In vivo base editing of post-mitotic sensory cells

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Programmable nucleases can introduce precise changes to genomic DNA through homology-directed repair (HDR). Unfortunately, HDR is largely restricted to mitotic cells, and is typically accompanied by an excess of stochastic insertions and deletions (indels). Here we present an in vivo base editing strategy that addresses these limitations. We use nuclease-free base editing to install a S33F mutation in β-catenin that blocks β-catenin phosphorylation, impedes β-catenin degradation, and upregulates Wnt signaling. In vitro, base editing installs the S33F mutation with a 200-fold higher editing:indel ratio than HDR. In post-mitotic cells in mouse inner ear, injection of base editor protein:RNA:l lipid installs this mutation, resulting in Wnt activation that induces mitosis of cochlear supporting cells and cellular reprogramming. In contrast, injection of HDR agents does not induce Wnt upregulation. These results establish a strategy for modifying posttranslational states in signaling pathways, and an approach to precision editing in post-mitotic tissues.
**Results**

**Base editing prevents phosphorylation of Ser 33 in β-catenin.**

We hypothesized that base editing the β-catenin gene (CTNNB1 in humans) to replace an amino acid that is phosphorylated by GSK-3β with a residue that cannot be phosphorylated would impede β-catenin degradation and increase activation of T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors. Previous work suggests that mutation of β-catenin Ser 33 to Tyr, Pro, or Cys can abolish recognition by β-TrCP, preventing degradation of β-catenin. We designed a single-guide RNA (sgRNA) that when complexed with BE3, should introduce a C-to-T mutation at the DNA nucleotide encoding Ser 33 in β-catenin, resulting in the conversion of a TCT (Ser) codon to a TTG (Phe) codon (Fig. 1b). Murine S33-targeting sgRNA and human S33-targeting sgRNA share very similar protospears and both target the same codon. Since the phenylalanine side chain cannot be phosphorylated, this change should increase the cytosolic lifetime of β-catenin, increasing activation of TCF/LEF transcription factors and downstream signaling (Fig. 1a).

Next we tested if base editing is capable of installing the desired point mutation into β-catenin in cultured human cells. We transfected plasmids expressing BE3 and S33-targeting sgRNA or an unrelated control sgRNA into HEK293T cells. After a 3-day incubation, cells were harvested and the C-to-T conversion efficiency at nucleotide C8 of the β-catenin Ser 33 codon (counting the PAM as nucleotide positions 21–23) was measured by high-throughput DNA sequencing (Fig. 1b). We observed efficient mutation of the target codon from TCT to TTT (31% ± 0.9%) together with the efficient conversion of another cytosine within the base editing window, C6–T6 (28% ± 0.7%) (all efficiencies listed are mean ± S.E.M. for three biological replicates with no enrichment for transfected cells). Editing at C6 was expected given the 5-base editing window of BE3 (C4–C8). In addition, C1–T1 (1.7% ±
The resulting S33F phosphorylation-dependent manner. Base editing with BE3 precisely mutates the Ser 33 codon to instead encode Phe, which cannot be phosphorylated.

sequencing reads (with no enrichment for transfected cells) with C8 converted to T8 (resulting in the S33F mutation) was measured with high-throughput HEK293T cells were transfected with plasmids expressing BE3 and S33-targeting sgRNA, or BE3 and an unrelated sgRNA. The percentage of total sequencing (HTS).

best-performing ratio of Cas9:sgRNA:ssDNA template and BE3. Values and error bars re

per well of a 48-well plate. C-to-T conversion ef

To test if the S33F mutation in

Effect of S33F β-catenin mutation on Wnt signaling in vitro.

To test if the S33F mutation in β-catenin increases the amount of non-phosphorylated β-catenin in cells, we measured the amount of total and non-phosphorylated β-catenin using cell fractionation followed by Western blotting (Fig. 2b). We observed 6-fold greater levels of non-phosphorylated β-catenin in nuclear extracts of cells transfected with plasmids encoding BE3 and S33-targeting sgRNA compared to control cells transfected with plasmids encoding BE3 and an unrelated sgRNA (Fig. 2b and Supplementary Fig. 3a). The total amount of β-catenin, including both phosphorylated and non-phosphorylated forms, was 7-fold higher in nuclear protein extracts from cells treated with BE3 and the S33-targeting sgRNA than in control cells treated with BE3 and an unrelated-sgRNA (Fig. 2b and Supplementary Fig. 3a), consistent with stabilization and enhanced translocation of β-catenin into the nucleus.

To assay the effects of installing the β-catenin S33F mutation on Wnt signaling, we used an established Wnt reporter system in HEK293T cells. This assay requires co-transfection with three plasmids: (i) Topflash, which contains TCF/LEF operators that activate a luciferase reporter gene; (ii) a β-catenin-specific sgRNA to target exon 3 of the CTNNB1 gene; and (iii) a control sgRNA.

0.1% and C15–T15 (0.90% ± 0.05%) conversions were also observed at much lower frequencies, consistent with our previous studies. Importantly, unlike editing of the target C8 to T8, conversion of C1, C6, or C15 to T does not change the predicted amino acid sequence of the resulting protein, as all three of the other C-to-T mutations are silent.

We observed low (2.0% ± 0.3%) indel frequencies from BE3 and S33-targeting sgRNA plasmid transfection. Control samples treated with plasmids expressing BE3 and an unrelated sgRNA were also analyzed, resulting in no C-to-T mutation at the target locus above our limit of detection (~0.025% mutation; see Methods). Taken together, these observations validate a base editing strategy that converts the wild-type β-catenin gene to the S33F mutant in mammalian cells efficiently and with a high degree of product selectivity.

Correcting for a β-catenin mutation

The results from Figs. 1 and 2 were used to determine the degree of product selectivity. Treatment with BE3 and S33-targeting sgRNA resulted in a 15.5-fold increase in the ratio of S33F:indels relative to control samples treated with BE3 and an unrelated sgRNA (Fig. 2b and Supplementary Figs. 3a and 3b).

Table 1: S33F mutation efficiency % Indels

| Treatment | S33F mutation efficiency % Indels | Ratio of S33F:indels |
|------------|-----------------------------------|---------------------|
| No treatment | 5.4 ± 0.59 | 49 ± 6.8 | 0.11 |
| BE3 | 31 ± 0.88 | 2.0 ± 0.27 | 15.5 |

Fig. 1 Base editing strategy and comparison of HDR and base editing following plasmid delivery. a Schematic representation of the canonical Wnt pathway and a base editing strategy to stabilize β-catenin. In the absence of Wnt signaling, β-catenin is phosphorylated at Ser 33 by GSK-3β and degraded in a phosphorylation-dependent manner. Base editing with BE3 precisely mutates the Ser 33 codon to instead encode Phe, which cannot be phosphorylated. The resulting S33F β-catenin has an extended half-life and can activate target gene transcription by binding with TCF/LEF transcription factors. b HEK293T cells were transfected with plasmids expressing BE3 and S33-targeting sgRNA, or BE3 and an unrelated sgRNA. The percentage of total sequencing reads (with no enrichment for transfected cells) with C8 converted to T8 (resulting in the S33F mutation) was measured with high-throughput sequencing (HTS).

c Plasmid delivery of Cas9 and BE3 (750 ng) with S33-targeting sgRNA (250 ng) into HEK293T cells using 1.5 μg ssDNA. Cas9:sgRNA: 0.7 μg ssDNA, Cas9:sgRNA: 1.3 μg ssDNA, Cas9:sgRNA: 2.7 μg ssDNA, BE3:sgRNA. No treatment.

d Product selectivity analysis. Corrected mean ± S.E.M. of three biological replicates performed on separate days.
activate expression of firefly luciferase when bound by β-catenin, or Fopflash, a negative control plasmid that contains mutated TCF/LEF sites that cannot be activated by β-catenin; (ii) Renilla, which expresses renilla luciferase to enable normalization for transfection efficiency; and (iii) a β-catenin cDNA plasmid that expresses either the wild-type (Ser 33) or mutated (Phe 33) β-catenin. 3 days post-transfection, the level of TCF/LEF-mediated Wnt signaling was quantified by the luminescence ratio of the Topflash and Fopflash reporters (Fig. 2a). HEK293T cells transfected with a plasmid expressing mutated S33F β-catenin showed a time-dependent increase in the Topflash:Fopflash luminescence ratio (Supplementary Fig. 1c), reaching a maximum ratio of 180 ± 1.2 3 days post-transfection, 4.3-fold higher than the Topflash:Fopflash luminescence ratio in cells transfected with a plasmid expressing wild-type β-catenin (42 ± 0.7) (Fig. 2a). These results support a model in which mutating Ser 33 to Phe in β-catenin increases Wnt signaling activity in mammalian cells.

Next, we used base editing to install the β-catenin S33F mutation in human cells and assayed the effect on Wnt signaling. We co-transfected HEK293T cells with four plasmids: a BE3-expression plasmid, an sgRNA-expression plasmid (either targeting β-catenin Ser 33, or encoding an unrelated control sgRNA), the transfection efficiency reporter Renilla, and either the Topflash or Fopflash reporter plasmid. HEK293T cells transfected with plasmids expressing the reporters and BE3 + S33-targeting sgRNA exhibited a time-dependent increase in Topflash:Fopflash luminescence ratio (Supplementary Fig. 1b). After 3 days, cells treated with the highest dose of base editor (750 ng BE3 plasmid and 250 ng sgRNA plasmid per well of a 48-well plate) exhibited a much higher Topflash:Fopflash ratio (310 ± 31) than cells transfected with BE3 and an unrelated sgRNA (1.0 ± 0.02) (Fig. 2c). The C-to-T conversion efficiency at the target Ser 33 codon was analyzed by high-throughput DNA sequencing (Fig. 2d). At the highest BE3 dose, the C-to-T conversion efficiency at position 33 was 16 ± 1.0% (Fig. 2d). Lower doses of BE3 and sgRNA resulted in markedly lower base editing efficiency and lower Wnt signaling levels (Figs. 2c and 3d). Wnt signaling levels were strongly correlated with S33F base editing efficiency (R² = 0.97, p < 0.0001 for non-zero slope, linear regression analysis, Supplementary Fig. 1a) suggesting that treatment with BE3 and the S33-targeting sgRNA strongly enhances Wnt signaling levels in a base editing-dependent manner.

Finally, we assayed the ability of β-catenin base editing to increase the expression of known endogenous Wnt-responsive genes in HEK293T cells, including Axin-related protein (AXIN2), cyclin D1 (CCND1), cyclin dependent kinase inhibitor 1A (CDKN1A), and fibroblast growth factor 20 (FGF20). Transfection of HEK293T cells with BE3 and β-catenin S33-targeting
sgRNA resulted in 1.8 ± 0.21-fold higher expression of AXIN2, 1.7 ± 0.17-fold higher expression of CCND1, 1.7 ± 0.7-fold higher expression of CDKN1A, and 12 ± 2.2-fold higher expression of FGF20 compared with cells treated with BE3 and an unrelated sgRNA (Supplementary Fig. 3b). Taken together, these results demonstrate that treatment of cells with the β-catenin S33F base editor converts the endogenous β-catenin gene into the S33F mutant allele, substantially increases levels of non-phosphorylated β-catenin, increases expression of a reporter gene downstream of TCF/LEF operators in a base editing-dependent manner, and also increases expression of endogenous Wnt-responsive genes.

Comparison of base editing and HDR. A commonly used approach to introduce precise modifications into genomic DNA is to harness HDR using a targeted nuclease and a donor DNA template that contains the desired modification and is homologous to the target locus. We compared the outcomes of base editing to Cas9 nuclease-mediated HDR to install the S33F mutation in β-catenin in HEK293T cells. For this comparison we used the recently described CORRECT HDR method, which introduces desired mutations into target loci more efficiently than previous HDR methods.

We recently demonstrated delivery of Cas9 nuclease ribonucleoprotein (RNP) complexes to the inner ear to mediate spatially localized genome editing in vivo with enhanced DNA specificity relative to plasmid transfection. In light of these advantages, we also compared RNP delivery of base editors with RNP delivery of HDR agents. We optimized RNP delivery-mediated CORRECT HDR by treating cells with different stoichiometries of Cas9 protein, S33-targeting sgRNA, and a donor ssDNA template. Optimized cationic lipid-mediated delivery of Cas9, guide RNA, and donor DNA template resulted in an average HDR efficiency of 4.9 ± 0.8% S33F mutation using a 1:1:1:0.5 ratio of Cas9:sgRNA:ssDNA.

Comparison of efficiency and product selectivity of HDR vs. base editing following RNP delivery. RNP delivery into HEK293T cells of 200 nM Cas9 or 200 nM BE3 pre-complexed with the S33-targeting sgRNA or an unrelated sgRNA and delivered in a cationic liposome. a Frequency of S33F mutation (blue) and indels (green) from treatment with CORRECT HDR agents (Cas9, sgRNA, and ssDNA donor template) in the ratios shown. b Product selectivity ratio, defined as the ratio of S33F modification to indel modification, resulting from treatment with the CORRECT HDR agents used in a. c Comparison of target C to T conversion efficiency following RNP delivery of BE3 (blue) or CORRECT HDR (purple) and the S33-targeting sgRNA. The control corresponds to cells treated with BE3 protein and unrelated sgRNA. d Product selectivity of base editing and CORRECT HDR. Values and error bars reflect mean ± S.E.M. of three independent biological replicates performed on different days.
molar ratio of Cas9:S33-targeting sgRNA:donor ssDNA template (Fig. 3a). In contrast, the efficiency of installing the S33F mutation using BE3, the same guide RNA, and the same cationic lipid averaged 13 ± 0.3% (Fig. 3c). Consistent with the DNA transfection results above, the CORRECT HDR method was also accompanied by a much higher indel frequency than that of the base editing approach; we observed 37 ± 1% indels from CORRECT HDR, but only 0.52 ± 0.03% indels from base editing (Fig. 3b,d). Therefore, the product selectivity ratio of S33F editing: indels was 200-fold higher for base editing than for CORRECT HDR. Taken together, these results indicate that base editing either by DNA delivery or RNP delivery enables more efficient installation of the S33F mutation in β-catenin with fewer indels and thus much higher product selectivity than the CORRECT HDR method.

**Off-target analysis of β-catenin S33F base editing.** Genome editing agents can induce unintended DNA modifications at off-target genomic loci that are similar in sequence to the target locus. Recent studies have shown that off-target base editing mediated by BE3 is generally a subset of off-target loci modified by the corresponding Cas9 nuclease and guide RNA, as expected given that the DNA-binding capability of BE3 is derived from Cas9. We investigated the potential off-target genome editing by Cas9 nuclease programmed by the S33F β-catenin sgRNA used herein with two methods. First, we used GUIDE-Seq, an unbiased genome-wide method that has been extensively used to identify off-target loci in mammalian cells following Cas9sgRNA exposure (See Methods). We performed GUIDE-Seq on murine NIH/3T3 cells treated with Cas9:S33-targeting sgRNA. The on-target β-catenin locus was identified with 1108 GUIDE-Seq reads, corresponding to an on-target modification frequency of 23%. Despite robust detection of on-target modification, we observed zero GUIDE-Seq reads corresponding to off-target modification following treatment with Cas9:S33-targeting sgRNA (Supplementary Table 1), suggesting little or no Cas9-mediated off-target modification by this guide RNA in NIH/3T3 cells.

As a complementary approach, we used the Cutting Frequency Determinant (CFD) algorithm to predict off-target loci in the mouse genome associated with S33-targeting sgRNA. Following nucleofection of plasmids encoding Cas9 and S33-targeting sgRNA into NIH/3T3 cells, we performed deep sequencing to measure indel frequency at the top ten computationally predicted off-target loci (Supplementary Table 1). Consistent with the GUIDE-Seq results, we observed no detectable indel formation (<0.05%) at any of the ten predicted off-target loci (Supplementary Table 1).

These data collectively suggest that the S33-targeting sgRNA used to modify the β-catenin locus mediates few, if any, off-target editing events by Cas9 in murine cells. Since off-target base editing is typically a subset of Cas9 off-target modification for a given sgRNA, these findings suggest that the changes in β-catenin phosphorylation state and Wnt signaling activity are unlikely to arise from off-target base editing.

**In vivo base editing induces post-mitotic cell reprogramming.** Base editing relies on cellular mismatch repair machinery, which is expressed in most cells, in contrast to the cellular DSB repair and recombination machinery that mediates HDR, which is poorly expressed in non-mitotic cells. This difference raises the possibility that base editing may be effective in post-mitotic cells in vivo, even though HDR in post-mitotic cells remains a major challenge.

We previously discovered that local in vivo injection of cationic lipid reagents normally used for nucleic acid transfection could potently deliver negatively charged proteins or protein:nucleic acid complexes, including Cas9:sgRNA ribonucleoprotein (RNP) complexes, into the organ of Corti. Because activation of Wnt signaling outside the inner ear can promote oncogenesis, such a local delivery platform that minimizes exposure of other cells to the editing agent is ideally suited for in vivo base editing of β-catenin.

We tested different ratios of base editor:sgRNA:lipid in vivo. The optimal ratio combined purified BE3 (57 µM), the β-catenin S33-targeting sgRNA or an unrelated sgRNA (100 µM) and 2.0 µL cationic lipid in a total volume of 12.0 µL. After a 5-min incubation, we injected 1.0 µL of the resulting mixture into the cochlea of postnatal day 1 (P1) wild-type CD1 mice (Fig. 4a). The intracochlear injection was followed by subcutaneous injection of 5-ethyl-2′-deoxyuridine (EdU) daily throughout the 5 days to enable detection of proliferating cells. Cochlear tissues were harvested at P7 and EdU, Myo7a (a marker for cochlear hair cells), and Sox2 (a marker for supporting cells) were detected by chemical staining and immunofluorescence.

Confocal microscopy of cochlear tissue harvested from mice treated with BE3 and the β-catenin S33-targeting sgRNA revealed cells positive for both EdU and Sox2, consistent with newly divided supporting cells (Fig. 4h). The post-mitotic status of the postnatal day 7 (P7) cochlear sensory epithelium was confirmed by the lack of EdU incorporation in the organ of Corti, in contrast with EdU incorporation in mesenchymal cells (e.g., tympanic border cells), which are known to be mitotic (Supplementary Fig. 2, orange arrowhead). A cochlea treated with BE3 and the β-catenin S33-targeting sgRNA displayed multiple EdU-positive supporting cells in the apical turn (n = 3, EdU and Sox2 double-positive cells = 16 ± 5.3, Fig. 4h). We also observed EdU and Sox2 double-positive cells expressing the hair cell marker Myo7a (Fig. 4m,n, blue arrows). Indeed, all EdU and Myo7a double-positive cells observed were also Sox2-positive (Fig. 4e,h,m,n), consistent with transdifferentiation of supporting cells into hair cells. The expansion of supporting cells (EdU and Sox2 double-positive cells) was within the inner pillar cell region and likely represented Lgr5-positive cells, consistent with previous reports.

In contrast, treatment of the cochlea with optimized CORRECT HDR reagents (1:1.1:0.5 molar ratio of Cas9: sgRNA: donor ssDNA), resulted in no evidence of newly divided supporting hair cells (EdU- and Sox2-positive) or newly divided hair cells (EdU- and Myo7a-positive) (Fig. 4d,g,k,l), consistent with the inefficiency of HDR in these post-mitotic cells. A control cochlea treated with BE3 and an unrelated sgRNA also showed no newly divided supporting cells or hair cells (Fig. 4c,f,i,j). The lack of EdU-positive cells in the sensory epithelium excludes the possibility of cell division resulting from lipid-mediated BE3 protein delivery. Together, these results suggest that base editing of Ser 33 to Phe in β-catenin, in contrast with Cas9 nuclease-mediated HDR, can induce cell division and transdifferentiation of supporting cells into hair cells in post-mitotic cells in vivo. This difference can be attributed to mechanistic differences between base editing and HDR-mediated editing, as the cellular machinery that mediates HDR is inactive or poorly expressed in non-dividing cells such as the target cells in the sensory epithelium.

To visualize the location and distribution of cationic lipid-mediated protein and RNP delivery following intracochlear injection into the mouse inner ear of P1 mice, we performed analogous intracochlear injections of lipid complexes with proteins. Cre-mediated recombination in Ai9 tdTomato mice results in tdTomato fluorescence. We injected (~30) GFP–Cre complexed with lipid into these mice, and observed tdTomato fluorescence in supporting cells (Supplementary Fig. 4). We then performed injections into wild-type CD1 mice of lipid complexed...
High-throughput DNA sequencing of genomic DNA from bulk cochlear tissue of treated mice confirmed base editing of β-catenin Ser 33 to Phe in three regions of the cochlea: the organ of Corti (2.8% of total sequencing reads containing S33F β-catenin), the stria vascularis (3.0%), and the modiolus (0.7%), with low indel formation (averaging 0.4% across all tissues) (Fig. 5). We note that samples of cochlear cells from treated mice included cells that were not exposed to base editor, and thus we expect the percentage of dissected tissue containing the S33F mutation to be substantially less than the frequency of base editing observed in cultured cells, consistent with previous reports. In contrast, we observed no substantial C-to-T conversion (≤0.25%) or indels (≤0.1%) in three regions of the cochlea injected with optimized CORRECT HDR agents (Fig. 5), consistent with the known ineffectiveness of HDR in post-mitotic supporting cells. Collectively, these results confirm that base editing, in contrast to HDR, can mediate local installation of the β-catenin S33F mutation in post-mitotic sensory cells in vivo.

**Discussion**

This study establishes in vivo base editing of post-mitotic sensory cells through the local injection of a base editor RNP:lipid complex into the inner ear of mice. Here, the resulting base editing event precisely introduced a S33F mutation into β-catenin, altering its ability to be phosphorylated and decreasing its degradation rate, thereby enhancing Wnt signaling in vitro and in vivo. This single amino acid change was installed in cultured cells more efficiently and with far fewer undesired genome modifications using base editing with BE3 than using HDR with Cas9 nuclease and a donor DNA template. Our observations in vivo also reveal that base editing, but not HDR, can be used to...
effect physiological changes in the post-mitotic mammalian inner ear, consistent with the lack of dependence of base editing on homologous recombination machinery.

In contrast with the use of base editing to directly restore the sequence of a mutated gene to that of the corresponding wild-type allele, this study establishes that base editing the potential of a protein to undergo post-translational modification, in this case to block its ability to be phosphorylated and degraded, can also achieve a desired physiological outcome. Such an approach offers a potential advantage over simple gene correction in cases in which a low level of protein alteration can exert an amplified physiological effect. In this application, the effects of β-catenin S33F base editing, amplified through the ability of a persistent transcription factor to mediate multiple transcriptional events, greatly augments Wnt signaling, leading to detectable changes in cell proliferation and cell state.

Our work establishes that local delivery of base editor as an RNP complex into the cochlea enables a high degree of specificity both for the target DNA locus and for the target cells in the cochlea. These features are distinct from the delivery of diffusible small molecules, which can perturb the activity of homologous protein targets and have a greater potential to affect the homeostasis of other tissues in vivo. Another key feature of the RNP delivery approach used in this study is that precise genome alterations are made without exposing cells to exogenous agents, the observed genome modification frequency in these samples is less than the editing efficiency of treated cells. Values and error bars reflect mean ± S.E.M. of four mice injected with BE3 and four mice injected with CORRECT HDR agents. Control samples in a, b are organ of Corti from three contralateral un.injected ears.

Cloning of plasmids. The sgRNA plasmids were generated by USER cloning. Phusion U Hot Start DNA Polymerase (Thermo Fisher) was used to replace desired protosensors from the sgRNA template plasmid. The cDNA plasmids were generated by site directed mutagenesis (New England Biolabs). Primers were designed with overhang containing the desired point mutation sequence and used to amplify from a previously reported β-catenin cDNA construct. PCR products were carried out using NEB Stable Competent cells (New England Biolabs). See Note S2 for a full list of primers used in this study.

Expression and purification of BE3 protein. BE3 protein was prepared by co-expression in BL21 Star (DE3)-competent E. coli cells using a plasmid encoding the bacterial codon-optimized base editor with a His6 N-terminal purification tag. Detailed purification steps are described in our previous study and the expression plasmid is available on Addgene (Note S1). After protein expression, bacteria cells were lysed by sonication and the lysate was cleared by centrifugation. The cleared lysate was incubated with His-Pur nickel nitroacetic acid (nickel-NTA) resin. The resin was washed before bound protein was eluted with elution buffer. The resulting protein fraction was further purified on a 5 mL Hi-Trap HP SP (GE Healthcare) cation exchange column using an AktA Pure PPLC. Protein-containing fractions were concentrated using a column with a 100,000 kDa cutoff (Millipore) centrifuged at 3,000 g and the concentrated solution was sterile filtered through an 0.22 µm PVDF membrane (Millipore).

In vitro transcription of sgRNA. PCR was performed using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) and primers as listed in the Note S2 to linearize DNA fragments containing the T7 RNA polymerase promoter sequence upstream of the desired 20 bp sgRNA protosensor and the sgRNA backbone. HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) was used to transcribe sgRNA at 37 °C for 14–16 h with 1 µg of linear template per 20 µl reaction. Fluorescein-labeled sgRNA was transcribed by adding 10% v/v Fluorescein RNA Labeling Mix (Sigma Aldrich) to the transcription system. Purification of sgRNA was performed with MEGAGear Transcription Cleanup Kit (Thermo Fisher), following the manufacturer’s instructions. Purified sgRNAs (100 µM) were stored in aliquots at −80 °C.

Protein extraction and western blotting. Total proteins were extracted with radiomune precipitation assay buffer from whole cells. Cytoplasmic and nuclear protein extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher) according to the manufacturer’s instructions. The protein lysates were separated on 4–12% NuPAGE Bis-Tris gels (Invitrogen) and electrotransferred to 0.2 µm PVDF (polyvinylidene difluoride) membranes (BioRad). The membranes were probed with rabbit anti-Non-phospho β-catenin (1:1000, Cell signaling 4270), rabbit anti-β-catenin (1:2000, Sigma C2260), and mouse anti-GAPDH (1:800, Millipore MAB374) followed by horseradish peroxidase-conjugated anti-rabbit (Chemicon), or anti-mouse (Chemicon) antibodies. The blots were detected with ECL Plus Western Blotting Substrate (Thermo Fisher) (See Supplementary Fig. 5). As noted in the Millipore data sheet, GAPDH resides in both the cytosol and nucleus, where GAPDH is translocated to the nucleus when cells respond to the initial stages of apoptosis or oxidative stress.

Real-time quantitative PCR (RT-qPCR). For each biological replicate, total RNA was extracted from HEK293T and reverse-transcribed into cDNA with oligo(dT) primers (Thermo Fisher) and Superscript III reverse transcriptase (Life Technologies).
Plasmid transfection into cell lines. HEK293T cells were seeded on 48-well collagen-coated BioCoat plates (Corning) in an antibiotic-free medium. After 12 h, HEK293T cells were transfected at ~70% confluency. For BE3 or HDR-CORRECT plasmid transfection, 750 ng of editing agent plasmid and 250 ng of sgRNA plasmids, with or without ssDNA (0.7, 1.3, 2.7 µg) were transfected using 1.5 µl of Lipofectamine 2000 (Thermo Fisher) per well. For Wt activity, unless otherwise noted, 200 ng Topflash, 20 ng Renilla, 750 ng of BE and 250 ng of sgRNA expression plasmids were transfected using 1.5 µl of Lipofectamine 2000 (Thermo Fisher) per well according to the manufacturer’s protocol. Balancing pUC19 plasmid (New England Biolabs) was transfected to make constant total DNA amount across conditions. For GUIDE-seq, 500 ng of Cas9, 250 ng of sgRNA encoding plasmids, and 100 pmol dsODN were transfected into NIH/3T3 cells using LONZA 4D-Nucleofector with the EN-158 program according to the manufacturer’s protocols.

Protein transfection into cell lines. HEK293T cells were seeded on 48-well collagen-coated BioCoat plates (Corning) in 250 µl of an antibiotic-free medium. After 12 h, HEK293T cells were transfected at ~70% confluency. Base editor protein was incubated with 1.1 times molar excess of the necessary sgRNA at room temperature for 5 min. In parallel, Cas9 protein was incubated with 1.1 times molar excess of the necessary sgRNA at room temperature. The complex was then incubated with 1.5 µl Lipofectamine 2000 (Thermo Fisher) and transfected according to the manufacturer’s protocol for plasmid delivery. BE3 and Cas9 protein were added to a final concentration of 200 nM (based on a total well volume of 275 µl).

High-throughput sequencing. Genomic DNA was isolated using the Agencourt DNAAdvance Genomic DNA Isolation Kit (Beckman Coulter) according to the manufacturer’s instructions. First DNA amplification was performed by quantitative PCR with Phusion U Hot Start and SYBR Gold Nuclear Acid Stain (Thermo Fisher) using the Agencourt RapidTips (Definity Genomics). The second PCR was performed to attach Indexing Adapters (Illumina). The products were gel-purified and quantified using the KAPA Library Quantification Kit-Illumina (KAPA Biosystems). Samples were sequenced using a single-end read from 200–250 bases (depending on the amplicon size) on the MiSeq (Illumina) according to the manufacturer’s instructions.

Intracochlear delivery of ribonucleoprotein. Intracochlear delivery was performed in the post-natal day one (P1) CD1 mice or P5-TdTomato mice as described previously. Briefly, mice were anesthetized by lowering the body temperature before the surgical procedure. A postauricular incision was made near the right ear, and the bulla was lifted to expose the cochlea. BE3 protein (57.7 µM stock concentration) was pre-complexed with the sgRNA or fluorescein-sgRNA (100 µM stock concentration) in a 1:1 molar ratio and then mixed with Lipofectamine 2000 (Thermo Fisher) at a 1:1 volumetric ratio. For delivery of ~30 GFP-Cre in P5-TdTomato mice, 3 µl of 45 µM protein was mixed with 3 µl of Lipofectamine 2000. The resulting solution (1.0 µl) was injected with a glass pipette (end diameter, 5 µm) attached to a nanolitre micropump (WPI, UMP3 + Micro4 + NanoF4) at the rate of 150–200 nL min⁻¹ through the cochlear capsule into scala media at the cochlear basal turn. Controls included the contralateral side of the un.injected cochlea, pups that received BE3 protein pre-mixed with an unrelated sgRNA, or pups that received sgRNA only. After injection, the incision was closed and the mice were brought onto a heating pad to recover.

EdU incorporation for cell proliferation. Pups received 10 µl of 5-ethyl-2-deoxyuridine (EdU, 10 mg ml⁻¹) by subcutaneous injection once daily for 5 days. EdU incorporation was detected with Click-iT EdU Imaging Kit (Invitrogen) according to the manufacturer’s instructions.

Immunohistochemistry. The temporal bones of P7 pups with BE3 and EdU delivery were fixed with 4% paraformaldehyde at 4°C overnight. Cochlear tissues were either dissected into apical, mid, and basal turn for whole-mounts or embedded in optimal cutting temperature medium (OCT) for frozen serial sections with cryostat (LEICA CM3050). After EdU detection, tissues were incubated with rabbit anti-MYO7A (1:500, Proteins Biomedical 25-6790) and goat anti-Sox2 (1:100, Santa Cruz Biotechnology sc-17320) antibodies followed by secondary antibodies conjugated with Alexa Fluor 488, and 648 (Invitrogen A11055, A10042). Images were taken with a confocal microscope (Leica TCS SP8) and processed with Image J (NIH).

Tissue dissection for HTS. Three to five days after the BE3: sgRNA delivery, cochlear tissues were collected by microdissection for high-throughput sequencing. Tissues were dissected into the organ of Corti, stria vasculo and modiolus. Each tissue was further dissected into between five and ten separate pieces, and DNA extraction was performed separately for each sample, followed by high-throughput sequencing as described above. The data presented in Fig. 5 shows sequencing data resulting from the extraction of one microdissected sample from each cochlear region.

Data analysis. Sequencing reads were demultiplexed using MiSeq Reporter (Illumina). Indel frequencies were assessed using a previously described MATLAS script4, which counts indels of ±1 base occurring in a 30-base window around the BE3 nicking site. Indels were defined as detectable if there was a significant difference (Student’s two-tailed t-test, p < 0.05) between indel formation in the treated sample and untreated control. Base editing frequencies were further assessed using a previously described MATLAS script. In brief, reads which did not contain insertions or deletions were aligned to an appropriate reference sequence via the Smith-Waterman algorithm. Individual bases with an Illumina quality score less than or equal to 30 were converted to the placeholder nucleotide (N). This quality threshold results in nucleotide frequencies with an expected theoretical error rate of 1 in 1000. This ensures that reads containing both base edits and indels are not counted as successful base-edits, and only analyzes the non-indel containing population of reads. To calculate the number of edited reads as a percentage of the total number of successfully generated sequencing reads, the percentage of non-indel containing edited reads as measured from the alignment algorithm were multiplied by (1 - fraction of reads containing an indel).

Data availability. High-throughput sequencing data that support the findings of this study have been deposited in the NCBI Sequence Read Archive database under Accession number SRP136325. All other data are available upon reasonable request.

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