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Report

Differential Regulation of Germline mRNAs in Soma and Germ Cells by Zebrafish miR-430

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Summary

Early in development, primordial germ cells (PGCs) are set aside from somatic cells and acquire a unique gene-expression program [1]. The mechanisms underlying germline-specific gene expression are largely unknown. Nanos expression is required during germline development [2–5] and is posttranscriptionally restricted to PGCs [4, 6–8]. Here we report that the microRNA miR-430 targets the 3′ untranslated region (UTR) of *nanos1* during zebrafish embryogenesis. A miR-430 target site within the *nanos1* 3′ UTR reduces poly(A) tail length, mRNA stability, and translation. Repression is disrupted in maternal-zygotic *dicer* mutants (MZ*dicer*), which lack mature miRNAs [9], and is restored by injection of processed miR-430. Although miR-430 represses other genes equally in germline and soma, specific regions in the *nanos1* 3′ UTR compensate for microRNA-mediated repression in PGCs and allow germline-specific expression. We show that the 3′ UTR of an additional PGC-specific gene, *TDRD7*, is also targeted by miR-430. These results indicate that miR-430 targets the 3′ UTRs of germline genes and suggest that differential susceptibility to microRNAs contributes to tissue-specific gene expression.

Results

nanos1 3′ UTR Induces mRNA Deadenylation

Zebrafish *nanos1* mRNA is maternally supplied to the early embryo [4]. Multiple posttranscriptional mechanisms function via the *nanos1* 3′ UTR to ensure protein expression specifically in PGCs. First, *nanos1* mRNA is localized to the germ plasm at the cleavage furrows [4, 10, 11]. Second, the *nanos1* 3′ UTR represses *nanos1* translation in somatic cells. Third, *nanos1* mRNA is rapidly degraded in somatic cells but is stably maintained in PGCs [4]. These mechanisms lead to protein expression specifically in PGCs [4, 12]. A GFP reporter mRNA containing the *nanos1* 3′ UTR injected at the one-cell stage is stabilized in PGCs but is translationally repressed and degraded in somatic cells [4] (Figure 1A). We took advantage of this injection assay to elucidate the molecular basis of posttranscriptional regulation of *nanos1*.

The poly(A) tail is a key determinant of mRNA stability and translation efficiency [13–15]. We therefore asked whether the posttranscriptional repression activity of the *nanos1* 3′ UTR in somatic cells is mediated by the control of poly(A) tail length (Figure 1B). We compared the poly(A) tail dynamics of GFP-*nanos1* mRNA to two control mRNAs that contained either GFP ORF alone (GFP mRNA) or GFP ORF with the *vasa* 3′ UTR (GFP-*vasa* mRNA). We used the latter reporter because the *vasa* 3′ UTR restricts the mRNA to PGCs but does not repress protein synthesis in somatic cells as efficiently as the *nanos1* 3′ UTR (Figure 1A and [16]). This suggests that the *nanos1* and *vasa* 3′ UTRs mediate posttranscriptional regulation by different mechanisms. Analysis of the poly(A) tail dynamics revealed that GFP-*nanos1* mRNA was initially polyadenylated but almost completely lost its poly(A) tail at 4–6 hr post fertilization (hpf). In contrast, GFP and GFP-*vasa* mRNA retained an approximately 150 nucleotide (nt) poly(A) tail (Figure 1C). The injected GFP reporters recapitulated the poly(A) tail dynamics of endogenous *nanos1* mRNA and *vasa* mRNAs (Figure S1). These results indicate that the bulk of *nanos1* mRNA lost its poly(A) tail during embryogenesis as a result of a sequence element within its 3′ UTR. Similar results were obtained when the poly(A) tail was added in vitro prior to injection (Figure 1D), suggesting that the *nanos1* 3′ UTR induces deadenylation and might also reduce de novo polyadenylation.

Deadenylation of *nanos1* mRNA Is Not Caused by Translational Repression

Deadenylation could be either the cause or the result of translational repression [17]. To distinguish between these possibilities, we analyzed the poly(A) length of GFP reporter mRNAs primed with A-cap. A-cap does not interact with the translation-initiation factor eIF4E and inhibits translation initiation [18]. A-capped GFP mRNA was not translated in zebrafish embryos (Figure S2A) and had the same poly(A) profile as the m^7G-capped GFP mRNA (Figure S2B, lower panel). Conversely, A-capped GFP-*nanos1* mRNA was rapidly deadenylated, similarly to m^7G-capped GFP-*nanos1* mRNA (Figure S2B, upper panel). These results indicate

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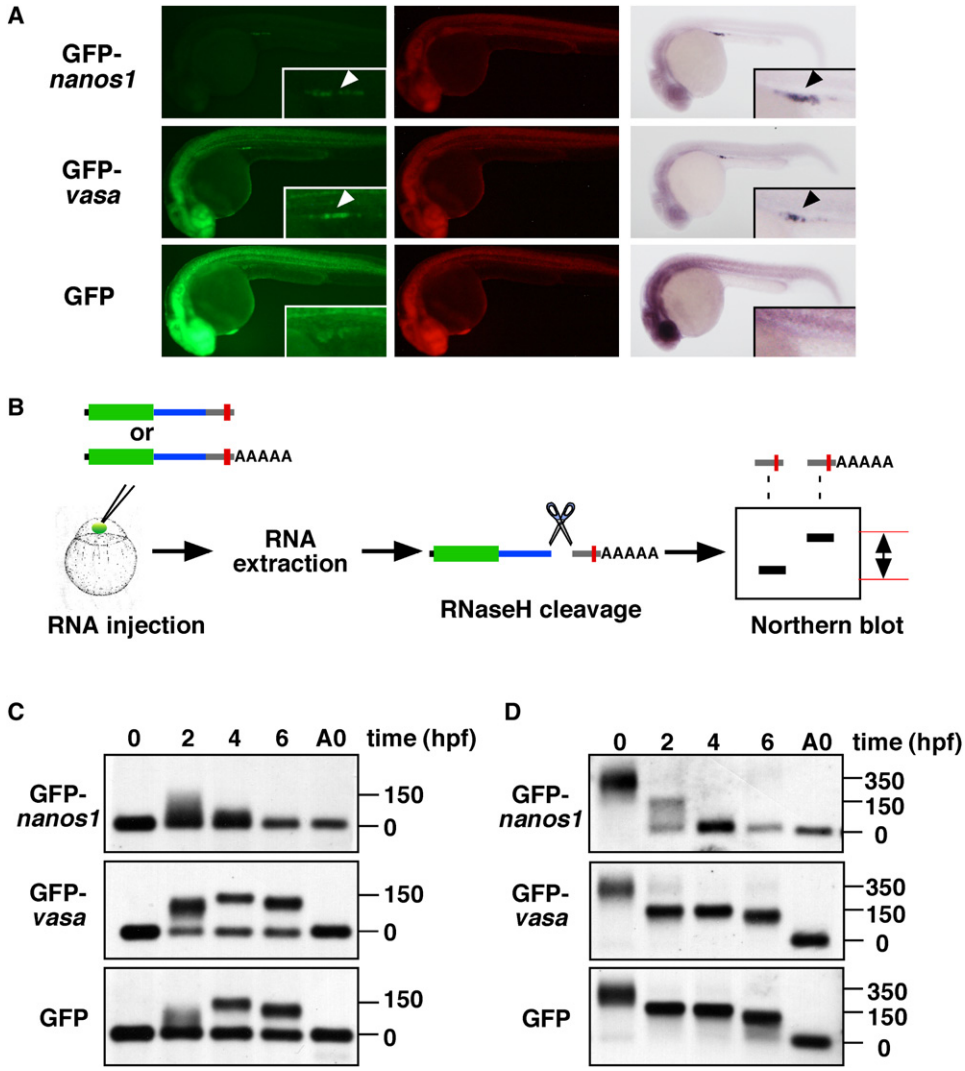

Figure 1. The *nanos1* 3′ UTR Induces Deadenylation

(A) GFP mRNAs with different 3′ UTR fragments and control DsRed mRNA were coinjected at the one-cell stage. GFP (green) and DsRed (red) expression were analyzed at 26 hr post-fertilization (hpf). The levels of GFP mRNA were examined by in situ hybridization (dark purple). Insets show the gonad region. Arrowheads indicate PGCs.

(B) Schematic representation of the poly(A)-tail assay for GFP reporter mRNA. See Experimental Procedures for details.

(C) Poly(A)-tail profile of GFP reporter mRNAs with SV40 poly(A) signal. Time after fertilization is indicated above each lane. (A0) shows completely deadenylated fragments. Poly(A) tail length is shown on the right of each panel.

(D) Poly(A)-tail profile of GFP reporter mRNAs with SV40 poly(A) signal+poly(A) tail.

that deadenylation promoted by the *nanos1* 3′ UTR is not caused by the lack of translation initiation and does not require active translation.

A Short *cis*-Regulatory Element Controls Deadenylation and Translational Repression

To identify the *cis*-regulatory element within the *nanos1* 3′ UTR required for deadenylation, we examined the poly(A) tail dynamics and GFP expression of a series of reporters that contained deletion mutants of the *nanos1* 3′ UTR (Figure 2A). This analysis led to the identification of a 79 nt deadenylation element (Ib). A GFP reporter mRNA bearing the fragment Ib underwent deadenylation and translational repression. Conversely, deletion of the fragment Ib from the *nanos1* 3′ UTR (ΔIb) blocked rapid deadenylation and repression of the GFP reporter in somatic cells (Figures 2A and 2B and data not shown). Thus, the fragment Ib is necessary and sufficient to induce rapid deadenylation and repress GFP reporter expression in somatic cells.

To identify the core sequence elements responsible for rapid deadenylation, we used a series of mutants within region Ib (Figure 2C). Base substitutions within regions D and E strongly inhibited deadenylation of the GFP reporter mRNA, whereas other substitutions only affected deadenylation weakly (B, F, G, and H) or had no effect (A, C, I, J, and K) (Figure 2D). Concomitantly, repression of the GFP reporter was lost by substitutions D and E (Figure 2E and data not shown). These experiments indicate that the sequence elements D and E

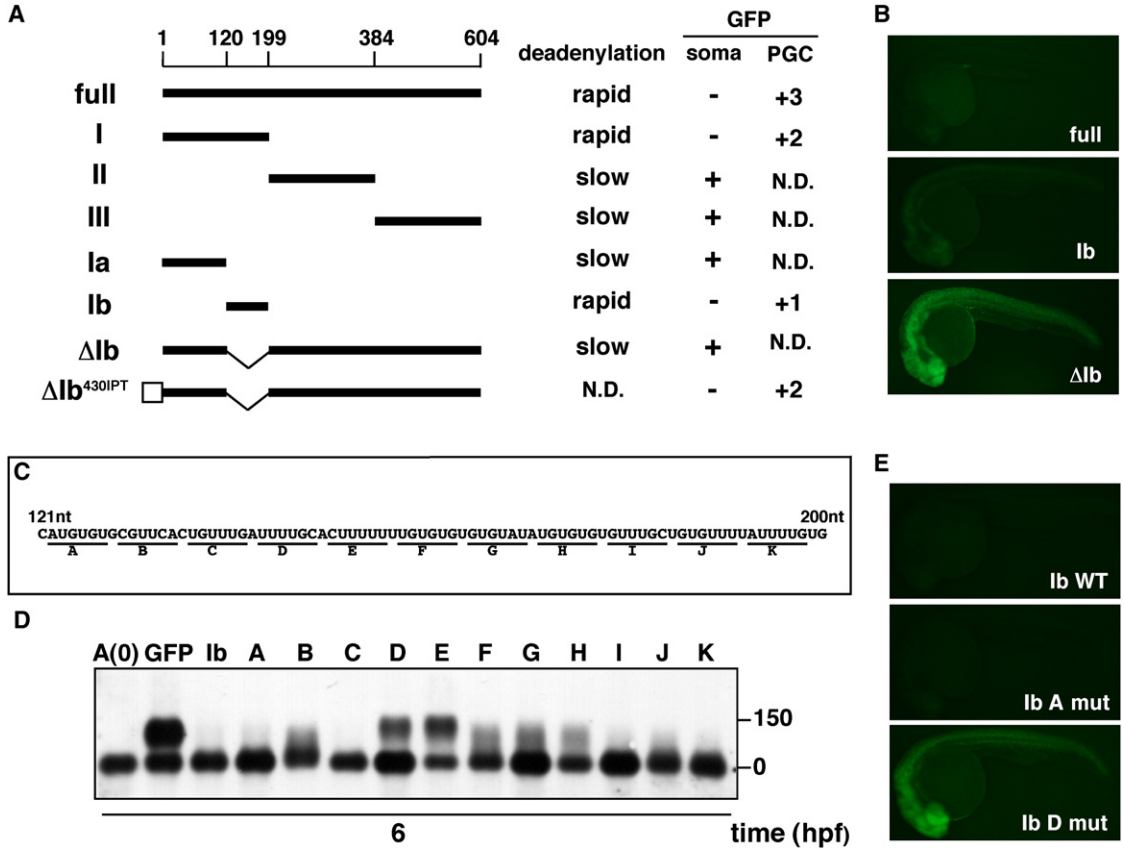

Figure 2. Identification of the Deadenylation Element

(A) Schematic representation of *nanos1* 3′ UTR deletion mutants. Deadenylation activity (rapid or slow) and GFP expression levels in somatic cells (+ or −) and PGCs (+3 represents the GFP expression of full-length *nanos1* 3′ UTR) are indicated on the right. The white box indicates miR-430 site. N.D.: not determined. The positions relative to the stop codon are shown above.

(B) GFP fluorescence from *nanos1* 3′ UTR deletion mutant reporters at 26 hpf.

(C) Sequence of the Ib region. Base substitutions to the BamHI site (A–K) are shown below.

(D) Poly(A) profile of Ib base-substitution mutant reporters at 6 hpf.

(E) GFP fluorescence of Ib mutant reporters at 26 hpf.

within the *nanos1* 3′ UTR are necessary for the rapid deadenylation and repression of the GFP reporter in somatic cells.

MiR-430 Induces Deadenylation and Translational Repression in Somatic Cells

We hypothesized that a microRNA (miRNA) might bind to the D-E element and induce mRNA deadenylation and repression. Although their mechanism of action is not fully understood, miRNAs regulate target mRNA deadenylation [19–21] and inhibit protein synthesis by repressing translation [22–24]. We found that the sequence GCACUU in site D-E in the *nanos1* 3′ UTR is complementary to miR-430 nucleotides 2–7 (Figure 3A), the miRNA "seed" sequence important for target mRNA recognition [25–28]. MiR-430 is expressed ubiquitously during early embryogenesis and is required for normal morphogenesis during gastrulation and brain development [9]. To determine whether miR-430 induces target deadenylation and repression through the D-E region in the *nanos1* 3′ UTR, we used three approaches. First, we injected the GFP-*nanos1* reporter into wild-type, MZ*dicer* mutants, and MZ*dicer* mutants injected with miR-430 duplex (MZ*dicer*$^{+miR\text{-}430}$) (Figure 3B). The RNaseIII enzyme Dicer is required for miRNA processing, and loss of maternal and zygotic *dicer* (MZ*dicer*) results in loss of mature miRNAs, including miR-430 [9]. We found that repression and deadenylation of the GFP-*nanos1* reporter were reduced in MZ*dicer* mutants. Conversely, injection of the miR-430 duplex into MZ*dicer* mutants restored regulation of the *nanos1* reporter mRNA (Figures 3C and 3D). In contrast, expression of the GFP-*vasa* reporter, which neither contains a GCACUUU sequence nor promotes deadenylation, is similar in wild-type and MZ*dicer* embryos (Figure S4). Second, we mutated two nucleotides that are located in the predicted target site and that disrupt the pairing with the miR-430 seed (G**CA**CUU to G**GU**CUU; Figure 3A). We found that these mutations lead to higher GFP expression levels in somatic cells and to delayed deadenylation in comparison to those with the wild-type reporter (Figures 3E and 3F). Third, we inserted three copies of the BCDE element downstream of the GFP ORF (3×BCDE) (Figures 3G and 3H). 3×BCDE recapitulated the deadenylation and repression activity of the *nanos1* 3′ UTR, whereas a mutated BCDE sequence (G**CA**CUU to

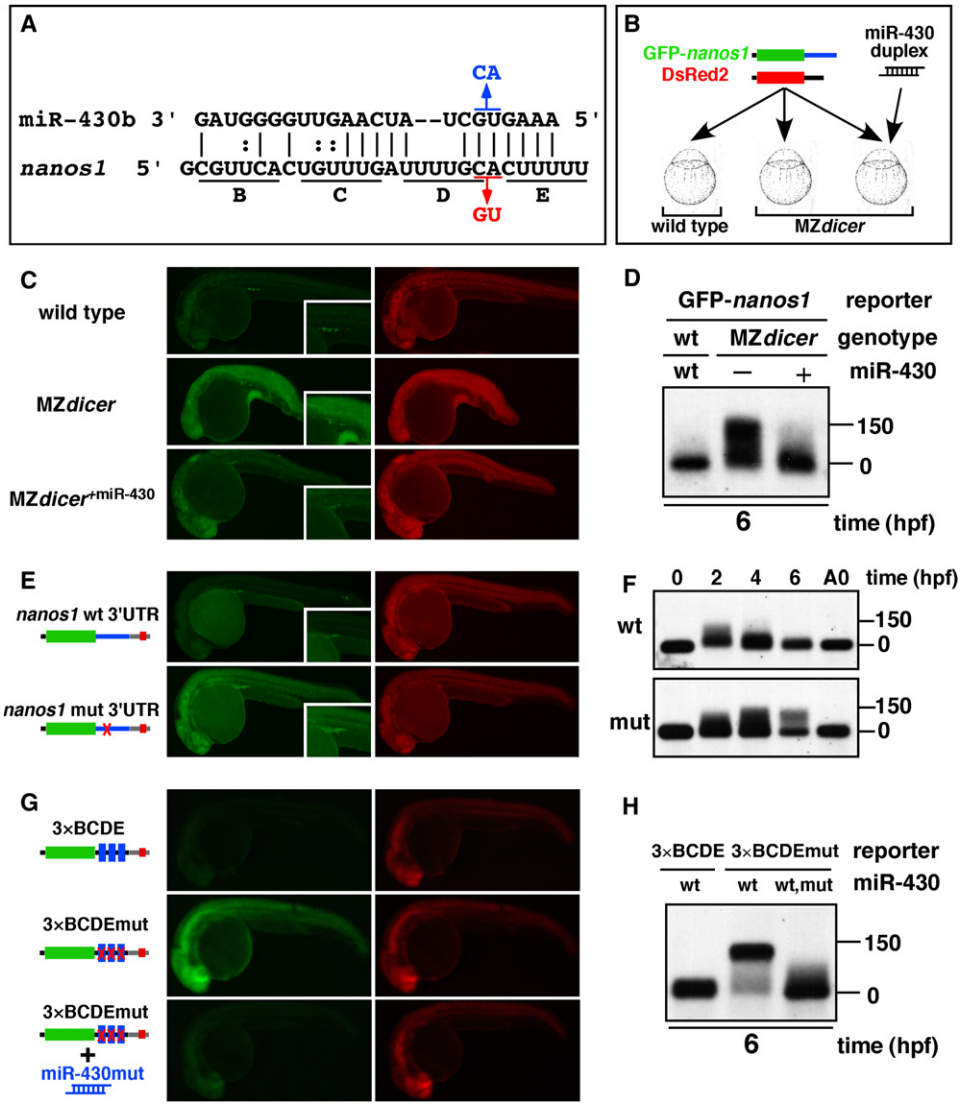

Figure 3. miR-430 Mediates Deadenylation and Repression of Protein Synthesis Induced by the *nanos1* 3′ UTR

(A) Putative base pairing of miR-430b with the *nanos1* 3′ UTR BCDE site. Base substitutions whose presence in the BCDE site disrupted the putative miR-430b binding are shown in red. Mutated nucleotides whose presence in miR-430b restored base pairing with the mutated *nanos1* 3′ UTR are shown in blue. (B) Experimental set-up to test the effect of the miR-430 on GFP-*nanos1* reporter with MZ*dicer* mutants. (C, E, and G) GFP reporter expression (green) and control DsRed expression (red) at 26–30 hpf in wild-type (C, E, and G), MZ*dicer* (C), and MZ*dicer*$^{+miR-430}$(C). Inset shows the enlarged view of gonad region. (D, F, and H) Poly(A) length of GFP reporter mRNAs used in (C), (E), and (G) at different times after injection. The mutant reporters (E–H) (*nanos1* mut) include the base substitutions shown in red(A).

GGUCUU, 3×BCDEmut) did not. Furthermore, coinjection of a mutant miR-430b duplex with compensatory mutations that base pair with BCDEmut restored deadenylation and repression. These experiments indicated that the BCDE site is a *bona fide* miR-430 target site and induces deadenylation and repression of the GFP reporter.

MiRNAs not only cause translational repression but also cause degradation of target mRNAs [19, 29, 30]. We therefore asked whether miR-430 is also responsible for the clearance of the GFP-*nanos1* reporter mRNA ([4] and Figure 1A). In situ hybridization showed that GFP-*nanos1* mRNA accumulates in somatic cells in the absence of miR-430 regulation (Figure S5). These experiments indicate that miR-430 accelerates GFP-*nanos1* mRNA decay in somatic cells.

Cis Elements within the *nanos1* 3′ UTR Allow Protein Expression in PGCs in the Presence of miRNAs

The differential regulation of the *nanos1* 3′ UTR by miR-430 contrasts with previous studies of miR-430 targets. In particular, miR-430 is uniformly expressed during early embryogenesis, and previously analyzed miR-430 target mRNAs and synthetic miR-430 reporters are repressed equally in somatic cells and PGCs [9, 19]. Indeed, in the absence of additional sequences from the *nanos1* 3′ UTR, the 3×BCDE reporter was repressed in the soma and germline (Figures 3E and 4C). Thus, miR-430 can be active both in the soma and in PGCs, but the *nanos1* 3′ UTR is more susceptible to repression in the soma. To determine the mechanistic basis for the differential repression by miR-430, we performed four experiments. First, we asked whether differential

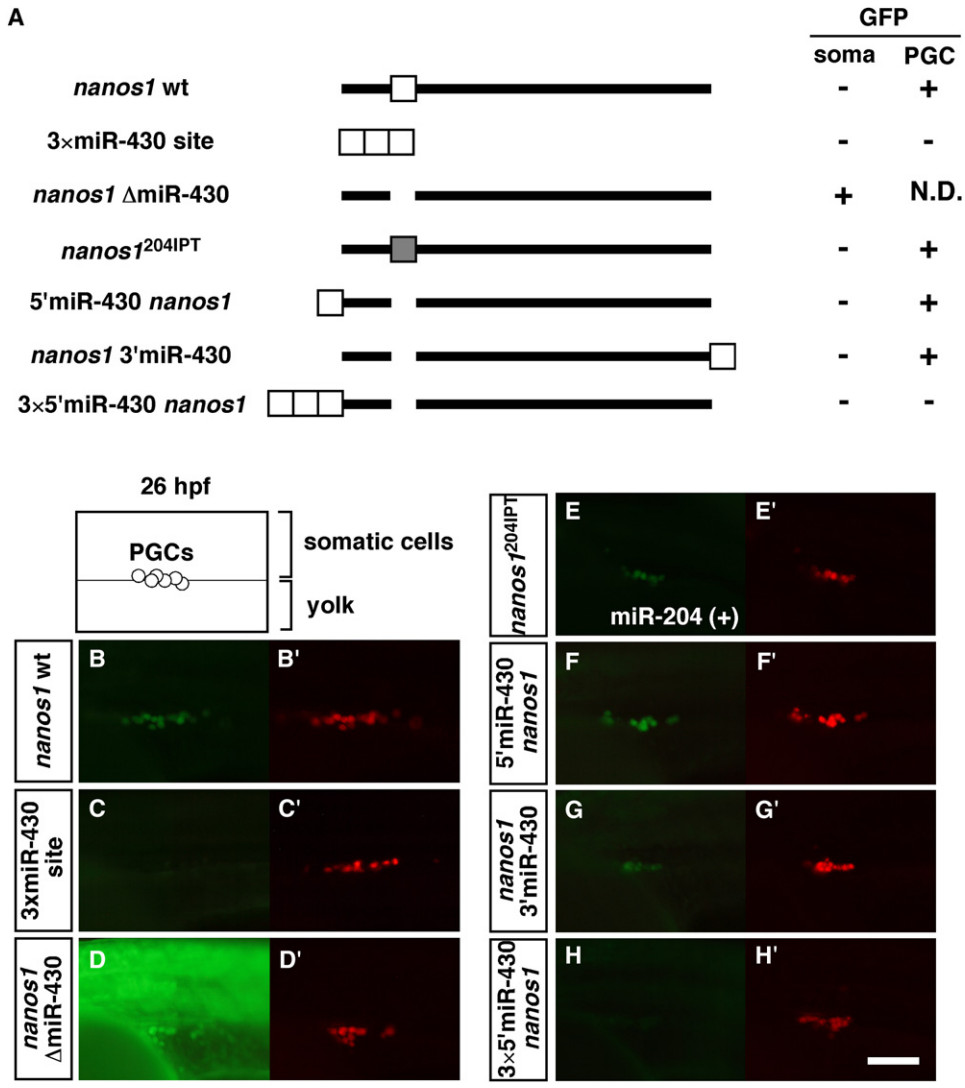

Figure 4. The *nanos1* 3′ UTR Allows Protein Expression in PGCs in the Presence of miRNAs

(A) Schematic of GFP-*nanos1* 3′ UTR reporter constructs. The black bar indicates the *nanos1* 3′ UTR. The open box indicates the miR-430 target site, and the gray box indicates the miR-204 site. GFP reporter mRNA was injected at the one-cell stage, and GFP expression in PGCs was assayed at 26 hpf. For visualization of PGCs, DsRed mRNA containing the *nanos1* 3′ UTR was coinjected. GFP expression levels in PGCs and somatic cells are indicated on the right. N.D.: not determined. (B-H) Expression of the GFP reporter in the gonad region (green) at 26 hpf. (**B′**–**H′**) Control DsRed expression in PGCs (red). The scale bar in (H′) represents 100 μm.

susceptibility is unique to miR-430 or whether other miRNAs act similarly. We replaced the miR-430 target site in the *nanos1* 3′ UTR with a miR-204 imperfect target site (IPT) (GFP-*nanos1*$^{204\ IPT}$ mRNA). Injection of intermediate levels of the miR-204 duplex strongly repressed the GFP-*nanos1*$^{204\ IPT}$ reporter in somatic cells but not in PGCs (Figure 4E). This experiment suggests that miRNAs have differential effects on the *nanos1* 3′ UTR in the soma and germline. Second, we asked whether the location of the miR-430 target site within the *nanos1* 3′ UTR affects its activity. We placed the miR-430 site at the 5′ or 3′ end of the *nanos1* 3′ UTR, 127 nt or 450 nt away from its original location (Figure 4A). The *nanos1* 3′ UTR promoted protein synthesis in PGCs in the presence of the displaced miR-430 site. This result indicates that the local environment of the miR-430 site is not required for differential expression in the soma and germline (Figures 4F and 4G). Third, we determined whether *cis* elements in the *nanos1* 3′ UTR allow PGC expression in the presence of miR-430. We examined GFP expression of the *nanos1* 3′ UTR deletion constructs in PGCs (Figure 2A and Figure S3) and found that fragment Ib, which contains the miR-430 site, promoted GFP expression in PGCs. Similarly, the remainder of the *nanos1* 3′ UTR (Δ1b) also promoted GFP expression in PGCs. Addition of the miR-430 site (ΔIb^{430IPT}) showed that the ΔIb fragment directed protein synthesis in PGCs in the presence of miR-430 (Figure S3). Hence, the *nanos1* 3′ UTR contains at least two *cis*-regulatory regions that contribute to the differential repression in soma and germline. Fourth, we asked whether the *nanos1* 3′ UTR allows expression in PGCs by sequestering the target mRNA from miR-430. In this scenario (e.g., storage in a specific subcellular compartment), *nanos1*

reporter mRNA would be inaccessible to miRNAs in germ cells. To test this model, we introduced three copies of the miR-430 target site in the 3′ UTR. Strikingly, this derivative of the *nanos1* 3′ UTR was repressed by miR-430 in both PGCs and somatic cells (Figure 4H). Similarly, injection of high amounts of miR-204 inhibited the GFP expression of *nanos1*$^{miR\text{-}204}$ mRNA in PGCs and somatic cells (data not shown). Taken together, these experiments indicate that the *nanos1* 3′ UTR is accessible to miRNAs in PGCs and that the *nanos1* 3′ UTR contains elements that allow posttranscriptional activation in PGCs even in the presence of miR-430.

The *TDRD7* 3′ UTR Is Targeted by miR-430 and Directs Protein Expression in PGCs

To determine whether the regulation of germline genes by miR-430 is a general phenomenon, we asked whether miR-430 regulates the expression of other PGC-specific mRNAs. Using the ZFIN gene expression database (http://zfin.org), we found that zgc:56669, which encodes Tudor-domain-containing protein 7 (TDRD7), includes two GCACUU sequences in its 3′ UTR. In situ hybridization showed that, similar to *nanos1*, zgc:56669/*TDRD7* is expressed maternally and is restricted to PGCs (Figures 5A–5E and data not shown). Reporter assays revealed that, similar to the *nanos1* 3′ UTR, the zgc:56669/*TDRD7* 3′ UTR repressed protein synthesis in somatic cells (Figure 5G). Two lines of evidence suggest that repression of GFP-zgc:56669/*TDRD7* in somatic cells depends on miR-430. First, GFP expression of the *TDRD7* reporter was higher in somatic cells that lack miR-430 (MZ*dicer* mutants) than in wild-type embryos (Figures 5G and 5H). Second, mutations in the predicted target sites (Figure 5F) abolished repression of the GFP reporter in somatic cells (Figures 5I and 5J). These results indicate that both the *nanos1* and zgc:56669/*TDRD7* 3′ UTRs mediate miR-430-induced repression in somatic cells.

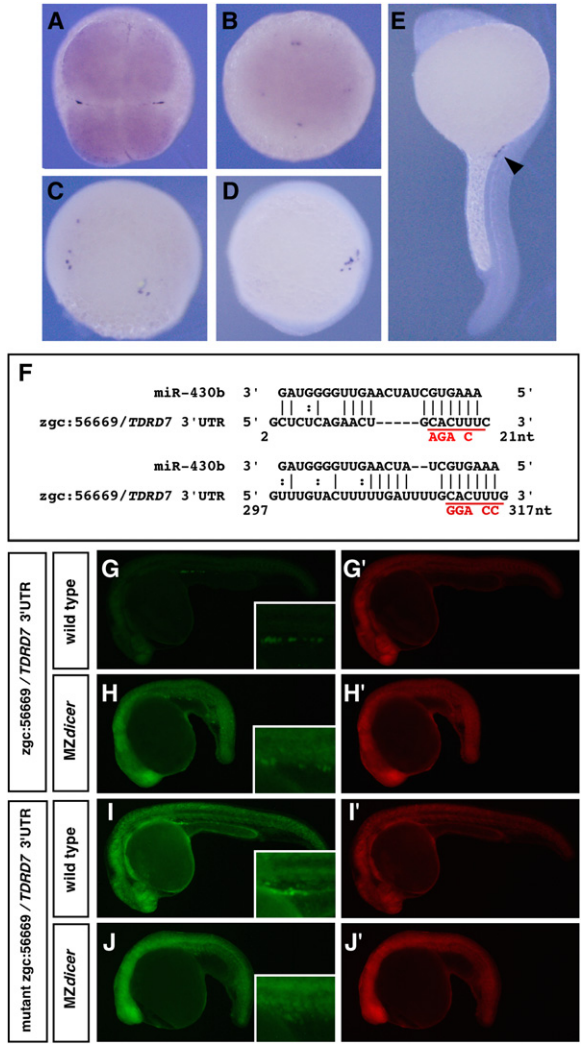

Figure 5. The *TDRD7* 3′ UTR Is Targeted by miR-430 in Somatic Cells and Allows Protein Expression in PGCs

(**A–D**) In situ hybridization of zgc:56669/*TDRD7* at (A) four-cell, (B) sphere, (C) 80% epiboly, and (D) three-somite stages and (E) 24 hpf (dark purple). An arrowhead indicates PGCs. (A) and (B) are animal-pole views, whereas (C), (D), and (E) are lateral views. (F) Putative base pairing of miR-430b with zgc:56669/*TDRD7* 3′ UTR. Base substitutions that disrupt the putative miR-430b binding are shown in red. The positions relative to the stop codon are shown. (G–I) GFP mRNA with wild-type or mutant zgc:56669/*TDRD7* 3′ UTR were injected into wild-type (G and I) or MZ*dicer* (H and J) embryos at the one-cell stage. GFP expression patterns at 26 hpf are shown. The inset shows an enlarged view of the gonad region. (G′–J′) Control DsRed expression at 26 hpf.

Discussion

Our study indicates that miR-430 targets the 3′ UTR of zebrafish *nanos1* to induce mRNA deadenylation, mRNA degradation, and translational repression in somatic cells (Figure 3). Conversely, the *nanos1* 3′ UTR also includes *cis*-acting elements that allow activity in PGCs even in the presence of miRNA-mediated repression (Figure 4). Because another germline gene, *TDRD7*, is also targeted by miR-430, our results suggest that the differential effects of miR-430 in somatic cells and PGCs contribute to germline-specific gene expression.

Although miR-430 is an important regulator of the *nanos1* 3′ UTR, two observations suggest that there are additional mechanisms that regulate *nanos1* expression. First, miR-430 is expressed at the onset of zygotic transcription (MBT) [9], whereas maternally provided GFP-*nanos1* mRNA is already posttranscriptionally repressed prior to MBT [12]. Second, the degradation of endogenous *nanos1* mRNA still occurs in MZ*dicer* mutants (data not shown). Therefore, we propose that miR-430 contributes to, but is not the sole determinant of, the soma-specific decay and translational repression of *nanos1* mRNA. Indeed, studies in *Drosophila* have shown that *nanos* mRNA is regulated by multiple posttranscriptional mechanisms, including deadenylation, mRNA decay, and translational repression [6, 31–35].

Previous reporter studies have shown that miR-430 target mRNAs are equally susceptible to repression in somatic cells and PGCs [9, 19]. The results presented here identify a novel class of miR-430 targets that are differentially regulated between soma and germline. In the case of *nanos1*, differential repression is due to *cis*-acting elements in the 3′ UTR. The exact role of these sequences is not known, but our study excludes several

simple models. First, local masking of the miR-430 binding site is unlikely to be responsible for PGC-specific activation because the *nanos1* 3′ UTR can promote protein synthesis in PGCs independently of the sequence and location of the miRNA target site (Figures 4E, 4F, and 4G). Second, it is unlikely that the *nanos1* mRNA is sequestered from miR-430 because extra copies of the miR-430 target site make the *nanos1* reporter susceptible to repression in PGCs (Figure 4H). Excluding these models, we speculate that PGCs promote the expression of *nanos1* and other germline-specific mRNAs not by inactivation of the miRNA or its associated machinery but by recruiting other factors to the 3′ UTR that increase mRNA stability or translation.

In addition to revealing a role for microRNAs in soma versus germline gene expression, our results have wider implications for the regulation of mRNAs by miRNAs. We found that miRNAs can be effective regulators of a target mRNA in one tissue but ineffective in another tissue. Hence, the presence of a miRNA target site and repression in one tissue (e.g., somatic cells) does not necessarily result in repression in another tissue (e.g., PGCs). Analogously, it has been shown that some miRNA targets have differential susceptibilities under stress and normal conditions [36]. Hence, miRNA-mediated regulation is conditional on tissue-specific or cell-state-specific factors. Recent studies have also shown that some predicted miRNA targets are expressed at high levels in cells that express the cognate miRNA [19, 29, 37, 38]. These observations and our results suggest that there are not only "anti-targets," which have evolved 3′ UTRs that lack miRNA target sites [37, 38], but also "antagonistic targets," which have evolved mechanisms to counteract the effects of miRNAs.

Supplemental Data

Supplemental Data include Experimental Procedures and six figures and are available online at http://www.current-biology.com/cgi/content/future/16/21/2135/DC1/.

Acknowledgments

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