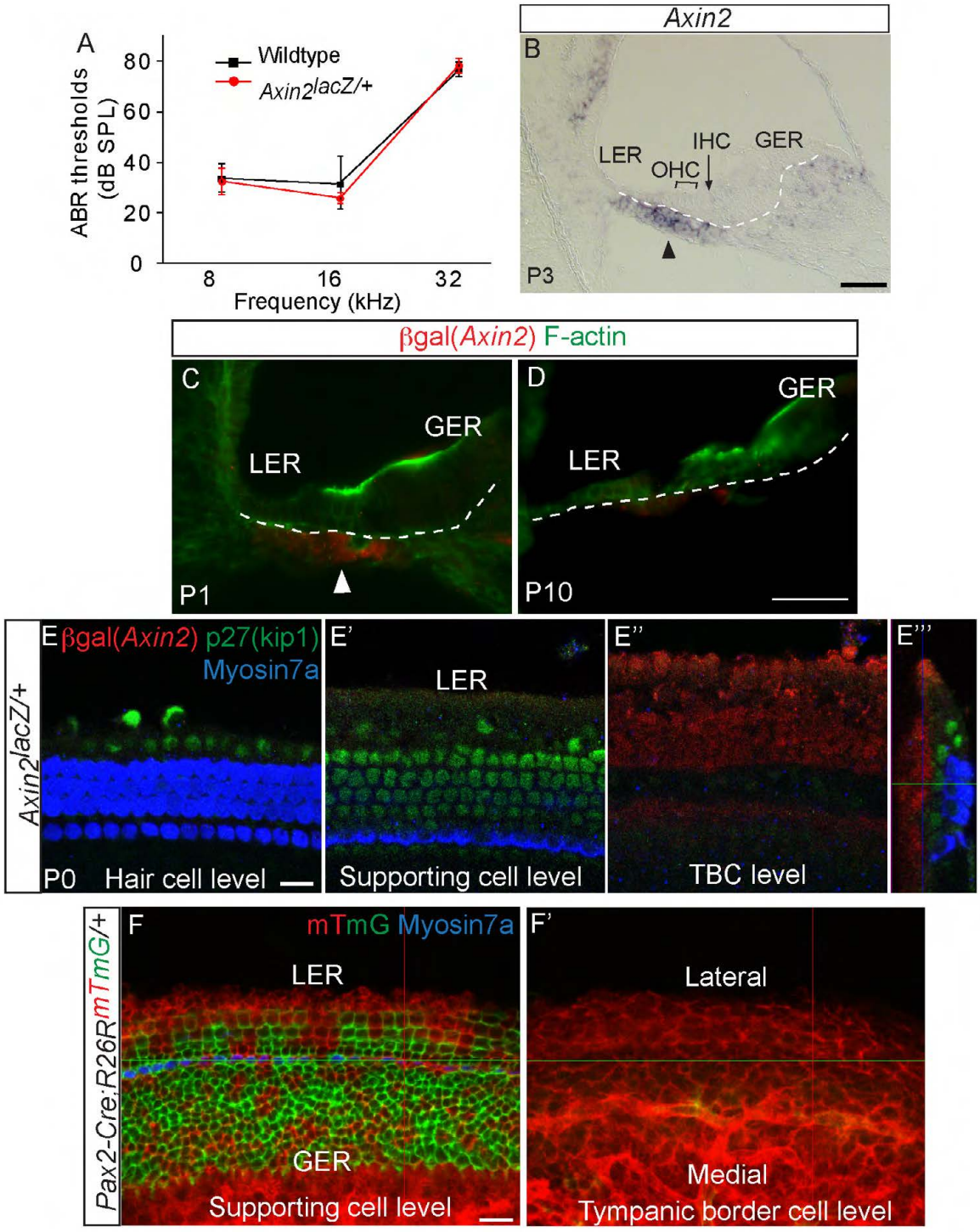


Fig. S1. *Axin2* expression and auditory function of *Axin2*$^{lacZ/+}$mice. (**A**) Thresholds of auditory brainstem responses (ABRs) were measured in P30 *Axin2*$^{lacZ/+}$ mice (*n*=6) and their wild-type (*n*=5) littermates (both CD1 background). Pure tone auditory stimuli were presented at 8, 12 and 32 KHz, and no significant threshold shift was detected. (**B**) Representative image of a cryosection of the cochlea from a P3 wild-type mouse. *In situ* hybridization experiments demonstrated robust *Axin2* expression in tympanic border cells (TBCs) (arrowhead) beneath the sensory epithelium (dotted line). A low level of *Axin2* expression was also noted on the lateral cochlear wall and spiral limbus. (**C**,**D**) Anti-β-galactosidase and FITC-conjugated phalloidin were used to label cryosections of cochleae from P1 and P10 *Axin2*$^{lacZ/+}$mice. In the P1 cochlea, intense *Axin2*^{lacZ} expression was noted in TBCs (arrowhead), corroborating the *in situ* hybridization and X-gal staining results (Fig. 1A, Fig. 2H-J). When P1 and P10 cochleae were stained and imaged in identical fashion, a reduction in *LacZ* expression in TBCs was observed. (**E-E**′′′) Whole-mount preparation of P0 *Axin2*$^{lacZ/+}$ cochlea demonstrates β-gal expression specific to TBCs. E′′′ shows a reconstructed side view of *z*-stack confocal images. (**F**,**F**′) Representative confocal images of whole-mount cochleae from P1 *Pax2-Cre;R26R*$^{mTmG/+}$ mice are shown. Many, but not all, supporting cells exhibit mGFP labeling, thus deriving from the *Pax2* lineage. This pattern of tracing is similar to that of sensory hair cells (Fig. 1J). No mGFP-positive cells were observed in the TBC region. LER, lesser epithelial ridge; GER, greater epithelial ridge; OHC, outer hair cells; IHC, inner hair cells. Scale bars: 50 μm in B-D; 25 μm in E-F′. Data are mean±s.d.

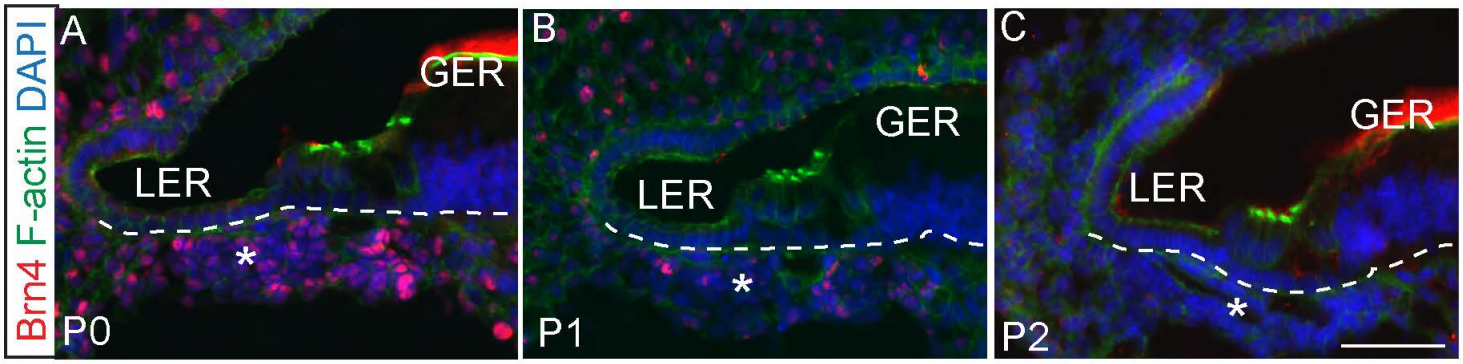


Fig. S2. Expression of Brn4 in the postnatal cochlea. (**A-C**) Robust expression of Brn4 in tympanic border cells (TBC, asterisk) is noted in the P0 cochlea, and expression is remarkably downregulated in the P1 cochlea and undetectable in the P2 cochlea. GER, greater epithelial ridge; LER, lesser epithelial ridge. Scale bars: 50 μm in A-C.

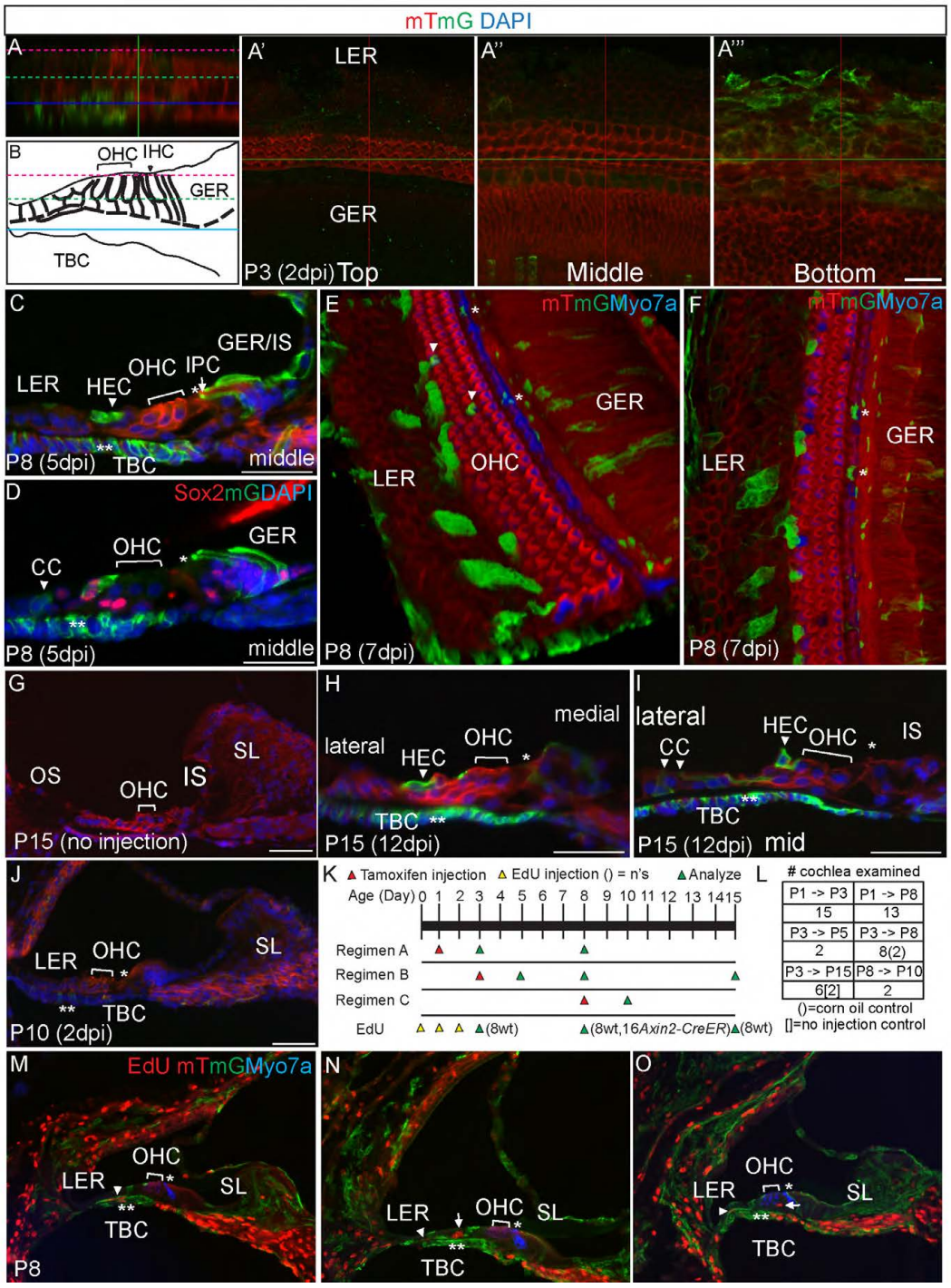


Fig. S3. Lineage tracing of *Axin2*-positive cells. (**A**,**B**) A single dose of tamoxifen administered to *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ pups at P1 resulted in restricted mGFP-positive expression among TBCs 2 days later. Shown are confocal images at three different focal planes, as well as the reconstructed cross-sectional image of whole-mount cochlea. (**C**,**D**) Cryosections of traced cochleae. At 5 DPI, traced cells included various supporting cells, a subset of which expressed Sox2. (**E**,**F**) mGFP-positive traced cells included outer (arrowheads) and inner (asterisks) hair cells in the P8 cochlea, 7 days after tamoxifen injection. Shown are 3D reconstruction of confocal images of whole-mount cochleae. (**G**) No mGFP reporter activity was seen without tamoxifen injection or in corn oil-only control (Fig. 3G). (**H**,**I**) mGFP-positive traced cells remain integrated in the organ of Corti in the P15 cochlea. Traced TBCs (double asterisks) have transitioned into a one- or two-cell layer (also see Fig. 2A-C). (**J**) Tamoxifen injection at P8, when *Axin2* expression level decreased from early ages (Fig. 2H-J), yielded mGFP reporter activity among TBCs, but none in the SE, in the P10 cochlea. (**K**) Experimental paradigms employed for lineage tracing and label retention experiments. (**L**) Table summarizing the number of cochlea analyzed in various tracing paradigms. (**M-O**) To track proliferating *Axin2*-positive cells, we injected EdU (P0-2) and tamoxifen (P1) into *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ pups. Approximately 67% of animals survived this regimen. Injected mice were analyzed at P8 mice and their cochleae rarely showed mGFP-positive, EdU-positive cells (arrowheads) in the SE. We also noted EdU-positive, mGFP-negative cells (arrows in N,O) in the SE, which could have derived from untraced *Axin2*-positive TBCs or other proliferating cell types. CC, Claudius cells; HEC, Hensen's cells; DC, Deiters' cells; PC, pillar cells; IPC, inner phalangeal cells; LER, lesser epithelial ridge; OS, outer sulcus; SL, spiral limbus; OHC, outer hair cells; wt, wild type. Single asterisk indicates IHC; double asterisks indicate TBCs. Scale bars: 15 μm in A; 25 μm C-J,M-O.

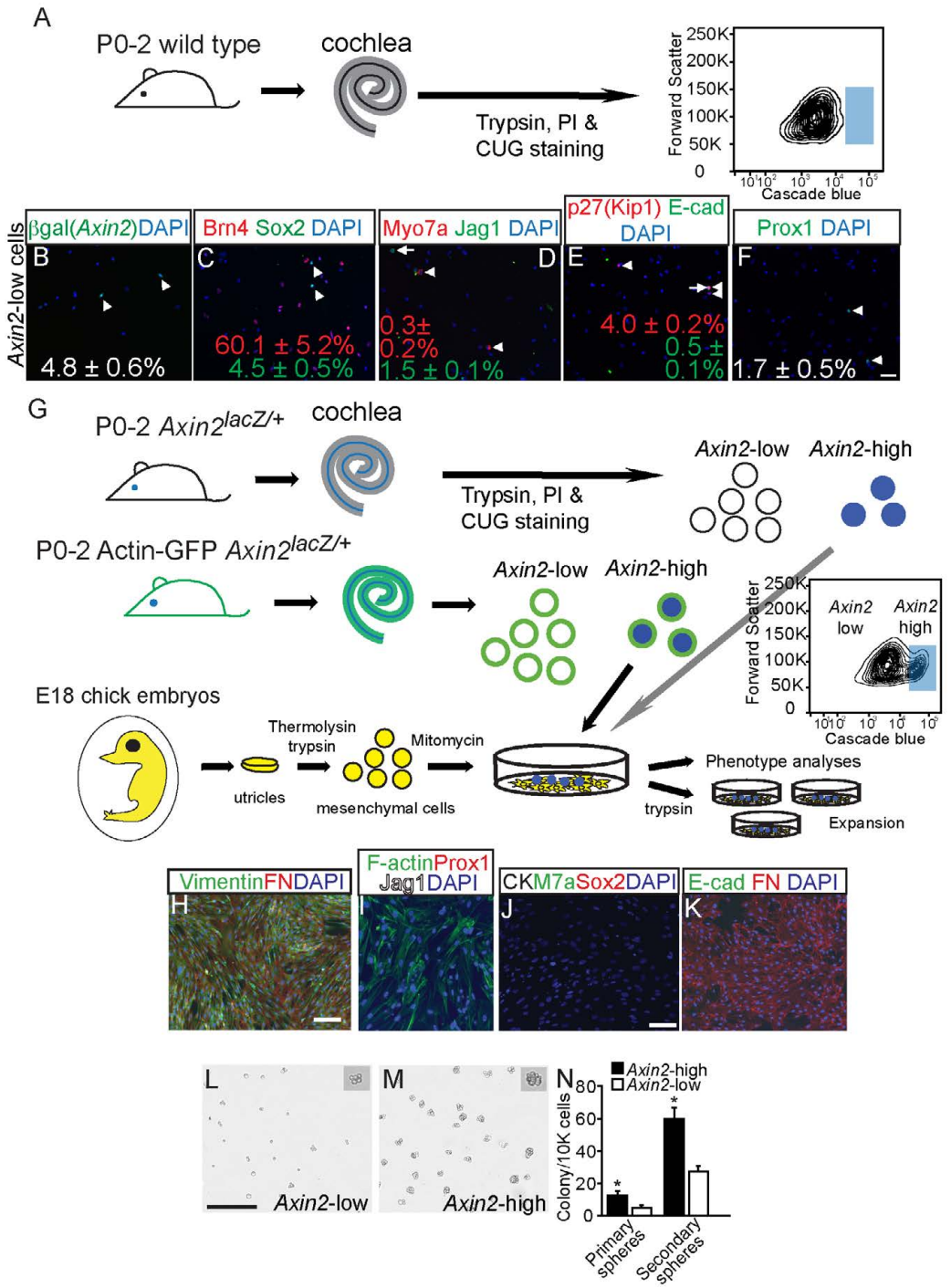


Fig. S4. Colony formation assays and expression profile of chicken mesenchymal feeder cells. (**A**) Schematic diagram of cochlear dissociation and flow cytometry. To isolate the *Axin2*-positive cochlear cells, cochleae from P0-2 *Axin2*$^{lacZ/+}$ mice were harvested, dissociated and stained. The top 16.1±0.6% and bottom 23.2±2.3% CUG-labeled cells were selected and analyzed for relative *Axin2* expression via quantitative RT-PCR, which found that the CUG-positive cells had 46-fold higher *Axin2* expression levels than the CUG-negative cells (Fig. 4H). (**B-F**) Immunostaining of isolated *Axin2*^{hi} cells shows that they are highly pure (Fig. 4C-F) and that the *Axin2*^{lo} cells represent a mixed population of fewer than 5% *LacZ*-positive cells (arrowhead in B), 60% Brn4-positive cells and SE cells [Sox2- (arrowhead in C), myosin 7a- (arrowhead in D), Jag1- (arrow in D), p27 (Kip1)- (arrowhead in E), E-cadherin- (arrow in E) or Prox1-positive (arrowhead in F)] (n=4 with 2500-5000 DAPI-positive cells analyzed). (**G**) Schematic representation of the flow cytometry experimental design as described in detail in the Materials and methods section. Flow cytometry plot on the right is representative of CUG-labeled cells from *Axin2*$^{lacZ/+}$ with the blue gate demonstrating the *Axin2*-positive cell population. (**H-K**) Chicken mesenchymal cells prepared as in G expressed vimentin and fibronectin (FN), but not cytokeratin, myosin 7a, Prox1, Sox2, jagged 1 or E-cadherin. (**L-N**) Using an established floating sphere formation assay, we found that the *Axin2*^{hi} cells consistently formed approximately three times more primary spheres than the *Axin2*^{lo} population after 5 days *in vitro* (P<0.001, n=3). When primary spheres derived from the *Axin2*^{hi} cells were dissociated and re-cultured, they expanded with greater efficiency than those formed from the *Axin2*^{lo} cells, with the rate of increase twofold higher for the *Axin2*^{hi} than the *Axin2*^{lo} cells (n=4). Scale bars: 25 μm in B-F; 50 μm in H-K; 250 μm in L,M. Data are mean±s.d. Asterisks denote statistical significance.

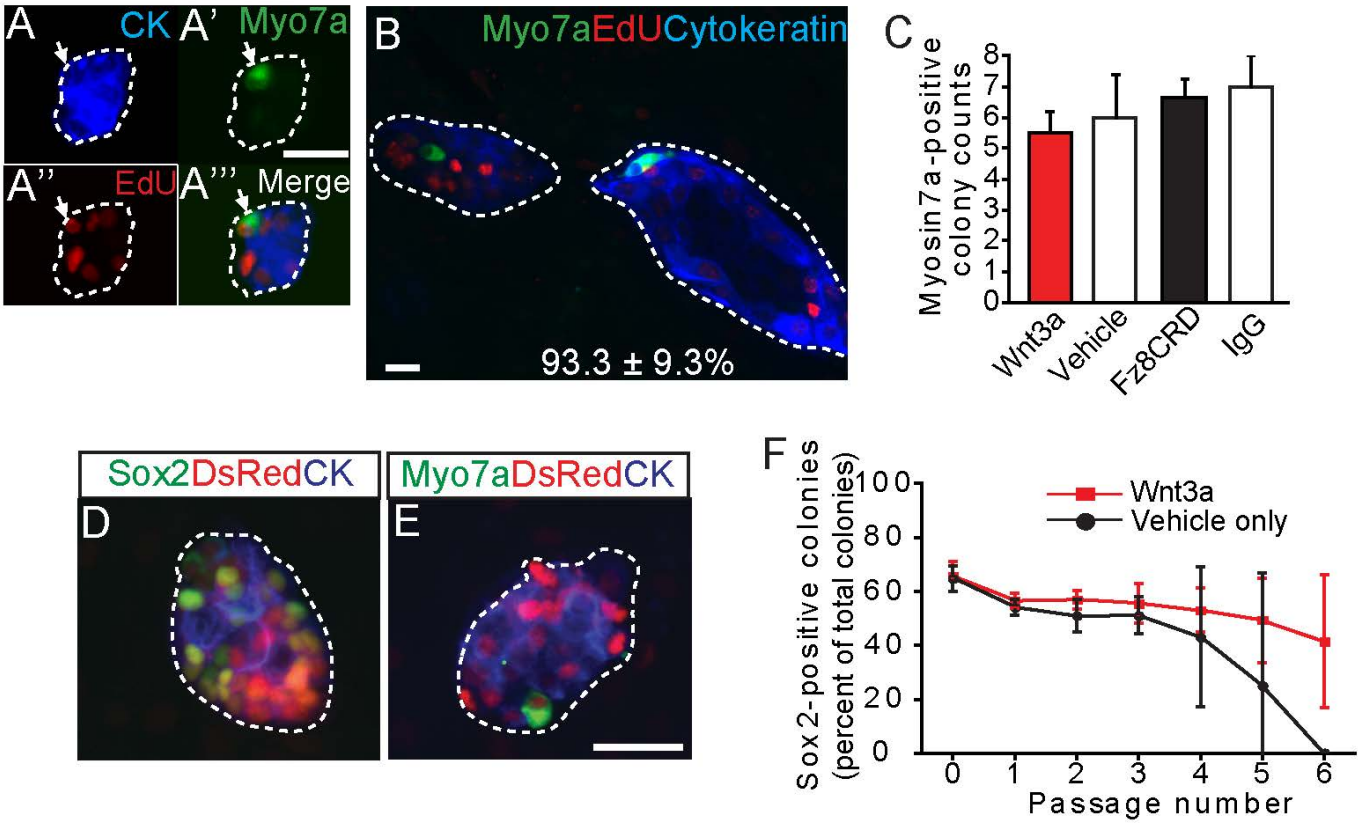


Fig. S5. Characteristics of colonies of *Axin2*^{hi} cells. (**A-B**) When isolated *Axin2*^{hi} cells were cultured in the presence of EdU (first 3 of 10 days of culture), most myosin 7a-positive hair cells were EdU negative (n=11). (**C**) Purified *Axin2*^{hi} cells were cultured in the presence of Wnt3a, drug vehicle, Fz8CRD or IgG on feeder cells for 10 days. The number of colonies containing myosin 7a-positive hair cells was not significantly different among treatment groups (n=3 for each treatment condition). (**D**,**E**) When *Axin2*^{hi} cells isolated from *Actin-DsRed*-positive *Axin2*$^{lacZ/+}$cochleae were cultured on chicken mesenchymal cells for 7 days, all Sox2-positive and myosin 7a-positive cells were also *Actin-DsRed*-positive, indicating that they are derived from mouse tissues. (**F**) Colonies from the *Axin2*^{hi} cells were incubated in Wnt3a or drug vehicle and passaged for multiple generations. Although the percentage of Sox2-positive expanded colonies did not significantly differ between the two treatment groups (P=0.19, one-way ANOVA; n=4), that of the vehicle treated group declined after several passages (median Sox2-positive cell number in *Axin2*^{hi} colonies = 21 after one passage, 22 after two passages, 32 after three passages, 29 after four passages, 31 after five passages and 13 after six passages). A decline in myosin 7a-positive colonies was also observed after passaging with or without Wnt supplementation. Specifically, we observed 8.9±0.4% and 10.0±2.1% myosin 7a-positive colonies in Wnt3a- and vehicle-treated groups, respectively, after 0 passage. These percentages tapered to 5.0±1.2 and 5.3± 2.5% after one passage, and 1.2±1.5% and 1.6±3.1% after two passages (median myosin 7a-positive cell number in *Axin2*^{hi} colonies = one after both 1 and 2 passages). We did not observe any myosin 7a-positive colonies in generations 4 through 7. Scale bars: 25 μm. Data are mean±s.d.

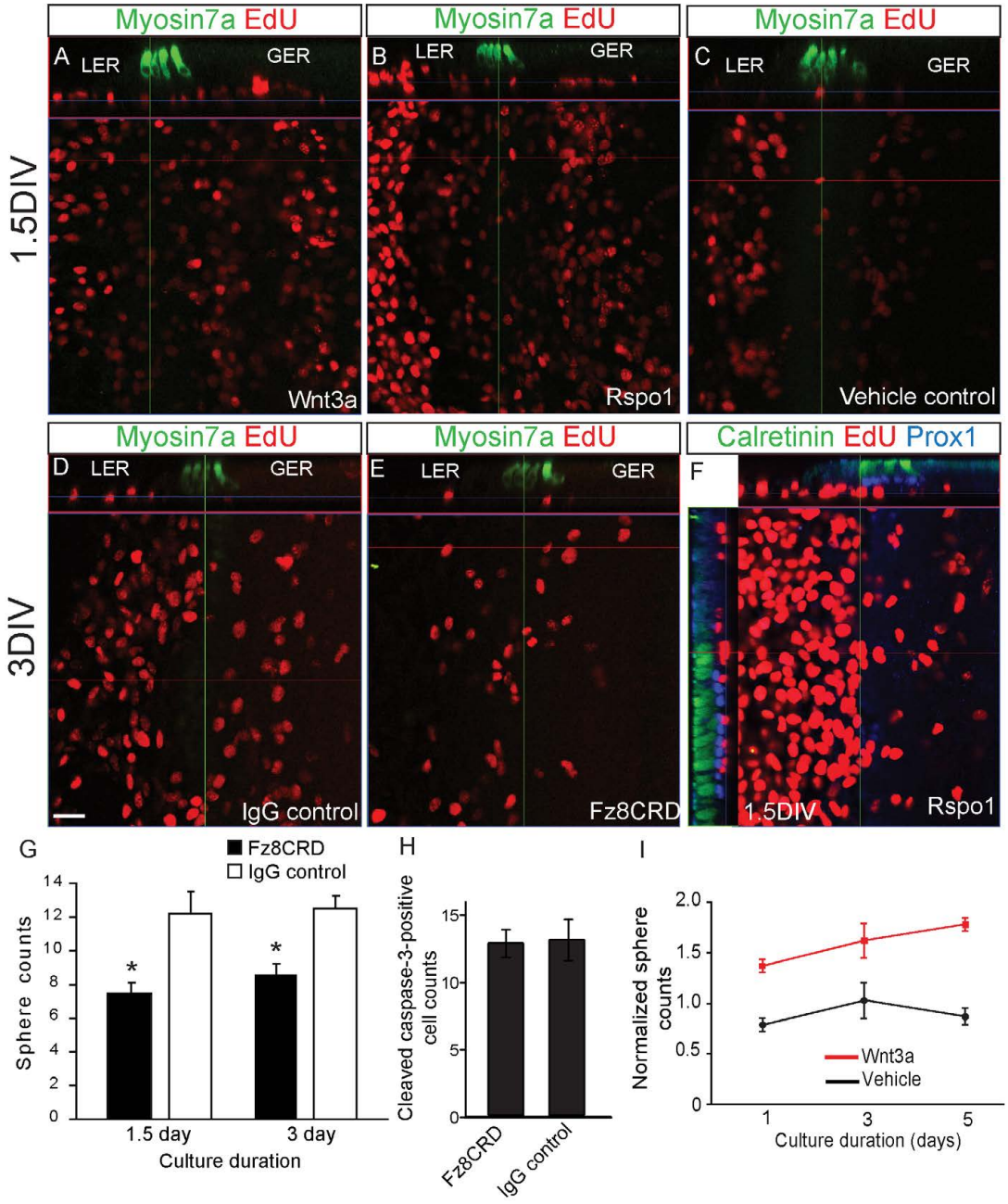
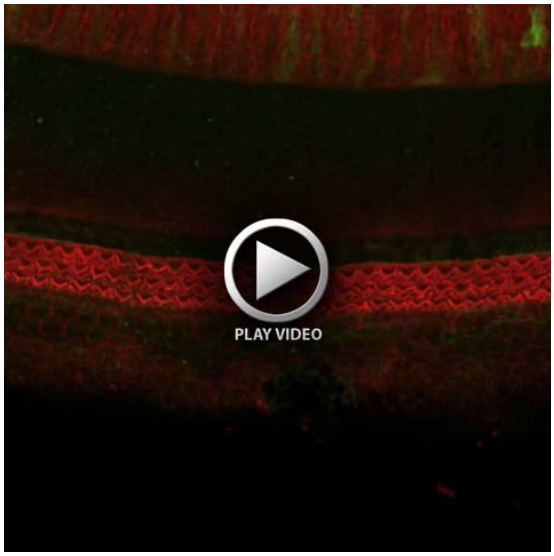
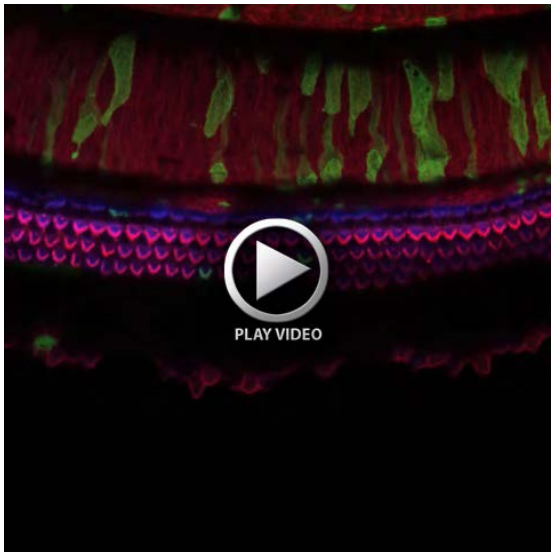


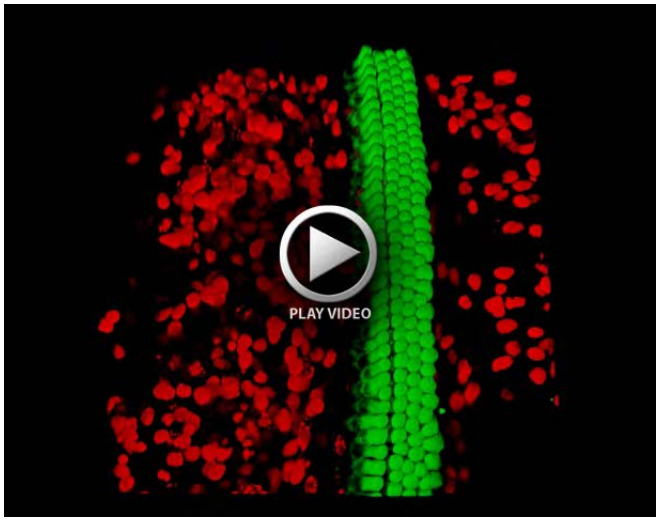
Fig. S6. Wnt proteins selectively increased proliferation among cochlear TBCs. (**A-C**) Representative confocal images of P2 wild-type cochleae cultured for 36 hours with EdU present during the last 12 hours. Both reconstruction of *z*-stack images (top) and images captured at the level of TBCs are shown. Purified Wnt3a or R-spondin 1 dramatically upregulated proliferating cells in this region. Vehicle-only control demonstrated a level of proliferation comparable with that seen *in vivo* (Fig. 2A). (**D**,**E**) Shown are representative confocal images of P2 wild-type cochleae cultured for 3 days with EdU present during the last 12 hours. Proliferation among TBCs was dependent on endogenous Wnt signaling, as treatment with Fz8CRD suppressed EdU labeling among TBCs after a 3-day culture period. (**F**) Whole cochleae from P2 wild-type mice were incubated with R-spondin 1 for 36 hours with EdU present during the last 12 hours, and then immunostained for the hair cell marker calretinin, supporting cell marker Prox1 and EdU. Two different views of reconstructed *z*-stack confocal images along with the image captured at the level of TBCs are shown. As in native cochlear tissues where proliferation was restricted to the TBC region, upon treatment with Wnt agonists, only TBCs were competent to upregulate proliferation. (**G**) P0-2 wild-type cochleae were treated with the Wnt inhibitor Fz8CRD or IgG control for 1-3 days, then dissociated into single cells and analyzed for floating sphere formation. Significantly fewer spheres were generated from cochleae treated with Fz8CRD ($P<0.0001$, one-way ANOVA; $n=21$-28). (**H**) Whole cochleae (P0-2) treated with Fz8CRD for 3 days were examined for expression of cleaved caspase 3, a marker of apoptosis. Labeled cells in the TBC region were quantified. No significant difference was observed between the Fz8CRD and IgG-treated cochleae ($n=3$). (**I**) P0-2 wild-type cochlear cultures treated with Wnt3a or vehicle only for 1 to 5 days were dissociated and assessed for their floating sphere-forming capacity. Sphere counts were normalized to untreated media controls in corresponding culture durations. Wnt3a treatment exerted a significant effect on the ability of the cochleae to proliferate through floating sphere formation ($P<0.01$; $n=12$). When this treatment was lengthened to 5 days, we noted an increasing proliferative capacity, whereas corresponding vehicle-only control remained constant. DIV, days *in vitro*; LER, lesser epithelial ridge; GER, greater epithelial ridge. Scale bars: 30 μm. Data are mean±s.d. Asterisk indicates statistical significance.



Movie 1. *Z*-stack of confocal images of whole-mount cochlea from P3 *Axin2*$^{CreERT2/+}$; *R26R*$^{mTmG/+}$ mice, 2 days post tamoxifen injection. Red, mTomato; green, mGFP.



Movie 2. *Z*-stack of confocal images of whole-mount cochlea from P8 *Axin2*$^{CreERT2/+}$; *R26R*$^{mTmG/+}$ mice, 7 days post tamoxifen injection. Red, mTomato; green, mGFP; blue, myosin 7a.



Movie 3. 3D reconstruction of confocal images of whole-organ cochlea from P3 wild-type mice cultured in the presence of Wnt3a and EdU for 36 hours. Red, EdU; green, myosin 7a.

Table S1. Wnt proteins promote proliferation among *Axin2*^{hi} but not *Axin2*^{lo} cells‡

	Axin2^{hi} colonies				*Axin2*^{lo} colonies			
Treatment	**Wnt3a**	**Vehicle**	**Fz8CRD**	**IgG only**	**Wnt3a**	**Vehicle**	**Fz8CRD**	**IgG only**
Cytokeratin^{+} colonies	57.3±8.0	58.3±3.1	44.6±8.7	48.5±8.5	9.7±2.9	9.3±1.2	10.3±1.7	11.0±1.4
Ki67^{+} colonies	35.7±5.7*	22.7±2.5	12.0±2.7**	21.5±4.4	5.7±0.4	6.3±0.6	4.3±1.0	4.8±0.5
Total Ki67^{+} cells	323.4±0.9**	168.1±0.7	84.7±8.7**	203.7±12.4	15.5±0.4	17.9±0.2	12.5±1.5	14.5±0.8

‡Shown values represent mean±s.d. of three to five independent experiments. *Axin2*^{hi} and *Axin2*^{lo} cells isolated through flow cytometry were incubated in the above reagents (Wnt3a 200 ng/ml and Fz8CRD 25 μg/ml) for 10 days *in vitro*.

*P<0.05, **P<0.001 (comparisons between drug and vehicle controls).

Table S2. Wnt agonists increased Ki67-marked proliferating tympanic border cells in cochlear explants[‡]

	Wnt3a (200 ng/ml)	Vehicle control	R-spondin1 (1 μg/ml)	Control
1 DIV	146.3±7.0*	81.3±8.0	152.3±10.0*	84.0±6.2
3 DIV	152.7±12.0*	78.3±5.5	158±10.1*	80.3±9.0
5 DIV	132.3±9.9*	64.3±7.6	135.3±8.6*	67.3±7.0

[‡]Shown values represent mean±s.d. of triplicate experiments.

*P<0.001; DIV=days *in vitro*.