Development/Plasticity/Repair

Hey1 and *Hey2* Control the Spatial and Temporal Pattern of Mammalian Auditory Hair Cell Differentiation Downstream of Hedgehog Signaling

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Mechano-sensory hair cells (HCs), housed in the inner ear cochlea, are critical for the perception of sound. In the mammalian cochlea, differentiation of HCs occurs in a striking basal-to-apical and medial-to-lateral gradient, which is thought to ensure correct patterning and proper function of the auditory sensory epithelium. Recent studies have revealed that Hedgehog signaling opposes HC differentiation and is critical for the establishment of the graded pattern of auditory HC differentiation. However, how Hedgehog signaling interferes with HC differentiation is unknown. Here, we provide evidence that in the murine cochlea, *Hey1* and *Hey2* control the spatiotemporal pattern of HC differentiation downstream of Hedgehog signaling. It has been recently shown that HEY1 and HEY2, two highly redundant HES-related transcriptional repressors, are highly expressed in supporting cell (SC) and HC progenitors (prosensory cells), but their prosensory function remained untested. Using a conditional double knock-out strategy, we demonstrate that prosensory cells form and proliferate properly in the absence of *Hey1* and *Hey2* but differentiate prematurely because of precocious upregulation of the pro-HC factor *Atoh1*. Moreover, we demonstrate that prosensory-specific expression of *Hey1* and *Hey2* and its subsequent graded downregulation is controlled by Hedgehog signaling in a largely FGFR-dependent manner. In summary, our study reveals a critical role for *Hey1* and *Hey2* in prosensory cell maintenance and identifies Hedgehog signaling as a novel upstream regulator of their prosensory function in the mammalian cochlea. The regulatory mechanism described here might be a broadly applied mechanism for controlling progenitor behavior in the central and peripheral nervous system.

Key words: ***Atoh1*; hair cells; Hedgehog signaling; *Hey1*; *Hey2*; inner ear cochlea**

Introduction

Auditory hair cells (HCs) and their surrounding supporting cells (SCs) derive from a common pool of postmitotic prosensory cells within the developing cochlear duct (Chen and Segil, 1999). *Atoh1*, which encodes for a basic helix-loop-helix (bHLH) transcriptional activator, is necessary for the generation of inner ear HCs (Bermingham et al., 1999) and is among the earliest known markers of auditory HCs (Chen et al., 2002b; Cai et al., 2013). Unique to the mammalian cochlea, ATOH1-mediated HC differentiation follows a distinct basal-to-apical gradient, whereby HCs located near the cochlear base differentiate first and the most apically located HCs differentiate last. In addition to the steep basal-to-apical differentiation gradient, a less steep gradient exits, with medially located inner HCs (IHCs) differentiating before laterally located outer HCs (OHCs) (Sher, 1971; Chen et al., 2002b). This unique pattern of differentiation is thought to ensure the precise arrangement of HCs, with one row of IHCs and three rows of OHCs spanning the length of the cochlear duct. Recent findings have shown that Hedgehog signaling negatively regulates ATOH1-mediated HC differentiation and is critical for the establishment of the basal-to-apical gradient of HC differentiation in the mammalian cochlea (Driver et al., 2008; Bok et al., 2013; Tateya et al., 2013). However, how Hedgehog signaling exerts its negative influence on *Atoh1* expression and subsequently HC differentiation is unresolved. HES and HES-related HEY factors, which belong to the subfamily of bHLH transcriptional repressors, are known to interfere with bHLH activators at the transcriptional and post-transcriptional levels (Fischer and Gessler, 2007; Kageyama et al., 2008). In the CNS, gain-of-function studies have suggested that HEY and HES proteins cooperate with each other in suppressing bHLH activator-driven neuronal differentiation and in maintaining the neural stem cell fate (Ishibashi et al., 1994; Sakamoto et al., 2003). In the developing cochlea, two highly redundant *Hey* genes, *Hey1* and *Hey2*, are highly expressed in prosensory cells but are rapidly downregulated upon their differentiation and only persist in a subset of SC

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precursors (Hayashi et al., 2008b; Li et al., 2008; Doetzlhofer et al., 2009). In SC precursors, HEY1 and HEY2 cooperate with the coexpressed HES factors, HES1 and HES5, to repress a HC-specific gene program (Li et al., 2008; Tateya et al., 2011). Their functions in prosensory cells are unknown, but based on their responsiveness to Notch signaling, it has been proposed that *Hey1* and *Hey2* function as Notch effectors in prosensory cell specification (Hayashi et al., 2008b). However, more recent findings suggest that in prosensory cells *Hey1* and *Hey2* expression is controlled by additional unidentified Notch-independent signaling mechanisms (Basch et al., 2011). Here, we show that *Hey1* and *Hey2* are dispensable for prosensory cell specification and proliferation but are critical for maintaining prosensory cells undifferentiated. Moreover, we identify Hedgehog signaling as a critical upstream regulator of their prosensory-specific expression and graded downregulation during cochlear differentiation. Together, our findings indicate that *Hey1* and *Hey2* control the spatial and temporal pattern of auditory HC differentiation downstream of Hedgehog signaling.

Materials and Methods

Mouse breeding and genotyping. *Atoh1/nGFP* transgenic mice (Lumpkin et al., 2003) were obtained from Jane Johnson (University of Texas Southwestern Medical Center, Dallas). *Hey1 floxed* (Fischer et al., 2005) and *Hey2* knock-out (Gessler et al., 2002) mice were obtained from Manfred Gessler (University of Wuerzburg, Wuerzburg, Germany). *Atoh1 floxed* mice (Shroyer et al., 2007) were obtained from The Jackson Laboratory (Stock #008681). *Pax2-Cre BAC* transgenic mice (Ohyama and Groves, 2004) were obtained from Andrew Groves (Baylor College, Houston). Mice were genotyped by PCR, and genotyping primers are available upon request. *Hey1 floxed* and *Hey2 knock-out* and *Pax2-Cre tg/+* mice were used to produce *Hey1–Hey2* double mutants (*Pax2-Cre tg/+; Hey1* $^{fl/fl}$*; Hey2* $^{-/-}$), designated *Hey1* $^{\Delta/\Delta}$ *Hey2* $^{-/-}$, and *Hey2* homozygous (*Hey1* $^{fl/fl}$ *Hey2* $^{-/-}$) and *Hey2* heterozygous mutant (*Hey1* $^{fl/fl}$ *Hey2* $^{-/+}$) littermates were used as experimental controls. *Atoh1 floxed* and *Pax2-Cre tg/+* mice were used to generate *Atoh1* mutant mice (*Pax2-Cre tg/+; Atoh1* $^{fl/fl}$), designated *Atoh1* $^{\Delta/\Delta}$. These mice were maintained on a C57BL/6; CD-1 mixed background. Mice of both sexes were used in this study. Embryonic development was considered as E0.5 on the day a mating plug was observed. The day of birth was considered P0. All experiments and procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committees protocol, and all experiments and procedures adhere to National Institutes of Health-approved standards.

Tissue harvest and processing. Embryos and early postnatal pups were staged using the EMAP eMouse Atlas Project (http://www.emouseatlas.org) Theiler staging criteria. Inner ear cochleae were collected in Hanks buffer (Invitrogen). To free the cochlear epithelial duct from surrounding tissue, dispase (1 mg/ml; Invitrogen) and collagenase (1 mg/ml; Worthington) mediated digest was used as previously described (Doetzlhofer et al., 2009). To prevent overdigestion, tissue was refixed after enzyme digestion with 4% para-formaldehyde (PFA). To expose the auditory sensory epithelium (Stage P0–P4), the cochlear capsule, spiral ganglion, and Reissner's membrane were removed, and the remaining tissue (cochlear surface preparation) was briefly fixed in 4% PFA. To obtain cochlear sections, whole heads (Stage E13.5–E15.5) or isolated inner ears (Stage P0-P4) were fixed in 4% PFA in PBS, cryoprotected using 30% sucrose in PBS, and embedded in OCT (Sakura Finetek) for cryosectioning.

Histochemistry and in situ *hybridization (ISH).* Immunostaining was performed as described previously (Doetzlhofer et al., 2009). Primary antibodies used were anti-Myosin VI (1:500, Proteus), anti-SOX2 (1:500, Santa Cruz Biotechnology), and anti-p75 (1:1000, EMD Millipore). Cell nuclei were fluorescently labeled with Hoechst-33258 dye (Sigma). Actin filaments were labeled with AlexaFluor (488 or 546) conjugated phalloidin (1:1000, Invitrogen). AlexaFluor (488 or 546) labeled secondary antibodies (1:1000, Invitrogen) were used. For ISH, pBluescript II (Stratagene) and pGem-T easy (Promega) vectors containing full-length mouse *Atoh1*, *Sox2*, *Hey1*, and *Hey2* cDNA were used as templates to synthesize digoxigenin-labeled antisense RNA probes according to the manufacturer's specifications (Roche). The ISH procedure was modified from a protocol from Domingos Henrique (Henrique et al., 1995).

Organotypic cochlear culture. E13.0-E13.5 embryos were screened for *Atoh1/nGFP* expression and staged (see Tissue harvest and processing). Embryos of inappropriate stage and nontransgenic embryos were discarded. *Atoh1/nGFP* transgenic inner ear cochleae were harvested in Hanks media (Invitrogen), and collagenase/dispase enzyme digest was used to free the cochlear duct from the surrounding mesenchyme. Remaining tissue, including the cochlear epithelial duct, the vestibular sacculus, and the innervating spiral ganglion, was placed onto filter membranes (SPI Supplies, Structure Probe) and cultured in DMEM-12 (Invitrogen), 1% FBS (Atlanta Biologicals), 5 ng/ml EGF (Sigma), 100 U/ml penicillin-streptomycin (Sigma), and 1× B27 supplement (Invitrogen). All cultures were maintained in a 5% CO_2/ 20% O_2 humidified incubator. Hedgehog ligand SHH (R&D Systems) was used at 50 nM final concentration. Hedgehog inhibitor cyclopamine-KAAD (EMD Millipore) was used at 5 μM final concentration. FGFR inhibitor SU5402 (3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl) methylene]-2-indolinone, Tocris Bioscience) was used at 10 μM final concentration. Stock solutions for SHH (5 μM in PBS 0.1% BSA), SU5402 (10 mM in DMSO), and cyclopamine-KAAD (5 mM in DMSO) were stored at −80°C. SHH, cyclopamine-KAAD, and SU5402 were added at plating, and control explants received 0.1% DMSO as vehicle control.

X-gal assay. To preserve *LacZ* encoded β-galactosidase activity, *Hey2* $^{LacZ/+}$ cochlear explant cultures were fixed for 10 min with 2% PFA on ice. After washing in PBS, tissue was incubated at 35°C overnight in X-gal reaction buffer containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-Dgalactopyranoside, Corning, Cellgro), 5 mM $K_3Fe(CN)_6$ (Sigma), 5 mM $K_4Fe(CN)_6$ (Sigma), and 2 mM $MgCl_2$ (Sigma) in PBS.

RNA extraction and qPCR. Before RNA extraction, cochlear epithelia were isolated from cultured cochlear explants using dispase/collagenase treatment, and three to four cochlear epithelia were pooled per condition. RNeasy Micro kit (QIAGEN) was used to isolate total RNA, and mRNA was transcribed into cDNA using iScript kit (Bio-Rad). qPCR was performed with a SYBR Green kit (Invitrogen) and gene-specific primer sets on a StepOne Plus PCR Detection System (Applied Biosystems/Invitrogen). Each PCR was performed in triplicate. Relative gene expression was analyzed by using the ΔΔCT method (Schmittgen and Livak, 2008). The comparative Ct study feature of StepOne plus software (Applied Biosystems/Invitrogen) was used to aggregate biological replicate data. C-DNA from freshly isolated cochlear epithelia, Stage E15.5 was used as a calibrator, and the ribosomal gene *Rpl19* was used as endogenous reference gene. The following primers were used for qPCR: *Atoh1* forward, ATG CAC GGG CTG AAC CA; *Atoh1*-R TCG TTG TTG AAG GAC GGG ATA; *Sox2* forward, CTG TTT TTT CAT CCC AAT TGC A; *Sox2* reverse, CGG AGA TCT GGC GGA GAA TA; *Hey1* forward, CAC TGC AGG AGG GAA AGG TTA T; *Hey1* reverse, CCC CAA ACT CCG ATA GTC CAT; *Hey2* forward, AAG CGC CCT TGT GAG GAA A; *Hey2* reverse, TCG CTC CCC ACG TCG AT; *Hes1* forward, GCT TCA GCG AGT GCA TGA AC; *Hes1* reverse, CGG TGT TAA CGC CCT CAC A; *Fgf20* forward, CAC GGG TCG CAG GTA TTT TG; *Fgf20* reverse, CCT GGC ACC ATC TCT TGG A; *Isl1* forward, CGG AGA GAC ATG ATG GTG GTT; *Isl1* reverse, AGG GCG GCT GGT AAC TTT G; *Id1* forward, GAA CGT CCT GCT CTA CGA CAT G; *Id1* reverse, TGG GCA CCA GCT CCT TGA; *Id2* forward, AAG GTG ACC AAG ATG GAA ATC CT; *Id2* reverse, CGA TCT GCA GGT CCA AGA TGT; *Id3* forward, GAG CTC ACT CCG GAA CTT GTG; *Id3* reverse, CGG GTC AGT GGC AAA AGC; *Ptch1* forward, CTG GCT CTG ATG ACC GTT GA; *Ptch1* reverse, GCA CTC AGC TTG ATC CCA ATG; *Rpl19* forward, GGTCTGGTTG-GATCCCAATG; *Rpl19* reverse, CCCGGGAATGGACAGTCA.

Proliferation assay. EdU (5-ethynyl-2′–deoxyuridine, Invitrogen) was reconstituted in PBS and administered at 50 μg per gram of body weight to time-mated pregnant dams by a single intraperitoneal injection. Click-iT AlexaFluor-488 or -546 Kit (Invitrogen) was used to detect incorporated EdU according to the manufacturer's specifications. EdU incorporation in prosensory cells, HCs, and SCs was quantified in co-

chlear sections and cochlear surface preparations, which have been stained with a nuclear dye (Hoechst-33258) and immunostained for SOX2 and myosin VI (MYO6) to visualize prosensory cells, SCs, and HCs, respectively. Confocal images were assembled and analyzed in Photoshop CS3 (Adobe), and ImageJ software (National Institutes of Health) was used to measure the length of counted segments.

Quantification of basal to apical extent of HC differentiation. HC differentiation in E13.0–E13.5 *Atoh1/nGFP* transgenic cochlear explants was monitored over 48-h culture period, and green fluorescent images of native GFP expression were captured using fluorescent stereo-microscopy (Leica) every 12 h. HC differentiation in E15.0 DKO and control cochlear tissue was analyzed in enzyme-purified cochlear epithelial preparations immunostained for HC marker MYO6. Fluorescent images were analyzed in Photoshop CS3 (Adobe), and lengths of *Atoh1/nGFP*-positive and MYO6-positive domains were measured using ImageJ software (National Institutes of Health).

Quantification of HCs and SCs. Cochlear surface preparations obtained from P0–P2 DKO ($Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$) pups and control littermates ($Hey2^{-/+}$ and $Hey2^{-/-}$) were immunostained for MYO6 and SOX2. High-power confocal images of SC layer ($SOX2^{+}$) and HC layer ($MYO6^{+}$) at defined apical-basal positions were used for HC and SC counts. Low-power fluorescent images of the HC layer were used to reconstruct the entire cochlear sensory epithelium. Images were assembled and analyzed in Photoshop CS3 (Adobe). ImageJ software (National Institutes of Health) was used to measure length of counted segments and total length.

Statistical analysis. Values are presented as mean ± SEM; n = biological replicates analyzed. Two-tailed Student's t tests were used to determine the confidence interval. $p \leq 0.05$ were considered significant. p values > 0.05 were considered not significant.

Results

Downregulation of *Hey1* and *Hey2* during cochlear differentiation occurs independently of *Atoh1*

Hey1 and *Hey2* are highly expressed by prosensory cells in the undifferentiated cochlea. As differentiation progresses along the developing cochlear duct, their expression becomes restricted to distinct subtypes of SCs (Hayashi et al., 2008b; Li et al., 2008; Doetzlhofer et al., 2009). The mechanism responsible for the graded downregulation of *Hey1* and *Hey2* during cochlear differentiation is currently unknown. One possibility is that *Hey1* and *Hey2* downregulation is a result of HC differentiation. To examine the dynamics of *Hey1* and *Hey2* expression in prosensory cells and to address whether *Hey1* and *Hey2* downregulation depends on HC differentiation, *Hey1*, *Hey2*, and *Atoh1* mRNA expression was analyzed in embryonic day 13.5 (E13.5) and E15.5 wild-type and E15.5 *Atoh1* mutant cochlear tissue using RNA in situ hybridization (ISH) assays. ISH staining for *Sox2* was used to mark the prosensory/sensory domain (bracket). *Sox2* encodes for a high-mobility group transcription factor, known to be highly expressed in prosensory cells and embryonic HCs and SCs (Kiernan et al., 2005). At E13.5, *Hey1* and *Hey2* were coexpressed with *Sox2* in prosensory cells throughout the cochlear duct (Fig. 1*A*,*C*,*D*, bracket), whereas *Atoh1* was beginning to be upregulated in prosensory cells at the base of the cochlea (Fig. 1*B*, bracket). 48 h later, at E15.5, *Atoh1* was highly expressed in both IHCs (arrowhead) and OHCs (bar) in the cochlear base. In the cochlear mid-turn *Atoh1* was highly expressed in IHCs and was beginning to be upregulated in future OHCs (Fig. 1*F*, arrowhead and bar). In the undifferentiated cochlear apex, *Atoh1* was faintly expressed in future IHCs at the medial border of the prosensory domain (Fig. 1*F*, arrowhead, bracket). At E15.5, *Hey1* and *Hey2* were highly expressed in the *Sox2*-positive prosensory domain in the undifferentiated cochlear apex, but in the cochlear mid-turn *Hey1* and *Hey2* expression domain narrowed to the undifferentiated OHC domain (Fig. 1*E*,*G*,*H*). In the cochlear base, which already contained *Atoh1* expressing IHCs and OHCs, *Hey1* and *Hey2* expression was confined to differentiating SC precursors (Fig. 1*F–H*). Previous studies showed that in the *Atoh1* mutant cochlea, prosensory cells form and express *Sox2* and *Hey2* but fail to differentiate into HCs and SCs (Woods et al., 2004; Kiernan et al., 2005; Li et al., 2008). Consistent with these findings, prosensory-specific expression of *Sox2*, *Hey1*, and *Hey2* in the undifferentiated cochlear apex appeared to be unaffected by the loss of *Atoh1* (Fig. 1*I–L*). Moreover, the initial downregulation of *Hey1* and *Hey2* appeared to be equally unaffected by the loss of *Atoh1*. In the mid-turn of both the E15.5 wild-type (Fig. 1*G*,*H*) and *Atoh1* mutant (Fig. 1*K*,*L*) cochleae, *Hey1* (Fig. 1*G*,*K*) and *Hey2* (Fig. 1*H*,*L*) were downregulated in the IHC domain but continued to be weakly expressed in the OHC domain. However,

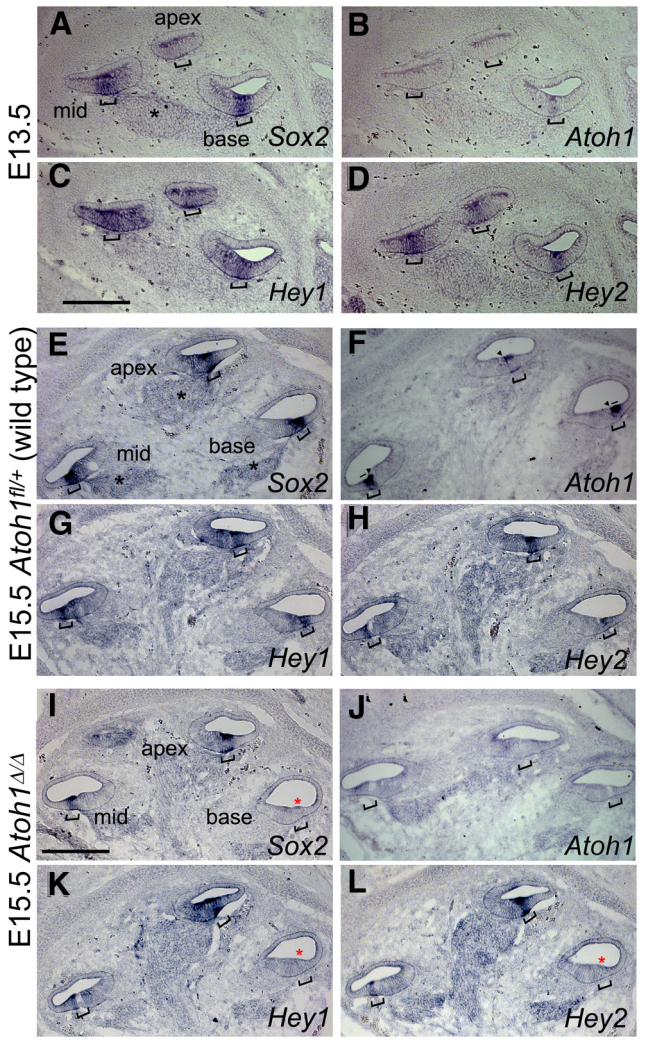

Figure 1. *Hey1* and *Hey2* downregulation in prosensory cells occurs independently of *Atoh1*. ***A–D***, *Hey1* and *Hey2* transcripts are highly expressed in prosensory cells. At E13.5, *Hey1* (***C***) and *Hey2* (***D***) are coexpressed with *Sox2* (***A***) in the prosensory domain (bracket) and *Atoh1* (***B***) begins to be upregulated in the cochlear base. ***A***, **Sox2* expression in spiral ganglion neurons. Scale bar, 200 μm. ***E–L***, Downregulation of *Hey1* and *Hey2* occurs in the absence of HC differentiation. In E15.5, $Atoh1^{fl/+}$ (wild-type) cochlear tissue, basal-to-apical upregulation of *Atoh1* (***F***) in IHCs (arrowhead) and OHCs (bar) coincides with the basal-to-apical downregulation of *Hey1* (***G***) and *Hey2* (***H***) in *Sox2*-positive HC and SC precursors (***E***, bracket). ***E***, **Sox2* expression in ganglion neurons. In E15.5, *Atoh1* mutant ($Atoh1^{\Delta/\Delta}$) cochlear tissue initial downregulation of *Hey1* (***K***) and *Hey2* (***L***) in *Sox2*-positive HC and SC precursors (***I***, bracket) occurs in the absence of *Atoh1* (***J***). Red asterisks indicate degenerated sensory epithelia in the *Atoh1* mutant cochlear base. Scale bars, 200 μm.

in the *Atoh1* mutant cochlear base, no *Sox2*, *Hey1*, or *Hey2* expression was detected (Fig. 1*I*,*K*,*L*). Previous studies showed that basally located prosensory cells in the *Atoh1* mutant cochlea start to undergo apoptosis at E15.5 (Chen et al., 2002b; Cai et al., 2013). The observed loss of *Sox2*, *Hey1*, and *Hey2* expression in the base of the *Atoh1* mutant cochlea is likely to be a secondary effect caused by the death of HC and SC precursors. Together, our data demonstrate that the initial downregulation of *Hey1* and *Hey2* in prosensory cells occurs independently of *Atoh1*, suggesting that *Hey1* and *Hey2* might act upstream of *Atoh1* and might negatively regulate *Atoh1* induction during cochlear differentiation.

Loss of *Hey1* and *Hey2* results in premature onset of HC differentiation

To determine the function of *Hey1* and *Hey2* in the developing cochlea, *Hey1–Hey2* double mutant animals were generated. Conventional *Hey1–Hey2* double-mutant animals die during early embryonic development because of severe vascular defects (Fischer et al., 2004). To prevent early embryonic lethality, a conditional knock-out strategy was applied in which, in a *Hey2* mutant background (Gessler et al., 2002), *Hey1* floxed allele (Fischer et al., 2005) was ablated using the inner ear-specific *Pax2-Cre* line (Ohyama and Groves, 2004). As previously reported, *Hey1* single knock-out mice show no obvious changes in both the number and organization of HCs or SCs (Hayashi et al., 2008b; Doetzlhofer et al., 2009). *Hey2* single knock-out mice have, dependent on their strain background, either no defects in the number or organization of HCs or SCs (Hayashi et al., 2008b) or only very mild HC and SC patterning defects (Li et al., 2008; Doetzlhofer et al., 2009). Moreover, a previous study revealed no defects in prosensory cell proliferation or differentiation, allowing the use of *Hey2* single mutants as experimental controls (Li et al., 2008). To confirm conditional *Hey1* deletion, qPCR experiments were performed on enzyme-purified cochlear epithelial ducts at Stage ~E13.5. Our analysis revealed that *Hey1* as well as *Hey2* mRNA expression in the DKO cochlear epithelia was reduced 10-fold compared with cochlear epithelia obtained from control littermates with one intact allele of *Hey1* or *Hey2*, respectively. This suggests that our strategy successfully ablated *Hey1* and *Hey2* in the developing cochlea (Fig. 2*A*, red and white bar). Furthermore, our analysis revealed no significant difference in *Sox2* mRNA expression in DKO cochlear epithelia compared with control epithelia ($Hey1^{\Delta/\Delta}$ $Hey2^{-/+}$, $Hey1^{+/+}$ $Hey2^{-/+}$, $Hey1^{+/+}$ $Hey2^{-/-}$) (Fig. 2*A*, gray bar), suggesting that prosensory cell formation was not impacted by the loss of *Hey1* and *Hey2*. However, we observed that *Atoh1* was noticeably higher expressed in DKO cochlear epithelia, compared with cochlear epithelia obtained from control littermates (Fig. 2*A*, dark gray bar). To address whether HC differentiation might have occurred prematurely in the absence of *Hey1* and *Hey2*, the pattern of *Atoh1* transcript, ATOH1 activity, and HC-specific marker expression was analyzed in DKO embryos and their littermate controls ranging in stages from E14.0 to E15.5. At E14.5 in $Hey2^{-/-}$ control littermates, *Atoh1* mRNA was only expressed in the cochlear base in a narrow band of cells, which will give rise to IHCs (Fig. 2*B*). However, in DKO embryos, *Atoh1* mRNA was readily detected in both the IHC and OHC domains in the cochlear base and in the future IHC domain in the mid-turn of the cochlear duct (Fig. 2*C*). At E15.0 in control littermates, *Atoh1* mRNA was expressed in both IHCs (arrowhead) and OHCs (bar) in the cochlear base and in IHCs in the cochlear mid-turn, but no *Atoh1* expression was detected in the cochlear apex (Fig. 2*D*). However, in DKO animals, *Atoh1* was readily detected in IHCs and OHCs

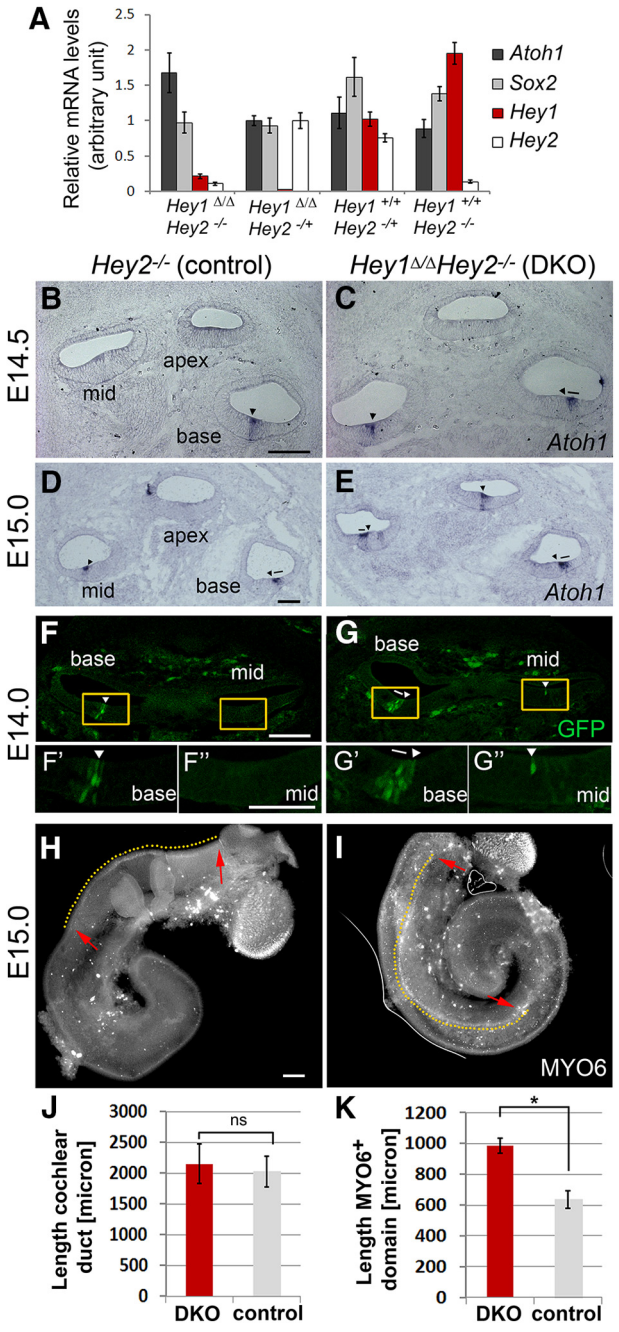

Figure 2. Loss of *Hey1* and *Hey2* results in accelerated auditory HC differentiation. ***A***, qPCR analysis of relative *Atoh1*, *Sox2*, *Hey1*, and *Hey2* mRNA levels in $Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$ (DKO) and control ($Hey1^{\Delta/\Delta}$ $Hey2^{-/+}$, $Hey1^{+/+}$ $Hey2^{-/+}$ and $Hey1^{+/+}$ $Hey2^{-/-}$) cochlear epithelia, Stage E13.5. Data are mean ± SEM. ***B–E***, ISH-based analysis of *Atoh1* expression pattern in $Hey2^{-/-}$ (control) (***B***, ***D***) and $Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$ (DKO) (***C***, ***E***) cochlear sections, Stage E14.5 (***B***, ***C***) and Stage E15.0 (***D***, ***E***). At both E14.5 and E15.0, *Atoh1* expression extends further apically in DKO (***C***, ***E***) than control (***B***, ***D***) cochlear sections. Scale bar, 100 μm. ***F–G″***, Low-power (***F***, ***G***) and high-power (***F′***, ***F″***, ***G′***, ***G″***) confocal images of HC-specific *Atoh1/nGFP* reporter expression (green) in E14.0 $Hey2^{-/-}$ (control; ***F***, ***F′***,***F″***) and E14.0 $Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$ (DKO; ***G***, ***G′***, ***G″***) cochlear sections. ***F***, ***G***, Yellow boxes represent location of the high-power images (***F′***, ***F″***, ***G′***, ***G″***). Scale bar, 100 μm. ***H–I***, Low-power images of E15.0 $Hey2^{-/-}$ (***I***) and E15.0 DKO (***H***) cochlear epithelial ducts immunostained for myosin VI (MYO6, white). Yellow dotted line indicates MYO6-positive sensory domains; red arrows indicate beginning and end. Sale bar, 100 μm. ***J***, ***K***, Quantification of cochlear length and extent of HC differentiation in E15.0 $Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$ (DKO) and $Hey2^{-/+}$ and $Hey2^{-/-}$ (control) littermates. Graphs represent cochlear duct length (***J***) and length of MYO6-positive sensory domain (***K***) for control (gray bar) and DKO (red bar) cochlear ducts. Data are mean ± SEM. $n = 4$ or 5 cochlear explants from three independent experiments. $^*p \leq 0.05$. n.s., Not significant.

both in the cochlear base and cochlear mid-turn and *Atoh1* mRNA started to be upregulated in the cochlear apex (Fig. 2*E*). To determine whether ATOH1 activity was also prematurely upregulated in the absence of *Hey1* and *Hey2*, we crossed the well-characterized HC-specific *Atoh1/nGFP* transgenic line into our *Hey1–Hey2* double mutant line. Previous studies demonstrated that the *Atoh1* enhancer used to drive GFP reporter expression is positively regulated by ATOH1 itself, making *Atoh1/nGFP* transgene expression a readout for ATOH1 activity (Helms et al., 2000; Lumpkin et al., 2003). At E13.5–E14.0, HC differentiation initiates at the cochlear mid-base, and in E14.0 $Hey2^{-/-}$ littermates (control) a narrow band of GFP-positive cells was only present in the cochlear base, representing future IHCs (Fig. 2*F*,*F*′). No GFP-positive cells were observed further apically in the cochlear mid-turn (Fig. 2*F*,*F*″). However, in E14.0 DKO embryos, GFP was broadly expressed in future IHC and OHCs in the cochlear base (Fig. 2*G*,*G*′), and GFP-positive cells were already observed in the cochlear mid-turn (Fig. 2*G*,*G*″). To quantify the basal-to-apical extent of HC differentiation, cochlear epithelial preparations of Stage E15.0 DKO (Fig. 2*I*) and their $Hey2^{-/-}$ control littermates (Fig. 2*H*) were stained with the HC marker myosin VI (MYO6), and the length of the sensory domain containing MYO6-positive HCs was measured. As expected, both DKO and control cochlear epithelia were similar in length (Fig. 2*J*); however, HC differentiation, as judged by the basal-to-apical extent of HC-specific MYO6 expression, was more advanced in the *Hey1–Hey2* double mutant (DKO) cochlea than in the *Hey2* single mutant cochlea (control) (Fig. 2*K*). In summary, our data suggest that *Hey1* and *Hey2* are critical for preventing premature upregulation of *Atoh1* in prosensory cells and loss of *Hey1* and *Hey2* results in premature HC differentiation along the developing cochlear duct.

Loss of *Hey1* and *Hey2* results in abnormal distribution of OHCs along the cochlear duct

To determine how loss of *Hey1* and *Hey2* might impact HC patterning, cochlear surface preparations and cochlear sections were prepared from neonatal $Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$ (DKO) pups and $Hey2^{-/-}$ and $Hey2^{-/+}$ littermate controls and stained for HC markers. Immunostaining for MYO6 was used to visualize HC soma, and phalloidin staining was used to visualize the HC-specific actin-rich apical protrusions called stereocilia. It has previously been shown that, in *Hes/Hey* compound mutants, in which either *Hey1* or *Hey2* were codeleted with *Hes1* and or *Hes5*, HC density and ectopic HCs are significantly increased compared with control tissue (Li et al., 2008; Tateya et al., 2011). Surprisingly, in *Hey1–Hey2* double mutants (DKO), HC density as well as the total length of the sensory epithelium (52.8 ± 2.7 HCs/100 μm, 6083.8 ± 117.6 μm, $n = 6$) was not significantly different from $Hey2^{-/+}$ (48.6 ± 1.2 HCs/ 100 μm, 6206.8 ± 89.8 μm, $n = 5$) and $Hey2^{-/-}$ (51.8 ± 0.6 HCs/100 μm, 5863.7 ± 178.2 μm, $n = 5$) littermate controls, indicating that HCs were not overproduced in the absence of *Hey1* and *Hey2*. However, the precise cellular pattern of one row of IHCs and three rows of OHCs seen throughout the length of the auditory sensory epithelium in *Hey2* single mutant (control) neonatal pups (Fig. 3*A*,*C*,*E*) was significantly altered in *Hey1–Hey2* double mutant (DKO) pups (Fig. 3*B*,*D*,*F*). In the absence of *Hey1* and *Hey2*, ectopic IHCs were most frequently observed in the cochlear mid-turn (Fig. 3*D*,*I*), whereas ectopic OHCs were most frequently observed in the cochlear base (Fig. 3*F*,*J*). Strikingly, in DKO cochleae, OHCs were frequently missing in the cochlear apex, and the sensory epithelium consisted only of two rows of OHCs instead of three (Fig. 3*B*,*K*). Moreover, mispatterned HCs in DKO cochleae frequently had deformed and misoriented stereocilia (Fig. 3*B*,*D*,*F*). In mice, patterning of auditory HCs as well as stereocilia orientation is not fully completed at birth, and both processes undergo refinement in the first postnatal days (Anniko, 1983). To determine whether the defects persist until later stages of postnatal refinement, we analyzed the HC patterning and stereocilia orientation in postnatal day 4 (P4) $Hey2^{-/-}$ (Fig. 3*G*) and DKO cochlear tissue (Fig. 3*H*). Our examination revealed that both HC patterning defects and stereocilia defects were largely retained as exemplified by the OHC patterning defects and stereocilia defect seen in the cochlear apex of DKO pups at P4 (Fig. 3*H*). Next, we examined the SC phenotype in the *Hey1–Hey2* double mutant cochlea (DKO). SOX2 immunostaining was used to mark all subtypes of SCs. Moreover, SC subtypes were further subdivided into inner SCs (inner border cells, inner phalangeal cells) and outer SCs (pillar cells and Deiters cells) based on nuclear morphology and relative position within the sensory epithelium. Outer SC (oSC) density and total length of the sensory epithelium were not significantly changed in neonatal DKO pups (68.4 ± 1.2 oSCs/100 μm, 5945 ± 298 μm, $n = 3$) compared with $Hey2^{-/+}$ (65.7 ± 1.0 oSCs/100 μm, 6170.58 ± 212 μm, $n = 3$) and $Hey2^{-/-}$ (70.9 ± 2.1 oSCs/100 μm, 5706.5 ± 145.5 μm, $n = 3$) littermate controls, suggesting that the oSCs were not overproduced in the absence of *Hey1* and *Hey2*. However, oSCs were similar to OHCs unevenly distributed along the *Hey1–Hey2* double mutant cochlear duct, with ectopic oSCs in the cochlear base (Fig. 3*O*,*O*′,*U*, white and yellow arrows) and missing oSCs in the cochlear apex (Fig. 3*M*,*M*′,*Q*, white and yellow dashed line). Moreover, the occasional ectopic IHCs were accompanied by ectopic inner SCs (iSCs) as exemplified by supernumerary SOX2-positive iSCs in the cochlear mid-turn (Fig. 3*S*, white and yellow asterisk). In addition, we found evidence for late SC-to-HC conversion in DKO cochlea. In the late embryonic and early postnatal cochlea, *Hey1* and *Hey2* are coexpressed in outer pillar cells, suggesting a role in the differentiation or maintenance of outer pillar cells. To analyze the outer pillar cell phenotype, p75 immunostaining was used. In the early postnatal cochlea, the low-affinity neurotrophin receptor p75 (NGFR) is highly expressed on the apical surface of both outer and inner pillar cells (von Bartheld et al., 1991). Similar to $Hey2^{-/+}$ control tissue, in DKO cochlear surface preparations, p75-positive cells were clearly present in the in the pillar cell region, suggesting that pillar cells differentiate normally in the absence of *Hey1* and *Hey2* (Fig. 3*V*–*W*″). However, we observed occasional gaps in the p75-positive stripe, in which p75-positive pillar cells were replaced by HCs, suggesting a late pillar cell-to-HC fate switch in the absence of *Hey1* and *Hey2* (Fig. 3*W*,*W*″, white asterisk). In summary, our analysis revealed that, in the absence of *Hey1* and *Hey2*, the normal cellular pattern of the auditory sensory epithelium is severely disrupted. Some of the patterning defects, such as the frequently observed ectopic IHCs, are shared with *Hes* single and *Hes/Hey* compound mutants and are likely due to defects in partition of the prosensory cell pool into HCs and SCs. However, the uneven distribution of OHCs and oSCs along the cochlear duct and the ectopic HCs within the pillar cell region is unique to *Hey1–Hey2* double mutants and is consistent with a function of *Hey1* and *Hey2* in prosensory cell maintenance and a later function in maintaining a pillar cell fate.

Loss of *Hey1* and *Hey2* does not alter the timing or pattern of prosensory cell cycle withdrawal

In the developing brain, HES and HEY transcription factors are critical for maintaining proliferation of neural stem cells and

progenitor cells, and loss of *Hes/Hey* function results in premature cell cycle withdrawal (Sakamoto et al., 2003; Kageyama et al., 2008; Fujitani et al., 2010). Similarly, in the developing cochlea, loss of *Hes1*, which is expressed at low levels at the prosensory stage, results in premature cell cycle withdrawal (Murata et al., 2009). In the murine cochlea, cell cycle exit of prosensory cells occurs within a 48 h time window and follows an apical-to-basal gradient, with apical progenitors exiting first ~E12.5 and very basal progenitors exiting as late as E14.5 (Ruben, 1967; Lee et al., 2006). To determine whether loss of *Hey1* and *Hey2* might have caused premature cell cycle withdrawal, the rate of prosensory cell proliferation was analyzed at E13.5, the peak of cell cycle withdrawal in *Hey2*$^{-/-}$ (control) and *Hey1*$^{\Delta/\Delta}$ *Hey2*$^{-/-}$ (DKO) mice. Timed-mated dams received a single injection of the thymidine analog EdU at E13.5 and EdU incorporation was analyzed 2 days later in cochlear sections using a chemical assay (Salic and Mitchison, 2008). SOX2 immunostaining was used to mark prosensory cells and their progenies (HC and SC precursors). We reasoned that, if HEY1 and HEY2 positively regulate prosensory cell proliferation, loss of *Hey1* and *Hey2* should reduce the number of cycling SOX2-positive HC and SC precursors. However, an EdU pulse at E13.5 revealed no difference in overall proliferation, timing, or pattern of cell cycle withdrawal. SOX2-positive prosensory cells in control (Fig. 4*A*,*C*,*E*) and DKO (Fig. 4*B*,*D*,*F*) animals incorporated EdU at similar rates, showing a similar pattern of apical-to-basal cell cycle withdrawal with very low EdU incorporation in the cochlear apex and highest incorporation in the cochlear base (Fig. 4*G*). Next, we examined whether the onset of cell cycle withdrawal of HC and SC precursors occurs prematurely in the absence of *Hey1* and *Hey2*. Timed-mated dams received a single injection of EdU at E12.5, and EdU incorporation in IHCs and OHCs and oSCs was analyzed in cochlear surface preparations of DKO animals and *Hey2*$^{-/+}$ and *Hey1*$^{\Delta/\Delta}$ *Hey2*$^{-/+}$ control littermates, Stage E18.5. In littermate controls, EdU incorporation in MYO6-positive IHCs and OHCs declined from base to apex with the lowest frequency of EdU incorporation in the most apical region (Fig. 4*H–L*,*R*,*S*, light and dark gray bar). Similarly, in the absence of *Hey1* and *Hey2*, EdU incorporation in IHCs and OHCs gradually declined from base to apex, with the lowest frequency of EdU incorporation in the

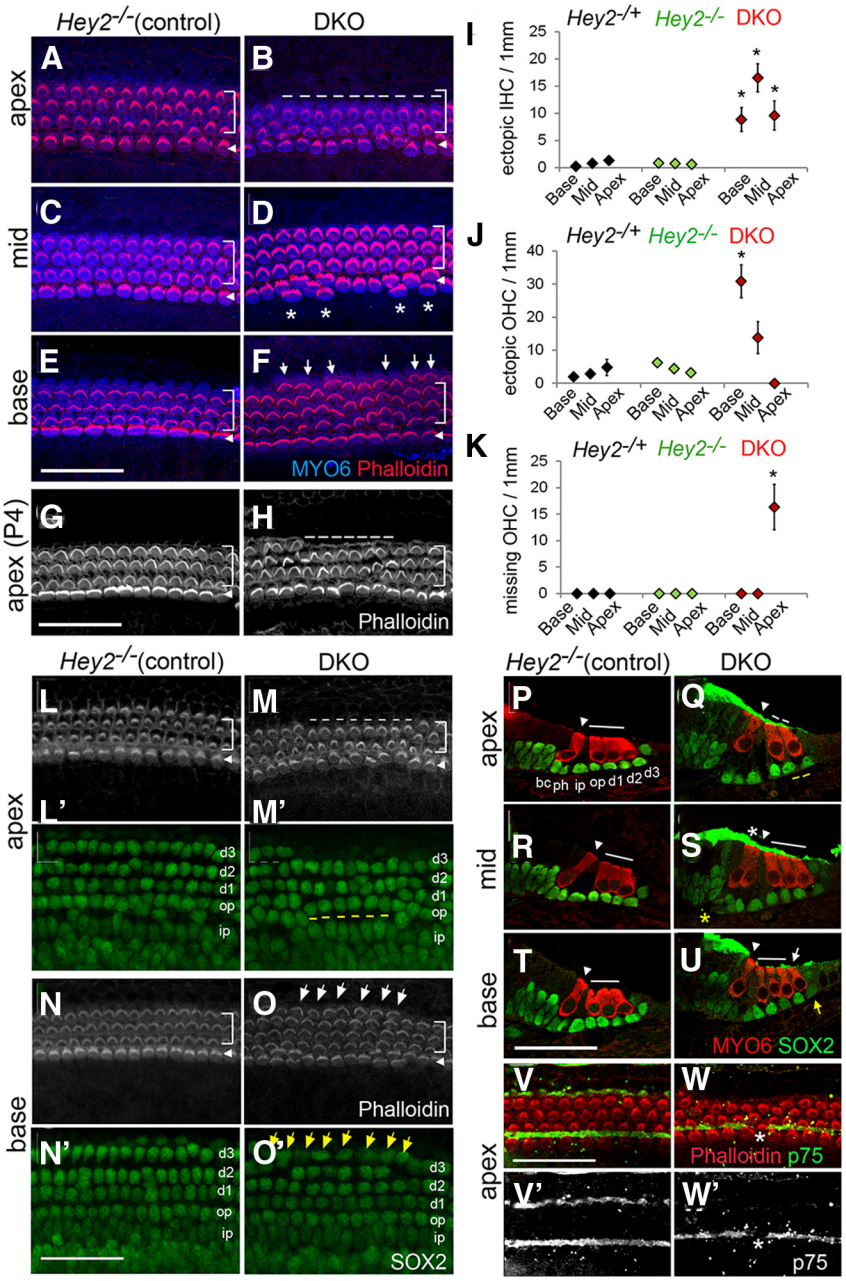

Figure 3. Loss of *Hey1* and *Hey2* results in complex HC and SC patterning defects. ***A–W′***, HC and SC phenotype was analyzed in the early postnatal control (*Hey2*$^{-/-}$ and *Hey2*$^{-/+}$) and DKO cochlea in whole mounts (surface preparations) and sections. HCs were labeled using MYO6 and phalloidin staining, and SCs were labeled using SOX2 staining. HC and SC subtypes were identified by morphology and their relative location within the sensory epithelium. Arrowheads indicate IHC domain; brackets indicate OHC domain. Dashed line indicates missing OHCs (white) and missing oSCs (yellow). Asterisks label ectopic IHCs (white) and ectopic iSCs (yellow). Arrows indicate ectopic OHCs (white) and ectopic oSCs (yellow). bc, inner border cell; ph, inner phalangeal cell; ip, inner pillar cell; op, outer pillar cell; d1–3, Deiters cells. ***A–F***, HC phenotype in *Hey2*$^{-/-}$ (***A***, ***C***, ***E***) and DKO (***B***, ***D***, ***F***) cochlear surface preparations, Stage P0. Shown are high-power confocal images of MYO6 and phalloidin-positive HC layer at apical (***A***, ***B***), mid (***C***, ***D***), and basal (***E***, ***F***) positions. Scale bar, 50 μm. ***G***, ***H***, HC phenotype in *Hey2*$^{-/-}$ (***G***) and DKO (***H***) cochlear surface preparations, Stage P4. Shown are high-power confocal images of the HC layer (phalloidin, white) at an apical location. Scale bar, 50 μm. ***I–K***, Quantification of ectopic IHCs (***I***), ectopic OHCs (***J***), and missing OHCs (***K***) in DKO (red diamonds) and *Hey2*$^{-/-}$ (green diamonds) and *Hey2*$^{-/+}$ (black diamonds) cochlea Stage P0 –P2. Cochlear surface preparations were divided into three segments (base, mid, apex), and ectopic and missing HCs were counted for each segment per 1 mm. Data are mean ± SEM ($n = 7$, three independent experiments). *$p \leq 0.05$. ***L–O′***, HC and SC phenotype in DKO (***M***, ***M′***, ***O***, ***O′***) and *Hey2*$^{-/-}$ (control) (***L***, ***L′***, ***N***, ***N′***) cochlear surface preparations, Stage P0. Shown are confocal images of the HC layer (phalloidin, white) and corresponding (′) SC layer (SOX2, green) of apical (***L–M′***) and basal segments (***N–O′***). Scale bar, 50 μm. ***P–U***, SC phenotype in *Hey2*$^{-/-}$ (control) (***P***, ***R***, ***T***) and DKO (***Q***, ***S***, ***U***) cochlear sections, Stage P0. Shown are confocal images of apical (***P***,***Q***), mid (***R***, ***S***), and basal (***T***, ***U***) cochlear sections immunostained with HC marker MYO6 (red) and SC marker SOX2 (green). Scale bar, 50 μm. ***V–W′***, Pillar cell phenotype in *Hey2*$^{-/+}$ (control) (***V***, ***V′***) and DKO (***W***, ***W′***) cochlear surface preparations, Stage P0. Shown are merged and single (′) channel confocal images of HC layer in cochlear apex. Phalloidin-positive HC bundles are shown in red, and p75 (NGFR)-positive pillar cell heads are shown in green and white (′). The pillar cell-specific p75 staining is disrupted by an ectopic HC (white asterisk) in the DKO cochlea (***W***, ***W′***). Scale bar, 50 μm.

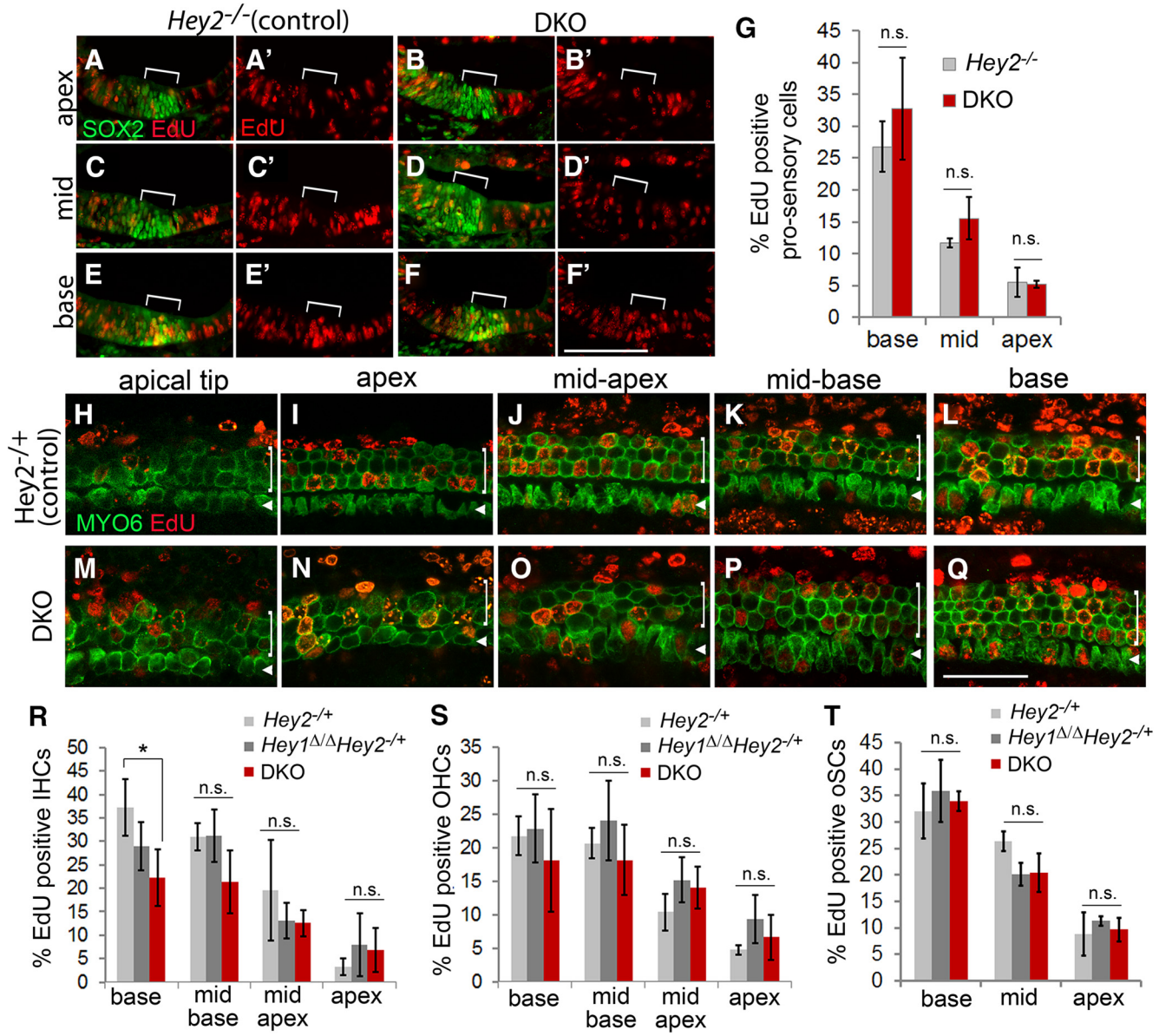

Figure 4. Loss of *Hey1* and *Hey2* does not alter prosensory cell proliferation. ***A–G***, Loss of *Hey1* and *Hey2* does not alter the apical-to-basal gradient of prosensory cell cycle exit. Incorporation of EdU in prosensory cells was analyzed in $Hey2^{-/-}$ (control) (***A***, ***C***, ***E***) and $Hey1^{\Delta/\Delta}$ $Hey2^{-/-}$ (DKO) (***B***, ***D***, ***F***) animals. EdU was injected at E13.5, and E15.5 cochlear sections were immunostained for prosensory marker SOX2 (green) and processed for EdU labeling (red). Shown are both merged and EdU only images (′) for the cochlear apex (***A–B′***), mid (***C–D′***), and base (***E–F′***). White bracket indicates prosensory domain. Scale bar, 100 μm. ***G***, Quantification of EdU incorporation in $Hey2^{-/-}$ control (gray bar) and DKO (red bar) prosensory cells in the cochlear apex, mid, and base. Data are mean ± SEM ($n = 3$, two independent experiments). n.s., Not significant. ***H–T***, Loss of *Hey1* and *Hey2* does not alter proliferation rate of HC and SC precursors. EdU was injected at E12.5 and EdU incorporation in HCs and SCs was analyzed at E18.5 in control ($Hey2^{-/+}$ and $Hey1^{\Delta/\Delta}$ $Hey2^{-/+}$) and DKO cochlear surface preparations. ***H–Q***, EdU incorporation in E18.5 $Hey2^{-/+}$ (control) (***H–L***) and DKO (***M–Q***) cochlear sensory epithelium. Shown are high-power confocal images of MYO6-positive HC layer (green) at the cochlear apical tip (***H***, ***M***), apex (***I***, ***N***), mid-apex (***J***, ***O***), mid-base (***K***, ***P***), and base (***L***, ***Q***). Arrows point to IHCs; brackets indicate OHCs. EdU-positive nuclei are shown in red. Scale bar, 50 μm. ***R–T***, Quantification of EdU incorporation in IHCs (***R***), OHCs (***S***), and oSCs (***T***) in E18.5 $Hey2^{-/+}$ (light gray), $Hey1^{\Delta/\Delta}$ $Hey2^{-/+}$ (dark gray), and DKO (red). Data are mean ± SEM ($n = 3$, two independent experiments). *$p \leq 0.05$. n.s., Not significant.

most apical region (Fig. 4*M–Q*, *R*, *S*, red bar). Moreover, loss of *Hey1* and *Hey2* did not alter the spatiotemporal pattern of cell cycle withdrawal of SC precursors, as evidenced by the similar rate of EdU incorporation in control (Fig. 4*T*, light and dark gray bar) and DKO oSC precursors (Fig. 4*T*, red bar). The only differences we noted were that *Hey1–Hey2* double mutant IHCs located in the cochlear base incorporated EdU at lower levels than $Hey2^{-/+}$ control (Fig. 4*R*, red and light gray bar). The lower than normal EdU incorporation of basally located IHCs is likely to be a secondary effect due to forced cell cycle exit caused by their premature differentiation. In summary, our EdU incorporation data suggest that loss of *Hey1* and *Hey2* does not significantly alter the overall proliferation rate of prosensory cells or the apical-to-basal pattern of prosensory cell cycle withdrawal.

Hedgehog signaling maintains *Hey1* and *Hey2* expression in prosensory cells

Hey1 and *Hey2* are well-characterized transcriptional targets of Notch signaling. However, the regulation of *Hey1* and *Hey2* expression in cochlear prosensory cells appears to be more complex, as loss of canonical Notch signaling has no effect on *Hey1* and *Hey2* mRNA expression in prosensory cells (Basch et al., 2011). A candidate pathway for positively regulating *Hey1* and *Hey2* transcription at the prosensory stage is Hedgehog signaling.

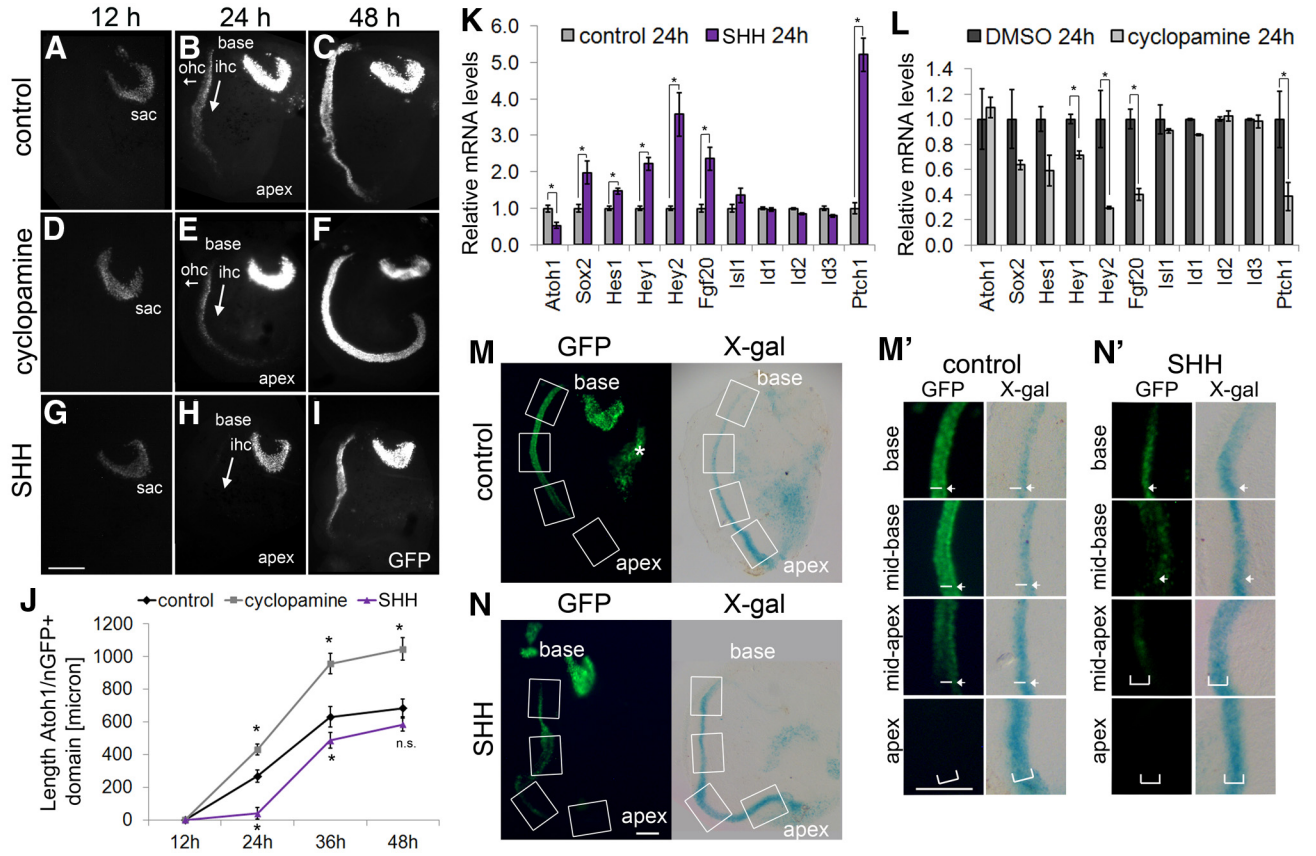

Figure 5. Hedgehog signaling positively regulates *Hey1* and *Hey2* expression in prosensory cells. ***A–I***, Effects of Hedgehog signaling on HC differentiation. HC-specific *Atoh1/nGFP* reporter expression was monitored in control (***A–C***), cyclopamine (***D–F***), and SHH-treated (***G–I***) E13.0 cochlear explants over a 48 h time period. Shown are low-power fluorescent images *of Atoh1/nGFP* (GFP, white) at 12 h (***A***, ***D***, ***G***), 24 h (***B***, ***E***, ***H***), and 48 h (***C***, ***F***, ***I***). Long arrows indicate basal-to-apical gradient; short arrows indicate medial-to-lateral gradient. Locations of IHCs (ihc) and OHCs (ohc) and basal (base) to apical (apex) extent of cochlear duct are indicated. *Atoh1/nGFP* is also expressed in the vestibular sacculus (sac). Scale bar, 200 μm. ***J***, Quantification of basal-to-apical extent of *Atoh1/nGFP* expression in control (black diamond), cyclopamine (gray square), and SHH (purple triangle) treated cochlea explants after 12, 24, 36, and 48 h. Data mean ± SEM ($n = 5$). *$p \leq 0.05$. n.s., Not significant. ***K***, ***L***, Hedgehog signaling positively regulates *Hey1* and *Hey2* mRNA expression. ***K***, qPCR-based analysis of cochlear epithelial-specific gene expression in SHH-treated (Shh, purple bar) and untreated (control, gray bar) E13.5 cochlear explants after 24 h *in vitro*. Data are mean ± SEM ($n = 4$, two independent experiments). *$p \leq 0.05$. ***L***, qPCR based analysis of cochlear epithelial-specific gene expression in cyclopamine (light gray bar) and vehicle control (DMSO, dark gray bar) treated E13.5 cochlear explants after 24 h *in vitro*. Data are mean ± SEM ($n = 4$, two independent experiments). *$p \leq 0.05$. ***M–N'***, Hedgehog signaling maintains *Hey2* expression in prosensory cells. E13.5 *Atoh1/nGFP* transgenic *Hey2*$^{LacZ/+}$ cochlear explants were cultured with (***N***, ***N'***) and without SHH (***M***, ***M'***) for 48 h. Shown are low-power (***M***, ***N***) and high-power (***M'***, ***N'***) images of HC-specific *Atoh1/nGFP* reporter expression (GFP, green) and *Hey2-LacZ* reporter expression (X-gal, blue). ***M***, ***N***, White boxes represent the fields along the cochlear duct shown in ***M'*** and ***N'***. ***M***, *Auto-fluorescent debris. ***M'***, ***N'***, White brackets indicate undifferentiated prosensory domain; white arrowheads indicate location of GFP-positive IHCs; white bars indicate location of GFP-positive OHCs. Scale bar, 100 μm.

Overactivation of Hedgehog signaling in the undifferentiated cochlea blocks *Atoh1* induction and subsequently HC generation (Driver et al., 2008; Tateya et al., 2013), and there is mounting evidence that a Hedgehog activity gradient controls the gradient of HC differentiation. Hedgehog signaling in prosensory cells is thought to be activated by the Hedgehog ligand SHH, which is transiently produced by spiral ganglion neurons during cochlear outgrowth (Driver et al., 2008; Liu et al., 2010). Spiral ganglion-specific deletions of *Shh* result in severe to moderate cochlear outgrowth defects dependent on the time of ablation. In addition, deletion of *Shh* causes prosensory cells to drop out of the cell cycle prematurely and to differentiate precociously (Bok et al., 2013). Similarly, the cochlear-specific genetic ablation of *Smoothened* (*Smo*), which encodes for a critical transduction component of Hedgehog signaling, results in precocious HC differentiation (Tateya et al., 2013). To examine the relationship among Hedgehog signaling, *Hey1* and *Hey2* expression, and HC differentiation, we made use of a well-characterized cochlear explant system in which the developing cochlear epithelial duct and its innervating spiral ganglion are cultured in the presence of mesenchyme (Montcouquiol and Kelley, 2003; Doetzlhofer et al., 2009). Native nuclear GFP expression of the HC-specific *Atoh1/nGFP* line was used to monitor the dynamics of HC differentiation. Exogenous SHH protein was added to stimulate Hedgehog signaling, and cyclopamine, a steroidal alkaloid known to inhibit SMO (Chen et al., 2002a), was added to inhibit Hedgehog signaling in Stage E13.5 cochlear tissue. At plating and after 12 h *in vitro*, untreated (control), SHH-treated, and cyclopamine-treated explants showed no HC-specific *Atoh1/nGFP* expression in the developing cochlear duct (Fig. 5*A*,*D*,*G*). However, after 24 h *in vitro*, striking differences in the basal-to-apical progression of HC differentiation emerged. In cochlear explants treated with cyclopamine, IHC differentiation was further progressed (Fig. 5*E*,*J*) than in untreated control cultures (Fig. 5*B*,*J*). However, cochlear explants treated with exogenous SHH contained no or only few GFP-positive HCs after 24 h *in vitro* (Fig. 5*H*,*J*). After 48 h *in vitro*, differences in the extent of OHC differentiation (medial-to-lateral gradient) emerged, with OHC differentiation being more advanced in cyclopamine-treated explants (Fig. 5*C*,*F*) and less advanced in SHH-treated

explants compared with control explants (Fig. 5*C*,*I*). To determine whether *Hey1* and *Hey2* might be targets of Hedgehog signaling, we analyzed the transcript levels of *Hey1* and *Hey2* in enzyme-purified cochlear epithelia after 24 h of SHH-mediated Hedgehog overactivation (Fig. 5*K*) or cyclopamine-mediated Hedgehog inhibition (Fig. 5*L*). To confirm successful overactivation or inhibition of Hedgehog signaling in our experimental paradigm, we included in our qPCR analysis the known Hedgehog target gene *Ptch1* (*Patched homolog 1*) (Vokes et al., 2007). Our qPCR-based analysis revealed that *Hey1*, *Hey2*, and *Ptch1* transcripts were significantly increased in SHH-treated cochlear explants compared with untreated control explants (Fig. 5*K*). Conversely, in the presence of Hedgehog inhibitor cyclopamine, *Hey1, Hey2*, and *Ptch1* transcripts were significantly reduced compared with explants treated with vehicle control DMSO (Fig. 5*L*). The modest but significant reduction of *Hey1* transcript levels in cyclopamine-treated cultures suggests that Hedgehog-independent mechanisms might compensate for the loss of Hedgehog signaling. A significant increase in transcript levels in response to Hedgehog overactivation was also observed for *Sox2*, *Hes1*, and *Fibroblast growth factor 20* (*Fgf20*) (Fig. 5*K*). Similar to *Hey1* and *Hey2, Hes1*, *Sox2*, and *Fgf20* are positively regulated by Notch signaling in the developing cochlea (Takebayashi et al., 2007; Dabdoub et al., 2008; Hayashi et al., 2008a, b), suggesting cooperation between Notch and Hedgehog signaling in maintaining their expression in prosensory cells. We also examined the mRNA expression of the prosensory-specific HLH factors and known ATOH1 antagonists ID1, ID2, ID3, and the prosensory-specific transcription factor ISLET1 (ISL1) (Radde-Gallwitz et al., 2004; Jones et al., 2006). However, none of these transcripts showed Hedgehog dependency, suggesting that modulation of Hedgehog signaling does not globally alter progenitor-specific gene expression (Fig. 5*K*,*L*). To determine how Hedgehog signaling affects the spatial and temporal pattern of *Hey2* expression, we made use of our *Hey2* knock-out line, in which exon 2 of the *Hey2* gene was knocked out by *LacZ* insertion ($Hey2^{LacZ/+}$) (Gessler et al., 2002). E13.0 *Atoh1n/GFP* transgenic $Hey2^{LacZ/+}$ cochlear explants were cultured in the absence (control) or presence of exogenous SHH, and after 48 h *in vitro Hey2-LacZ* expression was analyzed using the X-gal assay (Fig. 5*M–N′*; X-gal). GFP expression marked HCs and IHCs (arrowhead) and OHCs (bar) were identified by their location within the sensory epithelium (Fig. 5*M–N′*; GFP). In control cochlear explants, broad *Hey2* expression in the undifferentiated cochlear apex narrowed to the OHC domain in the cochlear mid-apex. In the differentiated cochlear mid-base and base, which already contained both IHCs and OHCs, *Hey2* expression was limited to the pillar cell region (Fig. 5*M*,*M′*). However, in cochlear explants treated with exogenous SHH, *Hey2* remained broadly expressed throughout most of the length of the cochlear duct, strongly correlating with the delayed upregulation of GFP in HCs (Fig. 5*N*,*N′*). Together, our analysis identifies *Hey1* and *Hey2* as targets and likely effector genes of Hedgehog signaling in the developing cochlea.

FGF signaling functions downstream of Hedgehog signaling in *Hey1* and *Hey2* regulation

A recent study has shown that forced expression of a constitutively active *Smo allele* (*Smo-M2)* results in prolonged *Fgf20* expression in prosensory cells and a permanent block of OHC differentiation. Interestingly, interference with FGF20-activated FGFR signaling reverses the inhibitory effect of Hedgehog overactivation in E17.5 *R26-Smo-M2* transgenic cochlear explants and restores OHC differentiation (Tateya et al., 2013). We recently have shown that FGFR signaling cooperates with Notch signaling in the transcriptional regulation of *Hey2* in pillar cells (Doetzlhofer et al., 2009), and an intriguing scenario is that FGFR signaling might similarly contribute to the regulation of *Hey2* in prosensory cells. To determine whether FGFR signaling is required for the positive effects of Hedgehog signaling on *Hey1* and *Hey2* expression, E13.5 *Atoh1/nGFP* transgenic cochlear explants were cultured in the presence of FGFR kinase inhibitor SU5402 (Mohammadi et al., 1997), SHH and SU5402, SHH and DMSO, or DMSO for 24 h (Fig. 6*A–H*). Compared with vehicle control (DMSO), treatment with SU5402 did not alter the onset of HC differentiation (Fig. 6*A*,*B*), nor did it alter the rate at which HC differentiation progressed toward the cochlear apex (Fig. 6*E*,*F*,*I*). However, cotreatment with SHH and FGFR inhibitor (SHH + SU5402) reversed the inhibitory effect of Hedgehog overactivation (SHH + DMSO) on HC differentiation (Fig. 6*G–I*). Next, we analyzed whether FGF signaling is required for Hedgehog mediated activation of *Hey2* and or *Hey1* transcription. Treatment of E13.5 cochlear explants with exogenous SHH (SHH + DMSO) increased *Fgf20*, *Hey2*, and *Ptch1* expression by >3-fold and increased *Hey1* expression ~2-fold (Fig. 6*J*). However, cotreatment of cochlear explant cultures with SHH and FGFR inhibitor (SHH + SU5402) largely reversed the positive effects of SHH treatment (SHH + DMSO) on *Hey2* and *Hey1* expression (Fig. 6*J*). Importantly, cotreatment with SHH and SU5402 did not alter expression of the known Hedgehog target gene *Ptch1,* nor did it alter the expression of *Fgf20*, suggesting that cotreatment with SU5402 did not globally attenuate SHH-activated Hedgehog signaling. However, in contrast to cyclopamine treatment, which reduced both *Hey1* and *Hey2* levels (Fig. 5*L*), SU5402 treatment did not reduce *Hey1* and *Hey2* mRNA levels compared with the control (Fig. 6*J*). This could be in part due to incomplete inhibition of FGFR signaling in our experimental paradigm. Alternatively, FGFR-independent Hedgehog signaling mechanisms might maintain *Hey1* and *Hey2* expression in the absence of FGFR signaling. Further studies are needed to determine the mechanisms of FGFR-dependent regulation of *Hey1* and *Hey2* expression in prosensory cells.

Discussion

Hey1 and *Hey2* prevent premature *Atoh1* upregulation in prosensory cells

Hey1 and *Hey2*, which are highly expressed in prosensory cells in the developing cochlea, have been recently implicated in prosensory cell specification downstream of Notch signaling (Hayashi et al., 2008b). However, our loss-of-function data suggest that *Hey1* and *Hey2* are dispensable for prosensory domain specification but are critical components of the molecular machinery that negatively controls the graded upregulation of *Atoh1* in HC precursors during cochlear differentiation. In the murine cochlea, *Atoh1* promoter activity can be detected in prosensory cells as early as E12.5 (Woods et al., 2004; Yang et al., 2010; Driver et al., 2013). In contrast, *Atoh1* transcript and protein levels are exceedingly low at this stage. Starting at E13.5–E14.0, *Atoh1* transcript and protein are rapidly induced in nascent HCs following the basal-to-apical and medial-to-lateral gradient of HC differentiation, suggesting the existence of a regulatory mechanism that negatively regulates the graded upregulation of *Atoh1* in HC precursors (Chen et al., 2002b; Groves et al., 2013). Our expression and functional data identify *Hey1* and *Hey2* as an integral part of this negative regulatory mechanism. We show that *Hey1* and *Hey2* downregulation occurs independently of *Atoh1*, indicating

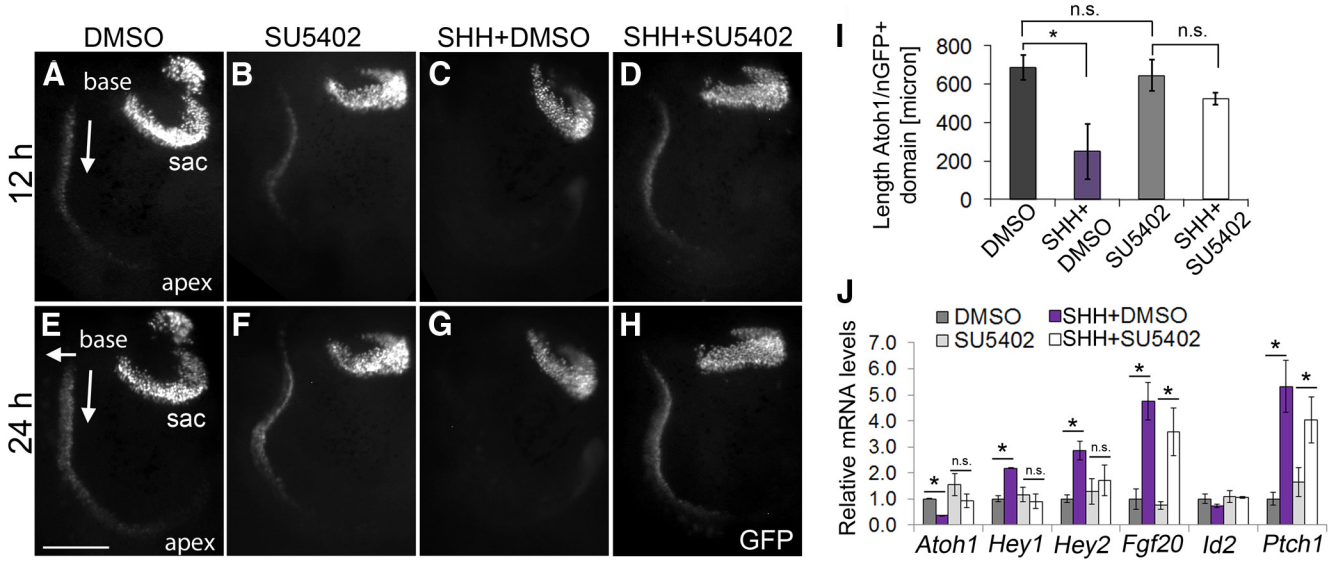

Figure 6. FGFR signaling acts downstream of Hedgehog signaling in *Hey1* and *Hey2* regulation. ***A–I***, FGFR signaling is required for SHH-mediated inhibition of HC differentiation. HC-specific *Atoh1/nGFP* reporter expression was monitored in E13.5 cochlear explants treated with vehicle control DMSO (***A***, ***E***), FGFR inhibitor SU5402 (***B***, ***F***), SHH + DMSO (***C***, ***G***), and SU5402 + SHH (***D***, ***H***) over a 24 h time period. Shown are low-power fluorescent images of HC-specific *Atoh1/nGFP* expression (GFP, white) at 12 h (***A–D***) and 24 h (***E–H***). Long arrow indicates basal-to-apical gradient; short arrow indicates medial-to-lateral gradient. ***I***, Quantification of basal-to-apical extent of HC-specific *Atoh1/nGFP* expression in DMSO (dark gray bar), SHH + DMSO (purple bar), SU5402 (light gray bar), and SHH + SU5402 (white bar). Error bars indicate mean ± SEM ($n = 5$). *$p \leq 0.05$. n.s., Not significant. ***J***, FGFR signaling is required for SHH-mediated activation of *Hey1* and *Hey2* transcription. Shown is qPCR-based analysis of *Hey1* and *Hey2* mRNA levels in control (dark gray bar), SHH (purple bar), SU5402 (light gray bar), and SHH + SU5402 (white bar) treated cochlear epithelia after a 24 h treatment. Scale bar, 200 μm. Errors bars indicate mean ± SEM ($n = 3$, two independent experiments). *$p \leq 0.05$. n.s., Not significant.

that *Hey1* and *Hey2* act upstream of *Atoh1* in the differentiating cochlea. Moreover, we demonstrate that genetic inactivation of *Hey1* and *Hey2* results in premature upregulation of *Atoh1* transcript and *Atoh1* enhancer activity (*Atoh1/nGFP*) in IHC and OHC precursors. Similarly, a decrease in expression of *Hey1* and *Hey2* due to loss of Hedgehog signaling results in premature induction of HC-specific *Atoh1/nGFP* in the developing cochlea, whereas an increase in *Hey1* and *Hey2* expression due to overactivation of Hedgehog signaling delays HC-specific *Atoh1/nGFP* expression. How do HEY1 and HEY2 inhibit *Atoh1* expression? It is likely that HEY1 and HEY2 interfere with the ability of ATOH1 to auto-regulate its own expression. Such interference could take place on a posttranscriptional level, as both HEY1 and HEY2 are known to bind to ATOH1, which would render ATOH1 inactive (Sakamoto et al., 2003; Doetzlhofer et al., 2009). In addition, HEY1 and HEY2 could bind to *Hey* binding motifs present in the *Atoh1* auto-regulatory enhancer element and interfere with *Atoh1* auto-regulation on a transcriptional level (Helms et al., 2000; Lumpkin et al., 2003; Heisig et al., 2012).

Hey1 and *Hey2* are not required for maintaining prosensory cell proliferation

In the developing brain, HES/HEY factors are critical for maintaining neuronal stem cells/progenitor cells in the cell cycle, and their continued expression prevents premature differentiation (Sakamoto et al., 2003; Kageyama et al., 2008). In contrast to the dual role of HEY1 and HEY2 in neuronal stem cell/progenitor cell proliferation and differentiation, our data indicate that loss of *Hey1* and *Hey2* only affects HC and SC differentiation without altering prosensory cell proliferation or cell cycle withdrawal. Interestingly, a recent study by Murata et al. (2009) revealed that HES1, which is broadly expressed in the developing cochlear epithelium before differentiation, is critical for prosensory cell proliferation, and loss of *Hes1* results in premature cell cycle withdrawal of prosensory cells. However, loss of *Hes1* does not alter the timing of HC and SC differentiation. These findings suggest that, in the developing cochlea, HES1 and HEY1/HEY2 play divergent roles at the prosensory stage, with HES1 being required for keeping prosensory cells proliferating and HEY1/HEY2 being required for maintaining prosensory cells undifferentiated.

Hey1 and *Hey2* are required for proper HC and SC patterning

Previous work demonstrated that HEY1 and HEY2, in cooperation with HES1 and HES5, play a central role in patterning the auditory sensory epithelium (Li et al., 2008; Tateya et al., 2011). In contrast to *Hes*/*Hey* compound mutants in which both HC density and the total number of HCs are significantly increased (Li et al., 2008; Tateya et al., 2011), the total number of HCs is not significantly changed in the absence of *Hey1* and *Hey2*. Instead, OHCs and oSCs are unevenly distributed along the cochlear duct, with ectopic OHCs and ectopic oSCs being frequently found in the cochlear base and OHCs and oSCs are frequently missing in the cochlear apex. Our analysis did not reveal any defects in the prosensory cell formation, proliferation, or cell cycle withdrawal in the absence of *Hey1* and *Hey2*, suggesting that this unique defect in OHC patterning arises during cellular differentiation. In parallel with cellular differentiation, the auditory sensory epithelium undergoes dramatic changes in both width and length. Convergence and extension (CE) under the control of the planar cell polarity (PCP) pathway (Wang et al., 2005) is thought to drive these changes. In CE/PCP mutants, HCs are misaligned, HC bundles are misoriented, and the number of OHC rows varies (Etheridge et al., 2008; Narimatsu et al., 2009). It is plausible that the premature onset of HC differentiation in the absence of *Hey1* and *Hey2* alters or disrupts CE of the auditory sensory epithelium. Alternatively, loss of *Hey1* and *Hey2* might deregulate genes directly or indirectly involved in CE/PCP. Future studies will have to determine whether and how the CE/PCP pathway is affected in *Hey1–Hey2* double-mutant cochlea. The continued expression of *Hey1* and *Hey2* in a subset of SC precursors and, later, in outer

pillar cells suggests that defects in SC maintenance contribute to the complex patterning defect seen in the absence of these factors. Consistent with a role for *Hey1* and *Hey2* in SC maintenance, p75-positive pillar cells are occasionally replaced by ectopic HCs in *Hey1–Hey2* double mutant cochlea, which suggests a late pillar cell-to-HC fate switch.

Hedgehog signaling positively regulates *Hey1* and *Hey2* expression in prosensory cells

Hey1 and *Hey2* are well-characterized Notch target genes and function as Notch effectors in many tissues. Canonical Notch signaling requires the function of transcription factor RBPJ (Kopan and Ilagan, 2009). Similar to *Hes1* and *Hes5*, both *Hey1* and *Hey2* contain multiple RBPJ binding sites (Maier and Gessler, 2000); and, based on *in vitro* γ-secretase inhibition experiments, it has been suggested that prosensory-specific expression of *Hey1* and *Hey2* is under the control of Notch signaling (Hayashi et al., 2008b). However, conditional ablations of *Rbpj* have resulted in either no change (Basch et al., 2011) or only modest reduction in prosensory *Hey1* and *Hey2* expression (Yamamoto et al., 2011), suggesting that, in addition to Notch signaling, Notch-independent mechanisms control *Hey1* and *Hey2* expression in prosensory cells.

Here we identify Hedgehog signaling as a critical positive regulator of *Hey1* and *Hey2* expression in prosensory cells. Furthermore, our regulatory data identify *Hes1* and *Sox2* as novel Hedgehog targets in the developing cochlea and confirm a recent report that has indicated *Fgf20* as a novel Hedgehog target gene (Tateya et al., 2013). Similar to *Hey1* and *Hey2*, *Hes1*, *Fgf20*, and *Sox2* have been shown to be positively regulated by Notch signaling in the developing cochlea (Takebayashi et al., 2007; Dabdoub et al., 2008; Hayashi et al., 2008a, b). Interestingly, in various neuronal stem/progenitor cell populations, a similar Hedgehog-dependent regulation of canonical Notch target genes has been reported (Takanaga et al., 2009; Wall et al., 2009; Wu et al., 2012), suggesting that cooperation between Notch and Hedgehog signaling might constitute a broadly applied mechanism for maintaining neuronal stem cells and progenitor cells.

Previous loss-of-function studies have revealed that FGF20-activated FGFR signaling is required for specification/survival of OHC precursors (Hayashi et al., 2008a; Huh et al., 2012). In addition, a recent study has demonstrated that FGF20-activated FGFR signaling mediates the OHC blocking function of excessive Hedgehog signaling, suggesting that FGF20-activated FGFR signaling, initially required for OHC specification, needs to be turned off or turned down for OHC differentiation to proceed (Tateya et al., 2013). Here we confirm the importance of FGFR activity in mediating the HC-repressive function of Hedgehog signaling and identify *Hey1* and *Hey2* as targets and potential effectors of this FGFR-dependent Hedgehog activity. It should be noted that the difference in phenotypes in Hedgehog pathway mutants, *Fgf20* mutants, and *Hey1–Hey2* double mutants suggests that SHH may be regulating other pathways in the cochlea and that FGF20 is not solely functioning through regulating *Hey1* and *Hey2* (Huh et al., 2012; Bok et al., 2013; Tateya et al., 2013). Interestingly, FGFR inhibition by itself did not alter *Hey1* and *Hey2* expression or HC differentiation, whereas inhibition of Hedgehog signaling resulted in a significant reduction of *Hey1* and *Hey2* expression and a significant acceleration of HC differentiation. How can this be explained? The most likely explanation is that FGFR-independent Hedgehog signaling mechanisms may have compensated for the loss of FGFR signaling. In addition, it is likely that FGFR activity was not completely shut down in our experimental paradigm but was rather reduced, which in case of Hedgehog overactivation reset FGFR signaling back to baseline levels, allowing *Hey1* and *Hey2* downregulation and OHC differentiation to proceed. Additional investigations will be necessary to determine the mechanisms of FGFR-dependent regulation of *Hey1* and *Hey2* in prosensory cells.

References

Anniko M (1983) Postnatal maturation of cochlear sensory hairs in the mouse. Anat Embryol 166:355–368. CrossRef Medline

Basch ML, Ohyama T, Segil N, Groves AK (2011) Canonical Notch signaling is not necessary for prosensory induction in the mouse cochlea: insights from a conditional mutant of RBPjkappa. J Neurosci 31:8046–8058. CrossRef Medline

Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY (1999) Math1: an essential gene for the generation of inner ear hair cells. Science 284:1837–1841. CrossRef Medline

Bok J, Zenczak C, Hwang CH, Wu DK (2013) Auditory ganglion source of Sonic hedgehog regulates timing of cell cycle exit and differentiation of mammalian cochlear hair cells. Proc Natl Acad Sci U S A 110:13869–13874. CrossRef Medline

Cai T, Seymour ML, Zhang H, Pereira FA, Groves AK (2013) Conditional deletion of atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. J Neurosci 33:10110–10122. CrossRef Medline

Chen JK, Taipale J, Cooper MK, Beachy PA (2002a) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev 16:2743–2748. CrossRef Medline

Chen P, Segil N (1999) p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. Development 126:1581–1590. Medline

Chen P, Johnson JE, Zoghbi HY, Segil N (2002b) The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. Development 129:2495–2505. Medline

Dabdoub A, Puligilla C, Jones JM, Fritzsch B, Cheah KS, Pevny LH, Kelley MW (2008) Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. Proc Natl Acad Sci U S A 105:18396–18401. CrossRef Medline

Doetzlhofer A, Basch ML, Ohyama T, Gessler M, Groves AK, Segil N (2009) Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. Dev Cell 16:58–69. CrossRef Medline

Driver EC, Pryor SP, Hill P, Turner J, Rüther U, Biesecker LG, Griffith AJ, Kelley MW (2008) Hedgehog signaling regulates sensory cell formation and auditory function in mice and humans. J Neurosci 28:7350–7358. CrossRef Medline

Driver EC, Sillers L, Coate TM, Rose MF, Kelley MW (2013) The Atoh1-lineage gives rise to hair cells and supporting cells within the mammalian cochlea. Dev Biol 376:86–98. CrossRef Medline

Etheridge SL, Ray S, Li S, Hamblet NS, Lijam N, Tsang M, Greer J, Kardos N, Wang J, Sussman DJ, Chen P, Wynshaw-Boris A (2008) Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. PLoS Genet 4:e1000259. CrossRef Medline

Fischer A, Gessler M (2007) Delta-Notch- and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res 35:4583–4596. CrossRef Medline

Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M (2004) The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev 18:901–911. CrossRef Medline

Fischer A, Klattig J, Kneitz B, Diez H, Maier M, Holtmann B, Englert C, Gessler M (2005) Hey basic helix-loop-helix transcription factors are repressors of GATA4 and GATA6 and restrict expression of the GATA target gene ANF in fetal hearts. Mol Cell Biol 25:8960–8970. CrossRef Medline

Fujitani M, Cancino GI, Dugani CB, Weaver IC, Gauthier-Fisher A, Paquin A, Mak TW, Wojtowicz MJ, Miller FD, Kaplan DR (2010) TAp73 acts via the bHLH Hey2 to promote long-term maintenance of neural precursors. Curr Biol 20:2058–2065. CrossRef Medline

Gessler M, Knobeloch KP, Helisch A, Amann K, Schumacher N, Rohde E, Fischer A, Leimeister C (2002) Mouse gridlock: no aortic coarctation or

deficiency, but fatal cardiac defects in Hey2 $^{-/-}$ mice. Curr Biol 12:1601–1604. CrossRef Medline

Groves AK, Zhang KD, Fekete DM (2013) The genetics of hair cell development and regeneration. Annu Rev Neurosci 36:361–381. CrossRef Medline

Hayashi T, Ray CA, Bermingham-McDonogh O (2008a) Fgf20 is required for sensory epithelial specification in the developing cochlea. J Neurosci 28:5991–5999. CrossRef Medline

Hayashi T, Kokubo H, Hartman BH, Ray CA, Reh TA, Bermingham-McDonogh O (2008b) Hesr1 and Hesr2 may act as early effectors of Notch signaling in the developing cochlea. Dev Biol 316:87–99. CrossRef Medline

Heisig J, Weber D, Englberger E, Winkler A, Kneitz S, Sung WK, Wolf E, Eilers M, Wei CL, Gessler M (2012) Target gene analysis by microarrays and chromatin immunoprecipitation identifies HEY proteins as highly redundant bHLH repressors. PLoS Genet 8:e1002728. CrossRef Medline

Helms AW, Abney AL, Ben-Arie N, Zoghbi HY, Johnson JE (2000) Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. Development 127:1185–1196. Medline

Henrique D, Adam J, Myat A, Chitnis A, Lewis J, Ish-Horowicz D (1995) Expression of a Delta homologue in prospective neurons in the chick. Nature 375:787–790. CrossRef Medline

Huh SH, Jones J, Warchol ME, Ornitz DM (2012) Differentiation of the lateral compartment of the cochlea requires a temporally restricted FGF20 signal. PLoS Biol 10:e1001231. CrossRef Medline

Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R (1994) Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J 13:1799–1805. Medline

Jones JM, Montcouquiol M, Dabdoub A, Woods C, Kelley MW (2006) Inhibitors of differentiation and DNA binding (Ids) regulate Math1 and hair cell formation during the development of the organ of Corti. J Neurosci 26:550–558. CrossRef Medline

Kageyama R, Ohtsuka T, Kobayashi T (2008) Roles of Hes genes in neural development. Dev Growth Differ 50 [Suppl 1]:S97–S103.

Kiernan AE, Pelling AL, Leung KK, Tang AS, Bell DM, Tease C, Lovell-Badge R, Steel KP, Cheah KS (2005) Sox2 is required for sensory organ development in the mammalian inner ear. Nature 434:1031–1035. CrossRef Medline

Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137:216–233. CrossRef Medline

Lee YS, Liu F, Segil N (2006) A morphogenetic wave of p27Kip1 transcription directs cell cycle exit during organ of Corti development. Development 133:2817–2826. CrossRef Medline

Li S, Mark S, Radde-Gallwitz K, Schlisner R, Chin MT, Chen P (2008) Hey2 functions in parallel with Hes1 and Hes5 for mammalian auditory sensory organ development. BMC Dev Biol 8:20. CrossRef Medline

Liu Z, Owen T, Zhang L, Zuo J (2010) Dynamic expression pattern of Sonic hedgehog in developing cochlear spiral ganglion neurons. Dev Dyn 239:1674–1683. CrossRef Medline

Lumpkin EA, Collisson T, Parab P, Omer-Abdalla A, Haeberle H, Chen P, Doetzlhofer A, White P, Groves A, Segil N, Johnson JE (2003) Math1-driven GFP expression in the developing nervous system of transgenic mice. Gene Expr Patterns 3:389–395. CrossRef Medline

Maier MM, Gessler M (2000) Comparative analysis of the human and mouse Hey1 promoter: Hey genes are new Notch target genes. Biochem Biophys Res Commun 275:652–660. CrossRef Medline

Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, Schlessinger J (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276:955–960. CrossRef Medline

Montcouquiol M, Kelley MW (2003) Planar and vertical signals control cellular differentiation and patterning in the mammalian cochlea. J Neurosci 23:9469–9478. Medline

Murata J, Ohtsuka T, Tokunaga A, Nishiike S, Inohara H, Okano H, Kageyama R (2009) Notch-Hes1 pathway contributes to the cochlear prosensory formation potentially through the transcriptional down-regulation of p27Kip1. J Neurosci Res 87:3521–3534. CrossRef Medline

Narimatsu M, Bose R, Pye M, Zhang L, Miller B, Ching P, Sakuma R, Luga V, Roncari L, Attisano L, Wrana JL (2009) Regulation of planar cell polarity by Smurf ubiquitin ligases. Cell 137:295–307. CrossRef Medline

Ohyama T, Groves AK (2004) Generation of Pax2-Cre mice by modification of a Pax2 bacterial artificial chromosome. Genesis 38:195–199. CrossRef Medline

Radde-Gallwitz K, Pan L, Gan L, Lin X, Segil N, Chen P (2004) Expression of Islet1 marks the sensory and neuronal lineages in the mammalian inner ear. J Comp Neurol 477:412–421. CrossRef Medline

Ruben RJ (1967) Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otolaryngol Suppl 220:221–244. Medline

Sakamoto M, Hirata H, Ohtsuka T, Bessho Y, Kageyama R (2003) The basic helix-loop-helix genes Hesr1/Hey1 and Hesr2/Hey2 regulate maintenance of neural precursor cells in the brain. J Biol Chem 278:44808–44815. CrossRef Medline

Salic A, Mitchison TJ (2008) A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc Natl Acad Sci U S A 105:2415–2420. CrossRef Medline

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108. CrossRef Medline

Sher AE (1971) The embryonic and postnatal development of the inner ear of the mouse. Acta Otolaryngol Suppl 285:1–77. Medline

Shroyer NF, Helmrath MA, Wang VY, Antalffy B, Henning SJ, Zoghbi HY (2007) Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. Gastroenterology 132:2478–2488. CrossRef Medline

Takanaga H, Tsuchida-Straeten N, Nishide K, Watanabe A, Aburatani H, Kondo T (2009) Gli2 is a novel regulator of sox2 expression in telencephalic neuroepithelial cells. Stem Cells 27:165–174. CrossRef Medline

Takebayashi S, Yamamoto N, Yabe D, Fukuda H, Kojima K, Ito J, Honjo T (2007) Multiple roles of Notch signaling in cochlear development. Dev Biol 307:165–178. CrossRef Medline

Tateya T, Imayoshi I, Tateya I, Ito J, Kageyama R (2011) Cooperative functions of Hes/Hey genes in auditory hair cell and supporting cell development. Dev Biol 352:329–340. CrossRef Medline

Tateya T, Imayoshi I, Tateya I, Hamaguchi K, Torii H, Ito J, Kageyama R (2013) Hedgehog signaling regulates prosensory cell properties during the basal-to-apical wave of hair cell differentiation in the mammalian cochlea. Development 140:3848–3857. CrossRef Medline

Vokes SA, Ji H, McCuine S, Tenzen T, Giles S, Zhong S, Longabaugh WJ, Davidson EH, Wong WH, McMahon AP (2007) Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. Development 134:1977–1989. CrossRef Medline

von Bartheld CS, Patterson SL, Heuer JG, Wheeler EF, Bothwell M, Rubel EW (1991) Expression of nerve growth factor (NGF) receptors in the developing inner ear of chick and rat. Development 113:455–470. Medline

Wall DS, Mears AJ, McNeill B, Mazerolle C, Thurig S, Wang Y, Kageyama R, Wallace VA (2009) Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity. J Cell Biol 184:101–112. CrossRef Medline

Wang J, Mark S, Zhang X, Qian D, Yoo SJ, Radde-Gallwitz K, Zhang Y, Lin X, Collazo A, Wynshaw-Boris A, Chen P (2005) Regulation of polarized extension and planar cell polarity in the cochlea by the vertebrate PCP pathway. Nat Genet 37:980–985. CrossRef Medline

Woods C, Montcouquiol M, Kelley MW (2004) Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat Neurosci 7:1310–1318. CrossRef Medline

Wu SM, Tan KS, Chen H, Beh TT, Yeo HC, Ng SK, Wei S, Lee DY, Choo AB, Chan KK (2012) Enhanced production of neuroprogenitors, dopaminergic neurons, and identification of target genes by overexpression of sonic hedgehog in human embryonic stem cells. Stem Cells Dev 21:729–741. CrossRef Medline

Yamamoto N, Chang W, Kelley MW (2011) Rbpj regulates development of prosensory cells in the mammalian inner ear. Dev Biol 353:367–379. CrossRef Medline

Yang H, Xie X, Deng M, Chen X, Gan L (2010) Generation and characterization of Atoh1-Cre knock-in mouse line. Genesis 48:407–413. CrossRef Medline