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Supplemental Information

Otic Mesenchyme Cells Regulate Spiral Ganglion Axon Fasciculation through a Pou3f4/EphA4 Signaling Pathway

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SUPPLEMENTAL FIGURES

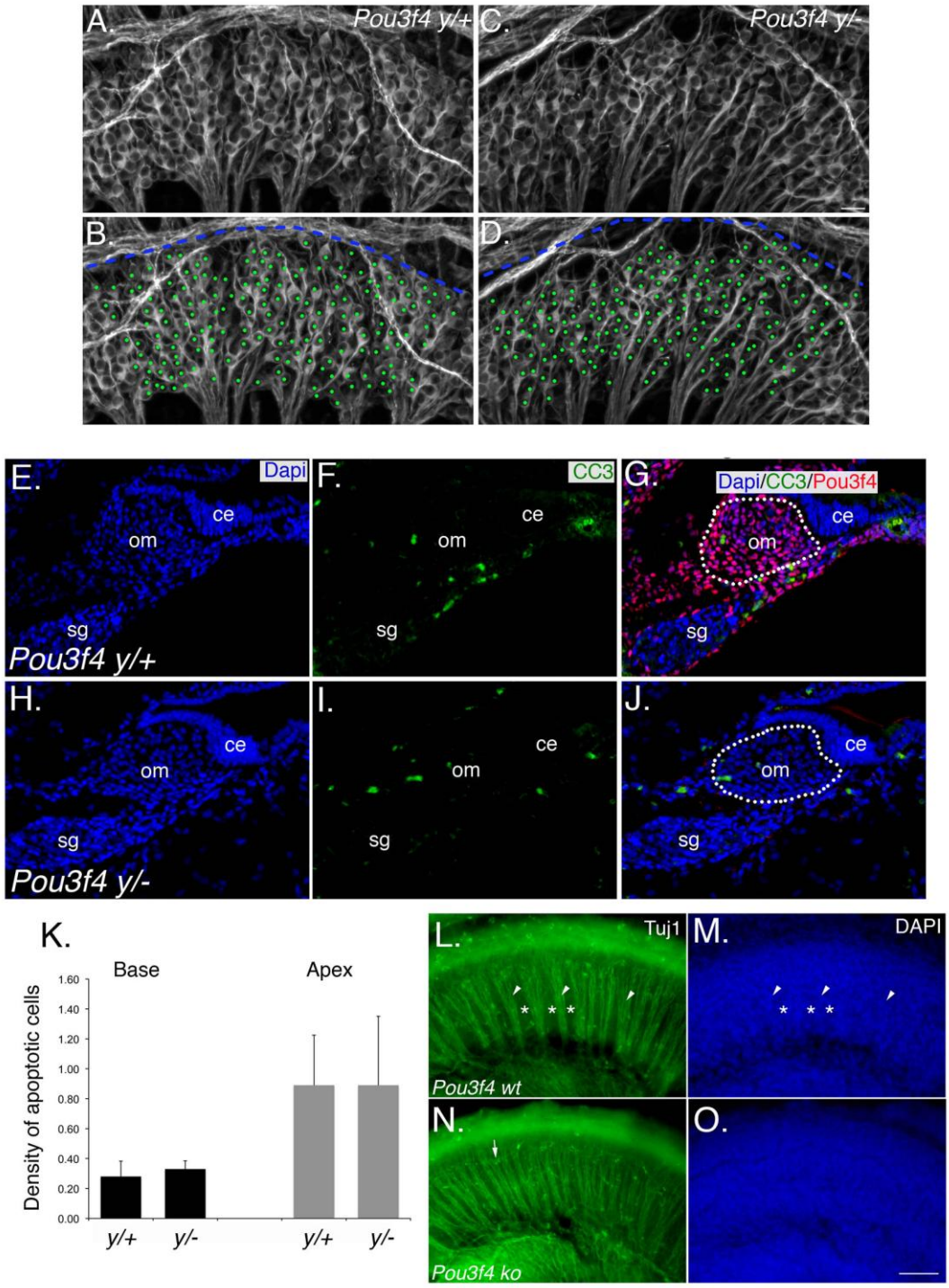

Figure S1, related to Figure 2. No change in SGN density or apoptosis between *Pou3f4* wild type and null cochleae.

(A and B) Whole mount image of the SGN soma from a *Pou3f4*$^{y/+}$ cochlea at E17.5 stained with anti-Tuj1 antibodies. As illustrated in the histogram in Figure 2M, the density of the SGNs was quantified by

dividing the number of SGNs (green dots) by the length of the region along the longitudinal axis of the cochlea (marked by the blue dashed line).

(C and D) SGNs from a *Pou3f4*$^{y/-}$ cochlea. Scale bar in C pertains to A-D, 20 μm.

(E-J) Representative sections from the base of a *Pou3f4* $^{y/+}$ and a *Pou3f4*$^{y/-}$ cochlea at E16.5. sg, spiral ganglion; om, otic mesenchyme; ce, cochlear epithelium; CC3: cleaved caspase 3. The dotted line indicates the region for quantification of CC3-positive cells.

(K) Histogram showing the density of mesenchyme cells (mean +/- SEM) undergoing apoptosis in *Pou3f4* $^{y/+}$ and *Pou3f4*$^{y/-}$ cochleae. There was no significant difference between the two groups at either the base or apex at this developmental stage.

(L-O) Representative whole-mount preparations of *Pou3f4* wild type and knockout cochleae at post-natal day 0 (P0). *Pou3f4* knock-out cochleae show no appearance of necrotic lesions. The tissue used in these photographs was from a *Pou3f4* mutant line previously reported (Phippard et al., 1999) and does not show any differences to the Cre knock-in line used throughout this study. Scale bar in O pertains to E-J and L-O, = 75 μm.

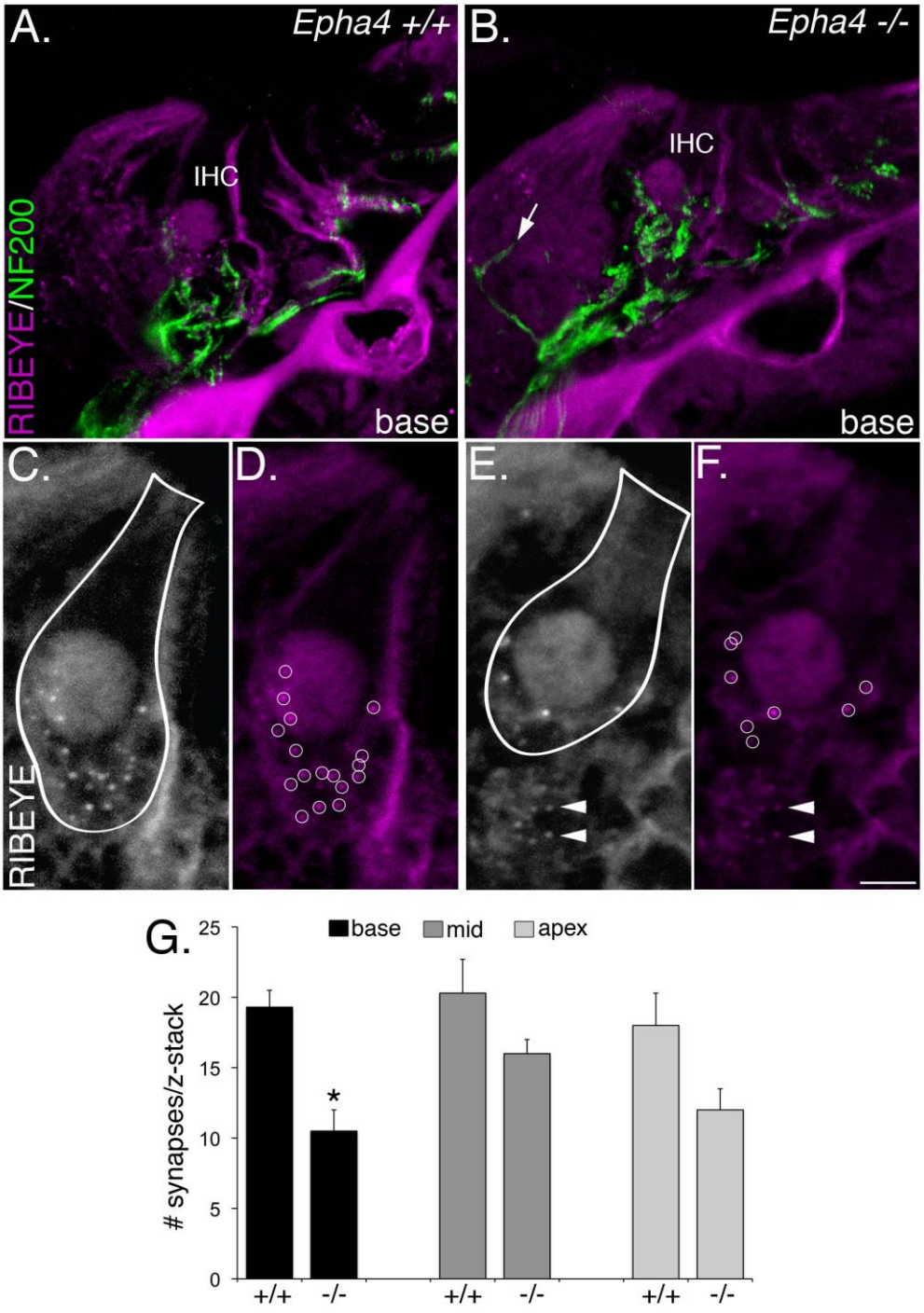

Figure S2, related to Figure 5. Ribbon synapse formation is impaired in *Epha4* null cochleae.

(A and B) Representative sections from *Epha4*$^{+/+}$ and *Epha4*$^{-/-}$ cochleae stained with Ribeye and neurofilament 200 antibodies. (B) Compared to the *Pou3f4*$^{y/-}$ mice, the *Epha4* mutants showed more severely defected axon routing toward the hair cells (see arrows), and, for reasons unknown, showed faint ectopic Ribeye-positive puncta in areas between the basement membrane and the inner hair cell (see arrowheads in E and F).

(C-F) High magnification views from A and B. The grayscale images show ~500 nm ribeye-postive puncta (ribbon synapses) at the base of the inner hair cell (outlined). The magenta image shows the ribbon synapses circled. The arrows in E and F point to ribeye-positive puncta that do not appear to be associated with the inner hair cell. Scale bar in F = 10 μm, A and B; 3.5 μm, C-F.

(G) Histogram comparing inner hair cell synapse numbers from sections from *Epha4*$^{+/+}$ and *Epha4*$^{-/-}$ mice at P7. Mean +/- SEM.

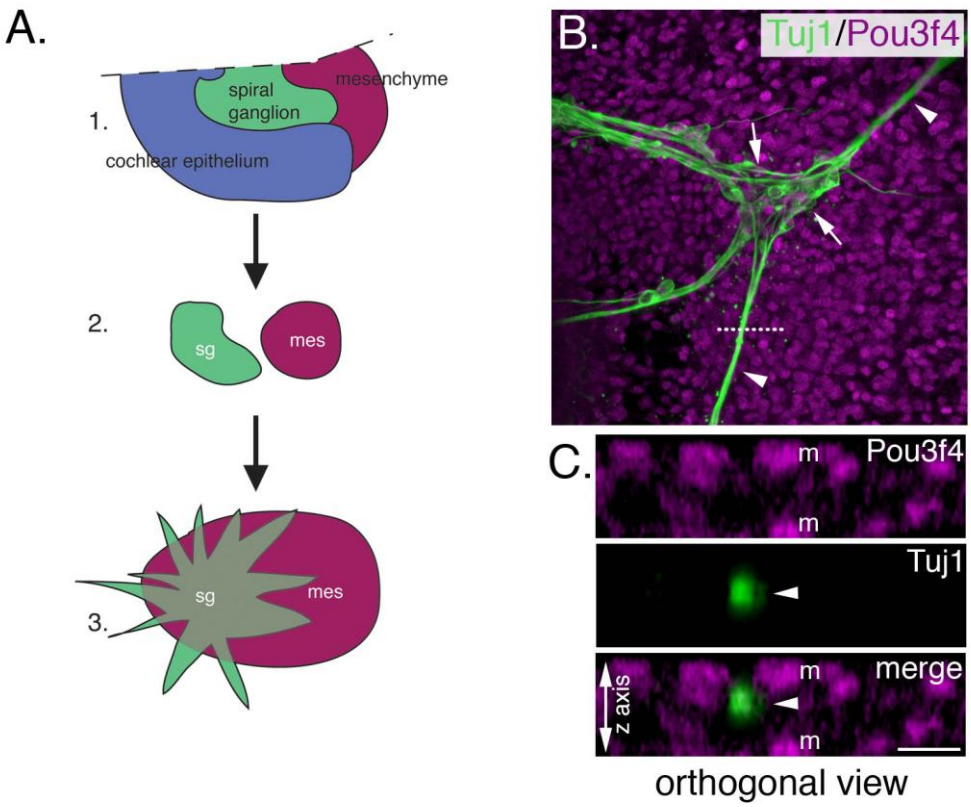

Figure S3, related to Figure 6. Spiral ganglion neuron and mesenchyme co-culture methods.

(A) Illustration of the culture scheme used for the experiments in Figure 6. Step 1: the cochlea including the cochlear epithelium (blue), spiral ganglion (green) and mesenchyme (red), is removed from the inner ear. Step 2: the spiral ganglion (sg) and mesenchyme (mes) are removed and cultured on Matrigel with approximately 1 mm of separation. Step 3: spiral ganglion neurons disperse and extend axons while the two cell populations intercalate.

(B) Representative image from a control culture stained with Tuj1 (neurons; green) and Pou3f4 (mesenchyme; magenta) antibodies. The arrows point to SGN soma and the arrowheads point to fasciculated axons.

(C) An orthogonal reconstruction from the dotted line in B demonstrating that the SGNs and mesenchyme cells interact in three dimensions in this assay. Note that Pou3f4-positive mesenchyme cells and the SGN axons interact in the Z axis; mesenchyme cells (m) are on top and below the SGN fascicle (arrowhead). Scale bar = 60 μm in B; 15 μm in C.

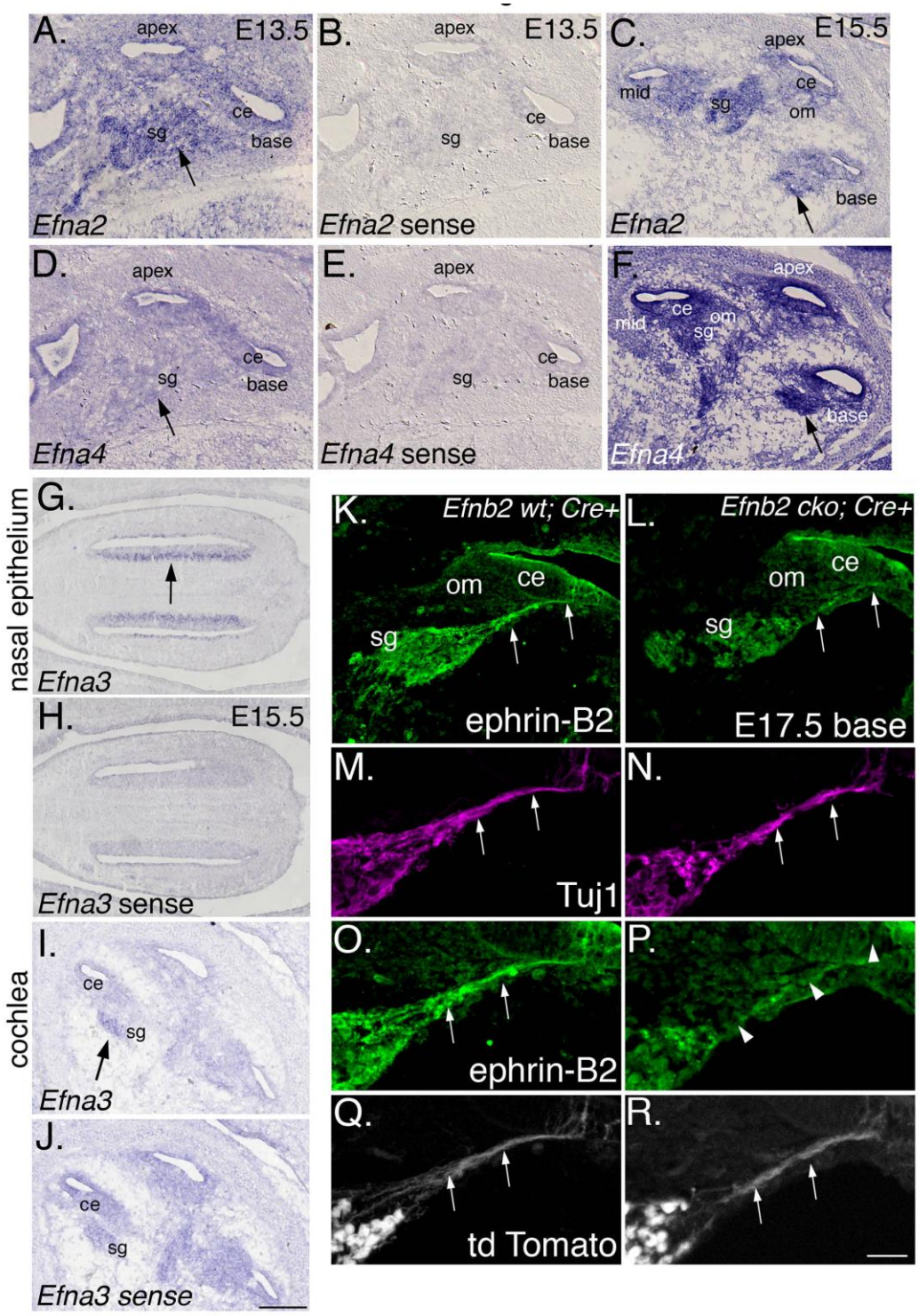

Figure S4, related to Figure 7. *Efna2, Efna3,* and *Efna*4 mRNA expression patterns in the cochlea by *in situ* hybridization and demonstration of ephrin-B2 reduction in conditional knock-out mice.

(A-C) *Efna2* mRNA expression at E13.5 and E15.5. Panel B, sense control probe. *Efna2* is expressed broadly at E13.5 (A) and is then expressed by a subset of cells in the spiral ganglion, cochlear epithelium and mesenchyme at E15.5.

(D-F) *Efna4* expression at E13.5 and E15.5. Panel E, sense control probe. *Efna4* is faintly detectable over controls at E13.5 in the cochlear epithelium (D) and then is expressed at high levels in the spiral ganglion, cochlear epithelium and mesenchyme E15.5 (F). At E15.5, *Efna4* appears to mark both spiral ganglion neurons and glia.

(G-J) *Efna3* expression at E15.5. Panels H and J, sense control probes. Panels G and H show *Efna3* expression in the nasal epithelium as a positive control. *Efna3* appears to be faintly detectable (I) over controls (J) in the spiral ganglion at E15.5 with little detectable expression elsewhere. Scale bar = 200 μm. ce, cochlear epithelium; sg, spiral ganglion; om, otic mesenchyme.

(K and L) Ephrin-B2 staining in *Efnb2 wt; Cre+* and *Efnb2 cko; Cre+* cochleae. ce: cochlear epithelium; sg: spiral ganglion; om, otic mesenchyme.

(M and N) Tuj1 staining in both genotypes showing the position of the SGNs and their peripheral axons (arrows).

(O and P) A high magnification view from K and L. The arrowheads in P point to the SGN axons where ephrin-B2 is almost completely absent.

(Q and R) td Tomato reporter expression showing equivalent Cre activity in both genotypes and a relatively equivalent presence of Cre+ SGN peripheral axons. Scale bar = 40 μm for K and L, 20 μm for M-R.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies used. Primary antibodies used in this study were as follows: mouse-anti-Tuj1, 1:500 (Covance); goat-anti-Sox10, 1:200 (Santa Cruz Biotechnology, Inc.), rabbit-anti-Pou3f4 (from Dr. Aimee K. Ryan, McGill University); rabbit-anti-cleaved caspase 3, 1:200 (Cell Signaling Technologies); rabbit-anti-Glur2/3, 1:150 (Millipore); mouse-anti-Ctbp2/Ribeye (BD Biosciences); goat-anti-EphA4 extracellular domain, 1:200 for expression studies and 1:500 for comparing wild-type and *Pou3f4* hemizygous tissues (R&D systems); chicken-anti-neurofilament 200, 1:5000 (Aves Labs); goat-anti-Ephrin-B2, 1:200 (Novus Biological); chicken-anti-GFP, 1:1000 (Aves Labs); rat-anti-Integrin α6, 1:250 (Serotec). 4',6-diamidino-2-phenylindole (DAPI; Roche) was used at 1:5,000. Alexa-633-phalloidin (Invitrogen) was used at 1:100. We also raised a chicken-anti-Pou3f4 antibody using amino acids 158-179 from the mouse Pou3f4 protein sequence (Aves Labs). This antibody was used at 1:10,000.

***In situ* hybridization probes.** The following cDNA templates were used to generate antisense *in situ* hybridization probes: *Epha4*, bases 2782-4242 (NM_007936); *Efnb2*, bases 136-1188 (NM_010111); *Efnb3*, bases 747-1137 (BC052001); *Efna1*, bases 51-744 (NM_010107.4) *Efna2*, bases 824-1804 (NM_007909.3); *Efna3*, bases 483-1221 (BC125003.1); *Efna4*, bases 16-763 (U90663.1); and *Efna5*, bases 185-886 (NM_207654.2).

Quantitative PCR primers. Quantitative PCR was performed as described (Driver et al., 2008) using cDNA derived from whole cochleae at E14.5, but with the following *Epha4*-specific primer sets: ATGTGATCAAAGCCATCGAG (forward) and GTCCAGCATGTTGACGATCT (reverse). 36B4 primers (ribosomal protein) were used as an internal control: CACTGGTCTAGGACCCGAGAAG (forward) and GGTGCCTCTGGAGATTTTCG (reverse). The following forward and reverse genomic primers were used after ChIP: for *β-actin*, GCCCTCTTTTGTGCCTTGATAG and TAAAGGGTCACTTACCTGGTGC; for *Neurogenin-1*, TGGTGTCGTCGGGGAACGAG and

AAGGCCGACCTCCAAACCTC. For *Epha4*: site 1, TTAGCTTTGCACTCAACACG and CAATAAGCCTGAGCCAACAG; site 2, AATGTGCACAACCCAGAACT and TTCTCATCACCCCCTCTCTT; site 3, AGATGCTCTTCTGTCGATGC and ACTGGAAGAGCACAGGGTAA; site 4, CTTCCTTCCTTCTTCCCTGA and GTTTCAAGGCAGTGATGCTC; site 5, GACTGTGTCCAGCAACCATT and GATGTCGGTTTTAATCCTAACAG. Primers for the *Epha4* negative control site (between exons III and IV) that does not contain the putative ATTATTA consensus region were: GACGTTAGCACGGACCTAGC and GCGCTCCACACATAATGCT.

Quantifications. Student's two-tailed t-tests were used throughout this study. To quantify fasciculation in *Pou3f4*, *Epha4*, and *Efnb2 cko* mutant lines, 5 μm confocal Z-stacks were processed using NIH-ImageJ. For each sample, the fluorescence intensity threshold was adjusted such that the space occupied by axons was distinguishable from adjacent areas. The areas of segments unoccupied by axons were quantified as individual particles, and the cumulative value for all particles were subtracted from the total area. The quantification was restricted to the area between the lateral edge of the SGN soma and the medial edge of the epithelium. In addition, we counted the number of processes that crossed between fascicles. To compare SGN density between $Pou3f4^{y/+}$ and $Pou3f4^{y/-}$ embryos, whole mount preparations stained with anti-Tuj1 antibodies and numbers of SGN soma were counted manually and then normalized to the length of the region of interest along the longitudinal axis of the cochleae. For the 24 h Matrigel outgrowth assays, bundle diameter was measured manually; for consistency, measurements were taken at the narrowest point of the bundle before the first branch. Staining, acquisition and threshold settings were identical within all groups of samples in this study. Sample number (n) was $\geq$ 6 throughout.

To compare apoptosis at E16.5, 12 μm sections were stained with both DAPI and cleaved caspase 3 (CC3) antibodies. Numbers of CC3-positive cells were normalized to the counted area. To quantify ribbon synapses, cross sections were acquired from embedded postnatal cochleae then stained with anti-Ribeye and anti-neurofilament antibodies. Immunostaining on sections rather than whole mounts was used in this

study because it most easily allowed the visualization of the cochlear base. 14 X 0.3 μm confocal z-stacks were acquired and synapse numbers were tabulated manually. For each group, at least three different animals were used, and three-to-five sections per animal were counted.

To quantify fascicle diameter in the SGN/otic mesenchyme culture experiments, confocal z-stacks of fixed and immunostained SGNs were acquired at 20X then projected using ImageJ. At the top, middle and bottom of each image, a line was drawn and the diameter of any fascicle crossing those lines was measured. For each treatment group, n = 10; 10-100 fascicles per n. With minor exception: for the 1 μM Pou3f4 MO group, n = 5.

cDNA Microarray comparing *Pou3f4* wild type and null mesenchyme (data not shown). For these experiments, mesenchyme was mechanically removed from E15.5 *Pou3f4* wild type and null cochleae. From the left and right ears combined, total RNA was generated using the Arcturus Pico-pure kit, then cDNA was synthesized using Superscript III (Invitrogen). cDNA samples from 2 wild type and 2 null embryos were then used to probe individual mouse whole genome expression arrays (Affymetrix). The Genomics Core Facility of the National Institute of Diabetes and Digestive and Kidney Diseases performed the hybridization and analysis of these arrays. Results from this array study were confirmed using quantitative PCR.