Auditory Hair Cell-Specific Deletion of p27^{Kip1} in Postnatal Mice Promotes Cell-Autonomous Generation of New Hair Cells and Normal Hearing

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Hearing in mammals relies upon the transduction of sound by hair cells (HCs) in the organ of Corti within the cochlea of the inner ear. Sensorineural hearing loss is a widespread and permanent disability due largely to a lack of HC regeneration in mammals. Recent studies suggest that targeting the retinoblastoma (Rb)/E2F pathway can elicit proliferation of auditory HCs. However, previous attempts to induce HC proliferation in this manner have resulted in abnormal cochlear morphology, HC death, and hearing loss. Here we show that cochlear HCs readily proliferate and survive following neonatal, HC-specific, conditional knock-out of p27^{Kip1} (p27CKO), a tumor suppressor upstream of Rb. Indeed, HC-specific p27CKO results in proliferation of these cells without the upregulation of the supporting cell or progenitor cell proteins, Prox1 or Sox2, suggesting that they remain HCs. Furthermore, p27CKO leads to a significant addition of postnatally derived HCs that express characteristic synaptic and stereociliary markers and survive to adulthood, although a portion of the newly derived inner HCs exhibit cytocauds and lack VGlut3 expression. Despite this, p27CKO mice exhibit normal hearing as measured by evoked auditory brainstem responses, which suggests that the newly generated HCs may contribute to, or at least do not greatly detract from, function. These results show that p27^{Kip1} actively maintains HC quiescence in postnatal mice, and suggest that inhibition of p27^{Kip1} in residual HCs represents a potential strategy for cell-autonomous auditory HC regeneration.

Key words: Cdkn1b; Cip/Kip; cochlea; cyclins; regeneration; retinoblastoma

Introduction

The mammalian auditory organ generally consists of one row of inner hair cells (IHCs), three rows of outer HCs (OHCs), and several subtypes of underlying supporting cells (SCs). These HCs and SCs permanently cease dividing during gestation (Ruben, 1967), which limits HC regeneration and the rehabilitation of sensorineural hearing loss. More broadly, quiescence occurs via cyclin/cyclin-dependent-kinase (CDK) inhibition and subsequent hypophosphorylation of the retinoblastoma protein (Rb; Polyak et al., 1994). Several CDK inhibitors (CDKIs), including p27^{Kip1}, are expressed in the cochlea and, along with Rb, promote quiescence therein (Chen and Segil, 1999; Löwenheim et al.,

1999; Chen et al., 2003; Mantela et al., 2005; Sage et al., 2005, 2006; Laine et al., 2007, 2010; Yu et al., 2010). The p27^{Kip1} protein becomes detectable in the prosensory epithelium by embryonic day 14, coincident with cell cycle exit (Chen and Segil, 1999; Lee et al., 2006). Upon differentiation, p27^{Kip1} protein is downregulated in HCs, but persists at readily detectable levels in SCs (Chen and Segil, 1999; Löwenheim et al., 1999). Despite apparent downregulation of p27^{Kip1} protein, p27^{Kip1} mRNA is detectable in isolated auditory HCs at postnatal and adult ages (Laine et al., 2007; H. Liu et al., 2014), suggesting that p27^{Kip1} protein may still be expressed. Unfortunately, the issue of whether p27^{Kip1} persists at a level sufficient to promote quiescence in postnatal cochlear HCs remains unresolved. For example, deletion of Rb in mice readily promotes proliferation of cochlear HCs and SCs (Mantela et al., 2005; Sage et al., 2005, 2006; Weber et al., 2008; Yu et al., 2010), but nonspecific deletion of p27^{Kip1} causes proliferation preferentially in prosensory cells and SCs, rather than HCs (Chen and Segil, 1999; Löwenheim et al., 1999; Kanzaki et al., 2006; Oesterle et al., 2011).

Furthermore, it is unknown whether auditory HCs can provide a cell-autonomous source for regeneration. Specifically, while cell cycle can be reinitiated in cochlear HCs when CDKIs or Rb are deleted, these manipulations have hitherto resulted in cochlear disorganization, extensive cell death, and severe hearing impairment (Chen and Segil, 1999; Chen et al., 2003; Mantela et al., 2005; Kanzaki et al., 2006; Sage et al., 2006; Laine et al., 2007, 2010; Weber et al., 2008; Huang et al., 2011). Therefore, inhibit-

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Table 1. Antibodies used for immunostaining

Target antigen	Clonality	Host	Conjugate	Dilution (immunohistochemistry)	Labels	Provider
BrdU	Monoclonal	Mouse	Alexa-488	1:25–1:50	S phase	Invitrogen, catalog #B35130
Calbindin D28K	Polyclonal	Rabbit	Unconjugated	1:500	HCs	Millipore, catalog #AB1778
CldU	Monoclonal	Rat	Unconjugated	1:200	S phase	AbD Serotec, catalog #0BT0030G
Ctbp2 (Ribeye)	Monoclonal	Mouse	Unconjugated	1:500	HC synapse	BD Transduction, catalog #612044
Espin	Polyclonal	Rabbit	Unconjugated	1:2000	stereocilia	Gift from Stefan Heller, Stanford University
IdU	Monoclonal	Mouse	Unconjugated	1:200	S phase	BD Biosciences, catalog #347580
Myosin VI	Polyclonal	Rabbit	Unconjugated	1:200	HCs	Proteus Bioscience, catalog #25-6791
Myosin VIIa	Polyclonal	Rabbit	Unconjugated	1:200	HCs	Proteus Bioscience, catalog #25-6790
Parvalbumin	Monoclonal	Mouse	Unconjugated	1:1000	HCs	Sigma-Aldrich, catalog #P3088
Phospho-histone H3	Monoclonal	Rabbit	Alexa-488	1:25–1:50	M phase	Cell Signalling, catalog #3465
Prox1	Polyclonal	Rabbit	Unconjugated	1:500	Progenitor cells, SCs	Millipore, catalog #AB5475
Sox2	Polyclonal	Goat	Unconjugated	1:500	Progenitor cells, SCs	Santa Cruz Biotechnology, catalog #sc-17320
Tuj1	Monoclonal	Mouse	Unconjugated	1:1000	Neurons	Covance, catalog #MMS-435P
VGlut3	Polyclonal	Rabbit	Unconjugated	1:500	IHCs	Synaptic Systems, catalog #135203

ing the Rb pathway in such a way as to preserve cell proliferation, but minimize cell death, is critical. Here, we have found that HC-specific, conditional deletion of $p27^{Kip1}$ at neonatal ages results in HC proliferation and survival of supernumerary HCs to adulthood. Furthermore, HC-specific $p27^{Kip1}$ deletion does not result in any loss of hearing as measured by evoked auditory brainstem responses (ABRs). These findings demonstrate that $p27^{Kip1}$ is expressed in postnatal cochlear HCs at a level sufficient to promote quiescence, and that its disruption promotes HC proliferation without significant cell death or hearing loss. These studies further suggest that HCs can be used as a potential cell-autonomous source to generate new HCs, and that $p27^{Kip1}$ inhibition may be a viable therapeutic strategy for HC regeneration.

Materials and Methods

Animals and procedures. All mice used in the experiments described were housed, bred, and cared for in the animal resources center at the St. Jude Children's Research Hospital according to institutional animal care and use guidelines and approved protocols. $p27^{loxP/loxP}$ mice were generously provided by Dr. Matthew L. Fero (Fred Hutchinson Cancer Research Center; Chien et al., 2006; Liu et al., 2012d). Deletion of $p27^{Kip1}$ in the organ of Corti was restricted to HCs by breeding $p27^{loxP/loxP}$ mice with Atoh1-CreERTM transgenic mice courtesy of Dr. Suzanne Baker (St. Jude Children's Research Hospital) and inducing the CreER with tamoxifen (75 mg/kg, i.p.) at postnatal day (P) 0 and P1 [conditional knock-out (CKO) of $p27^{Kip1}$ ($p27^{CKO}$)]. To parallel the $p27^{CKO}$ experiments, Atoh1-CreERTM mice were also bred with Rb^{loxP/loxP} mice (Marino et al., 2000), generously provided by Dr. Anton Berns (Netherlands Cancer Institute) and induced similarly at P0 and P1 (hereafter, RbCKO). Three different populations of control mice were used: (1) tamoxifen-injected littermates that were Atoh1-CreERTM positive, but wild type for $p27^{Kip1}$ or Rb; (2) tamoxifen-injected littermates that were homozygous for $p27^{loxP/loxP}$ or Rb^{loxP/loxP} alleles, but negative for Atoh1-CreERTM; and (3) nonlittermates that had identical genotypes to experimental animals, but were not induced with tamoxifen (injections of corn oil only). Sample sizes were $N = 4$ mice of either sex for each group (control and experimental) at each time point for all experiments except for the ABRs and cell counts in the adult $p27^{CKO}$ mice, where sample sizes were $N = 5$ for each group. Genotypes for each mouse were verified by PCR amplification and visualization by gel electrophoresis using previously published primers and cycling conditions (Chien et al., 2006; Weber et al., 2008).

To test for cell-cycle entry, mice were injected with the thymidine analogs 5'-ethynyl-2'-deoxyuridine (EdU) at 12.6 mg/kg intraperitoneally (Invitrogen), bromodeoxyuridine (BrdU) at 50 mg/kg intraperitoneally (Sigma Aldrich), 5-chloro-2'-deoxyuridine (CldU) at 50 mg/kg intraperitoneally (Sigma Aldrich), or 5-iodo-2'-deoxyuridine (IdU) at 50 mg/kg intraperitoneally (MP Biomedical). To assess multiple rounds of division following $p27^{CKO}$, mice ($N = 4$) were injected twice with EdU

at P4, followed by three injections of CldU at P5, and three injections of IdU at P6. The EdU injections at P4 were spaced 4 h apart, while the injections of thymidine analogs at P5 and P6 were spaced 2 h apart, with the mice being killed and cochleae fixed for staining 2 h after the final IdU injection at P6. To assess multiple rounds of division following RbCKO, mice were injected once with EdU at P4 followed by three injections of BrdU at P6 (2 h apart), and then killed and cochleae fixed for staining 2 h after the final BrdU injection. To test hearing function, ABRs were conducted at 6 weeks of age using previously described methods (Wu et al., 2004).

Immunohistochemistry. After overnight fixation in 4% paraformaldehyde, whole mounts of the organ of Corti were blocked and permeabilized (10% normal serum, 1% Triton X-100, and 1% BSA in 10 mM PBS) for 1 h at room temperature, then incubated at 4°C overnight in primary antibodies (Table 1). Where needed, whole mounts were placed into secondary antibodies conjugated to Alex-Fluor fluorophores (Invitrogen) at 1:1000 for 1.5–3 h at room temperature. EdU staining was performed using Click-iT EdU imaging kits from Invitrogen according to the manufacturer's instructions (Salic and Mitchison, 2008). Stained cochleae were mounted to slides using Prolong Gold (Invitrogen), and analyzed using a Zeiss LSM 700 confocal microscope (Carl Zeiss). Due to the degradation of actin by the copper sulfate required to catalyze EdU labeling, colabeling of EdU with phalloidin generally cannot be accomplished without specially designed reagents (Kaiser et al., 2009; Uttamapinant et al., 2012). Here, colabeling of phalloidin and EdU was accomplished by correlative light microscopy. Samples were first immunostained for HC markers and exposed to Alexa Fluor-conjugated phalloidin (1:200), then coverslipped and imaged. Next, the coverslip was removed, samples were washed and reblocked while remaining mounted on the slide, and then exposed to the Click-iT EdU detection reagents before being washed, coverslipped, and reimaged. Images were then superimposed to reveal phalloidin and EdU staining within the same cells.

Data analysis. For experiments where mice were ages P2–P8, total numbers of myosin VI (Myo6)-positive HCs and total numbers of proliferative HCs [i.e., those that were double positive for Myo6 and either EdU or phosphorylated histone H3 (PH3)] were counted manually from confocal z-stack images. The total lengths of the mounted cochlear tissues were measured and images were pooled into groups to represent the apical, middle, and basal turns of approximately equal lengths. Data were first normalized to cochlear length and the total numbers of proliferative HCs (either EdU or PH3 positive) were compared. Then, proliferative indices (PIs) were generated by calculating the percentages of EdU-positive or PH3-positive IHCs ($\frac{\text{Proliferative IHCs}}{\text{Total IHCs}} \times 100$) and OHCs ($\frac{\text{Proliferative OHCs}}{\text{Total OHCs}} \times 100$). In this way, levels of proliferation could be compared independently of any effects that cell addition and/or cell death may have had on the total numbers of HCs at any given time. Since no EdU-positive or PH3-positive cells were detected in any of the control samples, nor at the P2 time points, statistics for EdU and PH3 measures

Figure 1. p27CKO causes cochlear HCs to re-enter the cell cycle with EdU and PH3 staining being greatest at P4, in apical turns, and in IHCs compared with OHCs. **A**, Myo6-positive (white) IHCs and OHCs in p27CKO cochleae readily incorporate EdU (red) after a single injection given at P4 (shown), P6, or P8, but not P2. **B**, No EdU-positive HCs were observed in any control mice at P4 (shown) or at any other age investigated. **C**, There were significantly greater numbers of EdU-positive HCs at P4 compared with P6 and P8 (** $p < 0.005$), and significantly more EdU-positive HCs at P6 compared with P8 (* $p = 0.05$). There were also significantly more EdU-positive HCs in apical compared with basal turns at each age (P4, P6, and P8) individually ($tp < 0.05$). **D**, The PI for EdU was significantly higher in the apex versus middle (x vs y , $p < 0.005$) and basal turns (x vs z , $p < 0.0001$), and the PI for middle turns was significantly higher than that for basal turns (y vs z , $p < 0.05$). While the overall main effect suggested a greater EdU PI in IHCs compared with OHCs, there was also an interaction that was likely driven by the significant difference between IHCs and OHCs in apical turns (* $p < 0.05$). **E**, p27CKO mice exhibit PH3-positive mitotic figures (green) in the nuclei of Myo6-positive HCs (white) at P4 (shown), P6, and P8, but not at P2. **F**, No PH3-positive HCs were observed in control mice at P4 (shown) or at any other age investigated. **G**, Consistent with the EdU data, PH3 labeling revealed significantly more HCs in M phase at P4 than at P8 (* $p < 0.05$). While there was again a main effect of cochlear turn. The effect was most pronounced at P6, where the numbers of PH3-positive HCs in the apical turn were significantly greater than those in the basal turn ($tp < 0.05$). **H**, For the PH3-based PI, the overall percentage of PH3-positive IHCs was greater than that for OHCs, but there was also an interaction with cochlear turn, likely driven by the pronounced effect in the middle turns (* $p = 0.05$). Again, apical turns had significantly higher PIs than middle (x vs y , $p < 0.05$) and basal turns (x vs z , $p < 0.005$), and middle turns also had significantly higher PIs than basal turns (y vs z , $p < 0.01$). Scale bars, 20 μ m. Data is presented as mean $\pm$ SEM.

were run only on experimental samples from ages P4, P6, and P8. For EdU-positive and PH3-positive HC counts, four separate repeated-measures ANOVAs were conducted, one each for EdU and for PH3 and for each genotype (p27CKO and RbCKO). These two-way repeated-measures ANOVAs tested for differences in HC proliferation across cochlear turn (apical, middle, base) and age (P4, P6, P8). For EdU-based and PH3-based PIs, a three-way repeated-measures ANOVA was conducted for each measure and for each genotype to look for differences in rates of HC proliferation across cell type (IHC vs OHC), cochlear turn (apical, middle, basal), and age (P4, P6, P8). Wherever the sphericity assumption was violated, a Greenhouse–Geisser correction was applied to the degrees of freedom. Following significant main effects, *post hoc* analyses were conducted using Bonferroni-corrected repeated-measures contrasts and Tukey’s honestly significant difference (HSD). Finally, where appropriate, Bonferroni-corrected pairwise comparisons (two-tailed Student’s *t* test) were conducted to compare values across cochlear turn (apex, middle, base) at each level of age (P4, P6, P8), across age at each level of cochlear turn, or across cell type at each level of cochlear turn.

For mice at age P42, HCs were counted from sample images corresponding to 160 μ m in cochlear length. From each semicircular cochlear turn (apex, middle, and base), three sample z-stacks were taken at positions correlating to 30, 90, and 150°, so that all of the images were equidistant from one another along the whole length of the cochlea. Counts from these images were averaged together to obtain each data point for each turn. These counts were then compared by independent sample Student’s *t* tests comparing across the experimental condition (p27CKO vs control or RbCKO vs control) at each level of cochlear turn for IHCs and OHCs separately (six total comparisons). ABR threshold and wave-one amplitude values obtained from p27CKO and control mice at P42 were again analyzed by separate two-way repeated-measures ANOVAs, which revealed no main effects or interactions for any of the variables. ABR thresholds in RbCKO mice were not compared statistically due to undetectable thresholds (Bryda et al., 2012; but see Akil et al., 2012). All statistics were performed using SPSS 17 software.

Results

Neonatal CKO of p27^{Kip1} by Atoh1-CreERTM results in HC proliferation, which occurs preferentially in IHCs in the apical turn of the cochlea and declines rapidly with age

We generated a HC-specific p27CKO mouse line by crossing p27^{loxP/loxP} mice with the HC-specific Atoh1-CreERTM mouse line and inducing the CreER with tamoxifen at P0 and P1. The Atoh1-CreERTM was chosen because it has been characterized pre-

viously with several different floxed-stop reporters and found to be HC specific when Cre activity is induced with the same tamoxifen dose at P0 and P1 (Chow et al., 2006; Weber et al., 2008; Cox et al., 2012, 2014; Liu et al., 2012a). To investigate whether p27^{Kip1} promotes quiescence in cochlear HCs, p27CKO mice were injected with the S-phase marker EdU at P2, P4, P6, or P8 and then killed 6 h after injection. Colabeling of cochlear whole mounts for EdU and the HC-specific protein Myo6 revealed EdU-positive HCs in all of the p27CKO mice investigated at P4, P6, and P8 (Fig. 1A). No EdU-positive HCs were detected in any of the p27CKO mice 1 d after tamoxifen administration (P2) or in control mice at any age between P2 and P8 (Fig. 1B).

A repeated-measures ANOVA of EdU-positive HCs across age (P4, P6, P8) and cochlear turn (apical, middle, basal) revealed a significant main effect of age ($F_{(2,9)} = 49, p = 1 \times 10^{-5}$), a significant effect of cochlear turn ($F_{(2,18)} = 93.6, p = 3 \times 10^{-10}$), and a significant interaction term for the two variables ($F_{(4,18)} = 20.5, p = 2 \times 10^{-6}$). *Post hoc* Tukey's HSD (Fig. 1C) showed that there was significantly greater EdU incorporation at P4 compared with P6 ($p = 4 \times 10^{-4}$) and P8 ($p = 1 \times 10^{-5}$) and significantly greater EdU incorporation at P6 compared with P8 ($p = 0.01$). Bonferroni-corrected pairwise contrasts demonstrated that there was also greater EdU incorporation in the apical turn of the cochlea compared with middle ($F_{(1,9)} = 81, p = 3 \times 10^{-5}$) and basal ($F_{(1,9)} = 133.8, p = 3 \times 10^{-6}$) turns, and significantly more EdU-positive HCs in middle compared with basal turns ($F_{(1,9)} = 17.8, p = 0.006$). Bonferroni-corrected pairwise comparisons at each age revealed significant differences between apex and base at P4 ($t_{(3)} = 8.2, p = 0.04$), P6 ($t_{(3)} = 7.5, p = 0.045$), and P8 ($t_{(3)} = 17.4, p = 0.004$), and a significant difference between the apex and the middle turn at P8 ($t_{(3)} = 17.4, p = 0.004$). The interaction term was likely driven by a greater degree of change with age in the apical and middle turns compared with the basal turns. Indeed, when comparing changes with age, the numbers of EdU-positive HCs in the apical turn declined significantly between P4 and P8 ($t_{(3)} = 8, p = 0.04$) and between P6 and P8 ($t_{(6)} = 5.6, p = 0.009$), while in the middle turn, EdU-positive HCs only differed significantly between P6 and P8 ($t_{(6)} = 5.5, p = 0.02$). In the basal turn, there were no significant differences with age at all.

To determine whether differences in proliferation might exist between IHCs and OHCs, we generated a PI as the percentage of EdU and Myo6 double-positive cells divided by the total number of Myo6-positive HCs. More specifically, the number of EdU-positive IHCs was divided by the total number of IHCs and the number of EdU-positive OHCs was divided by the total number of OHCs. A repeated-measures ANOVA of the EdU-based PIs revealed significant main effects across age ($F_{(2,9)} = 49.6, p = 1 \times 10^{-5}$), cochlear turn ($F_{(2,18)} = 87.6, p = 1 \times 10^{-9}$), and HC type (IHC > OHC, $F_{(1,9)} = 100.4, p = 4 \times 10^{-6}$), as well as significant interactions between age and turn ($F_{(4,18)} = 24.7, p = 4 \times 10^{-7}$), age and cell type ($F_{(2,9)} = 45.6, p = 2 \times 10^{-5}$), turn and cell type ($F_{(2,18)} = 10.5, p = 0.001$), and among all three variables ($F_{(4,18)} =$

Figure 2. p27CKO causes proliferative expansion of cochlear HCs via multiple rounds of cell division. To determine whether cells were completing multiple rounds of the cell cycle, p27CKO mice were injected in duplicate with EdU at P4 followed by triplicate injections of CldU at P5 and IdU at P6. **A**, EdU (red), CldU (blue), and IdU (green) were separately incorporated in Myo6-positive HCs (white), suggesting cell-cycle entry at P4, progression through cycle, and S-phase entry again at P5 and P6. **B–D**, Single-channel images reveal the individual EdU (**B**), CldU (**C**), or IdU (**D**) labeling. Arrows denote nuclei that are double positive for EdU and CldU, suggesting that these cells were in S phase at P4 and re-entered the cell cycle at P5. The arrowhead denotes a HC that is positive for EdU, CldU, and IdU, suggesting that it completed two rounds of cell division between P4 and P6. **E**, EdU and Myo6 double-positive cells were counted 6, 48, or 96 h (hr) after a single injection of EdU at P4 and revealed a significant expansion of total numbers of EdU-positive HCs between 6 and 96 h after injection ($*p < 0.05$). At all time points characterized, the apical turn of the cochlea had significantly more EdU-positive HCs, suggesting greater proliferation or survival of proliferative cells in the apical turn compared with middle and basal turns ($\dagger p < 0.05$). Scale bars, 20 μ m. Data is presented as mean $\pm$ SEM.

3.1, $p = 0.03$). Tukey's HSD and Bonferroni-corrected pairwise contrasts revealed similar results for the PI values as the EdU counts. Specifically, the PI was greater at P4 than at P6 ($p = 2 \times 10^{-4}$) and at P8 ($p = 1 \times 10^{-5}$) and greater in apical compared with middle ($F_{(1,9)} = 111.9, p = 6 \times 10^{-6}$) and basal ($F_{(1,9)} = 196.9, p = 6 \times 10^{-7}$) turns (Fig. 1D). The significant main effect of HC type suggests that IHCs may be generally more proliferative than OHCs. To explore this possibility, we used Bonferroni-corrected pairwise comparisons to compare PIs for IHCs and OHCs at each level of cochlear turn. Our findings involving EdU-based PIs suggested that IHCs were significantly more proliferative than OHCs in the apical turn ($t_{(11)} = 3.7, p = 0.01$).

In addition to EdU incorporation, cochleae from p27CKO mice revealed many HCs that were immunopositive for a phosphorylated form (phosphor-serine 10) of histone 3 (PH3), which is a hallmark of mitosis (Gurley et al., 1973; Van Hooser et al., 1998). Again, all of the p27CKO mice at P4 and P6, and 50% of the p27CKO mice at P8 exhibited cells that were positive for both PH3 and Myo6 (Fig. 1E). Again, no PH3-positive HCs were observed in any of the p27CKO mice at P2, or in any of the control mice at any of the ages investigated (Fig. 1F).

Consistent with the EdU data, comparisons of PH3-positive HCs (Fig. 1G) also revealed a significant effect of age ($F_{(2,11)} = 18.59, p = 0.03$), a significant effect of cochlear turn ($F_{(1,12)} = 15.7, p = 0.002$), and a trend toward an interaction ($F_{(2,12)} = 3.589, p = 0.06$). Again, a *post hoc* Tukey's HSD suggested that HCs in p27CKO mice were more proliferative at P4 than at P8 ($p = 0.02$), and Bonferroni-corrected pairwise contrasts suggested greater numbers of PH3-positive HCs in the apical turn compared with both middle ($F_{(1,11)} = 13.8, p = 0.009$) and basal turns ($F_{(1,11)} = 17.1, p = 0.006$), and greater numbers in middle compared with basal turns ($F_{(1,11)} = 14.1, p = 0.009$). Bonferroni-corrected pairwise comparisons at each level of age revealed a significant difference between apical and basal turns at P6 ($t_{(4)} = 5.6, p = 0.02$), but not at P4 or P8 (Fig. 1G). For the PH3-based PIs, there was a significant effect of cell type (IHCs > OHCs, $F_{(1,11)} = 9.03, p = 0.01$), a significant effect of turn ($F_{(1,14.5)} = 17.7, p = 3 \times 10^{-5}$), and a significant effect of age ($F_{(2,11)} = 8.3, p = 0.006$), with only the age–turn interaction being significant

Figure 3. Postnatally derived HCs exhibit espin-positive and phalloidin-positive stereocilia bundles. Cochleae from p27CKO mice were analyzed at P21 after being injected with EdU at P4. **A, B**, Phalloidin staining (red) reveals an overall pattern of stereocilia bundles that is largely consistent between control samples (**A**) and p27CKO samples (**B**), although a small number of phalloidin-positive cytotriads, characterized by long processes, can be seen medial to the organ of Corti in apical (apex) and middle (mid) turns of p27CKO cochleae (**B**, insets in apex and mid). Scale bars, 100 μ m. Widths of the inset images represent 62 μ m (apex) and 57 μ m (mid). **C**, High-magnification projection images show a representative parvalbumin (Parv)-positive OHC (green) that exhibits an EdU-positive nucleus (white) and phalloidin-stained (Phal) stereocilia (red). **D**, Similarly, high-magnification images revealed calbindin (Calb)-positive IHCs that exhibited both EdU-positive nuclei (white) and phalloidin-positive stereocilia (red). **E**, At high magnification, a representative phalloidin-positive (red) cytotriad (arrow) can be seen to traverse the length of the Calb-positive (green) HC body. Scale bars: **C–E**, 10 μ m. Arrowheads indicate stereociliary bundles corresponding to EdU-expressing cells. **F, G**, Consistent with the presence of actin-positive stereocilia bundles, projection and orthogonal images of cochlear whole mounts show both OHCs (**F**) and IHCs (**G**) that triple label for Parv (green), EdU (white), and the stereocilia bundle-specific protein espin (red). Espin positive stereociliary bundles that correspond to EdU positive cells are denoted by yellow arrows. Scale bars: **F, G**, 20 μ m.

($F_{(2,6,14,5)} = 4.4$, $p = 0.03$). Again, apical turns had a significantly higher PH3-based PI than middle ($F_{(1,11)} = 11.2$, $p = 0.02$) and basal turns ($F_{(1,11)} = 22.5$, $p = 0.003$), and middle turns also had a significantly higher PI than basal turns ($F_{(1,11)} = 17.2$, $p = 0.006$). Bonferroni-corrected pairwise comparisons of IHCs versus OHCs for each level of cochlear turn revealed a significant difference in the middle turn ($t_{(13)} = 2.7$, $p = 0.05$), but not the apical or basal turns (Fig. 1H). Together, these data suggest that

proliferation of HCs following neonatal CKO of p27^{Kip1} occurs preferentially in IHCs compared with OHCs, most prominently in the apical and then middle turns of the cochlea, and in a wave pattern that peaks at P4 and declines to near zero by P8.

HCs complete and re-enter the cell cycle in response to p27CKO

To determine whether proliferative HCs in this model were able to complete the cell cycle or undergo multiple rounds of division, p27CKO mice were injected with EdU in duplicate at P4, followed by triplicate injections of the S-phase markers CldU at P5 and IdU at P6. Mice were killed at P6, 2 h after the final IdU injection, and their cochleae quadruple stained for EdU, CldU, IdU, and Myo6 (Fig. 2A). Among the Myo6 and EdU double-positive HCs, $66 \pm 5.3\%$ were also positive for CldU, and $8.7 \pm 2\%$ were colabeled with IdU. These data suggest that many of the HCs that entered S phase at P4 had completed and then re-entered the cell cycle by P5, and that a smaller number of these cells were competent to enter S phase at P6. In addition, $2.4 \pm 0.6\%$ of the EdU-positive HCs were colabeled for both CldU and IdU, suggesting that at least some of the p27CKO HCs completed multiple rounds of cell division between P4 and P6.

Barring a large degree of cell death, the completion of multiple rounds of cell cycle between P4 and P6 in p27CKO mice would suggest that the numbers of proliferative HCs should increase over time. To assess this potential expansion of proliferative HCs, p27CKO cochleae were collected either 6, 48, or 96 h after a single EdU injection given at P4. Between 6 and 96 h after EdU injection, the number of EdU and Myo6 double-positive cells increased significantly (main effect of time after injection: $F_{(2,9)} = 6.1$, $p = 0.02$; Tukey's HSD for 6 h vs 96 h: $p = 0.02$), suggesting that at least a portion of the HCs that were in S phase at P4 expanded via proliferation (Fig. 2B). Consistent with the greater degree of proliferation in apical turns noted above, there was a significant main effect of cochlear turn for this dataset ($F_{(2,18)} = 104.2$, $p = 1 \times 10^{-10}$) and a significant interaction

between cochlear turn and time after injection ($F_{(4,18)} = 4.2$, $p = 0.01$). Bonferroni-corrected pairwise comparisons at each time point revealed significantly more EdU-positive HCs in apical turns compared with middle turns (6 h: $t_{(3)} = 6.2$, $p = 0.03$; 48 h: $t_{(3)} = 8.7$, $p = 0.009$; 96 h: $t_{(3)} = 5.1$, $p = 0.045$) and basal turns (6 h: $t_{(3)} = 29.3$, $p = 3 \times 10^{-4}$; 96 h: $t_{(3)} = 6.5$, $p = 0.02$). Combined with the data presented above, these findings suggest that, after an

initial peak in cell cycle entry at P4, a diminishing number of HCs progress through multiple rounds of the cell cycle, which leads to a more sigmoidal, rather than exponential, increase in the numbers of EdU-positive HCs over the 96 h period following a single EdU injection at P4.

Daughter HCs derived from postnatal p27CKO-induced proliferation express synaptic and stereociliary HC markers

The mechanotransducing HCs of the inner ear require certain attributes necessary for their function that include, but are not limited to, stereociliary bundles and synaptic machinery. Furthermore, there is some evidence to suggest that HCs generated in postnatal cochleae fail to express markers of terminal differentiation (Liu et al., 2012c; 2014b; Cox et al., 2014). To ascertain the presence of stereociliary and synaptic proteins in p27CKO cochleae, experimental mice were injected with EdU at P4, aged to P21, and stained for EdU, myosin VIIa (Myo7a) or parvalbumin, and either phalloidin (actin cytoskeleton/stereocilia), espin (stereociliary bundles), C-terminal binding protein-2 (Ctbp2; ribbon synapses), or class III β -tubulin (Tuj1; innervating nerve fibers). We also stained samples for the vesicular glutamate transporter VGlut3, which is essential to synaptic transmission and is expressed in IHCs beginning at P0 (Seal et al., 2008; Peng et al., 2013). Numerous EdU-positive HCs (labeled with either Myo7a or parvalbumin) coexpressed phalloidin and espin (Fig. 3), as well as Ctbp2 and VGlut3 (Fig. 4), suggesting that many of the postnatally generated cells express markers that are expressed in endogenous IHCs. Also, Tuj1-positive nerve fibers were readily visible throughout the cochlea, and could be seen clearly adjacent to EdU-positive HCs (Fig. 4E–G), suggesting that p27CKO daughter HCs are likely innervated. However, a portion of the postnatally derived IHCs were VGlut3 negative, and several examples could be seen that lacked stereociliary bundles and instead exhibited phalloidin-positive cytauds (Flock et al., 1979; Kanzaki et al., 2002). Specifically, we noted several cells that were located ≥ 1 cell diameters medial to the endogenous IHC position, were positive for either Myo7a, parvalbumin, or calbindin; and had long, phalloidin-positive processes that traversed the length of the cell body (Fig. 3B). The presence of such cells may support the hypothesis that proximity to the organ of Corti correlates with proper hair bundle formation (Gubbels et al., 2008) and may also inform our understanding of cytauds, which have been linked to improper development of the stereocilia (Goodyear et al., 2012). Also, we observed that there were occasional parvalbumin-positive IHCs medial to the tunnel of Corti that

Figure 4. Postnatally derived HCs express markers of synaptic function and innervation. Cochleae from p27CKO mice were analyzed at P21 after being injected with EdU at P4. **A, B**, The majority of EdU (white) and parvalbumin (Parv, red) double-positive IHCs (arrows) also expressed VGlut3 (green). As expected, EdU and Parv double-positive OHCs were VGlut3 negative (arrowhead), although there were also occasional Parv-positive and EdU-positive IHCs that were VGlut3 negative (arrowhead). **C, D**, Myo6-positive (red) IHCs that were positive for EdU (white, arrows) were also positive for the Ctbp2 protein (green), a critical part of the ribbon synapse that is characteristic of mature IHCs. **E, F**, Consistent with the presence of synaptic proteins, Myo7a (blue) and EdU (red) double-positive HCs in p27CKO mice appear to be contacted and surrounded by Tuj1-positive neuronal processes (green), suggesting postsynaptic innervation. **G**, An orthogonal section from a sample different from that in **E** and **F** demonstrates clear Tuj1 staining (arrows) that is most intense toward the basal portion of EdU-positive HCs. Scale bars, 20 μ m.

did not express VGlut3 (Fig. 4A, B). These results suggest that while many of the newly generated cells on the neural side of the tunnel of Corti exhibit markers of terminally differentiated IHCs by P21, some of the new HCs may require >3 weeks to become terminally differentiated or may not do so at all. However, given that most of the postnatally derived HCs do exhibit all of the markers tested, the data suggest that many postnatally generated HCs could be mature and contribute to hearing function.

p27CKO does not result in the dedifferentiation of cochlear HCs to a progenitor cell state

Given that some of the HCs in the p27CKO mice downregulated VGlut3, we wondered to what extent HCs could be dedifferenti-

Figure 5. p27CKO does not cause SC proliferation or the upregulation of Sox2 or Prox1 in HCs. **A**, Immunostaining of p27CKO cochleae at P4 (6 h after EdU injection) revealed no colabeling of EdU-positive (red) or Myo6-positive (white) HCs with the prosensory cell and SC marker Sox2 (green). Myo6-positive HCs with binuclear appearance (arrowhead) were often observed. **B**, In the SC layer underneath the HCs, Sox2 readily labeled the SC nuclei and the pillar cells (PCs) and Deiters cells (DCs) are clearly delineated. However, we did not see any Sox2-positive SCs in the organ of Corti that colabeled with EdU in any of the P4 (shown) or P6 samples. **C**, Orthogonal images show that EdU-positive nuclei (red) were detectable in Myo6-positive HCs (white), in tympanic border mesenchymal cells (arrow), and in Boettcher or Claudius cells (asterisks), but not in any of the Sox2-positive SCs (green). **D**, After EdU injections at P4 (shown) and P6, EdU (red) was readily detectable in Myo6-positive HCs (white). However, Prox1 was never seen to colabel with either EdU or Myo6 in any of the samples. Again, binucleated HCs were often observed (arrowhead). **E**, In the SC layer underneath the HCs, Prox1 readily labeled PCs and DCs, though none of these cells was ever observed to colabel with either EdU or Myo6. **F**, Orthogonal images illustrate the presence of EdU (red) in the nuclei of Myo6-positive HCs (white), but not in Prox1-positive SCs (green) that sit beneath the HCs. Scale bars, 20 μ m.

ating in response to the loss of p27. To test this, we performed immunocytochemistry for the sex-determining region Y-box 2 (Sox2) and prospero-related homeobox 1 (Prox1) proteins, since both Sox2 and Prox1 are expressed in cochlear prosensory progenitor cells and in developing HCs and SCs before becoming restricted to SCs postnatally (Kiernan et al., 2005; Bermingham-McDonogh et al., 2006). Additionally, both Sox2 and Prox1 can be upregulated in postnatal cochlear HCs following reactivation of Notch signaling (Liu et al., 2012b). In p27CKO samples from mice that were injected with EdU at either P4 ($N = 4$) or P6 ($N = 4$) and killed 6 h after injection, both Prox1 and Sox2 robustly labeled SC nuclei. However, we did not observe any instances of colabeling of either Prox1 or Sox2 with Myo6 or with EdU in the organ of Corti (Fig. 5). Additionally, in all of the p27CKO samples that we analyzed, for all of the ages and experiments where cell cycle markers were examined, we did not see a single EdU-positive, CldU-positive, IdU-positive, or PH3-positive cell within the organ of Corti that did not also express either Myo6, Myo7a, or calbindin. Furthermore, we often observed Myo6-positive HCs that were binucleated (Fig. 5A,C), as well as doublets of Myo6 and EdU double-positive HCs, suggesting that p27CKO HCs likely retain Myo6 expression as they progress through telophase and complete cell division. Together, these data suggest that, within the organ of Corti, only HCs, and not SCs, were seen to proliferate in response to p27CKO. Furthermore, while some of the cochlear HCs in the p27CKO model may dedifferentiate to

an immature HC state (i.e., one that lacks VGlut3), they are likely not dedifferentiating into a SC-like or progenitor-like state where one would expect the down-regulation of HC markers, such as Myo6, and/or the upregulation of prosensory cell/SC markers, such as Sox2 and Prox1.

Supernumerary IHCs in the p27CKO mouse cochlea survive until ≥ 6 weeks of age and normal hearing function is preserved

To determine whether postnatally generated HCs in p27CKO cochleae could survive to adulthood, p27CKO mice were analyzed at P42 and the total numbers of calbindin-positive HCs were counted (Fig. 6A–D). Since results from the previous experiments suggested that the bulk of postnatally generated HCs were IHCs in the apical turn of the cochlea, IHCs and OHCs from each turn were analyzed separately. The p27CKO mice exhibited a significant increase in the numbers of IHCs in the apical ($t_{(4,9)} = 8.4$, $p = 4 \times 10^{-4}$) and middle ($t_{(4,2)} = 2.9$, $p = 0.04$) turns compared with control mice (Fig. 6C), suggesting that supernumerary, postnatally derived IHCs did indeed survive to P42. Consistent with lower levels of proliferation in OHCs and basal turns, the numbers of OHCs in all three turns, and the numbers of IHCs in basal turns, did not differ from controls (Fig. 6C,D), suggesting that significant numbers of new HCs were not added to these regions, or that they did not survive until P42. Still,

the survival of a normal contingent of HCs in all regions, and even supernumerary IHCs in the apical and middle turns, suggests that p27^{Kip1} is not necessary for the survival of postnatal cochlear HCs. This is consistent with results from p27^{Kip1} germline mutants where supernumerary HCs survive to young adult ages (P21; Löwenheim et al., 1999; Kanzaki et al., 2006), but differs from germline mutants of p19^{Ink4d} and p19^{Ink4d}/p21^{Cip1} compound mutants where HCs do not survive (Chen et al., 2003; Laine et al., 2007).

To determine whether the addition of supernumerary cochlear HCs after p27CKO would result in any changes to hearing function, ABRs were recorded from p27CKO mice at P42 and compared with littermate controls that received the same tamoxifen paradigm and either had wild-type p27^{Kip1} alleles or were Atoh1-CreERTM negative. Free-field evoked ABRs were recorded at frequencies ranging from 4 to 44 kHz and delivered at sound pressure levels of 75 dB and below. A repeated-measures ANOVA did not reveal any significant effects of p27CKO ($F_{(1,8)} = 0.003$, $p = 0.96$), suggesting that postnatally derived HCs resulting from p27CKO do not disrupt normal hearing sensitivity and may even contribute to normal function (Fig. 6E). We similarly compared wave-one amplitudes at 65 dB (4–44 kHz) and again found no difference between p27CKO and control mice ($F_{(1,8)} = 0.008$, $p = 0.93$), suggesting that hearing is normal in 6-week-old mice after HC-specific CKO of p27^{Kip1} (Fig. 6F).

IHC versus OHC differences in the p27CKO model are caused by factors upstream of Rb, while differences in proliferation along the tonotopic axis are influenced by factors downstream of Rb

In response to HC-specific p27CKO, we observed that HC proliferation declines with age after P4, occurs along a gradient from the apical to the basal turn of the cochlea, and occurs preferentially in IHCs compared with OHCs. Since p27^{Kip1} deletion is known to result in inactivation of the Rb protein (Polyak et al., 1994), we hypothesized that these patterns of proliferation would be similar in a RbCKO mouse model if they are caused by factors that are downstream or independent of the Rb pathway. Indeed, several results in the RbCKO model were consistent with the proliferative phenotypes observed in the p27CKO model. Specifically, EdU-positive (Fig. 7*A*) and PH3-positive (Fig. 7*E*) HCs were readily detectable in all of the RbCKO mice investigated at P4, P6, and P8, while no EdU-positive or PH3-positive HCs were detected in any of the controls or in any of the RbCKO mice at P2. A repeated-measures ANOVA revealed significant main effects of cochlear turn (EdU: $F_{(2,18)} = 12.4$, $p = 4 \times 10^{-4}$; PH3: $F_{(1,11)} = 10$, $p = 0.007$) and age (EdU: $F_{(1,9)} = 10.8$, $p = 0.009$; PH3: $F_{(2,9)} = 4.4$, $p = 0.046$), and a significant interaction between turn and age (EdU: $F_{(4,18)} = 5.3$, $p = 0.005$). *Post hoc* Tukey's HSD revealed that both EdU incorporation ($p = 0.03$) and PH3 immunostaining ($p = 0.046$) were significantly greater at P4 than P8 and thus both measures indicate that HC proliferation declined with age (Fig. 7). Similarly, the apical turn was more proliferative than the basal turn (EdU: $F_{(1,9)} = 55.2$, $p = 1 \times 10^{-4}$; PH3: $F_{(1,9)} = 97.8$, $p = 4 \times 10^{-6}$). Bonferroni-corrected pairwise comparisons revealed this effect to be driven by greater numbers of EdU-positive HCs in the apical compared with basal turn at P4 ($t_{(3)} = 5.8$, $p = 0.03$) and P8 ($t_{(3)} = 7.6$, $p = 0.02$), though not at P6 (Fig. 7*C*). Similar comparisons for PH3-positive HCs also showed more PH3-positive HCs in apical versus basal turns at P4 ($t_{(3)} = 8.4$, $p = 0.01$) and apical compared with middle ($t_{(3)} = 5.1$, $p = 0.042$) and basal turns ($t_{(3)} = 5.1$, $p = 0.045$) at P6 (Fig. 7*G*). These data suggest that the decline of proliferation from apical to basal turns is common to both p27^{Kip1} and Rb inactivation. Since p27^{Kip1} is upstream of Rb (Polyak et al., 1994), it is likely that whatever is causing this gradient of responsiveness along the tonotopic axis of the cochlea is either downstream or independent of Rb.

Despite some seeming similarity, there were a couple of key differences between the p27CKO and RbCKO models. Specifically, the RbCKO model exhibited a much larger degree of HC proliferation, with the numbers of EdU-positive and PH3-

Figure 6. Postnatally derived, supernumerary IHCs survive to 6 weeks of age and may contribute to normal hearing function. *A*, Supernumerary calbindin-positive (red) IHCs are readily apparent in the IHC region (bracket) of apical turns in cochleae from 6-week-old p27CKO mice. *B*, In contrast, age-matched controls that were Cre negative and expressed wild-type p27^{Kip1} exhibit a discrete single row of IHCs (bracket). Hoechst (blue) was used to counterstain cell nuclei. *C*, *D*, Quantification of calbindin-positive HCs from all three turns of the cochlea revealed a significant increase in the density of IHCs in apical and middle cochlear turns (*C*), but no significant increases in the numbers of OHCs in any of the three turns (*D*). *E*, *F*, Evoked ABRs suggest no difference in hearing sensitivity between p27CKO and control mice, as measured by pure tone thresholds (*E*) and wave-one amplitudes (*F*). Scale bar, 20 μm. Data are presented as mean ± SEM. ** $p < 0.005$, * $p < 0.05$.

positive HCs in the RbCKO at P4 being >10-fold and 15-fold higher than in the p27CKO, respectively (Figs. 1, 7). Also, while the PIs generated for the RbCKO mice did recapitulate the effect of cochlear turn (EdU: $F_{(2,18)} = 3.6$, $p = 0.048$; PH3: $F_{(1,11.8)} = 7.4$, $p = 0.01$), with greater proliferation in apical versus basal turns (EdU: $F_{(1,9)} = 11.5$, $p = 0.02$; PH3: $F_{(1,9)} = 42.1$, $p = 3 \times 10^{-4}$), they did not reveal a significant main effect of age (EdU: $F_{(2,9)} = 3.7$, $p = 0.07$; PH3: $F_{(2,9)} = 2.6$, $p = 0.13$) or of HC type (EdU: $F_{(1,9)} = 0.05$, $p = 0.82$; PH3: $F_{(1,9)} = 0.08$, $p = 0.78$). These data (Fig. 7*D*, *H*) suggest that whatever is causing the differential proliferation between IHCs and OHCs in the p27CKO model is likely upstream of Rb, since the PIs for OHCs and IHCs do not differ following complete inactivation of Rb by RbCKO.

Figure 7. Patterns of EdU and PH3 staining in RbCKO mice reveal greater proliferation at P4 than P8, in apical turns compared with basal, but no difference between IHCs and OHCs. **A**, Myo6-positive HCs (white) in RbCKO cochleae readily incorporate EdU (red) after a single injection given at P4 (shown), P6, and P8. **B**, Cells double positive for Myo6 and EdU were not observed in any control mice at P4 (shown) or any other age. **C**, Numbers of EdU-positive HCs were highest at P4, declining to P8 (* $p < 0.05$). Apical turns had significantly more EdU-positive HCs than basal turns at P4 ($t_p < 0.05$). At P8 apical turns had significantly more EdU-positive HCs than both middle ($t_p = 0.05$) and basal ($t_p < 0.05$) turns. **D**, EdU-based PIs suggested no significant difference between IHCs and OHCs in any cochlear turn. *Post hoc* comparisons did, however, recapitulate a significant difference between apical and basal turns (x vs y , $p = 0.05$). **E**, PH3 immunostaining (green) of Myo6-positive HCs (white) suggests cochlear HCs in RbCKO mice enter M phase of the cell cycle at P4 (shown), P6, and P8. **F**, No PH3-positive HCs were observed in control mice at P4 (shown), P2, P6, or

While the EdU and PH3 data clearly suggested a decline in the numbers of cycling HCs from P4 to P8, the data from both EdU-based and PH3-based PIs were more equivocal. It is possible that this discrepancy in the RbCKO model could be due to loss of cells rather than, or in addition to, a decrease in the rate of proliferation. Indeed, while double labeling for EdU and BrdU reveals that some RbCKO HCs are completing and re-entering the cell cycle between P4 and P6 (Fig. 8A), the numbers of EdU-positive cells exhibited a sharp decline between 6 and 96 h after a single EdU injection at P4 (main effect of time after injection: $F_{(2,9)} = 5.9$, $p = 0.02$; *post hoc* Tukey's HSD for 6 vs 96 h: $p = 0.02$; Fig. 8B). While in the p27CKO model, proliferation and completion of cell cycle contributes to an expansion of HC number, here, RbCKO HCs that were in S phase at P4 are clearly being lost, which is consistent with previous reports of HC death following Rb inactivation (Weber et al., 2008; Huang et al., 2011). Together, these data suggest that deletion of p27^{Kip1} does not result in the complete inactivation of Rb. Otherwise the degree of proliferation and of cell death would be much greater in the p27CKO model and match the RbCKO model where Rb is completely inactivated.

Finally, the survival of so many HCs in the p27CKO model starkly contrasts with the RbCKO model. Indeed, in the RbCKO model, there are almost no detectable cochlear HCs remaining at P42 (Fig. 9A,B), where there are significantly fewer HCs in all of the cochlear turns and for both IHCs (apex: $t_{(6)} = 65.7$, $p = 1 \times 10^{-9}$; middle: $t_{(3)} = 36.9$, $p = 3 \times 10^{-5}$; base: $t_{(3)} = 64.9$, $p = 1 \times 10^{-5}$) and OHCs (apex: $t_{(3)} = 55.7$, $p = 1 \times 10^{-5}$; middle: $t_{(3)} = 34.4$, $p = 1 \times 10^{-4}$; base: $t_{(3)} = 67.5$, $p = 1 \times 10^{-5}$) compared with controls (Fig.

P8. **G**, Similar to EdU staining, the numbers of PH3-positive HCs in the RbCKO model were greatest at P4, which differed significantly from P8 (* $p < 0.05$). The number of PH3-positive HCs was also greater in apical compared with basal turns at P4 ($t_p < 0.05$), and in apical compared with both middle and basal turns at P6 ($t_p < 0.05$). **H**, Similar findings were revealed by analysis of the PI based on PH3 staining, where the apex was significantly greater than base (x vs y , $p < 0.0005$), but there was no detectable difference between IHCs and OHCs at any level. Together, these data suggest that, like in the p27CKO model, CKO of Rb results in proliferation of HCs that is greatest in apical turns and declines toward the base, and that proliferation is greatest at P4, declining to P8. However, unlike the p27CKO model, there were no differences in either PI measure between IHCs and OHCs. Data are presented as mean $\pm$ SEM.

Figure 8. RbCKO cochlear HCs can progress through and complete the cell cycle, but exhibit overall cell loss rather than proliferative expansion. **A**, RbCKO mice were treated with EdU at P4 followed by triplicate injections of BrdU and analysis at P6. HCs that were triple positive (arrowhead) for BrdU (green), EdU (red), and calbindin (Calb, white) were in S phase at P4, progressed through the cell cycle, and entered S phase again at P6. **B**, Total numbers of EdU and Myo6 double-positive cells were also counted at several different time points after a single injection of EdU at P4. A significant decrease in total numbers of EdU-positive HCs between 6 and 96 h after EdU injection is consistent with profound HC death, as has been previously reported (see Discussion). Scale bars, 20 μ m. ****** p < 0.005. Data are presented as mean $\pm$ SEM.

9C,D). Thus, contrasting the loss of HCs in the RbCKO with the surviving and supernumerary HCs in the p27CKO mouse reveals that Rb is clearly essential for HC survival at neonatal ages, while p27^{Kip1} is not. Furthermore, the near complete loss of HCs in all turns of the cochlea suggests that the Atoh1-CreERTM is effective along the entire length of the cochlea, which is not only consistent with previously published reports using various reporter lines and the same induction paradigm (Chow et al., 2006; Weber et al., 2008; Cox et al., 2012, 2014; Liu et al., 2012a), but also suggests that the observed gradient of proliferation from apical to basal turns of the cochlea results from Rb or p27^{Kip1} inactivation rather than loss of CreER activity toward the base. As expected given such a dramatic loss of HCs, hearing loss in the RbCKO model is very severe and recognizable waveforms are undetectable by evoked ABRs at P42 (Fig. 9E). This again is in stark contrast to the p27CKO model and conforms to the notion that Rb is necessary for cochlear HC survival, but p27^{Kip1} is not.

Discussion

Previous studies have left questions remaining as to whether p27^{Kip1} is expressed in cochlear HCs at a level sufficient to exert antiproliferative activity. Here we show that p27CKO specifically from neonatal cochlear HCs results in proliferation of both IHCs and OHCs, generating a significant number of postnatally derived HCs that survive to adult age. This demonstrates that cochlear HCs do express a biologically relevant amount of p27^{Kip1}, which plays a direct role in maintaining their postmitotic state. This is consistent with reports that p27^{Kip1} mRNA is expressed in cochlear HCs (Laine et al., 2007; H. Liu et al., 2014), and suggests that immunohistochemical and transgenic reporter methods are likely not sensitive enough to detect functional levels of p27^{Kip1} in cochlear HCs (Chen and Segil, 1999; Lee et al., 2006; Liu et al., 2012d). Indeed, immunostaining of p27^{Kip1} in SCs, where its expression is high, requires both antigen retrieval and tyramide-

based amplification (Liu et al., 2012d), which suggests a low affinity for the native protein.

The patterns of cochlear HC proliferation in response to p27CKO, both longitudinally and radially, raise interesting questions about the regulation of quiescence within different HC types and along the length of the cochlea. Specifically, IHCs were more proliferative in response to p27CKO than OHCs, a pattern that was not replicated in RbCKO cochleae. These data suggest that a cell cycle regulator upstream of Rb likely mediates the differential responsiveness of the two HC types to p27CKO. For example, CDKs with overlapping function to p27^{Kip1} could be preferentially expressed in OHCs, thus compensating for the loss of p27^{Kip1} in OHCs, but not in IHCs. Conversely, the expression of cyclins could be lower in OHCs compared with IHCs. Indeed, CDKs and cyclin D1 exhibit dynamic patterns of expression during the postnatal maturation of the cochlea (Laine et al., 2007, 2010), suggesting that some CDKs or cyclins could be differentially expressed in IHCs versus OHCs. Also, we noted a gradient of proliferation in the p27CKO cochlea, being greatest in the apical turn and diminishing toward middle and basal turns. This pattern was replicated in the RbCKO model, suggesting that factors either downstream or independent of Rb are likely responsible for the decreasing proliferative capacity toward the basal turn. For example, E2F transcription factors, which are known cell cycle activators downstream of Rb (Chellappan et al., 1991), could be expressed at higher levels in apical versus basal turns of the cochlea. Indeed, many genes exhibit gradient patterns of expression along the cochlea at postnatal ages (Sato et al., 2009; Son et al., 2012), suggesting that a number of factors could be at work in the current phenotype. Other possible candidates could include extracellular matrix (ECM) and cell adhesion proteins, which can inhibit proliferation and may prevent regenerative phenotypes in mammalian sensory epithelia (Henriet et al., 2000; Stockinger et al., 2001; Burns et al., 2008; McClatchey and Yap, 2012). Indeed, several ECM and cell adhesion genes exhibit increasing expression along the tonotopic axis of the cochlea, which could help explain decreased proliferation in the basal turn in both p27CKO and RbCKO models (Son et al., 2012). In sensorineural hearing loss, the greatest HC loss occurs in OHCs rather than IHCs and in basal turns rather than apical turns (McGill and Schuknecht, 1976), underscoring the importance of characterizing regenerative phenotypes along the longitudinal and radial axes of the cochlea, as well as in uncovering the cause of the apical-to-basal gradient seen here.

One of the more important findings of the current work is that a significant portion of postnatally generated HCs were able to survive into adulthood and express markers found in mature, functioning HCs. Many of these cells expressed the synaptic proteins Ctip2 and VGlut3, and were innervated by Tuj1-positive neurites. Also, these cells exhibited stereociliary bundles that were readily detectable by phalloidin and espin immunostaining. These data suggest that many of the p27CKO daughter HCs could be functional, although individual cell measurements will be required to confirm this. Still, the presence of VGlut3-positive, postnatally derived IHCs represents a significant advance for HC regeneration since neither Atoh1-mediated transdifferentiation of SCs to IHCs, nor the endogenous regeneration of HCs from SCs in neonatal cochleae yields any VGlut3-positive, terminally differentiated IHCs (Cox et al., 2014; Z. Liu et al., 2014). Nor do HCs derived from pluripotent stem cells express all of the markers or attributes of mature cochlear HCs (Oshima et al., 2010; Ronaghi et al., 2014). This discrepancy between autologous p27CKO-mediated generation of IHCs in the postnatal cochlea,

Figure 9. Neonatal, HC-specific RbCKO results in the almost complete loss of cochlear HC and auditory function as measured at 6 weeks of age. **A**, Unlike what is seen in the p27CKO model, immunostaining for the HC-specific marker calbindin (red) reveals a near complete loss of cochlear HCs by 6 weeks of age in RbCKO cochleae. **B**, In contrast, age-matched control samples exhibit a normal contingent of IHCs and OHCs. Nuclei were counterstained with Hoechst (blue). **C, D**, Quantification of IHCs (**C**) and OHCs (**D**) confirm that there are significantly fewer IHCs and OHCs in RbCKO cochleae compared with controls (** $p < 0.0005$). **E**, As expected with such a dramatic loss of both IHCs and OHCs, evoked ABRs could not be obtained from RbCKO mice at any of the frequencies tested between 4 and 44 kHz. In contrast, age-matched control mice had readily measurable hearing thresholds. Scale bar, 20 μ m. Data are presented as mean $\pm$ SEM.

and noncell-autonomous generation of IHCs, suggests that cell-autonomous HC regeneration may provide a more direct route for obtaining mature HCs than approaches that rely on transdifferentiation of SCs or stem cells.

Finally, the neonatal, HC-specific deletion of p27^{Kip1}, unlike models of conditional Rb or p19^{Ink4d} deletion, did not result in widespread HC death (Chen et al., 2003; Weber et al., 2008). This is likely due to the inability of p27CKO to completely abolish Rb activity, thus resulting in less proliferation and less cell death than RbCKO itself. Similarly, there was no hearing loss phenotype in p27CKO mice as measured by evoked ABRs. Again, this is in contrast to what is seen in models of Rb or p19^{Ink4d} deletion (Chen et al., 2003; Sage et al., 2006; Fig. 9). This is also an improvement upon some noncell-autonomous methods for the generation of HCs, where new IHCs either do not survive (Cox et al., 2014) or the addition of immature, transdifferentiated IHCs results in a significant loss of hearing function (Z. Liu et al., 2014). Here, the neonatal CKO of p27^{Kip1} demonstrates that it is not only possible to generate additional HCs from existing HCs in the postnatal mouse cochlea, but that these new HCs can also receive innervation, express markers of maturity, and contribute to, or at least not detract from, hearing function. This is of critical importance because survival of newly generated HCs is an obvious necessity for any regenerative approach, but also because, to our knowledge, it has yet to be accomplished in any model where HCs were produced via proliferation in postnatal animals. Also, it suggests that p27^{Kip1}, which, based on the efficiency of the Atoh1-CreERTM, should have been deleted in >80% of IHCs and >95% of OHCs (Chow et al., 2006), is not critical to mechanotransduc-

tion, cochlear amplification, or other functions of HCs necessary for auditory sensation. While further validation is needed to determine the extent to which these new cells contribute to hearing on an individual level or to discover how much, if at all, they can actually improve hearing in a model of damage, the mere fact that hearing is preserved after postnatal p27CKO represents a significant step forward for cochlear regenerative medicine.

Perhaps the most intriguing aspect of the findings presented here is that they provide a proof of principle for the notion that auditory HC regeneration may not need to follow the SC-to-HC conversion strategy established by nonmammalian vertebrates. Rather, cochlear HC regeneration could be cell autonomous. Indeed, the current data demonstrate that it is possible to make new HCs from existing HCs, and that these HCs can express markers of maturity, survive to adult ages, and remain incorporated into functioning cochleae. Furthermore, recent successes in generating HCs from SCs in the mammalian cochlea generally achieve only partial recovery of HC numbers and function (Kawamoto et al., 2003; Izumikawa et al., 2005, 2008; Kraft et al., 2013; Mizutani et al., 2013; Z. Liu et al., 2014). These approaches, however, could be potentiated by HC-specific p27CKO, which could further contribute to HC number

and functional regeneration by using residual HCs and/or newly transdifferentiated HCs as a cell source. Indeed, cell-autonomous replenishment can occur in other systems, such as the heart and kidney, and has been induced in several models to achieve therapeutic regeneration (Dor et al., 2004; Eulalio et al., 2012; Senyo et al., 2013; Kusaba et al., 2014). Furthermore, transdifferentiation-based approaches generally require the targeting of multiple factors, whereas cell-autonomous regeneration can often be accomplished by the targeting of only one factor, e.g., p27^{Kip1}. Additionally, transdifferentiation approaches often have low efficiencies of conversion, requiring large numbers of progenitor cells, and generally result in incomplete phenotypic conversion (Oshima et al., 2010; Liu et al., 2012c, 2014a; Ronaghi et al., 2014). In contrast, cell-autonomous regeneration has been successfully carried out from reduced numbers of cells in several systems with mature gene expression and preservation of function following proliferative expansion (Dor et al., 2004; Eulalio et al., 2012; Kusaba et al., 2014). In this context, the findings presented here not only help us better understand the role played by p27^{Kip1} in cochlear HCs, but also suggest that p27^{Kip1} may be a viable therapeutic target for HC regeneration in the cochlea and that HCs themselves may be used as a cell source for their own regeneration.

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