



# Sufu- and Spop-mediated regulation of Gli2 is essential for the control of mammalian cochlear hair cell differentiation

Tianli Qin<sup>a,b</sup>, Chin Chung Ho<sup>b</sup>, Boshi Wang<sup>b</sup>, Chi-Chung Hui<sup>c,d</sup>, and Mai Har Sham<sup>a,b,1</sup>

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Development of mammalian auditory epithelium, the organ of Corti, requires precise control of both cell cycle withdrawal and differentiation. Sensory progenitors (prosensory cells) in the cochlear apex exit the cell cycle first but differentiate last. Sonic hedgehog (Shh) signaling is required for the spatiotemporal regulation of prosensory cell differentiation, but the underlying mechanisms remain unclear. Here, we show that suppressor of fused (Sufu), a negative regulator of Shh signaling, is essential for controlling the timing and progression of hair cell (HC) differentiation. Removal of *Sufu* leads to abnormal *Atoh1* expression and a severe delay of HC differentiation due to elevated *Gli2* mRNA expression. Later in development, HC differentiation defects are restored in the *Sufu* mutant by the action of speckle-type PDZ protein (Spop), which promotes *Gli2* protein degradation. Deletion of both *Sufu* and *Spop* results in robust *Gli2* activation, exacerbating HC differentiation defects. We further demonstrate that *Gli2* inhibits HC differentiation through maintaining the progenitor state of Sox2<sup>+</sup> prosensory cells. Along the basal–apical axis of the developing cochlea, the Sox2 expression level is higher in the progenitor cells than in differentiating cells and is down-regulated from base to apex as differentiation proceeds. The dynamic spatiotemporal change of Sox2 expression levels is controlled by Shh signaling through *Gli2*. Together, our results reveal key functions of *Gli2* in sustaining the progenitor state, thereby preventing HC differentiation and in turn governing the basal–apical progression of HC differentiation in the cochlea.

Sufu | Shh signaling | Gli | hair cell | cochlea

Cochlear hair cells (HCs) are mechanoreceptor cells responsible for hearing. One row of inner HCs (IHCs) and three rows of outer hair cells (OHCs) together with different subtypes of supporting cells (SCs) form the organ of Corti (oC) along the entire cochlea. Both HCs and SCs originate from the Sox2<sup>+</sup> progenitor (prosensory) pool (1). Sox2 is an essential factor for the development of cochlear sensory HCs and is regulated both positively and negatively during HC differentiation (2–5). Sox2 is required to confer sensory competence to the prosensory cells; without Sox2, both HCs and SCs are absent (2, 3). However, Sox2 must be down-regulated during HC differentiation (4, 5). The transcription factor *Atoh1* is necessary and sufficient for specifying HC lineage in the cochlea (6–9). Within the Sox2<sup>+</sup> prosensory domain, activation of *Atoh1* initiates HC differentiation and down-regulates Sox2 expression level, whereas cells retaining Sox2 expression become SCs and greater epithelial ridge (GER) (Fig. 1A). The expression level of Sox2 is therefore tightly regulated in the formation and patterning of the developing oC.

During cochlea development, prosensory cells in the apical region first exit from mitosis with P27<sup>Kip1</sup> activation at embryonic day 12.5 (E12.5), and subsequently cell cycle exit spreads toward the base around E14.5 (10, 11). Remarkably, although progenitor cells in the apex exit the cell cycle first, they remain undifferentiated in neonatal mice. Around E13.5, progenitor cells in the midbase region start showing *Atoh1* activation (12). IHCs in the medial side of the oC at the cochlear midbase are specified first, and then differentiation extends laterally and apically (12) (Fig. 1A). In the mature oC, IHCs are the primary sensory cells, whereas OHCs adjust sound perception (13). Positional variations of HCs along the basal–apical axis establish the tonotopic map, where sounds of high frequencies are processed in the base, and the cochlear apex decodes low-frequency sounds (14, 15). Therefore, precise differentiation and patterning of HCs are crucial for hearing functions.

Sonic hedgehog (Shh) from the spiral ganglia (SG) has been implicated in controlling the basal–apical progression of HC differentiation. Expression of *Shh* in the SG gradually declines from base to apex, which is similar to the HC differentiation pattern (16). In mice with *Shh* inactivation in the SG, HC differentiated prematurely and exhibited reversed apical-to-basal direction, which resembled the progression of cell cycle exit (17), supporting the idea that Shh signaling negatively regulates cochlear HC differentiation. Similarly, genetic ablation of *Smo*, the transducer of Shh signaling, in

## Significance

Sonic hedgehog (Shh) is responsible for the temporal and directional control of mammalian cochlear hair cell (HC) differentiation. We demonstrate that restriction of *Gli2* expression, the major transcriptional activator of the Shh signaling pathway, is a prerequisite for cochlear HC differentiation. *Gli2* blocks HC differentiation by maintaining the progenitor state of Sox2<sup>+</sup> prosensory cells. The spatiotemporal differentiation pattern of HCs along the basal–apical axis of the cochlea is achieved by Sufu- and Spop-mediated control of *Gli2* transcription in the sensory progenitor cells; cells expressing low *Gli2* could differentiate, whereas cells with high *Gli2* were locked in the progenitor state. This study unravels the mechanism underlying Shh-mediated control of sensory HC differentiation in the mammalian cochlea.

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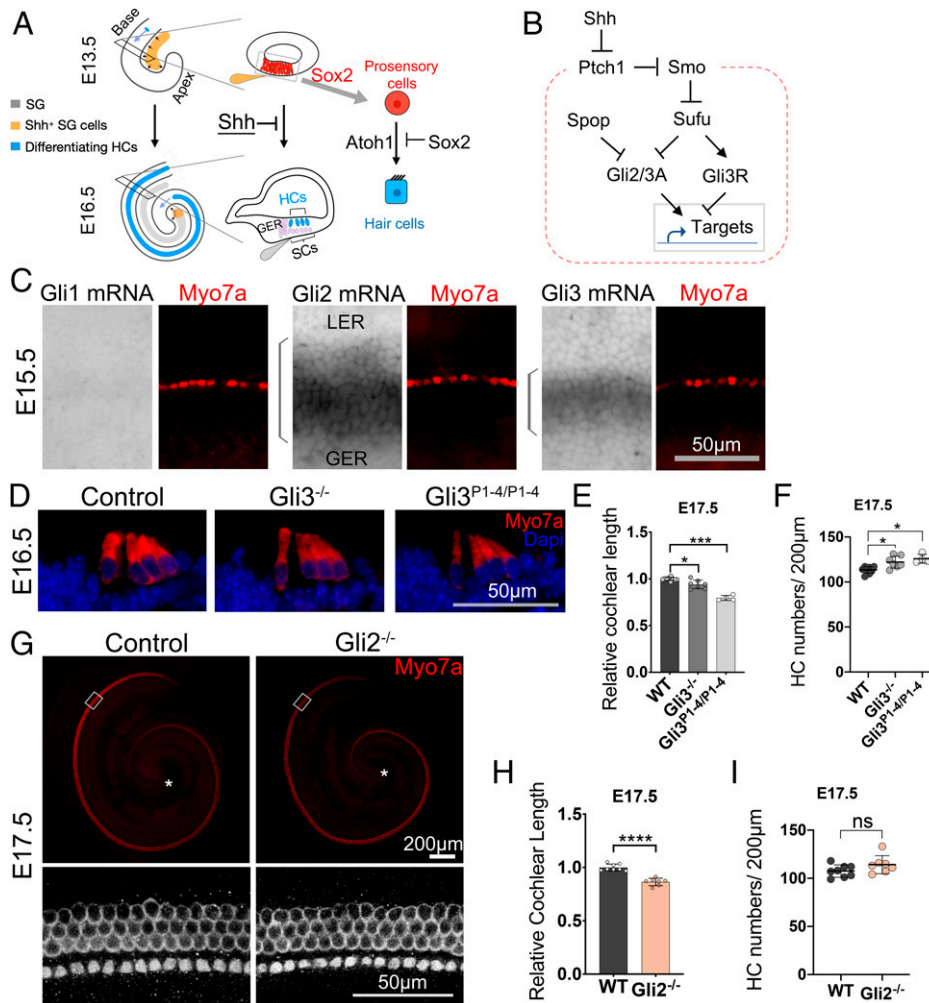
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<sup>1</sup>To whom correspondence may be addressed. Email: mhsham@cuhk.edu.hk.

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**Fig. 1.** Compensation between Gli2 and Gli3 in regulating cochlear HC differentiation. (A) *Left*, Schematic diagram of basal–apical progression of cochlear HC differentiation. *Right*, Schematic diagram of the cross-section view of the development of the oC. Both HCs and SCs are originated from Sox2<sup>+</sup> prosensory cells. Shh signaling inhibits HC differentiation. However, the underlying mechanism is not clear. Sox2 defines cochlear sensory progenitor cells. Sox2 expression level should be down-regulated during HC differentiation. (B) Schematic diagram of the Shh signaling pathway. Hh ligands bind to and inactivate the receptor Ptch1, which leads to the derepression of Smo. Activation of Smo initiates the signaling cascade by disassociating the Sufu–Gli complex. Sufu sequesters full-length Gli2/Gli3 protein in the cytoplasm, safeguards them from Spop-mediated degradation, and promotes their transition to truncated repressor form. Released full-length GliAs translocate to the nucleus and activate downstream targets. Gli2 is the major activator, whereas Gli3 is the major repressor of the pathway. Gli3 also contributes minor activator function. (C) *Gli1*, *Gli2*, and *Gli3* whole-mount in situ hybridization counterstained with Myo7a antibody in the medial part of the E15.5 cochlea ( $n \geq 3$  for all samples). All pictures are oriented with the lesser epithelial ridge (LER) on Top and the GER on Bottom. *Gli1* is absent in the differentiating cochlear epithelium. *Gli2* is expressed in the differentiating oC and GER. *Gli3* is expressed in the GER and medial part of the oC, which overlaps with IHCs. (D) Immunostaining of Myo7a showed the presence of IHCs and OHCs in E16.5 *Gli3*<sup>-/-</sup> ( $n = 3$ ) and *Gli3*<sup>P1-4/P1-4</sup> ( $n = 3$ ) cochlear sections. (E) Quantification of *Gli3*<sup>-/-</sup> ( $n = 7$ ) and *Gli3*<sup>P1-4/P1-4</sup> ( $n = 4$ ) cochlear length relative to littermate controls ( $n = 10$ ) at E17.5 (SI Appendix, Fig. S1). *Gli3*<sup>-/-</sup> cochlear length varied between samples; some were normal and some were slightly shorter. *Gli3*<sup>P1-4/P1-4</sup> cochleae are 20% shorter than in littermate controls; WT, wild type. (F) Quantification of E17.5 control ( $n = 10$ ), *Gli3*<sup>-/-</sup> ( $n = 7$ ), and *Gli3*<sup>P1-4/P1-4</sup> ( $n = 4$ ) HC numbers (SI Appendix, Fig. S1). (G) *Top*, whole-mount Myo7a immunostaining of E17.5 control and *Gli2*<sup>-/-</sup> cochleae. White asterisks indicate the end of the cochlear apex. *Bottom*, high magnification of Myo7a<sup>+</sup> HCs in the basal region indicated by a white box. (H) Quantification of *Gli2*<sup>-/-</sup> ( $n = 7$ ) cochlear length relative to littermate controls ( $n = 8$ ) at E17.5. *Gli2*<sup>-/-</sup> cochleae are around 15% shorter than in littermate controls. (I) Quantification of E17.5 control ( $n = 8$ ) and *Gli2*<sup>-/-</sup> ( $n = 7$ ) HC numbers. Scale bars are indicated. Data are shown as mean  $\pm$  SD; \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ; ns, not significant ( $P > 0.05$ ).

the cochlear epithelium leads to premature HC differentiation, whereas constitutive activation of *Smo* inhibits HC differentiation (18). Activation of Shh signaling also blocks HC differentiation in ex vivo cochlea culture (19). Furthermore, *Gli3* <sup>$\Delta$ 699/ $\Delta$ 699</sup> mice with constitutive expression of a truncated repressor form of Gli3 protein exhibit expanded sensory regions and formation of ectopic HCs (19). However, it remains unclear how Shh signaling is interpreted by prosensory cells during cochlea development to achieve the spatiotemporal differentiation pattern (Fig. 1A).

Gli transcription factors (Gli1, Gli2, and Gli3) are transducers of Shh signaling (20). While Gli1 acts as a transcriptional activator, Gli2 and Gli3 are bifunctional; they contain a transcriptional activation domain in the C terminus and a transcriptional repression domain in the N terminus (21, 22).

Genetic and cell-based experiments revealed that Gli2 is the major activator of mammalian Shh signaling, whereas Gli3 functions as a major repressor of the pathway (21, 23–27). Upon pathway activation, full-length Gli2/Gli3 activate Shh target genes, including *Gli1*, which serves to amplify the signaling output (28, 29). Suppressor of fused (Sufu) negatively regulates Shh signaling through retaining full-length Gli activator (GliA) in the cytoplasm and facilitating the formation of truncated Gli repressor (GliR) (30–33). Speckle-type PDZ protein (Spop) promotes proteasome-mediated degradation of full-length Gli proteins. Sufu competes with Spop for binding to Gli2/Gli3 full-length protein (34). Deletion of *Sufu* would render Gli2/Gli3 vulnerable to Spop-mediated ubiquitination and degradation (34, 35). Therefore, Sufu also plays an important role in

preventing the degradation of Gli2/Gli3. Shh signaling outcomes depend on the balance of GliA and GliR (Fig. 1B). Thus, Sufu–Gli regulation provides a fine-tuning system for cells to respond to Shh signaling.

Here, by using genetic mouse models, we show that inactivation of *Sufu* in the cochlear epithelium leads to a delay of HC differentiation. *Sufu* regulates HC differentiation by modulating Gli2 levels. Restoration of HCs in *Sufu*-mutant cochleae at a late developmental stage is due to Spop-mediated Gli2 degradation. Deletion of both *Sufu* and *Spop* causes a robust activation of *Gli2* and *Gli1* and severe disruption of HC formation. Furthermore, we unveil that Shh signaling controls the expression level of the progenitor maintenance factor Sox2 along the cochlear basal–apical axis. Together, these results suggest that Shh/Gli2 signaling maintains cochlear prosensory cells in the progenitor state, and down-regulation of Gli2 is a prerequisite for HC differentiation.

## Results

**Compensation Between Gli2 and Gli3 in Regulating Cochlear HC Differentiation.** Gli factors are transcriptional mediators of the Shh signaling pathway. To understand how Shh signaling is interpreted by cochlear epithelial cells, we examined the mRNA expression of *Gli1*, *Gli2*, and *Gli3* in the developing cochlea. At E15.5, whole-mount in situ hybridization counterstained with myosin7a (*Myo7a*) antibody showed that *Gli1* expression was absent in the differentiating cochlear epithelium, *Gli2* was expressed in the differentiating oC as well as GER, and *Gli3* was expressed in the GER and medial side of the oC, which overlaps with IHCs (Fig. 1C). Activated Shh signaling negatively regulates HC differentiation and would lead to elevated Gli2A levels and reduced Gli3R levels. A previous study showed that *Gli3*<sup>Δ699/Δ699</sup>-mutant (which only express Gli3R) cochleae contain ectopic HCs (19). To investigate whether reduction of Gli3R would inhibit cochlear HC differentiation, we analyzed the cochleae of *Gli3*<sup>−/−</sup> and *Gli3*<sup>P1-4/P1-4</sup> mice. In *Gli3*<sup>P1-4</sup> mice, the introduction of four nonphosphorylatable amino acid residues rendered the mutant Gli3 protein incapable of processing into Gli3R (36). We found that *Gli3*<sup>P1-4/P1-4</sup> cochleae were about 20% shorter than those in littermate controls (Fig. 1E and *SI Appendix*, Fig. S1), whereas *Gli3*<sup>−/−</sup> cochlear lengths were variable, with some similar to the littermate controls and others slightly shorter (Fig. 1E). Quantification of HC numbers revealed a small increase of HC density in both *Gli3*<sup>−/−</sup> and *Gli3*<sup>P1-4/P1-4</sup> mutants (Fig. 1F). Nonetheless, the stereotypical “1 + 3” pattern, as shown by *Myo7a* immunostaining, was unaffected in both *Gli3*<sup>−/−</sup> and *Gli3*<sup>P1-4/P1-4</sup> cochleae (Fig. 1D and *SI Appendix*, Fig. S1).

We next analyzed *Gli2*<sup>−/−</sup> cochleae to explore whether removal of Gli2 would recapitulate the reversed apical–basal HC differentiation pattern in *Shh* conditional knockout (CKO) mutants (17). Noticeably, *Gli2*<sup>−/−</sup> cochleae were about 15% shorter than those in littermate controls (Fig. 1H). *Myo7a* staining showed that HC differentiation still progressed normally from base to apex, as *Myo7a*<sup>+</sup> HCs were absent in the apex at E17.5, and HC patterning was also unaffected in *Gli2*<sup>−/−</sup> cochleae (Fig. 1G and I). Taken together, analysis of *Gli2*- and *Gli3*-mutant cochleae suggests a compensatory effect between Gli2 and Gli3 in regulating HC differentiation.

**Deletion of *Sufu* Leads to a Delay of Cochlear Hair Cell Differentiation.** Due to functional compensation between Gli2 and Gli3, we aimed to make use of other regulators to modulate the activities of both Gli2 and Gli3 to gain insight of their

roles in HC development. We used *Emx2Cre* to generate a CKO of *Sufu* in the developing cochlea to perturb the availability of Gli factors in the cochlear epithelium. Deletion of *Sufu* did not lead to significant changes of cochlear length at E16.5. At E18.5, *Emx2Cre;Sufu*<sup>ff</sup> cochleae were slightly longer (around 8%) than those in littermate controls (Fig. 2C). Whole-mount immunostaining of *Myo7a* showed that in E16.5 control cochleae, differentiated HCs displayed a stereotypical “1 + 3” organization of IHCs and OHCs in the oC in the basal region (Fig. 2A). However, in E16.5 *Emx2Cre;Sufu*<sup>ff</sup> cochleae, OHCs were completely absent (Fig. 2A and A′). We also examined HC differentiation defects in E18.5 *Emx2Cre;Sufu*<sup>ff</sup> cochleae. In control cochleae, a “1 + 3” structure could be detected in both the base and apex (Fig. 2B). Interestingly, while OHCs were still missing in the apex of *Emx2Cre;Sufu*<sup>ff</sup> cochleae, differentiation of OHCs was largely restored in the basal region (Fig. 2B and B′). These results illustrate that *Sufu* is required for the timing and progression of cochlear HC differentiation, and deletion of *Sufu* leads to a delay of HC differentiation.

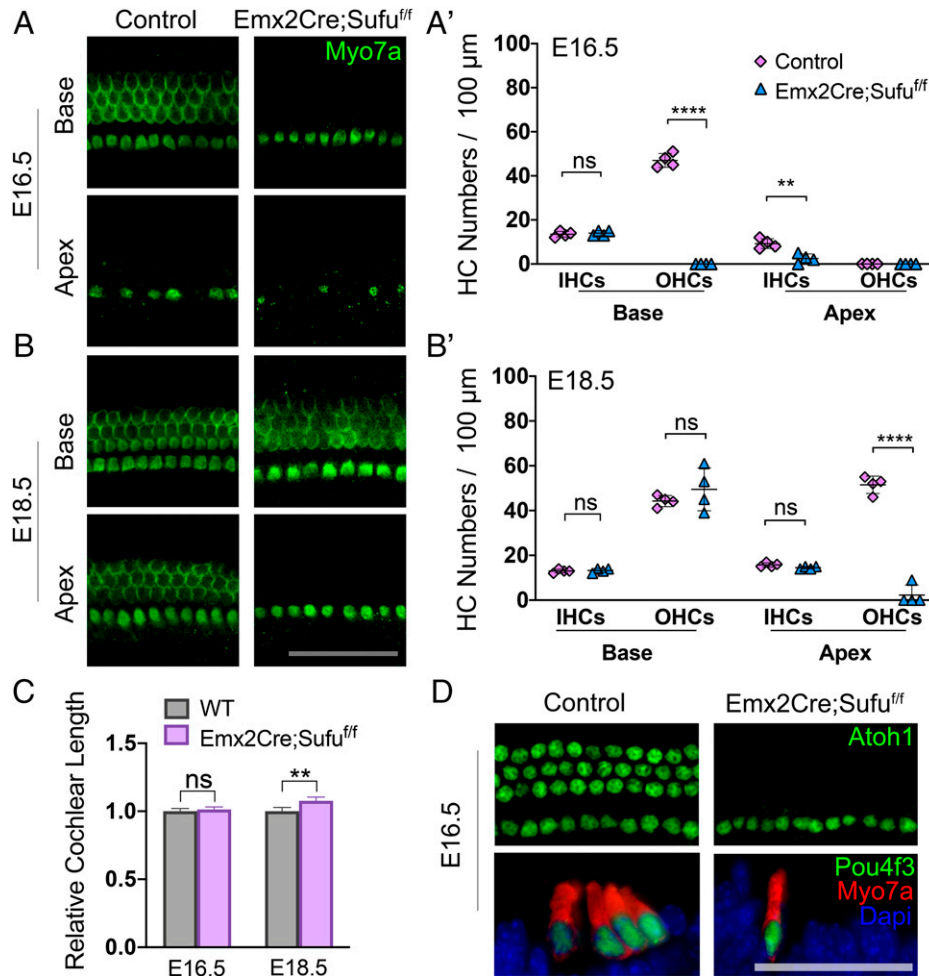
As *Atoh1* is an essential factor for HC differentiation, we examined the expression of *Atoh1* in the oC by immunostaining. At E16.5, while *Atoh1* expression was detected in all HCs of the control cochleae, it was lost in the presumptive OHC region, but only present in the IHCs, of *Emx2Cre;Sufu*<sup>ff</sup> oCs (Fig. 2D). Furthermore, expression of the *Atoh1* downstream target *Pou4f3* (37) was also lost specifically in the outer compartment of *Emx2Cre;Sufu*<sup>ff</sup> oCs (Fig. 2D). These observations further illustrate the differentiation defect of OHCs in the *Sufu* mutant.

### Gli2 Regulates HC Differentiation in *Sufu*-Deficient Cochleae.

It is expected that deletion of *Sufu* would lead to elevated Gli2A levels and reduced Gli3R levels. Given that complete loss of Gli3R did not lead to significant HC differentiation defects, we asked whether elevated Gli2A may be the primary cause of abnormal HC development in *Sufu* CKOs. To address this, we first examined mRNA expression of *Gli2* as well as its downstream target *Gli1* in the *Sufu* mutant by whole-mount in situ hybridization at E16.5. In the control cochleae, *Gli2* was expressed in the GER region on the medial side to the differentiating oC (Fig. 3A), whereas *Gli1* was not expressed in the differentiating cochlear epithelium (Fig. 3B and C). However, in the *Emx2Cre;Sufu*<sup>ff</sup> mutant, *Gli2* was ectopically expressed in the oC, where OHCs were absent (Fig. 3D). *Gli1* was ectopically expressed in the GER region but not in the oC (Fig. 3E and F). We reasoned that the GER expression of *Gli1* likely reflects the elevated activities of Gli2A in the *Sufu* mutant. To examine whether elevated Gli2 (major GliA) contributes to the HC differentiation defects in the *Sufu* mutant, we generated *Emx2Cre;Sufu*<sup>ff</sup>;*Gli2*<sup>ff</sup> mice to reduce the level of Gli2 in *Sufu*-mutant cells. In E16.5 *Emx2Cre;Sufu*<sup>ff</sup>;*Gli2*<sup>ff</sup> cochleae, OHCs were partially restored (Fig. 3G and H), demonstrating that deletion of *Gli2* could partially rescue HC differentiation defects in *Sufu* mutants. These results support the notion that elevated Gli2 levels in the *Sufu* mutant inhibit HC differentiation.

At E18.5, the OHC differentiation defect in the *Sufu* mutant was restored in the basal region but remained in the apex. To address whether this phenomenon was consistent with Gli2 levels, we further examined *Gli2* and *Gli1* expression in the E18.5 *Sufu* mutant. By E18.5, ectopic expression of both *Gli2* and *Gli1* in the *Emx2Cre;Sufu*<sup>ff</sup> cochlear base was diminished to an extent comparable to the control (Fig. 3I–L). However, *Gli2* was ectopically expressed in the *Sufu*-mutant cochlear apex where OHCs were still absent (Fig. 3M and O). Absence of *Gli1* expression in the *Sufu*-mutant cochlear apex suggested that the level of Gli2A





**Fig. 2.** *Sufu* is required for timing of cochlear HC differentiation. (A) Whole-mount immunostaining of Myo7a showed significantly reduced HC numbers in both the base and apex of E16.5 *Sufu*-CKO cochleae ( $n = 4$ ) compared to controls ( $n = 4$ ). (A') Quantification of IHCs and OHCs in the base and apex of E16.5 control and *Emx2Cre;Sufu<sup>ff</sup>* cochleae. (B) Whole-mount immunostaining of Myo7a showed that HC differentiation is recovered in the base of E18.5 *Sufu*-CKO cochleae ( $n = 4$ ) compared to controls ( $n = 4$ ). (B') Quantification of IHCs and OHCs in the base and apex of E18.5 control and *Emx2Cre;Sufu<sup>ff</sup>* cochleae. (C) Quantification of *Emx2Cre;Sufu<sup>ff</sup>* cochlear length relative to littermate controls at both E16.5 and E18.5 ( $n \geq 3$  for all samples). (D) Immunostaining of Atoh1 and downstream targets Pou4f3 and Myo7a showed that essential differentiation factors are absent in the outer compartment of E16.5 *Emx2Cre;Sufu<sup>ff</sup>* cochleae ( $n = 3$ ) compared to in controls ( $n = 3$ ). (Scale bar, 50  $\mu\text{m}$ .) Data are shown as mean  $\pm$  SD; \*\* $P \leq 0.01$ , \*\*\*\* $P \leq 0.0001$ ; ns, not significant ( $P > 0.05$ ).

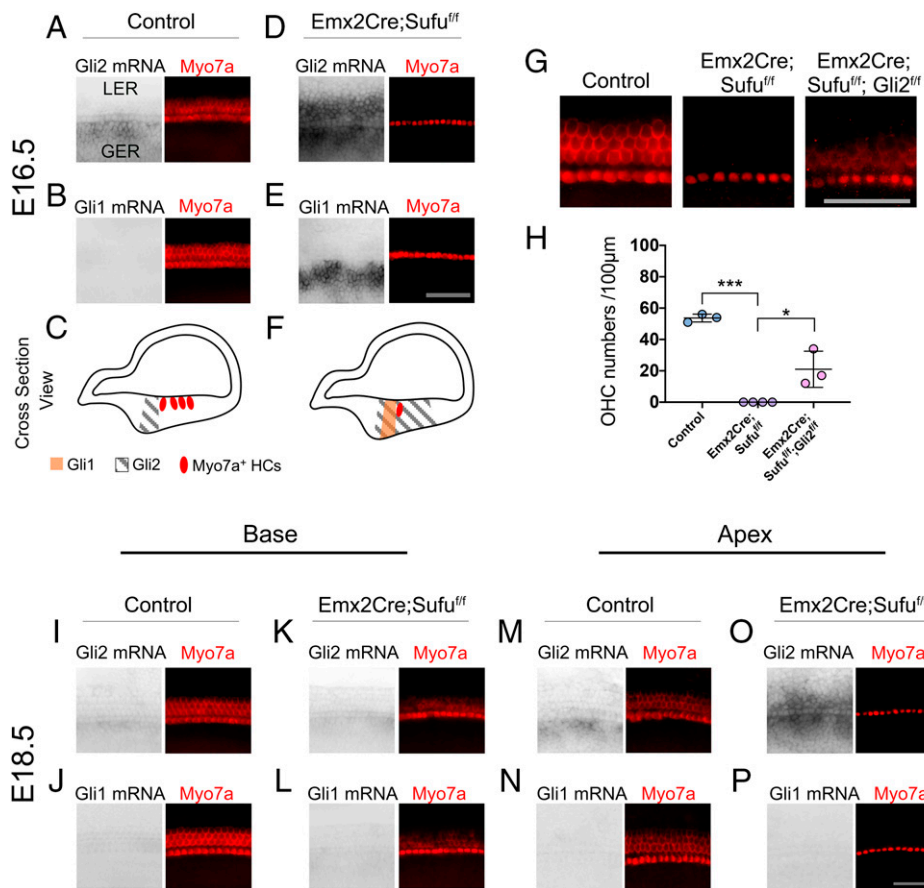
was insufficient to activate *Gli1* but could still inhibit OHC differentiation in the apical region (Fig. 3 *N* and *P*), resembling the status of E15.5 control cochleae (Fig. 1 *C*). These results coincide with the delay of HC differentiation in *Emx2Cre;Sufu<sup>ff</sup>*-mutant cochleae. Taken together, gene expression analysis and genetic rescue experiments highlight the inhibitory role of Gli2(A) in the regulation of cochlear HC differentiation.

#### Inhibition of Gli2 Is a Prerequisite for HC Differentiation.

Next, we asked how reduction of both *Gli2* and *Gli1* levels from E16.5 to E18.5 may be achieved in the basal region of *Emx2Cre;Sufu<sup>ff</sup>*-mutant cochleae. Deletion of *Sufu* leads to Spop-mediated proteasomal degradation of Gli2 (34, 35). We hypothesized that Spop could be involved in the down-regulation of Gli2 in E18.5 *Sufu*-mutant cochleae. To test this, we deleted both *Sufu* and *Spop* in the developing cochlea by generating *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>* embryos. While *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>* cochlear length was comparable to that of the littermate controls (Fig. 4 *S*), we found widespread expression of both *Gli2* and *Gli1* in the *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>* cochlear epithelium at E16.5 (Fig. 4 *A–F* and *SI Appendix, Fig. S2*). Consistent with the notion that Gli2 inhibits HC differentiation, HC differentiation was severely disrupted in *Emx2Cre;*

*Sufu<sup>ff</sup>;Spop<sup>ff</sup>* cochleae; only few Myo7a<sup>+</sup> HCs could be found in the mutant cochleae (Fig. 4 *D–F* and *T* and *SI Appendix, Fig. S4*). Noticeably, at E18.5, ectopic expression of both *Gli2* and *Gli1* was maintained in the mutant cochleae, and HC differentiation was largely compromised in *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>* cochleae (Fig. 4 *J–O* and *T* and *SI Appendix, Figs. S2* and *S4*). This ectopic *Gli2* and *Gli1* expression was detected along the entire length of the mutant cochleae (*SI Appendix, Fig. S3*). We reasoned that the expanded expression of *Gli2* and *Gli1* in both E16.5 and E18.5 *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>* cochleae could be due to further elevation of Gli2A activities at both RNA and protein levels. These results suggest that Spop contributes to the degradation of Gli2 and restoration of HC differentiation in E18.5 *Emx2Cre;Sufu<sup>ff</sup>* cochleae. Nonetheless, removing one allele of *Spop* did not result in a more severe phenotype in the *Sufu*-mutant cochleae (Fig. 4 *U* and *V*). Furthermore, HC differentiation was not affected by the deletion of *Spop* in the *Sufu* heterozygous mutant background (Fig. 4 *U* and *V*). Therefore, although *Spop* plays regulatory roles in modulating Gli2 levels in the *Sufu*-mutant cochleae, it appears dispensable for cochlear HC differentiation in the normal context.

In addition to its role as the receptor, Patched1 (Ptch1) is also a negative regulator of Hh signaling. After binding with Hh ligands,



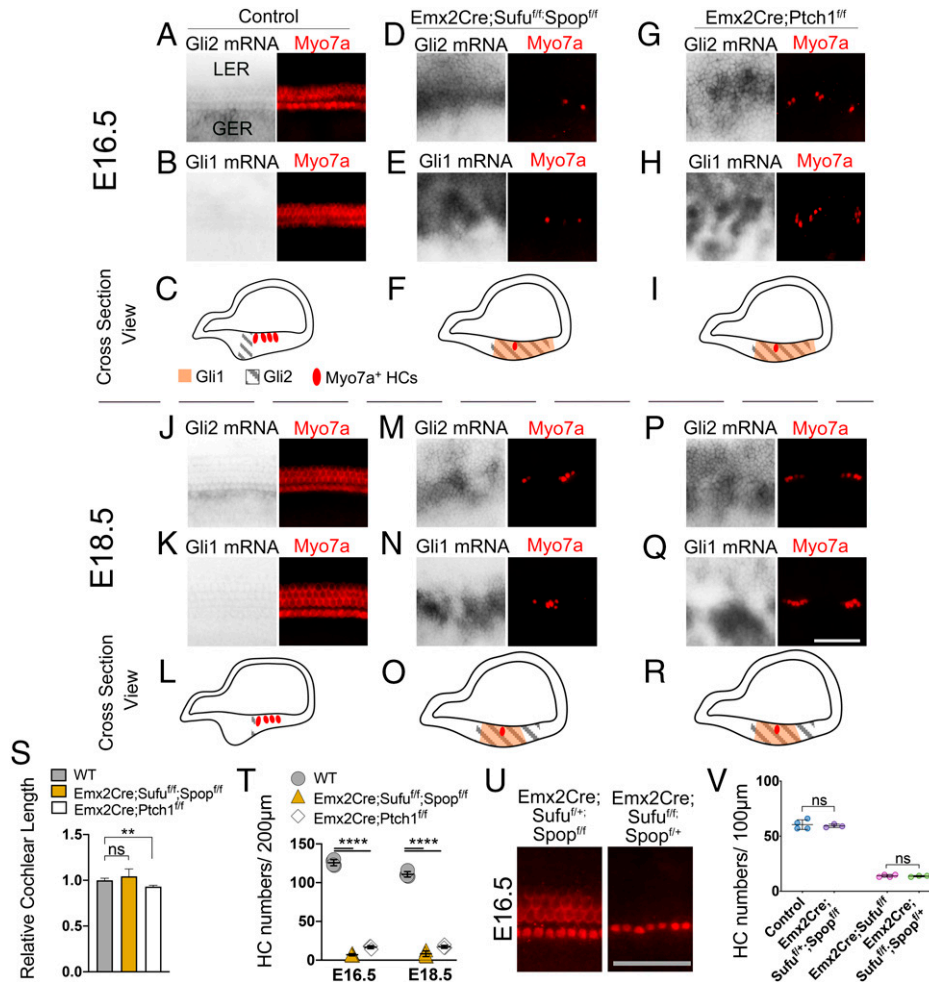
**Fig. 3.** Gli2 regulates HC differentiation in Sufu-deficient cochleae. (A, B, D, and E) *Gli2* and *Gli1* whole-mount in situ hybridization counterstained with Myo7a antibody in the basal region of E16.5 control and *Emx2Cre;Sufu<sup>ff</sup>* cochleae ( $n \geq 3$  for all samples). (C and F) Diagrams of the cross-section view summarizing expression patterns of both *Gli2* and *Gli1* relative to the HCs in E16.5 control and *Emx2Cre;Sufu<sup>ff</sup>* cochleae. (G) Whole-mount immunostaining of Myo7a in the cochlear basal region show that the OHC differentiation defect is partly rescued in *Emx2Cre;Sufu<sup>ff</sup>;Gli2<sup>ff</sup>* mice ( $n = 3$ ) compared to in *Emx2Cre;Sufu<sup>ff</sup>* mice ( $n = 4$ ) at E16.5. (H) Quantification of OHC numbers in E16.5 control ( $n = 3$ ), *Emx2Cre;Sufu<sup>ff</sup>* ( $n = 3$ ), and *Emx2Cre;Sufu<sup>ff</sup>;Gli2<sup>ff</sup>* ( $n = 3$ ) cochleae. (I–P) *Gli2* and *Gli1* whole-mount in situ hybridization counterstained with Myo7a antibody in E18.5 control and *Emx2Cre;Sufu<sup>ff</sup>* cochleae ( $n \geq 3$  for all samples). Base and apex are indicated. All whole-mount in situ hybridization and Myo7a staining images in Fig. 3 are oriented with LER on top and GER on bottom. (Scale bar, 50  $\mu\text{m}$ .) Data are shown as mean  $\pm$  SD; \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$ ; ns, not significant ( $P > 0.05$ ).

*Ptch1* is inactivated, which results in derepression of Smoothed (Smo). Smo promotes dissociation of the Sufu–Gli complex. Full-length Gli proteins then activate downstream targets (20). Therefore, removal of *Ptch1* would lead to a significant activation of Gli2. We hypothesized that *Ptch1* CKO cochleae may also exhibit ectopic Gli2 activation together with HC differentiation defects. We examined expression of *Gli2* and *Gli1* as well as HC differentiation in the *Emx2Cre;Ptch1<sup>ff</sup>*-mutant cochleae. At E16.5, both *Gli2* and *Gli1* were widely expressed in the *Emx2Cre;Ptch1<sup>ff</sup>* cochlear epithelium, and few differentiating HCs could be detected (Fig. 4 G–I and T and SI Appendix, Figs. S2 and S4). At E18.5, both *Gli2* and *Gli1* were enriched in the cochlear epithelium, and ectopic expression was persistent along the entire cochlea; HC differentiation was severely disrupted (Fig. 4 P–R and T and SI Appendix, Figs. S2–S4). While *Emx2Cre;Ptch1<sup>ff</sup>* cochleae were slightly shorter (7%) than those of littermate controls (Fig. 4S), robust elevation of *Gli2* and *Gli1* expression as well as severe defects of HC differentiation in the *Emx2Cre;Ptch1<sup>ff</sup>*-mutant cochleae were similar to that observed in *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>* cochleae. Taken together, these results show that restriction of Gli2 levels by negative regulators of the Shh signaling pathway, such as *Sufu*, *Spop*, and *Ptch1*, is essential for cochlear HC differentiation.

**Sufu/Gli2 Control the Dynamic Spatiotemporal Expression of Sox2 in the Developing Cochlea.** The down-regulation of *Gli2* levels and restoration of HC differentiation in the E18.5

*Emx2Cre;Sufu<sup>ff</sup>* mutant prompted us to address whether the prosensory cells were locked in the progenitor state upon *Sufu* deletion. Given the essential functions of Sox2 as a progenitor factor in the oC (2–5), we examined Sox2 expression in *Sufu*-CKO cochleae. At E16.5, Sox2 expression could be clearly detected in the differentiating HCs, SCs, and GER in the control. However, Sox2<sup>+</sup> cells appeared disorganized in the *Emx2Cre;Sufu<sup>ff</sup>* oC (Fig. 5A), resembling the Sox2 expression pattern at E14.5 (SI Appendix, Fig. S5A). At E14.5, there was a small increase in Sox2<sup>+</sup> cell numbers in the *Emx2Cre;Sufu<sup>ff</sup>* cochleae (SI Appendix, Fig. S5 A and B). At E18.5, while Sox2 expression was down-regulated in the HCs of control oCs, it was still maintained in the differentiating HCs of *Emx2Cre;Sufu<sup>ff</sup>* oCs (Fig. 5A). Moreover, in *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>*-mutant cochleae, there was a significant expansion of the Sox2<sup>+</sup> domain compared to the control (Fig. 5A and SI Appendix, Fig. S5D). This broader Sox2 expression is not due to cochlear outgrowth defects, as *Emx2Cre;Sufu<sup>ff</sup>;Spop<sup>ff</sup>*-mutant cochleae are not shorter than the control cochleae (Fig. 4S). Together, these results demonstrate that elevated Gli2 levels in the cochlea cause prolonged maintenance of Sox2 expression and expansion of the Sox2<sup>+</sup> domain.

The expression level of Sox2 is critical for cell differentiation in the oC. Loss of Sox2 leads to depletion of HCs, whereas high levels of Sox2 inhibit HC differentiation (2–5). It remains unclear how Sox2 expression is spatially and temporally regulated, as



**Fig. 4.** Low level of Gli2 is a prerequisite for HC differentiation. (A–R) *Gli2* and *Gli1* whole-mount in situ hybridization counterstained with Myo7a antibody in E16.5 and E18.5 control, *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/fl</sup>*, and *Emx2Cre;Ptch1<sup>fl/fl</sup>* cochleae ( $n \geq 3$  for all samples). (C, F, I, L, O, and R) Diagrams of the cross-section view summarizing expression patterns of both *Gli2* and *Gli1* relative to the HCs in the E16.5 and E18.5 control, *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/fl</sup>*, and *Emx2Cre;Ptch1<sup>fl/fl</sup>* cochleae. (S) Quantification of E16.5 *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/fl</sup>*, and *Emx2Cre;Ptch1<sup>fl/fl</sup>* cochlear length compared to in littermate controls ( $n \geq 3$  for all samples). (T) Quantification of E16.5 and E18.5 control, *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/fl</sup>*, and *Emx2Cre;Ptch1<sup>fl/fl</sup>* HC numbers ( $n \geq 3$  for all samples). (U) Whole-mount immunostaining of Myo7a in E16.5 *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/fl</sup>* cochleae ( $n = 3$ ) showed that HC differentiation is normal when *Spop* is deleted. Removing one allele of *Spop* only in the *Sufu*-mutant background (E16.5 *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/+</sup>* [ $n = 3$ ]) cochleae does not exacerbate the HC differentiation defect. (V) Quantification of HC numbers in E16.5 control, *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/fl</sup>*, *Emx2Cre;Sufu<sup>fl/fl</sup>*, and *Emx2Cre;Sufu<sup>fl/fl</sup>;Spop<sup>fl/+</sup>* cochleae. All whole-mount in situ hybridization and Myo7a staining images in Fig. 4 are oriented with LER on top and GER on bottom. (Scale bar, 50  $\mu\text{m}$ .) Data are shown as mean  $\pm$  SD; \*\* $P \leq 0.01$ , \*\*\*\* $P \leq 0.0001$ ; ns, not significant ( $P > 0.05$ ).

HCs differentiate from base to apex of the cochlea. We hypothesized that *Sox2* is differentially expressed along the basal–apical axis, which is consistent with the directional progression of HC differentiation. To further investigate the dynamic spatiotemporal regulation of *Sox2* along the basal–apical axis, we analyzed enhanced green fluorescent protein (EGFP) signals driven from the *Sox2* locus using *Sox2<sup>EGFP/+</sup>* cochleae. Higher EGFP intensity was detected in the undifferentiated apex than in the differentiating base at E16.5. Moreover, differentiating cells from base to midapex of E16.5 *Sox2<sup>EGFP/+</sup>* cochleae exhibited lower EGFP intensity than undifferentiated progenitors from base to midapex of E13.5 (Fig. 5 B and C). These results showed that *Sox2* expression level is higher in the undifferentiated prosensory cells than in the differentiating oC, and the level of *Sox2* is down-regulated from cochlear base to apex.

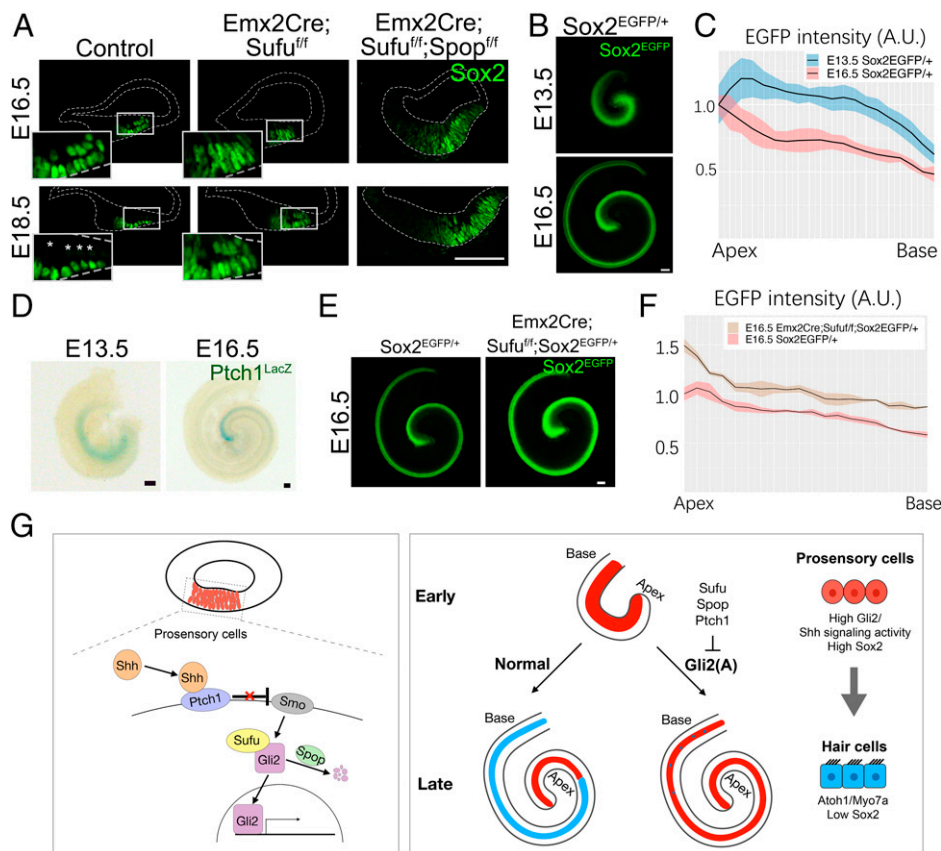
The decreasing *Sox2* level from base to apex is consistent with the diminishing pattern of *Shh* expression in the SG (16). *Ptch1* is the receptor and also a downstream target of Shh signaling. We examined the expression pattern of *Ptch1* at E13.5 and E16.5 by using *Ptch1<sup>LacZ/+</sup>* reporter mice. *LacZ* expression revealed that *Ptch1* was expressed along the entire length of the cochlea at E13.5 and became restricted to the apical region at

E16.5 (Fig. 5D). The basal–apical *Ptch1* expression pattern mimics the dynamic changes of *Sox2* expression. We hypothesized that down-regulation of Shh signaling activity is responsible for the down-regulation of *Sox2* expression from base to apex in the developing cochlea. To address this notion, we analyzed EGFP intensity of *Emx2Cre;Sufu<sup>fl/fl</sup>;Sox2<sup>EGFP/+</sup>* cochleae. EGFP intensity at E16.5 revealed elevated *Sox2* expression levels throughout the cochlea in *Emx2Cre;Sufu<sup>fl/fl</sup>;Sox2<sup>EGFP/+</sup>* mice compared to the littermate *Sox2<sup>EGFP/+</sup>* controls (Fig. 5 E and F). These results showed that Shh signaling/*Gli2* maintain high *Sox2* expression levels and enhance progenitor maintenance in the oC throughout the entire cochlea and suggested that differential expression of *Sox2* along the basal–apical axis of developing cochlea is modulated by Shh signaling.

## Discussion

Shh signal from the SG has been shown to regulate the basal–apical differentiation pattern of cochlear HCs, thereby separating the cell cycle exit process from subsequent differentiation (17). Nevertheless, it is not clear how Shh signal is decoded by the prosensory cells to control temporal and spatial





**Fig. 5.** *Sufu*/*Gli2* control the spatiotemporal dynamic expression of *Sox2* in the developing cochlea. (A) Immunostaining of *Sox2* on sections of E16.5 and E18.5 control, *Emx2Cre*;*Sufu*<sup>fl/fl</sup>, and *Emx2Cre*;*Sufu*<sup>fl/fl</sup>;*Spop*<sup>fl/fl</sup> cochleae ( $n \geq 3$  for all samples). *Left Bottom*, magnified view of the white rectangle circled areas. White asterisks indicate down-regulation of *Sox2* in HCs of E18.5 control cochleae. (B) EGFP expression of E13.5 and E16.5 *Sox2*<sup>EGFP/+</sup> cochleae ( $n = 6$  for both stages). (C) Quantification of EGFP intensity of *Sox2*<sup>EGFP/+</sup> cochleae from apex to base at both E13.5 and E16.5 showed that the *Sox2* expression level is higher in the undifferentiated progenitor cells (E16.5 apex and E13.5 midbase to apex) than in differentiating cells (E16.5 base to medial part). Data are shown as mean  $\pm$  SD; A.U., arbitrary units. (D) X-gal staining of E13.5 ( $n = 12$ ) and E16.5 ( $n = 8$ ) *Ptch1*<sup>LacZ/+</sup> cochleae revealed restriction of *Ptch1* to the cochlear apex during development. (E) EGFP expression of E16.5 *Sox2*<sup>EGFP/+</sup> and *Emx2Cre*;*Sufu*<sup>fl/fl</sup>;*Sox2*<sup>EGFP/+</sup> cochleae ( $n = 4$ ). (F) Quantification of EGFP intensity of E16.5 *Sox2*<sup>EGFP/+</sup> and *Emx2Cre*;*Sufu*<sup>fl/fl</sup>;*Sox2*<sup>EGFP/+</sup> cochleae from apex to base showed elevated *Sox2* level throughout cochlea when *Sufu* is removed. Data are shown as mean  $\pm$  SD. (Scale bar, 100  $\mu$ m.) (G) Summary diagram. *Left*, in the prosensory cells, *Sufu* sequesters *Gli2* full-length activator (*Gli2A*) in the cytoplasm. At the same time, *Sufu* secures a pool of *Gli2A* from preventing them from *Spop*-promoted degradation. Binding of *Shh* ligands to *Ptch1* leads to derepression of *Smo* activity. Activated *Smo* separates *Gli2A* from *Sufu*. *Gli2A* translocates into the nucleus and activates downstream targets. Prosensory cells are thus maintained in their progenitor state. *Right*, *Gli2*, subjected to a tight regulation of *Shh* signaling modulators, maintains the progenitor state of prosensory cells and thereby inhibits HC differentiation. Down-regulation of *Shh* signaling activity/*Gli2* from base to apex releases the cells from the progenitor state, and HC differentiation progresses from base to apex. When *Shh* signaling/*Gli2* is elevated, cells are locked in the progenitor state, and HC differentiation is severely disrupted.

progression of differentiation. Due to functional compensation between *Gli2* and *Gli3* in HC differentiation, delineating their regulatory roles in this process has been challenging. The functions of the intracellular factors *Sufu* and *Spop* in modulating *GliA* and *GliR* levels in the prosensory cells were not previously known. Through analysis of *Sufu* and *Spop* genetic mutants, this study provides mechanistic insight into the regulatory roles of *Gli* factors in HC differentiation. We have demonstrated that *Gli2(A)* promotes progenitor maintenance of cochlear prosensory cells, and down-regulation of *Gli2(A)* by multiple regulators (*Sufu*, *Spop*, and *Ptch1* in this study) is a prerequisite for HC differentiation in the oC.

At around E13.5, *Shh* is broadly expressed in the SG. During development, *Shh* expression gradually declines from base to apex in the SG (16). Consistently, *Ptch1*<sup>LacZ/+</sup> reporter shows that *Ptch1* expression is down-regulated from cochlear base to apex. As a negative regulator of the *Shh* signaling pathway, *Ptch1* represses the activity of *Smo*. Without *Shh* ligands, *Gli* full-length activators are transformed into *GliR*. In the presence of *Shh*, *Ptch1* is inactivated, and *Smo* facilitates separation of *Gli* full-length activators (*GliA*) and *Sufu*. *GliA* would

translocate into the nucleus and activate downstream targets. Indeed, removal of *Ptch1* results in robust activation of both *Gli2* and *Gli1* and severe HC differentiation defects at both E16.5 and E18.5. However, deletion of *Sufu*, encoding another negative regulator of the pathway, causes milder HC differentiation defects and weaker ectopic *Gli1/Gli2* expression at E16.5 than *Ptch1* CKO. Moreover, defects of HC differentiation and *Gli1/Gli2* expression in *Sufu* CKO cochleae are gradually alleviated at E18.5. We attributed these differences between *Sufu* and *Ptch1* CKOs to the *Spop*-mediated degradation of full-length *Gli2* activators. Without *Sufu*, *Gli2* is susceptible to *Spop*-mediated degradation so that *Gli2* level is mildly up-regulated at E16.5 and then diminished at E18.5. Indeed, when *Spop* is deleted in the *Sufu*-mutant background, expression of both *Gli2* and *Gli1* is significantly up-regulated, and HC differentiation is severely compromised at both E16.5 and E18.5, which are similar to the *Ptch1* CKO. Interestingly, removal of *Spop* alone does not affect cochlear HC differentiation. Given that *Sufu* and *Spop* competitively bind to full-length *Gli2* proteins (34), it is likely that *Sufu*-mediated regulation of *Gli* is sufficient to tune down the level of full-length *Gli2* activators

when *Spop* is absent. The following is the model that we present here (Fig. 5G): in the prosensory cells, Sufu binds to full-length Gli2A to prevent Spop-mediated degradation and maintains a sufficient amount of Gli2A in the cytoplasm.  $Ptch1^{+}/Sox2^{+}$  prosensory cells receive Shh ligands secreted from the SG. Binding of Shh to Ptch1 leads to activation of Smo and the subsequent dissociation of Sufu and Gli2A. Gli2A then accumulates in the nucleus to activate downstream targets, including Gli1 and Ptch1. Therefore, the prosensory cells are maintained in the progenitor state. Without Shh ligands, Sufu sequesters Gli2A in the cytoplasm, and prosensory cells are released from their progenitor state. Hence, HCs differentiate from cochlear base to apex, following the pattern of *Shh* decline.

While the absence of Gli3(R) does not affect cochlear HC differentiation,  $Gli3^{P1-4/P1-4}$ -mutant cochleae are significantly shortened (20% shorter than in littermate controls), which is a phenotype also observed in  $Gli3^{\Delta 699/\Delta 699}$  cochleae (50% shorter than in littermate controls) (19, 38). In  $Gli3^{P1-4/P1-4}$  mutants, Gli3 proteins only exist in the full-length form, and no Gli3R could be generated. By contrast,  $Gli3^{\Delta 699/\Delta 699}$  mutants only express Gli3R. This revealed that the ratio of Gli3A to Gli3R may be important for normal outgrowth of the cochlear duct. Unlike  $Gli3^{-/-}$  or  $Gli3^{P1-4/P1-4}$  mutants, there are ectopic HCs in the  $Gli3^{\Delta 699/\Delta 699}$  cochleae, which could result from an expanded prosensory domain. Therefore, it is possible that excessive Gli3R could affect cochlear prosensory specification in an Shh-independent manner, as also seen in the limb bud where Gli3R establishes anteroposterior asymmetry independently of Shh (39). Moreover, excessive Gli3R might affect normal development of the GER region such that some of the nonsensory cells in the GER were not able to maintain their identity and switched to a sensory cell fate.

Strikingly,  $Gli2^{-/-}$  cochleae do not exhibit an apical–basal HC differentiation pattern observed in the *Shh* CKO (17), and removal of *Gli2* in the *Sufu*-mutant background at E16.5 does not completely rescue the defects. Given that Gli3 could also act as a weak activator, these observations suggest that Gli3 could partly compensate for the function of GliA when Gli2 is absent in the cochlea.

While Sufu regulates Gli protein posttranslationally, it is interesting to note that *Gli2* mRNA is ectopically expressed in the E16.5 *Sufu*-mutant oC. This indicates that *Gli2* may autoregulate itself to sustain high Gli2 protein expression in the oC. In the kidney, Gli2 could bind to its own promoter (40). Autoregulation of *Gli2* may help to ensure sufficient supply of Gli2 protein for an acute response to Shh signaling. Due to the lack of robust antibody for the detection of Gli2 protein localization and expression pattern in the cochlea, we analyzed expression of *Gli1*, a transcriptional target of Gli2 protein, to indirectly assess Gli2 protein expression and function. Intriguingly, ectopic *Gli1* mRNA expression could only be found in the GER region but not in the oC of the *Sufu* mutant. It is possible that in the *Sufu*-CKO oC, Gli2 protein expression level can inhibit HC differentiation but is insufficient to activate *Gli1*. Consistent with this notion, we showed that *Gli1* expression is broadly detected in the floor of the cochlear epithelium when Gli2 is stabilized from protein degradation in the *Sufu;Spop*-mutant cochleae. Of note, OHCs are specifically lost in the E16.5  $Emx2Cre;Sufu^{ff}$  cochleae, and some IHCs still form in the  $Emx2Cre;Sufu^{ff};Spop^{ff}$  and  $Emx2Cre;Ptch1^{ff}$  mutants, suggesting that OHCs are more susceptible to elevated Shh signaling activity/Gli2 than IHCs, and there is differential regulation of IHCs and OHCs during development.

Sox2 is a transcription factor essential for cochlear sensory development. Absence of Sox2 causes loss or significant reduction of prosensory regions and HC differentiation (2, 3). Sox2 is required to confer competence to the sensory progenitor cells. Importantly, Sox2 has to be tuned down during subsequent differentiation processes, as continued Sox2 expression inhibits HC differentiation (4, 5). Due to the essential roles of Sox2 in HC development, a tight control of Sox2 level is critical in ensuring proper HC differentiation. Despite that several factors/signaling pathways (e.g., Fibroblast growth factor signaling [41], Notch signaling [42], and Gata3 [43], etc.) have been identified to regulate Sox2 expression in the prosensory cells, the spatiotemporal mechanisms of tuning down Sox2 expression during HC differentiation remain unclear. Although forced expression of Atoh1 would turn off Sox2 expression (4), removal of Atoh1 does not affect Sox2 down-regulation in the oC (44), suggesting the involvement of other signaling pathways or factors in the suppression of Sox2. By analyzing EGFP intensity of  $Sox2^{EGFP/+}$  cochleae, we found that Sox2 expression level is higher in the undifferentiated progenitor and is gradually down-regulated from base to apex during the time window of HC differentiation (Fig. 5). Through inactivation of *Sufu* in the  $Sox2^{EGFP}$  background, we showed that elevated Shh signaling activity prevents the down-regulation of Sox2 during HC development. Our results indicate that decline of Gli2/Shh signaling activity mediated by Sufu and other modulators of the Shh pathway triggers the down-regulation of Sox2 as the progenitors commit to differentiation in a spatial-specific manner. Although a high level of Gli2 is capable of boosting Sox2 expression level in the oC, it is not a prerequisite for Sox2 expression, as Sox2 is still expressed, and HCs form in *Shh*-CKO ( $Foxg1Cre;Shh^{lox}$ ) cochleae (17). Thus, the function of Gli2/Shh signaling in the oC is to adjust the expression level of Sox2 and activate other progenitor genes to maintain the progenitor state, thereby preventing HC differentiation (Fig. 5G). Indeed, restoration of HC formation in *Sufu* mutants over time indicates that those cells are kept in the progenitor state at E16.5 and are capable of differentiation once the Gli2 level is reduced at E18.5.

It has been shown by Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) analysis of Sox2<sup>+</sup> cells in the cochlea that the Gli-binding motif is enriched in the peaks with decreased accessibility from E12 to E16 (45). Consistently, in several other systems and cancers, Sox2 could also be activated by Gli2 or Shh (46–52). In telencephalic neuroepithelial cells, Gli2 is capable of binding to the enhancer of *Sox2* and activating *Sox2* expression (51). Gli and Sox2 could cooperatively regulate Shh target genes in the developing neural tube (52). It is possible that in the cochlear prosensory cells, Gli2 may also regulate *Sox2* expression directly or cooperatively. Therefore, identification and characterization of functional Sox2 enhancers in the cochlear prosensory cells are essential to decipher how Sox2 is precisely regulated during HC development. Comprehensive profiling of Gli2- and Sox2-binding regulatory regions in the cochlear prosensory cells would be required to address the molecular mechanisms.

In summary, by using genetic approaches in this study, we have established the inhibitory functions of Gli2A in cochlear HC differentiation. Gli2, modulated by Sufu, Spop, and Ptch1, is the key transcriptional regulator of the Shh signaling pathway in preventing HC differentiation. High levels of Gli2 activate Sox2 and maintain the progenitor state of the prosensory cells, and HC differentiation is stalled consequently. Therefore, Gli2 levels are rigorously controlled to allow proper HC differentiation to occur. Given that hearing loss was observed in human patients with *Ptch1* mutation (53), our



study in deciphering the mechanisms of Shh-mediated regulation of HC development has shed light on the understanding of the etiology of hearing impairment in human patients. As demonstrated here, Shh signaling/Gli2 promote the maintenance of the progenitor cell state in the cochlea, suggesting that Gli2 is involved in the gene regulatory networks responsible for maintaining prosensory identity and could be deployed for regeneration of HCs in therapeutic applications.

## Materials and Methods

**Mice.** *Emx2Cre* (54), *Sufu<sup>fl/fl</sup>* (55), *Spop<sup>fl/fl</sup>* (56), *Ptch1<sup>fl/fl</sup>* (57), *Gli2<sup>fl/fl</sup>* (58), *Sox2<sup>EGFP/+</sup>* (59), *Gli2<sup>+/-</sup>* (60), *Gli3<sup>+/-</sup>* (61), *Gli3<sup>SP1.4/+</sup>* (36), and *Ptch1<sup>LacZ/+</sup>* (62) mice were described before. All experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong (CULATR 5025-19 and 4771-18). The day vaginal plug was observed was considered as E0.5. PCR primers for genotyping are listed in the *SI Appendix, Table S1*.

**Immunohistochemistry and Whole-Mount Immunostaining.** Embryo heads were bisected and fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Heads were then incubated in 15% sucrose solution overnight at 4 °C and embedded in gelatin. Samples were sectioned at 10 μm and subjected to immunohistochemistry. For whole-mount immunostaining, fixed inner ears were dissected to remove bony capsule and vestibule. Cochleae were further dissected to remove the cochlear roof epithelia, and the oC was exposed. Sections or whole-mount tissues were blocked in 10% horse serum (Gibco, 26050-088) with 0.1% Triton X-100 (Sigma-Aldrich, ×100) at room temperature for 1 h and incubated with diluted primary antibody in 10% horse serum with 0.1% Triton X-100 overnight at 4 °C. The following primary antibodies were used: rabbit anti-Atoh1 (Proteintech, 21215-1-AP), rabbit anti-Myo7a (Proteus, 26-6790), mouse anti-Pou4f3 (Santa Cruz, sc-81980), and goat anti-Sox2 (NeuroMics, GT15098). Samples were then washed and incubated with Alexa Fluor-labeled secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature.

**Whole-Mount In Situ Hybridization.** Embryo heads were bisected and fixed in 4% PFA overnight at 4 °C and dehydrated through a methanol gradient. After rehydration, cochleae were dissected from the bony capsule, treated with proteinase K (Sigma-Aldrich, P6556), postfixed in PFA/glutaraldehyde, and incubated with DIG-labeled riboprobes at 65 °C overnight. After washing in maleic acid buffer containing 0.1% Tween 20 (MABT), samples were blocked in MABT supplemented with 10% blocking reagent (Roche, 11585614910) and 20% horse serum, incubated with anti-digoxigenin antibody conjugated to alkaline

phosphatase (Roche, 11093274910) at 4 °C overnight, and washed for 5 h at room temperature and overnight at 4 °C. BM purple (Roche, 11442074001) was applied to the samples until the signal was fully developed. Probes for *Gli1* and *Gli2* have been described before (56).

**Imaging.** Images were acquired using an Olympus BX53 fluorescence microscope, Leica MZ10F fluorescence stereomicroscope, or Carl Zeiss 700/780 laser scanning confocal microscope. All Sox2-EGFP images were taken using an Olympus BX53 fluorescence microscope under the same settings and the same exposure times for signal intensity comparison. Images were analyzed using Photoshop CC 2015 or ImageJ software (version 1.53).

**X-gal Staining.** β-galactosidase activity of *Ptch1<sup>LacZ/+</sup>* reporter mice was analyzed as described previously (63). Briefly, inner ears were dissected from fresh embryos in cold phosphate-buffered saline, fixed for 30 min at 4 °C, washed at room temperature, and incubated with X-gal (Sigma-Aldrich, 7240906) in the dark at room temperature overnight.

**Statistics.** Statistical analyses were performed using GraphPad Prism 8. Two-tailed *t* tests with Welch's correction were used in comparing two independent groups of samples. For Figs. 2A and 3H, one-tailed *t* tests with Welch's correction were used in comparing numbers of OHCs, as cell numbers would not be less than the value of zero (*SI Appendix, Table S2*). Statistical data are included in *SI Appendix, Table S2*; \*, *P* ≤ 0.05; \*\*, *P* ≤ 0.01; \*\*\*, *P* ≤ 0.001; \*\*\*\*, *P* ≤ 0.0001; ns, not significant (*P* > 0.05). *n* represents the numbers of cochleae in each group.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*.

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Author affiliations: <sup>a</sup>School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China; <sup>b</sup>School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China; <sup>c</sup>Program in Developmental & Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, M5G 0A4, Canada; and <sup>d</sup>Department of Molecular Genetics, University of Toronto, Toronto, ON, M5S 1A8, Canada

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