Lef1 is required for progenitor cell identity in the zebrafish lateral line primordium

Hillary F. McGraw, Catherine M. Drerup, Maya D. Culbertson, Tor Linbo, David W. Raible and Alexei V. Nechiporuk

SUMMARY

The zebrafish posterior lateral line (pLL) is a sensory system that comprises clusters of mechanosensory organs called neuromasts (NMs) that are stereotypically positioned along the surface of the trunk. The NMs are deposited by a migrating pLL primordium, which is organized into polarized rosettes (proto-NMs). During migration, mature proto-NMs are deposited from the trailing part of the primordium, while progenitor cells in the leading part give rise to new proto-NMs. Wnt signaling is active in the leading zone of the primordium and global Wnt inactivation leads to dramatic disorganization of the primordium and a loss of proto-NM formation. However, the exact cellular events that are regulated by the Wnt pathway are not known. We identified a mutant strain, lef1nl2, that contains a lesion in the Wnt effector gene lef1. lef1nl2 mutants lack posterior NMs and live imaging reveals that rosette renewal fails during later stages of migration. Surprisingly, the overall primordium patterning, as assayed by the expression of various markers, appears unaltered in lef1nl2 mutants. Lineage tracing and mosaic analyses revealed that the leading cells (presumptive progenitors) move out of the primordium and are incorporated into NMs; this results in a decrease in the number of proliferating progenitor cells and eventual primordium disorganization. We concluded that Lef1 function is not required for initial primordium organization or migration, but is necessary for proto-NM renewal during later stages of pLL formation. These findings revealed a novel role for the Wnt signaling pathway during mechanosensory organ formation in zebrafish.

KEY WORDS: Lef1, Lateral line primordium, Progenitor cells, Zebrafish

INTRODUCTION

Collective cell migration is defined as a directional migration of interconnected groups of cells. This process is important during organogenesis and is also displayed by invasive groups of metastatic cells in some cancers (Friedl et al., 2004; Yilmaz and Christofori, 2010). The lateral line (LL) system of zebrafish has proven to be an attractive model for the study of collective cell migration, as it is formed by a migrating group of cells close to the surface of the animal, which makes it accessible to various experimental manipulations (Dambly-Chaudiere et al., 2003; Perlin and Talbot, 2007). The LL system, which senses changes in water currents, consists of discrete mechanosensory organs (neuromasts; NM) distributed across the surface of zebrafish. Each NM is comprised of mechanosensory hair cells and surrounding support cells (Aman and Piotrowski, 2009; Ma and Raible, 2009). The posterior LL (pLL) is formed during the first few days of zebrafish embryonic development by the placode-derived pLL primordium. The pLL primordium consists of ~100 cells that collectively migrate caudally along the trunk between 22 and 48 hours post-fertilization (hpf), depositing NMs every 5 to 7 somites (Ghysen and Dambly-Chaudiere, 2004; Ghysen and Dambly-Chaudiere, 2007; Sarrazin et al., 2010). During migration, cells in the trailing (rostral) region of the primordium are organized into polarized rosettes (proto-NMs) that will be deposited as NMs. After deposition, a new rosette is formed in the leading (caudal) region of the pLL primordium from a small population of proliferating progenitor cells (Nechiporuk and Raible, 2008). At this time the mechanisms regulating progenitor specification and renewal in the primordium are unknown.

The patterning and migration of the primordium are regulated by a network of signaling pathways, including canonical Wnt and FGF (Ma and Raible, 2009). Wnt signaling is active in the leading third of the primordium, which contains progenitor cells and newly forming rosettes. Direct Wnt targets, lef1 and axin2, are expressed in this leading region (Aman and Piotrowski, 2008). Expression of the Wnt inhibitor dkk1 in the middle region of the primordium, where polarized cells condense into rosettes, restricts Wnt activity to the leading zone. Both loss and overexpression of Wnt signaling lead to disruptions in rosette organization and NM deposition (Aman and Piotrowski, 2008). Active Wnt signaling is necessary for the expression of two FGF ligands, fgf3 and fgf10a, in the leading part of the primordium. The transcript encoding the FGF receptor, fgfr1, is expressed in the trailing zone, where activation of FGF signaling is required for proper patterning and migration of the primordium (Aman and Piotrowski, 2008; Lecadet et al., 2008; Matsuda and Chitnis, 2010; Nechiporuk and Raible, 2008). Overstimulation of Wnt pathway or loss of FGF signaling leads to abnormal primordium patterning, including expansions of leading zone markers (fgf10a, lef1 and axin2) and the loss of a trailing marker (pea3) (Aman and Piotrowski, 2008). However, the exact cellular events regulated by these pathways and how they coordinate to accomplish rosette renewal and NM deposition are not known.

Using a forward genetic approach, we sought factors required for rosette renewal during primordium migration. Mutagenesis screening yielded the lef1nl2 mutant, which exhibits premature truncation of the pLL and loss of terminal NMs. In lef1nl2 mutants, primordium migration and NM deposition begin normally, but over time the
primordium fails to generate new rosettes and becomes disorganized leaving a small trail of cells beyond the distal-most NM. Positional cloning revealed that left$^{nl2}$ is a mutation in left1, an effectors and target of Wnt signaling. Using live imaging, cell transplantation and lineage analyses, we show that left1 functions to regulate the identity of progenitor cells in the leading edge of the primordium.

**MATERIALS AND METHODS**

**Zebrafish strains**

Adult zebrafish were maintained under standard conditions. Embryos from AB and WIK adults were staged according to standard protocols (Kimmel et al., 1995). The pLL primordium and nerve were visualized using the Tg(–8.0cldhb:lynGFP)$^{2010}$ line (Haas and Gilmour, 2006) and the TgBAC(neurod:EGFP)$^{21}$ line (Olshozer et al., 2008), respectively. Wnt/β-catenin signaling was conditionally inhibited using the Tg(hsp70l:dkk1-GFP)$^{21}$ line (Stoick-Cooper et al., 2007).

**Mutagenesis screen and genetic mapping**

The left$^{nl2}$ mutation was identified in a three-generation N-ethyl-N-nitrosourea (ENU) mutagenesis screen (Mullins et al., 1994; Mullins and Nusslein-Volhard, 1993). Larvae were screened at 4 dpf for loss of function mutations using 2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI; Invitrogen) according to the established protocol (Harris et al., 2003).

**Heat-shock conditions and morpholinino injection**

The Tg(hsp70l:dkk1-GFP)$^{+}$-positive embryos were heat-shocked at 28 hpf for 30 minutes at 39°C using a Thermo Cycler (BioRad). For heat-shock conditions, a heat shock was administered to each embryo's left side, whereas the right side served as control.

**Time-lapse imaging and Kaede photoconversion**

For time-lapse imaging, embryos were anesthetized in 0.02% tricaine (MS-222; Sigma), embedded in 1.2% low-melting point agarose and imaged using a 200×/NA=0.95 dipping lens on an FV1000 (Olympus) confocal system for 12-14 hours with z-stacks collected at 6-minute intervals. Images were processed using ImageJ software. The progeny from left$^{nl2}$ heterozygous males that also contained the Tg(–8.0cldhb:lynGFP) and the TgBAC(neurod:EGFP) transgenes were injected into NLS-Kaede mRNA (Anido et al., 2002). The Kaede fluorophore was photo-converted between 22 and 24 hpf in one to four cells using 405 nm laser and 60×/NA=1.2 lens. Subsets of embryos were subjected to time-lapse imaging as described above. The remaining embryos were assessed for the number and location of red cells at 48 hpf.

**TUNEL labeling**

TUNEL labeling was carried out according to an established protocol modified for fluorescent detection (Nechiporuk et al., 2005). For global Wnt inactivation, Tg(hsp70l:dkk1-GFP)$^{+}$ embryos were heat-shocked at 28 hpf for 2 minutes at 38°C using a Thermo Cycler (BioRad) and fixed in 4% PFA at 34 hpf.

**Dermal bone and cartilage staining**

Adult left$^{nl2}$ mutants and wild-type siblings of comparable size were collected at 3 months post-fertilization. Alizarin Red and Alcian Blue staining was used to label cartilage according to established protocols (Elizondo et al., 2005).

**RESULTS**

**Loss of rosette renewal leads to the truncation of the pLL in the left$^{nl2}$ mutant**

The left$^{nl2}$ mutation was isolated in an ongoing ENU-based mutagenesis screen designed to identify recessive mutations with defects in pLL formation. The left$^{nl2}$ mutation was isolated based...
on a lack of caudal-most NMs as visualized by DASPEI, a vital dye that labels hair cells (see Fig. S1A,B in the supplementary material). Deposited NMs in the leftnl2 mutant contained similar numbers of hair and support cells when compared with wild-type siblings (see Fig. S1E,F in the supplementary material). Expression of the lateral line marker eya1 revealed that in leftnl2 mutants, the first 5 pLL NMs were deposited along the trunk, though at a more anterior axial level than wild-type NMs (Fig. 1A-D). However, the terminal cluster (tc) of NMs at the end of the tail was invariably absent in leftnl2 mutants and only a small trail of cells extended distally from the last deposited NM (Fig. 1A’). Because the loss of NMs often results from pLL primordium abnormalities, we examined primordium organization during migration through time-lapse imaging in leftnl2 mutants and wild-type siblings expressing the Tg(–8.0cldnb:lynGFP) transgene between 34 and 48 hpf (Fig. 1E). In a wild-type embryo, the migrating primordium deposited three trunk NMs and the tc at the end of the trunk (Fig. 1E’; see Movie 1 in the supplementary material). NM deposition was closely coupled to rosette renewal; a new rosette formed shortly after the deposition of each NM (Fig. 1E’’. Movie 1 in the supplementary material). In a leftnl2 mutant embryo, the pLL primordium contained three or four rosettes between 34 and 48 hpf and deposited the 4th and 5th NMs. The primordium became progressively smaller and failed to form new rosettes as the NMs were dropped off, leaving only a narrow trail of cells that migrated a short distance distal to the last NM (Fig. 1A’’. Movie 2 in the supplementary material). We conclude that leftnl2 is not required for the trunk NM deposition, but rather is required for formation of the tc, possibly by regulating rosette renewal during later stages of pLL formation.

**Adult leftnl2 mutants exhibit defects in dermal bone development and lateral line maturation**

Homozygous leftnl2 mutants develop into viable adults, although they were often malformed (see Fig. S2A,B in the supplementary material). Alizarin Red staining of calcified bone revealed that leftnl2 mutant adults had stunted lepidotrichia, leading to severely malformed pectoral, pelvic and caudal fins; dorsal and anal fins were less affected (see Fig. S2C,D in the supplementary material). leftnl2 mutants also showed a dramatic loss of teeth and short gill rakers (see Fig. S2E,F in the supplementary material and data not shown); other jaw structures appeared normal.

The leftnl2 mutant adults also showed defects in late pLL development. During the metamorphosis from larva to adult, the pLL undergoes a dramatic expansion in the number and location of NMs, that relies entirely on precursor cells deposited during the formation of the embryonic pLL (Nunez et al., 2009). In leftnl2 mutants, the pLL was able to assume the adult morphology of lines of NMs, called stitches, that are arranged dorsally along the trunk (see Fig. S3 in the supplementary material). In contrast to wild types, several posterior stitches were absent in leftnl2 mutants, probably owing to the failure of pLL extension during embryonic development. We conclude that Lef1 is required for the proper development of several organs in the adult.

**The leftnl2 mutation disrupts the left1 gene**

We used positional cloning to identify the genetic lesion in leftnl2. Initial meiotic mapping of 405 leftnl2 embryos and 188 wild-type siblings on an *AB/WIK* background placed the mutation between z43517b and z21408 on the distal arm of chromosome 1. Further

---

**Fig. 1. Abnormal primordium patterning leads to loss of terminal neuromasts in the left1 mutant.**

(A,B) eya1 expression in the pLL of wild-type sibling and left1nl2 mutant embryos at 2 dpf. The left1nl2 mutant lacks a tc. (A’). Distal limit of eya1 expression in wild-type and left1nl2 mutant (arrow) embryos. (C) Average number of NMs (excluding tc) in left1nl2 mutant and wild-type siblings (mean±s.d.) are not significantly different at 2 dpf (*n*=28 wild type, 11 left1nl2, *P*=0.49, Student’s *t*-test). (D) Axial positions of L1-L5 in left1nl2 and wild-type siblings at 2 dpf (mean±s.d.). The axial level of stalled primordium (prim) in left1nl2 mutants is indicated by the pink bar. Position of L1-L5 is significantly shifted anteriorly in left1nl2 mutants (*n*=18, *P*<0.001, two-way ANOVA with replication). (E-F”) Stills from time-lapse movies of primordium migration in wild-type sibling and left1nl2 mutant embryos that express the Tg(–8.0cldnb:lynGFP) transgene. Wild-type and left1nl2 embryos were imaged beginning at 34 hpf for 780 minutes and 840 minutes, respectively (see Movies 1 and 2 in the supplementary material). (E–F”) Over the course of 780 minutes, the primordium in the wild-type embryo migrated out of frame, deposited three NMs (red, blue and yellow asterisks) and generated two new rosettes (green and pink asterisks). (F–F”). Over the course of 840 minutes, the primordium in the left1nl2 mutant has slowed and became elongated (pink asterisk), having deposited four NMs (red, blue, yellow and green asterisks). Scale bars: 20 μm.
analyses narrowed the region that flanked the lef1nl2 mutation to ~460 kb (Fig. 2A). Within this region, the microsatellite marker z10888 showed tight linkage to the lef1nl2 mutation (0/1186 meioses). The start of the protein-coding region for lymphocyte enhancer binding factor 1 (lef1) lies 2.2 kb centromeric to z10888. We found that lef1nl2 mutants contained a guanine insertion at base pair 1120 (Fig. 2B) in the lef1 gene. This insertion led to a frame shift that produced a new stop site 29 amino acids downstream from the endogenous stop (Fig. 2C). Western blot analysis using an antibody raised against zebrafish Lef1 confirmed that in lef1nl2 mutants, the Lef1 protein had a higher molecular weight than wild-type (Fig. 2D). The mutant protein appears to be less stable, as we detected 60% less protein in the mutant when compared with wild-type (Fig. 2D). The mutant protein is not transcriptionally functional.

To confirm that disruption of the lef1 gene was indeed the cause of the lef1nl2 mutant phenotype, we used an antisense morpholine oligonucleotide to block lef1 splicing (lef1-MO) (Ishitani et al., 2005). Western blot analysis revealed that Lef1 protein localized predominantly to the cytoplasm and excluded from the nuclei (Fig. 2F,F′). By contrast, in lef1nl2 mutants, Lef1 protein was localized to the cytoplasm and excluded from the nuclei (Fig. 2F,F′), suggesting that the mutant protein is not transcriptionally functional.

To determine whether patterning of the pLL primordium is disrupted in lef1nl2 mutants, we examined expression of gene markers at 32 hpf. Both the expression and localization of axin2, sef, dkk1, fgf10a and pea3 appeared grossly normal in lef1nl2 mutants when compared with wild-type siblings (Fig. 3C-L). lef1 transcript is detectable in lef1nl2 mutants, indicating that the mutation does not cause mRNA decay (Fig. 3A,B). By contrast, the expression of these factors was lost following global inactivation of Wnt signaling using the Tg(hsp70l:dkk1-GFP) transgene (see Fig. S4A-F in the supplementary material), as previously reported (Aman and Piotrowski, 2008).

The chemokine receptors cxcr4b and cxcr7b are differentially expressed in the pLL primordium and are required for its migration (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Global inactivation of Wnt signaling by expression of Tg(hsp70l:dkk1-GFP) has been previously reported to expand the zone of cxcr7b expression (Aman and Piotrowski, 2008). By contrast, when we compared the expression patterns of cxcr4b and cxcr7b in wild-type siblings with those in lef1nl2 mutants (Fig. 3M-P) or Tg(hsp70l:dkk1-GFP) embryos, we did not observe any significant differences (see Fig. S4G-J in the supplementary material). Overall, these data indicate that loss of Lef1 activity does not affect primordium patterning.

Patterning of the pLL primordium is maintained in lef1nl2 mutants

To determine whether patterning of the pLL primordium is disrupted in lef1nl2 mutants, we examined expression of gene markers at 32 hpf. Both the expression and localization of axin2, sef, dkk1, fgf10a and pea3 appeared grossly normal in lef1nl2 mutants when compared with wild-type siblings (Fig. 3C-L). lef1 transcript is detectable in lef1nl2 mutants, indicating that the mutation does not cause mRNA decay (Fig. 3A,B). By contrast, the expression of these factors was lost following global inactivation of Wnt signaling using the Tg(hsp70l:dkk1-GFP) transgene (see Fig. S4A-F in the supplementary material), as previously reported (Aman and Piotrowski, 2008).

The chemokine receptors cxcr4b and cxcr7b are differentially expressed in the pLL primordium and are required for its migration (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Global inactivation of Wnt signaling by expression of Tg(hsp70l:dkk1-GFP) has been previously reported to expand the zone of cxcr7b expression (Aman and Piotrowski, 2008). By contrast, when we compared the expression patterns of cxcr4b and cxcr7b in wild-type siblings with those in lef1nl2 mutants (Fig. 3M-P) or Tg(hsp70l:dkk1-GFP) embryos, we did not observe any significant differences (see Fig. S4G-J in the supplementary material). Overall, these data indicate that loss of Lef1 activity does not affect primordium patterning.

Loss of Lef1 and Tcf7 does not replicate the pLL defects observed with the complete loss of Wnt signaling

In systems such as the mouse limb, the Wnt effector Tcf7 exhibits redundant functions with Lef1 during development (Galceran et al., 1999). Loss of Lef1 and Tcf7 does not replicate the pLL phenotype (Fig. 2D). Injection of lef1-MO into zygotes derived from a heterozygous intercross to determine whether combined loss of Lef1 function.

![Fig. 2. The lef1nl2 mutant contains a lesion in the lef1 gene. (A) The lef1nl2 mutation was mapped to a 460 Kb region on chromosome 1. Numbers of recombinants are indicated under each marker. (B) The lef1nl2 mutation is a single guanine insertion at position 1120 (red asterisk). The resulting frame shift is predicted to disrupt the nuclear localization signal (NLS) and extend the protein by 29 amino acids (aa; also shown as amino acids (aa). (C) Western blot of wild-type, lef1nl2 mutant and lef1 mutant whole embryo lysates probed with anti-Lef1 antibody. (D) Immunolabeling using anti-Lef1 antibody revealed nuclear labeling (red) in wild-type sibling and cytoplasmic labeling in lef1nl2 mutant embryos. Nuclei are labeled with DAPI. (G-I) Wild-type sibling, lef1nl2 mutant and lef1-MO injected embryos expressing the Tg(−8.0cldnb:lynGFP) transgene at 2 dpf. (G) The pL was truncated prematurely (white arrows) in the lef1nl2 mutant (H) and lef1 morphant (I). (J) The axial positions of the deposited NMs are shifted anteriorly in lef1nl2 mutants when compared with wild-type siblings and in lef1 morphants when compared with un.injected controls. (Data presented as means±s.d.; n=6–11 P<0.001, two-way ANOVA with replication.) Scale bars: 50 μm.](image-url)
Role of Lef1 in lateral line

**Lef1 is required in leading zone cells for primordium migration and terminal cluster formation**

The expression pattern of **lef1** in the leading zone of the primordium and the rosette renewal defects in the **lef1** mutants both suggest that Lef1 may be specifically required in cells within the leading zone. Using gastrula-stage transplants, we obtained 15 mosaic embryos in which rhodamine-labeled wild-type donor cells were localized to the leading region of the primordium by 48 hpf. In 14 of 15 cases, mosaic primordia that contained donor cells in the leading region at 48 hpf migrated to the end of the tail and formed the tc (Fig. 5B,B'). Primordia on the contralateral control sides of these embryos, which did not receive wild-type cells, did not show complete migration or form the tc (Fig. 5C). A small subset of these embryos (n=4) was followed using time-lapse imaging to trace the location of the donor cells between 24 and 48 hpf. In these embryos, donor cells resided within the caudal-most rosette and the leading zone (Fig. 5A); this distribution of donor cells was sufficient to rescue the formation of the tc in 3 out of 4 embryos (Fig. 5 and data not shown). In all chimeric embryos, the tc consisted predominantly of the donor cells (Fig. 5B and data not shown), supporting the idea that Lef1 activity is required in the leading, proliferative progenitor cells for primordium migration and tc formation.

**Loss of Lef1 function leads to reduced cell proliferation in the leading edge of the primordium**

As the overall patterning of the pLL primordium was not affected in **lef1** mutants, we reasoned that Lef1 might regulate a process necessary for rosette formation or renewal such as proliferation. In the migrating primordium, proliferation levels are high in the leading zone cells (Aman et al., 2010; Laguerre et al., 2009; Laguerre et al., 2005). This is consistent with the finding that a small population of cells in the leading edge acts as proto-NM progenitors (Nechiporuk and Raible, 2008). To analyze proliferation levels in **lef1** mutants and wild-type siblings, we used BrdU incorporation to mark cells in S phase. We found a significant reduction in the percentage of cells that incorporated BrdU in the primordia of mutants versus their wild-type siblings (26% reduction, Fig. 6A,B,D). The decrease in proliferative cells appeared to be confined to the leading region of **lef1** mutant primordia (Fig. 6A,B). To confirm this finding, we examined the BrdU incorporation index in leading edge cells. The leading edge was defined as cells caudal to most recently formed rosette. **lef1** mutants showed a significant reduction in the index of BrdU-positive cells in this region versus wild-type controls (45% reduction, Fig. 6F).

As inhibition of Wnt signaling by Dkk1 induction leads to a reduction in proliferation in the primordium (Aman et al., 2010), we asked how this reduction compared with that seen in **lef1** mutant primordia. Upon induction of **Tg(hsp70l:dkk1-GFP)** at 28 hpf, we saw a loss of BrdU-positive cells throughout the primordium (40% reduction, Fig. 6C,E) and a dramatic reduction of BrdU-positive cells in the leading region (68% reduction, Fig. 6G). These data indicate that in addition to the leading zone, Wnt activity regulates proliferation throughout the trailing region of the primordium.

To determine whether the decrease of proliferation in the primordia of **lef1** mutants and **Tg(hsp70l:dkk1-GFP)** embryos were due to increased in cell death, we performed TUNEL assays. There were few-to-no TUNEL-positive cells in the primordia of blocked in wild-type siblings, the pLL developed normally, as reported previously (Aman et al., 2010) (Fig. 4A). tcf7-MO/lef1**mut** embryos were identified by loss of the pectoral fin fold (see Fig. SS5D,E in the supplementary material) (Nagayoshi et al., 2008) and showed a loss of terminal cluster NMs similar to that of **lef1** mutants (Fig. 4B,C). Both the axial level of the deposited NMs and the axial level at which the pLL was truncated were shifted anteriorly when compared with un.injected **lef1** mutants (Fig. 4D). Furthermore, whereas NM number is not perturbed in **lef1** mutants or **tcf7** morphants, tcf7-MO/lef1**mut** embryos had fewer NMs (see Fig. S6A in the supplementary material). Thus, Tcf7 and Lef1 show a level of functional redundancy during development of the pLL.

The pLL defects caused by the combined loss of Lef1 and Tcf7 function, however, were not as severe as those caused by global inhibition of Wnt signaling. Following induction of **Tg(hsp70l:dkk1-GFP)** at 28 hpf, the primordium deposited only 1 or 2 NMs prior to disorganization and extension in a thin trail of cells that arrest midway along the trunk (Fig. 4E-F). There was a reduction in the number of additional NMs deposited following activation of the transgene (see Fig. S6B in the supplementary material). Taken together, these data show that Tcf7 is an effector of the canonical Wnt pathway in the pLL primordium in addition to Lef1, though global inhibition of Wnt signaling leads to more severe abnormalities in the pLL, suggesting the existence of other effectors.

![Fig. 3. Primordium patterning is maintained in lef1**mut** mutants.](image-url)

(A-P) RNA in situ hybridization of factors required for primordium patterning in wild-type siblings (left panels) and lef1**mut** mutants (right panels) at 32 hpf. Expression of lef1 (A,B), axin2 (C,D), sof (E,F), dkk1 (G,H), fgf10a (I,J), pea3 (K,L), ccr4b (M,N) and ccr7b (O,P) are similar in both wild-type and mutant embryos. Scale bars: 20 μm.
wild-type, lef1nl2 mutant or lef1-MO injected embryos (see Fig. S7A-D in the supplementary material). There was a low level of TUNEL-positive cells in the primordia of Tg(hsp70l:dkk1-GFP) embryos that were heat-shocked at 28 hpf (see Fig. S8A,B in the supplementary material). These data indicate that Wnt signaling is required for cell proliferation and/or survival in the pLL primordium. However, these cellular effects are at least partially mediated by factors other than Lef1.

**Leading edge cells in lef1nl2 mutants are preferentially sorted out of the primordium.**

Our mosaic analyses indicated that Lef1 activity is required in the leading region of the primordium, but is not solely responsible for regulating cellular proliferation. Thus, we reasoned that Lef1 might be necessary for identity of the primordium progenitor cells. Previous studies have indicated that progenitors reside in the leading edge of the primordium (Nechiporuk and Raible, 2008), probably immediately rostral to the leading tip cells. We used Kaede photoconversion to follow the fate of these leading cells in wild-type controls and lef1nl2 mutants. An average of two cells were photoconverted at 24 hpf; at this stage the primordium already contained three or four proto-NMs (Fig. 7A,B,E). The progeny of the labeled cells were assayed at 48 hpf (Fig. 7C-F). In wild-type embryos, converted cells primarily remained in the primordium, which formed the tc at 48 hpf (Fig. 7C,F). The positions of the red Kaede cells were significantly different in lef1nl2 mutants; we found fewer labeled cells in the primordium and more cells incorporated into deposited NMs versus controls (Fig. 7C,D,F). There was no significant difference in the number of red Kaede cells at 48 hpf between wild-type embryos and lef1nl2 mutant embryos.
Role of Lef1 in lateral line

Fig. 6. Wnt signaling regulates proliferation in the primordium. (A-C) Confocal projections of primordium following BrdU incorporation between 32.5 and 34.0 hpf in wild-type, lef1nl2 mutant and Tg(hsp70l:dkk1-GFP) embryos. All embryos express Tg(f–8.0cmdb:lynGFP). BrdU incorporation in the primordium of the wild-type (A–A”), the lef1nl2 mutant (B–B”) and the Tg(hsp70l:dkk1-GFP) transgenic embryos heat-shocked at 28 hpf (C–C”). Scale bar: 20 μm. (D,E) BrdU incorporation index for wild-type sibling and lef1nl2 mutants (D) and control and Tg(hsp70l:dkk1-GFP) embryos (E). (F,G) BrdU incorporation index for leading region in wild-type siblings and lef1nl2 mutants (F) and controls and Tg(hsp70l:dkk1-GFP) embryos (G) (n=10-22 embryos; data presented as mean±s.e.m., **P<0.009, ***P<0.002, Student’s t-test). The leading region is defined as the cells posterior to the leading rosette in the primordium.

We next asked whether a complete lack of Wnt activity altered donor cell behavior. We transplanted wild-type or Tg(hsp70l:dkk1-GFP) donor cells into wild-type hosts and heat-shocked the resulting chimeras at 28 hpf. Wild-type cells were incorporated into the primordium and migrated normally (Fig. 8E; see Movie 5 in the supplementary material). In contrast to the lef1nl2 donor cells, the Tg(hsp70l:dkk1-GFP)-positive cells remained in the leading edge and altered the behavior of the chimeric primordium (n=4, Fig. 8F,F’; see Movie 7 in the supplementary material). Shortly after the heat-shock, chimeric primordia lost organization and elongated. This result suggests that cells in which the diffusible factor Dkk1 has been ectopically expressed are able to exert non-autonomous effects on neighboring wild-type cells.

DISCUSSION

In the present study, we describe a novel zebrafish strain (lef1nl2), which contains a mutation in lef1, a downstream effector of canonical Wnt signaling. In contrast to a global loss of Wnt signaling, which severely disrupts patterning, proliferation and organization throughout the primordium, loss of Lef1 activity resulted in a distinct, late defect in rosette renewal. Fate mapping and mosaic analyses support the idea that this failure results from a loss of leading region progenitor cells and not reduced proliferation, defining a previously unrecognized role for Wnt signaling in pLL primordium organization.

Pattern of rosette renewal in the pLL primordium

One distinct feature of the lef1nl2 phenotype is the relatively normal deposition of the rostral NMs. Consistent with our observations, a recent study using morpholinos to block lef1 function found that NMs L1-L4 were deposited normally (Gamba et al., 2010). This suggests that Lef1 activity, which is necessary for proper specification and/or maintenance of the progenitor population, is not required during the initial patterning of proto-NMs. In both the lef1nl2 mutant and wild-type embryos, NMs L1-L4 correspond to first four proto-NMs that are initially specified within the primordium, whereas the L5 NM and terminal neuromasts arise from cells posterior to the last proto-NM. In the absence of Lef1 activity, cells leave the leading zone and prematurely incorporate into NMs, leaving an insufficient number of cells to generate new...
rosettes after the initial proto-NMs are deposited. We suspect that migration of the remaining cells continues only as long as the cohort remains in contact.

Multiple nuclear effectors mediate Wnt activity in the primordium

Our studies revealed that Lef1 mediates a novel Wnt-dependent role in primordium organization distinct from previously described Wnt functions such as patterning, proliferation and NM deposition (Aman et al., 2010; Aman and Piotrowski, 2008). We found that although global inhibition of the Wnt pathway disrupted expression of several factors, including fgf10a, pea3, lef1 and dkk1 (although not ccr7b as previously reported), expression patterns of these factors were grossly normal in the primordia when compared with wild type (mean±s.e.m., n=27 wild-type and 16 lef1nl2 mutant embryos. Cells in lef1nl2 mutants are significantly more likely to be localized in NMs and not in the primordium when compared with wild type (mean±s.e.m., n=27 wild-type and 16 lef1nl2 mutant embryos, *P<0.04, Student’s t-test). Specific time points were chosen to show a subset of labeled cells just before and after cell divisions. Leading zone divisions marked by blue arrows, whereas cell divisions in rosettes are marked by yellow arrowheads. Scale bars: 20 μm.

Lef1 is required for progenitor cell identity in the primordium

Previous work has shown that a small number of cells in the leading edge of the primordium act as a progenitor population to produce new proto-NMs (Nechiporuk and Raible, 2008), though the molecular and cellular mechanisms that regulate specification and renewal in primordium progenitor cells had not been

![Image](https://example.com/image.png)
established. It is also not clear whether this population is established before or after the onset of primordium migration (~22 hpf). Previous fate mapping demonstrated that the progenitors were already present at 24 hpf (shortly before the L1 is deposited) and gave rise to the progeny that populated the caudal NMs and the terminal cluster in the pLL (Nechiporuk and Raible, 2008). In the present study, we took advantage of this observation to investigate the fate of the progenitor cells in the \textit{lef1\textsuperscript{nl2}} mutant by lineage analyses. In the absence of Lef1 activity, labeled cells left the primordium and were incorporated into the caudal NMs in greater numbers when compared with wild-type embryos. This suggests that Lef1 activity is required for the presence of progenitor cells within the leading edge of the primordium. This was further confirmed by mosaic analyses, which revealed that \textit{lef1\textsuperscript{nl2}} mutant cells were excluded from the leading edge when placed in a wild-type environment. This is consistent with our observation that the cells in \textit{lef1\textsuperscript{nl2}} mutants tend to undergo cell division after exiting the leading zone, whereas the vast majority of wild-type cells divide in the leading zone of the primordium. This abnormal pattern of cell divisions explains the apparent contrast between the leading region (C; yellow bracket), whereas \textit{lef1\textsuperscript{nl2}} cells have moved out of the leading region (D; yellow bracket).

In conclusion, we have demonstrated a previously unreported role for Wnt signaling through Lef1 in regulating progenitor cells in the zebrafish pLL primordium. We also suggest that Wnt signaling requires multiple downstream effectors to mediate its functions during primordium migration and pLL formation. These results provide a model in which a signaling pathway can regulate multiple aspects of collective cell migration.

Acknowledgements
The authors thank the Piotrowski and Dorsky laboratories for reagents, and Jan Christian for her comments on the manuscript. This work was supported by the Basic Research Training in Embryonic Development NIH grant 5T32HD049309-05 to H.F.M. and by funds from March of Dimes (5-FY09-116), NICHD (HD055030) and Medical Research Foundation of Oregon to A.V.N. Deposited in PMC for release after 12 months.

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.062554/-/DC1

References

Fig. 8. Lef1 is required to maintain progenitor cell identity. (A-D) Confocal projections of Tg(–8.0cdlb:lynGFP)-positive, mosaic embryos containing either wild-type or \textit{lef1\textsuperscript{nl2}} donor cells (red) at 24 and 48 hpf. (A) Wild-type host primordium at 24 hpf with wild-type donor cells in the leading region (yellow bracket). (B) Chimeric primordium containing \textit{lef1\textsuperscript{nl2}} mutant cells in the leading region at 24 hpf (yellow bracket). At 48 hpf, wild-type donor cells remain in the leading region (C; yellow bracket), whereas \textit{lef1\textsuperscript{nl2}} cells have moved out of the leading region (D; yellow bracket). (E-F) Confocal projections of chimeric primordia containing wild type Tg(hsp70l:dkk1-GFP) donor cells. Embryos were heat-shocked at 28 hpf. (E) Primordium containing wild-type cells and a characteristic rounded morphology. (F) Wild-type primordium containing Tg(hsp70l:dkk1-GFP) donor cells shows loss of primordium organization. (F’) Contralateral side of the chimera shown in F is normal. Scale bars: 20 μm.


<table>
<thead>
<tr>
<th>Gene</th>
<th>Wildtype</th>
<th>tg(hs:Dkk1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>left1</td>
<td><img src="A" alt="image" /></td>
<td><img src="B" alt="image" /></td>
</tr>
<tr>
<td>fgf10a</td>
<td><img src="C" alt="image" /></td>
<td><img src="D" alt="image" /></td>
</tr>
<tr>
<td>pea3</td>
<td><img src="E" alt="image" /></td>
<td><img src="F" alt="image" /></td>
</tr>
<tr>
<td>cxcr4b</td>
<td><img src="G" alt="image" /></td>
<td><img src="H" alt="image" /></td>
</tr>
<tr>
<td>cxcr7b</td>
<td><img src="I" alt="image" /></td>
<td><img src="J" alt="image" /></td>
</tr>
</tbody>
</table>

32 hpf

lefl1 cxcr4bcxcr7 bgf10apea3

wildtype
wt sibling

<table>
<thead>
<tr>
<th>Merge</th>
<th>GFP</th>
<th>TUNEL</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A'</td>
<td>A''</td>
<td>A'''</td>
</tr>
<tr>
<td></td>
<td>A'''</td>
<td></td>
<td>40 hpf</td>
</tr>
</tbody>
</table>

lef1\textsuperscript{nl2}

<table>
<thead>
<tr>
<th>Merge</th>
<th>GFP</th>
<th>TUNEL</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>B'</td>
<td>B''</td>
<td>B'''</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 hpf</td>
</tr>
</tbody>
</table>

uninjected control

<table>
<thead>
<tr>
<th>Merge</th>
<th>GFP</th>
<th>TUNEL</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>C'</td>
<td>C''</td>
<td>C'''</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35 hpf</td>
</tr>
</tbody>
</table>

lef1-MO

<table>
<thead>
<tr>
<th>Merge</th>
<th>GFP</th>
<th>TUNEL</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>D'</td>
<td>D''</td>
<td>D'''</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>conversions</td>
<td>initial positions of converted cells</td>
<td>positions of cell divisions during primordium migration</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------------</td>
<td>------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>wt</strong></td>
<td><img src="image1.png" alt="Initial positions of converted cells" /></td>
<td><img src="image2.png" alt="Positions of cell divisions" /></td>
<td></td>
</tr>
<tr>
<td><strong>lef1n2</strong></td>
<td><img src="image3.png" alt="Initial positions of converted cells" /></td>
<td><img src="image4.png" alt="Positions of cell divisions" /></td>
<td></td>
</tr>
</tbody>
</table>