Jag1b is essential for patterning inner ear sensory cristae by regulating anterior morphogenetic tissue separation and preventing posterior cell death

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ABSTRACT
The sensory patches of the vertebrate inner ear, which contain hair cells and supporting cells, are essential for hearing and balance functions. How the stereotypically organized sensory patches are formed remains to be determined. In this study, we isolated a zebrafish mutant in which the jag1b gene is disrupted by an EGFP insertion. Loss of Jag1b causes cell death in the developing posterior crista and results in downregulation of fgf10a in the posterior prosensory cells. Inhibition of FGF activity in wild-type embryos also causes loss of the posterior crista, suggesting that Fgf10a mediates Jag1b activity. By contrast, in the anterior prosensory domain, Jag1b regulates separation of a single morphogenetic field into anterior and lateral cristae by flattening cells destined to form a nonsensory epithelium between the two cristae. MAPK activation in the nonsensory epithelium precursors is required for the separation. In the jag1b mutant, MAPK activation and cell flattening are extended to anterior crista primordia, causing loss of anterior crista. More importantly, inhibition of MAPK activity, which blocks the differentiation of nonsensory epithelial cells, generated a fused large crista and extra hair cells. Thus, Jag1b uses two distinct mechanisms to form three sensory cristae in zebrafish.

KEY WORDS: Jag1b, Inner ear, Cristae, Notch, FGF, Cell death, Zebrafish

INTRODUCTION
The mammalian inner ear comprises six distinct sensory regions: the organ of Corti, the saccular macula and utricular macula, and three cristae, located at the base of each semicircular canal. The sensory regions are responsible for hearing or detecting acceleration and keeping balance. During development, the vertebrate inner ear arises from thickened otic placodes, which invaginate to form otic vesicles, followed by a series of morphogenesis processes, including patterning of three anatomical axes and the prosensory domains. The prosensory domains must be specified precisely to form distinct sensory patches, which are composed of sensory hair cells and associated supporting cells (Kelley, 2006). How sensory patches are specified and patterned to form highly organized structures remains poorly understood.

The Notch signaling pathway plays important roles in inner ear development (Kiernan, 2013). In the classic Notch ‘lateral inhibition’ model, cells expressing high level of Notch ligands prevent their neighboring cells from producing the same ligands, which results in a mosaic pattern of ligand expression and eventually cell differentiation (Fortini, 2009). At late stages, lateral inhibition directs differentiation of hair cells and supporting cells (Bryant et al., 2002; Haddon et al., 1998; Lanford et al., 1999). One of the Notch ligands, Jag1, is expressed in the prosensory precursors and regulates specification of prosensory domains (Adam et al., 1998; Lewis et al., 1998; Morrison et al., 1999; Murata et al., 2012; Zecchin et al., 2005). In mice carrying point mutations impairing Jag1 function, posterior and sometimes anterior cristae are missing or mispatterned (Kiernan et al., 2001; Tsai et al., 2001). Moreover, prosensory domain markers are downregulated in FoxG1-Cre Jag1 conditional knockout (CKO) mice (Brooker et al., 2006; Kiernan et al., 2006; Pan et al., 2010). In Notch gain-of-function studies, Notch was shown to promote non-sensory regions of otocysts to form ectopic sensory patches and to express prosensory specific genes (Daudet and Lewis, 2005; Hartman et al., 2010; Neves et al., 2011; Pan et al., 2010). These results suggest that Jag1-dependent Notch signaling is essential for prosensory domain specification. However, how Jag1 precisely regulates prosensory domain formation and related morphogenesis remains to be fully defined.

Previous studies also showed that the FGF signaling pathway plays multiple roles during different stages of inner ear development (Schimmang, 2007). Fgf3 and Fgf8 were reported to be necessary for otic induction in zebrafish (Maroon et al., 2002; Phillips et al., 2004), chicken (Ladher et al., 2005; Vendrell et al., 2000) and mice (Alvarez et al., 2003; Ladher et al., 2005; Wright and Mansour, 2003). Fgfr2, a Fgf receptor (De Moerlooze et al., 2000), Fgf10 in mice (Alvarez et al., 2003; Wright and Mansour, 2003) and Fgf19 in chicken (Freter et al., 2008) are also involved in otic induction. In mouse, Fgfr1 deletion (Pirvola et al., 2002) and Fgfr2 inhibition (Hayashi et al., 2008) cause severe defects of both hair cells and supporting cells. In zebrafish, Fgf signaling is required for specification of sensory epithelia (Millimaki et al., 2007). Moreover, in a recent study, inner ear sensory epithelia were generated from embryonic stem cells in three dimensional culture by FGF activation (Koehler et al., 2013), suggesting that FGF signaling is crucially required for sensory epithelial development.

Once specified, prosensory regions also need go through stereotypical morphogenesis to form final functional structures. For example, each crista ampullaris is derived from prosensory patches and eventually positioned in the anterior, lateral and posterior regions of otic vesicles. Previous studies in chicken suggested that different sensory organs may be derived from a common prosensory domain (Knowlton, 1967; Neves et al., 2011; Oh et al., 1996; Sánchez-Guardado et al., 2013). Besides, in mice inner ear, the dynamic Bmp4 expression pattern also suggests that the anterior and lateral cristae may develop from a common
prosensory domain (Morsli et al., 1998). However, direct evidence supporting this model (Fritzsch et al., 2007) remains to be obtained.

Inner ear development is largely conserved between zebrafish and mammals (Whitfield et al., 2005). Cell tracing and in vivo imaging techniques in zebrafish offer a unique opportunity to address how inner ear structures develop morphogenetically (Haddon and Lewis, 1996). Here, we report a zebrafish gene-trap mutant, jag1b, in which jagged1 (jag1b, the homolog of mammalian jagged1) gene expression is disrupted by a Tol2 transposon insertion driving enhanced green fluorescent protein (EGFP) expression. We found that Jag1b is essential for maintaining prosensory cell viability in the posterior domain and separation of the anterior and lateral cristae. We also found that MAPK kinase activity is required for the formation of non-sensory cells in between the anterior and lateral cristae.

**RESULTS**

**jag1b expression is disrupted in the jag1b mutant**

In a Tol2 transposon-mediated gene trap screen in zebrafish, we identified a trapped line, jag1b, in which truncated Jag1b-EGFP fusion proteins are expressed in specific tissues, including lens and inner ear at 24 h post-fertilization (hpf) (Fig. 1A). The Tol2 transposon element is inserted into the second intron of jag1b gene (Fig. 1B), which is predicted to produce a fused transcript containing the first and second exon of jag1b and the EGFP-coding sequence (Fig. 1C). Because the translation start codon of jag1b is located in the first exon and is in frame with the EGFP-coding sequence, the fusion transcript should be translated into a fusion protein (Jag1b-EGFP) containing the first 125 amino acid residues of Jag1b and EGFP protein. The fusion transcript can be readily detected in the heterozygotes and homozygotes (Fig. 1E). The genotypes of wild type (jag1b+/+), heterozygotes (jag1b+/−) and homozygotes (jag1b−/−) can be visually identified by their respective EGFP fluorescence intensity (Fig. 1D). Among 2000 F2 embryos examined, 24.3% were wild type (GFP−), 52.1% were heterozygotes (GFP+) and 23.6% were homozygotes (GFP++), which agrees with Mendel’s law of segregation, suggesting that a single locus has been trapped. The expression pattern of jag1b revealed by RNA whole-mount in situ hybridization (supplementary material Fig. S1A) was highly similar to that of EGFP fluorescence, confirming that EGFP expression identifies Jag1b-expressing cells in the embryos. In addition, the expression of Jag1b-EGFP fluorescence in the otic vesicle (Fig. 1A, arrows) is consistent with a previous report that jag1b, rather than jag1a, is expressed in the zebrafish otic vesicle (Zecchin et al., 2005).

As expected, the full-length jag1b mRNA level was dramatically decreased in the homozygous mutants (Fig. 1F; supplementary material Fig. S1B). In addition, we detected full-length Jag1b protein of expected molecular weight in jag1b+/− and jag1b−/− embryos using a commercial antibody against the C-terminus of human JAG1 (the C-terminus of human JAG1 shares 82.6% amino acid identity with that of zebrafish Jag1b). Several smaller bands were also detected; however, a full-length Jag1b band was not seen in jag1b−/− embryos (Fig. 1G), supporting the notion that the trapped jag1b−/− line is very likely a Jag1b-null mutant.

**Jag1b-EGFP fusion protein labels prosensory regions and sensory cristae of the inner ear**

To investigate the role of Jag1b in the patterning of the prosensory domains, we first analyzed in detail Jag1b-expressing cells (EGFP expression is disrupted in the gene-trapping jag1b mutant. (A) EGFP expression patterns in jag1b−/− embryos. EGFP is expressed in the otic vesicle (arrows). Scale bar: 100 μm. (B) The zebrafish jag1b genomic locus. Primers used in D are indicated. Scale bar: 100 μm. (B) The zebrafish jag1b genomic locus. Primers used in D are indicated. Scale bar: 100 μm. (C) The mature wild-type (WT) jag1b transcript and the predicted mutant jag1b-EGFP fusion transcript. Primers used in E are indicated. (D) PCR genotyping confirms that the jag1b gene is disrupted in jag1b mutants (right). Wild type, heterozygotes and homozygotes show different GFP intensity (left). (E) RT-PCR analysis confirms the existence of the jag1b-EGFP fusion transcript in jag1b−/− and jag1b−/− embryos. (F,G) Real-time RT-PCR (F) and western blot (G) analyses indicate that jag1b mRNA and protein levels are decreased in jag1b mutant embryos. Error bars indicate s.e.m. of triplicated experiments.
positive) during inner ear development (Fig. 2A; supplementary material Movies 1, 3 and 4). In the jag1b+/− embryos, EGFP was detected ubiquitously in the otocyst as early as 19 hpf (Fig. 2A). From 24 to 36 hpf, EGFP was restricted to the ventral anterior and posterior regions of the otic vesicle. These EGFP-positive cells eventually gave rise to the anterior, lateral and posterior cristae, as indicated by distinct EGFP labeling of the cristae at 48 hpf and 72 hpf (Fig. 2A, arrowheads). We also used phalloidin staining to show that the EGFP-labeled cells indeed are the cristae at 72 hpf (Fig. 2C; supplementary material Fig. S2, arrows). The cristae in the jag1b−/− embryos are indistinguishable from those in the jag1b+/− embryos (supplementary material Fig. S2). In summary, Jag1b expression marks prosensory domains and sensory cristae during inner ear development in zebrafish.

**Jag1b is required for maintaining the cell survival of the posterior crista**

To investigate the roles of Jag1b in inner ear development, we examined sensory cristae in the jag1b−/− embryos. Compared with jag1b+/− embryos, jag1b−/− embryos (200/200) showed agenesis of the posterior crista (Fig. 2B,C, arrowheads). Ninety-five percent of jag1b−/− embryos also showed complete (134/200) or partial (56/200) loss of the anterior crista (Fig. 2B,C, arrows). These data indicate that full-length Jag1b protein is essential for the sensory cristae development. Jag1b is also expressed in the anterior and posterior maculae (supplementary material Fig. S3A,B). However, in 48 hpf and 72 hpf jag1b−/− embryos, the accumulation of macular hair cells is not affected, as indicated by phalloidin staining (supplementary material Fig. S3A,B), suggesting that Jag1b is not required for maculae development in zebrafish.

Previous studies suggested that Jag1 is required for prosensory domain specification in mice (Kiernan, 2013). In the inner ear of jag1 CKO mice, development of sensory patches is severely compromised, and Bmp4 expression in the patches is downregulated (Brooker et al., 2006; Kiernan et al., 2006; Pan et al., 2010). We found that at 24 hpf, there was no noticeable difference between jag1b+/− and jag1b−/− embryos in term of EGFP expression patterns and morphology of the otic vesicle (Fig. 3A). Furthermore, expression patterns of the early otic vesicle marker genes cldh4a, stm, pax5, pax2a, otx1a and fgfb4a, and the prosensory marker genes bmp4 and bmp2b, were also similar among all jag1b+/+, jag1b+/− and jag1b−/− embryos at 24 hpf (Fig. 3B; supplementary material Fig. S4A). To closely examine cell fates in the developing posterior crista, we used confocal live cell imaging to trace the EGFP-positive cells in the posterior region from 32 hpf to 40 hpf (supplementary material Movies 1 and 2). In the 32 hpf jag1b−/− embryos, EGFP was expressed in the ventral anterior and ventral posterior sides of the otic vesicle. EGFP-positive cells in the posterior region gave rise to the posterior crista (Fig. 3C; supplementary material Movie 1). At 32 hpf, morphology of the otic vesicle and EGFP expression pattern in the jag1b homozygous embryos were similar to those in the heterozygotes (Fig. 3C). However, most of the EGFP-positive cells of the developing posterior crista in the mutants were gradually lost during the following hours with two or three cells anterior to the crista remaining EGFP positive (Fig. 3C, supplementary material Movie 2). Consistent with this observation, mRNA expression of bmp4 and bmp2b, two markers of the prosensory cristae in zebrafish (Mowbray et al., 2001), was markedly decreased in the jag1b−/− embryos at 34 hpf (supplementary material Fig. S4B). This observation suggested that the prosensory cells in the developing posterior crista may undergo cell death or differentiation to other cell types. TUNEL assay results revealed apoptosis occurred only in the developing posterior crista region in jag1b−/− embryos (50/50), but not in jag1b+/− embryos (0/50) at 40 hpf (Fig. 3D). It should be noted that we did not detect apoptotic cells in other regions of the otic vesicle, including the anterior prosensory patch. Thus, these data indicated that Jag1b is required for maintaining the posterior prosensory domain.

**Jag1b is required for fgf10a expression in the posterior prosensory region**

The apoptosis at the posterior prosensory domain prompted us to investigate Jag1b downstream signaling mediators. Several studies have indicated that FGF signaling is essential for vertebrate inner ear
development (Schimmang, 2007). Fgf10 is expressed in all three cristae sensory epithelia and loss of Fgf10 results in complete agenesis of the posterior canal crista in mice (Pauley et al., 2003), a phenotype similar to that in jag1b−/− zebrafish embryos. In addition, it was reported that fgf10 is expressed in the zebrafish otic vesicle (Feng and Xu, 2010). We found that, at 48 hpf, fgf10a was expressed in the three sensory cristae in the zebrafish inner ear (supplementary material Fig. S5A, arrowheads). Through carefully analyzing expression patterns of fgf10a at early developmental stages, we found that fgf10a is expressed both in the anterior and posterior prosensory regions of the otic vesicle in wild-type embryos at 30 hpf and 34 hpf (Fig. 4A,D), which is similar to the jag1b expression pattern. In the jag1b−/− embryos, fgf10a expression was specifically downregulated in the posterior region (Fig. 4C,F, arrowheads) but not in the anterior region (supplementary material Fig. S5D-F).

Fig. 3. Jag1b is required for survival of the posterior prosensory cells.

(A) Confocal images of the otic vesicle of embryos at 24 hpf. Lateral views. Scale bar: 50 µm. (B) Whole-mount in situ hybridization of otic marker genes at 24 hpf. Scale bar: 20 µm. (C) Time-lapse live imaging of the developing posterior prosensory domain. The posterior sensory cells are gradually lost in jag1b−/− embryos. Scale bar: 10 µm. (D) TUNEL staining of embryos at 34 hpf and 40 hpf. At 40 hpf, apoptosis signals are detected only in the posterior prosensory domain of jag1b−/− embryos. Dorsolateral views. Scale bars: 40 µm (left) and 20 µm (right). Anterior towards the left and dorsal upwards. Boxed regions are enlarged on the right.

It should be noted that fgf10a expression in jag1b−/− embryos was also moderately downregulated compared with that in wild-type embryos (Fig. 4B,E, arrowheads), suggesting that full Jag1b activity is required for normal fgf10a expression in the posterior prosensory region. To examine whether FGF signaling is responsible for the loss of posterior crista development, we used a specific FGFR inhibitor SU5402 to block FGFR signaling (Mohammadi et al., 1997). Eighty percent of jag1b−/− embryos (n=100) treated with 10 µM SU5402 from 24 hpf to 26 hpf lost their posterior crista at 48 hpf (Fig. 4G-H″) accompanied with cell death in the posterior prosensory domain (Fig. 4I). Furthermore, the posterior crista was not detected by phalloidin staining in the FGF inhibitor-treated embryos at 72 hpf (74%, n=100) (Fig. 4J). Together, these results suggest that Jag1b may positively regulate expression of fgf10a, which is a survival signal for the posterior crista.

FGFs and their receptor tyrosine kinases control a multitude of developmental processes through three major pathways: the PKC pathway, Akt pathway and Erk (MAPK) pathway (Dorey and Amaya, 2010). The Akt pathway is reported to be important for cell survival. However, we found that the sensory cristae were not affected (supplementary material Fig. S5G) by treatment with Akt inhibitor Akti-1/2 (Schier and Talbot, 2005). These data suggest that inhibition of Akt activity alone was not sufficient to cause cell death in the developing cristae.

The anterior and lateral cristae primordia are separated by morphogenetic changes in the intervening epithelium

Although 95% jag1b−/− embryos showed complete or partial loss of anterior crista (Fig. 2B and C), there was no significant apoptosis in the developing anterior prosensory domain (Fig. 3D), suggesting that Jag1b is not required for cell survival in the anterior crista. We traced EGFP cells during development of the anterior crista using time lapse imaging (supplementary material Movies 3 and 4). In the jag1b−/− embryos, there was a single EGFP-positive prosensory patch at the ventral anterior side at 32 hpf. All EGFP-positive cells
of the patch showed similar cell shape, orientation and EGFP intensity (Fig. 5A). Subsequently, cells between the anterior crista primordia (ACP) and lateral crista primordia (LCP) (Fig. 5A′, arrowheads) gradually flattened and progressively lost EGFP expression (Fig. 5A-A′, arrowheads). At 48 hpf, those cells showed a distinct, flattened cell shape (Fig. 5A′, arrowheads) and separate the single anterior prosensory domain into the anterior crista (AC) and lateral crista (LC) (Fig. 5A″, brackets). We propose that cells between the ACP and LCP differentiate into non-sensory epithelial cells. During their differentiation, these cells may switch their cadherin expression and form new types of adhesion junctions to distinguish them from the flanking cells (Wheelock et al., 2008). To test this hypothesis, we examined expression of E-cadherin, an epithelial marker, by immunofluorescent staining. As expected, none of the EGFP-positive cells at 32 hpf in the single prosensory domain had E-cadherin staining (Fig. 5C,C′). However, at 40 hpf, cells in the middle region showed specific upregulation of E-cadherin (Fig. 5D,D′, arrowheads), suggesting these cells had undergone cell differentiation, while the ACP and LCP (Fig. 5D,D′, brackets) maintain potential to develop into sensory cristae.

Loss of the anterior prosensory crista raises a possibility that abnormal cell differentiation in the ACP of the early prosensory domain may be an underlying cause. We captured time-lapse images of morphogenesis of the anterior crista in jag1b+/- embryos (supplementary material Movies 5 and 6). At 32 hpf, cells in the anterior prosensory region in jag1b+/- embryos showed similar shape, orientation and EGFP intensity compared with those in jag1b+/+ embryos (Fig. 5A,B). However, starting from 40 hpf, the ACP in the mutant underwent morphological changes and reduced EGFP expression, similar to those in the middle segment (Fig. 5B-B′, arrows). Consistently, at 40 hpf, E-cadherin expression was detected in cells both in the ACP and middle region of the anterior prosensory domain (Fig. 5F,F′, arrows). Therefore, in the ACP, cells switched their fate to non-sensory epithelia and this resulted in the loss of the anterior crista. We conclude that selective differentiation of middle region of the prosensory domains contributes to the separation of anterior and lateral cristae.

Jag1b is required for restricting Erk activation to the middle region of the anterior prosensory domain

One mechanism to specifically regulate the cell shape change in the middle segment of the early anterior prosensory domain is for Jag1b to restrict a downstream activation signal in the segment. In addition to fgf10a expression in the prosensory regions, we found fgf8a expression in the anterior prosensory domain at 36 hpf (supplementary material Fig. S6A). Expression of pea3 and ern, both targets of Fgf8 signaling (Roehl and Nüsslein-Volhard, 2001), is upregulated in the middle of the anterior prosensory domain at 39 hpf (supplementary material Fig. S6B,C), suggesting FGF signaling may also mediate Jag1b functions in these cells. To test this possibility, we treated jag1b+/- embryos with 5 μM FGF inhibitor SU5402 from 32-34 hpf prior to the separation event. At 48 hpf, SU5402 treatment inhibited cell shape change in the middle region (82%, n=100) (Fig. 5G,H). Accordingly, cells in the entire region showed similar cell shape and orientation (Fig. 5H). Moreover, apoptotic cells were not detected in the developing anterior prosensory domain in SU5402-treated embryos (supplementary material Fig. S6D). This result suggests that FGF signaling is required for the separation of anterior and lateral cristae.

We speculated that FGF/Erk pathway mediated the morphological change of the cells in the middle of the anterior prosensory domain. If this is the case, then: (1) phosphorylated Erk, which represents activated Erk, should be expressed in the middle region of the anterior prosensory domain; and (2) direct inhibition of Erk activity should block E-cadherin expression in the middle segment of the prosensory domain and thus block the separation of anterior and lateral cristae. Because erk3 rather than erk1 and erk2 is expressed in the otic vesicle
(Krens et al., 2006), we examined Erk3 activation by examining phospho-Erk3 (p-Erk3) level by immunofluorescent staining. Indeed, in jag1b−/− embryos, cells in the middle region (Fig. 5I′, arrowheads) showed significantly higher p-Erk3 staining than in the ACP and LCP (Fig. 5I′, brackets) in the prosensory domain. Furthermore, in jag1b−/− embryos, ACP also underwent cell shape change (Fig. 5B−F, arrowheads). Consistent with this morphological change, p-Erk3 signal in these cells was significantly increased to a level similar to that in the middle segment (Fig. 5J′, arrows).

In agreement with our hypothesis, inhibition of FGF activity by SU5402 (supplementary material Fig. S6E) or direct inhibition of Erk phosphorylation by two highly specific MAPKK (MEK) inhibitors, CI-1040 (Allen et al., 2003) and PD0325901 (Kohno and Lewis, 2006), reduced p-Erk3 level and E-cadherin expression in the middle region of the anterior prosensory domain, which is essential for the separation of anterior and lateral cristae.

MAPK inhibition induces ectopic hair cells in a fused crista

As MAPK inhibition blocks differentiation of middle region of the anterior prosensory domain and resulted in uniform cell shape in the entire anterior domain, we investigated whether such inhibition would generate new hair cells in the middle segment. Each hair cell in zebrafish sensory cristae contains one extremely long kinocilium (Haddoon and Lewis, 1996; Whitfield et al., 2002) and can be detected under DIC microscopy (Nicolson et al., 1998). Indeed, compared with the DMSO control, CI-1040 treatment caused various degrees of failed separation of anterior and lateral cristae at 72 hpf. We divided the CI-1040-treated embryos into three categories based on shapes of the fused large crista and phalloidin staining of stereocilia bundles. In type (I) (22%, n=95) and type (II) (24%, n=95) embryos, cells between AC and LC showed similar morphology to the flanking cells (Fig. 7A-C), but kinocilia (Fig. 7A-C) and stereocilia bundles are not detected in the middle cells (Fig. 7E-G′, arrowheads), suggesting that they represent an intermediate state between crista cells and non-sensory epithelial cells. In type (III) (54%, n=95) embryos, along the entire fused crista, kinocilia were clearly detected under DIC confocal microscopy (Fig. 7D, arrowheads) and stereocilia bundles were detected by phalloidin staining (Fig. 7H′-H″). Similar results were also observed in PD0325901-treated jag1b−/− (supplementary material Fig. S8) or jag1b+/− (supplementary material Fig. S9) embryos. Moreover, the crista identity of the fused structure was confirmed by whole-mount in situ hybridization using the crista maker msxC (Fig. 7I). By carefully counting kinocilia in the fused crista using z-stacks of confocal images, we found that the fused
indicate staining of E-Cadherin or p-Erk3. (M-M
Kiernan et al., 2006; Neves et al., 2011; Pan et al., 2010). All six
development. Previous studies suggested that Jag1/Notch signaling
signaling pathway is involved in multiple steps of inner ear
development. Sensory patches specification, morphogenesis
Patterning fine structures in organs is fundamentally important in
DISCUSSION
MAPK is essential for separation of anterior and lateral cristae.
treatment, further supporting the observation that upregulation of
AC and LC were converted into hair cells after the MAPK inhibitor
Fig. 7J) in DMSO-treated embryos, suggesting some cells between
Fig. 7J) than anterior and lateral cristae combined (21.4±9.56,
Jag1b+/− 39 hpf
DMSO (32-39 hpf)
Jag1b+/− 39 hpf
CI-1040 (32-39 hpf)
Jag1b+/− 39 hpf
PD0325901 (32-39 hpf)
jag1b+/-
48 hpf
jag1b−/−
48 hpf
DMSO
CI-1040
PD0325901
Fig. 6. MAPK pathway inhibition blocks the separation of the anterior and lateral cristae. (A-L) MAPKK inhibition represses expression of p-Erk3 and E-cadherin in both jag1b+/− and jag1b−/− embryos. Arrows and arrowheads indicate staining of E-Cadherin or p-Erk3. (M-M) In jag1b−/− embryos, treatment with the MAPKK inhibitors CI-1040 (M) and PD0325901 (M′) blocks separation of the anterior and lateral cristae. (N-N′) In jag1b−/− embryos, the MAPKK inhibitors CI-1040 (N) and PD0325901 (N′) block the morphological change of cells in the anterior prosensory domain. Dorsolateral views. Scale bars: 20 µm. Anterior towards the left and dorsal upwards.
crista of CI-1040-treated embryos had more hair cells (27.8±8.79,
Fig. 7J) than anterior and lateral cristae combined (21.4±9.56,
Fig. 7J) in DMSO-treated embryos, suggesting some cells between
AC and LC were converted into hair cells after the MAPK inhibitor
Fig. 7J in MAPK pathway inhibition. Further supporting the observation that upregulation of
MAPK is essential for separation of anterior and lateral cristae.

Prior to the current study, it is unclear how all three sensory
cristae are formed from the prosensory domains. Based on Bmp4
expression pattern in the developing chicken and mouse inner ear,
previous work has suggested that the anterior and lateral cristae
may derive from a common prosensory domain (Morsli et al., 1998;
Oh et al., 1996). However, the direct evidence supporting this
model and the mechanism are still missing. We have provided live
imaging evidence that anterior and lateral cristae are derived from a
single anterior prosensory domain. The MAPK signaling is
specifically upregulated in the middle part of the anterior
prosensory domain, resulting in specific cell differentiation to
establish a boundary between the anterior and lateral cristae (Fig. 5).
One of the roles of Jag1b could be inhibition of Erk
activation in the ACP, permitting Erk upregulation only in the
middle segment. This is partially supported by the observation that
loss of Jag1b causes ectopic Erk activation in ACP of the anterior
prosensory domain and subsequent loss of the anterior crista.
Formation of the lateral crista is apparently not dependent on Erk
inhibition. How Jag1b precisely restricts Erk activity remains to be
elicitated. We found FGF signaling is required for the separation
of anterior prosensory domain (Fig. 6G,H). However, mRNA
expression of FGF genes such as fgf8a did not show restricted
advantage of the jag1b EGFP gene-trapping mutant, we studied the
detailed differentiation and morphogenesis of crista precursor cells.
We found that, in zebrafish, Jag1b, the homolog of mammalian
Jag1, is not required for the prosensory domain specification.
However, at later stages, anterior and posterior cristae are lost in the
mutant. The inner ear phenotype of the jag1b mutant is similar to that
of Jag1 heterozygote mutant mice, but is less severe than that of the
Jag1 inner ear CKO mice (Kiernan et al., 2001, 2006). Genomic
redundancy (there is a jag1a gene in the genome) cannot explain the
less severe phenotype because jag1a is not expressed in the
zebrafish otic vesicle (Zecchin et al., 2005). In the inner ear of Jag1
CKO mice, Sox2 and Bmp4 expression is downregulated (Hartman
et al., 2010; Kiernan et al., 2006; Pan et al., 2010). Sox2 mutant
mice fail to establish prosensory domain and show severe sensory
defects (Kiernan et al., 2005), indicating that Sox2 is required for
prosensory domain specification (Dabdoub et al., 2008; Kiernan
et al., 2005). However, in zebrafish, Sox2 is not required for sensory
epithelium establishment (Millimaki et al., 2010). Thus, factors
required for specification of the prosensory domain in zebrafish
remain to be identified.

Because early steps of sensory domains development are largely
unaffected in zebrafish jag1b mutant, we were able to uncover the
roles of Jag1b in sensory domains maintenance and morphogenesis.
We found that Jag1b is essential for maintaining the posterior
prosensory patch by preventing cell apoptosis. Conditional deletion
of RBPjk/CSL, a necessary transcriptional mediator of canonical
Notch pathway (Kopan and Ilagan, 2009), causes severe vestibular
defects and a shortened cochlear duct in the mouse inner ear (Basch
et al., 2011; Yamamoto et al., 2011). This is similar to the
phenotype in Jag1 CKO mice. Cell death was increased in the
cochlea in mice, suggesting canonical Notch signaling is required
for maintaining sensory cells (Basch et al., 2011; Yamamoto et al.,
2011). We also found that Jag1b is required for fgf10a expression,
suggesting Fgf10a may be a signal that mediates cell survival of
posterior prosensory patches. In agreement with our finding, a
previous study indicated that Notch functions upstream of Fgf20
and Notch-mediated regulation of prosensory formation in the
cochlea occurs via Fgf20 (Munnamalai et al., 2012). In addition,
Fgf10 expression was also reduced in the inner ear of RBPj CKO
mice (Yamamoto et al., 2011).

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early development. Sensory patches specification, morphogenesis
and maintenance are essential steps in inner ear development. Notch
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Kiernan et al., 2006; Neves et al., 2011; Pan et al., 2010). All six
sensory patches are affected in Jag1 CKO mice (Kiernan et al.,
2006), but it is unclear how Jag1 regulates sensory patch
maintenance and morphogenesis. In this study, we investigate
how inner ear sensory cristae are formed in zebrafish. Taking
expression pattern (supplementary material Fig. S5). In fact, 
fgf8a mRNA expression was not affected in jag1b−/− embryos 
(supplementary material Fig. S5). Whether regulation of FGF 
activity is at translational or post-translational level remains to 
be investigated. Previously, Notch pathway was shown to be 
essential for vertebrate somitogenesis, in which Notch signaling 
synchronizes presomitic mesoderm (PSM) but is not required for 
 somite boundary set-up (Jiang et al., 2000; Özbudak and Lewis,
2008). Instead, FGF signaling is shown to be required for 
segmentation (Pourquié, 2011). One possibility is that in wild-
type embryos Jag1b-mediated Notch signaling might also 
synchronize anterior prosensory domain cells. Loss of Jag1b 
might lead to synchronization of anterior prosensory cells, which results in ectopic MAPK activation. As the separation of anterior prosensory domain takes at least 4 hours, inner ear sensory domain morphogenesis might represent an ideal system with which to study dynamic interaction between Notch and FGF signaling.

We also showed that inhibition of MAPK signaling blocked 
the separation of anterior and lateral cristae (Fig. 6). Some of the non-
 sensitivity precursors were converted into sensory hair cells in MAPK 
inhibitor-treated embryos (Fig. 7). Further study along this line may 
help develop an approach to reprogram non-sensory epithelial cells 
directly into prosensory cells, which may be implicated in hair cell 
regeneration in the future.

Fig. 7. Inhibition of MAPK signaling generates a larger crista. (A-D) DIC confocal imaging of CI-1040-
treated jag1b−/− embryos at 3 dpf. Kinocilia are indicated 
by arrowheads. AC, anterior crista; LC, lateral crista. 
 (E-H) Phalloidin staining of MAPK inhibitor CI-1040-
treated jag1b−/− embryos at 3 dpf. Lateral views. Anterior 
towards the left and dorsal upwards. Scale bars: 40 µm. 
Arrows and arrowheads indicate cells between AC and LC (E–G”). (I) Whole-mount in situ hybridization of 
DMSO-and CI-1040-treated embryos. The probes 
used is msxC antisense. Arrowheads indicate cristae. 
(J) Total kinocilia number in the anterior and lateral 
cristae of DMSO-treated embryos and kinocilia number 
in the fused crista of CI-1040-treated embryos. Data are 
shown as mean±s.e.m. Statistical analysis was 
performed using a t-test. n indicates the number of inner 
ears for each experimental group.
MATERIALS AND METHODS
Zebrafish husbandry and embryo manipulations were performed as described previously (Westerfield, 2000). The T2ASAd gene trap vector has been described previously (Liu et al., 2012). T2ASAd plasmid (25 ng/µl) and Tol2 transposase mRNA (50 ng/µl) were injected (1 nl each) into one-cell embryos. Over 200 injected embryos were raised and outcrossed with wild-type fish. EGF expression was examined at different developmental stages until 72 hpf. Gene trap fish lines with obvious EGF expression were identified, including the jag1b mutant.

Tail-PCR and genotyping
To identify genes trapped in mutants, we used Tail-PCR (Liu and Chen, 2007). Jag1b genomic sequence (GenBank NC_007124.5) was used to design primers p1s used for genotyping. The primers used in Tail-PCR and genotyping are shown in supplementary material Table S1.

RT-PCR and RT-qPCR
For RT-PCR, total RNA was isolated from 10 embryos of jag1b+/−, jag1b+/+ and jag1b−/− at 24 hpf using TRIzol reagent (Life Technologies). The first-strand cDNA was synthesized by reverse transcription with M-MLV reverse transcriptase (Life Technologies). RT-qPCR was performed in Bio-Rad CFX96 system. jag1b mRNA sequence (GenBank NM_131863.1) was used to design primers, which are shown in supplementary material Table S1.

Inhibitor treatment
To inhibit FGFR activity, Jag1b−/- embryos were treated with 10 µM (24 hpf to 26 hpf) or 5 µM (32 hpf to 34 hpf) SU5402 (Sigma) then extensively washed in E3 medium. To inhibit Akt activity, jag1b−/- embryos were treated with Akti-1/2 (10 µM) from 24 to 48 hpf. To inhibit Erk activation, embryos were treated with MAPKK (MEK) inhibitor 10 µM Ci-1040-treated embryos at 3 days post-fertilization (dpf). The step size between two sequential z-stack sections was no more than 1 µm, so that none of the visible kinocilia would be missed in the z-stack images. Kinocilia numbers were counted manually based on the z-stack images.

Confocal microscope imaging and kinocilia number counting
For live embryos at desired stages, dechorionated embryos were anesthetized in 0.016% tricaine (Sigma). Live embryos as well as stained embryos (immunofluorescent staining and phallloidin staining) were mounted on glass bottom cell culture dish (NEST) and adjusted into lateral or dorsolateral orientation to capture prosensory domains or sensory cristae. Typical section or z-stack images were captured. For kinocilia number counting, z-stack images were captured to document all of the visible kinocilia in the anterior and lateral cristae in DMSO-treated embryos or in the fused crista of CI-1040-treated embryos at 3 days post-fertilization (dpf). The focal plane was located on the anterior prosensory domain (supplementary material Movies 3-6) or posterior prosensory domain (supplementary material Movies 1 and 2).

Acknowledgements
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Competing interests
The authors declare no competing or financial interests.

Author contributions
W.-R.M. and J.Z. conceived and designed the experiments, and analyzed and wrote the paper. W.-R.M. performed the experiments.

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Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.113662/-/DC1

References


**Movie 1.** Confocal time lapse live imaging of the posterior prosensory domain from 32 hpf to 40 hpf in *jag1b*+/− embryo.

**Movie 2.** Confocal time lapse live imaging of the posterior prosensory domain from 32 hpf to 40 hpf in *jag1b*−/− embryos.
Movie 3. Confocal time lapse live imaging of the anterior prosensory domain from 32 hpf to 36 hpf in \textit{jag1b}^{+/−} embryo.

Movie 4. Confocal time lapse live imaging of the anterior prosensory domain from 34 hpf to 46 hpf in \textit{jag1b}^{+/−} embryo.
**Movie 5.** Confocal time lapse live imaging of the anterior prosensory domain from 32 hpf to 40 hpf in jag1b<sup>−/−</sup> embryo.

**Movie 6.** Confocal time lapse live imaging of the anterior prosensory domain from 34 hpf to 46 hpf in jag1b<sup>−/−</sup> embryo.
Figure S1. Endogenous *jag1b* mRNA expression in wild type embryos detected by whole-mount in situ hybridization. (A) The endogenous *jag1b* mRNA expression pattern is similar to the EGFP expression pattern in *jag1b* gene trapping heterozygous. (B) *jag1b* mRNA is significantly reduced in *jag1b* homozygous.
Figure S2. EGFP labels three sensory cristae in the inner ear. Phalloidin staining of jag1b+/+ and jag1b+/− embryos at 72 hpf stage. EGFP labels the anterior, lateral, and posterior sensory cristae in jag1b+/− embryos (arrows). The cristae in heterozygous embryos show no difference compared to WT embryos (arrow heads). AC: anterior crista. LC: lateral crista. PC: posterior crista. Lateral views with anterior to the left and dorsal up.
**Figure S3. The anterior and posterior maculae are normal in jag1b⁻/⁻ embryos.** (A) Phalloidin staining of the anterior macula at 48 hpf and 72 hpf. Compared to jag1b⁺/+ and jag1b⁺⁻ embryos, the anterior macula in jag1b⁻/⁻ embryos is normal. Lateral views with anterior to the left and dorsal up. (B) Phalloidin staining of the posterior macula at 48 hpf and 72 hpf. Compared to jag1b⁺/+ and jag1b⁺⁻ embryos, the posterior macula in jag1b⁻/⁻ embryos is normal. Dorsal views with anterior to the left. Scale bar: 40 μm.

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Figure S4. The expression of otic marker genes are similar among jag1b<sup>+/+</sup>, jag1b<sup>+-</sup>, and jag1b<sup>-/-</sup> embryos. (A) Whole-mount in situ hybridization of otic marker genes at 24 hpf stage. The expression of otic maker genes: stm, bmp2b, pax2a, pax5 are similar among jag1b<sup>+/+</sup>, jag1b<sup>+-</sup>, and jag1b<sup>-/-</sup> embryos. (B) Compared to wild type embryos, the expression of bmp4 (arrows) and bmp2b (arrowheads) is downregulated in jag1b<sup>+-</sup> embryos and dramatically reduced in jag1b<sup>-/-</sup> embryos. Scale bar: 20 μm.
Figure S5. fgf10a is expressed in the sensory cristae. (A-C) Whole-mount in situ hybridization of fgf10a in jag1b\(^{+/+}\), jag1b\(^{+-}\), and jag1b\(^{-/-}\) embryos at 48 hpf stage. Probe: fgf10a anti-sense. fgf10a is expressed in the sensory cristae (arrow heads). In jag1b\(^{-/-}\) embryos, the anterior and posterior cristae are lost. (D-E) Whole-mount in situ hybridization of fgf10a in jag1b\(^{+/+}\), jag1b\(^{+-}\), and jag1b\(^{-/-}\) embryos at 30 hpf stage. Probe: fgf10a anti-sense. In jag1b\(^{-/-}\) embryos, fgf10a expression is not affected in the anterior prosensory domain. (G) Sensory cristae development is not affected by Akt inhibitor treatment. Lateral views with anterior to the left and dorsal up.
Figure S6. *fgf8a* is expressed in the anterior pro-sensory domain. (A) Whole-mount in situ hybridization, Probe: *fgf8* anti-sense. *fgf8* is expressed in the anterior prosensory domain at 36 hpf stage. *jag1b*<sup>+/+</sup>, *jag1b*<sup>+/−</sup>, and *jag1b*<sup>−/−</sup> embryos show similar *fgf8a* expression pattern. (B,C) Whole-mount in situ hybridization of *pea3* (B) and *erm* (C) in WT embryos at 39 hpf stage. Probe: *pea3* and *erm* anti-sense. *pea3* and *erm* expression (arrowheads) is up-regulated in the middle region of the anterior prosensory domain. (D) TUNEL assay of DMSO and SU5402 treated embryos. (E) p-Erk3 immunostaining of DMSO and SU5402 treated embryos. Dorsolateral views with anterior to the left and dorsal up.
Figure S7. TUNEL assay of embryos at different stages. Cell death was not detected in the developing anterior prosensory domain of MAPK inhibitor treated embryos. Dorsolateral views with anterior to the left and dorsal up.
Figure S8. MAPK pathway inhibition generates a large crista in jag1b<sup>+/−</sup> embryos. (A-D) DIC confocal imaging of PD0325901 treated jag1b<sup>+/−</sup> embryos at 3 dpf stage. Kinocilia are indicated by arrow heads. AC: anterior crista. LC: lateral crista. (E-H′′) Phalloidin staining of the MAPKK inhibitor PD0325901 treated jag1b<sup>+/−</sup> embryos at 72 hpf stage. The cells in the middle of the anterior prosensory domain of DMSO treated jag1b<sup>+/−</sup> embryos becomes non-sensory epithelial cells (E-E′′′, arrow heads). The cells in the middle of the anterior prosensory domain of PD0325901 treated jag1b<sup>+/−</sup> embryos show similar cell shape with the flanking sensory cells (F-G′′′, arrows), or some of them become sensory hair cells. Lateral views with anterior to the left and dorsal up. Scale bars: 40 μm.
**Figure S9.** MAPK pathway inhibition generates a large crista in *jag1b*+/+ embryos. (A) DIC imaging and phalloidin staining of the MAPKK inhibitor CI-1040 or PD0325901 treated *jag1b*+/+ embryos at 72 hpf stage. Scale bars: 40 μm. Lateral views with anterior to the left and dorsal up.
Supplementary Table 1: Sequence information of primers used in this study

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