The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear

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Summary

The mammalian auditory sensory epithelium, the organ of Corti, contains sensory hair cells and nonsensory supporting cells arranged in a highly patterned mosaic. Notch-mediated lateral inhibition is the proposed mechanism for creating this sensory mosaic. Previous work has shown that mice lacking the Notch ligand JAG2 differentiate supernumerary hair cells in the cochlea, consistent with the lateral inhibitory model. However, it was not clear why only relatively modest increases in hair cell production were observed in *Jag2* mutant mice. Here, we show that another Notch ligand, DLL1, functions synergistically with JAG2 in regulating hair cell differentiation in the cochlea. We also show by conditional inactivation that these ligands probably signal through the NOTCH1 receptor. Supernumerary hair cells in *Dll1/Jag2* double mutants arise primarily through a switch in cell fate, rather than through excess proliferation. Although these results demonstrate an important role for Notch-mediated lateral inhibition during cochlear hair cell patterning, we also detected abnormally prolonged cellular proliferation that preferentially affected supporting cells in the organ of Corti. Our results demonstrate that the Notch pathway plays a dual role in regulating cellular differentiation and patterning in the cochlea, acting both through lateral inhibition and the control of cellular proliferation.

Key words: Notch signaling, Lateral inhibition, Hair cell differentiation, Cochlea

Introduction

The Notch signaling pathway plays myriad roles in both developing and adult multicellular organisms (for reviews, see Baron, 2003; Gridley, 2003; Lai, 2004; Schweisguth, 2004). One of its best known roles, originally studied in *Drosophila melanogaster* and *Caenorhabditis elegans*, is in mediating lateral inhibition, a patterning mechanism in which a cell differentiating as one type prevents its neighboring cells from doing the same, thus creating a mosaic of cell types from an initially equivalent epithelium (Bray, 1998; Lai, 2004). The cellular arrangement of the sensory epithelium in the inner ear, in which each hair cell is surrounded by supporting cells, is suggestive of this type of patterning mechanism (Corwin et al., 1991; Lewis, 1991; Kiernan et al., 2002). Moreover, hair cells and supporting cells have been shown to arise from a common progenitor in lower vertebrates (Fekete et al., 1998), consistent with a lateral inhibitory mechanism in which different cell types arise from an initially equivalent epithelium. Expression studies have shown that the genes encoding the Notch ligands JAG2 and DLL1 are both expressed in nascent hair cells as they begin to differentiate (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999). Several different Notch receptors are also expressed in the ear, supporting a role for Notch-mediated lateral inhibition in the inner ear (Lindsell et al., 1996; Lanford et al., 1999). Direct functional evidence for lateral inhibition in the ear is supported by studies of Notch pathway mutants in zebrafish, mouse and chicken (Haddon et al., 1998; Lanford et al., 1999; Riley et al., 1999; Zhang et al., 2000; Zine et al., 2001; Daudet and Lewis, 2005). In the mouse, a deletion of the *Jag2* gene resulted in extra rows of both inner and outer hair cells in the cochlea (Lanford et al., 1999). Similarly, mice deleted for *Hes1* and *Hes5*, downstream targets of Notch signaling, as well as mice lacking one copy of the *Notch1* gene, demonstrated increased hair cell numbers (Zhang et al., 2000; Zine et al., 2001). Although consistent with the model, it was unclear why only relatively modest increases in hair cell numbers in the cochlea were observed. Furthermore, it was not known whether supporting cell numbers were concomitantly reduced, indicating a cell fate switch. Thus, it was not clear whether Notch signaling played only a minor role in cell patterning in the organ of Corti, or whether the milder phenotype was observed due to genetic redundancies in the pathway.

Because both the *Dll1* and *Jag2* genes are expressed in nascent hair cells (Lanford et al., 1999; Morrison et al., 1999), the prospect of redundancy was a real possibility, as both ligands may be necessary to fully deliver a lateral inhibitory signal. To test whether the *Dll1* gene also plays a role in lateral inhibition and to investigate potential genetic interactions with *Jag2* mutations, we generated embryos that carried various

combinations of *Jag2* and/or *Dll1* mutant alleles. Our results show that *Dll1* functions synergistically with *Jag2*, demonstrating that both ligands are required to regulate the numbers of hair cells that form in the mammalian cochlea. Using conditional gene inactivation, we also show that both the JAG2 and DLL1 ligands are likely to signal through the NOTCH1 receptor. Consistent with the proposed lateral inhibition model, most supernumerary hair cells in the *Dll1/Jag2* double mutant cochleae did not arise through excess proliferation, suggesting instead a switch in cell fate. However, supporting cells did exhibit abnormal proliferation, implicating a novel role for the Notch pathway in regulating cellular proliferation in the ear.

Materials and methods

Mice

Targeted disruptions of the *Jag2* and *Dll1* loci were described previously (Hrabe de Angelis et al., 1997; Jiang et al., 1998). The construction and characterization of the $Dll1^{hyp}$ allele will be described in detail elsewhere (R.C. and A.G., unpublished). Briefly, the full-length *Dll1* cDNA along with a neomycin phosphotransferase selection cassette was inserted into the *Dll1* locus by gene targeting. Mouse embryos doubly heterozygous for this allele and the *Dll1* null allele (i.e. $Dll1^{hyp/-}$ embryos) survived past the period of vascular lethality at E10.5 that causes the death of $Dll1^{-/-}$ embryos. The $Dll1^{hyp/-}$ embryos exhibit segmentation defects consistent with the previously described role for the *Dll1* gene during somite formation (Hrabe de Angelis et al., 1997). All data collected to date support the model that the $Dll1^{hyp}$ allele is a hypomorphic *Dll1* mutant allele. *Jag2* and *Dll1* mice were maintained on a mixed 129S1/SvImJ; C57BL/6J and C57BL/6J backgrounds, respectively. To produce *Foxg1-Cre* $Notch1^{flox/-}$ mice, $Notch1^{flox/flox}$ (Yang et al., 2004) mice were mated to mice heterozygous for both *Foxg1-Cre* (Hebert and McConnell, 2000) and *Notch1* (Swiatek et al., 1994). The *Foxg1-Cre* mice were maintained on an outbred Swiss Webster background, whereas the $Notch1^{+/-}$ mice and the $Notch1^{flox/flox}$ mice were maintained on a C57BL/6J background.

Immunocytochemistry

For whole-mount preparations, inner ears were dissected and fixed overnight in 4% paraformaldehyde. The bony shell and the stria vascularis were removed, and the ears were incubated with a biotinylated lectin (Griffonia simplifonia I, Vector Laboratories) diluted 1:100. A FITC-labeled avidin secondary reagent was used to visualize the hair cells. All other immunocytochemistry was performed on standard 7-μm paraffin-embedded sections. Antibodies used included anti-myosin VIIa (1:1000, a gift from Drs A. EL-Amraoui and C. Petit, Institut Pasteur, Paris, France), anti-$p27^{kip1}$ (1:100, Neomarkers) and anti-BrdU (1:500, Roche). For anti-$p27^{kip1}$ labeling, an antigen-retrieval step was performed by boiling the sections for 10 minutes in 10 mM citric acid. For BrdU labeling, two different protocols were used: in the first, antigen retrieval was performed as described previously for $p27^{kip1}$, followed by pepsin digestion (100 μg/ml for 20 minutes at 37°C) and acid treatment (2N HCl for 30 minutes at 37°C). For doubly labeled sections, heat-activated antigen retrieval was performed followed by DNAse I digestion (5 U/ml for 30 minutes at 37°C). Cell death was examined by TUNEL staining of paraffin-embedded sections, using the TMR in situ cell detection kit (Roche).

Cell counts

Hair cell counts from lectin-stained wholemounts

Hair cell counts were performed on mid-basal regions of the lectin-stained cochleae, extending between 800 and 1400 μm. Each genotype contained counts from three or four different embryos. After capture of high-resolution images of the cochleae, counts and measurements were performed using Zeiss Axiovision software.

Hair and supporting cell counts from myosin VIIa/$p27^{kip1}$ immunostained sections

For each ear, images from 32 sections through the mid-modiolar region of the cochlea that were triply labeled for 4′-6-Diamidino-2-phenylindole (DAPI; to stain nuclei), myosin VIIa (to label hair cells) and $p27^{kip1}$ (to label supporting cells) were captured using Zeiss Axiovision software. Nuclei from cells that were doubly labeled with DAPI and either myosin VIIa (hair cells) or $p27^{kip1}$ (supporting cells) in the basal and middle turns of the organ of Corti were counted.

Cell proliferation

To examine cell proliferation, pregnant female mice were injected with a BrdU solution (10 mg/ml in PBS; final dose, 50 μg per gram of body weight), three times daily (at 4 hour intervals), between E14.5 and E17.5. Animals were euthanized and half heads were fixed and embedded for paraffin sectioning.

Electron microscopy

Inner ears were prepared for scanning electron microscopy, as described previously, using a version of the osmium tetroxide-thiocarbohydrazide (OTOTO) method (Kiernan et al., 1999). Specimens were examined with a Hitachi 3000N scanning electron microscope.

In situ hybridization

For sample preparation, inner ears were dissected from the head and fixed overnight in 4% paraformaldehyde. After washing in PBS, the bony shell and stria were removed from the cochleae and the samples were dehydrated in methanol. In situ hybridization was performed as described (Stern, 1998), with the exception of the post-hybridization washes, which were done according to Rau et al. (Rau et al., 1999). After the reactions were judged to be complete, cochleae were flat mounted on glass slides in 70% glycerol. Probes for α-tectorin and β-tectorin (gifts from Drs K. Legan and G. Richardson, University of Sussex, UK) were as described (Rau et al., 1999).

Results

Absence of cochlear hair cell defects in *Dll1* hypomorphic mutants

The *Dll1* gene is expressed in nascent hair cells in the inner ear (Morrison et al., 1999), but a role for the *Dll1* gene in regulating cochlear hair cell differentiation has not been examined because of the early embryonic lethality of embryos homozygous for a targeted $Dll1(Dll1^{-/-})$ null mutation (Hrabé de Angelis et al., 1997). However, mouse embryos that carried both the *Dll1* null allele and a newly constructed *Dll1* hypomorphic allele ($Dll1^{hyp/-}$ mice) survive until birth (R.C. and A.G., unpublished; see Materials and methods). We therefore examined cochlear hair cell differentiation and patterning in $Dll1^{+/-}$, $Dll1^{+/hyp}$ and $Dll1^{hyp/-}$ mutant embryos. Embryos were harvested at embryonic day 18.5 (E18.5), and hair cell patterning in their cochleae was examined by staining with a lectin that binds to hair cell stereocilia. Similarly to $Jag2^{+/-}$ cochleae, $Dll1^{+/-}$ and $Dll1^{+/hyp}$ cochleae did not show a significant increase in hair cells, although occasional second row inner hair cells and fourth row outer hair cells were observed (Fig. 1B-D). Surprisingly, cochleae from $Dll1^{hyp/-}$ mutant embryos did not show a statistically significant increase in hair cell numbers (Table 1), although supernumerary inner

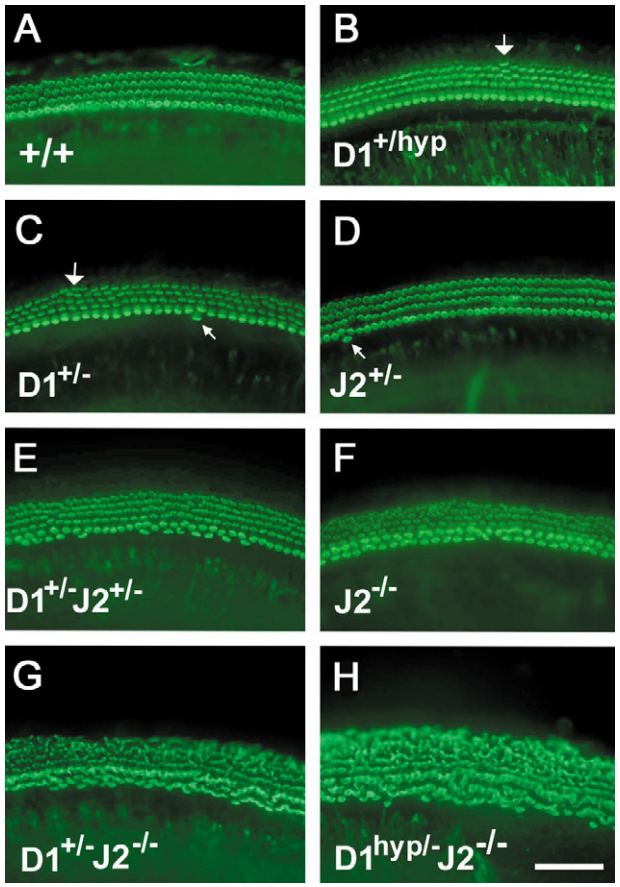

Fig. 1. Graded increase in hair cell formation with a reduction in Notch ligand gene dosage. (A-D) Lectin-stained cochleae with only single null or hypomorphic mutations in the *Dll1* or *Jag2* genes show relatively normal hair cell numbers and patterning, although occasional extra inner or outer hair cells are observed (arrows). (E-H) Double heterozygous $Dll1^{+/-}$ $Jag2^{+/-}$ cochleae (E) demonstrate a similar increase in outer hair cells but not as large an increase in inner hair cells as $Jag2^{-/-}$ cochleae (F). $Dll1^{+/-}$ $Jag2^{-/-}$ (G) or $Dll1^{hyp/-}Jag2^{-/-}$ (H) cochleae show a large increase in hair cell formation when compared with $Jag2^{-/-}$ cochleae (F). The hair cells in these cochleae are extremely disorganized. Scale bar: 50 μm.

and outer hair cells were observed more frequently than in wild-type embryos.

Mutations in *Dll1* and *Jag2* interact during cochlear development

We then tested whether the *Dll1* and *Jag2* genes functioned synergistically during cochlear hair cell differentiation. As neither $Jag2^{-/-}$ homozygous mice nor $Dll1^{hyp/-}$ compound heterozygous mice survive postnatally, we set up crosses between $Dll1^{+/-}$ $Jag2^{+/-}$ and $Dll1^{+/hyp}$ $Jag2^{+/-}$ mice to generate embryos with varying *Dll1* and *Jag2* gene dosages. Cochleae that were mutated for only a single allele of a Notch ligand (either $Dll1^{+/-}$, $Dll1^{+/hyp}$ or $Jag2^{+/-}$) demonstrated no significant increases in hair cell numbers when compared with wild-type embryos, although occasional extra inner and outer hair cells were observed (Fig. 1A-D). However, cochleae that were doubly heterozygous for both *Dll1* and *Jag2* ($Dll1^{+/-}$ $Jag2^{+/-}$) showed many extra inner hair cells and a nearly complete fourth row of outer hair cells, indicating a genetic interaction between mutations in these two genes (Fig. 1E). Hair cell counts from the middle turn of the cochleae demonstrated that $Dll1^{+/-}$ $Jag2^{+/-}$ double heterozygous cochleae have significantly more hair cells than wild type (Table 1). $Dll1^{+/-}$ $Jag2^{+/-}$ cochleae had nearly as many supernumerary hair cells as $Jag2^{-/-}$ cochleae, although there were not as many extra inner hair cells. This indicates that the *Dll1* gene may not play as large a role in the inhibition of inner hair cell development as the *Jag2* gene does. Counts of $Dll1^{+/hyp}$ $Jag2^{+/-}$ cochleae revealed a more modest increase in hair cell numbers, reflecting the hypomorphic nature of the $Dll1^{hyp}$ allele and demonstrating the graded response of the cochlea to the dosage of genes encoding Notch ligands.

Cochleae that lacked both copies of *Jag2*, and that carried either a single null allele of *Dll1* ($Dll1^{+/-}$ $Jag2^{-/-}$) or a null allele combined with the *Dll1* hypomorphic allele ($Dll1^{hyp/-}$ $Jag2^{-/-}$), showed even larger increases in hair cell numbers than $Jag2^{-/-}$ cochleae (Fig. 1G,H). In $Dll1^{hyp/-}$ $Jag2^{-/-}$ cochleae, two to four rows of inner hair cells were present and four to six rows of outer hair cells could be identified. However, unlike the $Dll1^{+/-}$ $Jag2^{+/-}$ or $Jag2^{-/-}$ cochleae, the rows of hair cells were more disorganized and the hair cells were very densely

Table 1. Synergistic effects of *Dll1* and *Jag2* mutations on hair cell numbers

Genotype			Counts/100 μm (mean±s.e.m.)			
Dll1	*Jag2*	*n*	IHCs	OHCs	HCs	OHC/IHC
+/+	+/+	3	13.6±0.6	44.3±2.3	57.9±2.6	3.3
+/–	+/+	3	13.8±0.3	45.5±0.6	58.8±0.8	3.3
+/hyp	+/+	3	13.2±0.9	44.1±1.6	57.3±2.5	3.3
+/+	+/–	4	14.2±0.4	44.0±1.2	58.2±1.6	3.2
+/hyp	+/–	3	15.0±0.1	46.8±0.8	61.7±1.2	3.1
hyp/–	+/+	3	14.3±0.4	46.6±0.2	60.9±0.5	3.3
+/–	+/–	4	17.4±0.5***	50.9±0.9*	68.9±1.0**	2.9
hyp/–	+/–	3	18.9±1.0***	52.0±3.6*	70.9±4.4***	2.8
+/+	–/–	3	22.8±0.7***	50.6±2.6*	73.4±3.3***	2.2

Inner hair cells (IHCs) and outer hair cells (OHCs) were counted separately for each indicated genotype, and were then combined to get total numbers of hair cells (HCs). Counts were expressed in numbers of hair cells per 100 μm, to take into account the slightly different lengths that were counted for each cochlea. Counts were also expressed as outer hair cell versus inner hair cell ratios (OHC/IHC). Significant increases in hair cells numbers were not observed until more than one copy of a Notch ligand (*Dll1* or *Jag2*) was deleted or reduced. Significance was determined by comparing each of the mutant groups with the control group (+/+; +/+), using Dunnett's method.

*P<0.05; **P<0.01; ***P<0.0001.

packed, making accurate determination of hair cell numbers difficult in whole-mount preparations.

We used scanning electron microscopy to examine in more detail the morphological defects in the double mutant cochleae (Fig. 2). *Dll1*$^{hyp/-}$ *Jag2*$^{-/-}$ mutant cochleae were extremely disorganized, both in the patterning of the sensory cell rows and, at a single hair cell level, in the loss of polarity and disorganization of many hair cell stereocilia bundles (Fig. 2F). When compared with *Jag2*$^{-/-}$ and *Dll1*$^{+/-}$ cochleae (Fig. 2A-D), it is clear the amount of disorganization correlates with the amount of Notch ligand that is present, as some disorganization is present even in *Jag2*$^{-/-}$ cochleae. However, it is not clear from these data whether the Notch pathway plays a direct role in planar polarity signaling (Barald and Kelley, 2004) and/or patterning of the hair cells, or whether the observed disorganization may be a secondary effect of the increased numbers of hair cells present in the mutant cochleae.

Supporting cell numbers are only modestly reduced in *Dll1/Jag2* double mutant cochleae

One prediction of the lateral inhibition model is that additional sensory hair cells would be generated at the expense of nonsensory supporting cells. In order to examine whether supporting cells were decreased in *Dll1/Jag2* double mutant cochleae, hair and supporting cell counts were performed on mid-modiolar sections of *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ mutant and control cochleae that had been labeled with both a hair cell marker (Myosin VIIa) and a supporting cell marker (p27^{kip1}) (Fig. 3A-D). Hair cell counts revealed a 1.7-fold increase in *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ cochleae when compared with controls. However, supporting cell counts revealed a milder 1.2-fold decrease in supporting cells (Fig. 3I).

p27^{kip1} immunostaining of the *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ mutant and control cochleae indicated that many of the missing cells resided beneath the outer hair cells and were presumably Deiter's cells (Fig. 3C,D). In order to assess which supporting cell populations were decreased, *Dll1/Jag2* double mutant and control cochleae were processed for whole-mount in situ hybridization using probes that mark specific populations of supporting cells, α-tectorin and β-tectorin (Rau et al., 1999). Expression of α-tectorin in wild-type cochleae marks the supporting cell populations surrounding the organ of Corti proper (including Koelliker's organ), the supporting cells surrounding the inner hair cells (inner phalangeal cells), and Hensen's cells. In *Dll1*$^{+/-}$ *Jag2*$^{-/-}$ cochleae, α-tectorin expression appeared to be similar to in controls, suggesting that none of these surrounding supporting cell populations were converted to hair cells (Fig. 3E,F). β-Tectorin marks several supporting cell regions that lie in the organ of Corti proper, including the inner and outer pillar cells, the last row of Deiter's cells, and a smaller domain of cells in Koelliker's organ. In *Dll1*$^{+/-}$ *Jag2*$^{-/-}$ cochleae, most of these expression domains appeared to be similar to those in controls, with the exception of the Deiter's cell expression domain (Fig. 3G,H).

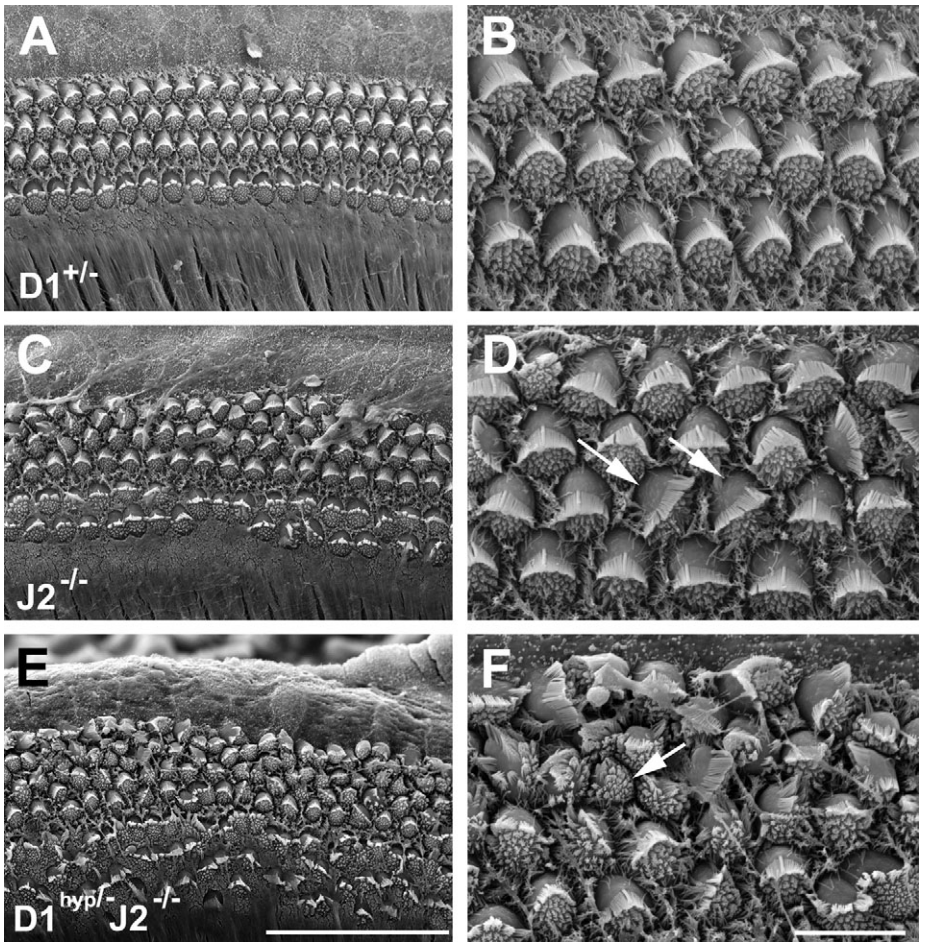

Fig. 2. Disorganization and loss of planar cell polarity in *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ cochleae. Scanning electron microscopy of the basal region of cochleae of the indicated genotypes. (A,C,E) Low-power views. (B,D,F) Higher power views of the outer hair cell rows. Note the extremely disorganized and disoriented state of the hair cell bundles in the *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ cochleae (F). In addition, some of the hair cells appear immature (arrow), with stereocilia covering the entire apical surface and not displaying the graded heights they would normally have acquired by this time. Some of this disorientation is also apparent in *Jag2*$^{-/-}$ cochleae (D), where some of the stereocila bundles on the outer hair cells do not orient properly (arrows). Scale bar: 50 μm in A,C,E; 10 μm in B,D,F.

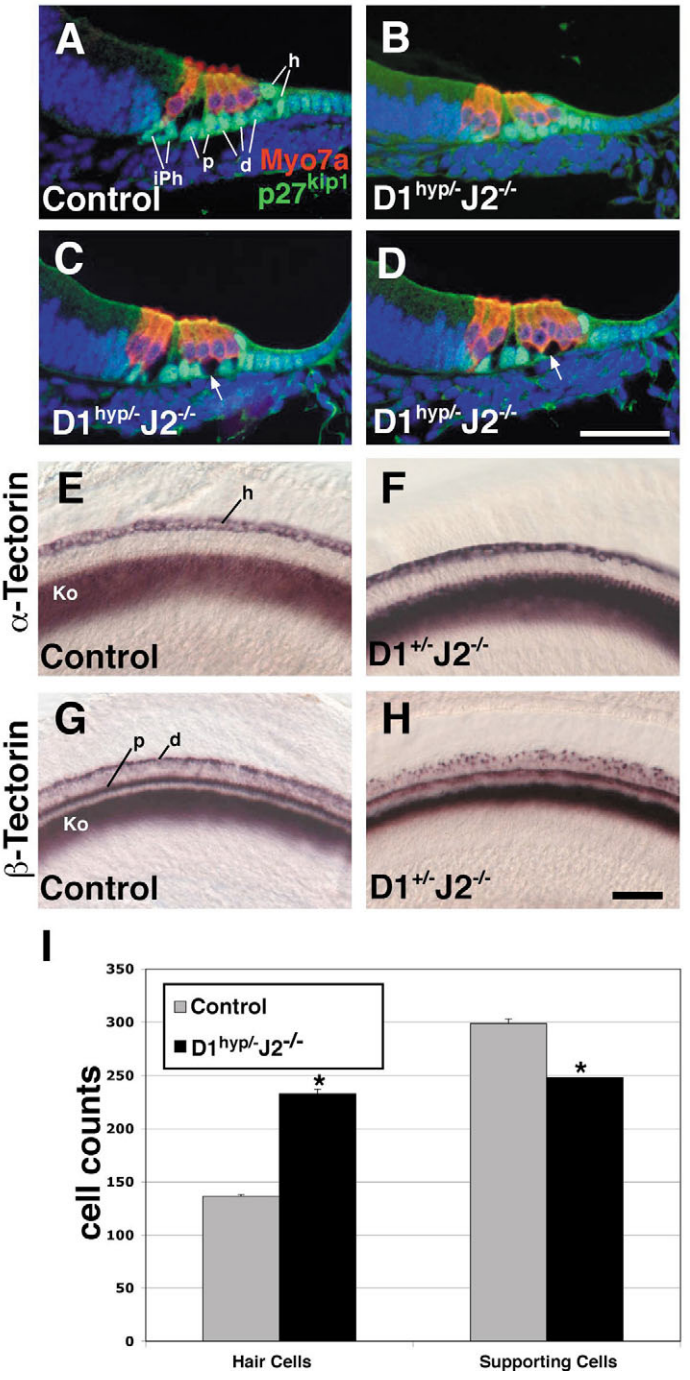

Fig. 3. Hair cells are dramatically increased in *Dll1/Jag2* double mutant cochleae, whereas supporting cells are only modestly reduced. (A-D) Myosin VIIa (red), $p27^{kip1}$ (green) and DAPI (blue nuclear stain) immunostained sections from E18.5 $Dll1^{hyp/-}Jag2^{-/-}$ mutant and control cochleae. Supporting cells are clearly still present in $Dll1^{hyp/-}Jag2^{-/-}$ mutant cochleae, as shown by the $p27^{kip1}$-stained cells. However, some $p27^{kip1}$-stained cells (presumably Deiter's cells) appeared to be missing in some sections (C,D, arrows). (E-H) E18.5 cochleae processed for in situ hybridization using the indicated probes. Expression of α-tectorin or β-tectorin in the various supporting cell populations did not appear to differ between controls and mutants, with the exception of β-tectorin expression in Deiter's cells (H), which appears to be disorganized and reduced. (I) Hair and supporting cell counts from sections, as shown in A-D. Sections from three ears were counted for each group, either control or $Dll1^{hyp/-}Jag2^{-/-}$. Controls were either wild type, or *Dll1* or *Jag2* single heterozygotes. Counts of both hair cells and supporting cells were significantly different between $Dll1^{hyp/-}Jag2^{-/-}$ mutant and control cochleae (*P<0.001, Student's t-test). However, the increase in hair cells did not equal the supporting cell losses (P<0.0001; one-way ANOVA). d, Deiter's cells; h, Hensen's cells; Ko, Koelliker's organ; iPh, inner phalangeal cells; p, pillar cells. Scale bars: in D, 50 μm for A-D; in H, 50 μm for E-H.

Here, the β-tectorin expression was punctate rather than being maintained in a continuous stripe, and appeared to be reduced, suggesting that many of the missing supporting cells were derived from the Deiter's cell population.

Abnormal proliferation in $Dll1^{+/-}$ $Jag2^{-/-}$ and $Dll1^{hyp/-}$ $Jag2^{-/-}$ cochleae

Because we could only detect mild supporting cell losses, we next examined whether any of the hair and/or supporting cells were produced via excess cell division. The majority of hair cells and supporting cells in the organ of Corti undergo their final division between E12 and E14 in a spatio-temporal gradient from apex to base (Ruben, 1967). The *Dll1* and *Jag2* genes are not expressed in the cochlea until approximately E14.5, when cells in the organ of Corti have exited the cell cycle and are beginning to differentiate (Lanford et al., 1999; Morrison et al., 1999). One possibility is that Notch signaling is important for the suppression of continued cell division in the cochlea, rather than for mediating lateral inhibition. Therefore, we examined whether there was continued proliferation occurring between E14.5 and E17.5 in *Dll1/Jag2* double mutants. Pregnant $Dll1^{+/hyp}$ $Jag2^{+/-}$ females that had been mated to $Dll1^{+/-}$ $Jag2^{+/-}$ males were injected with bromodeoxyuridine (BrdU) between E14.5 and E17.5. Embryos were taken at E18.5 and their cochleae were processed to detect BrdU incorporation. Between E14.5 and E17.5, there was little proliferation in the control mouse ventral cochlear epithelium with the exception of cells in Koelliker's organ and scattered cells in the lesser epithelial ridge (Ruben, 1967), although occasionally labeled cells were observed in the organ of Corti (Fig. 4 and Table 2). However, mutant cochleae (either $Dll1^{+/-}$ $Jag2^{-/-}$ or $Dll1^{hyp/-}$ $Jag2^{-/-}$) frequently displayed labeled cells in the organ of Corti. The most commonly labeled cell types were supporting cell types; amongst these, pillar cells were most often labeled, followed by Deiter's cells and Hensen's cells (Fig. 4B-D and Table 2). Hair cells were only rarely labeled (Table 2). These data show that the majority of the supernumerary hair cells are not arising through continued proliferation, consistent with the model of Notch-mediated lateral inhibition. However, supporting cells were frequently labeled, which may explain why only modest decreases in supporting cell numbers were observed in $Dll1^{hyp/-}$ $Jag2^{-/-}$ mutant cochleae.

Similar phenotypes suggest that the JAG2 and DLL1 ligands signal through the NOTCH1 receptor during hair cell formation and patterning

Previously, we demonstrated that mice heterozygous for a *Notch1* null allele exhibited increased numbers of outer hair cells, suggesting that the NOTCH1 protein was required for

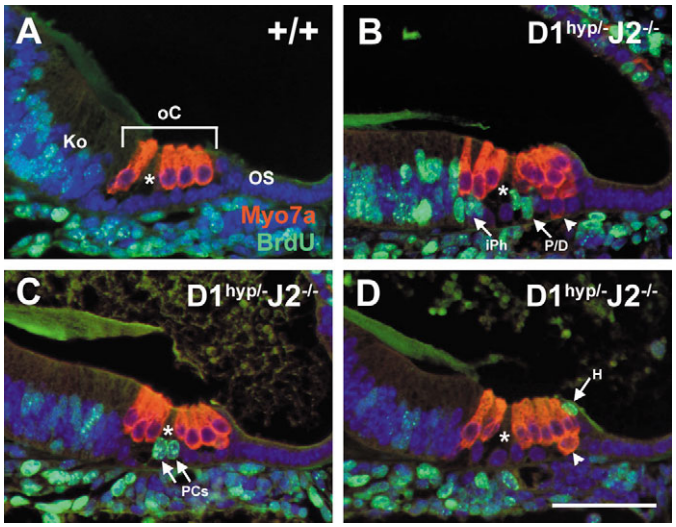

Fig. 4. Continued proliferation occurs in *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ mutant cochleae. (A-D) Immunohistochemistry on sections from E18.5 embryos was used to detect proliferating cells (green) in the organ of Corti after injections of BrdU from E14.5-E17.5. Hair cells are labeled with an antibody to detect myosin VIIa (red) and nuclei are stained with DAPI (blue). (A) Control section through the cochlea, demonstrating that there is normally no proliferation after E14.5 in the organ of Corti, although BrdU-labeled cells can been detected in Koelliker's organ and stromal cells beneath the organ of Corti. (B-D) Sections from *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ cochleae, demonstrating examples of unexpected proliferating cells in the organ of Corti. The probable identities of these labeled cells based on the position of their nuclei are indicated. Some myosin VIIa-positive nuclei are located near the basement membrane in the position of supporting cell nuclei in the mutant cochleae (arrowheads in B and D). Asterisks indicate the tunnel of Corti in A-D. H, Hensen's cells; iPh, inner phalangeal cells; Ko, Koelliker's organ; oC, organ of Corti; OS, outer sulcus; pc, pillar cells; P/D, pillar/Deiter's cells. Scale bar: 50 μm.

proper differentiation in the organ of Corti (Zhang et al., 2000). However, we were unable to assess the ear phenotype of *Notch1*$^{-/-}$ embryos as they die at midgestation (Swiatek et al., 1994). Therefore, we conditionally inactivated *Notch1* function in the otic epithelium using the *Foxg1-Cre* mouse line (Hebert and McConnell, 2000; Pirvola et al., 2002) and a *Notch1*^{flox} allele (Yang et al., 2004). Embryos with the genotype *Foxg1-Cre Notch1*$^{flox/-}$ and littermate controls were isolated at E18.5, and whole-mount preparations of the cochleae were stained with lectin. These experiments showed a large increase in the hair cell population in the *Foxg1-Cre Notch1*$^{flox/-}$ cochleae (Fig. 5B,D,E) that resembled the hair cell increases observed in *Dll1/Jag2* double mutant cochleae. Measurements of the lengths of the entire cochleae in *Foxg1-Cre Notch1*$^{flox/-}$ mutants (mean=5359 μm±260; *n*=3) versus controls (mean=5311 μm±492; *n*=3) were not significantly different (*P*=0.92), indicating that the supernumerary hair cells are a result of real increases in hair cell numbers and not simply a redistribution of the cells due to a shortened cochlea. Interestingly, the increase in hair cells in *Foxg1-Cre Notch1*$^{flox/-}$ cochleae was far more dramatic than that in the *Dll1*$^{hyp/-}$ *Jag2*$^{-/-}$ cochleae. In fact, *Foxg1-Cre Notch1*$^{flox/-}$ cochleae displayed about a 3-fold increase in the number of hair cells (compared with the 1.6-fold increase in the *Dll1*$^{hyp/-}$ *Jag2*$^{-/-}$ double mutants), whereas the decrease in supporting cell numbers was only slightly more severe than in the double mutants (1.6-fold in the *Foxg1-Cre*

Table 2. BrdU-labeled cell counts

Ear	Genotype		Number of sections	IHC	OHC	Total hair cells	Pillar	Deiter	Inner phalangeal	Hensen	Total support cells	Total
Controls	*Dll1*	*Jag2*										
190	+/+	+/+	39	0	1	1	0	0	0	0	0	1
219	hyp/+	+/+	41	0	0	0	0	1	0	2	3	3
191	+/+	+/–	38	0	0	0	0	0	0	0	0	0
193	+/hyp	+/–	58	0	0	0	0	0	0	0	0	0
220	+/+	+/+	40	0	0	0	0	0	1	0	1	1
181	+/+	+/–	38	0	0	0	0	0	0	0	0	0
	Total		254	0	1	1	0	1	1	2	4	5
	Totals/section			0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.02	0.02
Mutants												
195 (1)	+/–	–/–	39	0	2	2	1	0	0	2	3	5
195 (2)	+/–	–/–	39	1	1	2	2	1	0	0	3	5
182	+/–	–/–	41	0	0	0	2	1	0	0	3	3
	Total		119	1	3	4	5	2	0	2	9	13
	Totals/section			0.01	0.03	0.03*	0.04	0.02	0.00	0.02	0.08**	0.11**
Mutants												
184	hyp/–	–/–	38	0	0	0	26	11	3	12	52	52
217 (1)	hyp/–	–/–	70	1	2	3	15	9	3	0	27	30
217 (2)	hyp/–	–/–	39	1	0	1	5	1	5	0	11	12
198 (1)	hyp/–	–/–	41	2	6	8	3	5	3	7	18	26
198 (2)	hyp/–	–/–	25	0	1	1	4	1	0	0	5	6
	Total		213	4	9	13	53	27	14	19	113	126
	Totals/section			0.02	0.04	0.06**	0.25	0.13	0.07	0.09	0.53***	0.59***

Total numbers of dividing cells for each group were normalized by dividing the number of labeled cells by the total number of sections that were counted. Fisher's exact tests were used to determine whether the numbers of dividing cells was significantly different in either mutant group compared with the control group (*P<0.05; **P<0.01; ***P<0.0001). Statistical tests were only applied to total hair cell, total supporting cell and total cell counts, and not to individual cell types.

Notch1$^{flox/-}$ cochleae versus 1.2-fold in the *Dll1/Jag2* double mutant cochleae; Fig. 5E). The difference in the severity of the phenotypes could be explained by the fact that the *Dll1*$^{hyp/-}$ *Jag2*$^{-/-}$ cochleae still retained some residual DLL1 function due to the hypomorphic nature of one of the alleles, or, alternatively, there may be yet another Notch ligand, such as JAG1, that plays a role in the lateral inhibitory process. However, the overall similarity between the *Dll1*$^{hyp/-}$ *Jag2*$^{-/-}$ double mutant and *Foxg1-Cre Notch1*$^{flox/-}$ phenotypes strongly suggests that both the DLL1 and the JAG2 ligand signal through the NOTCH1 receptor during sensory cell differentiation in the cochlea. Our genetic data is supported by experiments demonstrating that both the DLL1 and the JAG2 ligand can bind cell lines expressing the NOTCH1 receptor and activate a Notch reporter construct (Hicks et al., 2000; Shimizu et al., 2000).

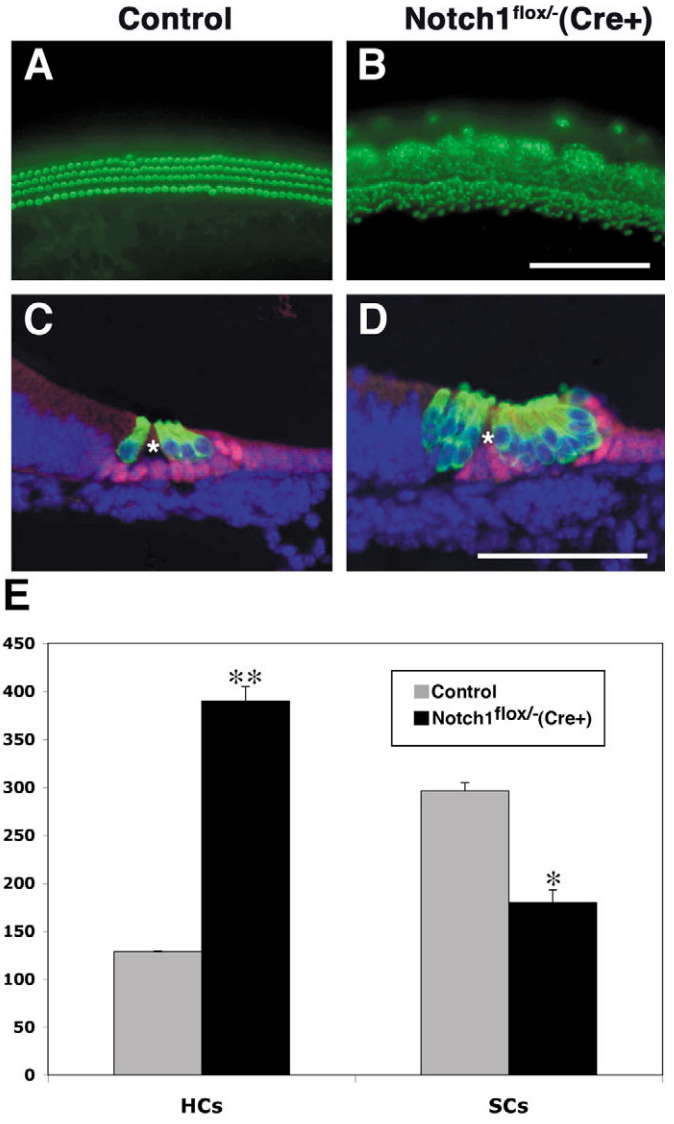

Fig. 5. Conditional *Notch1* deletion in the ear causes supernumerary hair cell formation in the cochlea that resembles the *Dll1*$^{hyp/-}$*Jag2*$^{-/-}$ mutant phenotype. (A,B) Lectin-stained whole-mount cochleae demonstrating the large increase in both inner and outer hair cells in *Foxg1-Cre Notch1*$^{flox/-}$ cochleae (B) when compared with *Notch1*$^{flox/-}$ cochleae (A), which appear normal. (C,D) Examples of the organ of Corti in sections from control (C) and *Foxg1-Cre Notch1*$^{flox/-}$ mutant (D) cochleae stained for myosin VIIa (hair cells, green) and p27kip1 (support cells, red). (E) Hair and supporting cell counts from 32 mid-modiolar sections, as shown in C and D (counts were done as for the *Dll11/Jag2* double mutants). Sections from three ears were counted for each group, either control or *Foxg1-Cre Notch1*$^{flox/-}$. Counts of both hair cells (HCs) and supporting cells (SCs) were significantly different between mutant and control cochleae (*P<0.001, **P<0.0001; Student's t-test). Similar to the *Dll1/Jag2* double mutant cochleae, the increases in hair cells did not equal the supporting cell losses (P<0.0001; one-way ANOVA). Scale bars: in B, 100 μm for A,B; in D, 50 μm for C,D.

Discussion

We demonstrate here a role for the Notch ligand DLL1 in patterning of the inner ear, where, along with the JAG2 ligand, it regulates the numbers of sensory hair cells that form in the organ of Corti. We also show that both ligands are likely to signal through the NOTCH1 receptor. Cochleae that lack both copies of *Jag2* and have a reduction in *Dll1* show an even greater increase in hair cell numbers than *Jag2*$^{-/-}$ mutant cochleae do, indicating that their roles are synergistic. Interestingly, although there were increased numbers of hair cells in *Dll1*$^{hyp/-}$ mutant cochleae, these increases were not statistically significant (Table 1). This could be due to the fact that, because a *Dll1* hypomorphic allele was used rather than a complete null, DLL1 protein levels may not have been reduced sufficiently to affect cochlear patterning. Thus, it was only when the mutant *Dll1* alleles were combined with either one or two copies of the *Jag2* null allele that significant effects on hair cell formation were achieved. These data suggest there is a threshold of Notch ligand expression (either *Dll1* or *Jag2*) that must be achieved for proper patterning to take place.

The standard model of lateral inhibition predicts that, if supernumerary sensory cells are produced via a cell fate switch, a concomitant loss of nonsensory supporting cells should be observed along with the increase in sensory hair cells. However, an examination of supporting cell populations revealed only a modest loss of p27^{kip1}-positive supporting cells that was significantly different from the increase in hair cell numbers (only about a 1.2-fold loss in supporting cells compared with a 1.7-fold increase in hair cells; Fig. 3I). This affect was even stronger in *Foxg1-Cre Notch1*$^{flox/-}$ mutant cochleae, where there was a dramatic 3-fold increase in hair cells accompanied by only about a 1.6-fold drop in p27^{kip1}-positive supporting cells (Fig. 5E). Deiter's cells appeared to be the most dramatically reduced supporting cell population, suggesting a cell fate switch from Deiter's cells to outer hair cells. Cell death does not appear to account for the missing supporting cells, as the number of apoptotic profiles was not increased in either *Dll1/Jag2* double mutant cochleae at E15.5 or *Foxg1-Cre Notch1*$^{flox/-}$ mutant cochleae at E18.5, as determined using the TUNEL assay (data not shown). BrdU incorporation studies in *Dll1/Jag2* double mutant cochleae revealed ectopic proliferation of supporting cells with very few labeled hair cells in the double mutant cochleae. Interestingly, pillar cell numbers were neither decreased nor increased in any of the Notch mutants described here, despite the fact that they were frequently observed to be dividing. These data suggest that pillar cells may have unique stem cell-like properties in

the developing organ of Corti. Stem cell-like cells have been identified in the adult mammalian utricle (Li et al., 2003), although it is not known whether similar cells are present in the cochlea. Taken together, these data indicate that the majority of the supernumerary hair cells arise via a cell fate switch, and not through continued cell proliferation. However, continued proliferation of the remaining nascent supporting cells compensates for the loss of supporting cell precursors, resulting in only modest decreases in the supporting cell population. These data support a role for the Notch pathway in mediating lateral inhibition in the inner ear, and also reveal a role for Notch signaling in the control of cell proliferation within the developing organ of Corti (Fig. 6).

A previously described role for the Notch pathway in regulating cell proliferation has been to maintain cells in an undifferentiated state, thereby promoting cell proliferation in many contexts. However, it has become clear in recent years that the effects of Notch signaling on cell proliferation are complex and context dependent (Weng and Aster, 2004). For example, an emerging role for the Notch pathway in promoting

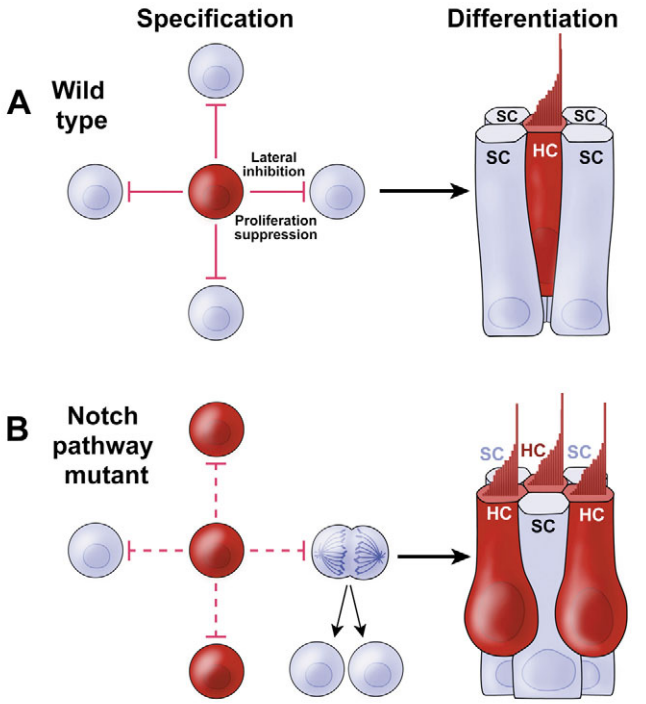

Fig. 6. Model for the role of Notch signaling during hair and supporting cell differentiation in the cochlea. The diagram depicts on the left the signaling that takes place between precursor cells (Specification), and shows on the right the resulting cell fates (Differentiation) that are adopted as a result of this signaling in both wild-type (A) and Notch pathway mutant (B) cochleae. Red cells have reduced Notch activation and will ultimately differentiate as hair cells. The blue cells are those receiving sufficient Notch activation to prevent them from adopting the hair cell fate. The Notch-mediated inhibitory signal acts in two ways: (1) to inhibit the surrounding cells from adopting the hair cell fate; and (2) to suppress continued proliferation of the surrounding cells. In the Notch mutant cochleae precursor cells, the inhibitory signal is reduced. This leads to the production of supernumerary hair cells and the continued cell division of some precursor cells that then preferentially differentiate into supporting cells. HC, hair cell; SC, supporting cell.

differentiation and cell cycle withdrawal has been revealed by studies in the skin and nervous system. Conditional *Notch1* deletion in mouse skin causes hyperplasia and deregulation of differentiation, leading to the development of basal cell carcinoma-like tumors (Rangarajan et al., 2001; Nicolas et al., 2003). Similarly, specific Notch1 downregulation is found in aggressive cervical cancers (Talora et al., 2002). These results have led to the suggestion that, in some contexts, Notch signaling may act as a tumor suppressor by promoting differentiation and cell cycle withdrawal. In the nervous system, the Notch pathway has been implicated in promoting the glial cell fate (Furukawa et al., 2000; Hojo et al., 2000; Morrison et al., 2000). Given that inner ear supporting cells share some characteristics with glia, these results raise the possibility that the Notch pathway plays an instructive role in supporting cell differentiation in the cochlea. When Notch signaling is downregulated, as in this study, some progenitor cells may fail to differentiate properly and continue dividing. Thus, Notch signaling may play a dual role in sensory differentiation in the inner ear, preventing adoption of the hair cell fate through a lateral inhibitory mechanism while promoting cell cycle withdrawal and supporting cell differentiation. Alternatively, the continued progenitor/supporting cell proliferation may be an indirect effect caused by the loss of contact-mediated inhibition due to the supporting cell fate conversion or other cellular changes in the epithelium. Regeneration studies using ototoxic drugs or acoustic trauma in the avian inner ear and in the mammalian vestibular regions have shown that hair cell death triggers proliferation of the supporting cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Warchol et al., 1993; Matsui et al., 2002). This suggests that, under normal circumstances, the hair cell exerts an anti-proliferative effect on the surrounding support cells, although the molecular identity of this signal is not known. It is interesting to note that analysis of the zebrafish Mind bomb mutant, a mutation in a gene encoding a ubiquitin ligase involved in Notch signaling, demonstrated a 10-fold excess hair cells in the inner ear, far more than could be explained by a simple cell fate conversion (Haddon et al., 1998). These results suggest that excess cell division may also occur in these mutant ears, although proliferation was not specifically examined. Taken together, these data suggest there may be a direct role for Notch signaling in the control of cell proliferation in the organ of Corti.

Previous studies have shown that control of cell proliferation in the organ of Corti involves the cyclin-dependent kinase inhibitors, $p27^{kip1}$ and $p19^{Ink4d}$ (Chen and Segil, 1999; Lowenheim et al., 1999; Chen et al., 2003), and the retinoblastoma protein (Rb) (Mantela et al., 2005; Sage et al., 2005). Studies of mice that lack these proteins in the mouse inner ear have shown that both $p19^{Ink4d}$ and Rb primarily appear to play a role in maintaining the postmitotic state of the hair cells, whereas $p27^{kip1}$ appears to be more involved in preventing the continued proliferation of the progenitor cells and supporting cells. Because we observed mainly dividing supporting/progenitor cells, we were interested in determining whether $p27^{kip1}$ expression was affected in the Notch mutants. We examined $p27^{kip1}$ expression in both *Dll1/Jag2* double mutant and *Foxg1-Cre Notch1*$^{flox/-}$ mutant cochleae at E18.5 (Fig. 3B-D, Fig. 5D) but found no differences in expression between mutants and controls.

Both *Dll1/Jag2* double mutant cochleae and *Foxg1-Cre Notch1$^{flox/-}$* cochleae displayed severely disorganized hair cell rows, and loss of organization and polarity of the hair cell stereociliary bundles. It is not clear whether this disorganization is a direct consequence of reduced Notch signaling, or whether it is a secondary event resulting from the abnormal cellular composition of the cochlea. Recent work has shown that an evolutionarily conserved mechanism for generating cell polarity within epithelial cell layers, termed planar cell polarity (PCP), is involved in regulating the polarity of inner ear hair cells and the orientation of their stereocilia bundles (Barald and Kelley, 2004). A role for Notch signaling in PCP has not been reported in any vertebrate system. However, a role for the Notch pathway in planar polarity has been shown during eye development in *Drosophila*, where Notch signaling specifies the R4 photoreceptor cell fate (McNeill, 2002). Proper specification of the R3 and R4 cell fates is essential for the loss of symmetry within the ommatidial clusters and thus the proper genesis of PCP within the eye. Similarly, correct specification of the hair cell and supporting cell fates may be required to establish PCP within the inner ear. Further insights into a possible role for Notch signaling in planar polarity in vertebrates may come from gene expression or genetic interaction studies with recently identified planar polarity genes in the inner ear (Curtin et al., 2003; Montcouquiol et al., 2003; Lu et al., 2004). However, it should be noted that, unlike the previously identified PCP mouse mutants where hair cell bundles are intact but disoriented, many of the hair bundles in the Notch mutants appear to have lost their organization completely. This suggests that reduced Notch signaling may affect a more basic level of bundle organization than PCP. Moreover, similarly disorganized bundles have been observed in mutant cochleae that lack the *Rb* gene, which exhibit a large overproliferation of hair cells (Mantela et al., 2005; Sage et al., 2005). Taken together, these data suggest that the disorganization may be an indirect effect of having too many hair cells in the epithelium, leading to a disruption in the signals that polarize and organize the stereocilia bundles.

These results highlight the complexity of Notch signaling in the inner ear, and demonstrate that the Notch pathway plays a dual role in regulating cellular differentiation and patterning in the cochlea, acting both through lateral inhibition and the control of cellular proliferation (Fig. 6). Unlike birds and amphibians (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Corwin and Oberholtzer, 1997), mammals demonstrate little regenerative potential in the inner ear (Johnsson et al., 1981; Forge, 1985), which may be related, at least in part, to a failure of supporting cell proliferation after injury (Roberson and Rubel, 1994). Our results suggest that modulation of the Notch pathway may represent an important avenue for regeneration studies in the mammalian inner ear.

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