Title

Reprogramming by Drug-like Molecules Leads to Regeneration of Cochlear Hair Cell-Like Cells in Adult Mice

Authors

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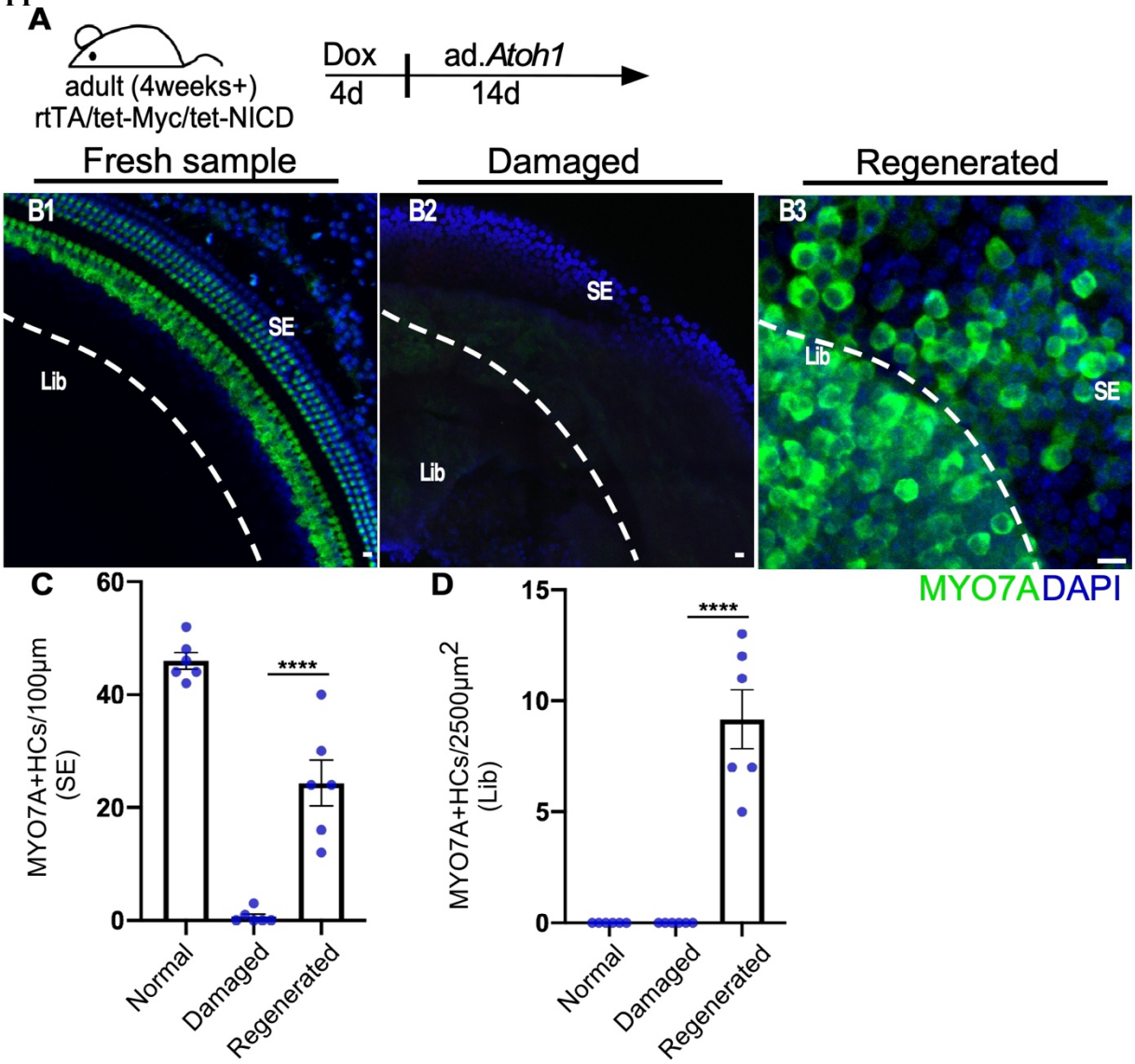
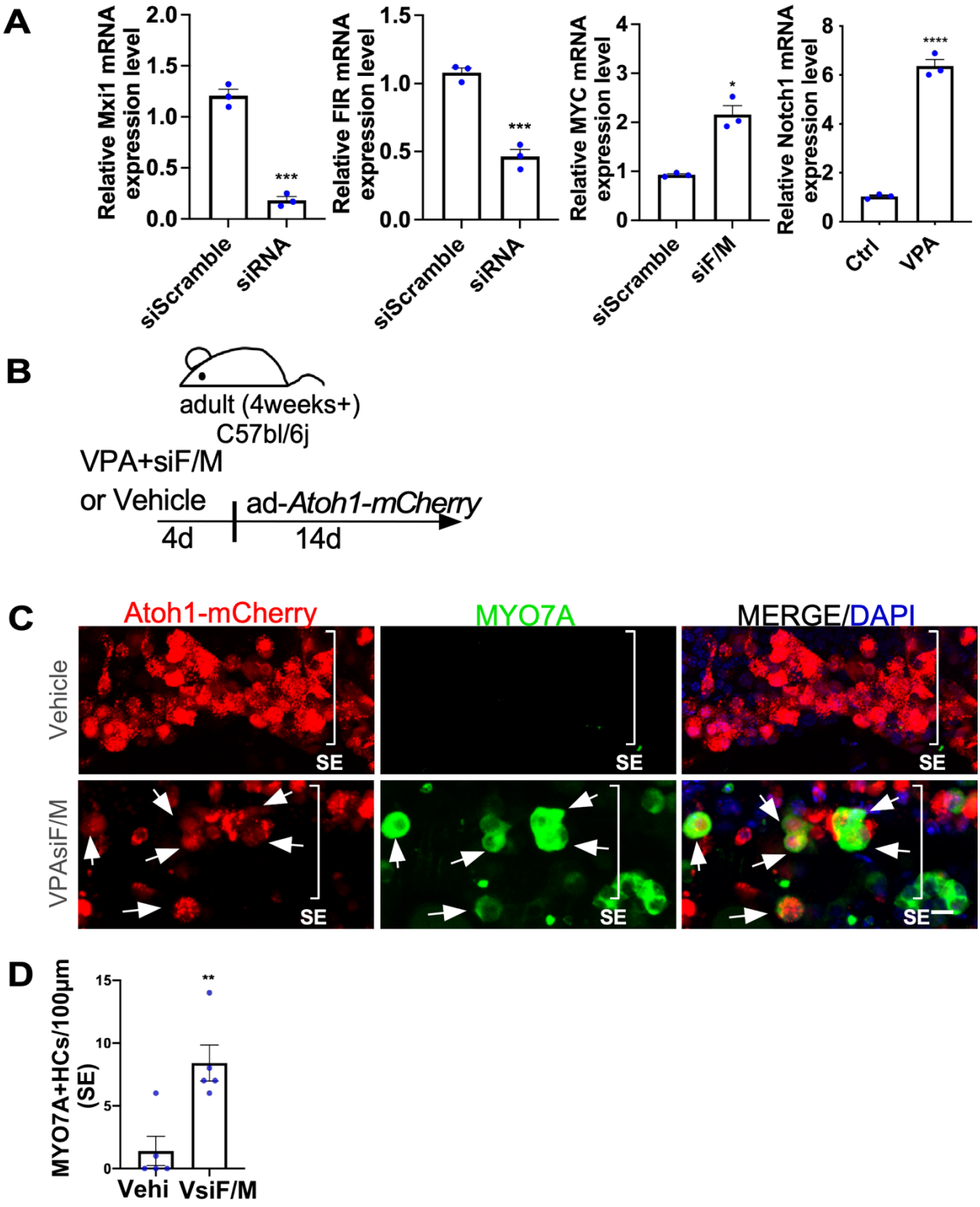
Supplemental information



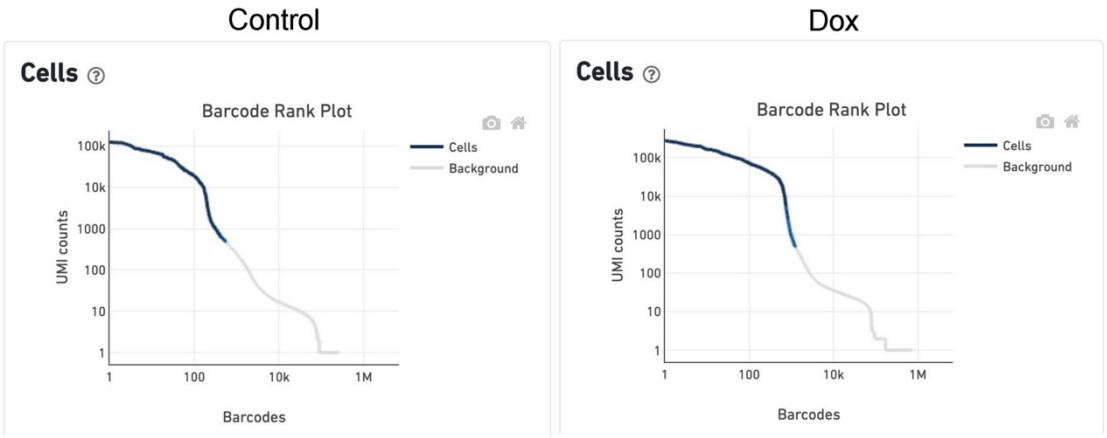
Supplemental Figure 1. Efficient HC regeneration by Myc/NICD reprogramming in adult transgenic rtTA/tet-*Myc*/tet-*NICD* cochlea *in vitro*. A. A schematic diagram illustrating the experimental procedure of transient *Myc/NICD* induction by Dox and HC regeneration induced by Ad.*Atoh1 in vitro*. **B1-3.** Freshly dissected (Fresh sample), cultured untreated (Damaged), and cultured Dox/Ad.*Atoh1* treated (Regenerated) adult (P30) rtTA/tet-*Myc*/tet-*NICD* mouse cochlea samples *in vitro*. HCs were regenerated in the Dox/Ad.*Atoh1* treated sample. **C.** Quantification and comparison of regenerated HC-like cells ($MYO7A^+$) in the sensory region (SE) and the limbus region (Lib) of the apical turn of the cultured cochlea. ****$p < 0.0001$, two-tailed unpaired Student's t-test. Error bar, mean ± SEM; n=5 for each group. Source data are provided as a Source Data file. SE: Sensory region; Lib: Limbus region. D: Dox. Scale bars: 10 μm.



Supplemental Figure 2. VPA/siFir/siMxi1 reprograms WT adult cochlear cells to regenerate hair cells *in vitro*. A. In cultured WT adult cochlea treated with different siRNAs, qPCR showed that the *Fir* siRNA reduced *Fir,* the *Mxi1* siRNA reduced *Mxi1* and *Fir/Mxi1* siRNAs upregulated *Myc;* VPA upregulated *Notch1*. **B.** A schematic diagram illustrating the experimental procedure

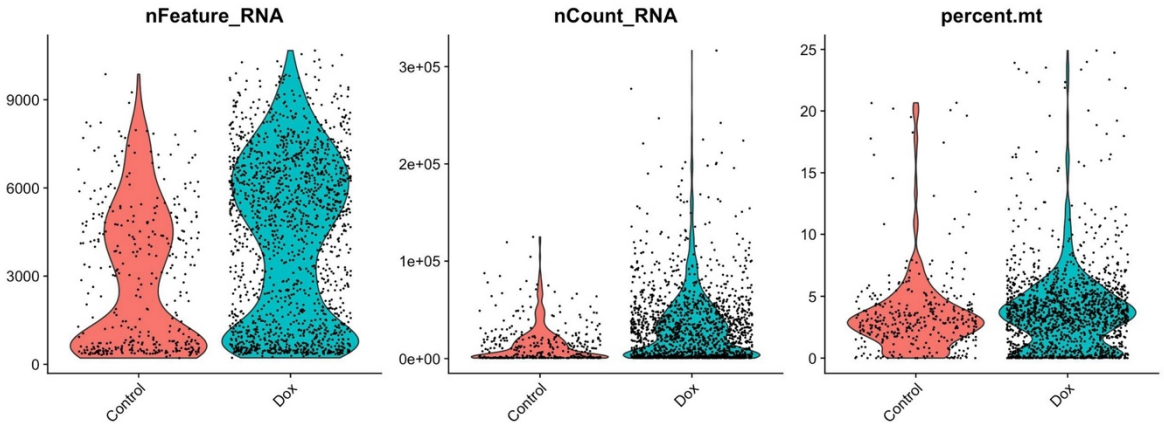
of *Notch1* induction by VPA and *MYC* induction by siFIR/siMxi1 in WT mice, followed by Ad.*Atoh1.mCherry in vitro*. **C.** Upon VPA/siFM- or sterile water (Vehicle)-treatment followed by Ad.*Atoh1.mCherry* infection in cultured adult (P30) WT mouse cochlea, HCs were regenerated only in the VPA/siFM/Ad-*Atoh1*-mCherry treated samples shown by $MYO7A^{+}/mCherry^{+}$ by labeling (arrows) but not in the Vehicle/Ad.*Atoh1.mCherry* treated samples. **D.** Quantification and comparison of regenerated HCs in the apical turn of the cultured cochleae between VsiFsiM/Ad.*Atoh1* treated or Vehicle/Ad.*Atoh1* treated groups. *$p < 0.05$, Student's t-test. Error bar, mean ± SEM; n=5 for each group. Source data are provided as a Source Data file. SE: Sensory region; Lib: Limbus region. V: VPA; siF: siFIR; siM: siMxi1. N=5 in each group. Scale bars: 10 μm.

A



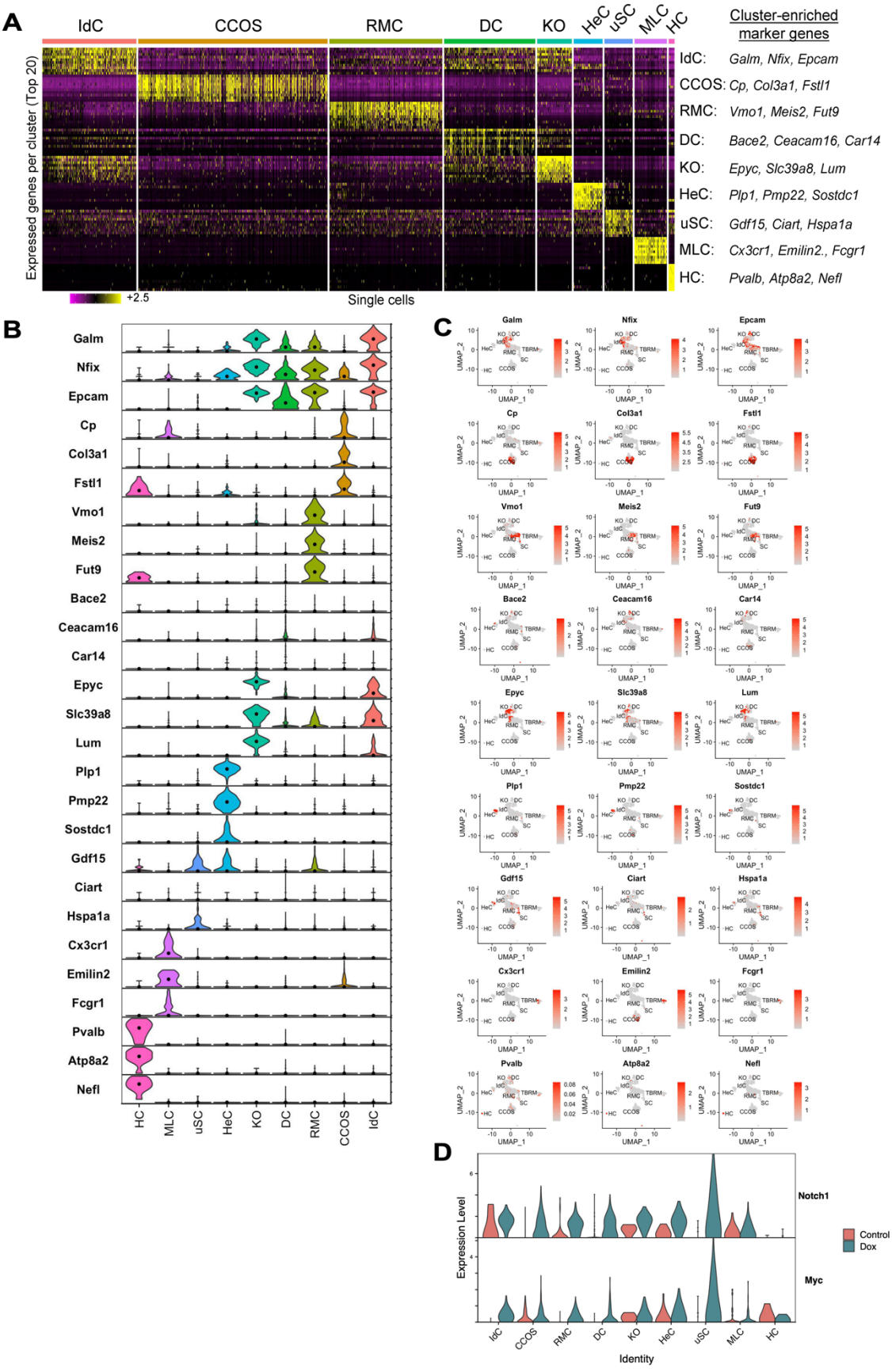
	CONTROL	DOX
Sequencing		
Number of Reads	154,757,202	168,573,104
Sequencing Saturation	91.0%	76.2%
Valid Barcodes	96.6%	96.9%
Mapping (against GRCm38/mm10-v3.0.0)		
Reads Mapped Confidentially to Genome	87.1%	89.2%
Reads Mapped Confidentially to Transcriptome	49.9%	60.4%
Cells		
Estimated Number of Cells	512	1,883
Fraction Reads in Cells	86.0%	89.9%
Mean Reads per Cell	302,260	174,687
Median Genes per Cell	700	3,574
Total Genes Detected	17,558	19,245
Median UMI Counts per Cell	1,420	15,510

B

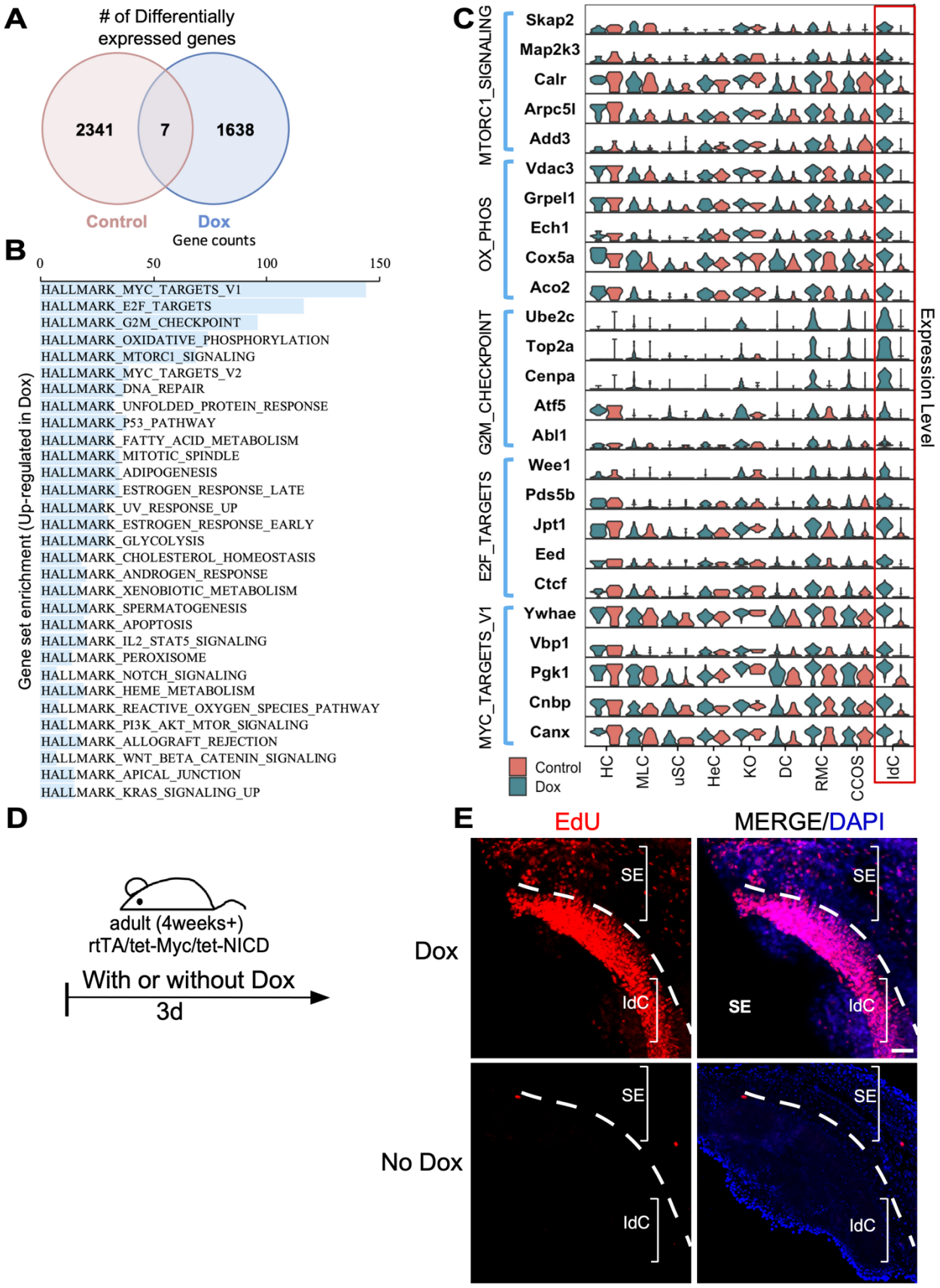


Supplemental Figure 3. Cell Ranger metrics and filtering criteria for single-cell RNA sequencing datasets. A. Knee plots were generated with the Cell Ranger analysis based on the number of UMIs/barcode (cell) and quality control parameters for single cell libraries prepared with the 10X Genomics platform. A similar number of genes were detected in the Dox treated and control groups. **B.** Number of genes (nFeature_RNA), UMIs (nCount_RNA) and percentage of

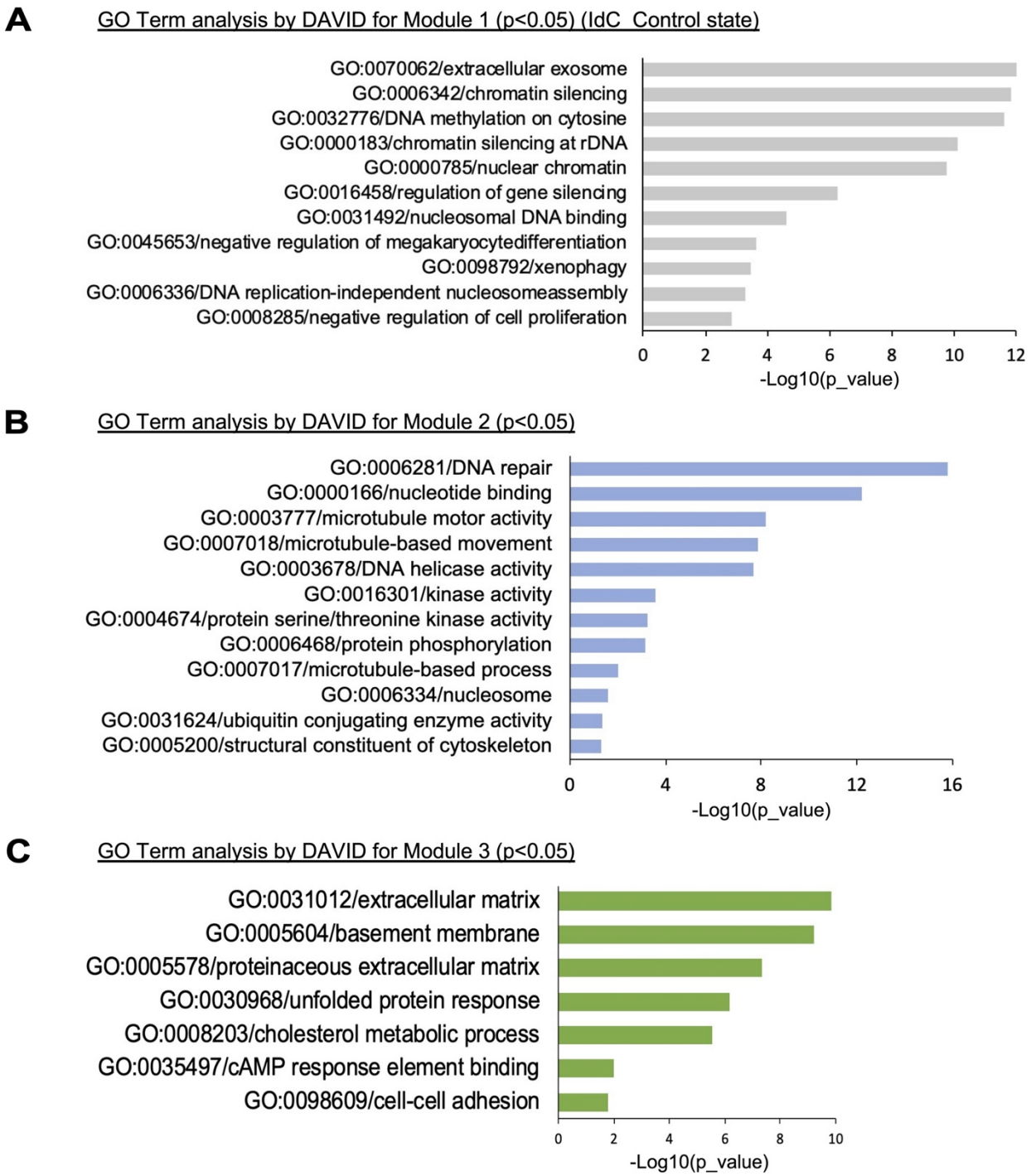
mitochondrial gene reads per cell across libraries after filtering by Seurat package that were used for downstream analyses. Each dot represents a single cell.


A
IdC
CCOS
RMC
DC
KO
HeC
uSC
MLC
HC
Cluster-enriched marker genes
IdC: Galm, Nfix, Epcam
CCOS: Cp, Col3a1, Fstl1
RMC: Vmo1, Meis2, Fut9
DC: Bace2, Ceacam16, Car14
KO: Epyc, Slc39a8, Lum
HeC: Plp1, Pmp22, Sostdc1
uSC: Gdf15, Ciart, Hspa1a
MLC: Cx3cr1, Emilin2., Fcgr1
HC: Pvalb, Atp8a2, Nefl
Expressed genes per cluster (Top 20)
+2.5
Single cells
B
Galm
Nfix
Epcam
Cp
Col3a1
Fstl1
Vmo1
Meis2
Fut9
Bace2
Ceacam16
Car14
Epyc
Slc39a8
Lum
Plp1
Pmp22
Sostdc1
Gdf15
Ciart
Hspa1a
Cx3cr1
Emilin2
Fcgr1
Pvalb
Atp8a2
Nefl
HC
MLC
uSC
HeC
KO
DC
RMC
CCOS
IdC
C
UMAP_1
UMAP_2
D
Notch1
Myc
Control
Dox
Expression Level
Identity

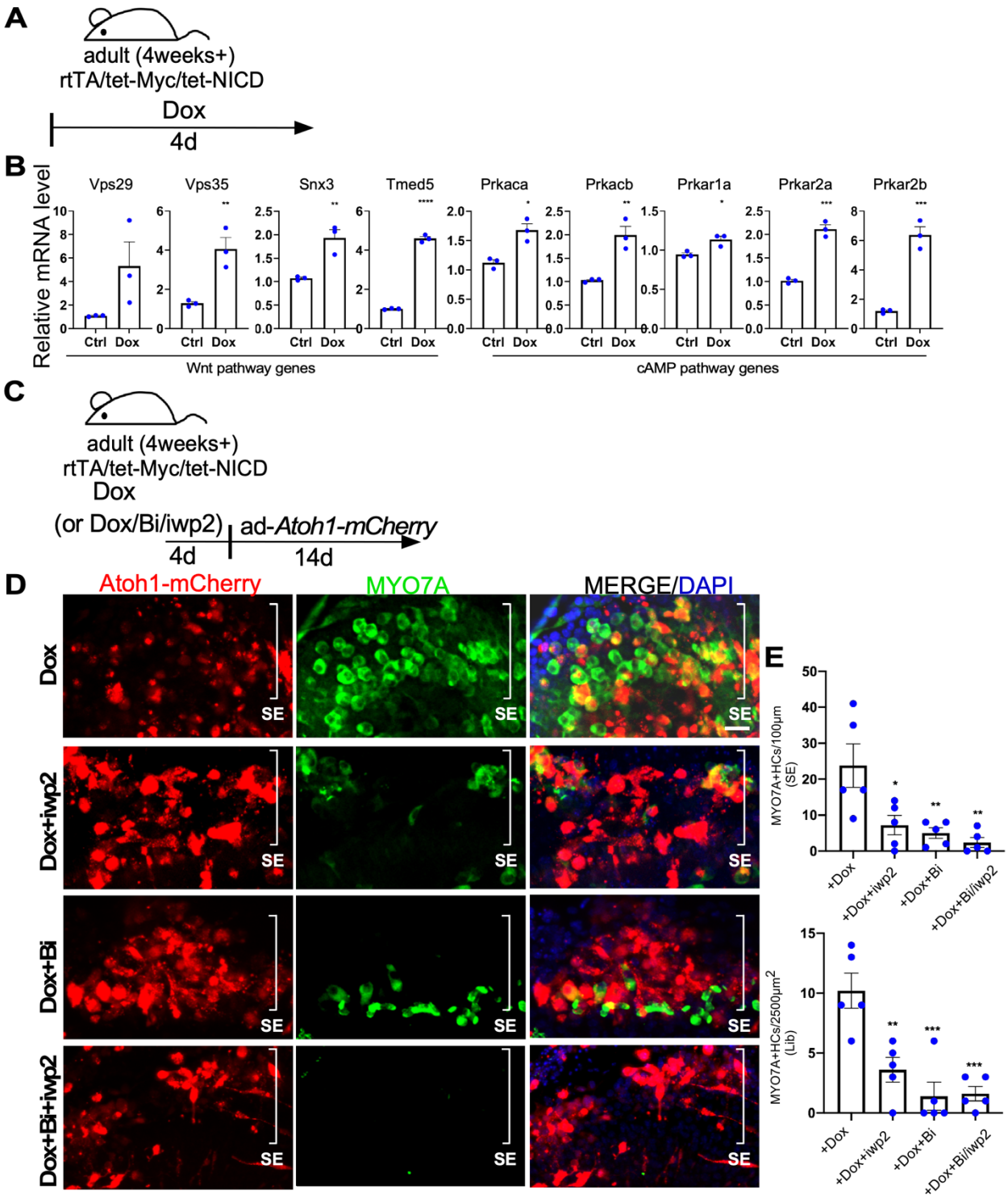
Supplemental Figure 4. Identities of cochlear cell types based on scRNA seq analysis. A. A heatmap highlighting key marker genes that were used to infer putative identities compared to all other cell-type clusters. The rows correspond to the top 20 genes most selectively upregulated in individual clusters ($p < 0.01$, LogFC > 0.25), and the columns show individual cells that were grouped into distinct clusters. Color scale represents log-transformed and normalized counts scaled to a maximum of 2.5 per row. The assignment cell-type cluster is indicated by a color bar at the top of the heatmap. **B.** Violin plot showing the distribution of normalized and log-transformed expression levels of representative cell-type-enriched marker genes across cell types. Points correspond to the median expression level. **C.** Expression levels of representative markers for each cell type are plotted onto the UMAP plot. Color key from gray to red indicates relative expression levels from low to high. **D.** Notch1 and Myc co-activations across all cell clusters.


A
# of Differentially expressed genes
2341
7
1638
Control
Dox
B
Gene counts
0
50
100
150
Gene set enrichment (Up-regulated in Dox)
HALLMARK_MYC_TARGETS_V1
HALLMARK_E2F_TARGETS
HALLMARK_G2M_CHECKPOINT
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_MTORC1_SIGNALING
HALLMARK_MYC_TARGETS_V2
HALLMARK_DNA_REPAIR
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_P53_PATHWAY
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_MITOTIC_SPINDLE
HALLMARK_ADIPOGENESIS
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_UV_RESPONSE_UP
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_GLYCOLYSIS
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_SPERMATOGENESIS
HALLMARK_APOPTOSIS
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_PEROXISOME
HALLMARK_NOTCH_SIGNALING
HALLMARK_HEME_METABOLISM
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_APICAL_JUNCTION
HALLMARK_KRAS_SIGNALING_UP
C
MTORC1_SIGNALING
Skap2
Map2k3
Calr
Arpc5l
Add3
OX_PHOS
Vdac3
Grpel1
Ech1
Cox5a
Aco2
G2M_CHECKPOINT
Ube2c
Top2a
Cenpa
Atf5
Abl1
E2F_TARGETS
Wee1
Pds5b
Jpt1
Eed
Ctcf
MYC_TARGETS_V1
Ywhae
Vbp1
Pgk1
Cnbp
Canx
Expression Level
Control
Dox
HC
MLC
uSC
HeC
KO
DC
RMC
CCOS
IdC
D
adult (4weeks+)
rtTA/tet-Myc/tet-NICD
With or without Dox
3d
E
EdU
MERGE/DAPI
Dox
No Dox
SE
IdC

Supplemental Figure 5. Gene set enrichment analysis for MSigDB Hallmark gene set collection in Dox-treated samples. A. A Venn diagram depicting global patterns of differentially expressed genes across each sample reveals genes that are highly enriched in control or Dox-treated samples (MAST analysis, FDR adjusted p value <0.01). Only seven genes were found to be expressed in both samples. **B.** Top enriched GO terms for the 1638 up-regulated genes (Dox vs. Control) (FDR adjusted p value ≤ 0.01). Numbers indicate counts of genes in each GO term that were measured by MSigDB including the MYC target genes being the most highly enriched. **C.** Violin plot depicting differential expression profiles of the genes across cell clusters from each sample that are represented by the top 5 Hallmark gene sets. The red box demarcates the interdental cell (IdC) cluster showing a heightened sensitivity to Dox-induced MYC/NICD activation than the control. **D.** A schematic diagram of the experimental procedure of cochlea samples treated with Dox *in vitro*. **E.** In the Dox treated WT adult (P30) mouse cochlea, high proliferation activity (EdU^+) was detected in the interdental cells (IdC) of the limbus region, whereas relatively lower proliferation was seen in other cochlear regions. In the cochlea without Dox treatment, virtually no EdU^+ cells were detected. IdCs: interdental cells; SE: Sensory region. The dashed line demarcates the SE from Lib regions.; Scale bars: 100 μm.

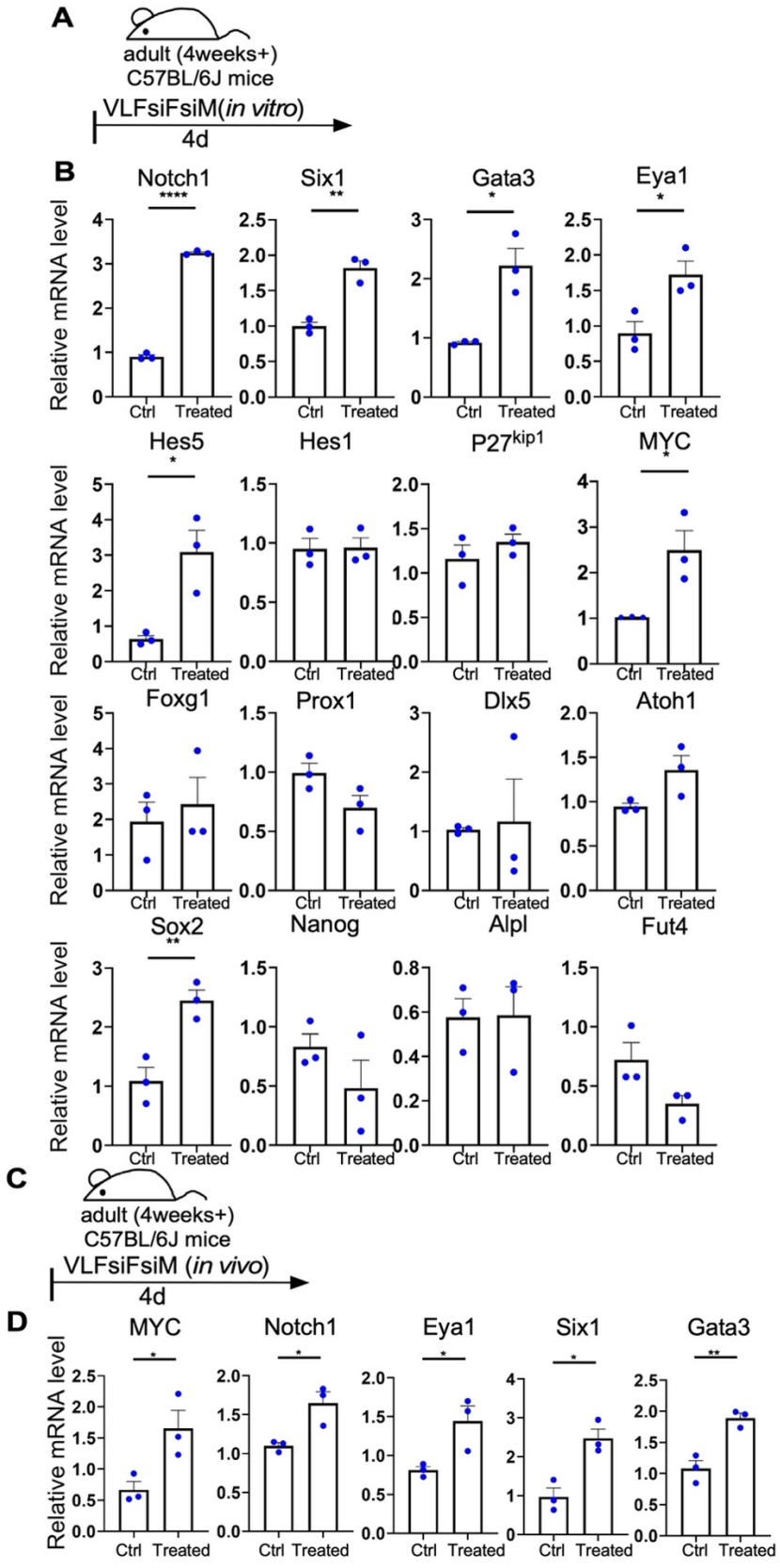


Supplemental Figure 6. Enriched GO categories by the genes with significant expression change over pseudotime of Module 1 (21 genes), Module 2 (77 genes) and Module 3 (123 genes). GO terms of each Module's genes were assessed by DAVID. The GO terms with p value < 0.05 were considered significantly enriched by differentially expressed genes.

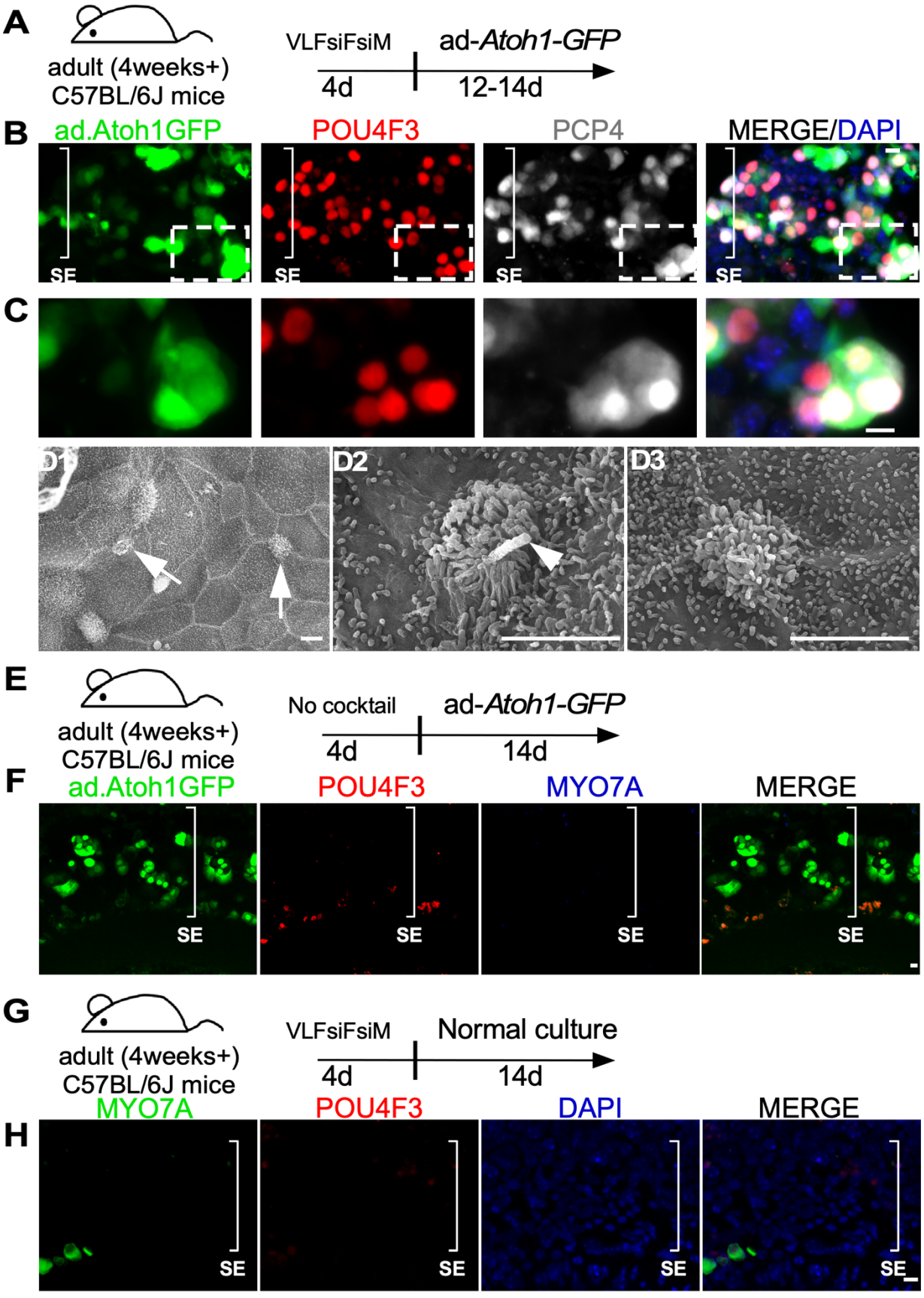


Supplemental Figure 7. Wnt and cAMP are necessary for Myc/NICD mediated reprogramming. A. A schematic diagram to illustrate the experimental design. **B.** By qRT-PCR, Wnt/Adenylyl cyclase-pathway genes were upregulated in cultured adult rtTA/tet-*Myc*/tet-*NICD* mouse cochleae fours days after Dox treatment compared to untreated control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-tailed unpaired Student's t-test. Error bar, mean ± SEM, n=3. n is the number of biologically independent samples. **C.** A schematic diagram to show the

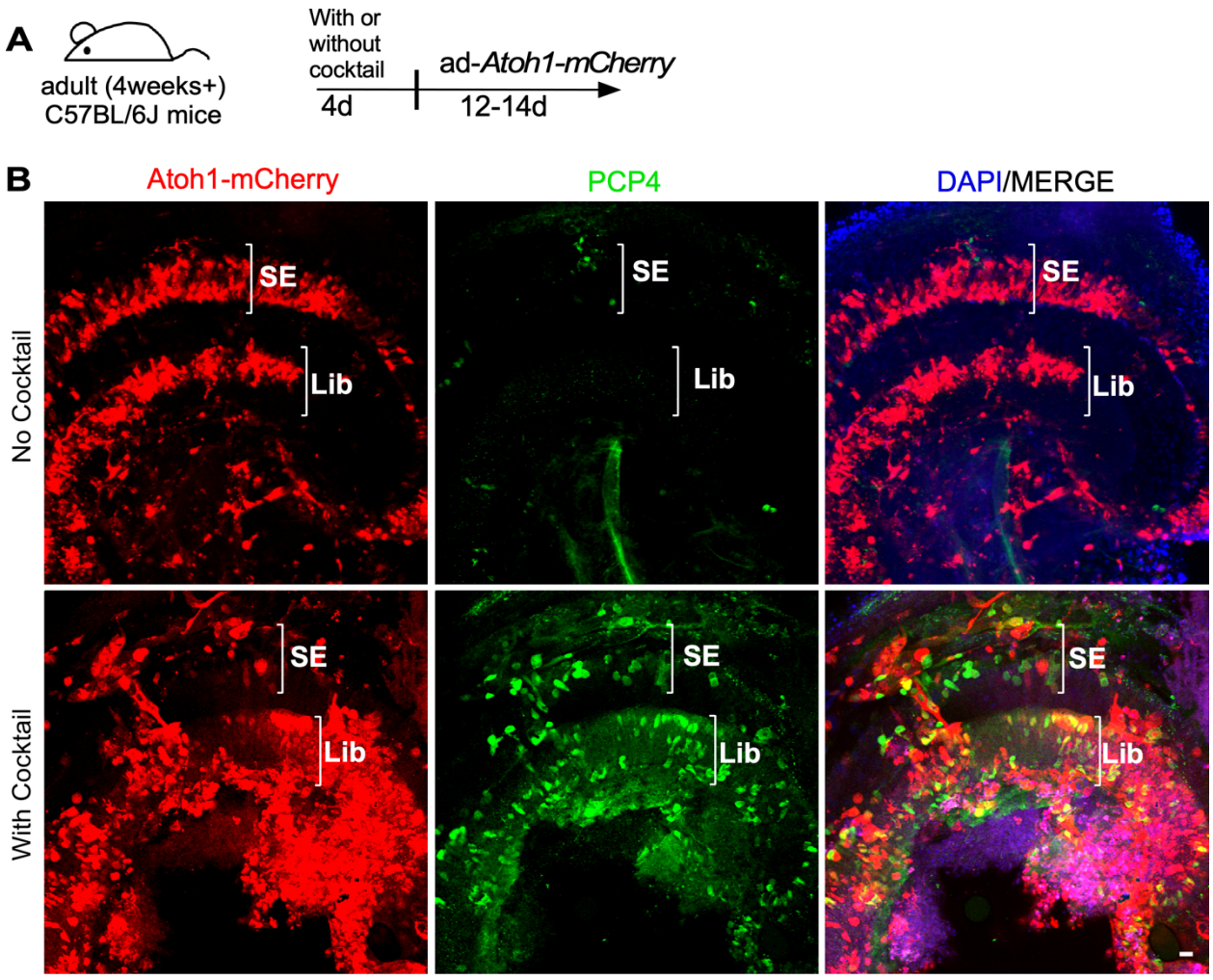
experimental design by comparing HC regeneration efficiency in the cultured adult rtTA/tet-Myc/tet-NICD cochleae treated with Dox, Dox+Bi(an adenylyl cyclase inhibitor Bithionol) and Dox+iwp2 (a Wnt inhibitor) followed by Ad.*Atoh1.mCherry* infection. **D**. Fluorescent immunohistochemistry showed more HCs (MYO7A+) were regenerated in the Dox treated samples compared to the samples with Dox+Bi, Dox+iwp2 or Dox+Bi+iwp2 treatment. **E**. Quantification and comparison of regenerated HCs in the apical turn of the cultured cochleae among different treatment groups showed a significant decrease in regenerated HCs in the samples treated with Bi, iwp2 or Bi+iwp2 in the SE and Lib. SE: Sensory region; Lib: Limbus region. *$p < 0.05$, **$p<0.01$, Student's t-test. Error bar, mean ± SEM; n=5 for each group. Source data are provided as a Source Data file. Scale bars: 20 μm.


A
adult (4weeks+)
C57BL/6J mice
VLFsiFsiM(in vitro)
4d
B
Notch1
Six1
Gata3
Eya1
Hes5
Hes1
P27kip1
MYC
Foxg1
Prox1
Dlx5
Atoh1
Sox2
Nanog
Alpl
Fut4
Relative mRNA level
Ctrl
Treated
C
adult (4weeks+)
C57BL/6J mice
VLFsiFsiM (in vivo)
4d
D
MYC
Notch1
Eya1
Six1
Gata3
Relative mRNA level
Ctrl
Treated

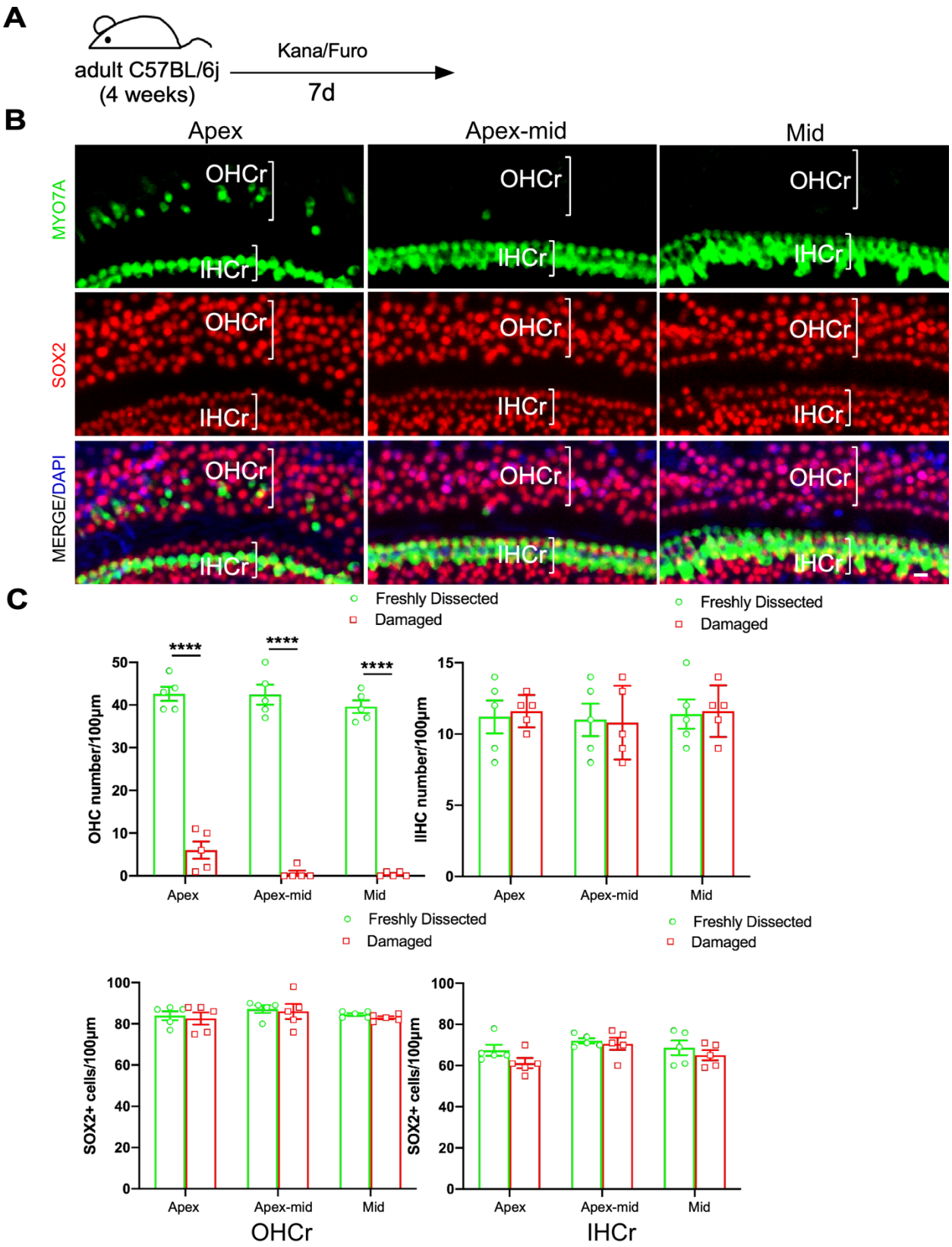
Supplemental Figure 8. The cocktail elicits inner ear reprogramming effect similar to that induced by MYC/NICD co-activation *in vitro.* **A.** A schematic diagram illustrating the experimental procedure. **B**. Selected genes, which have been tested previously after MYC/NICD co-activation in the rtTA/tet-Myc/tet-NICD mice, were studied by qRT-PCR in cultured adult WT mouse cochlea after 4-day cocktail (VLFsiFsiM) treatment. Up-regulated inner ear progenitor genes included *Six1*, *Eya1*, *Gata3* and *Sox2*, as well as *Notch1* and its target *Hes5*. Some inner ear progenitor genes (*Hes1, Foxg1 and Dlx5*) showed little expression level change. Stem cell genes (*Fut4, Nanog and Alpl*) or differentiation genes (*Prox1*, $P27^{kip1}$ or *Cdkn1b*) were not up-regulated. **C.** A schematic diagram illustrating the experimental procedure by trans-tympanic membrane injection. **D**. qRT-PCR study of the adult WT mouse cochlea after 4-day cocktail (VLFsiFsiM) treatment *in vivo*. *p<0.05, **p<0.01, ****p<0.0001, two-tailed unpaired Student's t-test. Error bar, mean ± SEM, n=3. n is the number of biologically independent samples. V: VPA; L:LiCl; F:FSK; siF: siFIR; siM: siMxi1.


A
adult (4weeks+)
C57BL/6J mice
VLFsiFsiM
4d
ad-Atoh1-GFP
12-14d
B
ad.Atoh1GFP
POU4F3
PCP4
MERGE/DAPI
SE
C
D1
D2
D3
E
adult (4weeks+)
C57BL/6J mice
No cocktail
4d
ad-Atoh1-GFP
14d
F
ad.Atoh1GFP
POU4F3
MYO7A
MERGE
SE
G
adult (4weeks+)
C57BL/6J mice
VLFsiFsiM
4d
Normal culture
14d
H
MYO7A
POU4F3
DAPI
MERGE
SE

Supplemental Figure 9. The cocktail efficiently reprograms WT adult cochlea to regenerate hair cells *in vitro.* **A.** A schematic diagram illustrating the experimental procedure of VLFsiFsiM treated adult WT mice, followed by Ad.*Atoh1-GFP* infection *in vitro*. **B, C.** Ad.*Atoh1-GFP* infected reprogrammed cells that transdifferentiated into HC-like cells ($POU4F3^+/PCP4^+$) were in the in the apex of the cocktail treated cochlea. **C**. Enlarged image from (**B),** to show regenerated HC-like cell cluster ($PCP4^+/POU4F3^+$). **D1-2.** SEM images of regenerated HCs in WT adult mouse cochlea after the cocktail/Ad.*Atoh1-GFP* treatment *in vitro*. **D1.** An overview of regenerated ectopic HCs (arrows) from a VLFsiFsiM treated cochlea sample. **D2.** Enlarged images from D1 (arrows) to show an HC with immature stereocilia and kinocilium (arrowhead). **D3.** Enlarged images from D1 (arrow) to show an HC with immature stereocilia and without kinocilium. **E**. A schematic diagram illustrating the experimental procedure of Ad.*Atoh1-GFP* infection WT cochlea *in vitro*. **F.** No existing HC was preserved after Ad.*Atoh1-GFP* infection in the sensory epithelial region. **G**. A schematic diagram illustrating the experimental procedure of cocktail treated WT cochlea *in vitro*. **H.** No existing HC was preserved after cocktail treatment in the sensory epithelial region. V: VPA; L:LiCl; F:FSK; siF: siFIR; siM: siMxi1. Scale bars: 10 μm.

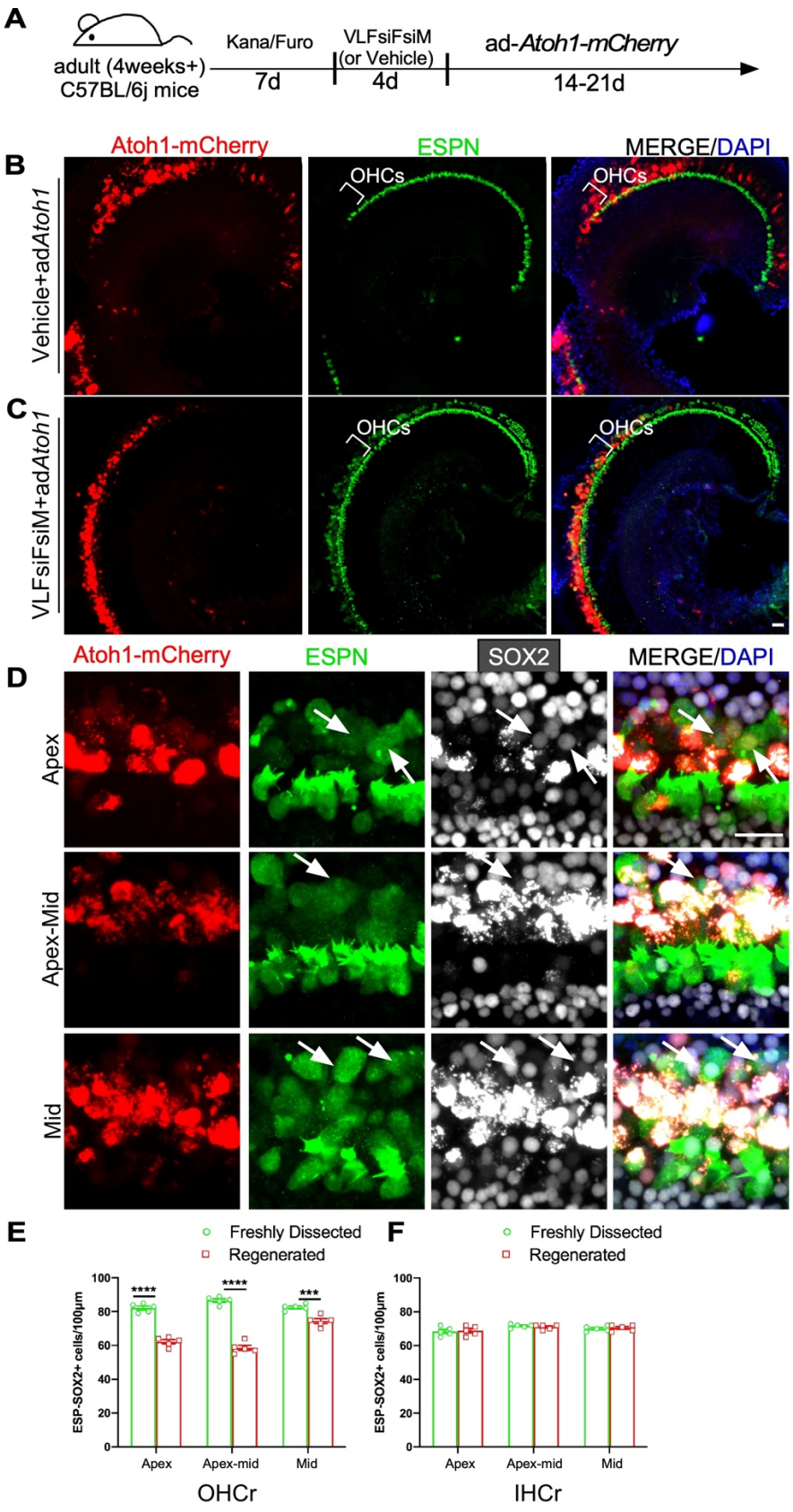


Supplemental Figure 10. Low magnification images delineating the HC-like cells regeneration status. A. A schematic diagram illustrating the experimental procedure of using WT mouse model with HC-like cell regeneration from reprogramming by the cocktail/ad.Atoh1 *in vitro*. **B.** Abundant HC-like cells ($PCP4^{+}$/$Atoh1mCherry^{+}$) in the SE and Lib were seen after the cocktail treatment but not in control without cocktail treatment. SE: Sensory Epithelial Region; Lib: Limbus Region. Scale bars: 20 μm.



Supplemental Figure 11. A mouse model with hair cell loss induced by Kanamycin/Furosemide treatment. A. A schematic diagram illustrating the experimental

procedure of C57BL/6j mice treated with Kanamycin/Furosemide *in vivo*. **B.** 7 days after the treatment by Kana/Furo, a complete OHC loss was observed from the Apex-mid to the mid-turn and over 90% OHCs loss in the Apex. IHCs and SCs ($SOX2^{+}$) were preserved during the period. **C.** Quantification showed the complete OHC loss (Apex-mid to mid-turn) and the preservation of IHCs and SCs after Kana/Furo treatment.****$p < 0.0001$, two-tailed unpaired Student's t-test. Error bar, mean ± SEM; N=5 in each group. Source data are provided as a Source Data file. Scale bars: 10 μm.


A
adult (4weeks+)
C57BL/6j mice
Kana/Furo
7d
VLFsiFsiM
(or Vehicle)
4d
ad-Atoh1-mCherry
14-21d
B
Atoh1-mCherry
ESPN
MERGE/DAPI
Vehicle+adAtoh1
OHCs
C
VLFsiFsiM+adAtoh1
D
SOX2
Apex
Apex-Mid
Mid
E
Freshly Dissected
Regenerated
ESP-SOX2+ cells/100μm
****
***
Apex
Apex-mid
Mid
OHCr
F
IHCr

Supplemental Figure 12. Efficient HC regeneration in the mouse model with HC loss *in vivo*. **A**. A schematic diagram illustrating the experimental procedure of using WT C57BL/6j mouse model with HC loss for reprogramming by the cocktail and HC regeneration *in vivo*. **B, C.** Confocal images of wholemount of Kana/Furo treated WT adult cochleae that were subsequently treated with the cocktail (VLFsiFsiM) or Vehicle and infected by Ad.*Atoh1.mCherry.* Abundant HCs ($ESPN^+$) in the OHC region (OHCr) were seen after the cocktail treatment (c), but not in the Vehicle treated sample (b) despite of Ad.*Atoh1.mCherry* infection (tdT+). Few cells were infected in the IHC region. **D.** Confocal z-stack images of regenerated HCs ($ESPN^+$) from the mid-Mid turn to the Apex in the cocktail treated adult WT cochlea *in vivo*. Many new HCs were positive for SOX2 (arrows, $ESPN^+/SOX2^+/tdT^+$), indicating their SC origin. **E.** Quantification and comparison of non-transdifferentiated SCs ($ESPN^-$/SOX2+) showed a significant decrease in the SC number in the OHC region (OHCr) in the treated ears compared to the freshly dissected ears. **F**. In the IHC region (IHCr), the SC number was similar between the freshly dissected or the cocktail treated inner ears. ****$p < 0.0001$, two-tailed unpaired Student's t-test. Error bar, mean ± SEM; N=5 in each group. Source data are provided as a Source Data file. Scale bars: 40 μm (B, C), 20 μm (D).

Material and Methods

Animals

Rosa-rtTA (rtTA), Sox2Cre transgenic mice and Ai14 tdTomato reporter mice were from Jackson Laboratory (Stock# 006965; 017593; 007914); tet-on-Myc mice were from Dr. M. Bishop of the University of California, San Francisco; tet-on-NICD mice were from Dr. D. Melton of Harvard University. For the transgenic rtTA/tet-on-Myc/tet-on-NICD mice, the background was mixed C57/129SvJ/CD1, with roughly equal numbers of sexes; Atoh1-nGFP mice were from Dr. Jane Johnson (University of Texas Southwestern Medical Center, Dallas, TX); the wild type mice were C57BL/6 from Jackson Laboratory. All experiments were performed in compliance with ethical regulations and approved by the Animal Care Committees of Massachusetts Eye and Ear and Harvard Medical School.

Adult cochlear culture and viral infection *in vitro*

The adult mouse cochleae culture system has been well established (1, 2). Different from the neonatal cochlear culture method, in which the cochleae were disassociated from the bone, adult mouse whole cochleae (4-6 weeks old) were dissected with the bone attached. The bulla was first removed from the skull and dipped in 75% ethanol for 3 mins before being placed in HBSS. The vestibular region was also removed. Under a dissecting microscope, the middle ear, vessels and the debris were removed from the bulla. The bone covering the apical turn was removed, and round window and oval window membranes were opened to allow media exchange with the cochlear fluids. The ligament portion and Reissner's membrane at each end of the cochlea were also removed to facilitate the access of medium to the sensory epithelial region. The cochleae were maintained in DMEM/F12 (Invitrogen) supplemented with N2 and B27 (both from Invitrogen) for 14-18 days. Ad.*Atoh1*/Ad.*Atoh1-mCherry*/Ad.*Atoh1-GFP* adenoviruses were purchased from the SignaGen Laboratories, Rockville, MD, with titers of 6×10^{12} pfu/ml.

Immunohistochemistry

Mouse cochleae were fixed in 4% paraformaldehyde at 4°C overnight, followed by decalcification in 120 mM EDTA for 24 hours. The decalcified cochlea was used for whole

mount immunohistochemistry following a standard procedure. All specimens were incubated at room temperature for 30 min in 10% donkey/goat serum with 0.5% Triton X-100 for blocking. Primary antibodies used in this research were ESPN (1:100, Sigma-Aldrich), Sox2 (1:50, Thermo Fisher Scientific), Myo7a (1:500, Proteus BioSciences), PCP4 (1:100, Sigma-Aldrich), Prestin (1:100, Sigma-Aldrich), NF-H (1:1000, Millipore), CtBP2 (1:200 BD Transduction Laboratories), Parvalbumin (1:100, Cell Signaling) and Pou4f3 (1:50, Santa Cruz biotechnology). Species- specific Alexa Fluor-conjugated secondary antibodies were used for detection. 1-10 μg/ml DAPI was used for nuclear staining.

Single-cell preparation

Sensory epithelium from cochlea explants were dissected, and then dissociated immediately incubating with 0.25% trypsin-EDTA for 15 min at 37ºC. Tissues were gently singularized by P1000 pipette in complete medium (DMEM/F12 medium containing 10% FBS). Cells were strained twice through 40 μm mesh into 5 ml medium, spun down for 5 min at 300 *g* to pellet and resuspend in 50 ml of complete medium. Roughly 1600 cells were loaded onto a 10X Chip-G for a target recovery of 1000 cells. Single cells from explant cochlear cultures of control (n=4) and Dox-treated (n=8) groups were captured separately to prepare two libraries.

Single-cell RNA library preparation and sequencing

Single cell suspensions were immediately transferred to individual channels of chip (Chromium Next GEM Chip G) to generate gel beads in emulsion together with reverse transcription master mix on the droplet-based high-throughput Chromium Controller (10X Genomics) allowing cell lysis, individual cell barcoding, and reverse transcription by Chromium Next GEM Single Cell 3’ Kit v3.1 (10X Genomics, PN-1000128). Briefly, single cell library preparation was carried out by emulsion breakage, PCR amplification, cDNA fragmentation, oligo adapter, and Illumina sample index addition following the manufacturer's protocol. The single-cell derived cDNA libraries were then assessed with bioanalyzer (High Sensitivity DNA Kit, Agilent 2100 Bioanalyzer) for quality control. Following library preparation, next-generation sequencing was performed with paired-end sequencing of 150 bp each end using Illumina HiSeqXTen (Novogene Inc.).

Computational analysis of single-cell data

Transcriptome sequencing analysis and read mapping were performed using CellRanger pipeline (version 5.0.0; 10X Genomics) according to the manufacturer's guidelines. Raw sequencing reads processed by CellRanger were demultiplexed and mapped to the mouse reference genome (mm10, GRCm38) using STAR. CellRanger-generated count matrices were loaded into Seurat v3.2.2 (3) for downstream analysis. Outlier cells were first identified based on three metrics (library size, number of expressed genes and mitochondrial proportion). Cells with less than 200 unique molecular identifiers or high mitochondrial content were filtered out. Datasets from each sample were integrated into a single matrix for comparative analysis with the integration workflow from Seurat using {Merge} function. After this, we performed global-normalization using the {SCTransform} function embedded in Seurat R package for normalization and scaling of UMI and mitochondrial content. For the unsupervised clustering, we chose 0.5 as the resolution parameter. Briefly, we performed the downstream analysis steps including {SelectIntegrationFeatures}, {PrepSCTIntegration}, {FindIntegrationAnchors}, {IntegrateData}, {RunPCA}, {RunUMAP}, {FindNeighbors} and {FindClusters} functions on the integrated dataset. Identification of differentially expressed cell type-specific markers was carried out in Seurat utilizing the {FindAllMarkers} function. Using this function, cells from each cluster were compared against one another to detect uniquely expressed genes. Based on default parameters, only genes that were enriched and expressed in a minimum of 10% of each population (min.pct = 0.1) and with a log fold difference larger than 0.25 (logfc.threshold = 0.25) were considered. We used a Wilcoxon rank sum test to perform the analysis and confirmed with another algorithm called Model-based Analysis of Single-cell Transcriptomics (MAST) using the {FindAllMarkers} function on default settings. A differentially expressed gene (DEG) is defined as any gene expressed in at least 10% of the cells, which has a p-value <0.001 and a >1.5x average fold change from all other clusters being tested. Additionally, cell clusters were defined to have at least 10 DEGs that were unique when compared to other clusters. These DEGs per cluster were plotted using {DimPlot} and {FeaturePlot} functions in Seurat for visualization in UMAP plot. Using the {VlnPlot} function, we generated Violin plots to look at gene expression across clusters, and {DoHeatmap} to generate heatmaps of the top 20 DEGs. Finally, we annotated each cell type by extensive literature reading and searching for the specific gene expression patterns.

Single-cell trajectory analysis

We used Monocle3 (v.0.2.3.0) R package (https://cole-trapnell-lab.github.io/monocle3/) (4) to investigate inferred developmental trajectories between interdental cell subsets and order cells in pseudotime based on their transcriptional similarities, in an unbiased manner. The subset data, previously scaled, normalized and clustered by the Seurat tool, were imported into Monocle3 using {new_cell_data_set} function. Monocle3 was run on our normalized counts matrix for the subclustered interdental cell data (IdC_Control and IdC_Dox). The data were subject to UMAP dimensional reduction and cell clustering using the {cluster_cells} function. A principal graph was plotted through the UMAP coordinates using the {learn_graph} function that represents the path from IdC_Control to IdC_Dox. This principal graph was further used to order cells in pseudotime using the {ordercells} function in Monocle3. Following that, we defined the population of IdC_Control cells as the root cell state (the starting point) to further investigate genes allowing IdC_Dox cells the ability to divide and regenerate. DEGs between IdC_Control and IdC_Dox clusters used to generate hypothetical developmental relationships were determined using {graph_test} function. Monocle3 identified modules of co-regulated genes within our selected clusters using the function graph test to identify variable genes in the data based on Moran's I statistic. We retained the genes that had a significant q-value (< 0.001) from the auto-correlation analysis, grouped these genes into modules using UMAP and Louvain community analysis, and then genes with the greatest q-values were plotted on a heatmap using a proposed function {pheatmap}.

Pathway analysis

DAVID v6.8 (https://david.ncifcrf.gov/) (5, 6) was applied using default settings to identify enriched pathways based on the DEGs between two groups or clusters. We used pathway gene sets from the biological processes of Gene Ontology (http://www.geneontology.org/) or the Hallmark pathway collection from Molecular Signatures Database (MSigDB; http://www.gsea-msigdb.org/gsea/).

Data availability

Raw and pre-processed single-cell sequence data that support the findings of this study are available through the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) (GSE205187). R scripts for data processing are available through https://github.com/ZYChenLab/HC_Regeneration**.** There is no restriction on the use of the code or data for non-profit academic organizations.

Chemical reprogramming *in vitro* for HC regeneration.

In the rtTA/tet-*Myc*/tet-*NICD* mouse model, cochleae were treated with Dox (Sigma, 2 μg/ml final concentration); in the rtTA/tet-*Myc* and rtTA/tet-*Myc*/Atoh1-GFP mouse models, cochleae were treated with Dox (Sigma, 2 μg/ml final concentration) and VPA (1 mM); in the Atoh1-GFP and WT mouse models, cochlea were treated with VPA (1 mM), LiCl (8mM), FSK (20μM), siFIR (0.02 μM), and siMxi1 (0.02 μM) for 4 days, followed by Ad.*Atoh1 (or* Ad.*Atoh1.mCherry or* Ad.*Atoh1GFP)* (6×10^{10} pfu/ml) infection overnight. siRNAs were delivered following the manufacturer's instructions (Polyplus-transfection; 89129-920). The controls were cultured adult cochleae of the same genotype treated with vehicle (sterile water with 0.1% DMSO) plus Ad.*Atoh1.mCherry or* Ad.*Atoh1GFP*. The culture was placed into fresh medium for an additional 10-14 days. Cochleae were harvested and decalcified before immunohistochemistry.

Lineage tracing

For in vivo lineage tracing studies, 4-6-week-old Sox2-CreER/tdTf/f mice were injected with tamoxifen (Sigma, 200 mg/kg) daily for three days before i.p. injected with Kanamycin/Furosemide (Kana/Furo) to kill hair cells, waited for 7 days, with the subsequent delivery of the cocktail (VLFsiFsiM) into the middle ear space, followed by the injection of Ad.Atoh1-GFP into the inner ear by cochleostomy. For in vitro lineage tracing studies, 4-Hydroxytamoxifen (20 ng/mL) was added to cultures on day 0 to activate Cre for lineage tracing studies. Ad.Atoh1 virus was added to the medium for 16 to 24 hours at a concentration of $6x10^{10}$ pfu/ml.

Trans-tympanic Injection of chemicals *in vivo.*

All adult mice used were between 4 and 6 weeks old. Trans-tympanic injections were performed 7 days after the subcutaneous injection of Kanamycin (1mg/g; Sigma) followed by intraperitoneal injection of Furosemide (0.3mg/g; Hospira Inc) 30 min later. Mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). Trans-tympanic injections were conducted with 5µl chemical combinations of VPA (5mg/ml), LiCl (40mM), FSK (50µg/ml), siFIR (0.6µg/10µl), and siMxi1 (0.6µg/10µl), or vehicle (sterile water with 0.5% DMSO). Chemicals or vehicle were injected into one ear through the tympanic membrane (TM) in mice. Microforceps were used to retract the skin and visualize the medial superior fold adjacent to the TM. A Hamilton syringe with 33G needle was used to inject drugs trans-tympanically.

Viral Injection *in vivo*.

All surgical procedures were done in a clean, dedicated space. Instruments were thoroughly cleaned with 70% ethanol and autoclaved before surgery. Mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). For viral injection, cochleostomy was performed on the anesthetized mice by opening the bulla, and adenovirus with a titer of 5×10^{12} pfu/ml was injected into the middle turn of the scala media by a pressure-controlled motorized microinjector at a speed of 3 nl/sec. A total of 1 µl of adenovirus was injected into each cochlea.

Confocal microscopy

Confocal microscopy was performed using a Leica TCS SP8 with Leica Application Suite Advanced Fluorescence (LAS AF) software V2.6.0. Sequential scanning with different laser channels was used for image acquisitions. Confocal images were processed using ImageJ package (www:\\imagej.nih.gov\ij). For Z-stacks, equal numbers of images of adjacent optical sections, 0.1 mm in thickness, were used for processing with identical parameters, including median filtering and adjustment of brightness and contrast, between experimental and control groups.

qRT-PCR

The cochlear tissues including the organ of Corti, spiral limbus, and lateral wall were dissected out from the temporal bones of cultured adult cochleae. Total RNA was extracted from cochleae using RNeasy Mini Kits from Qiagen (Valencia, CA). cDNA was synthesized using reverse transcriptase (Takara) with random primers. Real-time quantitative PCR amplification reactions were carried out using QuantiTeck SYBR Green PCR kit (Takara) on an ABI StepOnePlus system (Applied Biosystem) with StepOne software V2.3. All reactions were carried out in duplicates with the expression of the gene normalized using *Gapdh* as the endogenous housekeeping control gene.

FM1-43 uptake

To study the presence of functional channels in HC-like cells regenerated *in vivo,* 4- week-old C57bl/6j cochleae were treated as described. 2 weeks after viral infection, cochleae were incubated with FM1-43FX (5 μM)(Life Technologies) for 30 seconds before wash and fixation with 4% PFA. The cochleae were subsequently decalcified and processed for immunolabeling.

Scanning Electron Microscopy.

Following dissection, the tissue was harvested and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (EMS) supplemented with 2 mM $CaCl_2$ at room temperature for 1.5-2 hours on a tissue rotator. Samples were then washed with distilled water 3 times and additional dissection was performed post-fixation, as needed. The samples were then rinsed with 0.1 M sodium cacodylate buffer 3 times for 10 min and treated with 1% osmium tetroxide (O) for 1 h, followed by a thorough rinse with distilled water 3 times for 10 min each, and treated with saturated thiocarbohydrazide (T) in distilled water for 30 min. This treatment was repeated, followed by an additional 1% osmium tetroxide step, yielding a treatment order of O-T-O-T-O. Following the final treatment and another round of washes, samples were transferred to 20 ml scintillation vials in 2 ml distilled water for dehydration with ethanol by adding 50 μl of 100% ethanol to the vial, then doubling the added volume every 10 min. Once the vial was full, samples were transferred to a 100% ethanol, then critical point dried from liquid CO_2 (Tousimis Autosamdri 815). The

samples were then mounted on aluminum specimen stubs with a carbon tape, sputter-coated with 4.5 nm of platinum (Leica EM ACE600) and observed with a field emission SEM (Hitachi S-4700).

Statistical analysis

The Prism 8 statistical package (GraphPad Software, Inc) was used in data processing. To count HCs upon partial-reprogramming followed by activation of Atoh1 *in vitro*, multiple regions from the apex (80-100% of the length of cochlear duct from the hook) were included. Briefly, new HC-like cells generated from Hensen cell region, Deiters' cell region, pillar cell region, inner phalangeal and inner sulcus cell region were all included in the "SE" region; the cells generated from limbus region were included in the "Lib" region.

To count the number of HCs upon VLFsiFsiM *in vivo*, the apex (80-100% of the length of cochlear duct from the hook); the apex-mid (60-80% of the length of cochlear duct from the hook) and mid (50-60% of the length of cochlear duct from the hook) were included. The injection sites were close to the mid turn. ESPN+ HCs were counted and the HC density was calculated as the average number of HCs per 100 μm for each cochlea. Controls were sterile water plus 0.5% DMSO-injected cochleae. Multiple cochleae were used for each counting and in statistical analysis.

Data were presented as mean ± SEM. Two-tailed Student’s t-test was used to compare two groups ($p<0.05$ was considered significant). ANOVA analysis with Turkey’s multiple comparisons test was used to compare three or more groups ($p<0.05$ was considered significant).

Other Supplementary Materials for this manuscript include the following:

Tables S1 to S6
Movies S1, 2
Supplementary Data Source (Excel)

Legend for Movies S1, and S2

Confocal images of wholemount of Kana/Furo treated WT adult cochleae that were subsequently treated with the cocktail (VLFsiFsiM) and infected by Ad.*Atoh1.mCherry.* Abundant HCs ($ESPN^{+}$) (green) in the OHC region (OHCr) were seen (Movies S1, 2). Few cells were infected in the IHC region.

Reference

1. Y. Shu *et al.*, Renewed proliferation in adult mouse cochlea and regeneration of hair cells. *Nature communications* **10**, 5530 (2019).
2. W. Li *et al.*, A Novel in vitro Model Delineating Hair Cell Regeneration and Neural Reinnervation in Adult Mouse Cochlea. *Front Mol Neurosci* **14**, 757831 (2021).
3. T. Stuart *et al.*, Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-1902 e1821 (2019).
4. J. Cao *et al.*, The single-cell transcriptional landscape of mammalian organogenesis. *Nature* **566**, 496-502 (2019).
5. W. Huang da, B. T. Sherman, R. A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4**, 44-57 (2009).
6. W. Huang da, B. T. Sherman, R. A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research* **37**, 1-13 (2009).