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Supplemental Information

Greater epithelial ridge cells are the principal organoid-forming progenitors of the mouse cochlea

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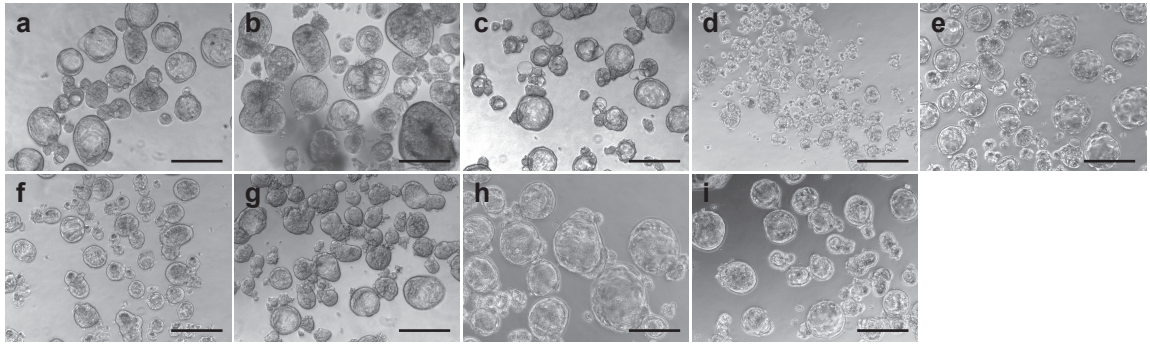
A

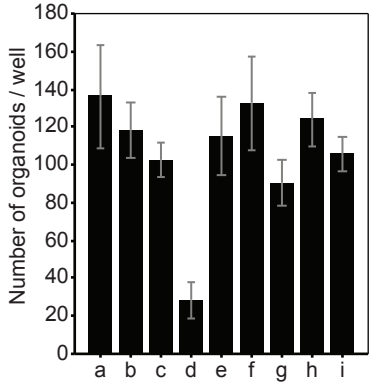
Media supplements

a	**EFI**
b	**EFI**, **C**
c	**EFI**, Trichostatin A
d	**EFI**, Reversine
e	**EFI**, NXT1219
f	**EFI**, Paullone
g	**EFI**, DAPT
h	**EFI**, SAG
i	**EFI**, Vismodegib

B



C



D

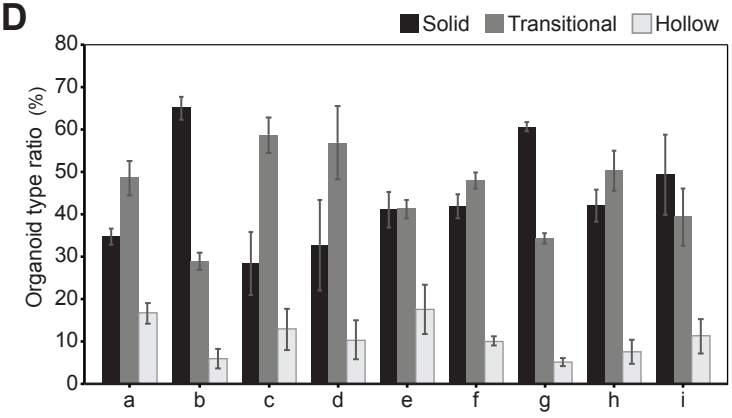


Figure S1 Further Optimization of Organoid Culture Conditions. Related to Figure 1. (A) Media supplements used for conditions a-i. (B) Representative organoids that formed after seven days in culture in conditions a-i. Scale bars = 200 μm. (C, D) The number of organoids (C), and the ratio of solid, transitional, and hollow organoid morphologies (D) after seven days culture. Shown are the means ± standard deviations (SD) of triplicate experiments.

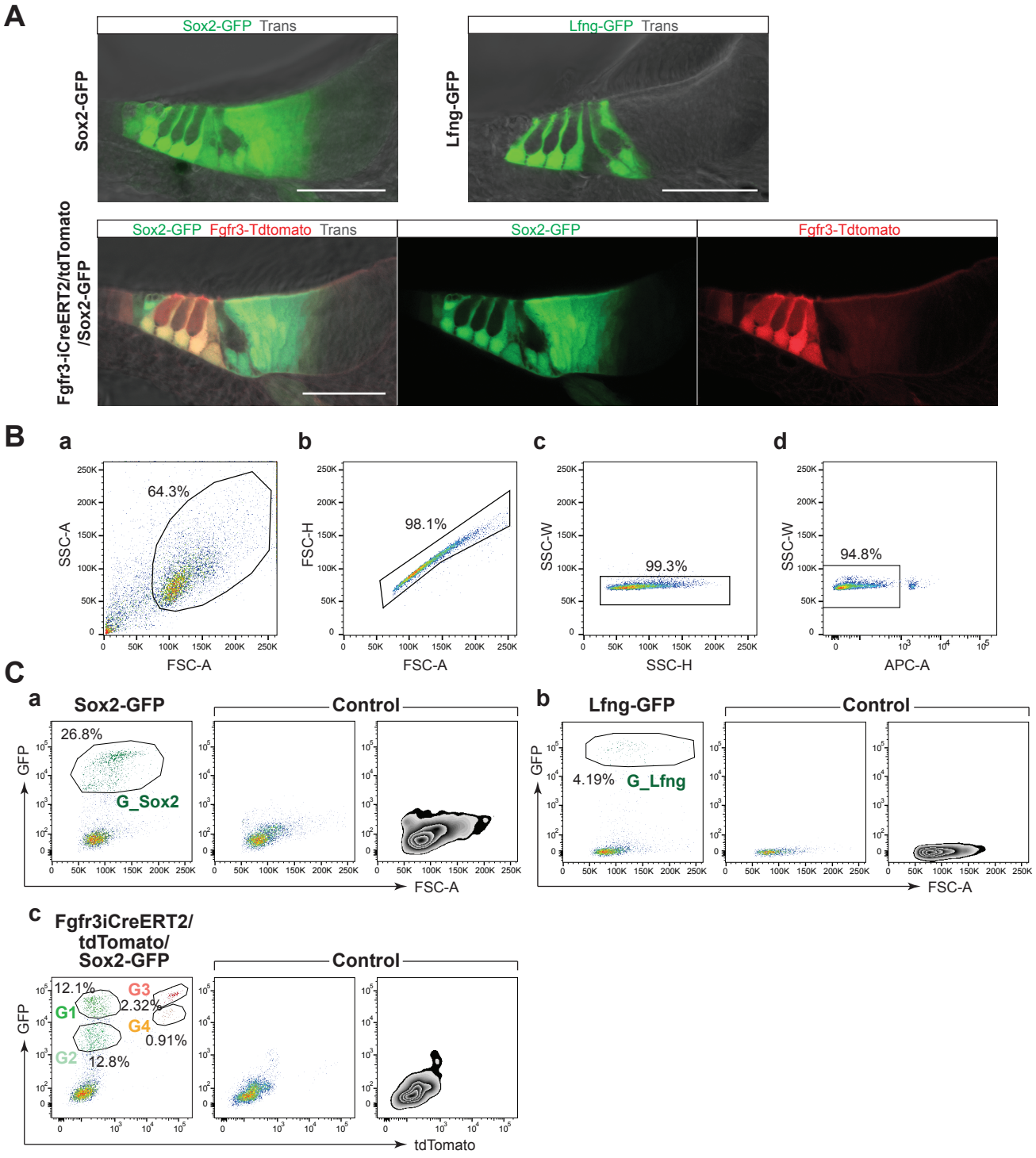
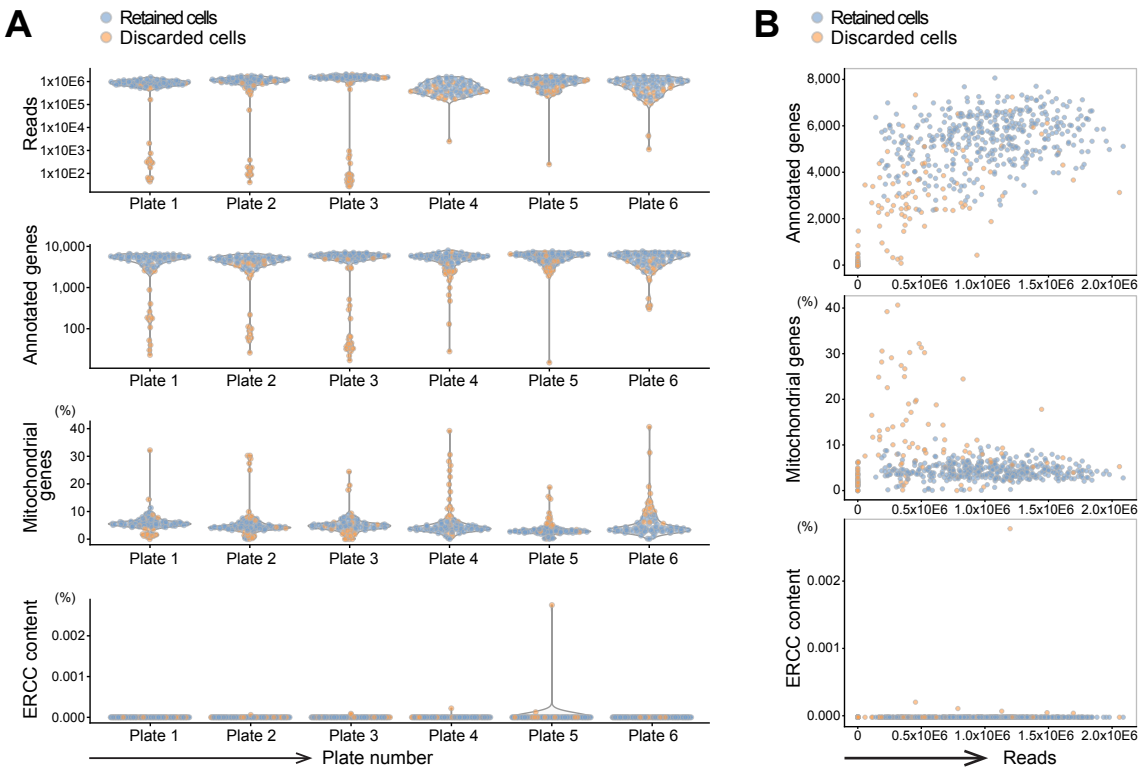
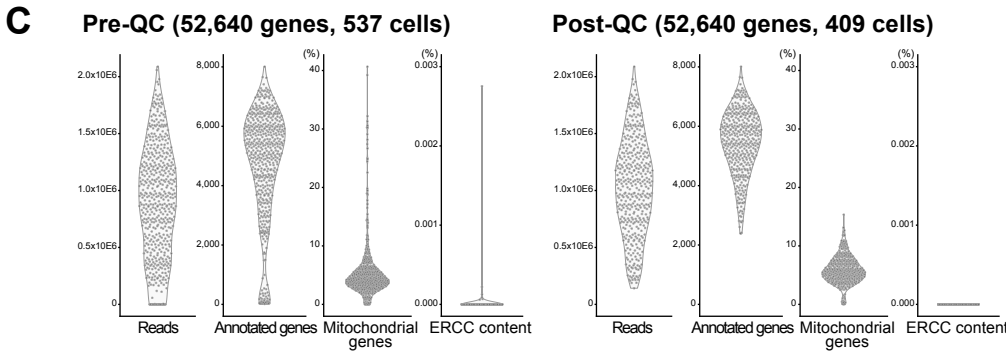


Figure S2 Flow Cytrometric Isolation of Cochlear Cell Subgroups. Related to Figure 2. (A) Fluorescent reporter gene expression in P2 organ of Corti vibratome sections of the Sox2-GFP, lunatic fringe (Lfng)-GFP, and Fgfr3-iCreERT2/Ai14tdTomato/Sox2-GFP mouse strains. Scale bars = 50 µm. (B) FACS plots and gating methods for isolating viable single cells. (a) Debris exclusion. (b and c) Isolation of singlets. (d) Live cell isolation using dye exclusion (SYTOX Red dead cell stain). The fraction of cells per gate is shown in percent.
(C) FACS plots of cochlear duct cells harvested from transgenic mice (left: dot plots, as shown in Figures 2D–2F) and control mice (middle: dot plots; right: zebra plots). Wild type (a and b) and non-recombined Fgfr3-iCreERT2/Ai14tdTomato (c) mouse strains were used as controls. Identical FACS settings were used for control and FACS harvest experiments. The fraction of cells per gate is shown in percent.





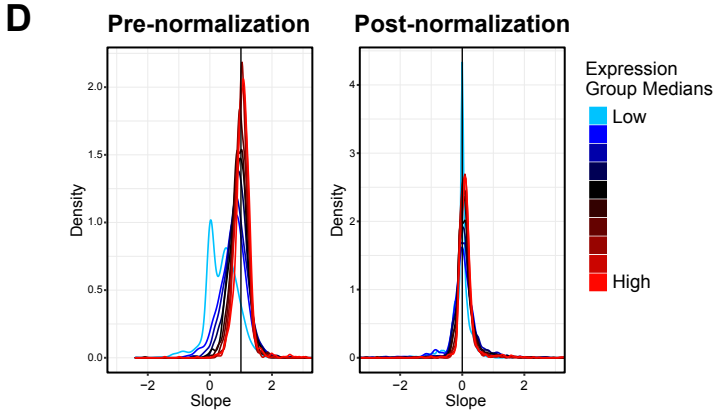


Figure S3 Quality Control of Single-cell RNA-Seq Results. Related to Figure 3. (A) Per-cell library size in reads, the number of annotated genes, mitochondrial gene content, and spike-in control (ERCCs) contents are shown for each different collection plate used for single-cell collection. Retained cells and discarded low-quality cells are colored blue and orange, respectively. (B) The number of annotated genes, mitochondrial gene content, and ERCCs content were plotted against per-cell library size. Retained cells and discarded low-quality cells are colored blue and orange, respectively. (C) Pre-quality control (QC) (left) and post-quality control (right) library profiles are shown by per-cell library size, the number of annotated genes, mitochondrial gene content, and ERCCs content. Post QC, we note a median number of 1,039,374 reads per cell and a median number of 5,561 genes per cell. (D) Slope normalization for different abundant gene groups, determined based on their unnormalized median expression, shown for pre- (left) and for post-normalization with SCnorm (right).

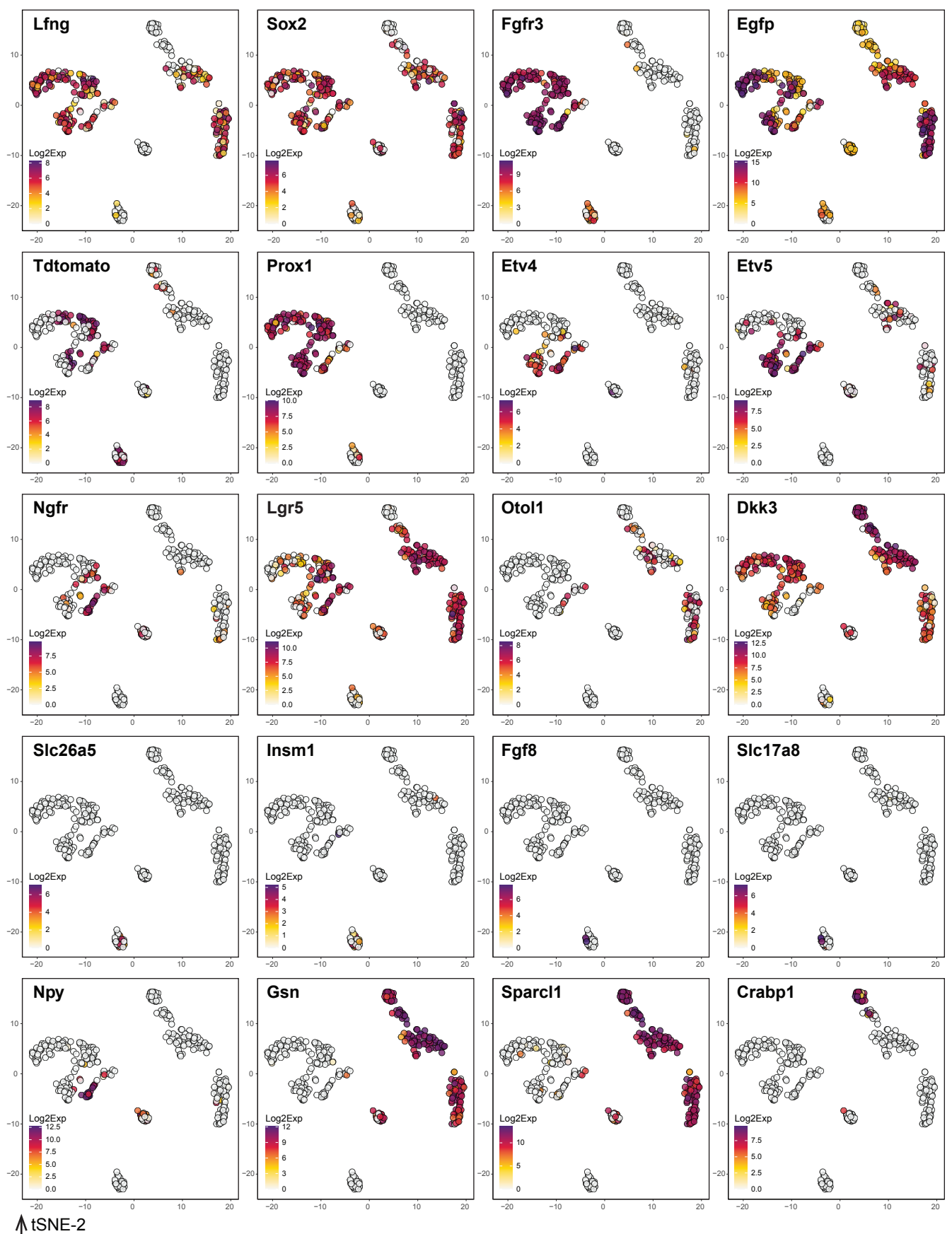


Figure S4 Expression of Known Markers and Reporter Genes. Related to Figure 3. The expression of each gene is projected onto the tSNE plot in reference to Figure 3A. In addition to marker gene expression, detected expression levels for promoter alleles for Lfng, Sox2, and Fgfr3, as well as reporter genes Egfp and tdTomato, are shown.

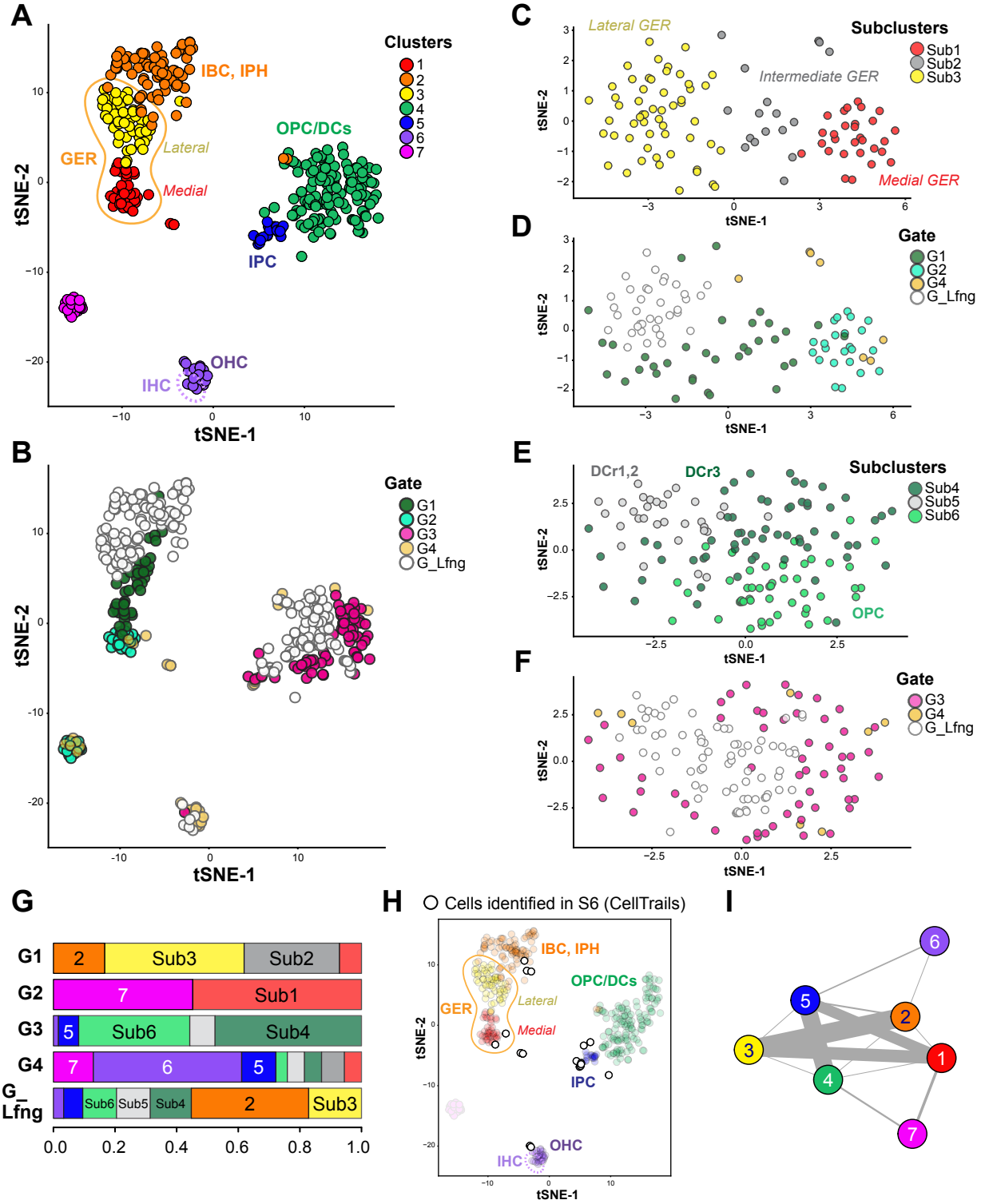


Figure S5 Alternative Clustering Analysis. Related to Figure 3. (A) The transcriptomic data derived from FACS-isolated cochlear cells were subjected to graph-based clustering and visualized in tSNE plots. Seven distinct major clusters were identified. (B) tSNE plot for the FACS gates used for cell isolation. (C) GER clusters 1 and 3 were extracted, and re-clustering revealed three groups, here labeled with Sub1, Sub2, and Sub3, representing medial, intermediate, and lateral GER cells, respectively. (D) tSNE plot for the FACS gates used for cell isolation, confirming that G2 is associated with medial GER cells. (E) Reclustering of cluster 4 discriminated between first and second row Deiters' cells (Sub 5), third row Deiters' cells (Sub 4), and outer pillar cells (Sub 6). (F) tSNE plot for the FACS gates used for cell isolation. (G) Representation of the different clusters in G1-G4, and G_Lfng groups. Cluster affiliations of the cells in G1-G4, and G_Lfng are shown by proportion and colored by cluster designation. (H) The cells identified in cluster S6 using CellTrails clustering were projected into the tSNE plot shown in A to illustrate the distribution of S6 cells. Projected S6 CellTrails cells are represented by black-stroked white circles. (I) Partition-based graph abstraction shows the relationship between clusters. The thickness of the lines encodes the log-ratio weights among clusters; thicker lines represent a closer relationship.

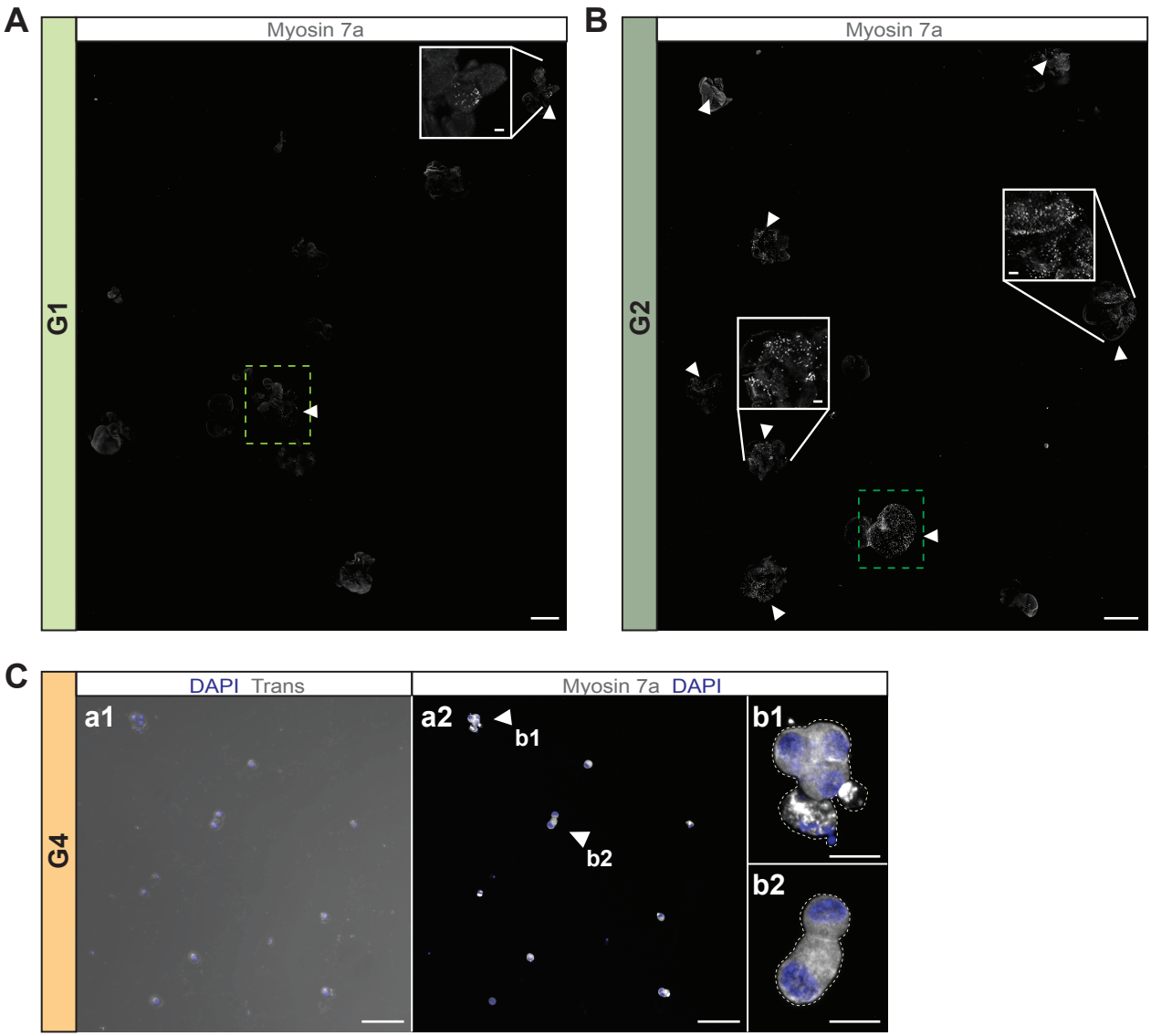


Figure S6 Organoid Colonies Generated from Different Cochlear Cell Subtypes. Related to Figure 4. (A) Colonies generated from G1-derived organoids after 14 days of substrate-attached culture in media continuously supplemented with EFI_CVPM. The micrograph is taken from the same culture chamber as in Figure 4A. The dotted square surrounds the colony presented in Figure 4A. The solid square highlights another example. The arrowheads indicate the areas where Myosin7a-expressing cells are abundantly observed. Scale bars = 500 μm and 50 μm (solid square). (B) Colonies generated from G2-derived organoids, using the same experimental conditions as A. The micrograph is taken from the same culture chamber as in Figure 4B. The dotted square surrounds the colony presented in Figure 4B. The solid squares highlight other examples. The arrowheads indicate the areas where Myosin7a-expressing cells are abundantly observed. Scale bars = 500 μm and 50 μm (solid squares). (C) Colonies generated from G4-derived organoids, same experimental conditions as A. (a1) Small colonies, consisting of only a few cells formed from G4-derived organoids. (a2) Myosin7a-expressing cells are visible. Scale bars = 50 μm (a1 and a2). (b1, b2) Higher magnification of the colonies labeled in (a2). Dotted lines outline the colonies. Scale bars = 10 μm.

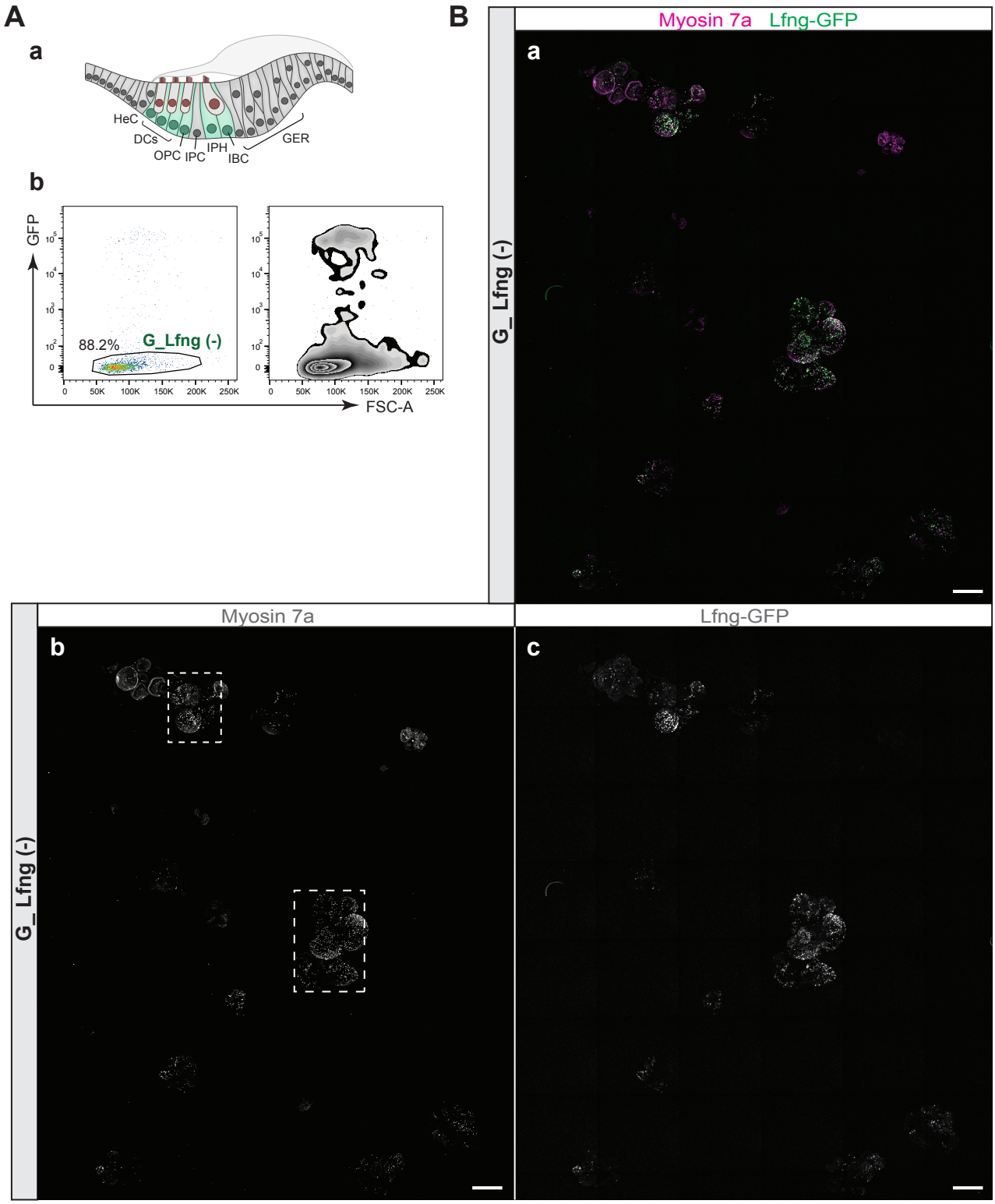


Figure S7 Hair Cells and Supporting Cells Differentiate in G_Lfng (-) GER Organoid-Derived Colonies. Related to Figure 6. (A) (a) Illustration of the cell types labeled in the Lfng-GFP mouse strain at P2. HeC: Hensen's cells, DCs: Deiters' cells, OPC: outer pillar cell, IPC: inner pillar cell, IPH: inner phalangeal cell, IBC: inner border cell, GER: greater epithelial ridge cells. (b) FACS plot (left: dot plot; right: zebra plot) and the location of the G_Lfng (-) gate applied for GFP-negative cell isolation containing GER cells. The fraction of cells per gate is shown in percent. (B) Example of colonies generated from G_Lfng (-)-derived organoids after 14 days of substrate-attached culture in media continuously supplemented with EFI_CVPM. The micrograph is taken from the same culture chamber as in Figure 6A. Myosin7a and Lfng-GFP expression is closely associated and abundant. The dotted squares surround the colonies presented in Figures 6B and 6C. Scale bars = 500 μm (a-c).