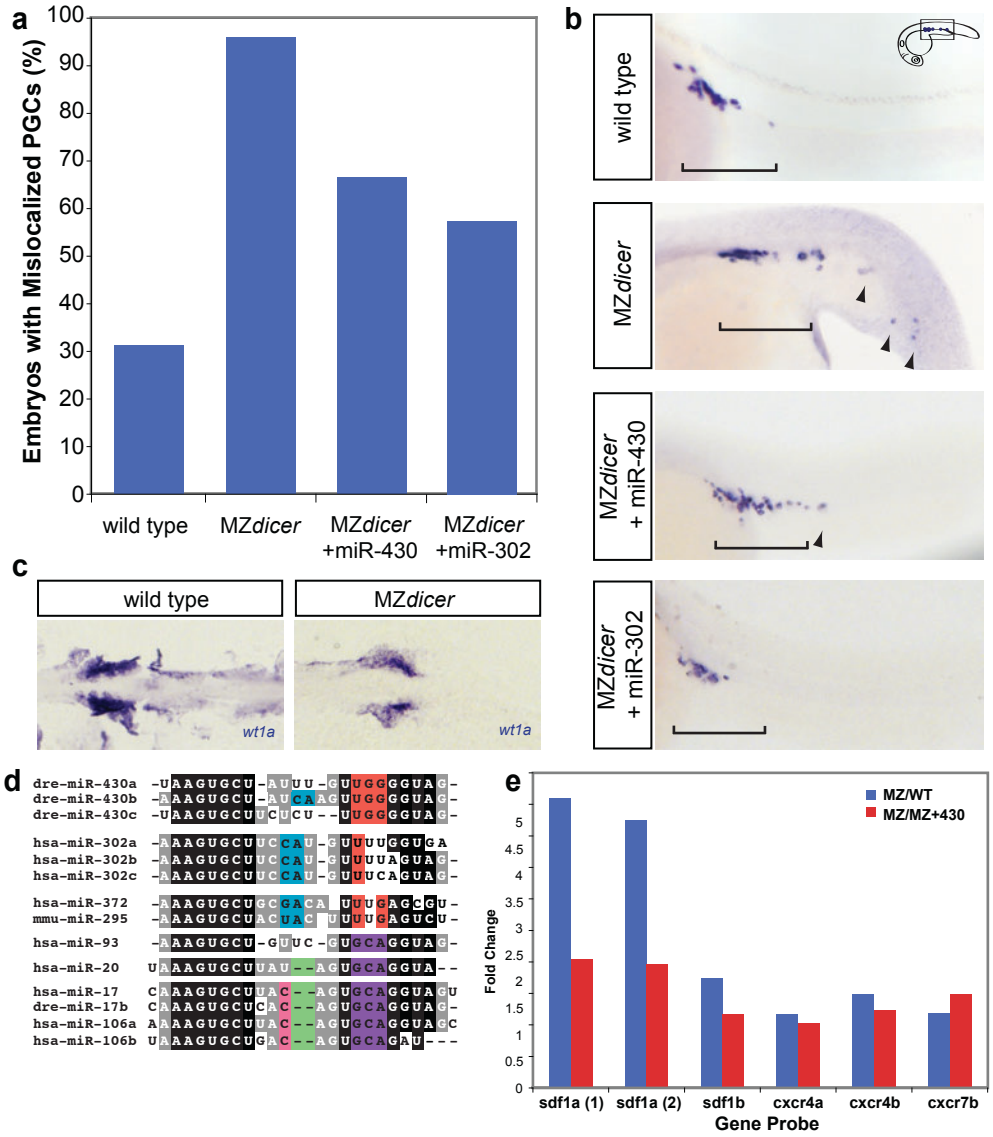
Supplementary Information

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

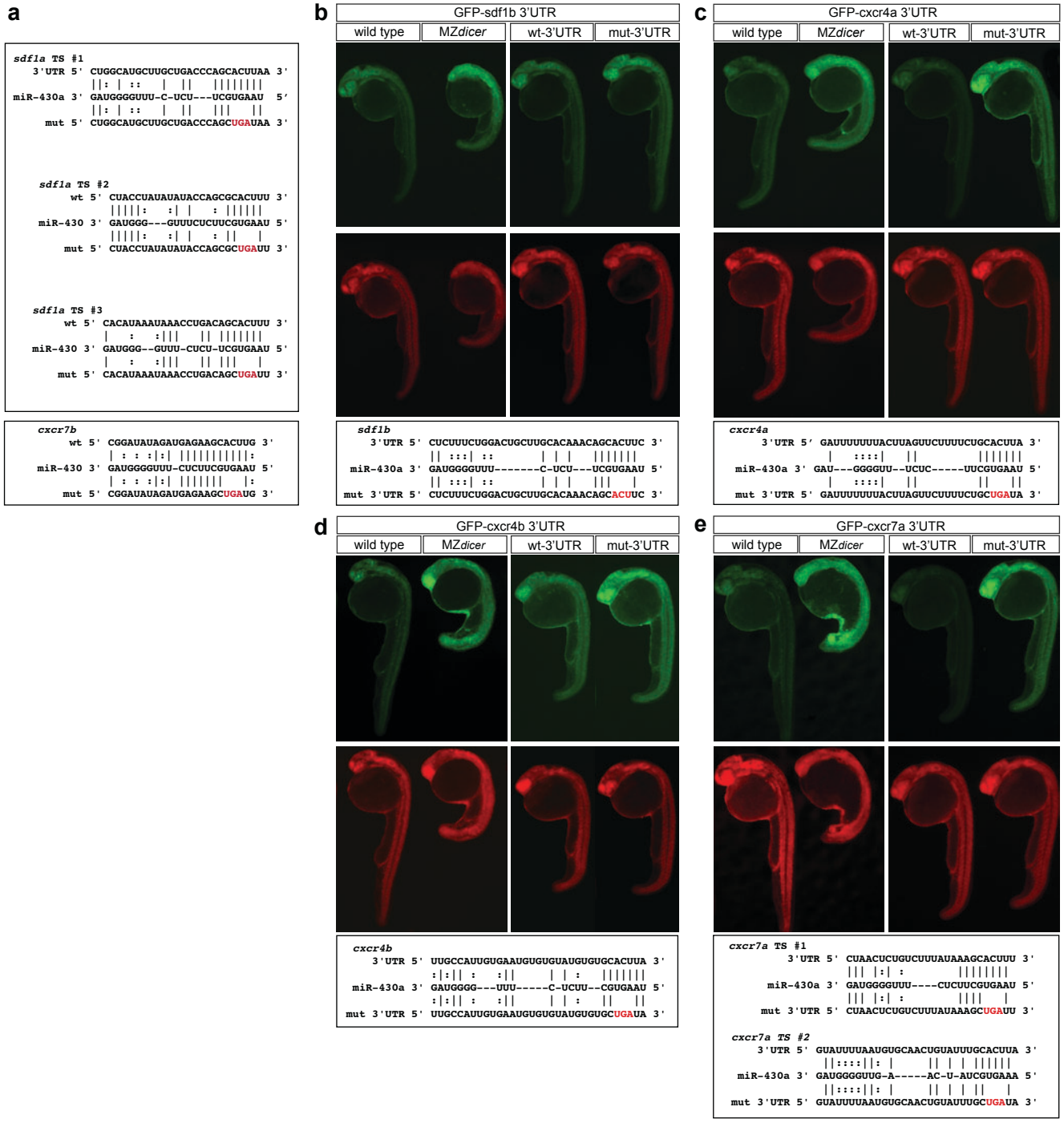
Alison A. Staton[1], Holger Knaut[2], and Antonio J. Giraldez[1, 3]

1) Department of Genetics, Yale University School of Medicine, New Haven, CT 06510
2) Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016
3) Yale Stem Cell Center, Yale University School of Medicine, New Haven, CT 06520

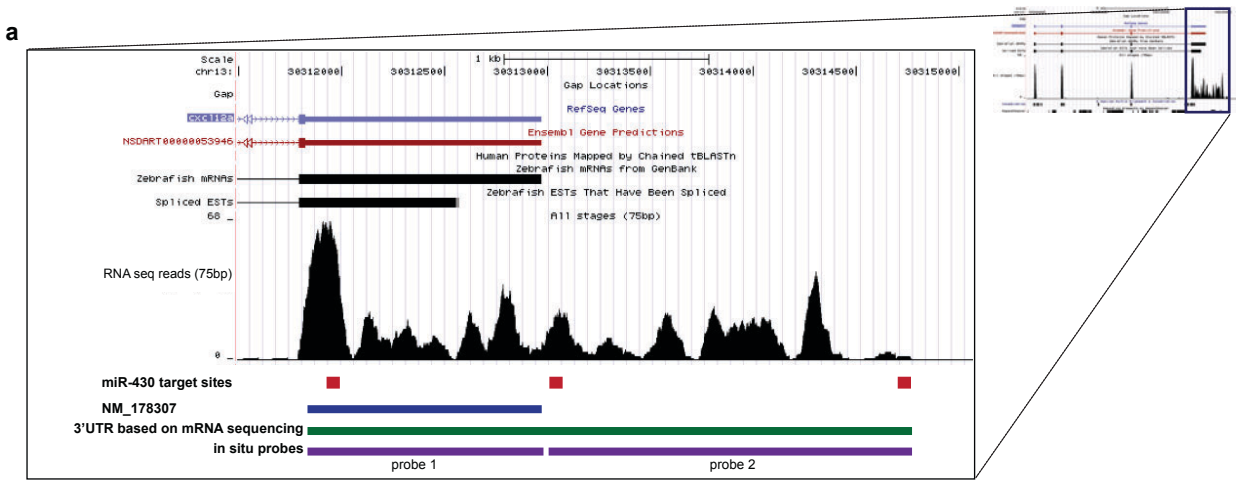
To whom correspondence should be addressed
E-mail: antonio.giraldez@yale.edu
Tel: 203.785.5423
Fax: 203.785.4415

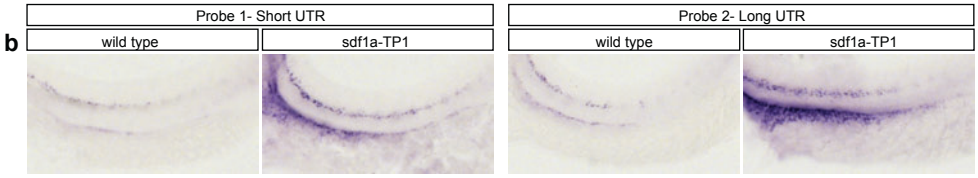


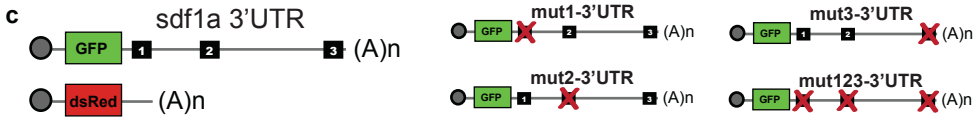
Supplementary Figure 1. PGCs are mislocalized in the absence of miRNAs. (a) Quantification of the percentage of embryos with mislocalized PGCs. Injecting the miRNA duplex for miR-430 or miR-302 partially rescues the mislocalization phenotype seen in MZ*dicer*. (b) Whole mount in situ hybridization to detect *nanos* mRNA labels germ cells. The inset in the upper panel illustrates the region of the embryo shown. Arrowheads indicate mislocalized cells. (c) Alignment of miR-430 and a subset of related miRNAs. miR-430 belongs to a group of miRNAs that share a AAGUGC seed motif in the 5' end. This seed sequence is thought to play a major role in pairing with targets, so these miRNAs are likely evolutionarily related and able to regulate common transcripts[1, 2]. (d) In situ hybridization for *wt1a* in wild type and MZ*dicer* embryos at the 14 somite stage. Dark staining shows the pronephric mesoderm, which is formed in both wild type and MZ*dicer*. This agrees with *pax2a* staining previously showing formation of the pronephric mesoderm in MZ*dicer*[1]. (e) Microarray results for embryos at 8.5 hpf. Expression of *sdf1a* is increased in the absence of miR-430. The change in expression in MZ*dicer* embryos compared to wild type is shown in blue. Two probe sets for *sdf1a* were present on the array, labeled as (1) and (2). To determine whether miR-430 is required for this increase, the difference in expression of MZ*dicer*+miR-430 compared to MZ*dicer* was examined (red). Both probes for *sdf1a* show a miR-430-dependent increase in expression. The other chemokine signaling genes examined do not show significant differences at the RNA level in wild type vs. MZ*dicer*.

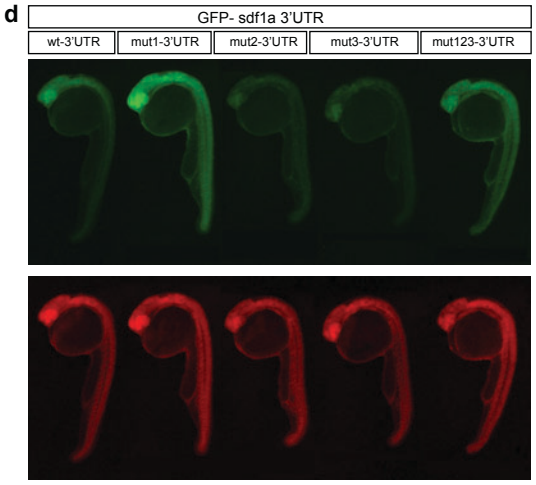


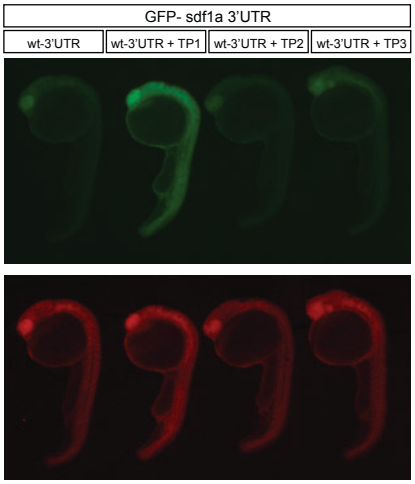
Supplementary Figure 2. The 3'UTRs of *sdf1b*, *cxcr4a*, *cxcr4b*, and *cxcr7a* confer post-transcriptional regulation through miRNAs. (a) Predicted Watson-Crick pairing of each putative miR-430 target site in the *sdf1a* 3'UTR and *cxcr7b* 3'UTR with miR-430a. GFP mRNAs with the 3'UTR of *sdf1b* (b), *cxcr4a* (c), *cxcr4b* (d), or *cxcr7a* (e) were injected in wild type and MZ*dicer* embryos. dsRed mRNA was co-injected as a control. Fluorescent micrographs show GFP target expression (green) and dsRed expression (red) at 24-28 hpf. The seed sequence of each miR-430 target site was mutated (mut-3'UTR) to test the requirement of the miR-430 target site for miRNA-mediated regulation by comparing the expression of GFP in embryos injected with the wild type or the mutant reporter. Predicted pairing of each wild type or mutated target site with miR-430 are shown below.

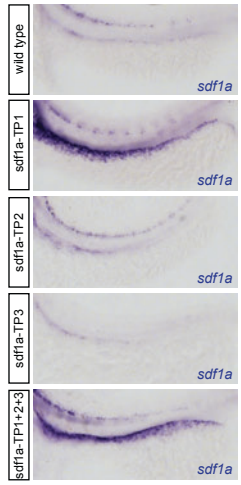








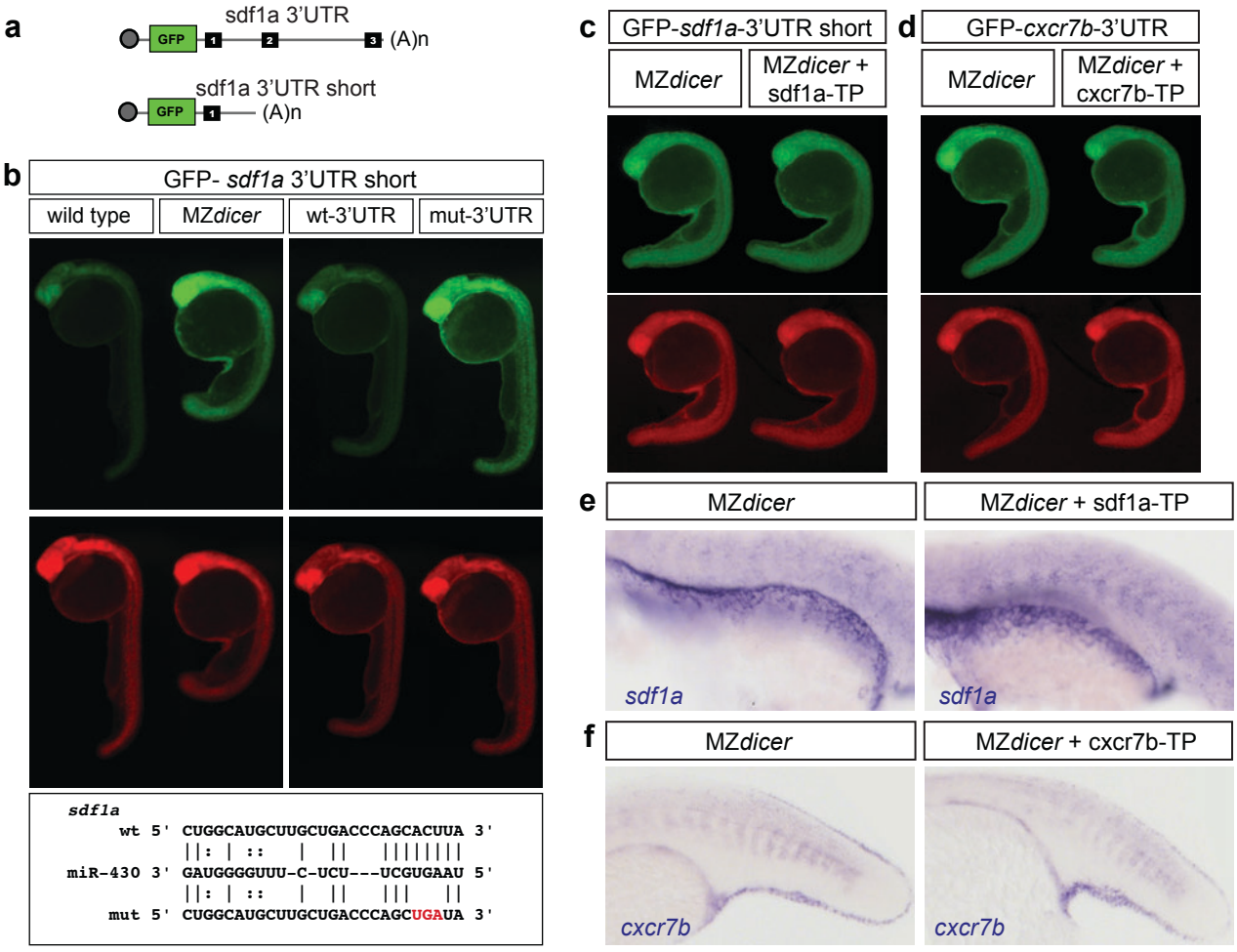




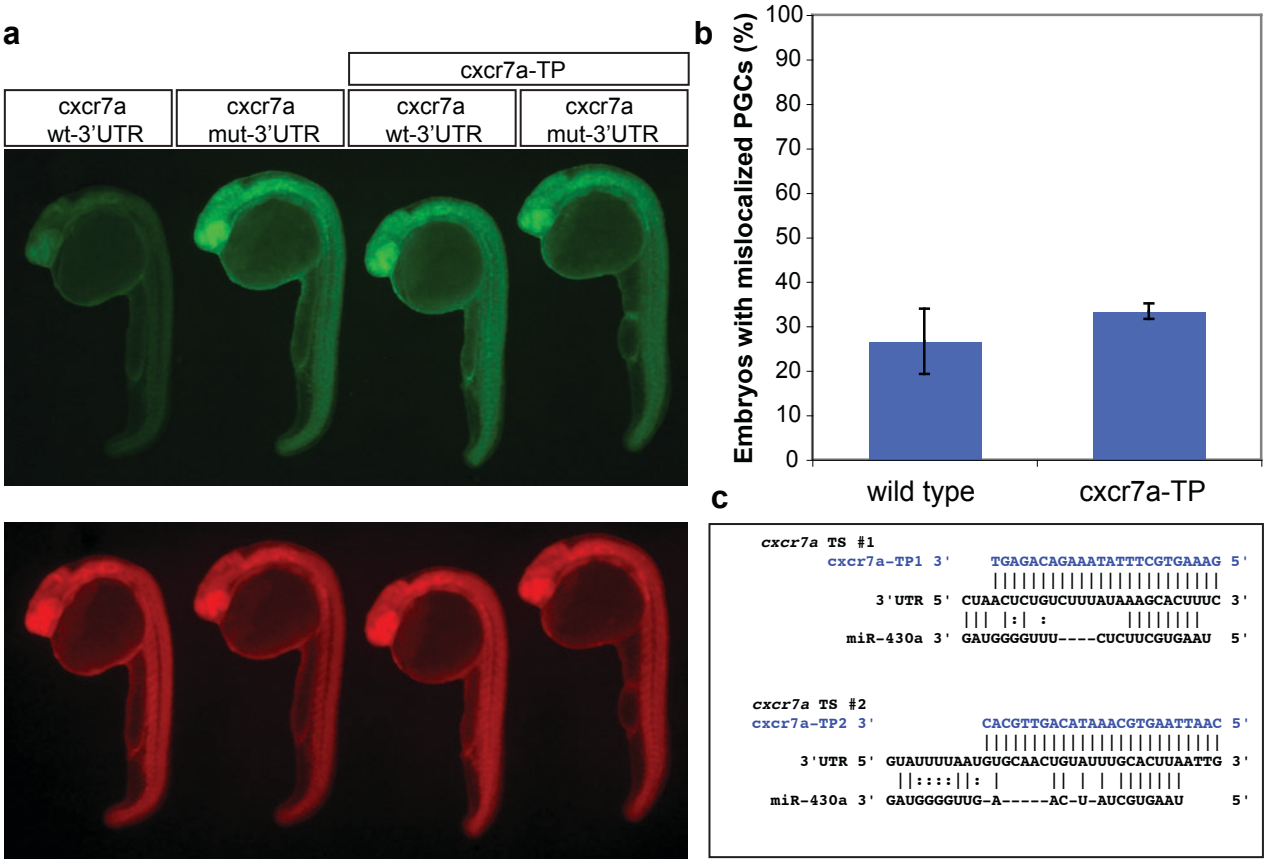

```
sdf1a TS #1
   TP-1 3'                    GGTCGTGAATTGGACGGTTTATACG 5'
                              ||||||||||||||||||||||||||
 3'UTR 5' CUGGCAUGCUUGCUGACCCAGCACUUAACCUGCCAAAUAUGC 3'
          ||: | ::   |  ||   ||||||||
miR-430a 3' GAUGGGGUUU-C-UCU---UCGUGAAU               5'
          ||: | ::   |  ||   |||   ||
    mut 5' CUGGCAUGCUUGCUGACCCAGCUGAUAACCUGCCAAAUAUGC 3'


     sdf1a TS #2
        TP-2 3'       TATATATGGTCGCGTGAAATATTGG 5'
                      |||||||||||||||||||||||||
          wt 5' CUACCUAUAUAUACCAGCGCACUUUAUAACC 3'
                |||||:   :| |   : ||||||
    miR-430 3' GAUGGG---GUUUCUCUUCGUGAAU       5'
                |||||:   :| |   : ||   |
         mut 5' CUACCUAUAUAUACCAGCGCUGAUUAUAACC 3'

   sdf1a TS #3
      TP-3 3'            TGGACTGTCGTGAAAAGCCTAAATG 5'
                         |||||||||||||||||||||||||
        wt 5' CACAUAAAUAAACCUGACAGCACUUUUCGGAUUUAC 3'
              |   :   :|||   || |||||||
  miR-430 3' GAUGGG--GUUU-CUCU-UCGUGAAU           5'
              |   :   :|||   || |||   |
     mut 5' CACAUAAAUAAACCUGACAGCUGAUUUUCGGAUUUAC 3'
```

```
cxcr7b 3'UTR
   cxcr7b-TP 3' CAGCCTATATCTACTCTTCGTGAAC  5'
                ||||||||||||||||||||||||||
      3'UTR 5' GUCGGAUAUAGAUGAGAAGCACUUG  3'
               | : : :|:| ||||||||||:
  miR-430a 3'   GAUGGGGUUU-CUCUUCGUGAAU  5'
               | : : :|:| |||||||   |:
  mut 3'UTR 5'  CGGAUAUAGAUGAGAAGCUGAUG  3'
```

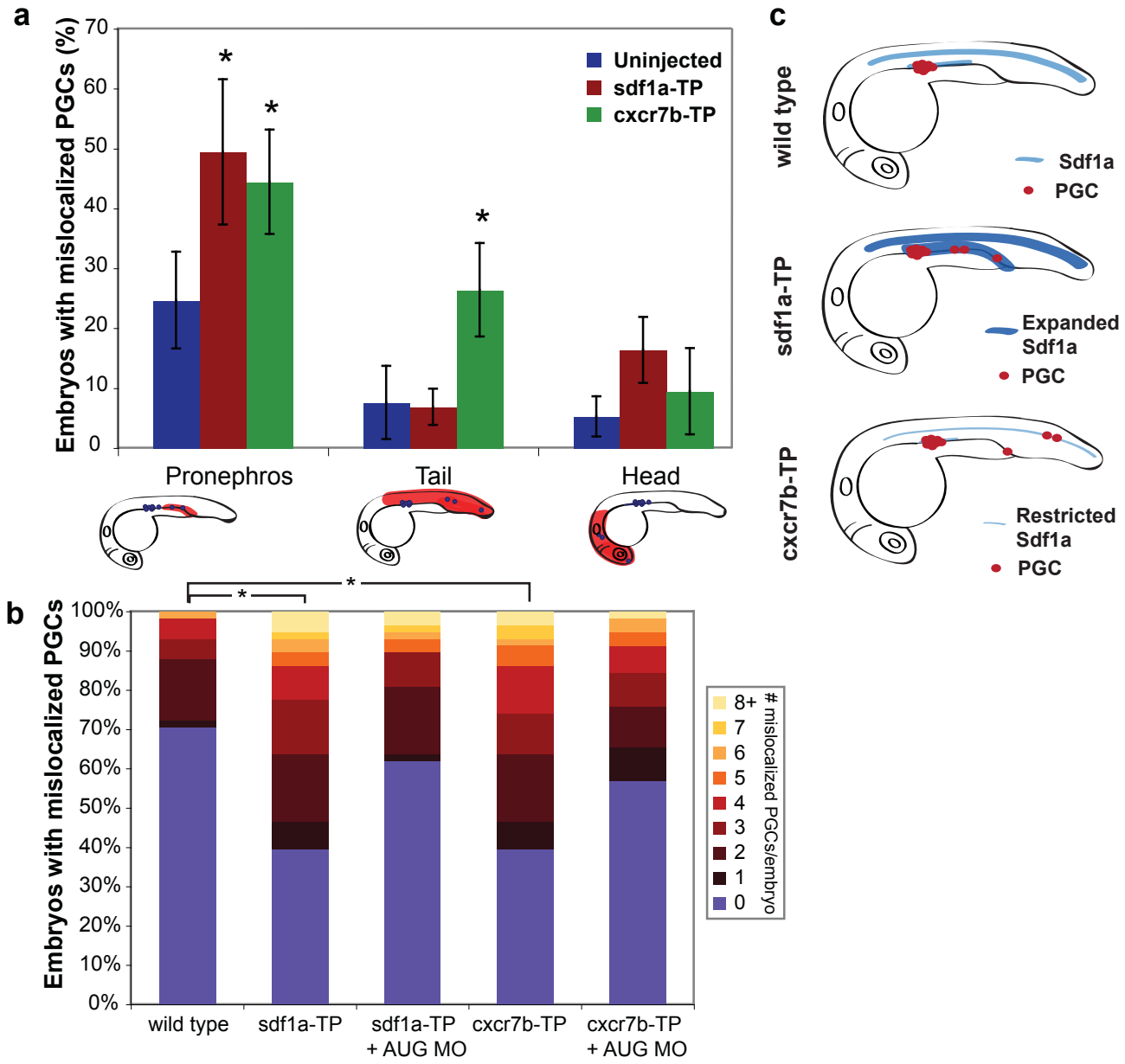
Supplementary Figure 3. Analysis of target sites in the *sdf1a* 3'UTR. (a) Snapshot of the UCSC genome browser showing the region 3' to the *sdf1a* ORF including RNA seq reads that map to this region. The 3'UTRs based on the ref_seq NM_178307 (*sdf1a*-short) is shown in blue and the 3'UTR based on the RNA seq data is shown in green. Shown in purple are two different probes to detect expression of the 5' and 3' regions of the 3'UTR. The *sdf1a* 3'UTR contains 3 putative miR-430 target sites. Target site 1 is in the region overlapping probe 1, while targets 2 and 3 are downstream of the ref_seq NM_178307 in the region marked by probe 2. (b) In situ hybridization to detect *sdf1a* mRNA using either a probe complementary to the 5' (probe 1) or the 3' (probe 2) region of the 3'UTR in wild type and embryos injected with sdf1a-TP1. Note that protection of the first miR-430 target site in *sdf1a* (present in the 5' region) also stabilizes transcripts with the long 3'UTR, indicating that both regions of the 3'UTR are present in the same transcript mRNA. (c) Schematic representation of the GFP reporters with the wild type *sdf1a* 3'UTR and each of the reporters with the first miR-430 target site mutated (mut1-3'UTR), with the second target site mutated (mut2-3'UTR), with the third target site mutated (mut3-3'UTR), and with all three target sites mutated (mut123-3'UTR). The co-injected dsRed control mRNA is shown below. (d) Wild type embryos injected with the GFP reporter mRNAs including the wild type or the mutated *sdf1a* 3'UTR as indicated in (c), DsRed was co-injected as a control. (e) Target protectors for each of *sdf1a* 3'UTR target sites were co-injected with the wild type GFP reporter. The target protector for the first target site (sdf1a-TP1) relieves repression while sdf1a-TP2 and sdf1a-TP3 do not have a strong impact on the expression of the GFP reporter mRNA. The sequences of the target sites and corresponding TPs are shown below. (d) In situ hybridization for *sdf1a*. Embryos were injected with TP1, TP2, TP3, or all three TPs for *sdf1a*. Images show the expression of *sdf1a* in the pronephric region in 24 hpf embryos. Note that mutating the first miR-430 target site relieves miRNA-mediated repression of the reporter similar to mutating all three sites together, indicating that the first miR-430 target site confers most of the regulation. Consistent with this observation, only the target protector complementary to the first site is able to relieve miRNA-mediated repression of the reporter or the endogenous *sdf1a* mRNA.



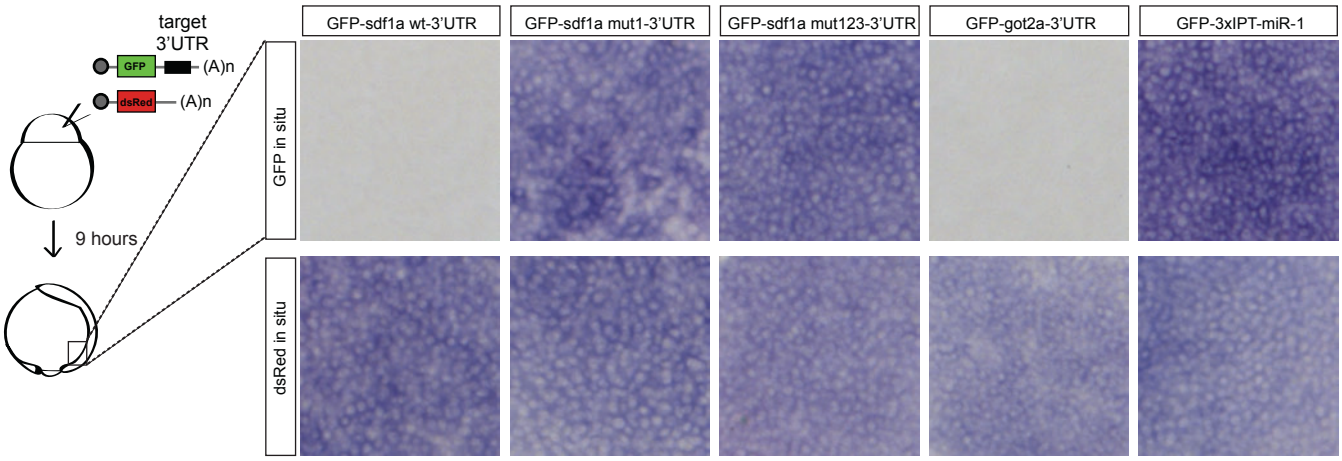
Supplementary Figure 4. Target Protectors do not alter the expression of the target in the absence of miRNA-mediated regulation. (a) Schematic representation of two *sdf1a* GFP reporter mRNAs containing either the *sdf1a* full length 3'UTR or a short 3'UTR that includes the first miR-430 target site and coincides with the ref_seq NM_178307 (*sdf1a* 3'UTR short). (b) Fluorescence microscopy shows GFP (green) and dsRed (red) expression in 24-28 hpf embryos injected with GFP reporters with *sdf1a* 3'UTR short. Endogenous miR-430 represses the expression of the reporter in wild type but not MZ*dicer* embryos. Similarly wild type embryos injected with the mut-3'UTR reporter fail to repress GFP. The sequence of the wild type or the mutant target site and miR-430 are shown below. (c, d) The GFP reporter for *sdf1a*-short (c) or *cxcr7b* (d) was injected into MZ*dicer* with or without the corresponding TP. dsRed mRNA was co-injected as a control. Fluorescent micrographs show GFP target expression (green) and dsRed expression (red) at 24-28 hpf. No difference in GFP expression was seen between MZ*dicer* and MZ*dicer*+TP. (e, f) In situ hybridization to detect endogenous *sdf1a* or *cxcr7b* mRNA in MZ*dicer* and MZ*dicer*+TP. sdf1a-TP protected the endogenous mRNA against miRNA-mediated degradation in wild type embryos (Fig. 3) but not in MZ*dicer* mutants devoid of miR-430. This result suggests that the TP does not confer upregulation in the absence of miRNA-mediated repression. The expression level of *cxcr7b* RNA does not change in MZ*dicer* compared to wild type (Supplementary Fig. 8) or upon injection of target protector, suggesting that *cxcr7b* is likely regulated at the level of translation.



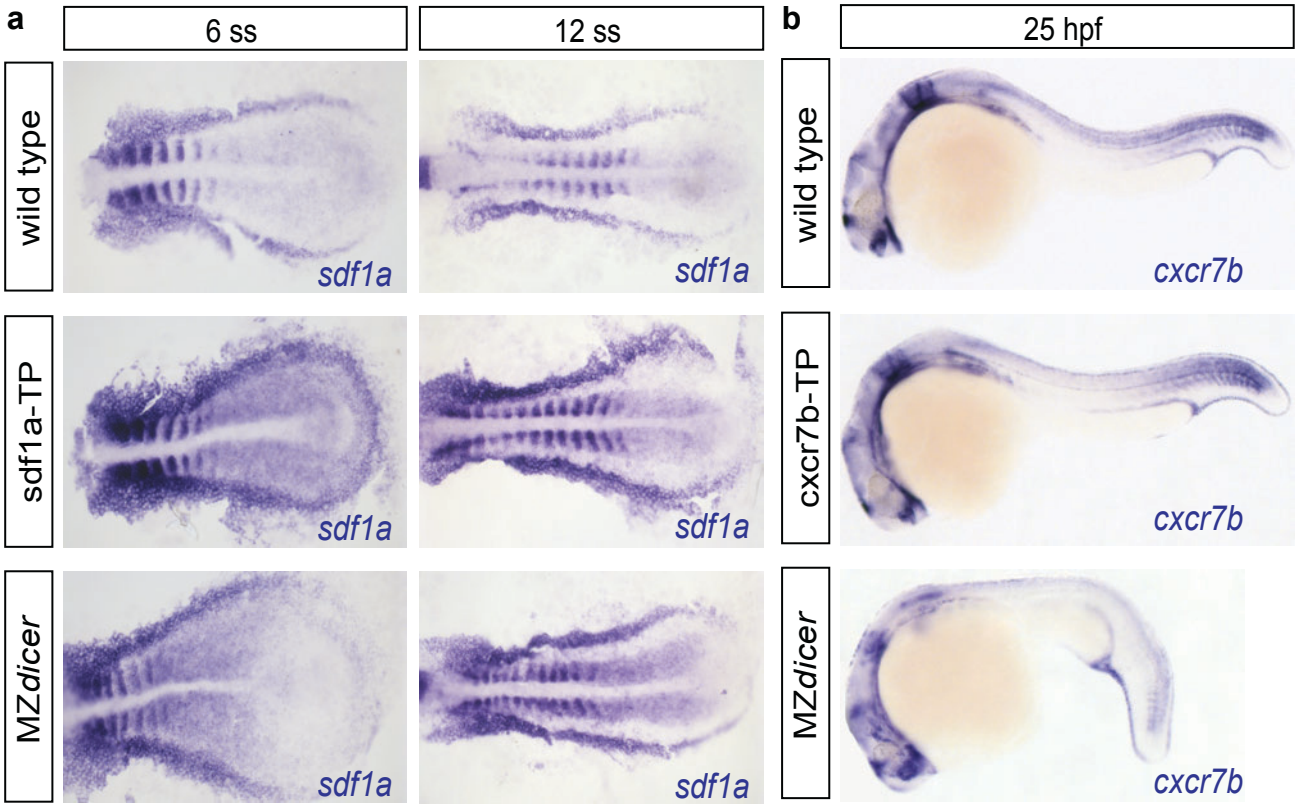
Supplementary Figure 5. Blocking miR-430-mediated regulation of *cxcr7a* does not affect PGC migration. (a) 0.1 pmol of TP for each target site in the *cxcr7a* 3'UTR was co-injected with the *cxcr7a* GFP reporter. These TPs are able to interfere with repression of the reporter by miR-430. (b) Upon injection of cxcr7a-TPs, no significant increase in PGC migration was observed. (c) Predicted Watson-Crick pairing of each target site with miR-430 and the corresponding TP.



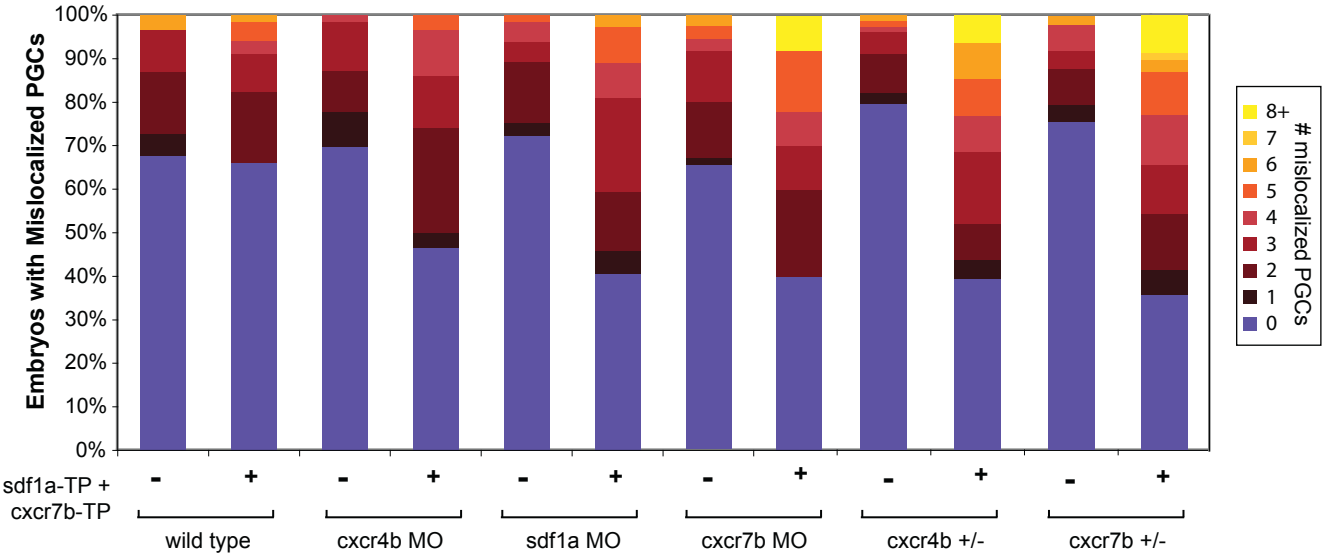
Supplementary Figure 6. Characterization of the mislocalized PGCs when miR-430 mediated regulation of *sdf1a* or *cxcr7b* is blocked. (a) Percentage of embryos with PGCs mislocalized to different parts of the embryo: lower pronephros, tail, and head. sdf1a-TP causes an increase in the percentage of embryos with PGCs along the lower pronephros (*, p=3.6x10-9, two-tailed Fisher's exact test). cxcr7b-TP increases the percentage of embryos with PGCs both along the lower pronephros and in the tail (*, pronephros, p=1.683 x10-5; tail, p=1.137x10-7, two-tailed Fisher's exact test). (b) Quantification of the number of mislocalized PGCs per embryo in wild type, TP-injected, and embryos injected with the TP and the cognate AUG MO (*, p=3.4x10-5, Wilcoxon rank sum test). (d) Schematic model representing the predicted expression of Sdf1a and the resulting location of PGCs. Sdf1a-TP increases *sdf1a* expression, leading to a stronger and expanded domain of signal expression and permitting cells to remain in these ectopic zones (See Fig. 3). Protecting *cxcr7b* from miR-430 regulation is predicted to increase expression of the decoy receptor, further restricting the domain of ligand expression such that PGCs may lose the migratory path and stray into other regions such as the tail.



Supplementary Figure 7. miR-430 promotes degradation of RNA containing the *sdf1a* 3'UTR. In situ analysis (dark blue) to detect the levels of co-injected GFP reporter mRNA or a control dsRed mRNA 9 hours post injection. The GFP reporters include the wild type *sdf1a* 3'UTR, a mutant 3'UTR where the first target site has been mutated (mut1), a mutant 3'UTR where all three target sites have been mutated (mut123), a 3'UTR of a known miR-430 target (*got2a*)[3], and a 3'UTR with three partially complementary sites to miR-1, which is not expressed at the time of the assay (3xIPT-miR-1)[1]. The photos show a detail of the dorsal-lateral margin of the embryo (black rectangle, left). Note the higher reporter levels (dark blue) when the miR-430 target sites have been mutated or in the case of the control miR-1 reporter (3xIPT-miR-1) when compared to the wild type *sdf1a* reporter or *got2a* reporter. In contrast, the levels of the control dsRed mRNA that was co-injected with each GFP reporter are largely unchanged between the different experiments.

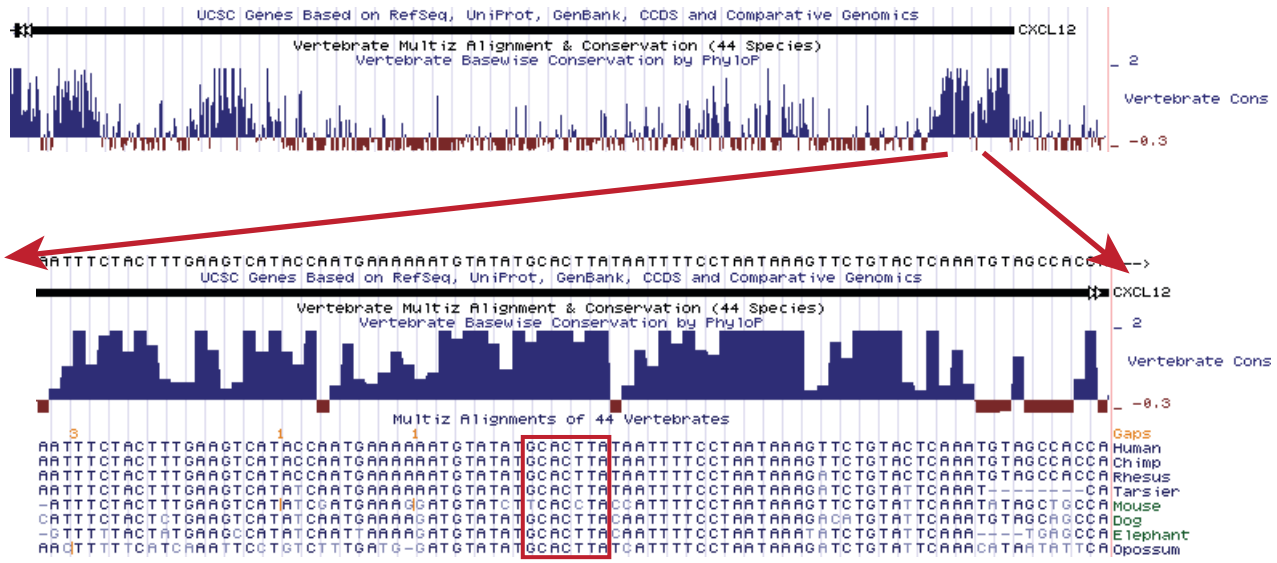


Supplementary Figure 8. miR-430 mediated repression restricts the expression of *sdf1a*. (a) Flat mount in situ to detect *sdf1a* mRNA at 6 somite stage (ss) and 12ss. Dorsal view of TP-injected and MZ*dicer* embryos show stronger and extended expression in the lateral mesoderm. (b) Whole mount in situ hybridization to detect *cxcr7b* mRNA at 25 hpf. No increase in RNA levels is observed in MZ*dicer* or upon inhibiting miR-430 targeting of the *cxcr7b* 3'UTR, suggesting that *cxcr7b* is likely regulated at the level of translation.



Supplementary Figure 9. Regulation by miR-430 buffers against alterations in gene dosage that can lead to PGC mislocalization. Analysis of mismigrated cells shows that in embryos injected with an AUG MO (for either *cxcr4b*, *sdf1a* or *cxcr7b*), heterozygous for *cxcr4b*, or heterozygous for *cxcr7b*, injection of both TPs (for *sdf1a* and *cxcr7b*) causes an increase in the number of mislocalized cells per embryo (see also Fig. 6).

Conservation of miR-302 target site in mammals



miR-427 target site in Xenopus

5′-gaaaactttcttctaatctttccaactttgcttcctgtcagatg**GCACTTA**aggcctgcacatttgttttcaatagggaagagcacaggcaca- 3′

miR-430 target site in Zebrafish

5′-tcacagatatgtaccatatagtcctctggcatgcttgctgaccc**AGCACTTA**acctgccaaatatgcgtcccagtaaatgaaaagaccaccag- 3′

Supplementary Figure 10. Putative target sites for miR-302, a miR-430 homolog, are found in a conserved region of the human *sdf1a* 3'UTR. Although this region is not conserved in Xenopus or zebrafish, both genes have the target site sequence in their 3'UTRs.

Supplementary Table 1. Oligonucleotide sequences

Primers

sdf1a-shortutr-F	5' AAACTCGAGTGTCTGCCAGATGACAAGGA 3'
sdf1a-shortutr-R	5' CAATCTAGATTGAGGCT CGACAGTCTCTG 3'
sdf1a-utr-F	5' CCAGGCTAGCAAGAACTCAATTAACAAAGA 3'
sdf1a-utr-R	5' CAGGTCTAGACATTCAT GTAAATCCGAAAAGTGCTGTC 3'
sdf1b-utr-F	5' AAACTCGAGAACGCTCAGAGTAAACAGACGA 3'
sdf1b-utr-R	5' AAAGCTAGCCATCTGCC ATTCTGCGATTA 3'
cxcr4a-utr-F	5' AAACTCGAGGCGCACCTTGGACTATGAAC 3'
cxcr4a-utr-R	5' CAATCTAGATTG TTGTAAAGACTTCAAATTGACAAA 3'
cxcr4b-utr-F	5' AAACTCGAGCCACATGGATGATTAAGC TTATAAAAA 3'
cxcr4b-utr-R	5' CAATCTAGATGGAAGTT AAAATGCGTCAAAAATG 3'
cxcr7a-utr-F	5' AAACTCGAGAAAAGTGCCAAAGGCTGATG 3'
cxcr7a-utr-R	5' CAATCTAGACGGTGTTC CATCAGAACTCA 3'
cxcr7b-utr-F	5' AAACTCGAGGGTGTTGACCATCACCAACT 3'
cxcr7b-utr-R	5' CAATCTAGAGGCACATT TCAGACATTACTCC 3'
sdf1a-orf-F	5' CCAGGATCCATGGATCT CAAAGTGATCGTAGTAGTCGC 3'
sdf1a-orf-R	5' CCACTCGAGTTAGACCTGCTGCTGTTGGGCTT 3'
cxcr7b-orf-F	5' GGATCCGCCACCATGAG TGTGAACGTGAATGATTTC 3'
cxcr7b-orf-R	5' CTCGAGTCATAATGGTC CCTGGTTTTCCACG 3'
sdf1a-qpcr-F	5' CAGATGACAAGGAAATCTGACAG 3'
sdf1a-qpcr-R	5' GCGCTAACAGAGAAGACTACATGG 3'
eif1a-qpcr-F	5' TGATCTACAAATGCGGT GGA 3'
eif1a-qpcr-R	5' CAATGGTGATACCACGC TCA 3'

Target Protectors

sdf1a-TP-1	5' GCATATTT GGCAGGTTAAGTGCTGG 3'
sdf1a-TP-2	5' GGTTATAAAGTGCG CTGGTATAT AT 3'
sdf1a-TP-3	5' GTAAATCCGAAAAGTGCT GTCAGGT 3'
cxcr7b-TP	5' CAAGTGCTTCTCAT CTATATCCGAC 3'
cxcr7a-TP-1	5' GAAAGTGCTTTATAAAG ACAGAGTT 3'
cxcr7a-TP-2	5' CAATTAAGTGCAAATAC AGTTGCAC 3'
sdf1a control-TP	5' CTGCCTAACCCAAGATA ACACGACA 3'

AUG Morpholinos

sdf1a AUG MO	5' TTGAGATCCATGTTTGCAGTGTGAA 3'
cxcr7b AUG MO	5' ATCATTCACGTTCACAC TCATCTTG 3'
cxcr4b AUG MO	5' AGTGTGCTCAAAAAGGCGCAATAAG 3'

Mature miRNA duplex

miR-430c	5' UAAGUGCUUCUCUUUGGGGdTdA 3'
	5' CCCCAAAGAGAAGCACUAAdTdT 3'
miR-302b	5' UAAGUGCUUCCAUGUUUUAdGdT 3'
	5' UAAAACAUGGAAGCACUAAdTdT 3'

Supplementary Note

The MZ*dicer* mutant is useful for examining the importance of miRNAs early in development because it offers a genetic loss of miRNAs. However, as miRNAs are known to have many targets, these embryos have severe morphological defects resulting from the misregulation of numerous targets. Target Protectors offer a method for dissecting the importance of individual targets. In our experiments, more MZ*dicer* embryos have mislocalized PGCs than TP-injected embryos, a phenotype which correlates with the higher levels of *sdf1a* RNA seen in MZ*dicer* in situs (Fig. 3). There are several possible explanations for the difference between MZ*dicer* and TP-injected embryos. First, other miRNAs may be targeting *sdf1a* to act cooperatively with miR-430 in providing regulation. This is supported by injection of mature miR-430 duplex into MZ*dicer* embryos, which was unable to completely rescue the PGC mismigration (Supplementary Fig. 1). Repression by tissue-specific miRNAs, such as miR-133 in the muscle, may allow clearance from particular tissues once *sdf1a* is no longer needed there. TPs only interfere with miR-430 targeting, and therefore the phenotype would only be a portion of what is seen when all miRNAs are lost. Alternatively, MZ*dicer* embryos lack buffering of chemokine signals as well as a wide range of other miRNA targets. Thus, the more severe mislocalization phenotype may be in part due the perturbation caused by the transcripts upregulated in a background lacking miRNA-mediated buffering of chemokine signaling.

References:

1. Giraldez, A.J. et al. MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308**, 833-8 (2005).
2. Svoboda, P. & Flemr, M. The role of miRNAs and endogenous siRNAs in maternal-to-zygotic reprogramming and the establishment of pluripotency. *EMBO Rep.* **11**(8):590-7 (2010).
3. Giraldez, A.J. et al. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75-9 (2006).