

Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear

Rachael Brooker^{1,2}, Katsuto Hozumi³ and Julian Lewis¹

Each of the sensory patches in the epithelium of the inner ear is a mosaic of hair cells and supporting cells. Notch signalling is thought to govern this pattern of differentiation through lateral inhibition. Recent experiments in the chick suggest, however, that Notch signalling also has a prior function – inductive rather than inhibitory – in defining the prosensory patches from which the differentiated cells arise. Several Notch ligands are expressed in each patch, but their individual roles in relation to the two functions of Notch signalling are unclear. We have used a Cre-LoxP approach to knock out two of these ligands, Delta1 (*Dll1*) and Jagged1 (*Jag1*), in the mouse ear. In the absence of *Dll1*, auditory hair cells develop early and in excess, in agreement with the lateral inhibition hypothesis. In the absence of *Jag1*, by contrast, the total number of these cells is strongly reduced, with complete loss of cochlear outer hair cells and some groups of vestibular hair cells, indicating that *Jag1* is required for the prosensory inductive function of Notch. The number of cochlear inner hair cells, however, is almost doubled. This correlates with loss of expression of the cell cycle inhibitor p27^{Kip1} (*Cdkn1b*), suggesting that signalling by *Jag1* is also needed to limit proliferation of prosensory cells, and that there is a core part of this population whose prosensory character is established independently of *Jag1*-Notch signalling. Our findings confirm that Notch signalling in the ear has distinct prosensory and lateral-inhibitory functions, for which different ligands are primarily responsible.

KEY WORDS: Notch, Jagged1 (*Jag1*), Delta1 (*Dll1*), p27^{Kip1} (*Cdkn1b*), Ear, Hair cells, Lateral inhibition, Lateral induction, Mouse, Cochlea

INTRODUCTION

The Notch pathway serves for communication between cells that are next-door neighbours: both the receptor Notch and its ligands Delta and Serrate are transmembrane proteins, so that signalling depends on direct cell-cell contact. Binding of ligand triggers proteolytic cleavage of Notch, releasing an intracellular fragment, N^{ICD}, that enters the nucleus and regulates transcription of specific target genes (for reviews, see Baron, 2003; Lai, 2004). In this way, Notch signalling gives each cell the means to control gene expression in its immediate neighbours. Feedback in this control machinery, such that the strength of the signal a cell receives affects the strength of the signal it delivers, can generate striking spatial and temporal patterns of gene expression within a multicellular population (Lewis, 1998). Thus, in the phenomenon of lateral inhibition, activation of Notch in a given cell diminishes the ability of that cell to produce functional ligands that can activate Notch in the neighbours (Heitzler and Simpson, 1991). A cell that signals more strongly thereby causes its neighbours to signal more weakly, and the effect is to amplify differences between adjacent cells. If the feedback is sufficiently steep, the predicted outcome is a mosaic of cells in sharply different states (Collier et al., 1996): cells that express functional Notch ligands alternate with cells that do not; the cells that express ligands escape Notch activation because their neighbours do not express ligands, and vice-versa.

Completely different behaviour is predicted if Notch activation regulates ligand expression in an opposite way – that is, if Notch activation in a given cell increases the ability of that cell to produce

functional Notch ligands, as occurs, for example, at the *Drosophila* wing margin (Bray, 1998; de Celis and Bray, 1997) and in some vertebrate cells (Ross and Kadesch, 2004). In this case, which we have called lateral induction (Eddison et al., 2000; Lewis, 1998), the effect is cooperation between neighbours, instead of competition. A cell that expresses Notch ligands strongly will make its neighbours do the same. Notch signalling will tend to intensify, maintain and extend the domain where Notch ligands are produced and Notch is activated.

The sensory patches in the vertebrate inner ear seem at first sight a beautiful example of lateral inhibition. They consist of a mosaic of sensory hair cells and supporting cells, generated from prosensory precursor cells that express Notch and have the ability to differentiate in either way (Fekete et al., 1998). At the onset of differentiation, the nascent hair cells switch on expression of two Notch ligands, Delta1 (*Dll1*) and Jagged2 (*Jag2*, in mammalian terminology) (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999), thereby apparently inhibiting the adjacent prosensory cells from becoming hair cells and forcing them to become supporting cells instead. Loss of *Jag2* in the mouse allows hair cells to be produced in excess (Lanford et al., 1999), though the effect is mild, suggesting that *Jag2* and *Dll1* cooperate, in quasi-redundant fashion, to deliver lateral inhibition to the neighbours of hair cells. Similar but stronger effects are seen in zebrafish with a mutation in the *deltaA* gene (Riley et al., 1999). In the zebrafish *mind bomb* mutant, where Notch signalling is still more severely defective (Haddon et al., 1998; Jiang et al., 1996; Schier et al., 1996), all the cells in the prosensory patches differentiate as hair cells instead of supporting cells and the expression of the homologs of *Dll1* and *Jag2* is greatly increased, as the lateral inhibition hypothesis would predict.

At the same time, in the same tissue, however, another Notch ligand, *Jag1* (*Serrate1* in the chick, *SerrateA* or *Jagged1b* in the zebrafish), shows completely opposite behaviour, suggestive of lateral induction rather than lateral inhibition. Expression of this

¹Vertebrate Development Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK. ²Hereditary Hearing Group, The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK. ³Department of Immunology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan.

Author for correspondence (e-mail: julian.lewis@cancer.org.uk)

ligand begins (in mouse and chick) several days before hair-cell differentiation, and appears to mark the prosensory patches, within which its level of expression is uniform from cell to cell (Adam et al., 1998; Morrison et al., 1999). Subsequently, Jag1 becomes restricted to the supporting cells, and its expression in this subpopulation is strong, uniform, and sustained (Stone and Rubel, 2000), even though the supporting cells are in contact with one another and exposed to the influence of Dll1 (transiently) and Jag2 (persistently) from neighbouring hair cells. All this suggests that *Jag1* expression is positively regulated by activated Notch, so that the effects of Dll1 and Jag2 in neighbouring hair cells combine with the effects of Jag1 in neighbouring supporting cells to keep Jag1 levels high in each supporting cell. Experiments in the chick embryo in which the Notch signalling pathway is artificially blocked (Eddison et al., 2000) or overactivated (Daudet and Lewis, 2005) indicate that activated Notch does indeed stimulate expression of *Jag1*.

What then is the function of Jag1 in the inner ear? There are at least two attractive suggestions, and they conflict. The procrastination hypothesis proposes that Jag1, by keeping the level of Notch activation high in the prosensory cells and, later, in the supporting cells, serves to prevent them from differentiating prematurely or inappropriately into hair cells (Eddison et al., 2000). Indeed, in the zebrafish *mind bomb* mutant, where Notch signalling is defective, the whole population of the prosensory patch differentiates into hair cells prematurely (Haddon et al., 1998). By contrast, the prosensory induction hypothesis proposes that activation of Notch by Jag1 serves to put cells into a prosensory state and is needed to make hair cell differentiation possible (Adam et al., 1998; Eddison et al., 2000; Kiernan et al., 2001). Support for this latter idea comes from our recent experiments in the chick. By expressing N^{1CD} ectopically, we have found that Notch activity has two different roles in the inner ear: at early stages it can induce formation of a sensory patch (converting cells to a prosensory character), whereas at late stages it mediates lateral inhibition, limiting the proportion of cells within a prosensory patch that actually differentiate as hair cells (Daudet and Lewis, 2005).

Homozygous knockout mutations of *Jag1* and *Dll1* in the mouse are both lethal at early embryonic stages, before the ear has developed (Hrabé de Angelis et al., 1997; Xue et al., 1999). Heterozygous *Dll1* mutants have apparently normal ears (Morrison et al., 1999). Heterozygous *Jag1* mutants show a mild reduction of the number of outer hair cells in the cochlea but a mild increase in the number of inner hair cells, as well as defects in their semicircular canals (Kiernan et al., 2001; Tsai et al., 2001).

To discover which, if any, of our hypotheses are correct, we need to see the homozygous knockout phenotypes in the ear. We have used a Cre-Lox strategy to achieve this. Loss of *Jag1* does not cause premature differentiation but does lead to a severe deficit of sensory tissue, as predicted by the prosensory induction hypothesis. Loss of *Dll1* has effects of an opposite type, though less severe, consistent with the predicted role as a mediator of lateral inhibition. Neither phenotype, however, is quite as we expected; in both cases, in different ways, it seems that the Notch ligands influence patterns of cell division as well as choices of differentiated fate.

MATERIALS AND METHODS

Production of mice carrying conditional *Dll1^{fllox}* and *Jag1^{fllox}* alleles

We produced mice carrying conditional alleles of *Dll1* (*Dll1^{fllox}*) and *Jag1* (*Jag1^{fllox}*), in which LoxP sites flanked exons encoding the DSL (Delta-Serrate-Lag2) domain, required for binding to Notch (Jacobsen et al., 1998; Lai, 2004). Details for *Dll1^{fllox}* are given by Hozumi et al. (Hozumi et al.,

2004). The same strategy was used for *Jag1^{fllox}* (see Fig. S1 in the supplementary material). Recombination of *Jag1^{fllox}* by Cre is predicted to knock out gene function by removing the DSL exons (exons 3 and 4 for *Dll1*, 4 and 5 for *Jag1*) and introducing a frame shift that generates a stop codon shortly after the start of exon 5 (for *Dll1*) or 6 (for *Jag1*).

Mouse breeding

Mice were bred on a C57BL/6J background. The *Foxg1^{Cre}* strain, with Cre knocked into the *Foxg1* locus in place of the *Foxg1*-coding region, was a gift from Jean Hébert and Susan McConnell, and was maintained as a heterozygous colony, as homozygotes are not viable; genotyping was by PCR as described by Hébert and McConnell (Hébert and McConnell, 2000). Both *Jag1^{fllox}* and *Dll1^{fllox}* mice were homozygous viable. They were genotyped by PCR as follows: for unrecombined *Jag1^{fllox}*, the primers were TGAAGCTCAGGACAGTGCTC and GTTTCAGTGCTGCCATTGC (flanking the 5' LoxP site); for the recombined allele, *Jag1^{Δfllox}*, primers were TGAAGCTCAGGACAGTGCTC and a primer targeted downstream of the 3' LoxP site, ATAGGAGGCCATGGATGACT. For unrecombined *Dll1^{fllox}*, primers were CACACCTCTACTTACCTGA and GAGAGTACTGGATGGAGCAAG (flanking the 5' LoxP site); for the recombined allele, *Dll1^{Δfllox}*, primers were CACACCTCTACTTACCTGA and a primer targeted downstream of the 3' LoxP site, GGCGCTCAAAGGATATGGGA.

Mice carrying the conditional alleles were crossed with *Foxg1^{Cre}* heterozygotes, and doubly heterozygous progeny were identified by PCR. These were then crossed with mice carrying one copy of a conditional allele, to produce litters containing conditional knockout mice (*Foxg1^{Cre/+}; Dll1^{fllox/fllox}* or *Foxg1^{Cre/+}; Jag1^{fllox/fllox}*) and sibling controls. For *Jag1*, out of 499 genotyped embryos collected at E16.5 to P0 from such matings, 37 had the conditional knockout genotype, implying some intrauterine mortality, as Mendelian ratios would give 62. For *Dll1*, 26 out of 214 embryos had the conditional knockout genotype – close to the Mendelian proportion.

Immunohistochemistry

Embryos were staged by age, taking the day of the vaginal plug as E0.5, and were checked for morphological stage at the time of collection (Kaufman, 1992; Theiler, 1989). For serial sections at E17.5, embryos were decapitated and their heads were fixed in 4% paraformaldehyde in PBS for 1 hour, then embedded in 1.5% Lennox agar (Gibco-BRL) with 3% sucrose and cryosectioned at 15 μm. For whole-mount immunostaining of the cochlea, inner ears were dissected from the temporal bone and a small opening was made in the apex of each cochlea before fixation in 4% formaldehyde in PBS for 1 hour. The tissue was then permeabilised by immersion for 1 hour in PBS with 0.3% Triton-X and 10% foetal calf serum, followed by incubation overnight at 4°C with primary antibody in PBS with 0.1% Triton-X and 10% fetal calf serum.

Antibodies and reagents used were: Jag1 (C-20, Santa Cruz; 1/100), p27^{Kip1} (Cell Signalling; 1/100), calretinin (Chemicon; 1/1000) and Alexa A488-conjugated rabbit anti-goat and goat anti-rabbit as secondaries (Molecular Probes; 1/500). Alexa 594- and 633-conjugated phalloidin (Molecular Probes; 1/100) and DAPI were applied to the sections with the secondary antibody.

For flat-mounting, whole-mount cochleas from E17.5 mice were cut into three, giving apical, middle and basal regions. Those from the younger embryos were left whole. Whole-mount and sectioned specimens were mounted in SlowFade (Molecular Probes) and imaged with a Zeiss LSM510 confocal microscope.

E10.5 embryos were formalin fixed, embedded in wax, serially sectioned at 8 μm and stained with 3A10 neurofilament antibody (generated by T. M. Jessell and J. Dodd, obtained from the Developmental Studies Hybridoma Bank, Iowa), with a biotin-conjugated rabbit anti-mouse secondary antibody (Jackson Immunoresearch Labs) and Haematoxylin counterstain.

Cell counts

Auditory hair cells and supporting cells were counted from whole mounts of the cochlea stained with fluorescent phalloidin and (except in *Jag1* knockouts) with Jag1 antibody. Hair cells were identified by their phalloidin-stained hair bundles and the apical location of their nuclei in the epithelium, and supporting cells by their Jag1 staining and the basal location of their

nuclei, as seen in z-series of confocal optical sections spanning the full depth of the sensory epithelium. For each specimen and each portion of cochlea, counts were made from three 100 μm lengths of the organ of Corti, and the average was calculated.

Vestibular hair cells were counted on immunostained cryosections cut perpendicular to the plane of the saccular macula; three samples of 200 μm length were scored per individual. Hair cells were identified by the apical location of their nuclei in the epithelium and by their staining for calretinin.

For measurements of cochleovestibular ganglion volume at E10.5, the outlines of the ganglion were traced and cross-sectional areas measured (using ImageJ) on every second serial section. The areas were then added together and multiplied by twice the section thickness.

RESULTS

We present our *Jag1* findings first, beginning with effects in the cochlea when the gene is conditionally knocked out, and then describing more briefly the effects in the vestibular system. We then report the corresponding data for the *Dll1* knockout. Last, we present some further experiments that reveal an unexpected effect of *Jag1* on expression of the cell-cycle regulator p27^{Kip1} (Cdkn1b – Mouse Genome Informatics); these findings may help to explain the *Jag1* knockout phenotype.

Cre expressed under *Foxg1* control recombines *Jag1*^{fllox} early in ear development

For our conditional knockout experiments, we generated lines of mice carrying floxed alleles of *Dll1* (Hozumi et al., 2004) and *Jag1* (see Materials and methods). We crossed these lines with mice that had the *Cre* recombinase gene knocked into the *Foxg1* locus, to produce *Foxg1*^{Cre/+};*Jag1*^{fllox/fllox} embryos. The *Foxg1* promoter has been reported to drive expression of *Cre* recombinase in the developing inner ear from E8.75, at the early otic placode stage, 1 day earlier than the earliest reported expression of *Jag1* in the ear, resulting in efficient recombination of floxed constructs throughout the otic vesicle at subsequent stages (Hébert and McConnell, 2000; Mitsiadis et al., 1997; Pirvola et al., 2002). To check that recombination had occurred as expected, we stained sections of *Foxg1*^{Cre/+};*Jag1*^{fllox/fllox} conditional knockout embryos and their control littermates with antibody against *Jag1* at E10.5, the earliest time at which expression of *Jag1* is normally detected with immunostaining. Complete sets of serial sections through the left and right otocysts were analysed in this way for three *Jag1* conditional knockout mice and two littermate controls. In all the conditional knockout mice, the patch of *Jag1* antibody staining in the ventral otocyst was completely lost (Fig. 1A,B). Loss of *Jag1* antibody staining was confirmed at later embryonic stages, at E15.5 and E17.5 ($n=3$), in wholemount cochlea preparations (Fig. 1C,D) and in cryosections of inner ears. We conclude that our conditional knockout strategy efficiently eliminated expression of the targeted protein in the inner ear, starting from a very early stage in ear development.

Sensory cells do not differentiate prematurely in the *Jag1* conditional knockout cochlea

We wished first of all to test the procrastination hypothesis: that *Jag1* serves to prevent the premature production of hair cells by activating Notch in all the cells of the prosensory patch. For this, we focused on the cochlea. Hair-cell differentiation in the normal cochlea begins around E14.5 in the mid-basal region, and a wave of differentiation proceeds basally and apically over the next few days (Lim and Anniko, 1985). By E17.5 hair cells are seen in the basal and middle regions, but have not yet differentiated in the apex. Effects on the timing of hair-cell production can be sensitively detected as changes in this pattern. In addition, the final arrangement of the auditory hair

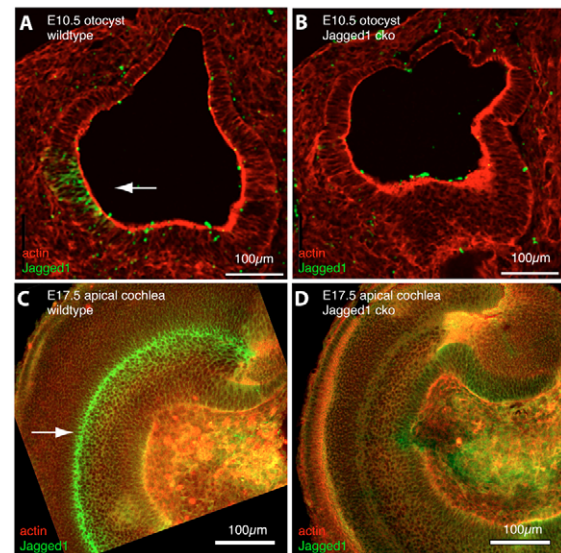


Fig. 1. Loss of *Jag1* antibody staining in the inner ear of *Jag1* conditional knockout mice. (A,B) Confocal images of sagittal cryosections at E10.5 (dorsal to the left). (A) Littermate control. The *Jag1* antibody stains a patch of cells (arrow) in the ventral part of the otocyst. (B) *Jag1* conditional knockout. The patch of *Jag1* staining is lost. (C,D) Confocal optical sections of apices of whole-mount E17.5 cochleas. (C) Littermate control. *Jag1* is detected in a narrow band of cells (arrow) – the future sensory patch. (D) *Jag1* conditional knockout. The *Jag1* staining is lost.

cells and supporting cells is precise and predictable, making it easy to detect even a small abnormality in the numbers of hair cells or supporting cells produced.

We collected *Jag1* conditional knockout embryos and littermate controls at E17.5 and stained the cochleas as whole mounts with fluorescence-tagged phalloidin. In the controls, differentiated hair cells with hair bundles were clearly visible in the basal and middle regions of the cochlea, while in the apical region hair bundles were not yet visible although their appearance was foreshadowed by a local upregulation of actin (Fig. 2A). In the *Jag1* knockout, the same was true: we did not see any sign of a change in the timing of hair cell differentiation, in any of the three individuals analysed (Fig. 2B). This argues strongly against the idea that *Jag1* is required to prevent premature hair cell differentiation. We shall see that the phenomena are very different for *Dll1* knockouts.

Outer hair cells are missing but inner hair cells are overproduced in the *Jag1* conditional knockout cochlea

Although the timing of hair-cell differentiation appeared normal in the *Jag1* knockouts, the number and distribution of hair cells was greatly altered. In the middle part of the cochlea at E17.5, the littermate controls showed the normal stereotyped pattern of three rows of outer hair cells and one row of inner hair cells (Fig. 2C); by contrast, the *Jag1* knockouts showed just two rather disorganized rows of hair cells (Fig. 2D). The total number of hair cells per 100 μm length of cochlea in this middle region was reduced to 25.8 ± 0.5 (mean \pm s.e.m., $n=8$) as compared with 63.5 ± 1.5 (mean \pm s.e.m., $n=10$) hair cells in littermate controls.

The reduction in the number of hair cells seen in the *Jag1* conditional knockout cochlea did not, however, reflect a simple general decrease of hair cell production. Outer hair cells appeared to

be lost completely, whereas the number of inner hair cells per 100 μm was almost doubled, to 25.8 ± 0.5 ($n=10$) compared with 15.3 ± 0.35 ($n=8$) for the controls. Two criteria indicated that the hair cells in the conditional knockout were all of inner hair cell character. First, they all expressed calretinin, which is normally seen in inner but not outer hair cells at this stage (Fig. 3A,B). Second, like normal inner hair cells, they all lay on the inner side of a distinctive band of supporting cells – the inner pillar cells (Fig. 3C,D). This was clear in all five cases for which appropriate z -series of images were collected.

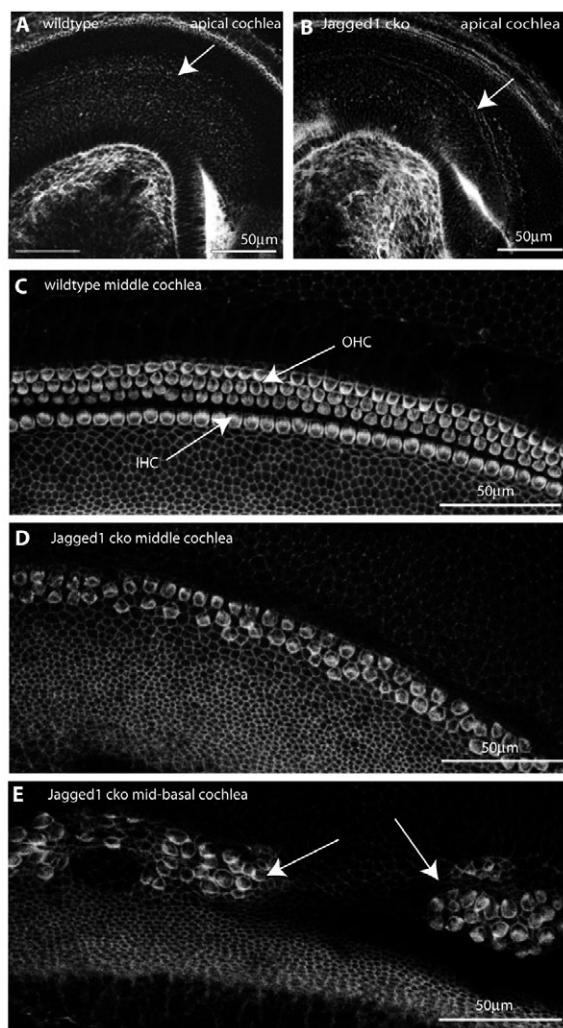


Fig. 2. Defective sensory patch development in the *Jagged1* conditional knockout cochlea. Confocal images of whole-mount E17.5 cochleas stained with fluorescent phalloidin. (A) In the apex of the control cochlea, faint lines of actin staining prefigure the appearance of the inner hair cells (arrow); no hint of outer hair cells is yet visible. (B) In the *Jagged1* conditional knockout littermate, the appearance of the cochlear apex is similar, with no sign of premature differentiation. (C) In the middle part of the control cochlea, the standard pattern of one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) is seen. (D) In the middle part of the *Jagged1* conditional knockout cochlea, approximately two disorganized rows of hair cells are produced. (E) In the mid-basal region of the *Jagged1* conditional knockout cochlea, hair cells lie in islands (arrows) separated by gaps of bare epithelium; the mid-basal region of the control cochlea (not shown) has the same pattern of four rows of hair cells as in the middle region (C).

Further towards the base of the cochlea, the number of hair cells was more severely reduced. The continuous band of two inner hair cell rows was broken up, giving way to irregular islands of hair cells amid naked epithelium (Fig. 2E).

The deficit of cochlear hair cells in the *Jagged1* conditional knockout reflects a failure of initial production, not subsequent degeneration

The mid-basal region of the cochlea is normally the earliest region of hair-cell differentiation (Lim and Anniko, 1985). Thus, one interpretation for the gaps seen in the band of hair cells in this part of the mutant cochlea at E17.5 is that some of the hair cells initially produced had degenerated. To test this idea, we examined the pattern of hair cells in the basal region two days earlier in development, at E15.5 (data not shown). In control cochleas at this stage, when hair cell differentiation has just begun, a single row of cells with upregulated actin, the inner hair cells, could be seen in the most basal regions, while multiple rows of hair cells were already visible in mid-

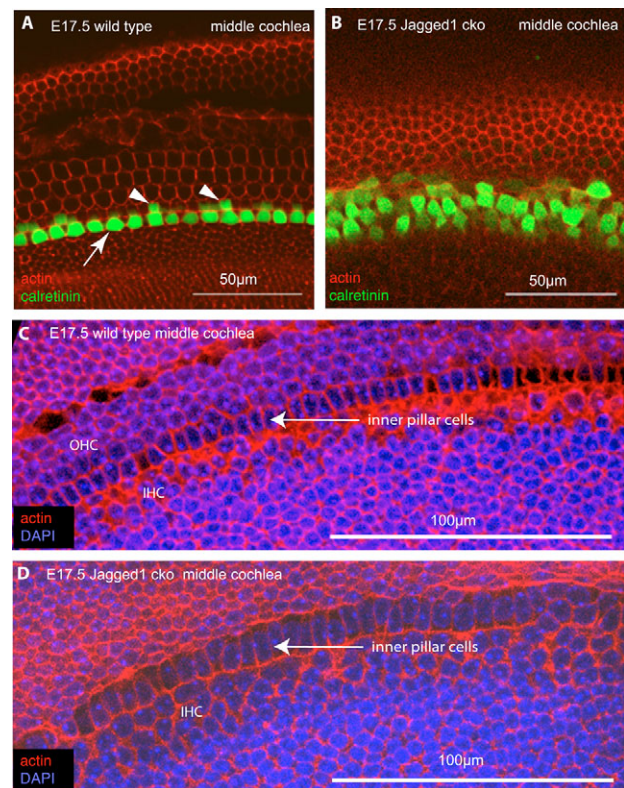


Fig. 3. The hair cells in the cochlea of the *Jagged1* conditional knockout have characteristics of inner hair cells. Confocal optical sections of whole mounts of the middle region of the cochlea at E17.5 are shown. (A) Control, stained with calretinin antibody. At this stage the inner hair cells (arrow) but not outer hair cells are calretinin-positive, and so are occasional inner pillar cells (arrowheads). (B) *Jagged1* conditional knockout cochlea. All the hair cells produced are stained for calretinin, as well as some adjacent cells (possibly inner pillar cells). (C) Control cochlea, stained with the nuclear dye DAPI and with phalloidin. A row of inner pillar cells with characteristic rectangular profiles can be seen in the wild type between the rows of inner (IHC) and outer hair cells (OHC). (D) In the *Jagged1* conditional knockout, cells with this morphology can be seen outside the disorganised rows of hair cells. The cells appear larger than in C merely because the plane of section is slightly different.

basal regions. In the mutant cochlea, there was no sign of hair cells in the most basal region, while hair cells appeared already grouped in islands in the mid-basal region. The deficit of hair cells is therefore unlikely to be due to hair-cell loss following normal initial production.

Loss of *Jag1* causes loss of some but not all vestibular sensory patches

Although we have not analysed the vestibular regions of the *Jag1* conditional knockout ears in as much detail as the cochleas, abnormalities were clearly visible. All three semicircular canals were truncated in all cases examined ($n=37$) (Fig. 4A,B). Correspondingly, in each of two knockout embryos that we serially cryosectioned and stained with phalloidin and/or calretinin, we observed a complete loss of the anterior and posterior cristae and corresponding ampullae. The epithelium at the sites where one would normally see the horizontal crista and utricular macula was identifiable by its thickened appearance but lacked hair cells in both cases (Fig. 4C,C',D,D'). By contrast, the saccular macula appeared entirely unaffected by the loss of *Jag1* (Fig. 4E,F), with a normal density of hair cells [37.3 ± 3.8 (mean \pm s.e.m., $n=3$) per 200 μm length of saccular epithelium in the *Jag1* conditional knockout mouse, compared to 32.7 ± 5.3 ($n=3$) in a control littermate]. Thus, in the vestibular system, as in the auditory system, *Jag1* is required for the production of some but not all of the hair cells.

Auditory hair cells are produced prematurely and in excess in the *Dll1* conditional knockout

We analysed the conditional knockout phenotype for *Dll1* in the same way as for *Jag1*, focusing mainly on effects in the cochlea. In the *Dll1*^{*Afllox/Afllox*} conditional knockout cochlea, in contrast to *Jag1*^{*Afllox/Afllox*}, hair cells differentiated prematurely and were clearly visible already in the apex at E17.5, although none could yet be seen in this region in control littermates (Fig. 5A-D). Moreover, the hair cells in the apical cochlea were present in a huge excess. Instead of the normal pattern of one row of inner hair cells and three rows of outer hair cells, which become visible postnatally in the apex of the wild-type cochlea, between 6 and 8 rows of outer hair cells had already formed at E17.5 in the mutant, and the inner hair-cell row also appeared to have been duplicated.

Similar but less extreme effects were seen in the rest of the cochlea. In the middle and basal regions, there was one extra row of outer hair cells and an increase in the number of inner hair cells (Fig. 5E,F).

Detailed counts in the middle turn gave an average of 62 ± 5.1 outer hair cells per 100 μm length of cochlea (mean \pm s.e.m., $n=7$) in the *Dll1* conditional knockout, arranged in four orderly rows, compared with 46 ± 1.7 (mean \pm s.e.m., $n=6$) in control mice, constituting the normal three rows. The number of outer hair cells in this region per 100 μm was thus increased in the *Dll1* conditional knockout by ~35%.

The premature and excessive production of hair cells was associated with a reduction or delay in the growth of the cochlea, which, viewed as a flat mount at E17.5, showed slightly fewer than the normal number of turns (about 1.5 turns in the mutant, as against 1.75 in the controls) and had a shorter length as measured along its outer curvature [3144 ± 78 μm ($n=6$) when compared with 4454 ± 56 μm ($n=6$) for littermate controls]. The extreme broadening of the sensory patch at the apex may thus be a result of premature differentiation, shortening the period of growth preceding differentiation and preventing the prosensory patch from elongating and narrowing in the normal way.

Supporting cells are also produced in excess in the *Dll1* conditional knockout

The overproduction and premature differentiation of hair cells in the *Dll1* conditional knockout are effects one would predict if there was a partial loss of lateral inhibition. One would then also expect the extra hair cells to be produced at the expense of supporting cells: supporting cell numbers should be decreased. Yet we saw no obvious deficit (Fig. 5G,H). To check this impression, we counted the numbers of hair cells and supporting cells in the middle region of cochleas from E17.5 individuals (see Materials and methods). Seven conditional knockout mice and six littermate controls were analysed in this way. For the sake of precision, we focused on the outer hair cell region, as this had well-marked borders – the outer one defined by the outer margin of *Jag1* expression, the inner defined by the row of pillar cells. (It was difficult to make precise counts of supporting cells in the inner hair cell region because the inner margin of *Jag1* expression is rather diffuse.) The number of *Jag1*-positive outer supporting cells – Deiters' cells plus outer pillar cells – was 80 ± 7.4 per 100 μm (mean \pm s.e.m., $n=7$) in the *Dll1* conditional knockout cochleas, compared with 70 ± 4.8 ($n=6$) in the littermate controls – an increase of 14%. The ratio of supporting cells to hair cells was nevertheless reduced, from 1.52 ± 0.03 (mean \pm s.e.m., $n=6$) for the controls to 1.29 ± 0.04 (mean \pm s.e.m., $n=7$) for the knockouts. This difference is statistically significant ($P<0.001$, *t*-test).

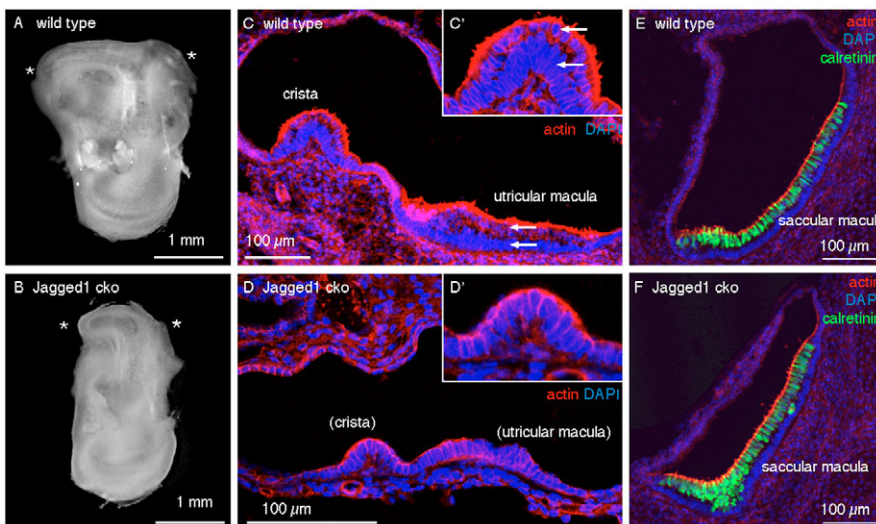


Fig. 4. The vestibular apparatus is defective in the *Jag1* conditional knockout. Specimens at E17.5. (A,B) Freshly dissected inner ears under the dissecting microscope; medial view, anterior towards the left. The *Jag1* conditional knockout (B) has a truncated anterior semicircular canal (left, asterisk) and a missing posterior semicircular canal (right, asterisk). **(C,D)** Confocal optical sections through the horizontal crista and utricular macula, stained for actin and DNA. In the wild-type control (C), nuclei of hair cells and supporting cells are arranged in two distinct layers (arrows in C and C'); in the *Jag1* conditional knockout (D), the corresponding patches are smaller and there is only one layer of nuclei, reflecting absence of hair cells. (C',D') Enlargements of the horizontal crista. **(E,F)** Confocal optical sections of the saccular macula, stained for actin, DNA and calretinin. The mutant macula appears to be unaffected by loss of *Jag1*.

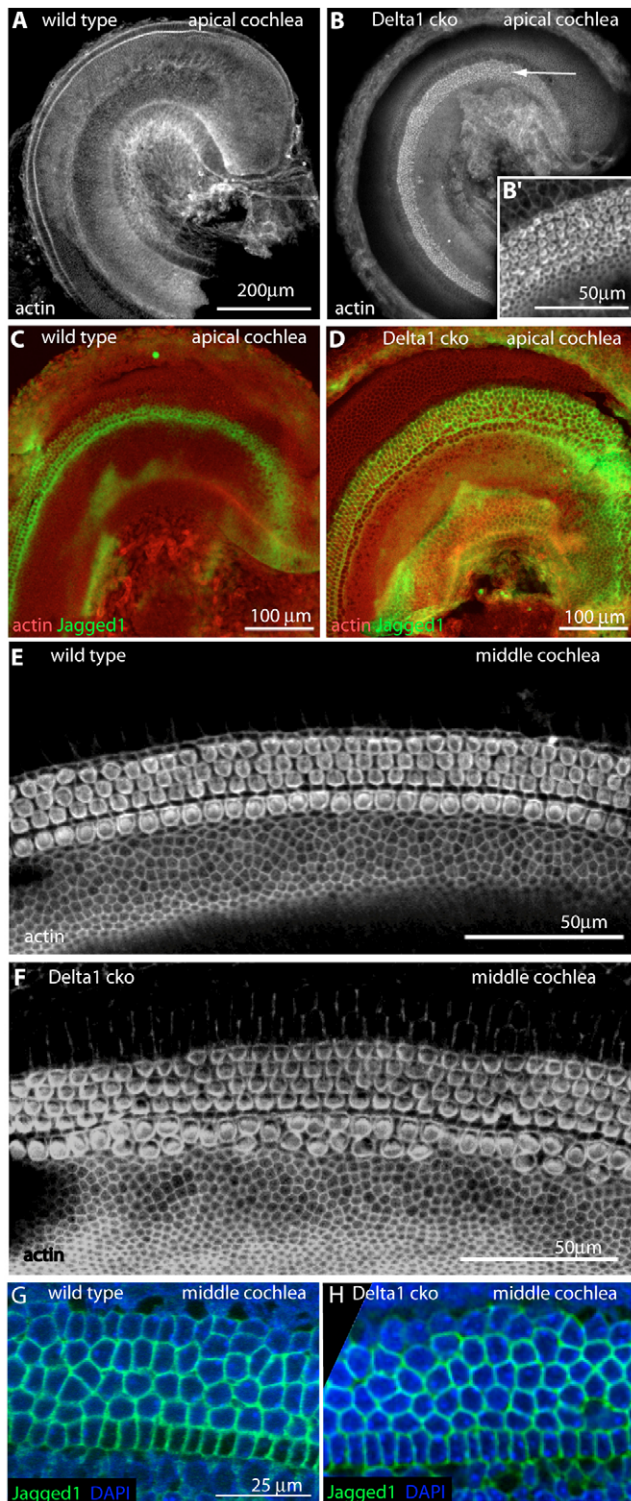


Fig. 5. Hair cells are produced early and in excess in the *Delta1* conditional knockout cochlea. Confocal optical sections of whole mounts of cochleas at E17.5. (A) In the apex of the wild-type mouse cochlea, stained with phalloidin, no hair cells are visible as yet. (B,B') In the apex of the cochlea of a *Delta1* conditional knockout littermate, hair cells are already plainly visible and present in excess (arrow; B' shows detail). (C) Middle region of wild-type cochlea shows a narrow band of Jag1 staining, corresponding to the future sensory patch. (D) *Delta1* conditional knockout shows a wider band of Jag1 staining, corresponding to the enlarged and precocious sensory patch, and suggesting an increase in the numbers of supporting cells (which are Jag1-positive) as well as hair cells (which are not). (E,F) A milder excess of hair cells is seen in the middle and basal parts of the *Delta1* conditional knockout cochlea (F), with one extra row of outer hair cells and many more inner hair cells than in the littermate control (E). (G,H) Confocal optical sections of the middle region of the cochlea in a plane passing through the bodies of the supporting cells at a deeper level than the hair cells. The membranes of the supporting cells are stained with Jag1 antibody, and the mutant has slightly more of these cells per unit length of cochlea.

counts imply that the additional hair cells are not produced at the expense of a proportionate reduction in the number of supporting cells.

Loss of *Dll1* results in a loss of the sensory epithelium of the maculae

The gross structure of the inner ears of *Dll1* conditional knockout mice appeared almost normal in the intact specimens. Sagittal sections of the entire inner ear at E17.5 revealed that the semicircular canals and cristae were all present and of the normal morphology, in all three specimens analysed. The maculae, however, were severely affected, with the saccular macula and the utricular macula being either lost or severely reduced; when present, these sensory patches had the normal arrangement of hair cells and supporting cells (Fig. 6).

This deficit of sensory epithelium could be the result of a failure of lateral inhibition during the genesis of neuroblasts from the early otocyst: loss of *Dll1* at an early stage is expected to cause an excessive proportion of vestibular prosensory cells to adopt a neuronal fate and delaminate from the otic epithelium (see Discussion). To investigate this possibility, we examined the size of the rudiment of the cochleovestibular ganglion in *Dll1* conditional knockout embryos and littermate controls at E10.5. From serial sections stained with neurofilament antibody, we estimated that this ganglion had a volume of 7.0 ± 1.3 nl (mean \pm s.d., $n=3$) in the knockouts, compared with 4.2 ± 0.2 nl (mean \pm s.d., $n=3$) in the controls. These figures are rough and should be viewed with caution, because at this stage the rudiments of the facial and cochleovestibular ganglia form a single confluent mass, and it was difficult to distinguish the cochleovestibular component. Nevertheless, they tend to support the suggestion that loss of *Dll1* caused excessive numbers of cells to be diverted from an epithelial to a neuronal fate.

Loss of *p27^{Kip1}* does not underlie the *Dll1* conditional knockout phenotype

The pattern of four rows of outer hair cells and almost two rows of inner hair cells seen in the *Dll1* conditional knockout mice is strikingly similar to the pattern of supernumerary hair cells reported

In the apex of the cochlea, where the overproduction of both hair cells and supporting cells was more obvious, the Jag1 stain was expanded to encompass the population of supernumerary hair cells (Fig. 5D). We found 84 ± 7 hair cells per 100 μ m and 170 ± 15 supporting cells per 100 μ m (mean \pm s.e.m., $n=3$) – a much larger proportion of supporting cells to hair cells than in the middle turn of the cochlea in either wild-type or mutant. No comparison could be made with control littermates as hair cell production has not yet occurred in apex of the normal cochlea at E17.5. Nevertheless, these

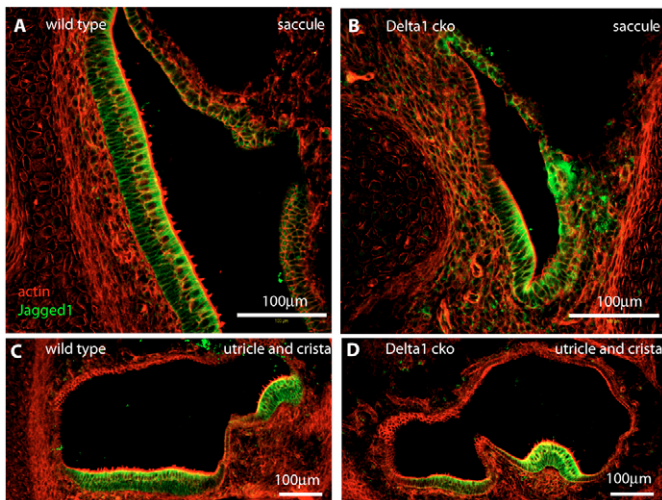


Fig. 6. *Delta1* conditional knockout mice have vestibular defects. Coronal cryosections of vestibular regions of the inner ear of mutants and littermate controls stained with Jag1 antibody and fluorescent phalloidin. (A,B) Sacculle. The saccular macula is much smaller (and sometimes missing) in the *Delta1* conditional knockout (B), when compared with the *Foxg1-Cre* littermate control (A). (C,D) Utricle and horizontal crista. The utricular macula is also drastically reduced in the mutant (D) compared with the control (C), though the crista is less affected.

in cochleas with null mutations of *p27^{Kip1}* (Chen and Segil, 1999; Lowenheim et al., 1999). *p27^{Kip1}* is a cyclin-dependent kinase inhibitor (CKI) that regulates progress through the cell cycle (Sherr and Roberts, 1999). It is expressed in the cochlea in the region of the developing sensory patch before hair cell differentiation, as cells of the patch exit the cell cycle (Chen et al., 2002; Chen and Segil, 1999; Ruben, 1967). When *p27^{Kip1}* is deleted, cells of the sensory patch continue to proliferate beyond E14.5, after the time of their normal terminal mitosis (Chen and Segil, 1999; Lowenheim et al., 1999). We wondered, therefore, whether a failure of *p27^{Kip1}* expression, secondary to a defect of Notch signalling, might explain the overproduction of hair cells and supporting cells in the *Dll1*

knockout. In the wild-type cochlea at E14.5, a broad band of *p27^{Kip1}*-positive cells is seen by immunofluorescence in the future hair-cell region. In the *Dll1* conditional knockout cochlea at this stage, the pattern of *p27^{Kip1}* immunofluorescence, in all three specimens that we analysed, appeared almost exactly the same (Fig. 7A,B). The abnormality of hair-cell and supporting-cell numbers is therefore not likely to be the consequence of a loss of *p27^{Kip1}*. As we discuss below, it remains possible nevertheless that altered patterns of proliferation play some part in the *Dll1* knockout phenotype.

Expression of *p27^{Kip1}* is lost or reduced in the *Jag1* conditional knockout cochlea

The distribution of *p27^{Kip1}* in the *Jag1* conditional knockouts, however, was surprising. Antibody staining of whole-mount E14.5 cochleas showed that expression of this cell-proliferation inhibitor was completely or almost completely lost (Fig. 7C-E). The effect was clear in all four specimens examined. Loss of the inhibitor would be expected to cause an increase of sensory cell numbers; yet what we saw was a loss of sensory cells. There is, however, a way of looking at these observations that makes them seem less paradoxical. Although the total number of hair cells and supporting cells was reduced, the number of inner hair cells and supporting cells was increased. The absence of outer hair cells in the *Jag1* knockout may be ascribed to a failure of prosensory induction; the gain of inner hair cells may reflect increased proliferation resulting from the loss of *p27^{Kip1}*. A more detailed analysis of cell proliferation and the role of *p27^{Kip1}* in the *Jag1* knockout will be the subject of a future paper.

DISCUSSION

We have shown that *Dll1* and *Jag1* can be effectively knocked out in the inner ear from a very early stage, several days before the appearance of the first hair cells, and that the consequences are very different according to which of the two Notch ligands is lost. In the *Dll1* knockout, cochlear hair cells are produced early and in excess. In the *Jag1* knockout, cochlear hair cells are produced at the normal time, but in reduced total numbers: although the number of inner hair cells is increased, the outer hair cells are completely absent. Loss of *Jag1* leads to a loss of cochlear expression of the cell proliferation inhibitor *p27^{Kip1}*; loss of *Dll1* does not. Contrasting

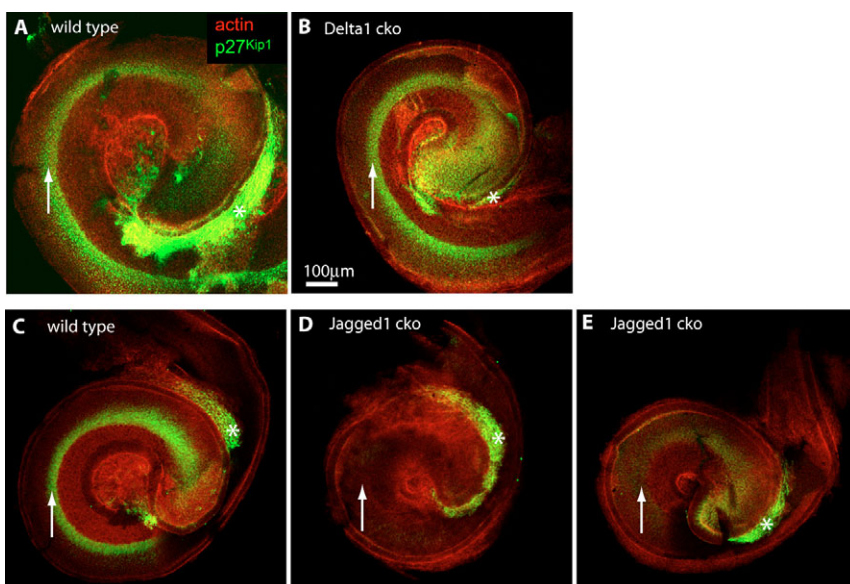


Fig. 7. *p27^{Kip1}* expression is drastically reduced in the *Jag1* but not the *Delta1* conditional knockout cochlea.

Confocal optical sections of whole-mount E14.5 cochleas stained with anti-*p27^{Kip1}* antibody and fluorescent phalloidin. The bright staining (asterisks) in tissue outside the sensory epithelium in the apex of the cochlea is non-specific and occurs in an area damaged during dissection. (A) Wild-type control. *p27^{Kip1}* is expressed in a band of cells (arrow) corresponding to the future organ of Corti. (B) *Delta1* conditional knockout littermate. There is little change in the expression of *p27^{Kip1}* in the developing sensory patch, except that it extends further towards the apex in accordance with the premature differentiation of this region. (C) Wild-type littermate control for the *Jag1* conditional knockout. (D) *Jag1* conditional knockout, showing complete loss of expression of *p27^{Kip1}* in the developing sensory patch (arrows in C,D). (E) Another *Jag1* conditional knockout specimen, showing some weak residual expression of *p27^{Kip1}* (arrow).

effects are seen in the vestibular region also. Cristae develop normally in the *Dll1* knockout, but are lost in the *Jag1* knockout; the saccular macula is lost in the *Dll1* knockout, but normal in the *Jag1* knockout.

What do these findings tell us about the role of Notch signalling and the multiple Notch ligands? Other experiments have shown that Notch signalling has two functions in the inner ear: first, to maintain cells in a prosensory state, making them competent to differentiate subsequently as hair cells or supporting cells; and second, through lateral inhibition, to limit the proportion of prosensory cells that go on to differentiate as hair cells (Daudet and Lewis, 2005). Our findings described here indicate that *Jag1* is responsible for activating the prosensory function of Notch, supporting previous suggestions from *Jag1* heterozygotes (Kiernan et al., 2001), while *Dll1* mediates the function of Notch in lateral inhibition. Moreover, it appears that the disturbances of Notch signalling may also alter the pattern of sensory cell proliferation.

Partial failure of lateral inhibition explains the *Dll1* knockout phenotype

In the case of *Dll1*, the finding that cochlear hair cells are produced early and in excess is strongly reminiscent of the *mind bomb* phenotype in zebrafish, where Notch signalling is defective and all cells in the sensory patch develop as hair cells, and do so prematurely (Haddon et al., 1998). This is what one would expect if lateral inhibition is reduced (Goriely et al., 1991). Taken with what we know about *Dll1* and Notch signalling in other systems, a failure of lateral inhibition seems much the most likely cause of the *Dll1* knockout phenotype. The overproduction of hair cells is relatively slight, but this is easily accounted for by the presence of *Jag2*, which has been shown to have similar expression in hair cells and similar action, creating redundancy in the lateral inhibition mechanism (Lanford et al., 1999).

More puzzling, however, is the finding that supporting cells are also produced in excess. This seems to clash with the simple lateral inhibition hypothesis. On the other hand, the ratio of supporting cells to hair cells is reduced, as the lateral inhibition hypothesis would predict. There are several possible interpretations.

(1) By occurring prematurely, differentiation in the *Dll1* knockout may hinder normal elongation and narrowing of the cochlear prosensory patch, so that it appears broader, with more rows of cells and more cells of both types per 100 μm length. Our observations suggest that something of this sort occurs in the apex of the cochlea, at least. Within the broadened prosensory region, partial loss of lateral inhibition will give rise to a modest increase of hair cells at the expense of supporting cells, accounting for the decreased ratio of supporting cells to hair cells.

(2) An excess of hair cells through loss of lateral inhibition might cause additional supporting cells to be recruited secondarily from the adjacent non-sensory region of the epithelium. Indeed, there is evidence that signals from hair cells can convert adjacent non-sensory cells to a *Jag1*-positive supporting-cell character: this is seen when hair cells are produced ectopically by artificial expression of *Math1* (Woods et al., 2004), and it could be followed by some cell rearrangement (Goodyear and Richardson, 1997).

(3) An excess of hair cells might cause the residual supporting cells to undergo extra rounds of cell division. Kiernan et al. (Kiernan et al., 2005) have found evidence of this in the cochlea of mice that are homozygous for a knockout mutation of *Jag2* combined with a hypomorphic allele of *Dll1*: they see a phenotype that is similar to the one we have described above, but more extreme, and they find by BrdU labelling that cell proliferation is

prolonged abnormally in the supporting-cell population. Thus, in both our mutants and theirs, it seems likely that an initial failure of lateral inhibition is followed by some compensatory proliferation of the residual supporting cells.

The above suggestions are not mutually exclusive, and all of them postulate that the primary effect of loss of *Dll1* is a reduction of lateral inhibition. How, then, are we to explain the loss of sensory maculae in the vestibular part of the inner ear? In the zebrafish *mind bomb* mutant, hair cells in the absence of supporting cells are extruded from the otic epithelium and rapidly disappear (Haddon et al., 1999); a similar process could have occurred in the *Dll1* mutants, leading to loss of one or both of the maculae. Another interpretation invokes the known early role of *Dll1* in otic neurogenesis (Adam et al., 1998; Alsina et al., 2003). Early in ear development, a subset of cells in the anteroventral region of the otocyst become determined as neuroblasts and delaminate from the epithelium; when lateral inhibition fails, as a result of failure of Notch signalling, an excessive proportion of cells undergo this fate (Haddon et al., 1998). Our measurements of the volume of the cochleovestibular ganglion suggest that this indeed occurred in our *Dll1* knockouts. The diversion of cells towards a neuronal fate would be expected to deplete the prosensory population in the anteroventral otic epithelium, which is thought to give rise to the maculae (Adam et al., 1998; Morsli et al., 1998; Satoh and Fekete, 2005).

Jag1 mediates a prosensory function of Notch

At the outset, we considered the hypothesis that *Jag1*, by keeping Notch activated throughout each prosensory patch, served to prevent premature differentiation. We can now rule out this possibility: where hair cells were produced in the *Jag1* knockout cochlea, they were produced at the normal time. This contrasts with the behaviour seen in *Dll1* knockouts, where hair cells at the apex of the cochlea differentiated at least a day prematurely. It seems, therefore, that the role of *Jag1*, in the cochlea at least, is not the inhibition of hair cell differentiation, but something else.

Previous studies have described the heterozygous *Jag1* loss-of-function phenotype in the ear: numbers of outer hair cells were somewhat reduced, and numbers of inner hair cells somewhat increased (Kiernan et al., 2001; Tsai et al., 2001). Our findings for homozygous *Jag1* knockouts are similar but more extreme: outer hair cells are lost entirely, while the number of inner hair cells is roughly doubled. How is this curious combination of effects to be explained?

We have given evidence against interpretations in terms of hair-cell degeneration or conversion of outer hair cells to an inner hair-cell character. The obvious suggestion, rather, is that the observed loss of outer hair cells in the *Jag1* knockout reflects a need for *Jag1* as activator of the prosensory function of Notch in the outer hair cell region. The effects of loss of *Jag1* in the vestibular system can be explained in a similar way. Some sensory patches, the anterior and posterior cristae, were entirely missing in the *Jag1* conditional knockout. Others, the horizontal crista and the utricular macula, appeared to be present as epithelial thickenings but lacked hair cells. Finally, the saccular macula was apparently unaffected by loss of *Jag1*. This pattern of vestibular defects is quite different from that seen in the *Dll1* conditional knockouts, and it can be easily explained if we consider that the role of *Jag1* is to activate the prosensory function of Notch and thereby maintain or extend the set of cells competent for sensory differentiation. Some prosensory regions are evidently dependent on this action of *Jag1*, others are not.

Jag1, like Notch, may have distinct early and late functions

If Jag1 contributes to the induction of prosensory patches, how are we to explain the increase of inner hair cell numbers when Jag1 is lost? One possibility is that Jag1, like Notch, has a late function in lateral inhibition that is distinct from its early, inductive, prosensory function: by activating Notch at later stages, during hair cell determination, it may contribute to lateral inhibition in concert with Dll1 and Jag2. Although loss of the early (inductive) effect would lead to loss of sensory epithelium, loss of the late (inhibitory) effect could lead to overproduction of hair cells in the patches that remain.

Another interpretation is suggested, however, by our unexpected finding that loss of Jag1 leads to a failure of *p27^{Kip1}* expression in the cochlea. As *p27^{Kip1}* is an inhibitor of cell cycling, its loss is predicted to permit increased numbers of cell divisions, leading to increased production of both hair cells and supporting cells. Indeed, the *p27^{Kip1}* knockout (Chen and Segil, 1999; Lowenheim et al., 1999) has an excess of both inner and outer hair cells. Thus, our observations may perhaps be most plausibly explained by the hypothesis that Jag1 is needed, first, to extend the prosensory state by lateral induction beyond the inner hair cell region into the outer hair cell region, and, second, to enable expression of *p27^{Kip1}*, thereby helping to terminate cell proliferation within the prosensory patch when the time comes for differentiation.

One of the key issues for future research will be to determine whether the early (prosensory induction) and late (lateral inhibition and proliferation control) functions of Jag1 and Dll1, with their very different consequences for hair-cell production, are mediated by the same or different members of the Notch protein family (Shimizu et al., 1999; Shimizu et al., 2000). Other Notch genes besides *Notch1* may be expressed in the mouse ear – indeed, we have preliminary evidence that *Notch3* is expressed there (data not shown) – and they may be specialized as between these two roles. Certainly at least two different members of the Hes family of downstream mediators of Notch signalling – *Hes1* and *Hes5* – are expressed in the ear, in subtly different patterns, and with different mutant phenotypes that could reflect differential involvement in signals delivered by Jag1 and Delta; the *Hes1* knockout, for example, has an excess of inner hair cells in the cochlea, while the *Hes5* knockout has an excess of outer hair cells (Zheng et al., 2000; Zine et al., 2001).

Our observations show that the different Notch ligands have radically different but interlocking functions in the ear, and that these functions can be interpreted in terms of the different roles of Notch signalling at successive stages in the induction and internal patterning of the sensory patches and the different rules by which Notch activity regulates the expression of each ligand. In all sorts of other tissues, from the central nervous system to the vasculature and the epidermis, multiple Notch ligands are expressed in closely correlated but distinctive patterns. The lessons learnt from the ear may thus be important for Notch signalling in other systems.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/7/1277/DC1>

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