



# Follistatin regulates the specification of the apical cochlea responsible for low-frequency hearing in mammals

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The cochlea's ability to discriminate sound frequencies is facilitated by a special topography along its longitudinal axis known as tonotopy. Auditory hair cells located at the base of the cochlea respond to high-frequency sounds, whereas hair cells at the apex respond to lower frequencies. Gradual changes in morphological and physiological features along the length of the cochlea determine each region's frequency selectivity, but it remains unclear how tonotopy is established during cochlear development. Recently, sonic hedgehog (SHH) was proposed to initiate the establishment of tonotopy by conferring regional identity to the primordial cochlea. Here, using mouse genetics, we provide *in vivo* evidence that regional identity in the embryonic cochlea acts as a framework upon which tonotopy-specific properties essential for frequency selectivity in the mature cochlea develop. We found that follistatin (FST) is required for the maintenance of apical cochlear identity, but dispensable for its initial induction. In a fate-mapping analysis, we found that FST promotes expansion of apical cochlear cells, contributing to the formation of the apical cochlear domain. SHH, in contrast, is required both for the induction and maintenance of apical identity. In the absence of FST or SHH, mice produce a short cochlea lacking its apical domain. This results in the loss of apex-specific anatomical and molecular properties and low-frequency-specific hearing loss.

frequency discrimination | cochlea | tonotopy | follistatin

Complex environmental auditory stimuli comprise multiple sound frequencies, and precise frequency discrimination is critical for extracting information essential to animal communication and survival. The peripheral auditory organ, which is commonly referred to as the cochlea, achieves frequency discrimination via a specialized topography along its longitudinal axis known as tonotopy. Auditory hair cells in the basal (proximal) cochlea, closer to the vestibular organs, are tuned to high-frequency sounds, whereas hair cells in the apical (distal) cochlea are more sensitive to lower frequencies (1, 2).

Cochlear tonotopy arises from gradients in the mechanical properties of the basilar membrane along the length of the cochlea. Hair cells also exhibit graded changes in morphological and physiological characteristics, which facilitate the cochlea's frequency selectivity (1, 2). Each hair cell has a bundle of stereocilia protruding from its surface that is responsible for detecting sound vibrations. These stereocilia are shorter in the hair cells at the cochlear base and longer toward the apex (3–5). In terms of number, hair cells at the base have more stereocilia per bundle than those at the apex (4, 6). In mammals, the angle of the V-shaped stereocilia on outer hair cells is wider at the base, growing gradually narrower toward the apex (7, 8). In addition, non-sensory cochlear structures including the spiral ligament, the tunnel of Corti, and the tectorial membrane display gradual changes along the tonotopic axis (9–11).

The expression of genes critical to auditory function also varies along the tonotopic axis. *Kcna4* and *Kcna10*, which encode voltage-gated potassium channels, are expressed in opposing gradients along the length of the cochlea (12, 13). *Cacna1d*, which encodes the calcium channel CaV1.3a1, and *Calb2*, which encodes the calcium-binding protein calbindin, are expressed at higher levels at the apex than at the base (14). Genes associated with non-syndromic hearing loss, such as *Slc26a5*, *Tectb*, and *Otof*, are also expressed at higher levels at the apex of the mouse cochlea, gradually tapering to lower levels at the base (13).

Although various morphological and molecular features associated with frequency discrimination have been identified, the underlying mechanisms by which tonotopy is established during development remain poorly understood. Recently, sonic hedgehog (SHH) signaling from the ventral midline (i.e., the notochord and floor plate) was proposed to initiate the establishment of tonotopy in both birds and mammals (4). An increasing gradient of SHH from base to apex confers regional identity to the primordial cochlea (4, 5). In chicken embryos, SHH establishes a gradient of bone morphogenetic protein 7 (BMP7) and subsequently of retinoic acid (RA) along the basilar papilla, which is analogous to the

## Significance

The frequency discrimination important for animal communication and survival is achieved via a special functional arrangement of cells along the length of the cochlea. Hair cells at the base are tuned to high-frequency sounds, whereas those at the apex are tuned to lower frequencies. Gradual anatomical and physiological changes along the cochlea facilitate this frequency selectivity, but it remains unclear how this functional arrangement is established. Using mouse genetics, we found SHH specifies apical identity in the cochlear primordium. Then, SHH and FST together maintain cochlear cells with an apical fate. In the absence of FST or SHH, mice produce short cochleae lacking the apical domain, leading to low-frequency hearing loss.

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The authors declare no competing interest.

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mammalian organ of Corti (4, 15, 16). Activation of SHH, BMP7, or RA in the embryonic basilar papilla promotes gene expression and hair cell morphology characteristic of the apex, whereas inhibition of BMP7 or RA induces basal phenotypes (4, 15, 16). Thus, BMP7 and RA appear to act downstream of SHH in tonotopic patterning in birds. It remains to be confirmed, though, whether tonotopic changes in the developing basilar papilla affect the ability of adults to respond to specific sound frequencies.

Unlike in chickens, *Bmp7* expression in the developing mouse cochlea is neither activated by SHH nor expressed in a gradient (4). This suggests a divergence in the mechanisms that establish tonotopy between birds and mammals. It remains unclear, though, whether, and how regional identity specified by SHH in the developing mouse cochlea contributes to the tonotopic organization of the mature cochlea. Using a mouse genetic approach, we show in this study that once SHH specifies apical identity in the cochlear primordium, both SHH and follistatin (FST), an antagonist of TGF $\beta$ /BMP/activin signaling, play critical roles in the establishment of tonotopy by maintaining apical identity. Our observations of cochlear gene expression, hair bundle morphology, and hearing function indicate that the regional identity of the primordial cochlea provides a framework upon which the adult cochlea acquires the tonotopic properties essential for frequency selectivity.

## Results

**Apical Identity in the Developing Mouse Cochlea is Lost in the Absence of FST.** To determine whether FST plays a role in the tonotopic organization of the mammalian cochlea, we first asked whether the regional identity specified by SHH is compromised in the developing cochleae of *Fst*<sup>-/-</sup> embryos. We assessed regional identity via genes displaying an increasing or decreasing cochlear base-to-apex expression gradient throughout mouse embryogenesis (4, 5). In E15.5 cochleae, we found expression of *Fst*, *Msx1*, *Slitrk3*, and *Efnb2* was strong at the apex and weaker toward the base, so we used these genes as apical cochlear markers (Fig. 1 *A–D*, arrows and arrowheads) (4). Then, because we found expression of *A2m* and *Inhba* was restricted to the base, we used these genes as basal cochlear markers (Fig. 1 *I* and *J*, arrows) (4). We visualized the graded expression patterns of these regional markers by performing in situ hybridizations on serial cochlear sections and plotting their signal intensities against the corresponding section numbers from base to apex (Fig. 1 *T* and *SI Appendix, Fig. S1*) (5).

When we examined the cochleae of E15.5 *Fst*<sup>-/-</sup> embryos, we found the overall inner ear structure and cochlear length were similar to those of controls (*Fst*<sup>+/-</sup>), except for a subtle malformation of the apical end (Fig. 1 *Q* and *R*). *Fst*<sup>-/-</sup> cochleae had undetectable *Msx1* expression at the apex and mid-apex (Fig. 1 *F* and *T*, *b* and *SI Appendix, Fig. S1*). Mutant cochleae also showed greatly reduced *Slitrk3* and *Efnb2* expression compared to controls (Fig. 1 *G*, *H*, and *T*, *c* and *d* and *SI Appendix, Fig. S1*), although their expression gradients were maintained. In contrast, *Fst*<sup>-/-</sup> cochleae showed normal basal expression of *A2m* and *Inhba* (Fig. 1 *M*, *N*, and *T*, *e* and *f* and *SI Appendix, Fig. S1*). These results indicate a selective down-regulation of apical but not basal markers in the developing cochleae of *Fst*<sup>-/-</sup> mutants, suggesting a loss of apical identity. This was particularly true in the most apical region.

Since apical cochlear identity is specified by strong SHH activity (4, 5, 17), we asked whether the loss of apical identity in *Fst*<sup>-/-</sup> cochleae was due to abnormal SHH signaling. We found expression of *Ptch1* and *Gli1*, indicators of SHH activity, was comparable between *Fst*<sup>+/-</sup> and *Fst*<sup>-/-</sup> cochleae (Fig. 1 *K*, *L*, *O*, *P*, and *T*, *g* and *h* and *SI Appendix, Fig. S1*). This suggests the loss of

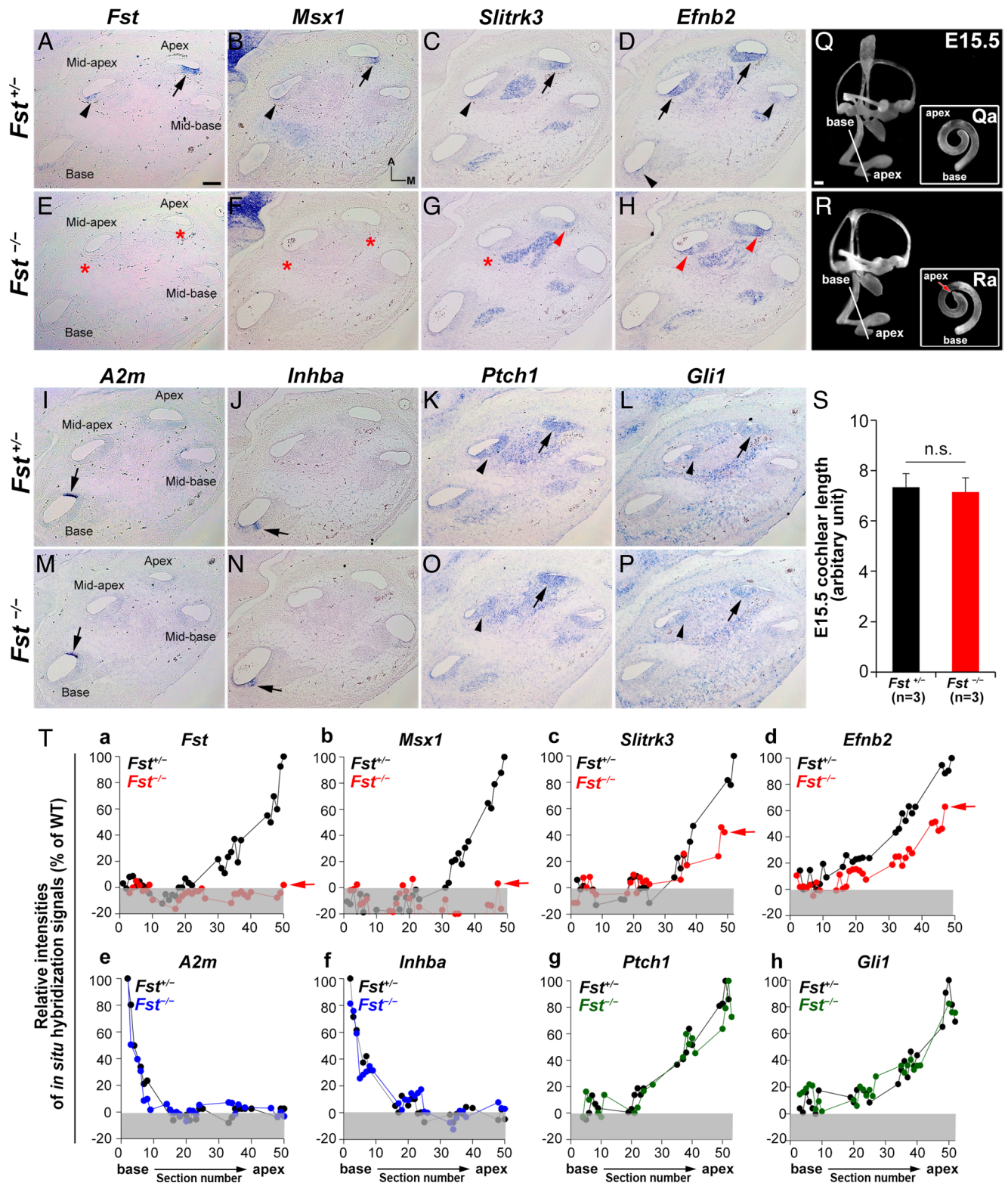
apical identity in *Fst*<sup>-/-</sup> cochleae was not due to defective SHH signaling. Together, these results indicate FST is critical for apical cochlear specification.

**FST is Insufficient to Induce Apical Identity or Inhibit Basal Identity.** Because *Fst* expression is regulated by SHH signaling (4, 18), and because both SHH and FST are required for apical cochlear specification (Fig. 1) (4), we hypothesized that FST functions downstream of SHH. To test this idea, we asked whether ectopic FST expression promotes apical identity at the expense of basal identity, like ectopic SHH signaling (4). To this end, we used transgenic mice (*R26-FST*) that express the human *FST* transgene under the control of the ubiquitous R26 promoter in the presence of doxycycline (*SI Appendix, Fig. S2A*) (19). We administered doxycycline to timed pregnant female mice starting at E10.5 (*SI Appendix, Fig. S2B*) and confirmed *FST* overexpression in the entire cochlea at E15.5 (*SI Appendix, Fig. S2H*, red arrows). We noted that the expression gradients of the apical markers *Msx1*, *Slitrk3*, and *Efnb2* were maintained (*SI Appendix, Fig. S2 D–F and I–K*), as was normal expression of the basal marker *Inhba* (*SI Appendix, Fig. S2 G–L*). These results indicate ectopic *FST* expression is insufficient to induce apical identity or inhibit basal identity. Thus, although SHH and FST are both required for apical cochlear specification, only SHH is sufficient to induce apical identity.

*FST* overexpression in *R26-FST* embryos inhibits activin signaling in the developing cochlea, leading to delayed hair cell differentiation (19). Since *FST* overexpression in *R26-FST* embryos does not disrupt regional identity, it is possible that activin A signaling is not required for regional specification in the cochlea. We tested this idea by examining the expression of regional markers in *Inhba*<sup>-/-</sup> mutants, which lack activin signaling. We found *Inhba*<sup>-/-</sup> cochleae at E18.5 exhibited delayed hair cell differentiation and ectopic inner hair cell production (*SI Appendix, Fig. S3 A–H*), as previously reported (19). We did not see any change, however, in the expression of the apical markers *Fst* and *Msx1* or the basal marker *A2m* (*SI Appendix, Fig. S3 I–P*). These results suggest activin A signaling is not required for the specification of regional cochlear identity.

**FST is Essential for Maintaining Apical Cochlear Identity.** Although our loss- and gain-of-function experiments indicated a requirement for FST in apical cochlear specification, FST, unlike SHH, proved insufficient to promote apical identity or inhibit basal identity (Fig. 1 and *SI Appendix, Figs. S1 and S2*). To better understand FST's role in apical specification, we examined the cochleae of *Fst*<sup>-/-</sup> mutants at earlier embryonic stages, starting from E11.75, when expression of the apical marker *Msx1* is first detected at the tip of the cochlear primordium (4, 17). In control (*Fst*<sup>+/-</sup>) embryos at E11.75, we found strong expression of the apical markers *Fst* and *Msx1* and the SHH signaling indicator *Ptch1* at the apex but not the base of the cochlear primordium (*SI Appendix, Fig. S4 A, a–c and i–k*). In the cochlear primordia of E11.75 *Fst*<sup>-/-</sup> mutants, although *Fst* expression was absent (*SI Appendix, Fig. S4 A, m*), the apical expression of *Msx1* (*SI Appendix, Fig. S4 A, n*) and *Ptch1* was unaffected (*SI Appendix, Fig. S4 A, o*). These results indicate FST is not required for the initial specification of apical identity. *Msx1* expression in *Fst*<sup>-/-</sup> cochleae began to drop at E12.5 (*SI Appendix, Fig. S4 B, f*) and was almost completely absent by E13.5 (*SI Appendix, Fig. S4 C, f*). We also found that the *Ptch1* expression gradient—an indicator of the SHH signaling gradient—was similar between *Fst*<sup>+/-</sup> and *Fst*<sup>-/-</sup> cochleae during these stages (*SI Appendix, Fig. S4 B, c and g and C, c and g*). We did not observe any terminal





**Fig. 1.** Specification of apical cochlear regional identity is compromised in *Fst*<sup>-/-</sup> cochleae. (A–P) In situ hybridization analysis with apical (*Fst*, *Msx1*, *Slitrk3*, and *Efnb2*) and basal (*A2m* and *Inhba*) regional markers and readouts of SHH signaling (*Ptch1* and *Gli1*) in *Fst*<sup>+/-</sup> and *Fst*<sup>-/-</sup> embryos at E15.5. Arrows and arrowheads indicate relatively strong and weak expression, respectively. Red asterisks and red arrowheads indicate absence or down-regulation of expression, respectively. (Q–S) Paint-fill analysis of the *Fst*<sup>-/-</sup> inner ear (Q, R) and cochlear lengths of control and *Fst*<sup>-/-</sup> at E15.5 (S). (T) Relative in situ hybridization signal intensities along the cochlear duct in *Fst*<sup>+/-</sup> and *Fst*<sup>-/-</sup> embryos. Red arrows indicate the apical end of the cochleae of *Fst*<sup>-/-</sup> embryos. Gray boxes below 0% in each graph indicate background signal. Representative graphs are presented from one *Fst*<sup>+/-</sup> and one *Fst*<sup>-/-</sup> cochlea for each gene. Additional samples are in *SI Appendix*, Fig. S1. The scale bar in A, 100  $\mu$ m, also applies to B–P. The scale bar in Q, 100  $\mu$ m, also applies to R.

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the apical region where *Msx1* was down-regulated during these stages (*SI Appendix*, Fig. S4 A, p; B, b; and C, b).

This suggests the *Msx1* down-regulation we observed was not secondary to a loss of *Msx1*-expressing cells via cell death. These results indicate that although FST is dispensable for the initial

specification of apical cochlear identity, it is required for its maintenance (Fig. 1 and *SI Appendix, Fig. S4*).

**Both SHH and FST Are Required for the Maintenance of Apical Cochlear Identity.** Genetic ablation of SHH signaling after specification of apical identity leads to low-frequency-specific hearing loss (20). This suggests SHH may also have a maintenance function in the apical cochlea in addition to its role in initial specification. Thus, we analyzed gene expression in *Emx2<sup>Cre/+</sup>; Smo<sup>lox/lox</sup>* mutants, in which the SHH transducer *Smo* is deleted starting at E11.5 after the specification of regional identity (20). In *Emx2<sup>Cre/+</sup>; Smo<sup>lox/lox</sup>* cochleae, we found *Ptch1* expression was reduced and almost completely abolished by E16.5, indicating a progressive loss of SHH activity (*SI Appendix, Fig. S5 A, f, B, f; C, f; F, and G*). At E12.5, *Fst* and *Msx1* were specifically expressed at the apex but not at the base in both control (*Smo<sup>lox/lox</sup>*) and *Emx2<sup>Cre/+</sup>; Smo<sup>lox/lox</sup>* mutant mice (*SI Appendix, Fig. S5A*), indicating normal specification of apical identity. *Msx1* expression, however, was greatly reduced at E15.5 (*SI Appendix, Fig. S5 B, e and D*) and completely lost by E16.5 (*SI Appendix, Fig. S5 C, e and E*). Interestingly, *Fst* expression was not reduced in *Emx2<sup>Cre/+</sup>; Smo<sup>lox/lox</sup>* mutant cochleae at these stages (*SI Appendix, Fig. S5 A, d; B, d; and C, d*), indicating that apical identity was progressively lost despite normal FST expression. These results, along with the fact that *Fst<sup>-/-</sup>* mutants showed progressive loss of apical identity with intact SHH signaling (Fig. 1 and *SI Appendix, Fig. S4*), indicate that both FST and SHH are required to maintain apical cochlear identity, with neither alone being sufficient.

**SHH's Capacity to Induce Ectopic Apical Identity Requires FST.** Since both FST and SHH are essential for the maintenance of apical identity, we next asked whether FST participates in SHH's ability to ectopically induce apical identity at the base of the cochlea. To this end, we examined regional markers in the cochlea of *Pax2-Cre; SmoM2* mutant mice expressing the constitutively active SHH transducer *R26-SmoM2* in the presence or the absence of FST (*Fst<sup>+/+</sup>* or *Fst<sup>lox/lox</sup>*) (4). As previously demonstrated (4), *Pax2-Cre; SmoM2* cochleae exhibited up-regulation of the SHH target genes *Ptch1* and *Gli1* (*SI Appendix, Fig. S6 C and D*, red arrows), down-regulation of the basal markers *A2m* and *Inhba* (*SI Appendix, Fig. S6 I and J*), and up-regulation of the apical markers *Fst*, *Msx1*, *Slitrk3*, and *Efnb2* across the entire cochlea (*SI Appendix, Fig. S6 Q–T*).

We next asked whether SHH's ability to induce apical identity depends on FST. In *Pax2-Cre; SmoM2; Fst<sup>lox/lox</sup>* mutant cochleae, strong SHH activity is maintained along the entire cochlea even in the absence of *Fst* expression (*SI Appendix, Fig. S6 E and F–U*). The basal markers *A2m* and *Inhba* remained completely down-regulated, indicating that strong SHH activity inhibits basal identity independent of FST (*SI Appendix, Fig. S6 K and L*). Surprisingly, however, in the absence of FST, the ectopic expression of the apical markers *Msx1* and *Slitrk3* at the cochlear base was restored to the wild-type gradient increasing from base to apex (*SI Appendix, Fig. S6 V and W*). This indicates that SHH's ability to induce ectopic apical identity at the base depends on FST. Interestingly, *Pax2-Cre; SmoM2; Fst<sup>lox/lox</sup>* cochleae continued to exhibit ectopic up-regulation of the apical marker *Efnb2* (*SI Appendix, Fig. S6X*, red arrows), suggesting that, unlike *Msx1* and *Slitrk3*, *Efnb2* expression depends solely on SHH.

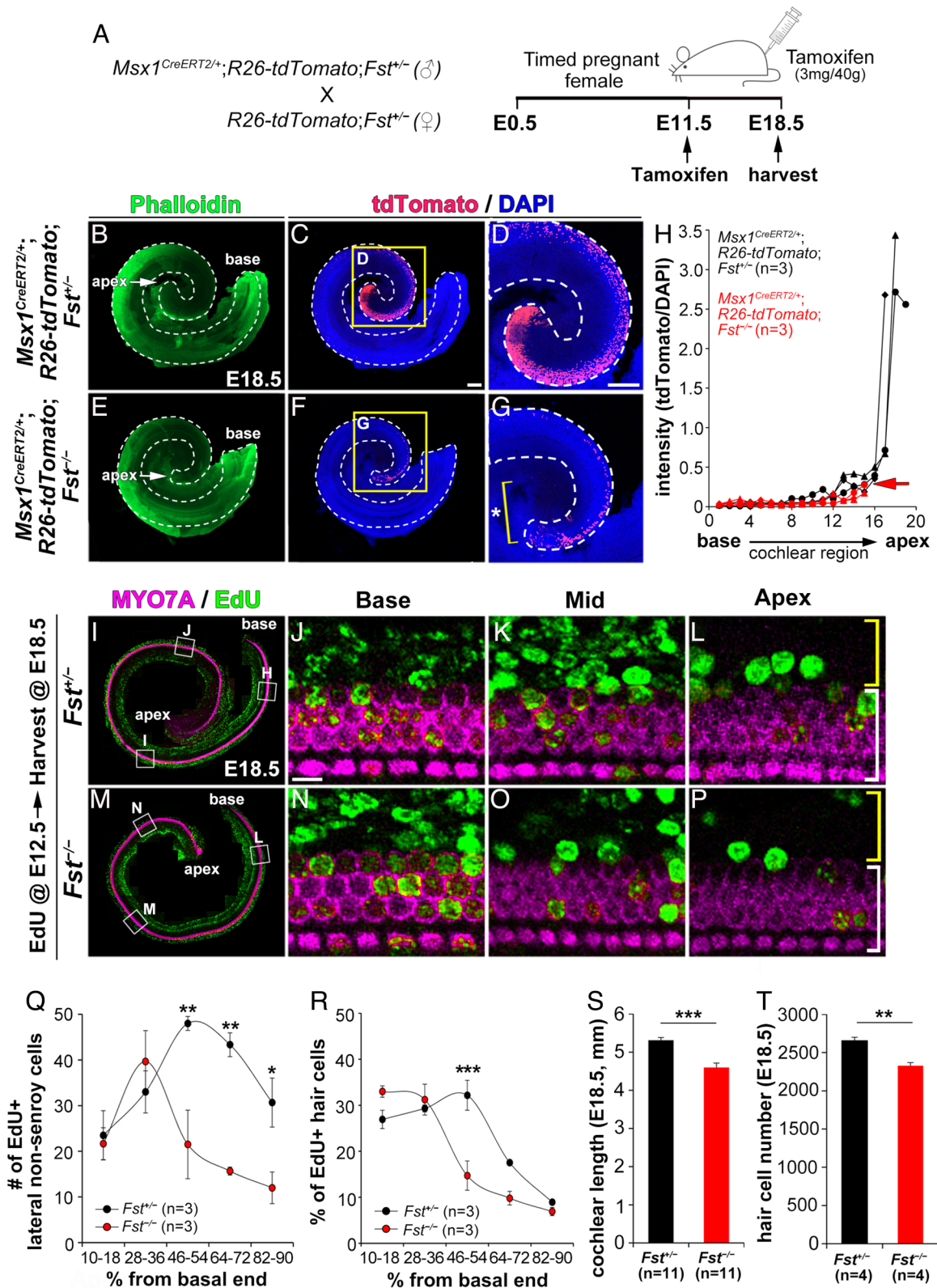
**FST Supports the Expansion of an Apical Cochlear Cell Population.** Our results indicate that FST plays an essential role in maintaining apical cochlear identity (Fig. 1 and *SI Appendix, Fig. S4*). To clarify the consequences of the loss of apical identity, we fate-

mapped the lineage of the apical cell population (*Msx1*-positive) by crossing *Msx1<sup>CreERT2/+</sup>* mice with *R26-tdTomato* reporter mice in the presence or the absence of *Fst* (*Fst<sup>+/-</sup>* or *Fst<sup>-/-</sup>*) (21, 22). After injecting tamoxifen into pregnant female mice at E11.5, when *Msx1* expression is comparable between *Fst<sup>+/-</sup>* and *Fst<sup>-/-</sup>* embryos (*SI Appendix, Fig. S4*), we examined *Msx1*-lineage cell fate at E18.5 (Fig. 2*A*). In control *Msx1<sup>CreERT2/+</sup>; R26-tdTomato; Fst<sup>+/-</sup>* embryos, we found a dense population of *Msx1*-lineage cells in the lateral compartment of the apical cochlea, with fewer cells spread along the cochlear duct toward the base (Fig. 2*C and D*). The loss of FST (in *Msx1<sup>CreERT2/+</sup>; R26-tdTomato; Fst<sup>-/-</sup>* embryos), however, greatly reduced the *Msx1*-lineage cell population (Fig. 2*F and G*) and shortened the cochlear duct because of the loss of the apical domain in which the dense *Msx1*-lineage population would have otherwise resided (Fig. 2*G*, asterisk). When we plotted tdTomato-fluorescence intensity along the cochlear duct for control mice, we found the strongest signal clustered in the apical cochlear regions, with the remaining regions exhibiting only weaker signals (Fig. 2*H*, black lines). *Msx1<sup>CreERT2/+</sup>; R26-tdTomato; Fst<sup>-/-</sup>* mutant mice did not exhibit any strong fluorescence at the apex, showing only weak signal along the cochlear duct (Fig. 2*H*, red lines and red arrow). These results suggest that FST is crucial for the formation of the apical cochlear domain because of its role in expanding the apical (*Msx1*-positive) cell population.

**FST Promotes Cell Proliferation in both Sensory and Non-Sensory Cochlear Cells.** MSX1 controls cell lineage development by promoting cell proliferation in the dental and limb mesenchyme (23, 24). We therefore asked whether loss of *Msx1* in *Fst<sup>-/-</sup>* cochleae affects cell proliferation. After injecting timed pregnant female mice with 5-ethynyl-2'-deoxyuridine (EdU) at E12.5, which is when *Msx1* expression begins to fall in *Fst<sup>-/-</sup>* embryos (*SI Appendix, Fig. S4*), we harvested the resulting embryos at E18.5. *Fst<sup>-/-</sup>* mutants showed far fewer EdU-positive cells in the sensory and lateral non-sensory compartments of the apical and middle cochlea than *Fst<sup>+/-</sup>* controls (Fig. 2*I–P*). We quantified EdU-positive cells by dividing the length of the cochlea into five regions from the basal end (0%) to the apical end (100%) in both the lateral non-sensory cell region and in the hair cell region. Fewer EdU-positive cells appeared in the lateral compartment of the middle-to-apical cochlear regions than in controls (Fig. 2*Q*). EdU-positive hair cells were also significantly reduced in the middle cochlear region (Fig. 2*R*). Less than 10% of the hair cells in the apex of both genotypes were EdU-positive, probably because the cells at the apex had already exited the cell cycle before EdU injection (25). This reduced cell proliferation resulted in a significant reduction of cochlear length and total hair cell number in *Fst<sup>-/-</sup>* embryos (Fig. 2*S and T*). Together, these results indicate FST plays a crucial role in the expansion of the apical cochlear domain by promoting cell proliferation.

We next asked whether the reduced cell proliferation of *Fst<sup>-/-</sup>* mutants also affects hair cell differentiation (*SI Appendix, Fig. S7*). In E15.5 controls (*Fst<sup>+/+</sup>*), we confirmed the base-to-apex progression of hair cell differentiation by noting the presence of cortical condensation in inner and outer hair cells at the base (*SI Appendix, Fig. S7B*, brackets) but not the apex (*SI Appendix, Fig. S7D*, asterisks). In *Fst<sup>-/-</sup>* mutants, cortical condensation was evident across the entire cochlea, including the apex (*SI Appendix, Fig. S7E–H*, brackets). At E18.5, *Fst<sup>-/-</sup>* mutants exhibited more organized cortical condensation in the apex than controls (*SI Appendix, Fig. S7L–P*). *Fst<sup>-/-</sup>* cochleae also frequently possessed an extra row of outer hair cells at the apex (*SI Appendix, Fig. S7P*, arrowhead). This has been observed before in mutant cochleae exhibiting premature cell cycle exit (5, 26). These results are consistent with a





**Fig. 2.** Failure of apical cochlear expansion caused by reduced cell proliferation in *Fst<sup>-/-</sup>* cochleae. (A–H) Fate-mapping of *Msx1*-lineage cells using *Msx1<sup>CreERT2/+</sup>; R26-tdtomato* mice in the presence (*Fst<sup>+/-</sup>*) or absence (*Fst<sup>-/-</sup>*) of FST. After tamoxifen was injected into pregnant female mice at E11.5, the embryos were harvested at E18.5 (A). Quantification of tdTomato-fluorescence intensity along the cochlear duct (H). (I–R) Comparing EdU-labeled cells in *Fst<sup>+/-</sup>* and *Fst<sup>-/-</sup>* embryos. After EdU was injected into pregnant female mice at E12.5, EdU-labeled cells (green) were counted among the lateral non-sensory cells (yellow brackets) and MYO7A-positive hair cells (white brackets) (I–P). The number of EdU-positive cells in the lateral compartment (Q) and the percentage of EdU-positive hair cells (R) were plotted against the distance along the cochlear duct divided into five regions from base to apex. (S–T) Comparisons of cochlear length and hair cell number between E18.5 *Fst<sup>+/-</sup>* and *Fst<sup>-/-</sup>* embryos. Data in Q–T are presented as means ± SE. Statistical comparisons were determined via two-way ANOVA for the EdU analysis and unpaired *t* tests with Bonferroni corrections for the cochlear length measurements (\**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001). The scale bar in C, 200 μm, also applies to B–C, E–F, and I–M. The scale bar in D, 200 μm, also applies to G. The scale bar in J, 10 μm, also applies to K–L and N–P.

previous report that FST maintains pro-sensory cells in a proliferative and undifferentiated state (19). Our results also indicate that FST's regulation of cell proliferation extends to non-sensory cells in the lateral cochlear compartment (Fig. 2 *I–R*). Together, these results confirm FST contributes to apical cochlear expansion by regulating cell proliferation.

**Loss of Apical Identity Results in Loss of Apical Hair Bundle Morphology.** Our developmental gene expression and fate-mapping analyses suggested that FST plays a role in maintaining and expanding apical cell populations (Figs. 1–2 and *SI Appendix*, Fig. S4). We next asked how this early role of FST in the embryonic cochlea contributes to the tonotopic organization of the mature cochlea. Because the *Fst*<sup>-/-</sup> mutation is neonatal lethal in mice, we generated an inner ear-specific *Fst* conditional knockout (*Fst* cKO) by crossing *Pax2-Cre* mice with *Fst*<sup>lox/lox</sup> mice (27, 28). We confirmed that *Fst* cKO (*Pax2-Cre*; *Fst*<sup>lox/lox</sup>) cochleae exhibit the same changes as *Fst*<sup>-/-</sup> cochleae in the expression of apical (*Fst*, *Msx1*, *Slitrk3*, and *Efnb2*) and basal markers (*A2m* and *Inhba*) (*SI Appendix*, Fig. S8). *Fst* cKO mice were viable, fertile, and survived to at least 3 mo of age. In 4-wk-old *Fst* cKO mice, although we did not observe any apparent hair cell disorganization or degeneration, we frequently observed an extra row of apical outer hair cells (Fig. 3 *A–F*) like those we observed in embryonic cochleae (*SI Appendix*, Fig. S7).

Next, we evaluated tonotopic changes in outer hair cell morphology, noting stereocilia length, angle of the stereocilia V-shaped arrangement, and number of stereocilia per hair cell. Each of these characteristics gradually changes along the tonotopic axis (3, 5, 7, 8). First, we divided the cochlear duct into five tonotopic regions—base, mid-base, mid, mid-apex, and apex—with each defined as a percentage of the overall length. In 4-wk-old control mice (*Fst*<sup>lox/lox</sup>), stereocilia length increased gradually from base to apex (Fig. 3 *G–I* and *S*). Although *Fst* cKO cochleae maintained this gradient, stereocilia length was significantly reduced along the length of each cochlea, except at the base (Fig. 3 *J–L* and *S*). In control mice, the angle of the stereocilia V-shaped arrangement is widest at the base, gradually narrowing toward the apex (Fig. 3 *M–O* and *T*). In *Fst* cKO cochleae, this angle was significantly wider along the length of the cochlea, except at the base (Fig. 3 *P–R* and *T*). The base of control cochleae has more stereocilia per hair cell than those at the apex, and *Fst* cKO cochleae show significantly higher numbers than controls across their entire length (Fig. 3 *U*). These results suggest the tonotopic organization of cochlear stereocilia morphology in *Fst* cKO mice is shifted basally.

Because *Fst*<sup>-/-</sup> mutants have shorter cochleae due to apical truncation (Fig. 2), we compared stereocilia morphology at equivalent distances from the base in control and *Fst* cKO mice (*SI Appendix*, Fig. S9A). Interestingly, we only found a significant difference in stereocilia length and angle at the apex of the cochlear duct (*SI Appendix*, Fig. S9B and C). Control and *Fst* cKO outer hair cells also had similar numbers of stereocilia along the cochlear duct, although there were some regions with differences (*SI Appendix*, Fig. S9D). These results suggest the failure to maintain apical identity in embryonic *Fst* cKO mutant cochleae results in a loss of the morphological properties characteristic of apical stereocilia.

We next asked whether the loss of apical identity secondary to abnormal SHH signaling in *Emx2*<sup>Cre/+</sup>; *Smo*<sup>lox/lox</sup> mutants (*SI Appendix*, Fig. S5) was also responsible for the similar phenotypes we observed in *Fst* cKO mutants. When we compared stereocilia length, angle, and number along the tonotopic axis of control (*Smo*<sup>lox/lox</sup>) and mutant (*Emx2*<sup>Cre/+</sup>; *Smo*<sup>lox/lox</sup>) cochleae at P15, we observed a similar tonotopic shift toward more basal properties like we saw in *Fst* cKO mutant cochleae (*SI Appendix*, Fig. S10).

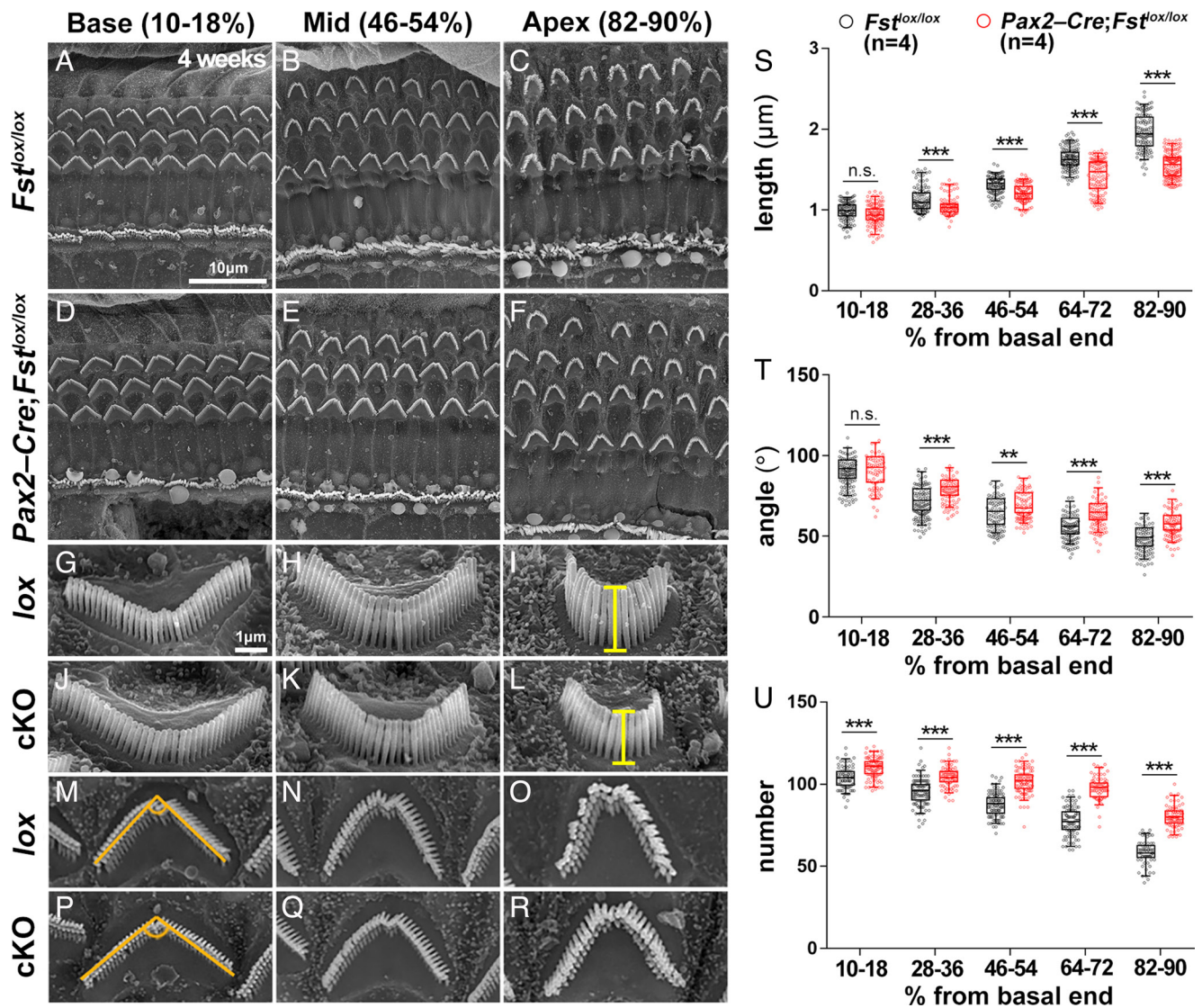
Similar to what we observed in *Fst* cKO mice, control and *Smo* cKO mice showed similar hair bundle morphology at equivalent distances from the base. In *Smo* cKO mice, however, the apical end was absent (*SI Appendix*, Fig. S11). Together, these results indicate that loss of apical identity in the embryonic cochlea due either to loss of FST or SHH leads to a loss of apical tonotopic characteristics in the mature cochlea.

**Loss of the Apical Cochlea is Accompanied by Changes in Gene Expression.** We next used RNA sequencing to investigate changes in gene expression in *Fst* cKO mutants associated with the loss of the apical cochlear domain. After isolating basal, middle, and apical regions from the cochleae of 4-wk-old control and *Fst* cKO mice (*SI Appendix*, Fig. S12A), we examined their global gene expression profiles and compared them via a principal components analysis. In control, cochleae, basal, middle, and apical gene expression profiles were distinctly separate along the PC1 axis, which accounted for 55.18% of the overall variability (*SI Appendix*, Fig. S12B, black circles). In *Fst* cKO cochleae, however, while the basal and middle gene expression profiles were clearly separate, the apical and middle profiles clustered close to one another (*SI Appendix*, Fig. S12B, red circles). This suggests that in *Fst* cKO mutants, the gene expression profile of the apical cochlea has shifted toward that of the middle cochlea. This most likely reflects the fact that the portion of the cochlea collected as the “apex” contains more of the middle cochlear region since *Fst* cKO mutants lack a true apical end.

Next, we looked for genes and pathways affected by apical loss in *Fst* cKO mutants. To focus on genes differentially expressed along the tonotopic axis, we selected genes that increased or decreased by at least 1.5-fold from the base to the apex in control cochleae. We classified these genes as the UP (612 genes) or DOWN (730 genes) groups, respectively (*SI Appendix*, Fig. S12C and D, and *Datasets S1–S2*). We found *Fst* was included in the UP group, indicating that it was expressed in a graded pattern from E10.5 to 4 wk of age (*Dataset S1*) (8). Most of the gene ontology (GO) terms enriched in the UP group were related to the inner ear and hearing. These terms included sensory perception of sound, inner ear morphogenesis, stereocilium, and potassium ion transport (*SI Appendix*, Fig. S13 and *Dataset S3*). In contrast, the GO terms enriched in the DOWN group were associated with broad developmental and biological processes, including extracellular matrix organization, cell adhesion, and multicellular organism development (*SI Appendix*, Fig. S13 and *Dataset S3*). Interestingly, the UP and DOWN groups from experiments with chicken basilar papillae showed enrichment of similar GO terms (29). These results suggest genes whose expression increases from the base to the apex of the cochlea are important for the functional aspects of tonotopy, such as sound perception, whereas genes whose expression decreases toward the apex are associated more with the structural properties of tonotopy, such as the extracellular matrix in the basilar membrane (29).

Next, we further classified genes in the UP group that were significantly down-regulated at the apex or had a less steep gradient of expression in *Fst* cKO mice than controls, referring to them as UP-1 genes (*SI Appendix*, Fig. S12C and *Datasets S1* and *S2*). We reasoned that UP-1 genes would be the most affected by the loss of the apical cochlea in *Fst* cKO mutants. Similarly, we further classified genes in the DOWN group that were significantly up-regulated at the apex or that had a less steep gradient of expression in *Fst* cKO mice than controls, referring to them as DOWN-1 genes (*SI Appendix*, Fig. S12D and *Dataset S2*). Plotting heatmaps of the expression of 128 UP-1 genes and 269 DOWN-1 genes in *Fst* cKO cochleae revealed changes in gene expression levels at the





**Fig. 3.** Loss of apical region stereocilia morphology in 4-wk-old *Fst* cKO mutants. (A–R) Scanning electron micrographs of the organ of Corti from control (*lox*) (*Fst*<sup>lox/lox</sup>) and inner ear-specific *Fst* cKO (*Pax2-Cre; Fst*<sup>lox/lox</sup>) mice. The lengths of the outer hair cell stereocilia were measured at the vertex of the V-shaped hair bundles from the lateral side (G–L). The angle of the V-shaped stereocilia and the number of stereocilia per outer hair cell were measured using a top-down view (M–R). The scale bar in A, 10 μm, also applies to B–F. The scale bar in G, 1 μm, also applies to H–R. (S–U) Quantification of the length, angle, and number of outer hair cell stereocilia along the tonotopic axis in each of the five cochlear regions beginning with the basal end. These represent the base (10 to 18%), mid-base (28 to 36%), mid (46 to 54%), mid-apex (64 to 72%), and apex (82 to 90%) regions. Statistical comparisons were determined via two-way ANOVA with Bonferroni corrections for multiple comparisons (n.s., non-significant, \*\**P* < 0.01, and \*\*\**P* < 0.001).

apex that were still associated with an expression gradient (SI Appendix, Fig. S12 E and F and Datasets S1 and S2). As expected, we found a significant reduction in *Fst* expression in *Fst* cKO cochleae (SI Appendix, Fig. S12 E, a and Dataset S1). The UP-1 group included genes like *Tectb*, *Otof*, and *Otogl* that are associated with hearing loss, the potassium ion channel-related genes *Kcnip4* and *Kcna10* and the calcium-binding protein gene *Calb2*. The DOWN-1 group also included genes related to hearing loss (e.g., *Slc4a10* and *Gjb6*), as well as genes like *Car2*, *Adra2a*, *Smpd3*, *Tnmd*, and *Dcn*, which are known to be expressed in a gradient (SI Appendix, Fig. S12 F, a and Dataset S2). To identify the cascade of upstream regulators that may cause the gene expression changes we observed in the UP-1 and DOWN-1 groups, we performed an upstream regulator analysis using Ingenuity Pathway Analysis tools (SI Appendix, Fig. S12 G and Dataset S4). For the DOWN-1 group, we noted TGFβ signaling components such as TGFβ1, SMAD3, and INHA among the identified upstream

regulators (SI Appendix, Fig. S12 G and Dataset S4). Together, these results suggest the global gene expression profile of the apex of *Fst* cKO cochleae shifts toward that of the middle cochlea via a mechanism that may be downstream of aberrant TGFβ/BMP signaling.

### Loss of the Apical Cochlea Leads to Low-Frequency-Specific Hearing Loss.

The loss of apical identity in embryonic cochleae we observed leads to tonotopic changes in stereocilia morphology and gene expression in mature cochleae. We next asked whether these structural and molecular changes in tonotopy affect the cochlea's ability to respond to specific sound frequencies. Because *Fst* cKO mutants lack the cochlear apex, we expected a deficiency in low-frequency hearing. We therefore assessed hearing sensitivity by measuring auditory brainstem response (ABR) thresholds in 4-wk-old control and *Fst* cKO mice in response to broadband click stimuli or

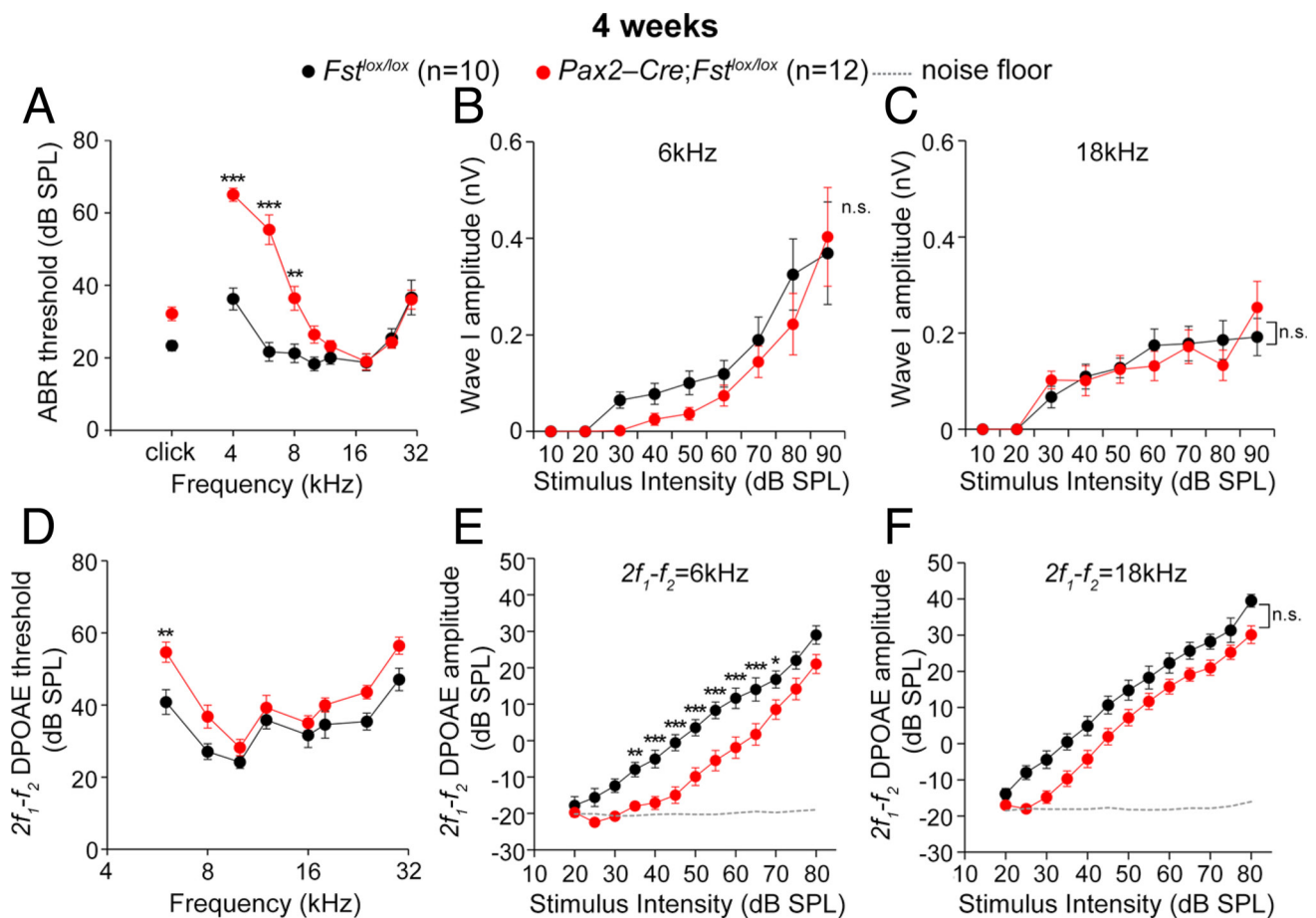
pure tones. Although we did not observe significant changes in the ABR thresholds to broadband click stimuli (Fig. 4A), we found that the ABR thresholds to pure tone stimuli were significantly higher at low frequencies (4 to 8 kHz) but not high frequencies (Fig. 4A). These results demonstrate that *Fst* cKO mutant mice are less sensitive to low-frequency sounds than high-frequency sounds.

Last, we asked whether these elevated ABR thresholds were caused by a defect in the inner hair cells activating spiral ganglion neurons or in the outer hair cells acting as the cochlear amplifier. The ABR wave I amplitudes, which represent the summed auditory nerve fiber response, were similar in control and *Fst* cKO mice to low (6 kHz) and high (18 kHz) frequency sounds at 4 wk (Fig. 4B and C). In contrast, distortion-product otoacoustic emission (DPOAE) thresholds, which reflect the cochlear amplifier function of the outer hair cells, were significantly higher at 6 kHz than 18 kHz (Fig. 4D). We confirmed this reduction in *Fst* cKO mice at low frequencies via an input/output (I/O) function analysis of the DPOAE amplitudes (Fig. 4E and F). These results suggest the low-frequency-specific hearing loss we observed in *Fst* cKO mice is likely caused by defective outer hair cell function disrupting the cochlear amplifier for low-frequency sounds. When we tracked this low-frequency hearing loss of *Fst* cKO mice at later time points, we found that it continued out as far as 12 wk of age (SI Appendix, Fig. S14). Together, these results suggest FST is

crucial for the establishment of tonotopy via its role in establishing and patterning the features necessary for low-frequency hearing in the cochlear apex.

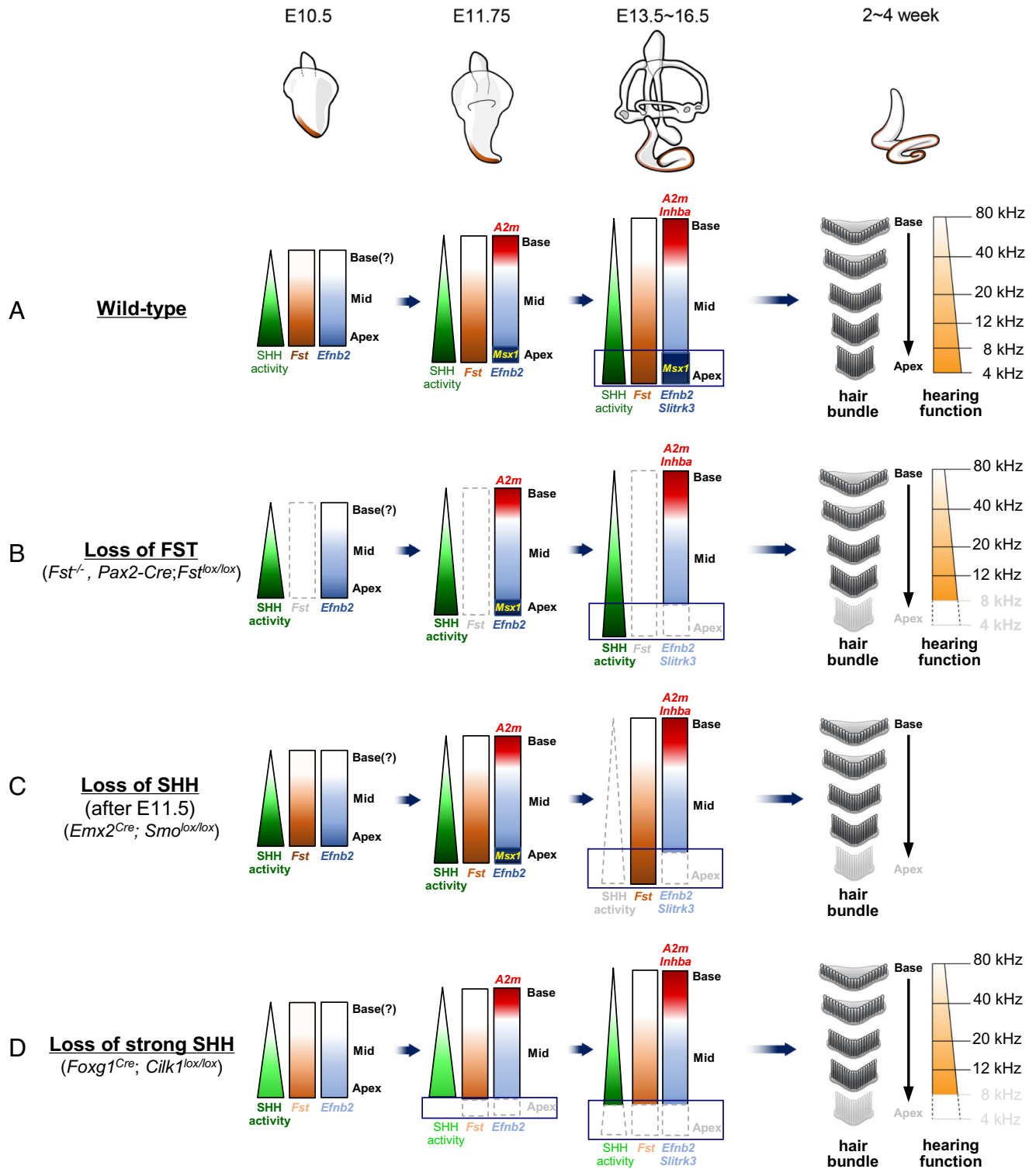
## Discussion

**Sequential Progression of the Establishment of Tonotopic Organization in the Mammalian Cochlea.** Observations from our current study and from previous studies indicate that the establishment of tonotopic organization proceeds sequentially during cochlear development (Fig. 5A). An extrinsic SHH signaling gradient confers regional identity to the developing cochlea by promoting apical identity and suppressing basal identity (4, 5, 17). This regional identity is evident in increasing base-to-apex expression gradients of the apical markers *Fst* and *Efnb2* as soon as an out-pocketing of the cochlear primordium becomes visible at the ventral otocyst in E10.5 mice (Fig. 5A). By E11.75, the slightly elongated cochlear primordium expresses *Msx1* and *A2m* in the apical and basal-most regions, respectively (Fig. 5A). Then, two more expression gradients emerge: an increasing base-to-apex gradient of *Slirrk3* and an increasing apex-to-base gradient of *Inhba* (Fig. 5A). These differential gradients are maintained during cochlear elongation throughout embryogenesis independent of the apex-to-base wave of cell cycle exit and the base-to-apex wave of hair cell differentiation (4, 5).



**Fig. 4.** Low-frequency-specific hearing loss in 4-wk-old *Fst* cKO mutants. (A–C) ABR analyses of 4-wk-old *Fst*<sup>lox/lox</sup> and *Pax2-Cre; Fst*<sup>lox/lox</sup> mice. ABR thresholds of *Fst*<sup>lox/lox</sup> and *Pax2-Cre; Fst*<sup>lox/lox</sup> mice to click stimuli and individual frequencies (A). I/O function analyses of wave I amplitudes from the ABRs to low (6 kHz) and high (18 kHz) frequencies (B, C). (D–F) DPOAE analyses of 4-wk-old *Fst*<sup>lox/lox</sup> and *Pax2-Cre; Fst*<sup>lox/lox</sup> mice.  $2f_1-f_2$  DPOAE thresholds of *Fst*<sup>lox/lox</sup> and *Pax2-Cre; Fst*<sup>lox/lox</sup> mice (D). DPOAE I/O function analyses at 6 kHz and 18 kHz (E, F). Data are presented as means  $\pm$  SE. Statistical comparisons were determined via two-way ANOVA with Bonferroni corrections for multiple comparisons (n.s., non-significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).





**Fig. 5.** Summary diagram illustrating tonotopic organization in the mammalian cochlea. (A) The tonotopic organization of the cochlea is established sequentially. First, apical-to-basal regional identity is established. This then provides a framework upon which the basal-to-apical tonotopic characteristics tuned to specific frequencies appear along the mature cochlea. (B–D) Loss or gain of FST or SHH function perturbs this framework in the developing cochlea, resulting in changes in the tonotopic characteristics of the mature cochlea. See the *Discussion* section for details on each genotype.

This regional identity of the cochlear primordium provides a framework for the tonotopic organization of the mature cochlea (Fig. 5A).

Once the tonotopic framework is established in the cochlear primordium, both FST and SHH work to maintain apical identity as indicated by *Msx1* expression. This apical identity is

progressively lost in the absence of either FST or SHH during cochlear development (Fig. 5B and C). FST also plays a role in the expansion of the apical cell population by promoting cell proliferation in both the sensory and non-sensory compartments (Fig. 2) (19). This increase in apical cells contributes to the extension of the apical cochlear domain (Fig. 2). In this extended

apical cochlea, hair cells differentiate to adopt apical tonotopic characteristics optimized for responses to low-frequency sounds (Fig. 5A). In the absence of FST, the cochlear duct is shortened because it lacks the apical domain. This leads to the loss of the apical hair bundles and apical gene expression that supports low-frequency hearing (Fig. 5B).

It should be noted that failure to maintain apical identity in the absence of FST results in tonotopic changes nearly identical to those observed in the ciliary mutant *Foxg1<sup>Cre</sup>; Cilkl1<sup>lox/lox</sup>* (Fig. 5D), which result from a failure to induce apical identity secondary to abnormal SHH signaling (5). The phenotypes these mutants share include loss of the apical cochlear region, apical hair bundle characteristics, and low-frequency-specific hearing (Fig. 5 B–D) (5). Using a mouse genetic approach, we have clearly demonstrated that a change in regional identity in the embryonic cochlea influences tonotopy in the corresponding region of the adult cochlea, leading to a frequency-specific hearing defect.

**The Functional Relationship between SHH and FST in the Development of Tonotopy.** Although *Fst* expression is activated by SHH signaling (SI Appendix, Fig. S6) (4), our analyses of the cochlea of mice with single or compound mutations of SHH and FST suggest a complex functional relationship rather than a simple signaling cascade (Fig. 5 and SI Appendix, Fig. S15). Unlike SHH, FST is neither sufficient (as tested in *R26-FST* mice) nor necessary (as tested in *Fst<sup>-/-</sup>* mice) to induce apical identity (SI Appendix, Figs. S2 and S4). Nor is the activin A signaling antagonized by FST required for regional cochlear patterning (as tested in *Inhba<sup>-/-</sup>* mice) (SI Appendix, Fig. S3). Thus, it is unlikely that SHH specifies regional identity in the mouse cochlea by regulating TGFβ/BMP signaling. The situation is somewhat different, though, in the chicken basilar papilla. There, SHH regulates regional patterning by establishing an expression gradient of BMP7 (4, 15). While BMP7 promotes apical identity, BMP inhibition via *CHRDLL1* (chordin-like 1) promotes basal identity (15). *Bmp7* is not, however, activated by SHH signaling, nor are *Bmp7* and *Chrdll1* expressed in gradients in the mouse cochlea (4, 8). If SHH function is mediated by other TGFβ/BMP ligands or other signaling pathways, it remains unclear. We hope in the future to explore the effects on the mouse cochlea of the conditional loss of common mediators of TGFβ/BMP signaling like SMAD4. We also expect that transcriptomic analyses along the tonotopic axis during cochlear development will provide further insight into this phenomenon.

Instead of acting in a signaling cascade, both SHH and FST are required for apical cochlear patterning. Loss of FST (*Pax2-Cre; Fst<sup>lox/lox</sup>*) or SHH (*Emx2<sup>Cre</sup>; Smo<sup>lox/lox</sup>*) causes similar tonotopic changes, namely apical cochlear truncation and loss of apical tonotopic characteristics (Fig. 5 B and C). It remains unclear whether SHH and FST pattern the apical cochlea cooperatively or independently. The expression of *Msx1*—a marker of apical identity—is abolished by E13.5 in *Fst<sup>-/-</sup>* mutants (SI Appendix, Fig. S4) but remains until E16.5 in the absence of SHH function (*Foxg1<sup>Cre</sup>; Shh<sup>lox/-</sup>* and *Emx2<sup>Cre</sup>; Smo<sup>lox/lox</sup>*) (SI Appendix, Fig. S5) (4). Thus, SHH and FST's maintenance of apical identity may have different temporal requirements. Furthermore, although both FST and SHH regulate cell proliferation and contribute to apical cochlear extension (Fig. 2) (5, 19, 26), SHH activity is stronger in the medial cochlear compartment and FST expression is stronger in the lateral compartment (Fig. 1 and SI Appendix, Fig. S4). Thus, SHH and FST do seem to contribute to cochlear cell proliferation via distinct roles in the medial and lateral compartments, respectively.

**Signals Promoting Basal Cochlear Identity.** In contrast to our progress with apical tonotopic patterning, we do not yet understand how the basal cochlea is specified and patterned. Basal cochlear identity is effectively suppressed by strong SHH activity, so it is specified in the region where SHH activity is low (SI Appendix, Fig. S6) (4). It is possible that low SHH activity is sufficient to induce basal identity, but it is also possible that another signaling pathway either actively promotes basal identity, inhibits apical identity, or both via a gradient opposing that of SHH. Indeed, the presence of such a signal is implied from the phenotypes of cochlea that combined gain of SHH function and loss of FST function (SI Appendix, Figs. S6 and S15). We found a restoration of increasing base-to-apex expression gradients of apical markers such as *Msx1* and *Slitrk3* under constitutive SHH activation (*Pax2-Cre; SmoM2*) in the absence of FST (*Pax2-Cre; SmoM2; Fst<sup>lox/lox</sup>*) (SI Appendix, Figs. S6 and S15). This was somewhat surprising, because *Pax2-Cre; SmoM2; Fst<sup>lox/lox</sup>* cochleae activate SHH ubiquitously and lack FST, meaning both the SHH and FST gradients that are so important for establishing regional identity are essentially abolished. Yet, regional patterning of the cochlea appears to occur in these mutant mice, indicating that signals other than SHH and FST are involved in regional patterning. As this unknown signal inhibits SHH-induced gene expression at the base but not the apex, it is likely expressed in a gradient that decreases from base to apex (SI Appendix, Fig. S15C). In addition, since its inhibitory action on SHH-induced gene expression is relieved by FST, this signal is likely related to TGFβ/BMP/activin signaling. Although activin A signaling falls into this category (19), activin A cannot be the unknown signal because we were able to confirm normal regional specification in the absence of activin A signaling (*Inhba<sup>-/-</sup>* mice) (SI Appendix, Fig. S3). We hope soon to identify this ligand expressed in a decreasing base-to-apex gradient that inhibits apical gene expression and is antagonized by FST. This and any other signals that induce basal identity and suppress apical identity independent of SHH or FST will give insight into basal identity specification.

In summary, our present study provides in vivo evidence that tonotopic organization in the mammalian cochlea is established sequentially after the initial formation of a framework in the cochlear primordium. This framework provides positional information for the development of the molecular, structural, and functional characteristics that underlie tonotopy in the mature cochlea. Perturbation of regional identity in the embryonic cochlea affects the ability of the mature cochlea to discriminate the corresponding sound frequencies. It remains unclear, though, how cochlear cells and tissues with specific regional identities acquire their respective tonotopic properties in concert with complex and seemingly unrelated developmental processes, such as continuous cochlear extension and opposing waves of cell cycle exit and hair cell differentiation.

## Materials and Methods

The mice used in this study were generated as previously described (4, 19, 20, 27, 28, 30, 31). Immunofluorescence staining, scanning electron microscopy, in situ hybridization, paint-fill injections, and EdU and TUNEL staining were performed as previously described (5, 26, 32–34). ABRs and DPOAEs were measured in a sound-proof chamber using Tucker-Davis Technologies (TDT) RZ6 digital signal processing hardware and the BioSigRZ software package as previously described (33). A more detailed account of the materials and methods used in this study appears in the SI Appendix, Materials and Methods section.

**Data, Materials, and Software Availability.** All study data are included in the article and/or SI Appendix. All the raw data of our RNA-seq analyses have been deposited in the Gene Expression Omnibus data repository under accession code: GSE203228.



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