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Control of Chemokine-Guided Cell Migration by Ligand Sequestration

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SUMMARY

Primordial germ cell (PGC) migration in zebrafish is directed by the chemokine SDF-1a that activates its receptor CXCR4b. Little is known about the molecular mechanisms controlling the distribution of this chemoattractant in vivo. We demonstrate that the activity of a second SDF-1/CXCL12 receptor, CXCR7, is crucial for proper migration of PGCs toward their targets. We show that CXCR7 functions primarily in the somatic environment rather than within the migrating cells. In CXCR7 knocked-down embryos, the PGCs exhibit a phenotype that signifies defects in SDF-1a gradient formation as the cells fail to polarize effectively and to migrate toward their targets. Indeed, somatic cells expressing CXCR7 show enhanced internalization of the chemokine suggesting that CXCR7 acts as a sink for SDF-1a, thus allowing the dynamic changes in the transcription of *sdf-1a* to be mirrored by similar dynamics at the protein level.

INTRODUCTION

The generation of positional information during development and adult life is crucial for processes ranging from global patterning of the embryo to building of tissues and organs and their maintenance. Positional information is often established by factors that spread from a defined source, thereby providing the basis for differential cellular response along a gradient. The graded distribution of molecules can dictate different fates in response to the signal level at the position where the responding cells reside (e.g., (Driever and Nüsslein-Volhard, 1988; Ferguson and Anderson, 1992; Nellen et al., 1996)). Alternatively, cells can respond to the distribution of signaling molecules in the environment by migration directed toward or away from the source of an attractant or a repellent, respectively (e.g., (Berg, 1975; Charest and Firtel, 2006; Eisenbach and Giojalas, 2006; Franca-Koh et al., 2006; Raz and Reichman-Fried, 2006; Renault and Lehmann, 2006).

A particularly challenging task is controlling cell migration during early development, a phase during which a large-scale reorganization of embryonic structures results in displacement of sources of guidance cues as well as tissues that serve as substrate on which cells migrate. Determining the strategies that allow cells to reach their target during these stages is thus a major biological question, the answer to which is likely to shed light on the mechanisms governing directed cell migration under less demanding conditions at other time points in the life of the organism.

A useful model for studying guided migration in vivo during early embryogenesis is that of primordial germ cells (PGCs), cells that typically migrate from their site of specification to the position where the gonad develops where they differentiate into gametes (reviewed in Kunwar et al., 2006). PGC migration in zebrafish is well understood, particularly since the guidance cue (the chemokine SDF-1a [Doitsidou et al., 2002]) and its receptor (CXCR4b [Doitsidou et al., 2002; Knaut et al., 2003]) are known. Specifically, the migration route of the PGCs is tightly correlated with dynamic changes in the mRNA expression pattern of the chemoattractant SDF-1a (Doitsidou et al., 2002; Reichman-Fried et al., 2004).

The demonstration that CXCR7 is a receptor for SDF-1 (Balabanian et al., 2005; Burns et al., 2006) ended the seemingly monogamous relationships between this chemokine and its first-to-be-identified receptor CXCR4. In vitro studies aimed at determining the function of CXCR7 yielded conflicting results. Whereas one study suggested that CXCR7 activation promotes cell migration (Balabanian et al., 2005), a more recent study argues that activation of this receptor is not involved in migration. According to the latter work, CXCR7 does not induce calcium mobilization, which normally characterizes the biochemical response to chemokine binding (Burns et al., 2006). Last, in agreement with the results presented above, both CXCR7 ligands, CXCL11 and CXCL12, failed to induce calcium signaling, nor could they promote phosphorylation of either ERK1/2 or Akt/PKB (Proost et al., 2007).

Irrespective of its biochemical activity, CXCR7 function was found to be essential for proper migration of the posterior lateral line primordium, proving that this receptor indeed participates in controlling cell migration in vivo (Dambly-Chaudière et al., 2007; Valentin et al., 2007). Yet, the precise contribution of the receptor to the control of guided cell migration is not known. To determine the role CXCR7 plays in vivo, we examined its function in the context of zebrafish PGC migration.

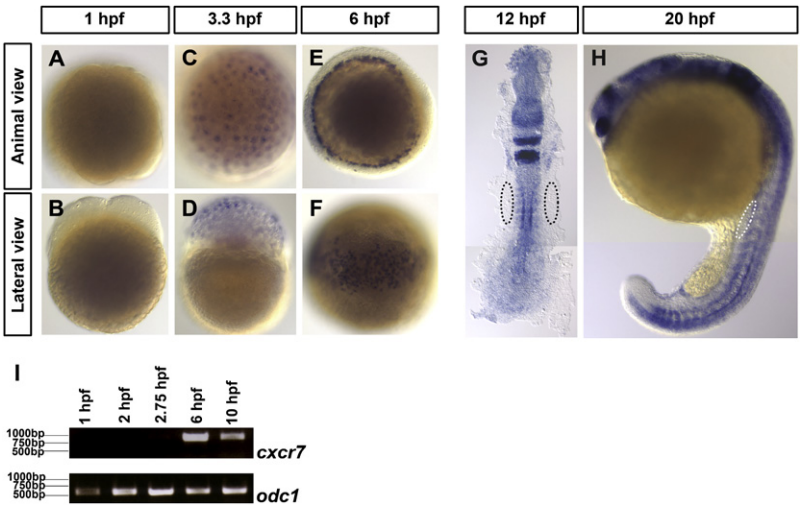

Figure 1. Expression Pattern of *cxcr7*

(A–H) Distribution of *cxcr7* mRNA in wild-type embryos during the first 20 hr of development. In situ hybridization using a *cxcr7*-specific probe shows no staining in four-cell stage embryos (A and B), weak uniform expression at 3.3 hpf (C and D), and enhanced *cxcr7* expression in a ring of deep cells at 6 hpf (E and F). At later stages of development (G and H), uniform *cxcr7* expression with enhanced expression in mesoderm derivatives and in the nervous system is detected, but no expression is observed at the region where the PGCs are located (encircled domains).

(I) Absence of maternally provided *cxcr7* mRNA as determined by RT-PCR at the indicated stages. Control reactions are presented in which primers specific for the maternally provided *ornithine decarboxylase1* (*odc1*) mRNA were used.

In this work we show that in embryos in which CXCR7 function is compromised, PGCs exhibit strongly impaired cell polarity and faulty migration. Strikingly, unlike the other SDF-1a receptor, CXCR4b, whose function is required within the migrating cells, CXCR7 function is exerted primarily in somatic cells. We provide evidence suggesting that CXCR7 effectively reduces the level of SDF-1a available for PGCs by binding and internalizing the chemokine and thus serves to sequester it. In this way, CXCR7 lowers the level of the chemoattractant in the environment, thereby permitting rapid dynamic changes in the distribution of the protein in response to alterations in the transcription pattern of *sdf-1a*.

RESULTS

cxcr7 Is Expressed in Zebrafish Embryos during the Time When PGCs Migrate toward Their Targets

Zebrafish PGCs initiate their active migration toward SDF-1a at 4.5 hr postfertilization (hpf) (Blaser et al., 2005) and reach their final target within the next 15 hr (Weidinger et al., 1999, 2002). In situ hybridization and RT-PCR revealed that *cxcr7* RNA is not provided maternally (Figures 1A, 1B, and 1I) and is transcribed throughout the embryo just before the onset of PGC migration (3.3 hpf, Figures 1C and 1D). During gastrulation stages, the receptor is expressed in a ring of deep cells (6 hpf, Figures 1E and 1F), followed by low-level expression and uniform distribution along with stronger expression in specific structures in the nervous system and in mesodermal derivatives (Figures 1G and 1H). Unlike the distinct expression of *cxcr4b* in PGCs (Doitsidou et al., 2002), we did not observe specific prominent expression of *cxcr7* in migrating PGCs. Thus, while it is possible that *cxcr7* RNA is expressed in PGCs during early stages of development when its expression domain overlaps the location of the PGCs, it is not detectable in the germ cells at later stages (Figures 1G and 1H, encircled domains).

CXCR7 Function Is Essential for Proper PGC Migration

To determine whether CXCR7 plays a role in the migration of PGCs, we have knocked down its activity using morpholino antisense oligonucleotides (Nasevicius and Ekker, 2000) (Figure S1). Strikingly, PGC migration was severely affected in embryos compromised for the receptor function. The CXCR7 loss-of-function phenotype is reminiscent of that of CXCR4b-deficient embryos such that by the end of the first day of development, PGCs failed to form cell clusters at the region where the gonad develops (Figures 2A–2C). This phenotype is likely to reflect defects in cell migration rather than an adverse effect on cell differentiation or survival as the PGCs in manipulated embryos show normal expression of various RNA markers such as *nanos*1 and *h1m* (Köprunner et al., 2001; Müller et al., 2002) (Figure 2C and data not shown). In addition, PGCs in treated embryos were able to protect and translate specific mRNAs (Köprunner et al., 2001) (Figure 2F), formed the typical perinuclear granules (Wolke et al., 2002) (Figure 2F), and exhibited normal proliferation (Figure 2I). Importantly, by reducing the activity of CXCR7, the distribution of *sdf-1a* transcripts was not altered, but the PGCs appeared to be uncharacteristically located outside of the RNA expression domains of the chemokine (Figures 2G and 2H).

To verify that the observed PGC migration phenotype indeed resulted specifically from knockdown of CXCR7 function, we have injected the antisense oligonucleotides, while uniformly co-expressing *cxcr7* mRNA mutated in a way that rendered it resistant to the inhibition. The introduction of *cxcr7* mRNA into embryos by injection could not mimic the endogenous expression pattern or level of the receptor and therefore did not result in a complete suppression of the phenotype. Nevertheless, this treatment led to a dramatic reduction in the severity of the phenotype, demonstrating that the CXCR7 function is required for proper migration of the PGCs (Figures 2K–2L).

CXCR7 Function Is Required in Somatic Cells

The finding that *cxcr7* is not specifically expressed in the PGCs, coupled with the fact that the severity of the knockdown phenotype is significantly reduced by uniform expression of the receptor in the embryo, could indicate that the receptor function is required in the somatic environment rather than in the PGCs themselves. To examine this possibility, we transplanted PGCs

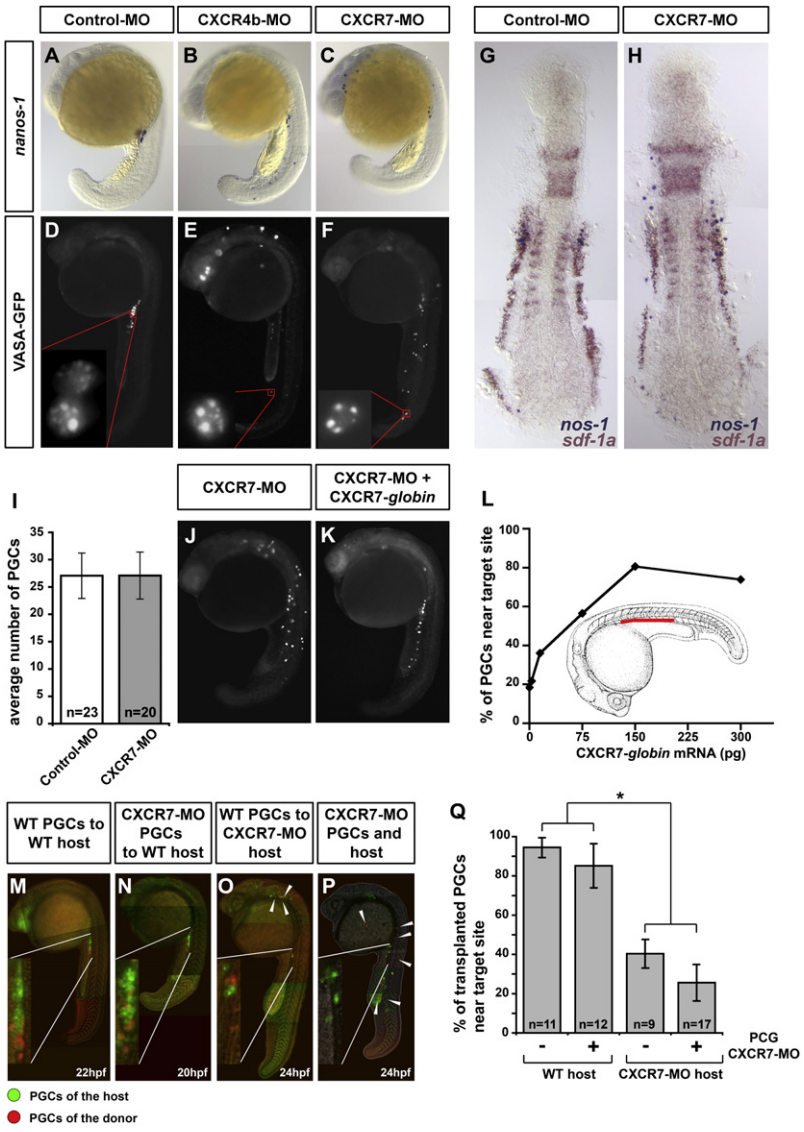

Figure 2. CXCR7 Is Essential for Normal PGC Migration and Is Required in the Somatic Environment of the Embryo

(A–C) Reduction of CXCR7 activity leads to aberrant PGC migration as demonstrated by in situ hybridization using a germ cell-specific *nanos1* probe. PGCs in control embryos cluster at the region where the gonad develops after 20 hr of development (A). Similar to reduction of CXCR4b activity (B), knockdown of CXCR7 results in a pronounced germ cell migration phenotype (C).

(D–F) CXCR7 knockdown does not affect PGC specification. Images of 22 hpf embryos injected with Vasa-GFP-*nos1*-3′UTR mRNA are shown. Germ cell-specific mRNA protection and proper localization of the Vasa-GFP fusion protein to germinal granules is observed in embryos injected with control morpholino (D), CXCR4b morpholino (E), and CXCR7 morpholino (F).

(G and H) General embryonic patterning and expression of *sdf-1a* are not affected by CXCR7 knockdown. Two-color in situ hybridization using *nanos1* (blue) and *sdf-1a* probes (red) of embryos injected with control (G) or CXCR7 (H) morpholino is shown.

(I) CXCR7 knockdown does not affect PGC number as counted at 12 hpf. n signifies the number of embryos examined. Error bars represent standard error of the mean (SEM).

(J–L) The effect of CXCR7 morpholino on PGC migration is reversed by CXCR7 expression. The severe migration phenotype induced by the CXCR7 antisense oligonucleotide (J) is reversed by global expression of RNA encoding CXCR7 (K). A graph demonstrating the dose-dependent rescue of the CXCR7 morpholino-induced phenotype by global expression of CXCR7 (L). For all injections the total amount of injected mRNA was identical (300 pg) by addition of control mRNA (mCherry-F-*globin* mRNA). The red bar indicates the correct target for the migrating PGCs.

(M–Q) PGC migration depends on the activity of CXCR7 in somatic tissues. PGCs expressing DsRedExpress (red) were transplanted into embryos with PGCs expressing EGFP-F (green). Wild-type and CXCR7 knocked-down PGCs arrived at the region of the gonad in wild-type hosts (M and N). A large proportion of wild-type and CXCR7-depleted PGCs does not arrive at the correct target in CXCR7-depleted host embryos (O and P, arrowheads). In (Q), the percent of transplanted PGCs reaching their target after the first day of development is shown. PGCs in CXCR7-depleted hosts embryos show a significant reduction of migration fidelity as compared to PGCs in wild-type hosts ($p < 0.001$, t test, marked with an asterisk). PGCs deficient for CXCR7 do not show a significant difference in arriving at the target as compared to control PGCs ($p > 0.17$, t test). n signifies number of embryos examined. Error bars represent SEM.

from embryos knocked down for CXCR7 into wild-type embryos and compared their ability to reach the target with that of wild-type cells transplanted into CXCR7-depleted embryos. As shown in Figures 2M–2Q, the ability of PGCs to reach their target depends on CXCR7 function in somatic cells. Specifically, PGCs transplanted into a wild-type somatic environment exhibited a high rate of arrival at the target (Figures 2M, 2N, and 2Q). In contrast, most of the PGCs transplanted into a CXCR7-depleted environment failed to reach the region where the gonad develops (Figures 2O, 2P, and 2Q). Importantly, CXCR7 knockdown within PGCs (Figures 2N, 2P, and 2Q) did not significantly affect the efficiency with which they arrived at their target ($p > 0.17$, two-sided t test). Together, these findings support the idea that CXCR7 function is required in somatic cells rather than in the PGCs.

CXCR7 Promotes Internalization of SDF-1a and Clearing of the Chemokine from the Extracellular Space

To determine the role CXCR7 plays in somatic cells, we have studied the subcellular localization of the protein and compared it with that of CXCR4b and SDF-1a. To this end, we have tagged these molecules with fluorescent proteins without affecting their normal activity (see Minina et al., 2007 for CXCR4b, Figure S2 for CXCR7, and Movie S1 for SDF-1a) and followed their distribution within the cells. Interestingly, we found that in contrast to CXCR4b that is largely localized to the plasma membrane (Figure 3A), CXCR7 is enriched in intracellular structures (Figure 3B). These findings raised the possibility that CXCR7 binds SDF-1a and, as a result of internalization, sequesters the chemokine in the cell. CXCR7 could thus affect the shape of

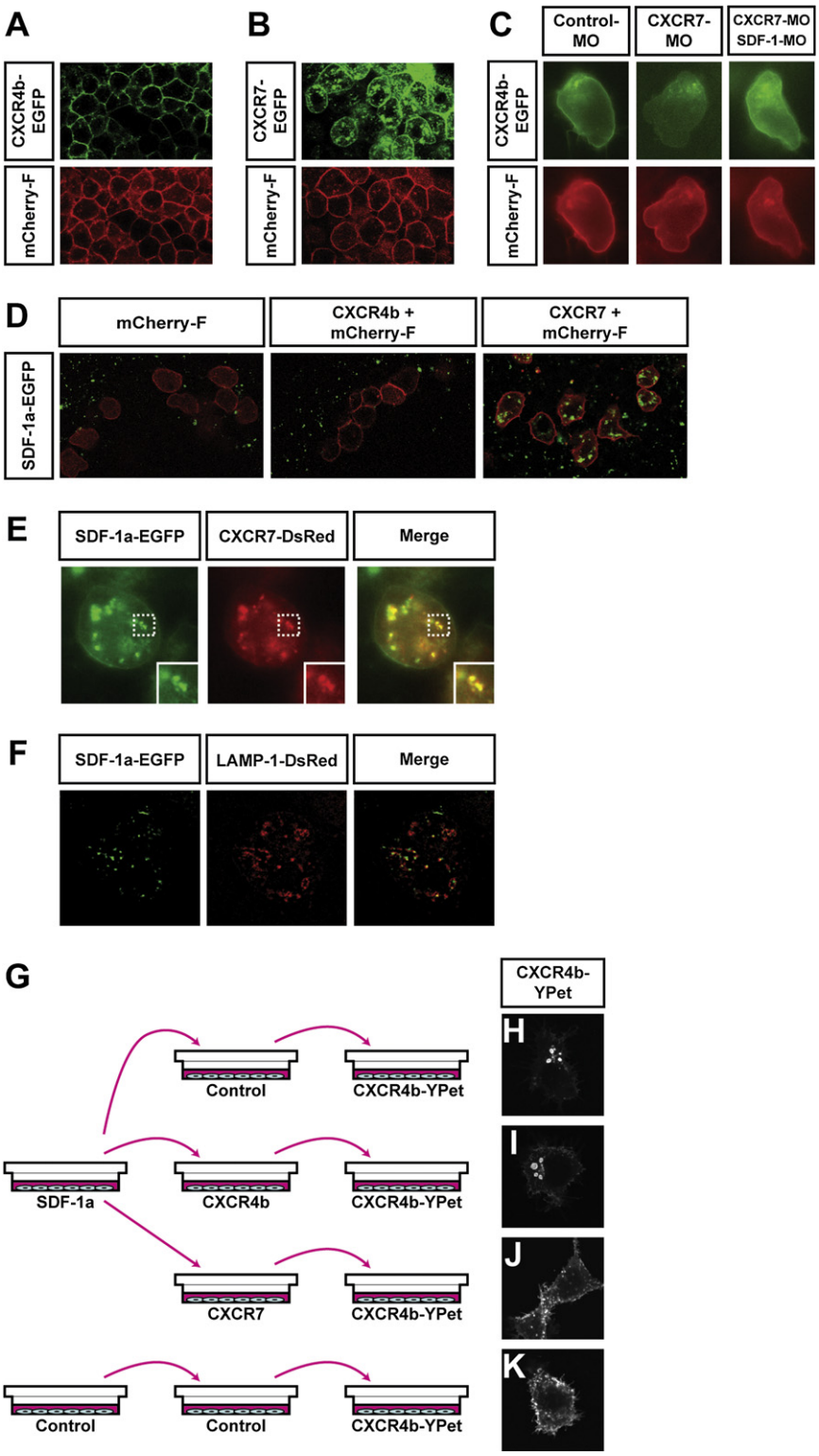

Figure 3. CXCR7 Is an SDF-1a Receptor that Promotes the Internalization of the Chemokine

(A and B) Subcellular localization of CXCR4b and CXCR7 (green) in somatic cells of the embryo. CXCR4b (green) is predominantly found on the membrane of cells (red label of farnesylated mCherry) (A), while CXCR7 (green) is found on the plasma membrane and intracellularly (B).

(C) CXCR7 knockdown increases extracellular SDF-1a levels as judged by internalization of CXCR4b in PGCs. In control embryos (left panel), CXCR4b (green) localizes to the plasma membrane of PGCs (red). CXCR7 knockdown leads to a reduction of CXCR4b on the membrane (middle panel). Membrane localization of CXCR4b in CXCR7 knockdown embryos is restored by SDF-1 knockdown (right panel).

(D) SDF-1a is internalized by CXCR7-expressing cells. Somatic cells (red membrane) expressing CXCR7, CXCR4b, or a control protein were transplanted into host embryos that globally expressed SDF-1a-EGFP. Confocal images were taken 1 hr after transplantation. Transplanted cells (red) expressing either control protein or CXCR4b (left and middle panel, respectively) do not show uptake of SDF-1a (green). In contrast, cells expressing CXCR7 showed intracellular accumulations of SDF-1a protein (right panel).

(E) SDF-1a and CXCR7 colocalize in vesicular structures. Images were taken 1 hr after transplantation of cells expressing CXCR7-DsRedMonomer into SDF-1a-EGFP-expressing hosts. The inset shows a magnification of the dotted box.

(F) SDF-1a accumulates in lysosomes upon CXCR7-mediated internalization. Deconvoluted images were taken 1 hr after transplantation of cells expressing untagged CXCR7 and the lysosomal marker LAMP-1 fused to DsRedMonomer into SDF-1a-EGFP-expressing host embryos.

(G–K) CXCR7-expressing cells reduce extracellular SDF-1a levels. In (G) is a graphic illustration of the experiments designed to examine the depletion of SDF-1a from conditioned medium by CXCR7-expressing cells. The conditioned medium was incubated with cells transfected with the different DNA constructs and subsequently transferred to reporter cultures expressing CXCR4b-EGFP. The extent of CXCR4b-EGFP internalization was then determined. In (H), strong CXCR4b internalization is observed in cells exposed to medium treated with control cells. In (I), medium depleted by CXCR4b-expressing cells induced CXCR4b internalization in 87.5% of all reporter cells, compared to control. CXCR4b internalization was only observed in 56.3% of cells exposed to medium depleted by CXCR7-expressing cells (J). Medium from cells transfected with empty pCDNA3 vector did not induce CXCR4b internalization (K).

the SDF-1a gradient by reducing the level of the chemokine in the extracellular space. We have previously shown that high levels of SDF-1a in the environment trigger the internalization of CXCR4b in germ cells (Minina et al., 2007). Using the subcellular localization of CXCR4b as a measure for the amount of SDF-1a outside of the cells, we determined the distribution of this receptor under conditions where CXCR7 levels were reduced. Indeed, knocking down CXCR7 significantly lowered the level of CXCR4b on the

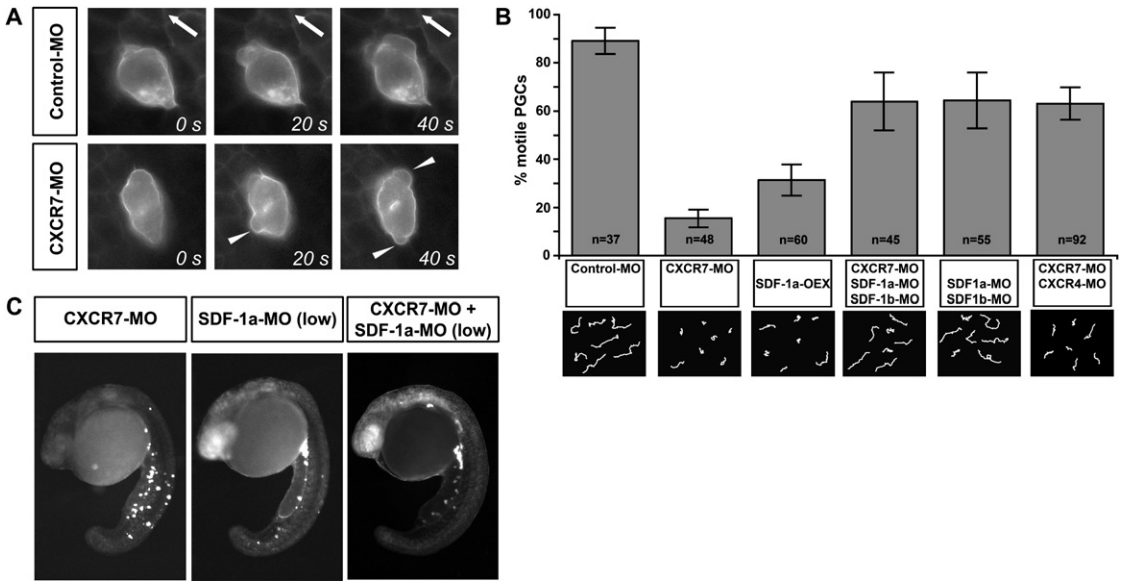

Figure 4. CXCR7 Controls PGC Polarity by Regulating SDF-1 Levels

(A) CXCR7 knockdown reduces the polarity of migrating germ cells. Wild-type PGCs show a typical polarization of the cells with protrusions at the leading edge in the direction of migration (upper panel, arrows). PGCs in CXCR7-depleted embryos exhibit reduced polarity with protrusions extended in opposite directions (lower panel, arrowheads). Cells labeled with EGFP-F.

(B) CXCR7 depletion reduces the motility of PGCs in an SDF-1a-dependent manner. The motility of PGCs was followed in time-lapse movies. Error bars represent SEM. Examples for 70 min long migration paths of germ cells are shown. PGCs in CXCR7 morphants exhibit low motility with short tracks that are reminiscent of PGCs migrating in embryos with high-uniform SDF-1a expression (SDF-1a-OEX). Removal of SDF-1 in CXCR7-depleted embryos restores PGC motility to a level that is similar to that in SDF-1-depleted embryos. Similarly, knocking down CXCR4 restores PGC motility in CXCR7 morphants.

(C) Reduction of SDF-1a expression suppresses the CXCR7 knockdown phenotype. The migration phenotype of embryos knocked down for CXCR7 (left panel, 66.4% ± 3% ectopic cells per embryo, n = 30 embryos) is suppressed by coinjection of low levels (0.02 pmol) of SDF-1a morpholino (middle panel: SDF-1a-MO, 29.4% ± 3% ectopic cells per embryo, n = 22 embryos; right panel: CXCR7-MO and SDF-1a-MO, 36.0% ± 2% ectopic cells per embryo, n = 63 embryos).

plasma membrane (Figure 3C). In addition, introducing antisense oligonucleotides directed against *sdf*-1 reduced the level of CXCR4b internalization observed in CXCR7-deficient embryos (Figure 3C, right panel). These findings support the notion that the enhanced CXCR4b internalization in CXCR7 morphants results from higher levels of extracellular SDF-1. To directly address this idea, we followed the fate of GFP-tagged SDF-1a protein when it encounters cells expressing CXCR7 (Figure 3D). Indeed, a marked internalization of SDF-1a by somatic cells expressing CXCR7 was observed (right panel), whereas nontreated cells or cells overexpressing CXCR4b did not exhibit internalization of the tagged SDF-1a (left and middle panels, respectively). Importantly, in a similar experimental setting, we observed strict colocalization of SDF-1a-EGFP and CXCR7-DsRedMonomer (Figure 3E), supporting the idea that SDF-1a internalization is mediated by CXCR7. To examine the fate of the internalized SDF-1a protein, we compared the localization of the protein with that of the lysosomal marker LAMP-1. For this purpose we transplanted cells expressing CXCR7 and DsRed-tagged LAMP-1 into SDF-1a-EGFP-expressing hosts and detected SDF-1a accumulation in many of the labeled lysosomes (Figure 3F). Similar results were obtained when the lysosomes were labeled using the LysoTracker reagent (Figure S3).

These results suggest that CXCR7 could reduce the time the chemokine is present in the extracellular space, thus permitting the formation of a gradient as well as dynamic alterations in the distribution of the molecule during development. To test this hypothesis more directly, we have assayed the potency of cells expressing CXCR7 in depleting SDF-1a from their environment. In this experiment, SDF-1a-conditioned medium was incubated with human cells expressing zebrafish CXCR7, and the activity level in inducing CXCR4b internalization served as a measure for the remaining amount of the chemokine. Indeed, cells expressing CXCR7 effectively depleted SDF-1a from the medium as compared with the cells transfected with an empty vector or cells expressing CXCR4b (Figures 3G–3K).

CXCR7 Function Is Important for Cell Polarity and Migration

The results presented above suggest that CXCR7 reduces the level of SDF-1a in the embryo allowing proper generation of the chemokine gradient. Detailed morphological analysis of PGCs migrating within the CXCR7-depleted environment strongly supports this idea. Specifically, in contrast with their morphology in wild-type embryos (Figure 4A, upper panels, and Movie S2), PGCs in CXCR7 knocked-down embryos appear less polarized, such that protrusion formation is not focused at the leading edge of the cell, but rather could be observed simultaneously at two opposite sides of the cell (Figure 4A, lower panels, and Movie S3). Further evidence for the reduced polarity

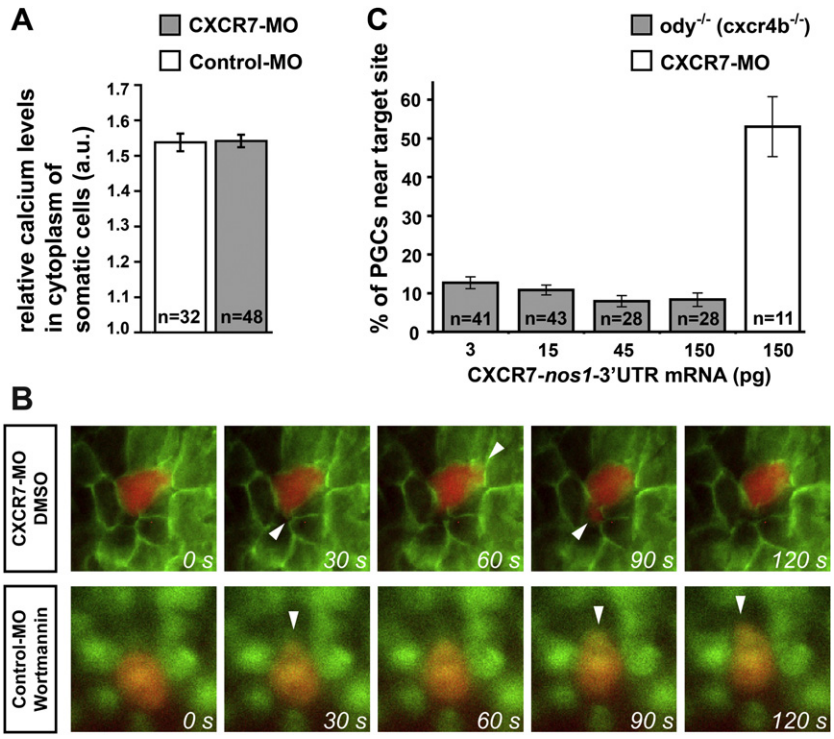

Figure 5. CXCR7 Does Not Activate Major Pathways Downstream to Chemokine Signaling

(A) CXCR7 depletion does not alter calcium levels in the cytosol of somatic cells in control and CXCR7-depleted embryos ($p > 0.1$, t test). n signifies the number of cells examined. Error bars represent SEM. a.u., arbitrary units.

(B) CXCR7 knockdown phenotype is not caused by absence of PI3K function. Shown are PGCs expressing DsRed (red) migrating in embryos globally expressing Akt-PH-EGFP. Migration was monitored in CXCR7-depleted embryos and compared with the migration of PGCs in embryos in which PI3K was inhibited. In CXCR7-depleted embryos PGCs display multiple protrusions in opposing directions (upper panel, arrowheads), typical of CXCR7 inhibition. By contrast, PGCs treated with the selective PI3K inhibitor Wortmannin (25 μM) are polar and migrate with the protrusions, forming in the direction of migration (lower panel, arrowheads). Effective inhibition of PI3K function was monitored by the localization of Akt-PH-EGFP. In DMSO-treated embryos (upper panel), the PH domain localizes to the plasma membrane, whereas PI3K inhibition by Wortmannin induces translocation of the sensor to the cytosol (lower panel). Movies of control cells not treated with the drug and cells in CXCR7-depleted embryos treated with Wortmannin are provided in Movies S8 and S9, respectively.

(C) Germ cell-specific expression of CXCR7 does not substitute for CXCR4b function. CXCR7 expression does not revert the effect of CXCR4b-deficient fish (gray bars), but rescues CXCR7 morpholino-treated embryos (white bar). n signifies the number of embryos examined. Error bars represent SEM.

of PGCs in CXCR7-depleted embryos was obtained by interfering with calcium polarity in the cells. Expressing an activated form of the STIM1 protein in PGCs elevates calcium levels in the rear of migrating cells and challenges their calcium polarity (Blaser et al., 2006). Whereas wild-type PGCs expressing mutated STIM1 are still able to polarize and migrate (Movie S4) (Blaser et al., 2006), a similar manipulation in CXCR7 knocked-down embryos resulted in a dramatic loss of PGC morphological polarity and motility (Movie S5). We consider this finding a further indication for the reduced polarity of PGCs migrating in manipulated embryos.

To validate the notion that the basis for the phenotype of CXCR7 knockdown lies with higher levels of SDF-1 in the environment, we examined PGC behavior in manipulated embryos. PGCs in CXCR7 knocked-down embryos exhibited strong inhibition of motility manifested in short migration tracks (Figure 4B). This phenotype could be mimicked by global SDF-1a expression in otherwise wild-type embryos (Figure 4B). A striking reversal of the CXCR7 knockdown phenotype was observed when CXCR7 and both ligands (SDF-1a and SDF-1b) were simultaneously knocked down; experimental cells exhibited motility similar to cells lacking the guidance cue (Figure 4B) (Doitsidou et al., 2002; Reichman-Fried et al., 2004). Suppression of the CXCR7 phenotype was similarly achieved by concomitant knockdown of CXCR4b (Figure 4B). Although PGCs in these experiments regained motility, they were nevertheless dispersed throughout the embryos (data not shown), since by knocking down CXCR4b or SDF-1 (along with CXCR7), the guidance signal was eliminated. We reasoned that a mild reduction in SDF-1a level might permit cell motility in embryos lacking CXCR7, while preserving the function of the chemokine as a guidance cue. In such a case, one would predict that treating CXCR7 morphants with low levels of *sdf-1a* morpholino should allow many PGCs to reach their target. Indeed, such a manipulation reduced the severity of the migration phenotype observed in 24 hpf embryos (Figure 4C).

To determine whether the effect of CXCR7 on SDF-1a distribution is accompanied by signaling through the ligand-bound receptor, we have tested the possible involvement of two key pathways acting downstream of chemokine receptors, namely elevation in calcium levels and PI3K activation (e.g., Andrews et al., 2007; Blaser et al., 2006; Bleul et al., 1996; Sotsios et al., 1999; Vicente-Manzanares et al., 1999). We found that CXCR7 knockdown had no effect on the level of calcium in somatic cells (Figure 5A). In addition, in embryos in which PI3K activity was inhibited using Wortmannin (as evident by membrane-to-cytoplasm translocation of Akt-PH-EGFP), PGCs maintained their polarity and migrated actively, unlike PGCs in CXCR7 knocked-down embryos, which exhibit defects in motility and cell polarity (Figure 5B and Movies S6 and S7). Last, despite the apparently high-binding affinity of CXCR7 to SDF-1 in the mammalian system (Balabanian et al., 2005), the zebrafish CXCR7 cannot substitute for CXCR4b. Specifically, preferential expression of CXCR7 in PGCs did not suppress the CXCR4b knockdown phenotype (Figure 5C). Consistent with previous findings (Burns et al., 2006; Proost et al., 2007), our results support the idea that at least in the context of PGC migration in zebrafish, CXCR7 is a silent receptor that does not signal.

CXCR7 Affects the Direction of PGC Migration In Vivo

To demonstrate that somatically expressed CXCR7 affects the distribution of SDF-1a in the tissues within which the PGCs migrate, we have generated embryos in which SDF-1a was uniformly expressed and superimposed an uneven distribution of CXCR7 (Figure 6A). We found that the PGCs were preferentially located within domains lacking CXCR7, suggesting that the receptor affected the local SDF-1a concentration, promoting PGC migration toward regions where higher levels of SDF-1a are present (Figure 6B). To visualize the dynamic response of the PGCs to cells expressing CXCR7, we have examined the effect of CXCR7-expressing cells on the migration of PGCs toward an SDF-1a source (Figures 6C and 6D and Movies S10 and S11). PGCs rapidly and effectively migrated toward cells expressing the chemokine, while ignoring control cells transplanted on their way to the source (Figures 6C, upper panels, and 6D, left panel). In a striking contrast, transplantation of CXCR7-expressing cells (red cells in Figure 6C) between the PGCs and the chemokine source (blue cells in Figure 6C) dramatically affected the migration path of the PGCs such that they rarely crossed the CXCR7 expression field (Figures 6C, lower panels, cells 2 and 3, white tracks; and 6D, right panel). Consistently, expression of high levels of CXCR7 in the germ cells themselves affected their migration, presumably by reducing the effective level of SDF-1a around the cells (Figure S4).

To examine the possibility that CXCR7 plays a similar role in other contexts in embryonic development, we investigated the potential function for the gene in the nervous system, where it exhibits a dynamic expression pattern. At 12 hpf *cxcr7* is most prominently expressed in two broad stripes close to the head-trunk border (Figure 1G). Interestingly, the posterior *cxcr7* stripe overlaps with *sdf-1*a, while the anterior *cxcr7* stripe shows a largely complementary pattern with respect to *sdf-1a* (Figure 6E, upper panel). To determine whether the distribution of SDF-1a is altered in a manner that is consistent with the expression pattern of *cxcr7*, we have examined the exact position of PGCs abnormally found in this location in *spadetail* mutants (Weidinger et al., 1999, 2002). This analysis revealed that PGCs were never found in a region where *cxcr7* was expressed (Figure 6E, lower panel). Accordingly, the cells usually ignored the broad *sdf-1a* mRNA expression that is partially overlapping with that of *cxcr7* mRNA (Figure 6E, large brackets) and would settle in an *sdf-1a*-expressing clustering point away from the anterior *cxcr7* stripe (Figure 6E, small brackets).

Together, the results presented in this section are in agreement with the notion that CXCR7 can shape the SDF-1a gradient, thereby affecting the migration path of PGCs within the embryo. As CXCR7 is expressed in a broad range of tissues, it could function in controlling the distribution of SDF-1 in those locations, thereby regulating processes other than PGC migration.

DISCUSSION

During their migration, zebrafish PGCs arrive at locations where *sdf-1*a RNA is expressed (e.g., Figure 2G) (Blaser et al., 2005; Doitsidou et al., 2002; Reichman-Fried et al., 2004). Dynamic alterations in the expression pattern of *sdf-1a* are followed by a rapid migration response of germ cells to maintain their position in close proximity to tissues that express the RNA encoding the chemokine (Figure 7, left panels) (Reichman-Fried et al., 2004). Two processes could account for the observed tight association of PGCs with *sdf-1a*-transcribing cells. First, the responding cells could be capable of detecting minute differences in the level of the attractant and would therefore continuously migrate to remain within domains of *sdf-1a* transcription, where slightly higher levels of the secreted SDF-1a would be found. In addition to the sensitivity and effective response of PGCs to the signal, processes in the environment could cooperate by controlling the shape of the SDF-1a gradient. For example, continuous clearing of the ligand from somatic tissues would constitute a useful mechanism for achieving migration precision. In this study, we provide evidence consistent with the idea that CXCR7 activity is essential for attaining a distribution of SDF-1a that is capable of polarizing the PGCs and directing their migration toward cells expressing the RNA of the attractant. In contrast to CXCR4b, whose internalization regulates the signaling level of the receptor by removing it from the membrane (Minina et al., 2007), CXCR7 regulates the signaling level of CXCR4b by reducing the level of SDF-1a in the extracellular environment. In the absence of CXCR7 activity, an increase in the absolute level of SDF-1a and a decrease in gradient steepness would thus interfere with proper directed migration despite the correct RNA expression pattern (Figure 7, right panels). Whereas anti-SDF-1a antibodies are currently not available, such a reagent would provide interesting insights into the precise effect of CXCR7 loss of function on the distribution of the chemoattractant in the embryo.

While the molecular details differ, a mechanism for controlling cell migration reminiscent to the one proposed here has been suggested to account for guidance of germ cell migration in *Drosophila* (Renault and Lehmann, 2006; Renault et al., 2004). *Drosophila* PGCs are thought to migrate along a gradient of lipid phosphate that acts as a chemoattractant. Analogous to sequestration of SDF-1a, dephosphorylation of the lipid phosphate in specific somatic tissues of the *Drosophila* embryo renders those regions repulsive, thereby directing the migration of the cells toward their target.

The process in which zebrafish PGCs effectively avoid domains where the attractive molecule ceases to be expressed is reminiscent of that observed in the resolution of inflammatory response. In this case, tissue homeostasis depends on migration of cells that were originally attracted to the site of an inflammatory stimulus, away from that location. Different mechanisms that promote clearing of the attractive signal were identified, and these allow efficient resolution and reduction of tissue damage (reviewed in Hansell and Nibbs, 2007; Mantovani et al., 2006; Serhan and Savill, 2005). Of particular relevance for this study is the chemokine depletion without signaling that was proposed to account for the function of the antiinflammatory cytokine IL-10 (D'Amico et al., 2000). In this case, it has been shown that IL-10 maintains the expression of inflammatory chemokine receptors (CCR1, CCR2, and CCR5) on mature dendritic cells (DCs) that act as molecular sinks for the proinflammatory chemokines CCL3 and CCL5. The receptors on the DCs function exclusively in sequestering the chemokine, as they do not induce signaling or chemotaxis. Similarly, CCL3 and CCL5 clearing during the

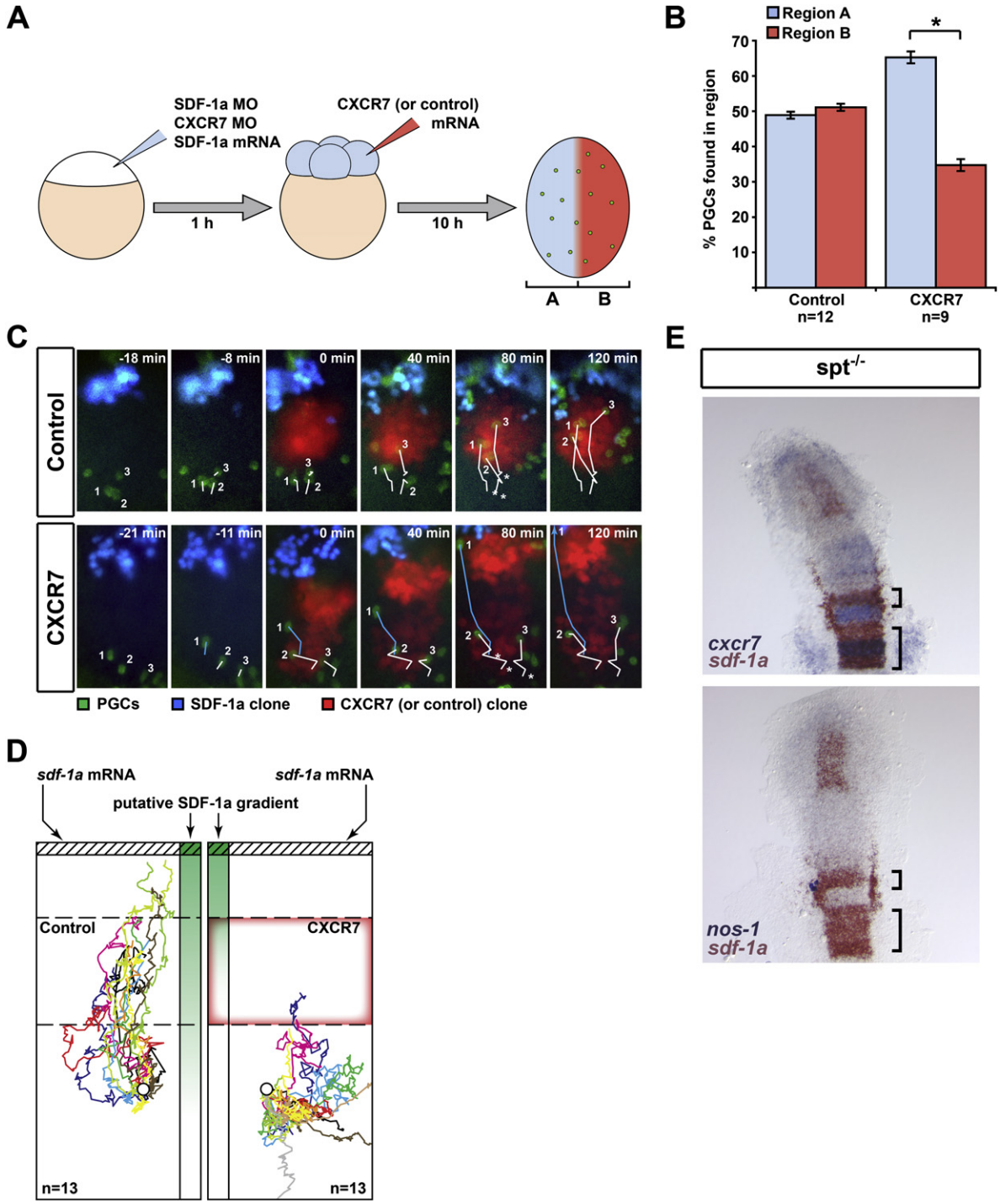

Figure 6. CXCR7 Affects the Direction of Germ Cell Migration In Vivo

(A) A schematic representation of the experimental manipulations generating a CXCR7 expression domain (red, Region A) superimposed on uniform SDF-1a expression (blue, Region B). PGCs are depicted in green.

(B) In contrast to control experiments, PGCs vacated the CXCR7-expressing B region (p value < 0.001, t test). n signifies the number of embryos examined, and error bars represent SEM.

(C) Snapshots of representative time-lapse movies with germ cells (green) migrating toward a transplanted source of SDF-1a (blue) in SDF-1-deficient embryos. A transplant of cells (red) expressing either CXCR7 or control protein was placed at the migration path. In control experiments (upper panel), germ cells (white tracks labeled 1–3) readily traverse the transplant toward the source of SDF-1a. Asterisks denote the starting points. When encountering a CXCR7-expressing transplant

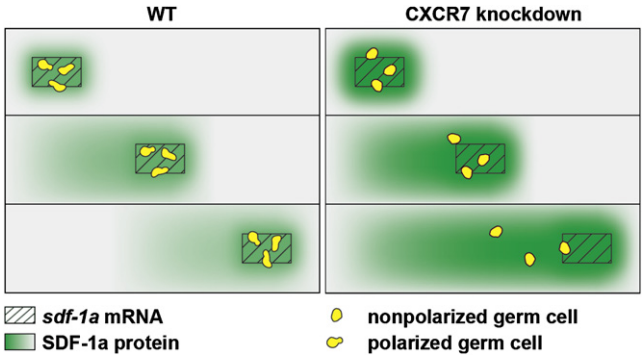

Figure 7. A Model for the Role of CXCR7 in PGC Migration

Morphogenetic movements and changes in expression pattern cause dynamic shifts of *sdf-1a* expression sites (hatched box). CXCR7-mediated removal of SDF-1a facilitates the generation of a sharp gradient (green), allowing the PGCs (yellow) to polarize and migrate toward the site of *sdf-1a* transcription (left panel). In the absence of CXCR7 function (right panel), SDF-1a is not cleared efficiently (extended green gradient), resulting in abnormally high SDF-1a levels and inability of germ cells to establish polarity. Consequently, germ cells lose their close association with *sdf-1a* transcription domains.

resolution of peritonitis depends on their receptor CCR5 that sequesters these chemokines in apoptotic leukocytes (Ariel et al., 2006). As SDF-1 has been implicated in rheumatoid arthritis and in acute lung injury inflammatory responses (e.g., De Klerck et al., 2005; Matthys et al., 2001; Nanki et al., 2000; Petty et al., 2007), it would be interesting to examine whether CXCR7 is involved in regulation of inflammation in these tissues. Furthermore, since the SDF-1/CXCR4 pair is involved in other pathological conditions (in particular cancer, e.g., Muller et al. [2001] and Orimo et al. [2005]) and controls a wide range of developmental and homeostatic activities (e.g., Aiuti et al., 1997; Peled et al., 1999; Zou et al., 1998), examining the role of CXCR7 in these processes would be an important avenue for future research.

Whereas our results provide strong evidence that CXCR7 is a nonsignaling receptor that functions as a sink for SDF-1a in the case of PGC migration, it could be that this molecule functions differently in different contexts. For example, the results of Valentin et al. (2007) are compatible with the idea that in the case of the zebrafish lateral-line primordium, CXCR7 activation plays an instructive role in dictating cell behavior in the posterior part of the migrating organ. The question of whether CXCR7 acts as a professional or a part-time decoy receptor would thus require detailed examination of the biochemical and cellular responses in different settings in which this receptor functions.

Although this study reiterates the central role of SDF-1a and CXCR4b in guiding PGC migration, our results highlight the importance of regulation by other molecules. It would therefore be important to examine additional parameters that could influence SDF-1a function in vivo, especially those relevant for its spread within the embryo. Exploring the role of components of the extracellular matrix that are known to bind SDF-1 as well as enzymes modifying SDF-1 in the extracellular environment would be especially informative in this context.

EXPERIMENTAL PROCEDURES

Zebrafish Strains

Fish of the AB background or transgenic fish carrying the Tol-kop-EGFP-F-*nos1*-3′UTR transgene (Blaser et al., 2006) or a similar line with a Tol-kop-DsRedExpress-F-*nos1*-3′UTR transgene served as wild-type fish. The transgenes direct EGFP-F or DsRedExpress expression to the PGCs. *ody*$^{-/-}$ mutant embryos were used to analyze the migration in the absence of CXCR4b function (Knaut et al., 2003).

Cloning and RT-PCR of *cxcr7*

The zebrafish *cxcr7* open reading frame (accession number XM682279) was amplified from midsomitogenesis cDNA and cloned into expression vectors for expression in germ cells (CXCR7-*nos1*-3′UTR) and for global expression (CXCR7-*globin*).

For RT-PCR, total RNA was isolated from 1 hpf, 2 hpf, 2.75 hpf, 6 hpf, and 10 hpf using TRIZOL (Invitrogen) and cDNA was synthesized using oligo(dT) primers. Primer sequences are provided in the supplemental material.

RNA Expression Constructs and Injections

Capped sense mRNA was synthesized using the mMessageMachine kit (Ambion). RNA was microinjected into the yolk of one-cell stage embryos unless stated otherwise.

A description of the constructs used is provided in the Supplemental Data.

Knockdown of CXCR7, CXCR4b, SDF-1a, and SDF-1b

Knockdown of CXCR7 function was achieved by injection of 1.2 pmol CXCR7-MO morpholino antisense oligonucleotides (5′-ATCATTCACGTTCACACTC ATCTTG-3′) into one-cell stage embryos. A second oligonucleotide (5′-GAA ATCATTCACGTTCACACTCATC-3′) also impaired PGC migration, albeit with lesser effectiveness. Knockdown of CXCR4b and both zebrafish SDF-1 homologs was achieved using 0.4 pmol of either oligonucleotides against *cxcr4b*, *sdf-1a* (Doitsidou et al., 2002), and *sdf-1b* (5′-TTGCTATCCATGCCAAGAGCG AGTG-3′). Control experiments were performed using equal concentrations of irrelevant oligonucleotides.

Fluorescence Microscopy

Epifluorescence images were captured using a Zeiss microscope controlled by the Metamorph Software (Visitron Systems). Time-lapse movies were generated for imaging cell morphology and behavior as well as for track analysis. Frames were captured at 5 s or 10 s intervals for high-magnification movies and at 1 min intervals for low-magnification movies.

Confocal fluorescence images were obtained with the Leica TCS SL confocal microscope.

Measurement of Calcium Levels

Calcium measurements were performed as previously described (Blaser et al., 2006). Somatic cell measurements were performed on the cytosol of cells in the vicinity of migrating PGCs.

Germ Cell Transplantation

Germline chimeras were produced by transplantation of PGCs from Tol-*kop*-DsRedExpress-*nos1*-3′UTR transgenic embryos into Tol-*kop*-EGFP-F-*nos1*-

(lower panel), the migration toward the SDF-1a source is inhibited (cells 2 and 3). Cells that do not encounter CXCR7-expressing cells on their migration path (cell 1) are not affected (blue track).

(D) Multiple migration tracks of germ cells encountering a control transplant (dashed box) or a transplant expressing CXCR7 (red box outline). Tracks have been corrected for morphogenetic movements and were given a common starting coordinate (circle) with the SDF-1a transplant positioned to the top (hatched box). The putative SDF-1a gradient drawn in green. n signifies the number of cells examined. Tracks represent 150 min of PGC migration.

(E) Regions expressing *sdf-1a* fail to attract PGCs if the expression overlaps with that of *cxcr7*. Two-color in situ hybridization on 13 hpf *spt*$^{-/-}$ embryos using *cxcr7* (blue) and *sdf-1a* (red) probes (top panel) and *nanos1* (blue) and *sdf-1a* (red) probes (lower panel).

3′UTR transgenics. CXCR7-depleted donor cells were transplanted into a control morpholino-injected host or vice versa. Control experiments were performed by injecting control or CXCR7 morpholino into both donor and host embryos.

Transplanted cells were obtained from the germ ring of 4 hpf donor embryos and transplanted into embryos of the same stage and their location determined in 24 hpf embryos.

In Vitro SDF-1a Internalization Assays

SDF-1a conditioned media was obtained from HEK293 transfected with pCS2-SDF-1a-FLAG plasmid. Forty-eight hours after transfection, the medium was replaced with a serum-free medium, and SDF-1a conditioned media was collected 48 hr later. The presence of SDF-1a in the medium was confirmed by immunoblotting. For depletion experiments, conditioned medium was subjected to two rounds of 30 min incubation on HEK293 cells transfected with either pCDNA3-CXCR7, pCDNA3-CXCR4b, or with an empty pCDNA3 vector. To check for SDF-1a presence in the treated medium, HEK293 cells were transfected with pCDNA3-CXCR4b-YPet, starved for 48 hr, and stimulated with SDF-1a conditioned media or depleted media at 37°C for 30 min. Cells were washed in PBS and fixed in 4% PFA for confocal microscopy. To quantify the percentage of internalization, 100 cells were counted.

In Vivo SDF-1a Internalization Assays

Cells from 4 hpf embryos injected with 150 pg CXCR7-*globin* and mCherry-F-*globin* mRNA were transplanted into 6 hpf host embryos expressing SDF-1a-EGFP. Following 1 hr incubation, confocal microscopy was performed at an elevated pinhole diameter of 250 nm. As controls, cells expressing 150 pg CXCR4b-*globin* and mCherry-F-*globin* mRNA or only mCherry-F-*globin* mRNA were transplanted.

For colocalization studies, cells from donor embryos that were injected either with CXCR7-DsRedMonomer-*globin* mRNA or with CXCR7-*globin* mRNA and LAMP-1-DsRedMonomer-*globin* mRNA were used. High-magnification epifluorescent Z series were obtained and deconvoluted using the Nearest Neighbor method of the Metamorph software suite.

Mosaic CXCR7 Expression in Zebrafish Embryos

Tol-kop-EGFP-F-nos1-3′UTR transgenic embryos were injected with SDF-1a and CXCR7 morpholino, 30 pg morpholino-resistant SDF-1a-*globin*, and zH1m-GFP-*globin* mRNAs to generate embryos with uniform expression of SDF-1a, uniform nuclear labeling and germ cell-specific membrane labeling. At the four-cell stage, one of the blastomers was injected with CXCR7-*globin* and mCherry-F-*globin* (or with mCherry-F-*globin* alone as control) mRNA. At 11 hpf, the position of the PGCs was determined with respect to the red fluorescent domain that signified CXCR7 expression. Embryos containing five or more labeled germ cells were included in the analysis.

In Vivo Attraction Assays

CFP-labeled, SDF-1a-expressing cells from a 4 hpf donor embryos were transplanted into animal positions of 6 hpf Tol-kop-EGFP-F-nos1-3′UTR embryos in which SDF-1a and SDF-1b were knocked down. After the PGCs initiated directional migration toward the SDF-1a secreting transplant, mCherry-F-labeled, CXCR7-expressing cells from 4 hpf embryos (injected with 150 pg CXCR7-*globin* mRNA at the one-cell stage or mCherry-F alone as a control) were transplanted between the migrating PGCs and the SDF-1a transplant. Cell movement was tracked for up to 150 min using the manual tracking plugin of the ImageJ software. Tracks were corrected for morphogenetic cell movements and aligned into one starting coordinate with the SDF-1a-secreting cells positioned upwards.

Supplemental Data

Supplemental Data include 4 figures, 11 movies, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/132/3/463/DC1/.

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