

Figure S1. Pre-selecting promising Atoh1 sgRNAs by zygote injection. (A) Cartoon figure illustrating how ‘promising’ Atoh1-sgRNAs were pre-selected by directly testing their base editing efficiency *in vitro* in zygotes. Zygotes were cultured *in vitro* for 3 days (until blastocyst stage) before performing nest-PCR and DNA sequencing. (B-D) Example sequencing data of three promising Atoh1 sgRNAs (#1-#3). Our standard for ‘promising’ was that above 40% of samples experienced base editing C to T. sgRNA-2 had relatively lower efficiency than sgRNA-1 and 3. (E) One example of ‘unpromising’ Atoh1-sgRNA (#4) which failed all base editing among 5 samples.

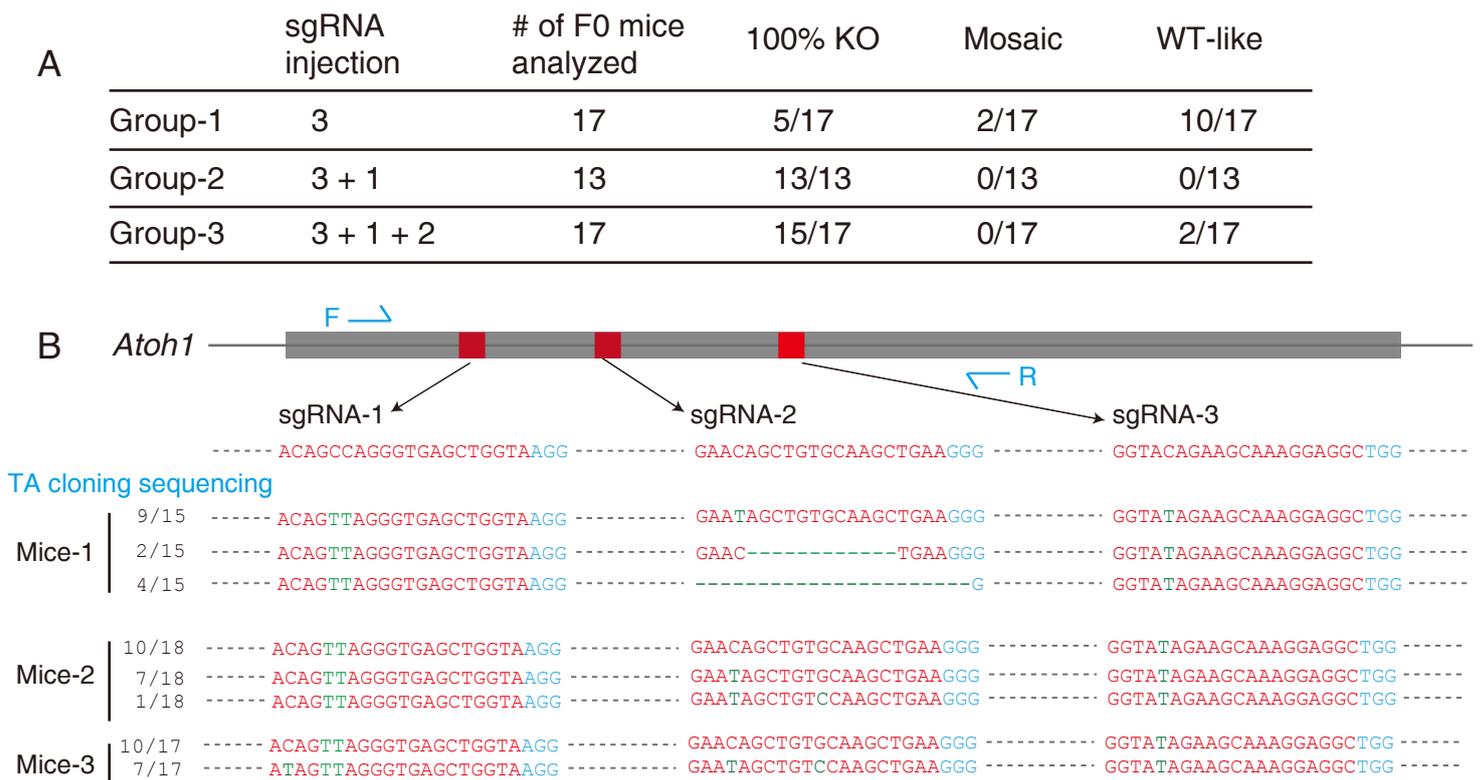


Figure S2. Atoh1 base editing efficiency comparison between three

different conditions. (A) Detailed information about the three experimental groups in which different combinations of sgRNA were tested. The combination of sgRNA-3 and 1 (group 2) showed the best results. (B) TA clone for Sanger sequencing of Group-3's inner ear DNA (n=3, mice number). The target regions of the three sgRNAs were sequenced simultaneously; this allowed us to verify that base editing occurred in the same allele.

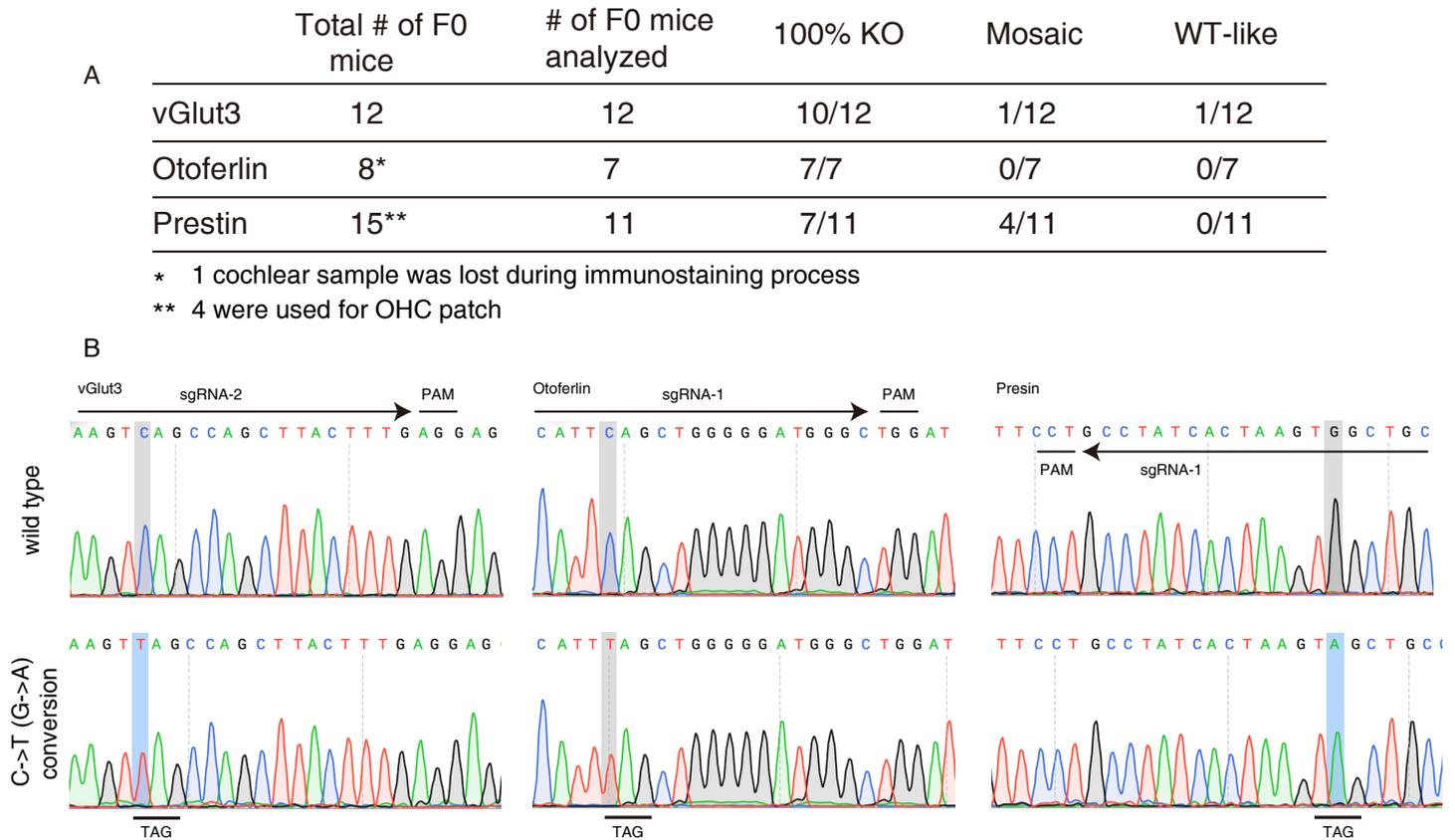


Figure S3. Individual base editing in mouse *vGlut3*, *Otoferlin* and

***Prestin* genes.** (A) Detailed information concerning base editing efficiency of each gene. Two pre-tested effective sgRNAs were used in combination per gene. (B) Examples of DNA Sanger sequencing results for each gene. Upper panels represent wild type alleles, lower panels are the mutant alleles that underwent targeted C to T conversion (highlighted). Note that in *Prestin* gene, sgRNA-1 is in opposite direction and the PAM sequence AGG is located on the opposite strand. Indeed, in this case it was a G to A conversion.

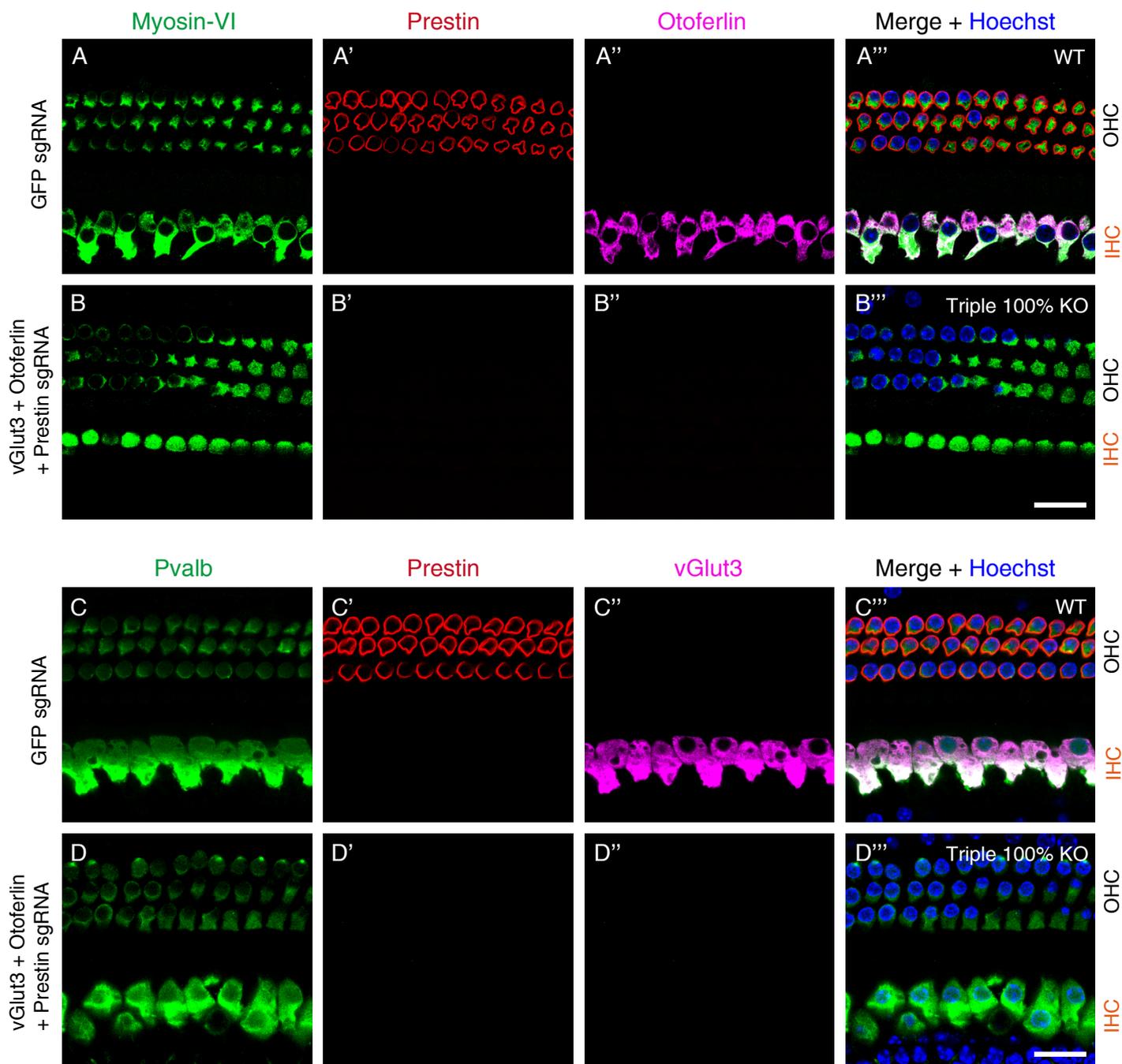


Figure S4. Simultaneous inactivation of vGlut3, Otoferlin and Prestin does not cause HC death. (A-B''') Triple staining of Myosin-VI, Prestin and Otoferlin in WT group (A-A''') and triple mutant (B-B'''). Myosin-VI staining was performed to confirm the presence of both OHCs and IHCs. **(C-D''')** Triple staining of Parvalbumin (Pvalb), Prestin and vGlut3 in WT group (C-C''') and triple mutant (D-D'''). Pvalb staining also served to confirm the presence of both OHCs and IHCs. Note that (B-B''') and (D-D''') depicted two portions of the same cochlea. This allowed us to conclude that vGlut3, Otoferlin and Prestin had been successfully inactivated, and that nevertheless, both OHCs and IHCs survived. Scale bars: 20 μ m.

A

| | | Mice # 1 | | Mice # 5 | | Mice # 8 |
|--------------------------|----------------|-----------------------|-------|--|----------------|-----------------------|
| <i>Vglut3</i> sgRNA-1 | WT | AACCCAGAAAAAGATCCA | | AACCCAGAAAAAGATCCA | | AACCCAGAAAAAGATCCA |
| | base editing → | AACCTCAGAAAAAGATCCA | 5/5 | AACCTTAGAAAAAGATCCA AACCTCAGAAAAAGATCCA | 15/31 16/31 | AACCTTAGAAAAAGATCCA |
| <i>Vglut3</i> sgRNA-2 | WT | AAGTCAGCCAGCTTACTTTG | | AAGTCAGCCAGCTTACTTTG | | AAGTCAGCCAGCTTACTTTG |
| | → | AAGTTAGCCAGCTTACTTTG | 31/31 | AAGTTAGCCAGCTTACTTTG | 19/19 | AAGTTAGCCAGCTTACTTTG |
| <i>Prestin</i> sgRNA-1 | WT | GCAGCCACTTAGTGATAGGC | | GCAGCCACTTAGTGATAGGC | | GCAGCCACTTAGTGATAGGC |
| | → | GCAGCTATTAGTGATAGGC | 8/8 | GCAGCTATTAGTGATAGGC | 7/7 | GCAGCTATTAGTGATAGGC |
| <i>Prestin</i> sgRNA-2 | WT | TCTCGAAGCCTTGTTTCAGGA | | TCTCGAAGCCTTGTTTCAGGA | | TCTCGAAGCCTTGTTTCAGGA |
| | → | TTTGAAGCCTTGTTTCAGGA | 23/23 | TTTGAAGCCTTGTTTCAGGA | 17/17 | TTTGAAGCCTTGTTTCAGGA |
| <i>Otoferlin</i> sgRNA-1 | WT | CATTCAGCTGGGGGATGGGC | | CATTCAGCTGGGGGATGGGC | | CATTCAGCTGGGGGATGGGC |
| | → | CATTAGCTGGGGGATGGGC | 14/18 | CATTAGCTGGGGGATGGGC | 32/32 | CATTAGCTGGGGGATGGGC |
| <i>Otoferlin</i> sgRNA-2 | WT | CATTCAGCTGGGGGATGGGC | | CATTCAGCTGGGGGATGGGC | | CATTCAGCTGGGGGATGGGC |
| | → | CATTAGCTGGGGGATGGGC | 24/24 | CATTAGCTGGGGGATGGGC | 15/21 | CATTAGCTGGGGGATGGGC |

B

| | Mice # 1 | | Mice # 5 | | Mice # 8 | |
|---|----------|--------|----------|--------|----------|--------|
| | SNVs | Indels | SNVs | Indels | SNVs | Indels |
| Variants called by mutect2 | 7067 | 950 | 8920 | 959 | 8750 | 1035 |
| After filtering variants located in genome complex regions ^a | 2444 | 456 | 3205 | 474 | 3113 | 468 |
| Exons | 84 | 5 | 85 | 5 | 88 | 5 |
| Protein coding ^b | 57 | 3 | 53 | 2 | 54 | 3 |
| Variants in all samples | 6 | 2 | 6 | 2 | 6 | 2 |
| Predicted off-targeted sites (24705) | 0 | 0 | 0 | 0 | 0 | 0 |

^aAll UCSC repeats and microsatellites

^bNonsynonymous SNVs and frameshift indels

Figure S5. On-target and off-target analysis of whole genome

sequencing (WGS) results. (A) WT (blue color) and mutant sequences (red arrow pointed) of the targeted sites by WGS. Red T highlights C to T conversions in mutant sequences. The numbers before slashes indicate the number of reads containing C to T conversions, and numbers after slashes represent the total read count. The numbers in rectangles highlight the cases in which not all sgRNA-targeted sites in *Otoferlin* experienced base editing. **(B)** Summary of variant calls from WGS data.

Table S1. Primers for targeted deep sequencing

[Click here to Download Table S1](#)

Table S2. Pre-tested and promising sgRNAs used in this study

| sgRNA Names | 20bp sequence (5'-3', without PAM) |
|--------------------|---|
| Tyr-sgRNA | ACCTCAGTTCCCCTTCAAAG |
| Atoh1-sgRNA-1 | ACAGCCAGGGTGAGCTGGTA |
| Atoh1-sgRNA-2 | GAACAGCTGTGCAAGCTGAA |
| Atoh1-sgRNA-3 | GGTACAGAAGCAAAGGAGGC |
| vGlut3-sgRNA-1 | AACCCCAGAAAAAAGATCCA |
| vGlut3-sgRNA-2 | AAGTCAGCCAGCTTACTTTG |
| Otoferlin-sgRNA-1 | CATTCAGCTGGGGGATGGGC |
| Otoferlin-sgRNA-2 | TGTACAGGAGATGATCAAAA |
| Prestin-sgRNA-1 | GCAGCCACTTAGTGATAGGC |
| Prestin-sgRNA-2 | TCTCGAAGCCTTGTTTCAGGA |