Use of target protector morpholinos to analyze the physiological roles of specific miRNA-mRNA pairs *in vivo*

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MicroRNAs (miRNAs) regulate gene expression by pairing with complementary sequences in the 3′ untranslated regions (UTRs) of transcripts. Although the molecular mechanism underlying miRNA biogenesis and activity is becoming better understood, determining the physiological role of the interaction of an miRNA with its target remains a challenge. A number of methods have been developed to inhibit individual miRNAs, but it can be difficult to determine which specific targets are responsible for any observed phenotypes. To address this problem, we use target protector (TP) morpholinos that interfere with a single miRNA-mRNA pair by binding specifically to the miRNA target sequence in the 3′ UTR. In this protocol, we detail the steps for identifying miRNA targets, validating their regulation and using TPs to interrogate their function in zebrafish. Depending on the biological context, this set of experiments can be completed in 6–8 weeks.

INTRODUCTION

miRNAs are short RNAs (~22 nt) that specifically regulate gene expression at the mRNA level[1]. Developmental, homeostatic and disease processes are modulated by miRNA-mediated repression of transcripts[2,3]. This selective repression by miRNAs is generally conferred by bases two through eight of the mature miRNA. This region, known as the 'seed', binds complementary sequences in the 3′ UTRs of mRNA transcripts[4]. A single miRNA may regulate hundred of targets. In addition, miRNA genes are abundant in the genomes of animals, from *Caenorhabditis elegans* to humans, with hundreds identified in the genomes of mice and humans[5]. Because of the vast number of miRNAs and the limited sequence required for targeting, miRNAs are estimated to regulate between 25% and 60% of human transcripts, on the basis of the evolutionary pressure to maintain the target sequences in evolution[6–8]. Thus, to understand the role of each miRNA in an organism's development and physiology, it is fundamental to know which putative miRNA-target regulatory interactions are physiologically relevant in the biological context under study. This protocol describes the methods available to test whether a target is regulated by a miRNA and to interrogate the physiological role of this regulation *in vivo* using target protectors (TPs). This approach has provided insight into the role of miR-430 in targeting the morphogen *nodal*[9] and the chemokine signal *sdf1a*[9,10].

To further dissect the roles of individual miRNAs while avoiding nonspecific effects, many studies conducted since then have removed individual miRNAs. The first of these mutants, lin-4 and let-7, were identified in *C. elegans* genetic screens for defects in developmental timing[11,12]. In mice and *Drosophila*, mutants have been made through deletion or targeted mutation of the miRNA gene Bantam (for instance, miR-17 (ref. 13) and miR-451 (ref. 14) in mice; and miR-1 (ref. 15), miR-278 (ref. 16), miR-309 (ref. 17) and miR-8 (ref. 18) in *Drosophila*). In other systems in which knockout technology is not as advanced, transient techniques are used (**Fig. 1**). Morpholinos (MOs) have been used extensively in zebrafish and *Xenopus* to inhibit translation of mRNAs[19,20]. By designing a MO to bind the mature miRNA, the hairpin structure of the pre-miRNA is disrupted, preventing the processing required to make a mature miRNA[21]. This is particularly useful in studying early embryonic phenotypes because the MO is injected at the one-cell stage and is effective for the first 5 d of development[22–24]. Other antisense oligonucleotides, such as antagomirs, have been optimized for use in cell lines[25,26]. An alternate approach, rather than impeding processing, aims to bind all mature miRNAs. By stably overexpressing an mRNA with multiple miRNA-binding sites, the miRNA binds this ectopic transcript rather than its endogenous target. Because they are used to soak up the mature miRNA, these mRNAs are called miRNA sponges[27–29].

Although deleting or blocking individual miRNAs provides a genetic way to dissect the overall function of a given miRNA, these approaches also have a number of shortcomings. Technically, generating these knockouts can be difficult because many miRNAs are members of large miRNA families and may be present in multiple copies in the genome. In addition, removing the miRNA causes upregulation of all the transcripts it regulates. Because a direct relationship cannot be drawn between an miRNA and a specific target, it can be difficult to gain insight into the molecular mechanism underlying a specific phenotype. Furthermore, the effects might be caused by secondary targets that are up- or downregulated. For instance, loss of regulation of a targeted transcription factor will also increase the expression of genes it controls, although these are not directly targeted by the miRNA.

To examine the importance of an miRNA repressing a particular target, we have developed TPs to specifically interfere with this interaction. TPs are antisense oligonucleotides designed to bind perfectly to the region of the 3′ UTR that is complementary to the miRNA[9]. We have successfully used this technology to examine the role of a particular miRNA, miR-430, in regulating the chemokine Sdf1 during primordial germ cell migration in zebrafish[10]. This approach has now been used in a number of other contexts including in cell culture[30], in zebrafish[10], in *Xenopus*[31,32] and in *Drosophila* (through transgenic delivery)[33]. In zebrafish and *Xenopus*, MOs have been used. The modified backbone of these oligos increases their stability[34] while preventing them from being loaded into the

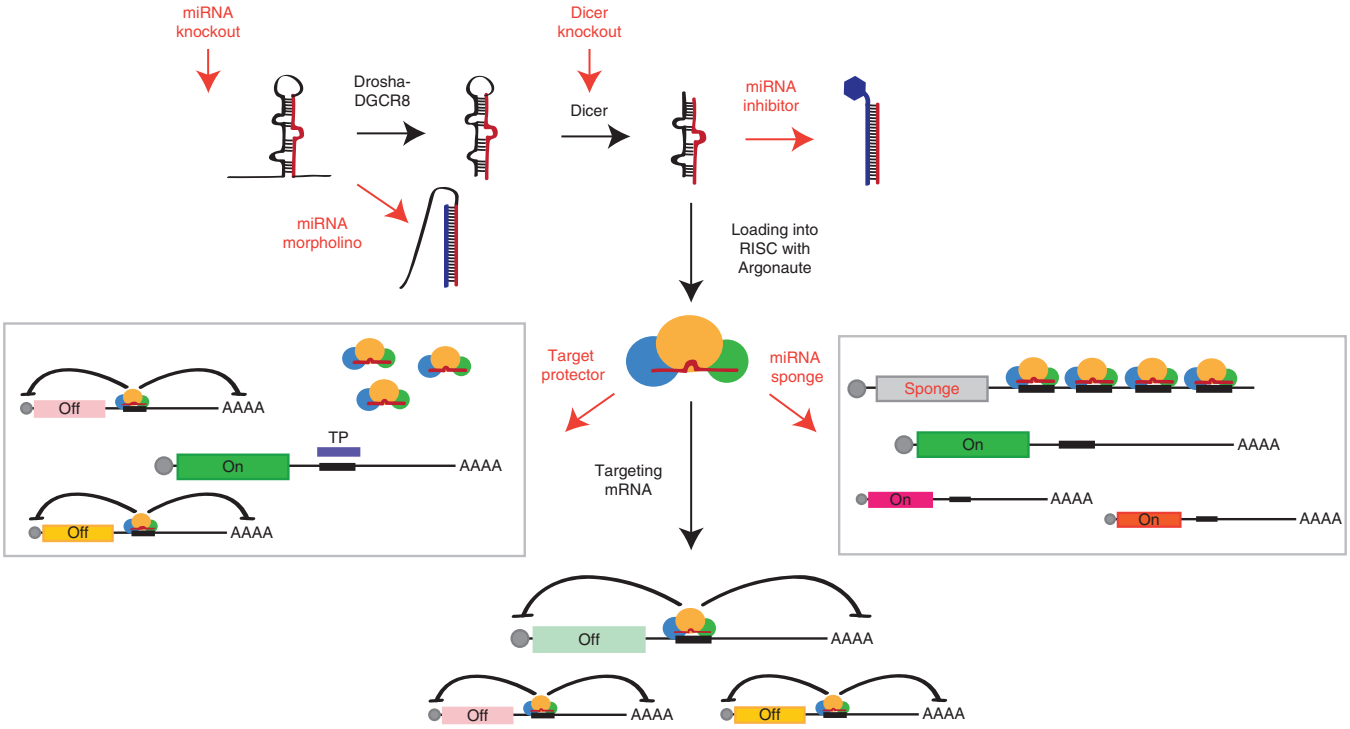

Figure 1 | Interfering with miRNA processing can help to reveal the functions of miRNAs. miRNA genes are transcribed by RNA polymerase II as long transcripts that can contain one or more miRNAs. These transcripts (pri-miRNAs) are cleaved first by Drosha-DGCR8 to produce a hairpin structure (pre-miRNA) and then by Dicer, generating a double-stranded RNA molecule. One of these strands is loaded into the RISC with Argonaute and guides the complex to target mRNAs[1]. Protein output from these genes is reduced, either due to deadenylation and accelerated degradation or translational repression. Tools that inhibit these steps (shown in red) are useful in understanding the roles of miRNAs.

RNA-induced silencing complex (RISC) and triggering an RNA interference response. Currently, the only other method for disrupting a specific miRNA-target pair is through creating a genetic mutation of the target site in the 3′ UTR using current mutagenesis techniques[35,36], which requires a great deal of time and resources.

The widespread regulatory role of miRNAs has become apparent in the last decade. Using TPs allows the examination of the effects miRNAs have on individual targets. This directed approach is broadly applicable. For instance, the use of TPs can aid those interested in examining the physiological role of individual miRNA targets. In addition, researchers who are new to the miRNA field can study the regulation of their genes or pathways of interest and test whether the regulation by different miRNAs has an important role in their biological system. From a therapeutic standpoint, this approach of interfering with the miRNA-mediated repression of a single target can have a number of applications. If a disease is caused by lowered expression of a gene, using a TP to interfere with its regulation by miRNA could restore expression to the normal level. This strategy takes advantage of the ability of TPs to increase expression by a modest amount and only in cells where the gene is already expressed. Similarly, TPs could be used to increase the expression of a gene with a beneficial or disease prevention role. Finally, if a mutation creates a new miRNA-binding site that results in lower expression of an important gene, treatment could occur by protecting this new target site from the miRNA[9].

Several potential limitations of TPs should be considered. Although MOs bind with a high level of specificity to their target sequences, if similar sequences are present in the genome, it is possible that off-target effects could be observed. This would be particularly problematic if the sequence surrounding the miRNA seed is similar to that of other targets of that miRNA. In addition, if the TP does not bind efficiently to the target sequence, interference with miRNA targeting may be incomplete, thus masking the full effect of miRNA regulation. Finally, MOs have the limitations that they are generally only active for the first 5 d of development, and they can cause nonspecific effects due to toxicity.

In this protocol, we describe the process for (i) determining whether a 3′ UTR confers miRNA-mediated repression, (ii) designing TPs and (iii) confirming the specificity of the TP-induced phenotype. Although the protocol described here is focused on zebrafish, many of the principles can be easily adapted to other organisms or even to cell culture.

Experimental design

After identifying a putative target mRNA, the ability of its 3′ UTR to confer regulation *in vivo* is determined using a reporter vector. This reporter is injected in zebrafish embryos in the presence or absence of the miRNA of interest. In addition, a reporter is made in which the putative target site in the 3′ UTR is mutated. Once regulation is confirmed, a TP and control TP are designed and ordered. The TP is injected in embryos to determine an appropriate concentration and then co-injected with the reporter mRNA to confirm that it relieves miRNA-mediated repression. The TP is then injected in wild-type embryos and the resulting phenotype is characterized. The specificity of this phenotype is confirmed by comparison with the control TP phenotype and by rescue with a translation-blocking MO. Below, we describe a few topics to consider when designing the experiments.

Prediction of target sites. Several approaches can be taken to identify the 3′ UTR of a gene of interest. The publicly available UCSC (http://genome.ucsc.edu/) or Ensembl (http://www.ensembl.org/index.html) genome browsers can be used to locate the coding sequence. In many cases, an annotated 3′ UTR based on EST data is available. It is important that the entire UTR be identified to ensure that functioning regulatory sites are not missed. Three ways of identifying the UTR are looking at RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/) sequences, examining mRNA sequencing data and using 3′ rapid amplification of complementary DNA ends. In most cases, RefSeq is sufficient, but it is important to note that a stretch of As in the 3′ UTR sequence may cause mispriming by oligo dT and can result in an underestimation of the 3′ UTR length.

Predicting which miRNAs target a 3′ UTR remains a challenge in the field. Many software programs are available to predict which miRNAs might target a given UTR (PicTar, TargetScan and so on; reviewed in refs. 37,38). These algorithms consider many factors including match to the seed sequence, 3′ compensatory binding and conservation among species. In deciding which miRNAs to study, it is also important to focus on those that are expressed in the tissue and developmental stage being studied. MiRNA expression data in zebrafish are available[39].

If a particular miRNA is being studied, the 3′ UTRs of potential targets can first be searched for 7- or 8-nt matches to the seed sequence. However, it is important to note that the seed is not always sufficient for repression[40], and noncanonical miRNA seeds can also be targeted[12,41–44]. Because of these difficulties in predicting miRNA targets, a number of approaches have been used to identify targets experimentally. Several studies have used gene expression analysis to find mRNAs that are upregulated when miRNA function is blocked or downregulated upon miRNA overexpression[6,45–47]. These expression studies have been followed by experiments examining the changes in protein expression that occur in the presence and absence of miRNAs[48,49]. In addition, knowledge of how target regulation occurs has allowed biochemical experiments to pull down RISC proteins and determine which miRNAs and mRNAs are associated[50–57]. By using a combination of these *in silico* predictions and available data sets, putative miRNA-target interactions can be identified. Initial experiments using TPs demonstrated that protection of a particular miRNA site in the 3′ UTR does not affect the regulation of a separate site in the same 3′ UTR[9]. This is important to keep in mind, as 3′ UTRs may be regulated by more than one miRNA, or the same miRNA may target a transcript at several sites. In either case, separate TPs, one for each target site, would be required to completely relieve this regulation.

Primer design. Once the 3′ UTR is identified, design primers to amplify the entire sequence. Primer3 (http://primer3.sourceforge.net/) or other primer design software can be used to find a forward primer that begins immediately after the end of the coding sequence. Include an XhoI restriction site at the beginning of this primer to allow cloning into the expression vector (5′-caactcgag-template sequence-3′). The reverse primer should be as close to the end of the 3′ UTR as possible and should include an XbaI restriction site (5′-CAATCTAGA-sequence complementary to end of UTR-3′). It is important to check whether XhoI or XbaI restriction sites are present in the 3′ UTR sequence. If these sites are encoded, other restriction sites (SalI in the place of XhoI and NheI in the place of XbaI) can be used to clone into the expression vector.

To mutate the miRNA target site, design a set of primers to the region including the seed. One primer binds 3′ to 5′ and acts as a reverse primer, and the other binds 5′ to 3′ and will act as a forward primer. When aligned, at least 10 nt of these primers should overlap. In these primers, change 3 nt in the middle of the seed (for instance, TGA to ACT). Amplify the 3′ UTR using the original forward primer with the mutated reverse primer and the original reverse primer with the mutated forward primer. Thereafter, combine the PCR products of these reactions for a PCR with the original forward and reverse primers. This product can be cut and cloned as above. More details on this mutagenesis can be found in reference 58.

Validation of miRNA-mediated regulation. To study the regulation of a particular target, it is important to first establish that the 3′ UTR confers regulation by a particular miRNA. miRNAs may speed degradation or slow translation of their targets, but repression caused by either mechanism can be assessed by measuring protein output of a reporter[12,45,59,60]. Reporter assays were initially designed with β-galactosidase but have more recently been adapted to use a fluorescent protein (GFP or luciferase). GFP allows the direct visualization of downregulation, which can be particularly useful in examining tissue-specific regulation. On the other hand, luciferase allows for more precise quantification of expression levels, making it more suitable for measurement of subtle differences and for high-throughput applications[46]. Both types of reporters share the same basic design, using a vector encoding the reporter gene followed by the 3′ UTR sequence (**Fig. 2a**,**b**). For comparison, a second reporter that lacks a regulated 3′ UTR is expressed in the same cells. In zebrafish, these reporters are frequently *in vitro* transcribed and injected as mRNA. This allows for ubiquitous expression of the reporter.

DNA reporter constructs have also been used[46,61]. These constructs contain both the reporter and the control in the same plasmid, driven by separate promoters (**Fig. 2c**). Expression of the control but not the reporter indicates repression by the miRNA. Because inheritance of this construct is uneven, embryos express the DNA reporter in a mosaic pattern and individual cells can be evaluated. This type of reporter can be particularly useful for addressing tissue-specific regulation of a target. For instance, in looking at the regulation provided by muscle-specific miR-1, muscle cells will only express the green control, whereas nearby nonmuscle cells will be both green and red.

In this protocol, we will describe the use of a GFP reporter and dsRed control mRNA. The protocol can easily be adapted for luciferase mRNA, as shown in **Box 1**. For more details about the dual-promoter DNA reporter, refer to references 46 and 61. Once the regulation of a reporter has been established, predicted target sites can easily be mutated to verify that the target sequence is required for repression. Two or three nucleotides in the sequence complementary to the miRNA seed are mutated using site-directed mutagenesis.

Design and test target protectors. To block the regulation of an endogenous miRNA target, TPs are designed to bind the target site in the 3′ UTR. We design our TPs to be 25 nt long, which is the standard length for MOs. We follow several parameters to create a TP that binds well, which includes the entire sequence (7–8 nt) complementary to the miRNA seed, contains a G or C at the 3′ end and has an overall 40–60% of GC. We consider recommendations for effective MOs, such as avoiding sequence self-complementarity

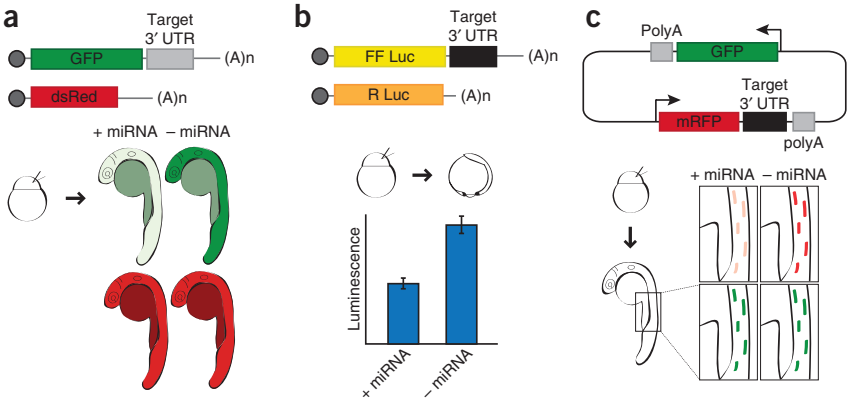

Figure 2 | Several different reporters can be used for validating miRNA targets. (**a**) Injection of GFP mRNA with the 3′ UTR of interest. In the presence of the miRNA, GFP expression is lower than when the miRNA is absent. dsRed mRNA is injected as a control. (**b**) Firefly (FF) luciferase with the 3′ UTR of the putative target is injected. Luminescence, a measure of luciferase expression, is higher in the absence of the miRNA. Renilla (R) luciferase is used as a control. (**c**) A DNA construct containing both red fluorescent protein (RFP) and GFP is injected. The 3′ UTR is after RFP. Integration of the plasmid is mosaic, so only some cells will be fluorescent. The lower expression of RFP compared with that of GFP indicates repression by the miRNA.

and stretches of four or more contiguous Gs. We do not typically focus on including sequences upstream of the seed, even though this may be an area of partial binding for the miRNA. We have successfully developed TPs that extend upstream or downstream from the seed. However, our experience includes a limited number of examples, and we have not carried out an exhaustive analysis to determine whether binding the sequence complementary to the 3′ end of the miRNA affects the efficiency of the TP. Finally, once a suitable TP sequence is identified, we run a BLAST search to look for other regions of the genome where binding could occur. Upon finding a unique sequence, the MO can be ordered from Gene Tools.

Some 3′ UTRs may contain multiple miRNA target sites. This may include multiple sites targeted by one miRNA or single target sequences for several miRNAs. In either case, reporter assays can be used to determine whether all sites confer repression, as described above. This will narrow down the number of TPs to be designed. Individual TPs for each validated target site can then be co-injected to achieve complete protection of the target, as we did in protecting the two miR-430 target sites in the cxcr7a 3′ UTR[10]. The limitation of this approach is that the concentration of the TPs must be adjusted so that they do not have a toxic effect.

Controls. Before examining the phenotypic effect of using the TP, it is important to test whether the designed TP can relieve miRNA-mediated repression without causing off-target effects. To this end, we first inject the TP at a range of concentrations and determine the highest concentrations that do not cause nonspecific defects such as general cell death or embryo lethality. This sets the concentration range for the following experiments. Second, we co-inject the TP with the GFP reporter mRNA and determine the lowest concentration of TP required to relieve GFP reporter repression to a level similar to that of the mutant reporter or the reporter in the absence of the miRNA (MZ*dicer* mutants). Third, we test that the injection of the TP does not cause an increase in expression of the GFP reporter in the absence of the miRNA. This indicates that a change in wild-type reporter expression is due to interference with miRNA targeting and not to general stabilization of the 3′ UTR. Fourth, to confirm that the TP was indeed functioning in this manner, we design a control TP that binds part of the 3′ UTR sequence that lacks any predicted miRNA target sites. If the TP is functioning simply by blocking miRNA-mediated repression of the target, the control TP, which binds outside the target site, should not alter the expression of the wild-type reporter.

Box 1 | Measuring luciferase expression in zebrafish embryos ● TIMING 1 d

1. Inject firefly luciferase reporter and Renilla luciferase control RNAs into the cell of a one-cell embryo. Inject 100 pl at a concentration of 10 ng μl^{-1}.
2. Split the injected embryos into two equal groups. In one group, inject the mature duplex of the miRNA of interest. Leave the other group without the miRNA.
3. Transfer the embryos to an agarose-coated plate with blue water and incubate at 28 °C for at least 8 h. Measurement should be taken after 8 h.p.f. but before the endogenous miRNA is expressed.
4. Transfer 5–10 embryos into a transparent 1.5-ml tube. Make triplicates for each injection group. Remove as much water from the tube as possible.
5. Use the Dual-Glo luciferase assay system and prepare reagents according to the kit instructions. Perform the assay at room temperature.
6. Add 50 µl of Dual-Glo luciferase reagent to each tube.
7. Homogenize embryos by vortexing for 5 s.
8. Wait for 10 min.
9. Measure firefly luciferase luminescence.
10. Add 50 µl of Dual-Glo Stop & Glo and mix briefly and wait for at least 10 min.
11. Measure Renilla luminescence.
12. Normalize the luminescence of the firefly luciferase to the Renilla luciferase for each sample. Average the triplicates for each group and compare the expression in the presence and absence of the miRNA.

Phenotype analysis. To assess the role of a particular miRNA-target interaction, we inject the TP in wild-type embryos and determine the resulting phenotype. This part of the protocol can be adapted for the process being examined. This may include *in situ* hybridization, antibody staining or live imaging[62–65]. Previous studies examining the target of interest can provide insight into the potential phenotypic effect. For instance, in studying *ndr1* and *lft1*, (also known as *squint* and *lefty*, respectively), expression of downstream signaling genes and changes in morphology known to accompany disturbance of the levels of these transcripts were assayed[9]. Similarly, overexpression and knockdown of *sdf1a* (official name: *cxcl12a*) and *cxcr7b* had previously been shown to disrupt germ cell migration[66,67]. In examining regulation of *sdf1a* and *cxcr7b*, we used *in situ* hybridization for *nanos3* to examine the location of germ cells[10]. Alternatively, if the physiological effects of altering gene expression levels are unknown, the endogenous expression can be used to identify cell types or tissues affected by inhibiting miRNA-mediated repression.

Once a phenotype is identified, it can be compared with the phenotype observed in the absence of the miRNA. Two additional experiments can test the specificity of the observed phenotype. First, injection of a control TP outside the miRNA complementary region should not cause a phenotype different from the wild type. Second, we test whether the phenotype observed is a direct consequence of increasing the translation of the endogenous gene by asking whether reducing the translation of the target in the presence of the TP restores the wild-type phenotype. To this end, we inject low amounts of a MO that blocks the translation of the target mRNA (directed against the translation start codon, AUG MO). To design the AUG MO, the same criteria used to create the TP (described above) can be used. Alternatively, if ordering through Gene Tools, the sequence including the start site can be provided and a recommended MO sequence will be returned. Reducing the overall translation of the transcript can counteract the upregulation that was caused by removing the miRNA-mediated repression, thereby rescuing the TP-induced phenotype. This rescue experiment requires careful titration of the AUG MO, as injecting too much will produce a knockdown phenotype. In addition, this experiment is likely to be most effective when examining the effects of a broadly expressed miRNA. If the miRNA is only expressed in a subset of cells expressing the transcript, using an AUG MO that will knock down all transcripts may have additional phenotypes caused by the loss of function in domains in which the target is required and the miRNA is not expressed.

MATERIALS

REAGENTS

- Zebrafish wild-type strains can be obtained from Zebrafish International Resource Center (http://zebrafish.org/) **! CAUTION** Zebrafish lines should be maintained and bred in accordance with all relevant institutional ethical guidelines.
- Expression vector encoding GFP (pCS2 + GFP)[68]
- Expression vector encoding dsRed (pCS2 + dsRed)[68]
- Primers for amplifying 3′ UTR (see Experimental design for further information on primer design)
- Primers for mutating miRNA target site (see Experimental design for further information on primer design)
- AmpliTaq (Applied Biosystems, cat. no. N8080172)
- Buffer II (10×, provided with AmpliTaq)
- $MgCl_2$ (25 mM, provided with AmpliTaq)
- dNTPs (10 mM; Invitrogen, cat. no. 18427-013)
- XhoI (NEB, cat. no. R0146S)
- XbaI (NEB, cat. no. R0145S)
- NotI restriction enzyme (NEB, cat. no. R0189S)
- Alkaline phosphatase, calf intestinal (CIP; NEB, cat. no. M0290S)
- NEBuffer 4 (10×, provided with XhoI and XbaI enzymes)
- NEBuffer 3 (10×, provided with NotI enzyme)
- BSA (100×, NEB, provided with XhoI and XbaI enzymes)
- Agarose (American Bioanalytical, cat. no. AB00972-00500)
- Ethidium bromide solution (Sigma-Aldrich, cat. no. E1510)
  ! CAUTION Ethidium bromide is a hazardous substance and appropriate safety precautions should be followed during handling and disposal.
- Centrifuge tube (1.5 ml; USA Scientific, cat. no. 720678)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- Luria broth (LB)-ampicillin plates (see REAGENT SETUP)
- LB base (Invitrogen, cat. no. 12795-027)
- Agar (bacteriological; American Bioanalytical, cat. no. AB01185-00500)
- Ampicillin (Sigma-Aldrich, cat. no. A0166)
- Syringe filter (25 mm), 0.22 μM, nylon (Fisher Scientific, cat. no. 09-719c)
- One Shot TOP10/P3 chemically competent *Escherichia coli* (Invitrogen, cat. no. C5050-03) or other chemically competent *E. coli* cells
- SOC medium (Invitrogen, cat. no. 15544034)
- T4 DNA ligase (NEB, cat. no. M0202S)
- T4 DNA ligase buffer (supplied with ligase)
- Dual-Glo luciferase assay system (Promega)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27104)
- mMESSAGE mMACHINE SP6 kit (Ambion, cat. no. AM1340)
- RNeasy mini kit (Qiagen, cat. no. 74104)
- Nuclease-free water (Ambion, cat. no. AM9937)
- Solution of phenol red (0.5% (wt/vol), Gibco; cat. no. 15100-043)
- Glass capillaries with filament, 1 mm × 4 inch (World Precision Instruments, cat. no. TW100F-4)
- Fish water (water removed from the zebrafish facility system)
- Methylene blue (Sigma-Aldrich, cat. no. M9140)
- Blue water (see REAGENT SETUP)
- Protease (Roche, cat. no. 165921)
- Six-well plates (VWR, cat. no. 82050-842)
- Petri dishes (100 mm BD Biosciences, cat. no. F1029)
- Petri dishes (60 mm BD Biosciences, cat. no. F1007)
- Agarose-coated plate (see REAGENT SETUP)
- Agarose injection plate[69]
- Fire-polished wide-bore glass pipette
- Tris base
- Glacial acetic acid
- EDTA
- CDNA

EQUIPMENT

- Incubator, 28 and 37 °C
- Shaking incubator, 37 °C
- Filters, 0.22 μm pore
- Petri dishes
- Microcentrifuge (Eppendorf 5424, Eppendorf)
- Microcentrifuge tubes
- Razor blade for cutting gels
- Electrophoresis apparatus for agarose gels
- NanoDrop 2000 (Thermo Scientific) or other method of DNA quantification
- Microinjection apparatus with stereomicroscope (as shown in ref. 69)
- Micrometer (1 mm divided into 0.01-mm units; Meiji Techno, cat. no. MA285)

- Stainless steel forceps, no. 5 (Fine Science Tools, cat. no. 11252-20)
- Thermocycler
- Dissecting microscope (Zeiss Discovery V12, Carl Zeiss) with filters for GFP and dsRed
- Camera (Zeiss AxioCam MRc, Carl Zeiss) connected to a computer
- Adobe Photoshop or other imaging software
- Micrometer slide

REAGENT SETUP

TAE, 50× Add the following to 900 ml of distilled water: 242 g of Tris base, 57.1 ml of glacial acetic acid and 18.6 g of EDTA. Adjust the volume to 1 liter with additional distilled water. This buffer can be prepared ahead of time and stored at room temperature (~25 °C) for 1 year.

Agarose gel, 1% (wt/vol) Add 1 g of agarose to 100 ml of 1× TAE buffer. Microwave for ~1 min until all agarose is in solution. Add ethidium bromide to the hot agarose to a final concentration of 0.5 μg ml^{-1}. Pour this mix in a gel apparatus, add a comb to create wells and cool until it solidifies. Remove the comb and add 1× TAE buffer. Gels should be freshly prepared for each use.

Ampicillin stock solution Dissolve 1 g of ampicillin salt in 20 ml of water filtered with a 0.22-μm filter. Prepare 1-ml aliquots and store them at −20 °C for up to 1 year.

LB-ampicillin plates To prepare LB plates, add 25 g of LB base and 15 g of bacteriological agar to 1 liter of water. Autoclave for 30 min to sterilize. Cool to 55 °C, and then add 1 ml of 50 mg ml^{-1} ampicillin. Pour into 100-mm Petri dishes and cool until it solidifies. Store upside-down at 4 °C for 1–2 months.

Injection needles Pull glass capillary tubes to create needles with tapered tips that are strong enough to poke into a cell. It may be necessary to optimize conditions to pull the best needles. Needles can be pulled ahead of time and held in a Petri dish in clay.

Blue water Add 2 ml of 0.1% (wt/vol) methylene blue to 1 liter of fish water. Store at room temperature for 1 month.

Pronase Prepare a stock solution by dissolving 0.1 g of protease in 20 ml of water. Prepare 2-ml aliquots and store at −20 °C for several months. On the day of injection, add 2 ml of pronase stock to 8 ml of blue water to obtain a final concentration of 1 mg ml^{-1}.

Agarose-coated plates Dissolve 1 g of agarose in 100 ml of blue water. Microwave for ~1 min until all agarose is in solution. Pour the agarose in a Petri dish or in the wells of a six-well plate to cover the bottom surface (approximately 2–4-mm thick). Cool until the agarose is solid, and then fill the plate with blue water. Freshly prepare new plates for each use.

PROCEDURE

Generating fluorescent reporters ● TIMING 1–2 weeks

1| For each putative target being studied, set up a 50-μl PCR reaction as follows to amplify the 3′ UTR from a cDNA library (see Experimental design for further information on identification of the 3′ UTR and primer design). We use AmpliTaq, but any Taq DNA polymerase can be used.

	Volume (μl)	Final concentration
10× Buffer II	5	1×
$MgCl_2$ (25 mM)	5	2.5 mM
dNTPs (10 mM)	2	0.4 mM
Forward primer (10 μM)	1	0.2 μM
Reverse primer (10 μM)	1	0.2 μM
cDNA	2–3	
AmpliTaq polymerase (5 U $μl^{-1}$)	0.5	2.5 U
Sterile water	Up to 50	

2| Run the PCR using the following conditions.

Cycle number	Temperature (°C)	Time
1	95	2 min
2–34	95	30 s
	58	30 s
	72	1 min per kb amplified
35	72	7 min

3| Set up digestion of the PCR reaction with XhoI and XbaI. Mix the components and incubate at 37 °C for 2 h.

	Volume (μl)	Final concentration
10× NEBuffer 4	5	1×
10× BSA	5	1×
PCR (from Step 2)	25	Variable
XhoI (20 U $μl^{-1}$)	0.5	10 U
XbaI (20 U $μl^{-1}$)	0.5	10 U
Sterile water	14	

▲ **CRITICAL STEP** Check the sequence of the 3′ UTR PCR product for XhoI and XbaI sites. If either is present, use other cloning sites (as described in Experimental design).

4| While cutting the PCR product above, digest the reporter vector with XhoI and XbaI. Set up the reaction shown below and incubate at 37 °C for 2 h.

	Volume (μl)	Final concentration
10× NEBuffer 4	5	1×
10× BSA	5	1×
pCS2 + GFP	5	Variable
XhoI (20 U μl^{-1})	0.5	10 U
XbaI (20 U μl^{-1})	0.5	10 U
Alkaline phosphatase (CIP)	0.5	5 U
Sterile water	33.5	

5| Load 25 μl of each digestion from Steps 3 and 4 on a 1% (wt/vol) agarose gel in 1× TAE buffer. Discrete bands the size of the 3′ UTR PCR product and digested vector should appear in the gel. Use a razor blade to cut these bands out of the gel and place each into a 1.5-ml centrifuge tube.

! CAUTION Ethidium bromide is a mutagenic compound and appropriate precautions should be taken when handling the gel. Wear gloves and goggles when cutting out the gel.

? TROUBLESHOOTING

6| Use the QIAquick gel extraction kit to isolate DNA from gel slices according to the manufacturer's instructions. In the final step, elute the DNA in 30 μl of sterile water.

7| Combine the digested DNA in the ligation mix shown below. Incubate at room temperature for 15 min.

	Volume (μl)	Final concentration
10× T4 DNA ligase buffer	2	1×
pCS2 + GFP (XhoI, XbaI digested from Step 6)	1	Variable
3′ UTR PCR (XhoI, XbaI digested from Step 6)	3–10	Variable
T4 DNA ligase (400 U μl^{-1})	1	400 U
Sterile water	Up to 20	

8| Transform 3 μl of the ligation reaction in chemically competent DH5α cells, as described previously[70]. Recover cells in 250 μl of SOC medium and plate 100 μl of the transformation on an LB plate containing 50 μg ml^{-1} ampicillin. Incubate at 37 °C overnight.

9| Use a sterile pipette tip to pick 4–6 colonies and incubate in 2 ml of LB medium containing 50 μg ml^{-1} ampicillin. Shake at 250 r.p.m. at 37 °C overnight.

? TROUBLESHOOTING

10| Follow the manufacturer's instructions for the QIAprep spin miniprep kit to isolate the plasmid DNA. In the final step, elute the DNA in 30 μl of nuclease-free water.

11| Check for the presence of correct vector by digesting 3 μl of isolated plasmid DNA from Step 10 with XhoI and XbaI, as in Step 4. Run the reaction on an agarose gel and confirm that two bands are present: one that is the size of the 3′ UTR and another the size of the vector containing GFP.

■ PAUSE POINT The DNA plasmid can be stored at −20 °C for months.

? TROUBLESHOOTING

12| Once the reporter vector has been created, it can then be used to create a reporter with the miRNA target site mutated (mutated 3′ UTR). Any technique for site-directed mutagenesis may be used. We use the sequential PCR method, described in reference 58, to mutate 3 nt in the middle of the 3′ UTR sequence complementary to the seed, as shown previously[45].

■ PAUSE POINT The DNA plasmid can be stored at −20 °C.

Preparing reporter mRNAs for injection ● TIMING 2–3 d

13| Use a NanoDrop or other method of DNA quantification to determine the concentration of the plasmid DNA. Use the GFP reporter plasmids (wild-type and mutated), as well as the pCS2 + dsRed control plasmid.

14| Mix the following components to linearize the reporter plasmid. Incubate at 37 °C for 2 h.

	Volume (μl)	Final concentration
10× NEBuffer 3	5	1×
10× BSA	5	1×
Reporter plasmid	Variable	4 μg
NotI (10 U μl^{-1})	1	1 U
Sterile water	Up to 50	

▲ **CRITICAL STEP** Check the sequence of the 3′ UTR for NotI restriction sites. If this site is present, use another enzyme that has sites after the SV40 3′ UTR (such as SacII, ApaI or Asp718) and does not cut elsewhere in the vector. Change the buffer as necessary.

15| Load the entire reaction on a 1% (wt/vol) agarose gel with 1× TAE buffer. Run the gel at 100 V for ~1 h. Use a razor blade to cut out the band of the linearized plasmid. Collect the gel slice in an RNase-free 1.5-ml microcentrifuge tube.

▲ **CRITICAL STEP** From this point forward, all reagents should be kept free of RNase contamination.

16| Follow the manufacturer's instructions and use the QIAquick gel extraction kit to isolate DNA from gel slices. In the final step, elute the DNA in 30 μl of RNase-free water.

▲ **CRITICAL STEP** This gel extraction kit should be kept separate from that used in cloning and reserved for use in preparing *in vitro* transcript templates in order to keep it RNase free and prevent RNase contamination.

17| Use the mMESSAGE mMACHINE SP6 kit to *in vitro* transcribe capped RNA from the digested plasmid. Thaw the reaction buffer and NTP/CAP mix (from the kit) at room temperature. Add the following components to an RNase-free microcentrifuge tube. Incubate at 37 °C for 2 h.

	Volume (μl)	Final concentration
10× Reaction buffer	2	1×
2× NTP/CAP	10	1×
Linearized DNA template (from Step 16)	8	1 μg
Enzyme mix	2	

18| Add 80 μl of nuclease-free water to the reaction and follow the manufacturer's instructions for RNA cleanup using the RNeasy mini kit. In the final step, elute the RNA from the column with 30 μl of nuclease-free water.

19| Determine the concentration of the RNA using a NanoDrop or other method of RNA quantification. We typically obtain 15–25 μg per reaction. Split the RNA into 3-μl aliquots in nuclease-free tubes. Store at −80 °C.

▲ **CRITICAL STEP** RNA aliquots should be prepared to avoid freeze-thaw cycles that will accelerate the degradation of the RNA.

■ **PAUSE POINT** RNA can be stored at −80 °C for months.

? TROUBLESHOOTING

Injecting and analyzing of reporters ● TIMING 2–3 d

20| On the evening before injections, set up mating pairs of wild-type zebrafish, as described previously[71].

21| Obtain and dechorionate embryos as described in previous work[69,72].

▲ **CRITICAL STEP** For injection of zebrafish embryos, we dechorionate embryos and inject in the cell at the one-cell stage. In our experience, this reduces injection variability.

22| On the morning of injection, prepare the following mix and keep it on ice.

	Volume (μl)	Final concentration
GFP reporter mRNA (from Step 19)	Variable	100 ng μl^{-1}
dsRed control mRNA (from Step 19)	Variable	80 ng μl^{-1}
1% (wt/vol) phenol red	0.5	0.1%
Nuclease-free water	Up to 5	

23| Load the needle with 1–2 μl of the RNA injection mix. Place the needle in the microinjection apparatus and use forceps to break the tip of the needle.

24| Calibrate the injection volume by measuring the diameter of a drop expelled in mineral oil on a micrometer slide (**Fig. 3**). The micrometer divides 1 mm into 100 segments. Create a drop that has a diameter of 0.1 mm, producing a 500-pl drop. Adjust the injection volume by changing the injection time or breaking off more of the tip of the needle to make the opening slightly larger.
▲ **CRITICAL STEP** For accurate measurement, ensure that the drop does not collapse on the slide.

25| Use a polished glass pipette to place dechorionated, wild-type embryos at the one-cell stage in an injection plate. Insert the tip of the calibrated needle into the cell of a one-cell embryo. Inject 1 nl (two drops) in each embryo as described in previous work[69,72]. Inject groups of 15–20 embryos with either the wild-type or mutated reporter.
▲ **CRITICAL STEP** Dechorionated embryos must remain completely submerged in water. Because embryos without a chorion can adhere to plastic, all Petri dishes should be coated with agarose, as described in REAGENT SETUP.

26| Transfer the injected embryos to the wells of a six-well plate. Do not place more than 25–30 embryos per well. Place embryos in a 28 °C incubator. After 6–8 h, inspect embryos and remove those that are unfertilized or malformed.

27| Visualize reporter fluorescence in a dissecting microscope when embryos are either undergoing gastrulation (6–9 hours post-fertilization, h.p.f.) or at 24 h.p.f.
? TROUBLESHOOTING

28| Select three or four embryos from each group of injections that show similar expression of the control (dsRed). Place these embryos in separated areas of an agarose-coated plate.
? TROUBLESHOOTING

29| Use a 10-μl plastic pipette tip to punch a straight line of holes in the agarose, creating a holding place for each embryo. Place the embryos in these holes, allowing the yolk to rest in the hole with the rest of the embryo flat on the agarose. Be sure to keep all embryos from a group together so that they can be correctly identified. Turn embryos so that they are in the same orientation.

30| Take pictures of the red and green fluorescence of all embryos.

31| Compare pictures of the reporters with a wild-type 3′ UTR and a mutated 3′ UTR. Quantification of repression is described in **Box 2**.

Designing and validating the TP ● TIMING 2–3 weeks

32| Design and order the TP and control TP (see Experimental design).

33| Prepare a series of five dilutions of the TP in nuclease-free water (0.1–2 mM). Inject 15–20 wild-type embryos for each concentration (75–100 embryos total) with increasing amounts of the TP as described in Step 25. Leave a group of 15–20 embryos uninjected to function as a control.

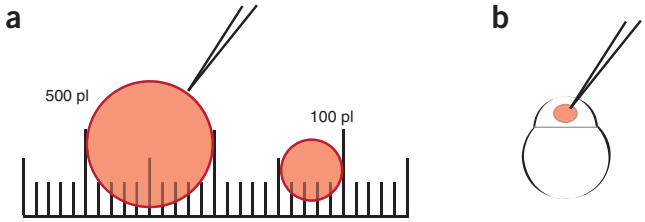

Figure 3 | Calibration of the injection needle. (**a**) The view through a microscope of a micrometer with injection bubbles of different volumes. The injection needle should be calibrated to allow accurate injection amounts. Use forceps to break the injection needle to the size required to create the desired volume. (**b**) Inject the reporter into the cell, not the yolk, of a one-cell-stage embryo. We find that this reduces variability.

Box 2 | Quantification of GFP reporter expression ● TIMING 1 d

1. Inject embryos with a GFP reporter and a dsRed control, as described in Steps 21–30.
2. Take pictures of the embryos to be compared side by side.
3. Measure the average pixel intensity for GFP and dsRed using Adobe Photoshop or other imaging software.
4. For each embryo, the intensity is the pixel intensity of the background area next to the embryo subtracted from the pixel intensity of the embryo.
5. To compare the difference in expression, use the following calculation: Fold increase in intensity = (GFP $Intensity_{No\ miRNA}$/GFP $Intensity_{miRNA}$) / (dsRed $Intensity_{No\ miRNA}$/dsRed $Intensity_{miRNA}$)

34| Transfer embryos to the wells of a six-well plate and store in a 28 °C incubator. After 6–8 h, remove all dead or unfertilized embryos. Note whether high levels of lethality occur in any group. Examine embryos at 24 and 48 h.p.f. for increases in embryo death or nonspecific defects such as increased cell death. This will help to determine which TP concentrations are toxic, and an amount just below this can be used for subsequent experiments.
? TROUBLESHOOTING

35| Inject the fluorescent wild-type and mutated 3′ UTR reporter (each with dsRed control) in wild-type embryos as described in Steps 22–25. Inject 40–50 embryos per reporter.

36| Split the embryos injected with a given reporter into three groups, equal in number. Inject one of these subgroups (as described in Step 25) with the amount of TP determined above in Step 34, inject another with the same amount of the control TP and leave one subgroup that is not injected with any TPs.

37| Place these groups of embryos in separate wells of an agarose-coated six-well plate. Store them in an incubator at 28 °C and remove dead or unfertilized embryos after 6–8 h. Compare the fluorescence of each subgroup as shown in Steps 24–28. Look for an amount of TP that relieves repression of the wild-type 3′ UTR reporter so that its expression is comparable to that of the reporter with the mutated 3′ UTR (**Fig. 4**).
▲ CRITICAL STEP The wild-type 3′ UTR reporter with TP should not show more fluorescence than the mutated 3′ UTR reporter. Injecting the TP with the mutated 3′ UTR reporter should not cause a change in expression. In addition, injecting

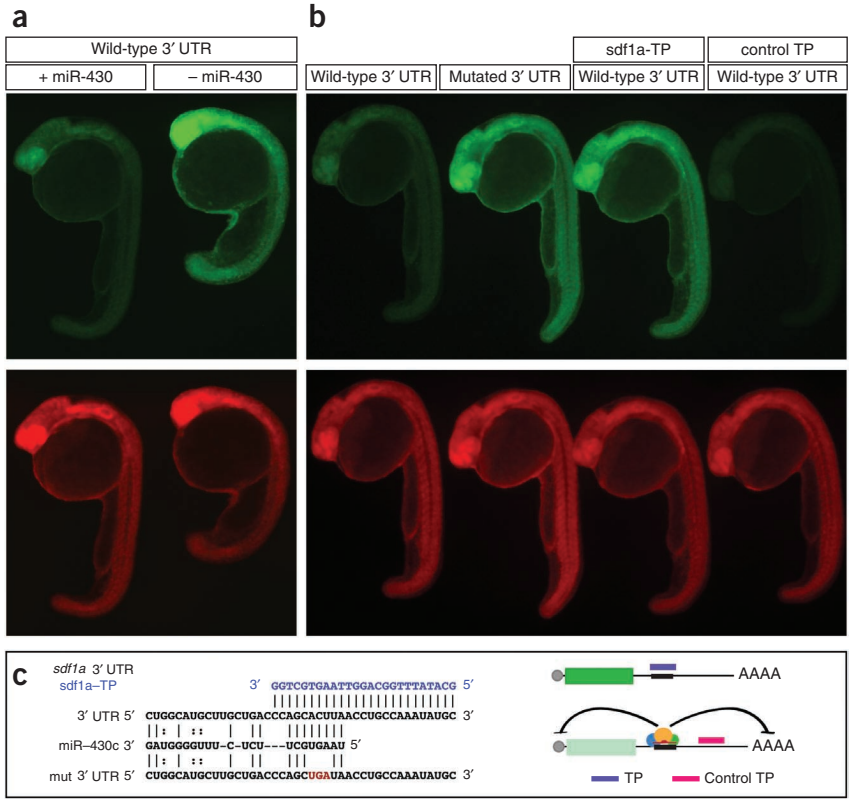

Figure 4 | Reporters demonstrating that the *sdf1a* 3′ UTR confers repression by miR-430. (**a**) Expression of GFP is reduced in the presence of miRNAs. GFP with part of the *sdf1a* 3′ UTR was injected in wild-type (+ miRNA) and MZ*dicer* (– miRNA) embryos. dsRed was co-injected as a control. Note that there is no change in dsRed expression. (**b**) GFP with the *sdf1a* 3′ UTR (wild-type 3′ UTR) or with the same 3′ UTR with the miR-430 site mutated (mutated 3′ UTR) were injected in wild-type embryos. There is a strong increase in GFP expression when the miR-430 target site is mutated. The wild-type reporter was then co-injected with a target protector designed to bind the miR-430 site (sdf1a-TP) or a control target protector (control TP). Only sdf1a-TP relieves repression of GFP expression. Note that the expression of GFP with the mutated 3′ UTR or in the presence of sdf1a-TP is similar. dsRed is used as an injection control. (**c**) Sequence alignment of miR-430c and sdf1a-TP with the *sdf1a* 3′ UTR. The seed region at the 5′ end of the miRNA pairs perfectly with the 3′ UTR. Sdf1a-TP was designed to bind this sequence also, as illustrated in the schematic to the right. The control TP does not bind the sequence complementary to the miRNA. The mutations in the seed-binding site made to generate the mutated reporter are shown in red lettering. All zebrafish used for these experiments were cared for in accordance with university guidelines.

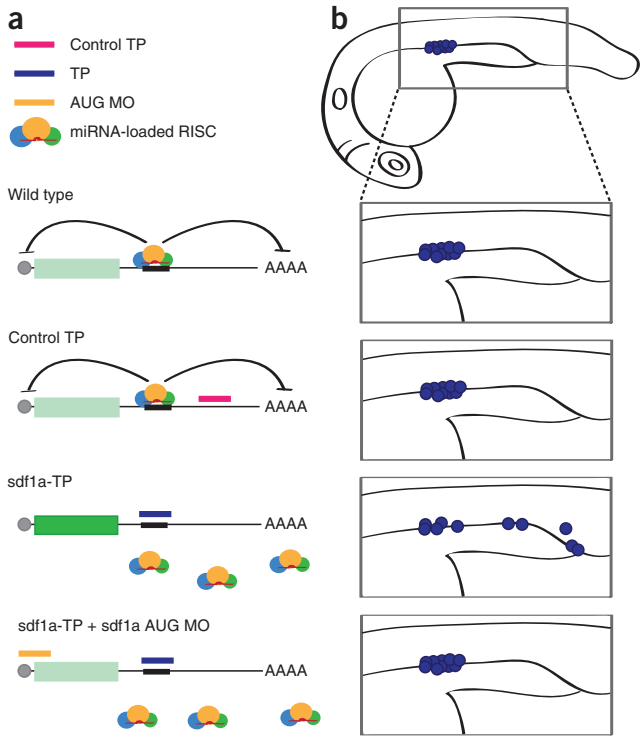

Figure 5 | Use of the sdf1a-TP reveals a role for miR-430 targeting in germ cell migration. (**a**) Scheme for injection of TPs and MOs. Injection of the TP, but not the control TP, should increase expression of the endogenous RNA. This increase is reduced by adding a low amount of the corresponding AUG MO. The endogenous transcript is shown in light green, with an increase in expression represented by a darker green. miRNA-mediated repression is shown by binding of RISC to the 3′ UTR with the resulting deadenlylation or translational repression (black lines). (**b**) Illustration of phenotypic results. In looking at *sdf1a* regulation, we examined germ cell migration. The cartoon shows localization of the primordial germ cells (blue) at 24 h.p.f. Injection of sdf1a-TP increases the number of germ cells that are located outside of the region where the germ cells should be. This mislocalization is rescued by injecting a low concentration of *sdf1a* AUG MO, indicating that the phenotype seen upon injection of the TP is specific to upregulation of *sdf1a*.

the control TP with either reporter should not change the expression of GFP. Any of these three results indicate that the TP is enhancing protein expression in an miRNA-independent manner.

? TROUBLESHOOTING

Identifying and quantifying the TP phenotype ● TIMING 1–4 weeks

38| Inject the amount of TP or control TP determined above in Step 37 into 40–50 wild-type embryos at the one-cell stage as described in Step 25. Save 25–30 uninjected embryos for comparison.

39| Analyze the injected embryos for phenotypic effects. This may involve analysis of morphology or labeling specific cell types using *in situ* hybridization or antibody staining (as described in Experimental design). As a starting point for analysis, the phenotype observed in the absence of the miRNA (miRNA knockdown or mutant) can be compared with the TP-injected embryos.

? TROUBLESHOOTING

Rescuing the TP phenotype to confirm specificity ● TIMING 2–3 weeks

40| Order a MO that binds the start site of the target gene (AUG MO). Prepare a series of five dilutions from 0.5 to 2 mM in nuclease-free water and inject them in the one-cell embryos as described in Step 25. Determine the concentration necessary to cause a complete knockdown. This can be accomplished by comparing the MO phenotype to published knockdown or mutant phenotypes. If nonspecific defects such as widespread cell death are observed, use a lower concentration.

41| Inject the diluted AUG MO in a wild-type embryo as described in Step 25. Inject at a concentration 10–20× lower than the amount required for a complete knockdown (as determined in Step 40). Identify the concentration range that produces a mild version of the phenotype observed in the complete knockdown. For instance, if the complete knockdown causes all germ cells to mislocalize, a milder phenotype may be embryos with only 50% of cells in the wrong location.

42| Co-inject (as described in Step 25) the TP at the concentration determined in Step 37 with a narrow range of low AUG MO concentrations as determined in Step 41. Collect embryos for analysis of the phenotype as described in Step 36. Compare the phenotypes of control TP, TP, control TP + low AUG MO and TP + low AUG MO. Because a low level of the AUG MO will reduce translation of the mRNA, co-injecting it with the TP should rescue the overexpression phenotype caused by protecting the mRNA from miRNA-mediated repression (**Fig. 5**).

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	No band is visible	PCR failed	Load undigested PCR on a gel. If a band is present, repeat the digest. If no band is visible, check primer sequences and repeat the PCR using a gradient of annealing temperatures
	Band is not the expected size	Restriction enzymes cut in the middle of the product	Check sequence for restriction enzyme sites. Redesign cloning to use enzymes that do not cut the product
9	No colonies	Ligation failed	Check the concentration of vector and insert used. Check that the ligation buffer and enzyme are not expired and have not been thawed too many times
		Wrong antibiotic selection	Ensure that transformation was plated on plates with ampicillin selection
	Too many colonies	No antibiotic selection	Make new LB plates with ampicillin selection. Check that ampicillin is not expired
		Too many cells were plated	Dilute the transformation and plate fewer cells
11	No insert	Vector recircularized without the insert	Prepare other colonies and check for the insert
			Repeat digest of the vector, making sure to treat with CIP, and then repeat cloning using a higher ratio of insert to vector
19	No RNA or low RNA concentration	Wrong vector was used	Sequence the vector to made sure that the SP6 promoter, coding sequence, and 3′ UTR are present
		RNA is degraded	Be sure that *in vitro* transcription kit is not contaminated and use all RNase-free products
27	No or little fluorescence is visible	RNA is degraded	Run the RNA on a gel. A smear or lack of a band indicates degradation. Repeat the *in vitro* transcription and be sure to aliquot the RNA to avoid freeze-thaw cycles, using RNase-free products
		Fluorescent protein is absent or mutated	Sequence the vector to ensure that the GFP or dsRed is present and free of mutations
28	Variability among embryos in an injected group	Variability in the amount of RNA injected	Calibrate the injection needle well. Be sure to inject the same volume in the center of the cell (not the yolk) of the embryo. Watch for any clogs in the needle
		Embryos are at different stages	Limit the number of embryos per well in 6-well plates to 20–25. Remove any dead embryos
34	High level of lethality or generalized cell death	Concentration of injected TP is too high	Repeat injections using lower amounts of TP. It is best to inject an amount of TP that is just below the threshold for nonspecific phenotypes. Necessary to relieve miRNA-mediated repression of the GFP reporter
		TP is toxic because of nonspecific binding	Redesign TP, changing most of the sequence but maintaining binding in the seed region
37	Variability among embryos in an injected group	Variability in the amount of RNA or TP injected	Calibrate the injection needle well. Be sure to inject the same volume in the center of the cell (not the yolk) of the embryo. Watch for any clogs in the needle
	TP does not increase expression	TP concentration is too low	Increase the amount of TP injected, staying below the threshold for nonspecific effects
		TP is not effective at relieving miRNA targeting	Confirm the sequence of the TP. Redesign TP so that it binds to a different sequence that still includes the seed

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
39	Control TP shows phenotype	Phenotype is not specific to relief of miRNA binding	Lower the amount of TP and control TP injected and test for the phenotype in both groups
			Analyze injected embryos for other phenotypes that are specific to the TP-injected group
		Control TP also stabilizes the transcript	Test whether the control TP stabilizes the GFP reporter. Confirm that the control TP does not bind to a potential miRNA-binding site
42	TP phenotype is not rescued	AUG MO concentration is too high/too low	Test a range of AUG MO concentrations. Use ranges that are near the threshhold of MO necessary to observe a phenotype
		TP phenotype is not specific to misregulation of this gene	Test lower concentrations of the TP to determine if they are sufficient to cause a phenotype that can be rescued with the AUG MO
			Analyze TP-injected embryos for other phenotypes that can be rescued by the AUG MO

● TIMING

Steps 1–12, Generating fluorescent reporters: 1–2 weeks
Steps 13–19, Preparing reporter mRNAs for injection: 2–3 d
Steps 20–31, Injecting and analyzing reporters: 2–3 d
Steps 32–37, Designing and validating the TP: 2–3 weeks
Steps 38–39, Identifying and quantifying the TP phenotype: 1–4 weeks
Steps 40–42, Rescuing the phenotype: 2–3 weeks
Box 1, Measuring luciferase expression in zebrafish embryos: 1 d
Box 2, Quantification of GFP reporter expression: 1 d
Note: For Steps 38–42, the timing can vary greatly depending on the nature of the phenotype. If the phenotype is subtle, it may take longer to identify. In addition, the nature of the assay used to examine the phenotype will affect the timing of these steps.

ANTICIPATED RESULTS

With this protocol, we have confirmed the targeting of a 3′ UTR by a particular miRNA. **Figure 4a** shows regulation conferred by the *sdf1a* 3′ UTR in the presence of miRNAs. We did not observe this repression when 3 nt in the miR-430 seed sequence were mutated (**Fig. 4b**). In addition, injecting a TP designed to bind the miR-430 target site relieved the repression of GFP (**Fig. 4b**). Finally, the physiological role of this interaction was determined by examining the location of germ cells (**Fig. 5**). Although each miRNA is predicted to target many mRNAs, only some are regulated *in vivo*. By using this procedure, it is possible to determine whether a transcript is regulated by a particular miRNA and to dissect the effect this interaction has on an organism's biology.

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AUTHOR CONTRIBUTIONS A.A.S. and A.J.G. designed experiments and interpreted results. A.A.S. performed all experiments and prepared the manuscript with input from A.J.G.

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