

Zebrafish MiR-430 Promotes Deadenylation and Clearance of Maternal mRNAs

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MicroRNAs comprise 1-3% of all vertebrate genes, but their *in vivo* functions and mechanisms of action remain largely unknown. Zebrafish miR-430 is expressed at the onset of zygotic transcription and regulates morphogenesis during early development. Using a microarray approach and *in vivo* target validation, we find that miR-430 directly regulates several hundred target mRNAs. Most targets are maternally expressed mRNAs that accumulate in the absence of miR-430. We also show that miR-430 accelerates the deadenylation of target mRNAs. These results suggest that miR-430 facilitates the deadenylation and clearance of maternal mRNAs during early embryogenesis.

Introduction

MicroRNAs (miRNAs) are small RNA molecules that basepair with target mRNAs to induce post-transcriptional gene repression (1, 2). MiRNAs have important roles during animal development. Interfering with miRNA processing using mutants for the RNaseIII enzyme Dicer, affects morphogenesis during zebrafish embryogenesis and mouse limb development (3, 4). Modulating the function of individual miRNAs leads to defects that range from aberrant cell fate specification and cell death to abnormal cell function (1, 2, 5, 6). Despite this progress, it is still poorly understood how miRNAs regulate developmental processes. In particular, the *in vivo* targets of miRNAs are largely unknown.

Several strategies have been employed to identify miRNA targets (7). Genetic approaches have identified several miRNA-mRNA target pairs that play important roles during *C. elegans* development (1, 2, 5, 6, 8–10). This approach preferentially identifies targets whose reduced activity suppresses miRNA loss-of-function phenotypes. *In silico* target predictions based on conserved complementarity between miRNAs and orthologous mRNAs have identified ~100-200 putative targets for a given miRNA (7, 11–18). It is unknown how many true, but non-conserved, targets are

missed in this approach. Cell culture studies have used microarrays to compare mRNA levels in the presence or absence of a transfected miRNA (19). This method has led to the identification of ~100 putative targets for miR-1 and miR-124. It is unclear how many of the *in silico* or cell culture targets are regulated *in vivo*. Hence, developing an *in vivo* system to identify a large fraction of biologically relevant miRNA targets could provide major insights into the roles of miRNAs during development.

Zebrafish embryos deficient for maternal and zygotic Dicer activity (*MZdicer*) cannot generate mature miRNAs (3). These mutants display defects during gastrulation and brain morphogenesis. These defects are rescued by injection of processed miRNAs belonging to the miR-430 family. MiR-430 is the most abundant miRNA family expressed during early zebrafish development (3, 20), conserved in other vertebrates (miR-302, miR-372) (3), and accumulates during the maternal-to-zygotic transition. This transition is a universal process in animal development, when the embryo activates zygotic gene expression and thus no longer solely relies on maternally provided transcripts (21). The activation of zygotic transcription coincides with the elimination of many maternal mRNAs by unknown mechanisms. The results presented here indicate that miR-430 accelerates the deadenylation and clearance of several hundred maternal transcripts during zygotic stages.

miR-430 accelerates target mRNA decay

Recent studies have suggested that miRNAs do not only inhibit productive translation but affect target mRNA levels (19, 22, 23). To distinguish between effects on gene transcription or mRNA decay, we investigated the stability of a miR-430 reporter mRNA in the presence or absence of miR-430. The reporter contained GFP and imperfect target sites for miR-430 in the 3' UTR (3xIPT-miR-430; Fig. 1, A and B) was injected shortly after fertilization. Reporter

mRNA started to decay after 4 hours post fertilization (hpf) in wild-type embryos, following miR-430 accumulation after the onset of zygotic transcription (~2.5 hpf; fig. S1). In contrast, reporter mRNA decay was delayed in the absence of miR-430 in *MZdicer* mutants (Fig. 1B and C). Injection of processed miR-430 into *MZdicer* mutants (*MZdicer*^{+miR-430}) restored accelerated decay. Similar results were obtained with miR-1 and its reporter mRNA (fig. S2)(3). These results indicate that miRNAs can accelerate the decay of target mRNAs *in vivo*.

Identification of putative miR-430 targets by microarray analysis

Since miR-430 can accelerate the decay of an artificial target mRNA, we speculated that *bona fide in vivo* targets accumulate in the absence of miR-430. Using gene expression microarrays, we compared mRNA levels in embryos that lack miR-430 (*MZdicer*) with embryos that have miR-430 (wild type and *MZdicer*^{+miR-430})(fig. S3, table S1). More than 750 mRNAs (represented by 811 probes in the array) were present at significantly higher levels (≥ 1.5 -fold) in *MZdicer* mutants (Fig. 2A, fig. S3, table S1). To determine if these mRNAs might be direct or indirect miR-430 targets, we analyzed whether upregulated mRNAs were enriched for putative miR-430 target sites. Such sites are complementary to the 5' seed region of miR-430, which is crucial for target recognition (14, 15, 24–26). We performed this analysis for the 328 upregulated mRNAs that had experimentally identified 3' UTRs (Fig. 2A, fig. S3; see SOM) and a control data set containing the 5219 genes on the array with defined 3' UTRs. Based on the rules developed by Lewis et al. (14), we searched for sequences complementary to a 6mer seed (positions 2-7 in the seed), a 7mer seed or an 8mer seed (with an adenosine at the 3' end of the target sequence)(14). mRNAs with increased levels in *MZdicer* mutants had significantly more predicted target sites/100kb in their 3' UTRs compared to the control set (Fig. 2B). This translated into a ~4 to ~10 fold enrichment compared to the control set (Fig. 2C) and a ~10 to ~30 fold enrichment when compared to the set of mRNAs that remained unchanged in the array (Fig. 2C). No enrichment was found for sequences complementary to the miRNA seeds of let-7 or miR-1 (fig. S4). These results suggest that the group of mRNAs whose levels were increased more than 1.5-fold in *MZdicer* mutants is greatly enriched for miR-430 targets.

miR-430 target validation

To validate putative targets, we tested miR-430-dependent regulation of reporters consisting of GFP and full-length 3' UTRs (Fig. 3A). First, we analyzed whether GFP expression from the reporter was higher in *MZdicer* mutants compared to wild type. Second, we assessed whether repression of the

GFP reporter in wild-type embryos depended on the miR-430 target sites in the 3' UTR. We co-injected GFP reporter mRNAs and control mRNA encoding the red fluorescent protein (dsRed) and compared the GFP levels in wild type and *MZdicer* mutants at 25-30hpf (Fig. 3, fig. S5, and SOM). We considered an mRNA to be regulated by miR-430 when the normalized GFP reporter expression in *MZdicer* was increased more than two-fold compared to wild type.

Between 92% and 71% of the tested putative targets were upregulated *in vivo* in the absence of miR-430 (n=37) (Fig. 3, B to D and figs. S5 and S6). For example, we validated 12/13 (92%) mRNAs with two or more target sequences complementary to miR-430 seeds, 6/8 (75%) mRNAs with one 8mer target site, and 5/7 (71%) mRNAs with one 6mer or 7mer target site. In contrast, none of the tested mRNAs that were unchanged in the array and lacked miR-430 sites were repressed in wild type compared to *MZdicer* (n=7).

To test for direct regulation by miR-430, we mutated two nucleotides in the predicted target site in the 3' UTR of 5 putative targets (GCACUU to GCAGAU; Fig. 3B, C, E and fig. S7). This strongly reduced or abolished repression in wild-type embryos in all cases (Fig. 3E and fig. S7), suggesting that most if not all the 3' UTRs validated in *MZdicer* mutants are likely to be direct miR-430 targets *in vivo*.

Combining the microarray data, target predictions, and validation results led to the identification of a group of 101 mRNAs that have a greater than 85% probability to be direct miR-430 targets (figs. S3 and S7). Members of this group were upregulated ≥ 1.5 fold in *MZdicer* mutants and had one 8mer target site or two target sites (6, 7 or 8mer) in their 3' UTRs. Even the 102 mRNAs that were upregulated ≥ 1.5 fold and had one 6mer or 7mer target site had a 71% probability to be miR-430 targets (figs. S3 and fig. S6). Hence, we estimate that miR-430 directly regulates ~160 mRNAs of a target set of 203 mRNAs with defined 3' UTRs (table S1, fig. S6). Since 3' UTRs were only available for roughly half (328/~750) of the upregulated mRNAs, we extrapolate that miR-430 might directly regulate more than 300 of these mRNAs (fig. S6). If we also take into account that the array covers less than half of all predicted zebrafish genes, we estimate that miR-430 directly regulates several hundred target mRNAs during early zebrafish development.

miR-430 targets are not preferentially conserved

Computational identification of miRNA targets often relies on the principle that conserved miRNAs have conserved targets (1, 7). We therefore asked how many of the 203 genes in the zebrafish miR-430 target set have a 6mer miR-430 target site in the 3' UTR of orthologous genes in two other teleosts, *Fugu rubripes* and *Tetraodon nigroviridis* (fig. S8 and table S1). As described in SOM, we estimate that ~50%

of our experimentally identified target sites are found in the orthologous gene in either Fugu or Tetraodon, and only ~25% are conserved in all three species (Fig. 2D, and fig. S8). Regulation mediated by the 3' UTR of conserved and non-conserved targets resulted in similar post-transcriptional regulation of the GFP reporter (fig. S8). These results suggest a very rapid evolution of the miR-430 target set and indicate that a large fraction of *bona fide* targets for miR-430 and other miRNAs (18) would be missed if conservation were used as the sole criterion.

Most miR-430 target mRNAs are expressed maternally

To investigate if the 203 mRNAs in the miR-430 target set share specific features, we analyzed their expression profile and gene ontology (GO). No significant differences in the distribution of GO classes were found for genes in the target set compared to the entire genome (fig. S9). We then compared the expression profile of genes in the miR-430 target set to genes in the control set that were expressed during early embryogenesis. ~40% of the target set mRNAs but only ~10% of the control set mRNAs were present at high levels at 1.5 hpf but at low levels at 5 and 9 hpf (Fig. 4A and fig. S10). These are mRNAs deposited into the egg by the mother before fertilization, since the genome is transcriptionally silent until ~2.5hpf (21, 27). In the absence of miR-430, the maternal transcripts in the target set accumulated to higher levels than in wild-type embryos (table S1). In addition to the 4-fold enrichment of predominantly maternal transcripts in the miR-430 target set, ~40% of predominantly maternal mRNAs (162 out of 402) were predicted to have miR-430 target sites compared to only 18% in the control set (Fig. 4C and fig. S10). Hence, not only was the miR-430 target set enriched for maternal mRNAs but maternal mRNAs were also enriched for miR-430 target sites. Another ~50% of the target mRNAs were expressed both maternally and zygotically. In particular, half of these targets maintained steady levels zygotically (Fig. 4B and fig. S10) but accumulated to higher levels in the absence of miR-430 (table S1). Taken together, the analysis of miR-430 targets indicates that miR-430 accelerates the clearance of several hundred maternal and maternal-zygotic mRNAs.

miR-430 promotes target mRNA deadenylation

The poly(A) tail stabilizes mRNAs and enhances cap-dependent translation (29–31). Upon fertilization, many maternally deposited mRNAs become polyadenylated and competent for translation, whereas deadenylation triggers translational silencing and decay (28–30). We therefore tested whether miRNA-induced target decay correlates with changes in the length of the poly(A) tail of reporter and endogenous target mRNAs. Time course analysis of injected target mRNAs revealed that polyadenylation is followed by rapid

deadenylation that is induced by miR-430 and results in complete deadenylation at about 6 hpf (~3 h after the onset of miR-430 accumulation; Fig. 5, fig. S11). Three controls indicated that rapid deadenylation is miRNA-mediated. First, mutation of miR-430 target sites in the 3' UTR delayed target deadenylation (Fig. 5, A, B, C and D). Second, deadenylation of target mRNAs is delayed in *MZdicer* mutants, similar to non-miRNA targets or GFP alone (Fig. 5, D, E and F; fig. S11). Third, injection of miR-430 into *MZdicer* mutants accelerates target deadenylation (Fig. 5, D and F). Similar results were observed for different endogenous and reporter miR-430 targets (fig. S11), as well as reporters partially complementary to miR-1 (Fig. 5G).

To test whether miRNA-induced target deadenylation is a secondary effect of non-productive translation, we repressed GFP reporter expression using morpholino antisense oligonucleotides that hybridize to the translational start site. This did not cause reporter mRNA decay or deadenylation to the same extent as the miRNA (Fig. 5G). Furthermore, miRNA-induced deadenylation still occurred in the absence of translation (Fig. 5G). Taken together, these results indicate that miR-430 accelerates target deadenylation and mRNA decay.

In summary, the study of miR-430 *in vivo* targets provides three major insights. First, miR-430 directly regulates several hundred target mRNAs during early embryogenesis. These targets are upregulated in *MZdicer* mutants, contain miR-430 target sites and can be validated *in vivo*. Although the number of identified miR-430 targets is high, it is likely that there are additional targets that were not identified in our approach. These targets might include mRNAs that are expressed at low levels, regulated by miR-430 during other developmental stages, or regulated at the level of translation rather than transcript stability. Our results provide *in vivo* support for *in silico* and cell culture studies that have predicted large sets of putative miRNA targets (7, 11–19, 31). However, 50% of the miR-430 target set would have been missed by relying on conservation, a criterion that is often used in computational approaches. Moreover, it remains unclear how many of the *in silico* or cell culture targets are biologically relevant targets *in vivo*. In contrast, *in vivo* approaches using miRNA loss-of-function combined with target validation identifies *bona fide* targets.

Second, our results suggest a critical role for miR-430 in the maternal-to-zygotic transition during embryogenesis. In all animals, the mother deposits mRNAs into the egg. Upon fertilization, maternal mRNAs are activated and translated, whereas the genome is transcriptionally silent until a later stage (21, 28–30). Since maternally provided transcripts cannot be repressed at the transcriptional level, post-transcriptional and post-translational mechanisms are required to regulate their activity (28–30). MiR-430 is

expressed at the onset of zygotic transcription and accelerates the deadenylation and decay of a large set of maternal mRNAs (fig. S12). The accumulation of these maternal transcripts is the likely cause of the developmental delay and gastrulation defects observed in *MZdicer* mutants (3). MiRNA-induced clearance of maternal mRNAs might be a universal mechanism to regulate the maternal-to-zygotic transition. Our analysis of miR-430 and its targets might also have wider implications for miRNA function during development. MiR-430 is involved in a developmental transition (maternal-to-zygotic), but even without miR-430 and despite the delayed clearing of maternal mRNAs, the embryo can still activate its zygotic program (e.g. embryonic patterning). Hence, lack of miR-430 does not arrest the embryo in a maternal state but results in a mixed maternal/zygotic state. This suggests that miR-430 and other miRNAs might not exclusively function as switches from one state (B) to the next state (C) but might sharpen developmental transitions and counteract the formation of mixed states (B/C) (fig. S12). This model has important implications for the role of miRNAs in disease. Premature expression of miRNAs might aberrantly target mRNAs required to specify state B, thus maintaining cells in an earlier state A (fig. S12). Such a mechanism might account for the miRNA-mediated induction of cancer (2). Our findings also provide a framework to interpret recent studies that have indicated that specific miRNAs and their predicted target mRNAs tend to be expressed in a mutually exclusive manner (17, 18). These and previous results (19, 32) have led to the speculation that miRNAs confer robustness by targeting erroneously transcribed genes or by preventing premature or prolonged mRNA accumulation. Our results provide *in vivo* support for the latter speculation, suggesting that miRNAs target mRNAs that might otherwise persist during later stages. In addition, a fraction of the target mRNAs continue to be co-expressed with miR-430 after the maternal-to-zygotic transition. These target mRNAs are not eliminated but modulated by miR-430. Thus, miR-430 accelerates the clearance of maternal mRNAs and dampens the levels of maternal-zygotic mRNAs.

Third, our study suggests that miR-430 accelerates deadenylation of target mRNAs and thus provides a potential mechanism for miRNA function. The poly(A) tail confers mRNA stability and stimulates translation via the interaction of poly(A) binding protein (PABP) with the 5' 7-mG cap (28–30). Previous studies have found that miRNAs inhibit Cap-dependent translation (33) and induce mRNA degradation (19, 22, 23, 34). We therefore postulate that miRNA-induced deadenylation is one of the mechanisms by which miRNAs enhance target decay and repress productive translation (fig. S12). Although it remains to be determined if deadenylation is the primary step in mRNA regulation, we observed that

block of translation does not accelerate mRNA deadenylation to the same extent as the miRNA. It is therefore conceivable that miRNAs induce the deadenylation of their targets, which results in the block of translation and the recruitment to processing bodies (P-bodies), where mRNAs are decapped and degraded (33–38). Taken together, our results suggest that miRNAs promote the deadenylation and decay of hundreds of target mRNAs and thus sharpen and accelerate the transition between developmental states.

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1108470/DC1
 Materials and Methods
 Figs. S1 to S12
 Table-S1(1.5fold).zip
 SOM-Targets-tested.pdf

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Fig. 1. miRNAs accelerate target mRNA decay. **(A)** Experimental approach to analyze mRNA target decay in presence or absence of microRNAs. GFP reporter contains three miRNA target sites and is injected at the one-cell stage. **(B)** Northern blot of miR-430 reporter mRNA in the presence or absence of miR-430. **(C)** *In situ* analysis (dark blue) of reporter mRNA in the presence or absence of miR-430 at 8 hpf. The panels show a detail of the dorsal-lateral margin of the embryo (black rectangle, left). Note the higher reporter levels in the absence of miR-430 (see fig. S2 for details).

Fig. 2. miR-430-regulated mRNAs are enriched for miR-430 target sites. **(A)** Comparison of mRNA expression levels between *MZdicer* mutants and [*MZdicer*^{+miR-430} and wild type]. Columns summarize the number of probes, number of mRNAs with known 3' UTRs, and number of mRNAs with known 3' UTRs containing a putative miR-430 target site

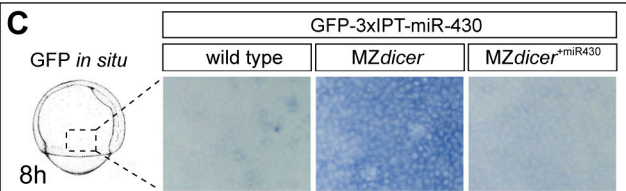
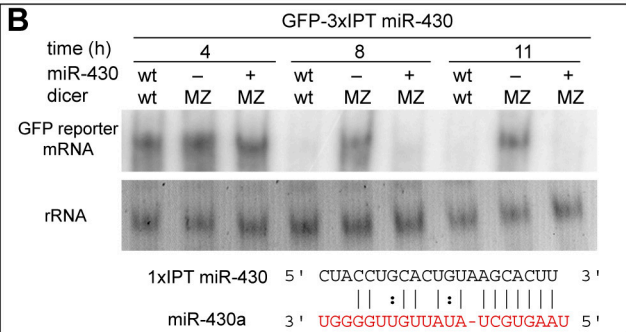
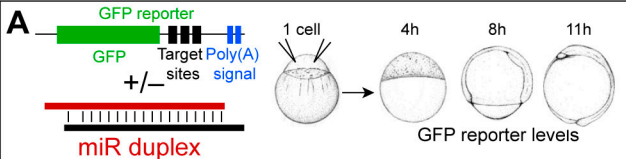
(defined by a 6mer (or more) sequence complementary to the miR-430 seed). (*) control set used for subsequent analyses. **(B, C)** Number (B) or fold enrichment (C) of 6mer or 8mer miR-430 target sites per 100 kb of 3' UTR in different groups (B) and compared to the control set (C). **(D)** Percentage of mRNAs that have a 6mer (or more) miR-430 target site in the orthologous genes in *Fugu rubripes* (Fugu) and *Tetraodon nigroviridis* (Tetra) (see fig. S3, S4, S8 for details).

Fig. 3. miR-430 target validation. **(A)** Experimental approach. GFP reporter mRNA is co-injected with control DsRed mRNA into wild type and *MZdicer* mutants. GFP reporter contains a 3' UTR that is wild-type or mutant for the miR-430 target sites. **(B)** GFP reporter expression (green) and control DsRed expression (red) at 25-30 hpf. Wild-type reporter is repressed in wild-type embryos compared to *MZdicer* mutants. Mutation of miR-430 target sites abolishes repression of the reporter in wild-type embryos. **(C)** Predicted pairing between wild type or mutant target 3' UTR and a member of the miR-430 family. **(D, E)** Fold increase of GFP expression in *MZdicer* mutants compared to wild type. Regulation was considered significant when the GFP reporter was upregulated ≥ 2 fold (solid color in C; see fig. S5, S7 and SOM for details).

Fig. 4. miR-430 facilitates the clearance of maternal transcripts. **(A, B)** Relative expression profile of mRNAs at 1.5 (maternally provided mRNAs), 5 and 9 hpf. **(A)** Expression profile of 50 mRNAs in the miR-430 target set. Predominantly maternal mRNAs are highlighted in red. The miR-430 target set is 4-fold enriched for predominantly maternal mRNAs when compared to a random set of mRNAs (also see fig. S10). **(B)** Expression profile of 50 mRNAs in the miR-430 target set that are present at different levels maternally but show stable levels during zygotic stages (blue). The mRNAs in the miR-430 target set accumulate to higher levels in the absence of miR-430 (table S1). **(C)** Fold enrichment of 8mer target sites for miR-430 and miR-1 in the control set, predominantly maternal mRNAs and predominantly zygotic mRNAs. 40% of the predominantly maternal mRNAs have a 6mer miR-430 target site (see SOM for details regarding maternal and zygotic mRNAs).

Fig. 5. miRNAs accelerate the deadenylation of target mRNAs. **(A)** Experimental set up to analyze poly(A) tail. Target mRNAs with either a polyadenylation signal [poly(A) signal] (B, D, E) or a poly(A) tail (C) were injected and assayed for poly(A) tail length using an RNase H/Northern assay (39)(see also SOM fig. S11). Incubation of mRNA with oligo dT and RNase H tests the mobility of the deadenylated RNA fragment. **(B-D)** Northern analysis of poly(A) tail of GFP reporter mRNA with wild-type (wt) or mutant (mut) target sites in the 3' UTR in the presence or absence of miR-430. GFP reporter mRNA is polyadenylated between 0-4hpf

(B). Deadenylation of wild-type GFP reporter is accelerated compared to GFP reporters with mutated miR-430 target sites (B,C) and in wild type compared to *MZdicer* mutants (D). Accelerated deadenylation can be partially rescued in *MZdicer* mutants by injecting miR-430 duplexes (D, fig. S11). (E) Method used to detect poly(A) tail length of endogenously expressed target mRNAs, RT-PCR PAT [poly(A) test]. The length of the smear reveals the approximate size of the poly(A) tail. (F) Time course RT-PCR PAT of endogenous miR-430 targets in the presence (wt, +) or absence of miR-430 (*MZdicer*, -). Note the shortening of the poly(A) tail in the presence of miR-430 (see SOM for details). (G) RNase H/Northern assay of a GFP reporter for miR-1 in the presence or absence of translational repression by antisense morpholino (MO). MiR-1 and MO repress GFP translation (green), but miR-1 preferentially accelerates deadenylation.

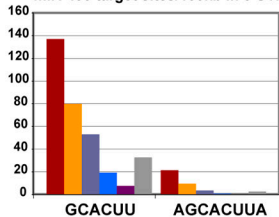


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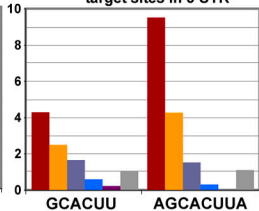
| | >2fold | 1.5-2fold | 1.2-1.5fold | Unchanged | <0.8 fold | Whole Array |
|--------------|--------|-----------|-------------|-----------|-----------|-------------|
| Probes | 275 | 536 | 330 | 2749 | 713 | 12754 |
| 3'UTR | 118 | 210 | 133 | 1293 | 359 | 5219* |
| Target sites | 88 | 115 | 50 | 139 | 17 | 952 |

■ a: ≥ 2 fold
 ■ b: 1.5-2fold
 ■ c: 1.2-1.5 fold
 ■ d: Unchanged
 ■ e: <0.8 fold
 ■ Control set*

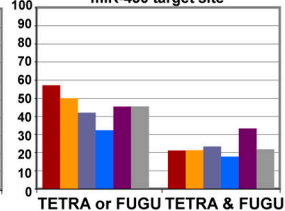
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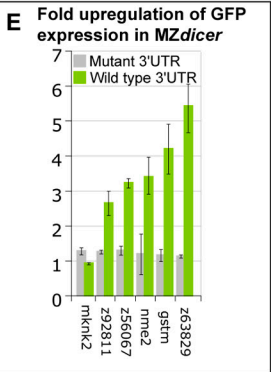
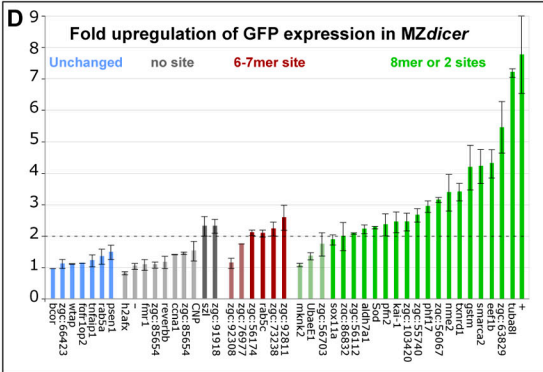
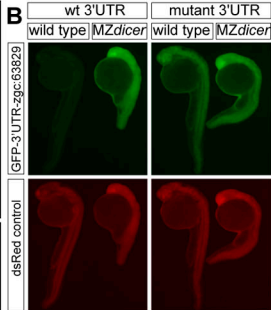
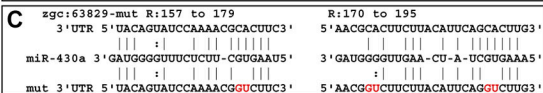
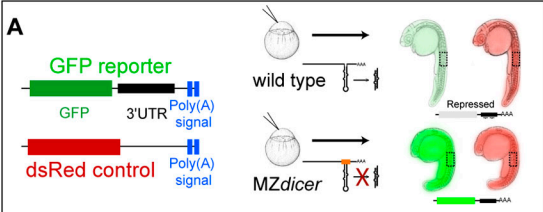


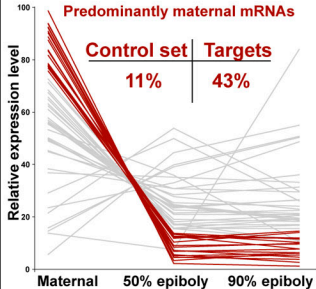
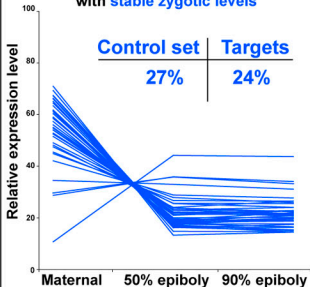
C Fold enrichment of miR-430 target sites in 3'UTR



D % of orthologues with conserved miR-430 target site





A Expression profile of miR-430 target set**B** Expression profile of miR-430 target set with **stable zygotic levels****C** Maternal genes are enriched for miR-430 target sites