Shroom3 is required downstream of FGF signalling to mediate proneuromast assembly in zebrafish

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SUMMARY
During development, morphogenetic processes require a precise coordination of cell differentiation, cell shape changes and, often, cell migration. Yet, how pattern information is used to orchestrate these different processes is still unclear. During lateral line (LL) morphogenesis, a group of cells simultaneously migrate and assemble radially organized cell clusters, termed rosettes, that prefigure LL sensory organs. This process is controlled by Fibroblast growth factor (FGF) signalling, which induces cell fate changes, cell migration and cell shape changes. However, the exact molecular mechanisms induced by FGF activation that mediate these changes on a cellular level are not known. Here, we focus on the mechanisms by which FGFRs control apical constriction and rosette assembly. We show that apical constriction in the LL primordium requires the activity of non-muscle myosin. We demonstrate further that shroom3, a well-known regulator of non-muscle myosin activity, is expressed in the LL primordium and that its expression requires FGF signalling. Using gain- and loss-of-function experiments, we demonstrate that Shroom3 is the main organizer of cell shape changes during rosette assembly, probably by coordinating Rho kinase recruitment and non-muscle myosin activation. In order to quantify morphogenesis in the LL primordium in an unbiased manner, we developed a unique trainable ‘rosette detector’. We thus propose a model in which Shroom3 drives rosette assembly in the LL downstream of FGF in a Rho kinase- and non-muscle myosin-dependent manner. In conclusion, we uncovered the first mechanistic link between patterning and morphogenesis during LL sensory organ formation.

KEY WORDS: Lateral line, Apical constriction, Rosette, Shroom3, Rho kinase, Collective cell migration, Morphogenesis, Zebrafish

INTRODUCTION
During morphogenetic processes in developing organisms, cells must simultaneously proliferate, change their shape, differentiate and, often, migrate. These biological processes must be tightly orchestrated in time and space in order for the cells to form an organ. Developmental patterning information, often in the form of morphogens, plays a major role in coordinating these different processes both within single cells and between neighbouring cells. Important efforts have been made in recent decades to understand the link between positional information and cell fate decisions. However, how signals and genes that control cell fate also affect the cytoskeletal and adhesion machinery that eventually drive morphogenesis is still poorly understood, in particular in vertebrates.

The zebrafish lateral line (LL) is a very good model system in which to study how cell migration, cell shape changes and differentiation are coordinated during organ formation. The LL is a mechanosensory system that allows the detection of water movements. It comprises mechanosensory organs, the neuromasts, which are distributed over the body surface both on the head (anterior LL) and along the trunk and tail (posterior LL). Neuromasts are clusters of ~25 cells, including hair cells in the centre surrounded by support cells (Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2004; Metcalfe et al., 1985; Winklbauer, 1989). During pLL development, a group of ~100 cells, the primordium of the pLL (pLLP), delaminates from a cranial placode posterior to the otic placode and migrates posteriorly along the myoseptum to the tip of the tail. As cells of the pLLP migrate collectively, they form radially organized subgroups, termed rosettes, which are then deposited repeatedly at the trailing end of the pLLP, where they fully differentiate into mechanosensory organs (Chitnis et al., 2012; Ghysen and Dambly-Chaudière, 2004; Ghysen and Dambly-Chaudière, 2007). The assembly of these two to three radially organized rosettes within the primordium is based on coordinated apical constriction (AC) of the cells in the cluster (Lecaudey et al., 2008), a process that requires Fibroblast growth factor (FGF) signalling activity (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). FGF signalling is active in approximately the back two-thirds of the pLLP where the FGF receptor 1 (Fgfr1) is present. Fgfr1 is activated by two FGF ligands, Fgf3 and Fgf10. Blocking FGF signalling pharmacologically or genetically leads to a loss of apically constricted rosettes and aberrant migration (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). The migration defect is the consequence, at least in part, of downregulation of the G protein-coupled receptor-encoding gene cxcr7b in the absence of FGF signalling (Aman and Piotrowski, 2008; Nechiporuk and Raible, 2008). Conversely, the morphogenetic defect has been proposed to result from the failure of cells in the trailing part to become epithelial in the absence of FGF activity (Lecaudey et al., 2008). However, the precise molecular mechanisms induced downstream of activated Fgfr1 that mediate apical constriction and proneuromast formation are not known.
AC refers to a change in cell shape, whereby epithelial cells constrict their apical side in a coordinated manner to adopt a wedge-like shape. AC thus underlies cell and tissue shape changes in many morphogenetic processes during development, including gastrulation, neural tube closure, lens pit invagination and gut morphogenesis (Pilot and Lecuit, 2005; Sawyer et al., 2010). In all cases, apical constriction requires the activity of non-muscle myosin (NMII), which contracts the apical meshwork of actin leading to a decrease in apical surface (Lecuit and Lenne, 2007; Sawyer et al., 2010). NMII comprises two heavy chains, two essential light chains and two regulatory light chains. Phosphorylation of the regulatory light chains by Myosin light chain kinase or Rho-associated kinase (Rock) is the main switch that activates NMII (Vicente-Manzanares et al., 2009). In vertebrates, the actin-binding protein Shroom3 has been reported to be involved in the regulation of apical constriction (Chung et al., 2010; Haigo et al., 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999; Lee et al., 2007; Nishimura and Takeichi, 2008; Plageman et al., 2011; Plageman et al., 2010). Shroom3 belongs to the Shroom family of proteins, which are characterized by conserved domains including a PDZ domain at their N-terminus, and two Apx/Shrm-specific domains, ASD1 and ASD2, separated by a poly-proline domain (Dietz et al., 2006; Hagens et al., 2006; Haigo et al., 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999; Lee et al., 2009). The ASD1 domain mediates direct binding to actin but is not essential to the apical constriction activity of Shroom3. By contrast, the ASD2 domain is essential for AC activity and has been shown to interact physically with and to localize Rocks (Nishimura and Takeichi, 2008), thus directly linking Shroom3 to NMII activation and AC.

In this study, we dissected the mechanisms downstream of Fgfr1 activation that lead to proneuromast formation in the pLLP. We first showed that the actin-binding protein Shroom3 localizes in the apical part of constricting cells. We then show that Shroom3 is essential for the apical constriction-based assembly of proneuromasts and that this process also requires NMII and Rho kinase activity. Finally, we demonstrate that Shroom3 is a transcriptional target of FGF signalling and that it mediates the activity of FGF in proneuromast assembly.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Zebrafish (Danio rerio) were raised and staged according to standard protocols (Kimmel et al., 1995). The Tg(fgf10:lyz::EGFP)z/ze (cldb::gfp), Tg(hsp70l:dnfgfr1-EGFP)pd1 and Tg(hsp70l:fgf3-Myc)zf115 transgenic lines have been described previously (Haas and Gilmour, 2006; Lecaudey et al., 2008; Lee et al., 2005).

Cloning of cDNAs, transgenic constructs and transgenic lines

Full-length Shroom3 was amplified from cDNA of 36 hours post-fertilization (hpf) zebrafish embryos and two splice variants, v1 and v2, were identified by 5’RACE (Roche). The sequences for shroom3v1 and shroom3v2 have been deposited in GenBank with accession numbers JX455752 and JX455753, respectively. All constructs were generated using the Tol2kit (Kwan et al., 2007) and Multisite Gateway (Invitrogen). JX455752 and JX455753, respectively. All constructs were generated using the SP6 mMessage mMachine Kit (Ambion). See supplementary material Table S1 for primer sequences.

Whole-mount in situ hybridization and immunohistochemistry

In situ hybridization (ISH) and immunofluorescence (IF) staining were performed according to standard procedures (Lecaudey et al., 2004). The following antibodies were used: mouse anti-γ-tubulin (1/100; Sigma), mouse anti-ZO1 (1/500; Invitrogen), rabbit anti-PMLC2 (1/50; Cell Signaling), rabbit anti-GFP (1/500; Torrey Pines Biolabs) and mouse anti-GFP (1/500; IL8, Clontech) and secondary Alexa dye-conjugated antibodies (1/500; Molecular Probes). Phalloidin stainings were performed with Rhodamine-Phalloidin (1/500; Molecular Probes).

Morpholino and rescue experiment

Mo_Shr3e6i6 sequence was 5’-CCCTAATAATTGTTACCTGACTAAC-3’ (Gene Tools). 4.2 pmol of morpholino were injected in the cell at the one-cell stage. Because morpholinos with a GC content >40% might work better at lower temperatures (Schuster et al., 2010), we kept Mo_shr3e6i6-injected embryos at a constant temperature of 23°C. For the rescue experiment, Mo_Shr3e6i6 was injected into hsp70l:shr3v1-tagRFP and hsp70l:shr3v2-tagRFP embryos. At 26 hpf, embryos were heat-shocked for 30 minutes at 39°C and fixed after 4 hours.

Heat-shock and inhibitor treatment

Tg(hsp70l:dnfgfr1-EGFP)pd1, Tg(hsp70l:fgf3-Myc)zf115, hsp70l:shr3v1-tagRFP and hsp70l:shr3v2-tagRFP embryos (28 hpf) were heat-shocked for between 15 minutes and 1 hour at 39°C. For inhibitor treatment, 28-hpf embryos were treated with 5 or 10 μM of SU5402 for 4-6 hours (Calbiochem), 50 μM of blebbistatin for 2-5 hours (Sigma), or 50 to 200 μM of Rockout for 30 minutes (Calbiochem).

For the experiment shown in Fig. 8, hsp70l:shr3v1-tagRFP and hsp70l:shr3v2-tagRFP embryos were stimulated 3 to 4 hours after the start of the heat-shock (HS) with 5 μM SU5402 for 4 hours before overnight fixation.

Imaging, image processing and statistical analysis

Live embryos were anesthetized in 0.01% MESAB (Sigma) and mounted in 1% low melting point agarose. Time-lapse movies were performed on an inverted Zeiss Spinning Disc or LSM510 confocal microscopes. z-stacks were captured at 3- to 5-μm intervals and flattened by maximum projection. Image processing and kymographs were performed with ImageJ 1.42Q software. Data from at least two experiments were pooled. Statistical analyses were performed using Student’s two-tailed t-test for unpaired values where appropriate. A P-value ≤0.05 was considered statistically significant.

Rosette number quantification using a trainable detector

The quantification of rosette numbers was based on a trainable detector. The core algorithm was adapted from the state-of-the-art detection methods in Computer Vision and Biomedical Image Analysis fields (Liu et al., 2012; Leibe et al., 2008). Similar approaches have been successfully applied to locate landmark structures like eyes/mouth in zebrafish embryos (Ronneberger et al., 2012) and to detect mitoses (Schlachter et al., 2010). In short, the detector uses densely computed scale-invariant feature transform (SIFT) descriptors ( Lowe, 2004) and an efficient filtering technique (Reisert and Burkhardt, 2008) for image patch description. The detection is carried out in a sliding-window fashion with a linear classifier.

First, the detector was trained with manually labelled data comprising 30 non- or DMSO-treated embryos and 17 embryos treated with SU5402. The performance of the detector was then validated by splitting the labelled data into a training set and a test set. Each set contained ~23 images, and the cross-validation was repeated five times with different random splitting. This led to a 91.7% Precision/Recall at the Equal-Error-Rate point, meaning that, with this threshold, a rosette had more than nine chances out of ten to be detected and a detected object had more than nine chances out of ten to be a true rosette. After training, the detector could find objects similar to the ones that were manually labelled in the training data in any test image, and give these objects a detection score indicating the extent of similarity. Using this detector, the rosette number could be counted automatically and, thus, in an objective manner. For more detailed explanations, we refer the readers to our supporting online material at http://www.bioss.uni-freiburg.de/cms/assets/files/professuren/organogenesis/rosette_detection.pdf. The trained detector (in Matlab) is available at http://lmb.informatik.uni-freiburg.de/people/liu/rosette-detection. The full Matlab package including the detector training algorithm is also available upon request.
RESULTS
Non-muscle myosin localizes and is active in the apical part of rosettes, and is required for their assembly

To form proneuromasts, groups of cells in the migrating pLLP coordinately constrict their apical side to form radially organized clusters or rosettes. This process is controlled by FGF signalling in the trailing cells that express fgfr1 (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). In epithelial cells, apical constriction is generally driven by activated NMII, which contracts the apical actin meshwork (Sawyer et al., 2010). In order to determine whether apical constriction in the pLLP requires NMII activity, we first determined its localization in the primordium. For this purpose, we injected mRNAs encoding the NMII heavy chain myh9 fused to gfp together with lynTdToma to visualize the cell membranes. GFP-myh9 localized at the very centre of the rosettes in the pLLP (Fig. 1A-D). The density of fusion protein increased from the most recently formed rosette (Fig. 1C–C’) to the ‘oldest’ one (Fig. 1B–B’). GFP-myh9 was also already detectable in the apical part of cells in the leading region, which had not yet fully constricted their apical membrane (Fig. 1D–D’). This suggests that the localization of NMII in the apical part of pLLP cells is a very early event that prefigures AC and rosette assembly. Using a phospho-specific antibody, we next examined levels of phosphorylated myosin light chain 2 (pMLC2), which reflect local actomyosin activation (Ámamo et al., 1996). pMLC2 signal was significantly stronger in the centre of each rosette (Fig. 1E-G). This showed that NMII is not only localized but also active in the centre of assembled and assembling rosettes. To determine whether NMII was required for AC during proneuromast assembly, we used the drug blebbistatin to block its activity (Kovács et al., 2004). Treatment of 28 hpf embryos, in which one proneuromast had already been deposited, with 50 μM blebbistatin led to disassembly of rosettes (Fig. 1H,I). Together, these data show that non-muscle myosin activity is required for cells to constrict their apical side and form radial rosettes in the trailing part of the pLLP.

Shroom3 is expressed in the rosette-forming region of the pLLP and localizes at the apical part of rosette cells

The actin-binding protein Shroom3 has been shown to drive AC of epithelial cells in different biological systems by activating non-muscle myosin (Chung et al., 2010; Haigo et al., 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999; Lee et al., 2009; Nishimura and Takeichi, 2008; Plageman et al., 2011; Plageman et al., 2010). We therefore decided to determine whether Shroom3 plays a role in the assembly of apically constricted rosettes in the zebrafish pLLP. We first cloned full-length zebrafish shroom3 by RT-PCR and 5’RACE. We isolated two splice variants, shr3v1 and shr3v2, which differ in their 5’ end and are likely to give rise to two different proteins, as in other vertebrates. The shorter cDNA, shr3v2, lacks the first two exons and, as a consequence, the N-terminal PDZ domain (Fig. 2A,B). Zebrafish shr3v1 and shr3v2 are thus orthologues of the mammalian Shroom3 isoform1 (ShroomL) and Shroom3 isoform2 (ShroomS), respectively (Hagens et al., 2006; Hildebrand and Soriano, 1999). Sequence analysis of the putative proteins indicates that they contain the two Shroom-specific domains, ASD1 and ASD2, as well as a poly-proline domain in between (Fig. 2A,B).

In order to determine whether Shroom3 could play a role in the assembly of apically constricted proneuromasts, we analysed its expression by in situ hybridization (ISH) between 20 and 48 hpf. We found that shroom3 was expressed at a very high level in the pLLP before the start of migration (not shown). Although the expression level decreased with time, it continued during the entire migration process (Fig. 2C,G,K). Interestingly, co-staining with a GFP antibody in the cldnb:gfp transgenic line showed that shroom3 is neither expressed in the leading cells nor in the most trailing cells but rather in the region where rosette assemble (Fig. 2D,H,L). shroom3 is also expressed in additional regions of the embryo, including the ventral part of the otic vesicle (Fig. 2E,I,M) and the distal part of the pronephros (Fig. 2F,I,N). Using RT-PCR with primers specific for each of the two splice variants, we showed that shroom3 is not expressed maternally (not shown). These results make Shroom3 a very good candidate to mediate cell shape changes underlying rosette assembly.

We therefore wanted to determine the intracellular localization of Shroom3 protein. For this purpose, we generated transgenic lines expressing shr3v1 or shr3v2 fused to tagRFP under the control of the heat shock-inducible promoter hsp70l. Upon heat shock (HS), shr3v1-tagRFP and shr3v2-tagRFP localized to the very centre of already assembled or assembling rosettes in the pLLP, showing a punctuate localization in the most apical part of the rosette.
of the cells (Fig. 2O-R). This localization was reminiscent of that of GFP-myh9 (Fig. 1A).

**Shroom3 is required for the assembly of apically constricted rosettes in the migrating pLLP**

To investigate the role of Shroom3 in LL morphogenesis, we used a splice-blocking morpholino targeting the exon6-intron6 boundary to simultaneously knock down both splice variants (Mo_shr3e6i6) (Fig. 3A). RT-PCR analysis of control and morpholino-injected embryos showed that Mo_shr3e6i6 led to an efficient retention of intron 6 (‘mis-spliced’ in Fig. 3B). The band corresponding to the wild-type mRNA had indeed almost completely disappeared at the injected concentration at all three time points analysed (Fig. 3B).

The retention of intron 6 was confirmed by sequencing of the PCR product. It leads to a premature STOP codon and, thus, to a putative truncated protein lacking the poly-proline and ASD2 domain (Fig. 3A). This strongly suggests that Mo_shr3e6i6 leads to a dramatic reduction of Shroom3 function.

Although the overall morphology of the embryos was indistinguishable from injected controls (Fig. 3C), knockdown of Shroom3 function significantly modified the internal organization of migrating pLLP. The organization in two to three radial rosettes, which characterize wild-type primordia, was lost in morphants between 28 hpf and 36 hpf (Fig. 3D-G). This phenotype was strongly reminiscent of pLLP lacking FGF activity (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Similar to *fgf3;fgf10*
double mutants, the absence of radially organized rosettes became less obvious after 36 hpf (data not shown). As a consequence, very few neuromasts were deposited from the trailing region of such primordia lacking apically constricted rosettes. Thus, the final pattern of neuromasts was aberrant with hardly any organs deposited along the first half of the migration path (supplementary material Fig. S1, Movies 1, 2). Interestingly, the migration speed of primordia lacking rosettes was not affected (supplementary material Fig. S1, Movies 1-4), confirming the idea that the internal organization of the primordium in rosettes is not a prerequisite for coordinated migration (Aman and Piotrowski, 2008).

To characterize further the phenotype resulting from Shroom3 knockdown, we looked at the localization of molecular markers showing a particular localization in the pLLP. The centrosome pattern, for example, reflected the internal organization of the primordium in rosettes. γ-Tubulin immunostaining indeed showed that whereas the centrosomes appeared scattered in the leading region, they formed organized rings around the rosette centres in the trailing part of the primordium (Fig. 3H-I). This organization is lost in shroom3 morphants, in which centrosomes fail to form rings and are instead aligned along the pLLP length (Fig. 3J-K). Actin and apical markers, such as the tight junction protein ZO-1, are strongly concentrated at the centre of rosettes in wild-type primordia (Hava et al., 2009; Lecaudey et al., 2008). In shroom3 morphants, by contrast, both actin and ZO1 staining were strongly reduced at the centre of the rosettes, as shown by phalloidin labelling and staining with a ZO1 antibody (supplementary material Fig. S2; Fig. 3L-O). To quantify the number of rosettes within the primordium in an unbiased manner, we developed a trainable detector to automatically analyse pictures of cldnb:gfp-expressing primordia (see Materials and methods). The results are displayed as detection response maps (Fig. 3D-H11032) and boxplot diagrams (Fig. 4E,F, two left-hand boxplots). This quantification showed that the difference in rosette numbers between injected controls and Mo_shr3e6i6-injected embryos is highly significant.

In order to confirm the specificity of our morpholino knockdown experiments, we injected Mo_shr3e6i6 in hsp70l:shr3v1-tagRFP or hsp70l:shr3v2-tagRFP embryos and heat-shocked the embryos at 26 hpf. Upon HS, both shr3v1-tagRFP and shr3v2-tagRFP were able to rescue the loss of apically constricted rosettes (compare Fig. 4C,E with 4A,B). Importantly, rosettes failed to form in heat-shocked, Mo_shr3e6i6-injected non-transgenic siblings (Fig. 4B), showing that the HS does not block the effect of the morpholino. We quantified the extent of the rescue using the rosette detector described previously. Induction of shr3v1-tagRFP or shr3v2-tagRFP in morphants significantly increased the number of rosettes per primordium from 0.5 to almost 1.5 (Fig. 4E,F). Embryos expressing shr3v1-tagRFP or shr3v2-tagRFP but non-injected with the morpholino had a number of rosettes similar to wild type, suggesting that shroom3 alone is not sufficient to induce AC and formation of an ectopic rosette in the leading region of the pLLP (Fig. 4E,F).
Altogether, this shows that Shroom3 function is required for the assembly of pLLP epithelial cells in radially organized rosettes. Based on the known activity of Shroom3 in other biological systems, this strongly suggests that Shroom3 activates non-muscle myosin activity to drive apical constriction and rosette assembly.

Rho kinase activity is required for apically constricted rosette assembly

Activation of non-muscle myosin is regulated by phosphorylation of either the heavy chain or the regulatory light chain (MLC). We have demonstrated that MLC2 is phosphorylated during rosette assembly (Fig. 1E-G). Phosphorylation of the RMLC depends on the activity of Myosin Light Chain Kinase (MLCK) and/or the Rho-associated kinase (Rock) (Vicente-Manzanares et al., 2009). As it has been shown previously that Shroom3 directly interacts with Rock1/2 and that this interaction is necessary for AC of neuroepithelial cells during neural tube closure (Nishimura and Takeichi, 2008), we wanted to determine whether Rock activity was also necessary for AC of the pLLP cells. We thus treated embryos with the Rock inhibitor Rockout (Provoost et al., 2007). Cldnb:gfp embryos were treated from 28 hpf onwards, once one neuromast had been deposited, with increasing concentrations of inhibitor for 30 minutes before image acquisition. Blocking Rock activity led to the disassembly of radially organized rosettes in the migrating pLLP in a dose-dependent manner (Fig. 5A-F). Quantification of these experiments with our rosette detector confirmed that the number of rosettes per primordium in embryos treated with 100 and 200 μM of Rockout was significantly reduced compared with DMSO treated embryos (Fig. 5G). These results show that Rock activity is required for the maintenance of apically constricted proneuromasts in the migrating primordium. Together with our previous findings, this strongly suggests that Shroom3 drives AC of the pLLP cells by activating non-muscle myosin in a Rho kinase-dependent manner.

FGF signalling is necessary and sufficient for expression of shroom3 in the pLLP

The FGF signalling pathway is required for cells of the pLLP to assemble into radially organized and apically constricted clusters that prefigure the neuromasts (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Our findings suggested that Shroom3 could act downstream of FGF signalling to drive AC in the trailing cells of the primordium. In order to test a potential link between FGF signalling and Shroom3, we checked whether FGF activity controlled shroom3 expression. We first analysed shroom3 expression in embryos treated with the FGF inhibitor SU5402. After treatment with 10 μM SU5402, the expression of shroom3 in the pLLP was completely abolished (compare Fig. 6C,D with 6A,B). By contrast, the expression of shroom3 in the otic vesicle and in the pronephros was not significantly affected (not shown). Similar results where obtained using the Tg(hsp70l:dnfgfr1-EGFP)ped1 transgenic line (Fig. 6E-J). Thus, FGF signalling is necessary for the expression of shroom3 in the pLLP. We then tested whether, conversely, FGF signalling was sufficient to induce shroom3 expression. Overactivation of FGF signalling using the Tg(hsp70l:fgf3-Myc)zf115 line led to increased shroom3 expression and an expansion of its expression domain to the leading region of the primordium (Fig. 6K-N). In addition, shroom3 was now expressed in the already deposited neuromasts in which it is normally downregulated (Fig. 6M, arrowheads). The expression, however, remained restricted to forming rosettes in the pLLP and was not induced in any ectopic region, suggesting that other factors restrict the competence to express shroom3 downstream of FGF. Here again, the expression in the otic vesicle and pronephros was not significantly affected (not shown). We quantified the expansion of shroom3 expression by measuring the size of the ‘shroom3-free’ domain in the leading region (Fig. 6O). The ‘shroom3-free’ domain was reduced by half in embryos overexpressing fgf3 compared with wild-type siblings. In conclusion, FGF signalling is both necessary and sufficient to activate shroom3 expression in the pLLP. This strongly supports a role for Shroom3 in translating FGF activity into AC and proneuromast formation.

Shroom3 function is required downstream of FGF for rosette assembly

To prove that Shroom3 mediates FGF activity in assembling apically constricted proneuromasts, we performed an epistasis analysis. Overactivation of FGF signalling by HS treatment of Tg(hsp70l:fgf3-Myc)zf115 embryos triggers the formation of an ectopic radially organized cell cluster in the leading region of the pLLP (Lecaudey et al., 2008). We have shown that shroom3

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**Fig. 4.** Induction of shroom3 expression rescues rosette assembly in Mo_Shr3e6i6-injected zebrafish embryos. (A–D′) Images of the pLLP in heat-shocked non-transgenic siblings (A–B′), hsp70l:shr3v1-tagRFP (C–C′) or hsp70l:shr3v2-tagRFP (D–D′) embryos injected with water (A–A′) or Mo_Shr3e6i6 (B–D′). A–D′ show the corresponding detection response images. Scale bars: 50 μm. (E,F) Boxplots displaying the results and statistics of confidence-weighted rosette numbers from the trained rosette detector (see Materials and methods). The red line and black diamond represent the median and mean value of each group, respectively. The edges of the box are the 25th and 75th percentiles. The black dashed line extends to the most extreme data points not considered to be outliers, and outliers are plotted individually as red plus signs. The medians of two groups are significantly different at the 5% significance level if their respective notch intervals do not overlap (interval end points represent the extremes of the notches). N is the number of independent experiments and n is the number of embryos per condition.

**DEVELOPMENT**

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**RESEARCH ARTICLE**
expression is ectopically induced in this additional proneuromast (Fig. 6N). We therefore reasoned that, if Shroom3 is mediating FGF activity, blocking Shroom3 activity in Tg(hsp70l:fgf3-Myc)zf115 should revert this phenotype. As expected, whereas control-injected Tg(hsp70l:fgf3-Myc)zf115 embryos had a normal pattern of rosettes in the absence of HS (Fig. 7A), they assembled an ectopic rosette in the leading region of the pLLP upon HS (Fig. 7B, white arrowhead). Formation of this ectopic rosette was blocked in heat-shocked Tg(hsp70l:fgf3-Myc)zf115 injected with the Mo_shr3e6i6 (Fig. 7C,D). This shows that Shroom3 is specifically required for the assembly of the ectopic rosette in the leading region following overactivation of FGF signalling. As a control, non-heat-shocked Mo_shr3e6i6-injected Tg(hsp70l:fgf3-Myc)zf115 embryos, as expected, failed to assemble any rosette (Fig. 7E). Importantly here again, rosettes also failed to form in heat-shocked, Mo_shr3e6i6-injected non-transgenic siblings (Fig. 7F), showing that the HS does not block the effect of the morpholino. We quantified the phenotype by measuring the distance between the tip of the primordium and the first rosette centre (Fig. 7, white arrowheads). This distance was significantly smaller in heat-shocked, water-injected Tg(hsp70l:fgf3-Myc)zf115 embryos (dark blue) compared with non-heat-shocked embryos (red). However, the same distance in heat-shocked Mo_shr3e6i6-injected Tg(hsp70l:fgf3-Myc)zf115 embryos (purple) was significantly increased compared with water-injected embryos (blue and red). The difference between heat-shocked and non-heat-shocked Mo_shr3e6i6-injected embryos was not significant (compare turquoise bars with purple bars) showing that the Mo_shr3e6i6 was able to totally block the strong FGF overactivation induced in Tg(hsp70l:fgf3-Myc)zf115 embryos. In conclusion, this experiment demonstrates that Shroom3 is required downstream of FGF to assemble apically constricted rosettes.

Fig. 5. Rho kinases are also required for the assembly of pLLP in apically constricted rosettes. (A–F) Live images of the pLLP of zebrafish embryos treated with DMSO (A), or 50 µM (B), 100 µM (C,D) or 200 µM (E,F) of the Rho kinase inhibitor Rockout. (G) Boxplot of the quantification of the Rockout treatment experiments. Scale bars: 50 µm.

Fig. 6. FGF signalling is necessary and sufficient for activating shroom3 expression in the pLLP. (A–N) Lateral views of zebrafish embryos stained by ISH with a shroom3 probe. Treatment of embryos with 10 µM of SU5402 (C–D’), or overexpression of dn-fgfr1 (H–I) totally inhibits shroom3 expression in the pLLP (compare C–D’ with A–B’, and H–I with E–G). Overexpression of fgf3 after heat-shock of Tg(hsp70l:fgf3-Myc)zf115 embryos leads to an increase and expansion of shroom3 expression (compare M–N’ with K–L’). (O) Quantification of the length of the ‘shroom3-free’ zone in the leading region in heat-shocked Tg(hsp70l:fgf3-Myc)zf115 (red) and non-transgenic siblings (blue). Statistical analyses were performed using the t-test. Error bars represent s.e.m. Scale bars: 200 µm in A,C,E,H,K,M; 20 µm in B,B’,D,D’,F,G,I,J,L’,N,N’.
Conversely, we wanted to test whether exogenously overexpressed shroom3 is sufficient to block the loss of rosettes resulting from inhibition of FGF activity. For this purpose, we first induced shroom3 expression using hsp70::shr3v1tagRFP or hsp70::shr3v2tagRFP transgenic embryos and then pharmacologically blocked FGF signalling using the FGF inhibitor SU5402. As expected, non-transgenic siblings treated with 5 μM SU5402 lost their apically constricted rosettes (compare Fig. 8B,F with 8A,E). By contrast, shr3v1 or shr3v2 overexpression in heat-shocked transgenic embryos was sufficient to block the loss of rosette formation following SU5402 treatment (Fig. 8D,H). In addition to the loss of rosettes, we checked the efficiency of the SU5402 treatment by ISH with the FGF target gene pea3. pea3 expression was very strongly reduced or totally absent upon SU5402 treatment and after HS (supplementary material Fig. S3). Together, these experiments show that Shroom3 acts downstream of FGF signalling to induce the assembly of apically constricted rosettes.

In conclusion, we have uncovered the mechanism by which FGF signalling translates into the formation of apically constricted rosettes in the pLLP. FGF signalling is necessary and sufficient to induce the expression of shroom3. In turn, Shroom3 drives the assembly of apically constricted rosettes. We show that AC in the pLLP also requires the activity of non-muscle myosin and ROCK. This strongly suggests that Shroom3 recruits Rho kinases to the apical side of the cells where they can activate non-muscle myosin to constrict the apical actin meshwork (Fig. 9).

**DISCUSSION**

**Shroom3 drives apical constriction and proneuromast formation in the pLLP**

Our results indicate that Shroom3 is required for the assembly of apically constricted epithelial clusters that prefigure the sensory organs in the zebrafish pLL downstream of the FGF signalling pathway. Shroom3 has been shown to interact physically with ROCK1/2 via its ASD2 domain and to localize ROCK1/2 to the apical side of epithelial cells where it can phosphorylate and activate NMII (Nishimura and Takeichi, 2008). Using pharmacological inhibitors, we showed that blocking the activity of NMII or ROCK leads to a loss of apically constricted rosettes similar to what is occurring in the absence of FGF or Shroom3 activity. It is therefore reasonable to speculate that Shroom3 regulates AC in the pLLP in a ROCK- and NMII-dependent manner. We therefore propose a model in which activation of Fgfr1 in the trailing part of the primordium induces expression of shroom3. Shroom3, in turn, would physically interact with, and localize, Rho kinases to the apical part of the cells where they phosphorylate and activate non-muscle myosin leading to AC of the cells and rosette formation (Fig. 9).

How Shroom3 itself gets localized to the apical part of the cells is still unclear. Shroom3 has been shown to bind actin directly via its ASD1 domain and a number of studies suggest that this interaction plays a role in Shroom3 apical localization (Dietz et al., 2006; Hildebrand and Soriano, 1999). Furthermore, the localization of dShrmA, the Drosophila Shroom variant that is most similar to Shroom3, has also recently been shown to require intact actin, as treatments with actin inhibitors lead to a mislocalization of dShrmA (Bolinger et al., 2010). These data suggest that the actin meshwork itself recruits the machinery that is necessary for its contraction and, thus, for cell shape changes. However, a number of other studies suggest, by contrast, that Shroom3 recruits actin (Haigo et al., 2003; Lee and Harland, 2007; Plageman et al., 2010). In support of this hypothesis, Shroom3 can, in addition to binding actin directly, interact with and apically localize proteins of the MENA/VASP family via its poly-proline domain (Plageman et al., 2010). MENA/VASP proteins are involved in apical accumulation and organization of actin (reviewed by Bear and Gertler, 2009; Krause et al., 2003), and thus could indirectly mediate the localization of actin by Shroom3. This shows that the exact cellular mechanism underlying Shroom3 localization and more generally Shroom3-mediated AC is still unclear. Whether MENA/VASP proteins are involved in lateral line morphogenesis is unknown. Finally, our data show that, like in other vertebrates (Haigo et al., 2003), the N-terminal PDZ domain of Shroom3 is neither required for its localization nor for its function as we showed that Shr3v2, which lacks this domain, shows the same localization and function as Shr3v1.

shroom3 is expressed specifically in the central part of the pLL and gets downregulated in assembled proneuromasts before deposition. This suggests that Shroom3 function is only required to set-up and activate the AC machinery at the apical side of the cells and that once the apical surface is constricted, Shroom3 seems not to be required anymore for its maintenance in mature rosettes.
Pharmacological treatment experiments support this idea as FGF, Rock and myosin inhibitors disassemble already-formed rosettes within the migrating pLLP, but not already-deposited proneuromasts (data not shown). This supports a model in which the AC module is required for rosette assembly and maintenance within the pLLP, but is dispensable after deposition. It is likely that cell-cell adhesion molecules including E- and N-cadherin or ZO-1, which are concentrated in the rosette centres, play an important role in this AC maintenance phase (Hava et al., 2009; Kerstetter et al., 2004; Lecaudey et al., 2008; Liu et al., 2003; Matsuda and Chitnis, 2010).

Internal organization of the pLLP in rosettes is not required for migration
We show here that Shroom3 function is required downstream of FGF to drive AC. Although the loss-of-rosettes phenotypes are very similar in Shroom3 and FGF loss-of-function conditions, the migration phenotypes are totally different. Whereas FGF-deficient primordia migrate in a very slow and uncoordinated manner (Lecaudey et al., 2008; Nechiporuk and Raible, 2008), loss of Shroom3 function does not affect migration. The migration defect in FGF-deficient primordia has been proposed to be a secondary consequence of the absence of rosettes, suggesting that the internal organization of the primordium is a prerequisite for proper migration (Lecaudey et al., 2008). However, a more recent study showed that the expression of the G protein-coupled receptor-encoding gene cxcr7b is downregulated in the absence of FGF signalling. This is likely to be the cause, at least in part, of the poor migration of FGF-deficient primordia (Aman and Piotrowski, 2008; Nechiporuk and Raible, 2008). Our results strongly support this model in which the internal organization in radially organized rosettes is not required for proper migration.

Shroom3 translates FGF signalling activation into apical constriction
We show that Shroom3 is required for the induction of an ectopic rosette in the leading region upon overactivation of the FGF
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**Development** 139 (24)

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[49x54]epithelial switch at the transition between the leading and the important function of FGF is to trigger such a mesenchymal-to-

sufficient to re-establish rosettes because the cells would have lost

presented in Fig. 8 but the other way around) would not be 

previously treated with SU5402 (similar to the experiment

think that the activation of

suggests that a prerequisite for AC is the acquisition of epithelial

properties, including apico-basal polarity. For this reason, we also

Shroom3 localizes to the apical part of the cells, possibly via its

activated and then in turn activates the expression of

is a model for the underlying mechanism. Upon Fgf10 binding, Fgfr1 is

leading them to apically constrict and form a rosette. The bottom panel

represents the pLLP with turquoise nuclei corresponding to cells

expressing fgf10 [adapted from Lecaudey et al. (Lecaudey et al., 2008)].

fgf10-expressing cells signal to their fgfr1 expressing neighbours

leading them to apically constrict and form a rosette. The top panel

presents the pLLP with turquoise nuclei corresponding to cells

expressing fgfr1 and dark blue nuclei corresponding to cells

showing that it takes several hours for the FGF inhibitor SU5402 to ‘disassemble’ rosettes. It also takes several hours after its

washout for apically constricted rosettes to reform (Lecaudey et al.,

2008; Nechiporuk and Raible, 2008). Thus, by inducing the expression of

shroom3, and potentially other epithelialization factors, FGF is priming the cells for the AC process, which can be

induced by Shroom3 as the main organizer through activation of

NMII.

While our manuscript was under revision, an interesting report

was published in *Development* showing that activated Fgfr signals

through Ras-MAPK to induce apical localization of Rock2a, thus

promoting apical constriction and rosette formation in the pLLP

(Harding and Nechiporuk, 2012). Our data both support and complemet these findings by identifying Shroom3 as the

mechanistic link between FGF signalling and apical constriction.

Together, these data suggests a model in which shroom3 expression

is activated downstream of Fgfr/Ras/MAPK to recruit Rock2a to the

apical side of the cells. Rock2a would in turn activate non-
muscle myosin to constrict the apical actin meshwork, leading to

rosette formation.

In conclusion, FGF independently controls at least three different

cell responses in the pLLP: (1) cell migration by, at least in part,

preventing the repression of

expression (this study). FGF signalling, therefore, has the

potential to coordinate different cellular responses within individual

cells and between neighbouring cells within the pLLP. Thus, we

provide here the first example of a mechanistic link between

patterning information and the cytoskeletal machinery during

proneuromast morphogenesis.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material available online at

http://dev.biologists.org/lookup/suppl;doi:10.1242/dev.083253/-/DC1

![Diagram of the Shroom3-driven apical constriction mechanism leading to rosette assembly in the pLLP.](Image 70x438 to 279x730)
References


Fig. S1. Shroom3 knockdown blocks apical constriction and rosette assembly in the migrating pLLP. (A-D) Images from a time-lapse movie of a control (A) and an Mo_Shr3e6i6-injected (B) embryo over 5 hours. A', B', C and D show kymographs of the time-lapse movies in A and B (A', B') and of two additional time-lapse movies of shroom3 morphants (C, D).
Fig. S2. Actin and the tight junction protein ZO1 are disorganized in Shroom3 morphants. (A-L) Side views of the primordium of 36 hpf cldnb:gfp embryos injected with water (A-D) or with Mo_Sh3e6i6 (E-L) stained with phalloidin (B,F,J) and for the tight junction protein ZO1 (C,G,K). The strong phalloidin and ZO1 staining present in the rosette centres in controls are totally absent in the morphants although the actin and tight junction organization is normal in the somites and somite boundaries, respectively. Scale bars: 50 μm.
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