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Chemokine signaling regulates sensory cell migration in zebrafish

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Abstract

Chemokines play an important role in the migration of a variety of cells during development. Recent investigations have begun to elucidate the importance of chemokine signaling within the developing nervous system. To better appreciate the neural function of chemokines in vivo, the role of signaling by SDF-1 through its CXCR4 receptor was analyzed in zebrafish. The SDF-1–CXCR4 expression pattern suggested that SDF-1–CXCR4 signaling was important for guiding migration by sensory cells known as the migrating primordium of the posterior lateral line. Ubiquitous induction of the ligand in transgenic embryos, antisense knockdown of the ligand or receptor, and a genetic receptor mutation all disrupted migration by the primordium. Furthermore, in embryos in which endogenous SDF-1 was knocked down, the primordium migrated towards exogenous sources of SDF-1. These data demonstrate that SDF-1 signaling mediated via CXCR4 functions as a chemoattractant for the migrating primordium and that chemokine signaling is both necessary and sufficient for directing primordium migration.

Keywords: Zebrafish; Chemokine; SDF-1; CXCR4; Migration; Lateral line; Transgenic; Morpholino knockdown; *Odysseus*

Introduction

Chemokines are a large family of molecules with important roles in both developmental and inflammatory processes (Rossi and Zlotnik, 2000). Stromal cell-derived factor-1 (SDF-1) and its CXCR4 receptor regulate movement by a variety of cell types including chemoattraction of leukocytes and migration of cerebellar, hippocampal, and neocortical interneurons (Lu et al., 2002; Stumm et al., 2003; Zhu et al., 2002). In zebrafish, there are two genes encoding SDF-1 ligands and two encoding CXCR4 receptors (Chong et al., 2001; Doitsidou et al., 2002). SDF-1a–CXCR4b signaling serves to guide primordial germ cells by chemoattraction (Doitsidou et al., 2002; Knaut et al., 2003) and regulates the differentiation of retinal ganglion cells (RGCs) including guiding their axons within the retina (unpublished data) of zebrafish embryos. SDF-1a–CXCR4b signaling is also critical for the development of a mechanosensory structure called the posterior lateral line in zebrafish (David et al., 2002). The lateral line sensory system is composed of neuromast sensory organs that are distributed over the surface of amphibians and fishes (Metcalfe, 1989; Stone, 1933). The neuromasts of the posterior lateral line are distributed along the trunk and tail of the embryonic zebrafish from the otocyst to the tip of the tail at the level of the horizontal myoseptum that separates the dorsal and ventral axial muscles (Gompel et al., 2001; Metcalfe, 1989). The posterior lateral line develops from a primordium of precursor cells, which migrates posteriorly along the horizontal myoseptum from its original position just posterior to the otocyst. As the primordium migrates, groups of cells are left behind periodically, which develop into sensory neuromasts. Migration of the primordium is dependent on SDF-1a–CXCR4b signaling (David et al., 2002). *sdf-1a* is expressed by the horizontal myoseptum, while *cxcr4b* is expressed by the primordium. Antisense knockdown of either the ligand or the receptor and mutations that affect the development of the horizontal myoseptum and eliminate expression of *sdf-1a* disrupt migration by the primordium. In this paper, we confirm the expression pattern of SDF-1a–CXCR4b and the disruption of primordium migration following antisense knockdowns of SDF-1a–CXCR4b signaling in zebrafish. Furthermore, we extend these findings by describing the expression pattern of *sdf-*

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1b, demonstrating that migration by the primordium is aberrant in *odysseus* (*ody*) embryos in which the *cxcr4b* gene is mutated (Knaut et al., 2003), showing that migration by the primordium is altered in transgenic embryos following ubiquitous induction of SDF-1b and showing directly that SDF-1a–CXCR4b signaling mediates chemoattraction of the primordium in vivo.

Materials and methods

Fish breeding and maintenance

Zebrafish were reared and maintained as described in Westfield (1995). Embryos were collected after natural spawns, kept at 28.5°C, and staged according to hour postfertilization (hpf). In some cases, embryos were transferred to water containing 0.2 mM of phenylthiourea at around 20 hpf to prevent pigmentation.

Generation of expression constructs and transgenic zebrafish

Construct *pHsp70/4-sdf-1b* was made by inserting the full-length *sdf-1b* cDNA (2.9 kb) into the *pHsp70/4-Egfp* vector (Halloran et al., 2000) between the KpnI and NotI sites to replace the EGFP coding sequence. To create construct *pHsp70/4:sdf1bEgfp*, the *sdf-1b* 5′UTR and coding region were amplified by PCR with primers: 5′-TGGTCGACAGAACACACACACTCGCTC-3′ and 5′-GTCTGTCGACTCTGAGCGTTTCTTCTTTATT-3′. (This PCR reaction removed the stop codon from *sdf-1b* coding sequence.) PCR fragment was cut with SalI and inserted into the *pHsp70/4 Egfp* vector at the SalI site. These constructs were heat-inducible (data not shown).

The method for generation of transgenic lines of zebrafish (*hsp70:sdf-1b*^{gfp} and *hsp70:sdf-1b*$^{5'utr}$) in which the transgene was heat-inducible was described by Xiao et al. (2003). Embryos were heat-induced by placing them in water at 37°C for 1 h as previously described (Halloran et al., 2000).

In situ hybridization and immunocytochemistry

Digoxigenin-labeled UTP (DIG-UTP) labeled sense and antisense RNA probes for *sdf-1a*, *sdf-1b*, and *cxcr4b* were generated by in vitro transcription from their cDNAs. The probes were hydrolyzed to 200 bases in 100 mM Na_2CO_3/$NaHCO_3$ pH 10.2. In situ hybridization to whole-mount embryos was carried out as previously described (Schulte-Merker et al., 1992).

The whole mount antibody labeling was performed as described previously (Westfield, 1995). The following antibodies and concentration were used for immunocytochemistry: anti-GFP (Chemokine), 1:25,000; anti-SV2 (Developmental Studies Hybridoma Bank, University of Iowa), 1:100; antiacetylated-α-tubulin (Sigma), 1:1000; MAb 4D9 (Developmental Studies Hybridoma Bank), 1:10; MAb Zn-5 (University of Oregon), 1:500.

Morpholino knockdown experiments

Morpholino oligonucleotides (MO; Gene Tools, LLC) were dissolved at 250 nM concentration in 1× Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) and injected into one- to four-cell stage embryos. The following antisense morpholinos were used to inhibit the translation of sdf-1a or cxcr4b mRNA. Numbers in parenthesis correspond to where on the target mRNA each morpholino was designed to bind; the **CAT** sequence in bold corresponds to the start ATG.

Anti-*sdf-1a* MO: 5′-ACTTTGAGATC**CAT**GTTTGGCAGTG-3′ (−10 to +14) Anti-*cxcr4b* MO: 5′-AAATGATGCTATCGTAAAATTC**CAT**-3′ (+1 to +25).

Standard control morpholino or a control MO for *cxcr4b* in which four bases were randomly changed (5′-AA*t*TTGA*a*GCTATCGTAA*t*ATT*g*CAT-3′ was injected at the same concentration). About 3–4 ng of morpholino was injected into each embryo.

The knockdown efficiency of the morpholinos was demonstrated by morpholino injection at 1- to 4-cell stage followed by injection of plasmid DNA of either *pHsp70/4-sdf-1a-Egfp*, *pHsp70/4-cxcr4b-Egfp* or *pHsp70/4-Egfp* at 8- to 32-cell stage. Injected embryos were heat-induced at 37°C for 1 h around 24 hpf and assayed for mosaic expression of SDF-1a^{GFP} or CXCR4b^{GFP} on a fluorescence microscope. Both the antisense *cxcr4b* and *sdf-1a* MO but not the control MOs efficiently knocked down translation of the expression constructs (data not shown).

Ectopic expression of SDF-1b^{GFP} in mosaic embryos

To ectopically express SDF-1b^{GFP} fusion protein and at the same time knock down the endogenous SDF-1a protein, antisense *sdf-1a* MO was injected into embryos at the one-cell stage followed by the injection of the expression construct *pHsp70/4-sdf-1b-egfp* or the control construct *pHsp70/4-egfp* into single blastomere of 4- to 32-cell stage embryos. Embryos were kept at 28.5°C, heat-induced at 20 and 32 hpf for 1 h each, and fixed at 36 hpf. The migrating primordium was assayed by in situ hybridization with a *cxcr4b* riboprobe and the axons of the posterior lateral line ganglion with antiacetylated-α-tubulin.

Results

Expression of SDF-1a and CXCR4b correlate with migration by the primordium of the posterior lateral line

As mentioned above, there are two *sdf-1* genes, *sdf-1a* and *sdf-1b*, in zebrafish (Doitsidou et al., 2002; Li et al.,

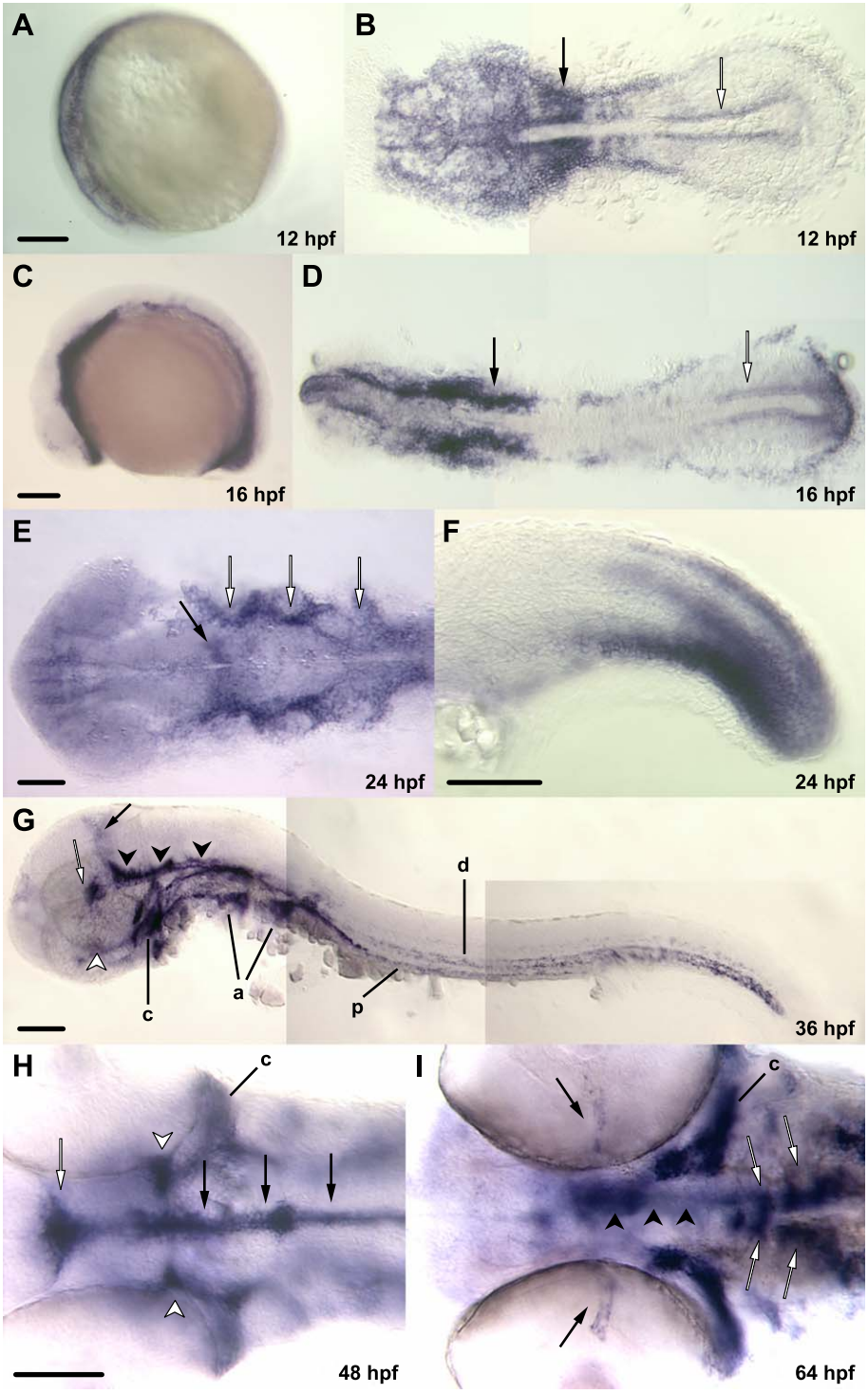


Fig. 1. Expression pattern of *sdf-1b* mRNA. A, C, F, and G are side views with anterior to the left and dorsal up. B, D, E, H, and I are dorsal views with anterior to the left. (A–D) During early somitogenesis, *sdf-1b* is expressed by the paraxial mesoderm (closed arrows) including the somites at 12 hpf and the adaxial cells (open arrow). (E) By 24 hpf, *sdf-1b* is expressed within the CNS at the midbrain or hindbrain boundary (closed arrow) as well as by the pharyngeal arches (open arrow). (F) *sdf-1b* is also expressed by the tail bud at 24 hpf. (G) By 36 hpf, expression within the CNS includes the floor plate in the midbrain and hindbrain (closed arrowheads), a group of midline cells anterior to the forebrain/hindbrain boundary (open arrow) and the optic stalk (open arrowhead) in addition to weaker expression at the midbrain/hindbrain boundary (closed arrow). Outside of the CNS, *sdf-1b* is expressed by presumptive head cartilage (c), the pharyngeal arches (a), dorsal aorta (d), and pronephros (p). (H) At 48 hpf, high expression is evident in the midbrain or hindbrain floor plate (closed arrows), the midline forebrain cells (open arrow), and the optic stalk (arrowheads) as well as the presumptive head cartilage (c) which is out of the plane of focus. (I) At 64 hpf, the optic stalk (closed arrow) maintains a low level of *sdf-1b* expression, whereas the ventral arch structures (open arrows) express *sdf-1b* at a very high level. Expression by the floor plate (arrowheads) can still be seen out of the focal plane. Bar scale, 50 μm.

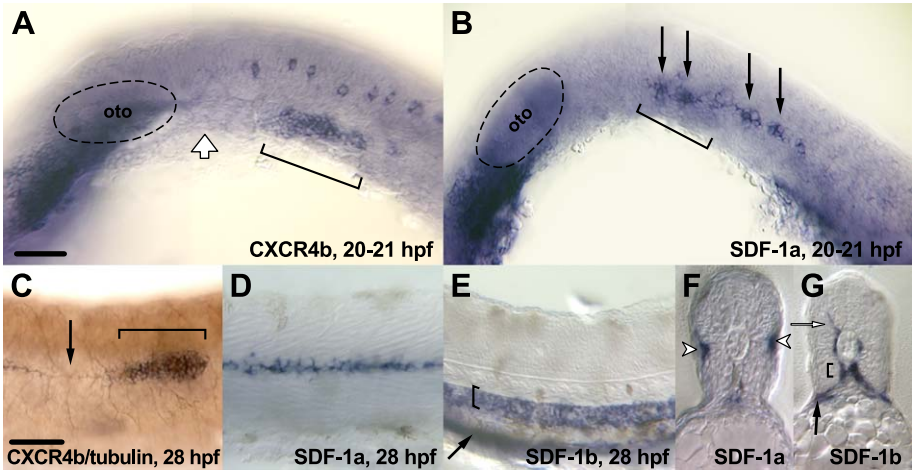


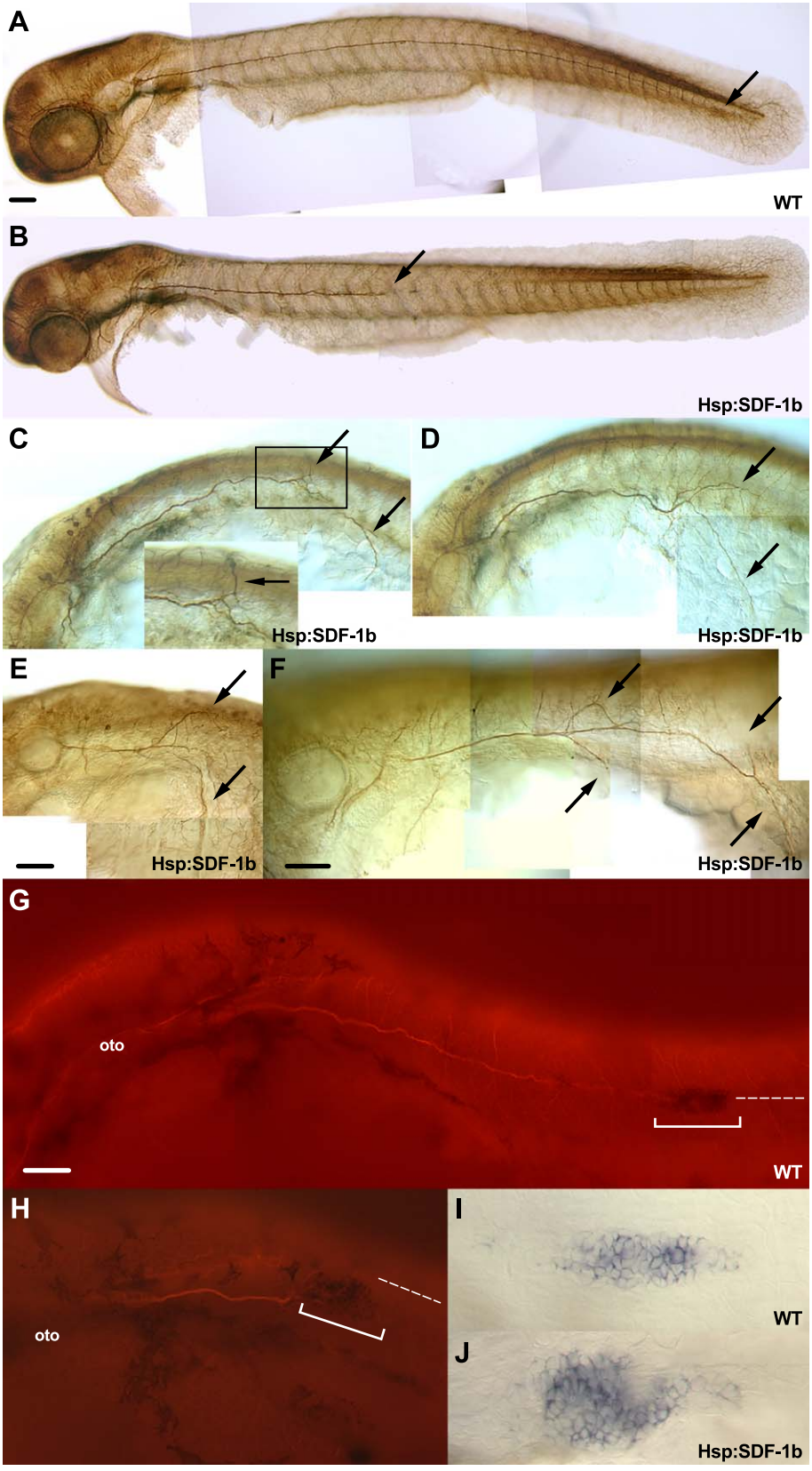
Fig. 2. Expression patterns of *sdf-1a* and *cxcr4b* correlate with the migration path of the posterior lateral line primordium. All panels are lateral views of the trunk of embryos probed with *sdf-1a*, *sdf-1b*, or *cxcr4b* riboprobes with anterior to the left and dorsal up. (A) *cxcr4b* is expressed by the posterior lateral line primordium (brackets) in a 20- to 21-hpf embryo. The leading margin of the primordium is at somite 1. Oto, denotes the otocyst. The approximate location of the ganglion of the posterior lateral line, which does not express *cxcr4b*, is marked by an open arrow. Rohon-Beard neurons within the spinal cord can also be seen to express *cxcr4b* dorsal to the primordium. (B) *sdf-1a* is expressed by groups of cells at the horizontal myoseptum (arrows) in the anterior myotomes in a 20- to 21-hpf embryo. Bracket denotes the location of the unlabeled primordium. (C) *cxcr4b* is expressed by the migrating primordium (brackets) at a midtrunk level in a 28-hpf embryo. The posterior lateral line axons (arrow) labeled with antiacetylated-α-tubulin extends in association with the primordium. (D and F) *sdf-1a* is expressed by the entire length of the horizontal myoseptum by 28 hpf. Shown are the *sdf-1a*-positive horizontal myoseptal cells in a side view (D) and cross section (F, open arrowheads) at midtrunk levels. (E and G) *sdf-1b* is expressed by the presumptive dorsal aorta (brackets in E and G) and pronephros (closed arrows in E and G) and the intersegmental vessels (open arrow in G) by 28 hpf. Shown are lateral view (E) and cross section (G) at the midtrunk levels of the embryo. Bar scale, 50 μm.

unpublished results). *sdf-1b* is expressed as early as 12 hpf in the anterior half of the embryo and by the lateral plate mesoderm at the margins of the embryo, anteriormost somites, and adaxial cells adjacent to the notochord in the posterior half of the embryo (Fig. 1). By 16 hpf, expression is evident in the pharyngeal arches and continues in the posterior lateral mesoderm and adaxial cells but is no longer seen in the somites. Expression within the CNS begins around 24 hpf and can be seen at the midbrain/hindbrain boundary. Additionally, *sdf-1b* is expressed in the tail bud at this stage. Several hours later, *sdf-1b* is beginning to be expressed by the midbrain and hindbrain floor plate (not shown) with strong expression evident by 36 hpf. Also at 36 hpf, one can observe strong expression in a group of midline cells in the forebrain just anterior to the midbrain floor plate. Furthermore, expression is evident outside the CNS in what appears to be head cartilage, dorsal aorta, and pronephros. Also evident is *sdf-1b* expression within the optic stalk.

Early on during development, *sdf-1a* is expressed to direct the migration of primordial germ cells (Doitsidou et al., 2002; Knaut et al., 2003). Later, *sdf-1a* is expressed by cells of the horizontal myoseptum where it acts to guide the migration of the posterior lateral line primordium which expresses the *cxcr4b* receptor (Fig. 2; David et al., 2002). In fact, *sdf-1a* is expressed by the anteriormost horizontal myoseptal cells and *cxcr4b* by the primordium as early as 20–21 hpf (Figs. 2A and B), which is about the onset of the migration by the primordium. Later on when the primordium is actively migrating, it expresses *cxcr4b*, and the entire length of the horizontal myoseptum expresses *sdf-1a*, while the dorsal aorta and pronephros but not the horizontal myoseptum express *sdf-1b* (Figs. 2C and E). Furthermore, when assayed with in situ hybridization, the ganglion of the posterior lateral line does not express *cxcr4b* (Fig. 2A) nor *cxcr4a* (not shown), which is highly and equally homologous to *cxcr4* in mammals as *cxcr4b* (Chong et al., 2001). Thus, *sdf-1a* and *cxcr4b* are expressed by the horizontal myoseptum and

Fig. 3. Ubiquitous misexpression of *sdf-1b* induces the posterior lateral line primordium and associated nerve to stall and follow aberrant pathways. (A) The lateral line nerve labeled with antiacetylated-α-tubulin is normal in a 48-hpf wild-type embryo following heat induction at 24 hpf. Arrow denotes the distal end of the nerve. (B–D) The lateral line nerve is stalled (B) or branches and follows aberrant pathways in 48-hpf *hsp70:sdf-1b*^{gfp} embryos following heat induction at 20 hpf (C and D) and in 44-hpf *hsp70:sdf-1b*^{gfp} embryos following heat induction at 20 and 33 hpf (E and F). Inset in C is a magnified view of the boxed area. Arrow in the inset marks a dorsal branch of the lateral line nerve. (G) Migration by the primordium (bracket), labeled with a *cxcr4b* riboprobe, and extension of the associated posterior lateral line axons (red fluorescence, labeled with antiacetylated-α-tubulin) is normal along the horizontal myoseptum (dashed line) in a non-heat-induced 32-hpf *hsp70:sdf-1b*^{gfp} embryo. Oto denotes the otocyst. (H) The primordium (bracket) and lateral line axons are stalled and ventral to the horizontal myoseptum (dashed line) in a 32-hpf *hsp70:sdf-1b*^{gfp} embryo following heat induction at 20 hpf. (I) The posterior lateral line primordium, labeled with a *cxcr4b* riboprobe, has an enlongated oval shape along the myoseptum in a 32-hpf control embryo. (J) Following ubiquitous misexpression of *sdf-1b*, the migrating primordia frequently takes on an irregular shape. Bar scale, 50 μm.

primordium, respectively, while no *cxcr4* receptor appears to be expressed by the posterior lateral line neurons before or during migration by the primordium and extension of the lateral line axons. This expression pattern suggests that SDF-1–CXCR4 signaling directly regulates migration by the primordium but not the extension of the lateral line axons.



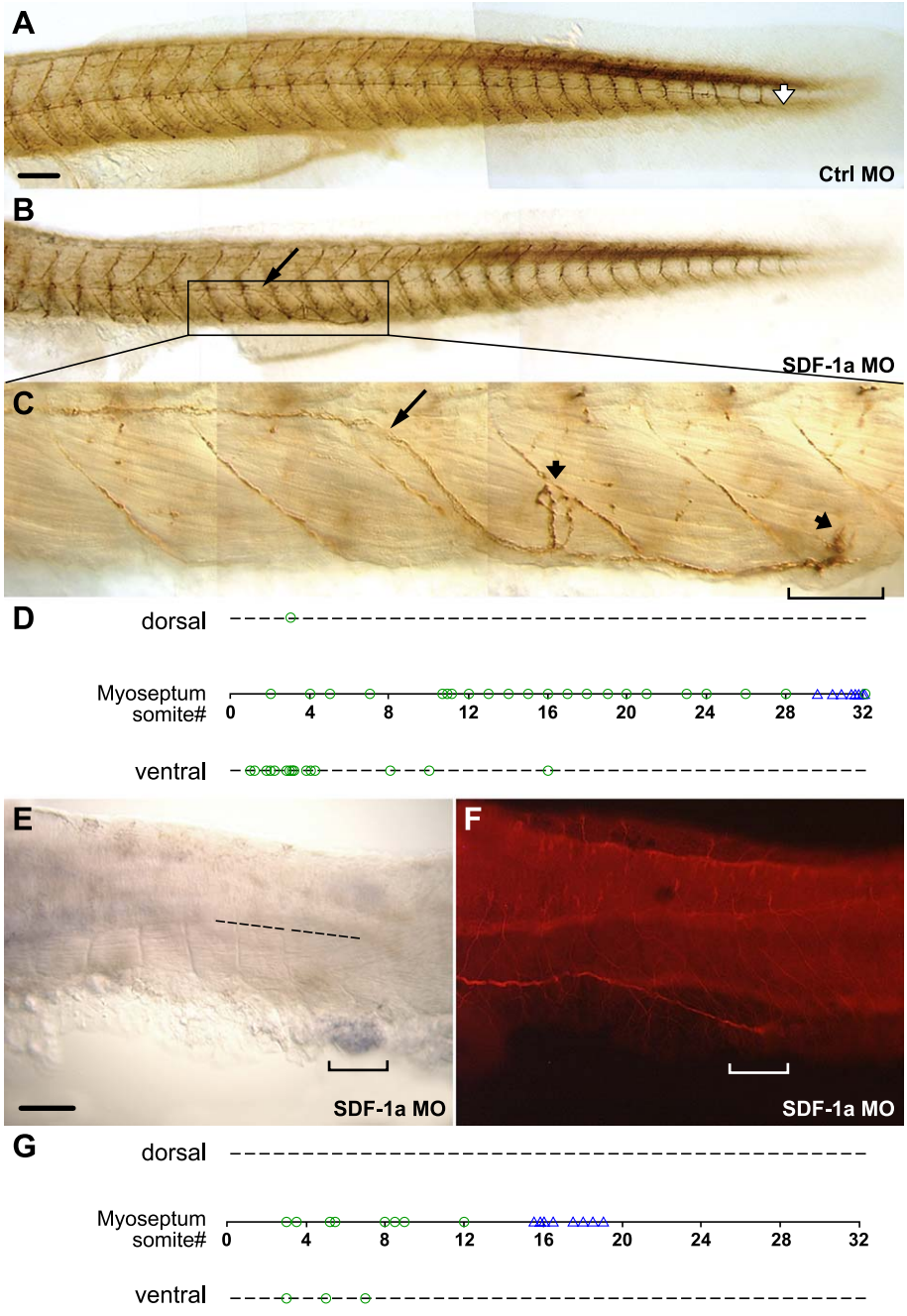


Fig. 4. The posterior lateral line primordium and nerve are stalled or misrouted when SDF-1a is knocked down by prior injection of antisense *sdf-1a* MO. (A) The lateral line nerve labeled with anti-SV2 is normal in 48-hpf control embryos injected with control MO. Open arrow denotes the distal tip of the nerve. The motor axons are also labeled by anti-SV2. (B) The lateral line nerve followed an aberrant pathway in a 48-hpf SDF-1a morphant embryo. Arrow denotes site at which the nerve strays from the horizontal myoseptum. (C) Magnified view of the boxed area in panel B showing that the nerve aberrantly extended ventrally. Arrows denote the ends of the lateral line nerve that appears to have branched. The unlabeled primordium is designated by brackets. (D) Schematic plot showing the locations of the distal ends of the lateral line nerve in SDF-1a morphant (○) and control MO (△) embryos at 48 hpf. (E) The primordium (brackets) labeled with a *cxcr4b* riboprobe is found in an aberrant location ventral to the horizontal myoseptum (dashed line) in a 32-hpf SDF-1a morphant embryo. (F) The posterior lateral line nerve labeled with antiacetylated-α-tubulin has followed an aberrant ventral pathway and remained associated with the misdirected primordium shown in panel E. The location of the primordium is bracketed. In the embryo shown in panels E and F, both the primordium was labeled for *cxcr4b* mRNA and the lateral line nerve labeled with antiacetylated-α-tubulin. (G) Schematic plot showing the locations of the primordium in SDF-1a morphant (○) and control MO (△) embryos at 32 hpf. Bar scale, 50 μm.

Misexpression of SDF-1b causes the primordium to stall and follow aberrant pathways

To examine the gain-of-function phenotype of SDF-1–CXCR4 signaling on migration by the primordium, *sdf-1b* was ubiquitously induced in transgenic zebrafish in which the heat-inducible zebrafish *hsp70* promoter regulated *sdf-1b* (Halloran et al., 2000; Xiao et al., 2003). Since the amino acid sequence of SDF-1a and SDF-1b are 91% similar and 82% identical to each other (unpublished data), we reasoned that SDF-1b may be similar to SDF-1a biochemically. Thus, misexpression of *sdf-1b* may mimic misexpression of *sdf-1a*. Lines in which SDF-1b was tagged with GFP or untagged were used and gave similar phenotypes. Transgenic embryos were heat-induced at 20 or 24 hpf and assayed with antiacetylated-α-tubulin to label the axons of the ganglion of the posterior lateral line (n = 30) at 48 hpf and/or in situ hybridization for *cxcr4b* to label the primordium (n = 24) at 32 hpf. In *hsp70:sdf-1b*^{gfp} and *hsp70:sdf-1b*$^{5'utr}$ embryos, ubiquitous expression of the transgene, tested by GFP fluorescence and *sdf-1b* in situ hybridization, respectively, was evident 1 h following heat induction (not shown). The lateral line axons normally extend embedded and in tandem with the primordium (Gompel et al., 2001). Following heat induction of *sdf-1b*, 100% of the embryos exhibited lateral line axons or primordia that were either stalled along the horizontal myoseptum or strayed from the horizontal myoseptum in the trunk (Figs. 3A–D; Table 1). Often, the primordia were misshapen with the mass of primordium cells appearing less cohesive (Figs. 3I and J). In cases in which the lateral line axons and the primordia were assayed simultaneously, they were seen to be in tandem in every case. Furthermore, when transgenic embryos were heat-induced at 20 and 33 hpf and then assayed at 44 hpf (n = 36), the lateral line nerve and primordia exhibited a higher level of stalling and misrouting (Figs. 3E–G; Table 1). The nerves or primordia stalled at segment 3.4 on average and misrouted in 23 of 36 cases in transgenic embryos heat-induced twice compared with stalled at segment 8.8 and misrouted in 11 of 30 cases in embryos heat-induced once. In heat-induced wild-type embryos (n = 50) and uninduced transgenic embryos (n = 50), the primordium or lateral line axons were normal in every case. Thus, ubiquitous induction of an *sdf-1* induced errors in migration by the primordium and extension of the associated lateral line growth cones in an apparent dose-dependent way.

Antisense knockdown of SDF-1a or CXCR4b causes the migrating primordium to stall and follow aberrant pathways

To see if SDF-1a–CXCR4b signaling is required for normal migration by the primordium, we injected antisense MOs against *sdf-1a* or *cxcr4b* into recently fertilized embryos that we had previously demonstrated were effective and specific for knocking down expression of the encoded proteins (unpublished data). Following injection of either *sdf-1a* or *cxcr4b* antisense MOs (n = 74 and n = 70, respectively), the primordium and associated posterior lateral line axons were stalled and/or strayed from the horizontal myoseptum (Figs. 4 and 5, Table 1). Injections of standard or *cxcr4b* control MOs (n = 36 and 40, respectively) or *sdf-1b* antisense MOs (n = 38) did not disrupt migration by the primordium or extension by the lateral line axons. Thus, SDF-1a–CXCR4b signaling is necessary for normal migration by the primordium and axon extension by the ganglion of the posterior lateral line and suggests that it mediates an attractive interaction to keep the primordium or lateral line axons on the horizontal myoseptum.

Interestingly, in *sdf-1a* morphants in which the primordium or the posterior lateral line nerve strayed from the horizontal myoseptum, the primordium or lateral line nerve did so in a ventral direction in nearly all cases (95%) (Table 1). On the other hand, in *cxcr4b* morphants, the primordium or lateral line nerve strayed equally in the dorsal versus ventral direction (Table 1). One explanation for the directionality bias seen following knockdown of the ligand but not following knockdown of the receptor is that a source of a second chemoattractive ligand may exist ventral to the horizontal myoseptum. In fact, *sdf-1b* is expressed by a longitudinal band of cells likely to be the dorsal aorta and pronephros which are located ventral to the horizontal myoseptum (Fig. 2E). Since SDF-1b and

Table 1
Posterior lateral line primordia or nerves were stalled or misrouted in 44 or 48 hpf embryos when SDF-1–CXCR4 signaling was perturbed

	n	Average segment[a]	Number of misrouted		
			Dorsal	Ventral	Both dorsal and ventral
Control	50[b] + 50[c]	32	0	0	0
SDF-1b overexpressed once[d]	30	8.8	2	9	0
SDF-1b overexpressed twice[e]	36	3.4	2	12	9
Control MOs	36[f] + 40[g]	32	0	0	0
SDF-1a MO	74	15.5	1	20	0
CXCR4b MO	70	20.2	5	5	0

[a] The segment the posterior lateral line primordium or nerve reached at 44 or 48hpf.
[b] Non-heat-induced *hsp:sdf-1b*^{gfp} embryos.
[c] Heat-induced wild-type embryos.
[d] The *hsp-sdf-1b*^{gfp} embryos heat-induced once at 20 or 24 hpf.
[e] The *hsp-sdf-1b*^{gfp} embryos heat-induced twice at 20 and 33 hpf. In all 23 cases of misrouting, the posterior lateral line nerve branched. Indicated are cases with only dorsal branches, only ventral branches, or both dorsal and ventral branches.
[f] Injected with standard control morpholino.
[g] Injected with a *cxcr4b* control morpholino in which four bases were randomly changed.

SDF-1a are highly homologous to each other and ubiquitous misexpression of SDF-1b results in aberrant migration of the primordium or lateral line nerve, it is possible that in the absence of SDF-1a, the primordium or lateral line axons move ventrally towards the source of SDF-1b.

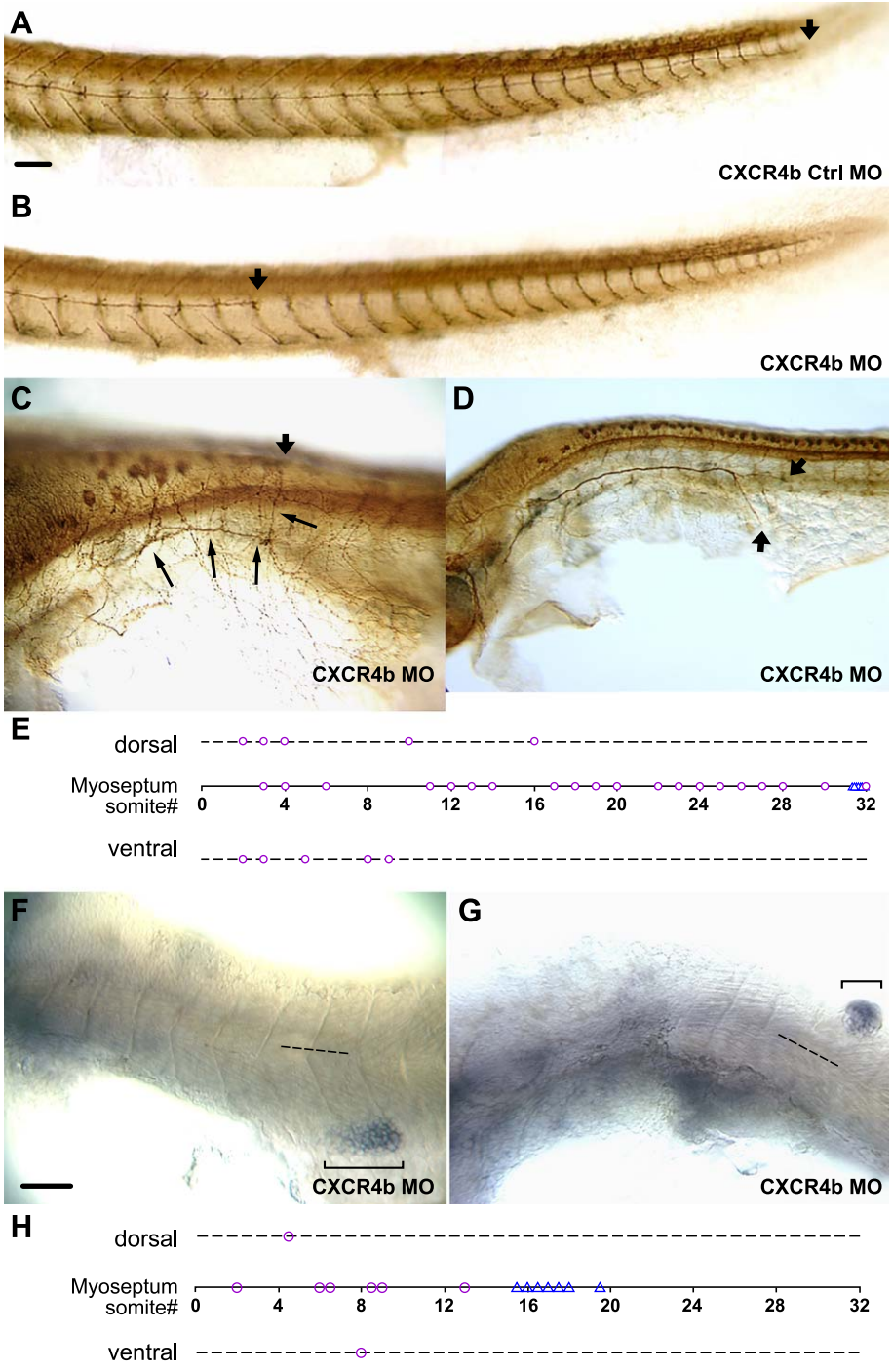


Fig. 5. The posterior lateral line primordium and nerve are stalled or misrouted when CXCR4b is knocked down. (A) The posterior lateral line nerve labeled with anti-SV2 is normal in a 48-hpf embryo previously injected with control *cxcr4b* MO. Distal tip of the nerve is denoted by the arrow. (B) The lateral line nerve is stalled in a 48-hpf CXCR4b morphant embryo. (C) The lateral line nerve aberrantly extended dorsally (arrows) in a 48-hpf CXCR4b morphant embryo. (D) The lateral line nerve aberrantly branched with one branch extending ventrally in a 48-hpf CXCR4b morphant embryo. (E) Schematic plot showing the locations of the distal ends of the lateral line nerve in CXCR4b morphant (○) and control MO (△) embryos at 48 hpf. (F) The primordium labeled with a *cxcr4b* riboprobe is located in an aberrant position ventral to the horizontal myoseptum (dashed line) in a 32-hpf CXCR4b morphant embryo. (G) The primordium is located in an aberrant dorsal position in a 32-hpf CXCR4b morphant embryo. (H) Schematic plot showing the locations of the primordium in CXCR4b morphant (○) and control MO (△) embryos at 32 hpf. Bar scale, 50 μm.

Migration by the primordium is aberrant in cxcr4b mutant embryos

Since *ody* is a mutation in the *cxcr4b* gene and one likely to be a loss-of-function mutation (Knaut et al., 2003), we examined migration by the primordium and extension by the lateral line axons in these mutants. As expected, primordium migration and lateral line axon extension were disrupted. The primordium and lateral line axons migrated or extended at most to segment 2 in 36-hpf *ody* embryos (n = 8) and segment 8 in 48-hpf *ody* embryos (n = 12) compared with segment 16 or greater for 36-hpf control embryos and segment 30 or greater for 48-hpf control embryos, respectively (Fig. 6). Comparison of 48-hpf embryos shows that the primordium or lateral line axons were more retarded in *ody* embryos compared with *sdf-1a* and *cxcr4b* morphants (Figs. 4–6), perhaps due to incomplete elimination of SDF1a–CXCR4b signaling in the morphants compared with *ody* mutants. However, the primordium and lateral line growth cones did not misroute in *ody* embryos, while in some *sdf-1a* and *cxcr4b* morphants, they did misroute as well. Overall, however, it is clear that migration or extension by the primordium or lateral line axons is aberrant in *ody* embryos and thus confirms that SDF-1a–CXCR4b signaling is required for normal migration by the primordium and extension of lateral line axons.

Erroneous primordium migration and lateral line axon extension following disruption of SDF-1a–CXCR4b signaling are likely a direct one rather than secondary to changes in the trunk and tail. In *sdf-1a* morphant, *cxcr4b* morphant, and *ody* embryos, the morphology of the trunk and tail and their major organs, the axial muscles, horizontal myoseptal region, and notochord, were normal (not shown). Additionally, muscle pioneer cells labeled with an *engrailed-1* riboprobe, MAb 4D9 that recognizes the engrailed protein, and MAb Zn5 that recognizes the DM-GRASP protein were normal in *sdf-1a* and *cxcr4b* morphants (Fig. 7). Furthermore, the primordium expresses *cxcr4b* mRNA normally in *sdf-1a*

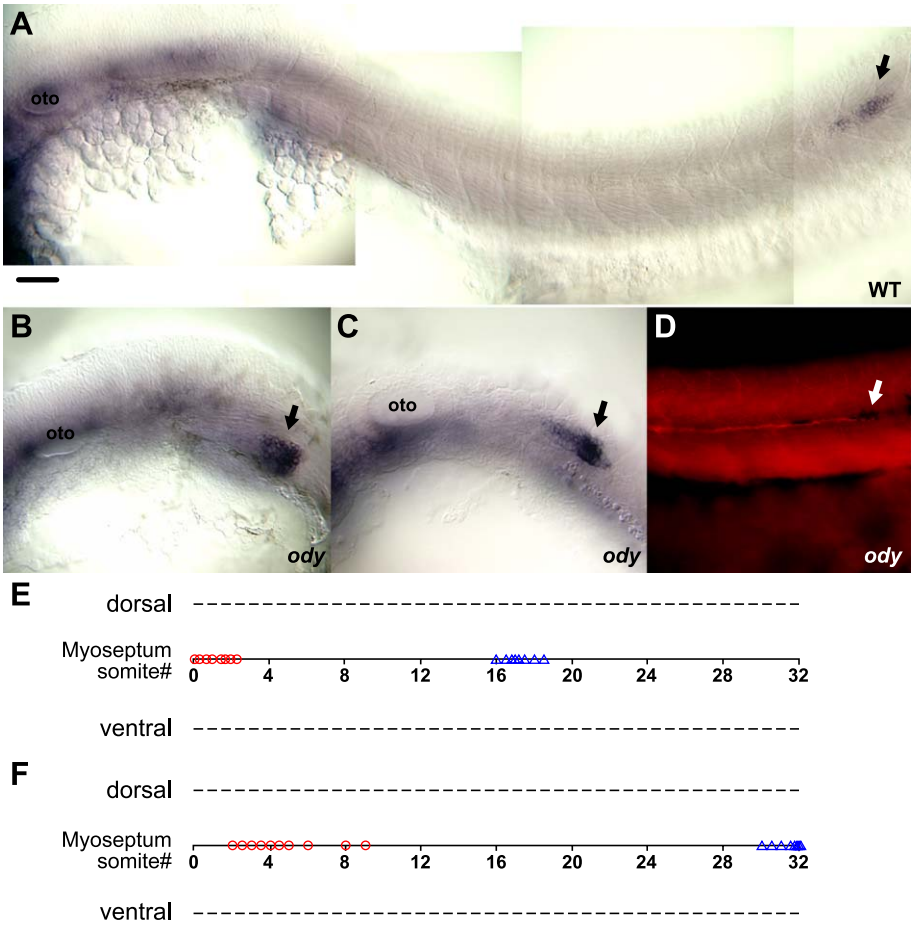


Fig. 6. The posterior lateral line primordium and axons are stalled in the *cxcr4b* mutant *odysseus* (*ody*). (A) The primordium (arrow) labeled with a *cxcr4b* riboprobe is located normally in somite 16 in a 32-hpf wild-type embryo. (B and C) The primordium is stalled in somite 3 in panel B and somite 1 in panel C in 32-hpf *ody* embryos. (D) The lateral line nerve labeled with antiacetylated-α-tubulin is stalled in somite 8 in a 48-hpf *ody* embryo. The distal tip of the nerve is marked by the arrow. In wild-type embryos, the lateral nerve reaches the posterior tip of the tail by 40 hpf. (E) Schematic plot showing the position of the primordium in *ody* (○) and wild-type (△) embryos at 32 hpf. (F) Schematic plot showing the position of the distal tip of the lateral line nerve in *ody* (○) and wild-type (△) embryos at 48 hpf. Bar scale, 50 μm.

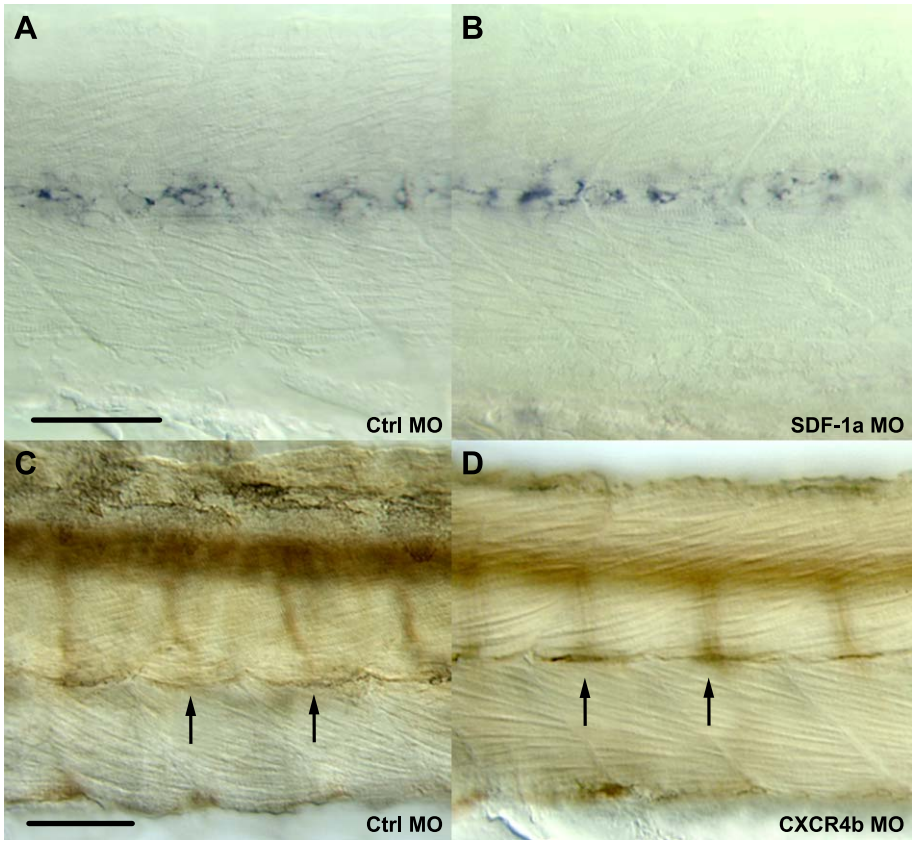


Fig. 7. Differentiation of the horizontal myoseptum is not affected in *sdf-1a* or *cxcr4b* morphants. (A and B) Lateral views of control (A) and *sdf-1a* morphant (B) 28-hpf embryos showing that muscle pioneer cells at the myoseptum properly express *eng-1*. (C and D) Control (C) and *cxcr4b* morphant (D) 48-hpf embryos showing that muscle pioneer cells (arrows) express DM-GRASP, as labeled by MAb Zn-5. Bar scale, 50μm.

and *cxcr4b* morphants, suggesting that the primordium has developed normally in the absence of SDF-1a and CXCR4b (Figs. 4D, 5E and F), and the horizontal myoseptum normally expresses *sdf-1a* in *ody* mutants, suggesting that the myoseptum is normal despite a lack of SDF-1a activity (not shown). Thus, SDF-1a–CXCR4b signaling appears to directly mediate migration by the primordium.

SDF-1 is a chemoattractant for the migrating primordium

Aberrant migration by the primordium and extension of the lateral line nerve in embryos that ubiquitously misexpress SDF-1b and in embryos deficient for SDF-1a or CXCR4b are consistent with a chemoattractive action of SDF-1 on the CXCR4b-bearing primordium. To directly test this hypothesis, migration of the primordium was examined when the primordium encountered sources of exogenous SDF-1b in wild-type embryos in which endogenous SDF-1a was knocked down. To do this, *sdf-1a* antisense MO was injected at the one-cell stage followed by injection of the *pHsp70-sdf-1b-Egfp* expression construct at the 4- to 32-cell stage, heat induction of *sdf-1b*^{gfp} at 20 and 32 hpf, and assayed at 36 hpf (n = 28; Fig. 8A). Control embryos (n = 33) were given the same treatment except that the *pHsp70-Egfp* expression construct was injected in place of the *pHsp70-sdf-1b-Egfp* construct. We had previously demonstrated that antisense *sdf-1a* MO did not affect *sdf-1b* translation, and injection of the *pHsp70-sdf-1b-Egfp* plasmid resulted in mosaic expression of SDF-1b due to random segregation of the expression plasmid in the dividing cells (data not shown). Under these conditions, the primordium often encountered muscle cells expressing exogenous *sdf-1b* in the absence of SDF-1a. In all the experimental and control embryos, both the primordia and the lateral line nerves were assayed.

The primordium and lateral line nerve were associated with muscles misexpressing SDF-1b^{GFP} much more frequently than with muscles misexpressing GFP (Fig. 8; Table 2). In each case, the primordium and the nerve were in tandem (Figs. 8F and G). Of the embryos misexpressing SDF-1b^{GFP}, 13 of 21 (62%) primordia or lateral line nerves that misrouted were in contact or within 2 μm from a muscle cell expressing SDF-1b^{GFP} with misrouting occurring dorsally (n = 4) as well as ventrally (n = 9). Of the primordia or lateral line nerves that misrouted but had not encountered an SDF-1b^{GFP}-misexpressing cell, all (n = 8) misrouted ventrally. In comparison, of the embryos misexpressing GFP, two of nine (22%) primordia or lateral line nerves that misrouted were in contact or within 2 μm from a muscle cell expressing GFP. All of the primordia or lateral line nerves

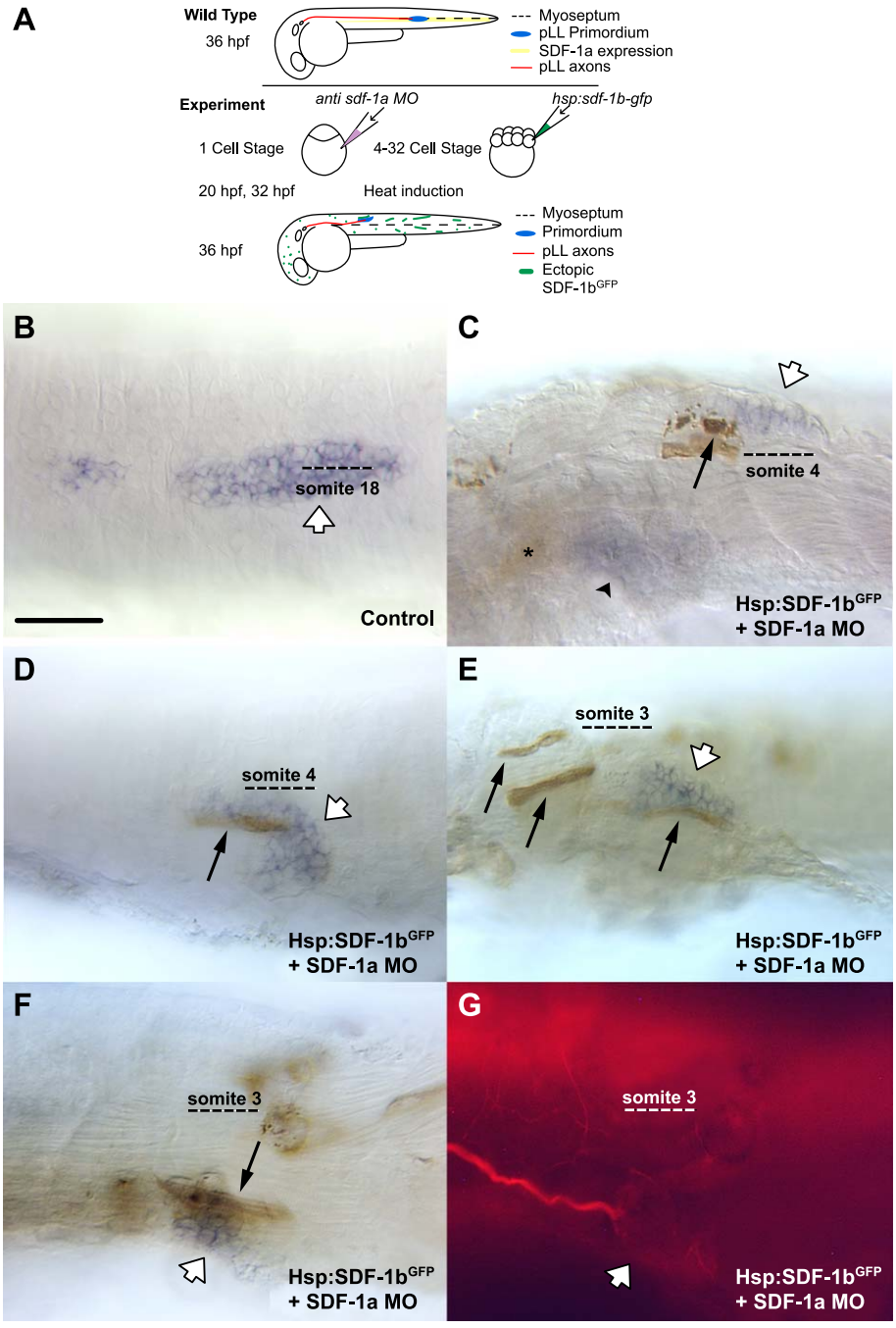


Fig. 8. The primordium and posterior lateral line axons are attracted by ectopic sources of SDF-1b in SDF-1a morphant embryos. (A) Schematic plot illustrating the experimental procedure. Embryos are injected with antisense *sdf-1a* MO at the one-cell stage followed by injection of the *hsp70:sdf*^{gfp} expression construct into a blastomere at the 4- to 32-cell stage. Subsequently, embryos are heat-induced at 32 hpf and assayed for the primordium with a *cxcr4b* riboprobe (blue), lateral line nerve with antiacetylated-α-tubulin (red flourescence), and the SDF-1b^{GFP}-expressing cells with anti-GFP (brown) at 36 hpf. (B) The primordium (arrow) is located normally along the horizontal myoseptum (dashed line) in somite 18 in a heat-induced control embryo. (C) The primordium (open arrow) is located in an aberrant dorsal position near several muscle and dermal cells (closed arrow) expressing SDF^{GFP} in somite 4 in an experimental embryo. The primordium on the other side (arrowhead) which is out of the focal plane is located in an aberrant ventral location near an SDF^{GFP}-expressing muscle fiber (asterisk). (D) The primordium (open arrow) appears to be wrapping around a SDF^{GFP}-expressing muscle fiber (closed arrow) in somite 4 of an experimental embryo. (E) The primordium (open arrow) is located in an aberrant ventral position near a SDF^{GFP}-expressing muscle fiber (closed arrow) in somite 3 in an experimental embryo. (F) The primordium (open arrow) is located in an aberrant ventral position near a SDF^{GFP}-expressing muscle fiber (closed arrow) in somite 3 of an experimental embryo. (G) The same view from the embryo seen in (F) showing that the posterior lateral line axons (labeled with antiacetylated-α-tubulin) have extended aberrantly in tandem with the misrouted primordium. The location of the primordium is indicated by the open arrow as in (F). Bar scale, 50 μm.

Table 2
Ectopic sources of SDF-1b tandemly chemoattract the migrating primordia[a] of the posterior lateral line and posterior lateral line nerves[b] in the absence of SDF-1a

		n	Average segment	Number of misrouted primordia	
				Dorsal	Ventral
SDF-1a MO + ectopic GFP	with GFP cells[c]	6	13.2	0	2
	without GFP cells[d]	27	12.8	0	7
SDF-1a MO + ectopic SDF-1b-GFP	with GFP cells	15	6.7	4	9
	without GFP cells	13	8.9	0	8

[a] Labeled with *cxcr4b* riboprobe.
[b] Labeled with antiacetylated-α-tubulin.
[c] Embryos in which the primordia are in close association (within 2 μm) with cells ectopically express GFP or SDF-1b^{GFP}.
[d] Embryos in which no GFP-positive cells were found near the primordia.

that misrouted that had encountered or not encountered GFP-expressing cells did so ventrally. The correlation of primordia or lateral line nerves with muscles expressing SDF-1b^{GFP} suggests that SDF-1b^{GFP} was a chemoattractant for the primordium. Presumably, in cases where the primordium or lateral line nerve did not encounter an SDF-1b^{GFP}-misexpressing cell, they were attracted by the endogenous SDF-1b secreted by the ventrally located dorsal aorta and pronephros in the absence of SDF-1a. These results suggest that normally SDF-1a derived from the horizontal myoseptum serves to guide the primordium via an attractive interaction.

Discussion

Disruption of SDF-1a–CXCR4b signaling clearly alters migration by the primordium and the posterior lateral line axons (David et al., 2002; this paper). Furthermore, the selective association of the primordium or lateral line axons and muscle cells misexpressing SDF-1b in embryos in which SDF-1a is knocked down strongly indicates that SDF-1 proteins are chemoattractive to the CXCR4-bearing primordium. This in turn suggests that normally SDF-1a expressed by the horizontal myoseptal cells acts to keep the migrating primordium on course via this attractive activity. The issue of how SDF-1 proteins are actually distributed in embryos, however, is unclear. SDF-1 is highly charged and known to bind glycosaminoglycans (Amara et al., 1999; Sadir et al., 2001) and thus presumably to the extracellular matrix which should act to limit the diffusion of the ligand and set up gradients of the ligand within close proximity to cells that secrete the ligand. When SDF-1a was knocked down, the primordium or lateral line axons sometimes did migrate tens of microns towards ventral sources of SDF-1b or migrate towards cells expressing ectopic SDF-1b, suggesting that SDF-1s can diffuse some distance within embryos. How endogenous SDF-1 proteins are distributed within zebrafish embryos, however, is unknown and awaits the identification of suitable antibodies.

The finding that exogenous SDF-1b can attract the primordium while endogenous SDF-1a regulates migration by the primordium suggests the hypothesis that SDF-1a and SDF-1b have similar activities and can functionally substitute for each other. The high level of homology between the two SDF-1s is certainly consistent with their putative functional similarity (data not shown). One corollary of their putative functional similarity is that ubiquitous misexpression of SDF-1b should be similar to that of SDF-1a. The fact that ubiquitous misexpression of SDF-1b and loss of function of SDF-1a have similar effects on migration by the primordium suggests that overexpression of SDF-1b could lead to massive desensitization of the CXCR4b receptors on the primordium cells and thus mimics a loss-of-function phenotype. In fact, the CXCR4 receptor is well known to desensitize and become internalized upon activation by agonists (Haribabu et al., 1997; Orsini et al., 2000). Alternatively, ubiquitous misexpression of exogenous SDF-1b may obscure the pathway demarcated by endogenous expression of SDF-1a by the horizontal myoseptum. If overexpression of SDF-1b leads to complete desensitization of the CXCR4b receptor, then one might expect that misrouting by the lateral line nerve or primordium should occur equally in the dorsal and ventral directions as was the case with the CXCR4b knockdowns. If overexpression leads to masking of the normal pathway and increased confusion of the primordium, then one might expect that there might be a ventral bias when misrouting occurs, since overall, there is more SDF-1 expression ventral to the horizontal myoseptum compared with dorsal to it. In fact, we did find that the proportion of misrouted lateral line nerve or primordia was higher in the ventral direction compared with dorsal. Whether this signifies that overexpression leads to masking of the normal pathway and/or to desensitization of the CXCR4b receptor is, however, unclear, since it is unknown whether the overexpression regimes effectively overexpress the ligand throughout the entire duration from time of heat induction to time of assay.

Disruption of SDF-1–CXCR4 signaling always leads to errors in migration of the primordium and pathfinding by the lateral line growth cones at the same time. Potentially, SDF-1–CXCR4 signaling could regulate the primordium and axons independently. Alternatively, SDF-1–CXCR4 signaling could control migration by the primordium, which in turn guides the lateral line growth cones or vice versa. Since the neurons in the posterior ganglion of the lateral line appear not to express *cxcr4a* or *cxcr4b* while the migrating primordium expresses *cxcr4b*, SDF-1 is likely acting on the primordium and not the growth cones. Thus, it appears that pathfinding by the lateral line growth cones is dependent on the migrating primordium. One possible mechanism that links the lateral line growth cones to the primordium might involve the HNK-1 glycoepitope (Becker et al., 2001). The lateral line growth cones express HNK-1, and antibody block of the HNK-1

epitope leads to errors in outgrowth by the lateral line axons but not migration by the primordium. Since the primordium expresses NCAM, it is possible that NCAM–HNK-1 binding may link the axons to the primordium and thus participate in the guidance of the axons by the primordium.

Despite a lack of a direct effect on the lateral line growth cones, SDF-1–CXCR4 signaling can directly regulate other growth cones. SDF-1–CXCR4 mediates repulsion or attraction of cerebellar growth cones in vitro (Xiang et al., 2002) and can interfere with the repulsive activity of Sema 3A, Sema 3C, and Slit-2 in vitro (Chalasani et al., 2003). In addition, ubiquitous misexpression of SDF-1b and knockdown of SDF-1a–CXCR4b signaling also disrupt pathfinding by retinal ganglion axons in zebrafish (data not shown), and knocking out CXCR4 induces errors by some dorsal root ganglion axons within the spinal cord in mice (Chalasani et al., 2003). Thus, it is likely that SDF-1 regulates pathfinding by a direct chemoattractant or chemorepellant activity on growth cones or via interference of repellants in vivo as well as guide migration by neurons and sensory cells.

Our results strongly suggest that SDF-1–CXCR4 signaling regulates migration by the lateral line primordium and that both SDF-1a and SDF-1b can act as a chemoattractant for the primordium. However, the horizontal myoseptum is not the only source of SDF-1 in the zebrafish trunk. The pronephros expresses *sdf-1a* (David et al., 2002) and *sdf-1b* and the dorsal aorta expresses *sdf-1b*. Given this situation, one wonders whether SDF-1a derived from the horizontal myoseptum is sufficient to keep the primordium on target. In fact, in most of the *sdf-1a* and *cxcr4b* morphants and all of the *ody* mutants, the primordium stalled but did not go off course. This suggests that other molecules may act in concert with SDF-1a to guide the primordium along the horizontal myoseptum. Two molecules that may also guide the primordium are Netrin-1a and Sema3A1. Netrin-1a, a putative chemoattractive molecule, is expressed by the muscle pioneers and the adaxial muscles including those at the horizontal myoseptum (Lauderdale et al., 1998), and Sema3A1, a repulsive molecule, is expressed by the axial muscles dorsal and ventral to the horizontal myoseptal region and so could restrict the primordium to the horizontal myoseptum (Shoji et al., 1998). Thus, it is possible that several molecular signaling systems may act in concert to insure correct migration of the lateral line primordium and associated axons.

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