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Anosmin1 Shuttles Fgf to Facilitate Its Diffusion, Increase Its Local Concentration, and Induce Sensory Organs

Highlights

- Fgf10 diffuses from front to back of migrating primordium to induce sensory organs
- Anosmin1 is required for front-to-back diffusion of Fgf10
- Anosmin1 increases the fraction of fast-diffusing Fgf10 molecules
- Anosmin1 co-diffuses with Fgf10 molecules in a complex

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In Brief

Wang et al. combine genetics with fluorescence microscopy to address how FGF diffuses across a migrating primordium during sensory organ formation in zebrafish. They show that the extracellular protein Anosmin1 shuttles Fgf10 from source to target cells, increasing the Fgf10 diffusion range and signaling activity to promote sensory organ formation.



Developmental Cell Article

Anosmin1 Shuttles Fgf to Facilitate Its Diffusion, Increase Its Local Concentration, and Induce Sensory Organs

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SUMMARY

Growth factors induce and pattern sensory organs, but how their distribution is regulated by the extracellular matrix (ECM) is largely unclear. To address this question, we analyzed the diffusion behavior of Fgf10 molecules during sensory organ formation in the zebrafish posterior lateral line primordium. In this tissue, secreted Fgf10 induces organ formation at a distance from its source. We find that most Fgf10 molecules are highly diffusive and move rapidly through the ECM. We identify Anosmin1, which when mutated in humans causes Kallmann Syndrome, as an ECM protein that binds to Fgf10 and facilitates its diffusivity by increasing the pool of fast-moving Fgf10 molecules. In the absence of Anosmin1, Fgf10 levels are reduced and organ formation is impaired. Global overexpression of Anosmin1 slows the fast-moving Fgf10 molecules and results in Fqf10 dispersal. These results suggest that Anosmin1 liberates ECM-bound Fgf10 and shuttles it to increase its signaling range.

INTRODUCTION

During development, tissues need to be patterned to give rise to different cell types. On a molecular level, this is often achieved through graded signals that induce distinct cell identities at different concentrations. The graded distribution is frequently the result of locally produced signaling molecules with finite half-lives that diffuse through the extracellular space to establish concentration gradients (Kiecker and Lumsden, 2012). While our understanding of patterning signals is advanced, little is known about the mechanisms employed by the components of the extracellular matrix (ECM) to regulate the movement of individual signaling molecules and, thus, their local concentrations. Fibroblast growth factors (FGFs) are a family of secreted signaling molecules, most of which bind to heparan sulfate (HS) in the ECM (Goetz and Mohammadi, 2013). During development, FGFs guide migrating cells, direct tissue patterning, induce cell proliferation, and regulate cell differentiation (Turner and Grose, 2010). In order to accomplish these tasks, the levels and distribution of FGFs and, thus, their mobility in the extracel-lular space need to be tightly regulated.

FGF signaling in humans and animals is modulated by the ECM protein Anosmin-1 (ANOS1) (de Castro et al., 2014). ANOS1 contains a whey acidic protein (WAP)-like domain and four fibronectin type III (FN[III]) domains and is often co-expressed with FGF ligands in the embryo. Human patients with mutations in ANOS1 are anosmic and fail to undergo puberty due to erroneous olfactory axon targeting and GnRH neuron migration (Hardelin and Dodé, 2008). Similarly, animals lacking Anos1 show reduced neurite branching and cranial defects (Rugarli et al., 2002; Tecle et al., 2013; Endo et al., 2012). In both humans and animals, these defects have been attributed to reduced FGF signaling due to the lack of Anos1-mediated FGF-FGF receptor complex formation based on in vitro studies (Díaz-Balzac et al., 2015; Hu et al., 2009; Yamada et al., 2012). Yet, how Anos1 regulates Fgf signaling in vivo remains largely elusive.

The development of the zebrafish posterior lateral line depends on FGF signaling. The posterior lateral line is formed by a migrating primordium of about 140 cells that deposits five to seven cell clusters along the body of the embryo during its migration (Dalle Nogare and Chitnis, 2017). Each of these clusters differentiates into a neuromast, a specialized organ that senses water flow around the embryo. The rosette-shaped neuromasts start to differentiate while in the primordium and bud off from the rear of the primordium at regularly spaced intervals. The cells in the front of the primordium co-express the FGF ligands fgf3 and fgf10a, whereas the cells in the back of the primordium express the FGF receptor fgfr1a (Lecaudey et al., 2008; Matsuda and Chitnis, 2010; Nechiporuk and Raible, 2008). It is thought that FGF secreted from the front of the primordium induces neuromast differentiation in the back of the primordium by diffusing to and concentrating in small, sealed-off extracellular spaces



Figure 1. FGF Ligand and Receptor Expression and Function in Sensory Organ Formation

(A) fgf3, fgf10a, fgfr1a, and fgfr1b mRNA expression (red) in the primordium (green) at 32 hpf. Scale bar represents 100 µm.

(B) Primordium position in embryos of indicated genotypes at 50 hpf. White arrows denote deposited neuromasts, arrowheads denote position of primordium, and red arrows indicate position of primordium at the beginning of the heat shock. Scale bar represents 500 μ m.

(C) Plot of the number of neuromasts for (E) and (B). Mean, SD, and individual data points shown. ** = p < 0.01, **** = p < 0.001, ns = not significant. ANOVA p < 0.0001. (D) Quantification of the number of neuromasts deposited after heat shock in embryos of the indicated genotypes. Mean, SD, and individual data points shown. ** = p < 0.01, *** = p < 0.01. ANOVA p < 0.0001.

(E) Neuromast deposition (arrows) and primordium migration (arrowhead) in the embryos of indicated genotype at 50 hpf. Scale bar represents 500 µm.

(F) Table of p values calculated with Sidak's multiple comparisons test with a single pooled variance for the pairwise comparisons shown. ANOVA p < 0.0001. See also Figure S1 and Video S1.

(microlumina) in the center of the forming neuromasts through an unknown mechanism (Durdu et al., 2014).

We used posterior lateral line development as a model system to examine the role of Anos1 in FGF signaling. Our results indicate that Anos1 regulates the range of FGF signaling in the primordium by promoting the diffusion of Fgf10a. Most Fgf10a molecules freely diffuse through the extracellular space, but a small fraction is severely retarded, probably because it is bound to heparin sulfates in the ECM. We found that Anos1 binds to and co-diffuses with Fgf10a and thus increases the number of freely diffusing Fgf10a molecules. As a result, more Fgf10a produced in the front of the primordium diffuses to the rear, where it accumulates locally, induces neuromast formation, and becomes trapped in the microlumina of these sensory organs. These results identify a role for Anos1 as a modulator of the diffusion range of FGF in the ECM.

RESULTS

Front-to-Rear FGF Diffusion Promotes Sensory Organ Formation

Sensory organ differentiation in the primordium depends on FGF signaling. Cells in the front of the primordium express

the FGF ligands fgf3 and fgf10a. Additionally, one to two central cells in each forming neuromast express fgf10a (Lecaudey et al., 2008; Matsuda and Chitnis, 2010; Nechiporuk and Raible, 2008) (Figures 1A and S1A). The FGF receptors fgfr1a and fgfr1b are expressed in the rear of the primordium (Figures 1A and S1A) (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Loss of Fgf3 and Fgf10a function in the primordium results in loss of FGF signaling, a failure to form microlumina, absence of neuromasts, and slower primordium migration (Figures 1B and S1D) (Durdu et al., 2014; Lecaudey et al., 2008; Matsuda and Chitnis, 2010; Nechiporuk and Raible, 2008). Partial block of FGF signaling by reducing FGF receptor activity or inducing the expression of a dominantnegative FGF receptor results in the formation of fewer neuromasts (Figures 1B and 1D; Video S1). Conversely, increasing FGF signaling by over-expressing Fgf10a results in the formation of extra neuromasts (Figures 1B and 1D; Video S1). Consistent with previous studies (Aman and Piotrowski, 2008; Durdu et al., 2014; Lecaudey et al., 2008; Matsuda and Chitnis, 2010; Nechiporuk and Raible, 2008), this indicates that FGF signaling induces neuromast formation in a dose-dependent manner.

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Figure 2. Secreted Fgf10a from the Front Diffuses to the Back of the Primordium

(A) Top left, immunostaining of intracellular Fgf10a-GFP in the primordium. Top right, Fgf10a-GFP only. Arrows indicate intracellular Fgf10a-GFP accumulating at the apical constrictions near the midline of the primordium. Middle and lower left, live image of Fgf10a-GFP and secGFP, respectively, with membrane marker. Middle and lower right, false coloring of Fgf10a-GFP and secGFP signal, respectively. Arrows indicate Fgf10a-GFP and secGFP signal in microlumen, and the arrowhead indicates Fgf10a-GFP in a patch surrounding an apical constriction before microlumen formation. Scale bar represents 100 μ m.

(B) Schematic of Fgf10a protein production and transport into the microlumina in the primordium.

(C) Live images of microluminal Fgf10a-GFP with membrane marker and false coloring of Fgf10a-GFP signal only in the embryos of indicated genotype. Arrows indicate Fgf10a-GFP signal in microlumina, and the arrowheads (left) indicate Fgf10a-GFP-producing central cells adjacent to the microlumen. Central cells are missing in embryos injected with *atoh1a* morpholino (right). Scale bar represents 10 μ m.

(D) Schematic representation of the mosaic analysis.

(E) Overview of mosaic primordium with cells of indicated genotypes on the left. Square indicates enlarged region shown on the right. Single slice of a z stack from Video S3 is shown. Scale bar represents 25 µm. Close up of forming neuromast on the right. Maximum x,y projection of z stack shown in Video S3 on the top right and maximum x,z projection of z stack shown in Video S3 on the bottom right. Scale bar represents 5 µm for x,y dimension and 10 µm for z dimension. Arrows indicate Fgf10a-GFP in microlumen, arrowhead indicates donor-derived lateral line nerve underneath the primordium, and double arrowhead indicates donor-derived skin cell on top of primordium. Three primordia with Fgf10a-GFP secreting cells in the front and 5 primordia with non-Fgf10a-GFP secreting cells in the front were analyzed.

See also Figure S2 and Videos S2 and S3.

Due to the opposing expression patterns of the FGF ligands and receptors, it is thought that FGF diffuses from the front of the primordium to signal to the cells in the rear of the primordium (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). To directly test this idea, we asked whether FGF ligand from the front of the primordium is sufficient to induce sensory organ differentiation in the rear of the primordium. We inhibited *fgf10a* expression in the central cells by reducing the activity of the transcriptional factor *atoh1a*. This blocks the differentiation of the central cells (Figure 2B) (Lecaudey et al., 2008; Matsuda and Chitnis, 2010). Loss of *fgf10a* expression in central cells was confirmed by in situ hybridization against *fgf10a* (Figures S1B and S1C). We also removed the activity of Fgf3 because the central cells compensate for the loss of Fgf10a by up-regulating *fgf3* expression (Matsuda and Chitnis, 2010). In these *fgf3^{-/-}; atoh1a MO* (morpholino oligomer-injected) embryos, sensory organs still differentiated similar to wild-type, *fgf3* mutant, or *atoh1a* MO-injected embryos (Figures 1C and 1E). Thus, Fgf10a from the front diffuses to the rear to promote neuromast formation.

We next assessed the distribution of Fgf10a in the primordium by expressing Fgf10a-GFP from a bacterial artificial chromosome/clone (BAC) transgene encompassing the *fgf10a* chromosomal locus (*fgf10a:GFP-fgf10a*) (Figure S2A). This transgene recapitulated the endogenous *fgf10a* expression pattern and rescued primordium migration and neuromast formation in *fgf3^{-/-}; fgf10a^{-/-}* embryos (Figure S2); *fgf10a^{-/-}* embryos do not show a defect in primordium migration and neuromast formation due to compensation through Fgf3 up-regulation (Lecaudey et al., 2008). As a control, we generated an identical transgene that expresses secreted GFP (secGFP) from the



Figure 3. Anos1 Enhances FGF Signaling in the Primordium

(A) Schematic of the cross-repression between Wnt and FGF signaling in the primordium.

(B) Top, anos1a and anos1b mRNA expression in the primordium outlined by membrane GFP. Bottom, anos1a and anos1b mRNA expression only (white). Scale bar represents 100 µm.

(C) dusp6:d2eGFP expression in the primordium and dusp6:d2eGFP expression only (fire heatmap) for the genotypes indicated. Scale bar represents 100 µm.

(D) Top, quantification of GFP intensity from *dusp6:d2EGFP* inside the primordium along the anterior-posterior axis. X axis represents distance from the front of the primordium. Mean and SEM are shown. n indicates number of embryos. Bottom, plot of p values comparing the two genotypes in the graph above for each position along the x axis.

(E) Wht reporter readout at 36 hpf in the primordium on the left and Wht reporter readout only (fire heatmap below). Scale bar represents 100 µm.

(F) Quantification of Wht reporter intensity. X axis represents distance from the front of the primordium. Mean and SEM and are shown. n = number of individual embryos measured.

(G) Neuromast size and number defects in *anos1* mutants. Neuromast 1 in embryos of the indicated genotype at 32 hpf (left). Scale bar represents 50 μ m. Quantification of neuromast 1 volume in embryos of indicated genotype at 32 hpf (middle). Neuromast number in 42-hpf embryos of indicated genotype (right). Mean, SD, and individual data points are shown. n.s.= p > 0.05, ** = p < 0.01, and **** = p < 0.0001. ANOVA p < 0.0001.

(H) (Left) ZO-1 and GFP immunostaining in the primordium of embryos of the indicated genotype at 36 hpf. (Right) ZO-1 only (fire look-up table). Arrows indicate ZO-1 signal at the center of apical constrictions. Scale bar represents 100 μ m.

(I) Quantification of microlumen volumes in forming neuromasts in embryos of indicated genotypes. Mean, SD, and individual data points are shown. n.s. = p > or = 0.05, * = p < 0.05., ** = p < 0.01. and **** = p < 0.0001. ANOVA p < 0.0001.

(J) Phenotype of embryos with reduced FGF signaling with or without mutations in *anos1a* and *anos1b* at 50 hpf. The arrow indicates the position of the primordium. Scale bar represents 500 μ m.

fgf10a locus (*fgf10a*:secGFP, Figure S2A). We found that Fgf10a-GFP was secreted apically from the front cells toward the midline of the primordium and concentrated above, forming apical constrictions and in the microlumina of the maturing neuromasts (Figure 2A; Video S2). secGFP also accumulated in the microlumina of control embryos (Figure 2A; Video S2).

To determine the contribution of the front source and central cell source to Fgf10a in the microlumina, we blocked central cell formation. We found that in embryos lacking central cells, the accumulation of Fgf10a-GFP in microlumina was reduced to a third (Figures 2B, 2C, and S2E). In controls, secGFP accumulation in microlumina was completely abolished since GFP secreted from the front is not trapped (Figure 2C). Using ubiquitously secreted mCherry, we confirmed that embryos with reduced Atoh1a function had intact microlumina (Figure S4D). To confirm that Fgf10a-GFP produced at a distance can diffuse to and concentrate in microlumina, we used blastomere transplantation to generate mosaic primordia that contain Fgf10a-GFP-secreting cells in the front but not in a forming or deposited neuromast where central cells are located (Figure 2E). In such mosaic primordia, Fgf10a-GFP from the front also accumulated in microlumina (Figure 2E; Video S3). Thus, luminal Fgf10a originates from two different sources - one source is the front of the primordium, where Fgf10a is secreted, diffuses to the rear, and becomes enclosed in the microlumina, and the second source is the central cells, where Fgf10a is secreted directly into the adjacent microlumen.

The Extracellular Matrix Protein Anosmin1 Enhances FGF Signaling

The extracellular protein ANOS1 modulates FGF signaling in many contexts (de Castro et al., 2014). In zebrafish, there are two ANOS1 paralogs, *anos1a* and *anos1b* (jointly referred to as *anos1*). Among other tissues, *anos1a* and *anos1b* are expressed in the migrating primordium (Ardouin et al., 2000; Yanicostas et al., 2008) (Figures 3B and S3A), indicating that Anos1 could play a role in regulating FGF signaling during sensory organ formation in the primordium. To test this idea, we generated *anos1* loss-of-function mutant alleles (Figure S3D). These alleles disrupt a critical disulfide bond, truncate the protein, and exhibit mRNA non-sense-mediated decay and thus are likely genetic null mutants (Figure S3B). We assessed FGF signaling in *anos1a*; *anos1b* double mutant primordia in several ways.

First, we used the FGF signaling reporter *dusp6:d2EGFP*. Consistent with previous FGF gain- and loss-of-function studies in other tissues (Molina et al., 2007), we found that the GFP fluorescence from the FGF reporter is absent in $fgf3^{-/-}$; $fgf10a^{-/-}$ mutant primordia and more than doubled in fgf10a over-expressing primordia (Figures 3C, 3D, 7E, 7F, and S3H). This suggests that this transgene also reports FGF signaling in the primordium. Quantification of the FGF reporter fluorescence in *anos1* mutant primordia showed a mild reduction of FGF

signaling in the rear of the primordium compared to wild-type embryos (Figures 3C, 3D, and S3H).

Second, we assessed FGF signaling through its effect on Wnt signaling. FGF signaling in the rear and Wnt signaling in the front of the primordium cross-repress each other such that reduced FGF signaling in the rear results in an expansion of the Wnt signaling domain in the front of the primordium (Figure 3A) (Aman and Piotrowski, 2008). To quantify Wnt signaling in the primordium, we used the Wnt reporter Tcf/Lef-miniP:dGFP (Shimizu et al., 2012). The Wnt reporter responded in a graded manner to FGF levels. The activation domain expanded from the front toward the rear of the primordium with decreasing fgf3 and fgf10a gene copy numbers until it was fully activated in the entire primordium in $fgf3^{-/-}$; $fgf10a^{-/-}$ double mutant embryos (Figures 3E, 3F, and S3E). The activation domain contracted to the first 50 µm of the front of the primordium in Fgf10a over-expressing embryos (Figure 7K). Using this reporter, we found that the Wnt signaling domain expanded about 10 µm from the front toward the rear of the primordium in anos1 mutant primordia compared to wild-type embryos (Figures 3E and 3F). This is similar to fgf10a mutants (Figure S3E).

Third, we assessed FGF signaling through sensory organ size and deposition, both of which depend on FGF signaling. Reduced FGF signaling leads to smaller and fewer neuromasts with delayed deposition, while increased FGF signaling results in larger and more neuromasts with more frequent deposition (Figures 1B and 1D) (Durdu et al., 2014; Lecaudey et al., 2008; Nechiporuk and Raible, 2008). We found that neuromasts are smaller in anos1 mutant embryos than in wild-type embryos (Figure 3G). We also found that microlumina are smaller in anos1 mutant embryos, similar to fgf3 and fgf10a mutant embryos, and that their formation is slightly delayed (Figures 3H and 3l). Finally, the spacing between the initial neuromasts was increased and frequently one neuromast was missing (Figures 3G, 3K, and S3C), while primordium migration and body size were only mildly affected in anos1 mutant embryos compared to wild-type embryos (Figure S3F). This is in contrast to anos1a morpholino knockdown experiments, in which the primordium stalled (Yanicostas et al., 2008). Since anos1a and anos1b single mutants did not display a primordium migration defect (Figure S3C) and anos1a^{-/-}; anos1b^{-/-} double mutants showed only a mild defect (Figures 3L and S3F; Video S4), this discrepancy is probably due to the error-prone nature of morpholinomediated knockdowns (Kok et al., 2015) rather than the absence of genetic compensation in morpholino-mediated knockdown (Rossi et al., 2015).

Next, we asked if *anos1* genetically interacts with *fgf3* and *fgf10*. We found that the mild primordium migration defect in $anos1a^{-/-}$; $anos1b^{-/-}$ double mutant embryos was enhanced when we further lowered FGF signaling by also reducing the copy numbers of *fgf3* and *fgf10a*. This frequently caused the primordium to stall prematurely (Figures 3J and 3L). However, loss

See also Figure S3.

⁽K) Primordium and neuromast position in embryos of indicated genotypes at 50 hpf (left). The arrow denotes the position of the primordium. The double-headed arrow indicates spacing between neuromast 1 and 2. Scale bar represents 500 μ m. Box and whisker plot of the distance between neuromast 1 and 2 for the genotypes indicated (right). n is the number of individual embryos. ns = p >0.05, ** = p < 0.01. ANOVA p < 0.01.

⁽L) Quantification of normalized distance migrated for the genotypes indicated. Mean, SD, and individual data points are shown. n.s. = p > 0.05, ** = p < 0.01, *** = p < 0.001. ANOVA p < 0.0001.

of Anos1 did not enhance the migration phenotype in embryos completely lacking FGF signaling ($fgf3^{-/-}$; $fgf10a^{-/-}$; $anos1a^{-/-}$; $anos1b^{-/-}$ quadruple mutants; Figure 3L), suggesting that Anos1 acts solely through FGF in this context. Importantly, the delayed deposition of the initial neuromasts and neuromast number in *anos1* mutant embryos was rescued when we added an extra copy of fgf10a from a transgene (Figures 3G and 3K). In wild-type embryos, adding an additional copy of fgf10a did not affect neuromast size, number, or deposition frequency, probably because FGF levels were not raised sufficiently to induce an FGF over-expression phenotype (Figure S3G). Consistent with the olfactory axon migration defect in humans with Kallmann Syndrome (Schwanzel-Fukuda et al., 1989; Shetty, 2015), we also observed mis-migrated olfactory axons in *anos1* mutants (Figures S3I and S3J).

Together, these data suggest that Anos1 enhances FGF signaling in the primordium to promote neuromast formation.

Anos1 Is Required for Luminal Accumulation of FGF from the Front of the Primordium

Our observations indicate that Anos1 enhances FGF signaling in the rear of the primordium. As indicated by previous reports (Díaz-Balzac et al., 2015; Hu et al., 2009; Yamada et al., 2012), Anos1 could enhance FGF signaling by facilitating the formation of the FGF ligand-receptor complex. Alternatively, Anos1 could affect how much FGF ligand reaches the rear of the primordium. Using a BAC transgene that encompasses the *anos1a* chromosomal locus (*anos1a:GFP-anos1a*) (Figure S4A), we found that Anos1a is expressed throughout the primordium and is concentrated in the microlumina of neuromasts (Figures 4A, S4B, and S4C; Video S5). This localization pattern is consistent with both the ideas that Anos1 interacts with the FGF receptors or regulates the accumulation of FGF ligand in the rear.

To distinguish between these possibilities, we first asked whether Anos1 activity is required to accumulate FGF from the front of the primordium in the microlumina in the primordium's rear. For this, we blocked fgf10a expression in the central cells by reducing Atoh1a activity (Figure 2C, arrowheads)-such that the only Fgf10a source is the Fgf10a secreted from the front of the primordium - and analyzed the distribution of Fgf10a-GFP in wild-type or anos1 mutant embryos lacking Atoh1a activity. In contrast to wild-type embryos in which Fgf10a-GFP from the front still accumulates in microlumina (Figure 2C), Fgf10a-GFP fails to accumulate in microlumina when Anos1 was genetically removed (Figures 4B and 4D). Also, Fgf10a-GFP accumulated to similar levels in microlumina of wild-type and anos1 mutant embryos when central cell production of Fgf10a-GFP was not blocked (Figure 4D). In these genetic scenarios, lumen integrity was not affected (Figure S4D), demonstrating that the absence of Fgf10a-GFP in anos1 mutants with reduced Atoh1a function was not due to Fgf10a-GFP leakage from incompletely sealedoff microlumina. Moreover, the accumulation of Fgf10a-GFP from the front of the primordium in microlumina did not require Fgfr1a (Figures 4B and 4D), indicating that Anos1 affects the transport of Fgf10a-GFP largely independently of the FGF receptors in the primordium.

Next, we asked whether loss of Anos1 activity on sensory organ formation is enhanced when Fgf10a secretion from the central cells and compensation through Fgf3 are both blocked. For this, we injected *anos1a^{-/-}; anos1b^{-/-}; fgf3^{-/-}* triple mutant embryos with *atoh1a* morpholino. In these embryos, we found that the primordia deposited fewer neuromasts compared to control embryos that have functional Anos1 but do not secrete Fgf10a from central cells (reduced *atoh1a* activity, Figures 1C and 1E) or that have functional Anos1 but do not secrete either Fgf3 or Fgf10a from central cells (reduced *atoh1a* activity and *fgf3* mutant, Figures 1C and 1E).

Together, these observations indicate that Anos1 is required for the microluminal accumulation of Fgf10a-GFP secreted from the front of the primordium, and if redundant Fgf sources are removed, lack of Anos1 results in impaired sensory organ formation. This implies an additional role for Anos1 in the regulation of FGF distribution besides its known role in facilitating FGF ligand-receptor complex formation (Díaz-Balzac et al., 2015; Hu et al., 2009; Yamada et al., 2012).

Anos1 Increases the Pool of Fast-Diffusing Fgf10a Molecules

Anos1 could regulate the distribution of Fgf10a to increase its microluminal accumulation in the rear of the primordium in three ways. First, it could increase the stability and half-life of Fgf10a and thus, its overall levels. Second, Anos1 could trap Fgf10a above forming neuromasts to locally enrich the growth factor. Third, it could facilitate the diffusion of Fgf10a from the front to the rear such that more Fgf10a reaches the rear of the primordium.

To test whether Anos1 stabilizes Fgf10a, we blocked secretion of Fgf10-GFP from central cells using brefeldin A and measured the degradation rate of luminal Fgf10a-GFP in embryos over-expressing *anos1b* from a heat shock promoter and in heatshocked control embryos. We found that the degradation rate of luminal Fgf10a-GFP was similar in *anos1b* over-expressing and control embryos (Figure 4C), indicating that Anos1 does not affect the stability of Fgf10a significantly.

To determine whether Anos1 traps Fgf10a or facilitates its diffusion, we asked how Anos1 affects the diffusivity of Faf10a-GFP using fluorescence correlation spectroscopy (FCS) (Figure 5A). FCS measurements yield information about the absolute concentration, the diffusion coefficients, and the number of species with different diffusivities (Ries and Schwille, 2012). These measurements were performed in the microlumina because the extracellular space above the primordium is too small for reliable measurements. It is likely that Fgf10a diffusion in the microlumina approximates Fgf10a diffusion above the primordium. Consistent with a previous study on Fgf8-GFP diffusion (Yu et al., 2009), we found that Fgf10a-GFP also diffused with two different mobilities in wild-type embryos. Most Fgf10a-GFP molecules (88.2%) diffused rapidly (40.2 μm²/s) as monomers, while a small fraction (11.8%) of Fgf10a-GFP diffused slowly (1.7 µm²/s) (Figures 5B, 5D, 5F, S5A, and S5E-S5K). The diffusion coefficient of the fast population is typical for a freely diffusing globular protein of Fgf10a-GFP's molecular weight (Phillips et al., 2012), suggesting that most Fgf10a molecules move freely through the extracellular space. In contrast, the 20-fold lower diffusion coefficient of the slow population suggests that about 10% of Fgf10a is bound to much larger complexes, such as HS, in the extracellular space (Bishop et al., 2007; Li et al., 2016; Makarenkova et al., 2009; Yu et al., 2009).



In the absence of Anos1 activity, the fraction of slow-moving Fgf10a-GFP molecules more than doubled (25.7%) at the expense of the fraction of fast-moving Fgf10a-GFP molecules (74.3%, Figures 5B, 5D, and 5F). The diffusivity of the two populations was little affected and Fgf10a-GFP remained

Figure 4. Anos1 Is Required for Luminal Accumulation of Fgf10a

(A) Left, Anos1a-GFP protein (green) in the primordium marked with *prim:lyn_mCherry* (red). Right, Anos1a-GFP only in false colors. Top, arrow indicates Anos1a-GFP in microlumen. Bottom, arrowhead indicates Anos1a-GFP signal above apical constrictions. Scale bar represents 100 μ m. (B) Live images of microluminal Fgf10a-GFP with membrane marker and false coloring of Fgf10a-GFP signal only in embryos of the indicated genotypes. Arrows indicate Fgf10a-GFP signal in microlumina and arrowheads (left) indicate Fgf10a-GFP-producing central cells adjacent to the microlumen. Central cells are missing in embryos injected with *atoh1a* morpholino (right). Scale bar represents 10 μ m.

(C) Total Fgf10a-GFP fluorescence intensity in the microlumen (left) and central cells (right) when secretion is blocked with brefeldin A. Error bars indicate the SD. Fgf10a-GFP degradation was fitted to a one-phase decay model with half-life values of 102 min (95% Cl 33 to 197 min) for heat-shocked control embryos and 79 min (95% Cl 25 to 157 min) for *anos1b*-over-expressing embryos. Fgf10a-GFP production rate of 75.2 \pm 8.2 min⁻¹ for heat-shocked control embryos and 89.2 \pm 11.8 min⁻¹ for *anos1b*-over-expressing embryos.

(D) Total Fgf10a-GFP intensity in mature microlumina at the fourth apical constriction from the front in uninjected embryos (left) and embryos injected with *atoh1a* morpholino (right). Mean, SD, and individual data points are shown. n.s. = p > 0.05, ** = p < 0.01. ANOVA p < 0.05. See also Figure S4 and Videos S4 and S5.

monomeric (Figures 5F and S5E-S5K). Control measurements showed that secGFP diffused as one species in microlumina with a diffusion coefficient of 56.0 µm²/s characteristic for freely diffusing GFP (Dayel et al., 1999; Yu et al., 2009), and its diffusivity was not affected much by Anos1 activity (Figures 5F and S5B). These observations suggest that Anos1 shifts the equilibrium between the fast- and slow-diffusing Fgf10a molecules toward faster-diffusing molecules and hence promotes overall Fgf10a diffusivity. Consistent with this idea, we found that most Anos1a molecules (75.6%) are also highly diffusive (31.1 μ m²/s), which suggests that most Anos1 molecules are not tethered to

the ECM (Figures 5C, 5F, S5F, and S5G). Importantly, the overall diffusivity of Fgf10a-GFP was not affected by Fgfr1a (Figures 5E and S5D). Together, these observations indicate that Anos1 facilitates the diffusion of Fgf10a rather than trapping it.



Figure 5. Anos1 Enhances Fgf10a Diffusion by Increasing the Pool of Fast-Diffusing Fgf10a Molecules

(A) Schematic of FCS measurements. Left, fluorescent molecules diffuse through a diffraction-limited spot. Arrows indicate diffusion. Middle left, fluorescence intensity fluctuations generated by molecules diffusing into and out of the confocal volume. Middle right, autocorrelation is calculated as the correlation between fluorescence intensity at time = t and time = t + τ . The x axis represents τ , the lag time, and the y axis represents the correlation G(τ). Right, crosshair indicating the position in the microlumen (white) where the FCS measurements were performed.

(B) Top, autocorrelation curves normalized to 50 µs and 2-component fits for Fgf10a-GFP in the indicated genotypes. Bottom, residuals of the fits.

(C) Top, autocorrelation curves normalized to 50 µs and 2-component fits for Anos1a-GFP in wild-type embryos. Bottom, residuals of the fits.

(D) Bar graph of the fraction of molecules in the slow component in the genotypes indicated. Error bars represent SD. *** = p < 0.001.

(E) Top, autocorrelation curves normalized to 50 μs and 1-component fits for Fgf10a-GFP in the indicated genotypes. Controls include *fgfr1a^{-/+}* and wild-type sibling embryos. Bottom, residuals of the fits.

(F) Table of fitted values. F represents the proportion of molecules in each component, and D is the diffusion coefficient.

The anos1 $a^{-/-}$; anos1 $b^{-/-}$ genotype is abbreviated as anos1 $^{-/-}$ in (D) and (F).

See also Figure S5.

Anos1 and Fgf10a Are in a Complex and Diffuse Rapidly Together

Anos1 could increase the pool of fast-diffusing Fgf10a molecules by competing with Fgf10a for binding sites in the ECM or by binding and shuttling Fgf10a. To distinguish between these possibilities, we asked whether Anos1a and Fgf10a diffuse together using fluorescence cross-correlation spectroscopy (FCCS). FCCS measurements provide information about the movement of two molecules moving together in a complex, the fraction of the molecules in the complex, and the diffusivity of the molecules in the complex (Bacia et al., 2006; Foo et al., 2012; Schwille et al., 1997; Shi et al., 2009; Sudhaharan et al., 2009; Jülich et al., 2015).

To increase the signal-to-noise ratio, we performed these measurements in the early embryo rather than in the microlumen,

and we used elevated laser intensities (Figure 6A). Because high laser intensities bleach fluorescent proteins as they traverse the confocal volume and thus overestimate the diffusion coefficients (Figure 6C) and underestimate the number of co-diffusing molecules (Figure 6B), we also performed these experiments at lower laser powers with a decreased signal-to-noise (Figures 6D and 6E).

This analysis showed that at least 12% of Fgf10a-GFP diffused together with Anos1a-mCherry for higher laser power FCCS measurements (Figures 6A and 6B). Noisier lower laser intensity FCCS measurements with reduced fluorescent protein bleaching indicate that the fraction of Fgf10a-GFP co-diffusing with Anos1a-mCherry is 28% (Figure 6D). Compared to the mobilities of the slow- and fast-moving Fgf10a-GFP and Anos1amCherry populations in the microlumen, the mobility of the Fgf10a-GFP/Anos1a-mCherry complex is more comparable to the fast-moving Fgf10a-GFP and Anos1a-mCherry populations (Figures 5F, 6C, 6E, and S6D). Importantly, control measurements showed that secGFP-mCherry dimers co-diffused rapidly in the early embryo (Figures 6A, 6C, S6A, and S6D) and that Fgf10a-GFP and secreted mCherry diffused freely and largely independently of each other (Figures 6A, 6C, S6B, and S6D). Thus, a fraction of Fgf10a is in a fast-diffusing complex with Anos1a, suggesting that Anos1 does not block Fgf10a binding sites in the ECM but rather binds to Fqf10a.

Excess Anos1 Slows the Diffusion of the Fast Fgf10a Population and Disperses Fgf10a

If Anos1 binds to a subset of Fgf10a molecules, liberates them from the ECM, and then locally increases the overall diffusivity of Fgf10a, then globally over-expressing Anos1 should increase the fraction of Anos1-bound Fgf10a, reduce the ECM-bound Fgf10a pool, and enhance Fgf10a diffusion and dispersal throughout the embryo. To test these predictions, we used FCS to measure the mobilities of Fgf10a-GFP and Anos1a-GFP in the microlumina of embryos ubiquitously over-expressing Anos1b or Fgf10a from a heat shock promoter. Compared to heat-shocked control embryos, the fraction of Fgf10a-GFP molecules in the fast-moving populations did not increase in Anos1b over-expressing embryos (92.8% and 92.2%, respectively, Figures 7A, 7C, and S7A). However, Anos1b over-expression caused the fast-moving Fgf10a-GFP molecules to move slower; the diffusion coefficient of the fast Fgf10a-GFP population was reduced from 49.9 μ m²/s to 28.2 μ m²/s (Figures 7B and S7D). Assuming a spherical shape, this reduction in diffusivity indicates a 5- to 6-fold size increase of the fast-moving Fgf10a-GFP particles. Anos1b over-expression had little effect on the diffusivity of the slow-moving population (Figure 7C). Together, this resulted in overall slower Fgf10a-GFP diffusion similar to the loss of Anos1 activity (Figure 7A). Intriguingly, the decrease in the diffusivity of Fgf10a-GFP in Anos1b over-expressing embryos was mirrored by a decrease of Anos1a-GFP diffusivity in Fgf10a over-expressing embryos. In Fgf10a over-expressing embryos, the Anos1a-GFP diffusion coefficient was reduced from 25.0 μm²/s to 15.7 μm²/s (Figures 7D-7F, S7B, and S7D). Assuming a spherical shape, this reduction in diffusivity indicates a 4-fold size increase of the Anos1a-GFP particles, which is similar to the size increase of the Fgf10a-GFP particles upon Anos1b over-expression (Figures 7B and 7E).

Next, we asked whether Fgf10a-GFP still localizes above forming neuromasts in embryos over-expressing Anos1b from a heat shock promoter. In most heat-shocked control embryos, Fgf10a-GFP was visible as a patch directly above forming but not enclosed microlumina of immature neuromasts (Figure 7D; Video S6). In contrast, Fgf10a-GFP was reduced or failed to concentrate above forming neuromasts in embryos that overexpressed Anos1b (Figure 7G; Video S6). In both scenarios, Fgf10a-GFP transcription in the central cell, Fgf10a-GFP secretion into the microlumen, Fgf10a-GFP luminal degradation, Fgf10a-GFP luminal levels, and lumen integrity—judged by retention of GFP secreted from the central cells—were not altered in microlumina in the rear of the primordium, which had already been enclosed at the time Anos1b over-expression was induced (Figures 4C and S7E–S7I).

Together, these observations suggest that excess Anos1 leads to clustering of freely diffusing Fgf10a into oligomeric, likely Anos1-containing, complexes. However, it does not decrease the pool of slow-moving, presumably ECM-bound, Fgf10a further than the wild-type levels of Anos1 do. Moreover, excess Anos1 blocks the accumulation of Fgf10a in patches above forming neuromasts but not in enclosed microlumina, consistent with the idea that excess Anos1 results in the dispersal of the growth factor in the embryo.

Excess Anos1 Blocks FGF Signaling and Neuromast Formation in the Front of the Primordium

Anos1 over-expression blocks the accumulation of Fgf10a above forming neuromasts, and therefore, it should block FGF signaling, microlumen assembly, neuromast formation in the front of the primordium and, as a consequence, primordium migration (Aman and Piotrowski, 2008; Lecaudey et al., 2008). We assessed FGF signaling in the primordium of Anos1b over-expressing embryos using the FGF-signaling reporter dusp6:d2EGFP and, as an indirect readout for FGF signaling, the Wnt reporter Tcf/Lef-miniP:dGFP. Compared to control embryos, the FGF signaling domain in the back of the primordium was shifted rearward by 35 µm in Anos1b over-expressing embryos, a distance roughly equal to the diameter of a forming neuromast (Figures 7I, 7J, and S3H). Complementary to the retraction of the FGF-signaling domain, the Wnt-signaling domain in the front expanded by a similar distance of 30 µm toward the rear of the primordium in Anos1b over-expressing embryos compared to control embryos (Figure 7K).

Next, we analyzed microlumen assembly in the primordium of Anos1b over-expressing embryos using the apically localized tight junction-associated protein and luminal marker ZO-1. In Anos1b over-expressing embryos, ZO-1 accumulation at the site of the first newly formed microlumen was shifted rearward by the length of one neuromast compared to control embryos, such that the first microlumen was positioned where the second microlumen is normally found (Figures 7L and S7C). Consistent with this, the size of the first, second, and third microlumen was increased in Anos1b over-expressing embryos compared to the size of the corresponding microlumen in control embryos (Figure 7L). This indicates that the first microlumen was lost in Anos1b over-expressing embryos.

We tested this idea further by asking whether Anos1b over-expression would dissolve forming microlumina using



Figure 6. Anos1 and Fgf10a Co-diffuse

(A) Normalized autocorrelation curves and cross-correlation curves for secGFP-mCherry dimers (left), Fgf10a-GFP and Anos1a-mCherry (middle), and Fgf10a-GFP and secreted mCherry (right) of FCCS measurements with high laser power. Bottom, residuals of the 1-component fits.

(B) Plot of the degree of cross-correlations of the individual FCCS measurements for secGFP-mCherry dimers (left), Fgf10a-GFP and Anos1a-mCherry (middle), and Fgf10a-GFP and secreted mCherry (right) of FCCS measurements with high laser power. Mean, SD, and individual data points are shown. * = p < 0.05, **** = p < 0.0001. ANOVA p < 0.0001.

(C) Plot of the diffusion coefficients of the individual FCCS measurements with high laser powers for secGFP-mCherry dimers (left), Fgf10a-GFP and Anos1a-mCherry (middle), and Fgf10a-GFP and secreted mCherry (right). Mean, SD, and individual data points are shown.

(D) Same plot as in (A) but for FCCS measurements with low laser power. Mean, SD, and individual data points are shown. * = p < 0.05, ns = p > 0.05. ANOVA p = 0.0002.

(E) Plot of the diffusion coefficients of the individual FCCS measurements with low laser powers for secGFP-mCherry dimers (left), Fgf10a-GFP and Anos1amCherry (middle), and Fgf10a-GFP and secreted mCherry (right). Mean, SD, and individual data points are shown. See also Figure S6.

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Figure 7. Anos1 Overexpression Blocks Fgf10a Accumulation and Signaling

(A) Top, normalized autocorrelation curves (gray and light blue) and 2-component fits (black and blue) for Fgf10a-GFP in the indicated genotypes. Bottom, residuals of the fits.

(B) Bar graph of the diffusion coefficient of the fast component in the genotypes indicated. Error bars represent SD. ** = p < 0.01.

(C) Table of fitted values. F represents proportion of molecules in each component, and D is the diffusion coefficient.

(D) Top, normalized autocorrelation curves (gray and light green) and 1-component fits (black and green) for Anos1a-GFP in the indicated genotypes. Bottom, residuals of the fits.

(E) Bar graph of the diffusion coefficient in the genotypes indicated. Error bars represent SD. *** = p < 0.001.

(F) Table of fitted diffusion coefficients (D).

(G) Maximum intensity projection of the apical half of two heat-shocked control (left) and two heat-shocked *hsp70:anos1b* (right) live embryos transgenic for *prim:lyn₂mCherry* and *fgf10a:GFP-fgf10*. Prim inset shows membrane outline of the primordium. Arrows in Fgf10a-GFP insets indicate patches of extracellular Fgf10a-GFP surrounding apical constrictions. These patches are present in 10 out of 10 heat-shocked control embryos but absent or strongly reduced in 5 out of 8 heat-shocked *hsp70:anos1b* embryos (bottom right) and slightly reduced in the remaining 3 out of 8 heat-shocked *hsp70:anos1b* embryos (bottom right). Warmer colors represent higher GFP fluorescence intensities. Scale bar represents 100 μm.

(H) Maximum projections of z stacks of primordia in embryos of indicated genotype 1 hr and 10 hr after heat shock. Images are stills from Video S7. Scale bar represents 100 µm.

(I) *dusp6:d2EGFP* expression (green) in the primordium (red) and *dusp6:d2EGFP* expression alone (fire heatmap) for the genotypes indicated. Scale bar represents 50 µm. Fire heatmap scale is shown below, with warmer colors indicating higher GFP fluorescence intensity.

(J) Top, quantification of GFP intensity from *dusp6:d2EGFP* in the primordium along the anterior-posterior axis. x axis represents distance from the front of the primordium. Mean and SEM shown. n = number of embryos. Bottom, plot of p values comparing the two genotypes in the graph above for each position along the x axis. (K) Left, Wnt reporter readout at 36 hpf in the primordium of heat-shocked embryos of the indicated genotypes. Middle, Wnt reporter readout only. Scale bar represents 100 μ m. Right, quantification of Wnt reporter intensity. X axis represents distance from the front of the primordium. Mean and SEM shown. n = number of embryos.

(L) Left, immunostaining against ZO-1 and GFP in the primordium of 36-hpf embryos. Images are maximum projections. Middle, ZO-1 only (fire look-up table). Arrows indicate ZO-1 signal at the center of apical constrictions. Scale bar represents 100 μ m. Right, quantification of microlumen volumes in forming neuromasts. Horizontal lines are the mean, vertical lines indicate the SD, and each data point is an individual embryo. n.s.= p > or = 0.05, * = p < 0.05. ANOVA p < 0.0001. See also Figure S7 and Videos S6, S7, and S8.

time-lapse microscopy of the luminal marker Cadherin-2-GFP (Cdh2-GFP). This analysis showed that upon Anos1b overexpression, assembling microlumina in the front of the primordium dissolved, while mature microlumina in the rear of the primordium were not affected (Figure 7H; Video S7). Microlumen maturation was not affected in control embryos (Figure 7H; Video S7). Consistent with the requirement of FGF signaling for migration (Aman and Piotrowski, 2008; Lecaudey et al., 2008; Nechiporuk and Raible, 2008), Anos1b over-expression also caused the primordium to stall (Figure S7J; Videos S7 and S8). *In vitro* studies suggested that excess Anos1 binds the FGF receptor and blocks its activation (Hu et al., 2009). This would imply that Anos1 over-expression could block FGF signaling and microlumen formation in the front of the primordium by blocking FGF receptor activation rather than dispersing the growth factor. To test this possibility, we analyzed the activation of the FGF-signaling reporter *dusp6:d2EGFP* in embryos over-expressing Anos1b, Fgf10a, or both Anos1b and Fgf10a together from heat shock promoters. We found that globally over-expressing Fgf10a alone or together with Anos1b increased FGF signaling



Figure 8. Computational Analysis of Model for FGF Shuttling by Anos1

(A) Schematic showing a cut through the three-dimensionally modeled primordium to indicate which cell positions express which surface receptors or secrete Anos1 or FGF.

(B) Computationally modeled density of the FGF/FGFR/HS complex on the primordium's surface as a function of different Anos1 concentrations.

(C) Computationally modeled concentration of free FGF in the extracellular space above the apical side of the primordium as a function of different Anos1 concentrations. 0 microns indicate the front of the modeled primordium in (A).

(D) Summary of how Anos1 affects Fgf10a ligand distribution. Black arrows indicate the progression of time. Green dots indicate Fgf10a, and red arrows indicate sites of Fgf10a secretion.

See also Figure S8.

in the rear of the primordium to similar levels. FGF signaling was not increased in heat-shocked control embryos and was decreased in embryos only over-expressing Anos1b (Figures 7I, 7J, and S3H). This suggests that Anos1 does not block FGF receptor activation.

Computational Modeling

Our experimental results suggest that localized production of FGF and Anos1 and their interactions with HS expressed on the surfaces of the cells of the primordium shape the spatial characteristics of these molecules' distributions as they diffuse in and around the primordium. To assess whether these effects can indeed account for the observed distribution of FGF and its dependence on Anos1 expression levels, we developed a computational model using Simmune software (Angermann et al., 2012; Cheng et al., 2014; Zhang et al., 2013) (Figures 8A and S8).

The biochemical model consists of FGF, the FGF receptor FGFR, surface-bound HS, and Anos1. The model assumes that FGF can bind to its receptor and, with a secondary binding site, to HS and that the trimeric complex FGFR:FGF:HS has a greater stability than the dimeric complexes FGFR:FGF and HS:FGF as suggested previously (Belov and Mohammadi, 2013). Since Anos1 does not seem to bind to HS (Figure 6), our model further assumes that Anos1 binds to the binding site of FGF that the growth factor uses for its interaction with HS (see STAR Methods and Figure S8).

In our simulations, we varied the production rate of Anos1 over six orders of magnitude and thus modulated the Anos1 concentration around the primordium. The simulation results illustrate that competition between Anos1 and HS for the shared binding site on FGF can influence the retention of FGF by HS and thus its spatial concentration profile and availability for complex formation with the FGF receptor (Figure 8B).

The assumption of cooperative binding between FGFR, FGF, and HS (Belov and Mohammadi, 2013) led to a biphasic behavior of the dependency of the concentration of receptor-bound FGF on the expression level of Anos1: in the absence or at very low levels of Anos1, FGF was retained strongly by HS in the front of the primordium, leading to very low levels of ligated FGF receptors in the rear (Figure 8C). When expressed at intermediate levels, Anos1 prevented this overly strong retention of FGF by HS in the front but still allowed FGF to form trimeric complexes with HS and FGFR. When strongly over-expressed, however, Anos1 suppressed most binding of FGF by HS, and FGF became almost uniformly distributed without becoming available for the formation of the stable FGFR:FGF:HS complexes. These simulations indicate that Anos1 can, depending on its concentration, play a dual role, enhancing or interfering with FGF signaling in the rear of the primordium.

DISCUSSION

Our analysis shows that Fgf10a secreted from the front will only enrich to detectable levels in microlumina in the rear of the primordium if Anos1 is present. Molecularly, wild-type levels of Anos1 halve the pool of slow-moving Fgf10a and thus increase the overall diffusivity of Fgf10. Intriguingly, at least 12% of Fgf10a also co-diffuses with Anos1 in the early embryo, suggesting that Anos1 liberates surface-bound, slow-moving Fgf10a

from the ECM and preserves it in the freely diffusing Fgf10a pool. In contrast, over-expression of Anos1 does not decrease the pool of slow-moving Fgf10a further, suggesting that Anos1 cannot access and liberate all ECM-bound Fgf10. Instead, excess Anos1 reduces the mobility of the pool of freely moving Fgf10, probably by binding to and clustering Fgf10. Similar to the loss of Anos1 activity, this also leads to an overall reduction of Fgf10a diffusivity, although by a different molecular mechanism. Together, these observations suggest that Anos1 liberates Fgf10a from the ECM and shuttles it to increase its signaling range.

Previous studies have shown that Anos1 acts on the FGF receptor (de Castro et al., 2014). At low concentrations, Anos1 enhances FGF-FGF receptor complex formation and thus signaling by either binding to FGF (Hu et al., 2009) or the preformed complex of FGF, FGF receptor, and the adhesion molecule L1CAM (Díaz-Balzac et al., 2015; Yamada et al., 2012). Intriguingly, this interaction requires a unique HS sulfation pattern (Díaz-Balzac et al., 2014; Tecle et al., 2013; Tornberg et al., 2011). At high concentrations, Anos1 binds unligated FGF receptor. Although this complex can still bind FGF, it cannot recruit the obligate co-factor HS and thus cannot signal (Hu et al., 2009). Our results indicate an additional function for Anos1 in regulating FGF signaling upstream of FGF ligand-receptor complex formation. We find that Anos1 increases the diffusivity of FGF and thus its signaling range. Importantly, excess levels of Anos1 affect the ability of endogenous FGF to activate FGF receptor signaling but do not block over-expressed FGF from FGF receptor activation. This is probably because over-expressed Anos1 binds and dissipates locally secreted, endogenous FGF but cannot efficiently sequester and dissipate ubiquitously over-expressed FGF to levels below that required for receptor activation.

Our computational modeling suggests that this is a reasonable scenario. If HS-bound FGF has a greater affinity for the FGF receptor than free FGF and if Anos1 and HS compete for FGF binding, then increasing Anos1 concentrations yield a biphasic FGF receptor response. At low Anos1 concentrations, FGF binds to HS and does not diffuse far from its source. At intermediate Anos1 concentrations, Anos1 liberates HS-bound FGF, and FGF diffuses further. At high Anos1 concentrations, most FGF is bound by Anos1, disperses evenly, and is not available for signaling. Together with previous reports (Díaz-Balzac et al., 2015; Hu et al., 2009; Yamada et al., 2012), these observations suggest that Anos1 modulates FGF signaling at the receptor and ligand level to ensure proper FGF receptor activation.

After Fgf10a is enriched in patches on the apical side of forming neuromasts, it becomes enclosed in sealed-off, extracellular lumina in the center of maturing neuromasts. While Anos1 facilitates the transport of Fgf10a from the front to the rear in the primordium, several of our observations suggest that it does not trap Fgf10a on the apical side above forming neuromasts. First, although Anos1 concentrates in microlumina together with Fgf10a, it does not localize in patches on the apical side of forming neuromasts like Fgf10a. Second, Anos1 does not decrease but instead increases the mobility of Fgf10a and is itself mostly mobile. Third, locally increased production of Anos1 in the primordium does not retain or enrich Fgf10a (data not shown). Fourth, Anos1-Fgf10a complexes do not diffuse slowly as expected for an Fgf10a trap but instead diffuse rapidly in the early embryo. Thus, another, unknown molecular mechanism traps Fgf10a on the apical side above forming neuromasts. The Fgf10a trap could entail immobile proteins or specifically sulfated HSPGs in the ECM that tether Fgf10a to the apical side of forming neuromasts, a notion that is consistent with the observation of less restricted FGF signaling in the primordium of embryos with defective HS synthesis (Venero Galanternik et al., 2015).

FGF is secreted from two sites in the primordium: the front and the central cells of the neuromasts (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Similar to the front cells, the central cells initially secrete FGF to the apical side of the primordium but later directly pump FGF into the microlumina. Previous studies have shown that FGF production is compensatory in the primordium. In the absence of Fgf10a from the central cells or the entire primordium, Fgf3 expression in the central cells increases and compensates for the loss of Fgf10a, resulting in normal neuromast development (Lecaudey et al., 2008; Matsuda and Chitnis, 2010; Nechiporuk and Raible, 2008). Our analysis shows that restricting FGF production in the primordium to only Fgf10a secreted from the front still results in almost normal neuromast development, indicating that Fgf10a secretion from the front alone is sufficient for sensory organ formation. However, when also removing Anos1 activity in this scenario, smaller and fewer neuromasts are deposited. This is presumably because, in the absence of Anos1, Fgf10a is less mobile and requires more time to diffuse from the front toward the rear of the primordium, where it takes longer to concentrate to levels that are sufficient to induce neuromast formation. These observations suggest a redundant sensory organ formation mechanism in which FGF secreted from the front is shuttled by Anos1 toward the rear of the primordium, where it becomes locally trapped on the apical side above the site of a future neuromast through an unknown mechanism. Once FGF accumulation reaches a certain threshold, central cells are induced. The central cells then start locally secreting more FGF toward the center of the forming neuromast, which induces lumen assembly and enclosure. Once the lumen is sealed off, the central cells secrete directly into it, and FGF becomes progressively more and more concentrated in the lumen of developing neuromasts. Such a feed-forward loop for local FGF accumulation by the central cells paired with the molecular redundancy of the FGF ligands provides a robust mechanism for sensory organ formation and may also underlie the robustness of other biological processes.

The affinity of FGF ligands for HS largely determines how far FGF ligands diffuse from their site of production through the ECM (Goetz and Mohammadi, 2013). Ligands with a high affinity for HS diffuse only over short distances and signal locally while ligands with a weak affinity for HS diffuse further and signal over a longer range. The FGF ligand affinity for HS is dictated by its protein sequence and cannot be altered to regulate the diffusion range (Makarenkova et al., 2009). In principle, however, a diffusible FGF-binding protein could compete with HS for FGF ligand binding and shuttle the growth factor through the ECM. Our results suggest that Anos1 is one such FGF shuttle protein. It moves together with Fgf10a through the extracellular space and increases Fgf10a's diffusivity and signaling range. Besides altering the affinity of HS for FGF through local modifications,

this is an elegant way to tailor the FGF signaling range in a context-dependent manner.

STAR*METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, one table, eight videos, and one data file and can be found with this article online at https://doi.org/10.1016/j.devcel.2018.07.015.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.W. and H.K.; Methodology, J.W. and H.K.; Software, Y.Y. and M.M.-S.; Formal Analysis, J.W., Y.Y., S.L., J.S., T.W., and H.K.; Investigation, J.W., S.L., and H.K.; Writing – Original Draft, J.W., M.M.-S., and H.K.; Writing – Review & Editing, M.M.-S. and H.K.; Visualization, J.W., S.L., M.M.-S., and H.K.; Supervision, E.R., T.W., M.M.-S., and H.K.; Funding Acquisition, J.W. and H.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-ZO-1	Thermo Fisher	Cat No. 33-9100; RRID: AB_87181
Mouse monoclonal anti-zns-2	DSHB	RRID: AB_531907
Rabbit polyclonal anti-GFP	Thermo Fisher	A-11122; RRID: AB_221569
Goat polyclonal anti-GFP	(Venkiteswaran et al., 2013)	N/A
Sheep polyclonal anti-mCherry	Covance, this paper	N/A
Donkey anti-Goat 488	Thermo Fisher	A-11055; RRID: AB_2534102
Goat anti-Rabbit 488	Thermo Fisher	A-11034; RRID: AB_2576217
Donkey anti-Rabbit Cy3	Jackson Immunoresearch	Cat No. 711-166-152; RRID: AB_2313568
Donkey anti-Goat Cy3	Jackson Immunoresearch	Cat No. 705-166-147; RRID: AB_2340413
Donkey anti-Sheep 647	Jackson Immunoresearch	Cat No. 713-606-147; RRID: AB_2340752
Donkey anti-Mouse Cy3	Jackson Immunoresearch	Cat No. 715-166-150; RRID: AB_2340816
Donkey anti-Mouse 647	Jackson Immunoresearch	Cat No. 715-606-150; RRID: AB_2340865
Sheep anti-DIG AP	Sigma Aldrich	SKU 11093274910; RRID: AB_2734716
Bacterial and Virus Strains		
NEB 5-alpha	New England Biolabs	Cat No C2987H
sw105	(Warming et al., 2005)	N/A
Chemicals, Peptides, and Recombinant Proteins		
NBT	Sigma Aldrich	SKU 11383213001
BCIP	Sigma Aldrich	SKU 11383221001
Digoxigenin-labeled nucleotides	Sigma Aldrich	SKU 11277073910
Trizol	Thermo Fisher	Cat No 15596026
Paraformaldehvde	Sigma Aldrich	SKU P6148-500G
Proteinase K	Sigma Aldrich	SKU P6556-1G
Bovine Serum Albumin	Sigma Aldrich	SKU A8022-50g
Methycellulose	Sigma Aldrich	SKU 1424506-1G
Glycerol	Sigma Aldrich	SKU G7893-1L
Tricaine (Ethyl 3-aminobenzoate methanesulfonate)	Sigma Aldrich	SKU E10521-50G
Low melt agarose (AguaPor HR)	National Diagnostics	Cat No EC-205 (100g)
Rhodamine 6G	Fischer Scientific	Cat No AC419010050
Alexa Fluor 594 Carboxylic Acid, tris(triethylammonium) salt	Thermo Fisher	A33082
Bsrl	New England Biolabs	R0527S
Hpych4v	New England Biolabs	R0620L
Acil	New England Biolabs	R0551S
Apol	New England Biolabs	R0566L
Taq [∞] i	New England Biolabs	R0149L
Brefeldin A	Sigma Aldrich	B7651-5MG
Critical Commercial Assavs		
Thermoscript Reverse Transcriptase	Thermofisher	11146-024
TOPO TA cloning	Thermofisher	Cat No K461020
mMessage mMachine T7 kit	Thermofisher	AM1344M
mMessage mMachine SP6 kit	Thermofisher	AM1340
Experimental Models: Organisms/Strains		
fqf3/lia ^{t24149}	(Herzog et al., 2004)	N/A
fgf10a/dae ^{tbvbo}	(Norton et al., 2005)	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
cldnb:lyn ₂ GFP	(Haas and Gilmour, 2006)	N/A
hsp70:dn-fgfr1	(Halloran et al., 2000)	N/A
dusp6:d2EGFP	(Molina et al., 2007)	N/A
Tcf/Lef-miniP:dGFP	(Shimizu et al., 2012)	N/A
cdh2:cdh2-sfGFP-TagRFP	(Revenu et al., 2014)	N/A
cxcr4b:cxcr4b-Kate2-ires-GFP-CAAX	(Venkiteswaran et al., 2013)	N/A
fgfr1a/spd ^{t3R705H}	(Rohner et al., 2009)	N/A
prim:lyn ₂ mCherry	this paper	N/A
hsp70:anos1b	this paper	N/A
hsp70:sec-mCherry	this paper	N/A
hsp70:fgf10a	this paper	N/A
fgf10a:GFP-fgf10a	this paper	N/A
fgf10a:secGFP	this paper	N/A
Anos1a:GFP-anos1a	this paper	N/A
Cxcr4b:H2A-mCherry	this paper	N/A
anos1a ^{d5}	this paper	N/A
anos1a ^{d7}	this paper	N/A
anos1b ^{d5}	this paper	N/A
anos1b ^{d79}	this paper	N/A
Oligonucleotides		
For DNA oligonucleotides, see Table S1	N/A	N/A
atoh1a morpholino: 5'- ATCCATTCTGTTGGTT TGTGCTTTT-3'	Gene Tools	Custom synthesis
Recombinant DNA		
pCRII	Thermo Fisher	Cat No K461020
pDest-hsp70-SV40pA-tol2	(Kwan et al., 2007)	N/A
CH211-262I10 (<i>fgf10a</i> BAC)	BACPAC Resources Center	N/A
CH211-267B13 (anos1a BAC)	BACPAC Resources Center	N/A
DKEY-169F10 (cxcr4b BAC)	Source Bioscience	N/A
Golden Gate TALEN and TAL Effector Kit 2.0	Addgene	ID 100000024
Software and Algorithms		
Simmune	https://www.niaid.nih.gov/research/ simmune-project	N/A
Prism 7	Graphpad	N/A
FIJI (ImageJ v1.48)	https://fiji.sc/	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Holger Knaut (holger.knaut@med.nyu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish Strains

Zebrafish care and husbandry and the use of live fish for experiments were approved and overseen by the New York University School of Medicine Institutional Animal Care and Use Committee. Zebrafish were raised and housed at 28 °C. 5 day old zebrafsih larvae were raised until 21 dpf on Cylop-eeze (Argent Laboratories, USA). Zebrafish older than 21 dpf were raised and maintained on brine shrimp (Brine Shrimp Direct, USA). For experiments, embryos were collected 15 min past egg laying, raised at 28 °C, unfertilized eggs were discarded and embryos were staged by comparing it to the staging series reporter by Kimmel and colleagues (Kimmel et al., 1995). Embryos were an approximately equal mix of male and female, as there does not exist a good method for distinguishing between the genders at the developmental stages presented in this paper. The *fgf3/lia*^{t24149} allele contains a missense

mutation (E138K) resulting in strong loss of function (Herzog et al., 2004). The *fgf10a/dae*^{tbvbo} allele contains a nonsense mutation that results in a premature stop codon at amino acid position 5 (Norton et al., 2005).

The *fgfr1a/spd*^{t3R705H} allele contains a missense mutation that changes arginine 705 in the second kinase domain to a histidine and results in a loss of function (Rohner et al., 2009). The *cldnb:lyn₂GFP* transgenic line contains an 8 kb fragment upstream of the *cldnb* start codon fused to a membrane-tethered GFP (Haas and Gilmour, 2006). The *hsp70:dn-fgfr1* transgenic line contains a 1.5 kb fragment from the zebrafish *hsp70* promoter (Halloran et al., 2000) driving the expression of a modified zebrafish Fgfr1 where the tyrosine kinase domain was replaced by EGFP (Lee et al., 2005). The *dusp6:d2EGFP* transgenic line contains a 10 kb fragment that includes the 5' UTR of *dusp6* driving the expression of a destabilized EGFP (Molina et al., 2007). The *Tcf/Lef-miniP:dGFP* transgenic line contains 6 tandem consensus Tcf/Lef binding sites fused to a minimal promoter derived from pGL4 (Promega Corporation) driving the expression of a destabilized EGFP (Shimizu et al., 2012). The *cdh2:cdf2-sfGFP-TagRFP* bacterial artificial chromosome (BAC) transgenic line expresses the sfGFP-TagRFP timer from the *cdh2* locus (Revenu et al., 2014). The *cxcr4b:cxcr4b:Kate2-ires-GFP-CAAX* bacterial artificial chromosome (BAC) transgenic line expresses Cxcr4b-Kate2 and membrane tethered GFP from the *cxcr4b* locus (Venkiteswaran et al., 2013). Other strains used in this manuscript were generated using the methods detailed in the Method Details section below.

METHOD DETAILS

Generation of Transgenic Strains

For the *prim:lyn₂mCherry* transgene, we cloned the *lyn₂-mCherry* coding sequence into pME and used Gateway recombination to assemble pME-lyn₂-mCherry, p5E-sox10(-7.2 kb) and p3E-polyA into pDestTol2pA. We verified the final construct by sequencing and co-injected it along with *tol2* transposase mRNA into zebrafish embryos at the one-cell stage. Stable transgenic fish were identified by out-crossing adults injected with the transgene and raising larvae from fish whose offspring expressed Lyn₂-mCherry. Note, this transgenic line does not recapitulate the *sox10* expression pattern but labels among other structures the posterior lateral line primordium. We termed this transgenic line *Tg(prim:lyn₂-mCherry)*.

For the *hsp70:anos1b* transgene, we used the Tol2 gateway cloning kit generated by the Chien lab (Kwan et al., 2007). We cloned the zebrafish *anos1b* coding sequence into pME and used Gateway recombination to assemble pME-hsanos1b, p5E-hsp70I and p3E-polyA into pDestTol2pA. We verified the final construct by sequencing and co-injected it along with *tol2* transposase mRNA into zebrafish embryos at the one-cell stage. Stable transgenic fish were identified by out-crossing adults injected with the transgene and raising larvae from fish whose offspring were identified to express *anos1b* upon heat shock as determined by RNA *in situ* hybridization. Initial founder fish with multiple insertions were outcrossed to obtain single-insertion lines as determined by the fraction of progeny carrying the *hsp70:anos1b* transgene. The full name of this transgenic line is *Tg(hsp70:anos1b)p2.4.17*.

For the *hsp70:sec-mCherry* transgene, the *pDestTol2pA-hsp70l-anos1b-sv40pA* plasmid used to generate the *hsp70:anos1b* line (see above) was used as a template. Using Gibson cloning (Gibson et al., 2009), we replaced the *anos1b* coding sequence with the coding sequence of *mCherry* fused to the 3' end of the *fgf3* secretion signal (amino acids 1 to 18). We verified the final construct by sequencing and co-injected it along with *tol2* transposase mRNA into zebrafish embryos at the one-cell stage. Stable transgenic fish were identified by out-crossing adults injected with the transgene and raising larvae from fish whose offspring were identified to express mCherry upon heat shock as determined by red fluorescence. Founder fish were verified to carry a single copy of the transgene by determining the fraction of progeny carrying the transgene. The full name of this transgenic line is *Tg(hsp70:secP-mCherry)p1*.

For the *hsp70:fgf10a* transgene, a genomic fragment spanning 1.5 kb upstream of the *hsp70* start codon (Halloran et al., 2000) was fused to the coding sequence of *fgf10a* followed by the *SV40pA* signal and cloned into a vector in which the multiple cloning site is flanked by I-Scel sites (Thermes et al., 2002). For transgenesis, $25 \text{ ng/}\mu$ l of this construct was co-injected with the I-Scel enzyme (New England Biolabs, Inc.) into one-cell-stage embryos. Stable transgenic fish were identified by out-crossing adults injected with the transgene and raising larvae from fish whose offspring were identified to express *fgf10a* upon heat shock as determined by *in situ* hybridization against *fgf10a*. Founder fish were verified to carry a single copy of the transgene by determining the fraction of progeny carrying the transgene. The full name of this transgenic line is *Tg(hsp70:fgf10a*).

For the *fgf10a:GFP-fgf10a* transgene, we used the bacteria artificial chromosome (BAC) clone CH211-262I10. This BAC clone spans 165 kb of genomic zebrafish DNA and encompass the entire *fgf10a* locus including 55 kb upstream and 75 kb downstream sequence based on the database ensembl and confirmed by PCR amplicons placed every 10 kb. We used recombineering to modify this BAC clone in three ways (Warming et al., 2005). First, we inserted the *Tol2(exon4)-FRT-galK-FRT-Tol2(exon1)-alpha-Crystallin-dsRed* cassette into the pTARBAC2 backbone (Fuentes et al., 2016) using *galK* as a selection marker, replacing nucleotides 3008-3052 of the pTARBAC2 backbone. We then removed galK by Flippase-mediated recombination. Second, we inserted a kanamycin resistance cassette to replace the sequence between the pTARBAC backbone and nucleotide 99078 of the genomic insert in the BAC, using kanamycin as a selection marker. This removed 65 kb of sequence that is 10 kb 3' to the last exon of the Fgf10a gene and reduced the total length of the BAC from 165 kb to 99 kb. The targeting cassette for this recombineering step was assembled by using 38 bp homologous to the 5' end of the pTARBAC backbone and 50 bp homologous to nucleotides 99029-99078 of the CH211-262I10 BAC. These arms of homology were added to the kanamycin resistance gene in the targeting cassette by PCR using primers with overhangs containing the regions of homology. Third, the EGFP coding sequence was inserted in-between the last codon of the Fgf10a signal peptide (amino acids 31 of Fgf10a) and the first codon of the Fgf10a mature protein using seamless galK-mediate recombineering. 577 bp upstream and 573 downstream of the end of the last codon of the Fgf10a signal peptide were added to the EGFP targeting cassette using Gibson cloning. The final BAC was characterized by restriction digest and PCR

amplification followed by sequencing of regions near the sites where the Kanamycin resistance and EGFP were inserted. BAC DNA was then purified with the nucleobond BAC 100 kit (Clonetech) and co-injected with *tol2* transposase mRNA into one-cell-stage zebrafish embryos. Stable transgenic larvae were identified by out-crossing adults injected with the transgene and by raising larvae with red fluorescence in the lens of the eye from the transgenesis marker *cryaa-dsRed* at 4 dpf. Initial founder fish with multiple insertions were outcrossed to obtain single-insertion lines as determined by the fraction of progeny carrying the transgene. The full name of this transgenic line is *TgBAC(fgf10a:EGFP-fgf10a)p7.2.br*.

For the *fgf10a:secGFP* transgene, we used the same strategy as described above for the *fgf10a:GFP-fgf10a* transgene, except the EGFP coding sequence followed by a stop codon and the *fgf10a* 3'UTR was inserted in-between the last codon of the Fgf10a signal peptide and the first codon of the Fgf10a mature protein using seamless galK-mediate recombineering (instead of only the *EGFP* coding sequence as for the *fgf10a:GFP-fgf10a* transgene). 577 bp upstream and 573 bp downstream of the end of the last codon of the Fgf10a signal peptide along with EGFP, a stop codon, and the *fgf10a* 3' UTR were assembled using Gibson cloning to obtain a pUC19 plasmid that harbors the *5'arm-EGFP-STOP-3'UTR-3'arm* targeting cassette. The final BAC was characterized by restriction digest and PCR amplification followed by sequencing of the regions across the site where the *EGFP-STOP-3'UTR* was inserted. The BAC was then purified with the nucleobond BAC 100 kit (Clonetech) and co-injected with *tol2* transposase mRNA into one-cell-stage zebrafish embryos. Stable transgenic larvae were identified by out-crossing adults injected with the transgene and raising larvae with red fluorescence in the lens of the eye from the transgenesis marker *cryaa-dsRed* at 4 dpf. Founder fish were verified to have a single copy of the transgene by determining the fraction of progeny carrying the transgene. The full name of this transgenic line is *TgBAC(fgf10a:sec-EGFP-STOP-3'UTR)p2a*.

For the anos1a:GFP-anos1a transgene, we used the CH211-267B13 BAC clone. This BAC clone spans 149 kb of genomic zebrafish DNA and encompass the entire anos1a locus including 45 kb upstream and 68 kb downstream of the first and last exon, respectively, based on the ensembl database (http://ensembl.org/index.html) and confirmed by PCR amplicons placed every 10 kb for 40 kb up- and downstream of the first and last exon, respectively. We used recombineering to modify this BAC clone in two ways. First, we inserted the Tol2(exon4)-FRT-galK-FRT-Tol2(exon1)-cryaa-Citrine cassette into the pTARBAC2 backbone of the CH211-267B13 BAC clone using galK as a selection marker, replacing nucleotides 3008-3052 of the pTARBAC2 backbone (Fuentes et al., 2016). We then removed galK by Flippase-mediated recombination. Second, the EGFP coding sequence was inserted in-between the last codon of the anos1a signal peptide (amino acid 34 of Anos1a) and the first codon of the Anos1a mature protein using seamless galK-mediated recombineering. For this, we first inserted galK using 904 bp upstream and 776 bp downstream of the end of the last codon of the Anos1a signal peptide as arms of homology. We then used the same arms of homology flanking the coding sequence of EGFP to replace galK with EGFP. Both targeting cassettes were assembled by stitching PCR. The final BAC was characterized by restriction digest and PCR amplification followed by sequencing of regions near the site where EGFP was inserted. BAC DNA was then purified with the nucleobond BAC 100 kit (Clonetech) and co-injected with tol2 transposase mRNA into one-cell-stage zebrafish embryos. Stable transgenic larvae were identified by out-crossing adults injected with the transgene and by raising larvae with yellow fluorescence in the lens of the eye from the cryaa-Citrine transgenesis marker at 4 dpf. Founder fish were verified to have a single copy of the transgene by determining the fraction of progeny carrying the transgene. The full name of this transgenic line is TgBAC(anos1a:GFP-anos1a)p1.

For the *cxcr4b:H2A-mCherry* BAC transgene, we used the BAC clone DKEY-169F10. This BAC clone contains the *cxcr4b* locus, and was modified in two ways by recombineering. First, the *Tol2* sites and the *cryaa:dsRed* transgenesis marker were inserted into the BAC backbone (Fuentes et al., 2016). Second, a cassette consisting of H2A-mCherry-FRT-galK-FRT flanked by 411 bp and 433 bp of homology upstream of *cxcr4b* exon 2, and downstream of the *cxcr4b* stop codon, respectively, was inserted to replace the *cxcr4b* coding sequence in *cxcr4b* exon 2 (amino acid 6–358, the last amino acid before the stop codon) using galK-mediated recombineering (Warming et al., 2005). The galK cassette was removed by Flippase-mediated recombination. This transgene expresses the first five amino acids from *cxcr4b* exon 1 fused to H2A-mCherry from the *cxcr4b* promoter. The final BAC transgene was characterized by EcoRI restriction digestion and sequencing of PCR amplicons of the modified locus. The DKEY-169F10 BAC clone was obtained from ImaGenes GmbH, Germany, (sales@imagenes-bio.de). The BAC was purified with the nucleobond BAC 100 kit (Clontech). We co-injected 1 nl of 50–250 ng/µl BAC transgene DNA, and 40 ng/µl *Tol2* mRNA into the lifting cell of the zygote of 0 to 20-minute-old embryos. The *Tol2* mRNA was transcribed from pCS2FA-transposase (Kwan et al., 2007) using the mMessage mMachine SP6 Transcription Kit (Thermo Fisher). Stable transgenic larvae were identified by out-crossing adults injected with the *cxcr4b:H2A-mCherry* BAC transgene, and by raising larvae positive for the red fluorescent transgenesis marker in the lens of the eye at 4 dpf. The full name of this transgenic line is *TgBAC(cxcr4b:H2A-mCherry*)p1.

Generation of Mutant Strains

We used the plasmid set described by Dahlem et al. to generate transcription activator-like effector nucleases (TALENs) targeting the codons for cysteine 163 in *anos1a* and cysteine 155 in *anos1b* (Dahlem et al., 2012). These cysteines are homologous to cysteine 172 in human ANOS1, which forms a crucial disulfide bond. In humans the C172R mutation leads to the loss of ANOS1 function, resulting in Kallmann Syndrome. TALEN sequences were designed using TALEN Targeter (https://tale-nt.cac.cornell.edu). The plasmids encoding the TALEN proteins were constructed by golden gate cloning and verified by sequencing. We used pTal3DD and pTal3RR instead of pTal3 as the destination vector. These plasmid variants encode a more efficient Fokl endonuclease that is also engineered to be a constitutive heterodimer to minimize off-target effects. To generate mutant lines, 400 pg of *in vitro* transcribed mRNAs encoding the TALENs targeting *anos1b* were injected into separate clutches of zebrafish embryos at the one-cell stage. Stable mutant lines were identified by outcrossing adults injected with TALEN mRNA and raising fish whose offspring had mutations

in *anos1a* or *anos1b* as determined by PCR followed by restriction enzyme digest. Alleles in stable mutant lines were characterized by sequencing. For *anos1a*, we recovered two alleles: a 5 nucleotide (nt) deletion and a 7 nt deletion (Figure S3B). For *anos1b*, we recovered four alleles: a 5 nt deletion (d5), a 79 nt deletion (d79), a 6 nt deletion (d6), and a 15 nt deletion (Figure S3B). *Anos1a* d7/d7; *anos1b* d5/d5 and *anos1a* d5/d5; *anos1b* d79/d79 were generated by first crossing anos1a+/- to anos1b+/- fish and then genotyping and in-crossing the anos1a+/-; anos1b+/- fish. All *anos1a* and *anos1b* alleles were outcrossed at least three times before generating *anos1a-/-; anos1b-/-* double mutants. All *anos1a-/-; anos1b-/-* fish described in the experiments are *anos1a* d5/d7; *anos1b* d5/d79 double compound heterozygotes in order to minimize any potential off-target effects from the TALEN mutagenesis. Note that all these alleles delete a critical cysteine in the whey-acidic protein (WAP) domain and result in a frame shift and premature stop codon (Figure S3D). *Anos1a* and *anos1a*; *anos1b* dnos1a; *anos1b* dnos1a; *anos1b* dnos1a; *anos1b* dnos1a.

Genotyping Primers and Restriction Enzymes

The following PCR primers and restriction enzymes were used to genotype the corresponding alleles. For a list of primers see also Table S1.

anos1a gene

Outer PCR primers: 5'-AGCTGTGTGCAAAGTGTTGTAG-3'/ 5'-CATATAGTTTGATAGAGCGCTTGGAC-3' Inner PCR primers: 5'-AGATGAGAGTGTGTGTACTATGGC-3'/5'-CCCAGAAGCCACTTTGTGTG-3' Restriction enzyme digest with Bsrl anos1b gene Outer PCR primers (all alleles): 5'-GTGTGACGAGCGCTGAGTTCCTG-3'/5'-ATTTCACCTGTTTCTTTAGTATG-3' Inner PCR primers (5 nt deletion allele): 5'-TGTGTGGAGAGCTGTGCGCGGGACCGCGAG-3'/5'-TAAATACCTTTATTGAAATTCCC CAGTATCATCCAGCCGTGCTCTCCAGTGTG-3' (these primers contain several mismatches to remove the additional Hpych4V sites surrounding the mutation) Restriction enzyme digest with Hpych4v Inner PCR primers (6, 15 and 79 nt deletion allele): 5'-TCGCTACGTGTGCAGAAGCAGGG-3'/5'-TAAATACCTTTATTGAAATTCC-3' faf3 gene Outer PCR primers: 5'-ATCCCGCCATGCCACAAT-3'/5'-TCTCGTACCCCACATAAAACTGAC-3' Inner PCR primers: 5'-CTGCTCTTGTTGTTACTGAGC-3'/5'-CTCAAATATCAAACGGTTTACTCAC-3' Restriction enzyme digest with Apol fgf10a gene Outer PCR primers: 5'-TGCATCACCCTTTCTCCCATCCAG-3'/5'-TCGTCCTTGCTTTTGGTGCCATTG-3' Inner PCR primers: 5'-GCTCTTCCCAGTTTTCCGAGCTCCAGGACAATGTGCAAATCG-3'/5'-TCCGTTCTTATCGATCCTGAG-3' (forward primer is dCAPS primer) Restriction enzyme digest with Taq^al fgfr1a gene PCR primers: 5'- TTTGCCGGTGAAATGGATGGCTCC-3'/5'- AGTCTTACAGCTCATGTGTGCATG-3' Restriction enzyme digest with Acil hsp70:anos1b transgene Outer PCR primers: 5'-TGAGCATAATAACCATAAATACTA-3/5'TCAGGCAGCGGGACACG-3' Inner PCR primers: 5'-AGCAAATGTCCTAAATGAAT-3'/5'-CGCAACGCTCACCTCAAAC-3' hsp70:fgf10a transgene Outer PCR primers: 5'-TGAGCATAATAACCATAAATACTA-3'/5'-TCGTCCTTGCTTTTGGTGCCATTG-3'

Inner PCR primers: 5'-AGCAAATGTCCTAAATGAAT-3'/5'-TCCGTTCTTATCGATCCTGAG-3'

Mosaic Analysis

At the 1,000-cell to sphere stage, 50 cells were transplanted from a *cldnb:lyn₂GFP; prim:lyn₂mCherry* or *cldnb:lyn₂GFP; prim: lyn₂mCherry*; *fgf10a:GFP-fgf10a donor embryo* into a *cxcr4b:H2A-mCherry* recipient embryo of an equivalent stage. Embryos were incubated at 28 degree C until 36 hpf, sorted for embryos with a mosaic primordium based on the membrane GFP expression from the *cldnb:lyn₂GFP* transgene and imaged on a Leica SP5 II confocal microscope using a 40x NA 1.1 objective. Forming and deposited neuromasts were analyzed from mosaic primordia that contained donor-derived cells in the front of the primordium and no donor-derived cells in the analyzed neuromasts to exclude the possibility of direct Fgf10a-GFP secretion from donor-cells into the microlumen.

Heat Shock

For confocal imaging experiments, *hsp70:anos1b*, *hsp70:fgf10a* and *hsp70:anos1b*; *hsp70:fgf10a* embryos were heat shocked twice at 39.5 degrees C for 1 hour at the 27-28 hpf and the 31-32 hpf stage in a water bath and imaged at 36-38 hpf. *hsp70:anos1b*; *cdh2-GFP* embryos were heat shocked at 39.5 degrees C for 1 hour at the 31-32 hpf stage and imaged starting at 34 hpf. *hsp70:dn-fgfr1* embryos were heat shocked at 39.5 degrees C for 20 minutes starting at the 31 hpf stage because longer heat shocks resulted in lethality.

For overview images, *hsp70:anos1b* and *hsp70:fgf10a* embryos were heat-shocked at 39.5 degrees C for 1 hour and *hsp70:dn-fgfr1* embryos were heat-shocked for 20 minutes at 28 hpf for overview images of the phenotype shown in Figure 1B and S7J. Images were taken at 50-52 hpf.

Morpholino Injections

1 nl of 0.05 mM antisense morpholino targeting *atoh1a* with the sequence 5'- ATCCATTCTGTTGGTTTGTGCTTTT-3' (Gene Tools, (Millimaki et al., 2007)) was injected into embryos at the one-cell stage.

Brefeldin Treatment

Embryos were incubated with 28 μ M brefeldin (Bfa, Sigma) dissolved in fish water (0.3g/l Instant Ocean Aquarium Sea Salt Mixture, Instant Ocean) and mounted in 0.5% low melt agar also containing 28 μ M Bfa. Z-stacks were taken every 30 minutes for a total duration of 3 hours using a Leica SP5 II confocal equipped with hybrid detectors in photon counting mode. We used a 40x NA 1.1 objective (HC PL APO 40x/1.10 W CORR CS2) with the pinhole set to Airy 3. The 488 nm laser was set to 150 μ W and the 561 nm laser was set to 57 μ W.

Whole-Mount In Situ Hybridization

Preparation of RNA probes and whole mount *in situ* hybridization were performed as previously described (Thisse and Thisse, 2008). Briefly, we cloned DNA templates with the primers listed below using a zebrafish 36 hpf cDNA library generated from polyA RNA extracted with Trizol (Invitrogen) and reverse transcribed with ThermoScript (Invitrogen). Probes were *in vitro* transcribed with digoxigenin-labelled nucleotides (DIG, Roche). They were detected with anti-DIG-AP antibody (1:2000, Roche) and NBT/BCIP (1:1000 each, Roche).

Embryos were fixed in 4% PFA (Sigma Aldrich) overnight at room temperature. Following fixation, they were permeabilized in methanol (Fisher Chemical) at -20°C, rehydrated, permeabilized with 10 μg/ml proteinase K (Sigma Aldrich) in PBST for 8 minutes at room temperature, and post-fixed in 4% PFA for 20 minutes at room temperature. Blocking and probe hybridization were performed at 68°C overnight. Following probe hybridization and washes, embryos were blocked in 2% BSA (Sigma Aldrich) in PBST and incubated with anti-DIG-AP antibody overnight at 4°C. Embryos were washed and developed in NBT/BCIP solution overnight at room temperature.

For assessing nonsense-mediated decay of mRNA transcripts for *anos1a* and *anos1b* mutant alleles, *anos1a-/-; anos1b-/-* double mutant embryos and wild-type embryos were stained and developed in the same tube. Wild-type embryos were marked by removing the tips of their tails after fixation and before staining.

Immunostaining was performed after stopping NBT/BCIP development with PBST supplemented with 1mM EDTA. Embryos were blocked in 2 % BSA in PBST and incubated with affinity-purified goat anti-GFP (1:100, (Venkiteswaran et al., 2013)) overnight at 4 degree C, followed by washes, incubation with donkey anti-goat-Alexa488 (1:1000, Invitrogen) overnight at 4 degree C, and additional washes.

Primers for Cloning RNA In Situ Probe Templates

The following primers were used to clone probe templates into the pCRII vector using TOPO TA cloning (Invitrogen).

- anos1a: 5'-GGTGGCACGGACATCAGAAGAACG-3', 5'-GAAGACCGGGAAGGCTGGCAAAAT-3'
- anos1b: 5'- TCCCGCTGAAGCCAAGAAGAGAC -3', 5'-CAGCAGGCCGGTAATCACAAAATG-3'

fgf3: 5'-ATGGTTATAATTCTGCTCTT-3', 5'-TTAAATGTCAGCCCTTCTGT-3'

- fgfr1a: 5'-AGGCGATGGGGATGGATAAAGAAA-3', 5'-TCCGGCGTCATGAGAAAACACT-3'
- fgfr1b: 5'-CTGGCGGAGTGATTTGTTTTGATT-3', 5'-ATGCTCCCGTATTCGTTCTCCACA-3'

The following primer pairs were used to amplify probe templates. The reverse primers contain the T7 promoter sequence, and purified PCR product was used directly as a template for the *in vitro* RNA transcription reaction using reagents from Roche.

- fgf10a: 5'-CTACAACCCCACCAAAGGGAAC-3', 5'-GAAATTAATACGACTCACTATAGGCATGTGTAACCGATAGAATAGC-3'
- EGFP: 5'-GTGAGCAAGGGCGAGGAGCTG-3', 5'-GAAATTAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC-3'

Whole Mount Immunostaining

Immunostaining was performed as previously described (Lewellis et al., 2013). Briefly, embryos were fixed in 4% PFA for two hours at room temperature. Following fixation, they were permeabilized in methanol at -20°C, rehydrated, permeabilized with 10 µg/ml proteinase K in PBST for 8 minutes at room temperature, and post-fixed in 4% PFA for 20 minutes at room temperature. Embryos were blocked in 2% BSA in PBST for 1 hour and incubated in primary antibody at 4 degree C overnight. Following PBST washes, they were incubated in secondary antibody overnight at 4 degree C. Rabbit anti-GFP (1:1000, Invitrogen), affinity-purified goat anti-GFP (1:100, (Venkiteswaran et al., 2013)), affinity-purified sheep anti-mCherry (1:1000, custom-made antibody generated against bacterially-produced, recombinant full-length mCherry protein by Covance), zns-2 (1:50, Developmental Studies Hybridoma Bank) and mouse anti-ZO-1 (1:500, ThermoFisher) were detected with goat anti-rabbit-Alexa488 (1:1000, Invitrogen), donkey anti-rabbit-Cy3 (1:1000, Jackson ImmunoResearch), donkey anti-sheep-Alexa647 (1:1000, Jackson ImmunoResearch), donkey anti-sheep-Alexa647 (1:1000, Jackson ImmunoResearch) secondary antibodies.

Wide Field Imaging

GFP from the cldnb:lyn2GFP Transgene

Embryos were imaged using a Leica M165 FC stereo microscope (fluorescent dissecting scope) with a 1x objective at 3.2x zoom. One-second exposures were taken using a GFP filter or two-second exposures were taken using a GFP/mCherry dual color filter. Images of the posterior lateral line in *hsp70:dn-fgfr1* embryos were taken with a Texas red filter with a two-second exposure to block out GFP signal from the *hsp70:dn-fgfr1* transgene.

Whole Mount RNA In Situ Hybridization

Intact embryos were mounted in 3 % methylcellulose in PBS and imaged on a Zeiss Discovery V8 stereo microscope using a 0.67x objective at 8x zoom.

Flat Mount RNA In Situ Hybridization

Embryo tails were dissected and mounted in 50 % glycerol on slides with thin wells made from a single reinforcement label (Avery). They were covered with a No. 1.5 coverslip and imaged using a Zeiss Axioplan equipped with a 20x NA 0.75 objective or Zeiss Apotome equipped with a 20x NA 0.8 objective.

Reflection Imaging

Reflection imaging of *in situ* hybridizations stained with NBT/BCIP was performed for *anos1a* and *anos1b* mRNA stainings. Tails were dissected and mounted in 50 % glycerol on slides with thin wells made from a single reinforcement label (Avery, Staples). Z-stacks of the primordium were taken on a Leica SP5 II confocal with a 40x NA 1.1 water immersion objective (HC PL APO 40x/1.10 W CORR CS2). The NBT/BCIP signal was captured with 633 nm laser reflection. The collection wavelength was set to 630-640 nm while illuminating with the 633 nm laser line. The GFP signal was collected simultaneously with a second detector, exciting with the 488 nm laser line and collecting signal in a 495-550 nm window.

Confocal Imaging

Still Images

All embryos were mounted in 0.5 % low melt agar dissolved in fish water and supplemented with 0.4 mg/ml MS-222 anesthetic.

The *Tcf/Lef-miniP:dGFP* Wnt reporter line was imaged using the Leica SP5 II confocal microscope with hybrid detectors set to photon counting mode and a Leica 40x NA 0.8 water dipping objective (HCX APO L 40x/0.80 W U-V-I). The pinhole was set to 1 airy unit. The 488 nm laser was set to 300 μ W and the 561 nm laser was set to 57 μ W.

The *dusp6:GFP*, *fgf10a:fgf10a-GFP*, *fgf10a:secGFP*, and *anos1a:anos1a-GFP* transgenic lines were imaged using the Leica SP5 II confocal microscope with hybrid detectors set to photon counting mode and a 40x NA 1.1 water immersion objective (HC PL APO 40x/1.10 W CORR CS2). The pinhole was set to 2 airy units (150 μ m). The 488 nm laser was set to 300 μ W and the 561 nm laser to 57 μ W.

The laser power was calibrated before each imaging session using an X-Cite Power Meter Model XR2100 (Lumen Dynamics), which measures the power of laser light emitted from the objective onto the stage.

MoviesEmbryos were mounted in 0.5 % low melt agar dissolved in fish water and anesthesized with MS-222 (0.4mg/ml). The embryos were imaged on a Leica SP5 II laser scanning confocal microscope equipped with two HyD hybrid detectors. For time-lapse imaging, we used a Leica 20x NA 0.5 objective (HCX APO L 20x/0.50 W U-V-I) for all movies except the *cdh2:cdh2-sfGFP*; *prim:lyn2mCherry* transgenic embryos, which were imaged with a Leica 20x NA 0.75 objective (HC PL APO 20x/.75 CS2). We acquired Z stacks every 10 or 15 minutes. Embryos were maintained at 28 degree C for the duration of imaging using a heated stage (Warner Instruments). For experiments requiring a heat shock, embryos were first mounted and then heat shocked immediately before imaging. The first image was collected 30-45 minutes after the end of the heat shock.

Light Sheet Imaging

Anos1a:anos1a-GFP; prim:lyn₂mCherry and fgf10a:fgf10a-GFP; prim:lyn₂mCherry embryos were mounted in 1% low melt agarose dissolved in fish water with 0.4 mg/ml MS-222 and imaged on the Zeiss Lightsheet Z.1 using a W Plan Apochromat 20x NA 1.0 water immersion detection objective and two PCO.Edge sCMOS detectors. Samples were illuminated from the dorsal side using a LSFM 10x NA 0.2 objective with pivot scan enabled. We used a 405/488/561 nm laser blocking filter combined with a LP 560 beam splitter to capture the red and green channels simultaneously. For anos1a:anos1a-GFP; prim:lyn₂mCherry embryos the 488 nm laser power was set to 5% and the 561 nm laser power was set to 2%, with exposure times of 400 ms for each plane and an optical zoom of 2.5. For fgf10a:fgf10a-GFP; prim:lyn₂mCherry embryos the 488 nm laser power was set to 2%, with exposure times of 800 ms for each plane and an optical zoom of 1.5.

mRNA Constructs

cDNA encoding *fgf10a-GFP*, anos1a-mCherry (N-terminus tag), sec-mCherry, secGFP-linker-mCherry and lynlyn-mCerulean were assembled into the *pDest-hsp70-SV40pA-tol2* plasmid backbone using Gibson cloning. For Fgf10a-GFP, the GFP was inserted between the secretion signal and mature Fgf10a protein. For Anos1a-mCherry, the mCherry was inserted after the Fgf3 secretion signal and immediately upstream of the mature Anos1a protein. Sec-mCherry and secGFP-linker-mCherry both consist of the respective

fluorescent proteins with an upstream Fgf3 secretion signal. For secGFP-linker-mCherry, the two fluorescent proteins are separated by a 46 amino acid glycine-alanine flexible linker. For *lynlyn-mCerulean*, *mCerulean* was cloned directly downstream of two tandem repeats of *lyn* transmembrane domain sequence.

Templates for *in vitro* transcription were generated by PCR using the plasmid constructs as a template. The forward primer included a SP6 transcription start site, and the reverse primer primed at the end of the SV40pA site. To generate mRNA, we transcribed these templates using the mMessage mMachine SP6 Kit (Thermo Fisher) and purified the mRNA with phenol-chloroform extraction followed by isopropanol precipitation.

Dual Color FCCS Sample Preparation

Wild-type zebrafish embryos were injected at the one-cell stage with 400 pg each of mRNAs encoding fgf10a-GFP, anos1a-mCherry, sec-mCherry and/or secGFP-linker-mCherry and 100 pg of mRNA encoding lynlyn-mCerulean to mark the cell membranes with CFP.

Embryos were mounted in 0.5 % low melt agar with the animal pole directly against the coverslip at the 2000-cell stage. Dual color FCS measurements were performed with the laser positioned in the extracellular space of the cell layer directly under the enveloping layer at the animal pole between the oblong and dome stage.

Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross Correlation Spectroscopy (FCCS) Imaging

FCS measurements were taken using a Zeiss 880 laser scanning confocal microscope equipped with a C-Apochromat 40x NA=1.2 water-immersion objective, a spectral detector (32 channel GaAsP photomultiplier tube array), and an incubation chamber set to 28 degrees C. For single-color measurements, the laser beam (488 nm from a 25 mW argon laser with attenuators set to 1.5 μ W (0.2 %) for Rhodamine 6G and 0.08 μ W (0.01 %) for GFP) was coupled into the objective via a 488/561/633 nm main beam splitter, and the emitted fluorescence between 490-552 nm was recorded on the spectral detector for GFP and Rhodamine 6G. For dual-color measurements with high laser intensities, the 488 nm 25 mW laser was set to 3.86 μ W (0.5 %) for Rhodamine 6G and GFP and the 561 nm 20mW laser set to 63 μ W (2 %) for mCherry and Alexa Fluor 594, with the same optical setup as the single-color measurements.

For dual-color measurements with low laser intensities, the 488 nm 25 mW laser was set to 1.9 μ W (0.25 %) for Rhodamine 6G and GFP and the 561 nm 20 mW laser set to 3.4 μ W (0.1 %) for mCherry and Alexa Fluor 594, with the same optical setup as the single-color measurements. We note that the dual-color measurements with high laser intensities (488 nm at 3.86 μ W, 561 nm at 63 μ W), yielded better signal-to-noise ratios but caused bleaching of the fluorescent proteins during measuring. This resulted in an overestimation of the diffusion coefficients and an underestimation of the number of co-diffusing particles. Dual-color measurements with low laser intensities (488 nm at 1.9 μ W, 561 nm at 3.4 μ W), did not result in significant fluorescent protein bleaching but in decreased signal-to-noise ratios. Emission wavelengths 499-552 nm were collected for GFP and Rhodamine 6G and 606-695 nm were collected for mCherry and Alexa Fluor 594. All samples were kept at 28 degree C during the measurements. The pinhole was set to 1 airy unit (31.8 μ m in radius for single-color and 34.2 μ m for dual-color measurements) and the confocal volumes of the two laser lines were calibrated using 10 nM Rhodamine 6G (R6G, ACROS Organics) and 5 nM Alexa Fluor 594 (A594, Thermo Fisher), respectively, in deionized water. All correlations of the FCS measurements were normalized to G(τ =1.6 μ s) except for the measurements of Anos1a-GFP which were normalized to G(τ =10 μ s)

Modeling Description

The spatial modeling platform Simmune builds computational representations of (multi-) molecular complexes and their reactiondiffusion dynamics based on the specification of pairwise interactions between molecular binding sites (Angermann et al., 2012; Cheng et al., 2014; Zhang et al., 2013). These specifications can be entered into the software through a visual interface that uses iconographic symbols for molecules, their subdomains and binding sites. All model assumptions about the biochemical properties of the interacting molecular species are accessible through this interface. There are thus no 'hidden' assumptions in the mathematical representation of the model.

To perform simulated experiments with Simmune, one needs to specify the molecular interactions of the simulated model. This information is stored in the model file. The supplementary model file (Data S1) can be loaded into the Simmune Modeler for facile inspection (and, if desired, modification) of model parameters and molecular interactions. In addition to the model file describing the biochemistry, one needs to define the geometry (for instance a single cell with a particular cell morphology or an arrangement of multiple cells). This spatial information is stored in a geometry file that can be created and modified with the Simmune Cell Designer. Simulations are run by loading the simulation description file (Data S1) into the Simulator. The simulation description file contains the name of the model file and the geometry file (Data S1) and the initial molecular concentrations in the volume elements of the simulation. Thus, in order to re-run the simulations the results of which are reported here, it suffices to run the Simmune simulator and load the description file FGF_Anos1_HS.sdc. As long as the name and location of the model file (FGF_Anos1_HS.dbf) and the geometry file (FGF_Anos1_HS.geo) are not changed (the model description file assumes that all three files are in one directory/folder) the model file can be modified to alter, for instance, Anos1 production parameters. This is described in the model file annotation as well.

Model Details

For the simulations of the reaction-diffusion system consisting of FGF, Anos1 and HS, we modeled the primordium as a 3D rectangular structure consisting of 126 individual cells arranged in a 3x3x14 shape (Figure 8A). Each cell is represented as a cube of

7 microns side length, giving the primordium in our model a length (x-axis) of 98 microns and a width (y- and z-axis) of 21 microns. To have more control over the localized secretion of FGF and Anos1 towards the top of the primordium, the cells were divided into three cells along the vertical axis (as along the depth, the y-axis) even though the actual primordium consists of only one layer of cells vertically. Three cells on top of the primordium produce FGF while Anos1 is being released by a stripe of cells (Figure S8B, right panel). All cells express HS on their surfaces while only the back half of the simulated primordium expresses the FGF receptor (Figures 8A and S8B). The extracellular space is 140 microns long and 35 microns wide. To account for loss of FGF into the surrounding space, FGF was assigned a tag that represents availability of FGF and switches from on to off with a transition time of 1 second for freely diffusing FGF while receptor bound FGF will remain available for 1000 seconds (16.7 minutes) reflecting internalization and eventual diffusional loss. During the simulation, intra- and extracellular concentrations can be assessed through a visual user interface (Figures 8A and S8B). All molecular reactions and rates for associations, dissociations and productions are shown in Figure S8C.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image Quantification

Wnt Signaling Reporter

We generated a mask of the primordium by thresholding the *prim:lyn_mCherry* channel and filling the holes to encompass the cytoplasm in addition to the membranes. We multiplied the GFP channel to this mask, removed the zeroes, and plotted the average GFP intensity at each anterior-posterior position against the distance from the front of the primordium. This procedure was automated using a custom-written imageJ script (Data S1).

FGF Signaling Reporter

We cropped a single Z-slice from the Z-stack that did not contain GFP signal from glial cells medial to the primordium and generated a mask of the primordium by thresholding the *prim:lyn₂mCherry* channel and filling the holes to encompass the cytoplasm in addition to the membrane. We multiplied the GFP channel by this mask, removed the zeroes, and plotted the average GFP intensity at each anterior-posterior position against the distance from the front of the primordium. This procedure was automated using a custom-written imageJ script (Data S1). We defined the rear of the primordium to begin at the inflection point of the plots of the average GFP intensity in the rear of each primordium.

Fgf10a-GFP and secGFP

To account for changes in microlumen intensity as proneuromasts matured, GFP intensity measurements were taken from the microlumen at the center of the fourth apical constriction from the front of the primordium. Confocal stacks were cropped in the axial dimension to only include planes within the boundary of each microlumen. These stacks were then sum projected, and an ROI for each microlumen was manually defined. Total GFP intensities were determined by measuring the integrated density (pixel area times mean pixel intensity) within each ROI.

Microlumina Volume Calculation

Embryos were fixed, stained with GFP and ZO-1 antibodies, and imaged using the Leica SP5 II confocal microscope with hybrid detectors set to standard mode and a Leica 40x NA 0.8 water-dipping objective (HCX APO L 40x/0.80 W U-V-I). Confocal stacks were cropped in the axial dimension to only include planes within the boundary of each microlumen. We generated a mask of the microlumen by thresholding the GFP channel and filling the holes. We multiplied the ZO-1 channel to this mask, removed zeroes, and sum projected the stacks. This procedure was automated using a custom-written imageJ script (Data S1). An ROI for each microlumen position was manually defined. Total volumes were determined by measuring the integrated density (pixel area times mean pixel intensity) within each ROI.

Neuromast Size Measurement

For the calculation of neuromast size, the region of the neuromast was manually defined by cropping. The GFP channel was thresholded to generate a mask that encompasses the neuromast. Then, the volume of the mask was determined by summing up the voxels in the mask. This procedure was automated using a custom-written ImageJ script (Data S1).

Fgf10a-GFP Production and Degradation Rate Measurement

To quantify the degradation of Fgf10a-GFP intensity in the microlumen, confocal stacks from each time point in embryos treated with Brefeldin were cropped in the axial dimension to only include planes within the boundary of each microlumen. These stacks were then sum projected, and a region of interest (ROI) for each microlumen was manually defined. Total GFP intensities were determined by measuring the integrated density (pixel area x mean pixel intensity) within each ROI. All these measurements were performed using ImageJ (NIH). The degradation of Fgf10a-GFP with time was fitted to a one phase decay model ($y=(y(0) - a)^*exp(-b^*x) + a$) using Prism 7 (GraphPad).

To quantify Fgf10a-GFP production, confocal stacks from each time point were cropped in the axial dimension to only include planes with intracellular GFP signal in the central cells. These were sum projected, an ROI was manually defined, and total GFP

intensity measured as integrated density. All these measurements were performed using ImageJ (NIH). The production of Fgf10a-GFP with time was fitted to a linear model ($y=a^{*}x + b$) using Prism 7 (GraphPad).

p Value Calculation

All statistical analysis for image quantification were performed with Graphpad Prism 7 software. P-values reported for T-tests were calculated using unpaired one-tailed two-sample t-tests assuming unequal sample variance (Welch's t-test). For experiments involving multiple testing, samples were first compared using ordinary one-way ANOVA. If this test was significant, then the relevant pairwise comparisons were made. Reported P-values were calculated using Sidak's multiple comparisons test with a single pooled variance.

FCS and FCCS Quantification

Auto-Correlation Functions (ACF) were fitted with a model accounting for one diffusive particle with possible photophysical fluctuations:

$$G(\tau) = \frac{1}{N_{app}} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_D}\right)^{-1/2} \left(1 + \frac{F_p}{1 - F_p} \exp\left(-\frac{\tau}{\tau_p}\right)\right) + G_{\infty},$$
 (Equation 1)

where N_{app} is the average number of fluorophores detected in the confocal volume, τ_p is the photophysics relaxation time, F_p is the fraction of the particles undergoing photophysical fluctuations, $K = \omega_z / \omega_{xy}$ where ω_{xy} and ω_z are the 1/e² intensity radii of the confocal volume in lateral and axial direction, respectively, and τ_D is the characteristic time that the observed molecules require to diffuse through the confocal volume. $\omega_{xy} = 2\sqrt{D\tau_D}$ was calibrated using the known Rhodamine 6G diffusion coefficient $D(301 \text{ K}) = 447 \mu m^2 \text{s}^{-1}$ (Müller et al., 2008), and the known Alexa A594 diffusion coefficient $D(301 \text{ K}) = 374 \mu m^2 \text{s}^{-1}$ (Nitsche et al., 2004). The apparent size of the overlapping volume between the two laser lines was calculated as:

$$V_{cross} = \left(\frac{\pi}{2}\right)^{\frac{3}{2}} \omega_{xy,corss}^2 \omega_{z,cross} = \left(\frac{\pi}{2}\right)^{\frac{3}{2}} \left(\omega_{xy,G}^2 + \omega_{xy,R}^2\right) \left(\omega_{z,G}^2 + \omega_{z,R}^2\right)^{\frac{1}{2}},$$

where $\omega_z/\omega_{xy,cross}$ and $\omega_{z,cross}$ are the apparent sizes of the overlapping volume in the lateral and axial directions, respectively; $\omega_{xy,G/R}$ and $\omega_{z,G/R}$ are the sizes of the illumination volume of the 488 nm/561 nm laser, in the lateral and axial directions, respectively (Schwille et al., 1997). From the Cross-Correlation Function (CCF), the diffusion coefficient was calculated following:

$$D_{cross} = \omega_{xy,cross}^2 / 4\tau_{D,cross}$$
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ACF measurements in fgf10a:fgf10a-GFP, fgf10a:fgf10a-GFP; anos1a-/-; anos1b-/-, fgf10a:fgf10a-GFP; hs:anos1b, fgf10a: fgf10a-GFP; fgfr1a-/-, fgf10a:sec-GFP,

fgf10a:sec-GFP; anos1a-/- and anos1b-/- , anos1a:anos1a-GFP embryos and anos1a:anos1a-GFP embryos; hs:fgf10a, and CCF measurements in embryos injected with mRNA coding for fgf10a-GFP+anos1a-mCherry; fgf10a-GFP+sec-mCherry and sec-GFP-linker-mCherry were performed by positioning the illumination volume at the center of the microlumen of the first neuromast trailing the primordium (for FCS) or in the extracellular space directly underneath the enveloping layer of 3.5 to 4.5 hpf embryos (oblong to dome stage, for FCCS), and the emitted fluorescence was recorded for 10 seconds and its ACF and CCF were calculated in situ using an online auto-correlator (Zeiss) for every 10 second interval. In general, 10 such 10 second-ACFs and 5 such 10 second-CCFs were collected and averaged for each measurement. However, measurements with evident photo-bleaching and/or stage-drifting or sample movement as judged by ACF/CCF curves with non-zero convergence values ($G(\infty)$) were discarded before averaging. All measurements were performed at 28 degree C.

The ACF in the GFP channel in both ACF and CCF measurements, and the ACF in the mCherry channel in CCF measurements was fitted using a custom-written MATLAB script (The MathWorks Inc.) (Data S1). The ACF measurements in the early embryo were fitted to Equation 1 (Figure S6). The ACF measurements in the mircolumen were fitted to Equation 1 (Figures S5A–S5C) and to a multiple-particle 3D free-diffusion model according to Equation 2.

$$G(\tau) = \frac{1}{N_{app}} \left[\sum_{i=1}^{N} f_i \left(1 + \frac{\tau}{\tau_{Di}} \right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_{Di}} \right)^{-1/2} \right] \left(1 + \frac{F_{\rho}}{1 - F_{\rho}} exp\left(-\frac{\tau}{\tau_{\rho}} \right) \right) + G_{\infty},$$
 (Equation 2)

$$\sum_{i=1}^{N} f_i = 1,$$
 (Equation 3)

where f_i is the fraction of particles with their characteristic diffusion time τ_{Di} . The fit to Equation 1 included short time-lags (starting at 1.6 µs). The fit to Equation 2 excluded short time-lags (starting at 50 µs) to avoid the relatively lower signal-to-noise ratio in short time-lags. For the fitting to Equation 2, each ACF was fitted to both single-particle (N = 1) and dual-particle (N = 2) models, and we performed an F-test to determine if the latter was a significantly better fit against the single-particle model (with the p-value < 0.05). Note

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that the fast and slow diffusing components were assumed of similar brightness (see below), otherwise the N_{app} and f_i should be weighed as $N_{app} = \sum_{i=1}^{N} Q_i N_i$ and $f_i = Q_i^2 N_i / \sum_{i=1}^{N} Q_i N_i$ where Q_i is the brightness of the *i*th component and needs to be carefully calibrated (Thompson, 2002). We note that there were unreasonable regression results for the dual-particle model (e.g. $f_i < 1$ or $\tau_{Di} < 10 \ \mu$ s) in 8/61 (8 out of 61 measurements) for *fgf10a:fgf10a-GFP*, 6/83 for *fgf10a:fgf10a-GFP*; anos1a-/-; anos1b-/-, 5/53 for *fgf10a:fgf10a-GFP*; heat-shock control, 5/42 for *fgf10a:fgf10a-GFP*; heat-shocked *hsp70:anos1b* and 9/79 for *anos1a:anos1a-GFP*. We considered these dual-particle fits as failed fits despite the F-test indicating that the dual-particle model was a better fit than the single-particle model for these ACFs.

For the fit to Equation 2 we note that there were unreasonable regression results (e.g. $F_p < 0$ or $\tau_D < 10\mu s$) in 30/61 (30 out of 61 measurements) for *fgf10a:fgf10a-GFP*, 38/83 for *fgf10a:fgf10a-GFP*; anos1a-/-; anos1b-/-, 16/53 for *fgf10a:fgf10a-GFP*; heat-shock control, 21/42 for *fgf10a:fgf10a-GFP*; heat-shocked *hsp70:anos1b* and 30/79 for *anos1a:anos1a-GFP*. We considered these fits as failed fits due to the poor signal-to-noise ratio in the time window shorter than ~ 50 µs.

The CCF measurements in the early embryo were fitted using a one-diffusive particle model (Figures S6A–S6C):

$$G(\tau) = \frac{1}{N_{app}} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_D} \right)^{-1/2} + G(\infty).$$
 (Equation 4)

The diffusion coefficient was then calibrated according to $D_{sample} = D_{fluor} \tau_{D,fluor} / \tau_{D,sample}$ with known diffusion coefficient of Rhodamine 6G or Alexa A594, or the calibrated overlapping confocal volume.

The CCF measurements using higher laser intensities did not yield unreasonable convergence during fitting (Figures 6B and 6C). The CCF measurements using lower laser intensities yield unreasonable convergence during fitting for the ACF of mCherry and CCF of 1 out of 4 measurements of Fgf10-GFP and Anos1a-mCherrry and for the ACF of mCherry and CCF of 2 out of 7 measurements of Fgf10-GFP and sec-mCherrry (Figures 6D and 6E).

The q-value was calculated as:

$$q = \frac{G_{cross}(0)}{\min(G_G(0), G_R(0))},$$
 (Equation 5)

where the $G_{cross}(0)$, $G_G(0)$, and $G_R(0)$ are the fitted value at $\tau = 0$ of CCF, ACF in GFP channel, and ACF in mCherry channel, respectively.

The concentration of GFP tagged protein was estimated via parameters fitted from the ACF in GFP channel:

$$C = \frac{N_{app}(F_{mean} - \beta)^2}{F_{mean}^2 N_A V_{eff}},$$
 (Equation 6)

where F_{mean} is the average fluorescence intensity, and β is the fluorescence background; N_A is the Avogadro constant, and $V_{eff} = (\pi/2)^{3/2} \omega_{xv}^2 \omega_z$ is the observation volume (Schwille et al., 1997).

In Figures 5B and 5C, we show the average of the ACFs for each genotype and the dual-particle fit of these averaged ACFs for the microlumen FCS measurements. Residuals for the dual-particle fit of these averaged ACFs are plotted in the graph below the ACFs. Individual ACFs for each genotype are plotted in Figures S5A–S5C and overlaid with the dual particle fit of the averaged ACFs. Residuals for two-particle fits or one-particle fits of individual ACFs and the averaged ACFs are plotted below the ACFs in Figures S5A–S5C.

In Figure 5F, we show the averaged results for dual-particle fits of individual ACFs for Fgf10a-GFP and Anos1a-GFP and the averaged results for single-particle fits of individual ACFs for secreted GFP expressed from *fgf10a*:sec*GFP* for the microlumen FCS measurements. Other FGF ligands have been shown to diffuse as two populations (Yu et al., 2009), and a significant portion of Anos1a ACFs were better fit by the dual-particle model than the single-particle model. For dual-particle fits, we only averaged fits where the F-test indicated that the dual-particle model fitted better than the single-particle model and where the dual-particle model did not produce unreasonable fits (see above). We assume that signal-to-noise limitations prevented us from detecting the second population in the subset of ACFs where the dual-particle model did not fit the data significantly better than the single-particle model. Likewise, for single-particle fits, we only averaged fits where the F-test indicated that the dual-particle fits of secGFP and Anos1a-GFP and Anos1a-GFP and dual-particle fits for secGFP are displayed in Figure S5D. In Figure S5E we show the single-particle fits for the FCS measurements in the microlumen when including the short time-lags.

We note that the FCS measurements in the microlumen are only best described by a two-component model when we exclude the noisier short time-lags (Figure S5D). When including shorter time-lags, the FCS measurements are best described by a one-component fit (Figure S5E). However, in both cases the overall diffusivity of Fgf10a decreases in the absence of Anos1 activity.

To determine the low intensities for the 488 nm laser line, we calibrated the laser intensities given in percentage to actual laser intensity measurements on the microscope stage (X-Cite Power Meter, Lumen Dynamics Group Inc.) and fitted these to a linear model ($y = a^*x$). The 0.01 % and 0.25 % laser intensities for the 488 nm laser line are extrapolated values.

Photon Counting Histogram Analysis

The open source Fiji plugin-"analysis PCH" by Jay Unruh1 (Stowers Institute, http://research.stowers.org/imagejplugins/ zipped_plugins.html) was used to read the Carl Zeiss confocor3 raw data (*.RAW files), to calculate the photon counting histogram and to fit the photon counting histogram to a 3D Gaussian point spread function model. A bin time of 17 µs was used to calculate the PCH from acquisitions each lasting 100 s.

DATA AND SOFTWARE AVAILABILITY

The Simmune software we used for modeling 'can be downloaded (without the need to register) from https://www.niaid.nih.gov/ research/simmune-project. The downloaded Simmune software package contains documentation and a tutorial which illustrate the process of model creation and simulation.