β-arrestin control of late endosomal sorting facilitates decoy receptor function and chemokine gradient formation

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SUMMARY
A crucial regulator of Cxcl12 is the decoy receptor Cxcr7, which controls the level of the chemokine in the tissue. The molecular mechanisms that enable Cxcr7 to function as an efficient molecular sink are not known. Using zebrafish primordial germ cells as a model, we identify a novel role for β-arrestins in controlling the intracellular trafficking of Cxcr7. β-arrestins facilitate the recycling of Cxcr7 from late endosomal compartments back to the plasma membrane, whereas the internalized ligand undergoes lysosomal degradation. β-arrestins thus function in regulating chemokine gradient formation, allowing responding cells to discriminate between alternative migration targets in vivo.

KEY WORDS: Primordial germ cells (PGCs), β-arrestin, Chemokine gradient, Chemotaxis

INTRODUCTION
The chemokine receptor Cxcr7 has been shown to bind Cxcl12 in vitro (Balabanian et al., 2005; Burns et al., 2006). Cxcr7 function is important for heart development (Sierro et al., 2007), interneuron migration (Sánchez-Alcañiz et al., 2011; Wang et al., 2011), leukocyte trafficking (Cruz-Orengo et al., 2011) and for promoting breast and lung tumorigenesis (Miao et al., 2007; Luker et al., 2012). Some of these in vivo studies, as well as those conducted in vitro (Luker et al., 2010; Naumann et al., 2010), are consistent with the idea that Cxcr7 serves as a receptor that effectively scavenges Cxcl12a and does not influence downstream signaling pathways (reviewed by Mantovani et al., 2006). Despite its importance in controlling Cxcl12 activity in the tissue, the molecular mechanisms promoting the decay function of Cxcr7 are unknown. A useful in vivo model for exploring this issue is the migration of primordial germ cells (PGCs) (Boldajipour et al., 2008) in zebrafish.

Here we demonstrate that the efficiency of Cxcr7b as a molecular sink relies on a novel role for β-arrestins, molecules previously suggested to be important for downstream signaling of the receptor (Rajagopal et al., 2010). Specifically, we show that the internalized Cxcr7b-Cxcl12 complex is targeted to late endosomes, the receptor (Rajagopal et al., 2010). Specifically, we show that the previously suggested to be important for downstream signaling of germ cells (PGCs) (Boldajipour et al., 2008) in zebrafish.

Weidinger et al., 1999).

Materials and methods
Zebrafish strains
Fish of the AB background and those carrying the Tol-kop-EGFP-F-nosI-3’UTR (Blaser et al., 2006) or the gsc-GFP transgene were used.

MATERIALS AND METHODS

Zebrafish strains
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Western blotting
Protein extracts from 8-hpf zebrafish embryos were subjected to standard western blotting analysis using primary antibodies against pErk1/2, Erk1/2 (Cell Signaling) and α-tubulin (Acris) at 1:1000 dilution.

Cloning and RT-PCR of zebrafish β-arrestins
Primers used for cloning and RT-PCR of zebrafish arrdc1, arrb2a and arrb2b (NM_001159822, NM_214681 and NM_201124) are listed in supplementary material Table S1.

Microinjection into zebrafish embryos
RNA and/or morpholino antisense oligonucleotides (MOs) were microinjected into the yolk of 1-cell stage zebrafish embryos, or into one blastomere at the 8- or 16-cell stage. The RNA expression constructs and MOs used are listed in supplementary material Table S1.

The specificity and efficiency of MOs targeting arrb2a and arrb2b were validated by blocking the translation of mRNAs encoding EGFP-tagged reporters (supplementary material Fig. S11A,B).

In situ hybridization
Whole-mount in situ hybridization was performed as previously described (Weidinger et al., 1999).

Image acquisition and analysis
Time-lapse imaging was performed on an AxioImager.M1 microscope (Zeiss) with a dual-view filter (MAG Biosystems), Cascade II and CoolSNAP ES2 cameras (Photometrics) and VS laser control. Confocal images were acquired on an LSM 710 microscope (Zeiss). Images were processed using Imaris (Bitplane), MetaMorph (Molecular Devices) and ImageJ (NIH) software. The ZEN colocalization coefficient module (Zeiss) was used for quantifying the colocalization between the Cxcr7b signal and that of endosomal markers.

RESULTS AND DISCUSSION
Partial overlap between cxcrr7b and β-arrestin expression
β-arrestins were originally shown to desensitize activated G protein-coupled receptors and to promote receptor endocytosis (Laporte et al., 1999; Laporte et al., 2000). By facilitating receptor internalization, β-arrestins allow adaptation to a persistent stimulus, thus effectively reducing the signaling level (Benovic et al., 1987). β-arrestins were also shown to be important for internalization and ligand-dependent depletion of Cxcr7 from the cell membrane...
Cxcr7 is unknown. The actual mechanism by which expressing the receptor (Luker et al., 2009). Nevertheless, the expression pattern of the zebrafish germ cell migration. We first monitored the RNA expression of other receptors, which require activated-arrb2a (arrdc1, arrb2b, cxcr7b and cxcr12a expression patterns in the anterior somites, freeing lateral Cxcr12a expression domains (magenta) from Cxcr7 and β-arrestin effects. The procedure for generating uniform Cxcr7b-EGFP expression (green) and cell clones knocked down for β-arrestins by injection with MOs targeting all β-arrestins (or control MO) and Histone H2B-mCherry for clone labeling (red). In control 7-hpf mosaic embryo (left), Cxcr7b is found in small vesicles. In cells compromised for β-arrestin function (red nucleus, right), Cxcr7b vesicles are often enlarged. (B) Average colocalization coefficient (see supplementary material Fig. S12) among Cxcr7b-ECFP and Rab7-mCherry (at 5 hpf), Lamp1-DsRedmonomer and Rab5-mCherry (at 8 hpf) in control and β-arrestin knockdown cells. Data were averaged among six measurements (one image per embryo, ~80 cells in the field of view). *P<0.05, **P<0.01, Student’s t-test. Error bars indicate s.e.m.

To determine the role that β-arrestins play in the context of Cxcr7b function in embryogenesis, we examined their function in germ cell migration. We first monitored the RNA expression pattern of the zebrafish β-arrestin homologs β-arrestin 1, β-arrestin 2a and β-arrestin 2b (arrdc1, arrb2a and arrb2b). We found that, similar to the cxcr7b expression pattern, these RNAs are initially ubiquitously expressed and become restricted to specific tissues at later stages of development (Fig. 1A,C,E,F; supplementary material Fig. S1).

β-arrestins control the endosomal sorting of Cxcr7b

We subsequently examined whether β-arrestins are involved in controlling the subcellular localization of Cxcr7b. We expressed an EGFP-tagged version of Cxcr7b in all cells of the embryo and generated cell clones in which β-arrestins were knocked down using morpholino antisense oligonucleotides (MOs) (Fig. 1G, cells with red nucleus). Unexpectedly, the level of the receptor on the cell membrane was not increased in β-arrestin knockdown cells (supplementary material Fig. S2), suggesting that receptor internalization is not inhibited under these conditions. Strikingly, the intracellular distribution of Cxcr7b was altered in cells knocked down for β-arrestin. Specifically, in manipulated cells, Cxcr7b protein accumulated in less dynamic intracellular structures that, in some cases, were significantly larger than in control cells (Fig. 1H; Fig. 2A,B; supplementary material Fig. S3 and Movies 1, 2). Thus, whereas Cxcr7b internalization per se does not depend on β-arrestins in this case, endosomal sorting of the internalized receptor is strongly affected. Interestingly, this role differs from that shown in the context of other receptors, which require β-arrestin for internalization (e.g. Ferguson et al., 1996; Goodman et al., 1996; Galliera et al., 2004), as well as in the case of Cxcr7, the internalization of which in vitro appears to be correlated with interaction with β-arrestin (Ray et al., 2012). β-arrestins can thus function in different phases of receptor trafficking depending on the receptor and the context.

To determine the basis for the abnormal distribution of Cxcr7b in cells knocked down for β-arrestin function, we monitored the localization of the receptor with respect to that of specific endocytic compartments (Huotari and Helenius, 2011). We monitored the subcellular localization of Cxcr7b-ECFP fusion protein relative to that of mCherry fused to Rab5, which labels early and recycling endosomes (Gorvel et al., 1991), Rab11, which is found in recycling endosomes (Ullrich et al., 1996), or Rab7, a late endosomal marker (Gorvel et al., 1991; Méresse et al., 1995), or relative to Lamp1-DsRedmonomer fusion protein, which is localized to late endosomes and lysosomes (Rohrer et al., 1996). Consistent with its suggested role as a molecular sink recycling between the membrane and intracellular compartments (Weber et al., 2004; Mantovani et al., 2006), we could identify Cxcr7b in Rab5-positive vesicles at stages when PGCs migrate (Fig. I). Interestingly, whereas β-arrestin knockdown did not change the distribution of Cxcr7b in Rab11 endosomes (supplementary material Fig. S4A), it reduced the proportion of Rab5 vesicles containing Cxcr7b (Fig. I), suggesting that β-arrestins regulate the level of Cxcr7b in early endosomes. Indeed, the opposite tendency was observed when monitoring the colocalization of Cxcr7b with Lamp1 vesicles in the absence of β-arrestins (Fig. I; supplementary material Fig. S5). Conducting the same measurements on a smaller microscopic field, the enhanced colocalization of Cxcr7b with Lamp1 vesicles in the absence of β-arrestin function was also observed (supplementary material Fig. S4B). These data suggest that β-arrestins increase the level of Cxcr7b in recycling compartments, thereby elevating its efficiency as a Cxcl12a sink. In the absence of β-arrestins, a higher proportion of the receptor is targeted to, and retained in, non-recycling endosomal compartments, interfering with efficient ligand uptake.

To confirm these statistical findings and observe the dynamics of Cxcr7b trafficking in wild-type and β-arrestin-deficient cells, we followed Cxcr7b-EGFP-containing vesicles in cells co-expressing the Lamp1-DsRedmonomer protein. In control cells, the Cxcr7b- and Lamp1-labeled foci showed fusion and splitting events (Fig. 2A; supplementary material Movie 1), consistent with the idea that Cxcr7b can exit from the late endosomal
compartment and recycle to the membrane. By contrast, in cells depleted of β-arrestins, the Cxcr7b- and Lamp1-labeled foci showed reduced dynamics (Fig. 2B; supplementary material Movie 2). Specifically, in the absence of β-arrestins, Cxcr7b-labeled foci fused with Lamp1-positive vesicles at a normal rate but separated less frequently from this compartment, resulting in fewer vesicles of increased size (Fig. 2B; supplementary material Fig. S3, Fig. S6A and Movie 2).

To observe the fate of internalized Cxcl12a with respect to its receptor, we transplanted cells expressing Cxcr7b-DsRed into embryos expressing Cxcl12a-EGFP. Interestingly, the EGFP signal was rapidly reduced following internalization, suggesting ligand degradation, whereas the signal of the receptor appeared stable (Fig. 2C; supplementary material Fig. S6B and Movie 3). The apparent degradation of Cxcl12a depends on Cxcr7b, which targets it to late endosomal compartments, as internalized ligand not in association with the decoy receptor appeared stable at the same time (supplementary material Movie 4). Notably, in cells knocked down for β-arrestin function, Cxcr7b that was trapped in the lysosomes was also subject to degradation (supplementary material Fig. S7), a possible contributing factor to the reduced level of recycled receptor on the cell membrane in this case (supplementary material Fig. S2).

Together, these observations are consistent with the idea that β-arrestins facilitate efficient chemokine scavenging activity of Cxcr7b by promoting its proper endosomal sorting (Fig. 2D). Consistent with the notion that β-arrestins function in receptor trafficking rather than actual signaling, we found that Erk1/2 (Mapk3/1 – Zebrafish Information Network) protein level and activation (phosphorylation, pErk1/2) are unaffected by Cxcr7b knockdown or overexpression (supplementary material Fig. S8). These results are consistent with the idea that, at the relevant stages of embryonic development, Cxcr7b does not activate MAPK signaling pathways, lending further support to the notion that the receptor functions as a decoy that regulates zebrafish PGC migration through signaling-independent mechanisms.

It is important to note that whereas β-arrestin knockdown affected the subcellular localization and presumably the function of Cxcr7b, we did not observe any effect on the distribution of the other Cxcl12a receptor Cxcr4b (supplementary material Fig. S9A,C,D).

**β-arrestins control the efficiency of Cxcr7b as a decoy receptor and the distribution of Cxcl12 in the tissue**

We next sought to determine whether the alterations in the subcellular distribution of Cxcr7b are relevant for Cxcl12 distribution and the directed migration of PGCs. We followed the position of the PGCs in response to manipulations of β-arrestin function. Cellular domains in which β-arrestins were knocked down were generated in embryos expressing uniform levels of Cxcr7b and Cxcl12a (Fig. 3A). Notably, we observed a strong bias of PGC localization to domains depleted of β-arrestins (Fig. 3A,B). In the absence of Cxcl12a, or of Cxcr7b, the apparent attractive activity of β-arrestin-depleted domains was abolished (Fig. 3A), supporting the idea that β-arrestin function is required for controlling Cxcl12a distribution in the tissue by promoting the sink activity of Cxcr7b.

To test this notion, we monitored the effect that β-arrestins have on the distribution of fluorescently tagged Cxcl12a in vivo. In these experiments, we uniformly expressed Cxcr7b and Cxcl12a-Venus in embryos lacking endogenous Cxcl12a. In such embryos, we generated mCherry-labeled cellular domains knocked down for β-arrestins (Fig. 3C). Strikingly, in contrast to the uniform Cxcl12a-Venus signal in control embryos (Fig. 3C, Control MO panels; Fig. 3D, upper panel), domains depleted of β-arrestins showed higher levels of the chemokine (Fig. 3C, β-arrestin MO panels; Fig. 3D, lower panel). This function of β-arrestins depended on Cxcr7b activity, as knocking down the receptor abolished the effect on Cxcl12a distribution (supplementary material Fig. S10). Together, these findings suggest that β-arrestins can control the chemokine level in the tissue by affecting the endosomal sorting of Cxcr7b.
To examine the role that β-arrestins play in the physiological context of the developing embryo, we examined their involvement in facilitating PGC migration away from the developing somites. Comparing the expression patterns of arrb2a and arrb2b with that of cxcl12a and cxcr7b at 12 hours postfertilization (hpf) revealed significant overlap of arrb2a, cxcr7b and cxcl12a in the forming somites and of cxcr7b and arrb2b close to and within the midline (Fig. 1B-F), both of which represent regions of the embryo that PGCs vacate (Weidinger et al., 1999). These findings raise the possibility that the level of Cxcl12a protein produced in the developing somites or diffusing to the midline is reduced at these locations by the action of Cxcr7b and the relevant β-arrestins. This scenario could account for the observed migration of PGCs that were born within the somite-forming region towards lateral locations of Cxcl12a expression, thereby approaching the site of the developing gonad (Fig. 4A; supplementary material Movie 5).

To test this possibility, we compared the number of PGCs located within the somites in embryos knocked down for a given β-arrestin versus a combination of the three. Indeed, embryos in which all β-arrestins are knocked down show, on average, 4.61 cells at forming somites as compared with 0.51 in control embryos (Fig. 4C). Using time-lapse microscopy to monitor the behavior of the PGCs, we find that the ectopic cells that did not vacate the somatic mesoderm in β-arrestin morphants showed a cellular phenotype reminiscent of that observed in embryos depleted for Cxcr7b function. Specifically, these cells fail to polarize or migrate (Fig. 4B; supplementary material Movie 6) (Boldajipour et al., 2008), consistent with the idea that they encounter high levels of Cxcl12a at this location.

The cellular phenotype observed in embryos knocked down for β-arrestins differs from that associated with defects involving the guidance receptor Cxcr4b, where motility per se is unaffected (Doitsidou et al., 2002). To further exclude a role for Cxcr4b in the evolution of the β-arrestin-dependent phenotype, we monitored the position of PGCs guided by a receptor mutated such that it cannot internalize (Minina et al., 2007). The ability of these PGCs to clear the somites and migrate laterally also depended on the function of β-arrestins (supplementary material Fig. S9B), showing that this migration step is independent of Cxcr4b internalization. Together, these results assign a novel role for β-arrestins in the modulation of Cxcl12a distribution in the tissue, ensuring proper target choice by PGCs (Fig. 4D).

The choice between conflicting chemotactic cues is a recurring theme in development and homeostasis. For instance, a migrating macrophage might have to direct its response in a field of cues that includes attraction signals originating from a wound, developmental guidance cues and signals emanating from apoptotic cells (Moreira et al., 2010). The potency of each of these competing signals depends, at least in part, on the absolute available level of the attractant. One of the mechanisms underlying the regulation of protein levels in discrete domains could involve molecules acting as sinks. Such a mechanism is exemplified in the context of PGC migration; the magnitude of attraction by Cxcl12a molecules acting as sinks. Such a mechanism is exemplified in the context of PGC migration; the magnitude of attraction by Cxcl12a

β-arrestins control the discrimination between alternative migration targets

PGCs follow the dynamics of Cxcl12a expression in the embryo. During early somitogenesis stages, cxcl12a RNA is expressed along the lateral plate mesoderm, which includes the site where the gonad develops, and the forming somites (Doitsidou et al., 2002) (Fig. 4C). Whereas both expression domains are inherently attractive for PGCs, the cells prefer the relevant lateral cxcl12a expression domain, while ‘ignoring’ the other.

![Diagram of β-arrestins control the level of Cxcl12a in the tissue and can direct PGC migration](image-url)
reduced severity of the β-arrestin phenotype could result from β-arrestin-independent Cxcr7b function and from masking of the early phenotype by maternally provided β-arrestin.

Finally, it is noteworthy that β-arrestins are expressed in other regions of the zebrafish embryo, such as in the posterior lateral line and the axial vasculature (see www.zfin.org), that engage in chemokine-controlled cell migration (David et al., 2002; Siekmann et al., 2009). Additionally, β-arrestin- and CXCR7-dependent accumulation of CXCL12 is observed in human breast cancer cells grown in vitro (Luker et al., 2010). It would be interesting to investigate the role of β-arrestins in these other contexts.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References

Fig. 4. Role of β-arrestins in discrimination between Cxcl12a expression domains. (A, B) Snapshots from supplementary material Movie 5 (A) and Fig. S6 (B). In early somitogenesis stages, PGCs (asterisks) specified close to the midline migrate laterally (A). In an embryo lacking β-arrestin function (B), this movement is not observed. (C) β-arrestins promote migration of PGCs away from the somites. Red bars (y-axis on left) indicate the average number of cells remaining in the somites; error bars indicate s.e.m. The distribution of the results is indicated by circles (y-axis on right); N, the number of embryos analyzed; ** P<0.05, *** P<0.001, Student's t-test. Bottom panels show examples of in situ hybridizations using nanos1 (nos1; blue, PGCs) and cxcl12a (brown) probes used to generate the graphs. (D) A model for PGC migration during early somitogenesis in wild type (left) and in embryos compromised for β-arrestins (right). The sink function of Cxcr7b (purple), aided by β-arrestins (red ellipsoid), converts uniform cxcl12a mRNA expression into a chemotactic Cxcl12a gradient, directing the PGCs to the right. In the absence of β-arrestins, Cxcr7b function is compromised, increasing Cxcl12a levels in the forming somites and hindering the migration of PGCs towards their target.


