Supporting Information

Shi et al. 10.1073/pnas.1219952110

SI Materials and Methods

TOPGAL Expression in the Developing Cochlear Duct. TOPGAL mice are used to detect β-catenin signaling by activation of a transgene in which the *LacZ* gene is under the control of a regulatory sequence with three consensus Tcf/Lef-binding motifs upstream of a minimal c-fos promoter (1). TOPGAL mice in a CD1 background were used in this study. β-Galactosidase was detected in cochleae of TOPGAL mice but not in the wild-type littermate (Fig. S1*A*). At E15, broad expression was seen in the stromal tissues, sensory epithelium, and cochlear wall (Fig. S1*B*). By E18, TOPGAL activity was restricted to the sensory epithelium and underlying stromal tissue (Fig. S1*C*). In postnatal animals, β-galactosidase expression largely decreased in the sensory epithelium but remained strong in the cochlear wall, basilar membrane, and spiral lamina (Fig. S1 *D* and *E*). By 2 mo, β-galactosidase could not be detected in the cochlea (Fig. S1*F*), correlating with the lack of bone remodeling in the cochlea.

Expression of β-Catenin in Sox2-Expressing Cochlear Cells. Tamoxifen was given at P1 to a *β-catenin*$^{flox(exon3)}$ mouse crossed to a *Sox2-Cre-ER* mouse to express *β-catenin* constitutively in all supporting cells. Cochleae were dissected at P5 and immunostained using an antibody to an activated form of β-catenin, phosphorylated on Y489 (2). The antibody detected β-catenin in Sox2-espressing cells but not in Sox2-negative hair cells (Fig. S2).

FM 1-43 Uptake by Cells Resulting from β-Catenin Overexpression. FM 1-43, a fluorescent dye, enters hair cells through mechanotransduction channels. FM 1-43 accumulated in both inner and outer hair cells of the wild-type organ of Corti. We used the *Sox2-Cre-ER* mice to activate β-catenin constitutively in all supporting cells by crossing to a *β-catenin*$^{flox(exon3)}$ mouse. We administered tamoxifen at P1 and dissected the cochlea at P5. FM 1-43 uptake was observed in hair cells generated in the expanded pillar cell region after β-catenin expression (Fig. S3 *A* and *B*).

Hair Cell Marker Expression in the Pillar Cell Region. New hair cells were analyzed at P9 for expression of outer and inner hair cell markers upon β-catenin activation at P1 in Sox2-expressing supporting cells. No ectopic hair cells were seen in wild-type littermates (Fig. S4*A*). At P9, in the β-catenin–expressing organ of Corti, cells in the pillar cell region proliferated and incorporated EdU, and ectopic cells adjacent to inner hair cells had incorporated EdU and were prestin-positive (Fig. S4*B*). Some ectopic cells adjacent to outer hair cells expressed both VGLUT3 and prestin (Fig. S4*C*).

β-Catenin Expression in Sox2-Expressing Cells. After overexpression of β-catenin by administration of tamoxifen at P44 and a 5 d survival, the cochlea from a *Sox2-Cre-ER*/*β-catenin*$^{flox(exon3)}$ mouse was examined and compared with an age-matched control cochlea. No ectopic hair cells or EdU incorporation were seen in a mouse overexpressing β-catenin in supporting cells.

β-Catenin Expression in Lgr5-Expressing Cells. We overexpressed β-catenin in postmitotic Lgr5-positive cells at E16.5 by administration of tamoxifen and examined the cochlea for Lgr5 expression at P0. After birth the *Lgr5-Cre-ER* mouse yielded lower proliferation than the *Sox2-Cre-ER* mouse when crossed to the *β-catenin*$^{flox(exon3)}$ mouse. Forced expression of β-catenin initiated an expansion of supporting cells between inner and outer hair cells. Only some of the pillar cells divided, likely due to incomplete Cre activity in this mouse (3), and as a result, expanded pillar cells appeared as clumps after Lgr5-Cre mediated overexpression (Fig. 4*A*). Lgr5-positive clumps containing 30–60 cells extended from the base to the apex of the cochlea (Fig. S6 *A* and *B*).

1. Vasioukhin V, Degenstein L, Wise B, Fuchs E (1999) The magical touch: Genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc Natl Acad Sci USA* 96(15):8551–8556.
2. Rhee J, Buchan T, Zukerberg L, Lilien J, Balsamo J (2007) Cables links Robo-bound Abl kinase to N-cadherin-bound beta-catenin to mediate Slit-induced modulation of adhesion and transcription. *Nat Cell Biol* 9(8):883–892.
3. Barker N, et al. (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449(7165):1003–1007.

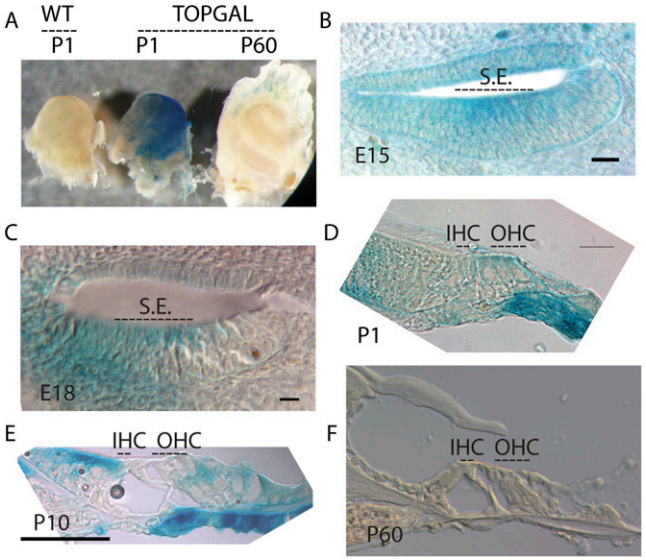

Fig. S1. TOPGAL expression in the developing and postnatal cochlea. (*A*) β-Galactosidase was detected with X-Gal staining. Cochleae of TOPGAL mice and wild-type littermates were stained with X-Gal. (*B*) At E15, broad expression was seen in the stromal tissues, sensory epithelium, and cochlear wall. (*C*) By E18, β-galactosidase expression was restricted to the sensory epithelium and underlying mesenchymal tissue. (*D*) At early postnatal stages, β-galactosidase expression largely decreased in the sensory epithelium. (*E*) At P10 β-galactosidase expression remained strong in mesenchymal tissue under the basilar member. (*F*) By 2 mo, no β-galactosidase could be detected in the cochlea. IHC, inner hair cell; OHC, outer hair cells; S.E., sensory epithelium. (Scale bar, 20 μm.)

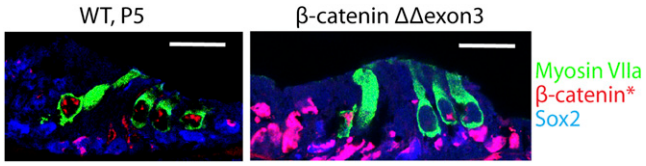

Fig. S2. Forced in vivo expression of β-catenin in Sox2-expressing cells. Administration of tamoxifen to *Sox2-Cre-ER:β-catenin*$^{flox(exon3)}$ mice was used to activate β-catenin in all supporting cells at P1. Detection with an antibody to Y489 phosphorylated (*) β-catenin (2) indicated that β-catenin was elevated in Sox2-expressing cells at P5 compared with the wild-type littermates. Hair cells were stained for myosin VIIa.

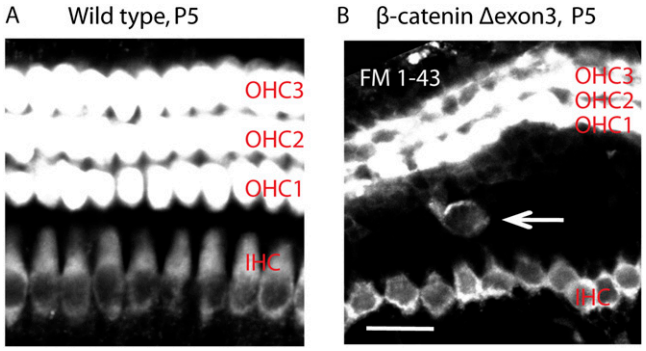

Fig. S3. Mechanotransduction channels in new hair cells. (*A*) FM 1-43 accumulated in both inner and outer hair cells in the freshly isolated organ of Corti from a wild-type mouse. (*B*) New hair cells in the expanded pillar cell region after induction of β-catenin overexpression took up FM 1-43 (arrow).

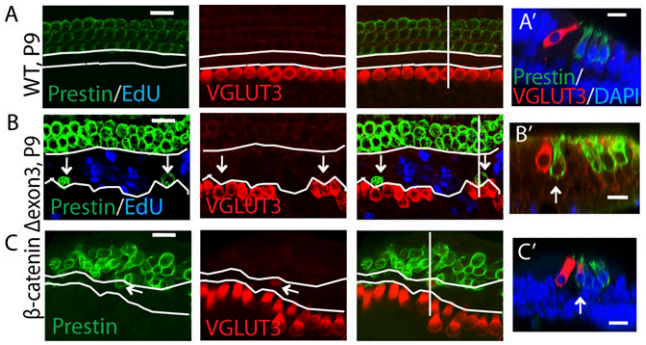

Fig. S4. Expression of hair cell markers in newly generated hair cells. (*A*) In the wild-type animal, prestin and VGLUT3 were expressed in outer and inner hair cells, respectively. No Edu incorporation was observed in hair cells or supporting cells at P9. (*A′*) An *x–z* scan of *A* at the white line showed inner and outer hair cells. (*B*) At P9, ectopic cells adjacent to inner hair cells induced by β-catenin expression had incorporated EdU and were prestin-positive (arrow). (*B′*) An *x–z* scan of *B* at the white line showed an ectopic, prestin-positive cell next to a VGLUT3-positive inner hair cell (arrow). (*C*) At P9, some ectopic cells adjacent to outer hair cells were VGLUT3-positive (arrow). (*C′*) An *x–z* scan of *C* at the white line showed an ectopic VGLUT3- and prestin-positive cell next to an outer hair cell (arrow). (Scale bar, 20 μm.)

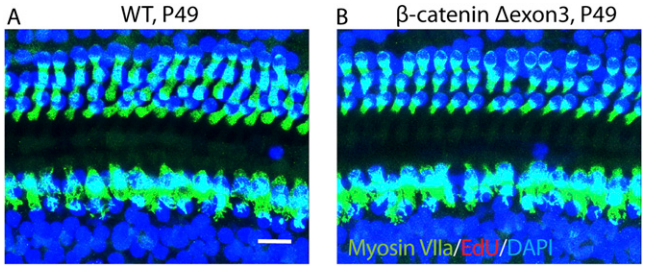

Fig. S5. Forced expression of β-catenin in adult mice. (*A*) A whole mount of the organ of Corti from a wild-type animal at P49 is shown. (*B*) A whole mount of the organ of Corti from a *Sox2-Cre-ER*/*β-catenin*$^{flox(exon3)}$ mouse was dissected 5 d after β-catenin was constitutively activated in all supporting cells by administration of tamoxifen. No Edu incorporation or ectopic hair cells were seen in the treated ears (n = 4). (Scale bar, 20 μm.)

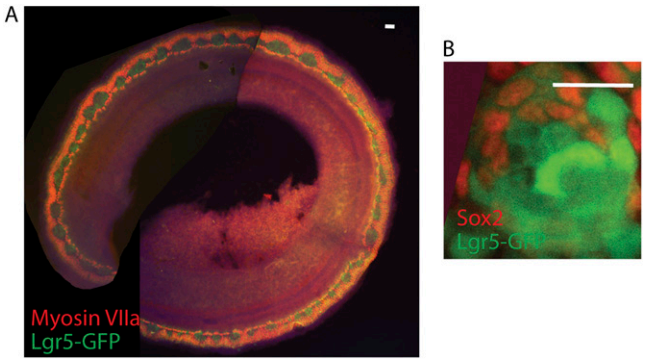

Fig. S6. Forced expression of β-catenin in Lgr5-positive cells. (*A*) Expansion of cells between inner and outer hair cells was apparent at P0 in cochleae from mice in which Wnt signaling was activated in Lgr5-positive cells at E16.5 using *Lgr5-Cre-ER* mice crossed to *β-catenin*$^{flox(exon3)}$ mice. The expanded clumps of cells extended from the base to the apex of the cochlea. (*B*) Each clump (30–60 cells) contained some cells that expressed the supporting cell marker Sox2. (Scale bar, 20 μm.)