Development/Plasticity/Repair

Deletion of *Notch1* during Cochlear Maturation Leads to Rapid Supporting Cell Death and Profound Deafness

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The sensory region of the mammalian hearing organ contains two main cell types—hair cells and supporting cells. During development, Notch signaling plays an important role in whether a cell becomes either a hair cell or supporting cell by mediating lateral inhibition. However, once the cell fate decisions have been determined, little is understood about the role Notch plays in cochlear maturation. Here, we report that deletion of *Notch1* from the early postnatal mouse cochlea in both male and female animals resulted in profound deafness at 6 weeks of age. Histologic analyses at 6 weeks revealed significant hair cell and supporting cell loss throughout the *Notch1*-deficient cochlea. Early analyses revealed a reduction in supporting cells in the outer hair cell region between postnatal day (P) 2 and P6, without a comparable increase in outer hair cell number, suggesting a mechanism other than lateral inhibition. Consistent with this, we found apoptotic cells in the outer supporting cell region of the cochlea at P1 and P2, indicating that *Notch1* is required for outer supporting cell survival during early cochlear maturation. Interestingly, inner supporting cell types were not lost after *Notch1* deletion. Surprisingly, we do not detect outer hair cell loss in *Notch1* mutants until after the onset of hearing, around P14, suggesting that hair cell loss is caused by loss of the supporting cells. Together, these results demonstrate that *Notch1* is required for supporting cell survival during early maturation and that loss of these cells causes later loss of the hair cells and cochlear dysfunction.

Key words: **cell death; cochlea; deafness; hair cells; Notch; supporting cells**

Significance Statement

During development, Notch signaling has been shown to be critical in regulating the cell fate choices between hair cells and supporting cells. However, little is known about how Notch functions after those cell fate choices are made. Here, we examine the role of *Notch1* in the maturing cochlea. We demonstrate that deletion of *Notch1* results in profound deafness by 6 weeks of age. Histologic analyses revealed rapid supporting cell death shortly after *Notch1* deletion, followed by eventual loss of the hair cells. These results reveal an unexpected role for Notch in supporting cell survival during cochlear maturation.

Introduction

The cochlea is a highly ordered structure containing precisely one row of inner hair cells and three rows of outer hair cells, surrounded by associated supporting cells in a pseudostratified epithelium. Each hair cell displays an array of mechanosensory stereocilia, which contain the channels critical for sensory transduction and hearing. The surrounding supporting cells are critical for both structural support and physiological functions of the cochlea (Ramírez-Camacho et al., 2006; Wan et al., 2013), even though many of their functions are unknown. Although lower vertebrates have some capacity to regenerate hair cells and supporting cells (Stone and Cotanche, 1994; Taylor and Forge, 2005; Brignull et al., 2009; Monroe et al., 2015; Franco and Malgrange, 2017), mammals, including humans, cannot replace damaged cochlear cells, leading to permanent hearing loss. A better understanding of the molecular mechanisms underlying the development and maintenance of the cochlear sensory region is important for developing strategies to preserve or regenerate these critical cell types.

Notch signaling is evolutionarily conserved in all metazoans and is required for cell fate decisions during development. Canonical Notch signaling occurs via interaction of a Notch ligand (JAGGED1-2, DLL1,3,−4) expressed on one cell with a transmembrane-bound Notch receptor (NOTCH1-4) on a neighboring cell. Interaction of a Notch ligand with its receptor results in cleavage of the Notch1 intracellular domain (NICD), which translocates to the nucleus and derepresses the CSL/RBPJk complex from Notch target genes, including those

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in the *Hes* family (Lanford et al., 1999; Mumm and Kopan, 2000; Zine et al., 2000; Kopan and Ilagan, 2009). Dysregulation and mutations in components of the Notch pathway have been linked to many diseases, including cancer (Lasky and Wu, 2005; Penton et al., 2012).

Notch signaling has been shown to be required for both the early development and subsequent differentiation of the cochlear sensory region during embryogenesis (Lanford et al., 1999; Zine et al., 2000). In a process known as lateral induction, JAG1/NOTCH are expressed in the prosensory region and act via a positive feedback mechanism to establish the inner ear sensory domains (Kiernan et al., 2001, 2006; Pan et al., 2010; Neves et al., 2011). Later in embryogenesis, Notch signaling is required for hair cell and supporting cell fate decisions in the cochlea via lateral inhibition. During this time, the Notch ligands JAG2 and DLL1 are expressed in the nascent hair cells and activate the NOTCH1 receptor in the surrounding cells, inhibiting their development as hair cells and resulting in their adoption of the supporting cell fate (Lanford et al., 1999; Zine et al., 2000; Kiernan et al., 2006). As predicted from the lateral inhibition model, loss of both *Jag2* and *Dll1* resulted in extra inner and outer hair cells, and conditional deletion of *Notch1* also exhibited extra hair cells at the expense of the supporting cells below, suggesting a change in cell fate (Kiernan et al., 2005). Previously, postnatal manipulations of Notch signaling indicated that NOTCH1 continues to play a role in lateral inhibition in the more apical regions of the cochlea and also inhibits proliferation (Li et al., 2015; Ni et al., 2016). However, the longer-term effects of Notch perturbations on cochlear function were unclear.

Here, we examined the role of Notch signaling during cochlear maturation by deleting *Notch1* in supporting cells at postnatal day (P)0/P1. We found that deletion led to profound hearing loss by 6 weeks, measured by auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE) auditory tests. Examination of the organ of Corti by immunohistochemical and scanning electron microscopy ultrastructural analyses revealed that the sensory region was void of almost all outer hair cells and supporting cells. Early postnatal analysis showed rapid loss of Deiters' and outer pillar cells by P2–P6 without a comparable increase in outer hair cell number, indicating the supporting cells were not becoming hair cells because of loss of lateral inhibition. Indeed, apoptotic cells were detected throughout the cochlea in these supporting cell layer as early as P1/P2. Interestingly, supporting cell types in the inner hair cell region, including border/phalangeal cells and Hensen's cells, were not similarly affected. Finally, loss of the outer hair cells did not occur until after the onset of hearing, almost 2 weeks after loss of the supporting cells. These results indicate the hair cell loss is a secondary consequence of loss of Notch signaling, likely because of missing supporting cells. Together these results demonstrate that *Notch1* is required for early supporting cell survival and the ultimate integrity of the organ of Corti during cochlear maturation.

Materials and Methods

Animals and tamoxifen treatment

All procedures were performed in accordance with guidelines and regulations of the University of Rochester Medical Center and the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All animal experiments were approved by the University of Rochester Committee on Animal Resources. The following mouse strains were used: Sox2-CreER (stock #017593, The Jackson Laboratory; Arnold et al., 2011) and Notch1^{flox} (a gift from Raphael Kopan; Yang et al., 2004). Animals were genotyped using the following primers: Cre1: 5'-TGA TGA GGT TCG CAA GAA CC and Cre2 5'-CCA TGA GTG AAC GAA CCT GG (yielding a 350 bp product) and Notch1F: 5'-TGC CCT TTC CTT AAA AGT GG and Notch1R: 5'-GCC TAC TCC GAC ACC CAA TA (mutants had a 281 bp product). Tamoxifen (Sigma-Aldrich) that was dissolved in corn oil was injected within 4 h of birth (P0) and 24 h later (P1). Pups were given intramuscular injections of tamoxifen (37.5 μg/g body weight) into the muscle on the left leg, parallel to vein. Animal pups that were used for uninjected controls came from litters that we born and aged the same as those that received tamoxifen injection. Both male and female mice were used in this study.

Auditory testing

ABR and DPOAE auditory testing was conducted using a Duet System (Intelligent Hearing Systems). Six-week-old mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg; Par Pharmaceuticals) in a sterile acepromazine/saline mixture (3 mg/kg). For ABR recordings, electrical responses were measured with three subdermal needle electrodes, one inserted beneath each pinna, and a ground electrode placed on the head. ABR stimuli were 5 ms tone pips presented at four frequencies between 8 and 32 kHz that began at 75 dB amplitude and decreased by 5 dB steps to 15–25 dB. Five hundred twelve sweeps were averaged for each frequency and amplitude. ABR thresholds for a particular frequency were determined by the last visible trace where waves 1 and 2 were seen for each stimulus (dB). For DPOAE recordings, a 10B+ high-frequency transducer/stimulator probe was placed at the opening of the ear canal. We measured the amplitude of evoked otoacoustic emissions to paired pure tones of frequencies f1 and f2, where f1/f2 = 1.2 and the f1 level was 10 dB above f2. Thirty-two sweeps were made in 5 dB steps starting with f1 at 10 dB and ending at 65 dB. Here, we report the f2 value at 55 dB measured at different frequencies between 8 and 32 kHz as this was the upper threshold tested.

Scanning electron microscopy tissue preparation and imaging

The bony labyrinth was dissected from 6-week-old mice and immediately fixed in 0.1 M phosphate buffer/2.5% glutaraldehyde overnight at 4°C. Bone surrounding the cochlea was carefully removed, exposing the organ of Corti. Samples were carried through the osmium-thiocarbohydrazide-osmium with repetition (OTOTO) procedure by the University of Rochester Electron Microscopy core facility. All cochlea were sputter coated in gold before imaging on a Zeiss Auriga scanning electron microscope, operating at 5 kV in secondary electron detector mode on the University of Rochester River Campus.

Cochlea fixation and immunostaining

Fixation. Cochlea were fixed overnight in 4% paraformaldehyde (Santa Cruz Biotechnology) at 4°C on a rocker. Tissue older than P10 was decalcified in 0.2 M EDTA, pH 7.4, for 3–14 d at 4°C with gentle rocking.

Whole mount and confocal imaging. Cochleae were dissected and immersed in 30% sucrose and flash frozen in liquid nitrogen for a freeze-thaw antigen retrieval. Cochleae were then washed in PBSTx (PBS with 0.2% Triton X-100) and blocked for 1–2 h in PBSTx/10% horse serum (Sigma-Aldrich). Primary antibody incubations were done overnight at 4°C in PBSTx/5% horse serum. The following day, cochleae were washed in PBSTx and incubated in secondary antibodies in PBSTx/5% horse serum for 2 h. After additional washes, cochleae were separated into apical, middle, and basal turns and mounted in Fluoro-Gel (Electron Microscopy Sciences). Confocal imaging was done using a Zeiss LSM 510 META confocal microscope with Zen 2009 software and a Nikon A1-R confocal microscope with Nikon NIS-Elements software.

Paraffin sections and immunofluorescence. After fixation and decalcification, tissue was dehydrated through a series of EtOH washes from 30% to 100%, cleared in xylene, embedded in paraffin, and sectioned at a thickness of 10 μm using a Microm HM310 microtome and dried on Superfrost Plus slides (Fisher Scientific). For antibody staining, slides were deparaffinized and rehydrated through a series of xylene/ethanol

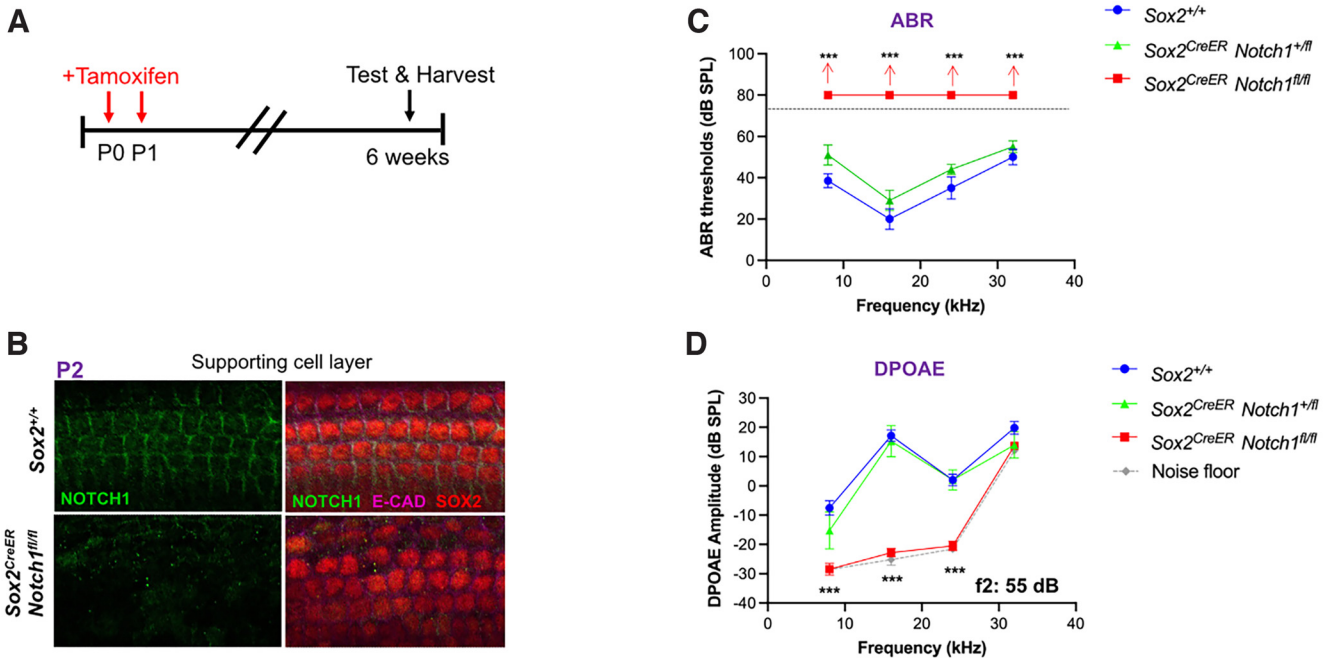

Figure 1. Deletion of *Notch1* at P0/P1 resulted in profound hearing loss at 6 weeks. ***A***, Experimental strategy. Tamoxifen was administered at P0 and P1, and animals underwent hearing tests before tissue collection and analysis 6 weeks later. ***B***, Deletion of *Notch1* at P0/P1 resulted in a significant loss of NOTCH1 protein by P2 in the supporting cell layer. ***C***, ABR thresholds measured from control ($Sox2^{+/+}$, blue circles), heterozygotes ($Sox2^{CreER}$ $Notch1^{+/fl}$, green triangles) and mutants ($Sox2^{ER}$ $Notch1^{fl/fl}$, red squares) at four frequencies between 8 and 32 kHz. Any mutant responses were above the highest value examined (75 dB, dashed line), and control and heterozygote responses were not significantly different from each other. ***D***, DPOAEs measured from control ($Sox2^{+/+}$), heterozygotes ($Sox2^{CreER}$ $Notch1^{+/fl}$), and mutants ($Sox2^{CreER}$ $Notch1^{fl/fl}$) between 8 and 32 kHz. The values plotted are the DPOAE amplitudes recorded at f2 = 55 dB, which is the highest sound level tested at each frequency. For both hearing tests, at least five mice were tested per genotype. Statistical significance was determined by two-way ANOVA and Bonferroni *post hoc* tests; ***$p < 0.001$. Error bars indicate SEM.

washes, and then antigen retrieval was performed by incubating in slides in boiling 10 mM sodium citrate buffer, pH 6, for 10 min. Tissue was blocked with 10% horse serum/PBS for 1–2 h and then incubated in primary antibodies in PBS/5% horse serum overnight at 4°C. The following day the slides were washed, tissue was incubated in secondary antibodies for 2 h at room temperature, and then slides were washed and mounted in Fluoro-Gel. All images were taken on a Zeiss Axio microscope using AxioVision SE64 software. For some antibodies used (Myo7a in particular), the strong antigen retrieval required for optimal staining resulted in tissue that appears slightly fuzzy in some images.

Antibodies. The following primary antibodies were used for confocal imaging: anti-Notch1 (rabbit, 1:250; Abcam), anti-E-cadherin (mouse, 1:400; BD Biosciences), anti-Sox2 (goat, 1:800; Santa Cruz Biotechnology), anti-Prox1 (rabbit, 1:1000; EMD Millipore), anti-Myo7a (rabbit, 1:1000; Proteus), anti-cleaved-Casp3 (rabbit, 1:1000; R&D Systems), anti-Fabp7 (rabbit, 1:500; Abcam), anti-Parvalbumin (mouse, 1:250; Sigma) and anti-Glast (rabbit, 1:500; Abcam). Secondary antibodies used for confocal analysis were Alexa Fluor 488 donkey anti-rabbit (1:500; Invitrogen), Alexa Fluor 546 donkey anti-goat (1:500; Invitrogen), DyLight 405 AffiniPure donkey anti-goat (1:250; Jackson ImmunoResearch), Alexa Fluor 647 donkey anti-mouse (1:500; Invitrogen), and Alexa Fluor 488 donkey anti-goat (1:500; Invitrogen). For immunofluorescence, the following antibodies were used: anti-Sox2 (goat, 1:800; Santa Cruz Biotechnology), anti-Myo7a (rabbit, 1:1000; Proteus), anti-Prox1 (rabbit, 1:2000; EMD Millipore), anti-CD44 (rat, 1:400; BD Pharmingen), anti-Parvalbumin (mouse, 1:500; Sigma-Aldrich), anti-Glast (rabbit, 1:700; Abcam), and anti-p75 (1:1000; Cell Signaling Technology). Secondary antibodies used with immunofluorescence were Alexa Fluor 488 donkey anti-rabbit (1:500; Invitrogen), Alexa Fluor 546 donkey anti-goat (1:500; Invitrogen), Cy3-conjugated donkey anti-rat (1:500; Jackson ImmunoResearch), Alexa Fluor 647 donkey anti-goat (1:500; Invitrogen), and Alexa Fluor 555 donkey anti-mouse (1:500; Invitrogen).

Quantification and statistical analysis. Significant differences in ABR and DPOAE thresholds were determined through two-way ANOVA tests, followed by Bonferroni *post hoc* analyses using Prism software. All p values <0.05 were considered significant. Quantification of all supporting cells and hair cells were performed by counting the number of cells in 200–300 micron regions of the apex, middle, and basal turns. Both one-way ANOVA with Tukey's *post hoc* test and two-way ANOVA with Bonferroni's *post hoc* tests were used to determine significant differences in cell counts. For all, a p value of <0.05 was considered significant.

Results

Notch1 deletion at P0/P1 resulted in profound hearing loss and substantial cellular loss throughout the organ of Corti of adult mice

Although the expression patterns and roles of Notch signaling components have been studied during embryogenesis, little is known about possible role(s) of Notch receptors postnatally. Both *Notch1* and *Notch3* transcript expression is widespread in the organ of Corti from P0 to P6 (Maass et al., 2015), and single-cell RNAseq has identified *Notch1* transcripts in supporting cells within the first week after birth (Cheng et al., 2019). Additionally, components of the Notch pathway have been shown to downregulate their expression during the first week after birth (Maass et al., 2015). To examine the role of *Notch1* during postnatal cochlear maturation, we deleted *Notch1* in most supporting cells of the cochlea by crossing an inducible $Sox2^{CreER}$ mouse line (Arnold et al., 2011) to $Notch1^{flox/flox}$ mice (Yang et al., 2004), which removes exon 1 of the *Notch1* gene. In our studies, a single tamoxifen injection within hours after birth (P0) and again 24 h later (P1) resulted in significant reduction of NOTCH1 protein levels in the cochlea (Fig. 1*B*).

Previously, Li et al. (2015) examined the effects of *Notch1* deletion in the postnatal cochlea and found that deletion at P0/P1

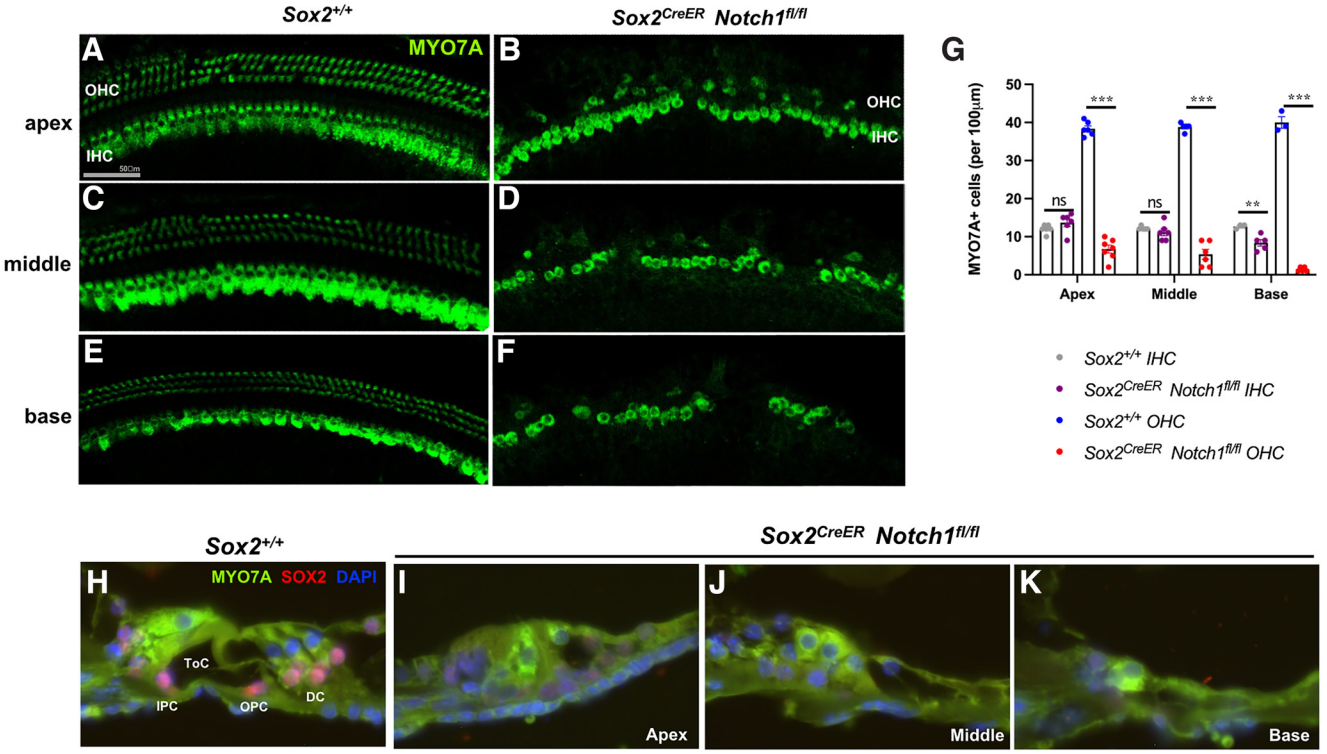

Figure 2. *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants had significant loss of supporting cells and outer hair cells at 6 weeks and disorganized inner hair cells. ***A–F***, Control cochlea (*Sox2*$^{+/+}$) had one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) in the apical (***A***), middle (***C***), and basal (***E***) turns of the cochlea, shown by MYO7A staining. In *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants (***B***, ***D***, ***F***), the row of inner hair cells was disorganized with some areas missing cells. There were more outer hair cells present in the apex (***B***) compared with the base (***F***) in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants. ***G***, Quantification of IHC and OHC numbers from each cochlear turn. Although the number of IHCs was only significantly different in the base in *Notch1* mutants compared with controls, the number of OHCs was highly significant between the two groups. Three to five cochleae were analyzed per genotype. Statistical significance was determined by a one-way ANOVA test followed by Tukey's *post hoc* test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns: not significant. Error bars indicate SEM. ***H–K***, Immunofluorescence staining of 6 week paraffin sections showed significant loss of both hair cells (MYO7A, green) and supporting outer pillar cells (OPC) and inner pillar cells (IPC) and Deiters' cells (DC; SOX2, red) below in all cochlear turns in *Sox2*^{ER} *Notch1*$^{fl/fl}$ mutants. The tunnel of Corti (ToC) was also absent from all cochlear turns in *Notch1* mutants.

caused excess hair cell production in the apical regions of the cochlea and some abnormal proliferation in the cochlea. However, these studies did not examine the effects of loss of *Notch1* on later maturation of the cochlea and function. To examine how loss of *Notch1* during cochlear maturation affected hearing in mice, we performed ABR and DPOAE auditory tests on mice 6 weeks after *Notch1* deletion. During the ABR tests, we found that *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mice (*Notch1* mutants; Fig. 1*C*, red) failed to show responses to sound in contrast to their control littermates (Fig. 1*C*, blue), indicating profound deafness. DPOAE amplitudes in *Notch1* mutants across these same frequencies were comparable to the noise floor, whereas control (*Sox2*$^{+/+}$) and heterozygous (*Sox2*^{CreER} *Notch1*$^{+/fl}$) mice had significantly higher DPOAE amplitudes at f_2 = 55 dB (Fig. 1*D*), indicating significantly compromised outer hair cell function. Together, these results show loss of *Notch1* during cochlear maturation resulted in auditory dysfunction by 6 weeks of age.

To investigate the cause of compromised auditory function in our *Notch1* mutants, we analyzed cochleae from these 6-week-old mice using confocal imaging and immunofluorescence (Fig. 2). We found that although *Sox2*^{CreER} *Notch1*$^{fl/fl}$ cochleae did have most of their inner hair cells, these cells had an abnormal, more rounded appearance compared with controls (Fig. 2*B*,*D*,*F* vs *A*,*C*,*E*). Quantification of inner hair cells using a MYO7A antibody in the apical and middle turns revealed no significant difference in *Notch1* mutants compared with controls, despite having a disorganized appearance (Fig. 2*G*). However, in the base of the cochlea, the number of inner hair cells in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ cochleae was significantly reduced compared with controls (Fig. 2*G*). Specifically, these mutant cochleae showed gaps in the inner hair cell row (Fig. 2*F*). In addition to inner hair cell differences, there was a striking absence of outer hair cells in all cochlear turns of *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mice, with ~80% of outer hair cells missing in the apical and middle turns, and >90% missing in the basal region (Fig. 2*B*,*D*,*F*,*G*). Immunofluorescence imaging of representative paraffin cross sections of all cochlear regions confirmed fewer MYO7A+ hair cells and also showed that most supporting cells in the organ of Corti were absent, indicated by a loss of DAPI and SOX2-positive nuclei compared with controls (Fig. 2*H–K*, red). In addition, the tunnel of Corti was not apparent in all cochlear turns at 6 weeks (Fig. 2*I–K*).

Further examination of the organ of Corti ultrastructure by scanning electron microscopy at 6 weeks confirmed that in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants most of the sensory region was void of outer hair cells (Fig. 3). The outer hair cells that remained had few or no stereocilia in the apical and middle turns (Fig. 3*D*,*E*). Although many of the inner hair cells had stereocilia with normal morphology, there were some stereocilia fusions in the apical and middle regions, as well as some inner hair cells with missing or elongated stereocilia (Fig. 3*D*,*E*, asterisks). In the base, some inner hair cells had reduced stereocilia (Fig. 3*F*, arrow). Additionally, as observed in the whole mount analysis (Fig. 2), there were regions missing inner hair cells altogether in the middle and base of the mutant cochlea. Together, these results show

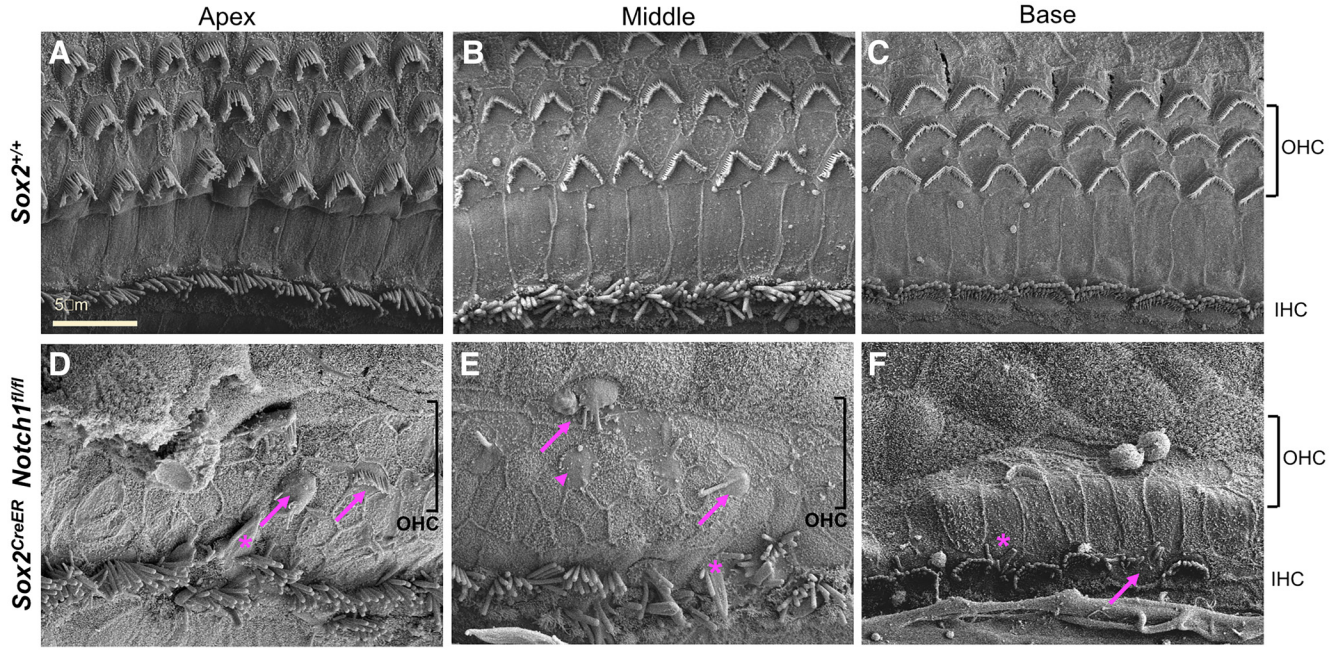

Figure 3. ***A–F***, Scanning electron microscopy of *Sox2*$^{+/+}$ control (***A–C***) and *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants (***D–F***) at 6 weeks. Control cochleae have one row of inner and three rows of outer hair cells with stereocilia of normal morphology in the apex (***A***), middle (***B***) and base (***C***). Although some of the *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant inner hair cells have stereocilia fusions that are found in all turns (***D–F***, asterisks), and some inner hair cells with fewer stereocilia were found in the base (***F***, arrow), the outer hair cells are largely absent from all turns in the mutants, with most having fewer stereocilia (***D***, ***E***, arrows) and some lacking all stereocilia (***E***, arrowhead). At least five cochleae were analyzed per genotype; representative images are shown.

that deletion of *Notch1* at P0/P1 resulted in loss of supporting cells, most outer hair cells, abnormal or missing inner hair cells, and profound deafness at 6 weeks.

Notch1 deletion at P0/P1 resulted in significant loss of outer pillar and Deiters' cells by P6

Because we observed a substantial loss of supporting cells and hair cells 6 weeks after *Notch1* deletion at P0/P1, we wanted to investigate when and how these cells were lost. We found at P6 there was significant loss of PROX1-positive cells—a marker for pillar and Deiters' cells (Bermingham-McDonogh et al., 2006)—throughout the cochlea of *Notch1* mutants (Fig. 4*A–C*, *G–I*, *O–T*). In the middle and basal cochlear turns, this loss of PROX1+ cells mostly consisted of Deiters' cells, whereas a majority of outer pillar cells were still present, seen by CD44 staining (Hertzano et al., 2010; Fig. 4*O–T*, red). Additionally, we examined whether the loss of supporting cells was caused by an increase in hair cells, indicating *Notch1* is important in maintaining lateral inhibition postnatally (Kiernan et al., 2005). However, although we observed some increase in outer hair cell numbers in cochleae (Fig. 4*K–L* vs *E–F*,*N*), this increase doesn't account for the number of supporting cells that were lost (Fig. 4*M*). In the apical region, there was ~40% increase in outer hair cells compared with controls (Fig. 4*D* vs *J*,*N*), but ~80% reduction in PROX1+ cells (Fig. 4*A* vs *G*,*M*). In the middle and basal regions, there is a <10% increase in outer hair cells (Fig. 4*K–L* vs *E–F*,*N*) but a 50–60% loss of supporting cells below (Fig. 4*H–I* vs *B*,*C*, *M*). Together, these results indicate that *Notch1* has a role outside lateral inhibition during cochlear maturation.

Notch1 is required for outer pillar and Deiters' supporting cell survival during early cochlear maturation

As there was already significant supporting cell loss observed at P6, we looked earlier at P2 to see whether there was any difference in hair cell and supporting cell numbers. Surprisingly, given that deletion had only taken place 24–48 h previously, by P2 there was already a significant difference in the number of PROX1+ cells in all regions of the cochlea, with the greatest number of PROX1+ cells absent from the apical region (Fig. 5*G–I* vs *A–C*,*M*). Several of these cells in the middle and basal regions, especially in the second and third row of Deiters' cells, had enlarged nuclei and were arranged abnormally (Fig. 5*H–I*). We next examined and quantified hair cells to see whether we saw an increase similar to that at P6 (Fig. 5*J–L* vs *D–F*,*N*). We did not see a significant increase in the number of inner or outer hair cells present at P2 in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mice in any region of the cochlea compared with controls (Fig. 5*N*), suggesting there is loss of supporting cells from the cochlear sensory region that is not because of a loss of lateral inhibition.

The observation that some Deiters' cells appeared abnormal and had larger nuclei suggested that these cells may be undergoing cell death. To this end, we stained for apoptotic cells in the cochlea using a cleaved-Caspase3 antibody (cCASP3), and found a significant number of cCASP3+ cells in the supporting cell layer in apical, middle, and basal regions of the *Notch1* mutant cochlea at P1 and P2 compared with controls (Fig. 6). Interestingly, although the overall number of cCASP3+ cells throughout the *Notch1* mutant cochlea was not different between the P1 and P2 time points (Fig. 6*C*), there were more cCASP3-positive cells in the apex of the cochlea 4 h after the P1 tamoxifen injection compared with the middle and base (Fig. 6*D*). At P2, more of the apoptotic cells were detected in the middle and basal regions (Fig. 6*E*). Additionally, no cCASP3+ cells were detected in hair cells in control or *Notch1* mutant cochleae. Together, these results indicate that NOTCH1 expression is required for supporting cell survival after birth, and in its absence cell death occurs rapidly.

Outer hair cell loss does not occur until the onset of hearing, despite earlier loss of supporting cells

Our results demonstrate a clear loss of PROX1+ supporting cells soon after *Notch1* deletion at P0/P1. However, although there

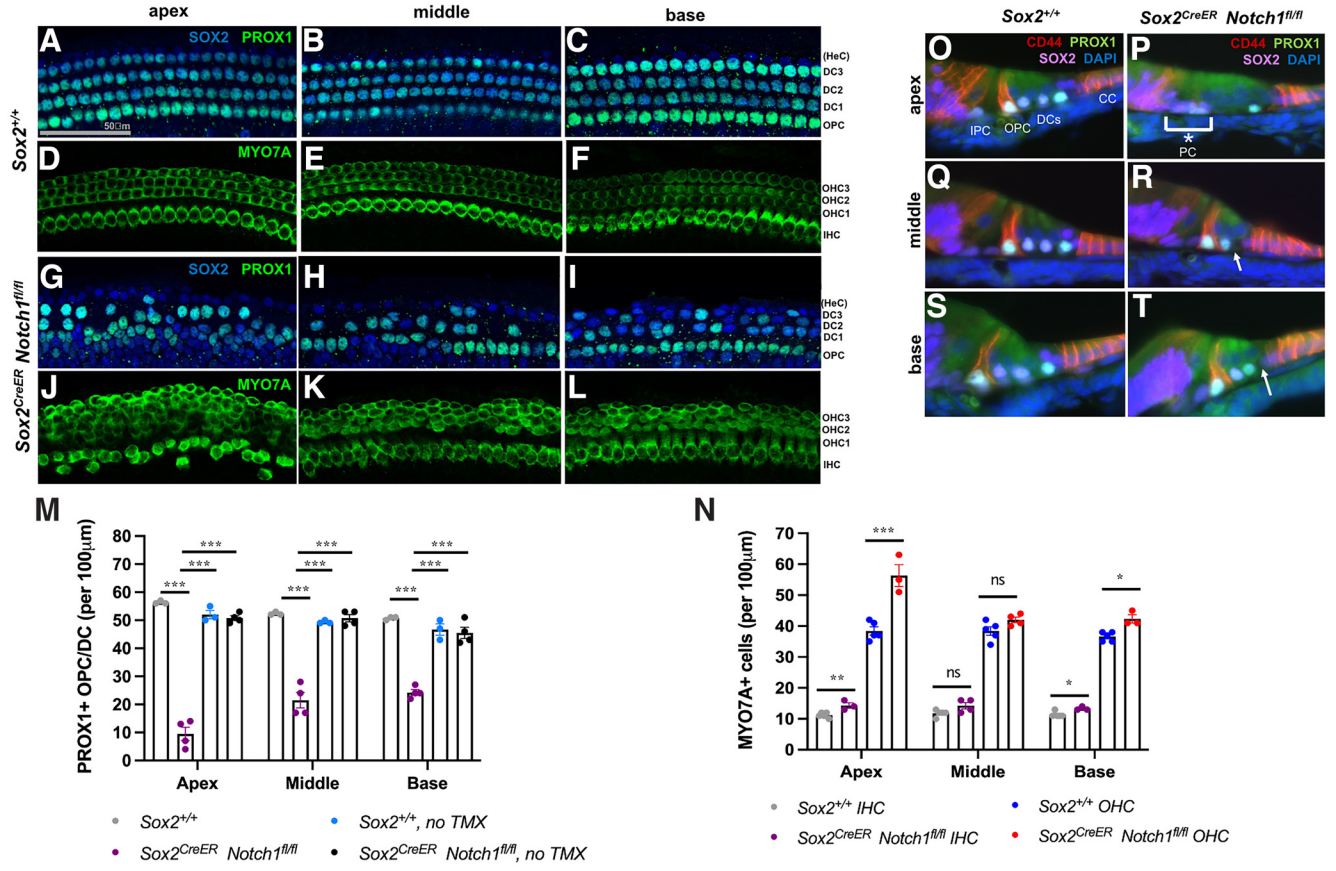

Figure 4. *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants show significant loss of outer pillar and Deiters' cells at P6. ***A–C***, *Sox2*$^{+/+}$ control cochleae had one row of outer pillar cells (OPC) and three rows of Deiters' supporting cells (DC) that were PROX1+ at P6. ***D–F***, Controls also had one row of inner hair cells (IHC) and three rows of outer hair cells (OHC). ***G–I***, *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant cochleae had fewer PROX1+ supporting cells in all cochlear turns, with the most missing in the apex. PROX1+ cells that remain were disorganized. ***J–L***, MYO7A staining revealed an increase in both IHCs and OHCs in the apex, as well as a minor increase in OHCs in the base in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants. OHCs in all turns appeared disorganized and clumped together. ***M***, ***N***, Quantification and statistical analysis of PROX1+ supporting cells (***M***) and hair cells (***N***) counted in the apex, middle, and basal cochlear regions of *Sox2*$^{+/+}$ and *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants and uninjected controls. Three to five cochleae were analyzed per genotype. Statistical significance was determined by a one-way ANOVA test followed by Tukey's *post hoc* test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant. ***O–T***, Immunofluorescence staining of P6 cochlear sections exhibit supporting cell loss and an increase of cells in the pillar cell region (as previously reported by Li et al., 2015). Representative cross sections of the apical (***O***, ***P***), middle (***Q***, ***R***), and basal (***S***, ***T***) turns of control and mutant cochlea. In *Sox2*$^{+/+}$ controls (***O***, ***Q***, ***S***), CD44 labels the outer pillar cell (OPC) and PROX1 labels the outer pillar and three Deiters' cells (DC). *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant cochlea exhibit a loss of almost all PROX1+ cells in the apex (***R***) and some PROX1+ cells—most prominently in the outer Deiters' cells—in the middle and base (***R***, ***T***, arrow). *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants also had an increased number of cells in the pillar cell region (***O*** vs ***P***, asterisk). HeC: Hensen's cells; CC, Claudius cells.

was no apparent hair cell loss observed at P6, at 6 weeks a majority of outer hair cells were absent. To examine when these hair cells were lost in our *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants, we quantified the number of MYO7A+ inner and outer hair cells in the apical, middle, and basal regions of cochlea before the onset of hearing (P10), shortly after the onset of hearing (P14), and at the end of cochlear maturation (P21) (Figs. 7, 8). We found that at P10, before the onset of hearing, there were significantly more MYO7A+ inner hair cells compared with controls in all cochlear turns, as seen in whole mount analysis (Fig. 7*A–D*). Although the number of outer hair cells was initially significantly higher in the apex of *Notch1* mutants (Fig. 7*D*), outer hair cells appeared disorganized and clumped together throughout the length of the cochlea, likely because of loss of supporting cells below (Fig. 7*A'–C'*). Interestingly, many of these outer hair cells, particularly those in the apex and middle cochlea, do not appear healthy and are likely on their way to being lost (Figs. 7*A',B'*, 8*D,E*). At P14, shortly after the onset of hearing, we observed ~40–50% of outer hair cell loss from the apical and middle cochlear regions, but no significant loss from the base (Figs. 7*E–H*, 8*L*), suggesting that hair cell loss in our *Sox2*^{CreER} *Notch1*$^{fl/fl}$ animals begins around the onset of hearing and progresses in an apical to basal direction. By P21, at the end of cochlear maturation, there was drastic outer hair cell loss from all cochlear turns, including the basal region (Figs. 7*I–L*, 8*R*). Additionally, the tunnel of Corti was still maintained in the basal region at the end of cochlear maturation, but was absent by 6 weeks, suggesting that there may be further cell loss that occurs after cochlear maturation. Also, there may be loss of inner hair cells as maturation progresses as there are significantly more inner hair cells in some regions at P6 and P10, but this significance is lost over time. By P21, the remaining hair cells exhibited a more rounded appearance, rather than their usual flask-shaped morphology (Fig. 8*P,Q*). Given the delay in outer hair cell loss, these results indicate it is likely a secondary consequence of loss of the underlying supporting cells.

Notch1 deletion caused expansion in the inner pillar cell region but did not alter Hensen's cells or inner phalangeal supporting cells

In our experimental design, *Notch1* was deleted from supporting cells throughout the cochlea in cells that express SOX2 at P0/P1, including Hensen's, Deiters', and pillar cells; inner phalangeal

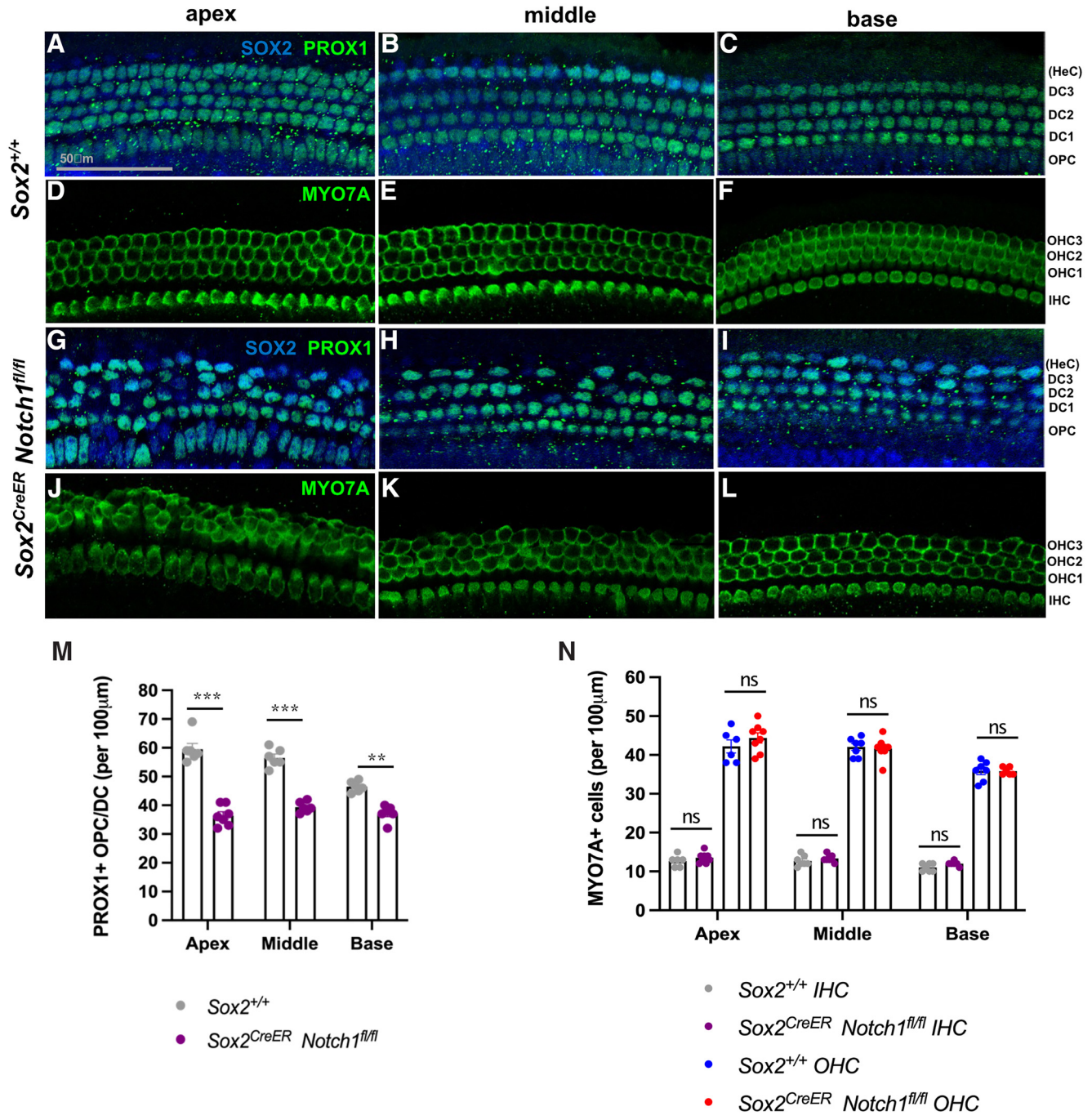

Figure 5. *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants have significant loss of PROX1+ cells with no apparent increase in hair cells at P2. ***A–C***, Control cochleae (*Sox2*$^{+/+}$) had one row of outer pillar cells (OPC) and three rows of Deiters' supporting cells (DC) that are PROX1+ at P2. ***D–F***, Controls also had one row of inner hair cells (IHC) and three rows of outer hair cells (OHC). ***G–I***, *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant cochlea had fewer Prox1+ supporting cells in all cochlear turns. Many nuclei in the middle and base regions appeared larger and misshapen. ***J–L***, MYO7A staining revealed no apparent increase in IHC or OHC throughout the cochlea of *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants, although OHCs clumped together in the apex. ***M***, ***N***, Quantification of and statistical analysis of PROX1+ supporting cells (***M***) and hair cells (***N***) counted in the apex, middle, and base regions of *Sox2*$^{+/+}$ and *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants. Five to six cochleae were analyzed per genotype. Statistical significance was determined by a one-way ANOVA test followed by Tukey's *post hoc* test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.

cells; and cells in Kölliker's organ (Arnold et al., 2011). *Notch1* is expressed throughout the organ of Corti during the first week of cochlear maturation (Maass et al., 2015), suggesting NOTCH1 may function in the other supporting cell types outside of outer pillar and Deiters' cells. To this end, we examined expression of markers known to be expressed in these different cell types during cochlear maturation. Previously, Li et al. (2015) reported increased numbers of hair cells in the apical regions and increased proliferation in the pillar cell region after *Notch1* deletion in the postnatal cochlea. Although they found that a small number of hair cells were EdU+ (~15 hair cells per cochlea), the majority of EdU+ labeled cells were supporting cells (SOX2+) in the pillar cell region. Similarly, we observed an increase of cells in the pillar cell region of the apex of the cochlea at P6 (Fig. 4*P*, asterisk). By P10–P14, there were excess inner pillar cells in the apex (Fig. 8*D*,*J*, asterisks), which persist until at least P21 (Fig. 8*P*, asterisks), although the cochlea is clearly undergoing cellular loss of both supporting cell

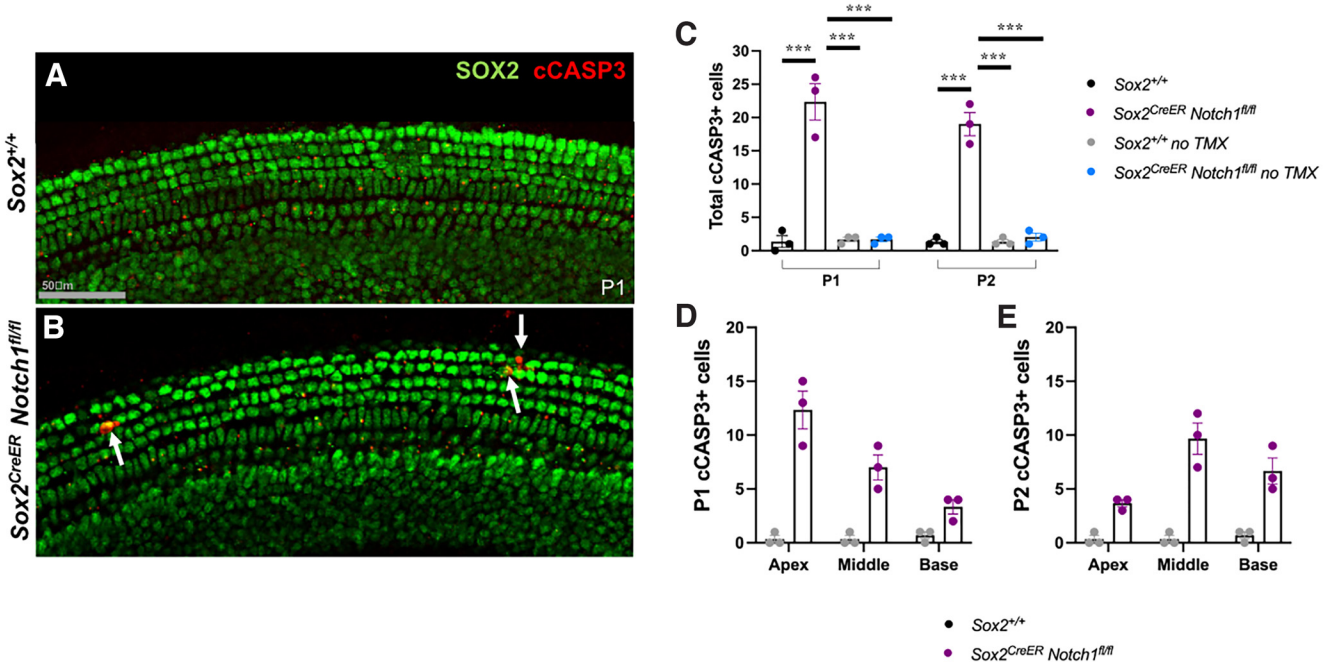

Figure 6. cCaspase3-positive supporting outer pillar and Deiters' cells were detected throughout *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant cochlea at P1/P2. ***A***, ***B***, Whereas *Sox2*$^{+/+}$ control cochlea had few to no cCASP3+ cells (***A***), cCASP3+ cells could be found in supporting cells in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant cochlea (***B***, arrows). ***C***, Quantification and statistical analysis of the total number cCASP3+ cells at P1 and P2 in control, *Notch1* mutant cochlea, and cochlea from animals that received no tamoxifen treatment. A representative image from the middle of the cochlea is shown in ***A***, ***B***. Three cochleae were analyzed for each genotype at each time point. Statistical significance was determined by a one-way ANOVA test followed by Bonferroni's *post hoc* test; ***$p < 0.001$. ***D***, ***E***, Distribution of cCASP3+ cells at P1 (***D***) and P2 (***E***) revealed that at P1 there was an apical to basal gradient in the number of cells undergoing apoptosis (***D***) and at P2 there were more apoptotic cells in the middle and base of mutant cochlea (***E***).

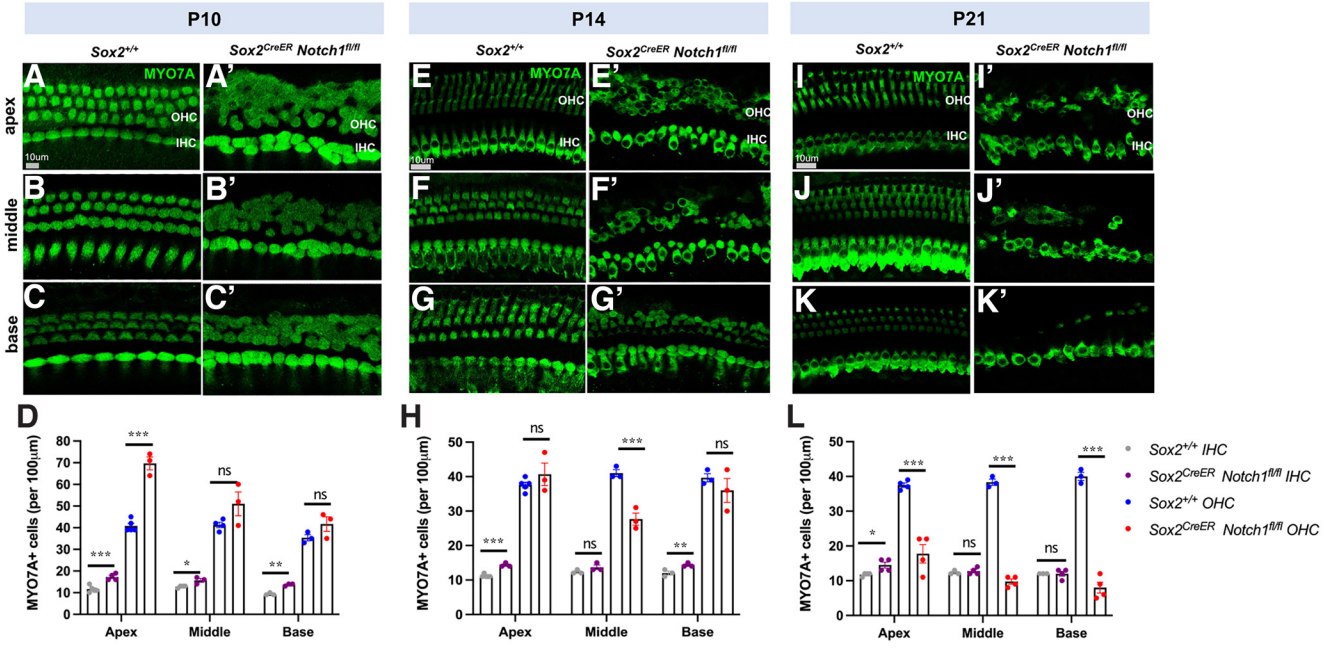

Figure 7. Hair cell loss occurs after the onset of hearing in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants. ***A–C′***, At P10, immunofluorescence and confocal microscopy showed that compared with controls (***A–C***), *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants had more outer hair cells in the apex (***A′***) and comparable numbers to controls in the middle (***B′***) and base (***C′***). ***D***, Quantification of IHC and OHC at P10 showed significance in IHC numbers between controls and mutants and OHC numbers in the apex. ***E–G′***, At P14, after the onset of hearing, there were less MYO7+ OHCs in the apex and middle cochlear regions (***E′***, ***F′***) compared with P10. The number of hair cells in the base of *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants was comparable to controls (***G′*** vs ***G***). ***H***, Quantification of hair cell numbers at P14 confirmed a reduction in OHC number from P10 in the apex and middle regions. ***I–K′***, By P21, the end of cochlear maturation, there were significantly less OHCs in all cochlear turns compared with controls, whereas IHCs were still present (***I–K*** vs ***I′–K′***). ***L***, Quantification of IHC and OHC at P21 in controls and *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants. Three to five cochleae were examined for each genotype at each time point. For all time points, statistical significance was determined by a one-way ANOVA test followed by Bonferroni's *post hoc* test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.

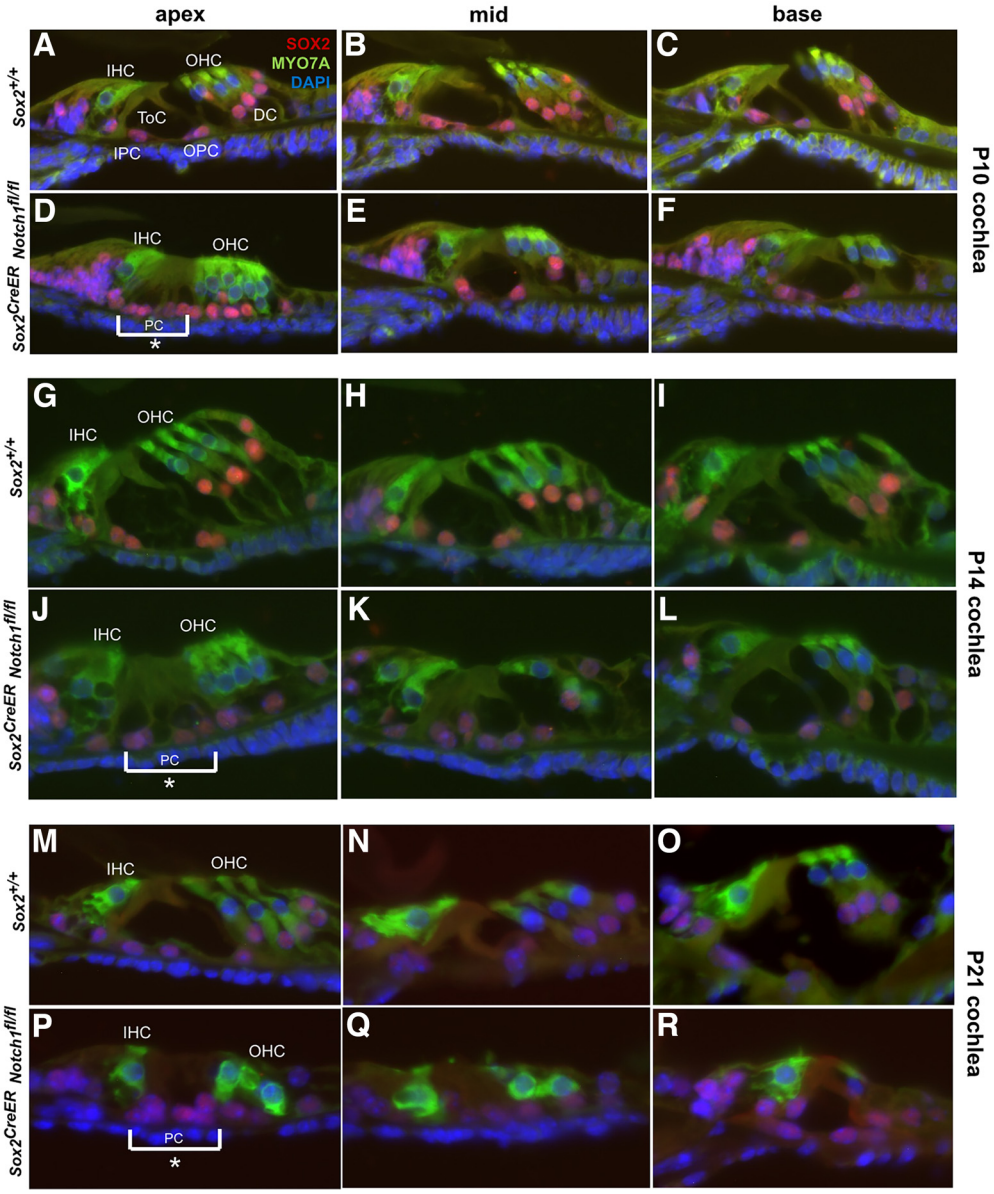

Figure 8. Immunofluorescence staining of P10, P14 and P21 cochlear sections showed OHC loss over time in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants. At P10, Notch1 mutants still had OHCs throughout the cochlea, with excess hair cells in the apex (***D–F*** compared to controls, ***A–C***). ***G–L***, By P14, OHC loss in the apex and middle regions was apparent (***J***, ***K***), while OHCs in the base remained (***L***) in Notch1 mutants compared to controls (***G–I***). At P21, there were missing OHCs from all cochlear regions in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants, and OHCs that did remain had an abnormal, rounded appearance (***P–R*** compared to controls, ***M–O***). We also found that supporting cells underlying the hair cells continue to disappear, and there is a progressive collapse of the tunnel of Corti as maturation progresses.

and hair cell populations by this time point. Thus, the excess proliferation between the inner and outer hair cell regions of the apical cochlea appear to mainly differentiate into pillar cells, which remain in the epithelium until at least 3 weeks after birth.

Although we found that *Notch1* deletion increased inner pillar cell numbers in the apical region while also affecting outer pillar and Deiters' cell survival throughout the cochlea, other supporting cell types showed no apparent alterations after *Notch1* deletion (Fig. 9). We found that expression of FABP7, which stains both the Hensen's cells and border/phalangeal cells (Saino-Saito et al., 2010), persisted at both P6 and P14 in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutant cochleae (Fig. 9*A–D*). The expression of FABP7 in Hensen's cells was more intense in mutants compared with controls at P6 (Fig. 9*B* vs *A*), but this may be because of Hensen's cells becoming disorganized when the neighboring Deiters' cells were lost. The persistent expression of FABP7 at P14 in *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants suggested that deletion of *Notch1* at birth does not directly affect Hensen's cell and border/phalangeal cell survival, in contrast to recent reports of loss of the JAG1 ligand (Chrysostomou et al., 2020). We confirmed the presence of the border and inner phalangeal cells in P10 paraffin sections with immunofluorescence using an antibody for GLAST (Jin et al., 2003; green) and found comparable expression in our controls and *Notch1* mutants (Fig. 9*E*,*F*). Additionally, we found that the border/phalangeal supporting cells surrounding the inner hair cells were still present at 6 weeks, although they had a disorganized appearance (Fig. 9*G*, *H*, green). These results show that deletion of *Notch1* at P0/P1 does not affect the survival of the border and inner phalangeal cells surrounding the inner hair cell or Hensen's cells during cochlear maturation.

Discussion

Although the roles that Notch signaling plays in cochlear sensory cell patterning and differentiation have been extensively studied during embryogenesis, there are limited studies examining the role of the NOTCH1 receptor during cochlear maturation. Although hair cells and supporting cells adopt their fate during late embryogenesis in the mouse, much of hair cell and supporting cell differentiation and maturation takes place postnatally (Walters and Zuo, 2013), and the role of Notch in these processes is less well understood. During this time, structural changes also occur in the cochlea, including the formation of the tunnel of Corti, as well as changes in tectorial membrane interactions with the surface of supporting cells and development of the basilar membrane (Walters and Zuo, 2013). There is also a decline in the expression of many genes and signaling molecules during the first week of cochlear maturation, including several members of the Notch family (Murata et al., 2006; Hartman et al., 2009; Maass et al., 2015), which have been correlated with the declining ability of the mammalian cochlea to promote hair cell regeneration (for review, see Walters and Zuo, 2013).

Previously, studies have shown that blocking Notch signaling causes an increase in hair cells, mainly in the apical regions of the cochlea, which are known to be less mature (Korrapati et al., 2013; Bramhall et al., 2014; Maass et al., 2015). Similar to embryogenesis, this hair cell increase is largely because of a conversion of supporting cells to hair cells, normally prevented by lateral inhibition mediated by Notch signaling. In addition, Li et al. (2015) have reported that postnatal loss of *Notch1* in supporting cells leads to increased cell proliferation in the apex of the cochlea. Here, we show that although we similarly observe increases of hair cells in the apex and inner pillar cell region (Figs. 4, 8), we also see rapid supporting cell loss even in regions of the cochlea not exhibiting increases in hair cell numbers (Figs. 4, 5). In addition, we observe hair cell loss several weeks later (Figs. 7, 8), likely as a secondary consequence of loss of supporting cells. Given the rapid supporting cell loss, we posit that the Notch1 receptor plays a critical role during cochlear maturation as a supporting cell survival factor.

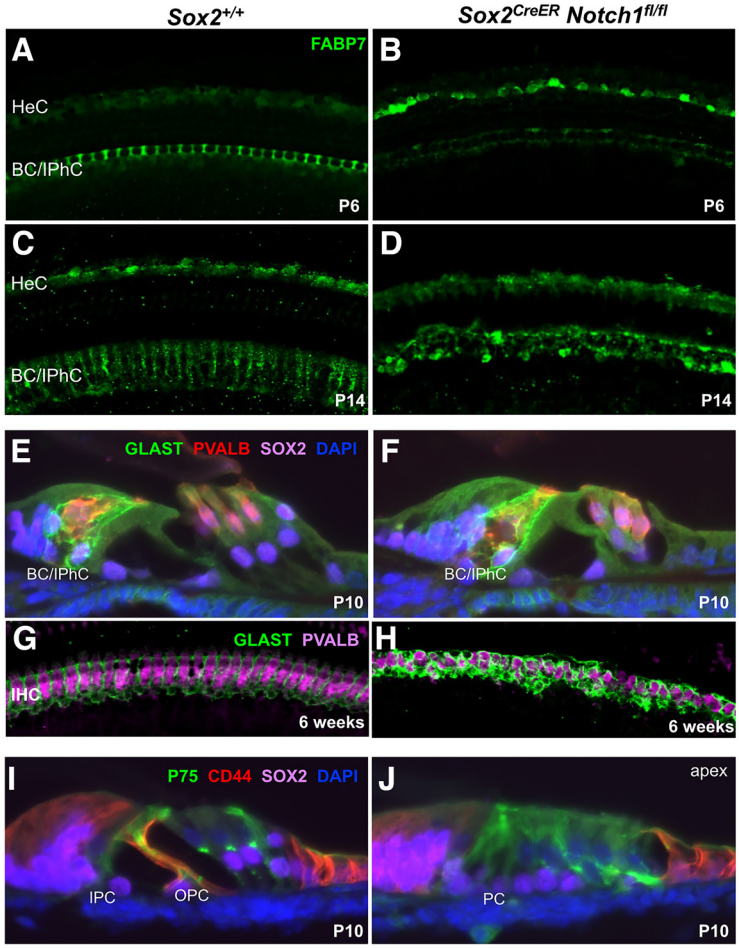

Figure 9. Other supporting cell subtypes in which *Notch1* was deleted were still present during cochlear maturation. ***A–D***, FABP7 expression in the border/phalangeal and Hensen's cells was detected both at P6 and P14 in controls (***A***, ***C***) and *Sox2*^{CreER} *Notch1*$^{fl/fl}$ mutants (***B***, ***D***). ***E–H***, GLAST, which is also expressed in the border/phalangeal cells showed similar staining patterns in controls and mutants at P10 (***E***, ***F***) and 6 weeks (***G***, ***H***). ***I***, ***J***, P75, a marker for inner pillar cells, showed an expansion of its expression domain in the apical region at P10 in mutants (***J***) compared with controls (***I***), as well as a loss of CD44 expression in the OPC region.

Loss of Notch1 causes rapid supporting cell death

We have shown that supporting cells, particularly Deiters' cells, are rapidly lost 24–48 h after deletion. This is in contrast to explant studies in which inhibition of Notch signaling is performed using the γ-secretase inhibitor DAPT, resulting in ectopic hair cells at the expense of supporting cells both during embryogenesis and postnatally (Takebayashi et al., 2007; Korrapati et al., 2013; Bramhall et al., 2014; Maass et al., 2015). Similarly, embryonic *Notch1* deletion using a Foxg1-Cre resulted in significant increases in hair cells (Kiernan et al., 2005). However, these studies mainly focused on short-term studies of the organ of Corti after Notch deletion/inhibition. In other studies, deletion of Notch components has affected cell survival. For example, Campbell et al (2016) showed that loss of Notch signaling, through both conditional deletion of the Notch regulator *Rbpj* in pillar and Deiters' cells and dnMAML throughout the ear at embryonic day (E)14.5/15.5, resulted in loss of Deiters' cells at E18.5 and soon after birth. Interestingly, Deiters' cell loss occurred in a basal to apical gradient when deleted this early, in contrast to the cell death and supporting cell loss we see in the postnatal cochlea (Figs. 4, 5). A study has also shown that deletion of RBPJ during cochlear differentiation resulted in rapid hair cell and supporting cell death in explant cultures; notably, deletion of RBPJ during cochlear differentiation did not result in an increase in hair cell numbers (Basch et al., 2011). However, loss of RBPJ/Notch signaling in P3 cochlear explants showed many ectopic hair cells (Yamamoto et al., 2006). It is difficult to make firm conclusions about the role of Notch signaling by examining RBPJ, the central Notch canonical transcription factor, given that its role in the absence of Notch activation is to act as a repressor; thus, deletion also leads to derepression of an unknown number of genes (Miele, 2011; Castel et al., 2013). Potentially, the differences in supporting cell survival versus conversion into hair cell may reflect differences in embryonic potential of the supporting cells versus their postnatal potential. This is consistent with our finding of excess hair cells only in the less

mature apical cochlear regions, similar to previous studies (Li et al., 2015). Additionally, these different outcomes may also reflect differences in culture versus *in vivo* studies, or Notch-independent effects of DAPT (Takebayashi et al., 2007; Korrapati et al., 2013; Bramhall et al., 2014; Maass et al., 2015).

Notch signaling has been shown to be important in cell survival in other cell types. In macrophages, Notch signaling is important in regulating apoptosis, which eliminates pathogens and other infections under normal conditions; Notch upregulation was found to be associated with the upregulation of the anti-apoptotic protein Mcl-1 and ability to reduce infection (Palaga et al., 2013). Many types of cancers have also been associated with increased Notch activity, including cervical cancer (Zagouras et al., 1995), laryngeal squamous cell carcinoma (Dai et al., 2015), breast cancer (Stylianou et al., 2006; Mittal et al., 2009), and leukemia (Weng et al., 2004), suggesting increased Notch activation results in cell proliferation and survival, rather than cell death. Efforts to unravel the anti-apoptotic function of Notch have shown that Notch acts via the Akt to maintain mitochondrial integrity (Perumalsamy et al., 2010). Importantly, this activity did not require RBPJ and proposes that activated Notch (NICD) acts directly to maintain the mitochondria and inhibit proapoptotic proteins. This finding is consistent with the rapid apoptosis of the supporting cells in our study, detected 24–48 h after tamoxifen delivery, a time frame that includes *Notch1* gene deletion, and reduction of existing Notch1 via protein turnover.

Hair cell loss is a secondary consequence of supporting cell loss

Our studies show that after deletion of *Notch1*, there was rapid loss of PROX1+ supporting cells throughout the cochlea (Figs. 4, 5). Surprisingly, the outer hair cells in *Notch1* mutants are not lost immediately when the supporting cells below are gone and remain in the sensory region until the onset of hearing, around P12–P14 in the mouse (Figs. 7, 8). This was similar to what Mellado Lagarde et al. (2013) reported after ablation of pillar and Deiters' cells in the postnatal cochlea using the diphtheria Toxin A (ROSA-DTA) crossed to the Prox1-Cre. This study showed a strikingly similar time frame for hair cell loss in an apical to basal gradient. These similarities indicate that the loss of outer hair cells is caused secondarily by early loss of the supporting cells. It is interesting that the loss of the outer hair cells coincides with the onset of hearing, around P12 in the mouse (Abe et al., 2007). It is possible that mechanical forces induced by the onset of hearing, including basilar membrane deflections and/or electromotility, cause outer hair cell loss in the absence of anchoring supporting cells. Alternatively, the loss of the functions of the supporting cells, such as ion uptake, leads to loss of the hair cells (Boettger et al., 2002; Rozengurt et al., 2003). We could not determine the exact mechanism of hair cell loss, although it should be noted that we did not detect cCASP3-positive hair cells during any time points examined in our studies, suggesting they are not lost through apoptotic cell death. It is possible the hair cells are simply extruded into the lumen, as has been described in birds (Mangiardi et al., 2004), and fish (Haddon et al., 1999).

Notch1 is required for hearing and the integrity of the organ of Corti after birth

To date, although a few studies have examined the role of different Notch pathway components in the postnatal cochlea (Takebayashi et al., 2007; Korrapati et al., 2013; Bramhall et al., 2014; Li et al., 2015; Maass et al., 2015; Ni et al., 2016), none have looked at the prolonged consequences of loss of NOTCH1 after birth. Here, we show that deletion of *Notch1* at P0/P1 from most supporting cell subtypes, including border cells, inner phalangeal cells, inner and outer pillar cells, Deiters' cells, and Hensen's cells, resulted in selective loss of specific supporting cell populations, particularly Deiters' and outer pillar cells. Similar to studies in which pillar and Deiters' subtypes of supporting cells are deleted (Mellado Lagarde et al., 2013), we observe outer hair cell loss and hearing impairment after *Notch1* deletion. Additionally, the phenotype resulting from loss of pillar and Deiters' cells also looked remarkably similar to that of our *Notch1* mutants, with inner hair cells present and a majority of outer hair cells missing. However, although Mellado Lagarde et al. (2013) reported elevated ABR responses (40–50 dB increase) in response to ablation of pillar and Deiters' cells, we observe no ABR responses at 6 weeks of age. These data indicate that deletion of *Notch1* also affects inner hair cells, which appear dysmorphic and display some stereocilia defects. Additionally, in some regions there were missing inner hair cells at 6 weeks. Examination of the supporting cells around the inner hair cells, including inner phalangeal cells and border cells, showed normal expression of subtype-specific markers, although that does not rule out more subtle defects in the supporting cells, and further investigation is warranted to determine how loss of *Notch1* affects the inner hair cells.

Here, we demonstrate that deletion of *Notch1* in the neonatal cochlea results in rapid apoptosis of supporting cells in the outer hair cell region, followed by loss of almost all sensory outer hair cells several weeks later. Although Notch signaling has been proposed as a way to regenerate hair cells through conversion of supporting cells through inhibition of lateral inhibition (Fujioka et al., 2015), our results highlight the complexities of Notch signaling and indicate a significant role in cell survival in the cochlea.

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