Supplementary Materials

Supplemental Figures and Figure Legends

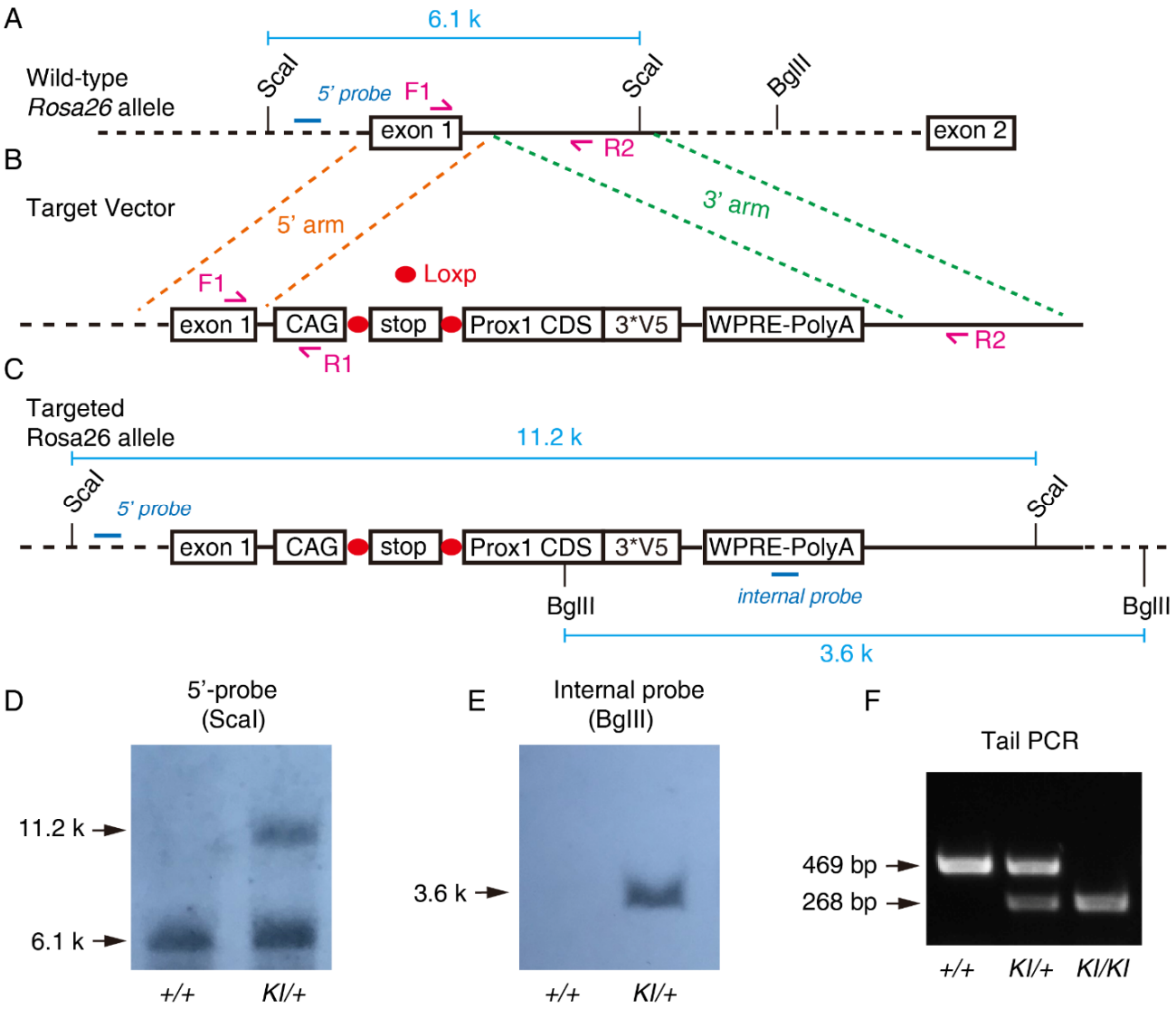

Fig. S1 Generation of *Rosa26-LSL-Prox1*/+ mouse strain. **A**–**C** Wild-type *Rosa26* locus (**A**), gene-targeting vector (**B**), and *Rosa26* locus after correct gene targeting (**C**). Prox1 is tagged with 3×V5 at its C-terminus; V5-tagged Prox1 is expressed only when the stop fragment is removed by Cre. **D**, **E** Southern blots of tail genomic DNA from F1 mice using a 5′-probe outside of the 5′-arm (**D**) and an internal probe

within WPRE sequences (**E**). **F** PCR-genotyping of tail DNA using three mixed primers, F1, R1, and R2 (locations marked in A and B). Amplicon size: knock-in (KI) allele, 268 bp; wild-type (+), 469 bp; KI/KI: homozygous; KI/+: heterozygous; +/+: wild-type.

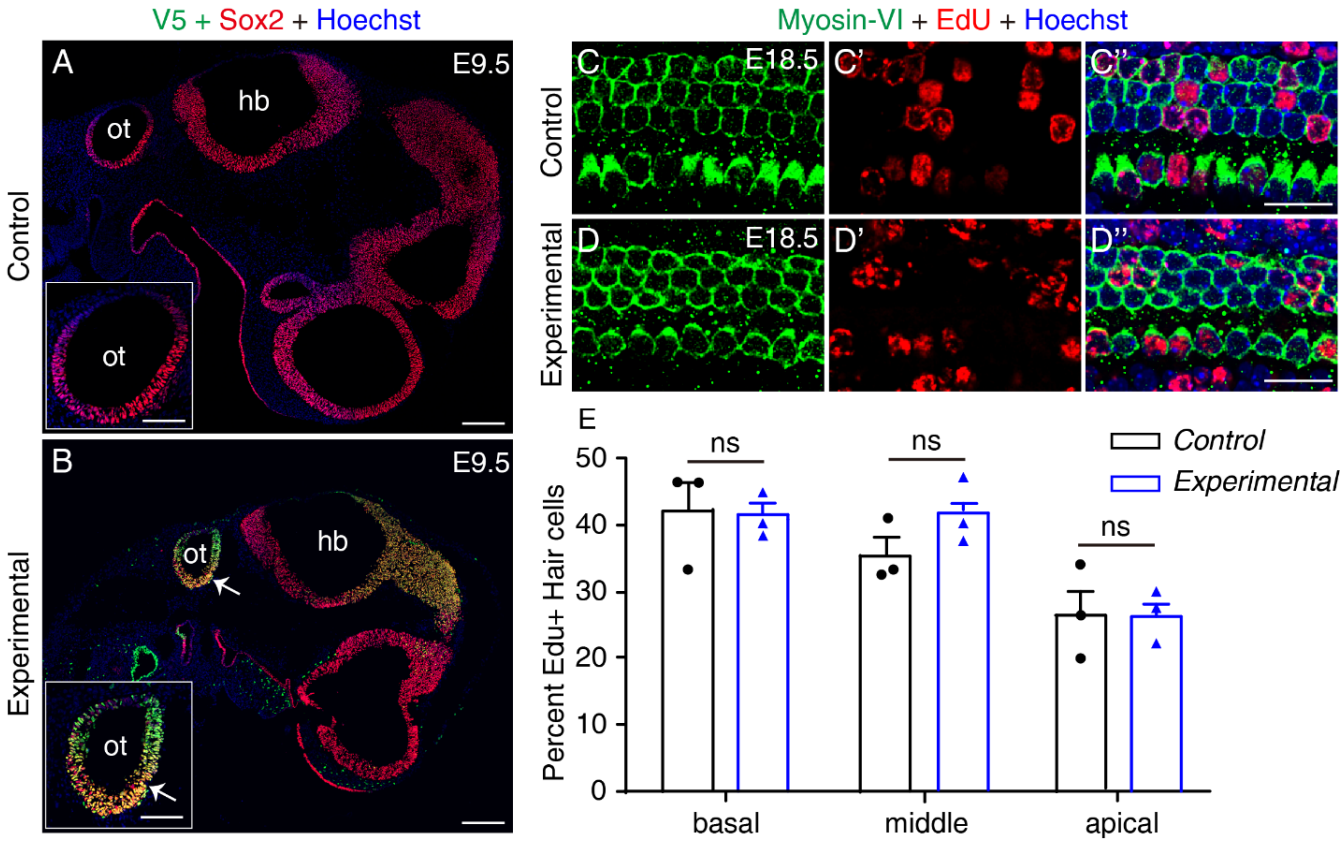

Fig. S2 Ectopic Prox1 causes a smaller otocyst but cochlear progenitor proliferative capacity is not affected. **A**, **B** Co-staining of Sox2 and V5 (Prox1) in the otocyst (ot) in a control (**A**) and a Prox1-overexpressing mouse (**B**). Ectopic V5-tagged Prox1 staining in experimental otocyst cells (Sox2+); overexpression of Prox1 leads to smaller otocysts at E9.5. **C–D"** Double staining of Myosin-VI and EdU in the cochlea in a control (**C–C"**) and a Prox1-overexpressing mouse (**D–D"**) at E18.5. **E** Percentages of EdU+ HCs in the control and experimental cochlea at E18.5. Quantification shows no significant difference between the two groups. Data are presented as means ± SEM**.** ot, otocyst; hb, hindbrain; scale bars, 200 μm (**A, B**), 100 μm (inset in A and B), 20 μm (C–D").

Schematic diagram of CUT&RUN in control group

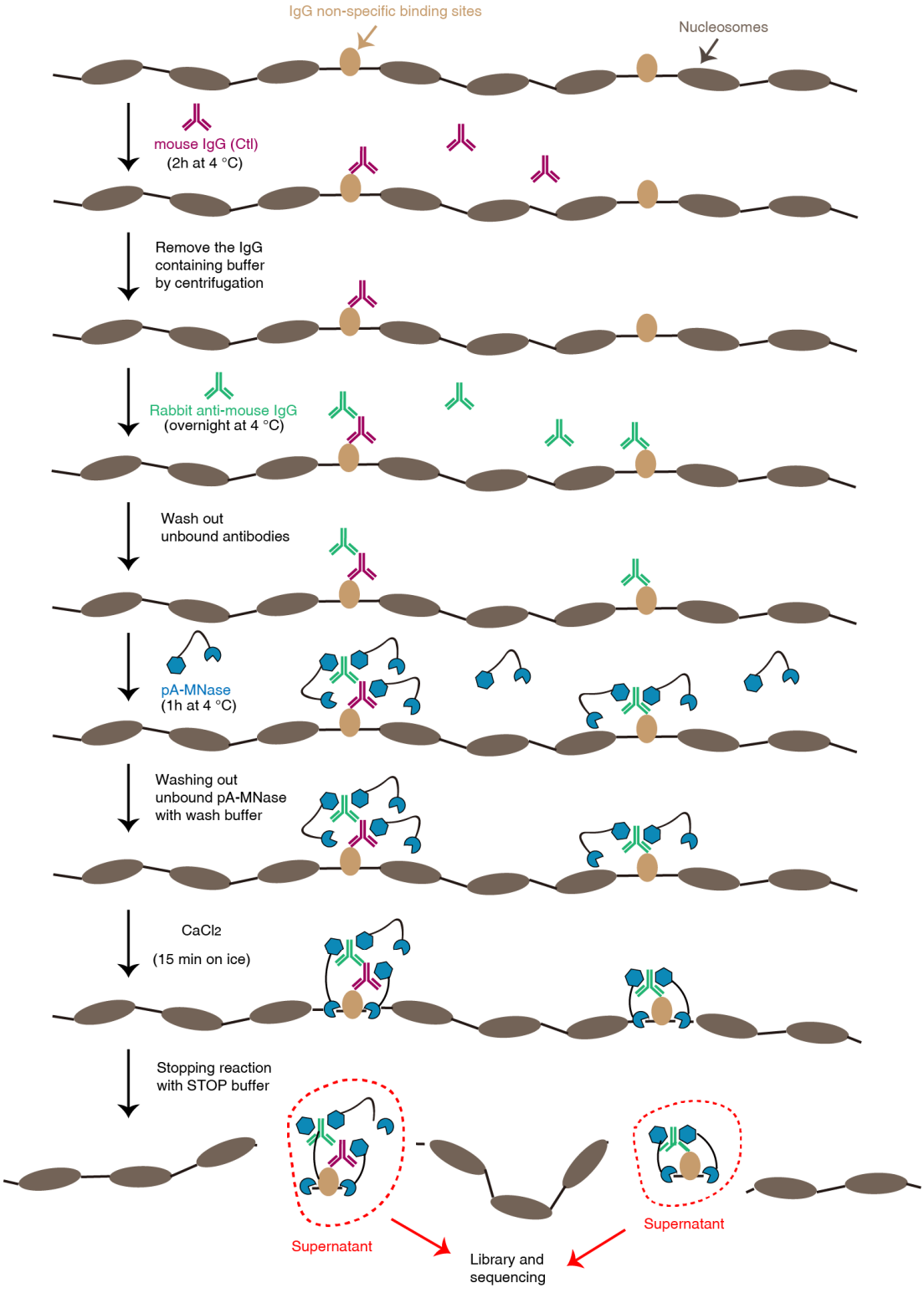

Fig. S3 Cartoon illustrates the detailed steps of CUT&RUN in the control group. Mouse IgG is used as the first and rabbit anti-mouse IgG as the secondary antibodies. Both non-specific binding sites of mouse and rabbit IgGs are included. The red dotted circles represent the DNA supernatant subjected to library construction and sequencing.

Materials and Methods

CUT&RUN Analysis

CUT&RUN experiments were conducted as described [1] with these modifications. Fresh cochlear tissue dissected from P1 mice was directly used in the experiments without capturing cells using ConA beads, and between successive steps, the tissue was separated from the buffer by centrifugation (5 min, 4°C, 100 × *g*). Samples were incubated with rabbit anti-Prox1 antibody (1:100; Cat# AB5475, Merck) for 2 h at 4°C in antibody-incubation buffer (20 mmol/L HEPES-NaOH, pH 7.5, 150 mmol/L NaCl, 0.5 mmol/L spermidine, 0.02% digitonin, 2 mmol/L EDTA, 1 × protease-inhibitor cocktail). For negative control samples, cochlear tissue samples were incubated with control IgG (mouse IgG, 1:100, sc-2025, Santa Cruz Biotechnology) for 2 h at 4°C in antibody-incubation buffer. The IgG-containing buffer was removed by centrifugation. Then rabbit anti-mouse IgG (1:100, A11059, Invitrogen) in wash buffer was added with additional overnight incubation at 4°C for efficient non-specific binding produced by IgG itself other than Prox1 antibody (the detailed steps are illustrated in Fig. S3). Unbound antibodies were washed out with wash buffer (20 mmol/L HEPES-NaOH, pH 7.5, 150 mmol/L NaCl, 0.5 mmol/L spermidine, 0.02% digitonin, 1 × protease-inhibitor cocktail). For each sample, pA-MNase was added to a final concentration of 500 ng/mL in wash buffer with 1 h incubation at 4°C. Subsequently, unbound pA-MNase was washed off with wash buffer, and after activating pA-MNase by adding $CaCl_2$ to a final concentration of 2 mmol/L, the samples were incubated on ice for 15 min. The reaction was stopped by adding 1/10 volume of 10× STOP buffer (1.7 mol/L NaCl, 100 mmol/L EDTA, 20 mmol/L EGTA, 0.02% digitonin, 250 μg/mL

glycogen, 1× protease-inhibitor cocktail).

The chromatin fragments released into the supernatant were extracted using phenol-chloroform and then ethanol-precipitated to collect the DNA fragments bound by Prox1. The NEBNext Ultra II DNA Library Prep Kit for Illumina (Cat# E7645S, New England Biolabs) was used for library construction as per the manufacturer's instructions, and then SPRI beads were used to perform size selection to enrich <300-bp libraries. Libraries were quantified using a Qubit dsDNA HS Assay Kit (Cat# Q32854, Thermo Scientific), quality-controlled using a Fragment Analyzer instrument (Agilent), and sequenced on an Illumina platform using the paired-end 150-bp mode. pA-MNase was kindly provided by Dr. Steve Henikoff [1]. The data generated in this study are available in the Gene Expression Omnibus under accession number GSE146191.

Bioinformatics Analysis of Sequencing Data

The raw sequencing reads were trimmed using fastp with default parameters to remove low-quality bases and adapters in paired-end reads [2]. The remaining reads were aligned to the mouse reference genome mm10 using Bowtie2 (version 2.3.4.1) with default parameters [3]. All unmapped reads, low mapping-quality reads (MAPQ <30), non-uniquely mapped reads, and PCR duplicates were removed by Samtools with parameter -F 1804 [4] and Picard (http://broadinstitute.github.io/picard/). For visualization in Integrative Genomics Viewer [5], read counts were normalized using bamCoverage in the Deeptools2 suite to compute the numbers of reads per kilobase per million reads sequenced on a 50-bp window [6]. MACS2 (v2.1.2) was used to call Prox1 CUT&RUN peaks from the BAM file using the narrowPeak setting and a p-value cutoff of 0.05 [7]. Peak location analysis of known mouse genes was performed using ChipSeeker [8] and the TxDb.Mmusculus.UCSC.mm10.knownGene database of murine transcripts available at Bioconductor. Peak-gene associations were analyzed using ChipSeeker, including closest genes from the Distal Intergenic category. Enrichment analysis for Gene Ontology (GO) among peak-

associated genes was performed using Metascape with default parameters [9].

Generation of the Conditional Prox1-overexpressing Mouse Model

The *Rosa26-CAG-Loxp-stop-Loxp-Prox1.3*V5/+* (*Rosa26-LSL-Prox1/+*) mouse was generated through CRISPR/Cas9-mediated gene targeting in mouse zygotes. The cDNA sequence of *Prox1* (gene ID: 19130) is CCDS35822.1 in the NCBI CCDS database. Donor vector DNA (Fig. S1B) was injected together with Cas9 and sgRNA (*5′-AAGGCCGCACCCTTCTCCGG-3′*) into C57BL/6 mouse zygotes. The positive F0 mice were crossed with wild-type C57BL/6 mice for germ-line transmission and generation of stable F1 mice, which were further screened using junction-PCR and Southern blotting analysis (Fig. S1D and E). The Southern blotting results confirmed the lack of random insertion of donor DNA into the mouse genome. Southern blotting was performed according to our previously described protocol [10].

For routine genotype analysis of F2 or later mouse progeny, PCR was performed on mouse-tail DNA with the following three primers (used concurrently) (Fig. 1): F1: 5′-*AGTCGCTCTGAGTTGTTATCAG*-3′; R1: 5′-*AGTCCCTATTGGCGTTACTATGG*-3′; R2: 5′-*TGAGCATGTCTTTAATCTACCTCGATG*-3′. The amplicon derived from primers F1 and R2 was expected to be 469 bp in the wild-type and 6999 bp in the knock-in (KI) allele; 6999 bp was more than the length that could be amplified using our PCR protocol, but the amplicon derived from primers F1 and R1 was expected to be present only in the KI allele at a length of 268 bp. The following PCR protocol was used: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s, and then 72°C for 10 min. *Pax2-Cre+* mice (010569-UNC) were obtained from the Mutant Mouse Resource & Research Centers (https://www.mmrrc.org/).

Sample Processing, Histology, Immunofluorescence, and EdU Labeling

Following our previous strategy [11], we used routine *in vitro* fertilization to ensure precise embryonic ages and eliminate potential false-negative vaginal-plug checks. E0.5 was defined as the time at which

embryos were transplanted into a pseudopregnant mother. Inner ear samples were dissected out and fixed in 4% PFA at 4°C for 4 h (E9.5 embryos) or overnight (E16.5 and E18.5 inner ears), washed three times with 1× PBS, and then labeled with these primary antibodies: anti-V5 (mouse, 1:500; MCA1360, Bio-Rad), anti-Sox2 (goat, 1:1000; sc-17320, Santa Cruz Biotechnology), anti-Myosin-VI (rabbit, 1:200; 25-6791, Proteus Bioscience), and anti-Prox1 (rabbit, 1:500; AB5475, Millipore). Lastly, samples were counterstained with Hoechst 33342 (1:1000; 62249, Thermo Scientific) in 1× PBS to visualize nuclei and were mounted with Prolong gold anti-fade mounting medium (P36930, Thermo Scientific). All images were captured using a Nikon NiE-A1 Plus or Nikon C2 confocal microscope and analyzed using ImageJ. Detailed protocols for inner ear histology are described in our previous report [12]. For whole-mount analysis, the cochlea was dissected and divided into basal, middle, and apical turns. Cochlear length was measured by drawing lines between the inner and outer hair cells, and Student's *t* test was used to compare differences in cochlear length. Edu labeling was performed using the Click-iT Edu labeling kit (Invitrogen, C10338) and Edu was injected intraperitoneally into pregnant mice at E12.5 at 10 μg/g body weight.

References

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