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Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode

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SUMMARY

The inner ear derives from a patch of ectoderm defined by expression of the transcription factor *Pax2*. We recently showed this *Pax2*$^+$ ectoderm gives rise not only to the otic placode but also to surrounding cranial epidermis, and that Wnt signaling mediates this placode-epidermis fate decision. We now present evidence for reciprocal interactions between the Wnt and Notch signaling pathways during inner ear induction. Activation of Notch1 in *Pax2*$^+$ ectoderm expands the placodal epithelium at the expense of cranial epidermis, while loss of Notch1 leads to a reduction in the size of the otic placode. We show that Wnt signaling positively regulates Notch pathway genes such as *Jag1*, *Notch1* and *Hes1*, and have used transgenic Wnt reporter mice to show that Notch signaling can modulate the canonical Wnt pathway. Gain and loss of function mutations in the Notch and Wnt pathways reveal that some aspects of otic placode development - such as *Pax8* expression and the morphological thickening of the placode – can be regulated independently by either Notch or Wnt signals. Our results suggest that Wnt signaling specifies the size of the otic placode in two ways – by directly up-regulating a subset of otic genes, and by positively regulating components of the Notch signaling pathway which then act to augment Wnt signaling.

Keywords

Mouse; Otic placode; Wnt; β-catenin; Notch1; Jagged1; Inner ear

INTRODUCTION

Inner ear development is an excellent example of how a Darwinian "organ of extreme perfection and complication" can arise from simple origins. The inner ear derives from a patch of thickened ectoderm, the otic placode, lying next to the posterior hindbrain. Signals that induce the otic placode are present in the hindbrain and cranial paraxial mesoderm, although the relative contribution of these tissues to the induction process varies between species (Barald and Kelley, 2004; Groves, 2005; Riley and Phillips, 2003; Torres and Giraldez, 1998). Members of the Fibroblast Growth Factor (FGF) family play a crucial role in inducing the otic placode in all vertebrates examined (Friesel and Brown, 1992; Ladher et al., 2005; Leger and Brand, 2002; Mackereth et al., 2005; Maroon et al., 2002; Phillips et al., 2001; Vendrell et al., 2000; Wright et al., 2004; Wright and Mansour, 2003). FGF signaling induces the expression

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of genes such as *Pax2* and *Pax8* in a broad region of cranial ectoderm stretching from rhombomeres 3 to 6 (Maroon et al., 2002; Martin and Groves, 2006; Wright and Mansour, 2003). Evidence from lineage tracing in chick (Streit, 2002) and mouse (Ohyama and Groves, 2004b; Ohyama et al., 2006) suggest that this broad *Pax2*$^+$ domain, which we have referred to as the "pre-otic field", contains cells fated to become otic and epibranchial placodes, as well as cranial epidermis.

We recently showed that Wnt signaling plays an important role in defining the size of the otic placode within this *Pax2*$^+$ pre-otic field. Wnt signaling is activated in a medial subset of the *Pax2*$^+$ domain closest to the hindbrain (Ohyama et al., 2006). Inactivation of Wnt signaling in *Pax2*$^+$ cells by conditional deletion of *β-catenin* leads to a large reduction in the size of the otic placode and a corresponding expansion of cranial epidermis. Conversely, activation of *β-catenin* in *Pax2*$^+$ cells expands the otic placode at the expense of cranial epidermis (Ohyama et al., 2006). To date, however, it is not clear how Wnt signals direct cranial ectoderm towards an otic fate. It is possible that Lef/Tcf/β-catenin transcriptional complexes activated by Wnt signaling directly regulate otic genes. Alternatively, Wnt signals might act indirectly by up-regulating short-range signals that partition cranial ectoderm into otic placode and epidermis.

There is growing evidence that Wnt and Notch signaling pathways co-operate during cell fate determination in many tissues (Crosnier et al., 2006; Estrach et al., 2006; Fre et al., 2005). Notch signaling plays various roles in patterning the inner ear, ranging from specification of neurons and prosensory patches to the generation of the stereotypical pattern of mechanosensory hair cells and supporting cells (Adam et al., 1998; Brooker et al., 2006; Daudet et al., 2007; Daudet and Lewis, 2005; Haddon et al., 1998; Kiernan et al., 2005; Lanford et al., 1999; Shi et al., 2005). Both the *Notch1* receptor and several of its ligands such as *Jagged1 (Jag1)* and *Delta-like1 (Dll1)* are expressed in the otic placode from very early stages (Abello et al., 2007; Adam et al., 1998; Daudet et al., 2007; Groves and Bronner-Fraser, 2000; Haddon et al., 1998). Notch signaling may therefore also have an early function during otic placode development. We now provide evidence that elements of the Notch pathway are positively regulated by Wnt signaling, and that Notch1 signaling can in turn modulate the canonical Wnt signaling pathway. We also show that while some aspects of otic placode identity are regulated only by Wnt signals, other features of placodal differentiation can be regulated independently by Wnt or Notch pathways.

MATERIALS AND METHODS

Genetically modified mice

The following lines of mice were used in this study: Pax2-Cre (Ohyama and Groves, 2004b; available from the Mutant Mouse Regional Resource Center; www.mmrrc.org/strains/10569/010569.html), conditionally activated Notch1 intracellular domain (*cN1ICD*; Murtaugh et al., 2003), *Notch1* null mutants (Conlon et al., 1995), conditional activated β-catenin *Catnb*$^{lox(ex3)}$ (*cAct*; Harada et al., 1999), conditional *β-catenin*^{floxed} mutants (*β-cat- CKO*; Brault et al., 2001) Tcf/Lef Wnt reporter (Mohamed et al., 2004); conditional *Rbpj/Rbsuh* mutants (Tanigaki et al., 2002) and a GFP-expressing Cre reporter (*Z/EG*; Novak et al., 2000). To generate *cN1ICD* animals, *N1ICD*^{floxed} homozygotes were crossed with Pax2-Cre animals. Age matched heterozygotes and wildtypes were used as controls for *Notch1* mutant embryos. Detailed mating strategies for *cAct* and *β-cat-CKO* mice have been described previously (Ohyama et al., 2006). To generate *Notch1; cAct* mutants, a line that was heterozygous for *Notch1; Pax2-Cre* was crossed to animals that were heterozygous for *Notch1; cAct*. To generate *cN1ICD; β-cat- CKO* mutants, a line that was heterozygous for *β-cat-null; Pax2-Cre* was crossed to animals that were heterozygous for *N1ICD* and homozygous for a floxed allele of *β-catenin*. For each mutant genotype at least 3 embryos were analyzed except for *Notch1; cAct* mutants (n = 2). All animal experiments were done in accordance with the guidelines of the institution's Animal Care and Use Committee.

Whole mount in situ hybridization, immunostaining and detection of β-galactosidase

Whole-mount in situ hybridization was performed as previously described (Ohyama et al., 2006). The following probes were used: Notch1 (Jeffrey Nye), Dll1 (Achim Gossler), Jag1 (Tim Mitsiadu), Hes1 and Hes5 (Ryoichiro Kageyama), Lunatic fringe (Lfng) (Thomas Vogt) and Wnt6 (Andrew McMahon). Probes for Pax2, Pax8, Foxi2, Dlx5, Krox20, Hoxb1, FGF3, and EphA4 have been previously described (Ohyama et al., 2006). Embryos were embedded in 15% sucrose and 7% gelatin in phosphate buffered saline (PBS) as previously described (Groves and Bronner-Fraser, 2000) and 15–30 mm thick sections cut using a Leica CM 1850 cryostat. Immunostaining and detection of β-galactosidase on cryostat sections and embryos was performed as previously described (Ohyama et al., 2006). The following primary antibodies were used: β-catenin (Zymed) at 1:200, activated Caspase-3 (R&D systems) at 1:1000, Green Fluorescent Protein (GFP) conjugated to fluorescein (Abcam) at 1: 250 to 1:500, β-galactosidase (ICN/MP Biochemicals) at 1:100, Jagged1 (Jag1; Santa Cruz) at 1:50 to 1:100, Pax2 (Zymed) at 1:500 and Phospho-Histone-H3 (PH3; Upstate/Millipore) at 1:1000. Secondary goat anti-rabbit antibody conjugated to Alexa 594 (Molecular probes) was used at 1:200. Sections were counterstained with the nuclear marker DAPI (Molecular probes). All images were captured using a Zeiss Axiocam digital camera and Axiophot2 or M^2 Bio microscopes and processed using Adobe Photoshop CS software.

Quantification of thickened placode and average placode cell density in *Notch1* mutants

The thickened otic placode was defined as the 2–3 cell layer of ectoderm located adjacent to rhombomere 5/6 (as identified morphologically with DAPI staining) and/or negative for *Foxi2* transcripts. Quantifications of placode size were made from 15 μm serial sections from *Notch1* mutants and age matched control embryos. Length measurements were made using Image J software. To allow for direct comparisons along the anterior-posterior (AP) axis of control and mutant mice, measurements were binned into 5 categories: 0–20 % (being the most anterior sections), 21–40 %, 41–60 %, 61–80 % and 81–100 % (being the most posterior sections). For a given genotype, each bin consisted of multiple sections from several embryos. The mean and standard error of the mean (SEM) were calculated for each bin. Non-parametric Mann-Whitney U-tests were performed to test for significance between genotypes. The cranial region of *Notch1* mutants was comparable in size to controls. To confirm this we measured the dorsal-ventral length of the neural tube adjacent to the otic placode. The measurements were processed as described for the otic placode. We found no differences in neural tube length between *Notch1* mutants and controls (data not shown). For average density measurements, serial 15 μm thick sections stained with DAPI and/or hybridized with *Dlx5* or *Foxi2* probes were used. Cell density for each section was calculated as follows: number of cells/$\mu m^2 \times 500$ and pooled for each genotype.

Quantification of cell proliferation, otic cup length and Wnt reporter domain length in *cN1ICD* mutants

Cell proliferation counts were performed as described previously (Ohyama et al., 2006). To account for variations in staging of embryos, the medio-lateral length of otic cup or Wnt domain was standardized against D-V neural tube length adjacent to the otic cup and expressed as a percentage. Only mid-sections from otic cups were used for quantitation and Student t-tests were performed to test for significance between genotypes.

RESULTS

Notch pathway genes are expressed during early otic placode development

The pre-otic field destined to give rise to the otic placode and surrounding epidermis is marked by *Pax2* expression from the 0 somite stage (0ss; E8 in the mouse; Ohyama and Groves, 2004b; Ohyama et al., 2006). *Pax2* expression later becomes restricted to the otic placode, which is morphologically visible as a thickening patch of ectoderm next to rhombomeres 5 and 6 from 8ss (E8.5) onwards (Ohyama and Groves, 2004b). To see if elements of the Notch pathway were expressed at an appropriate place and time to participate in otic placode induction, we compared the expression patterns of *Notch1, Jag1, Dll1, Hes1*, *Lfng*, and *Hes5* to *Pax2* (Fig. 1). At 0–1ss, no Notch pathway transcripts were detected in the pre-otic field (Fig. 1B–C). Onset of *Notch1* expression was observed as early as the 4ss, becoming stronger by 5–7ss (Fig. 1C). Scattered cells in the anterior *Pax2* domain adjacent to the neural tube expressed the Notch ligand *Jag1* from around 5ss, although posterior cells did not express *Jag1* until 8–9ss (arrowhead, Fig. 1C–D). *Dll1* was also expressed adjacent to the neural tube at 4–5ss (Fig. 1B–C) and restricted to the otic placode from 9ss (Fig. 1C). Between 12–14ss *Dll1* expression was gradually restricted to differentiating neuroblasts in the antero-ventral placode (data not shown; Adam et al., 1998). We were unable to detect *Lfng* at the pre-otic field and placode stages in whole mounts (data not shown), although at later otic vesicle stages, *Lfng* is expressed in the antero-ventral portion of the otic cup destined to produce the vestibuloacoustic ganglion and the utricular and saccular maculae (Morsli et al., 1998; Raft et al., 2004). *Hes1* and *Hes5* are effectors of the Notch pathway that function in many processes including regulation of cell fate decisions (Bray, 1998; Kageyama et al., 2007; Lai, 2004).

Hes1 expression was scattered throughout the pre-otic field and by 10–11ss was restricted to the otic placode (Fig. 1C). We found no evidence for *Hes5* expression in the pre-otic field (data not shown). These data suggest that at least some transcriptional targets of the Notch pathway are expressed during early phases of otic placode development.

We previously used a transgenic Wnt reporter mouse line (Mohamed et al., 2004) to show that the canonical Wnt signaling pathway is activated in the pre-otic field between 3–5ss (Ohyama et al., 2006). Several Wnt family members are expressed in an appropriate location to trigger the observed Wnt reporter activity – for example, *Wnt8* is expressed in rhombomere 4 (Ohyama et al., 2006). We also observed *Wnt6* expression in the *Pax2*$^+$ pre-otic field at 0ss. It continues to be expressed in the neural folds at 5–7ss and the dorsal-most region of the otic placode at 11ss (Fig. 1C; Lillevali et al., 2006). Since the onset of Notch pathway gene expression closely corresponded to Wnt reporter activity in the pre-otic field (Fig. 1B–C), we hypothesized that the Notch1 pathway may interact with the canonical Wnt signaling pathway in mediating the fate decision between otic placode and epidermis.

Notch pathway components are positively regulated by canonical Wnt signaling in the developing otic placode

Previous studies suggest that Notch pathway components can be regulated by β-catenin (e.g. Estrach et al., 2006; Katoh and Katoh, 2006). We therefore examined expression of Notch pathway genes in embryos carrying gain- or loss-of-function mutations of the canonical Wnt pathway in the *Pax2*$^+$ pre-otic field. We crossed Pax2-Cre transgenic mice (Ohyama and Groves, 2004b) with mice in which β-catenin is constitutively activated in Cre-expressing cells (*cAct*; Harada et al., 1999) and examined expression of *Jag1, Notch1* and *Hes1*. In *cAct* mutants, *Jag1* expression was expanded ventrally to the level of the pharynx at the 9–10ss (bracket, Fig. 2A) and this ectopic expression continued until at least E9.0. *Jag1* is thought to be a direct target of β-catenin, as its promoter region contains five, three and six consensus Tcf/Lef binding sites in mouse, human and rat respectively (Estrach et al., 2006; Katoh and Katoh, 2006). The domain of *Notch1* and *Hes1* expression also expanded, although only after a delay (from the 14–15ss; Fig. 2A, brackets). Such a delayed induction of *Notch1* and *Hes1* relative to *Jag1* has also been observed in epidermis in which *β-catenin* is activated (Ambler and Watt, 2007). Other Notch pathway genes such as *Dll1*, *Hes5* and *Lfng* were not expressed in *cAct* mutants (data not shown).

To determine if Wnt signaling is necessary for expression of Notch pathway components, we analyzed expression of *Jag1*, *Notch1* and *Hes1* in the *Pax2*$^+$ pre-otic field of mice lacking *β-catenin* (Brault et al., 2001; *β-cat-CKO*). The *Jag1* domain was significantly reduced at 10–11ss, as we previously reported for *Pax2* and *Pax8* (Ohyama et al., 2006). Many cells within the vestigial *β-cat-CKO* otic vesicle were β-catenin$^-$;Jag1$^-$, suggesting that Wnt signaling is directly responsible for *Jag1* induction in the placode (bracket; Fig. 2B). Close examination of the vestigial mutant vesicles at E9–9.5 revealed that cells expressing Jag1 were β-catenin$^+$ and had therefore failed to undergo Cre-recombination (Fig. 2B). The domains of *Notch1* and *Hes1* were also significantly reduced, although the resolution of the whole mount in situ technique made it difficult to determine whether all *Notch1* and *Hes1* expressing cells also expressed β-catenin protein (Fig. 2B).

Wnt and Notch signaling pathways differentially regulate expression of otic markers

The expression of Notch pathway genes in the pre-otic field and otic placode, together with the regulation of these genes by Wnt signaling suggested that Notch signaling may participate in the fate decision between otic placode and epidermis. To test this, we conditionally activated Notch1 in the pre-otic field using mice in which the active, intracellular domain of Notch1 receptor (N1ICD) was knocked into the ROSA26 locus with a transcriptional STOP cassette flanked by LoxP sites (Murtaugh et al., 2003). We drove expression of *N1ICD* in the *Pax2*$^+$ pre-otic field using Pax2-Cre mice (Ohyama and Groves, 2004b). The *Pax2-Cre* mouse line expresses Cre recombinase in the midbrain and rhombomere 1 (R1) of the hindbrain (Ohyama and Groves 2004b). Conditionally activated *N1ICD* (*cN1ICD*) mutants displayed an open neural tube phenotype at the level of the midbrain-R1 region, likely resulting from over-proliferation of precursor cells induced by Notch activation. However, the patterning of the posterior hindbrain next to the ear was normal at embryonic (E) day 8.5–9.5 based on the expression of *HoxB1* (rhombomere 4), *FGF3* (rhombomeres 5 and 6), *EphA4* and *Krox20* (rhombomeres 3 and 5; Fig. S1B), suggesting that any otic placode phenotype in *cN1ICD* mutants is not due to changes in the adjacent hindbrain.

We examined embryos inheriting both the Cre-inducible *N1ICD* and Pax2-Cre transgenes for otic placode and epidermal markers. The *N1ICD* transgene also harbors an IRES-nGFP sequence, allowing visualization of *N1ICD*-expressing cells by GFP fluorescence. GFP-expressing cells were observed throughout the pre-otic field from 5–6ss (data not shown). E9.5 *cN1ICD* mutant embryos displayed GFP expression throughout a thickened placode-like structure which expanded to the level of the ventral pharynx (Fig. S1A). Analysis of *cN1ICD* embryos at 10–11ss revealed that the *Pax8* domain was expanded ventrally (arrowheads and brackets, Fig. 3A, A′). We previously showed that *Foxi2* is an epidermal marker expressed in a complementary manner to *Pax2* and *Pax8* during otic placode development (Ohyama and Groves, 2004a). By E8.75–9, *Foxi2* expression was reduced dramatically in *cN1ICD* mutants compared to controls (dotted outline and brackets, Fig. 3C–C′), complementing the expansion of the thickened epidermis. To determine if cell proliferation was responsible for the expanded placode, we examined expression of the M-phase marker phosphohistone-3 (PH3) in *cN1ICD* embryos ($n = 10$ placodes) and control embryos produced by crossing the *Pax2-Cre* line with the Cre inducible *Z/EG GFP*-expressing line (Novak et al., 2000). We saw no significant differences in total PH3$^+$ or PH3$^+$;GFP$^+$ cell counts per section (Fig. S1D).

The expansion of *Pax8* at the expense of *Foxi2* in *cN1ICD* embryos is strikingly similar to that seen in embryos in which the canonical Wnt pathway is activated (*cAct* embryos; Ohyama et al., 2006). However, in contrast to *cAct* embryos, we saw only a modest expansion of the *Pax2* domain (bracket and arrowhead, Fig. 3B, B′; Fig. S1C) and no expansion of the otic markers *Gbx2* or *Sox9* (Fig. 3D). Finally, a marker of the dorso-lateral otocyst, *Hmx3*, which does not require either Wnt or Hedgehog signaling for its expression (Ohyama et al., 2006; Riccomagno et al., 2002) was also not expanded in *cN1ICD* mutants (Fig. 3D).

These results suggest that different aspects of otic placode development are differentially regulated by Wnt and Notch signaling. Placode markers such as *Pax2*, *Gbx2*, and *Sox9* appear to be regulated by Wnt signaling (Ohayama et al., 1996; Saint-Germain et al., 2004), but not Notch signaling, whereas markers such as *Pax8*, the morphological thickening of epithelium and the repression of the epidermal marker *Foxi2* can be regulated by both Notch and Wnt signals. To determine if Notch signaling can regulate these markers independently of Wnt signaling, we analyzed *β-cat-CKO;cN1ICD* mutant embryos in which β-catenin was inactivated and Notch1ICD was activated throughout the pre-otic field. Mutant embryos displayed greatly expanded regions of thickened placode-like epithelium that expressed both *Pax8* and *Jag1* (Figure 3E). This expanded region of thickened epithelium was also largely devoid of *Foxi2* expression (Fig. 3E), although occasional *Foxi2*$^+$ patches of cells could sometimes be detected. These results show that Notch and Wnt signals can independently regulate some aspects of otic placode development.

Inactivation of *Notch1* reduces the size of the otic placode

Our results show that Notch1 activation throughout the *Pax2*$^+$ pre-otic field expands some otic placode markers at the expense of epidermis. In complementary experiments, we examined *Notch1* mutants, in which a substantial portion of the *Notch1* gene is deleted (amino acids 1056–2049; Conlon et al., 1995). This deletion encompasses RAM and Ankyrin repeats required for RBPJk signaling (Conlon et al., 1995; Fortini and Artavanis-Tsakonas, 1994; Kurooka et al., 1998a; Kurooka et al., 1998b; Lamar et al., 2001; Nam et al., 2003; Tani et al., 2001). We confirmed that posterior hindbrain patterning was normal in *Notch1* mutants by assaying for *Hoxb1*, *FGF3* and *Krox20* expression (Fig. S2A). All three genes were expressed normally, suggesting that any defects observed in otic placode development are due to deficiency in Notch1 signaling in the placode, rather than in the hindbrain.

To determine if Notch signaling was necessary for the expression of otic markers, we examined *Pax2* and *Pax8* expression in *Notch1* mutants. By 9–11ss there was a dramatic down-regulation of *Pax2* expression in mutants in both the otic region and the epibranchial placodes (Fig. 4A). While the anterior-posterior limits of *Pax8* expression in the otic region was reduced, the expression in the hyoid arch was relatively unchanged (Fig. 4B). In *Notch1* mutant whole mounts, the limits of *Pax2/8* domains in the anterior-posterior axis were reduced (brackets, Fig. 4). Sections through *Notch1* mutants also revealed a reduction in the medial-lateral extent of *Pax2/8* expression (brackets, Fig. 4).

The reduction in the size of the otic placode in *Notch1* mutants may result from increased apoptosis, increased cell density or a change in cell fate. We measured the size of the placode by examining *Foxi2* expression, which is precisely excluded from the thickened placode region. The *Notch1* mutant otic placode was indeed smaller at 9–13ss on the basis of *Foxi2* expression (dotted outline, Fig. 5A–B). We compared the medial-lateral extent of the thickened otic placode in *Notch1* mutants and controls at 9–11ss and 12–13ss (see Methods: Fig. 5C, D) *Notch1* mutants (9–11ss, $n = 25$ placodes; 12–13ss, $n = 10$ placodes) had significantly smaller placodes compared to controls (9–11ss, $n = 13$ placodes; 12–13ss $n = 6$ placodes) regardless of the axial level of the section ($p < 0.05$–0.005; Fig. 5C–D). There were no significant changes in placode cell density at 9–11ss ($n = 10$ mutant placodes; $n = 6$ control placodes) and 12–13ss

($n = 5$ mutant placodes; $n = 4$ control placodes; $p > 0.05$; Fig. 5F), or in apoptosis when analyzed for activated caspase-3 expression (Fig. 5G; Conlon et al., 1995; Del Monte et al., 2007). We also confirmed that the smaller placode was not due to the precocious generation of neurons by analyzing *Ngn1* expression (data not shown).

Collectively, our data show that many otic placode precursors undergo a fate change to epidermis in *Notch1*-deficient embryos. It is possible that the other Notch receptors are active during otic placode development in addition to *Notch1*. We confirmed our results by examination of conditional mutants of *RBPJ*/*Rbsuh*, a transcriptional co-factor of NICD. The otic placode still forms in these mice (Fig. S2C; see also Oka et al., 1995; de la Pompa et al., 1997), confirming that Notch signaling can modulate the size of the otic placode but is not necessary for its induction.

Daudet and colleagues recently suggested that initiation, but not maintenance of *Jag1* expression in the chick otic placode is regulated independently of Notch1 signaling (Daudet et al., 2007). We confirmed this result in mice: *Jag1* continued to be expressed in the placode of *Notch1* mutants, but the intensity of expression was reduced compared to controls (Fig. S2B). It has been previously reported that *Jag1* continues to be expressed in a morphologically distinct otic placode in mice carrying mutations of *Pofut1*, an O-fucosyltransferase essential for Notch signaling (Shi and Stanley, 2003). We confirmed that *Jag1* and *Hes1* expression can be initiated in the absence of canonical Notch signaling by examination of conditional mutants of *RBPJ*/*Rbsuh*. Both genes continue to be expressed in a morphologically visible otic cup, although *Hes1* was expressed at significantly reduced levels compared to controls (Fig. S2C). This is consistent with *Hes1* expression being initiated by Notch signaling, but *Jag1* expression being initiated independently of Notch signaling.

Notch1 augments canonical Wnt signaling in the otic placode

Canonical Wnt signaling plays an important role in defining the size of otic placode by driving medial $Pax2^+$ pre-otic cells towards an otic rather than cranial epidermis fate (Ohyama et al., 2006). Similarly, conditional activation of Notch1 in the $Pax2^+$ pre-otic field expands some, but not all otic markers at the expense of epidermis (Fig. 3). Additionally, Notch pathway gene expression can be activated by canonical Wnt signaling (Fig. 2). These results suggested the possibility of reciprocal interactions between the Notch and Wnt pathways. To test whether Wnt signaling is modulated by the Notch pathway in the developing otic placode, we crossed Wnt reporter mice expressing a *β-galactosidase* reporter gene under the control of six Tcf/Lef DNA binding sites (Mohamed et al., 2004) to either *cN1ICD* or *Notch1* mutant lines.

Surprisingly, although the thickened $Pax8^+$placode was dramatically expanded to the level of the pharynx in *cN1ICD* embryos (Fig. 3A), Wnt reporter activity showed a much more modest expansion, extending a little beyond the lateral edge of the otic cup (Fig. 6A). We observed similar results with *Dlx5*, a known Wnt-responsive marker of the otic placode (bracket, Fig. 6A). To verify these results, we made use of the fact that *cN1ICD* mutants also express nuclear GFP after Cre recombination (Murtaugh et al., 2003). We co-immunostained *cN1ICD;Wnt* reporter embryos with anti-β-galactosidase and anti-GFP antibodies to mark the extent of the Wnt reporter and the expanded otic placode respectively (Fig. 6B). By E9–9.25, Wnt activity was elevated in the lateral regions of the mutant otic cup which normally demonstrate moderate or low Wnt activity (red arrowhead; Fig 6A). Furthermore, the otic cup region was larger in *cN1ICD* mutants compared to controls (Fig. S3, $n = 13$ mutant placodes, $n = 14$ control placodes; $p < 0.005$). However, the ectopic placode region lateral to the otic cup which expressed *N1ICD* and GFP did not express β-galactosidase (bracket, Fig. 6B). These results suggest that Notch signaling can augment Wnt signaling, but that the active Notch1 ICD does not directly regulate Wnt-responsive genes containing Tcf/Lef DNA binding sites.

To test if Wnt signaling can also be modulated by loss of Notch1 activity, we examined *Notch1* mutant mice crossed to a Wnt reporter mouse background. As expected, Wnt reporter activity was detected in *Notch1* mutant placodes (Fig. 6C). However, the intensity of Wnt activity, as measured by time-matched β– galactosidase reactions was weaker compared to controls. Additionally, the medio-lateral extent of the Wnt reporter and expression of the Wnt-responsive gene *Dlx5* was slightly reduced (Fig. 6C), reflecting the observed reduction in the placode size due to *Notch1* deficiency (Fig. 4). Taken together with our data showing that Wnt signaling can up-regulate Notch pathway components, our results are consistent with a model in which the Wnt pathway can positively regulate components of the Notch pathway, and can in turn be augmented by Notch signaling. One prediction of this model is that maximal activation of Wnt signaling by a constitutively activated β–catenin mutation will be unaffected by a *Notch1* mutation. To test this, we analyzed *Pax8* and *Foxi2* expression in *Notch1* mutant embryos that also carried the activated *β-catenin* (*cAct)* mutation. As expected, the size of the expanded *Pax8* domain seen in *cAct* embryos was not significantly different from *Notch1; cAct* mutants (Fig. 6D). Similarly, the reduced domain of epidermal *Foxi2* expression seen in *cAct* mutants was not significantly different in *Notch1; cAct* mutants (Fig. 6D).

DISCUSSION

Notch signaling plays multiple roles in inner ear patterning, from specification of neurons and prosensory patches to the generation of the stereotypical pattern of hair cells (Adam et al., 1998; Brooker et al., 2006; Daudet et al., 2007; Daudet and Lewis, 2005; Haddon et al., 1998; Kiernan et al., 2005; Lanford et al., 1999; Shi et al., 2005). Here, we have uncovered new roles for Notch and Wnt signaling in the early development of the ear. Conditional activation of *Notch1* in the *Pax2*$^{+}$ pre-otic field causes the expansion of some, but not all otic markers at the expense of epidermis. Conversely, in the absence of Notch1 signaling, the otic placode is significantly smaller. We have also shown that Wnt signaling regulates components of the Notch pathway, such as *Jag1*, and that Notch signaling positively regulates Wnt signaling. Our results suggest that Notch augments the Wnt signaling pathway to help define the size of the otic placode

The expression of Notch signaling pathway components in the otic placode – a role for Wnt signaling

Our expression data shows that several components of the Notch signaling pathway – *Notch1*, *Jag1*, *Dll1* and *Hes1* – are expressed in a medial subset of the mouse *Pax2*$^{+}$ pre-otic field from the 5ss onwards, and that Wnt signaling initiates expression of at least some Notch pathway components. *Notch1*, *Jag1*, *Dll1* and *Hes1* are all expressed in the pre-otic field after *Wnt6*, *Wnt8* and the first signs of Wnt reporter activity (Fig. 1C and Ohyama et al., 2006). Expression of these Notch pathway genes occurs only within the region of the medial Pax2^{+} pre-otic field that responds to Wnt signaling (Fig. 1C). Consistent with previous reports (Duncan et al., 2005;Espinosa et al., 2003;Estrach et al., 2006), we found that ectopic activation of the canonical Wnt pathway induced expression of *Jag1*, *Notch1* and *Hes1* (Fig. 2A), while conditional deletion of *β-catenin* greatly reduced their expression (Fig. 2B). Wnt signaling can control transcription of Notch pathway genes by directly acting on elements located in their promoters (Duncan et al., 2005;Espinosa et al., 2003;Estrach et al., 2006;Katoh and Katoh, 2006). In the case of *Jag1* promoter, there are multiple Tcf/Lef binding sites that are conserved between mouse and human (Estrach et al., 2006;Katoh and Katoh, 2006). Putative Tcf/Lef binding sites have also been identified in the *Notch1* promoter (Galceran et al., 2004), however, although the *Dll1* promoter also has Tcf/Lef binding sites (Galceran et al., 2004), its expression was not expanded in embryos expressing activated β-catenin. Recent evidence suggests that factors distinct from Notch signaling are required to initiate *Jag1* expression in the chick otocyst (Daudet et al., 2007), although maintenance of *Jag1* is Notch-dependent. Our results suggest that *Jag1* initiation in the developing ear may be directly regulated by Wnt signaling (Fig. 2B,7) while *Notch1* and *Hes1* expression may be initiated by Wnt signaling and possibly also by FGFs (Norgaard et al., 2003;Zhou and Armstrong, 2007), in addition to Notch signaling itself.

Overlapping and distinct functions of Notch and Wnt signaling in the otic placode

We recently showed that the mouse pre-otic field defined by expression of *Pax2* undergoes a fate decision to give rise to cranial epidermis and otic placode (Ohyama et al., 2006). The placode-epidermis fate decision is mediated by the canonical Wnt pathway, such that conditional deletion of *β-catenin* in *Pax2*$^+$ cells drastically reduces the otic placode and expands epidermis, whereas conditional activation of *β-catenin* in *Pax2*$^+$ cells expands the otic placode at the expense of epidermis (Ohyama et al., 2006). In the light of the expression of many components of the Notch signaling pathway in the developing otic placode (Fig. 1), we hypothesized that Notch signaling may act with the canonical Wnt pathway to specify otic placode identity.

Activation of Notch1 signaling in the pre-otic field leads to a massive expansion of thickened, placode-like epithelium expressing *Pax8* at the expense of *Foxi2*$^+$epidermis in a manner very similar to activation of β-catenin (Fig. 3). In contrast, although *Pax2* expression can be expanded by activation of Wnt signaling (Ohyama et al., 2006) it showed only modest expansion in *cN1ICD* mutants compared to *Pax8* (compare Fig. 3A and B). *Pax2* and *Pax8* are known to be differentially regulated by FGF signaling and the *foxi1* transcription factor during induction of the zebrafish ear (Hans et al., 2004;Nissen et al., 2003,Solomon et al., 2003; 2004), and our results suggests these genes may also be differentially regulated by Notch signaling. In particular, *Pax8* can be regulated by either canonical Wnt signaling, or by Notch signaling. However, it is not clear whether the two pathways regulate *Pax8* in entirely different ways or whether they converge on a nodal point, such as the binding of Lef/Tcf complexes to the *Pax8* promoter (Schmidt-Ott et al., 2007). *Pax8* expression correlates with epithelial thickening in all experiments in our study. However, further experiments are required to determine whether *Pax8* is directly responsible for regulating this morphological change in the otic placode.

Examination of *Notch1* mutants consistently showed a significant reduction in the size of the otic placode (Fig. 4B–C; 5). This small reduction is unlikely to be due to redundancy with other *Notch* genes, as there is no detectable expression of *Notch2–4* in the otic placode (Lewis et al., 1998; Williams et al., 1995). A similar persistence of the otic placode is seen after treating chick otic ectoderm with DAPT, a γ-secretase inhibitor that abolishes Notch signaling (Abello et al., 2007; Daudet et al., 2007), in mice carrying mutations in *Pofut1*, an O-fucosyltransferase that is an essential component of the Notch pathway (Shi and Stanley, 2003; C.J., unpublished), and in mice lacking *RBPJ/Rbsuh/CSL* (Oka et al., 1995; de la Pompa et al., 1997; Fig. S2C). In all these experiments, any reduction in placode size in the absence of Notch signaling is much more modest than that seen in mice in which Wnt signaling is blocked by conditional deletion of *β-catenin* (Ohyama et al., 2006; Fig. 2B).

Our results suggest a model (Fig. 7C) in which both Notch and Wnt signaling can specify the size of the epithelium destined to form the otic placode by virtue of their regulation of *Pax8*, *Foxi2, Jag1* and the induction of a thickened epithelial morphology. Our data from mice in which Wnt signaling is activated in the absence of *Notch1* (Fig. 6D), or Notch1 is activated in the absence of *β-catenin* (Fig. 3C) show that the two pathways can regulate these genes independently of each other. However, unlike the Wnt pathway, Notch signaling does not regulate the expression of otic placode-specific genes such as *Gbx2, Sox9* and *Hmx3*, as these are unchanged in *cN1ICD* mutants (Fig. 3D). In addition, our results, taken together with previously published studies, suggest that Notch signaling also acts to augment Wnt signaling during otic placode induction, rather than being absolutely necessary for placode induction

Notch signaling acts to augment Wnt signaling during otic placode induction

To integrate our gain- and loss-of-function experiments with the Notch and Wnt pathways, we propose a model in which some Notch pathway components such as *Jag1* are induced by Wnt signaling. Subsequently, activation of Notch1 by Jag1 feeds back to augment the Wnt response (Fig. 7B). This feedback activity has no effect on the most medial regions of the pre-otic field - which receive the highest levels of Wnt signaling - but acts to increase Wnt signaling in medio-lateral regions of ectoderm that receive modest to low levels of Wnt signaling. Thus, Notch-mediated feedback serves to sharpen and refine the initial medio-lateral gradient of Wnt activity during the pre-otic field (Ohyama et al., 2006) into a more binary pattern at the otic placode stage, where Wnt signaling is either active (giving rise to otic placode) or silenced (giving rise to epidermis; Fig. 7C).

Our data support this model in four ways. First, *Notch1* deficiency causes a reduction in the area and intensity of β-galactosidase activity in Wnt reporter mice and a reduction of the domain of the Wnt-responsive gene *Dlx5* (Fig. 6C). However, loss of *Notch1* does not abolish the expression of either marker, consistent with the notion that Notch1 signaling augments the Wnt response but does not initiate it. Second, the reduction in Wnt signaling resulting from loss of *Notch1* (Fig. 4; 5) causes a consistent reduction in the size of the otic placode, but does not eliminate it entirely. The otic placode also forms in mice lacking other crucial components of the Notch pathway, such as *Pofut1* or *Rbpj/Rbsuh/CSL* (Oka et al., 1995;de la Pompa et al., 1997;Shi and Stanley, 2003). Third, mutation of *Notch1* has no effect on the size of the otic placode in embryos also expressing constitutively active *β-catenin* in the entire pre-otic field (Fig. 6D), presumably because cells expressing artificially high levels of activated β-catenin are not dependent on Notch1 function for stabilization of otic fate. Finally, artificial N1ICD activation throughout the pre-otic field greatly expands *Pax8* to the ventral pharynx, but this is not the case for *Dlx5* or Wnt activity (Fig. 6A). This suggests that ectopic activation of N1ICD in regions of the pre-otic field that receive no Wnt signals is insufficient to augment or initiate the Wnt response (Fig. 7). Furthermore, Wnt reporter expression is enhanced in regions receiving moderate levels of Wnt activity in *cN1ICD* mutants (Fig. 6A). Although, the mechanism of how Notch signaling augments Wnt activity is not clear, this result suggests that it is unlikely that N1ICD can directly activate transcription of Wnt-responsive genes by itself. A growing body of evidence suggests that Wnt and Notch pathways interact during cell fate determination (Aoyama et al., 2007;Arias and Hayward, 2006;Crosnier et al., 2006;Estrach et al., 2006;Fre et al., 2005). Notch signaling can act upstream of the Wnt pathway (Balint et al., 2005;Johnston and Edgar, 1998;Neumann and Cohen, 1996), or downstream (Estrach et al., 2006). Stimulation of the Wnt pathway can either antagonize or activate the Notch pathway in different contexts – for example, *dishevelled* can antagonize Notch signaling (Axelrod et al., 1996), whereas down-regulation of GSK3 activity by Wnt signaling stimulates the Notch pathway (Espinosa et al., 2003). The Notch receptor is also able to antagonize β-catenin activity (Nicolas et al., 2003), sometimes in an NICD-independent manner (Hayward et al., 2006;Hayward et al., 2005).

Taken together, our current and previously published data suggest a model of otic placode induction where FGF signaling initially establishes a *Pax2*$^{+}$ pre-otic field that is then patterned by a gradient of Wnt signaling arising from the midline. Wnt signaling up-regulates components of the Notch pathway, which then act locally to augment the Wnt response and to mediate the placode-epidermis fate decision in the pre-otic field.

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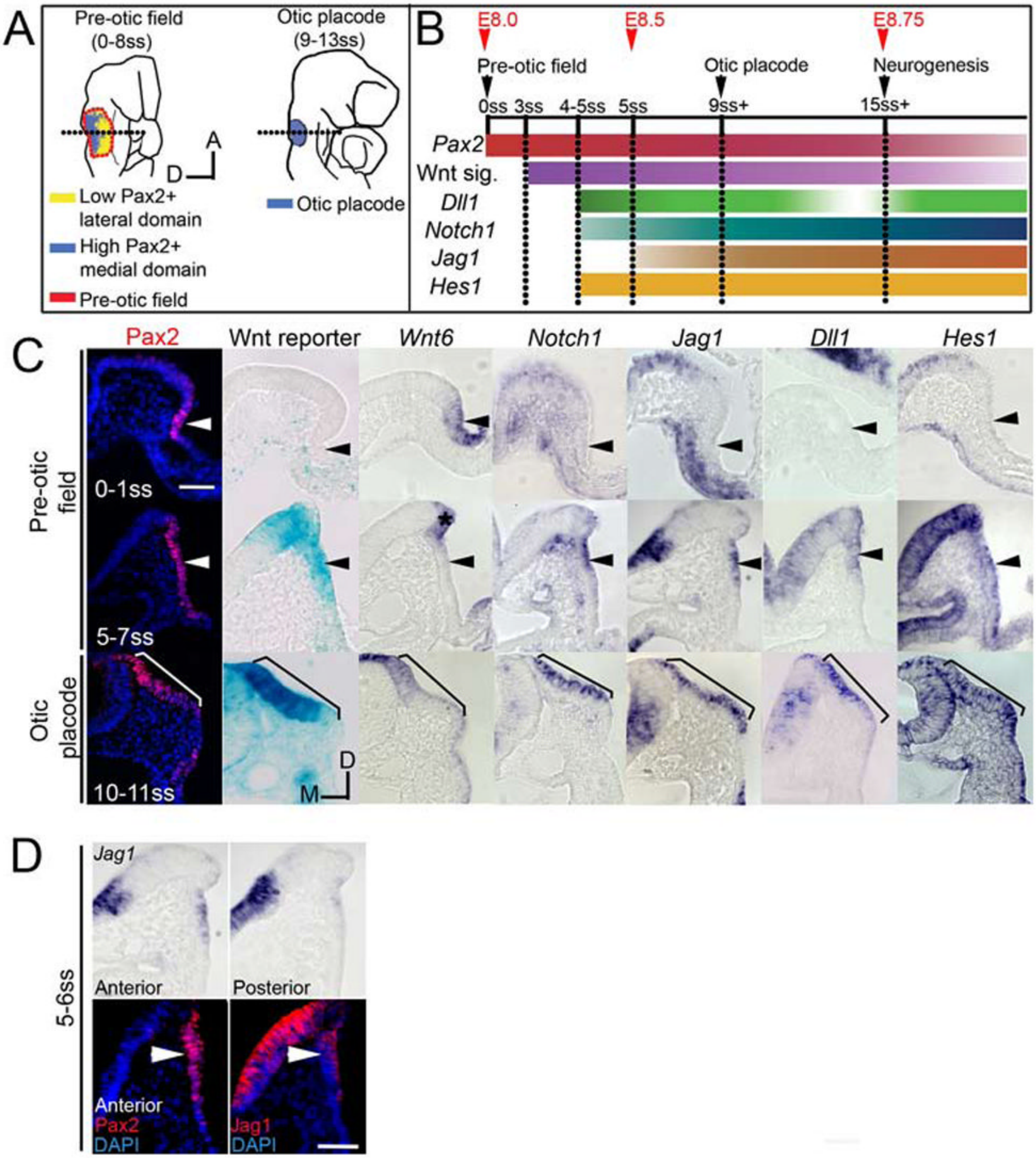

Fig. 1. Expression profile of Notch pathway genes during otic placode development

(A) Schematic views of embryos during otic placode development. The dotted line indicates the approximate level of sections in (C). (B) Summary showing the onset of *Notch1* (blue), *Jagged1* (*Jag1;* brown), *Delta-like1* (*Dll1*; green), and *Hes*1 (yellow-orange) with respect to *Pax2* (pink-red) and Wnt signaling (purple). Preceding neurogenesis, *Dll1* expression is high in the pre-otic field and progressively becomes weaker in the placode. During neurogenesis *Dll1* localizes to neuroblasts. (C) Sections comparing expression of *Wnt6*, *Notch1*, *Jag1*, *Dll1*, *Hes1* with respect to Pax2 (red) and Wnt reporter (blue). Arrowheads mark the pre-otic field. Brackets mark the extent of the otic placode. (D) Top row: *Jag1* expression in the anterior and posterior pre-otic field. Bottom row: consecutive serial sections through the pre-otic field immunostained with anti-Pax2 and anti-Jag1 antibodies respectively. Scale: 50 µm.

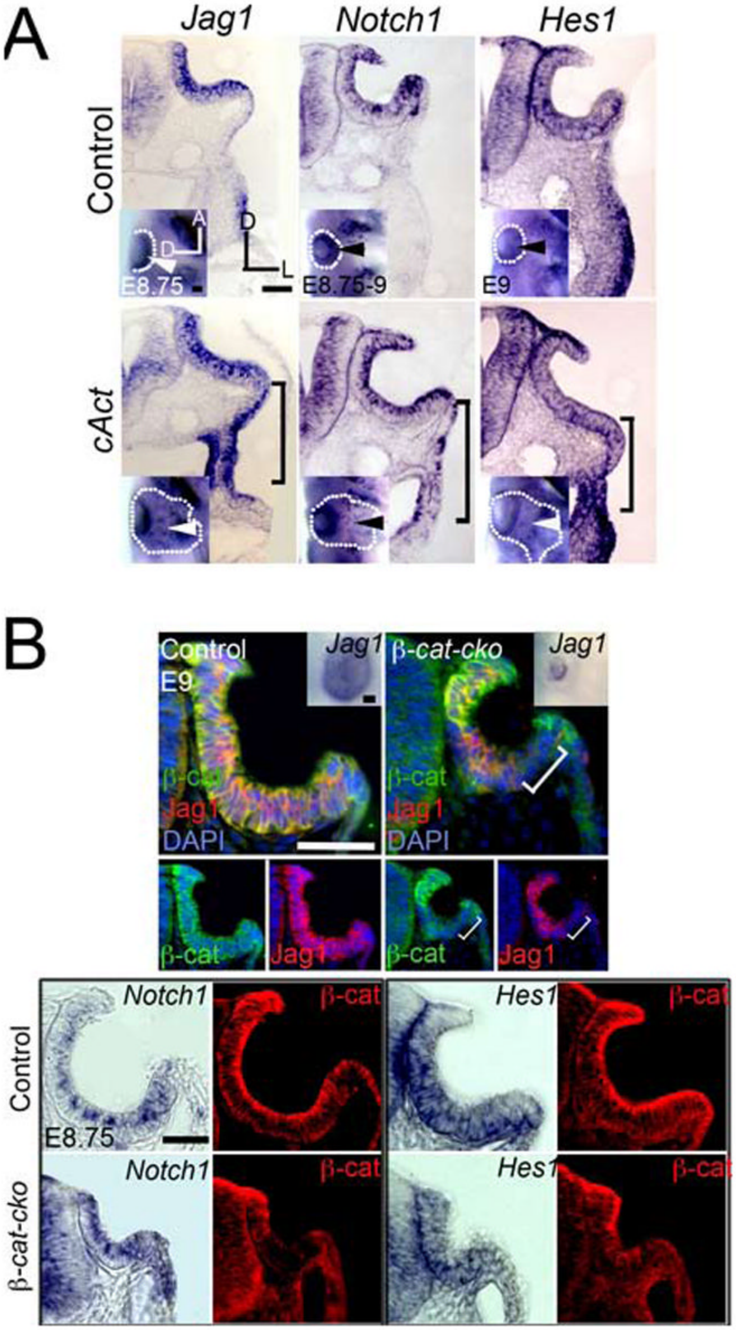

Fig. 2. The canonical Wnt pathway positively regulates components of the Notch pathway in the otic placode

(A) *Jag1*, *Notch1*, and *Hes1* domains are ectopically expanded in *cAct* embryos (bracket). Inset: corresponding whole-mounts. Arrowhead: normal (top row) or ectopic (bottom row) expression. Dotted outline: otic area. (B) Jag1, *Notch1* and *Hes1* domains are reduced in *β-cat-CKO* embryos. (Left panel) Anti-Jag1 (red) and β-catenin (β-cat, green) co-immunostaining. Bracket: β-catenin$^-$: Jag1$^-$ cells. Inset: *Jag1* expression at E9.5 in whole-mount. (Right panel) Whole-mount and (bottom panel) sections of *Notch1* and *Hes1* hybridized embryos. Scale: 50 μm.

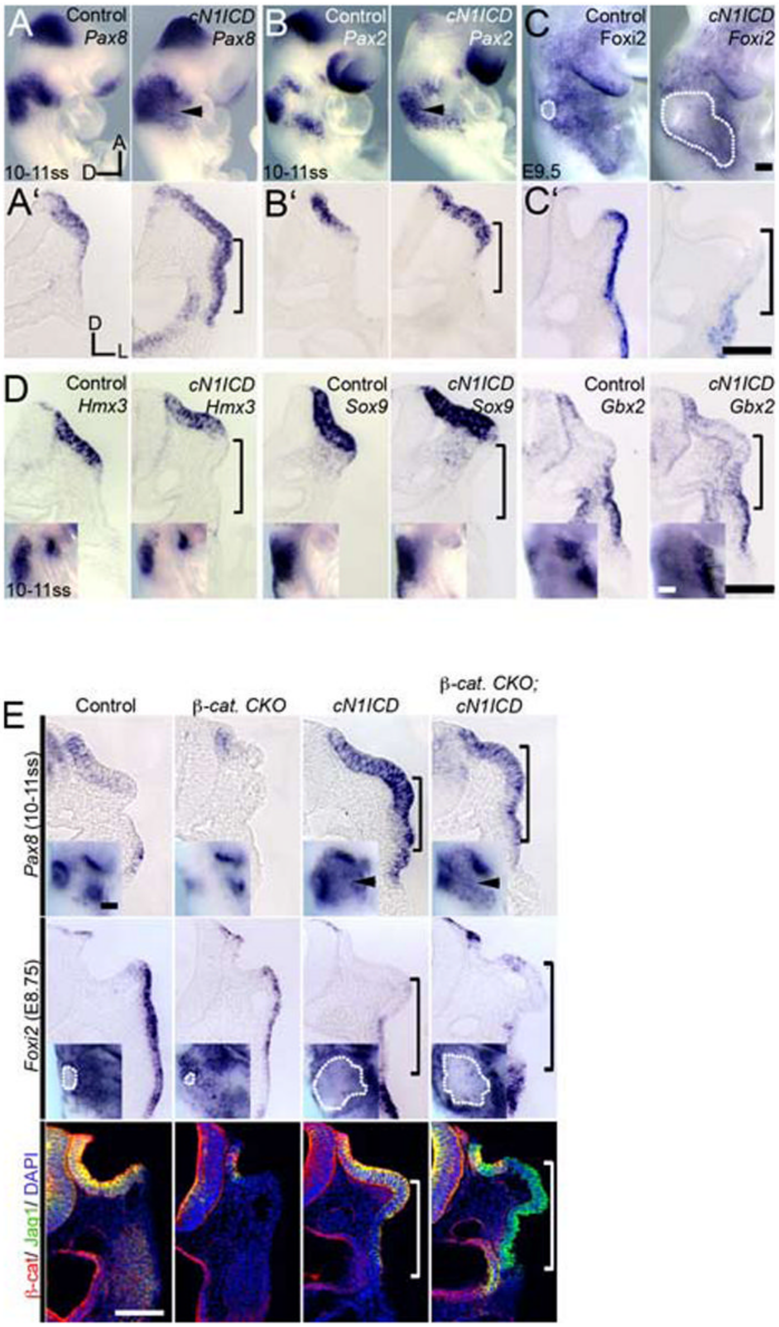

Fig. 3. Some, but not all otic markers are expanded at the expense of epidermis in conditionally activated Notch1 (*cN1ICD)* embryos

(A–C): Expanded otic placode in *cN1ICD* embryos hybridized with probes for *Pax2* (A, A′), *Pax8* (B, B′) and the epidermal marker *Foxi2* (C, C′). Arrowhead: ectopic expression. (C) Dotted outline indicates the invaginating otic cup (controls) or expanded otic region (cN1ICD mutants). (A′–C′) Corresponding transverse sections. Brackets: lateral ectopic placode region. Scale: 100 μm. (D) *Hmx3, Sox9* and *Gbx2* otic markers are not expanded in *cN1ICD* mutants. (E) In *β-cat-CKO; cN1ICD* mutants, *Pax8* (top row) and Jag1 (green; last row) are expanded at the expense of *Foxi2* (middle row). Last row; β-catenin (red) and Jag1 protein expression in *Foxi2* hybridized embryos. Scale: 100 μm.

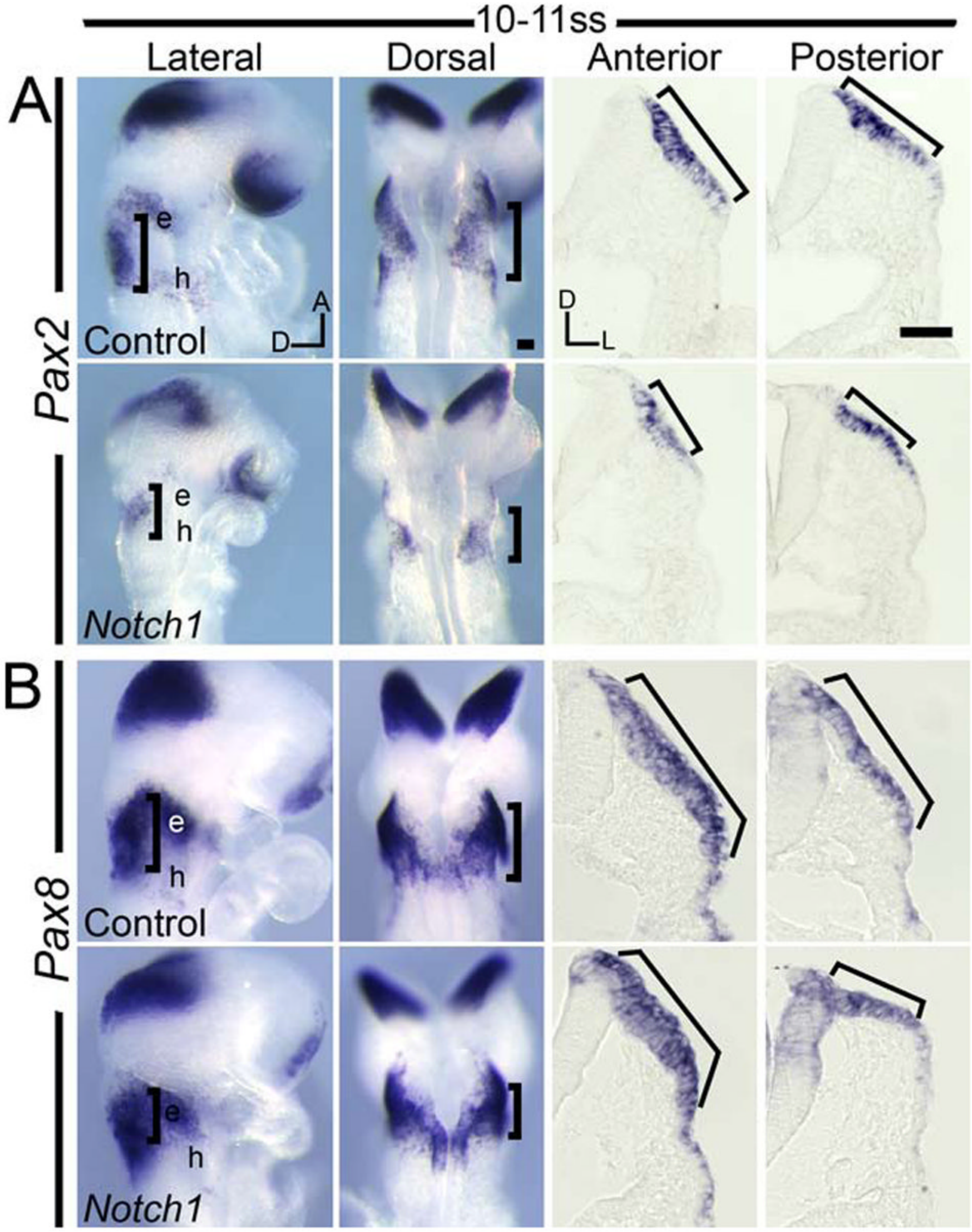

Fig. 4. Domains of *Pax2* and *Pax8* are reduced in *Notch1* mutants

(A–B) Dorsal and lateral whole-mount views of *Pax2* (A) and *Pax8* (B) expression. Anterior and posterior sections through control and *Notch1* mutant placodes are also shown. Brackets: otic expression. e, epibranchial placode; h, hyoid arch. Scale: 50 μm.

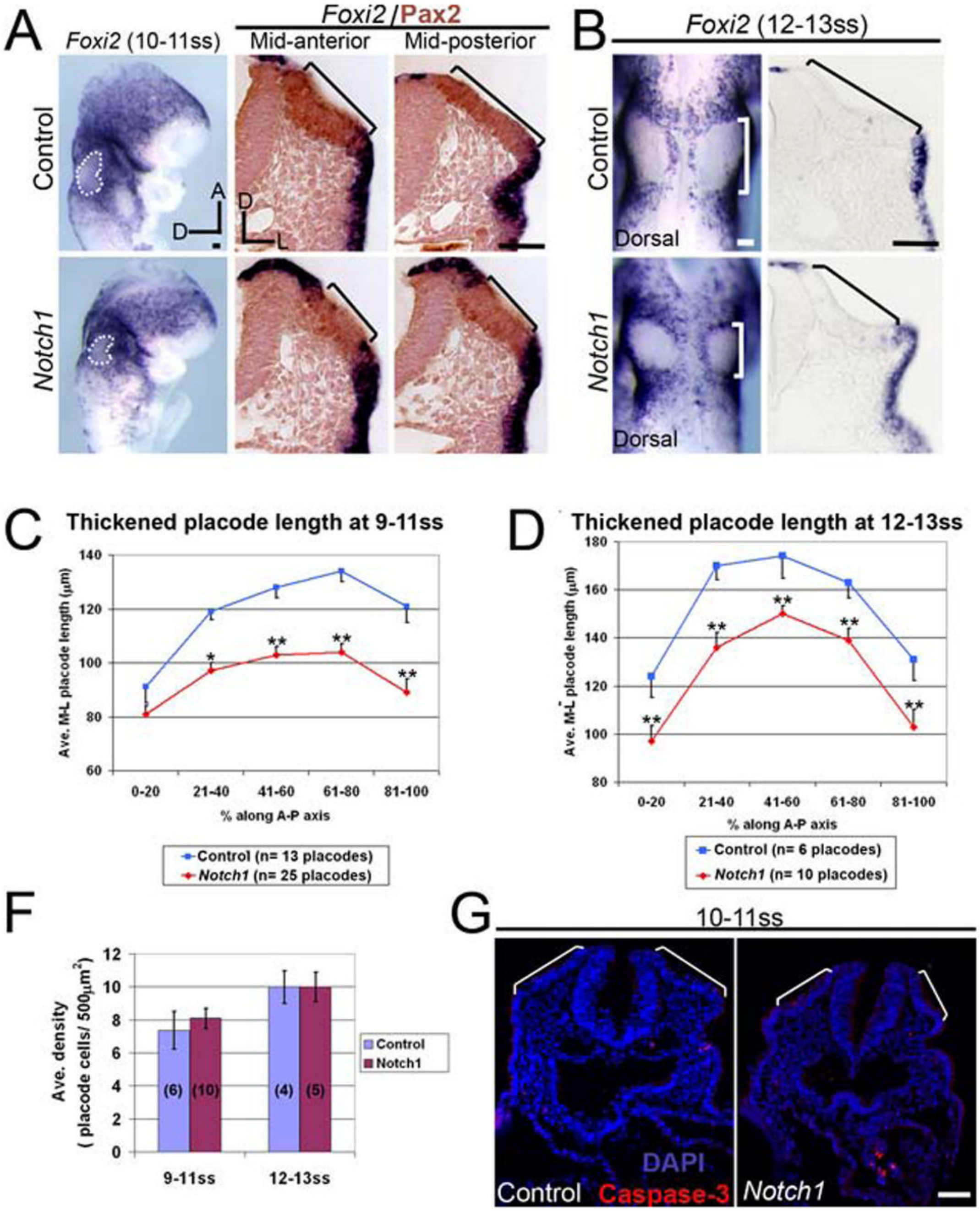

Fig. 5. The otic placode is significantly reduced in *Notch1* mutants

(A–B) *Foxi2* expression at 9–11ss (A) and at 12–13ss (B) in whole mounts and sections. Dotted outline/brackets indicate the thickened otic placode region that does not express Foxi2. (A) Corresponding sections showing *Foxi2* transcript (blue) and Pax2 protein expression (brown). (C–D) Quantitative comparison of medial-lateral placode length in control and Notch1 mutant embryos at 9–11 somite stage (ss) (C) and 12–13ss (D) (refer to Materials and Methods). (*) $p < 0.05$ and (**) $p < 0.005$. (F) Quantitation of average cell density in control and Notch1 mutant. Placode n values in parentheses. (G) Activated Caspase-3 expression (red) indicating lack of apoptotic cells within the Notch1 mutant otic placode at 10–11ss. Bracket: thickened placode. Error bars: SEM. Scale: 50 µm.

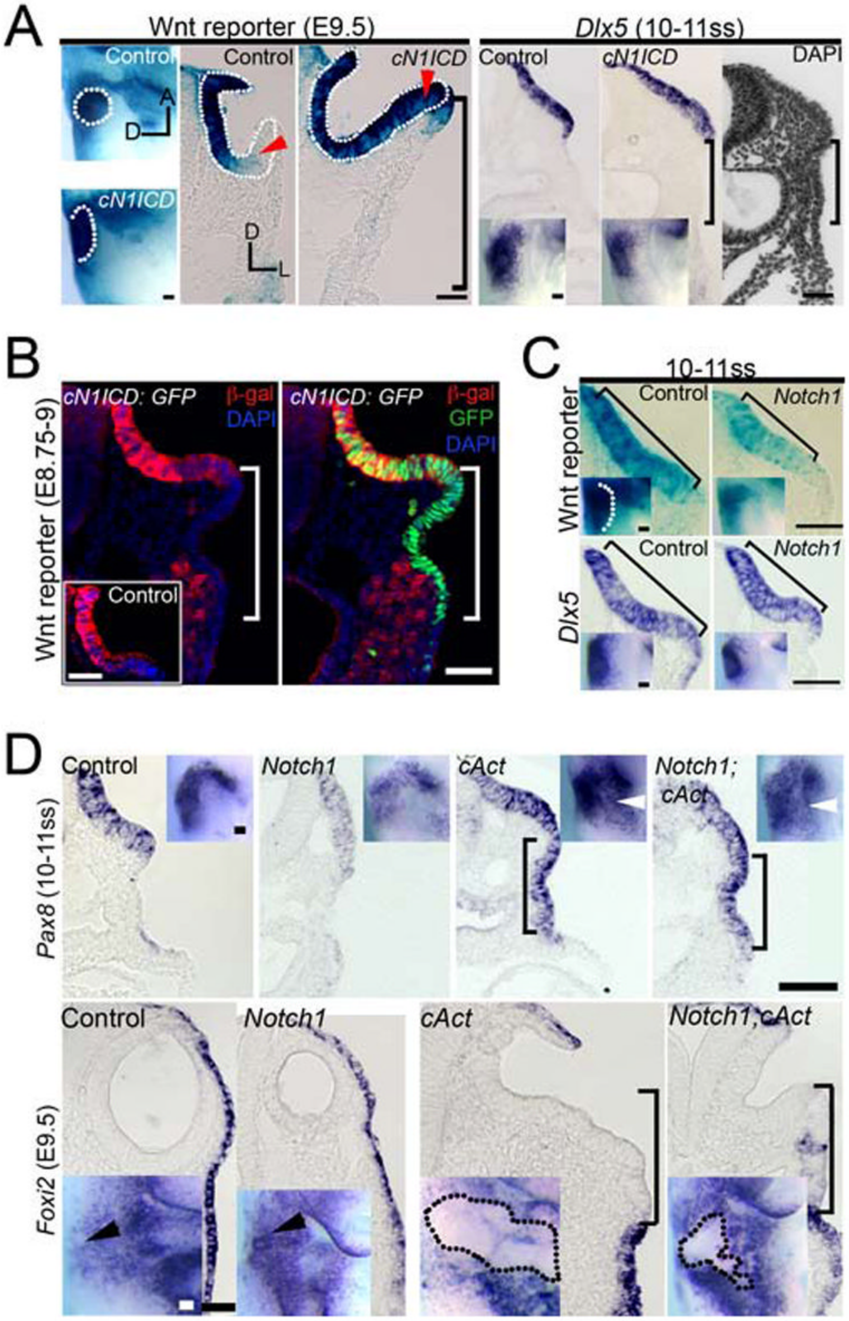

Fig. 6. Notch1 signaling augments Wnt signaling in the otic placode

(A) Wnt reporter and *Dlx5* mRNA expression is increased in conditionally over-expressing Notch-ICD (cN1ICD) embryos in whole-mount (top row) and corresponding mid-placode transverse sections (bottom row). Dotted outline: otic cup. Red arrowhead: medio-lateral otic region. (B) Wnt reporter expression in transverse sections of control and *cN1ICD;* Wnt reporter embryos; Wnt reporter mice co-immunostained with anti-β-galactosidase (β-gal; red) and anti-GFP (green) antibodies. Note that only the medial part of the expanded placode expresses the Wnt reporter. The inset shows anti-β-galactosidase staining in a normal Wnt reporter mouse. (A–B) Bracket: ectopic lateral placode region is negative for Wnt reporter and *Dlx5*. (C) Wnt reporter and *Dlx5* expression is diminished in *Notch1* mutants relative to controls. Brackets:

otic placode. (D) A comparison of *Pax8* and *Foxi2* expression in *Notch1* mutant, *cAct* and *Notch1; cAct* double mutant littermates. (A, C, D) Arrowhead: otic expression. Scale: 50 μm.

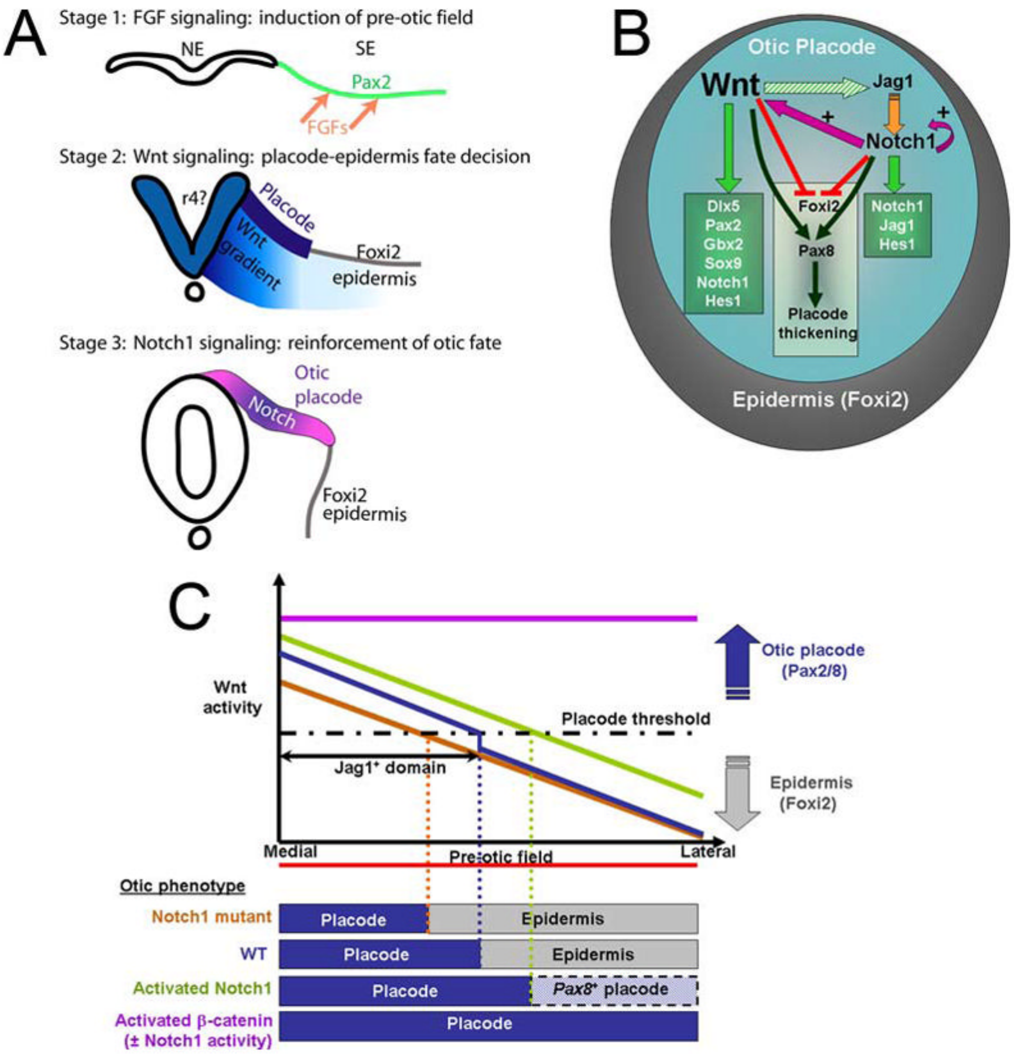

Fig. 7. Model of how Wnt and Notch pathways interact to regulate the size of the otic placode

(A) The generation of the otic placode can be divided into three stages. *Pax2* in the pre-otic field is induced by FGFs (arrows). A gradient of Wnts (light blue) determines the size of the otic field; above a certain threshold, Wnts drive cells towards an otic fate (dark blue) and below the threshold, cranial epidermis is formed (Foxi2) (Ohyama and Groves, 2003). Notch1 signaling is superimposed on the Wnt gradient (pink-blue) and acts to augment otic fate imposed by Wnts. NE; Neuroectoderm, SE; Surface ectoderm (this paper). (B) The Wnt pathway is the primary signal (denoted by bold lettering) that controls otic fate (blue region) by positively regulating (green arrows) the expression of *Dlx5, Sox9, Gbx2, Pax2/8* and components of the Notch1 pathway such as *Notch1* and *Hes1* (Figs. 1 and 2). *Jag1* expression is initiated by Wnts (dashed green arrow) (Fig. 2). Notch1 acts to: (1) augment Wnt and Notch1 activity within otic cells (pink arrow; plus sign) and (2) co-operate with Wnt to negatively regulate *Foxi2* (red) and positively regulate *Pax8* (dark green) and maintain a thickened otic placode. (C) A model summarizing the various otic placode phenotypes observed in this study. A gradient of Wnt activity emanating from the midline is established across the medio-lateral axis of the pre-otic field. Cells exposed to a certain threshold of Wnt signals express *Jag1* and differentiate as otic placode (blue). Below this threshold, cells differentiate as epidermis (grey). Jag1-Notch1 signaling augments Wnt signals in the medial region of the otic placode, whereas more lateral regions are not exposed to Notch1 signals and Wnt signaling is not augmented. In the absence of Notch1 (yellow line), the gradient of Wnt signaling becomes weaker, resulting in a smaller placode and more epidermis. When Notch1 is activated in the pre-otic field (green line), the Wnt gradient is augmented further. Some Wnt-dependent markers (*Dlx5*) are expressed only in the expanded Wnt domain, whereas markers such as *Pax8* are expressed throughout the pre-otic field (marked as lateral placode). When β-catenin is activated in the entire pre-otic field (purple line), all cells differentiate as otic placode (Ohyama et al., 2006).