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RESEARCH ARTICLE

# Notch-Wnt-Bmp crosstalk regulates radial patterning in the mouse cochlea in a spatiotemporal manner

**Vidhya Munnamalai[1,2] and Donna M. Fekete[1,2,3,*]**


## ABSTRACT

The sensory cells of the mammalian organ of Corti assume a precise mosaic arrangement during embryonic development. Manipulation of Wnt signaling can modulate the proliferation of cochlear progenitors, but whether Wnts are responsible for patterning compartments, or specific hair cells within them, is unclear. To address how the precise timing of Wnt signaling impacts patterning across the radial axis, mouse cochlear cultures were initiated at embryonic day 12.5 and subjected to pharmacological treatments at different stages. Early changes in major patterning genes were assessed to understand the mechanisms underlying alterations of compartments. Results show that Wnt activation can promote medial cell fates by regulating medially expressed Notch genes in a spatiotemporal manner. Wnts can also suppress lateral cell fates by antagonizing *Bmp4* expression. Perturbation of the Notch and Bmp pathways revealed which secondary effects were linked to these pathways. Importantly, these effects on cochlear development are dependent on the timing of drug delivery. In conclusion, Wnt signaling in the cochlea influences patterning through complex crosstalk with the Notch and Bmp pathways at several stages of embryonic development.


**KEY WORDS: Cochlea, Wnt, Radial axis, Notch, Bmp, Hair cells**

## INTRODUCTION

The detection of sounds over a wide range of frequencies and amplitudes in mammals depends on a precise arrangement, or patterning, of mechanosensory hair cells (HCs) and supporting cells (SCs) across the radial axis of the organ of Corti (OC). The outer hair cells (OHCs) of the lateral compartment are more abundant than inner hair cells (IHCs) of the medial compartment. This cellular organization emerges during development in the context of several key signaling pathways that can influence cell fates and whose ligands vary in a spatiotemporal manner across the cochlear duct (Fig. 1A). This includes *Bmp4*, *Fgf8*, *Fgf10*, *Fgf20* and Notch pathway components (Groves and Fekete, 2012; Hayashi et al., 2008b; Jacques et al., 2007; Ohyama et al., 2010; Urness et al., 2015). Transcripts for *Wnt7b*, *Wnt7a* and *Wnt5a* are asymmetrically and dynamically expressed within the prosensory domain of the cochlear duct as the OC begins to differentiate (Bohnenpoll et al., 2014; Dabdoub and Kelley, 2005; Qian et al., 2007). Such diversity suggests multiple roles for Wnt signaling that vary as cochlear development progresses (Munnamalai and Fekete, 2013). Wnt-mediated regulation of cell proliferation is well known in many organ systems, including the cochlea (Jacques et al., 2012). Whether the canonical Wnt signaling pathway intersects with the Notch, Bmp or Fgf pathways to regulate cochlear patterning remains relatively unexplored.

The Wnt and Notch pathways are known to crosstalk, a finding that coined the term 'Wntch' signaling. This interaction is context dependent and can be bi-directional (Collu et al., 2014; Zak et al., 2015). In the cochlea, Notch has a dual role in regulating lateral induction early (to induce prosensory fate) and lateral inhibition later (to block HC fate) (Kiernan, 2013). Studies have shown that Notch effector genes can be co-regulated by Wnts to influence cell fates (Petrovic et al., 2015; Romero-Carvajal et al., 2015; Zak et al., 2015).

Bmps and Wnts also have well-documented interactions/crosstalk in several developing systems (Itasaki and Hoppler, 2010). In some cases their interactions are synergistic (Basler et al., 1993; Galli et al., 2007; Hollyday et al., 1995; Lee et al., 1998). In others, Wnts and Bmps antagonize each other, creating counter-gradients across an organ (Baker et al., 1999; Jain et al., 2015; Theisen et al., 1996). Such crosstalk has not been examined in the cochlea.

To explore short-term changes in gene regulation immediately downstream of Wnt activation, we pharmacologically and transiently activated Wnt signaling before or during cell fate specification. The types of changes induced in the medial versus lateral OC compartments varied with the timing of drug treatment. To elucidate mechanisms underlying these Wnt-mediated phenotypes, we examined the differential expression of several patterning genes across the radial axis and blocked Wnt, Notch or Bmp signaling. Our results support a model in which Wnt signaling activates different downstream targets as the cochlea transitions from a field of progenitors into a patterned epithelium. Specifically, the effects of Wnts on Notch signaling vary as development progresses. We further show that Wnt and Bmp behave antagonistically to specify cell fates across the radial axis of the OC.

## RESULTS

### The use of explant cultures to investigate cochlear patterning

The spatial arrangement of IHCs and OHCs across the radial axis confers neural processing by distinct afferent and efferent synaptic partners. To evaluate the functions of different signaling pathways on this radial patterning, we cultured embryonic day (E) 12.5 mouse cochlear explants for 6 days *in vitro* (6DIV) to allow cell differentiation. This approach recapitulates development *in vivo* and permits us to manipulate signaling pathways in a temporal manner. For the purposes of this study, we define the medial compartment as giving rise to the greater epithelial ridge (GER), IHCs and their associated SCs. The lateral compartment gives rise to

[1]Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA. [2]Purdue Institute for Integrative Neuroscience, Purdue University, West Lafayette, IN 47907, USA. [3]Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN 47907, USA.

*Author for correspondence (dfekete@purdue.edu)

V.M., 0000-0002-4985-8265; D.M.F., 0000-0003-0662-0246

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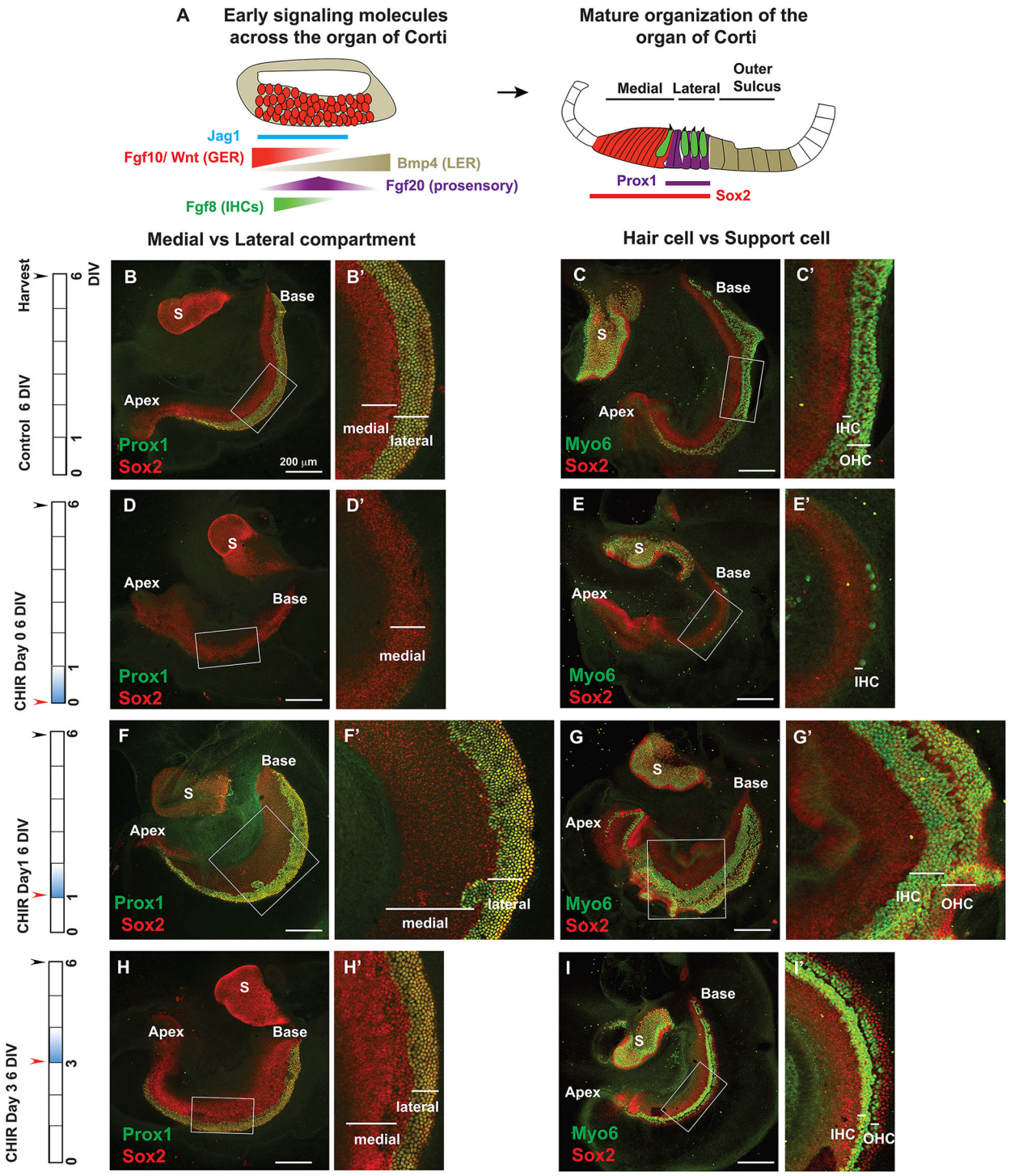


**Fig. 1. Cochlear patterning changes associated with activating Wnt signaling on different days in culture.** (A) At E12-12.5 the sensory domain consists of Sox2-positive progenitors. Over the next several days, genes that influence patterning and cell fate specification are expressed non-uniformly across the radial axis, with expected protein gradients shown schematically. (B-I′) On the time scale, each box represents 1DIV. The onset of CHIR addition (red arrowhead) is followed by blue shading to suggest a decline in CHIR potency over 24 h. The harvest time (black arrowhead) is indicated. Prox1 (green) and Sox2 (red) immunostaining are used to distinguish the lateral and medial compartments, while Myo6 (green) labels HCs. (B-C′) Representative controls ($n$=9). (D-E′) CHIR treatment on day 0 blocks lateral compartment formation and HC differentiation (each image represents $n$=6). (F-G′) CHIR treatment on day 1 expands the medial compartment and increases IHC s (F, $n$=11; G, $n$=9). (H-I′) CHIR treatment on day 3 reduces OHCs (each image represents $n$=6). Boxed regions are shown magnified to the right. GER, greater epithelial ridge; LER, lesser epithelial ridge; S, saccular macula.

Deiters cells, pillar cells and OHCs by E18.5 (Fig. 1A). To determine the status of medial versus lateral compartment formation, differentiated explants were immunolabeled for Sox2 (both compartments) and Prox1 (lateral compartment) (Fig. 1B). Colabeling with either an anti-Myo6 antibody or *Atoh1-GFP* transgene identified the HCs (Fig. 1C, Fig. 2A).

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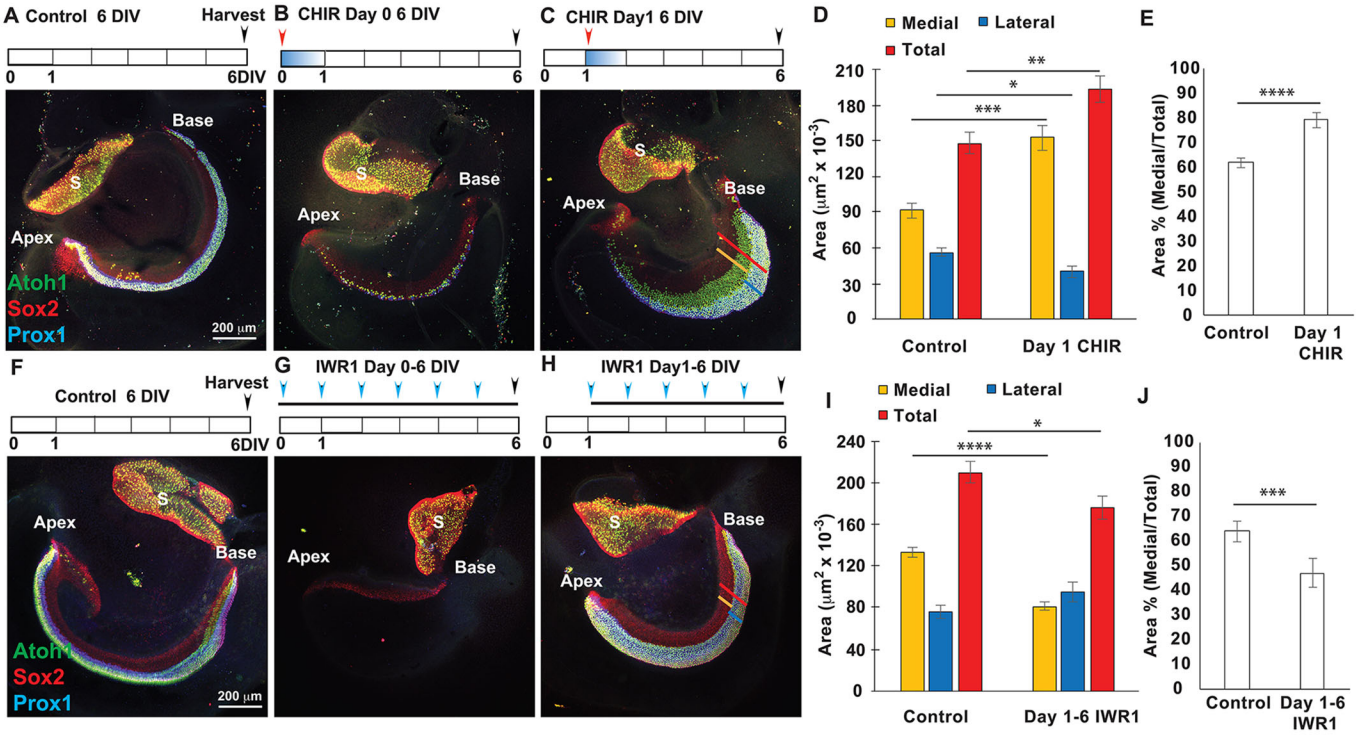


**Fig. 2. Compartment sizes correlate with Wnt perturbation.** All samples are from *Atoh1-GFP*$^{+/-}$ cochleas cultured for 6DIV and labeled for GFP, Sox2 and Prox1. (A-C) CHIR treatment on day 0 blocks formation of the lateral compartment and reduces HCs, whereas CHIR treatment on day 1 expands the sensory domain and the medial compartment. (D) Quantification of compartment sizes in day 1 CHIR-treated cochleas (*n*=11) compared with controls (*n*=9). (E) The percentage of the total area occupied by the medial compartment is increased by CHIR. (F-H) IWR1 treatment (blue arrowhead) on days 0-6 (*n*=6) blocks nearly all sensory formation, whereas IWR1 treatment on days 1-6 permits development of the sensory compartments. (I) Quantification reveals that the sensory domains of day 1-6 IWR1-treated cochleas (*n*=6) are smaller due to a reduced medial compartment, as compared with the control (*n*=6). (J) The percentage of the total area occupied by the medial compartment is reduced by IWR1. Two-tailed *t*-tests were performed between controls and the indicated drug treatments; **P*<0.05, ***P*<0.01, ****P*<0.0005, *****P*<0.00001. Error bars indicate s.e.m.

### Forced Wnt signaling influences OC patterning differently as development progresses

The goal of the first experiment was to transiently, yet robustly, activate the Wnt pathway in cochlear cultures using CHIR99021 (CHIR) as an inhibitor of glycogen synthase kinase 3β (GSK3β). A dose-response test identified that 10 μM CHIR altered *Axin2* transcripts over 4-fold at 6 h, with levels returning to near baseline at 24 h, despite the continuous exposure to CHIR (see below). All experiments used 10 μM CHIR, typically delivered as a 1-day pulse.

The results obtained from Wnt activation with CHIR differed drastically depending on the timing of drug addition. When explanted (day 0), E12.5 mouse cochlear cells are in a progenitor state, still mitotically active, and show no overt cellular differentiation. A 24-h EdU pulse-chase beginning on day 0 showed the presence of cycling progenitors across the radial axis (Fig. S1A). CHIR treatment resulted in a general suppression of cell fate specification. The lateral compartment failed to differentiate, as shown by the absence of Prox1-positive lateral domain SCs (Fig. 1D). Myo6-positive HCs in the remaining Sox2-positive sensory domain were significantly decreased (Fig. 1E). The *Atoh1-GFP*$^{+/-}$ transgene genotype facilitated quantification of IHCs and OHCs (Fig. 2, Fig. S2A). On day 0, CHIR treatment decreased HC formation overall, with a greater effect on OHCs, resulting in an OHC:IHC ratio of 0.3:1 compared with a ratio of 2.7:1 in controls (Table 1).

By day 1, the zone of non-proliferation is already established (Lee et al., 2006) (Fig. S1B), the medial compartment is specified and *Atoh1* expression has initiated in the first IHCs. In delaying the addition of CHIR until this stage, the medial compartment is most strongly affected: it is greatly expanded (Fig. 1F), with a large increase in IHCs. OHC numbers are unchanged, yielding an overall decrease in the OHC:IHC ratio to 0.9:1 (Fig. 1G, Table 1).

On day 3, there is no proliferation in the future OC (Fig. S1C). OHC formation has just initiated within the lateral compartment. Upon CHIR treatment, the lateral compartment was present at 6DIV (Fig. 1H). A moderate increase in IHCs combined with a significant decrease in OHCs along most of the cochlear length generated an OHC:IHC ratio of 0.7:1 (Fig. 1I, Table 1).

In summary, in response to Wnt activation at different times, the medial compartment was present but poorly differentiated (day 0) or enlarged (day 1 or day 3), whereas the lateral compartment was absent (day 0), reduced (day 1) or unchanged (day 3). IHCs and OHCs were reduced (day 0), increased and unchanged (day 1), or increased and reduced (day 3), respectively. Overall, different phenotypes emerged from forced activation of Wnt signaling across a narrow 4-day window of cochlear development.

**Table 1. HC quantification in CHIR-treated cochleas**

| Condition | # IHCs | # OHCs | OHC:IHC |
|---|---|---|---|
| Control (*n*=7) | 211±15 | 582±61 | 2.7±0.2 |
| Day 0 CHIR (*n*=6) | 61±16** | 28±17*** | 0.3±0.2*** |
| Day 1 CHIR (*n*=6) | 687±47*** | 618±73 (n.s.) | 0.9±0.1*** |
| Day 3 CHIR (*n*=6) | 326±42* | 226±47*** | 0.7±0.1*** |

One-way ANOVA was performed and statistical significance determined post-hoc by Dunnett's multiple comparison analysis between control and treatment groups. **P*<0.05, ***P*<0.01, ****P*<0.001; n.s., not significant.

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### Medial compartment size is altered in response to changes in Wnt signaling

Our data suggest that CHIR preferentially promoted the formation of IHCs and the medial compartment (Fig. 2A-C, Table 1). These data differ from a previous study using LiCl as a Wnt activator, in which the entire sensory domain was expanded and both HC types increased as a result of enhanced proliferation (Jacques et al., 2012). To confirm our results, we computed three sensory areas as indicated in Fig. S2B,C: total (Sox2 positive), lateral (Prox1 positive) and medial (Sox2 positive, Prox1 negative).

On day 0, there was no change in the total area upon CHIR treatment, whereas there was a significant reduction in lateral compartment size from 64±6 in controls to 15±3 (areas presented as $\mu m^2 \times 10^{-3}$). As a result, the medial area increased from 100±5 to 138±9 (Fig. S2D). Thus, the medial compartment occupied 90±2% of the sensory area upon CHIR treatment on day 0, versus 62±2% in control cochleas.

On day 1, the total area increased from 148±9 in controls to 193±11 in CHIR-treated cochleas. The medial area increased from 92±6 to 153±10 and the lateral area decreased from 56±3 to 40±5 (Fig. 2D). The medial compartment occupied 61±2% of the total area in control cochleas, whereas upon CHIR treatment the medial compartment occupied a significantly higher proportion of the sensory domain at 79±3% (Fig. 2E).

On day 3, the total area increased from 164±9 in controls to 201±9 in CHIR-treated cochleas. The medial area increased from 100±5 in controls to 147±7, whereas there was no change in the lateral area (Fig. S2D). This resulted in an increase in the proportion of the total area occupied by the medial compartment from 62±2% to 73±3%. None of these CHIR treatments disrupted cochlear lengths (Fig. S2E).

Since CHIR acts as a Wnt activator by inhibiting GSK3, we sought further evidence that the medial compartment is dependent on Wnt activity. We used the Wnt inhibitor IWR1 as previously described (Jacques et al., 2012). Differentiation of the sensory domain was repressed with IWR1 from day 0-6 (Fig. 2F,G). Since day 1 CHIR produced the largest increase (of 66%) in the medial compartment, we varied the onset of IWR1 to begin on day 1, and continued treatment until 6DIV. Differentiation of the two radial compartments was apparent; however, the total area ($\mu m^2 \times 10^{-3}$) of the sensory domain decreased from 210±15 to 176±29. The medial area decreased from 133±11 to 81±13, whereas the lateral area was unaffected (Fig. 2H,I). Upon IWR1 treatment, the medial compartment occupied a significantly lower proportion of the total area at 47±6% compared with 64±4% in controls (Fig. 2J). In summary, the medial and lateral compartments respond differently to activation/inhibition of Wnt signaling.

### Sensory cell proliferation is unchanged after 6 h and increased at 24 h of CHIR treatment

The Wnt dependence of medial compartment size might be related to the effect of Wnt on proliferation. To address this, we analyzed temporal proliferation in response to CHIR by simultaneously adding EdU. At 6 h, day 1 CHIR-treated cochleas showed no obvious difference compared with controls (Fig. 3A,B). However, by 24 h there was a homogeneous increase in proliferation across the sensory domain relative to controls, including the non-sensory cells on the lateral side of the epithelium (arrows, Fig. 3C′,D″). A similar response is seen 24 h after adding CHIR and EdU on day 0, revealing that proliferating cells acquire both sensory and non-sensory fates (Fig. S1D,E). Since proliferation was evenly distributed across the radial axis at 24 h, this leaves a window (of at least 6 h) during which upregulated Wnt-responsive genes may be acting on cell fates prior to cell proliferation, thereby leading to a preferential expansion of one compartment over the other.

### Gene expression changes differ in response to Wnt activation on day 0 versus day 1

To aid in understanding the different patterning outcomes induced on day 0 versus day 1 CHIR treatments, transcripts were probed by RT-qPCR or *in situ* hybridization (Fig. 4). Gene expression was analyzed 6 h after CHIR treatment to search for the earliest genes, reasoning that these might be responsible for initiating a cascade of signaling events that would manifest later as a change in pattern formation. At 6 h on day 0 CHIR treatment, there were significant increases in several presumed Wnt target genes (*Jag1*, *Axin2*, *Lgr5* and *Fgf20*), whereas no changes were detected for three other possible target genes (*Atoh1*, *Sox2* and *Ccnd1*) (Fig. 4A; see legend for statistics). Two genes (*Jag1* and *Axin2*) that were upregulated relative to controls at 6 h were statistically back to baseline by 24 h, revealing the transient nature of gene regulation (Fig. 4B). Although *Lgr5* was still elevated at 24 h compared with controls, the fold change was reduced compared with 6 h (Fig. 4A,B). By contrast, *Fgf20* continued to increase from 6-24 h. Curiously, RT-qPCR showed repression of *Sox2* and *Atoh1* transcripts at 24 h that were not apparent at 6 h (Fig. 4B), suggesting that downstream repressors are active between 6 and 24 h. *In situ* hybridization was performed 6 h after day 0 CHIR treatment to examine genes in the Notch pathway. *Jag1* expression was dramatically upregulated across the sensory domain compared with controls. *Lfng*, expressed on the medial side, remained unchanged in response to CHIR (Fig. 4C). At this early stage of development, *Mfng* and *Atoh1* were not detectable and were unchanged in response to CHIR (data not shown).

We saw very different gene expression changes with day 1 CHIR. Foremost, there was no increase in *Jag1* expression (Fig. 4D). *In situ* hybridization (Fig. 4E) provided spatial resolution to complement the RT-qPCR data for a number of key genes (Fig. 4D). A weaker *Jag1* signal was spread over a broader domain than in the controls. Unlike day 0, CHIR treatment on day 1 caused a marked decrease in *Lfng* expression, accompanied by a subtle increase in *Mfng* expression. After 6 h of day 1 CHIR, *Atoh1* expression increased in the mid-region of the cochlea. Thus, the regulation of Wnt-responsive target genes is context dependent at specific developmental time points. Surprisingly, there was no detectable change in *Sox2* transcripts that could explain the expanded medial compartment several days later (Fig. 1F′).

### Atoh1 can drive expansion of the medial compartment

*Atoh1* responded to CHIR before *Sox2* and before any increases in cell proliferation were observed. This has broader implications for cochlear patterning. *Atoh1* is first expressed in newborn IHCs (Bermingham et al., 1999) at the time of CHIR addition on day 1. Moreover, Atoh1 protein is reported to have non-cell-autonomous effects on *Atoh1* transcription to induce sensory fates (Kelly et al., 2012; Woods et al., 2004). To assess whether the excess Atoh1 could be influencing the medial compartment, we overexpressed *Atoh1* by electroporating *EGFP* and *Atoh1* expression plasmids into E13.5 cochlear explants (Fig. S3). Electroporation of the control EGFP plasmid did not alter sensory formation (Fig. S3A). As previously described, the presence of the *Atoh1* plasmid induces numerous ectopic HCs (Fig. S3B,B′) (Gubbels et al., 2008; Kawamoto et al., 2003; Liu et al., 2014). This was accompanied by an expansion of the Sox2 domain on the medial side of the OC 4 days later (Fig. S3B″). Thus, in day 1

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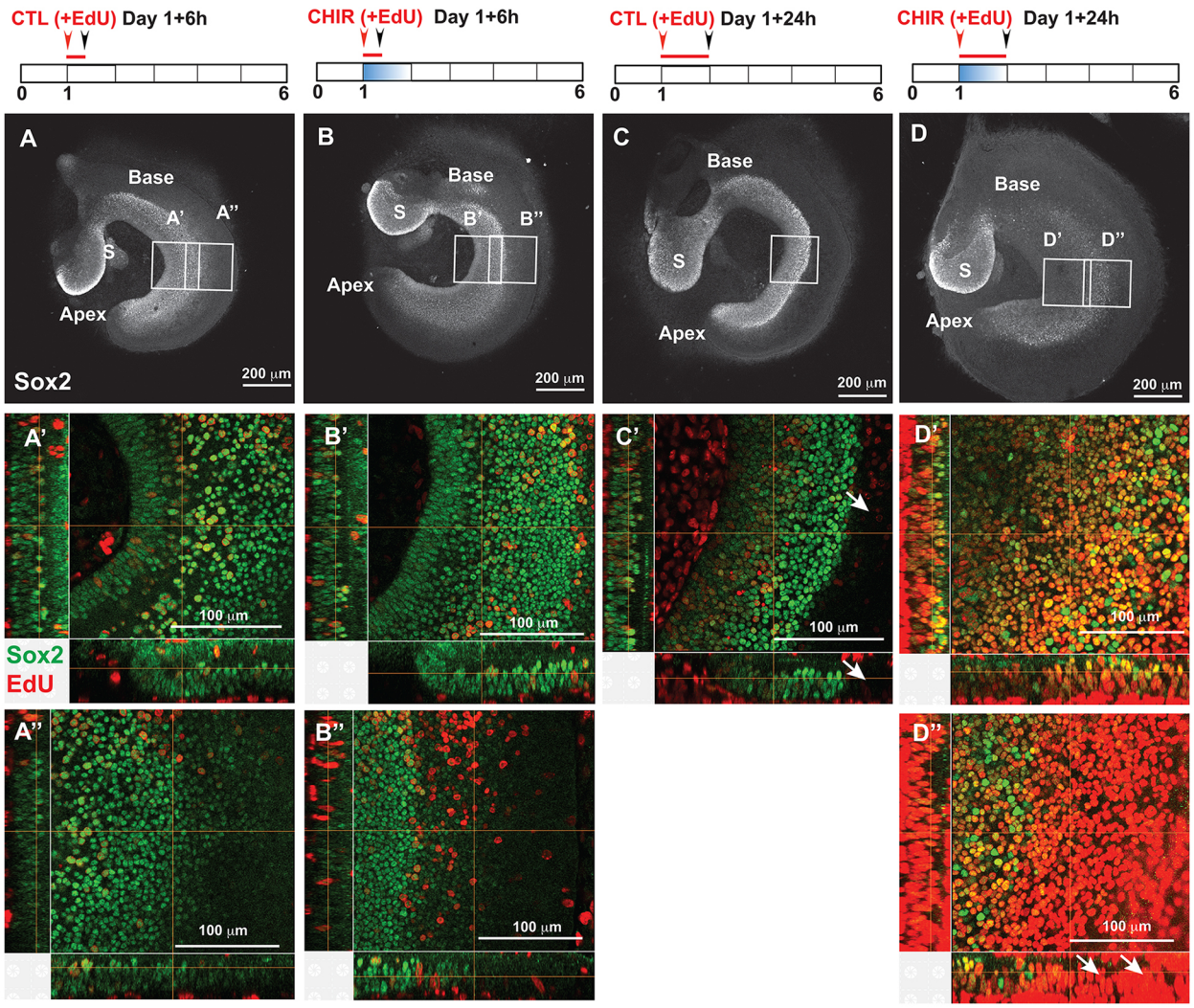


**Fig. 3. Proliferative responses to CHIR occur after a delay.** Controls and CHIR-treated cochleas were treated with EdU on day 1 for the indicated intervals (red bar), then fixed and labeled for EdU (shown only in the higher magnification images A′-D″, acquired with a 60× objective) and Sox2. Optical sections through these higher magnification images show colabeling (yellow) for EdU (red) and Sox2 (green) in the cochlear epithelium. (A-B″) After 6 h of CHIR treatment, there is no obvious difference in cell proliferation within the sensory domain. (C-D″) After 24 h of CHIR treatment, both medial and lateral sensory domains, and the lateral non-sensory domain, have dramatically increased proliferation (*n*=6). Arrows (C′,D″) indicate non-sensory area beyond the Sox2 domain.

CHIR-treated samples, *Atoh1* upregulation may be responsible for the eventual broadening of the medial compartment after the 24-h treatment window.

### Medial compartment expansion is partially mediated by Notch signaling

To tease apart the mechanisms that determine medial specification from the other Wnt-mediated effects in the cochlea, such as HC formation, we focused on a Notch-Atoh1 pathway. We hypothesized that the block on HC formation at day 0 was due to a Wnt-mediated repressor gene, and that CHIR addition on two successive days might feed forward to inhibit Wnt-activated *Atoh1* on day 1 due to the earlier upregulation of this putative repressor. In order to test this, we delivered two pulses of CHIR to E12.5 cochleas on day 0 and day 1 (Fig. 5). Control cochleas showed normal development (Fig. 5A), whereas the double dose of CHIR yielded a patterning defect that appeared to be a combination of the two distinct phenotypes resulting from either day alone (Fig. 5B). Specifically, cochleas displayed a wider medial domain, albeit with a disorganized border, and lost the lateral domain. Consistent with our hypothesis, there were fewer HCs, which were displaced towards the medial edge (Fig. 5C,D).

In order to test whether the Sox2-positive cells in this medial domain could give rise to HCs, cochleas were treated with DAPT, a γ-secretase inhibitor, from day 2-6 after the withdrawal of CHIR. As previously described, cochleas treated only with DAPT showed an increase in HCs at the expense of SCs, due to the block of lateral inhibition (Fig. 5E) (Hayashi et al., 2008a). However, cochleas treated with two pulses of CHIR followed by DAPT did not generate supernumerary HCs (Fig. 5F). Instead, DAPT inhibited prosensory formation. Prosensory inhibition due to DAPT treatment occurs comparably early (E12.5) and typically requires a higher dose of DAPT (30 μM) (Munnamalai et al., 2012; as compared with Fig. 5E).

Our current results suggest that an abnormally low level of Notch induction remains after two successive days of Wnt activation; although this is able to expand the medial sensory compartment, it is insufficient to support subsequent HC formation (Fig. 5F).

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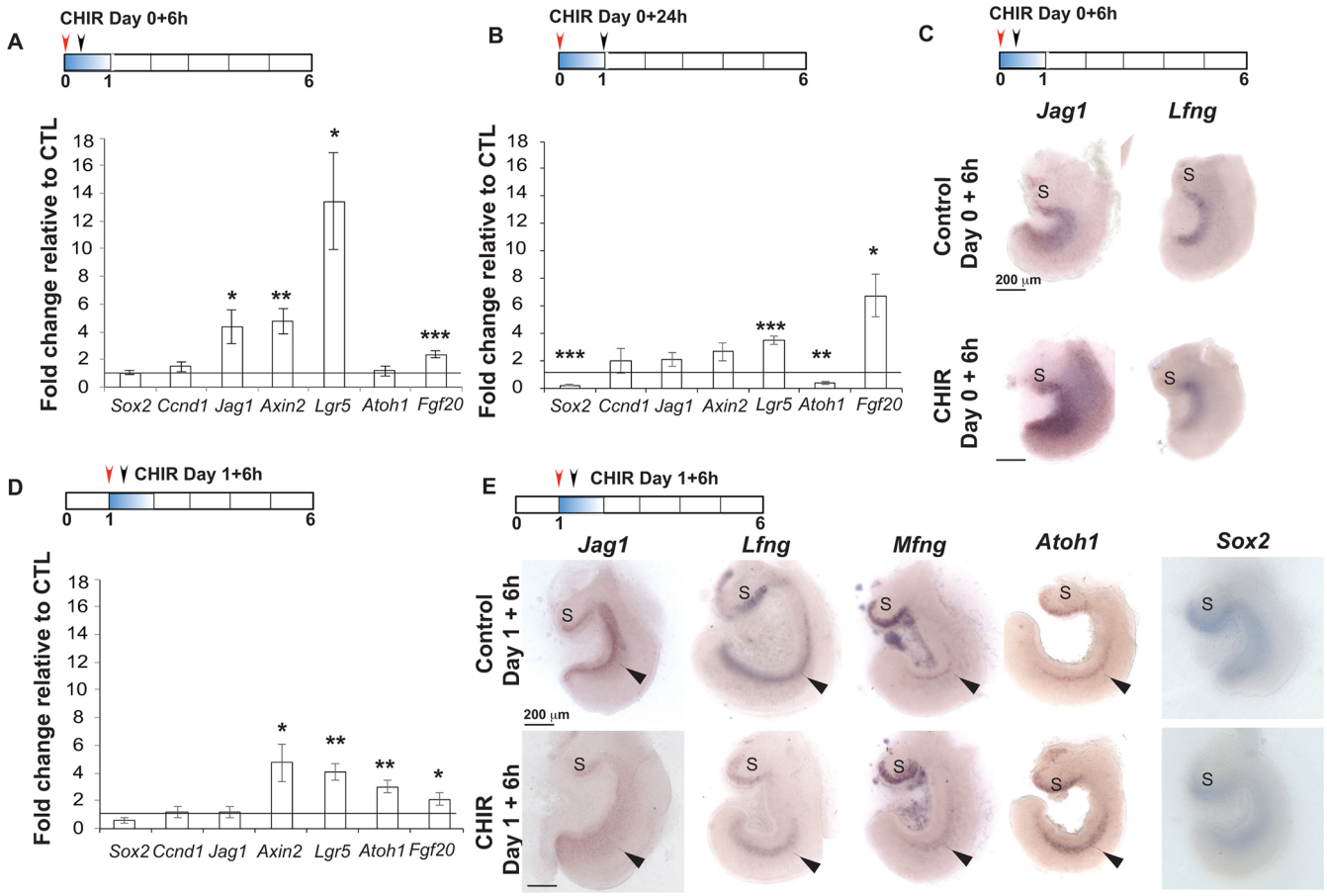


**Fig. 4. CHIR treatments on day 0 versus day 1 differentially alter gene expression.** (A) RT-qPCR for presumed Wnt target genes on cochleas treated with CHIR on day 0 for 6 h. Fold increases are quantified (one-tailed *t*-test) for *Jag1* (4.4±1.3), *Axin2* (4.8±0.9), *Lgr5* (13.4±3.5) and *Fgf20* (2.4±0.2). (B) RT-qPCR on cochleas treated on day 0 with CHIR for 24 h showing fold increases (two-tailed *t*-test) for *Lgr5* (3.5±0.9) and *Fgf20* (6.8±1.5) and decreases for *Sox2* (by 80±5%) and *Atoh1* (by 60±10%). (C) *In situ* hybridization of *Jag1* (*n*=6) and *Lfng* (*n*=6) reveals spatial and levels changes in response to CHIR treatment on day 0 for 6 h. (D) RT-qPCR on cochleas treated with CHIR on day 1 for 6 h (one-tailed *t*-test). CHIR treatment leads to fold increases for *Axin2* (4.7±1.4), *Lgr5* (4.1±0.6), *Atoh1* (3±0.5) and *Fgf20* (2.1±0.4). (E) *In situ* hybridization of *Jag1* (*n*=7), *Lfng* (*n*=10), *Mfng* (*n*=6), *Atoh1* (*n*=8) and *Sox2* (*n*=8) on cochleas treated with CHIR on day 1 for 6 h. **P*<0.05, ***P*<0.01, ****P*<0.005. Error bars indicate s.e.m. For the *in situ* hybridizations, arrowheads indicate qualitative changes between controls and CHIR-treated samples.

### Wnt activation antagonizes Bmp4 signaling

Changes in the medial cochlear duct were accompanied by effects on the lateral compartment (absent on day 0 and decreased on day 1). Therefore, we assessed the expression of a laterally expressed patterning gene, *Bmp4* (Fig. 6). On both day 0 and day 1, *Bmp4* expression was weak, and CHIR abolished *Bmp4* expression (Fig. 6A,B). However, day 3 CHIR did not completely repress the robust *Bmp4* expression (Fig. 6C), suggesting that Wnt activation could only repress a certain threshold of *Bmp4* transcripts at this later time point. Nonetheless, activation of the Wnt pathway resulted in consistent downregulation of *Bmp4* expression.

These data suggest that some of the lateral compartment defects resulting from Wnt activation might reflect reduced Bmp signaling. To explore this, we cultured *Atoh1-GFP*$^{+/-}$ cochleas for 6DIV with the Bmp receptor inhibitor dorsomorphin. Control cochleas showed normal development of sensory subdomains and HCs (Fig. 6D). When dorsomorphin treatment began prior to specification of the lateral compartment (day 0-6), the Sox2 domain was largely devoid of Prox1 compared with controls (Fig. 6E). To determine if the Bmp pathway also influenced the formation of the lateral compartment in a temporal manner, dorsomorphin treatment was initiated on successively later days to complement the experiments with CHIR. At each of these time points, Prox1 expression was reduced (Fig. 6F,G). Thus, Bmp signaling is required for the specification and maintenance of Prox1-positive SCs. All the *Atoh1-GFP*$^{+}$ cells resided outside the reduced Prox1 subdomain, suggesting they were IHCs rather than OHCs, as OHCs originate from a Prox1-positive lineage (Kirjavainen et al., 2008). Therefore, in the absence of Bmp signaling, the remaining sensory cells appear to assume medial fates.

Overall, the patterning defects observed in the lateral compartment following Wnt activation are consistent with an antagonistic effect on Bmp signaling.

### Both Wnt activation and Bmp inhibition increase IHCs

We sought to verify the identities of the HCs differentiating in CHIR- and dorsomorphin-treated cochleas using *Fgf8* as an IHC marker. We compared control cochleas (Fig. 7A) with those treated with either CHIR on day 1 (Fig. 7B) or dorsomorphin for days 1-6 (Fig. 7C). Both treatments generated more *Fgf8*-positive IHCs (Fig. 7B,C). However, the *Fgf8* levels in dorsomorphin-treated cochleas were noticeably lower than in untreated littermate controls processed simultaneously, suggesting that these HCs were immature.

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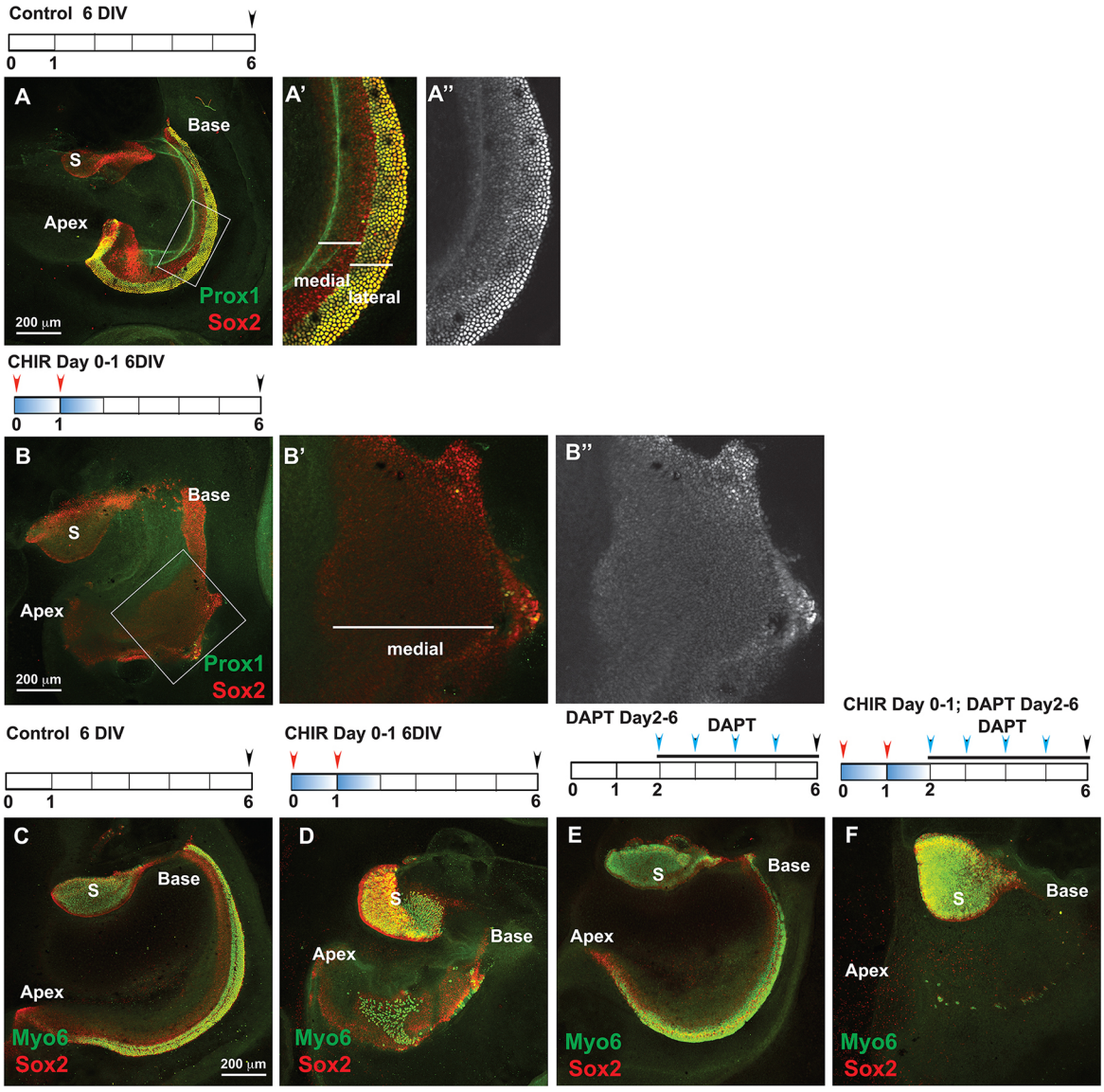


**Fig. 5. The medial compartment is partially specified by Notch signaling.** Cochleas were cultured for 6DIV under the indicated conditions and stained with the antibodies shown. Cochleas pulsed twice with CHIR show an expanded medial compartment and a missing lateral compartment when compared with controls (A-B″), with fewer presumed IHCs (C,D). Notch inhibition with 10 μM DAPT (blue arrowheads) on days 2-6 generates more HCs at the expense of Sox2-positive SCs (although difficult to see at this magnification, E). This fate switch is blocked if the cochleas are first pulsed twice with CHIR before the day 2-6 DAPT addition (F). *n*=6 per condition.

### Developmental expression patterns of Wnt ligands

The different temporal effects of Wnt activation raise the question of whether different Wnt ligand-receptor interactions might underlie some of these changes. Expression screening of E14.5 mouse embryos for Wnt family members detected only *Wnt5a*, *Wnt7a* and *Wnt7b* in the cochlear sensory epithelium (Diez-Roux et al., 2011). We performed a developmental time series for Wnt transcripts to fill in sampling gaps between E12.5 and E15.5 (Fig. S4) to complement previous studies (Bohnenpoll et al., 2014; Dabdoub and Kelley, 2005; Qian et al., 2007).

*Wnt7b* was expressed early but transiently, peaking at prosensory stages. At E12.5, *Wnt7b* expression was broad throughout the cochlear duct, including the prosensory domain marked by *Sox2* (Fig. S4A,B). At E13.5, *Wnt7b* expression was rapidly downregulated on the medial side and was restricted to the outer sulcus, beyond the *Sox2* domain (Fig. S4A′,B′). By E14.5, *Wnt7b* expression was absent in the cochlea (Fig. S4A″,B″). The onset of *Wnt5a* was later than of *Wnt7b* and was not detected in the sensory domain of the E12.5 cochlea (Fig. S4C) (Bohnenpoll et al., 2014). *Wnt5a* was first detected at E13.5 in the medial domain, marked by *Jag1* (Fig. S4C′,D′), and the hybridization signal stabilized through E15.5 (Fig. S4C″,D″). *Wnt7a* was rapidly upregulated from E12.5-15.5 (Fig. S4E-E″). In our studies, the developing reaction was stopped based on the strongest expression at any of the three time points, such that *Wnt7a* appeared to be absent at E12.5. However, slides developed for longer confirmed low-level expression of *Wnt7a* at E12.5 (data not shown), as previously shown (Bohnenpoll et al., 2014).

## DISCUSSION

The goal of this study was to explore the complexities of Wnt signaling in the mouse OC over time, a process that is confounded

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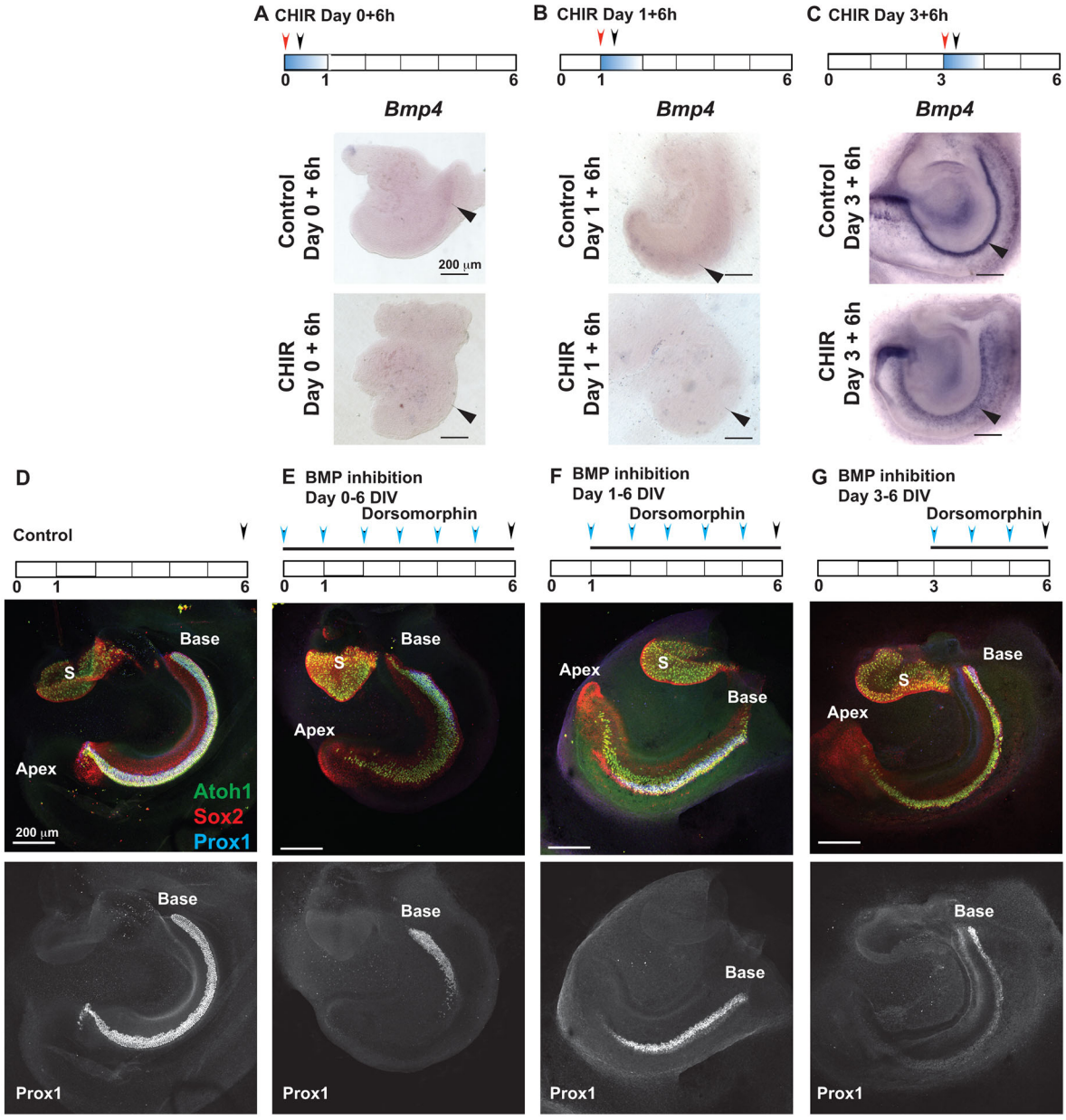


**Fig. 6. CHIR reduces *Bmp4* expression, and Bmp signaling is required for lateral compartment formation.** *In situ* hybridization of *Bmp4* on CHIR-treated cochleas on (A) day 0 ($n$=8), (B) day 1 ($n$=4) and (C) day 3 ($n$=6). Specimens ($n$=10 per group) from *Atoh1-GFP*$^{+/-}$ mice were labeled for GFP, Prox1 and Sox2 after 6DIV. Arrowheads indicate the presence and loss of Bmp4 in control and CHIR-treated cochleas, respectively. (D) Control. (E) Dorsomorphin (blue arrowheads) treated from day 0-6. IHCs are present throughout, whereas Prox1 and OHCs are found only in the base. (F) Dorsomorphin treated from day 1-6. (G) Dorsomorphin treated from day 3-6. In both F and G, the Prox1-positive compartment and associated OHCs are reduced compared with controls.

by the dynamic expression patterns of multiple Wnt ligands in the mid-gestation cochlea and crosstalk with multiple pathways. Genetic manipulations of the pathway had previously revealed that Wnts function at multiple stages in the embryonic inner ear, beginning with otic induction (Jayasena et al., 2008). Inducible Cre drivers allowed further development of the cochlea before β-catenin was knocked out or overexpressed (Shi et al., 2014). However, it is challenging to correlate phenotypic changes in the OC with specific Wnt ligands or their immediate target genes due to inherent delays between induction, Cre recombination and β-catenin turnover.

Organ cultures offer another approach to provide fine temporal control in the manipulation of Wnt signaling. Jacques and colleagues previously showed that pharmacological Wnt activation generated increases in both sensory domain size and HC numbers (Jacques et al., 2014, 2012). Their experimental paradigm differs significantly from the present study in using a different activator, LiCl, over a longer period, whereas we used a high concentration of CHIR pulsed on specific days. One notable difference is our day 0 phenotype, where we observed an increase in proliferation but the total area of the sensory domain was unchanged. Another difference is that on day 1, although the total sensory domain area increased we did not see increases in both medial and lateral compartments. Once again, on day 3, we observed an increase in the total area of the sensory domain and the medial compartment. At this high CHIR concentration, we can identify changes in gene expression as soon as 6 h, before the onset of enhanced

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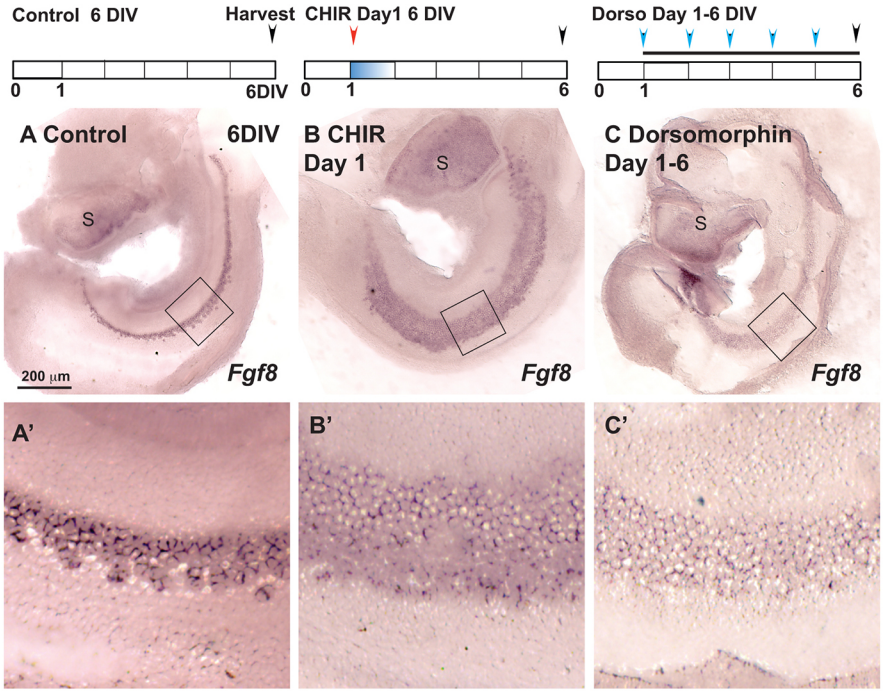


**Fig. 7. Wnt activation and Bmp inhibition each decrease IHC formation.** Cochleas cultured for 6DIV and probed for *Fgf8* transcripts by *in situ* hybridization in (A,A′) controls, (B,B′) day 1 CHIR treatment and (C,C′) day 1-6 dorsomorphin treatment (*n*=4 per condition).

proliferation or altered *Ccnd1* transcription. This dose may activate genes with higher thresholds of transcription that could not be regulated by a milder pharmacological treatment. One of the early upregulated genes, *Jag1*, can influence cell fates via the Notch pathway. Thus, we consider the possibility that control of compartment identity by Wnt-Notch signaling might be regulated independently of its effects on proliferation (on compartment size). Differential responses to Wnt activation observed for the medial versus the lateral compartments reinforce this interpretation.

We demonstrated that specification of medial versus lateral compartments was strongly, yet differentially, influenced by the timing of Wnt activation. This manifested as changes in the number of HCs associated with each compartment: a loss of nearly all HCs (day 0), an increase in IHCs (day 1 and day 3) and a decrease in OHCs (day 3). Wnt signaling promoted the medial compartment via the Notch pathway. We also showed that Wnt activation repressed *Bmp4* expression and that Bmp signaling is required for proper lateral fate specification. Fig. 8 summarizes these results in a

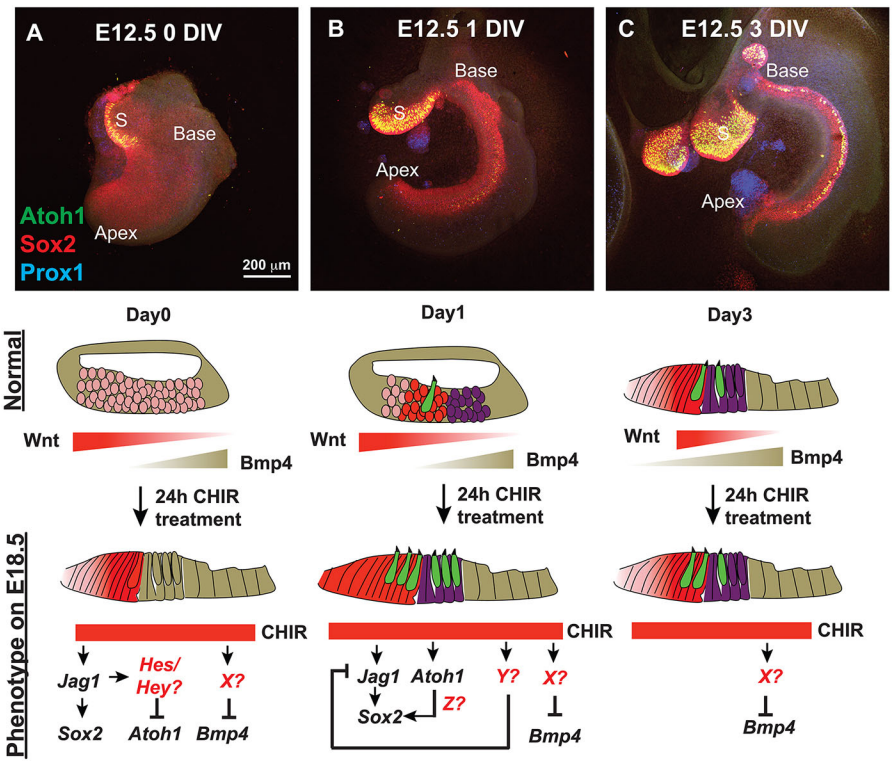


**Fig. 8. Model for OC patterning across the radial axis, and the changes induced by Wnt activation at different time points.** Top row shows normal development of E12.5 cultured cochleas on (A) day 0, (B) day 1 and (C) day 3. The middle row is a representation of normal cochlear development, which requires a balance of Wnt and Bmp signaling to pattern the cochlea at the three main time points. The bottom row shows Wnt signaling perturbed by CHIR, where this balance is affected, leading to different patterning phenotypes when assessed at E18.5. *X*, a hypothetical gene regulated by Wnts that represses *Bmp4*; *Y*, a hypothetical Wnt-mediated repressor of *Jag1*; *Z*, a hypothetical intermediate mediator of lateral induction (see text for details).

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working model that emphasizes crosstalk between either the Wnt-Notch or Wnt-Bmp pathways, based on correlations with CHIR-induced altered gene expression profiles. Our results emphasize that each of these signaling pathways must be constrained spatially and temporally to achieve proper radial patterning. Thus, the approach of temporal manipulation of Wnt signaling, combined with data from many previous reports, allows for more informed and detailed modeling of cochlear patterning.

Wnt activator added for the first 24 h *in vitro* suppressed differentiation of the OC when assayed after 6DIV. One interpretation is that the lateral compartment was selectively lost and that HC differentiation was repressed in the remaining medial compartment. We examined early changes in gene expression to try to understand the mechanism(s) underlying the subsequent changes in cochlear patterning. Within 6 h of Wnt activation, *Jag1*, a well-characterized Wnt target gene with TCF/LEF binding sites in its regulatory element (Estrach et al., 2006; Katoh and Katoh, 2006), was increased more than 4-fold and had spread across the floor of the cochlea to include the presumptive lateral compartment and beyond. Although *Jag1* is required for prosensory formation (Hartman et al., 2010; Kiernan et al., 2006; Pan et al., 2010), under these conditions the excess *Jag1* is detrimental to HC differentiation (Petrovic et al., 2014). When considering the combined effects of Wnt and Notch on HC differentiation, we note the reduction of *Atoh1* at 24 h after CHIR addition, but not at 6 h. Excess Jag1-Notch signaling can indirectly repress *Atoh1* via activation of downstream bHLH repressors of *Atoh1* (Fig. 8) (Kageyama and Ohtsuka, 1999; Tateya et al., 2011; Zheng et al., 2000). It is notable that *Sox2*, an important regulator of cochlear sensory fate, is also repressed 24 h after the onset of CHIR on day 0. Thus, some other mechanism, in addition to the onset of *Jag1* expression, must be required for lateral specification. Additional effects related to repression of Bmp signaling are discussed below.

On day 1, Wnt activation induced an extensive medial expansion of the Sox2 domain that was evident after 6DIV. Yet, after just 6 h, *Sox2* transcript levels were unaffected. In fact, by 24 h, Sox2 protein levels were decreased in hyperproliferating cells. The Wnt pathway is able to modulate Sox2 expression (at least indirectly) to maintain low levels in proliferating cells, as previously predicted (Munnamalai and Fekete, 2013). Although proliferation increases the size of the sensory domain, proliferation alone would not be expected to change the relative sizes of the compartments because CHIR treatment increased proliferation throughout the epithelium, including on the lateral side. Day 0 CHIR treatment also increased proliferation, yet this was insufficient to support lateral compartment formation. On day 3, when the medial compartment is no longer proliferating in controls, CHIR still induced an increase in the medial but not the lateral compartment. This reinforces that the differential effect on compartment size may reflect unique responses of the two domains to Wnt activation. To evaluate this, we performed the complementary experiment in which Wnt was inhibited from day 1-6. Although the total area of the sensory domain decreased, the decrease was specific to the medial compartment. Therefore, the size of the medial compartment is preferentially Wnt dependent. One model is that Wnt ligands emanating from the medial side of the prosensory domain impart medial identity to cells exposed to a specific threshold concentration, as expected for a morphogen. An alternative model that cannot be discarded based on available evidence is that the medial compartment expands (with Wnt activation) or shrinks (with Wnt inhibition) more robustly than the lateral compartment simply because the two regions are in different stages of specification for a critical time window during the first 2 days. Further analyses are required to determine whether relative changes in compartment sizes can be explained solely by inherent differences in the proliferative responsiveness of individual progenitors over time.

Two successive pulses of CHIR generated a combination of day 0 and day 1 phenotypes: suppression of both the lateral compartment and OHC formation (like day 0) and expansion of the medial compartment with increased IHCs (like day 1). Notch inhibition following treatment of CHIR on day 0 and day 1 blocked prosensory formation. Therefore, downstream Notch signaling was responsible for promoting a medial fate to these hyperproliferating cells after the withdrawal of CHIR.

An unexpected outcome of our study was the difference in responsiveness of Wnt target genes; for example, *Jag1* on day 1 (no change) compared with day 0 (strong upregulation). *In situ* hybridization after day 1 CHIR revealed that *Jag1* expression was weaker yet modestly expanded in the lateral direction. This contrasts with the robust elevation and complete lateral expansion of *Jag1* with day 0 CHIR. Therefore, we posit that an unknown Wnt-mediated repressor of *Jag1*, '*Y*', comes into play on day 1 (Fig. 8). This raises the question of why the transcriptional activation of *Jag1* observed on day 0 did not also increase the width of the medial compartment. We speculate that this might be related to the opposing effects that Wnt activation has on two other Notch pathway genes, namely *Lfng* and *Mfng*. Specifically, *Lfng* was unchanged in the future GER on day 0, whereas it was significantly decreased on day 1. *Mfng* was not expressed in the cochlea on day 0, but was modestly increased with Wnt activation on day 1. It remains to be addressed whether, or how, these Notch-related genes collectively affect the status of the medial compartment borders in our experiments, although there is evidence that the Fringe proteins determine the inside (medial) border of the IHC domain (Basch et al., 2013). Additionally, several genes were differentially regulated by CHIR after 6 h on day 0 versus day 1. These findings can be considered in the light of a recent report that β-catenin translocation to the nucleus alone is insufficient to activate gene transcription and is dependent on tissue- and age-specific contexts in early *Xenopus* embryos (Nakamura et al., 2016). An analogous requirement for coordination with other signaling pathways in the cochlea offers a possible explanation for the differences observed in Wnt responsiveness over time.

The increase in IHCs following day 1 CHIR treatment was likely to be caused by directly upregulating *Atoh1*, a documented Wnt target gene (Kuo et al., 2015; Shi et al., 2010, 2014). At the time of CHIR treatment, *Atoh1* expression was already ongoing and Wnt activation boosted it further. This response preceded any effect on *Sox2* expression. Overexpression of *Atoh1* not only induces ectopic HCs, but can also promote sensory formation (Kelly et al., 2012; Woods et al., 2004). Thus, the increase in *Atoh1* on day 1 might be a cause rather than a consequence of the increased size of the medial sensory domain. Since the observed effect suggests lateral induction, we hypothesize that this occurs through the action of an unknown intermediate '*Z*' (Fig. 8). Overexpression of *Atoh1* induced ectopic HCs and ectopic non-sensory patches of Jag1 expression in a previous study (Woods et al., 2004). Comparing *Atoh1* and *Jag1* expression on day 0 and day 1, we see an inverse regulation by Wnts. *Jag1* is upregulated on day 0, whereas *Atoh1* is not; and *Atoh1* is upregulated on day 1, whereas *Jag1* is not. This presents an interesting hypothesis for a dual mode of *Jag1* regulation: directly via Wnts and indirectly via *Atoh1*.

CHIR treatment also influenced the lateral compartment to different degrees on different DIV, which can be explained most

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readily by an indirect repression of *Bmp4*. Although Bmp regulation is context dependent, several studies report that Wnt activity can antagonize Bmp expression; our data are consistent with this scenario. Because the canonical Wnt pathway activates, rather than represses, transcription we hypothesize that Wnts regulate gene '*X*' (Fig. 8), which in turn represses *Bmp4* (Jain et al., 2015). The phenotype associated with Bmp inhibition alone (via dorsomorphin treatment) showed some similarities to Wnt activation on day 0, day 1 or day 3. When initiated on day 0, dorsomorphin caused a loss of the lateral compartment and increased IHCs. In the absence of Bmp signaling, HCs adopt the IHC fate, either by default or because this is the only option available when missing the lateral compartment. Previous work showed that an intermediate level of Bmp signaling is required for prosensory formation (Ohyama et al., 2010), in addition to the presence of Jag1 and Sox2 (Dabdoub et al., 2008; Kiernan et al., 2006; Puligilla et al., 2010). By day 3, in control cochleas IHCs have formed and OHC formation has just initiated (Fig. 8C). By activating the Wnt pathway at this stage, the downregulation of *Bmp4* coincided with a halt in OHC formation. Despite the presence of some Prox1-positive cells, it is possible that Bmp inhibition decreased the overall population of Prox1-positive cells, which would account for the loss of OHCs. A critical concentration of Bmp4 is required for HC formation (Ohyama et al., 2010), and the organ might have failed to reach this threshold in the context of excess Wnt signaling (Fig. 8). The phenotype induced by day 3 CHIR (too few OHCs) is complementary to knockouts of *Rspo2* (enhances Wnt) and *Nog* (Bmp inhibitor), which generate an extra row of OHCs (Hwang et al., 2010; Mulvaney et al., 2013). The data presented in this study show that Wnts and Bmp4 have antagonistic roles. We conclude that perturbing one pathway affects the other, with medial fates promoted by 'Wntch' signaling and lateral fates promoted by Bmp4 signaling.

In addition to crosstalk with the Notch and Bmp pathways, RT-qPCR analysis also showed an upregulation of *Fgf20* in response to CHIR. Others have shown that Wnts regulate *Fgf20* expression (Chamorro et al., 2005; Stoick-Cooper et al., 2007). In the cochlea, Fgf20-Fgfr1 signaling is required for specifying postmitotic sensory progenitors (Hayashi et al., 2008b; Munnamalai et al., 2012; Ono et al., 2014). This might help explain how the Wnt pathway, most commonly linked to cell proliferation, can also regulate sensory cell fate decisions within the cochlea.

In summary, this study examined the potential crosstalk between the Wnt pathway and other signaling pathways. We have shown that different mechanisms are likely to be operating downstream of Wnts on different days of development. Studies have shown that upon Wnt stimulation, regeneration is possible in the neonatal cochlea, but this potential is lost after postnatal day 3 (Chai et al., 2012; Cox et al., 2014). Such regenerative failure is linked to changes in the Notch pathway (Maass et al., 2015). Therefore, understanding the gene networks operating from early to late ages might prove informative in assessing feasible approaches to regeneration.

## MATERIALS AND METHODS

### Mice

*Atoh1*$^{tm4.1Hzo/J}$ (*Atoh1-GFP*) mice (Jackson Laboratory) and/or outbred Swiss Webster mice (Harlan Laboratories) were time-mated to obtain E12.5-15.5 embryos. Animal procedures were carried out in accordance with guidelines of the Purdue Animal Care and Use Committee (PACUC).

### Organotypic cultures

The date on which a vaginal plug was observed was assigned as E0.5. Only embryos conforming to expected E12.5 staging criteria (Theiler, 1989) were used for culturing. Dissected cochleas were placed on Millicell cell culture inserts (Millipore) as described (Munnamalai and Fekete, 2016). DMSO served as a vehicle control for parallel drug treatments. The Wnt pathway was temporally activated by shifting the day of 10 µM CHIR (Selleckchem) addition, with washing out 24 h later with DMSO medium, and cultured up to 6DIV. 10 µM DAPT (Peptides International), 20 µM dorsomorphin (Tocris Bioscience) and 150 µM IWR1 (Tocris Bioscience) were used to inhibit the Notch, Bmp and Wnt pathways, respectively. For proliferation assays using EdU incorporation, 1 µg/ml EdU was added to the culture medium and detected using the Click-IT reaction kit (Life Technologies). For electroporation of cochlea cultures with *Atoh1* and *EGFP* expression plasmids, see the supplementary Materials and Methods.

### Immunofluorescence

Cochleas were fixed with 4% formaldehyde in PBS with 0.1% Triton X-100 for 1 h at room temperature. Tissues were blocked with 2% donkey serum (Jackson ImmunoResearch) in PBS/Triton X-100, followed by overnight primary antibody incubation at 4°C. Primary antibodies (1:500): Myo6 (Proteus Biosciences, 25-6791), Sox2 (Santa Cruz, sc17320) and Prox1 (Millipore, AB5475). Alexa-labeled secondary antibodies (1:500; Molecular Probes) were incubated overnight. Immunolabeled explants were mounted in Fluoromount-G (Southern Biotech) prior to imaging. For details on quantification and statistics, see the supplementary Materials and Methods.

### Real-time quantitative PCR (RT-qPCR)

For each biological replicate, total RNA was extracted from four cochleas and reverse-transcribed into cDNA with oligo(dT) primers (Thermo Fisher) and Superscript III reverse transcriptase (Life Technologies). RT-qPCR was performed on a LightCycler 96 (Roche) for genes of interest with *Gapdh* as the housekeeping gene. Ct values for genes were averaged from three technical replicates. Three biological replicates were averaged for statistical analysis. Primers (5′-3′, forward and reverse): *Gapdh*, GGCATTGCTCT-CAATGACAA and CTTGCTCAGTGTCCTTGCTG; *Axin2*, TGACTC-TCCTTCCAGATCCCA and TGCCCACACTAGGCTGACA; *Sox2*, GCGGAGTGGAAACTTTTGTCC and CGGGAAGCGTGTACTTATC-CTT; *Atoh1*, TCCCGTCCTTCAACAACGAC and CTCTCCGACATTG-GGAGTCTG; *Fgf20*, CCTTGGGATGAATGACAAAGGA and CGACCCGTGTTTCCATGTTT; *Lgr5*, CCTACTCGAAGACTTACCCA-GT and GCATTGGGGTGAATGATAGCA; *Jag1*, CTGAGCATGCTTG-TCTCTCTGACC and ATCGATGTCTCTCTCACAGTGATCG; *Ccnd1*, GCGTACCCTGACACCAATCTC and CTCCTCTTCGCACTTCTGCTC.

### *In situ* hybridization

For whole-mount *in situ* hybridization, cochleas were fixed with 7.4% formaldehyde in RNase-free PBS and processed with DIG-labeled antisense probes as previously described (Munnamalai et al., 2012). Alkaline phosphatase activity was activated and developed with NBT/BCIP or BM purple (Roche). Samples were post-fixed and imaged. For *in situ* hybridization on serial sections and for details of imaging, see the supplementary Materials and Methods.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

V.M. and D.M.F. conceived the project, analyzed data and wrote the manuscript. V.M. performed the experiments.

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**Supplementary information**

Supplementary information available online at
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