored in yellow, CMV promoter colored in pink, hSpCas9 is colored in blue, SV40 NLS colored in dark blue, HA colored in green E2A colored in dark green, TagRFP is colored in red, T2A is colored in dark grey, PuroR is colored in grey, and bGH poly A is colored in brown.

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The coding sequence of U6-pRG2-sgRNA.

U6 colored in yellow, BsaI restriction enzyme cloning site is colored in pink, gRNA scaffold colored in dark green and pUC ori is colored in green

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The coding sequence of U6-sgRNA- split–N-Cas9 N-intein

AAV2 ITR is colored in red, U6 colored in dark green, Kcnq4-sgRNA T3 is colored in violet, gRNA scaffold is colored in grey, CMV enhancer colored in yellow, CMV promoter colored in pink, SV40 NLS colored in dark blue, FLAG-tag colored in green, split–N-Cas9 is colored in blue, N-Intein is colored in dark grey, and bGH poly A is colored in brown.

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Caption for Supplementary Movie 1

Ex vivo **imaging of outer hair cells using thallium-sensitive dye (FluxOR-Tl^+) in the apical turn (6 kHz) of the wild-type cochlea.** The videos were captured from 0 to 120 s at the reference time of the addition of thallium in the buffer and reframed in 30 s. Colors refer to the intensity of FluxOR-Tl^+, which is correlated with the membrane potential set by electro-chemical gradients with potassium, sodium, and chloride. Purple denote low intensity, whereas red denote high intensity.