The chemokine Sdf-1 and its receptor Cxcr4 are required for formation of muscle in zebrafish

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Abstract

Background: During development cell migration takes place prior to differentiation of many cell types. The chemokine receptor Cxcr4 and its ligand Sdf1 are implicated in migration of several cell lineages, including appendicular muscles.

Results: We dissected the role of sdf1-cxcr4 during skeletal myogenesis. We demonstrated that the receptor cxcr4a is expressed in the medial-anterior part of somites, suggesting that chemokine signaling plays a role in this region of the somite. Previous reports emphasized co-operation of Sdf1a and Cxcr4b. We found that during early myogenesis Sdf1a co-operates with the second Cxcr4 of zebrafish – Cxcr4a resulting in the commitment of myoblast to form fast muscle. Disrupting this chemokine signal caused a reduction in myoD and myf5 expression and fast fiber formation. In addition, we showed that a dimerization partner of MyoD and Myf5, E12, positively regulates transcription of cxcr4a and sdf1a in contrast to that of Sonic hedgehog, which inhibited these genes through induction of expression of id2.

Conclusion: We revealed a regulatory feedback mechanism between cxcr4a-sdf1a and genes encoding myogenic regulatory factors, which is involved in differentiation of fast myofibers. This demonstrated a role of chemokine signaling during development of skeletal muscles.

Background

Several cell movements are associated with somitogenesis, including the convergence of lateral mesodermal cells into presomatic mesoderm and later its segmentation. During somite epithelialization two types of cells are formed – epithelial border cells and inner mesenchymal cells. As somite matures, presumptive muscle cells start to express characteristic muscle-specific proteins (MSP) and elongate either actively or through fusion to form myofi-
sors migrate from their initial position adjacent to the notochord laterally through the paraxial mesoderm and become the most superficial muscle fibers [4,24]. Specification of most fast muscle in zebrafish does not show obvious signs of lineage-specific cell migration besides being involved in more general events of convergence, mesenchyme-to-epithelial transition (MET) during somite epithelialization followed later on by cell elongation during formation of myofibrils. The fast myofibrils differentiate specifically from the lateral aspect of somites and this process involves relatively short distance migration of prospective myoblasts. These cells express a subset of genes linked to cell migration and at certain A-P levels are capable of undergoing epithelial-mesenchymal transition (EMT), migrate and establish appendicular muscles [reviewed in [25,26]].

Chemokine receptors are members of the superfAMILY of seven-transmembrane domain, G-protein coupled receptors. The CXC chemokine receptor CXCR4 [27,28] is used by HIV-1 for binding to the cell membrane [27,29-31]. SDF-1α [chemokine (C-X-C motif) ligand 12; zebrafish gene nomenclature committee] and its receptor CXCR4 [chemokine (C-X-C motif) receptor 4; zebrafish gene nomenclature committee] bind only each other [32-35]. Importantly, a study of the expression of SDF-1α/CXCR4 in the mouse embryo demonstrated expression of CXCR4 in the presomites [36]. The knockout SDF-1α and CXCR4- mice are the only known chemokine/chemokine receptor mutants that display embryonic lethality [37]. They demonstrate defects of cell migration during formation of the neural tube and heart [38,39]. The Sdf1-Cxcr4 interaction also plays a role during the chemotaxis of primordial germ cells in zebrafish in mice [40-42], and sensory cells in zebrafish [43-47].

Our previous study demonstrated that the zebrafish Cxcr4 is encoded by two related genes expressed in a complex pattern, including somites [48]. Later on, it was shown that homologous genes are expressed in human muscle satellite cells and play a role in cell migration during tongue and limb myogenesis in mice [49-51]. While this suggests a role for Cxcr4 in late myogenesis, a role of Cxcr4 in early somitogenesis still remains to be elucidated. Since the zebrafish mutant of cxcr4b - ody does not show obvious defects in myogenesis [41], we analyzed the second receptor - cxcr4a.

Prior to segmentation in zebrafish, myoblasts initiate expression of myogenic regulatory factors (MRFs) [17] important for skeletal muscle commitment and myotube formation [52]. The highly related bHLH proteins MyoD, Myf5, Myogenin and Mrf4 have a pivotal function in muscle cell specification and differentiation [53-57]. They share a common dimerization domain and DNA binding domain (DBD), the basic helix-loop-helix (bHLH) motif. MRFs regulate myogenesis after forming heterodimers with ubiquitous E proteins. These bind to E box, with core consensus sequence of CANNTG, in the promoter of target genes [58]. How these proteins initiate the program of muscle cell differentiation remains to be deciphered explicitly. Recently, MyoD was shown to have repressive activity in presence of other cofactors [59].

In this report, we described the involvement of Cxcr4a and Sdf1α during formation of fast muscles in zebrafish and provided in vivo evidence of a role of cxcr4a-sdf1a in the regulation of MRFs during myogenic determination. The lack of Cxcr4a-Sdf1α-mediated signaling leads to reduction in expression of somitic markers and decrease in fast myofibrils. The lack of Cxcr4a-Sdf1α also affects migration of slow muscle. This effect could be indirect. In addition, we show that E12 and MyoD-Myf5 regulate cxcr4a. This suggested a possible feedback loop between cxcr4a-sdf1a axis and myoD/myf5. In addition, we discovered that ectopic Hh represses transcription of cxcr4a and sdf1a through a negative regulator of cell differentiation Id2. Taken together, our data connect MRFs and chemokines in a regulatory relationship during early myogenesis.

Results

cxcr4a and sdf1a are expressed in a dynamic manner during formation of fast muscles

Both cxcr4a and cxcr4b are expressed during somitogenesis [48]. To better understand the role of Cxcr4 and Sdf1 in the formation of somitic musculature, we re-evaluated the expression pattern of two sdf1a and two cxcr4 genes. sdf1a and sdf1b were cloned by PCR. As detected by WISH, transcripts of both cxcr4a and cxcr4b cover the newly formed somites almost completely, but the level of expression of cxcr4a is higher than that of cxcr4b (Figures 1A, B; Additional Figures A1A, B (see Additional file 1)) [reviewed in [60]]. The most posterior somite, which is still forming, weakly expresses cxcr4a. The next pair of somites that already formed expresses cxcr4a at a higher level (Figure 1A). As development proceeds, expression of both cxcr4a and cxcr4b become restricted to the anterior half of somite (Figure 1B; Additional Figure A1B (see Additional file 1)). It persists until about 22 h when it becomes restricted to the few posterior somites (data not shown). By end of segmentation, cxcr4a and cxcr4b transcripts are no longer detected by WISH.

Since SDF-1α is the only known ligand of CXCR4 [32,34], we examined the expression pattern of the two zebrafish sdf1 genes. Both sdf1α and sdf1b are expressed maternally (Figure 1L; Additional Figure A1H (see Additional file 1)). The level of sdf1a transcript increases rapidly from the onset of mid-blastula transition (MBT). In contrast, the level of sdf1b transcript increases from fertilization.
During early somitogenesis sdf1a is expressed in the lateral somitic mesoderm (Figure 1E). Later, sdf1a expression is restricted mainly to the posterior part of somite. However, in the anterior most three somites, sdf1a transcripts cover almost the entire somite (Figure 1D). At mid-somitogenesis, only the posterior somites express sdf1a (Figure 1H). During late segmentation the forming and newly-formed somites express sdf1a at low level (Figure 1I). By 24 h, sdf1a expression is no longer detected by WISH (data not shown).
The expression level of sdf1b in the somites is low (Additional Figure A1D (see Additional file 1)). It is first observed in the adaxial cells and later become restricted to the posterior part of somite similar to that of sdf1a. By mid-somitogenesis, sdf1b transcripts are restricted to the dorsal and ventral parts of somites (Additional Figures A1D-G (see Additional file 1)).

To define how cxcr4 or sdf1a are expressed in respect to other markers, we used the two-color WISH. The expression pattern of both cxcr4a and cxcr4b overlaps almost entirely with that of myoD in the forming somite and a few posterior-most somites (cxcr4a), but in more mature somites both cxcr4a are expressed in the anterior part of somite and myoD in the posterior part (Figure 1C; Additional Figure A1C (see Additional file 1)). In contrast to cxcr4a, expression pattern of sdf1a overlaps completely with that of myoD (Figures 1F–G). The expression patterns of cxcr4a and sdf1a are summarized in a diagram (Figures 1J–K). Therefore, cxcr4a and cxcr4b are co-expressed with sdf1a in the forming and newly formed somites, but not in more mature somites. This suggests that the chemokine and its receptor may have both early and late function during myogenesis.

**cxcr4a and sdf1a function is required for formation of fast muscles**

Based on the fact that cxcr4 is expressed during early somitogenesis, we hypothesized that deficiency of Cxcr4 or Sdf1 might affect early myogenesis. To test our hypothesis, we examined somite defects in the mutant odফ/+, which represents a loss of function of Cxcr4b [41]. There was no obvious somitic defect in odফ/+ (Additional Figures A2A-D (see Additional file 2)). This could be due to redundancy of cxcr4b and cxcr4a (Additional Figures A2E-J (see Additional file 2)) [48]. We therefore concentrated our study on Cxcr4a.

Different antisense morpholino oligonucleotides (MOs) designed to target non-overlapping regions of 5'-UTR of both cxcr4a and sdf1a were injected into one to two-cell stage embryos (morphants). The universal control MO and anti-cxcr4a/sdf1a MOs with 4–5 base mutations were injected into embryos used as controls. The morphological analysis or acridine orange staining to detect apoptosis or anti-phosphohistone H3 antibody staining to detect cell proliferation did not show obvious changes in somites of morphants (Additional Figure A3 (see Additional file 3) and data not shown). In contrast, myoD expression in cxcr4 morphants is much reduced in the paraxial cells (Figures 2A, C). Expression of another myogenic bHLH gene, myf5, was similarly affected in somites (Figure 2G; Additional Figures A2K-M (see Additional file 2)). Three MOs that targeted 5'UTR of cxcr4a caused a similar phenotype (data not shown). Taken together, these results suggested that Cxcr4a plays a role in early myogenesis.

Next we decided to evaluate which of the two ligands, Sdf1a or Sdf1b, co-operates with Cxcr4a during myogenesis. In Sdf1a morphants expression of myoD and myf5 was down-regulated in somites (Figures 2D, H). In contrast, overall expression of myoD and myf5 was unaffected in Sdf1b morphants even although in some of them somites were slightly elongated (data not shown). Furthermore, we designed sdf1a-EI-MO which targets the second intron of sdf1a causing missplicing of sdf1a transcripts as confirmed by electrophoresis and sequencing (Additional Figure A4B (see Additional file 4) and data not shown).
The phenotype of sdf1a-EI-MO morphants is similar to that of 5’UTR-sdf1a morphants. In addition, expression of genes encoding myosin light chain (mylz2) and myosin heavy chain (myhz1) decreased in cxcr4a and sdf1a morphants (Figures 2J–K), whereas expression of the early myocyte marker pax7 was relatively normal (data not shown). Taken together, these experiments showed that knockdown of Sdf1a causes reduction in myoD and myf5 transcription, a phenomenon similar to that of Cxcr4a knockdown. Thus, sdf1a is necessary for early myogenesis.

We then analyzed cxcr4a and sdf1a morphants in more details. Both cxcr4a and sdf1a morphants have reduced birefringence in myotomes (Figure 3A). In addition, transgenic mylz2-GFP morphants of cxcr4a and sdf1a show reduced GFP expression (Figures 3B–F). This prompted us to check the ultrastructure of muscle fibers in morphants using transmission electron microscopy (TEM). Both cross and sagittal sections illustrated that myofibrils were reduced in cxcr4a morphants (Figures 3G–J). Taken together, these results indicated that deficiency in Sdf1a-Cxcr4a mediated signaling caused abnormal development of skeletal muscles. The affected somitic cells most likely remained undifferentiated.

Loss of cxcr4a and sdf1a function affects slow muscle migration

It was previously shown that development of slow muscle is closely associated with that of fast muscle and that a change in adhesion within the myotome disrupts migration of slow myoblasts [1]. We tested whether perturbation of either Cxcr4a or Sdf1a affects slow muscle. To eliminate the possibility of early defects in slow myoblasts, we analyzed cxcr4a and sdf1a morphants at 19 hpf, when the posterior adaxial cells have not yet completed their migration. The adaxial cells in both control embryos and morphants (cxcr4a and sdf1a) were adjacent to the notochord (Figures 4A–C). A mild decrease in F59 anti-
Knockdown of E-box factors affects cxcr4a and sdf1a transcription. Dorsal views (A-F). Embryos between 13–14 h were analyzed. (A,D) Control showing cxcr4a (n = 20) and sdf1a (n = 16/20) transcription respectively. (B,E) Double myoD-myf5 morphants demonstrate reduction of cxcr4a (n = 15/20) and sdf1a (n = 16/20) transcription respectively. This indicates cooperative function of MyoD and Myf5. (C,F) e12 morphants have vast reduction of cxcr4a (n = 20/20) and sdf1a (n = 20/20) transcription, this confirms that E12 is a major regulating factor. Black arrow in F indicates sdf1a staining in the non somitic lateral mesoderm. Abbreviation: n – notochord.

Body staining in morphants is presumably due to a slight developmental delay. This correlates with the normal myoD staining in the adaxial cells (see Figures 2A–D). Normally by 25 hpf slow muscle cells migrate to the lateral edge of somite and align to form myofibrils (Figures 4D, G). In cxcr4a and sdf1a morphants this process was affected (Figures 4E–F, H–I). Taken together, these results show that while early specification of slow muscle in both cxcr4a and sdf1a morphants remain normal, the myofibrils were affected.

myoD and myf5 are required co-operatively for the expression of cxcr4a and sdf1a

In mammals, the primary MRFs, Myf5 and MyoD, are involved in both myoblast specification and differentiation [61]. The early expression of cxcr4a or sdf1a correlates with that of myoD or myf5. Therefore, we speculated that the knockdown of MyoD or Myf5 could cause a change in cxcr4a/sdf1a transcription. Neither injection of the two different myoD MO designed against distinct regions at 5’-UTR nor the splice site MO against the first intron of myoD (myoD-EI-MO), which effectively inhibits splicing of myoD (Additional Figures A4C-F (see Additional file 4)) caused significant changes in transcription of cxcr4a and sdf1a (data not shown).

Similarly, the splice site MO against the first intron of myf5 (myf5-EI-MO), which caused effective missplicing of myf5 (Additional Figures A4G-I (see Additional file 4)), did not affect expression of cxcr4a or sdf1a (data not shown). This could be due to redundancy of myf5 and myoD [62]. To explore whether these genes can regulate expression of cxcr4 or sdf1 cooperatively, we co-injected myoD and myf5 MOs. Analysis of myoD-myf5 double morphants using cxcr4a and sdf1a probes demonstrated that transcription of these two genes has decreased significantly (compare Figures 5A to 5B and 5D to 5E). The tissue-specific bHLH proteins act after forming dimers with ubiquitously expressed bHLH proteins such as E12. These dimers act as positive regulators of gene transcription. We knocked down E12 using a splice MO (Additional Figures A4K-N (see Additional file 4)). This resulted in strong decrease of cxcr4a and sdf1a transcription (Figures 5C, F). Other signaling cascades such as Delta-Notch were unaffected by this treatment (Additional Figures A2N-O (see Additional file 2)). Taken together, these results suggest that myogenic factors, MyoD and Myf5 may co-operatively contribute to the regulation of cxcr4a or sdf1a.

myoD and myf5 positively regulate cxcr4a transcription

To verify the idea that early MRFs could regulate expression of cxcr4a/sdf1a, we injected mRNA of myoD or myf5 into only one cell of the two-cell stage embryo and assayed for cxcr4a and sdf1a expression during somitogenesis (Figure 6A). To ascertain that mRNA is indeed asymmetrically distributed, the mRNA was co-injected with Fluorescein-Dextran. Only embryos with one-sided distribution of Fluorescein-Dextran were selected for analysis. Ectopic overexpression of myoD caused increased transcription of cxcr4a (Figures 6C, C’). For detailed analysis these embryos were carefully oriented and sectioned. Analysis of sections confirmed observations made on whole mounts (Figures 6J–J”). Image-Pro Plus software was used to evaluate the changes in transcriptional intensities over distance (Figure 6I). Similarly, ectopic overexpression of myf5 and e12 caused increased transcription of cxcr4a (Figures 6D, D’, E, E’, K, L–L””, M, N–N””).

The negative HLH proteins Id1-4 compete with the positive bHLH factors for dimerization with E12 and E47 by forming inactive dimers. This results in inhibition of transcription of genes – targets of positive MRFs [63,64]. Overexpression of id2 [65] in a one-sided fashion resulted in the downregulation of cxcr4a or sdf1a transcription (Figures 6F, F’, O, P–P”). Taken together, these data suggest that myoD or myf5 act in parallel with their co-factors to regulate transcription of cxcr4a or sdf1a.
Figure 6
MRFs and co-factors are required for cxcr4a transcription. (A) Schematic showing the outcome of two-cell stage injection. Blue represents a signal of cxcr4a in situ hybridization. Red circles represent fluorescein-dextran on injected side. In control uninjected embryos, transcription analysis with antisense probes will appear symmetrical on left and right sides of flat mount zebrafish embryo. Staining will not differ significantly between left and right sides in both uninjected embryos and control fluorescein-dextran injected embryos. All comparisons were done between opposing pairs of somites. For each set of experiment, a minimum of three embryos between 11–14 h were analyzed using cryosectioning. The uninjected side acts as the internal control. Embryos stained with cxcr4a riboprobe. (B-F') Dorsal views. (B'-F') Composite images of the bright-field and fluorescent image showing one sided distribution of mRNA expressing cells. Increased level of cxcr4a transcript can be seen after misexpression of myoD (n = 46) (C,C'), myf5 (n = 35) (D,D') and e12 (n = 36) (E,E'). Decreased expression of cxcr4a was observed after misexpression of id2 (n = 27) (F,F'). Black arrows indicate sites of effects. Embryos are carefully aligned for cross section. (H,J,L,N,P) Transverse sections at the level of somites. (H,J,L,N,P) Fluorescein-Dextran to indicate location and proper one-sided injection. (H',J',L',N',P') DAPI staining. (H'',J'',L'',N'',P'') Composite images of bright-field and fluorescent images indicate exact site of effect. An increase of cxcr4a transcript after misexpression of myoD (J), myf5 (L), E12 (N) and decrease after misexpression of id2 (P). White dotted lines demarcate area of staining while black dotted lines define where relative intensities of staining were measured. (G,I,K,M,O) Graphs from Image-Pro Plus software. Control, G. Changes of relative intensity, indicated by peaks in I,K,M and O respectively.
MyoD, Myf5 and Sdf1 act within a context of Hedgehog (Hh) signaling [22,66-69]. We asked what connection between Cxcr4a-Sdf1a and Shh exists during formation of skeletal muscles. Since transcripts of Cxcr4a-Sdf1a and Shh are present during formation of fast muscles [25,51,72]. In the gain-of-function (GOF) experiments, based on implantation of SDF1-containing beads into chick limb, the down-regulation of MyoD expression has been observed [51]. Such an outcome differs from that in our observations. This conflict probably demonstrates that an outcome of SDF1 signaling could be the context-and/or concentration-dependent as implied earlier [36]. Furthermore, the different methodological approaches have been used to collect data. We relied on the loss-of-function (LOF) approach, which similar to recent experiments with SDF1 inhibitor [73] demonstrated a positive regulatory role of SDF1-Cxcr4 axis upstream of MyoD. Taken together, results of LOF experiments in chick [73] and our results in zebrafish support the positive regulatory role of Cxcr4-Sdf1 upstream of MRFs during commitment of fast myocytes.

**Discussion**

We have demonstrated that expression of chemokine receptor Cxcr4a and its ligand Sdf1a in paraxial mesoderm is required during formation of fast muscles. This defines a much earlier role for these molecules in myogenesis comparing to what described in previous reports on the migration of progenitors of the appendicular muscles [25,51,72]. In the gain-of-function (GOF) experiments, based on implantation of SDF1-containing beads into chick limb, the down-regulation of MyoD expression has been observed [51]. Such an outcome differs from that in our observations. This conflict probably demonstrates that an outcome of SDF1 signaling could be the context-and/or concentration-dependent as implied earlier [36]. Furthermore, the different methodological approaches have been used to collect data. We relied on the loss-of-function (LOF) approach, which similar to recent experiments with SDF1 inhibitor [73] demonstrated a positive regulatory role of SDF1-Cxcr4 axis upstream of MyoD. Taken together, results of LOF experiments in chick [73] and our results in zebrafish support the positive regulatory role of Cxcr4-Sdf1 upstream of MRFs during commitment of fast myocytes.

**Functional differences within pairs of Sdf1s and Cxcr4s**

Our data show that the both pairs of genes encoding Sdf1 and Cxcr4 have overlapping expression in somites. Neither ccr4b nor sdf1b MO alone have obvious effect on formation of fast muscles. There are no defects in this tissue in ady mutant. Thus, it appears that Cxcr4a-Sdf1a axis alone is fully capable of supporting fast myogenesis, whereas Cxcr4b or Sdf1b perhaps can only partially compensate for the loss of this activity.

**Cxcr4a-Sdf1a signaling during myogenesis**

Both CXCR4 and SDF1 knockout mice exhibit a complex phenotype [74,75]. Despite the fact that they have been available for analysis for a long time, no defects in trunk muscles were detected [50]. Perhaps potential abnormalities in this tissue are too subtle compared to a plethora of more obvious defects elsewhere. Alternatively, an apparent change in regulatory machinery of Cxcr4 and Sdf1 expression between fish and mice, with respect to these proteins in fast muscle development of fish, could explain the differences that we have detected.

We never observed a complete absence of fast fibers in morphants. This could be due to other factors such as an incomplete knockdown or activity of paralogous genes, ccr4b and sdf1b, which might partially compensate for the reduction of function of Cxcr4a and Sdf1a. Our data suggest that Cxcr4a-Sdf1a signaling plays no role in tailbud mesoderm and starts to become necessary just before formation of somite border. It has been proposed that Cxcr4b in the lateral line primordium is involved in coor-
dition of internal cell movement [76]. Similarly, Cxcr4a-Sdf1a could be instrumental in coordinating a short distance movement of fast myocytes.

cxcr4a and sdf1a are not expressed in the adaxial cells and these cells express myoD and myf5 normally in the cxcr4a or sdf1a morphants. Thus, the defect of slow myofibrils observed in these morphants is likely an indirect one. It could be caused by abnormality of fast myocytes. This is in line with earlier data suggesting that migration of slow myocytes depends on fast myocytes [5].

**Cxcr4a-Sdf1a and regulation of MRFs expression**

The early expression pattern of sdf1a-cxcr4a in somites is very similar to that of myoD and myf5, but later on cxcr4a expression becomes restricted to the less differentiated anterior part of somite in contrast to MRFs expressed in the posterior more differentiated part of somite. This suggested that developmental events involving Cxcr4 precede induction of expression of myoD-myf5. Indeed, our functional analysis illustrated a requirement of Cxcr4 for regulation of expression of MRFs. Thus activation of MRFs expression by sdf1a-cxcr4a signaling during fast myogenesis occurs concurrently with the process of somite epithelialization and may play a role during this process.

**MRFs positively regulate expression of cxcr4a and sdf1a**

Until now there has been little evidence that MRFs could regulate expression of components of chemokine signaling [76,77]. Here for a first time we provided evidence that MRFs regulate transcription of cxcr4a and sdf1a. At the same time our results are consistent with previous reports that the MRFs are partially redundant as myogenic determinants [78]. And yet the double MyoD-Myf5 knockdown caused only partial reduction in the expression of cxcr4a and sdf1a. Perhaps some other regulatory factors such as Myocyte Enhancer Factor 2 (MEF2) act in parallel [54]. Alternatively, these data reflect an incomplete knockdown of MyoD-Myf5.

To act, MRFs form a dimer with E12 and E47, which are products of alternative splicing of E2A transcripts. They belong to a distinct class of ubiquitously expressed bHLH proteins of the E-protein family. The MRF/E protein heterodimers bind to a conserved DNA sequence, CANNTG, also known as the E-box, located in regulatory regions of many muscle-specific genes [80-82]. The promoter region of human CXCR4 contains an E-box sequence [83]. Similarly, we found two E-boxes in close proximity within a 2 kb stretch of cxcr4a 5’-untranslated region (our unpublished data). The upstream regulatory sequences of many muscle-specific genes, including MLC1/3 [84], acetylcholine receptor alpha [85], MCK [86] and myoD [87], contain multiple E-box sites. In general, at least two E-box sites are required for the activation of these genes by the MRFs [88,89]. These results support an idea that MRFs could directly regulate expression of cxcr4.

**Feedback loop between Cxcr4-Sdf1 and MRFs**

The expression pattern of Sdf1 genes overlaps with that of MRFs. Since Sdf1a expressed in the posterior part of somite probably interacts with Cxcr4a expressed in the anterior part of somite, this provides a missing link to complete the feedback regulation loop between Cxcr4-MRFs-Sdf1 that could be operating to link cascades of genes involved in chemokine signaling and myogenic differentiation. However, further investigation will be needed to understand the biochemical interactions.

**Conclusion**

In summary, our analysis of the developmental role of zebrafish Cxcr4-Sdf1 has led to the identification of the ligand-receptor pair essential for development of trunk muscles. This reveals a novel role of Sdf1-Cxcr4 in differentiation of fast myocytes of the trunk. Thus the chemokine signaling mediated by Sdf1-Cxcr4 emerged as an important regulatory pathway involved in myogenesis.

**Methods**

**Zebrafish strains and maintenance**

Adult zebrafish (*Danio rerio*) was maintained at 28.5°C as described [10]. The zebrafish AB line (ZIRC) was used as a wild-type fish. The *odysseus* (*ody*10049/cxcr4b) mutants and myosin light chain 2 (*mylz2*-GFP) transgenics were described [6,41]. Pigment formation was blocked with 1-phenyl-2-thiourea (PTU) [65].

**Whole mount in situ hybridization (WISH) and immunohistochemistry**

WISH was performed using single-stranded RNA probes labeled with digoxigenin-UTP or fluorescein-UTP (Boehringer Mannheim, Germany) by established protocol. The zebrafish probes cxcr4a, cxcr4b [48], *myoD* [90], *myf5* [17,91], *myhz1* and *myhz2* [13] have been described previously. Full-length sdf1a and sdf1b cDNA were obtained by RT-PCR using total RNA and primers designed based on sequence of the EST clones (BM184435 and BM070896), respectively. F59 Mab (1:25) [92] and secondary goat anti-mouse IgG antibody – Alexa Fluor 488 (1:1000; Molecular Probes, USA) was used to detect slow myosin heavy chain.

**RT-PCR**

Total RNA was isolated from 14 h embryos using RNeasy® Mini Kit (Qiagen, Germany). cDNA for reverse transcription (RT)-PCR analysis was synthesized using Qiagen® OneStep RT-PCR kit (Qiagen, Germany) and Peltier Thermal Cycler – 200 (MJ Research, USA) according to the manufacturer’s instructions. For mRNA splicing analysis, 25 ng of total RNA samples treated with DNase I was
used. RT-PCR conditions were as follows: Reverse transcription 50°C, 30 mins; PCR activation step 95°C, 15 mins; Denaturation 94°C, 1 min; Annealing 59°C, 1 min; Extension 72°C, 1 min; Cycles from Denaturation to Extension were repeated 39 times; Final Extension 72°C, 15 mins. 10 µl of RT-PCR products were loaded into each well for gel-electrophoresis. The sequences of primers for introns of well for gel-electrophoresis. The sequences of primers for
 AGE CCC GGC AGA C 3'; UMO or
 introns of

 Forward
 5' TTG CCA GCA CCA CCG ATT TTC 3'.

 Reverse
 CCC TGC CAA TGT AAC CA 3'; Reverse

 EF1alphaR1388
 TTG TAG CTA TTC C 3'; Forward
 EF1aphaF900 5' CGC CGC TGC ACC T 3'; Reverse

 GCT 3'; Reverse

 3'; Forward
 Reverse

 GCG TGA TTT T 3'; myoD-2-MO 5' GTA AGA CAA AGT CTT GGA A 3'; myoD-1-MO 5' TGC GAT AAC AAG GGG GCA 3' [43]; mS1a-2-MO 5' TT A3' [40]; S1a-3-MO 5' ATC ACT TTG AGA TCC ATG TTT C3'; S1a-1-MO 5' TGC AGT GTG AAG AAG AGA TCC GCA 3' 

 cxcr4a
 Expression constructs and RNA

 MORPHOLINO (MO) INJECTIONS

 MOs were obtained from Gene Tools, LLC (USA). The antisense sequences were designed to bind to the 5'UTR region including the initiation methionine or sequence between exon-intron (EI) junctions. To minimize the possibility of non-specific effects, we designed and used at least two MOs targeting non-overlapping sequences for each gene. MO sequences were as follows: Cr4a-1-MO 5' AATA AGC CAT CTC TAA AAG ACT CTC C 3'; Cr4a-2-MO 5' GAC TTC CCG TCC TTC AGT CTC C 3'; Cr4a-3-MO 5' ACA GTT TAA ATA CCT CTC TCG CGC G 3'; mCr4a-1-MO 5' ATA AAC CAT ATC TAA GAG ACC GTCT C3'; S1a-1-MO 5' TGGC AGT AAG AGA AAG AGA TCC GCA C3'; S1a-2-MO 5' TTA GAG TCA TGG TTA GTA TGG TGA A3' [40]; S1a-3-MO 5' ATC ACT TGT AGA AAG ACC TGT AGA C3' [43]; mS1a-2-MO 5' TTAAGA TAC ATG TGT GAA GTG TAA A3'; S1a-El-MO 5' GTCG AGA CAT CTC ACA TGA GTT CTA GGA A 3'; myoD-1-MO 5' TGC GAT AAC AAG GGG GCG TGA TTT T 3'; myoD-2-MO 5' GTA AGA CAA AGT CCT TCA GAT CCC G 3'; myoD-El-MO 5' GTT TCT CAC CAT GCC ATC AGA GCA C 3'; myf5-El-MO 5' GTC GTA TTAC CCC TCT TCT GAG C3'; e12-1EI-MO 5' GAA AAC AAG AGC GCC ACA TTA GAA G 3'; e12-3EI-MO 5' TTC ACA CTC ACC AGG CCC GGC AGA C 3'; UMO or control MO 5' CTT CTC ATG TAA CTC TGA ATG AAG C 3' [93]. Bold letters either represent base change or region in intron. MOs were diluted using 1× Danieau's solution to 0.46–1.5 mM stock solution or to proper concentration for injection. Since several MO give the same results only representative morphants were photographed.

 Expression constructs and RNA

cxcr4a (AY057095), cxcr4b (AY057094), sdf1a (BM184445), sdf1b (BM070896), myoD (Z36945), myf5 (AF270789), and id2 (DQ186992) were all cloned into PCScript (Clontech). Primers used for cloning: Forward
 cxcr4a (F) 5' ATG GAT CTC AAC GTG TTT T 3'; Reverse
 cxcr4a (R) 5' TTA ACT AGA GTG AAA GCT TGA

 GAT 3'; Forward cxcr4b (F) 5' ATG GAA TTT TAC GAT AGC A 3'; Reverse cxcr4b (R) 5' CTA ACT CTT GAG TAC ACT GGA 3'; Forward sdf1a (F) 5' ATG GAT GTC AAA GCT TAC GT 3'; Reverse sdf1a (R) 5' TTA GAC CTT GTC TCA TCC G 3'; Forward sdf1b (F) 5' ATG AGC AAA GTA GTA GCG C 3'; Reverse sdf1b (R) 5' TTA TCA GTC TCA TCT GTC TTC TTT C3'; Forward myoD (F) 5' ATG GAG TTG TCG CAT ATC CCC C 3'; Reverse myoD (R) 5' CCA CIT GAA TGG TTT TT TAC CA 3'; Reverse myf5 (F) 5' ATG AGC GTA TCC ACG GTCA A 3'; Forward myf5 (R) 5' TCA CAG TAC GTG GTA AAG TGG TGT TGG CCC 3'; Forward id2 (F) 5' TTA ACG CTA AAG GCC CCA ACG 3'; Forward id2 (R) 5' TTA ACG GAA GAA GGG GGA 3'.

 Cryosctioning and photography

 Cryosctioning of embryos was described [65]. Some sections were stained with 1.5 ml of diluted 3.5 µm DAPI (4', 6-diamidino-2-phenylidindethindione) for 20 min and washed in PBS 2× 20 min. Axiophot 2 compound microscope or laser scanning confocal microscope LSM 510 (Zeiss, Germany) with software supplied by the manufacturers or AX70 (Olympus, Japan) were used for photography. For image processing Adobe® Photoshop 5.5 and measuring of relative intensities Image-Pro® Plus 4.5.1 software was used.

 Light Microscopy

 For analysis of birefringency of the axial skeletal muscle the Olympus Light dissecting microscope equipped with polarizer (Olympus, Japan) was used as described [94]. A plane of polarization was standard in all analyses.

 Electron-microscopy

 Embryos were processed using standard protocols [95], embedded in 100% resin and polymerized at 65°C overnight. Ultra-thin sections were cut on a Reichert-Jung ultramicrotome (Germany) and examined under the transmission electron microscope JEM1010 (JEOL, Japan) at 100 kV.

 Authors' contributions

 SWC developed the concept, performed all the experiments and wrote the manuscript. LMN analyzed slow muscle. YJ drafted the manuscript. VK developed the concept, wrote the manuscript and approved the manuscript. All authors read and approved the manuscript.
Additional material

Additional file 1
Additional Figure A1 The dynamic expression of cxcr4b and sdf1b during segmentation. Dorsal views (A-C-F) and lateral views (B,G). (A) cxcr4b is expressed in the tailbud region, adaxial cells, paraxial mesoderm, 13.5 h. (B) Expression of cxcr4b is reduced as differentiation proceeds, strong expression is in forming and newly formed somites, 18 h. (C) Two color in situ for myoD (red) and cxcr4b (blue) reveals partial overlapping expression of cxcr4b and myoD, 14 h. White lines demarcate the somite boundaries. (E) sdf1b transcription starts early in the adaxial cells, 10 h. (D,F) Expression in somites is relatively weak; some part of adaxial and paraxial mesoderm express sdf1b at 14 h and 18 h respectively. (G) sdf1b transcription localizes in dorsal and ventral regions of somites as indicated by black arrowheads, 16.5 h. (H) Reverse transcription (RT)-PCR detects continuous presence of transcript of sdf1b during early development. sdf1b transcript is present before mid-blastula transition (MBT). To confirm results, products were sequenced. β-actin was used as a positive control. -RT control using β-actin primers without addition of reverse transcriptase, no band was detected (data not shown).

Abbreviations: ad – adaxial cells; d – dorsal; lm – lateral mesoderm; mn – mesoderm; ncc – neural crest cells; p – presomitic; pm – presomitic mesoderm; s – somite; S0 – forming somite; S1 – newly formed somite; tb – tailbud; v – ventral; ys – yolk sac. Scale bars = 50 μm.

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Additional file 2
Additional Figure A2 Control experiments. Dorsal views (A-O). (A,B) Embryos double stained with myoD (red) and vasa (blue), 14 h. vasa riboprobe ensures that ody+ was correctly identified since mutant embryos appear phenotypically normal. No significant change in myoD was observed in ody−. Arrows indicate the cluster of primordial germ cells (PGCs). (C,D) Embryos double stained with myf5 (blue) and vasa (red), 14 h. No significant change was detected in the paraxial mesoderm. Insets showing normal cluster of PGCs in ody+ and PGCs were found along midline in head region of ody−. (E,F) Two sets of experiment (E-G and H-J) demonstrating redundancy in function between cxcr4a and cxcr4b. A lower dosage of cxcr4a MO was used to obtain normal myoD staining but disrupted myoD in paraxial mesoderm in ody−. vasa (red) helps to identify ody−. White arrowheads in F indicate ectopic expression of myoD. (K-M) cxcr4a and sdf1a morphants show normal transcription of myf5 in tailbud domain, 14 h. (N,O) A representative e12 morphant stained with deltaC as a control for cxcr4a and sdf1a transcriptional analysis, 14 hpf. Somites are formed in these morphants and notch pathway is unaffected.

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Additional file 3
Additional Figure A3 Range of phenotypes in morphants. (A) Control. (B) cxcr4a morphants. (C) sdf1a morphants. cxcr4a and sdf1a MOs act in a dosage dependent manner.

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Additional file 4
Additional Figure A4 Efficiency of splice site MOs. (A,C,G,K) β-actin primers used in -RT control. Splice site MOs inhibit splicing in sdf1a (B), myoD (D), myf5 with degradation (H) and e12 (L). Total RNA from control (lane 1,5), 0.46 pmole/embryo (lane2,6), 0.92 pmole/embryo (lane 3,7), 2.3 pmole/embryo (lane4,8). Splice product sizes are indicated by white asterisks. (E,F) mylz2 riboprobe staining on control (n = 20/20) and myoD-E1 morphant (n = 19/20) respectively [96]. (L) myogenin riboprobe staining on control (n = 20/20) and myf5-E1 morphant (n = 18/20) respectively [97]. (M,N) sdf1a riboprobe staining on E12-E1 morphant (n = 20/20) and morphant rescued with e12 mRNA (n = 10/16).

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Additional file 5
Additional Figure A5 Control experiments for in vivo effects of PKI on the induction of myogenic transcription. (A,B) Fluorescent-dextran injected embryos stained with myoD (n = 10), 14 h. (C,D) PKI injected embryos with robust myoD (n = 10), 14 h. (E,F) Fluorescent-dextran injected embryos stained with myf5 (n = 10), 14 h. (G,H) PKI injected embryos with augmented myf5 (n = 10), 14 h.

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