Dll3 Is Expressed in Developing Hair Cells in the Mammalian Cochlea

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Notch mediates the process of lateral inhibition that controls the production of hair cells in the inner ear. Hair cells are known to express Notch ligands Dll1 and Jag2, which signal through Notch1 in adjacent supporting cells. However, recent genetic and pharmacological studies indicate that the level of Notch-mediated lateral inhibition is greater than can be accounted for by Dll1 and Jag2. Here, we report that another Notch ligand, Dll3, is expressed in developing hair cells, in a pattern that overlaps that of Dll1 and Jag2. We analyzed the cochleae of Dll3<sup>−/−</sup> mutant mice, but did not detect any abnormalities. However, earlier studies have demonstrated that there is functional redundancy among Notch ligands in cochlear development and loss of one ligand can be at least partially compensated for by another. Thus Dll3 may play a role in lateral inhibition similar to that of Dll1 and Jag2. Developmental Dynamics 236:2875–2883, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

The mammalian cochlea contains a specialized sensory epithelium called the organ of Corti, which mediates auditory function. The organ of Corti is composed of a highly ordered mosaic of mechanosensory hair cells and nonsensory supporting cells arranged in coiled rows. The orderly cellular arrangement and spatial and temporal gradients of differentiation in the organ of Corti make it a good model system to study sensory development and signaling. Of interest, development of the organ of Corti has been found to rely on several highly conserved mechanisms and much of our understanding of this model system has drawn on advances in the field of <i>Drosophila</i> neural development.

One of the key signaling pathways that regulate the development of the organ of Corti is the Notch pathway. In many model systems, from the <i>Drosophila</i> neuroblast to vertebrate sensory organs, Notch is best known for its role in lateral inhibition. In the process of lateral inhibition, cells that become committed to a particular cell fate express Notch ligands and activate the Notch receptor in neighboring cells, preventing them from adopting the same fate (Cabrera, 1990; Heitzler and Simpson, 1991). In the mammal, there are four Notch receptors and several ligands including Delta (Dll) 1, 3, and 4; and Jagged (Jag) 1 and 2 (Sparrow et al., 2002).

There is a good deal of evidence to show that lateral inhibition, mediated by Notch, controls cell fate determination in the cochlea; Notch ligands are expressed by the developing hair cells and activate Notch in the surrounding cells to prevent them from adopting a hair cell fate. In the mouse, Notch1 is expressed in the developing cochlea, including the presumptive sensory epithelium, while Dll1 and Jag2 are expressed in the developing hair cells (Lanford et al., 1999; Zhang et al., 2000; Kiernan et al., 2005). Loss of either Dll1 or Jag2 in the developing cochlea results in extra rows of hair cells.
cells (Lanford et al., 1999; Morrison et al., 1999; Kiernan et al., 2005; Brooker et al., 2006). When mutations inDll1 and Jag2 were combined in Dll1hyp/Jag2−/− mutant mice, cochleae displayed a greater increase in hair cell numbers, indicating that Dll1 and Jag2 act synergistically in hair cell development (Kiernan et al., 2005).

In addition to Jag2 and Dll1, recent evidence suggests that there is an additional ligand for Notch in the developing organ of Corti. First, conditional Notch1 mutations in the otic epithelium of FoxG1-Cre Notchflox/Notch1 mutant mice resulted in a far more dramatic increase in hair cell numbers than that observed in the Dll1hyp/Jag2−/− cochlea (Kiernan et al., 2005). Second, severe hair cell overproduction phenotypes were observed in cochlear explants treated with Notch1 antisense oligonucleotides or pharmacological inhibitors of Notch signaling (Zine et al., 2000; Yamamoto et al., 2006; Takebayashi et al., 2007). As noted above, there are additional Notch ligands, and their expression has not previously been described in the developing cochlea. Here we report for the first time that another Notch ligand, Dll3, is expressed in developing hair cells in a pattern that closely overlaps with Dll1 and Jag2 throughout cochlear development. Thus, Dll3 may play a role in lateral inhibition similar to that of Dll1 and Jag2.

RESULTS

Dll3 Is Coexpressed With Dll1 and Jag2 in Presumptive Auditory Inner Hair Cells at E15.5

We used in situ hybridization to determine whether Dll3 is expressed in the developing cochlea. We first detected this gene at embryonic day (E) 15.5 in a patch of one or two cells in the upper layers of the prosensory domain. To compare the temporal and spatial expression profiles of Dll3 with the other Notch ligands, Dll1 and Jag2, we probed similar E15.5 cochlear sections for these genes. Dll3, Dll1, and Jag2 are expressed in overlapping domains in the E15.5 mouse cochlea (Fig. 1A–I). We compared the expression of these Notch ligands with that of Math1, which is expressed in the differentiating hair cells (Fig. 1J–L). The expression of all three Notch ligands corresponds to the domain where inner hair cells will develop (Fig. 1K,L, arrowhead), rather than in the region of outer hair cell differentiation (Fig. 1K,L bracket).

We further confirmed that Dll3 is localized to the presumptive inner hair cell domain, by post-in situ immunolabeling for p27kip1 and Neurofilament-M (NF-M). The cell cycle regulator p27kip1 labels the zone of nonproliferating cells and marks the location of the sensory precursor domain (Chen and Segil, 1999). Immunolabeling of probed tissues confirms that Dll3 is expressed along the medial edge of the p27kip1 domain, in the two most basal turns of the cochlea at E15.5 (Fig. 1M–O). By immunolabeling with an antibody to NF-M, we also found that the peripheral processes of spiral ganglion neurons terminated near the Dll3-expressing, presumptive hair cells (Fig. 1M–O).

We found that Dll3 expression lags slightly behind expression of Dll1 during cochlear development. It was previously reported that Dll1 and Jag2 are expressed in cochlear hair cells beginning at approximately E14.5 (Lanford et al., 1999; Morrison et al., 1999). Similarly, we were able to detect strong expression of Dll1 in the basal turns of most E14.5 cochlea (Fig. 2B,D); however, Dll3 was not detectable at this age (Fig. 2A,C). Even at E15.5, Dll1 and Jag2 expression appeared stronger than Dll3 expression in similar sections (Fig. 1A–I), particularly in the mid-basal turn (Fig. 1B,E,H), which is less differentiated than the base. While early Dll1 and Jag2 expression was observed in columns extending through the thickness of the epithelium (Fig. 1E,H), Dll3 was restricted to cells near the lumen, which are presumably more differentiated. Thus, it appears that Dll3 is expressed slightly after Dll1 during the process of hair cell differentiation. To further define the timing of Dll3 expression, we probed similar E15.5 sections for MyosinVIIa; we found that this marker of hair cells is not yet expressed in the cochlea (data not shown). Therefore, Dll3 expression occurs after Math1 and Dll1, but before expression of MyosinVIIa in developing hair cells.

To investigate the temporal relationship between Dll3 expression and cell division, we labeled dividing cells at E14.5 with a 2-hr pulse of bromodeoxyuridine (BrdU) in utero. We then probed tissue for expression of Dll3 and Dll1 with in situ hybridization and used immunofluorescence to identify cells that had incorporated BrdU. Expression of Dll1 in the cochlea was usually one or two cell diameters away from the BrdU-positive region. However, we occasionally observed BrdU-positive cells within the region of Dll1 expression in the most basal portions of the E14.5 cochlea (Fig. 2F arrow). We never observed Dll3 expression to overlap with BrdU labeling.

Dll3 Is Expressed in Developing Inner and Outer Hair Cells in the E17 Mouse Cochlea

Dll3 continues to be expressed at later stages of cochlear development. In situ hybridization of cochleae at E17 revealed that Dll3 follows the wave of hair cell differentiation (Lim and Anniko, 1985; Chen and Segil, 1999) and overlaps with expression of Dll1, Math1, and MyosinVIIa (Fig. 3A–D,G–J). In the E17 cochlea, Dll3 is expressed in developing hair cells in a base-to-apex gradient that extends to the middle turn (Fig. 3A). Jag1 is expressed in supporting cells throughout most of the developing organ of Corti at this stage (Fig. 2E). Jag2 expression is also very similar to that of Dll3 at E17. Jag2 is expressed in developing hair cells, strongly in the basal turns and at a low level in the middle turn at E16.5 (Fig. 3M).

A close examination of the basal turn of E17 cochlear duct reveals that, in this region, Dll3 is expressed at a low level in inner hair cells and more strongly in outer hair cells (Fig. 3G). Dll1, Math1, and MyosinVIIa are also expressed at low levels in inner hair cells and higher levels in outer hair cells in the basal turn at this stage (Fig. 3H–J). This pattern is consistent with an inner hair cell to outer hair cell sweep of gene expression, where down-regulation has begun in inner...
Fig. 1. DLL3 is coexpressed with Dll1 and Jag2 in developing presumptive inner hair cells at embryonic day (E) 15.5. A–L: Similar sections of E15.5 cochlea were probed for expression of DLL3 (A–C), Dll1 (D–F), Jag2 (G–I), and Math1 (J–L) with in situ hybridization. On the right are high-magnification views of mid-basal (B,E,H,K) and basal (C,F,I,L) turns boxed in (A,D,G,J), respectively. A–C: Expression of DLL3 is observed in the presumptive inner hair cell domain strongly in the basal turn (C) and at a low level in the mid-basal turn (B). Expression patterns of Dll1 and Jag2 are similar to DLL3, but both are expressed more highly than DLL3 in the mid-basal turn (compare B to E, H). J–L: Strong expression of Math1 marks the column of presumptive inner hair cell precursors (arrowheads) and weaker, more lateral expression of Math1 marks the region of presumptive outer hair cell precursors (brackets). M–O: Post-in situ hybridization immunolabeling with p27 (green) and NF-M (red) confirms that expression of DLL3 is localized to the presumptive inner hair cell domain. Scale bar = 100 μm in A (applies to A,D,G,J), 50 μm in B (applies to B,C,E,F,H,I,K,L).
hair cells in the most basal region at this stage. In similar basal portions of the E17 cochlear duct, Jag1 is expressed at low levels in supporting cells and at higher levels in a subset of cells in the greater epithelial ridge medial to the region of developing inner hair cells (Fig. 3K). In the basal turn, Jag2 is expressed at a moderate level in inner hair cells and stronger in outer hair cells at this stage (Fig. 3N).

We used a combination of immunohistochemistry and in situ hybridization to examine the expression profile of Hes1 protein and Hes5 mRNA, two downstream targets of Notch signaling. In the E18 cochlea, Hes1 is expressed in supporting cells and a population of cells in the greater epithelial ridge, medial to the inner hair cell region (Fig. 3O). Hes1 is also expressed in presumptive glial cells associated with the processes of spiral ganglion neurons. At E17, Hes5 expression is detected in developing Dieters’ cells in the basal and middle turns of the cochlea (Fig. 3F,L).

**Dll3 Is Not Required for the Normal Development of the Cochlea**

To test whether the expression of Dll3 is required for normal cochlear development, we examined the postnatal cochlea of Dll3 Pudgy (Dll3pu) mutant mice (Kusumi et al., 1998). The Dll3pu mutation is a 4-bp deletion in the third exon of Dll3, which leads to a frame shift and early truncation of the predicted protein product, before the DSL (Delta/Serrate/Lag2) domain (Gruneberg, 1961; Kusumi et al., 1998). Dll3pu homozygous mutant mice are viable, but they exhibit several defects, including severe skeletal dysplasia and body truncation due to abnormal somite development (Kusumi et al., 1998). We crossed Dll3pu homozygous mutant males with C57BL/6J females to create heterozygous progeny, which we then intercrossed to produce litters of mixed genotype. Dll3pu homozygous mutant mice were identified by phenotype, and all mice were genotyped for the 4-bp pu mutation with the polymerase chain reaction (PCR).
Fig. 3. A–D: In situ hybridization of similar embryonic day (E) 17 sections shows that the pattern ofDll3 expression in developing inner and outer hair cells is highly overlapping with expression ofDll1, Math1, and MyosinVIIa, while Jag1 and Hes5 have distinct patterns of expression in support cells (asterisks indicate regions of expression). A–L: High-power differential interference contrast microscopy images (G–L) of the boxed regions in A–F. (Inner hair cells and outer hair cells are indicated with arrows and arrowheads, respectively.) G–J: Expression ofDll3, Dll1, Math1, and MyosinVIIa is observed in a low level in inner hair cells and a stronger level in outer hair cells. K: Jag1 is expressed in support cells as well as a population of cells in the greater epithelial ridge. L: Hes5 expression is detected in the region of presumptive Deiters cells. M: Jag2 expression at E16.5 is found in inner and outer hair cells. N: High-power view of boxed region in M. O: Hes1 antibody staining (blue) in E18 cochlea is found in supporting cells and a population of cells in the greater epithelial ridge. Scale bar = 200 μm in A (applies to A–F,M), 100 μm in G (applies to G–L,N,O).
We examined the inner ears from Dll3pu homozygous mutants and wild-type control littermates at P3. The gross morphology of the inner ears from mutant mice was normal (data not shown). We processed cochlea for whole-mount fluorescent immunostaining with antibodies to Myosin-VIIa, to label hair cells, and Prox1, to label developing Dieters' and pillar cells (Bermingham-McDonogh et al., 2006). We examined the cochlea from seven Dll3pu mutants and five control animals, obtained from three different P3 litters. The morphology and arrangement of hair cells and support cells was normal in Dll3pu mutants, as compared with wild-type controls (Fig. 5). The normal appearance of one inner and three outer hair cells (Fig. 5A,B,E,F) and three Dieters' and two pillar cells (Fig. 5C–F) was observed throughout the length of the cochlea in all animals, with only minor exceptions. There was an average of two to three double inner hair cells per cochlea and regions of mild support cell disorganization in both mutant and control animals, which is likely attributed to the strain background (JAX GEMM Strain–Mutant Stock descended from an X-rayed (101/Rl x C3H/Rl)F1 male).

DISCUSSION

Here, we have reported that Dll3 is coexpressed with Dll1 and Jag2 in developing auditory hair cells beginning at E15.5. Previous studies have shown that hair cell differentiation in the cochlea propagates from the base to apex and from the inner hair cell domain to the outer hair cell domain and is marked by the concentrated expression of the proneural basic helix–loop–helix transcription factor Math1 (Bermingham et al., 1999; Lanford et al., 2000; Chen et al., 2002; Woods et al., 2004; Hayashi et al., 2007). We find that expression of hair cell-specific Notch ligands follows closely behind Math1 expression in the developing cochlea. While the onset of Dll1 expression in developing inner hair cells at E14.5 closely coincides and occasionally overlaps with cell cycle exit (Fig. 2F), Dll3 expression occurs in the same population of cells approximately 1 day later. Lateral inhibition is thought to occur in cells surrounding Math1 expression, and possibly within the Math1-expressing population, beginning at E14.5. Therefore, although Dll3 is expressed slightly later than Dll1 in the developing hair cell domain, Dll3 is still expressed at the right time and place to play a role in lateral inhibition during cochlear development.

Dll3 continues to be coexpressed with Dll1 and Jag2 in developing inner and outer hair cells until birth and is down-regulated during the first postnatal week. Expression of Dll3 in the cochlea follows the wave of hair cell differentiation as it sweeps from base to apex and inner hair cell to outer hair cell from E15.5 to P0. The period of Dll3 up-regulation is followed by a wave of down-regulation that occurs between E17 and approximately P3. Of interest, this finding
indicates that the duration of expression of Dll3, as well as Dll1 and Jag2, in individual hair cell precursors is only approximately 3 or 4 days, although duration of expression in cochlear hair cells as a whole lasts approximately 8 days. Throughout this period of Dll3 expression, Dll1 and Jag2 are also expressed in hair cells, and undergo a similar down-regulation in the early postnatal period. The similarity in expression patterns among these three Notch ligands is consistent with an ongoing role for Dll3 in lateral inhibition.

The expression patterns of Hes1 and Hes5 indicate that they are potential downstream targets of Dll3/Notch signaling. Earlier studies have implicated Hes1 and Hes5 in lateral inhibition in the cochlea (Zine et al., 2001; Zine and de Ribaupierre, 2002). However, it is also likely that both Hes1 and Hes5 also mediate the Notch signal activated by Dll1 and Jag2. Another DSL ligand, Jag1 is expressed in the cochlea, but the expression pattern and results of knockout studies indicate that it does not function in lateral inhibition, but may have a role in prosensory specification that is still poorly understood (Fig. 3E,K; Kiernan et al., 2001, 2006).

Dll3 is likely redundant for lateral inhibition with Dll1 and Jag2, because mice mutant in Dll3 (Pudgy Dll3<sup>pu<sup>mut</sup></sup>) do not have a defect in hair cell patterning. This result is not surprising given the redundancy of expression of DSL ligands in developing hair cells. Earlier studies have shown that loss of Jag2 or Dll1 alone results in relatively modest increases in hair cell numbers (Kiernan et al., 2005; Brooker et al., 2006). More significant hair cell over-production phenotypes in cochlea that carried mutations in both Jag2 and Dll1 indicate that there is synergistic function between ligands, and loss of one Notch ligand in hair cells can be compensated by others (Kiernan et al., 2005). Therefore, Dll3 compensation could account for the mild phenotypes observed in Dll1 and Jag2 loss-of-function studies as compared with Notch1 mutation or Notch inhibition experiments (Lanford et al., 1999; Yamamoto et al., 2006; Takebayashi et al., 2007).

Dll3 is the most divergent DSL ligand. Understanding of Dll3 function has been complicated by conflicting reports (Dunwoodie et al., 1997; Ladi et al., 2005). DSL ligands are thought to function in two ways, first, in the well-characterized activation of Notch signaling through cell–cell interactions, and second, as cell-autonomous inhibitors of Notch signaling (Henrique et al., 1997; Jacobsen et al., 1998; Sakamoto et al., 2002). It has recently been reported that Dll3 does not activate Notch signaling like other DSL ligands, but that Dll3 does act as a cell autonomous inhibitor of Notch signaling (Ladi et al., 2005). Because we were unable to detect a phenotype in the Dll3 deficient mice, we are unable to determine whether Dll3 differs functionally from other DSL ligands. Further studies, such as the generation of Dll3/Dll1/Jag2 double or triple mutants, may provide insight into this issue and the role of Dll3 in cochlear development.

## EXPERIMENTAL PROCEDURES

### Animals

Mice were housed in the Department of Comparative Medicine, and all procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Washington. Embryonic and postnatal tissues from Swiss Webster mice were used for in situ hybridization and immunostaining. Dll3<sup>pu<sup>mut</sup></sup> mutant mice were obtained from Jackson Labs (stock no. 00306). We crossed Dll3<sup>pu<sup>mut</sup></sup> homozygous mutant males with C57BL/6J females to create heterozygous progeny, which we then intercrossed to generate homozygous Dll3<sup>pu<sup>mut</sup></sup> and wild-type littermates for phenotypic analysis. Dll3<sup>pu<sup>mut</sup></sup> mice were genotyped for the 4-bp pudgy deletion in Dll3 exon 3 as described previously (Kusumi et al., 1998), with minor changes. Briefly, tail DNA was collected from postnatal mice and subjected to PCR using the following primers (5’-ACGAGCGTCCCCGGTCTATAC-3’ and 5’-AGGTTGAGGTGAGGCAC-3’). The Dll3pu product (114 bp) and wild-type product (118 bp) were resolved using 4% MetaPhor Agarose gel (Cambrex).

### Paraffin In Situ Hybridization and Immunostaining

Embryos were collected from timed-pregnant Swiss Webster or C57BL/6J mice and staged according to Kaufman (1992). For postnatal mice, P0 was defined as the day of birth. For BrdU incorporation study, timed-pregnant E14.5 females received an intraperitoneal injection of BrdU (50 mg/kg) 2 hr before death. In situ hybridization was performed as previously described (Nelson et al., 2004; Hayashi et al., 2007). Briefly, embryonic whole heads or postnatal half-heads were fixed overnight at 4°C in a modified Carnoy’s solution (60% EtOH/30% formaldehyde/10% acetic acid), dehydrated though an EtOH series, prepared for paraffin embedding, and sectioned at 8 μm. Digoxigenin-labeled probes were in vitro transcribed from linearized cDNA clones corresponding to: Dll3 (BC052002, IMAGE:6404029), Dll1 (BC057400, IMAGE:6402691), Jag2 (BC009082, IMAGE:3598850), Jag1 (BC058675, IMAGE:6834418), Math1 (BC051256, IMAGE:6530849), Hes5 (BC103539, IMAGE:40039948), and MyosinVIIa (PCR clone corresponding to bp 1–1090 of NM_008663.1 cloned into pCRII-TOPO plasmid, O.BMcD.). Overnight hybridization and subsequent washes were carried out at 68°C. Hybridized probe was detected using anti-digoxigenin alkaline phosphatase-conjugated antibody (1:2,000 dilution, Roche Biochemicals, Indianapolis, IN) and visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for a blue precipitate. After in situ hybridization, sections were post-fixed in 4% parafomaldehyde and rinsed in phosphate buffered saline (PBS).

For immunostaining performed on paraffin sections after in situ hybridization, slides were blocked for 1 hr in 10% goat serum PBS with 0.1% Triton X-100 at room temperature. Primary antibodies were diluted in block and incubated overnight at 4°C: mouse anti-ti-p27 (1:500 dilution, BD Transduction Laboratories), rabbit anti-neurofilament-M (145 kDa; 1:1,000 dilution, Chemicon), rabbit anti-Calretinin (1:2,000 dilution, Swant). For BrdU labeling, rat anti-BrdU (1:100 dilution;
Accurate Chemical) was diluted in blocking solution supplemented with 100 Kunitz units/ml DNase1. Slides were washed and incubated with corresponding goat secondary antibodies at 1:500 for 1 hr at room temp. Secondary antibodies were species-specific Alexa Fluor 488 or 568 (1:500, Invitrogen).

For Hes1 immunostaining of parafin sections, antigen retrieval was performed by incubation in Revealit-Ag (Immunosolution, Jesmond, Australia) overnight at 37°C. Slides were washed in PBS then blocked for 1 hr at room temperature. Primary antibody, rabbit anti-Hes1 (H140; 1:250 dilution, SantaCruz Biotechnology), was diluted in block overnight at 4°C. Slides were washed and incubated with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (A9919; 1:200 dilution, Sigma) for 2 hr at room temperature. Slides were washed in PBS, equilibrated to alkaline pH in NTMT (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl2, 0.1% Tween 20) and incubated with NBT/BCIP liquid substrate (Sigma) to form a blue precipitate.

After immunostaining, slides were cover-slipped in Fluoromount G (Southern Biotechnology, Birmingham, AL). Images of stained sections were acquired on a Zeiss Axioplan 2 microscope equipped with differential interference contrast microscopy optics and a Spot camera. Images were compiled with Adobe Photoshop 7.0.

Whole-Mount Immunostaining

Inner ear tissues were harvested from postnatal day (P) 3 mice and fixed in 4% paraformaldehyde for 3 hr at 4°C, then washed in PBS, and the cochlear ducts were dissected from surrounding tissues. To expose the organ of Corti, the anlage of the stria vascularis was removed using fine forceps. Tissue was permeabilized in PBS/0.1% Triton X-100 for 1 hr at room temperature and blocked for 2 hr in 10% goat serum PBS with 0.1% Triton X-100 at room temperature. Primary antibodies were diluted in block and tissues were incubated overnight at 4°C: rabbit anti-Prox1 (AB5475; 1:1,000, Chemicon) and guinea pig anti-ti-MyosinVIIa (1:2,000 dilution, gift from Stefan Heller, Stanford University). Tissues were washed in block and incubated overnight at 4°C in secondary antibodies: chicken anti-rabbit AlexaFluor 488 and goat anti-guinea pig AlexaFluor 568 (both at 1:500, Molecular Probes). Tissues were washed in PBS and cover-slipped in PBS/glycerol. Confocal images of whole-mounts were captured on a Zeiss LSM Pascal confocal microscope. Images were processed using Improvion Velocity (3.0.2) and Adobe Photoshop (7.0).

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