Simultaneous zygotic inactivation of multiple genes in mouse through CRISPR/Cas9-mediated base editing

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ABSTRACT

In vivo genetic mutation has become a powerful tool for dissecting gene function; however, multi-gene interaction and the compensatory mechanisms involved can make findings from single mutations, at best difficult to interpret, and, at worst, misleading. Hence, it is necessary to establish an efficient way to disrupt multiple genes simultaneously. CRISPR/Cas9-mediated base editing disrupts gene function by converting a codon into a stop codon; this is referred to as CRISPR-stop. Its application in generating zygotic mutations has not been well explored yet. Here, we first performed a proof-of-principle test by disrupting Atoh1, a gene crucial for auditory hair cell generation. Next, we successfully disrupted vGlut3, Otof and prestin simultaneously. Our results show that CRISPR-stop can efficiently generate single or triple homozygous F0 mouse mutants, bypassing laborious mouse breeding. We believe that CRISPR-stop is a powerful method that will pave the way for high-throughput screening of mouse developmental and functional genes, matching the efficiency of methods available for model organisms such as Drosophila.

KEY WORDS: Inner ear, vGlut3, Prestin, Otoferlin, Base editing, CRISPR/Cas9

INTRODUCTION

The clustered regularly interspaced short palindromic repeat (CRISPR) system has been developed as a versatile genome editing tool in various organisms (Cong et al., 2013; Yao et al., 2015). CRISPR/Cas9 has been successfully used for normal hearing function. Finally, we successfully disrupted vGlut3, Otof and prestin simultaneously. Our results show that CRISPR-stop can efficiently generate single or triple homozygous F0 mouse mutants, bypassing laborious mouse breeding. We believe that CRISPR-stop is a powerful method that will pave the way for high-throughput screening of mouse developmental and functional genes, matching the efficiency of methods available for model organisms such as Drosophila.

CRISPR-stop (Billon et al., 2017; Kuscu et al., 2017). In addition, thanks to the specificity of altered base pairs, CRISPR-stop can target selectively the protein-coding or long non-coding RNAs, even if these happen to be at the same genomic locus (Billon et al., 2017). Recently, mutant zebrafish have also been generated using base-editing methods (Zhang et al., 2017). The power of using the CRISPR-stop approach in single- and multi-gene screening mouse developmental genes represents potential waiting to be harnessed by the community.

In vivo loss-of-function, rather than gain-of-function studies, are the gold standard for proving the necessity of the function of a gene in developmental biology. However, a given gene frequently has multiple homologs that can sometimes compensate for its absence. In this case, it is necessary to inactivate multiple related genes in order to uncover their function. It is therefore of interest to establish efficient ways to disrupt multiple genes simultaneously. We tested the use of the CRISPR-stop base editing method to meet this need. For ease of phenotypic and functional analysis, we used the mouse inner ear auditory organ, the cochlea, as a platform to demonstrate the capabilities of the CRISPR-stop method.

The mouse cochlea resides in the ventral region of the inner ear. Its auditory epithelium, the organ of Corti, contains hair cells (HCs) and neighboring supporting cells (SCs) (Kelley, 2006). Atoh1, a bHLH transcription factor, is required in HC fate specification; indeed, Atoh1−/− mice lack all HCs (Bermingham et al., 1999),
whereas overexpression of Atoh1 results in ectopic HCs (Gubbelts et al., 2008; Kelly et al., 2012; Liu et al., 2012; Zheng and Gao, 2000). Moreover, two subtypes of cochlear HCs exist: three rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs). Both OHCs and IHCs are crucial for hearing capacity but have different roles and express different proteins. For example, prestin, which is encoded by Slc26a5, is a lateral membrane protein exclusively expressed in OHCs (Liberman et al., 2002; Zheng et al., 2000). It powers the electromotility of OHCs, a central component of the mammalian cochlear amplification mechanism (Brownell et al., 1985; Liberman et al., 2002). Prestin-mediated electromotility can be readily assessed by the presence of nonlinear capacitance (NLC) using standard patch-clamp hardware (Bai et al., 2006). The nonlinear region of the NLC curve offers functional information about prestin, whereas the linear portion of the curve gives information about OHC size (Navarrete and Santos-Sacchi, 2006). Mutation of either vGlut3 or otoferlin leads to severe deafness in mice and human (Liu et al., 2003) and mice (Liberman et al., 2002). In contrast to the modulatory role of OHCs, IHCs are the primary sound receptors and express vesicular glutamate transporter 3 (vGlut3, also referred to as Slc7a8) and a calcium sensing protein, otoferlin (Li et al., 2018; Roux et al., 2006), both of which are not present in adult OHCs. Mutation of either vGlut3 or otoferlin leads to severe deafness in mice and humans (Ruel et al., 2008; Seal et al., 2008; Yasunaga et al., 1999). Because of their importance and well characterized function in the inner ear, we chose Atoh1, prestin, vGlut3 and otoferlin as targets for CRISPR-stop gene editing.

In a first step, we successfully optimized our experimental procedures using Atoh1 as proof-of-principle gene. Multiple sgRNAs for each gene were pre-tested through in vitro validation on injected zygotes; the most promising sgRNAs were chosen to be injected into zygotes that would eventually be implanted into pseudopregnant female mice. Our data suggest that the selection of two ‘promising’ sgRNAs (see Fig. S1 for quantitative definition of ‘promising’) together typically generate homozygous F0 mutant mice. Mosaic mutants did occur but at a low rate. Most importantly, we could also produce triple homozygous prestin, vGlut3 and otoferlin mutants in F0 mice, showing that targeting multiple genes simultaneously is possible using CRISPR-stop. Hence, CRISPR-stop presents a feasible approach for studying the effects of concurrent loss-of-function mutations in three, and potentially more, genes. Furthermore, as the sgRNA and base editor 3 (BE3) are injected into a one-cell stage zygote, which in turn gives rise to all tissues and organs of the body, the CRISPR-stop approach can be used to target any mammalian organ, making it a flexible and broadly applicable method. In conclusion, CRISPR-stop is a powerful tool that enables the rapid screening of candidate genes; this brings to mammalian research the equivalent of the transformative high-throughput RNAi screening technology available in some non-mammalian model organisms (Liu et al., 2015).

RESULTS
Base editing of the Tyr gene in mice using CRISPR-stop
In this study, we used the base editor 3 (BE3) complex (Komor et al., 2016). BE3 has three components fused together: Cas9 nickase (nCas9), cytidine deaminase (APOBEC1) and uracil glycosylase inhibitor (UGI), as shown in Fig. 1A. BE3 is guided to a target DNA sequence by gene-specific sgRNA; the complex catalyzes a base conversion in the target gene, converting C to T or G to A (Fig. 1B). If base editing occurs in-frame of a protein-coding sequence, a stop codon can be prematurely introduced and disrupt gene function (Fig. 1C); this approach is referred to as CRISPR-stop and works reliably in various cell lines (Billon et al., 2017; Kuscu et al., 2017). It is a powerful approach for loss-of-function studies, especially when the functional domains of the protein of interest are unknown.

To test whether the CRISPR-stop approach could be applied to mice, we initially focused on the tyrosinase gene (Tyr, for pigmentation), as a loss-of-function mutation in this gene produces mice with a white coat (Zuo et al., 2017), which is an easy and convenient phenotypic readout of successful gene inactivation. To achieve this, we injected BE3 mRNA together with a single design of Tyr sgRNA into one-cell stage zygotes (Fig. 1D,E). In total, 48 zygotes were implanted into pseudopregnant females and 18 F0 mice were obtained. Thirteen out of 18 (72.2%) displayed white coats, suggesting the occurrence of homozygous Tyr mutations (Fig. 1H). DNA sequencing confirmed the introduction of a premature stop codon in Tyr as a result of a C-to-T conversion (Fig. 1F,G). Nevertheless, in the same litters, five out of 18 (27.8%) bore black or brown coats, indicating the lack of, or heterozygous, Tyr mutations (Fig. 1H).

Proof-of-principle test of CRISPR-stop in mouse cochlea
Building on the positive results from the Tyr experiment, we next wanted to test whether CRISPR-stop could be used to affect in vivo organ function. We chose the cochlea as a model organ and the Atoh1 gene as an illustration. Atoh1 is required for generating cochlear HCs, as indicated by the complete loss of all HCs in Atoh1−/− mice (Bermingham et al., 1999). Such a severe phenotype provided us with a clear and rapid readout of the occurrence of loss-of-function mutations in Atoh1. As injecting a single sgRNA led to 72.2% homozygous mutants in the Tyr experiment, we tested whether injecting multiple designs of sgRNAs simultaneously would boost the chances of successful base editing. To achieve this, we designed seven sgRNA variants targeting Atoh1. DNA-sequencing data from injected blastocysts indicated that three of these sgRNAs were permissive to base editing and were further used for the subsequent steps outlined below (Fig. 2A and Fig. S1).

We injected BE3 mRNA together with sgRNA3 (group 1), sgRNA3+1 (group 2) or sgRNA3+1+2 (group 3), and compared the resulting base edits across the three groups. To rule out any effects caused by injecting BE3 mRNA and sgRNA themselves, we also injected GFP (green fluorescent protein) sgRNA with BE3 mRNA into zygotes that served as a control or wild-type group (Fig. 2B,C). Notably, in our study, base editing occurred equally well across all cochlear turns and no differences among different turns were identified. As Atoh1−/− mice die immediately after birth (Bermingham et al., 1999), we analyzed cochlear samples from each group at embryonic day 18.5 (E18.5). Detailed information about samples of each group can be found in Fig. S2A. Briefly, we used whole-mount immunostaining of the cochlea with myosin VI (a HC marker) to determine the effectiveness of the method at disrupting Atoh1. The complete absence of myosin VI was taken as supporting evidence for the presence of a homozygous loss-of-function mutation in the Atoh1 gene. Three categories of phenotypic outcomes were identified in these experiments. The first case consisted of complete HC loss, corresponding to an effective homozygous base editing, which we defined as 100% KO (knockout) (Fig. 2D). Cochlear sensory epithelium maintained Sox2 expression in spite of HC loss (Fig. 2E). In the second class of outcomes, HCs were observed but their numbers were greatly reduced; such a phenotype was defined as mosaic (Fig. 2F). This suggests that only a fraction of sensory progenitors experienced
base editing. Finally, in the third case, HCs were distributed as in the wild-type cochlea, i.e. in four rows consisting of one IHC row and three OHC rows, suggesting that no Atoh1 loss-of-function base editing had occurred in cochlear sensory progenitors. We defined such cases as wild-type like (Fig. 2G). Cochlear whole-mount myosin VI immunostaining demonstrated that group 2 (sgRNA3+1) produced consistent (100%) Atoh1 homozygous mutants, and that mosaic mutations occurred only in group 1 (sgRNA3) samples (Fig. 2H). Owing to a lack of reliable Atoh1 antibody at hand, Atoh1 protein expression was not assessed.

To further confirm that base editing had occurred in the Atoh1 gene, DNA Sanger sequencing of inner ear tissues was performed. The results showed that base C (arrow in Fig. 2I) had been converted to T (arrow in Fig. 2J), leading to the coding CAG being changed to TAG, a stop codon. We also performed next-generation deep sequencing to quantify the percentage of targeted C to T conversion among all cells dissected from inner ear tissues (Fig. 2K). The effectiveness of sgRNA3 in group 1 was 73.2±4.7% (n=18, mice number). In group 2, sgRNA3 caused 90±4.0% and sgRNA1 resulted in 44.4±16.2% base editing (n=7, mice number). In group 3, the effectiveness of sgRNA3 was 76.9±9.8%, sgRNA1 was 25.9±6.7% and sgRNA2 was 36.1±11.2% (n=10, mice number). Furthermore, for group 3 samples, we also performed TA cloning of PCR products covering all three sgRNAs-targeted sites. The data suggested that base editing occurred in the same allele (Fig. S2B). Taken together, the results show that introducing multiple sgRNAs can lead to non-additive gene disruption and increase the chances of loss-of-function gene mutations. Indeed, despite neither sgRNA alone being 100% effective, the combination of two sgRNAs in group 2 was sufficient to disrupt all Atoh1 translation.

**CRISPR-stop can efficiently generate different viable mouse models of severe deafness**

Next, we focused on determining whether CRISPR-stop could be used to generate various deafness mouse models. Prestin-, vGlut3- and otoferlin-null mice all survive to adulthood and exhibit severe hearing loss (Liberman et al., 2002; Roux et al., 2006; Ruel et al.,...
We therefore selected them as candidate genes to generate deaf mice using the CRISPR-stop approach. Based on the findings of the Atoh1 study above, for every gene targeted, the two most promising sgRNAs were injected together. Again, GFP sgRNA was used to generate wild-type control mice. Samples were analyzed at 4 weeks of age and three categories of

Fig. 2. Base editing in mouse Atoh1. (A) Atoh1 has a single exon; three sgRNAs were designed close to the ATG start codon. (B,C) Whole-mount (B) or cryosectioned (C) wild-type cochlear samples were dissected from mice (E18.5) derived from GFP-sgRNA/BE3-treated zygotes and stained for myosin VI alone or also for Sox2. Three rows of OHCs and one row of IHCs were observed. (D,E) Whole-mount (D) or cryosectioned (E) cochlear samples were dissected from mice (E18.5) derived from Atoh1-sgRNA/BE3-treated zygotes and stained for myosin VI alone or also for Sox2. 100% knockout phenotype samples were defined as lacking all hair cells (HCs) (D); Sox2 expression was maintained (E). (F) Mosaic phenotypes were defined as those with sparsely distributed HCs; in these samples, derived from Atoh1-sgRNA/BE3-treated zygotes, the total number of HCs was reduced. (G) Wild-type-like phenotypes consisted of intact HCs, implying that, in these mice, derived from Atoh1-sgRNA/BE3-treated zygotes, loss-of-function base editing had not occurred. (H) Distribution of different phenotypes, 100% knockout, mosaic and wild-type like from each experimental group. Group 1 mice were injected with sgRNA3 only, group 2 mice were given sgRNA1 and sgRNA3, and group 3 mice were injected with sgRNA1, sgRNA2 and sgRNA3. All samples were stained using myosin VI antibody to visualize and quantify HCs to determine the effectiveness of base editing. All cochlear samples in group 2 lost HCs, indicating that the combination of two sgRNAs was, in this case, optimal. (J) DNA Sanger sequencing comparison between Atoh1 wild-type (I) and mutant (Atoh1 KO) alleles (J). Base C (arrow in I, position 5 in sgRNA3) was converted into T (arrow in J), leading to the premature appearance of a TAG stop codon (underlined in J). (K) Quantification of targeted C-to-T editing efficiency of three groups. Each circle/square/triangle represents the percentage of targeted base edits in each mouse. Bars are mean and error bars indicate the s.e.m. Scale bars: 20 μm.
phenotype were defined as before: 100% knockout, mosaic and wild-type like.

We first confirmed that vGlut3 was exclusively expressed in IHCs (within the dotted lines in Fig. 3A,A') in wild-type group (n=3, mice number), consistent with our previous report (Li et al., 2018). vGlut3 has 12 exons and its endogenous stop codon TAA is located within exon 12. From the pre-tested sgRNAs targeting vGlut3, the two most effective ones were found to target exon 3 and exon 8, respectively. In total, 19 zygotes were injected with BE3 mRNA and these two sgRNAs, and transplanted into pseudopregnant mice; 12 mice were born (Fig. S3A). Double staining of parvalbumin (Pvalb) and vGlut3 showed that 10 mice (83.4%) were 100% knockout (Fig. 3B,B'), one (8.3%) was mosaic (inset in Fig. 3B) and one (8.3%) was wild-type like (Fig. 3G). Otoferlin, in similar fashion to vGlut3, is selectively expressed in IHCs in wild-type mice (dotted lines in Fig. 3C,C'). The otoferlin gene consists of 47 exons, and its endogenous stop codon, TGA, is located within exon 46. In light of this, we designed two sgRNAs targeting exons 7 and 19. We transplanted 20 zygotes and obtained eight mice (Fig. S3A). Co-staining for myosin VI and otoferlin showed that all of the seven mice analyzed were 100% knockout (Fig. 3D,D'). Taken together, this demonstrates the efficient use of the CRISPR-stop method to generate loss-of-function mouse lines for two IHC-specific genes: vGlut3 and otoferlin.

Contrary to vGlut3 and otoferlin, prestin is exclusively expressed in OHCs in wild-type mice (Fig. 3E,E'). The prestin gene consists of 20 exons; the last exon contains its endogenous stop codon, TAA. We designed two sgRNAs targeting exons 4 and 11. In total, 20 zygotes were transplanted and 15 mice were born (Fig. S3A). Co-antibody staining for myosin VI and prestin demonstrated that among all 11 mice that we could analyze, seven (63.6%) were 100% knockout (Fig. 3F,F') and four (36.4%) were mosaic (inset in Fig. 3F). To further confirm design-specific base-editing mutations, we amplified tail-DNA fragments covering the sgRNA target sites from each mutant model; in all cases, the sequencing data clearly showed a targeted C-to-T conversion (Fig. S3B).

To assess the hearing capacity of the mutant mice, we performed auditory brainstem response (ABR) analysis in the vGlut3, otoferlin

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**Fig. 3. Single gene base editing of mouse vGlut3, otoferlin and prestin.** (A–B') Whole-mount cochlear samples were stained for parvalbumin (Pvalb, a hair cell marker) and vGlut3. In the wild-type group (A,A'), Pvalb was expressed in both inner hair cells (IHCs) and outer hair cells (OHCs), while vGlut3 was expressed in only IHCs (within the dotted lines). In contrast, in cochleae (B,B', 100% knockout) derived from zygotes receiving two vGlut3 sgRNAs/BE3 mRNA, vGlut3 was absent from IHCs (within the dotted lines in B), while Pvalb expression was unchanged. The inset in B exemplifies the mosaic phenotype in which not all IHCs lost vGlut3. (C–D') Similarly to vGlut3, in the wild-type group (C,C'), otoferlin was, as expected, exclusively expressed in IHCs (within the dotted lines). Myosin VI was expressed in both IHCs and OHCs. In otoferlin mutants (D,D', 100% knockout), otoferlin was undetectable in IHCs (within the dotted lines); however, myosin VI expression pattern was left unchanged. (E–F') Prestin was expressed in OHCs (within the dotted lines) in the wild-type group as expected (E,E'). In prestin mutants (F,F', 100% knockout), all myosin VI+ OHCs (within the dotted lines) lacked prestin expression. The inset in F exemplifies the mosaic phenotype in which not all OHCs lost prestin. (G) Quantification of different phenotypes caused by CRISPR-stop in vGlut3, otoferlin and prestin mutants. (H) The auditory brainstem response (ABR) measurement of each mutant. Compared with the control wild-type group (black line), vGlut3, otoferlin and prestin mutants showed significant hearing impairment across all frequencies, with the exception of the prestin mutant at 45 kHz (P=0.1). ***P<0.001, **P<0.01, *P<0.05. Data are mean±s.e.m. Scale bars: 20 μm.
and prestin mutants. In order to pre-select mice belonging to the 100% knockout category, we sequenced tail DNA and chose animals who showed the most effective targeted C-to-T conversion (Fig. 3B,B’,D,D’,F,F’). Compared with control mice (n=5), each mutant mouse (n=5) displayed a significantly increased ABR threshold, indicating severe deafness (Fig. 3H). Compared with control mice (n=5), each mutant mouse (n=5) displayed a significantly increased ABR threshold, indicating severe deafness (Fig. 3H). In addition, vGlut3 and otoferlin mutants exhibited a higher ABR threshold than the prestin mutant mice, consistent with previous reports (Liberman et al., 2002; Roux et al., 2006; Ruel et al., 2008; Seal et al., 2008).

**Prestin and otoferlin mutants also have defective electrophysiological properties**

Besides ABR measurement, we also performed electrophysiological analysis of prestin and otoferlin mutant mice. As before, we selected mice in the 100% knockout category using tail-DNA sequencing. Nonlinear capacitance (NLC) is a surrogate measurement used to assess the electromotility function of OHCs (Santos-Sacchi, 1991). As expected, NLC was absent in prestin 100% knockout mice (Fig. 4A). In addition to lack of electromotility, OHCs in prestin mutants showed significantly reduced linear capacitance, Clin=4.01±0.26 (n=5, OHC numbers) when compared with control OHCs, Clin=6.75±0.23 (n=7 OHCs) (Fig. 4B). This suggests that the lack of prestin results in smaller OHCs, consistent with previous reports (Abe et al., 2007; He et al., 2010; Liberman et al., 2002).

Otoferlin is a calcium-sensor protein and controls membrane fusion and exocytosis (Pangrác et al., 2010). To test for any changes in exocytosis of otoferlin mutant IHCs, we performed whole-cell patch-clamp recordings of IHCs and applied voltage steps of 10 ms to 1 s duration to induce exocytosis. We calculated the change in capacitance before and after stimulation (ΔCm), and found that ΔCm was greatly reduced in otoferlin mutant mice (upper panel in Fig. 4C). For depolarization of 500 ms, ΔCm was significantly reduced (P<0.001) from 392±113 fF (n=12, IHC cell number, control) to 34.3±10.6 fF (n=9 IHCs, otoferlin mutant). Meanwhile, Ca2+ influx (QCa) was not significantly changed, being −55.7±5.36 pC for control and −41.5±7.73 pC for otoferlin knockout (lower panel in Fig. 4C). These results are highly consistent with previously published otoferlin mutant models (Moser and Beutner, 2000; Roux et al., 2006) and provide further evidence supporting the absence of prestin and otoferlin proteins in prestin and otoferlin mutant mice, respectively.

In summary, the DNA sequencing, immunostaining and functional analysis data show that CRISPR-stop successfully induced in vivo loss-of-function of two IHC-specific genes and one OHC-specific gene. The observed efficiency of the method 63.6-100% (Fig. 3G) is highly promising. Our results highlight the power of the CRISPR-stop method to rapidly and efficiently generate mouse deafness models.

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**Fig. 4. Patch-clamp measurement of OHCs and IHCs in prestin and otoferlin mutants, respectively, at 4 weeks of age.**

(A) Nonlinear capacitance (NLC) functions were measured in both prestin knockout and wild-type control (Ctrl) OHCs. The gray line is a representative example of a trace from a control OHC, which showed a typical nonlinear capacitance pattern. This was absent in prestin knockout OHCs (black line).

(B) Comparison of linear capacitance (Cclin) between control and prestin knockout. The ‘linear region’ used for this analysis is shown by the orange square in A. Cclin=6.75±0.23 (n=7, control); Cclin=4.01±0.26 (n=5, prestin knockout). OHCs in prestin knockout mice had a smaller Cclin, a previously reported indicator of reduced cell size. ****P<0.0001. Data are mean±s.e.m. Circles represent individual data points. Qmax=1.09±0.04; Vh=-61.5±5.5; Z=0.95±0.01. These three parameters are only applicable to control OHCs. (C) Step depolarization-evoked capacitance jump (ΔCm, upper) and Ca2+ influx (QCa, lower) in IHCs of control (black) and otoferlin knockout (red) mice. ΔCm was largely abolished in otoferlin knockout mice (**P<0.001) whereas QCa was not significantly changed (P>0.05, t-test). The data are mean±s.e.m. n.s., non-significant difference.
CRISPR-stop can lead to simultaneous inactivation of three genes

Encouraged by the high efficiency of CRISPR-stop at inactivating single genes in our pilot experiments described above, we next proceeded to determine whether we could inactivate vGlut3, otoferlin and prestin simultaneously. Control mice were derived from zygotes that were injected with GFP sgRNA and BE3 mRNA, and we confirmed the expression of vGlut3 and otoferlin in IHCs and prestin in OHCs in this control group (Fig. 5A-A‴). The same six sgRNAs (two per gene) used in the previous studies were injected together with BE3 mRNA into one-cell stage zygotes and produced triple homozygous mutant F0 mice (Fig. 5B-B‴). When compared with the dose of sgRNAs given during the individual gene mutation experiments (Fig. 3), we doubled the dose of each sgRNA in order to increase the probability of generating 100% triple knockout and decrease the incidence of mosaic and wild-type-like cases. In total, 40 zygotes were transplanted and 15 mice were born. All mice but one were found to be 100% triple knockout, showing no expression of vGlut3, prestin or otoferlin (Fig. 5B-C). One of the 15 exhibited mosaic prestin expression (inset in Fig. 5B′). To confirm that cells counterstained by Hoechst were HCs (within dotted lines in Fig. 5B‴), half of any single cochlea was stained for myosin-VI, prestin and otoferlin (Fig. S4A-B‴), while the other half was stained for parvalbumin (Pvalb), vGlut3 and prestin (Fig. S4C-D‴). This combinatorial approach permitted us to perform five-antibody staining in any single cochlea, allowing us to identify HC identity, via myosin-VI or Pvalb staining, while also being able to assess the presence of two other proteins of interest. These data also show that HCs (myosin-VI‴ or Pvalb‴) can survive regardless of the concurrent absence of vGlut3, otoferlin and prestin (Fig. S4B,D).

To further rule out the possibility that disrupting any one gene (e.g. vGlut3) could lead to the inactivation of the other two genes through pathways other than direct base editing, we stained single mutant mice with antibodies against the other two proteins (e.g. staining for otoferlin and prestin in vGlut3 mutants) (Fig. 5D-F‴).

**Fig. 5.** Simultaneous base editing of vGlut3, otoferlin and prestin. (A-B‴) Triple antibody staining against vGlut3, otoferlin and prestin in the wild-type group (A-A‴) and triple 100% knockout group (B-B‴). White dashed lines outline the OHCs; orange dashed lines outline the IHCs. The inset in B′ is one of the triple mutant cochlear samples that had mosaic prestin expression. (C) Quantification of protein expression of each gene in triple mutants. All but one of the 15 mice analyzed lost expression of vGlut3, prestin and otoferlin; the outlier exhibited mosaic prestin expression (inset in B′). (D-F‴) Triple co-staining against vGlut3, otoferlin and prestin in vGlut3 mutant (D-D‴), otoferlin mutant (E-E‴) and prestin mutant (F-F‴) groups. Mutations in either gene alone did not affect the expression pattern of the other two genes. (G) Auditory brainstem response (ABR) measurement of triple mutant F0 mice. Compared with the control wild-type group (black line), in all frequencies, triple mutants (blue line) showed significant hearing impairment. ***P<0.001, **P<0.01. Data are mean±s.e.m. Scale bars: 20 μm.
We found that disruption of any individual gene did not affect expression of the other two genes (Fig. 5D–F‴). Finally, we performed ABR measurement and vGlut3/otoferlin/prestin triple mutant mice were completely deaf (Fig. 5G). Taken together, these findings demonstrate that CRISPR-stop is effective at simultaneously disrupting vGlut3, otoferlin and prestin.

No off-target effects were observed in vGlut3, otoferlin and prestin triple mutants

To quantify on-target efficiency of base editing more precisely and also reveal any potential genome-wide off-target effects from the six injected sgRNAs (two sgRNAs per gene), we performed next-generation whole-genome sequencing (WGS) at an average depth of 20× on three triple mutant mice (numbers 1, 5 and 8) (Fig. S5A). With regards to the on-target analysis, we confirmed that the efficiency of vGlut3 and prestin sgRNAs targeted locations was 100% (all reads contained base editing) in all three mice (Fig. S5A). For the otoferlin-targeted positions, mouse number 8 base editing showed 100% efficiency. Mice 1 and 5 showed some heterogeneity in sgRNA target site base-editing efficiency: 14/18 (77.8%) and 100% at otoferlin sgRNA1 targeted position; and 100% and 15/21 (71.4%) at otoferlin sgRNA2 targeted position (Fig. S5A) for mice 1 and 5, respectively.

We next estimated off-target effects by identifying single nucleotide variations (SNVs) and insertions and deletions (indels) at these locations. After filtering variants located in genome complex regions, no SNVs or indels were detected within the predicted off-target sites, suggesting high specificity of the selected sgRNAs (Fig. S5B). Together, these results show that the silencing of three genes using two sgRNAs per gene was both efficient and specific.

In summary, we tested the CRISPR-stop approach in vivo and successfully generated five homozygous gene disruptions in F0 mice in Tyr, Atoh1, vGlut3, otoferlin and prestin. Importantly, we were also able to disrupt three genes simultaneously. The method is ideal to generate compound gene mutations; furthermore, three is unlikely to be an upper limit on the number of possible genes that can be simultaneously disrupted. As the F0 mutant mice produced homozygous mutants for one or multiple genes, laborious mouse colony breeding work can effectively be bypassed. We foresee that the CRISPR-stop method will become a game-changer for the development of high-throughput genetic screening of mice and potentially other mammals.

DISCUSSION

In this pilot in vivo study, we demonstrate, step-by-step, the powerful ability of the BE3-CRISPR-stop method to efficiently generate F0 homozygous loss-of-function mouse mutants for individual or combinations of different genes (Tyr, Atoh1, vGlut3, otoferlin and prestin). As detailed above, CRISPR-stop works by inducing targeted C-to-T or G-to-A conversion that can introduce a premature stop codon mutation, such as Duchenne muscular dystrophy, adenosine base editors (ABEs), which trigger A-to-G point mutation (3413T to C), CIB2 (368T to C), TMC1 (1543T to C), CDH23 (5663T to C) and GJB2 (269T to C) (Komor et al., 2018). For other types of mutations with a premature stop codon mutation, such as Duchenne muscular dystrophy, adenosine base editors (ABEs), which trigger A-to-G conversion, represent potential solutions (Ryu et al., 2018).

In summary, our data demonstrate that CRISPR-stop works robustly in mouse zygotes in vivo and can produce viable genetic loss-of-function studies, CRISPR-based technology is widely used for gene therapy. The CRISPR-stop method can also be potentially used for gene therapy, as exemplified in the case of TMC1, a putative mechanosensory transduction channel (MET) protein, the dominant-negative mutation of which impairs hearing (Vreugde et al., 2002). Inactivation of this dominant-negative mutation, using CRISPR paired with a sgRNA that can distinguish the wild-type from the mutant allele, results in reduced progressive hearing loss and higher HC survival rate (Gao et al., 2018). Moreover, one previous report estimated there to be 300-900 clinical relevant known human genetic diseases caused by point mutations, such as hearing impairment induced by an otoferlin point mutation (3413T to C), CIB2 (368T to C), TMC1 (1543T to C), CDH23 (5663T to C) and GJB2 (269T to C) (Komor et al., 2016). Fortunately, such T-to-C point mutation has an appropriate NNG PAM sequence nearby and can be rescued by CRISPR-stop. For point mutations without the appropriate NGG PAM sequence nearby, future x-Cas9-based CRISPR-stop may provide an alternative (Hu et al., 2018). For other types of mutations with a premature stop codon mutation, such as Duchenne muscular dystrophy, adenosine base editors (ABEs), which trigger A-to-G conversion, represent potential solutions (Ryu et al., 2018).

We also note that some gene mutations can be embryonically or perinatally lethal. Generating a mouse model with mosaic gene mutation through CRISPR-stop can potentially resolve lethality problems and avoid laborious mouse breeding when using tissue- or cell-specific Cre/LoxP-mediated conditional knockout approaches. Mosaic mouse mutants can be generated via two alternative approaches: the first approach consists of using either single sgRNA or multiple low-efficiency sgRNAs in order to achieve higher occurrence of mosaic mutations, as we described above; the second approach involves inducing base editing in only one cell of two-cell stage zygotes, instead of the usual one-cell stage zygotes. This ensures that organs will comprise cells of mixed progeny (wild type or mutant) derived from either of the two initial cells. The latter approach proved successful in a recent report where Tet3 functions were illustrated in the mosaic mutant (Wang et al., 2017). Although this is still an on-going study, we can obtain Atoh1−/−/mosaic mice that survive until at least postnatal day 7 (P7). Looking towards the future, we plan to further explore the potentials of mosaic mutant analysis to uncover yet-unknown functions of inner ear developmental genes.

Besides genetic loss-of-function studies, CRISPR-based technology is widely used for gene therapy. The CRISPR-stop method can also be potentially used for gene therapy, as exemplified in the case of TMC1, a putative mechanosensory transduction channel (MET) protein, the dominant-negative mutation of which impairs hearing (Vreugde et al., 2002). Inactivation of this dominant-negative mutation, using CRISPR paired with a sgRNA that can distinguish the wild-type from the mutant allele, results in reduced progressive hearing loss and higher HC survival rate (Gao et al., 2018). Moreover, one previous report estimated there to be 300-900 clinical relevant known human genetic diseases caused by point mutations, such as hearing impairment induced by an otoferlin point mutation (3413T to C), CIB2 (368T to C), TMC1 (1543T to C), CDH23 (5663T to C) and GJB2 (269T to C) (Komor et al., 2016). Fortunately, such T-to-C point mutation has an appropriate NNG PAM sequence nearby and can be rescued by CRISPR-stop. For point mutations without the appropriate NGG PAM sequence nearby, future x-Cas9-based CRISPR-stop may provide an alternative (Hu et al., 2018). For other types of mutations with a premature stop codon mutation, such as Duchenne muscular dystrophy, adenosine base editors (ABEs), which trigger A-to-G conversion, represent potential solutions (Ryu et al., 2018).
mutants. Single or compound F0 mutant mice can be efficiently generated, making them rapidly available for further analyses. This has the potential to significantly speed up in vivo genetic mouse screening, matching the efficiency of Droso phila screening technology. Injection of base editing complexes into neonatal cochlea for gene manipulation was recently reported (Rees et al., 2017; Yeh et al., 2018). In future, CRISPR-stop has the potential to be used to correct T-to-C point mutations in various human disease models, including deafness.

MATERIALS AND METHODS

Base editor 3 (BE3) mRNA and sgRNA synthesis

Three steps were needed for in vitro synthesis of BE3 mRNA. First, the PCR amplicon (5543 bp) was obtained by using pCMV-BE3 (Addgene, 73021) as template with forward primer (5′-3′) TCCGGCGGCCGCTTAATGACGT and reverse primer (5′-3′) CACACAGGAAACACCTAGACCATG, and KOD-plus-neo kit (TOYOBO, KOD-401), followed by purification with MinElute PCR purification kit (Qiagen, 25730). Second, the 5543 bp DNA amplicon (100 μg) was used as template for in vitro transcription with mMachine T7 Ultra kit (ThermoFisher Scientific, AM1345). Third, transcribed mRNA was further purified with MEGAclear Transcription Clean-Up Kit (Ambion, AM1908) and the concentration was adjusted to 500 ng/μl with RNase-free water (Thermo Fisher Scientific, AM9937). Finally, aliquots of BE3 mRNA (1 μl/tube) were saved and kept at −80°C.

A similar approach was used for each individual sgRNA synthesis. First, vector pX330 (Addgene, 42230) was used as template to obtain amplicon (120 bp) with KOD-plus-neo kit (TOYOBO, KOD-401). The same reverse primer (5′-3′) AAAAGACCCGACTGGTGC was used for all genes. However, the forward primer (5′-3′) was different for each gene and it was composed of three parts: TAATACGACTCACTATAGG+sgRNA (20 bp)+GGTTTTAGAGCTAGAAATAG. Only the middle part needed to be changed, depending on each specific gene. Second, the PCR amplicon, which was purified with MinElute PCR Purification Kit (Qiagen, 28004) and adjusted to 150 ng/μl, was transcribed in vitro with MEGA shortscript T7 kit (ThermoFisher Scientific, AM1354). Third, the transcribed sgRNAs were purified with MEGAclear Transcription Clean-Up Kit (Ambion, AM1908). The sgRNA concentration was adjusted with RNase-free water to 250 ng/μl (for a single gene mutant) or 1000 ng/μl (for triple gene mutants); 1 μl aliquots were dispensed and stored at −80°C. The quality of the in vitro transcribed mRNA is key for successful base editing. A clean PCR workstation (AirClean Systems, 600 model) is recommended, as well as the use of RNaseZap Decontamination Solution (ThermoFisher Scientific, AM9780).

The sgRNA optimization, mouse inner ear/tail genotyping, targeted deep sequencing and TA cloning sequencing

For each gene base editing, we chose four to seven sgRNAs and tested them individually in one-cell stage zygotes dissected out from super-ovaluted female C57BL/6 mice (4 weeks old) that were pretreated with PMSG and hCG, and crossed with C57BL/6 male mice. After injection of BE3 mRNA and sgRNAs, zygotes were cultured until blastocyte stage and DNA was extracted for rest PCR as the amount of blastocyte DNA was limited. Efficiency of the targeted base editing (C to T) was determined using Sanger DNA sequencing. All animal work conducted for this study was approved by the Institutional Animal Care and Use Committee at Institute of Neuroscience, Chinese Academy of Science and performed according to NIH guidelines.

The genotypes of gene-edited mice were determined by using PCR on genomic DNA extracted from mouse inner ears (Atoh1) or mouse tails (vGlut3, otoferlin and prestin). Both methods work well; in both cases, we found that the genotyping and phenotyping results matched perfectly. PCR primers were the same as the primers used in the sgRNA optimization step described above, with the exception that the nest PCR was no longer needed.

For Atoh1, targeted next-generation DNA-sequencing sites were amplified from genome DNA using Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech). The pair-end sequencing of PCR amplicons was completed using Illumina HiSeq X Ten platform in CloudHealth Genomics. All primers for targeted deep sequencing are described in Table S1.

For the TA cloning of Atoh1, inner ear genomic DNA from three mice with 100% knockout phenotypes was used. PCR amplicons covering sgRNA1-3 were subcloned into TA cloning vector pMD19T (Takara) for sequencing analysis. Per inner ear DNA, 20 clones in total were analyzed, and targeted base editing patterns are summarized in Fig. S2B.

Zygote injection and embryo transplantation

For the single-gene base-editing experiments, we used BE3 mRNA (100 ng/μl) and sgRNAs (50 ng/μl for each sgRNA). For triple mutant experiments, sgRNA concentration was doubled (100 ng/μl) and BE3 mRNA was kept the same. BE3 mRNA and sgRNAs were mixed well and centrifuged. Using a FemtoJet microinjector (Eppendorf) with constant flow settings, supernatant of the mixed solution was injected into the cytoplasm of fertilized eggs with well-recognized pronuclei, in a droplet of HEPE-S-CZB medium containing 5 μg/ml cytochalasin B (CB). The injected zygotes were cultured for 1.5 days (until two-cell stage) in KSOM medium with amino acids at 37°C. Finally, two-cell embryos were transferred into oviducts of pseudopregnant ICR females at 0.5 dpc. All pre-tested and promising sgRNAs used in this study are listed in Table S2.

Sample processing, histology and immunofluorescence

We followed our routine protocols described previously (Li et al., 2018; Liu et al., 2010). Briefly, after mice were perfused with 1×PBS and 4% PFA, inner ear tissue was dissected out and immediately placed into 4% PFA and kept at 4°C overnight. E18.5 mouse inner ear tissue was then washed with 1×PBS three times and then stored in 1×PBS at 4°C for cochlea dissection. Four-week-old mouse inner ear tissue was decalcified in EDTA for ~2 days and subsequently washed with 1×PBS three times.

The following primary antibodies were used: anti-myosin VI (rabbit, 1:200, 25-6791, Proteus Bioscience), anti-Sox2 (goat, 1:1000, sc-17320, Santa Cruz Biotechnology), anti-prestin (goat, 1:200, sc-22692, Santa Cruz Biotechnology), anti-vGlut3 (rabbit, 1:500, 135203, Synaptic System), anti-parvalbumin (mouse, 1:1000, P3088, Sigma) and anti-otoferlin (mouse, 1:200, ab53233, Abcam). All secondary antibodies that were compatible with different combinations of primary antibodies were purchased from Thermo Scientific. Following immunostaining, samples were embedded with Hoechst33342 solution in PBS (1:1000, 62249, ThermoScientific) to visualize cellular nuclei. Samples were mounted with Prolong gold antifade mounting medium (P36930, ThermoScientific). All immunofluorescence images were examined using either a Nikon Ni-E plus confocal microscope, a Nikon Ti-E plus confocal microscope or a Nikon C2 confocal microscope.

Whole-genome sequencing and off-target analysis

Triple mutants of vGlut3, otoferlin and prestin mouse tail DNA were sequenced at the BGI company using Illumina HiSeq X Ten and DNA sequencing. Coverage depth was over 20×. Qualified reads were mapped to the mouse reference genome (mm10) by BWA (v0.7.12) with default parameters. The BAM files were then sorted and duplicates were marked using Picard ‘MarkDuplicates’. Mutect2 was run on the aligned sequence files for variants detection. Single nucleotide variations (SNVs), and insertions and deletions (indels) were further filtered to exclude variants located in low-complexity regions, including UCSC repeat regions and microsatellite sequences. All of the mapped data are available from the Sequence Read Archive (SRA) under Accession Number SRP150150.

Potential off-target sites of sgRNAs were predicted as previously reported (Bae et al., 2014); further information can be found at www.rgenome.net/cas-offinder/. We extracted all off-target sites with no more than eight mismatches and two DNA or RNA bulges for each sgRNA.

Auditory brainstem response (ABR) measurement

The detailed protocols of ABR measurement have been described in our previous report (Li et al., 2018). For each condition, five mice (4 weeks of age) were tested at different frequencies: 4 kHz, 5 kHz, 8 kHz, 11 kHz,
16 kHz, 22 kHz, 32 kHz and 45 kHz. All frequencies were analyzed by two-way ANOVA followed by a Student’s t-test with a Bonferroni correction. Both male and female mice were used for ABR measurement.

**General patch-clamp preparation and procedure for preparing outer hair cells**

Inner ear temporal bones were excised and cochlea dissected in 1×PBS solution. Excised apical turns from the organ of Corti were used for recording. Individual OHCs were confirmed by visualizing stereocilia from their apical region under a 40× water immersion lens. Surrounding supporting cells were removed with a large tip pipette. OHCs were then approached by applying positive pressure in the pipette. Pipette pressure was released when targeted OHCs were isolated. The extracellular solutions contained 132 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, and 10 mM HEPES. Final solutions were adjusted to ~300 mOsm with D-glucose and to pH 7.2–7.3 with NaOH. The intracellular solution was the same as the extracellular solution except for the addition of 10 mM EGTA. Pipette impendence was ~5–6 MΩ.

OHCs were recorded under whole-cell patch-clamp configuration at room temperature. An axon 200B amplifier was used for data collection. Non-linear capacitance (NLC) was measured using a continuous high-resolution (2.56 ms sampling) two-sine stimulus protocol (10 mV peak at both 390.6 and 781.2 Hz) superimposed on the holding potential before and after stimulation, and 

\[
C_m = Q_{\text{max}} \frac{ze}{kT} \left(\frac{1}{1 + b}\right)^2 + C_{\text{lin}},
\]

where

\[
b = \exp\left(\frac{-ze(V_{pk} - V_{\text{h}}C_m)}{kT}\right).
\]

The following notations were used: \( Q_{\text{max}} \), maximum nonlinear charge moved; \( C_{\text{lin}} \), linear membrane capacitance; \( V_{\text{pkcm}} \) or \( V_h \), voltage at peak capacitance; \( V_m \), membrane potential; \( z \), valence; \( e \), electron charge; \( k \), Boltzmann’s constant; \( T \), absolute temperature. Averaged parameters were reported as mean±s.e.m. The t-test was used as statistical analysis to compare \( C_{\text{lin}} \) between wild-type control (Ctrl) and prestin knockout.

**Inner hair cell patch-clamp and capacitance measurements**

Apical cochlear turns were dissected out from control (Ctrl) and ofterlin knockout mice (pre-selected by tail-DNA sequencing at 4–5 weeks of age) in an external solution containing 130 mM NaCl, 2.8 mM KCl, 1.3 mM CaCl2, 1 mM MgCl2, 10 mM HEPES and 10 mM glucose (pH 7.40). Whole-cell patch-clamp recordings were performed in IHCs through a Heka EPC10/2 amplifier and the data were acquired on a PC running Heka Patchmaster. The pipette solution contained 155 mM Cs-methanesulfonate, 10 mM CsCl, 10 mM TEA-Cl, 10 mM HEPES, 2 mM EGTA, 3 mM MgATP and 0.5 mM NaGTP (pH 7.20). To increase the amplitude of Ca\(^{2+}\) currents, external Ca\(^{2+}\) was raised to 10 mM in experiments. IHCs were held at ~90 mV and excytosis was induced by depolarizing the membrane to ~10 mV. Sinewaves of 1 kHz and 70 mV peak-to-peak amplitude were superimposed on the holding potential before and after stimulation, and capacitance measurements were extracted under the Sine-DC mode in Patchmaster (Moser and Beutner, 2000). The liquid junction potential was calculated to be ~10 mV and was subtracted offline.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

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**Data availability**

All of the mapped data are available from the Sequence Read Archive (SRA) under Accession Number SRP150150.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.168906.supplemental

**References**


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.
**Table:**

<table>
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<th>Gene</th>
<th>Total # of F0 mice</th>
<th># of F0 mice analyzed</th>
<th>100% KO</th>
<th>Mosaic</th>
<th>WT-like</th>
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<td>10/12</td>
<td>1/12</td>
<td>1/12</td>
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<tr>
<td>Otoferlin</td>
<td>8*</td>
<td>7</td>
<td>7/7</td>
<td>0/7</td>
<td>0/7</td>
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<tr>
<td>Prestin</td>
<td>15**</td>
<td>11</td>
<td>7/11</td>
<td>4/11</td>
<td>0/11</td>
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</tbody>
</table>

* 1 cochlear sample was lost during immunostaining process
** 4 were used for OHC patch

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**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 μm.
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

<table>
<thead>
<tr>
<th>sgRNA Names</th>
<th>20bp sequence (5’-3’, without PAM)</th>
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<tbody>
<tr>
<td>Tyr-sgRNA</td>
<td>ACCTCAGTTCCCCTTCAAG</td>
</tr>
<tr>
<td>Atoh1-sgRNA-1</td>
<td>ACAGCCAGGGTGAGCTTGTA</td>
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