Atoh1α expression must be restricted by Notch signaling for effective morphogenesis of the posterior lateral line primordium in zebrafish

Miho Matsuda and Ajay B. Chitnis*

SUMMARY
The posterior lateral line primordium (pLLp) migrates caudally, depositing neuromasts to establish the posterior lateral line system in zebrafish. A Wnt-dependent FGF signaling center at the leading end of the pLLp initiates the formation of ‘proneuromasts’ by facilitating the reorganization of cells into epithelial rosettes and by initiating atoh1α expression. Expression of atoh1α gives proneuromast cells the potential to become sensory hair cells, and lateral inhibition mediated by Delta-Notch signaling restricts atoh1α expression to a central cell. We show that as atoh1α expression becomes established in the central cell, it drives expression of fgf10 and of the Notch ligand deltaD, while it inhibits expression of fgfr1. As a source of Fgf10, the central cell activates the FGF pathway in neighboring cells, ensuring that they form stable epithelial rosettes. At the same time, DeltaD activates Notch in neighboring cells, inhibiting atoh1α expression and ensuring that they are specified as supporting cells. When Notch signaling fails, unregulated atoh1α expression reduces Fgfr1 expression, eventually resulting in attenuated FGF signaling, which prevents effective maturation of epithelial rosettes in the pLLp. In addition, atoh1α inhibits e-cadherin expression, which is likely to reduce cohesion and contribute to fragmentation of the pLLp. Together, our observations reveal a genetic regulatory network that explains why atoh1α expression must be restricted by Notch signaling for effective morphogenesis of the pLLp.

KEY WORDS: mind bomb, Posterior lateral line, Neuromasts, Morphogenesis, Zebrafish, Cadherins, FGF signaling, Lateral inhibition

INTRODUCTION
The lateral line is a sensory system in fish and amphibians that is designed to sense directional water movements (reviewed by Ghysen et al., 2007). It consists of neuromasts, which contain sensory hair cells and surrounding supporting cells. The posterior lateral line primordium (pLLp) migrates under the skin and periodically deposits neuromasts to establish the primary pLL system in zebrafish (see Movie 1 in the supplementary material). The pLL presents an extraordinary system with which to study how a sensory system assembles itself. It is a relatively simple system, the morphogenesis of which is easy to monitor in live embryos. Furthermore, the ability to manipulate and monitor the function of individual signaling pathways in zebrafish embryos has led to a framework for understanding how interactions between Wnt, FGF, Notch and chemokine signaling pathways have a crucial role in directing the self-organization of this system (reviewed by Ma and Raible, 2009).

The migrating pLLp contains three to four ‘proneuromasts’ at various stages of maturation. As each proneuromast matures within the migrating pLLp, it forms a center-oriented epithelial rosette with a central atoh1α-expressing cell (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Eventually, a neuromast is deposited from the trailing end of the migrating primordium. Expression of atoh1α gives central cells the potential to become sensory hair cells, while surrounding cells are specified as supporting cells (reviewed by Ma and Raible, 2009). Supporting cells can proliferate and serve as progenitors that give rise to additional hair cells during growth and regeneration (Ma et al., 2008).

The formation of proneuromasts at the leading end of the migrating pLLp is initiated by two mutually antagonistic signaling centers: a Wnt signaling center in a leading domain and an FGF signaling center in an adjacent trailing domain (reviewed by Ma and Raible, 2009; Aman and Piotrowski, 2008). Establishment of the FGF signaling center is initiated by FGF ligand produced at the leading end in response to Wnt pathway activation. However, Wnt activation also drives expression of an FGFR antagonist, sef (il17rd – Zebrafish Information Network), which prevents local activation of the FGF signaling pathway. Meanwhile, activation of the FGF signaling pathway in the adjacent domain drives the expression of a diffusible Wnt antagonist, Dkk1. This prevents activation of the Wnt pathway from spreading towards the trailing end of the pLLp and establishes coupled, yet mutually inhibitory, Wnt-FGF systems at the leading end of the pLLp.

Once activated, FGF signaling has two crucial functions: it initiates the formation of center-oriented epithelial rosettes and the expression of the bHLH transcription factor Atoh1α (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Atoh1α drives expression of the Notch ligand Delta, which activates its receptor, Notch, in adjacent cells, which in turn inhibits atoh1α expression in these cells. In this manner, ‘lateral inhibition’ ensures that atoh1α expression and hair cell fate are restricted to a central cell, while inhibition of atoh1α in surrounding cells ensures that they are specified as supporting cells. Failure of Notch signaling in this context allows an expansion of atoh1α-expressing cells at the cost of supporting cells (Itoh and Chitnis, 2001).
Previous studies have shown that inhibition of Notch signaling with DAPT (Geling et al., 2002), which prevents γ-secretase-dependent cleavage of Notch, increases the number of atoh1a-expressing cells but does not interfere with the morphogenesis or deposition of neuromasts (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). In addition, inhibition of atoh1a function with morpholinos inhibits hair cell development without affecting rosette formation or neuromast deposition (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). These observations have suggested that neuromast morphogenesis is independent of atoh1a- and Notch-dependent determination of hair cell fate (Lecaudey et al., 2008; Nechiporuk and Raible, 2008).

MIND bomb 1 (Mib1; Mind bomb – Zebrafish Information Network) is a RING E3 ubiquitin ligase that ubiquitylates Notch ligands such as Delta. Ubiquitylation serves as a signal for endocytosis of Delta and is an essential step for effective activation of Notch in a neighboring cell (reviewed by D’Souza et al., 2008; Itoh et al., 2003). mib1 mutants have a broad and severe loss of Notch signaling and have an excess of atoh1a-expressing cells (Itoh and Chitnis, 2001). In this study, we show that Notch signaling has a previously unappreciated role in determining morphogenesis of the pLLp. By comparing mib1 mutants with embryos in which Notch signaling has been inhibited via other manipulations, we show that loss of Notch signaling clearly contributes to the aberrant morphogenesis of the pLLp in mib1 mutants. Our investigation of the mechanism by which Notch influences pLLp morphogenesis has revealed a role for Notch-restricted atoh1a expression in determining the pattern of FGF signaling in maturing neuromasts. It shows how unregulated atoh1a expression has the potential to influence rosette formation, migration and the cohesive properties of cells in the pLLp.

MATERIALS AND METHODS
Fish maintenance and mutant strains
Zebrafish were maintained under standard conditions. The embryos were staged according to Kimmel et al. (Kimmel et al., 1995). The mind bomb, mib6520, mib6512, mib6578 (Itoh et al., 2003; Jiang et al., 1996), notch1a, deadly seven, desb420 (Gray et al., 2001) and tg[cldnb:lynGFP] (Haas et al., 2006) mutant and transgenic strains were described previously. Treatment with 100 µM DAPT (Calbiochem) was started at 20 hpf. SU5402 (Calbiochem) was used at 20 µM.

Morpholino injections
All morpholino injections (1.5 ng each) were performed with co-injection of p53-MO (Robu et al., 2007). MOs were purchased from Gene Tools. Morpholinos used in this study were (5’ to 3’):
notch1a-MO, GAAAACGGTCTAATACTCCGG (Yeo et al., 2007);
notch3-MO, ATATCCCAAAGGCTGTAATTCCCCAT (Yeo et al., 2007);
ato1ha-MO, TCTCTTGTATCCGGTCTATTCCCAT (Millimaki et al., 2007); and
ato1h1b-MO, CATTGGTTGTTAGAATACTCAT (Millimaki et al., 2007).

Whole-mount in situ hybridization
Double in situ hybridization was performed as described previously (Jowett, 2001). RNA probes were synthesized using a DIG or FITC labeling kit (Roche). BM Purple (Roche) or BCIP/NBT substrate (Vector Laboratories) was used for single-labeled embryos. For double-labeled embryos, Fast-Red substrate (Roche) was used as the second chromogen after a 20-minute treatment with 0.1 M glycine pH 2.2. In situ hybridization results were imaged with a CCD camera mounted on a Zeiss Axiosplan2 microscope.

Immunohistochemistry
Immunohistochemistry was performed as described previously (Matsuda and Chitnis, 2009). Antibodies were: mouse anti-zebrafish DeltaD (zdD2, Abcam; dilution 1:400), mouse anti-ZO1 (Zymed; 1:500) and rabbit anti-Cldnb (kindly provided by Dr J. Hudspeth, Rockefeller University, NY, USA) (Lopez-Schier et al., 2004). Images were captured with a confocal microscope (LSM 510META, Carl Zeiss).

RESULTS
Neuromast deposition and pLLP migration are aberrant when Notch signaling is inhibited
We used tg[cldnb:lynGFP] transgenic fish (Haas and Gilmour, 2006), which express membrane-tethered GFP in the pLL system to examine pLLP migration and morphogenesis. Examination of deposited cell clusters in fixed embryos at 48 hours post fertilization (hpf) showed that in wild-type embryos, five neuromasts (L1 to L5) were deposited in a periodic pattern over the trunk, whereas the last three neuromasts (L6 to L8) were located at the tip of the tail where the pLLP normally stops migrating (Fig. 1A,E). In mib1 mutants, the distribution of deposited cell clusters was erratic and closely spaced after deposition of the second neuromast (Fig. 1B,F). Ultimately, pLLP migration stalled about half way to the tip of the tail (Fig. 1B,F). Deposited clusters were of variable size and sometimes two small, adjacent, partially fused rosettes were seen instead of a single, well-formed epithelial rosette (Fig. 1C,D).

Although no problems in initiation of rosette formation were seen at the early stage of pLLP migration (data not shown), by 32 hpf there was a delay in the central accumulation of Claudin b (Cldnb) expression with DAPT (N1a/N3MO/DAPT) eliminating her4 expression (see Fig. S2E in the supplementary material), suggesting that this is an essential step for effective activation of Notch signaling in maturing neuromasts. In wild-type embryos, five proneuromasts (L1 to L5) were deposited in a periodic pattern over the trunk, whereas the last three proneuromasts (L6 to L8) were located at the tip of the tail where the pLLP normally stops migrating (Fig. 1A,E). In mib1 mutants, the distribution of deposited cell clusters was erratic and closely spaced after deposition of the second neuromast (Fig. 1B,F). Ultimately, pLLP migration stalled about half way to the tip of the tail (Fig. 1B,F). Deposited clusters were of variable size and sometimes two small, adjacent, partially fused rosettes were seen instead of a single, well-formed epithelial rosette (Fig. 1C,D).

To determine whether loss of Notch signaling contributes to aberrant morphogenesis, mib1 mutants were compared with embryos in which Notch signaling was inhibited via other manipulations, including DAPT treatment and injection of morpholinos targeting Notch homologs. Three Notch receptors, notch1a, notch1b and notch3, are expressed in the migrating pLLP (see Fig. S1 in the supplementary material). As morpholinos to notch1b caused cell death, only morpholinos for notch1a and notch3 were used to knock down Notch function. To reduce minor side effects resulting from simultaneous injection of two morpholinos, the notch3 morpholinos were injected into notch1a loss-of-function mutants (deadly seven, desb420) (Gray et al., 2001).

Although DAPT treatment or knockdown of notch1a and notch3 function (N1a/N3MO) partially inhibited Notch signaling (Lecaudey et al., 2008; Nechiporuk and Raible, 2008) (data not shown), it did not reduce expression of her4 (see Fig. S2C,D in the supplementary material), a Notch target gene (Takke et al., 1999), the expression of which is lost in the mib1 pLLP (see Fig. S2B in the supplementary material). By contrast, exposing N1a/N3MO embryos to DAPT (N1a/N3MO/DAPT) eliminated her4 expression (see Fig. S2E in the supplementary material), suggesting that this combinatorial manipulation reduces Notch signaling in a manner that is more comparable to that in mib1 mutants. Whereas pLLP morphogenesis was not significantly affected when embryos were...
only treated with DAPT or had Notch1a and Notch3 function reduced (data not shown), phenotypes similar to those seen in mib1 mutants emerged when these manipulations were combined (Fig. 1G-I). These observations suggest that problems with pLLp morphogenesis can emerge when inhibition of Notch signaling is severe enough to eliminate her4 expression.

Time-lapse imaging starting at 28 hpf showed that the erratic distribution of cell clusters deposited relatively early during pLLp migration in mib1 embryos was in part due to poor cohesion of cells in depositing proneuromasts. Cells would often dissociate from a trailing proneuromast and then try to reassociate to form cell clusters with previously deposited cells (see Fig. S4B,E and Movie 2 in the supplementary material). This early defect in cell cohesion was not observed in N1a/N3MO/DAPT embryos (data not shown). However, time-lapse starting relatively late, at 32 hpf, showed that in both mib1ta52b and N1a/N3MO/DAPT embryos, ~3-5 hours after deposition of the second neuromast (L2), the migrating pLLp undergoes sudden fragmentation (Fig. 2B,C; see Movies 3, 4 in the supplementary material). This fragmentation accounts for the erratic distribution of cell clusters deposited relatively late during the migration. Following this fragmentation, a small fragment of the pLLp (Fig. 2B,C, bracket) continued to migrate as a much reduced collection of cells, until it too stopped migrating, suggesting that pLLp fragmentation contributes to the eventual failure of migration.

**Notch signaling regulates the expression of FGF signaling components**

In mib1 mutants, atoh1a expression is not effectively restricted to a central cell in maturing proneuromasts. Instead, its expression progressively expands to large cell clusters (termed 1° expansion) and then eventually to most of the cells within the migrating pLLp (termed 2° expansion) (Fig. 3A; see Fig. S5 in the supplementary material).

Expression of fgf10 overlaps with atoh1a in maturing proneuromasts at the trailing end of the pLLp, where expression is typically restricted to a central cell in the wild-type pLLp (see Fig. S6A,B in the supplementary material). Consistent with their correlated expression, mib1 mutants showed similar 1° and 2° expansion of fgf10 expression (Fig. 3B; see Fig. S5 in the supplementary material).
supplementary material). Furthermore, correlated expansion of atoh1a and fgf10 was seen in desb420/N3MO/DAPT embryos (see Fig. S7A-D in the supplementary material), confirming that expansion of both atoh1a and fgf10 can result from loss of Notch signaling. Their correlated expression is consistent with atoh1a being responsible for driving fgf10 expression in maturing neuromasts.

Knocking down atoh1a and atoh1b function with morpholinos reduced fgf10 expression at the trailing end of the pLP (see Fig. S6C,D in the supplementary material), confirming that, as shown previously (Nechiporuk and Raible, 2008), atoh1 determines fgf10 expression in maturing neuromasts.

**Expansion of atoh1a is accompanied by a complementary loss of fgfr1 and FGF signaling**

Although there is some overlap in their expression, fgf10 and fgfr1 are typically expressed in complementary patterns in the pLP. As fgf10 expression becomes restricted to a central cell in the maturing neuromasts, fgfr1 is expressed in surrounding cells and reduced in the central cell (Lecaudey et al., 2008). This complementary relationship becomes more obvious in mib1ta52b (Fig. 3B,C) and desb420/N3MO/DAPT embryos (see Fig. S7C-F in the supplementary material), in which expansion of central fgf10-expressing cells in maturing neuromasts is accompanied by a reduction of fgfr1-expressing cells. Initially, fgfr1 expression was reduced in small clusters of cells (1° reduction). Eventually, it was broadly reduced in the pLP and fgfr1 expression was restricted to a few cells at its edges (2° reduction) (Fig. 3C; see Fig. S5 in the supplementary material).

Progressive reduction in fgfr1 expression was accompanied by a reduction in expression of the FGF target gene pea3 (Lecaudey et al., 2008; Nechiporuk and Raible, 2008) in mib1ta52b (Fig. 3D; see Fig. S5 in the supplementary material) and desb420/N3MO/DAPT (see Fig. S7H in the supplementary material) embryos. This suggests that progressive expansion of atoh1a is associated with
loss of fgfr1 expression and progressive attenuation of FGF signaling centers, until they are both dramatically weakened and disorganized within the maturing neuromasts. As FGF signaling is required to maintain fgfr1 expression (Lecaudey et al., 2008; Nechiporuk and Raible, 2008), it is likely that loss of FGF signaling contributes to further loss of fgfr1 expression within maturing neuromasts.

**Loss of FGF signaling allows expansion of Wnt signaling towards the trailing end**

**dkk1** is expressed in an FGF signaling-dependent manner in the youngest proneuromast near the leading end of the pLLp. As a result, failure of FGF signaling was accompanied by a progressive reduction of **dkk1** expression in **mib1** mutants (Fig. 4A; see Fig. S8A in the supplementary material) and **des**/N3MO/DAPT (see Fig. S8A and Fig. S9A in the supplementary material) embryos between 27 and 35 hpf. Embryos were classified as having strong, weak or no **dkk1** expression in the pLLp. At 27 hpf, ~30% of the pLLps had no **dkk1** expression, by 31 hpf **dkk1** expression was lost in 45%, and by 35 hpf, which is close to the time when the pLLp typically falls apart, **dkk1** expression was lost in all the embryos examined.

To determine whether loss of the Wnt inhibitor **dkk1** causes expanded Wnt signaling, we examined **lef1** expression, which is dependent on Wnt signaling in the pLLp (Aman and Piotrowski, 2008). The size of the **lef1** domain was significantly larger in **mib1** mutants than in the wild type at 27, 31 and 35 hpf (Fig. 4B; see Fig. S8B in the supplementary material). Similar expansion of **lef1** expression was seen in **des**/N3MO/DAPT embryos (see Fig. S9B in the supplementary material).

**The altered balance of FGF and Wnt signaling reduces expression of Cxcr7b in the pLLp**

The polarized expression of two chemokine receptors, **cxcr4b** and **cxcr7b**, is essential for effective migration of the pLLp (Dambly-Chaudiere et al., 2007; Haas and Gilmour, 2006; Valentini et al., 2007). As previous studies suggested that Wnt signaling promotes **cxcr4b** expression and suppresses **cxcr7b** expression in the pLLp (Aman and Piotrowski, 2008), we hypothesized that the altered balance of Wnt and FGF signaling would alter their expression and contribute to aberrant migration of the pLLp. In both **mib1** mutants and **des**/N3MO/DAPT embryos there was a loss of **cxcr7b** expression (Fig. 4D; see Fig. S7 in the supplementary material), consistent with reduced FGF signaling and/or expanded Wnt activity contributing to reduced **cxcr7b** expression. At the same time, loss of **cxcr7b** expression was not accompanied by a significant change in **cxcr4b** expression (Fig. 4C), suggesting that additional factors prevent expansion of **cxcr4b** expression in the trailing end of the pLLp.

**Knockdown of atoh1 function can prevent progressive loss of FGF signaling in mib1 mutants**

As unregulated expansion of **atoh1** following loss of Notch signaling appears to have triggered a chain of events that reduces FGF activity, expands Wnt activation and alters expression of chemokine receptors, we hypothesized that inhibition of atoh1 function would prevent some of these changes. atoh1a and atoh1b morpholino (atoh1MOs) co-injection in mib1 mutants successfully prevented unregulated expansion of fgf10 expression (Fig. 5A) and allowed some recovery of fgfr1 and pea3 expression (Fig. 5B; see Fig. S10 in the supplementary material). It also prevented loss of **dkk1** expression (Fig. 5C; see Fig. S11 in the supplementary material) and allowed a partial recovery of **cxcr7b** expression (Fig. 5D).

**Inhibition of atoh1 function reduces defects in pLLp morphogenesis in mib1 mutants**

We next asked whether reduction of atoh1 function also prevents any of the problems with pLLp morphogenesis in mib1 mutants. atoh1 knockdown appeared to reduce all the defects that develop relatively late during migration in association with progressive expansion of atoh1a: it restored formation of the epithelial rosettes within the pLLp (compare Fig. 6B with 6D) and the pLLp migrated further without falling apart (Fig. 6F,H,K,L; see Fig. S3 in the supplementary material). Knockdown of atoh1 did not, however, restore cohesion of cells in depositing proneuromasts, a defect that develops in mib1 mutants relatively early during pLLp migration. Instead, it resulted in further loss of cohesion; cells that disassociated from depositing proneuromasts no longer showed a tendency to try and reassociate with previously deposited cells (see Fig. S4C,F and Movie 5 in the supplementary material). Importantly, knockdown of atoh1 also slightly reduced the size of the depositing neuromasts in wild-type embryos (see Fig. S12 and Movie 6 in the supplementary material). Although it remains unclear why deposited neuromasts are smaller in atoh1 morphants, one possibility is that atoh1a and mib1 independently contribute to cohesion, such that cohesion in depositing neuromasts is worse in mib1 mutants when atoh1a is knocked down.
Knockdown of atoh1 promotes Wnt-dependent lef1 expression

Although knockdown of atoh1 prevented loss of the FGF signaling system and restored FGF-dependent morphogenesis of epithelial rosettes and pLLp migration defects, it did not reduce the size of the lef1 expression domain in mib1 mutants. Instead, lef1 expression became even broader in atoh1MOs-injected embryos (Fig. 5E; see Fig. S13 in the supplementary material). This suggests that, although recovery of the pLLp system in atoh1MOs-injected mib1 mutants might have allowed some recovery of FGF signaling, there was no corresponding reduction in the size of the Wnt signaling domain.

As the atoh1-dependent FGF signaling system becomes a new source of FGF signals in maturing proneuromasts, it may inhibit persistence of the initial Wnt-dependent FGF signaling system. In atoh1 morphants, however, a failure to establish the atoh1-dependent FGF system might allow the initial Wnt-dependent FGF signaling systems to persist within maturing proneuromasts and account for the expanded lef1 expression. Consistent with this interpretation, fgf3, a component of the original Wnt-FGF system, continued to be expressed in the central cell of depositing and recently deposited neuromasts (Fig. 5F,G) when atoh1 function was knocked down. It is likely that in the absence of atoh1-dependent fgf10 expression, persistent fgf3 expression maintains FGF signaling in maturing proneuromasts.

Changes in pLLp cohesion correlate with changes in Cadherin gene expression

As the pLLp eventually undergoes fragmentation in the absence of Notch signaling, we asked whether this is related to changes in expression of epithelial cadherin (e-cadherin or cadherin 1 (cdh1)) and neural cadherin (n-cadherin or cadherin 2 (cdh2)), two cadherins that are expressed in the pLL system (Liu et al., 2003; Kerstetter et al., 2004) and are likely to contribute to cohesive interactions between cells.

In wild-type embryos, e-cadherin was at relatively low levels in the leading part of the pLLp, while expression became progressively higher at the trailing end and was relatively intense in depositing neuromasts (Fig. 7A,E). However, e-cadherin expression was relatively low in the central atoh1a-expressing cell in deposited neuromasts, suggesting that its expression is inhibited in these cells (Fig. 7L). Although there was no obvious change in e-cadherin expression at 33 hpf (see Fig. S14B in the supplementary material), e-cadherin expression was much reduced in the trailing domain of the mib1m178 pLLp by 37 hpf (Fig. 7B,F). In addition, the central e-cadherin-negative domain expanded in deposited neuromasts in mib1m178 mutants (Fig. 7J,M), suggesting that expanded atoh1a expression is eventually associated with reduced e-cadherin expression in both the pLLp and deposited neuromasts. Consistent with atoh1 being associated with inhibition of e-cadherin expression, atoh1MOs injection expanded e-cadherin expression to all the cells within the deposited neuromast in mib1 mutants (Fig. 7K,N). Whereas atoh1 knockdown did not significantly alter the overall e-cadherin expression pattern in the wild-type pLLp (Fig. 7C,G), it restored higher, wild-type-like expression of e-cadherin in the mib1 pLLp (Fig. 7D,H). The recovery of e-cadherin expression was accompanied by improved cell cohesion in the mib1 pLLp, suggesting that the reduction in e-cadherin might contribute to the eventual fragmentation of the pLLp.

In contrast to e-cadherin, n-cadherin was broadly expressed at the leading end of the pLLp, and its expression became restricted to a central domain in mature proneuromasts as they prepared for deposition (Fig. 7O,Q). It then continued to be expressed in a central cluster of cells in deposited neuromasts (Fig. 7S). Although n-cadherin expression overlapped with that of atoh1a in the central cell, it was generally broader and included surrounding cells, which were most likely to be prospective support cells that do not express atoh1a (Fig. 7T). Although the pattern of n-cadherin expression was little changed in mib1 and desb200/N3MO/DAPT embryos...
Distinct regulatory mechanisms regulate Delta and atoh1a expression at the leading and trailing ends of the pLLp

DeltaA and DeltaD contribute to the Delta-Notch-mediated lateral inhibition that maintains restricted atoh1a expression in maturing proneuromasts. Although their expression patterns are similar, we found that they are differentially regulated by atoh1a. Whereas knockdown of atoh1a dramatically reduced deltaD expression, it had much less effect on deltaA (compare Fig. 8A with 8B; see Fig. S1 in the supplementary material for deltaD expression in wild-type embryos), suggesting that atoh1a does not contribute as much to deltaA as to deltaD expression. Expression of deltaA was, however, progressively eliminated after 1-2 hours of exposure to the FGF signaling inhibitor SU5402 (Fig. 8C), suggesting that FGF signaling is essential for deltaA expression. These observations suggest that once atoh1a expression is established, it drives expression of deltaD, and then both DeltaA and DeltaD ensure that atoh1a expression remains restricted to the central cell.

As Atoh1a assumes control of fgf10 and deltaD expression and inhibits fgfr1 expression in maturing proneuromasts, we asked whether Atoh1a also becomes more responsible for its own expression and less dependent on FGF signaling. When embryos were exposed to SU5402, atoh1a expression was lost in the leading domain but retained in the trailing proneuromast following 15 minutes of exposure (Fig. 8D, arrowhead), suggesting that expression in the most mature proneuromast is less dependent on FGF signaling. However, atoh1a expression was eventually lost from both the leading and trailing ends of the pLLp by 30 minutes, revealing its continuing dependence on FGF signaling (Fig. 8D).

Mature proneuromasts at the trailing end of the pLLp express atoh1b, suggesting that atoh1b might help maintain atoh1a expression in these proneuromasts. Consistent with this, knockdown of atoh1b specifically reduced atoh1a expression at the trailing end of the pLLp (Fig. 8E, top). Furthermore, atoh1a expression was lost from both the leading and trailing ends of the pLLp within just 15 minutes following exposure to SU5402 when atoh1b function was also knocked down (Fig. 8E, bottom; compare with Fig. 8D, middle), showing that normally both FGF signaling and atoh1b contribute to maintaining atoh1a expression.

Consistent with atoh1a determining atoh1b expression, expanded atoh1a expression in mib1<sup>15626</sup> mutants was accompanied by expanded atoh1b expression in the trailing proneuromast (compare Fig. 8F with 8G, top), whereas atoh1b expression was completely lost in mib1<sup>15626</sup> embryos injected with atoh1aMO (Fig. 8G, bottom). Interestingly, knockdown of atoh1a broadened its own expression at the leading end of the pLLp (Fig. 8H), as loss of atoh1a-dependent deltaD expression reduced lateral inhibition. However, atoh1a expression was not maintained at the trailing end of the pLLp following atoh1a knockdown, as self-activation via atoh1b is required to maintain high levels of atoh1a expression.
DISCUSSION
In this study we have characterized defects in the morphogenesis of the pLLp system in mib1 mutants and shown that loss of Notch signaling allows unregulated atoh1a expression in the pLLp. This in turn eventually alters FGF, Wnt and chemokine signaling and Cadherin expression, all of which contribute in distinct ways to the aberrant morphogenesis of the pLL system.

Key to understanding how loss of Notch signaling affects pLLp morphogenesis was the discovery that two distinct FGF signaling systems operate in proneuromasts at the leading versus trailing end of the pLLp. Previous studies have shown that a Wnt-dependent FGF signaling system initiates proneuromast formation at the leading end of the pLLp by driving atoh1a expression and epithelial rosette formation (Fig. 9A). Our studies suggest that this...
FGF signaling system initiates expression of both atoh1a and the Notch ligand deltaA. In the context of this early expression, DeltaA activates Notch in neighboring cells, inhibits atoh1a expression and restricts initial atoh1a expression to a central cell. Atoh1a then initiates deltaD expression and together DeltaA and DeltaD mediate lateral inhibition to maintain restricted atoh1a expression.

Once atoh1a expression is stabilized in maturing neuromasts, we suggest that it establishes a second atoh1a-dependent FGF signaling system. Atoh1a drives expression of fgf10 and prevents fgfr1 expression (Fig. 9A,B, blue cells). Absence of atoh1a expression allows the surrounding cells to express fgfr1 (Fig. 9A,B, yellow cells). Fgf10 expressed by a central cell then activates Fgfr1 in its surrounding cells, ensuring that they maintain expression of Fgfr1 and Notch3, as expression of both is dependent on FGF signaling (data not shown). This ensures that surrounding cells remain competent to receive Delta and FGF signals from the central cell. By contrast, relatively low FGF signaling in the central cell (Fig. 9A,B, blue cells) prevents it from maintaining fgfr1 and notch3 expression, which is likely to reduce FGF and Notch pathway activation. As FGF signaling reduces in the central atoh1a-expressing cell, atoh1a expression becomes progressively less dependent on FGF signaling and more dependent on self-activation: atoh1a drives atoh1b expression, which in turn drives atoh1a expression (Fig. 9A,B, blue cells).

Formation of the atoh1a-dependent FGF signaling system in maturing neuromasts establishes a new vulnerability in the pLLp. Maintenance of stability in this system depends on effective Notch-mediated lateral inhibition. When Notch signaling is reduced, atoh1a expression can be induced in neighboring cells by FGF signaling (Fig. 9B, black cells). These cells express fgf10 (Fig. 9B, blue cells) and induce atoh1a in their neighbors, eventually resulting in progressive expansion of both atoh1 and fgf10 expression. This expansion is accompanied by a complementary loss of fgfr1 expression (Fig. 9B, yellow cells), a reduction in FGF signaling and a failure to maintain focal FGF signaling centers within maturing proneuromasts. It is important to note that the expansion of atoh1a expression and subsequent reduction of FGF signaling is progressive. Eventually, a tipping point is reached, resulting in breakdown of the pLL system. The timing of this event is variable and likely to depend on the severity of loss of Notch signaling. Inhibition of Notch signaling by DAPT alone or reduction of Notch function also results in expansion of atoh1a expression and reduction of fgfr1 expression (data not shown). However, we suspect that fragmentation of the system typically does not take place in this situation because the progressive event takes longer to develop and the pLLp might reach its destination before the ‘catastrophe’ develops. In principle, it is possible that DAPT contributes to the defects in pLLp morphogenesis by influencing the function of γ-secretase substrates other than Notch, such as Cadherins (Park et al., 2008). However, inhibition of Notch signaling is likely to be the most relevant mechanism, as development of severe morphological defects only accompanies manipulations that also cause loss of Notch-dependent her4 expression and many of the changes can be linked to the unregulated atoh1a expression that results from loss of Notch signaling.

Cells at the leading end actively direct caudal migration of the pLLp, and collective migration of pLLp cells critically depends on the cohesive properties of the cells. We suggest that the loss of e-cadherin expression that follows the unregulated expansion of atoh1a-expressing cells reduces cohesion and contributes to the eventual fragmentation of the pLLp. This is supported by the observation that knockdown of atoh1 is accompanied by both a recovery of e-cadherin expression and of cohesive integrity of the migrating pLLp. A decrease in e-cadherin expression, especially when accompanied by a relative increase of n-cadherin expression, has previously been associated with the epithelial-mesenchymal transition that takes place when cancer cells undergo a metastatic transformation (reviewed by Cavallaro and Christofori, 2004). In a similar manner, loss of e-cadherin may result in a loss of epithelial integrity and in a failure to maintain the cohesive interactions required for collective migration of the pLLp. Of course, additional adhesion factors similarly affected by atoh1a expression might also contribute to pLLp cohesion.

Whereas e-cadherin is specifically reduced/excluded from the central atoh1a-expressing cell (Fig. 7W, blue cells), n-cadherin is normally expressed in a central cell cluster (Fig. 7W, red cells), including both the central atoh1a-expressing cell and surrounding prospective support cells that are prevented from expressing...
This implies that support cells express both e-cadherin and n-cadherin (Fig. 7W, purple cells) and could potentially form effective adhesive junctions with both outer non-sensory cells that express only e-cadherin (Fig. 7W, blue cells) and inner sensory hair cell precursors that express only n-cadherin (Fig. 7W, red cells). In the absence of Notch signaling, as prospective support cells express atoh1a and are mis-specified as hair cell precursors, they would lose e-cadherin expression and no longer make effective junctional complexes with surrounding e-cadherin-expressing cells (Fig. 7W, no purple cells in maturing proneuromasts). This could ultimately contribute to pLLp fragmentation.

The possibility that support cells provide an essential adhesive link between sensory hair cells and surrounding non-sensory cells first emerged from ultrastructural analysis of the ear in zebrafish mib1 mutants, in which the sensory hair cell population expands at the cost of surrounding support cells (Haddon et al., 1999). This study showed that the expanded population of hair cells is expelled from the ear epithelium because effective junctional complexes do not form between hair cells and surrounding non-sensory cells in the absence of support cells. It was suggested that two distinct homophilic adhesion proteins might determine cohesive interactions between hair cells and between non-sensory cells. By expressing both of the homophilic adhesion proteins, support cells could form adhesive interactions with both hair cells and the surrounding outer non-sensory cells, and in this manner serve as the adhesive link between these two cell populations. Our analysis of e-cadherin and n-cadherin expression suggests a very similar role for support cells in the pLLp system and provides preliminary evidence for the predicted pattern of homophilic adhesion factor expression in hair cells, support cells and surrounding non-sensory cells.

Having shown that unregulated expansion of atoh1a expression can result in a cascade of defects that contribute to aberrant pLLp morphogenesis in mib1 mutants, we showed that inhibition of atoh1a function prevents many of these problems. This suggests that although atoh1a normally assumes significant control of FGF signaling in proneuromasts at the trailing end of the pLLp, the establishment of the atoh1a-dependent FGF signaling system is completely dispensable for many aspects of pLLp morphogenesis. What, then, is the significance of the atoh1a-dependent FGF signaling center?

Examination of hair cell precursors with DeltaD antibody shows that the specified sensory hair cell is always precisely at the center of the maturing neuromast (see Fig. S15 in the supplementary material). By determining both the sensory hair cell fate and morphogenesis of center-oriented epithelial rosettes, we suggest that the atoh1a-dependent FGF signaling system provides a robust mechanism to coordinate cell fate and morphogenesis in maturing neuromasts. The remarkable potential of neuromasts for regulated growth and

Fig. 9. Establishment of a focal FGF signaling center by atoh1 in maturing proneuromasts. (A) Wnt activation (red) drives FGF and sef expression, prevents local FGF activation and promotes FGF signaling (green) in an adjacent domain. FGF activation drives expression of the Wnt antagonist dkk1 and of atoh1a and deltaA. Delta activates Notch in neighboring cells to restrict atoh1a expression to a central cell (black). atoh1a drives expression of fgf10 (blue) and inhibits fgfr1 (yellow), establishing a focal FGF signaling system. (B) In the absence of Notch signaling, additional cells express atoh1a and fgf10, then shut off fgfr1. This results in progressive loss of active FGF signaling, loss of dkk1, and expansion of Wnt activation. (C) Detail of interactions between the central Atoh-expressing cell (blue) and surrounding cells (yellow). Atoh1a-Atoh1b cross-regulation helps maintain atoh1 expression in the central cell. Delta and FGF activate Notch and Fgfr1 in surrounding cells. Fgf10 expression is maintained by autoregulation. FGF activation maintains notch3 expression. Notch inhibits atoh1a expression in surrounding cells. (D) In the absence of Notch activation, surrounding cells (yellow) initiate atoh1a expression; this initiates FGF expression, suppresses Fgfr1 expression, reduces FGF signaling and eventually leads to loss of Notch3 expression.
regeneration emerges from the capacity of the central cells to serve as a source of FGF signals that can induce further atoh1a expression in neighbors. The specification of additional atoh1a-expressing cells remains, however, under tight regulation by Notch signaling, which determines when and how many atoh1a-expressing cells are induced by FGF signaling.

Simultaneous DAPT treatment and Notch gene knockdown does not recapitulate the very early defects in cohesion observed in cells of depositing proneuromasts in mihi^{65b} mutants (see Fig. S4 in the supplementary material). One interpretation is that these early defects reflect a quantitative difference in the severity of Notch signaling rather than a qualitatively distinct phenomenon. However, additional proteins that are functionally regulated by Mib1 might contribute to defects in cohesion. Additional Mib1-interacting proteins have been identified both by us and other groups (Jin et al., 2002; Choe et al., 2007; Ossipova et al., 2009) (our unpublished data). We are now investigating their function with a view to identifying additional mechanisms by which Mib1 might regulate cell cohesion during epithelial morphogenesis.

It should be noted that although this study focuses on changes within the pLP that contribute to its aberrant morphogenesis in mihi mutants, there are changes along its migratory path that are also likely to contribute. For example, there is an increase in the expression of the chemokine sdf1a (cxcl12a – Zebrafish Information Network) along its migratory path (see Fig. S16 in the supplementary material), which is also likely to influence pLP migration.

In summary, our study has identified a role for Notch-restricted atoh1a expression in establishing a focal FGF signaling center in maturing proneuromasts. It emphasizes the importance of understanding the interconnection of different signaling systems within a developing organ. It also illustrates how changes in the regulatory network within the pLP contribute to the self-organization of neuromasts as they form sensory organs with the potential for growth and regeneration, and how the failure of a key node in the genetic network can have wide-ranging effects on cell fate and cell behavior in the pLP system.

Acknowledgements
We thank James Hudspeth, Rockefeller University, NY, USA, for providing anti-Claudin b antibody, Motoyuki Itoh for preliminary exploration of these questions, and all members of the A.B.C. laboratory for their comments. This study was supported by the Intramural Research Program of the NIH/NINDS. Deposited in PMC for immediate release. This article is freely available online from the date of publication.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.052761/-/DC1

References
Abramoff, M. D., Magelhaes, P. J. and Ram, S. J. (2004). Image processing with ImageJ. Biophotonics Int. 11, 36-42.


Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. and Haass, C. (2003). Delta-associated protein kinase (DAPK)-interacting protein, DIP-1, is an E3 ubiquitin ligase that promotes tumor necrosis factor-induced apoptosis and regulates the cellular levels of DAPK. J. Biol. Chem. 277, 46980-46986.


