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# Notch-mediated lateral induction is necessary to maintain vestibular prosensory identity during inner ear development

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## Abstract

The five vestibular organs of the inner ear derive from patches of prosensory cells that express the transcription factor SOX2 and the Notch ligand JAG1. Previous work suggests that JAG1-mediated Notch signaling is both necessary and sufficient for prosensory formation and that the separation of developing prosensory patches is regulated by LMX1a, which antagonizes Notch signaling. We used an inner ear-specific deletion of the *Rbpjκ* gene in which Notch signaling is progressively lost from the inner ear to show that Notch signaling, is continuously required for the maintenance of prosensory fate. Loss of Notch signaling in prosensory patches causes them to shrink and ultimately disappear. We show this loss of prosensory fate is not due to cell death, but rather to the conversion of prosensory tissue into non-sensory tissue that expresses LMX1a. Notch signaling is therefore likely to stabilize, rather than induce prosensory fate.

### Keywords

Notch; Inner ear; Vestibular; Otic; Sensory; Development

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Appendix A. Supplementary data

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## 1. Introduction

The vertebrate inner ear evolved to detect sound, linear and angular acceleration. It does so with dedicated patches of mechanosensory hair cells and accessory supporting cells (Basch et al., 2016; Powles-Glover and Maconochie, 2018). Although the number and type of these sensory organs have been modified across the course of evolution (Beisel et al., 2005; Duncan and Fritzsch, 2012; Fritzsch and Elliott, 2017), the inner ears of all modern vertebrates contain between three and nine sensory patches – cristae, which detect angular acceleration, maculae, which detect linear acceleration (and in some specialized cases, magnetic fields (Wu and Dickman, 2012, 2011)), and a sensory papilla to detect sound, which in mammals has elaborated into the organ of Corti (Manley, 2017, 2012). The progenitors for each sensory organ arise in distinct regions of the developing inner ear at a stage where it is still essentially a sphere of cells (Groves and Fekete, 2012; Raft and Groves, 2015), and subsequently differentiate into their constituent hair cells and supporting cells. It is the mechanism of how these prosensory regions are established that we are concerned with in the present study.

Prosensory patches of the inner ear are characterized by their expression of the SOX2 transcription factor and the Notch ligand JAG1 (Alsina et al., 2009; Kiernan et al., 2001, 2005b; Neves et al., 2011). Both of the these genes are necessary for the differentiation of sensory patches: severe ear-specific hypomorphic mutations of *Sox2* in the *Lcc* mouse line cause an almost total absence of sensory organs, while an inner ear-specific deletion of *Jag1* causes a loss or severe reduction in many of the inner ear's prosensory patches (Brooker et al., 2006; Kiernan et al., 2006, 2001). These genes are also able to induce sensory fate when over-expressed in non-sensory regions of the inner ear, although *Jag1* is far more potent in this regard than *Sox2* (Neves et al., 2011; Pan et al., 2013). The action of Jag1 in inducing prosensory tissue has been termed *lateral induction* – a cell expressing *Jag1* and *Sox2* is able to induce the expression of *Jag1* and *Sox2* in neighboring cells in a Notch-dependent manner (Daudet and Lewis, 2005; Hartman et al., 2010; Neves et al., 2013; Pan et al., 2010). This mode of Notch signaling by JAG1 is distinct from the more familiar mode of *lateral inhibition*, where a cell expressing Notch ligands inhibits its neighbors from adopting the same fate (Brooker et al., 2006; Daudet and Lewis, 2005; Eddison et al., 2000; Haddon et al., 1998). This difference in signaling modality is thought to be at least partly contingent on the signaling strength of different Notch ligands (Delta versus Jagged) engaging a given Notch receptor (Petrovic et al., 2014).

Although JAG1-mediated lateral induction was originally proposed as the primary signaling mechanism that induces inner ear prosensory tissue, several lines of evidence necessitate a revision of this idea. Expression studies show that both JAG1 and SOX2 are initially expressed very broadly in the early inner ear and only later become refined to distinct patches (Adam et al., 1998; Gu et al., 2016; Hartman et al., 2010; Morrison et al., 1999; Pan et al., 2010) This has been confirmed recently by two independent lineage tracing studies that clearly demonstrate that SOX2 is initially expressed in the progenitors of both sensory *and* non-sensory regions of the inner ear and only later becomes restricted to the future sensory regions (Gu et al., 2016; Steevens et al., 2019). Moreover, the initial expression of SOX2 and JAG1appears to be regulated by signaling pathways other than Notch, such as

Wnt signaling (Ambler and Watt, 2007; Estrach et al., 2006; Jayasena et al., 2008). Finally, during the restriction of prosensory patches to particular regions of the ear, large regions of prosensory tissue are gradually sculpted and refined to form smaller individual patches (Bok et al., 2007; Kiernan, 2013; Mann et al., 2017; Morsli et al., 1998; Wu and Oh, 1996). This process is mediated in part by mutually antagonistic interactions between the Notch signaling pathway and the LMX1A transcription factor (Koo et al., 2009; Mann et al., 2017; Nichols et al., 2008). Over-expression of *Lmx1a* can attenuate Notch signaling, and ectopic activation of the Notch pathway can cause a down-regulation of *Lmx1a* from non-sensory regions of the ear (Mann et al., 2017). These observations suggest a model in which Notch signaling does not actively *induce* the prosensory patches, but rather *stabilizes* them in particular locations, whilst adjacent regions of the ear lose their prosensory identity through inhibition of *Lmx1a* (Mann et al., 2017).

In the present study, we tested this model by conditionally deleting *Rbpjκ*, a co-activator of the canonical Notch pathway (Kopan and Ilagan, 2009), after the initial induction of the inner ear. This allowed us to evaluate the effects of a loss of Notch signaling on extant prosensory patches. We show that all vestibular prosensory regions of the ear initially form, but rapidly disappear over a period of about 48 h as Notch signaling is lost. We show that these regions do not die, but rather appear to convert to non-sensory tissue expressing Lmx1a. We conditionally inactivated Jag1 in sensory regions to show it is the principal ligand in this stabilization process. We also show that Jag1 likely signals through multiple different Notch receptors to stabilize prosensory fate, as the vestibular system of Notch1 conditional mice develop essentially normally.

## 2. Results

### 2.1. Loss of Notch signaling by Rbpjκ deletion causes severe vestibular truncations

To examine the morphological effects of the loss of Notch signaling on early inner ear development, we used *Rbpjκ* conditional knockout mice (cKO (Han et al., 2002); to generate a complete loss of canonical Notch signaling in the inner ear. We used Pax2-Cre mice to delete the *Rbpjκ* allele, as they provide one of the earliest and most complete recombination events in the inner ear (Basch et al., 2011; Ohyama and Groves, 2004). We bred male Pax2-Cre; $Rbpj\kappa^{\pm}$ mice to $Rbpj\kappa^{flox/flox}$ female mice and collected embryos at embryonic day (E) 10.5, 11.5, 12.5, and 13.5. Paint filling (Kiernan, 2006) was performed on the inner ears of wild-type controls and their cKO littermates to observe the morphology of the inner ear at these stages. We found that at E10.5 and E11.5, *Rbpjκ* cKO otocysts appeared morphologically similar to those of their WT littermates, with no significant differences in size or shape of the otocyst (Fig. 1A). However, at E12.5, we noticed morphological differences between cKO and WT cKO inner ears, especially in the vestibular system. E12.5 *Rbpjκ* cKO inner ears showed variable truncations of the anterior and/or posterior semicircular canals as they differentiated from the epithelial canal plates. We never observed such truncations in wild-type control embryos (Fig. 1A). At E13.5, the *Rbpjκ* cKO truncations were even more apparent in the anterior and/or posterior semicircular canals, as well as in the lateral semicircular canal. The utricle and saccule also began to show a

reduction in size in the *Rbpjκ* cKO inner ear and were completely absent in some embryos (n =3).

The Notch1 receptor is known to play roles at multiple stages during inner ear development; it helps establish the size of the otic placode during inner ear induction (Jayasena et al., 2008), and it is necessary to regulate the correct proportion of differentiating hair cells and supporting cells by a process of lateral inhibition (Brooker et al., 2006; Daudet and Lewis, 2005; Eddison et al., 2000; Haddon et al., 1998). However, both Notch2 and Notch3 are known to be expressed in the developing otocyst at low levels (Lindsell et al., 1996). To determine if the defects in our *Rbpjκ* cKO mice were caused through a lack of signaling exclusively through the Notch1 receptor, we analyzed paint fills of the inner ears of mice in which Notch1 has been conditionally deleted with Foxg1-Cre mice (Kiernan et al., 2005a). Surprisingly, we found that unlike the vestibular system defects seen in *Rbpjκ* cKO, Notch1 cKO mutants did not demonstrate any obvious morphological vestibular defects. This suggests that additional Notch receptors are likely used during the early morphological development of the inner ear. Indeed, when we examined expression of Notch1, 2 and 3 in the otocyst at E9.5, we detected transcripts for all three genes (Supplementary Fig. 1). We found that at E9.5, all three Notch receptors are expressed in the otic epithelium. Interestingly, Notch1 and Notch2 appeared to have a differential expression pattern, with Notch1 more strongly expressed in the ventral otocyst and Notch2 more strongly expressed dorsally. Notch3 was expressed throughout the otocyst at low levels. This may account for the lack of obvious vestibular defects in the Notch1 cKO animals and emphasizes the need to look at the canonical loss of all Notch signaling in otic development.

Although the Pax2-Cre mice used to delete *Rbpjκ* from the ear begin to show recombination as early as E8.5 (Ohyama and Groves, 2004), it is likely that some residual RBPJ protein persists after inactivation of the *Rbpjκ* gene. To test this, we examined the otocysts of E9.5 *Rbpjκ* cKO and wild-type control embryos for the presence of RBPJk protein. We found that by E9.5 there was a clear loss of RBPJk protein in cKO otocysts compared to the surrounding mesenchyme and neural tube, as well as to wild-type littermates (Fig. 1C). This suggests that the Notch signaling pathway is initially active in the developing otocyst of *Rbpjκ* cKO embryos, but is almost completely lost by E9.5 at the latest. We therefore used our cKO embryos to study the consequences of a progressive loss of canonical Notch signaling occurring in the inner ear from E8.0 to E9.5.

## 3. Loss of Notch signaling results in a progressive loss or reduction of prosensory and neurosensory progenitors from the otocyst

The developing sensory patches of the ear are known to influence the formation of non-sensory tissue – for example, FGF and BMP signaling from the developing cristae regulate the outgrowth and formation of semicircular canals (Bok et al., 2007; Chang et al., 2004; Morsli et al., 1998; Wu and Oh, 1996). To determine if the progressive loss of semicircular canal tissue in *Rbpjκ* cKO embryos is due to defects in cristae, we examined sections of *Rbpjκ* cKO embryos for an early marker of cristae, *Bmp4* (Morsli et al., 1998). Starting at E10.5, BMP4 is expressed strongly in the anterior, lateral and posterior cristae prosensory

domains prior to hair cell differentiation and canal plate outgrowth (Fig. 2A). At E10.5, *Bmp4* expression was reduced or absent in the prospective crista regions of *Rbpjκ* cKO otocysts vestibular prosensory domains compared to wild-type controls. At E11.5, *Bmp4* was robustly expressed in all three vestibular cristae prosensory domains of wild-type embryos, but was almost completely absent in *Rbpjκ* cKO embryos (Fig. 2A). BMP4 is also expressed on the abneural side of the developing cochlear duct from a very early age (Morsli et al., 1998; Ohyama et al., 2010), but this expression is not regulated by Notch signaling (Basch et al., 2011). Accordingly, we still observed a small patch of *Bmp4* expression in the ventro-medial region of E10.5 and E11.5 otocysts in both wild-type and *Rbpjκ* cKO embryos (Fig. 2A).

The sensory epithelium of the utricular and saccular maculae develop from an antero-ventral region of the otocyst that also generates neurons of the cochleovestibular ganglion, a region known as the neurosensory domain (Basch et al., 2016; Raft et al., 2007). This neurosensory region expresses the Notch-modifying glycosyltransferase *Lfng* from E9.0 onwards (Burton et al., 2004; Morsli et al., 1998). We examined *Lfng* expression in the otocyst of wild-type and *Rbpjκ* cKO embryos between E9.5 and E11.5. At E9.5 we saw no significant difference in *Lfng* expression between wild-type and *Rbpjκ* cKO otocysts (Fig. 2B), but by E10.5 we began to see a marked reduction in the size and intensity of *Lfng* expression in the neurosensory domain. By E11.5, we could detect no *Lfng* expression in this region in *Rbpjκ* cKO embryos (Fig. 2B). Together, these observations suggest that a loss of Notch signaling in the otocyst between E8.5 and E9.5 causes a reduction and then a loss of developing prosensory regions destined to form the cristae and maculae.

### 3.1. Progressive loss of Notch signaling from the otocyst does not significantly affect neurogenesis in the VIII/VIIth ganglion

Notch-mediated lateral inhibition plays a key role during neurogenesis of the VIIIth ganglion from the early developing otocyst (Adam et al., 1998; Haddon et al., 1998; Raft et al., 2007, 2004) and other cranial ganglia (Lassiter et al., 2014). The anterior-ventral region of the otocyst labeled by *Neurogenin1* and *Lfng* uses Notch-mediated lateral inhibition to determine which cells will delaminate from the otic tissue and form the neural precursors of the VIIIth ganglion. Accordingly, disruption of Notch signaling leads to the production of excess neurons and a larger ganglion (Haddon et al., 1998; Ma et al., 1998; Raft et al., 2007). Neurogenesis in the neurosensory domain is gradually replaced by the generation of progenitors for the utricular and saccular maculae (Raft et al., 2007, 2004; Raft and Groves, 2015). Since this neuronal-sensory transition occurs as RBPJk is disappearing from the otocyst in our *Rbpjκ* cKO embryos, we predicted that any neurogenic phenotype in our mutant embryos would be less severe than that seen in null mutants of the Notch signaling pathway.

We measured the size of the VIII/VIIth ganglia in E9.5 *Rbpjκ* cKO and wild-type embryos by staining the ganglion with TuJ1 antibodies that label a neuron-specific tubulin (TUBB3; Fig. 3). At this stage, the vestibulo-acoustic and geniculate ganglia are still in close contact and appear as a single mass. We cleared the whole-mount stained embryos using the Sca*l*e clearing protocol (Hama et al., 2011) and collected images on a Zeiss LightSheet Z.1

microscope (see Supplementary Video 1 for a visualization of the rendering process). 3D reconstruction and rendering of the ganglion using the Imaris image analysis package allowed us to calculate the volume of the ganglion of *Rbpjκ* cKO and wild-type embryos. We saw no significant differences in the size of the *Rbpjκ* cKO ganglia at E9.5 (Fig. 3A and B), not at E10.5 (not shown), confirming that although loss of Notch signaling in our mutant embryos affects the development of the vestibular prosensory patches, it is likely occurring too late to significantly affect neurogenesis from the neurosensory domain of the otocyst and the geniculate placode.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ydbio.2020.02.015

### 3.2. Loss of Notch-mediated lateral induction results in a failure to maintain prosensory progenitor identity

Our data suggest that a loss of RBPJk protein from the developing otocyst leads to a reduction and then a loss of prosensory and neurosensory patches. These progenitor domains exhibit a mode of Notch signaling known as lateral induction (Brooker et al., 2006; Daudet et al., 2007; Daudet and Lewis, 2005; Neves et al., 2013; Petrovic et al., 2014). Lateral induction establishes a positive feedback loop among prosensory progenitors, as distinct from the negative feedback loop of Notch-mediated lateral inhibition and is uniquely facilitated by Jagged family Notch ligands (Neves et al., 2013, 2011). While the full set of targets in the lateral inductive process is not yet known, *Sox2* is induced in response to lateral inductive signals, and *Sox2*, like *Jag1*, is sufficient to induce prosensory tissue when ectopically expressed (Kiernan, 2013; Neves et al., 2011; Pan et al., 2013, 2010). We next examined the consequences of a loss of RBPJk protein on these two elements of lateral inductive signaling in the otocyst.

We examined JAG1 and SOX2 expression in *Rbpjκ* cKO embryos by immunostaining adjacent serial cryosections (Fig. 4). Consistent with our previous results showing a normally-sized neurosensory domain at E9.5, we saw no significant difference in the expression of JAG1 or SOX2 in the *Rbpjκ* cKO otocysts at that age compared to wild-type controls (Fig. 4). However, starting at E10.5 and continuing at E11.5, neither JAG1 nor SOX2 protein could be observed in the prosensory and neurosensory regions of the *Rbpjκ* cKO otocyst (Fig. 4). We were still able to see JAG1- and SOX2-expressing cells in the ventral-most regions of the otocyst associated with the developing cochlear anlagen, which develops independently of *Rbpjκ* (Basch et al., 2011). Taken together, these data suggest that the lateral inductive feedback loop can be broken by a reduction and then loss of Notch signaling, and that further suggests that despite early expression of prosensory and lateral induction markers, loss of Notch signaling and lateral induction results in a loss of prosensory character.

We noted that the normally thickened epithelial SOX2-and JAG1-expressing prosensory regions in wild-type embryos showed significant epithelial thinning in *Rbpjκ* cKO embryos (Fig. 4A and B). To determine whether this loss of epithelial thickening and prosensory character is simply due to defects in proliferation or to excessive cell death, we examined E10.5 *Rbpjκ* cKO serial cryosections for markers of proliferation and apoptosis.

Immunostaining for activated caspase-3 showed no differences between *Rbpjκ* cKO embryos and their wild type littermates (Fig. 5A). To quantify cell proliferation, we performed immunostaining for phosphor-histone H3 (pH3) on wild-type and *Rbpjκ* cKO otocysts (Fig. 5B). We divided each otocyst into four bins along the anterior-posterior axis and the average number of cells per section was calculated for each bin. We did not find any statistically significant differences between the wild-type and *Rbpjκ* cKO otocysts for any of the four bins, nor across the entire otocyst (Fig. 5C). This suggests that the epithelial thinning we observed may reflect a non-sensory character of the sort characterized by LMX1A expression that forms between developing sensory patches in mouse and chick (Mann et al., 2017).

### 3.3. The loss of prosensory identity after loss of Notch signaling results in a transformation to non-sensory tissue

Our results indicate that loss of RBPJK from the developing otocyst causes a reduction and then a loss in prosensory patches and in both JAG1 and SOX2 that are required for prosensory identity. We next tested whether the reduction of prosensory identity in otocyst was accompanied by an expansion of non-sensory identity. LMX1A is a transcription factor that has previously been demonstrated to be expressed in the non-sensory epithelium of the otocyst and is necessary for the correct segregation of prosensory patches in the otic epithelium (Koo et al., 2009; Mann et al., 2017; Nichols et al., 2008). We examined adjacent serial sections for SOX2 and LMX1A in *Rbpjκ* cKO and wild-type littermates at E10.5 and E11.5. As previously reported, SOX2 was expressed in the vestibular prosensory domains as in a complementary fashion to LMX1A, which is expressed in the surrounding non-sensory epithelium (Fig. 6A and B). At E10.5, *Rbpjκ* cKO otocysts lack SOX2 expression in vestibular prosensory patches. Instead, this thin epithelial territory expressed LMX1A. This spread of non-sensory LMX1A expression into regions normally expressing SOX2 was even more apparent at E11.5 (Fig. 6A and B). These results suggest that as lateral induction is diminished and then abolished in the otocyst of *Rbpjκ* cKO embryos, the tissue adopts a non-sensory fate.

To confirm that this prosensory to non-sensory transformation was due to a loss of lateral induction mediated by JAG1, we conditionally deleted *Jag1* in prosensory regions of the otocyst. We used *Jag1* conditional mice (Arnold et al., 2011; Kiernan et al., 2006) that carried a *ROSA Ai9* Cre reporter allele to both conditionally delete *Jag1* and mark cells undergoing Cre-mediated recombination with TdTomato. We used a *Sox2-CreER* allele to delete *Jag1* by administering tamoxifen at E8.5 or E9.5 (Fig. 7A and B). We have previously shown that this treatment leads to detectable recombination after 12 h and robust recombination after 24 h (Gu et al., 2016), occurring primarily in the lateral half of the otocyst (Fig. 7A). We examined *Jag1* cKO mutant embryos for expression of prosensory and non-sensory markers at E10.5 or E11.5, using the TdTomato Cre reporter to mark mutant cells. In control embryos, SOX2 and LMX1A expression was complementary in the otocyst (Fig. 7C and D). However, deletion of *Jag1* from the otocyst when tamoxifen was given at E8.5 resulted in reduced and patchy SOX2 expression and epithelial thinning in prosensory regions at E10.5, and an expansion of LMX1A expression into these regions (Fig. 7C). When tamoxifen was given at E9.5 and the mice analyzed at E11.5, we again saw a

significant reduction in SOX2 expression and saw evidence of LMX1A beginning to encroach into the former prosensory region. These results confirm that JAG1-mediated lateral induction is necessary to maintain prosensory identity in the otocyst, and that in its absence, prosensory regions transform to a non-sensory fate.

## 4. Discussion

As the vertebrate lineage evolved and diversified, the inner ear became more morphologically complex and added additional sensory organs to the anterior and posterior cristae and a central macula seen in agnathans (Beisel et al., 2005; Duncan and Fritzsch, 2012; Fritzsch and Elliott, 2017). These extra mechanosensitive organs evolved to convey more sophisticated vestibular information, to detect sound and even magnetic fields. The inner ear epithelium thus serves as a canvas on which extrinsic signals and cell-cell interactions allow the temporally and spatially regulated emergence of distinct sensory organs. In the present study, we show that JAG1 signaling, likely transduced by multiple Notch receptors, is absolutely required to maintain the identity of developing vestibular sensory organs in the mouse inner ear. We show that there is an ongoing equilibrium between prosensory and non-sensory fate in the ear, and that temporal inactivation of Notch signaling expands non-sensory identity at the expense of prosensory patches.

### 4.1. Notch signaling is active in the early ear, but does not specifically mark prosensory progenitors

The mouse inner ear arises from the otic placode, the first traces of which can be observed as a patch of *Pax2*-expressing cells, at approximately 8 days of gestation, concomitant with the appearance of the first pair of somites (Birol et al., 2015). Several hours later, at the 4–5 stage, *Notch1, Hes1* and *Jag1* mRNA, together with JAG1 protein, can be observed in a subset of *Pax2*-expressing cells, and by embryonic day 8.5, these Notch pathway components are expressed throughout the otic placode (Jayasena et al., 2008). The induction of these genes is regulated by canonical Wnt signaling from the hindbrain (Jayasena et al., 2008; Ohyama et al., 2006). Constitutive activation of β-catenin in the otic anlagen causes an expansion of *Notch1, Hes1* and *Jag1* (Jayasena et al., 2008), and previous work has shown *Jag1* to be a direct target of the canonical Wnt signaling pathway (Estrach et al., 2006). Although JAG1-NOTCH1 signaling subsequently feeds back to augment Wnt signaling in the otic placode (Jayasena et al., 2008), our previous studies and others examining null mutations of components of the core Notch pathway such as *Pofut1* (Shi and Stanley, 2003) and *Rbpjκ* (Oka et al., 1995) demonstrate that the initial expression of Notch pathway genes in the inner ear is regulated independently of Notch signaling itself and initiated instead by other signals, including the Wnt pathway.

By the time of otocyst formation around embryonic day 9, much of the mouse otocyst expresses JAG1 and SOX2 (Gu et al., 2016; Kiernan et al., 2005b; Lewis et al., 1998; Morrison et al., 1999), which will ultimately define prosensory patches, and *Lmx1a* (Koo et al., 2009) which ultimately defines non-sensory regions of the otocyst. Lineage tracing of *Sox2*-expressing progenitors at this age shows that large numbers of these cells form non-sensory tissue in addition to vestibular sensory organs (Gu et al., 2016; Steevens et al.,

2019). We predict that if similar experiments were carried out at this age with either *Jag1* or *Lmx1a* CreER lineage tracing mice, essentially the same result would be observed. Thus, at the time of otocyst formation, expression of *Lmx1a, Jag1* and *Sox2* do not distinguish between future prosensory and non-sensory populations of inner ear cells, and it is likely that all cells in this population are receiving at least some Notch signaling through Jag1-Notch interactions based on the expression of *Hes* family members at this stage (Abelló et al., 2007; Jayasena et al., 2008; Neves et al., 2013; Petrovic et al., 2014). It is possible that segregation of these two progenitor populations is already underway at this stage, but we currently have no biomarkers to differentiate between them. Indeed, the first indication of segregation of prosensory and non-sensory progenitors is seen at about E9.0, where some cells in the antero-ventral neurogenic domain of the otocyst that will give rise to the cochleovestibular ganglion and the utricular and saccular maculae begin to down-regulate *Lmx1a* and express *Lfng* (Koo et al., 2009). Shortly after this, LMX1A is down-regulated from the anterior and posterior poles of the otocyst, and JAG1 becomes expressed more strongly in these regions (Mann et al., 2017).

The observation that SOX2 is initially expressed in progenitors of sensory and non-sensory tissue, and that is necessary for the formation of both populations (Steevens et al., 2019) suggests that the segregation of prosensory and non-sensory regions of the otocyst is likely to be accompanied by a large-scale epigenetic remodeling of progenitor cells, with the transcriptional targets of SOX2 changing dramatically as this segregation occurs. Although SOX2 targets have been well-characterized in other tissues and pluripotent cell populations, identification of direct SOX2 targets in the otocyst has not been possible until relatively recently. The recent advances in performing ChIP- and ATAC-seq measurements of transcription factor binding and chromatin accessibility with small numbers of cells will be able to provide a better mechanistic underpinning of the segregation of sensory and non-sensory populations in the otocyst.

### 4.2. Notch signaling maintains, rather than induces prosensory fate in the inner ear by repressing Lmx1a

The data described above, together with our data reported here, suggest that Notch signaling is not used in the otocyst to induce prosensory fate. Instead, a large domain of cells expressing JAG1, SOX2 and LMX1A established early in the otic placode and otocyst gradually segregate into non-sensory LMX1A$^+$ cells and prosensory JAG1$^+$, SOX2$^+$ cells. In this model, Notch signaling is used to stabilize prosensory fate in particular locations. By using conditional knockout strategies that remove RBPJk or JAG1 protein from the otocyst after ear induction, we were able to show that prosensory regions gradually disappear and are replaced by non-sensory tissue expressing LMX1A over a period of several days (Fig. 6). Mann and colleagues have shown convincingly that this happens in a less exaggerated form in wild type mice and birds, where the segregation of large prosensory regions into smaller individual organs correlates with the appearance of LMX1A between these organs (Mann et al., 2017). We observed this prosensory-to-nonsensory conversion in the progenitors for all three cristae and both maculae (Fig. 2), and this conversion was presaged by a loss of SOX2 and JAG1 from each prosensory domain. We have previously reported that canonical Notch signaling is not necessary for the formation of the prosensory domain that gives rise to the

organ of Corti (Basch et al., 2011), and in the present study we confirmed that both prosensory (SOX2) and non-sensory (*Bmp4*, JAG1) markers of the cochlear duct primordium continued to be expressed in their normal location in the ventral otocyst.

Our results support an emerging model where antagonistic interactions between the Notch signaling pathway and LMX1A drive the segregation of prosensory and non-sensory tissue. Our evidence presented here, together with evidence from other groups show that the otocyst continues to experience these antagonistic interactions for at least several days after the segregation of prosensory patches. First, loss of RBPJk activity or JAG1 signaling cause prosensory patches to convert to $Lmx1a^{+}$, non-sensory regions over several days (Fig. 6). Second, ectopic expression of either *Jag1, Dll1* or the active intracellular domain of the Notch1 receptor (N1ICD) in non-sensory tissue that has already down-regulated JAG1 and SOX2 can lead to the re-establishment of prosensory fate (Hartman et al., 2010; Neves et al., 2011; Pan et al., 2013), the appearance of ectopic patches of hair cells and supporting cells (Pan et al., 2013), and the down-regulation of LMX1A (Mann et al., 2017). At present, the lack of known transcriptional targets of SOX2 means that it is not clear whether regions of non-sensory tissue that formerly expressed Sox2 are somehow primed to adopt a prosensory fate upon ectopic activation of Notch signaling. Third, ectopic activation of Notch signaling leads to an expansion of prosensory regions at the expense of LMX1A (Mann et al., 2017); our results now show that a progressive loss of Notch signaling has the opposite effect. Similarly, *Lmx1a* mutant mice fail to correctly segregate their sensory organs (Koo et al., 2009; Nichols et al., 2008; Mann et al., 2017), and ectopic expression of *Lmx1b* in chicken embryos causes diminution of sensory patches (Mann et al., 2017). Fate conversion of non-sensory epithelium to sensory tissue in Notch gain-of-function mice can be initiated in some parts of the ear as late as E14.5 (Pan et al., 2013). Determining for how long Notch signaling is necessary to stabilize the developing prosensory organs, will require conditional deletion of *Rbpjk* or *Jag1* at progressively later stages. It is nevertheless of interest that Mann and colleagues were able to identify $LMX1A^{+}$ cells abutting SOX2-expressing cells at the margin of the utricular macula in six-week-old mice, suggesting that elements of this antagonistic mechanism may persist in at least some regions of the ear in adult life.

The mechanisms underlying the mutual antagonism of Notch signaling and LMX1A expression are not clear. The most simple mechanism involves direct transcriptional repression: here, LMX1A would directly repress one or more components of the Notch pathway such as JAG1 or NOTCH1, and the NICD/RBPJk/MAML transcriptional complex or their downstream *Hes* or *Hey* gene targets would directly repress *Lmx1a*. Alternatively, either LMX1A or Notch signaling could cause inhibition by a less direct mechanism, such as initiating proteolytic degradation of antagonizing component. In either case, since prosensory identity is maintained by a JAG1-mediated positive inductive circuit, any factor that causes even a slight reduction in Notch signaling strength might be predicted to cause a rapid local loss of lateral induction and a de-repression of *Lmx1a*. However, although there is clearly evidence for local prosensory/non-sensory transformation at the margins of prosensory patches that involve a reduction in Notch signaling (Mann et al., 2017), some additional mechanism must act to maintain at least some Notch signaling to halt the continuing erosion of lateral induction that would otherwise be predicted to occur. FGF and BMP signaling are known to regulate both Notch pathway components and *Lmx1a* in the

otocyst (Abelló et al., 2010), although it is less clear how broad, diffusible signals emanating from within or outside the otocyst could lead to such finely-tuned and consistent sculpting of each prosensory/non-sensory interface. Moreover, as the number, type and location of inner ear sensory organs has varied considerably across vertebrate ear evolution (Fritzsch et al., 2013; Fritzsch and Elliott, 2017; Luo et al., 2011; Manley, 2000), it is hard to imagine how a small number of diffusible signals would be sufficient to establish such different arrangements of sensory organs in different species. It is also possible that once Notch signaling is activated in a prosensory region above a certain threshold, the tissue becomes immune to the encroachment of LMX1A. Such a model is hard to test experimentally due to difficulties in measuring precise levels of Notch signaling in vivo.

Our work demonstrates that Notch-mediated lateral induction is responsible for the maintenance of vestibular prosensory domains in the developing mouse otocyst through the Jag1 ligand and either independent of the Notch1 receptor or through multiple functionally redundant Notch receptors. This maintenance of prosensory character seem to be opposed by a non-sensory inducing signal that imposes a non-sensory identity as evidenced by the spread of LMX1A. Together with previous work, this suggests that the *Rbpjκ* cKO works as a model for the complete loss of Notch signaling from the point of vestibular prosensory domain development onward.

## 5. Materials and methods

### Mice:

For conditional inactivation of *Rbpjκ*, male Tg (Pax2-cre)$^{Akg1}$ (Ohyama and Groves, 2004) on an ICR background and heterozygous for the *Rbpjκ* null allele (*Rbpj*$^{tmHon1.1}$ (Han et al., 2002; Oka et al., 1995) were crossed to *Rbpj*$^{tmHon1}$ mice (Han et al., 2002) homozygous for the conditional RBPJ allele on an ICR background through timed matings. Mice were genotyped through PCR using the following primers: Cre = Cre1F (GCCTGCATTACCGGTCGATGCAACGA) and Cre1R (GTGGCAGATGGCGCGGCAACACCATT) produce a 700 bp band, RBPJ = RBPJ1 (AACATCCACAGCAGGCAA), RBPJ2 (GATAGACCTTGGTTTGTTTGG), and RBPJ3 (CCACTGTTGTGAACTGGCGTGG) produce a 500 bp floxed allele band, a 700 bp deleted allele band, and a 300 bp wild-type allele band. *Jag1* conditional knock out mice: Mice were generated by combining mice homozygous for *Jag1*$^{tm2Grind/tm2Grind}$ (Jax Stock # 010618 (Kiernan et al., 2006); and Gt (ROSA)26Sortm9(CAG-tdTomato)Hze (Jax Stock #007905) with Sox2$^{tm1(cre/ERT2)Hoch}$ (Arnold et al., 2011) heterozygous, Jag1$^{tm2Grind/tm2Grind}$ homozygous mice through timed matings. Mice were genotyped through PCR with the following primers: Cre = Cre1F (GCCTGCATTACCGGTCGATGCAACGA) and Cre1R (GTGGCAGATGGCGCGGCAACACCATT) produce a 700 bp band; Jag1 = Jag1F (GGCAACAAAACTTGCATGG) and Jag1R (GGGCACTAACA-GAATCTTCTACA) produce 220 bp WT and 250 bp mutant bands; tdTomato = tdTomwtF (AAGGGAGCTGCAGTGGAGTA), tdTomwtR (CCGAAAATCTGTGGGAAGTC), tdTomF (CTGTTCCTGTACGGCATGG), and tdTomR (GGCATTAAAGCAGCG TATCC) produce 196 bp WT and 297 bp mutant bands.

### Embryo collection:

After timed matings were set up, pregnant females were sacrificed when the embryos reached embryonic days 9.5, 10.5, 11.5, 12.5 and 13.5 and the litters collected, with tail clippings kept for genotyping purposes. The embryos were treated overnight in 4% paraformaldehyde at 4 °C and then washed in DEPC-treated PBS and prepared for use in immunofluorescence or RNA in situ hybridization.

### Tamoxifen Treatment:

Upon setting up timed matings for *Jag1* cKO mice, pregnant mothers were orally gavaged with a mixture of peanut oil and tamoxifen and progesterone. The tamoxifen and progesterone were each mixed at a concentration of 25 mg/ml. Gavaging was performed in the morning 8 days after successful plug detection using timed matings. The pregnant females were sacrificed either 10 or 11 days after plug detection, with tail clippings kept for genotyping and the embryos fixed in 4% paraformaldehyde then washed and stored in PBS at 4 °C until prepared for embedding and immunofluorescence.

### Paint filling:

*Rbpjκ* cKO mice were collected at E10.5, E11.5, E12.5 and E13.5 with tail clippings kept for genotyping. The embryos were treated and cleared according to the protocol described in (Kiernan, 2006). Cleared inner ears were injected with a solution of 0.1% white latex paint in methyl salicylate by hand using a Picospritzer III pressure injector.

### RNA *in situ* hybridization:

Embryos washed in DEPC-treated PBS the previous evening were washed in 0.1% Tween 20 in DEPC-treated PBS three times for 30 min and sunk overnight in 30% sucrose. Cryoprotected embryos were then sunk in OCT and frozen, after which they were stored at −80 °C until cryosectioned. Digoxygenin-labled RNA in situ hybridization probes were generated for *Bmp4*, and *Lfng*, using standard protocols. The in situ hybridization protocol used was as described in (Cai et al., 2015).

### RNAscope:

Embryos were washed, cryoprotected, embedded and sectioned as performed for standard RNA in situ hybridizations as mentioned above. Sections were then processed using the RNAscope Universal Pre-treatment kit and the RNAscope HD Detection RED kit from Advanced Cell Diagnostics according to the manufacturer’s instructions.

### Immunofluorescence:

Embryos washed in DEPC-treated PBS the previous evening were washed in 0.1% Tween 20 in DEPC-treated PBS three times for 30 min and sunk overnight in 30% sucrose. Cryoprotected embryos were then sunk in OCT and frozen, after which they were stored at −80 °C until cryosectioned. The primary antibodies used in this study were anti-activated caspase 3 (rabbit 1:200, R&D Systems AF835), anti-JAG1 (rabbit 1:75, Santa Cruz Biotechnology sc-8303), anti-phosphorylated histone 3 (rabbit 1:200, Cell Signaling Technology 9701S), anti-RBPJK (rat 1:100, Cosmo Bio 2ZRBP2), anti-SOX2 (rabbit 1:300,

EDM Millipore AB5603), Tuj1 (mouse 1:500, Covance LN# 14944102), and anti-LMX1A (rabbit 1:1000, EDM Millipore AB10533). The secondary antibodies used in this study were goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 594, and goat anti-mouse Alexa 488 from Invitrogen (1:1000). Embryos used for RBPJ staining were embedded in OCT and cryosectioned, then an Elite Rat IgG Vectastain ABC Kit from Vector Labs was used for avidin-biotin amplification of the anti-RBPJ antibody staining. A TSA-Plus Fluorescein System Kit from PerkinElmer was used to develop the slides after avidin-biotin amplification.

### Ganglion size calculation:

E9.5 embryos stained in whole mount with the Tuj1 antibody as described above were processed using the Sca*l*e A2/B2 clearing protocol (Hama et al., 2011). After clearing, embryos were then mounted in low-melting point agarose and suspended in columns for imaging using a Zeiss LightSheet Z.1 microscope courtesy of the Baylor College of Medicine OViM microscopy core. The resultant images were then processed in Imaris to create a 3D rendering and the VIIIth ganglion was then isolated and converted to a surface map. The volume of the VIIIth ganglion was then calculated from the surface map. The volumes were then statistically analyzed using a two-tailed Student's t-test for unequal variance.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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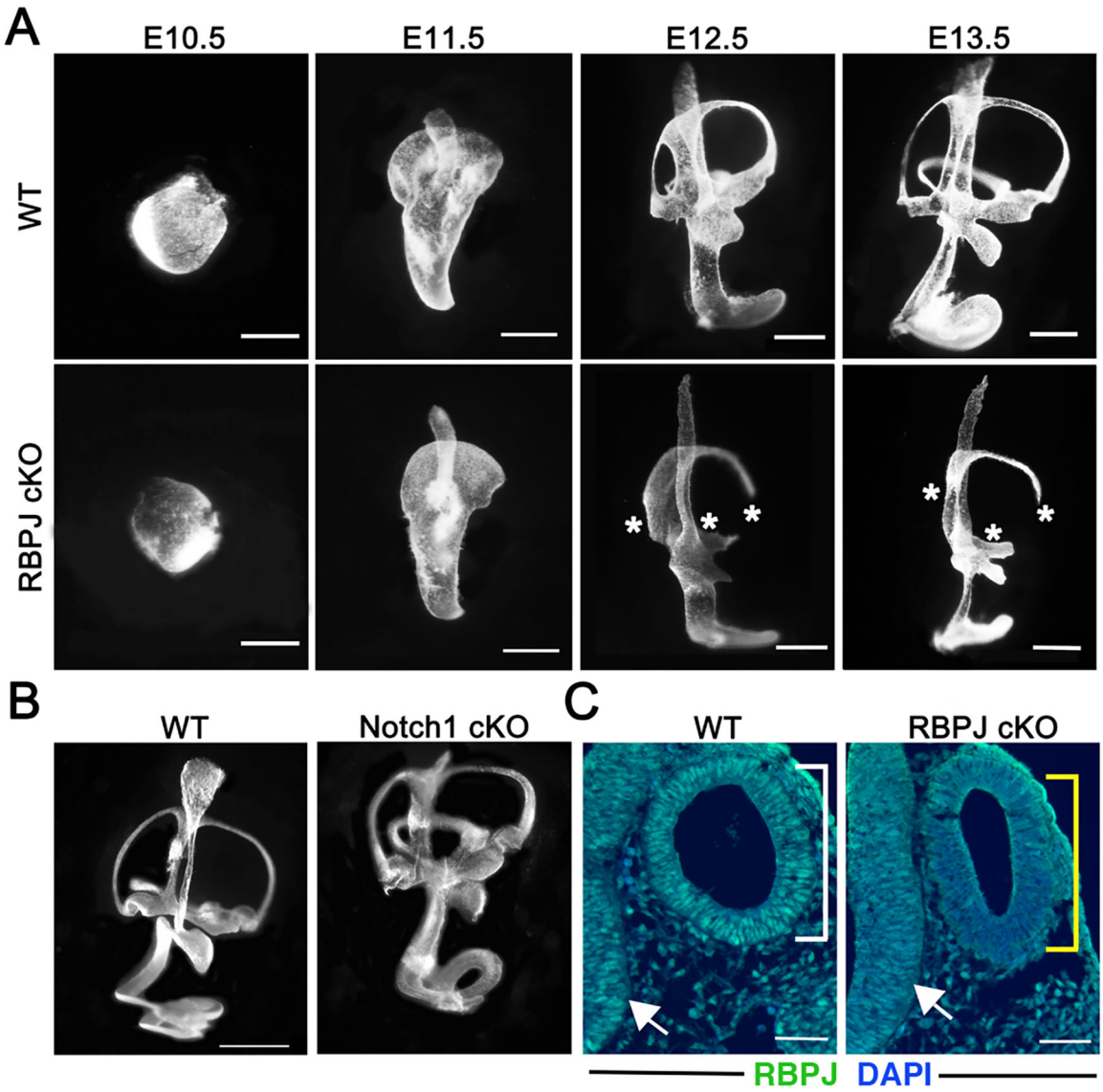


**Fig. 1. The complete loss of Notch signaling via the deletion of *Rbpjκ* causes severe truncations of the vestibular system.**

**A:** Paint-filling series of *Rbpjκ* cKO inner ears and their WT littermates from E10.5 to E13.5. Asterisks denote truncations of the semicircular canals of the vestibular system. Scale bars = 125μm for E10.5 and 250μm for E11.5 - E13.5. **B:** Paint-fillings for E14.5 *Notch1* cKO inner ears and WT littermate. Scale bar = 350μm **C**: Immunofluorescent staining for RBPJk (green) and DAPI (blue) in E9.5 *Rbpjκ* cKO and WT otocysts. White bracket denotes otic epithelium with RBPJk expression present, yellow bracket denotes otic epithelium without RBPJk expression. White arrows indicate RBPJk-positive staining in the neural tube of *Rbpjκ* cKO and WT embryos. Scale bar = 50μm.

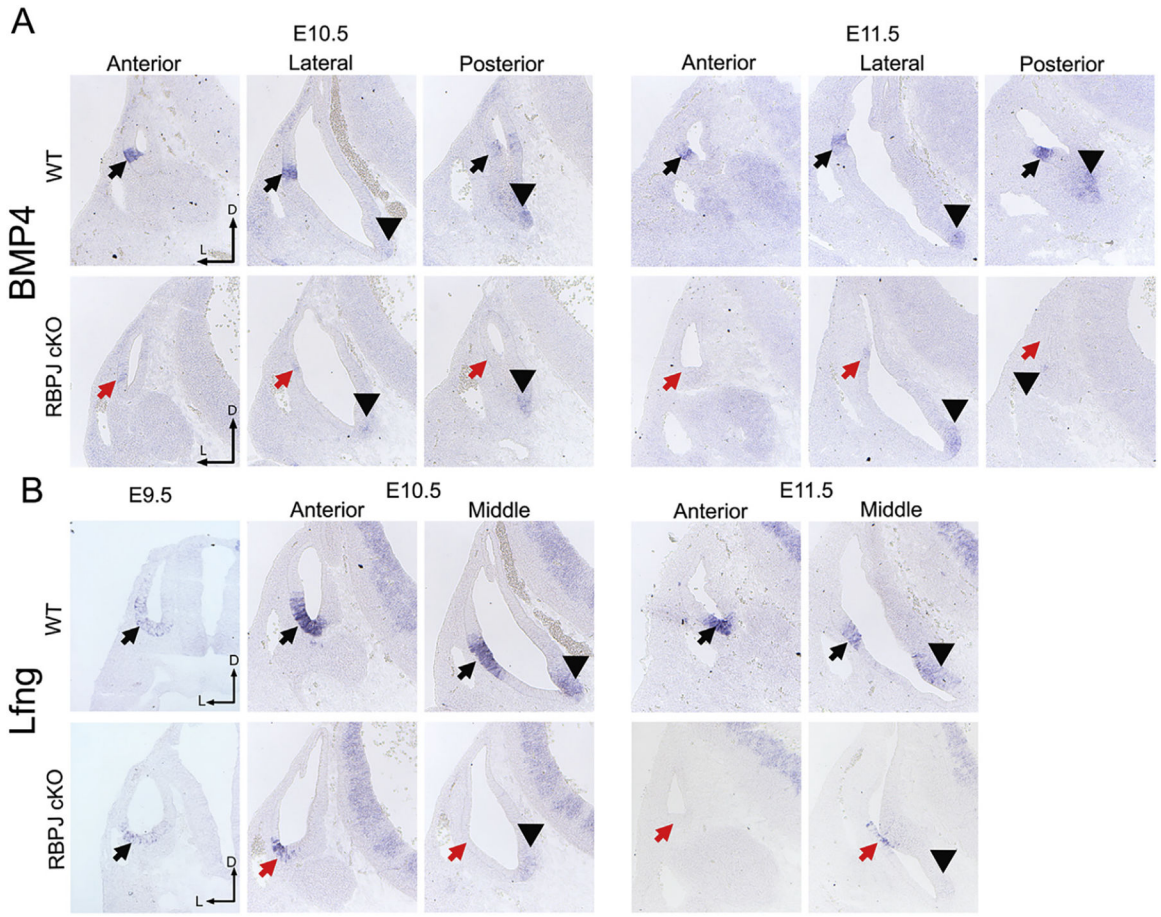


**Fig. 2. Loss of Notch signaling results in a reduction and eventual loss of prosensory markers for the cristae and maculae of the inner ear.**

**A:** RNA in situ hybridization for *Bmp4* in *Rbpjκ* cKO otocyst sections from E10.5 to E11.5. Black arrows indicate *Bmp4*-expressing cristae prosensory domains, black arrowheads indicate *Bmp4*-expressing cochlear prosensory domains, red arrows indicate *Bmp4* reduction or absence in *Rbpjκ* cKO cristae prosensory domains. **B:** RNA in situ hybridization for *Lfng* in *Rbpjκ* cKO otocyst sections from E9.5 to E11.5. Black arrows indicate *Lfng* expressing macular prosensory domains, black arrowheads indicate *Lfng* expressing cochlear prosensory domains, red arrows indicate *Lfng* reduction or absence in *RBPJ cKO* macular prosensory domains.

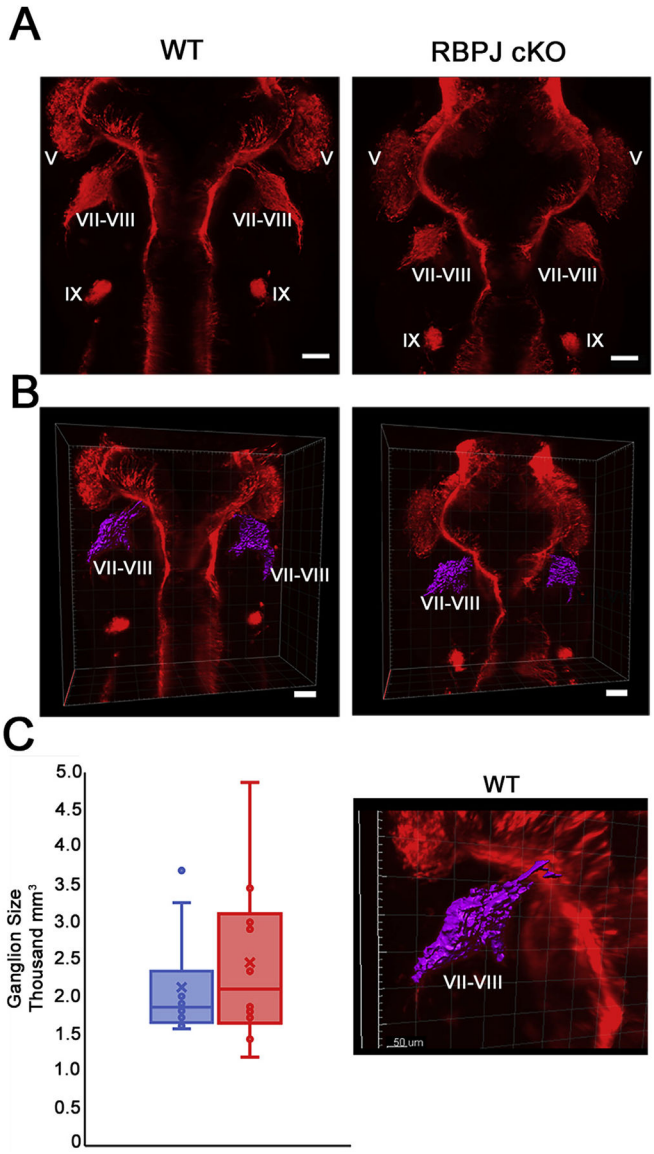


**Fig. 3. Deletion of *Rbpjκ* at the otocyst stage does not result in a significant increase in the size of the VIII/VIIth ganglion during neurogenesis.**

**A:** Whole mount immunofluorescence for βIII tubulin (Tuj1 antibody) in E9.5 *Rbpjκ* cKO and WT and littermate cleared with Sca*l*e and imaged using LightSheet microscopy. The Vth, VIIth, VIIIth and IXth ganglion are indicated with labels. **B:** Volume of Tuj1-labeled VIII/VIIth ganglia determined by surface mapping ganglion. **C:** Graph of data in box-plot format, bars represent maximum and minimum recorded values, with one value plotted as an outlier in WT graph. Mean represented by X, WT = 2.1 thousand millimeters$^3$, *Rbpjκ* cKO = 2.4 thousand milimeters$^3$. Student's two-tailed *t*-test was performed to determine statistical significance. P = 0.19, n = 10 for WT and *Rbpjκ* cKO. Scale bars in A and B = 100μm.

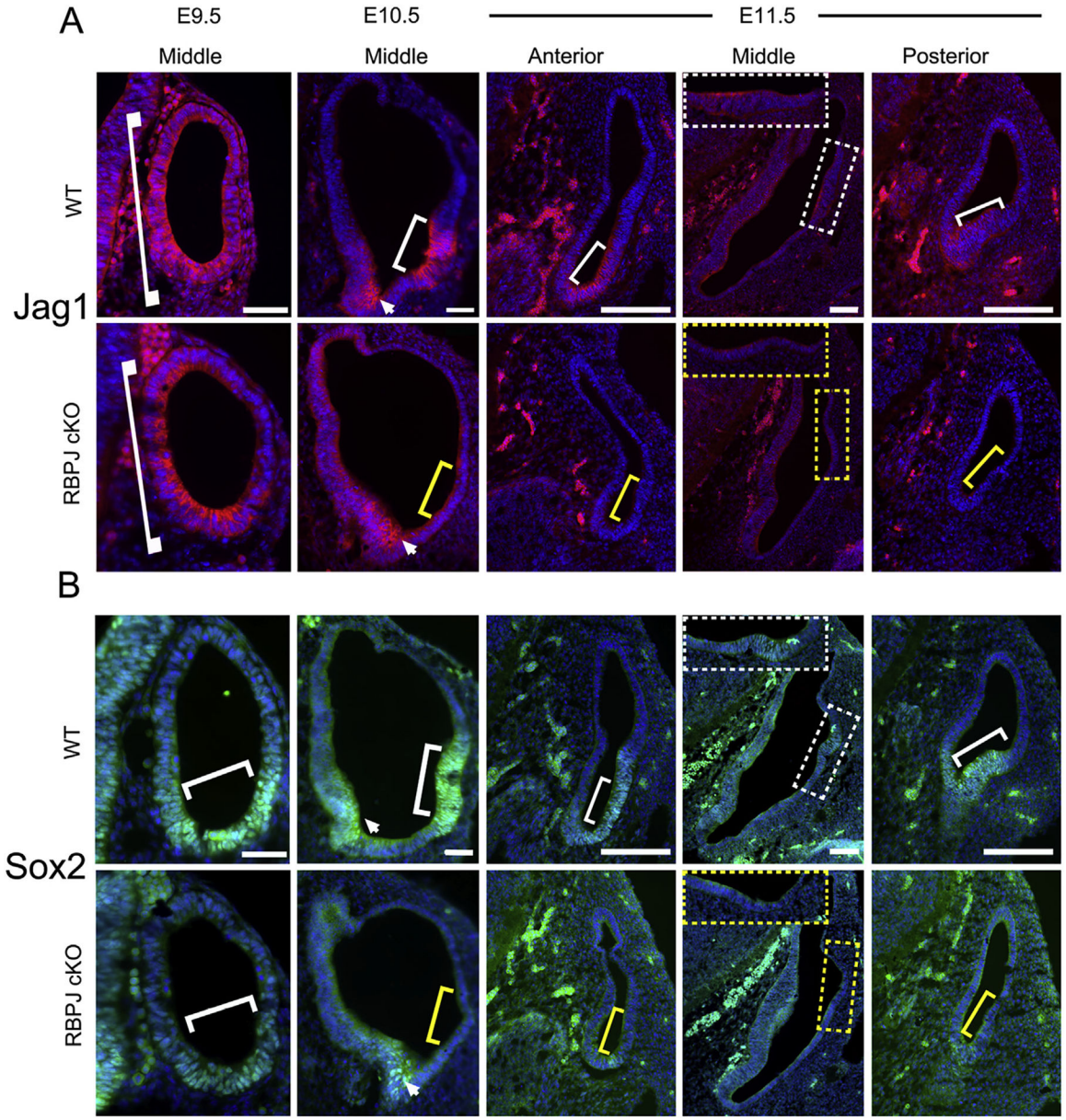


**Fig. 4. Loss of *Rbpjκ* results in a loss of some of the key components of lateral induction and vestibular prosensory character.**

**A:** Immunofluorescence staining for JAG1 (red) in *Rbpjκ* cKO and WT otic sections counterstained with DAPI (blue) from E9.5 to E11.5. White brackets indicate otic epithelium expressing JAG1 (E9.5) and JAG1 expression in vestibular prosensory domains (E10.5-E11.5), yellow brackets indicate vestibular otic epithelium that has lost Jag1 expression. White arrows indicate JAG1-expressing cochlear prosensory domains, yellow arrows indicate cochlear prosensory domains not expressing JAG1. **B:** Immunofluorescence staining for SOX2 (green) in *Rbpjκ* cKO and WT otic sections counterstained with DAPI (blue) from E9.5 to E11.5. White brackets indicate otic epithelium expressing SOX2 (E9.5) and SOX2 expression in vestibular prosensory domains (E10.5-E11.5), yellow brackets indicate vestibular otic epithelium that has lost SOX2 expression. White arrows indicate

SOX2-expressing cochlear prosensory domains, yellow arrows indicate cochlear prosensory domains with reduced SOX2. Scale bars = 50μm.

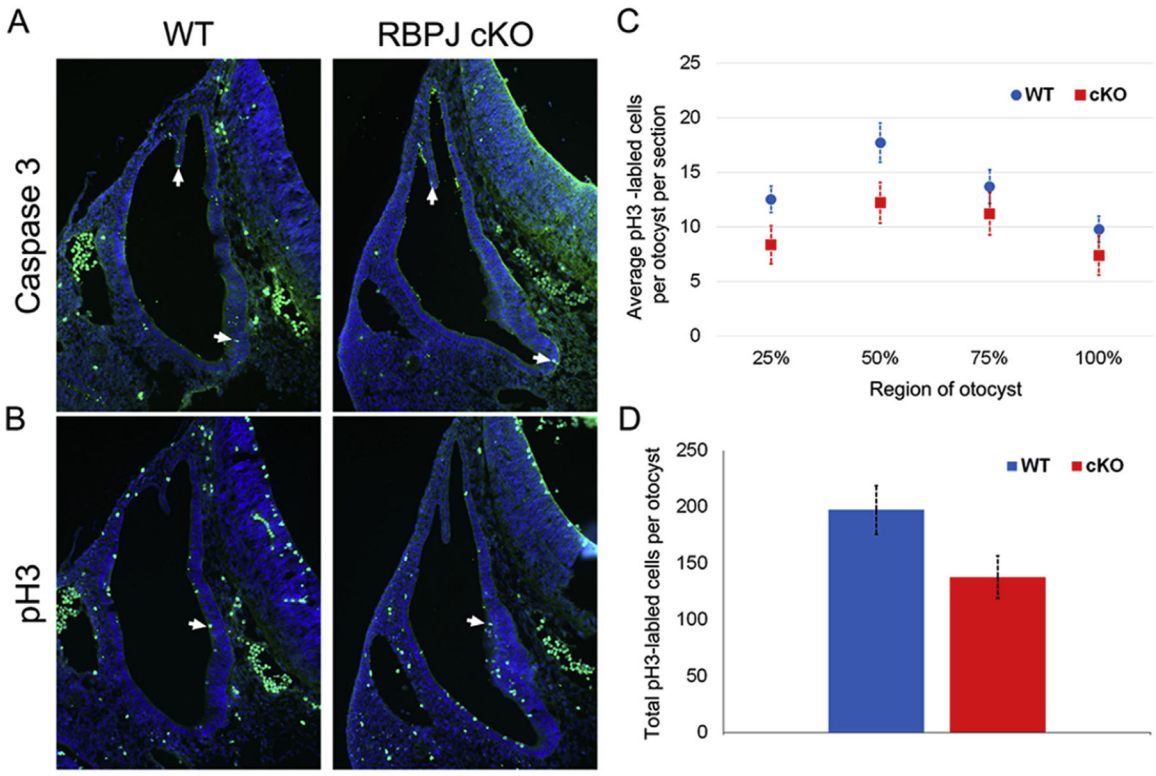


**Fig. 5. The loss of vestibular prosensory domains does not appear to be due to significant decrease in proliferation or an increase in apoptosis.**

**A:** Activated caspase 3 (green) immunofluorescence staining in E10.5 *Rbpjκ* cKO and WT otic sections counterstained with DAPI (blue). White arrows indicate typical instances of apoptosis detected in otic sections. B: Phosphorylated Histone H3 (green) immunofluorescence staining in E10.5 *Rbpjκ* cKO and WT otic sections counterstained with DAPI (blue). White arrows indicate examples of proliferating cells in the otic epithelium. Sections in A and B are adjacent serial sections from the same otocyst of a WT and mutant animal. **C:** Cell counts for proliferating cells for otocyst divided into quadrants. The 25% mark represents the anterior-most quarter of otic sections with 100% representing the posterior-most quarter. Bars indicate standard error of the mean. For 25% P = 0.08, for 50% P = 0.06, for 75% P = 0.34, for 100% P = 0.30, n = 5 WT and n = 6 cKO. **D:** Cell counts for proliferation in aggregate per otocyst. Bars indicate standard error of the mean, P = 0.07, n = 5 WT and n = 6 cKO. Student's two-tailed *t*-test was used for statistical analysis in C and D.

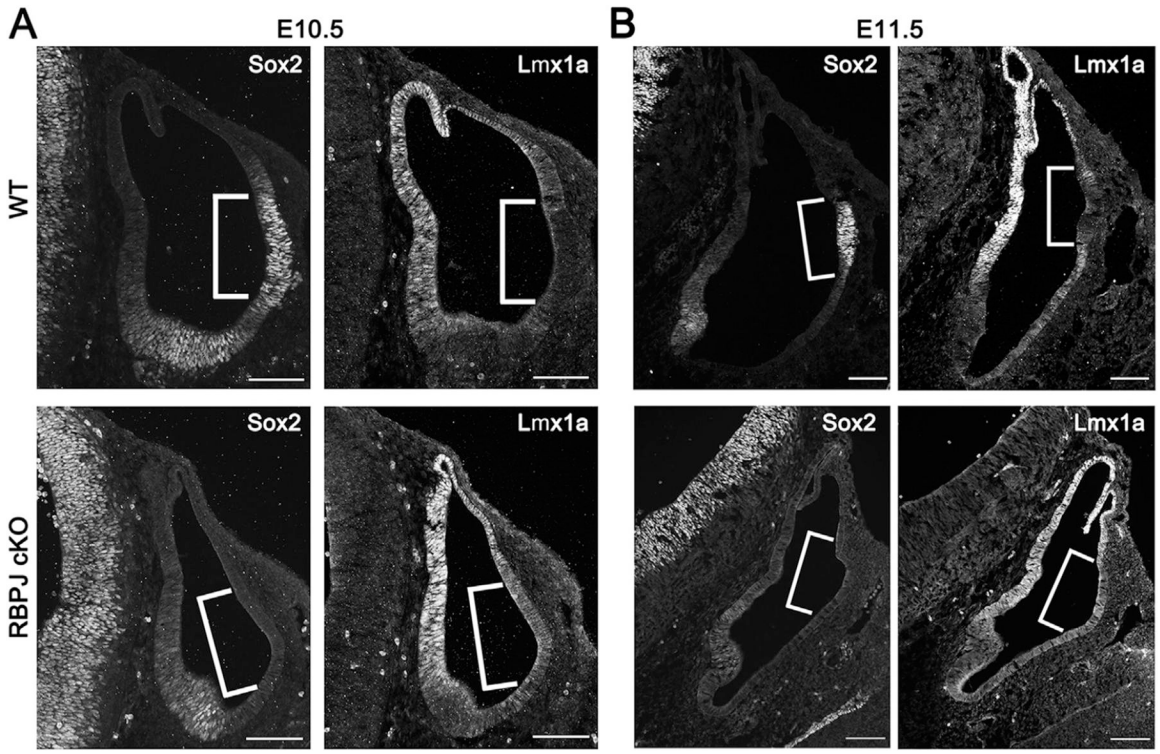


**Fig. 6. Loss of lateral induction by deletion of *Rbpjκ* results in a loss of vestibular prosensory domains and a corresponding spread of non-sensory character.**

**A:** SOX2 and LMX1A immunofluorescence staining on E10.5 *Rbpjκ* cKO and WT littermate otic sections with. Scale Bars = 100μm **B**: SOX2 and LMX1A immunofluorescence staining on E11.5 *Rbpjκ* cKO and WT littermate otic sections. At both ages, the domains of SOX2 and LMX1A are mutually exclusive in wild type embryos. However, in *Rbpjκ* cKO embryos, SOX2 staining is absent from the lateral region of the otocyst which is now a thin epithelium. LMX1A is beginning to be up-regulated in these regions. Scale bars = 100μm.

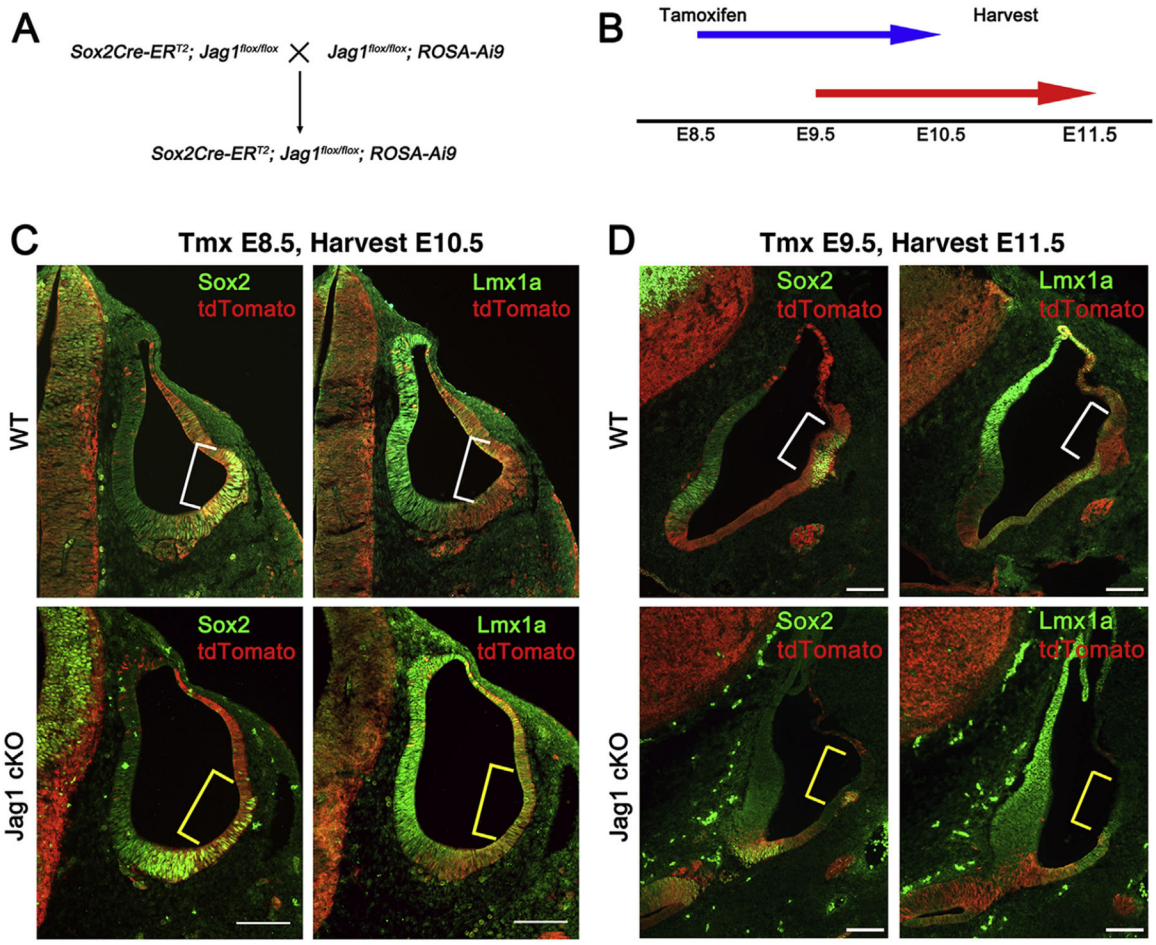


**Fig. 7. JAG1-mediated Notch signaling is required to maintain prosensory domains at the expense of non-sensory character.**

**A:** Mating scheme to conditionally delete *Jag1* in prosensory cells. *Sox2*-CreERT2 mice carrying two conditional alleles of *Jag1* (*Jag1$^{flox}$*) were mated with mice doubly homozygous for the *Jag1$^{flox}$* allele and the *Ai9* ROSA-tdTomato Cre reporter. **B:** Pregnant females received a dose of tamoxifen at a) E8.5 to initiate recombination and the mice were sacrificed at E10.5 for analysis, or b) E9.5 with sacrifice at E11.5. **C:** SOX2 and LMX1A immunofluorescence staining of E10.5 *Jag1* cKO and WT otic sections with tdTomato reporter activity showing cells in which *Jag1* was deleted. White brackets designate vestibular prosensory domains with typical SOX2 expression and corresponding absence of LMX1A expression. Yellow brackets denote vestibular prosensory domains in *Jag1* conditional mutants. Tissue in which *Jag1* was conditionally deleted are marked by the tdTomato reporter. In the mutant (tdTomato+) regions, SOX2 is staining is patchy and reduced, and LMX1A is has begun to spread into the tdTomato + domain. **D:** SOX2 and LMX1A immuno fluorescence staining of E11.5 *Jag1* cKO and WT otic sections with the tdTomato reporter. In mutant embryos, the expression of SOX2 staining is again reduced, with LMX1A spreading into the former prosensory area (yellow brackets). Scale bars = 100μm.