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Cxcl12 evolution – subfunctionalization of a ligand through altered interaction with the chemokine receptor

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SUMMARY

The active migration of primordial germ cells (PGCs) from their site of specification towards their target is a valuable model for investigating directed cell migration within the complex environment of the developing embryo. In several vertebrates, PGC migration is guided by Cxcl12, a member of the chemokine superfamily. Interestingly, two distinct Cxcl12 paralogs are expressed in zebrafish embryos and contribute to the chemotattractive landscape. Although this offers versatility in the use of chemokine signals, it also requires a mechanism through which migrating cells prioritize the relevant cues that they encounter. Here, we show that PGCs respond preferentially to one of the paralogs and define the molecular basis for this biased behavior. We find that a single amino acid exchange switches the relative affinity of the Cxcl12 ligands for one of the duplicated Cxcr4 receptors, thereby determining the functional specialization of each chemokine that elicits a distinct function in a distinct process. This scenario represents an example of protein subfunctionalization – the specialization of two gene copies to perform complementary functions following gene duplication – which in this case is based on receptor-ligand interaction. Such specialization increases the complexity and flexibility of chemokine signaling in controlling concurrent developmental processes.

KEY WORDS: Cxcr4, Cell migration, Chemokine, Evolution, Germ cell, Zebrafish

INTRODUCTION

Single-cell migration within the complex environment of the developing embryo is controlled by multiple cues, some of which are encoded by diffusible signaling molecules (Rorth, 2011) that can provide cells with conflicting directions (e.g. Moreira et al., 2010). Directed cell migration is often controlled by chemokines that form gradients in the extracellular space. These gradients are perceived by receptors presented on cells that respond by directed migration. This scenario is complicated by the fact that chemokines exhibit high degrees of structural similarity and can thus bind several receptors. Conversely, these receptors can be activated by more than one ligand (Viola and Luster, 2008). Cells have therefore had to develop mechanisms that allow them to distinguish between closely related chemokine signals in the environment.

Important chemokines include the isoforms of Cxcl12 (formerly SDF-1). Cxcl12 ligands bind the receptor Cxcr4 (Bleul et al., 1996; Oberlin et al., 1996) to control processes such as gastrulation (Nair and Schilling, 2008), the migration of groups of cells and vascular system formation (Tachibana et al., 1998; Zou et al., 1998; Siekmann et al., 2009), the homing of hematopoietic stem cells and leukocytes (Aiuti et al., 1997; Zou et al., 1998; Peled et al., 1999; Walters et al., 2010), neuronal development (Zou et al., 1998; Knaut et al., 2005; Lieberam et al., 2005), cancer progression and metastasis (Muller et al., 2001; Orimo et al., 2005).

A useful in vivo model for studying Cxcl12 function in the context of guided cell migration is the migration of primordial germ cells (PGCs) during embryonic development (Richardson and Lehmann, 2010). PGCs migrate from the location at which they are specified towards the developing gonads, where they differentiate into gametes (Wylie, 1999). We and others have shown that mouse, chicken and zebrafish germ cells express Cxcr4 and are guided towards the gonads by Cxcl12 (Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003; Stebler et al., 2004).

Interestingly, in the zebrafish, two Cxcl12 ligands and two Cxcr4 receptors are present and are expressed in the stages of development when PGC migration takes place [see Doitsidou et al. (Doitsidou et al., 2002) for Cxcl12a and Knaut et al. (Knaut et al., 2003) for Cxcl12b]. The PGCs that express Cxcr4b are therefore exposed to both ligands, offering the possibility to investigate the molecular basis for the potential discrimination between, and differential response to, the two signals.

Here we show that whereas the PGCs can effectively respond to both cues that are presented to them, in the course of their migration only Cxcl12a guides the cells towards their target. Using chimeric Cxcl12 molecules and point mutations, we studied the basis for the differential activity of the two chemokines and

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identified a domain in the molecule that dictates specificity for activation of either one of the two Cxcr4 receptors. These results provide a mechanism for the expansion of ligand-receptor families during evolution, thus facilitating the formation of increasingly elaborate signaling networks.

MATERIALS AND METHODS

Zebrafish strains

Fish of the AB, AB/TL genetic background, or carrying the Toll-kop-EGFP-F-nos1-3′UTR transgene (Blaser et al., 2005) served as wild-type fish.

In situ hybridization

In situ hybridization was performed as described (Thisse and Thisse, 2008). For comparative in situ hybridizations, length-matched *cxcl12a* and *cxcl12b* antisense probes were generated. Other probes used were *nos1* (Köprunner et al., 2001) and *egfp* (Blaser et al., 2005).

In vitro Cxcr4 internalization assay

Recombinant FLAG-tagged Cxcl12 proteins were purified from serum-free supernatants of HEK293T cultures (using Heparin HiTrap columns, GE Healthcare). For internalization experiments, starved HEK293T cells expressing Cxcr4b-YPet were exposed to 25 ng/ml Cxcl12a, Cxcl12b, Cxcl12a N33S or Cxcl12b S33N at 37°C for 30 minutes before fixation and analysis. One-hundred cells per treatment were counted.

Embryo microinjection

The open reading frames (ORFs) of genes of interest were fused to the *nos1*-3′UTR (for PGC-specific expression) or to that of *Xenopus globin* (for global expression) (Köprunner et al., 2001). mRNAs and morpholino antisense oligonucleotides (MOs) were microinjected into the yolk of one-cell stage embryos, unless stated otherwise.

For the in vivo chemokine activity screen, 150 pg *egfp-nos1* or *cxcl12-nos1* mRNA was used.

For gene knockdown, 0.4 pmol of the following MOs was injected: *cxcl12a* and *cxcr4b* (Doitsidou et al., 2002); *cxcl12b* and *cxcr4a* (Nair and Schilling, 2008). For *cxcr7* knockdown, 1.2 pmol was used (Boldajipour et al., 2008).

For transplantations, donor embryos were injected with *cxcl12-globin* and either *egfp-globin*, *ecfp-globin* or *mcherry-f-globin* (100 pg) mRNAs. Recipient wild-type or Toll-kop-EGFP-F-nos1-3′UTR transgenic embryos were injected with *cxcl12a* and *cxcl12b* MOs. Cells were transferred from a 4 hour post-fertilization (hpf) donor into a 6 hpf recipient. PGC migration was documented for 3 hours (time-lapse microscopy) or evaluated after 3 hours by in situ hybridization using *nos*1 and *egfp* probes.

For fluorescence correlation spectroscopy (FCS), embryos were injected with MOs against *Cxcr4* and *Cxcr7* and with mRNA encoding Cxcr4b-mDsRed (35 pg) or GPI-mRFP (5 pg). At the 64-cell stage, *cxcl12-egfp* mRNA (50 to 300 pg) was injected into one blastomere.

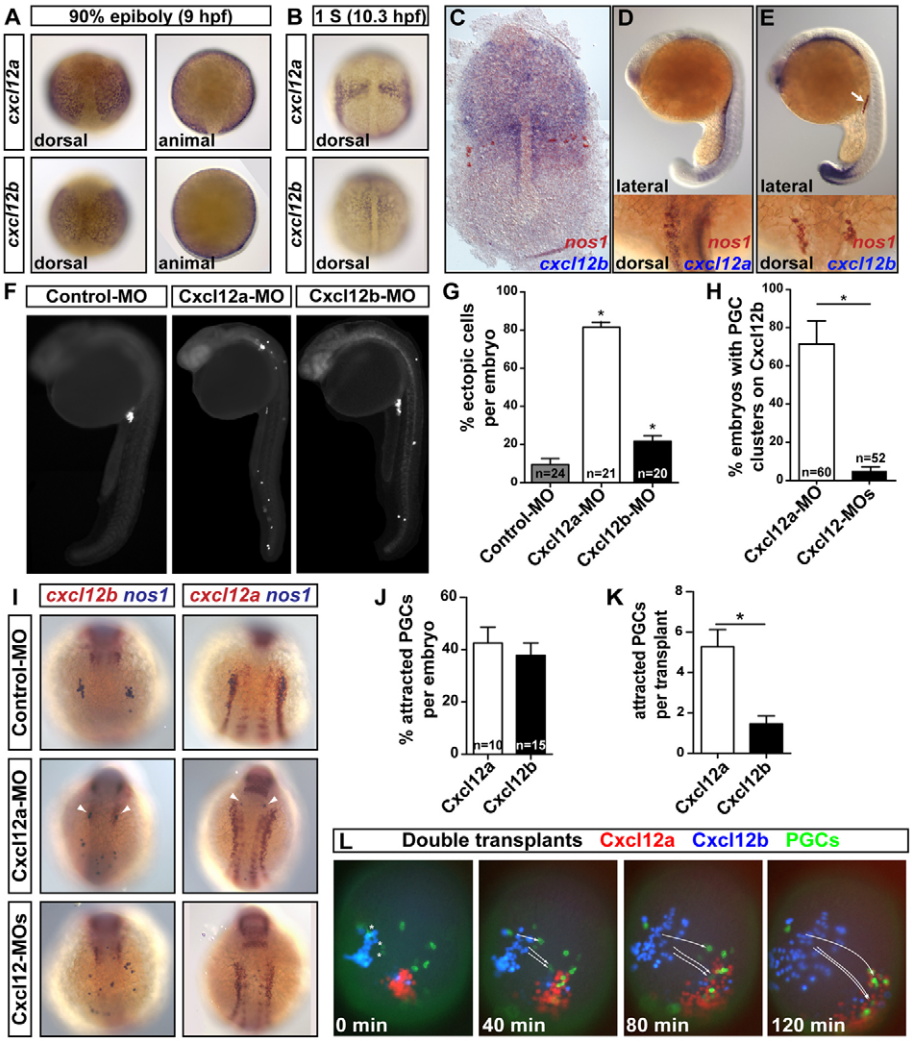

Fig. 1. Cxcl12 subfunctionalization affects gene expression and chemotactic activity of Cxcl12. (**A**,**B**) Zebrafish *cxcl12a* and *cxcl12b* mRNA expression patterns as detected by in situ hybridization using probes of identical length and identical staining duration to allow direct expression level comparison at late gastrula stage (A) and at the onset of somitogenesis (B). (**C**) During early somitogenesis stages, the primordial germ cells (PGCs, brown) clearly reside outside of regions of high *cxcl12b* expression (blue). (**D**,**E**) At 22 hpf, the PGC positions correlate only with *cxcl12a* expression. Insets show magnified views of the developing gonads. Arrow, gonad region. (**F**) Knockdown of Cxcl12a, but not of Cxcl12b, expression results in strong PGC migration defects. (**G**) Quantitation of the results presented in F. (**H**) *cxcl12b*-expressing tissues attract PGCs in embryos knocked down for Cxcl12a. Shown is the percentage of embryos with more than three PGCs at *cxcl12b*-expressing tissues. (**I**) PGCs (*nos1*, blue) normally reach *cxcl12a*-expressing domains (top), but arrive at regions expressing *cxcl12b* when Cxcl12a is knocked down (middle), or are located randomly when both chemokines are knocked down (bottom). Arrowheads, PGCs located in *cxcl12b* expression domains. (**J**) Percentage of PGCs attracted by Cxcl12a-expressing or Cxcl12b-expressing transplanted cells. (**K**,**L**) The percentage of PGCs attracted by Cxcl12a-expressing (red in L) or Cxcl12b-expressing (blue in L) co-transplanted cells. Arrows indicate the movement of the marked cells (asterisks) (see Movie 1 in the supplementary material). Error bars depict s.e.m.; *n*, the number of embryos analyzed. *, $P<0.05$ compared with control (*t*-test).

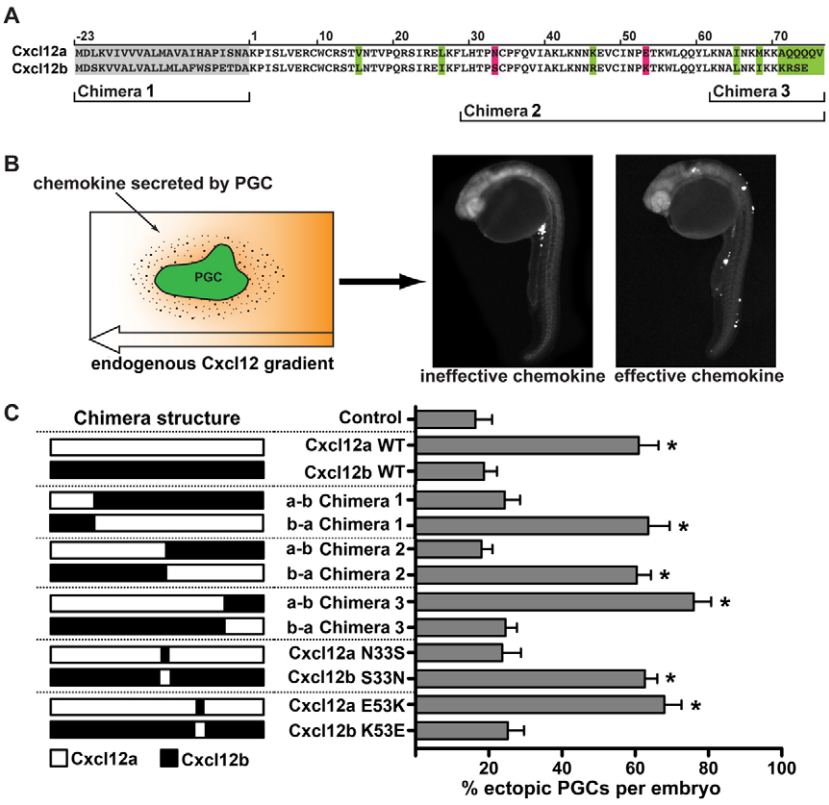

Fig. 2. In vivo functional analysis of PGC migration in Cxcl12 mutants. (**A**) Sequence alignment of zebrafish Cxcl12a and Cxcl12b. The signal peptide is marked in gray and residues that differ between the ligands are colored. Domain swaps in chimeric proteins used in C are indicated and the position of the analyzed point mutations highlighted in magenta. (**B**) Expression of a specific Cxcl12 form directed to the PGCs results in a local field of this protein that can interfere with the endogenous Cxcl12a gradient. Expression of an ineffective version of the ligand does not interfere with the migration of PGCs (left), whereas an effective ligand interferes with the gradient of the guidance cue (right). (**C**) The activity of chimeric and mutated Cxcl12 molecules in this assay. Bars show the mean percentage of ectopic PGCs per embryo. Error bars depict s.e.m. *, $P<0.05$ compared with control (ANOVA).

Microscopy

Standard and confocal microscopy (Kardash et al., 2010) and FCS (Ries et al., 2009; Yu et al., 2009) were performed as previously described. The fractional occupation of receptors was calculated as the ratio between the concentration of membrane-bound Cxcl12 ligands and that of Cxcr4 receptors. The results were calculated from 13 complete measurements on 4 hpf embryos (dual-color scanning on cell membranes and static FCS in the extracellular space) for Cxcl12a, 19 for the Cxcl12a mutant, 10 for Cxcl12b and 11 for the Cxcl12b mutant. Interaction of Cxcl12-EGFP with mRFP-labeled cells served as a background control and was subtracted from all calculations.

RESULTS AND DISCUSSION

Correlation between the expression pattern of *cxcl12* genes and the localization of PGCs

Two Cxcl12 ligands are expressed in the zebrafish embryo during the process of PGC migration (Doitsidou et al., 2002; Knaut et al., 2003), and both could in principle play a role in guiding PGCs towards their target. To investigate their relative contribution to PGC migration, we initially determined the mRNA expression pattern and expression level of the two genes using comparative mRNA in situ hybridization. We found that during early development, the mRNA expression patterns of *cxcl12a* and *cxcl12b* are almost indistinguishable (Fig. 1A and see Fig. S1 in the supplementary material), but that at 10 hpf, it is only the *cxcl12a* expression pattern that correlates with the position of the PGCs. For example, in 10 hpf embryos, *cxcl12b* expression is elevated close to the midline of the embryo (Fig. 1B lower panel, 1C), a position devoid of PGCs at this stage. Conversely, *cxcl12a* is highly expressed in the paraxial mesoderm and at the border between the head and the trunk where PGCs are found (Fig. 1B upper panel for *cxcl12a*, 1C for PGC location). Finally, at the end of the first day of development, the PGCs populate the region of the gonad where only *cxcl12a* is expressed (Fig. 1D,E). These results differ from those of Knaut et al., according to whom *cxcl12b* is expressed in a pattern that prefigures the route of PGC migration (Knaut et al., 2003).

PGCs show preference for the Cxcl12a paralog

The positioning of the PGCs relative to the expression of the Cxcl12 ligands indicates that, whereas the PGCs are initially exposed to both chemokines, the cells migrate towards tissues that express *cxcl12a*. Consistently, knockdown of Cxcl12a led to a dramatic loss of PGC migration fidelity in terms of arrival at the target (Fig. 1F,G). By contrast, Cxcl12b knockdown only mildly affected PGC migration (Fig. 1F,G), possibly as a consequence of the gastrulation defects associated with Cxcl12b knockdown (Nair and Schilling, 2008) rather than as the result of any direct role in migration (Knaut et al., 2003). Intriguingly, however, Cxcl12b does appear to be a potent guidance cue in the absence of Cxcl12a: in Cxcl12a-depleted embryos, PGCs clustered at *cxcl12b* expression sites (Fig. 1H,I). Knockdown of both ligands resulted in a random distribution of PGCs within the embryo (Fig. 1I, lower panels).

To directly compare the relative potency of the ligands as chemoattractants, we transplanted cells from embryos injected with equal amounts of *cxcl12a* or *cxcl12b* mRNA into Cxcl12-deficient embryos and followed the behavior of the PGCs at the stage during which they are normally guided by Cxcl12a. Consistent with the idea that both ligands can attract PGCs, cells expressing either Cxcl12a or Cxcl12b were equally potent (Fig. 1J). This finding suggests that, during early PGC migration, when both proteins are presented to PGCs, Cxcl12a is a more effective chemoattractant. To examine this possibility, we simultaneously transplanted cell clusters expressing equal amounts of either Cxcl12a or Cxcl12b into Cxcl12-deficient embryos. Under these conditions, the PGCs

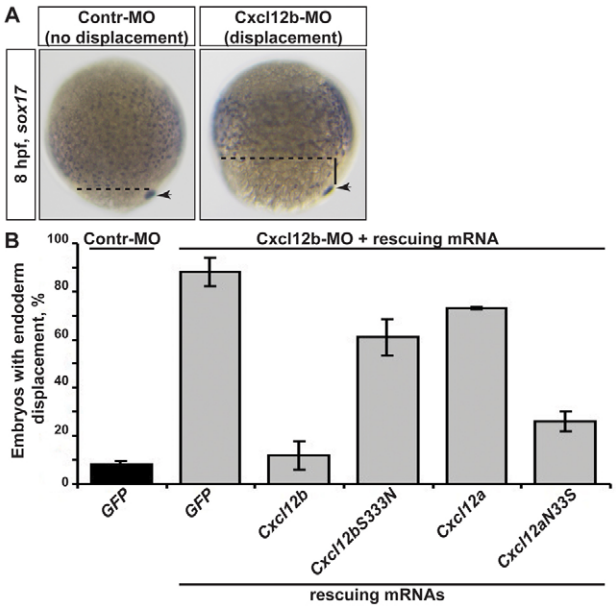

Fig. 3. Cxcl12 subfunctionalization affects the function of the Cxcl12 paralogs in gastrulation. (**A**) Knockdown of Cxcl12b slows down the migration of endodermal cells. Note the gap between the forerunner (arrowheads) and the endodermal cells. (**B**) The gastrulation phenotype induced by knockdown of Cxcl12b is effectively reverted by wild-type Cxcl12b as well as by the N33S Cxcl12a protein. Error bars depict s.e.m.

migrated towards the cells expressing Cxcl12a, mostly ignoring the cells expressing Cxcl12b (Fig. 1K,L and see Movie 1 in the supplementary material).

Together, these findings suggest that biochemical differences in the chemokines have evolved such that, despite their overlapping expression patterns, the correct guidance cue dominates in the response of the PGCs.

The molecular basis for the differential chemotactic activity of Cxcl12a and Cxcl12b

Despite the dramatic differences in their activity, Cxcl12a and Cxcl12b show a high degree of sequence similarity (Fig. 2A). To define the specific amino acids responsible for the apparent divergence in chemokine function, we developed a novel in vivo assay that allowed us to compare the potency of the Cxcl12 proteins. We directed the expression of Cxcl12a, Cxcl12b and that of mutated Cxcl12 forms to the PGCs themselves. This resulted in increased chemokine levels around the migrating cells, thus interfering with the cues that normally guide them to their target (Fig. 2B) (Doitsidou et al., 2002). The degree of such interference should reflect the extent of PGC responsiveness to the respective ligand. Indeed, we found that the majority of the PGCs overexpressing Cxcl12a failed to reach the gonads, whereas expression of Cxcl12b had no effect on PGC migration as compared with the control (Fig. 2B,C, Cxcl12a WT and Cxcl12b WT). We excluded a role for the secretion level of the two proteins, as exchanging the signal peptide between the ligands had no effect (Fig. 2A,C, Chimera 1). By contrast, exchanging the C-terminal half of the proteins reversed the specific activity of the ligands (Fig. 2A,C, Chimera 2), whereas the extreme C-terminus did not alter the function of the ligands (Fig. 2A,C, Chimera 3). Thus, we pinpointed the difference between Cxcl12a and Cxcl12b to amino acids 29-61, of which only those at positions 33 and 53 differ significantly in structure and charge. We found that exchanging asparagine 33 (which is conserved in mammalian Cxcl12) to serine (Cxcl12a N33S in Fig. 2C) abolished Cxcl12a activity, whereas a substitution at position 53 had no effect in this assay (Cxcl12a E53K in Fig. 2C). Conversely, the reciprocal exchange raised Cxcl12b activity to that of wild-type Cxcl12a (Cxcl12b S33N in Fig. 2C).

Whereas PGC migration relies exclusively on Cxcr4b and Cxcl12a, endodermal cell migration is controlled by Cxcr4a and Cxcl12b (Mizoguchi et al., 2008; Nair and Schilling, 2008). This raises the possibility that each of the two Cxcl12 ligands functions preferentially with one of the Cxcr4 receptors and that the amino acid exchange is key to the apparent specificity. We assayed the potency of the different Cxcl12 molecules in facilitating endodermal cell migration during gastrulation (Mizoguchi et al., 2008; Nair and Schilling, 2008). In this process, activation of Cxcr4a by Cxcl12b is required for proper integrin-dependent cell adhesion, such that Cxcl12b knockdown results in delayed gastrulation at 8 hpf (Fig. 3A) (Mizoguchi et al., 2008; Nair and Schilling, 2008). Indeed, expression of Cxcl12b, but not Cxcl12a, was able to revert the *cxcl12b* MO-induced defects (Fig. 3B). Strikingly, Cxcl12a mutated at position 33 was sufficient to restore normal gastrulation, whereas Cxcl12b with the reciprocal mutation failed to do so.

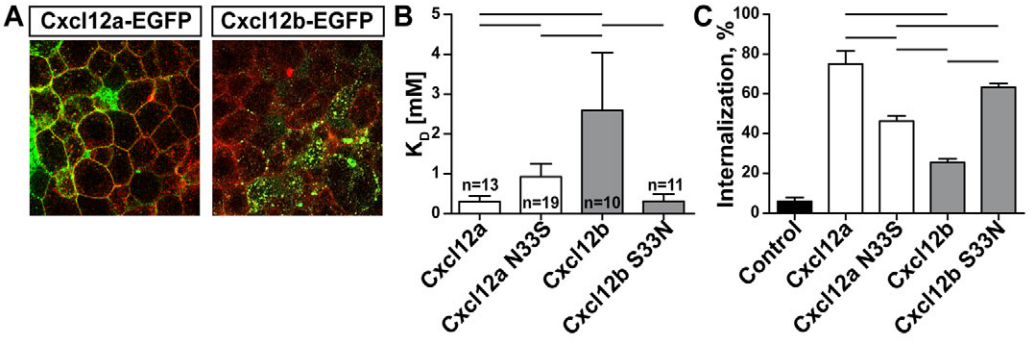

Fig. 4. Subfunctionalization of Cxcl12 affects the affinity towards and activation of Cxcr4. (**A**) The concentration of free ligand is compared with the concentration of ligand (green) on Cxcr4b-containing membranes (red). (**B**) Median apparent K_D values of dual-color FCS measurements. Error bars depict 95% confidence intervals and *n* is the number of measurements performed. Horizontal bars identify significant pairwise differences; $P<0.05$ (Kolmogorov-Smirnov test). (**C**) Internalization of Cxcr4b as a measure of Cxcl12 activity in vitro. Mean percentage of HEK293T cells expressing Cxcr4-YPet showing receptor internalization following incubation with recombinant Cxcl12 or with control ligand-free medium (see Fig. S2 in the supplementary material for representative results). Error bars represent s.e.m. Three experiments with 100 cells each were performed. Significant pairwise differences are identified by horizontal bars; $P<0.05$ (one-way ANOVA).

In summary, we identified a single amino acid responsible for the specialization, or subfunctionalization, of the two Cxcl12 copies, allowing them to perform complementary functions following gene duplication. This amino acid is located within the 30s flexible loop of the ligand, which has been suggested to facilitate intramolecular motions necessary for the proper positioning and cooperation of the receptor binding motifs (Baysal and Atilgan, 2001; Kofuku et al., 2009). This suggests that in the course of subfunctionalization (He and Zhang, 2005), the zebrafish Cxcl12 chemokines have diverged by functioning in concert with one Cxcr4 receptor, while reducing their interaction with the other. The idea that the two duplicated ligands and receptors co-evolved to generate the observed relative specificity is consistent with the finding that, in the case of mammalian CXCL12, for which only one CXCR4 receptor exists, the wild-type and the mutated N33S proteins exhibit equal potency in promoting directed migration as well as in inducing CXCR4 internalization (see Fig. S2 in the supplementary material).

The subfunctionalization of the Cxcl12 ligands occurred through altered binding and activation of the chemokine receptor Cxcr4

To determine the functional significance of the sequence divergence of the two ligands, we examined an array of biochemical properties that could influence the potency of the ligands. Specifically, we determined ligand affinity for the two receptors, their ability to activate the receptors, as well as ligand oligomerization and interaction with extracellular matrix components crucial to chemokine activity (Proudfoot et al., 2003; Handel et al., 2005).

To determine the affinity of Cxcl12 for Cxcr4 in vivo, we employed a dual-color scanning fluorescence correlation spectroscopy (FCS) setup originally developed for measuring the receptor binding constants of secreted morphogens (Ries et al., 2009; Yu et al., 2009). We engineered embryos that produced Cxcl12-EGFP from a restricted source, with mDsRed-tagged Cxcr4b expressed by all cells. Keeping the numbers of receptors in the membrane at similar levels, we compared the number of ligands bound to the membrane at a given extracellular concentration of free ligand (Fig. 4A). This allowed us to estimate the relative in vivo affinity of Cxcl12a for Cxcr4b, which we found to be an order of magnitude higher than that of Cxcl12b. In agreement with the idea that divergence of protein function occurred on the basis of the single amino acid substitution, Cxcl12b S33N exhibited a receptor affinity identical to that of Cxcl12a, whereas the affinity of Cxcl12a N33S for Cxcr4b was significantly reduced (Fig. 4B).

To assess receptor activation after chemokine binding, we monitored chemokine-mediated receptor internalization, which we had previously used as an indicator for Cxcl12/Cxcr4 activity (Minina et al., 2007; Boldajipour et al., 2008). Following the internalization of YFP-tagged Cxcr4b expressed by HEK293T cells in response to the different Cxcl12 proteins (see Fig. S3 in the supplementary material), we found that the internalization induced by medium containing Cxcl12b or Cxcl12a N33S was reduced compared with that promoted by Cxcl12a or Cxcl12b S33N (Fig. 4C and see Fig. S3 in the supplementary material).

Global changes in chemokine structure and oligomerization (Veldkamp et al., 2008), as well as binding to glycosaminoglycans, are known to influence chemokine function (Proudfoot et al., 2003; Handel et al., 2005). We tested these parameters in vitro and found that the position 33 point mutation did not affect ligand oligomerization (see Fig. S4A in the supplementary material), nor did it induce global changes in tertiary protein structure (see Fig. S4B in the supplementary material), nor influence binding to glycosaminoglycans (see Fig. S4C in the supplementary material).

In summary, the subfunctionalization of the *cxcl12* genes that allows the chemokines to carry out independent functions occurred through two processes: alterations in the expression pattern of the two genes and a change in the specificity of receptor binding. It is likely that similar mechanisms contributed, at least in part, to the current rich signaling repertoire of chemokines and their receptors as well as to the evolution of other receptor-ligand families.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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