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miRNA regulation of Sdf1 chemokine signaling provides genetic robustness to germ cell migration

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microRNAs (miRNAs) function as genetic rheostats to control gene output. Based on their role as modulators, it has been postulated that miRNAs canalize development and provide genetic robustness. Here, we uncover a previously unidentified regulatory layer of chemokine signaling by miRNAs that confers genetic robustness on primordial germ cell (PGC) migration. In zebrafish, PGCs are guided to the gonad by the ligand Sdf1a, which is regulated by the sequestration receptor Cxcr7b. We find that miR-430 regulates *sdf1a* and *cxcr7* mRNAs. Using target protectors, we demonstrate that miR-430–mediated regulation of endogenous *sdf1a* (also known as *cxcl12a*) and *cxcr7b* (i) facilitates dynamic expression of *sdf1a* by clearing its mRNA from previous expression domains, (ii) modulates the levels of the decoy receptor Cxcr7b to avoid excessive depletion of Sdf1a and (iii) buffers against variation in gene dosage of chemokine signaling components to ensure accurate PGC migration. Our results indicate that losing miRNA-mediated regulation can expose otherwise buffered genetic lesions leading to developmental defects.

Biological systems can compensate for genetic and environmental perturbations. Genetic buffering allows invariance of the phenotype in the face of perturbations. This endows the organism with reduced susceptibility to mutations and results in robustness[1,2]. External perturbations may arise from changes in the environment, whereas internal factors derive from inexact processes within a cell such as transcriptional bursts, changes in gene dosage and leaky transcription[3–5]. This variation in gene expression may lead to fluctuation in activity, limiting the accuracy of specific cellular processes. In order to prevent these fluctuations from having a severe impact on phenotype, several mechanisms for providing robustness have evolved. First, creating networks of feedback and feedforward loops can reduce the effects of variation by stabilizing different phenotypic states such that minor variation will not be sufficient to alter the current state[6]. Second, functional redundancy within regulatory networks provides stability because multiple changes need to occur to perturb the system[1]. Third, genetic buffering prevents small changes at the genetic level from influencing the phenotype. For example, the chaperone protein HSP90 facilitates proper folding of mutant proteins, masking the effects of these mutations by preventing variation in expression and activity[7,8]. Finally, additional mechanisms might be in place at the RNA level to provide robustness and guard against transcriptional fluctuations. It has been proposed that high levels of transcription coupled with inefficient translation can lower intrinsic noise in protein output[4,5]. This is supported both by theoretical models and experimental evidence in unicellular organisms[4]. Because of their ability to regulate a wide variety of genes[9], their function as rheostats that modulate the mRNA and protein output of their target genes[10,11], and their position within network motifs (reviewed in refs. 3,6,12), it has been postulated that miRNAs may play an important role in buffering biological systems against genetic variation. A prominent example in *Drosophila* is miR-7, which functions within a feedforward loop to guard against the consequences of temperature fluctuation to stabilize a phenotypic state[13]. However, few studies have experimentally addressed the role of miRNAs in genetic robustness in vertebrates.

RESULTS

Loss of miRNAs leads to mislocalized PGCs

In this study, we investigate the role of miRNAs in buffering against genetic variation in vertebrates using long-distance cell migration as a model. In this context, slight perturbations in protein levels or distribution of guidance cues might lead to erroneous cell migration because the chance that such small fluctuations will affect cell migration accumulates with the distance the cells need to migrate. PGC migration in zebrafish is a prominent example of such a long-distance migratory process that is likely evolutionarily reinforced (reviewed in ref. 14). PGCs express the chemokine receptor Cxcr4b and follow the shifting expression domain of its ligand Sdf1a (also known as Cxcl12a) as they migrate through the gastrulating embryo to reach the site of the future gonad. The dynamic expression of the ligand in the somatic tissues plays a crucial role in determining the migratory path and must be tightly regulated at the protein and the mRNA levels (reviewed in ref. 15). It has been proposed that the Sdf1a protein gradient is refined by Cxcr7, a 'decoy' receptor that is thought to act as an Sdf1a sink[16,17]. At the mRNA level, new transcriptional domains must be coupled with rapid removal of the transcripts that remain in previous domains of expression, yet the mechanisms underlying this regulation are

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elusive. One possibility involves rapid removal of *sdf1a* transcripts from tissues where they are no longer needed through miRNA-mediated degradation. To test this idea, we examined PGC localization in the absence of miRNAs. We generated mutant embryos lacking both the maternal and the zygotic contribution of the miRNA-processing enzyme dicer (termed here MZ*dicer*)[18,19]. Notably, in such embryos, we found that PGCs were frequently mislocalized. This mismigration was not due to disruption of the migratory path, as these somatic tissues are properly specified in MZ*dicer*. Importantly, reintroducing miR-430 or one of its mammalian homologs, miR-302, into MZ*dicer* mutants rescued the localization of PGCs (**Supplementary Fig. 1**). These results suggest that miR-430–mediated repression of specific target of mRNAs may play a role in PGC migration.

miR-430 can regulate expression of chemokine signaling genes

Because miRNAs accelerate the decay of their target mRNAs, we previously used microarrays to identify miRNA targets by comparing gene expression in wildtype, MZ*dicer* and MZ*dicer* embryos injected with miR-430 (ref. 19). Analysis of these data revealed that *sdf1a* and *cxcr4b* might be repressed by miR-430 (**Supplementary Fig. 1**). To determine whether additional genes in the chemokine signaling pathway are directly regulated by miR-430, we searched their 3′ untranslated regions (UTRs) for sequences complementary to the miR-430 'seed' sequence (reviewed in ref. 9). This analysis revealed that 3′ UTRs of both ligands (*sdf1a* and *sdf1b* (also known as *cxcl12b*)) and receptors (*cxcr4a*, *cxcr4b*, *cxcr7a* and *cxcr7b*) contain putative miR-430 target sites (**Supplementary Figs. 2** and **3**). If these transcripts are regulated by miR-430, then the target site in each 3′ UTR should influence their translation. To test this hypothesis, we co-injected reporter mRNAs encoding the GFP open reading frame and each full length 3′ UTR with a DsRed control mRNA (**Fig. 1a**). To analyze the level of regulation of each target, we quantified the fluorescence of the GFP reporter relative to the DsRed control in the presence or absence of miRNAs (**Fig. 1b**). These 3′ UTRs conferred repression of GFP in wildtype embryos but not in MZ*dicer* mutants that lack mature miR-430 (**Fig. 1c**,**d** and **Supplementary Fig. 2**). Next, we tested whether miR-430 sites play a direct role in regulation. Mutating the miR-430 sites in each 3′ UTR (GCACUU to GCUGAU) prevented miRNA-mediated repression of reporter mRNAs in wildtype embryos (**Fig. 1c**,**d** and **Supplementary Fig. 2**). To further characterize the regulation of the *sdf1a* 3′ UTR, we mutated individual target sites in this reporter. We found that mutation of the first target site was necessary and sufficient to relieve repression of the GFP reporter. However, mutating the second or third target site did not affect regulation of the reporter (**Supplementary Fig. 3**). These results indicate that the ligand (*sfd1a*) and the decoy receptors *cxcr7a* and *cxcr7b* are more strongly regulated by miR-430 (>twofold) than *cxcr4a* and *cxcr4b* and *sdf1b* (<twofold). On the basis of the regulation provided by the *sdf1a* and *cxcr7* 3′ UTRs and their essential role in PGC migration, we focused our analysis on these genes.

Target protectors specifically relieve miR-430–mediated repression

The analysis of individual miRNA-mRNA interactions in animals that are depleted of most miRNAs poses a challenge because many miRNA targets are misregulated[19,20]. To investigate the physiological function of miR-430–mediated regulation of *sdf1a* and *cxcr7a* and *cxcr7b*, we used target protectors. Target protectors are morpholino antisense oligonucleotides that are complementary to the miRNA recognition site in the transcript and interfere with miRNA-mRNA interactions, thus protecting a specific target from its miRNA (**Fig. 2a**)[21]. Three lines of evidence indicate that target protectors interfere with the regulation of *sdf1a* and *cxcr7* by miR-430. First, target protectors for *sdf1a*, *cxcr7a* and *cxcr7b* restore expression of the GFP reporter to a level similar to that observed in the absence

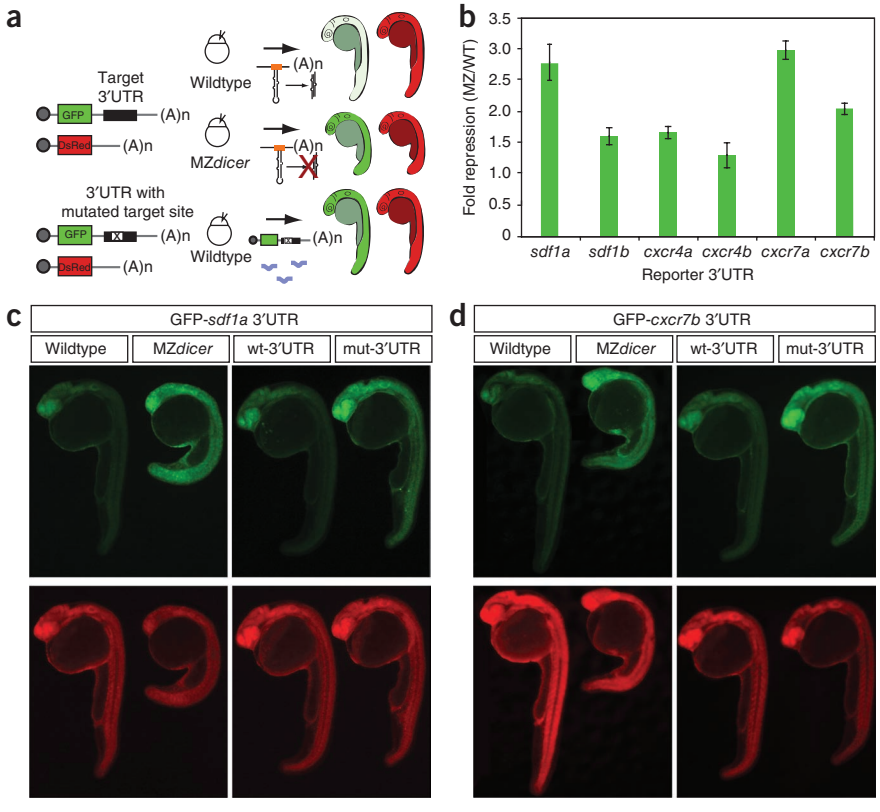

Figure 1 miR-430 target validation of chemokine signaling genes. (**a**) Schematic representation of the experimental setup. Expression of GFP reporters with the 3′ UTRs of putative targets is compared in wildtype and MZ*dicer* embryos. DsRed mRNA lacking a target site was co-injected as a control. To test whether the miR-430 target site plays a role in the regulation, wildtype embryos were also injected with wildtype reporters (wt-3′UTR) or mutant reporters where three bases in the miR-430 target site were mutated (mut-3′UTR). All three miR-430 target sites in the *sdf1a* 3′ UTR were mutated to generate the mutant reporter. (**b**) Quantification of the GFP fluorescence in MZ*dicer* compared to wildtype embryos injected with each GFP reporter mRNA and DsRed control. GFP fluorescence was normalized to the DsRed control. Data are shown as mean ± standard deviation (s.d.). (**c**,**d**) Fluorescence microscopy shows GFP (green) and DsRed (red) expression in 24–28 hpf embryos injected with GFP reporters with *sdf1a* or *cxcr7b* 3′ UTR. Endogenous miR-430 represses the expression of each reporter in wildtype but not in MZ*dicer* embryos. Similarly, wildtype embryos injected with the mut-3′UTR reporter fail to repress GFP. The sequences of the wildtype or the mutant target site and miR-430 are shown in **Supplementary Figure 2**.

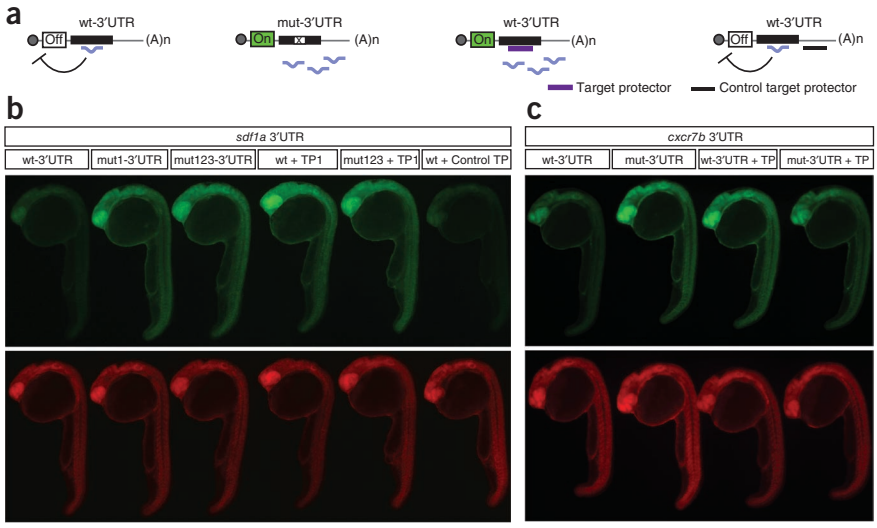

Figure 2 Target protectors prevent miRNA-mediated repression of target GFP reporters. (**a**) The schematic shows repression of targets by an miRNA and loss of repression of mutated reporters. GFP reporters are protected by target protectors (purple), whereas binding of the control target protector (black) downstream of the miR-430 target site does not prevent miRNA-mediated repression. (**b**,**c**) GFP and DsRed fluorescence in 24–28 hpf embryos injected with GFP reporters. Expression of a wildtype GFP *sdf1a* 3′ UTR reporter is repressed. Injection of *sdf1a* target protector (TP1), but not a control target protector, blocks miR-430–mediated repression of the GFP reporter. Expression of the mut-3′UTR GFP reporter with the first (mut1-3′UTR) or all three mutated target sites (mut123-3′UTR) is shown for comparison. (**c**) Derepression of the GFP-*cxcr7b* wt-3′UTR reporter was also observed upon injection of the *cxcr7b* target protector (TP). Predicted Watson-Crick pairing of the 3′ UTR target sites with each target protector and miR-430 are shown in **Supplementary Figure 3**.

of miR-430–mediated repression (mutated reporter; **Fig. 2b**,**c**, and **Supplementary Figs. 3** and **4**). We injected individual target protectors for each target site in the *sdf1a* 3′ UTR. Consistent with the results obtained during the mutagenesis of individual target sites, injection of a target protector complementary to the first site was sufficient to relieve repression to the level of the mutated reporter (**Fig. 2b** and **Supplementary Fig. 3**). Conversely, target protectors for the second and third sites did not have a noticeable effect, suggesting that the first miR-430 target site in *sdf1a* confers most of the regulation. Thus in all following experiments, *sdf1a*-TP refers to the target protector complementary to the first target site in *sdf1a*. Second, injection of the target protectors into MZ*dicer* mutants did not affect the expression level of the endogenous target mRNA compared to control MZ*dicer* mutants (**Supplementary Fig. 4**), suggesting that each target protector does not cause stabilization of the target mRNA independent of the miRNA. Third, a target protector designed to bind to a region of the *sdf1a* 3′ UTR approximately 200 bases downstream of the first miR-430 binding site (*sdf1a*-control-TP) did not affect repression of the reporter, indicating that target protection depends on the overlap with the miRNA target site (**Fig. 2b**). These results indicate that *sdf1a*-TP, *cxcr7a*-TP and *cxcr7b*-TP provide specific tools to interfere with miRNA-mediated regulation of endogenous *sdf1a* and *cxcr7a* and *cxcr7b*.

Regulation by miR-430 promotes accurate PGC migration

To investigate the physiological role of each miRNA-target interaction, we quantified PGC localization in target protector–injected embryos. Blocking miR-430 regulation of *sdf1a* or *cxcr7b* by injection of *sdf1a*-TP or *cxcr7b*-TP but not *cxcr7a*-TP increased the percentage of embryos with mislocalized germ cells at 24 h post fertilization (hpf) (from 30% in wildtype to more than 60% in injected embryos) (**Fig. 3** and **Supplementary Fig. 5**). Injection of *sdf1a*-TP resulted in an increase in germ cells that were outside the location of the future gonad (bracket in **Fig. 3d**) and remained in the posterior pronephric regions (**Fig. 3c**,**d** and **Supplementary Fig. 6**). Blocking the regulation of *sdf1a* by miR-430 increased both the number of mislocalized cells and the fraction of embryos with mislocalized cells posterior to the future gonad. Conversely, protecting *cxcr7b* from miR-430 regulation caused mislocalization of a significantly higher number of cells to ectopic positions in the tail (**Fig. 3e** and **Supplementary Fig. 6**). These results are consistent with a model in which misregulation of Cxcr7b depletes the Sdf1a protein, causing the PGCs to lose the correct migratory path. Two controls support the specificity of these effects. First, injection of *sdf1a*-control-TP did not significantly increase the percentage of embryos with mislocalized PGCs (**Fig. 3b**,**c**). Second, we asked whether the target protector phenotypes are caused by the binding of the target protectors to their cognate target 3′ UTR rather than off-targets. Because target protectors block the repression of the target by the miRNA, increasing the protein expression from the mRNA, we reasoned that if the target protector phenotype is due to an increase in expression of the target gene, it should be rescued by lowering the translation of this target (**Fig. 3a**). Indeed, a low level of a translation-blocking morpholino complementary to the *sdf1a* AUG start site[22] rescued the mislocalization seen after injection of *sdf1a*-TP alone (**Fig. 3c**,**d**,**f**). We observed similar results for *cxcr7b*-TP and its AUG-blocking morpholino[17] (**Fig. 3c**,**e**,**g**). These results suggest that miR-430 regulation of endogenous *sdf1a* and *cxcr7b* plays a role in PGC migration.

miR-430 promotes clearance of *sdf1a*

Germ cell migration depends on the dynamic expression of the ligand Sdf1a that elicits local attraction of the PGCs to a series of intermediate positions before reaching the future gonad[14,23]. Guidance is therefore achieved in part by modifying the position of the attractive domain. This process requires tightly controlled expression of *sdf1a* in new domains and rapid degradation of *sdf1a* transcripts lingering in old expression domains. Initially, *sdf1a* is expressed along the lateral mesoderm region and later becomes restricted to the anterior domains to attract the cells to the future gonad (**Fig. 3h**). However, the molecular mechanisms responsible for this dynamic expression are largely unknown. Based on the germ cell mismigration observed in *sdf1a*-TP–injected embryos, we hypothesized that miR-430 could facilitate clearance of previously expressed transcripts and, therefore, sharpen the domain of ligand expression. Using *in situ* hybridization, we found that the *sdf1a* 3′ UTR can enhance degradation of the GFP reporter mRNA, an effect that is dependent on the first miR-430 target site (**Supplementary Fig. 7**). We then examined the impact of miR-430 on endogenous *sdf1a* transcripts in MZ*dicer* and target protector–injected embryos. Throughout PGC migration, *sdf1a* expression was stronger in *sdf1a*-TP–injected and MZ*dicer* embryos (**Fig. 3h** and **Supplementary Fig. 8**). Consistent with our

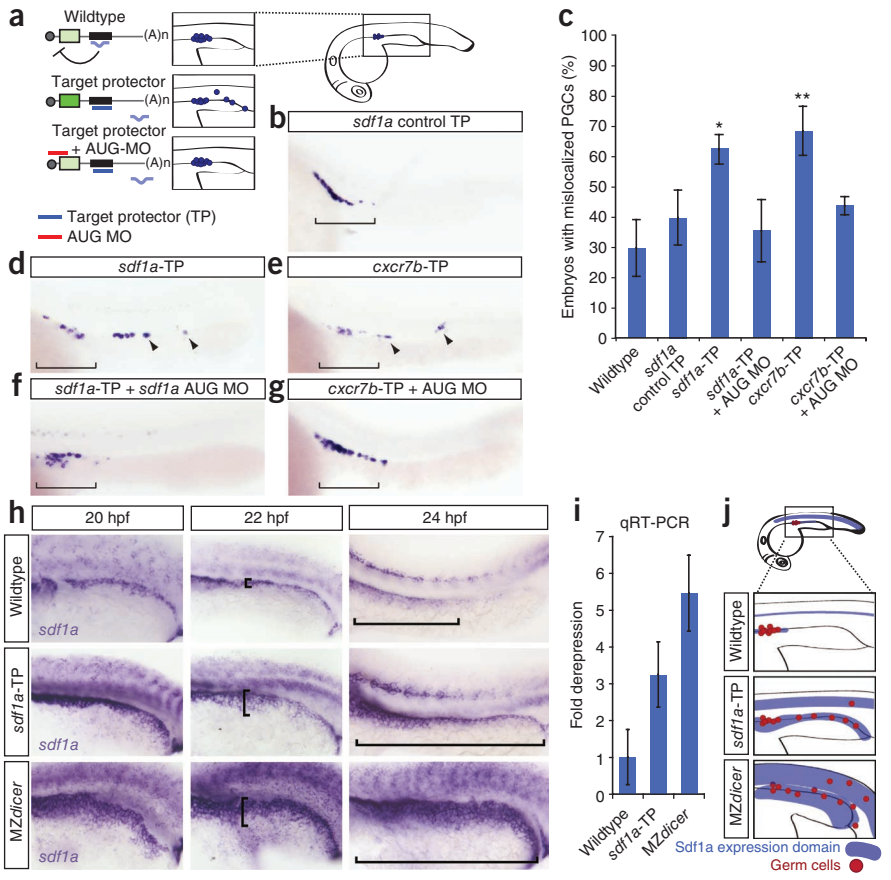

Figure 3 Blocking miR-430–mediated repression of *sdf1a* and *cxcr7b* causes PGC mislocalization and expanded *sdf1a* expression. (**a**) Schematic representation of the experimental setup. Injecting the target protector (purple) blocks miRNA-mediated repression, increasing mRNA expression and leading to mislocalization of cells. Co-injecting a morpholino to reduce translation of the target gene (red, AUG MO) rescues the mislocalization phenotype. The inset shows the region of the embryo depicted in **b** and **d**–**h**. (**b**,**d**–**g**) Whole mount *in situ* of nanos mRNA, labeling PGCs in 24 hpf embryos. Bracket shows correct localization of PGCs. Arrowheads identify mislocalized PGCs. (**c**) Quantification of the percentage of embryos with mislocalized PGCs in each experimental condition as indicated. A significantly increased number of target protector–injected embryos have mislocalized PGCs. $*P = 1.185 \times 10^{-7}$ for *sdf1a*-TP; $**P = 2.52 \times 10^{-7}$ for *cxcr7b*-TP; two-tailed Fisher's exact test. Error bars, ± s.d. (**d**,**e**) Representative images of PGC mislocalization are shown. (**f**,**g**) Co-injection of a low level of the corresponding AUG MO rescues the target-protector phenotype (*sdf1a* AUG MO, 0.01 pM; *cxcr7b* AUG MO, 0.045 pM). (**h**) *In situ* hybridization to detect *sdf1a* mRNA. The trunk of embryos at 20 hpf, 22 hpf and 24 hpf are wildtype embryos, embryos injected with *sdf1a*-TP or MZ*dicer* embryos. Brackets illustrate the extension of the *sdf1a* expression domain along the pronephric region. (**i**) qPCR for *sdf1a* in 24 hpf wildtype, *sdf1a*-TP–injected and MZ*dicer* embryos. We observed an increase in expression in the absence of miR-430–mediated repression. (**j**) Schematic summary of Sdf1a tail expression and the resulting PGC mislocalization.

previously published microarray data (**Supplementary Fig. 1**)[19], quantitative PCR (qPCR) showed that *sdf1a* mRNA levels are increased in MZ*dicer* embryos (**Fig. 3i**). Interfering with miR-430 regulation resulted in an expansion of the *sdf1a* expression domain, with a failure to clear it from more posterior regions of the trunk mesoderm (**Fig. 3h**). The regions that failed to clear *sdf1a* mRNA coincide with the positions of mislocalized germ cells in *sdf1a*-TP–injected embryos. Thus, miR-430 is required to modulate the amount of *sdf1a* RNA and to shape its expression pattern by clearing transcripts from old expression domains.

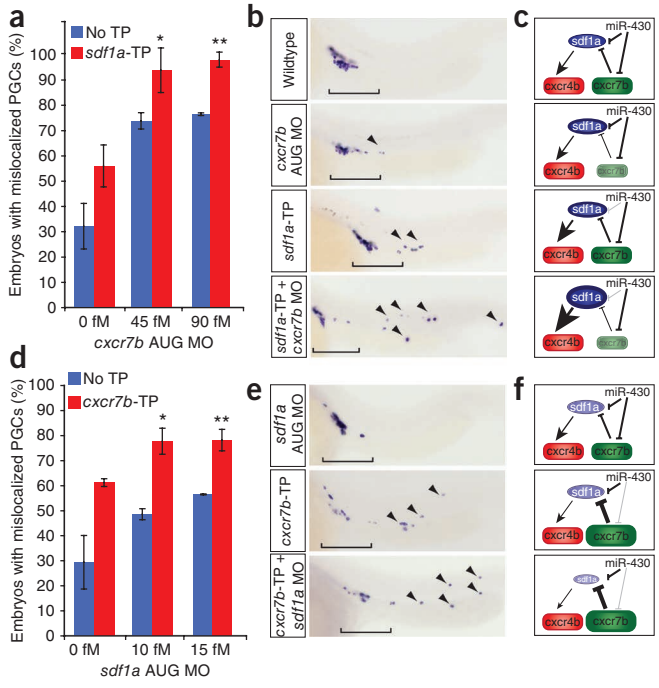

Figure 4 miR-430 and Cxcr7b act in a functionally redundant manner to refine Sdf1a expression. (**a**,**d**) Quantification of PGC mislocalization. We injected embryos at the one-cell stage with a morpholino targeting the start site (AUG MO) of *cxcr7b* (**a**) or *sdf1a* (**d**). We injected these AUG MOs at low concentrations, which were insufficient to completely knock down the transcript and which caused a weak mislocalization phenotype. (**a**) Co-injecting *sdf1a*-TP and *cxcr7b* AUG MO caused significantly more mismigration than injection of *sdf1a*-TP or the same amount of the AUG MO alone ($*P = 4.08 \times 10^{-3}$ for *sdf1a*-TP + 45 fM compared to 45 fM alone; $**P = 4.87 \times 10^{-3}$ for *sdf1a*-TP + 90 fM compared to 90 fM alone; two-tailed Fisher's exact test), suggesting that miR-430 regulation of *sdf1a* mRNA can partially compensate for a reduction of *cxcr7b*. Similarly, co-injecting *cxcr7b*-TP and *sdf1a* AUG MO significantly enhances the mislocalization phenotype ($*P = 8.93 \times 10^{-4}$ for *cxcr7b*-TP + 10 fM compared to 10 fM alone; $**P = 0.016$ for *cxcr7b*-TP + 15 fM compared to 15 fM alone; two-tailed Fisher's exact test), suggesting that regulation of *cxcr7b* by miR-430 prevents excessive clearance of the *sdf1a*. Data are shown as mean ± s.d. (**b**,**e**) nanos *in situ* at 24 hpf to visualize the location of germ cells. Brackets indicate correctly localized PGCs, and arrowheads show mislocalized cells. (**c**,**f**) Scheme representing the predicted effect of the experimental conditions on Sdf1a and Cxcr7b shown in **b** and **e**. The added effect of removing miR-430 targeting and modulation by Cxcr7b supports a functional redundancy of miR-430 and Cxcr7b.

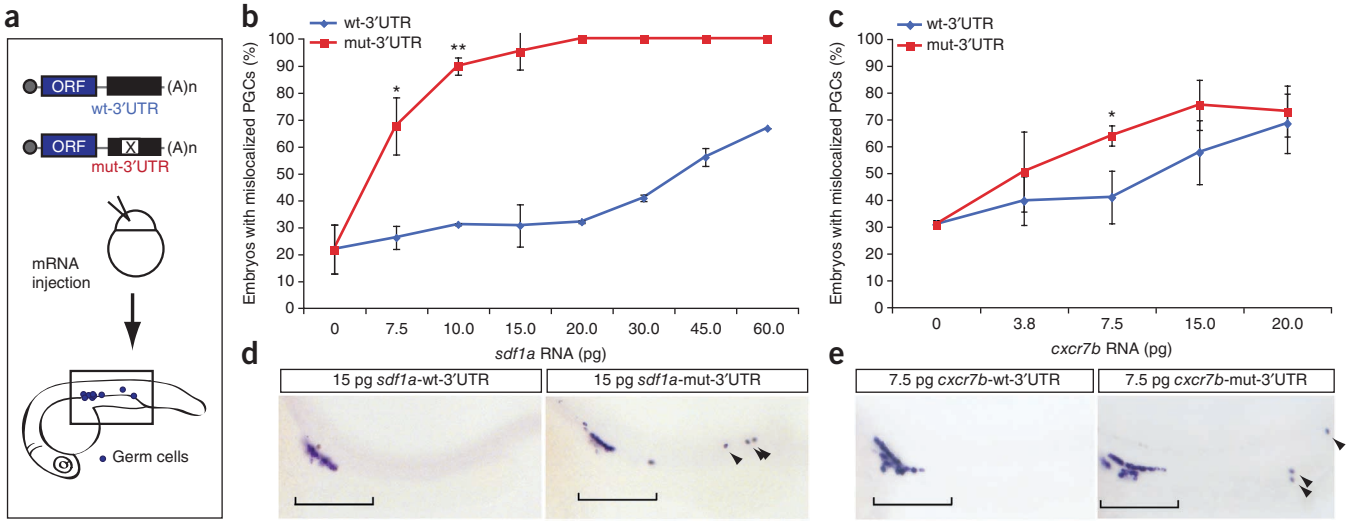

Figure 5 miR-430 buffers against overexpression of the chemokine signaling components. (**a**) Schematic representation of the experiment. mRNA encoding the open reading frame for the target gene with either the wildtype 3′ UTR (wt-3′UTR) or a mutant 3′ UTR (mut-3′UTR) with the miR-430 target site mutated was injected at the one-cell stage. PGC localization was assayed at 24 hpf using an *in situ* for nanos. The rectangle illustrates the region of the embryos shown in **d**, **e** and **g**. (**b**,**c**) Quantification of the percentage of embryos with PGCs outside of the gonad region upon injection of *sdf1a* mRNA (**b**) or *cxcr7b* mRNA (**c**). We saw a significant difference between transcripts with the wt-3′UTR (blue) and those with the mut-3′UTR (red) for *sdf1a* ($*P = 8.4 \times 10^{-4}$, $**P = 7 \times 10^{-6}$; two-tailed Fisher's exact test) and *cxcr7b* ($*P = 6.3 \times 10^{-3}$; two-tailed Fisher's exact test). (**d**,**e**) Representative images of injected embryos are shown. The bracket illustrates the PGCs that are correctly localized. Arrowheads indicate mislocalized PGCs. Error bars, ± s.d.

Cxcr7b and miR-430 negatively regulate *sdf1a*

To test whether Cxcr7b and miR-430 function to regulate *sdf1a* in a partially redundant manner, we asked whether *cxcr7b* interacted genetically with miR-430–mediated regulation of *sdf1a*. Decreasing the level of *sdf1a* regulation by miR-430 enhanced the germ cell migration defect observed in a *cxcr7b* hypomorphic condition caused by injecting subthreshold levels of a *cxcr7b* translation-blocking morpholino (*cxcr7b* AUG MO) (**Fig. 4a–c**). This phenotype was more severe than the *sdf1a*-TP alone phenotype in regards to the number of embryos affected as well as the mislocalization of the germ cells to the tail and head regions (**Fig. 4b**). We saw a similar result when injecting the *cxcr7b*-TP with an AUG MO for *sdf1a* (**Fig. 4d–f**). These results indicate that miR-430 and Cxcr7b act together to modulate *sdf1a* mRNA expression and protein levels, respectively.

miR-430 buffers against variation in gene dosage

Because miRNAs modulate the protein output of their target mRNAs, it has been hypothesized that they may guard against genetic variation[3]. By dampening the expression of both the ligand and the decoy receptor, miR-430 may provide robustness to chemokine signaling. To test this hypothesis, we assessed the ability of miR-430 targeting to buffer against higher levels of *sdf1a* and *cxcr7b* (**Fig. 5a**). Embryos injected with increasing amounts of *sdf1a* or *cxcr7b* mRNAs with the mutated 3′ UTR, but not the wildtype 3′ UTR, showed an increase in germ cell mismigration (**Fig. 5b–e**). For instance, injecting 15 pg of *sdf1a* mutated 3′ UTR significantly increased the percentage of embryos with mislocalized PGCs, but embryos receiving this amount of *sdf1a* wildtype 3′ UTR showed a wildtype phenotype (**Fig. 5b**,**d**). This suggests that repression by miR-430 is able to protect PGC migration from elevated levels of gene expression.

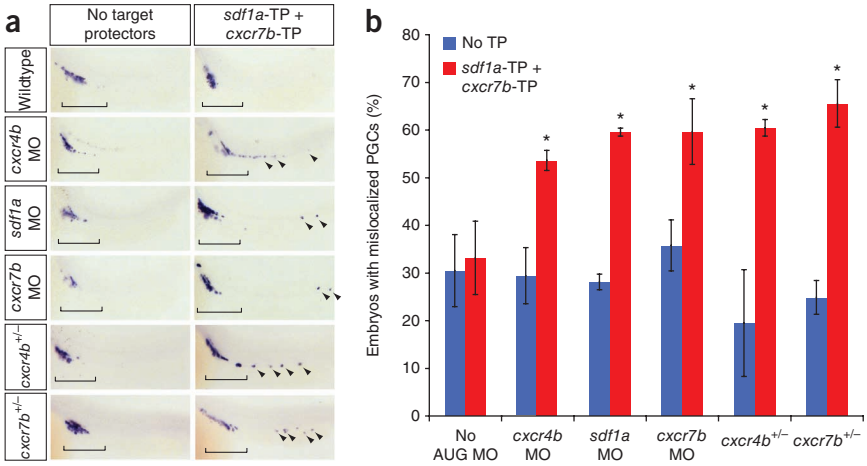

Figure 6 Regulation by miR-430 guards against variation in gene dosage. (**a**) Representative examples of PGC mislocalization, shown by nanos *in situ*. Brackets show correctly localized PGCs, and arrowheads indicate mislocalized cells. (**b**) Quantification of the percentage of embryos with mislocalized PGCs in different experimental conditions as shown. Co-injection of *sdf1a*-TP, *cxcr7b*-TP and a low level of translation-blocking morpholino (MO) against *cxcr7b* (0.02 pM), *sdf1a* (0.005 pM) or *cxcr4b* (0.005 pM) caused a significant increase in PGC mismigration. Asterisks denote $P < 0.05$ ($*P = 0.011$ for *cxcr4b* MO; $*P = 2.87 \times 10^{-3}$ for *sdf1a* MO; and $*P = 8.89 \times 10^{-3}$ for *cxcr7b* MO; two-tailed Fisher's exact test). A similar effect was seen upon injection of *sdf1a*-TP and *cxcr7b*-TP in *cxcr4b* heterozygous mutants ($*P = 7.50 \times 10^{-6}$) and *cxcr7b* heterozygous mutants ($*P = 1.64 \times 10^{-6}$) Error bars, ± s.d.

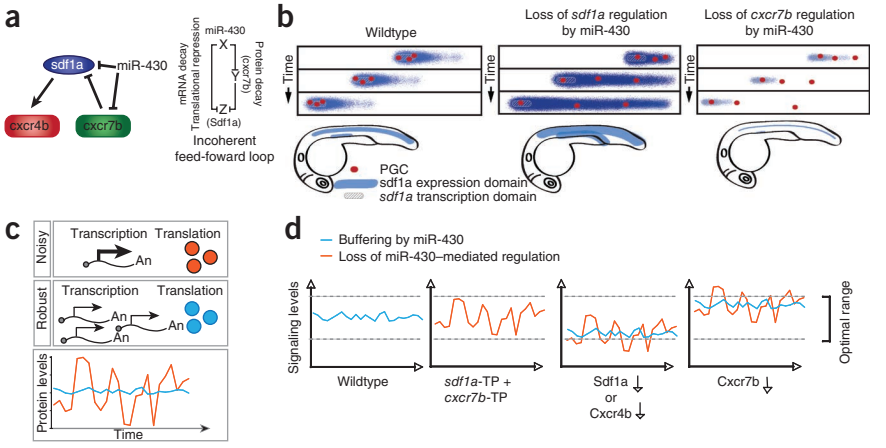

Figure 7 Model of miR-430–mediated repression of chemokine signaling. (**a**) Our results are consistent with a model in which miR-430 regulates *sdf1a* at the RNA level, whereas previous results indicate that Cxcr7b, a decoy receptor, restricts the spatial expression pattern of the Sdf1a protein. (**b**) A model adapted from reference 17 to represent how miR-430 regulates the dynamic expression of Sdf1a (blue gradient). miR-430–mediated regulation of *sdf1a* facilitates the formation of a sharp Sdf1a gradient by accelerating the clearance of *sdf1a* mRNA, concentrating expression on the actively transcribing domains (gray box) (middle). miR-430 modulates the levels of *cxcr7b* to prevent excessive clearance of the Sdf1a protein (right). (**c**) Model for generating robustness by regulating translation of abundantly transcribed genes. High levels of transcription coupled with inefficient translation can lower intrinsic noise in protein output[4,5]. (**d**) By dampening the expression of chemokine signaling genes, miR-430 buffers against changes in gene dosage (blue). We postulate that injecting target protectors to block miR-430–mediated repression of *sdf1a* and *cxcr7b* increases the variability of gene expression (red). This reduces the ability of the system to compensate for minor perturbations in expression (**Figs. 4** and **5** and **Supplementary Fig. 9**).

We then tested the role of miR-430 in compensating for decreases in expression. We began by eliminating miR-430–mediated regulation of chemokine signaling by co-injecting *sdf1a*-TP and *cxcr7b*-TP. This combination resulted in a wildtype phenotype, presumably because the increase in Cxcr7b can compensate for the increase in Sdf1a (**Fig. 6**, No AUG MO). To mimic genetic variation, we co-injected a low level of a morpholino against the chemokine signal or either of its receptors in the presence (No TP (no target protector)) or the absence of miR-430–mediated regulation (*sdf1a*-TP and *cxcr7b*-TP). Reducing the gene dosage of *sdf1a*, *cxcr7b* or *cxcr4b* increased PGC mismigration in the absence of miR-430 regulation (with 60% of the embryos presenting mislocalized germ cells) but not in wildtype embryos (**Fig. 6** and **Supplementary Fig. 9**). To support these experiments, we tested the effect of heterozygous mutations for *cxcr7b* and *cxcr4b* (refs. 24,25). The incidence of germ-cell mismigration for either of these heterozygous mutant embryos was similar to wildtype. However, blocking miR-430 regulation of *sdf1a* and *cxcr7b* in either a *cxcr4b*$^{+/-}$ or *cxcr7b*$^{+/-}$ mutant background increased the number of embryos with mislocalized PGCs (60%) (**Fig. 6** and **Supplementary Fig. 9**). Together, these experiments indicate that miR-430 lowers expression of *sdf1a* and *cxcr7b* such that minor perturbations in gene dosage do not cause mismigration (**Fig. 7**). Upon specific removal of the miRNA regulation, the system is more sensitive to small changes caused by a reduction in protein level or gene copy number. Together, these observations suggest that miR-430 provides genetic robustness and protects against alterations in gene dosage.

DISCUSSION

Secreted signaling molecules are potent developmental regulators, and therefore, their expression must be tightly controlled. This is achieved not only at the transcriptional level but also at the level of transcript degradation. This ensures that transcripts are made where they are needed and cleared from domains where they should no longer be present. Our results indicate that miR-430 functions in an incoherent feedforward loop to regulate chemokine signaling by targeting *sdf1a* and *cxcr7b* (**Fig. 7a**). In this network motif, a factor X (miR-430) represses Z (*sdf1a*) and Y (*cxcr7b*), which also represses Z. Indeed, mathematical models indicate that incoherent feedforward loops provide a mechanism for speeding transcriptional network response times that are generally slow[26,27] (reviewed in ref. 28). miR-430 is ubiquitously expressed[19], and it sharpens the *sdf1a* expression domain by accelerating degradation of the transcript in all cells. In this manner, only those cells actively transcribing the gene will express it, and once transcription is turned off, perduring transcripts are rapidly cleared. This regulation is necessary for accurate PGC migration, as loss of miR-430 (MZ*dicer* mutants) or blocking of miRNA target sites (target protectors) leads to an expansion of the *sdf1a* expression domain and mislocalized PGCs (**Fig. 3**). Although regulation of *cxcr7b* and *sdf1a* explains some of the phenotypes observed in MZ*dicer* embryos, the mismigration phenotype observed in these embryos is consistently stronger than that observed when the miRNA-mediated regulation of each individual target is blocked (**Fig. 3** and **Supplementary Fig. 1**), suggesting that there might be additional targets and other miRNAs that regulate PGCs migration (**Supplementary Note**).

mRNA degradation not only clears previous expression domains of *sdf1a* transcripts, but it also facilitates spatial separation of different Sdf1a expression domains. Sdf1 signaling is used to guide multiple migratory events during early development, including migration of the trigeminal sensory neurons[29], the posterior lateral line primordium[30], the vasculature[31] and the endoderm cells[32,33]. Employing the same guidance cue for each of these migratory processes is possible because the embryo maintains spatially restricted expression domains. The separation of these domains confines the cells to their respective paths so that one type of migrating cell does not follow the path intended for another cell type. Preserving distinct Sdf1a regions is in part accomplished by sharpening of the protein expression by Cxcr7b (ref. 17). Here we identify a previously unidentified regulatory mechanism, acting at the RNA level, in which miR-430 helps to maintain the separation between different migratory paths by (i) sharpening the expression domain of the ligand mRNA and by (ii) dampening the levels of the decoy receptor (Cxcr7b) to prevent excessive clearance of the guidance cue Sdf1a (**Fig. 7b**).

The interaction of Sdf1 with Cxcr4 and Cxcr7 is conserved among vertebrates, including mice and humans[16]. In addition to its roles in development, Sdf1 signaling is involved in tissue homeostasis by guiding immune cell migration[34], neovascularization[35], stem cell proliferation[36] and homing[37]. Because of their potent activities, mis-expression of these molecules has been associated with tumorigenesis and metastasis[38,39]. miR-430 is a member of a large family of miRNAs, many of which are expressed in mammals (reviewed in ref. 40). Notably, human SDF1 also contains a putative miRNA

target site for the homolog of miR-430, miR-302 (ref. 18), in its 3′ UTR (**Supplementary Fig. 10**). Indeed, our results indicate that miR-302 can rescue the germ cell migration in MZ*dicer* embryos (**Supplementary Fig. 1**), suggesting that this regulatory mechanism could be conserved in other vertebrate systems, where it may regulate cell migration and contribute to metastasis.

By dampening the expression of both the ligand and its decoy receptor, miR-430 fine tunes the amount of signaling. This interaction provides robustness by guarding against genetic variation that arises from increases or reductions in gene dosage. We observed that the embryo can withstand alterations in the levels of *sdf1a* and *cxcr7b* within a certain range, but this capacity is reduced when miR-430 function is compromised. miR-430 has previously been shown to regulate nodal signaling by dampening and balancing the expression of both the signal (*nodal*)[21] and its antagonist (*lefty*)[21,41]. As miR-430 plays a similar regulatory role in both of these contexts in the form of incoherent feedforward loops, a more general function for miR-430 may be to balance and lower the expression of positive and negative signaling molecules, reducing the ability of perturbations to have a deleterious effect. The evidence presented here suggests that miRNAs can act as effective elements to buffer genetic variation and maintain homeostasis. Although this role of miRNAs has been proposed, supporting experimental evidence is limited, particularly in vertebrates. Several studies in invertebrates have shown a role of specific miRNAs in providing robustness based on (i) the integration of the miRNA within a network motif (feedforward and feedback loops)[13,42,43], (ii) the stochastic nature of the loss-of-function phenotype[43,44] reviewed in reference 6 (for example, in *Drosophila*, miR-263a and miR-263b regulate the proapoptotic gene hid in sensory organs to protect against the stochastic cell death that is triggered throughout the eye[44]) and (iii) the protection against environmental factors, as seen in the buffering effect of miR-7 protecting against the effect of temperature fluctuations during eye development[13]. Here we show that in addition to the stochastic nature of the phenotype in the target protector–injected embryos, where few PGC fail to migrate to the gonad, miR-430–mediated regulation of *sdf1a* and *cxcr7b* buffers against genetic variation, providing robustness to the system.

Recent studies of the human genome have uncovered potential sources of genetic variation, most notably a large number of genetic lesions such as copy number variations and heterozygous and homozygous mutations. Because many of these lesions have no immediately appreciable phenotype[45], a regulatory mechanism must have evolved to maintain homeostasis. miRNAs are predicted to regulate over 70% of the protein-coding genes in mammals, making them prime candidates for this role[9,46]. Based on our results, we propose that miRNAs like miR-430 might play a widespread role in buffering the genetic variation borne by common genetic lesions, providing biological robustness to the organism.

URLs. Ensembl; http://uswest.ensembl.org/index.html.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

A.A.S. and A.J.G. designed the experiments and interpreted the results. A.A.S. performed all experiments except the genetic interactions in the *cxcr7b* and *cxcr4b* mutant backgrounds, which were performed by H.K. A.A.S. wrote the manuscript with input from H.K. and A.J.G.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Fish strains. MZ*dicer* fish were generated as previously described[18]. Embryos used were *dicer*$^{hu896/hu896}$ or *dicer*$^{hu896/hu715}$ (ref. 47). *cxcr4b* heterozygous fish were generated by crossing *cxcr4b*$^{t26035/t26035}$ (ref. 25) with wildtype fish. *cxcr7b* heterozygotes were a cross between *cxcr7b*$^{sa0016/sa0016}$ and wildtype fish[24].

Target prediction and microarray analysis. The 3′ UTRs of genes with a known role in PGC migration were analyzed for sites complementary to the miR-430 seed sequence (GCACTT). Both paralogs of *sdf1a* as well as their receptors contained complementary sequences. The expression of putative targets was examined using previously reported microarray data for complementary DNA (cDNA) from 8.5 hpf embryos hybridized to the Affymetrix GeneChip Zebrafish Genome Array[19].

GFP reporter constructs. The 3′ UTRs of *sdf1a*, *sdf1b*, *cxcr4a*, *cxcr4b*, *cxcr7a* and *cxcr7b* were identified using expressed sequence tag (EST) data available from Ensembl (see URLs). *cxcr7a* did not have a predicted 3′ UTR when this project was initiated, so a region 1.5 kb downstream of the predicted coding sequence was used. Currently, the estimated 3′ UTR based on the mRNA sequence is 1.3-kb long (RNASEQT00000016353, ZV9 ensemble genome assembly). Analysis of zebrafish RNAseq data at 6 h and 24 h revealed that reads in the *sdf1a* 3′ UTR extended beyond the published RefSeq mRNA NM_178307. This refseq lacks a polyA tail and instead has a genome-encoded stretch of seven adenosines, suggesting that the shorter 3′ UTRs for *sdf1a* might be due to mispriming in a genome-encoded A-rich region. Inspection of the zebrafish EST database indicate that there are multiple ESTs downstream of NM_178307, supporting a longer 3′ UTR. See *in situ* hybridization and **Supplementary Figure 3** for additional supporting information.

These 3′ UTRs were amplified from cDNA of 24 hpf embryos (**Supplementary Table 1**). 3′ UTR PCR products were digested and ligated into pCS2+GFP (xhoI-xbaI). The long Sdf1a 3′ UTR was constructed by cloning the product amplified by the *sdf1a* oligos and cutting the PCR fragment with nhe-xba and ligating downstream of the Sdf1a short UTR vector cut with xba. To clone mutant reporter constructs, the seed sequence in the wildtype reporter was changed from GCACTT to GCTGAT, creating three mismatches in the putative miRNA target site.

Constructs to express *sdf1a* (ENSDART00000053946, ZV8) and *cxcr7b* (ENSDART00000063665, ZV8) were generated by amplifying the open reading frame from cDNA of 24 hpf embryos. PCR products were digested and ligated into wildtype or mutated GFP reporter vectors, substituting GFP for the open reading frame of interest (BamHI-XhoI).

The oligonucleotide sequences used in this manuscript are shown in **Supplementary Table 1**.

mRNA and morpholino injection. For target validation, mRNA was transcribed from reporter constructs using mMessage mMachine kit (Ambion). One nanoliter of a solution of GFP reporter mRNA at 0.1 μg/μl and 0.08 μg/μl DsRed mRNA was injected into wildtype or MZ*dicer* embryos at the one-cell stage.

Target repression was quantified by comparing the average pixel intensity of GFP in injected wildtype and MZ*dicer* embryos, as previously described[19]. Briefly, pixel intensity in a rectangle of the trunk of a GFP-injected embryo (subtracting the background intensity from a rectangle next to the trunk) was normalized to DsRed intensity in the same area (subtracting the background). This intensity value in MZ*dicer* embryos was divided by the value in wildtype embryos to obtain fold repression in wildtype compared to MZ*dicer* mutants.

Target protector morpholinos were designed to bind with perfect complementarity to 25 nucleotides in the 3′ UTR including the miRNA seed sequence. The control target protector binds to a sequence 200 nucleotides downstream of the target site. Unless otherwise noted, 1 nl of 0.2 mM target protector was injected in one-cell–stage embryos.

All morpholinos were ordered from Gene Tools and dissolved in nuclease-free water. Morpholinos to bind the translation start site (AUG MOs) of *sdf1a* (ref. 22), *cxcr7b* (ref. 48) and *cxcr4b* (ref. 25) were injected at a concentration insufficient to completely knock down expression. These low levels were 1 nl of 0.02 mM (*cxcr7b* AUG MO) and 0.005 mM (*sdf1a* and *cxcr4b* AUG MO). Sequences of these AUG MOs are shown in **Supplementary Table 1**.

***In situ* hybridization.** *In situ* hybridization was performed as previously described[49]. For *sdf1a in situ*, wildtype, target protector–injected and MZ*dicer* mutant embryos were combined in the same tube to eliminate variability as described in reference 49. Before imaging, embryos were dehydrated in methanol after *in situ* and transferred to benzl benzoate/benzyl alcohol. *sdf1a in situs* were flat mounted in Permount. Images were taken on a Zeiss Axioimager M1.

To score germ cell mislocalization, embryos were fixed at 24 hpf in 4% paraformaldehyde, and PGCs were labeled by *in situ* for nanos. Locations of germ cells in each embryo were recorded as gonad, pronephros, tail or head. To quantify the mislocalization phenotype, embryos with at least one mislocalized PGC were counted. To determine the percentage of embryos with mislocalized PGCs, sets of 25–40 embryos were scored. Standard deviations were calculated for multiple repetitions of each experiment. Statistical significance was determined using a two-tailed Fisher's exact test or a Wilcoxon rank-sum test as indicated.

qPCR. RNA was extracted from ten embryos each of wildtype, Sdf1a-TP–injected, and MZ*dicer* at 24 hpf using Trizol reagent according to the manufacturer's instructions (Invitrogen). cDNA was made using an Invitrogen SuperScript III kit with oligo dT. FastStart SYBR Green Master Mix (Roche) was used to amplify *sdf1a* and *ef1a*. The fold change in *sdf1a* expression was calculated by ΔΔCt method using *ef1a* as a control.

Rescue of MZ*dicer* by injecting mature miRNA duplex. MZ*dicer* embryos were rescued by injecting at the one-cell stage 1 nl of 50 μM miR-430c duplex or 1 nl of 10 μM miR-302b (purchased from IDT). Rescue was assessed by ventricle inflation, mid-hind brain boundary formation and decreased tail curvature[18].

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