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

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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, *lef1*^{nl2}, that contains a lesion in the Wnt effector gene *lef1*. *lef1*^{nl2} 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 *lef1*^{nl2} 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; Lecaudey 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 *lef1*^{nl2} mutant, which exhibits premature truncation of the pLL and loss of terminal NMs. In *lef1*^{nl2} mutants, primordium migration and NM deposition begin normally, but over time the

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primordium fails to generate new rosettes and becomes disorganized leaving a small trail of cells beyond the distal-most NM. Positional cloning revealed that *lef1*^{nl2} is a mutation in *lef1*, an effector and target of Wnt signaling. Using live imaging, cell transplantation and lineage analyses, we show that Lef1 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(–8.0cldnb:lynGFP)*^{zf106} line (Haas and Gilmour, 2006) and the *TgBAC(neurod:EGFP)*^{nl1} line (Obholzer et al., 2008), respectively. Wnt/β-catenin signaling was conditionally inhibited using the *Tg(hsp70l:dkk1-GFP)*^{w32} line (Stoick-Cooper et al., 2007).

Mutagenesis screen and genetic mapping

The *lef1*^{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 NMs using 2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI; Invitrogen) according to the established protocol (Harris et al., 2003). Mature hair cells were labeled with FM1-43 (1:1000; Invitrogen). For genetic mapping, heterozygous carriers of *lef1*^{nl2} on a polymorphic *AB/WIK background were intercrossed to produce homozygous, heterozygous and wild-type progeny. Initial chromosome assignment was carried out by bulk segregant analysis of DNA pools from 20 wild-type and 20 mutant individuals. The following additional markers were designed to determine flanking regions: bx321GF, CAAAACCCTACTGACCC; bx321GR, GGAATTTTCCTTTATGGACA; bx537NF, GCGTTCT-GAAGTCTCCTCT; bx537NR, GTGATGGTGCCACTAAATGA.

Heat-shock conditions and morpholino 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). *Tg(hsp70l:dkk1-GFP)*-positive embryos were identified by GFP expression.

Antisense oligonucleotide morpholinos (MO) were microinjected into fertilized zygotes at the concentrations indicated. The *lef1*-MO, which blocks the splice donor site at exon 8/intron 8 (a gift from the Dorsky lab, University of Utah, Salt Lake City, UT, USA; TTTTAAGATACGAACC-CTCCGGCC) (Rai et al., 2010), was injected at 4 ng/nl. The *tcf7*-MO (a gift from the Dorsky lab) (Bonner et al., 2008) was injected at 2 ng/nl along with 3 ng/nl p53-MO (Robu et al., 2007).

In situ hybridization, immunolabeling, BrdU incorporation and image processing

RNA in situ hybridization was performed as described previously (Andermann et al., 2002). Digoxygenin- or fluorescein-labeled antisense RNA probes were generated for the following genes: *eya1* (Sahly et al., 1999), *fgf10a* (Grandel et al., 2000), *pea3* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), *lef1* (Dorsky et al., 1999), *tcf7* (Veien et al., 2005), *tcf7l1a* (Dorsky et al., 2003), *tcf71b* (Dorsky et al., 2003) *dkk1* (Aman and Piotrowski, 2008), *cxcr4b* and *cxcr7b* (Dambly-Chaudiere et al., 2007). Whole-mount immunolabeling was performed following established protocols (Ungos et al., 2003). The following antibodies were used: rabbit anti-GFP (1:1000; Invitrogen), rat or mouse anti-BrdU (1:100; Abcam or 1:20; Developmental Studies Hybridoma Bank); mouse anti-GFP (1:1000; Invitrogen), rabbit anti-Lef1 (1:200) (Lee et al., 2006), Alexa-488 (1:1000) and Alexa-568 (1:1000). Nuclei were visualized with DAPI. BrdU incorporation was carried out between 32.5 and 34.0 hpf using the protocol described by Laguerre et al. (Laguerre et al., 2005) followed by a published BrdU detection protocol (Harris et al., 2003; Laguerre et al., 2005). Fluorescently labeled embryos were imaged using Olympus FV1000 confocal system. Bright-field or Nomarski microscopy images were collected using Zeiss Lumar and Imager Z1 systems. Images were processed using ImageJ software (Abramoff et al., 2004). Brightness and contrast were adjusted in Adobe Photoshop.

Western blotting

For western blot analysis, protein was isolated from *Tg(–8.0cldnb:lynGFP)*, *lef1*^{nl2} and *lef1*-MO embryos. For each condition, 16 dechorionated (2 dpf) embryos were homogenized in sample buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM $NaHCO_3$) with proteinase inhibitors, run on a 12% SDS-PAGE gel and blotted onto a PVDF membrane. Anti-Lef1 antibody was used at 1:2000 (Lee et al., 2006). Anti-rabbit conjugated-HPR antibody (Invitrogen) was applied at 1:10,000 and visualized using SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's specification (Thermo Scientific). The blot was stripped with 25 mM glycine (pH=2.5) and 1% SDS and re-probed with rabbit anti-α-actin (1:10,000; Sigma) using the same secondary antibody.

Transplantation experiments

Transplantation experiments were carried out as previously described (Nechiporuk and Raible, 2008). All host embryos expressed the *Tg(–8.0cldnb:lynGFP)* and/or *TgBAC(neurod:EGFP)* transgenes. Donor zygotes were injected with fixable rhodamine dextran (Invitrogen). All host embryos receive donor cells in the left side, whereas the right side served as control. *lef1*^{nl2} donors were derived from heterozygous crosses of *lef1*^{nl2}/+ adults. Donors were individually assessed for the mutant phenotype at 3 dpf and correlated to their respective host embryos. Cells from heterozygous *Tg(hsp70l:dkk1-GFP)* donors were transplanted into wild-type hosts. Hosts were collected with their respective donors and heat-shocked as described above at 28 hpf.

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 20×/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 *lef1*^{nl2} heterozygous incrosses that also contained the *Tg(–8.0cldnb:lynGFP)* and the *TgBAC(neurod:EGFP)* transgenes were injected at the one-cell stage with 200 pg of *NLS-Kaede* mRNA (Ando 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 and fixed in 4% PFA at 34 hpf. *lef1*^{nl2} mutant and wild-type sibling embryos were fixed at 40 hpf.

Statistics

A two-sample Student's *t*-test assuming equal variance was used to compare total NM counts, BrdU incorporation indexes and pH3 labeling. Two-way ANOVA with replication was used to compare axial level of NMs in the different treatment conditions. Student's *t*-test and ANOVA were run using Excel software. To analyze *lef1*^{nl2} mutant to wild-type transplants, we employed Fisher's Exact Test (http://faculty.vassar.edu/lowry/fisher.html).

Dermal bone and cartilage staining

Adult *lef1*^{nl2} mutants and wild-type siblings of comparable size were collected at 3 months post-fertilization. Alizarin Red staining was used to label bone 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 *lef1*^{nl2} mutant

The *lef1*^{nl2} mutation was isolated in an ongoing ENU-based mutagenesis screen designed to identify recessive mutations with defects in pLL formation. The *lef1*^{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 *lef1*^{nl2} 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 *lef1*^{nl2} 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 *lef1*^{nl2} mutants and only a small trail of cells extended distally from the last deposited NM (Fig. 1A′,B′). Because the loss of NMs often results from pLL primordium abnormalities, we examined primordium organization during migration through time-lapse imaging in *lef1*^{nl2} mutants and wild-type siblings expressing the *Tg(–8.0cldnb:lynGFP)* transgene between 34 and 48 hpf (Fig. 1E,F). In a wild-type embryo, the migrating primordium deposited three trunk NMs and the tc at the end of the trunk (Fig. 1E-E‴; 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″,E‴). In a *lef1*^{nl2} 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. 1F′-F⁗; see Movie 2 in the supplementary material). We conclude that *lef1*^{nl2} 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 *lef1*^{nl2} mutants exhibit defects in dermal bone development and lateral line maturation

Homozygous *lef1*^{nl2} 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 *lef1*^{nl2} 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). *lef1*^{nl2} 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 *lef1*^{nl2} 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 *lef1*^{nl2} mutants, the pLL was able to assume the adult morphology of lines of NMs, called stitches, that are arranged dorsoventrally along the trunk (see Fig. S3 in the supplementary material). In contrast to wild types, several posterior stitches were absent in *lef1*^{nl2} 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 *lef1*^{nl2} mutation disrupts the *lef1* gene

We used positional cloning to identify the genetic lesion in *lef1*^{nl2}. Initial meiotic mapping of 405 *lef1*^{nl2} 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

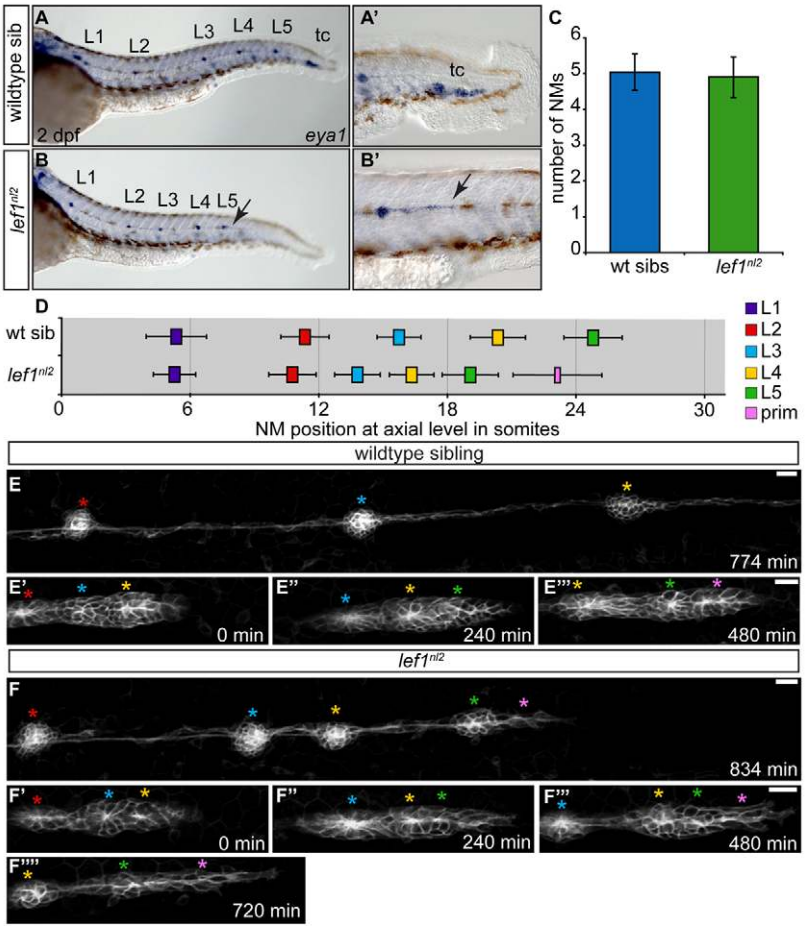

Fig. 1. Abnormal primordium patterning leads to loss of terminal neuromasts in the *lef1*^{nl2} mutant. (**A**,**B**) *eya1* expression in the pLL of wild-type sibling and *lef1*^{nl2} mutant embryos at 2 dpf. The *lef1*^{nl2} mutant lacks a tc. (A′,B′) Distal limit of *eya1* expression in wild-type and *lef1*^{nl2} mutant (arrow) embryos. (**C**) Average number of NMs (excluding tc) in *lef1*^{nl2} mutant and wild-type sibs (mean±s.d.) are not significantly different at 2 dpf (n=28 wild type, 11 *lef1*^{nl2}, P=0.49, Student's t-test). (**D**) Axial positions of L1-L5 in *lef1*^{nl2} and wild-type siblings at 2 dpf (mean±s.d.). The axial level of stalled primordium (prim) in *lef1*^{nl2} mutants is indicated by the pink bar. Position of L1-L5 is significantly shifted anteriorly in *lef1*^{nl2} 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 *lef1*^{nl2} mutant embryos that express the *Tg(–8.0cldnb:lynGFP)* transgene. Wild-type and *lef1*^{nl2} embryos were imaged beginning at 34 hpf for 780 minutes and 840 minutes, respectively (see Movies 1 and 2 in the supplementary material). (E-E‴) 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 *lef1*^{nl2} 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 *lef1*^{nl2} mutation to ~460 kb (Fig. 2A). Within this region, the microsatellite marker z10888 showed tight linkage to the *lef1*^{nl2} 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 *lef1*^{nl2} 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 *lef1*^{nl2} 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 extracts. Immunolabeling with the anti-Lef1 antibody revealed that, in wild-type siblings, Lef1 protein localized predominantly to nuclei in the leading portion of the primordium (Fig. 2E,E′). By contrast, in *lef1*^{nl2} 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 confirm that disruption of the *lef1* gene was indeed the cause of the *lef1*^{nl2} mutant phenotype, we used an antisense morpholino oligonucleotide to block *lef1* splicing (*lef1*-MO) (Ishitani et al., 2005). Western blot analyses revealed that Lef1 protein was completely lost in *lef1* morphants (Fig. 2D). Injection of *lef1*-MO produced embryos that had a pLL phenotype nearly identical to that of the *lef1*^{nl2} mutant (Fig. 2G-J). All together, these results demonstrate that the *lef1*^{nl2} mutation causes a complete or nearly complete loss of Lef1 function.

Patterning of the pLL primordium is maintained in *lef1*^{nl2} mutants

To determine whether patterning of the pLL primordium is disrupted in *lef1*^{nl2} 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 *lef1*^{nl2} mutants when compared with wild-type siblings (Fig. 3C-L). *lef1* transcript is detectable in *lef1*^{nl2} 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* without altering *cxcr4b* expression (Aman and Piotrowski, 2008). By contrast, when we compared the expression patterns of *cxcr4b* and *cxcr7b* in wild-type siblings with those in *lef1*^{nl2} 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). *tcf7* was expressed in the leading region of the primordium (see Fig. S5A in the supplementary material). By contrast, transcripts of two other Wnt effector genes, *tcf7l1a* and *tcf7l1b*, were excluded from the leading zone of the primordium (see Fig. S5B,C in the supplementary material). We injected a *tcf7* morpholino (*tcf7*-MO) into zygotes derived from a *lef1*^{nl2} heterozygous intercross to determine whether combined loss of Tcf7 and Lef1 affects pLL formation. When Tcf7 function was

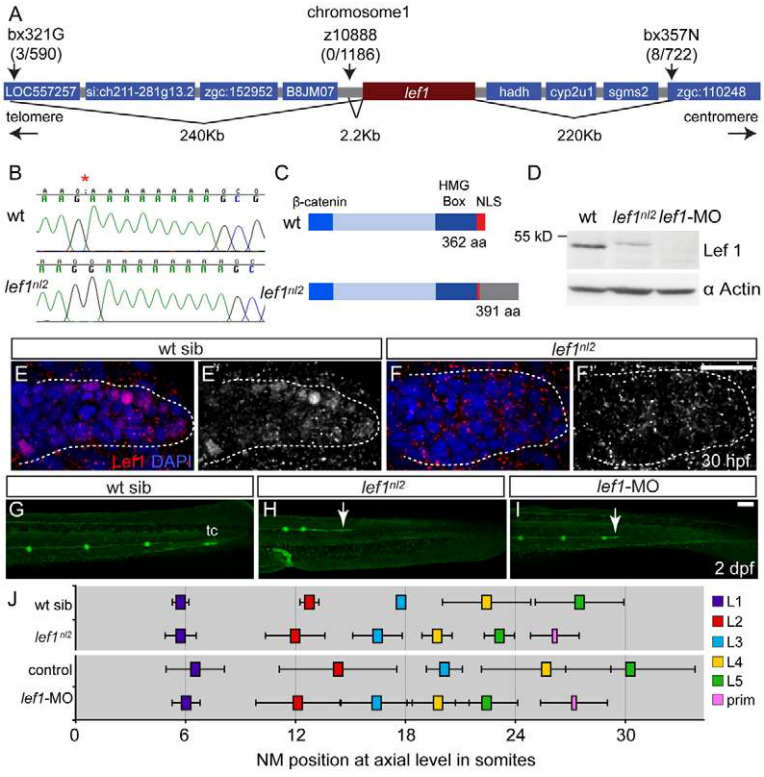

Fig. 2. The *lef1*^{nl2} mutant contains a lesion in the *lef1* gene. (**A**) The *lef1*^{nl2} mutation was mapped to a 460 Kb region on chromosome 1. Numbers of recombinants are indicated under each marker. (**B**) The *lef1*^{nl2} mutation is a single guanine insertion at position 1120 (red asterisk). (**C**) The resulting frame shift is predicted to disrupt the nuclear localization signal (NLS) and extend the protein by 29 amino acids (aa; also shown a β-catenin binding domain and HMG Box). (**D**) Western blot of wild-type, *lef1*^{nl2} mutant and *lef1* morphant whole embryo lysates probed with anti-Lef1 antibody and anti-α actin antibody. (**E-F′**) Immunolabeling using anti-Lef1 antibody revealed nuclear labeling (red) in wild-type sibling and cytoplasmic labeling in *lef1*^{nl2} mutant embryos. Nuclei are labeled with DAPI. (**G-I**) Wild-type sibling, *lef1*^{nl2} mutant and *lef1*-MO injected embryos expressing the *Tg(–8.0cldnb:lynGFP)* transgene at 2 dpf. (G) The pLL was truncated prematurely (white arrows) in the *lef1*^{nl2} mutant (H) and *lef1* morphant (I). (**J**) The axial positions of the deposited NMs are shifted anteriorally in *lef1*^{nl2} mutants when compared with wild-type siblings and in *lef1* morphants when compared with uninjected controls. (Data presented as mean±s.d.; *n*=6-11 *P*<0.001, two-way ANOVA with replication.) Scale bars: 50 μm.

DEVELOPMENT

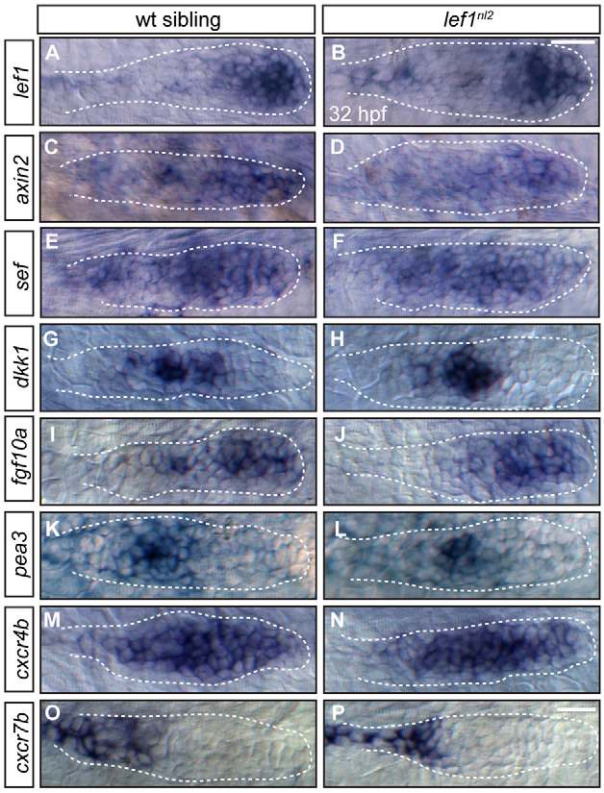

Fig. 3. Primordium patterning is maintained in *lef1*^{nl2} mutants. (**A-P**) RNA in situ hybridization of factors required for primordium patterning in wild-type siblings (left panels) and *lef1*^{nl2} mutants (right panels) at 32 hpf. Expression of *lef1* (A,B), *axin2* (C,D), *sef* (E,F), *dkk1* (G,H), *fgf10a* (I,J), *pea3* (K,L), *cxcr4b* (M,N) and *cxcr7b* (O,P) are similar in both wild-type and mutant embryos. Scale bars: 20 μm.

blocked in wild-type siblings, the pLL developed normally, as reported previously (Aman et al., 2010) (Fig. 4A). *tcf7*-MO/*lef1*^{nl2} embryos were identified by loss of the pectoral fin fold (see Fig. S5D,E in the supplementary material) (Nagayoshi et al., 2008) and showed a loss of terminal cluster NMs similar to that of *lef1*^{nl2} 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 uninjected *lef1*^{nl2} mutants (Fig. 4D). Furthermore, whereas NM number is not perturbed in *lef1*^{nl2} mutants or *tcf7* morphants, *tcf7*-MO/*lef1*^{nl2} 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.

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*^{nl2} mutant 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,C′). 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 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*^{nl2} 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*^{nl2} 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*^{nl2} 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*^{nl2} 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*^{nl2} 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*^{nl2} 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

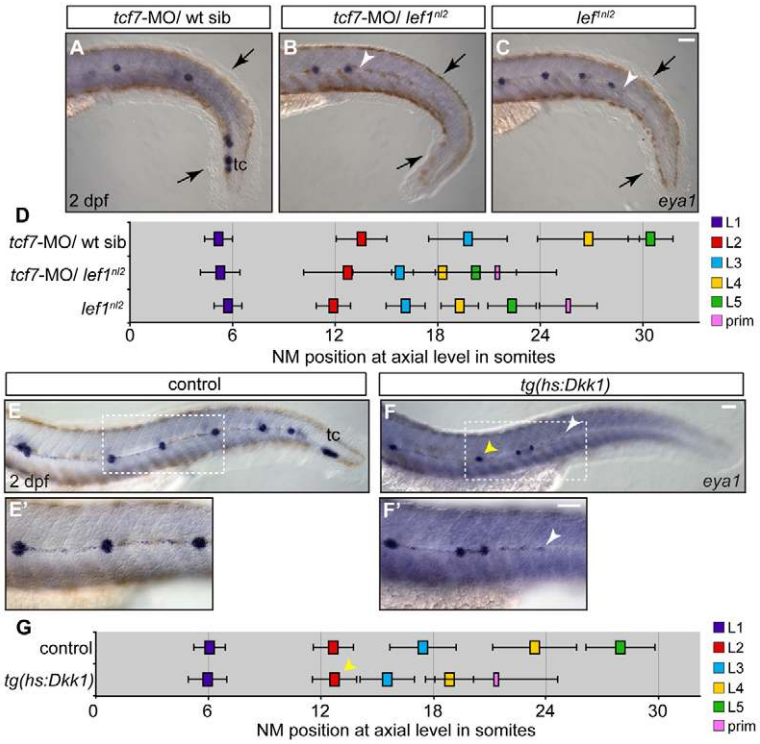

Fig. 4. Combined loss of Lef1 and Tcf7 does not recapitulate the pLL phenotype seen with the complete loss of Wnt signaling. (**A-C**) Zygotes from a *lef1*^{nl2}/+ incrosses were injected with *tcf7*-MO. NM distribution in the injected embryos was assessed by *eya1* expression at 2 dpf and compared with uninjected *lef1* mutant embryos. The loss of Tcf7 alone did not affect pLL patterning in *lef1*^{nl2}/+ sibling (A), the pLL is truncated more severely *lef1*^{nl2}/–; *tcf7*-MO (B; arrowhead) compared with *lef1*^{nl2} mutants alone (C; arrowhead). Note reduced fin fold (black arrows) caused by a combined loss of Lef1 and Tcf7 as reported previously (Nagayoshi et al., 2008). (**D**) Axial positions of the pLL NMs (L1-L5) and primordium (mean±s.d.; n=11, P<0.001, two-way ANOVA with replication). (**E-F′**) pLL formation in 2 dpf embryos following the induction of Dkk1 in the *Tg(hsp70l:dkk1-GFP)* line by a heat-shock at 28 hpf. Position of the primordium at the time of heat-shock is marked by the yellow arrowheads. Dashed rectangles indicate the regions shown at higher magnification in E′,F′. Following the heat-shock, one or two NMs are deposited and the pLL ends with a trail of *eya1*-positive cells (white arrowheads). (**G**) Axial positions of NMs deposited following heat-shock in control and *Tg(hsp70l:dkk1-GFP)* embryos (n=11). Scale bars: 20 μm.

wild-type, *lef1*^{nl2} 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 *lef1*^{nl2} 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 *lef1*^{nl2} 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 *lef1*^{nl2} 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 *lef1*^{nl2}

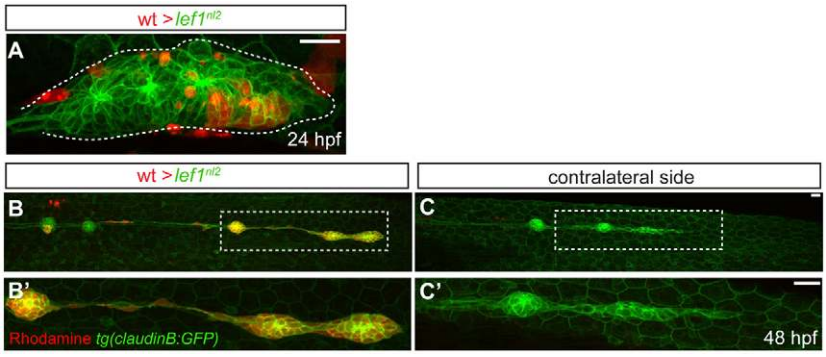

Fig. 5. Lef1 function is required in leading edge cells of the primordium for proper pLL formation. (**A-C′**) Confocal projection obtained from a *lef1*^{nl2} mutant host that received wild-type donor cells (rhodamine dextran, red). (A) At 24 hpf, the primordium contains donor cells in the leading zone and caudal-most rosette. (B) The same embryo as in A, showing complete primordium migration and tc formation at 48 hpf. (B′) High magnification of region outlined in B; the primordium is entirely composed of donor cells. (C) Lateral line is truncated on the contralateral side of the same embryo. (C′) High magnification of the region outlined in C. Both wild-type donors and mutant hosts expressed *Tg(–8.0cldnb:lynGFP)* transgene. Scale bars: 20 μm.

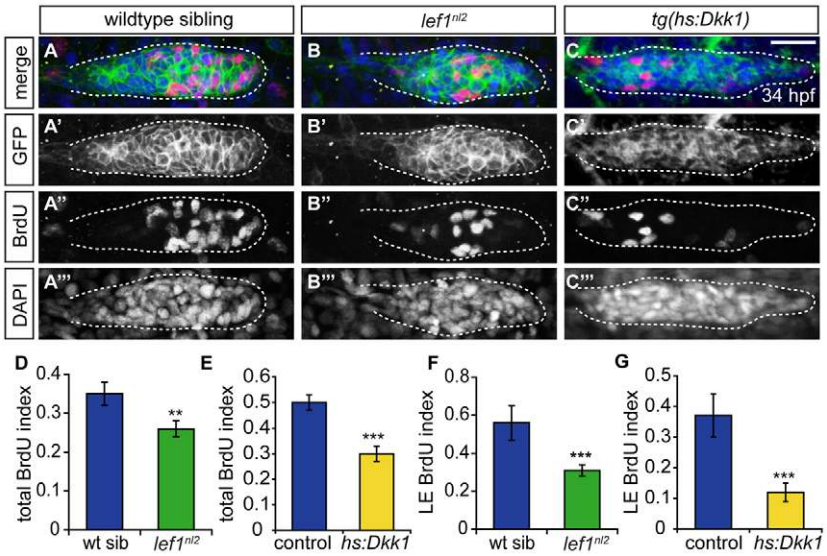

Fig. 6. Wnt signaling regulates proliferation in the primordium. (**A-C‴**) Confocal projections of primordia following BrdU incorporation between 32.5 and 34.0 hpf in wild-type, *lef1*^{nl2} mutant and *Tg(hsp70l:dkk1-GFP)* embryos. All embryos express *Tg(–8.0cldnb:lynGFP)*. BrdU incorporation in the primordia of the wild-type (A-A‴), the *lef1*^{nl2} 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 *lef1*^{nl2} mutants (D) and control and *Tg(hsp70l:dkk1-GFP)* embryos (E). (**F**,**G**) BrdU incorporation index for leading region in wild-type siblings and *lef1*^{nl2} 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.

mutants (Fig. 7E), indicating that the labeled cells in both groups divided an equal number of times. These results suggest that Wnt signaling through Lef1 is necessary to retain progenitor cells in the leading region of the primordium.

Together, our BrdU incorporation and lineage-tracing experiments indicate that the loss of Lef1 activity may affect a pattern of proliferation rather than an actual rate of cell division within the leading region. To address this question, we studied the cell division of converted Kaede cells in wild-type and *lef1*^{nl2} mutant embryos using live imaging (*n*=4; see Fig. S9 in the supplementary material). In wild-type embryos, the majority of cells divided in the leading zone of the primordium (12/15 cell divisions), although a small subset divided in rosettes (3/15 cell divisions; Fig. 7G-G‴; see Movie 3 in the supplementary material). By contrast, in the *lef1*^{nl2} mutants, we observed dividing cells in the rosettes (4/9 cell divisions) and deposited NMs (2/9 cell divisions) as well as in the leading zone (3/9 of cell divisions; Fig. 7H-H‴; see Movie 4 in the supplementary material). These experiments suggest that, although leading cells are able to proliferate in *lef1*^{nl2} mutants, they fail to remain in the leading zone and are preferentially incorporated into NMs. We suggest that this abnormal pattern of cell division is responsible for the reduction in BrdU incorporation levels observed in the *lef1*^{nl2} mutant primordia.

To explore further the role Lef1 plays in conferring progenitor identity, we compared the behavior of *lef1*^{nl2} mutant and wild-type cells transplanted into wild-type hosts. In chimeric embryos containing wild-type donor cells in their primordia, eight out of 28 (28.6%) had donor cells localized to the leading region at 48 hpf. By contrast, we never found cells derived from a *lef1*^{nl2} donor localized to the leading edge of wild-type primordia (*n*=24; $P<0.02$, Fisher's Exact Test). In a subset of these embryos, we examined mosaic primordia during the course of migration. At 24 hpf, donor cells from either wild-type or mutant donors were located in the leading zone of host primordia (*n*=4; Fig. 8A,B). At 48 hpf, wild-type donor cells remain in the leading region, whereas *lef1*^{nl2} mutant donor cells have left the leading region (Fig. 8C,D; see Movies 5, 6 in the supplementary material). Together with the lineage data, these results suggest that Lef1 is required to regulate progenitor identity and localization in the leading zone of 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 *lef1*^{nl2} 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 (*lef1*^{nl2}), 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 *lef1*^{nl2} 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 *lef1*^{nl2} 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

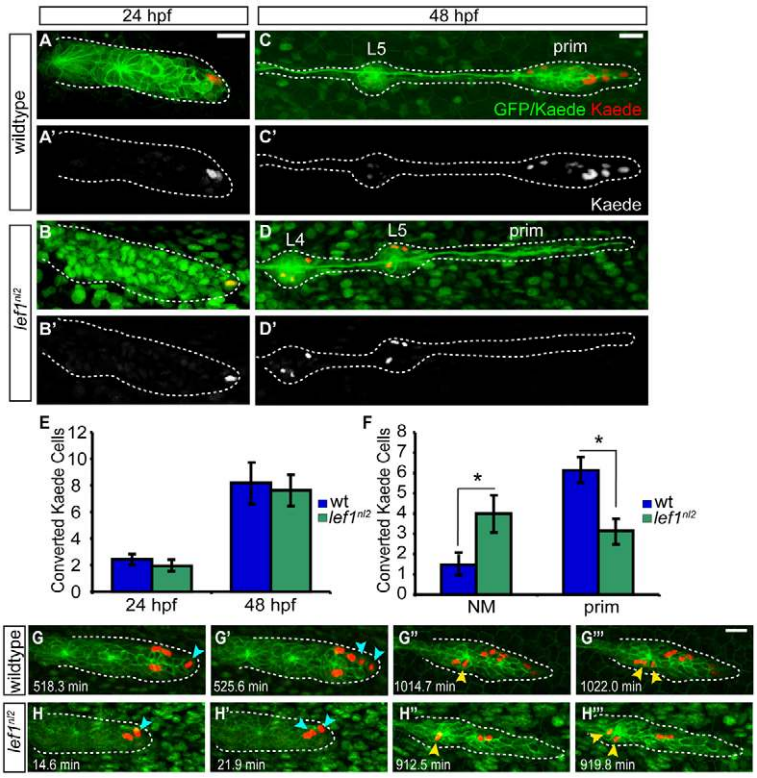

Fig. 7. Leading region cells change their fate in the absence of Lef1 function. (A-C′) *Tg(–8.0cldnb:lynGFP)* zygotes were injected with a nuclear-localized Kaede mRNA and an average of two cells were photoconverted at 24 hpf. (**A**,**B**) Wild-type and *lef1*^{nl2} mutant embryos immediately following photoconversion are shown. (**C-D′**) The embryos in A-B′ at 48 hpf showing the location of the progeny of the photoconverted cells. Note absence of the labeled (red) cells in the leading region of *lef1*^{nl2} mutant embryos. (**E**) Quantification of converted cells at 24 hpf and their progeny at 48 hpf. (**F**) Localization of converted cells at 48 hpf in wild-type and *lef1*^{nl2} mutants. Cells in *lef1*^{nl2} mutants are significantly more likely to be localized in NMs and not in the primordia when compared with wild type (mean±s.e.m., n=27 wild-type and 16 *lef1*^{nl2} mutant embryos, *P<0.04, Student's t-test). (**G-H‴**) Still images from time-lapse movies (see Movies 3 and 4 in the supplementary material) demonstrating division of Kaede-positive cells (red) in a wild-type (G-G‴) and a *lef1*^{nl2} embryos (H-H‴). 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.

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 *cxcr7b* as previously reported), expression patterns of these factors were grossly normal in the primordia of *lef1*^{nl2} mutants. These results suggest that Lef1 is not required for primordium patterning.

A second major difference between global Wnt inhibition and loss of Lef1 activity alone is in the respective numbers of NMs deposited. Embryos in which Wnt activity was blocked during early stages of primordium migration deposited only one or two additional NMs, and migration stalled at the level of the posterior trunk. Moreover, analyses of the chimeric embryos demonstrated that in contrast to the *lef*^{nl2} mutant cells, Dkk1-expressing cells were able to alter the behavior of their surrounding wild-type neighbors to produce a phenotype similar to that seen in our global Wnt inhibition experiments. Our observations of early primordium stalling following Wnt inhibition and a lack of expansion in *cxcr7b* expression differ from what had been observed previously. Aman and Piotrowski reported that primordium migration continued along the length of the trunk and that *cxcr7b* expanded throughout the primordium following activation of the *Tg(hsp70l:dkk1-GFP)* transgene (Aman and Piotrowski, 2008). It is possible that we used more stringent heat-shock conditions, as we also observed cell death with a concurrent loss in proliferation following activation of the *Tg(hsp70l:dkk1-GFP)* transgene, which had not been reported previously. Our observations indicate discrete roles for Wnt activity in cell proliferation and survival within the primordium. We hypothesize that this significant loss of cells results in early disruption of primordium organization and subsequent arrest in primordium migration.

These differences between global Wnt pathway inactivation and loss of Lef1 activity alone may reflect the requirement of multiple Lef1/Tcf factors to mediate canonical Wnt activity. For example, in mouse, loss of both Lef1 and Tcf7 were required to recapitulate the phenotype seen in the Wnt3a knockout (Galceran et al., 1999). Indeed, we observed a more severe truncation of the pLL when we blocked Tcf7 function in *lef1*^{nl2} mutant embryos. Although, this phenotype was still not as severe as the one that resulted from global Wnt inhibition, suggesting that additional Tcf factors may regulate primordium patterning and NM deposition. Alternatively, some of the phenotypes that result from activation of the *Tg(hsp70l:dkk1-GFP)* transgene may be due to disruption of a non-canonical Wnt pathway, as induction of Dkk1 can also inhibit non-canonical Wnt signaling (Cha et al., 2008). Finally, we cannot exclude the possibility that the leading progenitor cells are especially sensitive to decreased levels of Wnt activity in the absence of Lef1. Future work is needed to distinguish between these multiple possibilities of how Wnt signaling regulates identity of the leading progenitor cells.

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

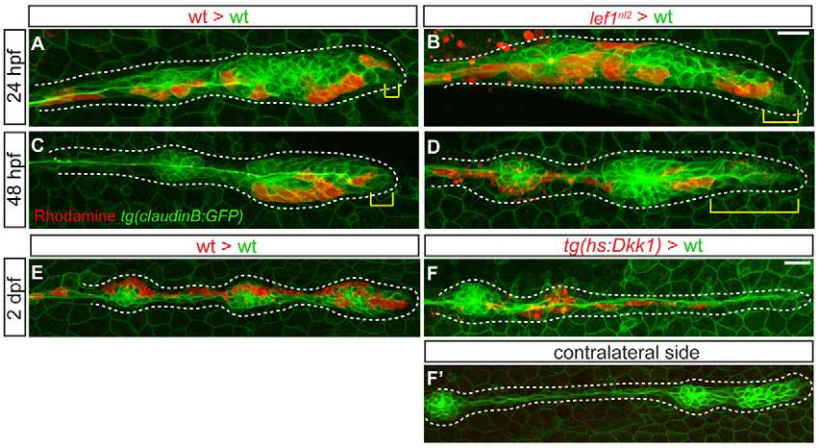

Fig. 8. Lef1 is required to maintain progenitor cell identity. (**A-D**) Confocal projections of *Tg(–8.0cldnb:lynGFP)*-positive, mosaic embryos containing either wild-type or *lef1*^{nl2} donor cells (red) at 24 and 48 hpf. (A) Wild-type host primordium at 24 hpf with wild-type donor cells in the leading region (yellow bracket). (B) Chimeric primordium containing *lef1*^{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 *lef1*^{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.

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 *lef1*^{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 *lef1*^{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 *lef1*^{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 loss of BrdU incorporation in the leading zone of *lef1*^{nl2} mutant primordia and the fact that lineage labeled cells in *lef1*^{nl2} mutants undergo the same number of cell division as those seen in wild-type embryos.

An unresolved issue about Lef1 is whether its function is required to specify progenitor cells or to maintain progenitor cell identity. There are a number of studies demonstrating that canonical Wnt signaling is involved in progenitor specification in multiple organ systems such as heart, hematopoietic and nervous systems (Freese et al., 2010; Gessert and Kuhl, 2010; Grigoryan et al., 2008; Staal and Luis, 2010). In addition, Wnt signaling has been implicated in maintaining progenitor cell self-renewal as well as in maintaining progenitor identity (Grigoryan et al., 2008; Nusse, 2008). Alternatively, Lef1 activity may regulate cell-cell adhesion in the primordium. However, levels of cadherin 2, which is prominently expressed in the leading region (Kerstetter et al., 2004; Liu et al., 2003; Matsuda and Chitnis, 2010), were not altered in *lef1*^{nl2} mutant primordia (data not shown). Distinguishing between these possible roles for Wnt/Lef1 signaling in the lateral line system will require generation of specific markers that identify the progenitor cells during various stages of development to define the dynamics of their behavior in wild-type and *lef1*^{nl2} mutants.

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.

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Competing interests statement
The authors declare no competing financial interests.

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