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Lef1-dependent Wnt/β-catenin signalling drives the proliferative engine that maintains tissue homeostasis during lateral line development

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SUMMARY

During tissue morphogenesis and differentiation, cells must self-renew while contemporaneously generating daughters that contribute to the growing tissue. How tissues achieve this precise balance between proliferation and differentiation is, in most instances, poorly understood. This is in part due to the difficulties in dissociating the mechanisms that underlie tissue patterning from those that regulate proliferation. In the migrating posterior lateral line primordium (PLLP), proliferation is predominantly localised to the leading zone. As cells emerge from this zone, they periodically organise into rosettes that subsequently dissociate from the primordium and differentiate as neuromasts. Despite this reiterative loss of cells, the primordium maintains its size through regenerative cell proliferation until it reaches the tail. In this study, we identify a null mutation in the Wnt-pathway transcription factor Lef1 and show that its activity is required to maintain proliferation in the progenitor pool of cells that sustains the PLLP as it undergoes migration, morphogenesis and differentiation. In absence of Lef1, the leading zone becomes depleted of cells during its migration leading to the collapse of the primordium into a couple of terminal neuromasts. We show that this behaviour resembles the process by which the PLLP normally ends its migration, suggesting that suppression of Wnt signalling is required for termination of neuromast production in the tail. Our data support a model in which Lef1 sustains proliferation of leading zone progenitors, maintaining the primordium size and defining neuromast deposition rate.

KEY WORDS: Lateral Line, Wnt signalling, Organogenesis, Tissue homeostasis, Zebrafish

INTRODUCTION

Tissue morphogenesis requires the coordination of proliferation, patterning, movement/migration and differentiation. Failure in any of these processes can lead to developmental abnormalities that affect the pattern, shape, size and cellular composition of the tissue. The developing posterior lateral line organ has proved to be an outstanding structure in which to explore these events (Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2007; Friedl and Gilmour, 2009).

The lateral line is a mechanosensory organ that is built through a coordinated process of collective cell migration, proliferation, epithelial morphogenesis and differentiation. The posterior lateral line (PLL) of the trunk and tail arises from placodal cells that undergo partial epithelial-mesenchymal transition and acquire migratory properties. A group of about 100 of these cells, the PLL primordium (PLLP), undergoes caudally directed collective cell migration along the myoseptum, regularly depositing groups of ~20 cells that will differentiate as the accessory and hair cells of the mature neuromast (Metcalfe et al., 1985; Ghysen and Dambly-Chaudière, 2004). Prior to deposition, cells in the trailing zone of the primordium become organised into rosette-like epithelial structures that mature into pro-neuromasts, which are reiteratively formed and deposited every 3-4 hours. When the primordium reaches the end of the tail, it fragments into two or three terminal neuromasts.

Despite the loss of cells that results from neuromast deposition, the PLLP maintains its size through compensatory homeostatic cell proliferation: only gradually diminishing in size as it approaches the tail tip. Indeed, it is estimated that the total number of cells increases threefold during migration (Gompel et al., 2001). Cell division occurs throughout the entire primordium, but is twice as high in the leading zone compared with the trailing zone (Laguerre et al., 2005). Consequently, precursors are continuously fed from the leading zone to the trailing zone as the primordium loses cells due to neuromast deposition, contributing to cell number homeostasis (Nechiporuk and Raible, 2008).

In addition to replenishing cells, the leading zone regulates the internal patterning and migration of the PLLP (Haas and Gilmour, 2006; Ghysen and Dambly-Chaudiere, 2007). The spatially polarised distribution of different chemokine receptors within the primordium is required for correct migration (David et al., 2002; Haas and Gilmour, 2006; Valentin et al., 2007; Dambly-Chaudiere et al., 2007) and this regionalisation of the primordium is dependent upon signalling from the leading zone. For example, Wnt activity in the leading zone is proposed to restrict Fgf pathway activation to the central and trailing zones (Aman and Piotrowski, 2008). Fgf signalling promotes epithelialisation, formation of pro-neuromasts and differentiation of neuromast cells (Lecaudey et al., 2008; Nechiporuk and Raible, 2008; Matsuda and Chitnis, 2010).

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Reciprocally, Fgf signalling induces expression of the secreted Wnt inhibitor Dkk1 that limits Wnt signalling to the leading zone (Aman and Piotrowski, 2008). Therefore, the leading zone is both a source of cells for proneuromast formation and a patterning centre that regulates the internal organisation of the primordium.

In this study, we explore how cell proliferation within the leading zone contributes to PLL development, primarily through analysis of the role of Lef1, a transcriptional effector of Wnt/β-catenin signalling. This pathway has been proposed to influence many events in PLL development including proneuromast formation, periodicity of neuromast deposition and proliferation (Aman and Piotrowski, 2008; Aman et al., 2011; Gamba et al., 2010). Lef1 has been suggested to regulate cell proliferation in the placodal field, early during the development of the PLL (Gamba et al., 2010). However, to date, no studies have definitively resolved what processes specific to Wnt/β-catenin signalling are required for PLL development, and a genetic dissection of the role of this pathway remains unexplored.

Lef1 is a Tcf family transcription factor that binds to β-catenin in response to Wnt pathway activation and mediates the expression of downstream genes. Although Wnt signalling is implicated in many developmental processes, there is often redundancy in the roles of individual Lef/Tcf factors such that mutations in individual genes result in milder phenotypes than those observed when the Wnt pathway is globally activated or repressed (Kim at el., 2000; Dorsky et al., 2003; Muncan et al., 2007; Nagayoshi et al., 2008). In the case of *lef1* mutant mice, defects are observed in tooth formation, mammary glands, dentate gyrus granule cells, body hair and whiskers, and in some neural crest-derived neurons (VanGenderen et al., 1994; Galceran et al., 2000). On other hand, overactivation of Wnt signalling is observed in cancerous cells of many origins (Clevers, 2006) and in this context, altered Lef1 function has been associated with acquisition of metastatic properties in melanomas (Larue and Delmas, 2006) and adenocarcinomas (Nguyen et al., 2009). It is intriguing that the PLLP and metastatic cells share several characteristics such as collective cell migration (Friedl and Gilmour, 2009) and expression of various signalling molecules and chemokine receptors (Ma and Raible, 2009; Ghysen and Dambly-Chaudiere, 2007; Gallardo et al., 2010).

In this study, we describe the isolation and characterisation of a zebrafish *lef1* mutant and show that in the absence of Lef1, the PLLP becomes depleted of precursor cells in the leading zone and, as a consequence, neuromast deposition terminates prematurely. Both inhibition of cell proliferation and ablation of proliferative leading zone cells phenocopy aspects of the loss of Lef1 function. We suggest that the PLLP in *lef1* mutants prematurely undergoes the terminal fragmentation that normally occurs in the tail tip upon downregulation of *lef1* expression/Wnt signalling. Although previous studies have suggested that the Wnt pathway regulates internal regionalisation and migration of the primordium, both of these processes are unaffected in *lef1* mutants. This allows us to conclude that Lef1 is a crucial regulator of proliferative cell renewal and tissue homeostasis during migration and differentiation of the PLLP.

MATERIALS AND METHODS

Fish strains and genotyping

Wild-type and mutant embryos were raised at 28.5°C and staged according to Kimmel et al. (Kimmel et al., 1995). Fish lines used were *Tg(-8.0cldnb:lynEGFP)^{zf106}* (Haas and Gilmour, 2006); *Tg(7xtcf-siam:eGFP)^{ia4}* (E.M. and F.A., unpublished); *apc^{CA50a}* (Paridaen et al., 2009). a*pc^{CA50a}* embryos were genotyped by PCR/digestion using primers F-5′-GTGTCCTGAGTGCCCTTTGG-3′ and R-5′-CTTGACACGTTTCTCA-GAATGCCAC-3′ following digestion with *Bpu*10I restriction enzyme.

ENU mutagenesis and lateral line screening

Mutations were induced in wild-type male AB/TL fish by four rounds of 3 mM ENU treatment as previously described (van Eeden et al., 1999). Homozygous F3 larvae were incubated for 5 minutes in a mixture of 4 μg/ml Diasp (2-Di-4-Asp, Sigma) and 0.3 μg/ml $DioC_6$ (3,3′-dihexyloxacarbocyanine iodide, Sigma), washed and observed with a dissecting fluorescence microscope to assess neuromast abnormalities or hair cell defects.

Genetic mapping and molecular analysis of *lef1* mutation

The *lef1^{u767}* mutation was mapped by bulked segregant analysis with SSLPs (Talbot and Schier, 1999). To obtain the full-length *lef1* coding sequence, we performed Trizol (Invitrogen) total RNA extraction from 3 dpf mutant and sibling larvae. cDNA was prepared by reverse transcription from total RNA with SuperScript-III Reverse Transcriptase (Invitrogen) using oligo(dT) primers (Invitrogen). Comparison of wild-type and mutant *lef1* full-length transcripts was carried out by direct sequencing of PCR products amplified using primers Lef1-F–5′-GTGCGCGAGCTGACCA-GAAAC-3′ and Lef1-R-5′-CCACTGGGGTCGAGAGGTGATG-3′.

The genomic lesion was identified by sequencing PCR product from individual embryo genomic DNA samples using primers Lef1 ex2-int2-F-5′-GGAGTTTATTTCCCGGCACT-3′ and Lef1 ex2-int2-R-5′-CATGGGTGCATTTGAAATGCAATGC-3′. Genotyping of *lef1^{u767}* was carried out by PCR/digestion using primers Lef1 Gen-F-5′-ACAGGGAC-CATCCGGATGATCG and Lef1 Gen-R-5′-TGGAATTGGTTTCTTC-ACACGTTTTACC, which incorporate a Hpy188I restriction site to the mutant amplicon.

Microinjections, BrdU incorporation, immunohistochemistry, in situ hybridisation, western blot and drug treatment

One-cell stage embryos were injected with 5 nl of *lef1* morpholino (5′-CTCCACCTGACAACTGCGGCATTTC-3′, Open Biosystems) at 0.05 pmol/nl or Kaede mRNA at 100 pg/nl. No apoptosis was detected with this dose of morpholino (not shown).

For BrdU incorporation, embryos were incubated in 10 mM BrdU 15% DMSO fish water at 4°C for 30 minutes and then returned to embryo medium at 28.5°C for 1 hour and fixed in 4% PFA for immunostaining. After BrdU incorporation, heads of fixed embryos were genotyped.

Cell division was blocked by incubating dechorionated embryos in 150 μM aphidicolin (Sigma, A0781) and 20 mM hydroxyurea (Sigma, H8627) in 2% DMSO fish water (Bonner et al., 2008). Wnt signalling was enhanced by incubating dechorionated embryos from 24-36 hpf in 5 μM BIO (Sigma, B1686) in 2% DMSO fish water.

Whole-mount immunohistochemistry was performed using: BrdU (1:200, Sigma); GFP (1:200, AMS Biotechnology); acetylated tubulin (1:1000, Sigma) using standard procedures (Wilson et al., 1990). Nuclei were counterstained using 1 μg/ml DAPI. Digoxigenin-labelled RNA probes were synthesised using a DIG labelling kit (Roche) and probes were detected with anti-DIG-AP antibody (1:5000, Roche) and NBT/BCIP substrate (Thisse and Thisse, 2008). For western blotting, we used a custom-made rabbit anti zebrafish Lef1 antibody (1:1000) kindly provided by the Dorsky lab (Lee at al., 2006).

Time-lapse imaging, photoconversion, cell ablation and image processing

Time-lapse and other imaging was performed on a Leica TCS SPE confocal microscope in an air chamber heated to 28.5°C. Live embryos were immobilised in 1.2% low melting point agarose (Sigma) and 0.016% Tricaine (Sigma) to anaesthetise. Image analysis of nuclei count and distance measurement were assessed using ImageJ software (http://rsb.info.nih.gov/ij/).

In Kaede-expressing embryos, the leading edge of the primordium was photoconverted at 24 hpf with a 405-nm laser on a Leica TCS SPE using 30% output in conventional scan mode, scanning the same area three times. Embryos were imaged with 488 nm and 543 nm excitation for green and red Kaede, respectively.

For cell ablation, *Tg(-8.0cldnb:lynEGFP)^{zf106}* embryos were anaesthetised in 0.016% tricaine and mounted in sealed agarose chambers. Two-photon laser excitation on a Zeiss-510 microscope was used to image and ablate the

caudal-most third of the primordium. A single 2D scan at 180 mW with a 40× ScanImage zoom was used to ablate at 24 hpf. Ablation efficiency was confirmed by analysing the absence of GFP expression and cell morphology.

RESULTS

Lef1 is required for development of the PLL primordium and fin growth

We screened zebrafish families carrying ENU-induced mutations to identify genes required for PLL development. Clutches of 72 hpf embryos from sibling crosses were stained with the vital dyes DiAsp and $DiOC_6$ to mark neuromast hair cells (red) and accessory cells (green) (Collazo et al., 1994; Koning et al., 1993). Using these dyes, wild types show the expected distribution of seven or eight PLL neuromasts spanning the trunk and tail (Fig. 1A).

We recovered the recessive mutation *sinfin*^{u767} (from the Spanish 'sin fin' meaning 'no end') in which mutants lack the most caudal PLL neuromasts displaying only the first four or five (4.6±0.9, n=15) (Fig. 1B). *sinf*^{u767} homozygotes are viable and no other overt phenotypes are apparent during embryonic stages (see Fig. S1A-D in the supplementary material). However, 30-day-old homozygous *sinf*^{u767} fish lack pectoral fins and other fins are reduced, a condition that persists throughout adulthood (see Fig. S1E-L in the supplementary material). Given that *sinf*^{u767} pectoral fins appear normal at 6 dpf, we infer that the mutation either affects the ability of the fins to grow or to maintain tissue homeostasis.

Bulked segregant analysis using SSLPs localised the *sinf*^{u767} mutation to chromosome 1 between z9704 and z11464 (see Fig. S2 in the supplementary material). Within this interval, we identified three genes expressed in the PLLP: *lef1*, *meis4.1* and *msx3b* (Gallardo et al., 2010) (http://zfin.org). Sequencing revealed that only in *lef1* did *sinf*^{u767} mutant cDNA differ from sibling cDNA, lacking the entire 2nd exon. RT-PCR using sibling and mutant cDNA with primers targeting the flanking 1st and 3rd exons confirmed the fully penetrant absence of the 2nd exon in the *lef1*^{u767} mutant transcript (Fig. 1D). This splicing aberration generates a shift in the reading frame leading to 19 missense codons followed by a stop codon (see Fig. S3 in the supplementary material). As a result, the shorter *lef1*^{u767} mutant transcript generates a truncated Lef1 protein containing only the first 71 residues. No wild-type Lef1 immunoreactivity was detected by western blot analysis of mutant embryos using a polyclonal antibody raised against wild-type Lef1 protein (Fig. 1E) (Lee et al., 2006). Sequencing of the genomic intron-exon-2 boundaries revealed a T to G base change that deletes the 5′ splice donor site in the 2nd intron (see Fig. S4 in the supplementary material). Expression of *lef1* in the leading edge of the PLLP of *lef1*^{u767} mutants is indistinguishable from wild types (Fig. 1F,G) (Aman and Piotrowski, 2008), showing that there is no obvious nonsense-mediated decay of the mutant transcript and suggesting that *lef1* expression does not depend upon Lef1 function in the PLLP. Knockdown of Lef1 in wild-type animals by an ATG-directed anti-*lef1* morpholino phenocopies the *lef1*^{u767} mutant in over 90% (n>100) of individuals (Fig. 1C). Therefore, although the *lef1*^{u767}mutant expresses a fragment of the Lef1 protein, its phenotype is indistinguishable from the translation start-site morpholino knockdown phenotype and, consequently, indicates that *lef1*^{u767} is a loss-of-function mutation.

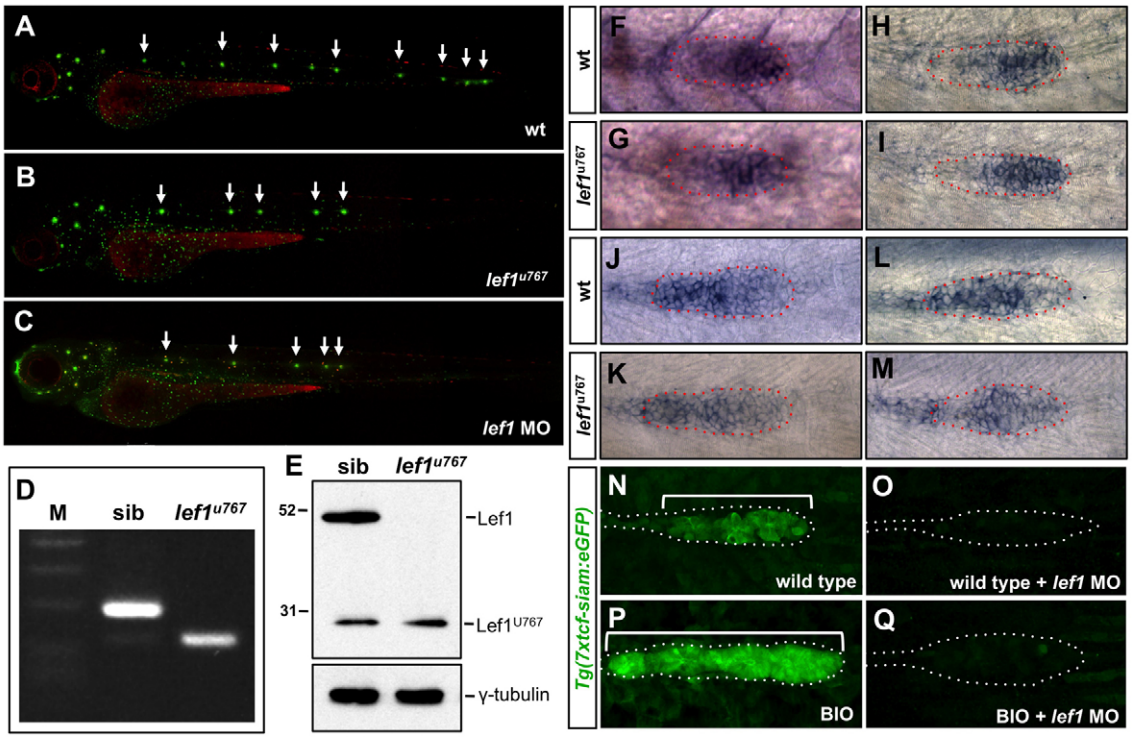

Fig. 1. Characterisation of the zebrafish *lef1*^{u767} mutant. (**A-C**) Lateral views of wild-type (A), *lef1*^{u767} (B) and *lef1* morphant (C) 3 dpf zebrafish embryos stained with DiAsp/$DiOC_6$. Arrows indicate positions of PLL neuromasts. (**D**) RT-PCR from sibling and *lef1*^{u767} cDNA using primers targeting exons 1 and 3. Mutant lane shows a 121 nucleotide band lacking 67 nucleotides of exon 2. DNA marker lane shows 400, 300, 200 and 100 bp bands. (**E**) Western blot showing absence of Lef1 band in *lef1*^{u767} mutant. (**F-M**) Expression of *lef1* (F,G), tcf7 (H,I), *tcf7l1a* (J,K) and *tcf7l2* (L,M) in wild-type (F,H,J,L) and *lef1*^{u767} mutant (G,I,K,M) 33 hpf embryos. Primordia are outlined. (**N-Q**) GFP expression in control *Tg(7Xtcf-siam:GFP)*^{ja4} Wnt reporter (N), *lef1* morphant (O), BIO incubated (P) and BIO-incubated/*lef1*-morphant (Q) 36 hpf embryos. PLLP is outlined in each case. Brackets indicate the length of the primordium.

We next assessed whether levels of Wnt signalling are affected in the PLLP of *lef1*^{u767} mutants. Indeed, we find that Lef1 is required for activation of the *Tg(7xtcf-siam:eGFP)*^{ia4} transgene reporter of Wnt signalling in the PLLP (Fig. 1N,O) but not in other tissues (see Fig. S5 in the supplementary material). Furthermore, exposure of *Tg(7xtcf-siam:eGFP)*^{ia4} embryos to the Wnt agonist BIO (Polychronopoulos et al., 2004) from 24 to 36 hpf, which generates a lateral line phenotype comparable with that of *apc* mutants, overactivates GFP expression (Fig. 1P; see Fig. S6 in the supplementary material). When *lef1* morphants were treated with BIO, GFP expression was still absent (Fig. 1Q), indicating that the transgene cannot be activated in the PLLP in the absence of Lef1 function. Supporting this, *gfp* mRNA expression in *Tg(7xtcf-siam:eGFP)*^{ia4} embryos follows that of *lef1* in PLLP leading zone (Fig. 6I-M). This suggests that Wnt/β-catenin driven activation of the reporter transgene is predominantly or exclusively dependent upon Lef1 function in the PLLP, but not in other tissues.

Although the *Tg(7xtcf-siam:eGFP)*^{ia4} reporter is dependent on Lef1 function, the Wnt pathway target genes *lef1* and *axin2* (not shown) are still expressed in the PLLP of *lef1* mutants, suggesting that the full extent of Wnt/β-catenin signalling in the PLLP may occur through Lef1 functioning together with other Tcfs. Expression of *tcf7l1a* (*tcf3a*) and *tcf7l2* (*tcf4*) is restricted to the trailing zone in both wild-type and *lef1*^{u767} mutants (Fig. 1J-M), suggesting that they are unlikely to function redundantly with Lef1. *tcf7* is expressed in the leading zone in wild type and *lef1*^{u767} mutants and so could function together with Lef1 (Fig. 1H,I). Although *tcf7* mutants have no phenotype in the PLLP (Aman et al., 2011), we did find that *lef1* mutants injected with a *tcf7* morpholino showed a slightly more severe phenotype with fewer neuromasts deposited more closely together (see Fig. S7 in the supplementary material). Removal of Lef1 therefore unmasks a subtle redundant role for Tcf7 in the leading zone of the PLLP. However, given the minor phenotypic consequences of abrogation of *tcf7* together with the dependence of the reporter transgene upon Lef1 function, it is clear that Lef1 is a major mediator of Wnt/β-catenin signalling in the leading zone of the PLLP.

Primordium patterning, migratory ability, neuromast morphogenesis and differentiation are unaffected in *lef1*^{u767} mutants

To assess the nature of the PLL defect in *lef1*^{u767} fish, we studied neuromast positioning relative to somite number in wild types, *lef1*^{u767} mutants and *lef1* morphants (Fig. 2A-C; see Table S1 in the

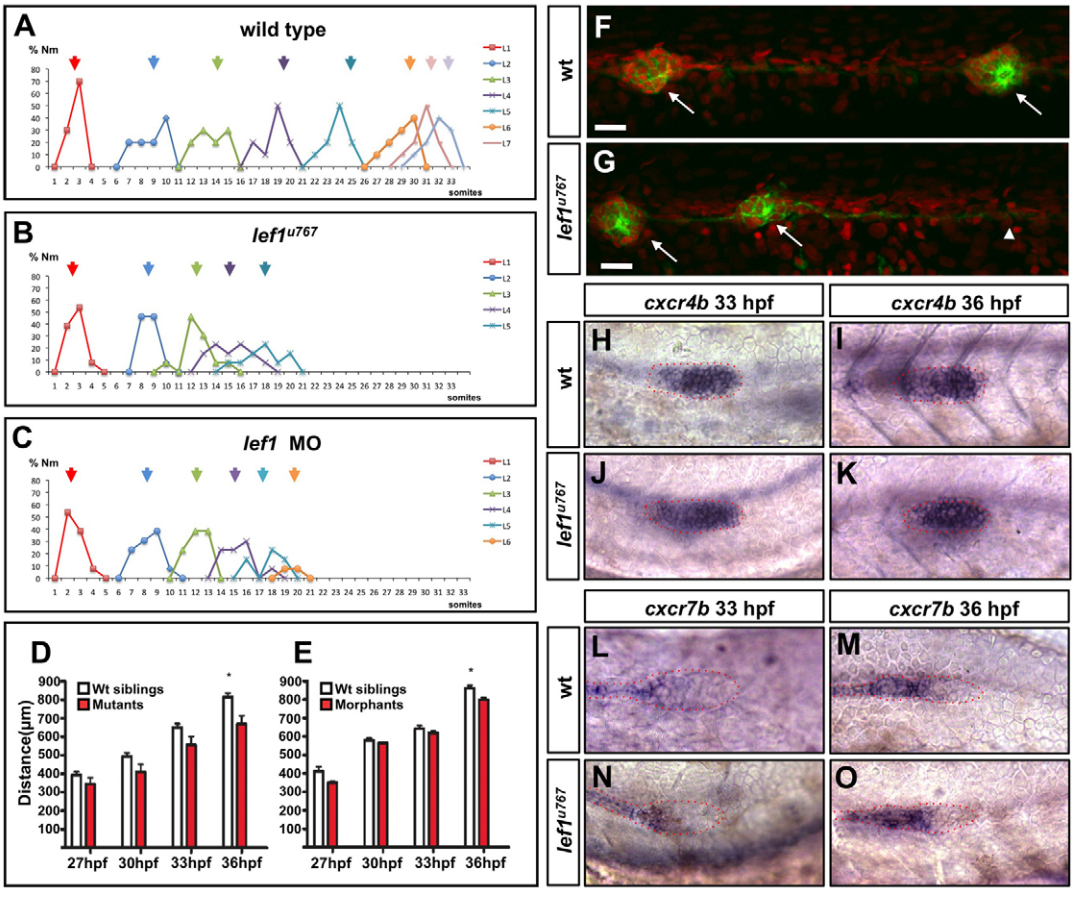

Fig. 2. Neuromast position, primordium kinetics and chemokine receptor expression are unaffected by loss of Lef1 function. (**A-C**) Histograms depicting the number/position of neuromasts in siblings (A) *lef1*^{u767} mutants (B) and *lef1* morphants (C) at 3 dpf. Arrows indicate the average position and colours denote the different populations of sequentially deposited neuromasts. (**D**,**E**) Plot of the distance (in μm) in *lef1*^{u767} mutants (D) and *lef1* morphants (E) between the caudal limit of the otic vesicle and the leading edge of the PLLP at 27, 30, 33 and 36 hpf (n=7 mutants/morphants and n=15 wild types in both cases). The only significant difference is observed at 36 hpf (*P<0.01, Wilcoxon rank-sum test). Data are mean+s.d. (**F**,**G**) GFP [*Tg(-8.0cldnb:lynEGFP)*^{zf106}]-positive cells extend beyond the terminal neuromast in 55 hpf *lef1*^{u767} embryos (arrowhead). Arrows indicate neuromasts L4 and L5. Nuclei are counterstained with propidium iodide. Scale bar: 20 μm. (**H-O**) Expression of *cxcr4b* (H-K) and *cxcr7b* (L-O) in wild type (H,I,L,M) and *lef1*^{u767} mutants (J,K,N,O) at 33 and 36 hpf.

supplementary material). The first three neuromasts (L1-L3) are always present in *lef1*^{u767} mutants, but neuromasts L4 and L5 are present in only 92.3% and 76% of cases respectively (n=15), and are also deposited at progressively shorter-spaced intervals compared with siblings. Plotting the average neuromast position for each consecutive neuromast show a tightly correlated linear relationship (r^2=0.998) in wild types, indicating regular spacing, whereas plots from mutants and morphants fit a parabolic curve (r^2=0.998), indicating that each consecutive neuromast is deposited at progressively reduced intervals (see Fig. S8 in the supplementary material).

The crowding of neuromasts in *lef1*^{u767} mutants suggests that there is either an increase in the neuromast deposition rate, or that the primordium decelerates during migration. To resolve this, we calculated primordium migration speed by measuring its position relative to the otic vesicle at four time points (Fig. 2D,E; see Table S2 in the supplementary material). The position of the primordium shows no significant difference between wild types and mutants or morphants at 27, 30 and 33 hpf, and only at 36 hpf did we observe a modest, but significant, difference in the position of the primordia (Fig. 2D,E). This corresponds to the time at which the terminal neuromasts are being deposited in the mutants/morphants. Consequently, mutant primordia are able to migrate at normal speed and show no tendency to decelerate until deposition of the final neuromast. Therefore, the crowding of neuromasts suggests that proneuromast deposition rate is accelerated in *lef1*^{u767} mutants (see Fig. 4 below).

Wnt/β-catenin signalling is thought to restrict the expression of the chemokine receptors Cxcr4b and Cxcr7b to the leading and trailing zones, respectively (Aman and Piotrowski, 2008). However, their expression patterns are unchanged in *lef1*^{u767} mutants (Fig. 2H-O), consistent with the absence of migration defects in *lef1*^{u767} mutant primordia.

Aman and Piotrowski (Aman and Piotrowski, 2008) also suggest that Wnt/β-catenin signalling regulates proneuromast formation through modulation of Fgf signalling, which is required for proneuromast rosette organisation and differentiation of neuromast cell types (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). However, the Fgf pathway genes *fgf3*, *pea3* and *sef1* show unaltered expression patterns in *lef1*^{u767} mutants (Fig. 3A-L), suggesting that Lef1 is not responsible for the induction, maintenance or suppression of Fgf signalling. Supporting this, apical/basal polarity and overall rosette morphology appear unaffected in *lef1*^{u767} mutants (Fig. 3M,N). Furthermore, the expression of *atoh1a*, a marker gene for cells fated to become neuromast hair cells (Itoh and Chitnis, 2001), is also normal in mutants (data not shown) as is acetylated tubulin and Diasp labelling of differentiated, functional mechanoreceptor hair cells (Fig. 3O-R).

In summary, our data show that Lef1-mediated Wnt signalling has no obvious role in PLLP migration, proneuromast formation/morphology and neuromast hair cell differentiation. Consequently, the failure to deposit caudal neuromasts in *lef1*^{u767} mutants must be due to another defect in the PLLP.

The *lef1*^{u767} PLL primordium collapses due to decreased proliferation and depletion of leading zone cells

To investigate the defective behaviour of the *lef1*^{u767} PLLP, we imaged *Tg(-8.0cldnb:lynEGFP)*^{zf106} transgene-labelled primordia (Haas and Gilmour, 2006) in *lef1* mutants/morphants. Migration of the primordium is not obviously affected until around 36 hpf when it collapses, depositing one or two neuromasts in quick succession (Fig. 4; see Movies 1-3 in the supplementary material). Subsequently, a small number of cells not incorporated into neuromasts continue to migrate caudally, forming a thin row of

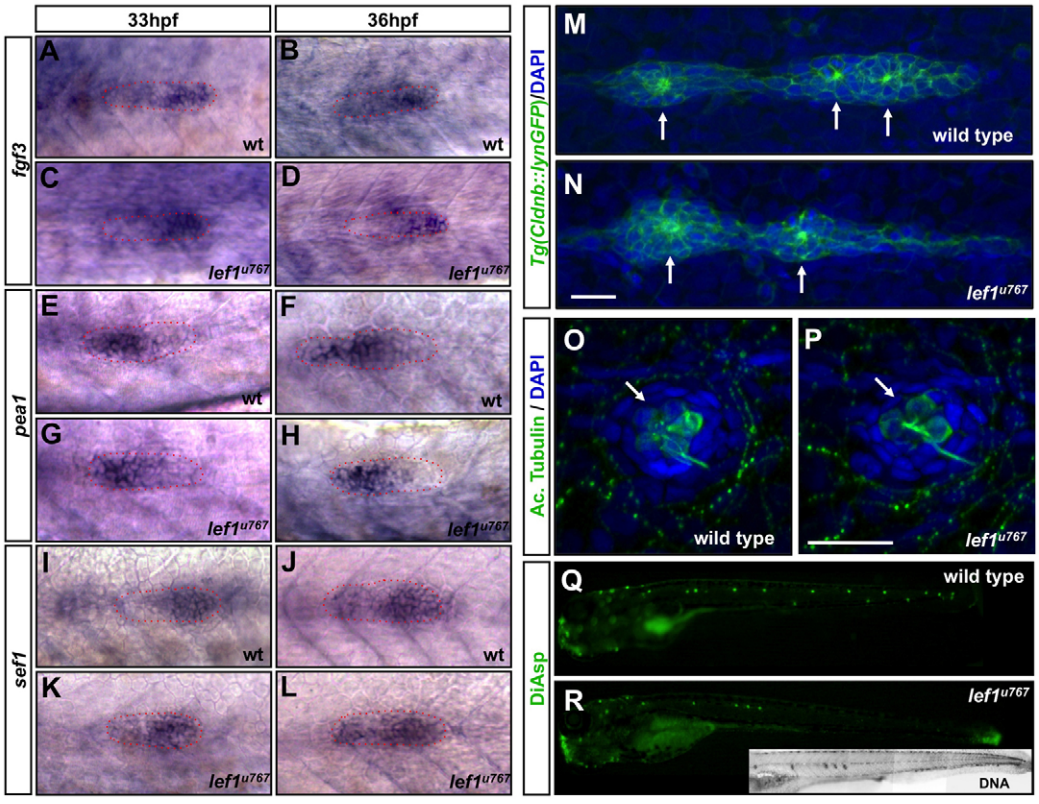

Fig. 3. Primordium organisation, neuromast morphology and differentiation are unaffected by loss of Lef1 function. (**A-L**) Expression of *fgf3* (A-D), *pea1* (E-H) and *sef1* (I-L) in wild type (A,B,E,F,I,J) and *lef1*^{u767} mutants (C,D,G,H,K,L). Primordia are outlined. (**M**,**N**) *Tg(-8.0cldnb:lynEGFP)*^{zf106}-labelled wild-type (M) and *lef1*^{u767} (N) primordia at 38 hpf. Membrane-anchored GFP reveals the polarisation of cells and their organisation into rosettes (arrows). (**O**,**P**) The last-deposited neuromast in a *lef1*^{u767} mutant (P) and wild type (O) labelled with anti-acetylated tubulin antibody (green). Nuclei are DAPI stained. Scale bar: 20 μm. (**Q**,**R**) Diasp staining in wild type (Q) and *lef1*^{u767}mutant (R) at 3 dpf. DAPI-labelling of nuclei (inset in R) shows no more neuromasts in the *lef1*^{u767} other than those labelled with Diasp).

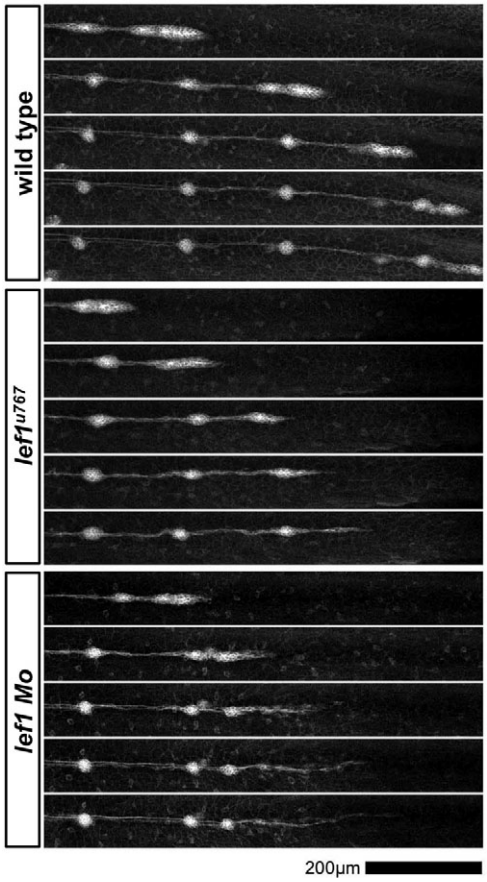

Fig. 4. Time-lapse analysis of primordium migration in *Tg(-8.0cldnb:lynEGFP)* embryos. Frames were taken from time-lapse movies of wild type (top panel), *lef1*^{u767} (middle panel) and *lef1* morphant (bottom panel). Frames are spaced at approximately 2-hour intervals.

cells stretching 100-200 μm. Therefore the lack of caudal neuromasts in *lef1*^{u767} mutants is not due to a cell migration defect but is instead due to the PLLP becoming depleted of cells following the premature deposition of a terminal pair of neuromasts, leaving only a few leading cells to continue migrating caudally (Fig. 2F,G).

The proliferation rate in the leading zone of the PLLP is twice as high as in the trailing zone (Laguerre at el., 2005) and, as *lef1* is expressed in the leading zone, we explored whether Lef1-mediated Wnt/β-catenin signalling regulates cell proliferation. *lef1*^{u767} mutants show a reduction of BrdU-labelled proliferative cells in the primordium at all time points tested (Fig. 5A-H). When we scored the percentage of BrdU-positive cells relative to total nuclei in the primordium, *lef1*^{u767} mutants showed an average of 48.7% compared with the number BrdU-positive cells in wild types (Fig. 5I; see Fig. S9C and Table S3 in the supplementary material). When we calculated the proliferation rates in the leading zone, mutants had only 37% as many BrdU labelled cells as wild types (see Fig. S9A,D in the supplementary material), whereas there was no significant difference in the proliferation rate in the trailing zone (see Fig. S9B, Table S3 in the supplementary material). There were also no differences in TUNEL-labelled cells between wild-type and *lef1*^{u767} mutant primordia (see Fig. S10 in the supplementary material), showing that apoptosis does not contribute to the depletion of cells in the mutants. These results suggest that the phenotype in *lef1*^{u767} mutants is a consequence of decreased proliferation in the PLLP leading zone with the consequent failure to replenish itself resulting in the eventual collapse of the primordium.

To test this hypothesis, we treated wild-type embryos from 24-48 hpf with the cell-cycle inhibitors aphidicolin and hydroxyurea (Bonner et al., 2008). In treated embryos, the primordium migrates, deposits a few neuromasts but fails to reach the tip of the tail and shows a few cells migrating caudally beyond the last neuromast (Fig. 5J,K). Although these features phenocopy aspects of the *lef1* mutant, the few deposited neuromasts in aphidicolin/hydroxyurea-treated embryos are further apart than in wild types (see Discussion).

Progressive depletion of *lef1*-expressing leading zone cells leads to collapse of the PLL primordium

The leading zone is a source of progenitors that adds cells to the trailing zone (Nechiporuk and Raible, 2008), contributing to the maintenance of the size of the primordium as it deposits neuromasts. Decreased proliferation in the leading zone of *lef1*^{u767} mutants could affect the ability of cells to translocate into the trailing zone.

To test whether Lef1 influences the ability of leading zone cells to move to the trailing zone, we photoconverted Kaede-expressing cells at the tip of the leading zone in 24 hpf *Tg(-8.0cldnb:lynEGFP)*^{zf106} wild-type and *lef1* morphant embryos. Eleven hours later, we observed that labelled cells had contributed to neuromasts in nine out of 10 *lef1* morphants, as they do in wild-type animals (Fig. 5L-O). Therefore, *lef1* morphant leading zone cells retain the ability to move into the trailing zone, which, together with its reduced proliferation, depletes the leading zone of cells.

To further test the hypothesis that depletion of leading zone cells underlies the *lef1*^{u767} PLLP defect, we laser-ablated leading zone cells (the caudal-most third of the primordium) in *Tg(-8.0cldnb:lynEGFP)*^{zf106} wild-type embryos at 24 hpf and followed the subsequent behaviour of the primordium. Leading zone-ablated primordia deposit an average of 5.2±1.8 neuromasts before collapsing, as occurs in *lef1*^{u767} mutants (Fig. 5P,Q, n=15). The terminal neuromasts of ablated primordia show a tendency to crowd and the few remaining primordium cells extend caudally, again as in the *lef1*^{u767} mutant. This approach supports the idea that the leading zone is required for the maintenance of overall primordium homeostasis and correct neuromast number and spacing.

Together, our data suggest that Lef1 regulates proliferation in the leading zone of the primordium, and that continued production of cells in this niche is required for the homeostatic maintenance of the primordium during its migration and morphogenesis.

Collapse of the *lef1*^{u767} primordium is analogous to the process by which the wild-type primordium terminates neuromast deposition

The PLLP reaches the end if its migration at the tip of the tail where it deposits two or three terminal neuromasts in close succession (Kimmel et al., 1995) (Fig. 6A,B). Time-lapse imaging using *Tg(-8.0cldnb:lynEGFP)*^{zf106} wild-type embryos shows that, as it reaches the tip of the tail, the primordium splits and simultaneously forms the terminal neuromasts, with a few remaining cells subsequently continuing migration (Fig. 6C; see Movie 4 in the supplementary material). This behaviour is

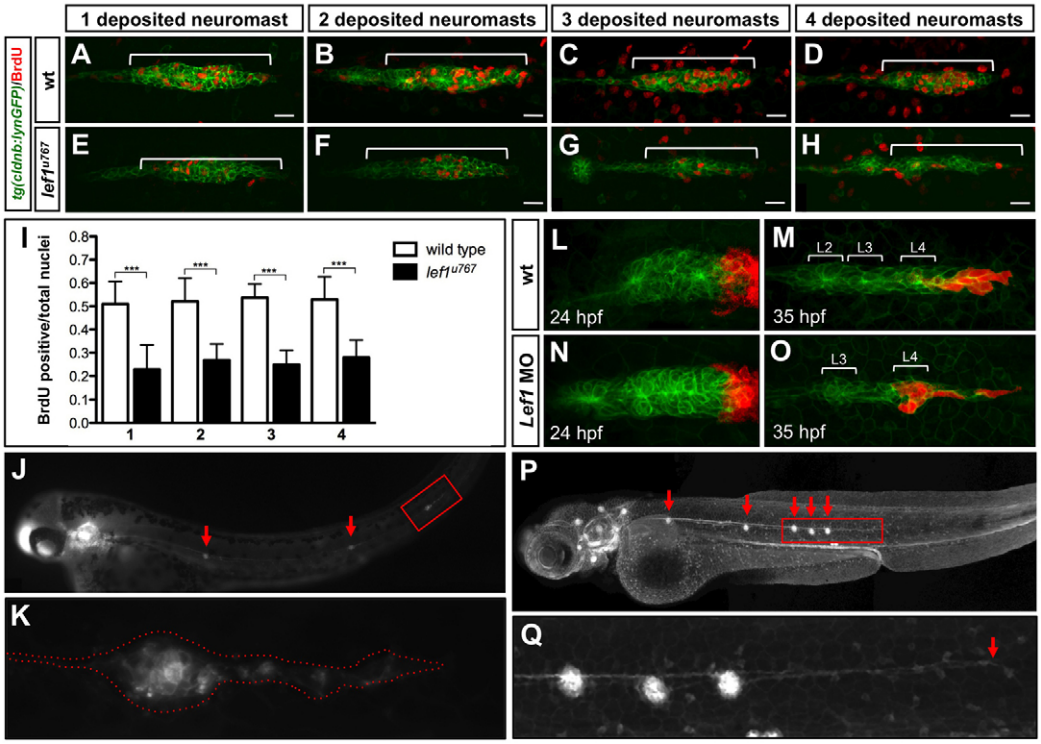

Fig. 5. Mutant PLL primordia collapse is due to decreased proliferation and depletion of leading zone cells. (**A-H**) Cell proliferation analysis in wild-type (A-D) and *lef1*^{u767} mutant (E-H) *Tg(-8.0cldnb:lynEGFP)*^{zf106} embryos. After BrdU pulse and chase, embryos were fixed after deposition of neuromast L1 (A,E), L2 (B,F), L3 (C,G) and L4 (D,H). Brackets show the primordium. Scale bars: 20 μm. (**I**) BrdU-positive cells/total cell ratio in wild type (white bars) and *lef1*^{u767} mutants (red bars) after one to four deposited neuromasts. Total cells where counted after DAPI staining nuclei. Significance was tested with Wilcoxon rank-sum test (***P<0.001). Data are mean+s.d. (**J**,**K**) 48 hpf aphidicolin/hydroxyurea-treated *Tg(cldnb:lynGFP)*^{zf106} embryos show three or four widely spaced neuromasts and a caudally extended row of cells (J). Red arrows indicate neuromasts. (K) Amplification of red rectangle in J. (**L-O**) Kaede photoconverted leading edge cells at 24 hpf in wild type (L) and *lef1*^{u767} (N) assessed at 35 hpf (M,O). Brackets indicate preformed neuromasts. (**P**,**Q**) Ablation of the caudal third of PLLP at 24 hpf leads to a *lef1*^{u767}-like phenotype with 20%, 33.3% and 46.6% of the embryos displaying 3, 5 and 7 neuromasts respectively at 72 hpf (15 ablated specimens). Red arrows indicate neuromasts. (Q) Inset from P with red arrow showing the remaining migrating cells.

reminiscent of what happens to the primordium further rostrally in *lef1*^{u767} mutants. Consequently, *lef1*^{u767} mutant primordia may be prematurely undergoing a terminal morphogenesis event that normally occurs in the tip of the tail in wild-type embryos. If correct, then terminal neuromast deposition in wild-type animals might be triggered by downregulation of *lef1* and reduced proliferation. In support of this, the primordium gradually decreases in size as it moves caudally (Kimmel et al., 1995) and we found that this is associated with a reduction in BrdU+ cells in the leading zone (from a 0.5±0.03 average ratio after the first four deposited neuromasts to 0.3±0.1 after the deposition of neuromast L6, n=13). Moreover, *lef1* mRNA levels progressively decrease to almost undetectable levels as the primordium approaches the tip of the tail (Fig. 6D-H) as does *gfp* mRNA in *Tg(7xtcf-siam:eGFP)*^{ia4} fish (Fig. 6I-M). Taken together, these data show that as the wild-type primordium reaches the tip of the tail, *lef1* expression and Lef1-dependent Wnt/β-catenin signalling is reduced, proliferation drops and the primordium fragments into terminal neuromasts.

Loss of Lef1 function partially rescues PLL primordium defects in *apc*^{CA50a} mutants

Based on analysis of *apc* mutants and overexpression of reagents that affect Wnt signalling, it has been proposed that Wnt/β-catenin signalling affects several aspects of PLL development, including regionalisation and migratory ability (Aman and Piotrowski, 2008). This is in contrast to our observation of a restricted role for Lef1 in the replenishment of progenitor cells that feed the PLLP as it undergoes migration and morphogenesis. It is possible that Wnt signalling directs alternative processes in the PLLP through different Lef/Tcf factors or that overactivation of the pathway may elicit phenotypes unrelated to the normal requirement for Wnt/β-catenin signalling. To begin to explore these possibilities, we assessed whether Lef1 is mediating the various PLLP defects observed in *apc* mutants.

Loss of *apc* function leads to overactivation of Wnt signalling and expression of *lef1* throughout the PLLP. This is accompanied by altered polarisation of the primordium, and migration arrest possibly due to disrupted expression of *cxcr4b* and *cxcr7b* (Aman and Piotrowski, 2008).

Abrogation of Lef1 in *apc* mutants did not fully rescue the primordium migration defect (Fig. 7A,B). However, the migratory capacity of the primordium did improve and there was a slight increase in the spacing between rostral neuromasts and a more caudal position for the final neuromast (Fig. 7A,B). We also observed the appearance of a long caudally extended trail of primordium cells in *apc* mutants lacking Lef1, similar to what happens in *lef1* mutants (Fig. 7C,D; see Fig. S11 in the supplementary material).

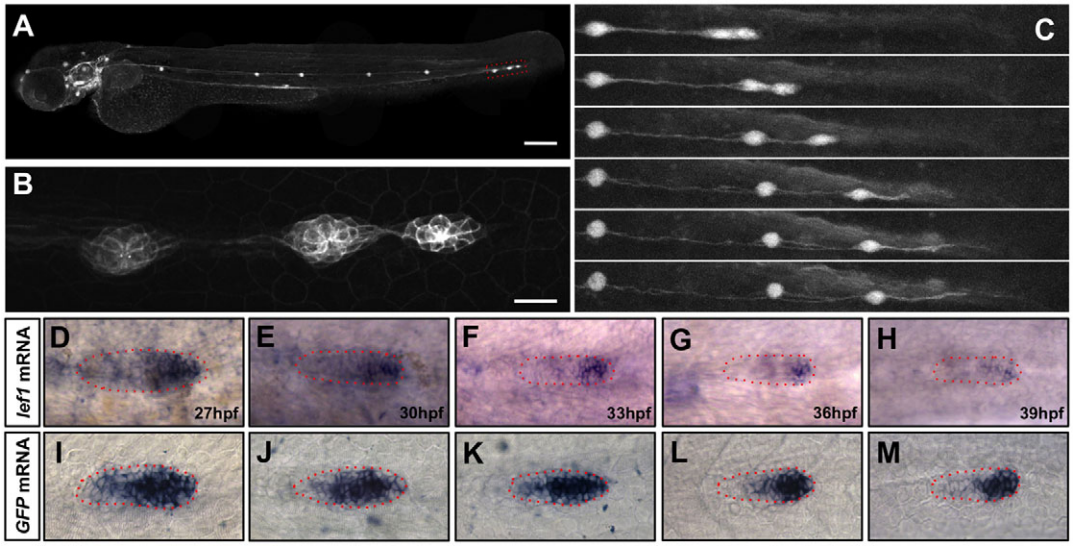

Fig. 6. Terminal neuromast formation in the wild-type PLL primordium. (**A**) Lateral view of a 72 hpf *Tg(-8.0cldnb:lynEGFP)*^{zf106} larvae. Scale bar: 200 μm. (**B**) The last three neuromasts from A. Scale bar: 20 μm. (**C**) Frames from Movie 4 in the supplementary material starting as the seventh neuromast is being deposited. Times between selected frames are 2, 5, 8, 5.3 and 4 hours. There is almost no difference between the last two frames, even though 4 hours have passed. (**D-H**) Expression of *lef1* at 27, 30, 33, 36 and 39 hpf in wild-type embryos. (**I-M**) mRNA expression of *Tg(7Xtcf-siam:GFP)*^{ia4} at 27, 30, 33, 36 and 39 hpf in wild-type embryos. Primordia are outlined in red.

The disrupted patterning of the PLLP in *apc* mutants was not restored by abrogation of Lef1 and there was no recovery of the patterned expression of Fgf pathway genes (data not shown). Consequently, Lef1 mediated Wnt/ß-catenin signalling only contributes in a relatively minor way to the range of PLLP defects observed in *apc* mutants.

DISCUSSION

Through a genetic screen, we have identified and characterised a null mutation in zebrafish *lef1* and have demonstrated that Lef1 has a crucial role in regulating cell proliferation in the leading zone of the migrating PLLP. We show that this role is uncoupled from primordium patterning, migration and neuromast formation. Nevertheless, the decrease of cell proliferation in the leading zone affects homeostasis of the primordium, resulting in accelerated neuromast deposition and finally to its collapse.

The *lef1*^{u767} mutation causes a phenotype that is indistinguishable from an ATG-morpholino loss-of-function phenotype (Fig. 1C). Consequently, any truncated Lef1 protein expressed in the mutant has no impact on the phenotype and is likely to be non-functional. As there is no overt lateral line phenotype in *tcf7* mutants (Aman et al., 2011), and abrogation of Tcf7 only subtly enhances the *lef1* mutant phenotype (see Fig. S7 in the supplementary material), we suggest that Lef1 is the main effector of Wnt signalling controlling cell proliferation in the leading zone.

Considering that other Tcf family genes are expressed in the primordium, it was surprising that loss of Lef1 function eliminates *Tg(7xtcf-siam:GFP)*^{ia4} reporter expression in the primordium, even when the pathway is activated with the Wnt agonist BIO. However, the transgene appears to be an accurate reporter of Lef1-mediated Wnt signalling activity in the primordium, with its expression mirroring the expression of *lef1* itself throughout PLLP development. In the absence of Lef1 function and transgene expression, we still observed some PLLP expression of genes that are often direct targets of Wnt signalling. It would appear, therefore, that within the PLLP, the transgene accurately reports Lef1 activity but may not show the full extent of Wnt pathway regulation of target genes. This is perhaps not surprising as the enhancers of endogenous target genes are probably subject to much more complex regulation than the artificial transgene enhancer.

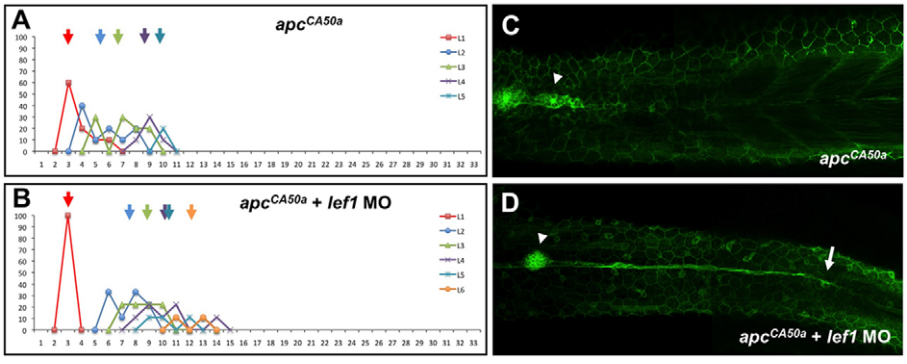

Fig. 7. Lef1 is epistatic to APC in controlling leading zone cell behaviour. (**A**,**B**) Histogram depicting the number/position of neuromasts in *apc*^{CA50a} (A) and *apc*^{CA50a} mutant/*lef1*-morphant (B) embryos. Arrows indicate the average position and colours denote the different populations of sequentially deposited neuromasts. (**C**,**D**) *apc*^{CA50a} (C) and *apc*^{CA50a} mutant/lef1-morphant (D) *Tg(-8.0cldnb:lynEGFP)*^{zf106} embryos showing groups of cells migrating caudally after the primordium has stalled (arrowheads). Arrow in D indicates the extended trail of cells in morphants.

Lef1-dependent proliferation in the leading zone maintains primordium homeostasis during migration and morphogenesis

The leading zone of the primordium has twice as many proliferating cells as the trailing zone (Laguerre et al., 2005) and is the source of progenitors whose daughters progress to the trailing zone to form new neuromasts (Nechiporuk and Raible, 2008). BrdU incorporation is strongly reduced in the *lef1*^{u767} mutant leading zone, whereas cells continue to move towards the trailing zone. Given that there is no significant change in the proliferation rate in the trailing zone, the most likely explanation for the *lef1* mutant phenotype is that the reduced proliferation leads to depletion of the leading zone and collapse of the primordium into terminal neuromasts.

Global inhibition of Wnt/β-catenin signalling through overexpression of Dkk1 reduces proliferation in the primordium but, unlike *lef1*^{u767} mutants, the primordium manages to migrate to the caudal tail (Aman and Piotrowski, 2008; Aman et al., 2011). We suspect that the reason for the difference is that Dkk1 overexpression additionally suppresses neuromast formation/deposition and, therefore, the trailing zone does not become depleted of cells and there is consequently no trigger for terminal neuromast formation. Given that gain of function of Dkk1 inhibits neuromast formation in the PLLP, the *lef1*^{u767} mutant provides a straightforward model for resolving the links between Wnt signalling, proliferation and neuromast deposition.

lef1^{u767} and wild-type primordia show similar morphologies and behaviours as they terminate their migration. We therefore suggest that in *lef1*^{u767} mutants, the primordium prematurely undergoes a normal termination event in an inappropriate rostral location and that in wild types, the inhibition of Lef1-mediated Wnt signalling terminates migration at the tip of the tail. Supporting this idea, expression of *lef1* and the *7xtcf-siam:GFP*^{ia4} transgene reporter is downregulated in the wild-type PLLP as it nears the tail tip. The reduced Lef1 activity would inhibit proliferation in the leading zone, depleting the trailing zone with the consequent fragmentation of the primordium into terminal neuromasts. The mechanism by which *lef1* is gradually downregulated is not known but the embryonic tailbud expresses a variety of candidate signalling molecules (Wilson et al., 2009). Consequently, it may produce a factor that suppresses *lef1* expression in the leading zone as the primordium approaches the tip of the tail, thereby terminating migration in the appropriate location.

Our data assign a role to leading zone proliferation in the morphogenesis of the PLLP. Lef1-dependent proliferation maintains tissue homeostasis of the primordium during migration and regulation of *lef1* expression provides a route to control the termination of migration and perhaps the frequency of neuromast deposition.

Neuromast deposition and primordium termination may depend on a mechanism by which the size of the leading zone determines cell behaviour in the trailing zone

Neuromasts are deposited increasingly closer together in *lef1*^{u767} mutants than in wild types and in the tail compared with the trunk, whereas neuromasts are more widely spaced when proliferation is reduced throughout the primordium (Aman et al., 2011) (this study). Termination of migration occurs prematurely in *lef1*^{u767} mutants and also when proliferation is reduced throughout the primordium. We have attempted to account for these various observations through a simple model in which trailing zone cells assess their location relative to a factor(s) produced by leading zone cells. We postulate that the amount of this factor(s) – and thereby, the distance at which it can influence trailing zone cells – is dependent on the size of the leading zone itself (Fig. 8).

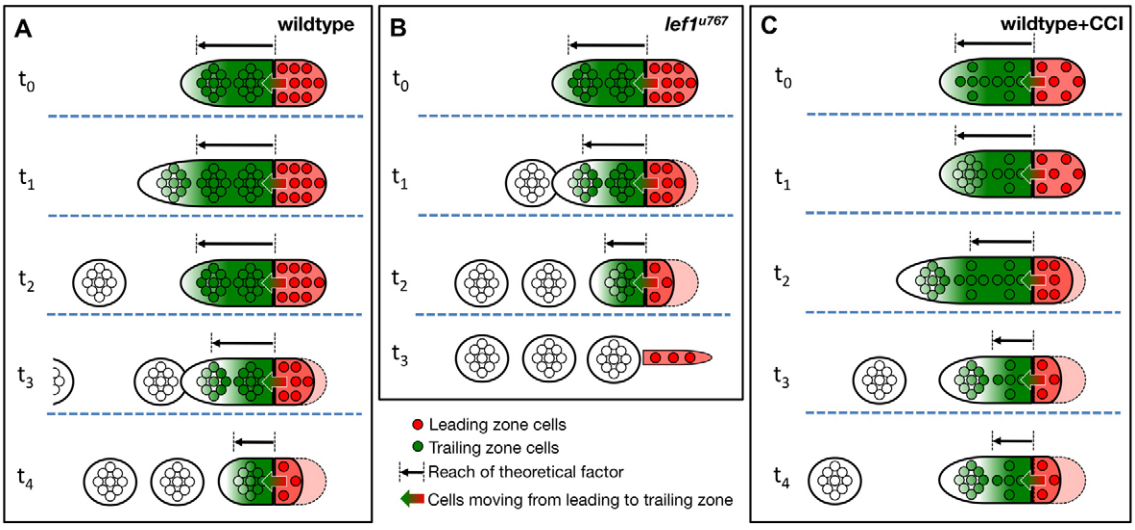

Fig. 8. Model describing the development of wild-type, *lef1* mutant and cell division-blocked PLLP. (**A**) The proportions of the wild-type primordium remain constant as it migrates from left to right (t_0-t_2), owing to high proliferation in the leading zone (red), feeding cells to the trailing zone (green) and leading to neuromast deposition. As the primordium reaches the tail (t_3-t_4), proliferation and size of the leading zone decreases. As a consequence, the reach of the postulated leading zone-secreted factor (black arrow) diminishes, accelerating and eventually depositing all neuromasts as in (B)-t_3. (**B**) In the *lef1* mutant, diminished proliferation in the leading zone depletes this region (t_0 to t_3), owing to passage of cells to the trailing zone and leading to premature termination of migration. (**C**) When cell division is inhibited (CCI, cell cycle inhibitors) in the whole primordium, neuromast deposition rate is reduced because the time required by the trailing zone to generate new neuromasts is increased. Eventually, the leading zone runs out of cells and displays a phenotype similar to the *lef1* mutant (B-t_3).

In wild types, proliferation in the trailing zone coupled with continuous influx of cells from the leading zone will move trailing primordium cells progressively further from the leading zone, triggering proneuromast formation and deposition. Lef1 maintains proliferation in the leading zone until the primordium reaches the tail tip, supplying the factor(s) from the leading zone throughout migration in the trunk (Fig. 8A). However, when the leading zone diminishes its levels of Lef1, and thereby decreases in size as it approaches the tail tip, the level of factor(s) from the zone will decrease and neuromast formation will be triggered in trailing zone cells closer to the leading zone (Fig. 8A, bottom row) eventually leading to primordium fragmentation and terminal neuromast formation. The size of the primordium decreases proportionally to the size of the *lef1*/*Tg(7xtcf-saim:GFP)*^{ia4}-expressing zone as it migrates caudally. This further supports a link between the size of the leading zone and the primordium as a whole. Our model is also consistent with data from an accompanying paper in this issue (McGraw et al., 2011), which shows that inhibition of *lef1* function modifies the identity of progenitor cells in the leading edge of the primordium.

In the *lef1*^{u767} mutant, the situation is initially as in wild type, but as the leading zone becomes prematurely depleted of cells, less factor(s) is produced, proneuromast formation occurs closer to the leading zone, neuromasts are deposited closer together and the primordium is depleted of cells terminating the process (Fig. 8B). The *lef1* mutant phenotype cannot simply be explained by an overall reduction of Lef1-mediated proliferation in the PLL placode as previously suggested (Gamba at el, 2010). We postulate that when proliferation is inhibited pharmacologically throughout the PLLP, more time is required for the trailing zone to accumulate cells to form neuromasts and, as migration speed is maintained, distance between deposition events increases (Fig. 8C). This model is consistent with the observation that the periodicity of neuromast deposition is not controlled by an endogenous clock mechanism (Aman et al., 2011) but rather depends on the size of the PLLP compartments, which is controlled in turn by their relative proliferation rates.

Fgf signalling promotes proneuromast formation: when inhibited, the trailing zone fails to organize into rosettes (Lecaudey et al., 2008; Nechiporuk and Raible, 2008), while overexpression of Fgf3 causes rosette formation in the leading zone (Lecaudey et al., 2008). Modulation of this pathway in the trailing zone by a factor(s) produced in the leading zone could provide a molecular basis for the model we propose. Matsuda and Chitnis (Matsuda and Chitnis, 2010) have proposed an elegant model in which Fgf signalling acts sequentially within the primordium. First, Wnt-dependent Fgf expression occurs in the leading zone (though signalling is locally repressed); second, *atoh1*-dependent Fgf signalling occurs in the maturing neuromasts. Lef1 is not required for either of these events and, instead, our model suggests that it regulates the position at which foci of Fgf activation occur relative to the leading edge cells. Although secreted proteins could be candidates for the factor we propose to emanate from the leading zone, this is not the only possibility. One could imagine that communication could be mediated through cell contacts or adhesive interactions.

Lef1-dependent Wnt/β-catenin signalling in the PLL primordium is not required for patterning, migratory ability or differentiation

Given that manipulation of the Wnt pathway can affect patterning, migration, proliferation and proneuromast formation, it is perhaps surprising that the *lef1*^{u767} phenotype is restricted to a proliferative deficit. This is intriguing but not without precedent as Wnt/β-catenin signalling is proposed to control patterning and cell proliferation independently within the spinal cord (Bonner et al., 2008).

Our data suggests that Lef1 mediates Wnt/signalling regulated proliferation in the leading zone; what, then, might be the roles for those Tcfs expressed in the trailing zone? Although single *tcf7l1a* (*tcf3a*) and *tcf7l2* (*tcf4*) mutants have no PLLP phenotypes (not shown), there maybe redundancy between these factors. One intriguing possibility is that interactions between Lef1 and Tcf7l1a/Tcf7l2 may help to establish or maintain the position of the boundary between leading and trailing zones. Indeed, if Tcf7l1a and Tcf7l2 act primarily as repressors of Wnt signalling in the PLLP, as shown in other contexts (Kim et al., 2000), this would be consistent with the lack of activation of the Wnt reporter *Tg(7xtcf-siam:GFP)*^{ia4} in the trailing zone. On other hand, the expression of other Tcfs in the primordium may contribute to the observation that abrogation of Lef1 only partially restores the consequences of enhanced Wnt signalling in *apc* mutants.

Lef1 may have similar roles in stem cell niches and metastasising cancers as in the PLL primordium

Other than the lateral line, the only other overt phenotype we observed in *lef1*^{u767} mutants is the reduction/absence of fins at post-embryonic stages. Although we have not investigated this phenotype, it is likely to be due to failure in fin growth or tissue homeostasis. In either case, a proliferative defect underlying the phenotype suggests a conserved role for Lef1-dependent Wnt/β-catenin signalling in different tissues. This pathway also impacts upon the viability of stem cell compartments in adult tissues suggesting analogies between proliferation control in developing/growing tissues and adult tissue homeostasis. For example, abrogation of *tcf7l2* depletes intestinal crypts due to loss of proliferating cells (Korinek at al., 1998; Muncan et al., 2007). Conversely, in *apc* mutants, the intestine grows polyps that eventually lead to hyper-proliferation of crypt cells and cancer.

Finally, *lef1* expression is enhanced in metastatic melanoma cell lines (Murakami et al., 2001) and its abrogation in metastatic adenocarcinoma abolishes the invasive properties of the cells (Nguyen et al., 2009). It will be interesting to determine whether this is due to proliferative defects that lead to stalled collective cell migration. Parallels between metastatic cells and the PLLP have been drawn based on shared morphological and molecular features (Friedl and Gilmour, 2009; Ilina and Friedl, 2009; Gallardo et al., 2010). We suggest that these tissues not only share the use of chemokine signalling for collective cell migration, but also may require Lef1-mediated Wnt/β-catenin signalling for tissue maintenance.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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