Cell, Vol. 120, 623–634, March 11, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2004.12.038

Involvement of MicroRNA in AU-Rich Element-Mediated mRNA Instability

Qing Jing,[1,4] Shuang Huang,[1] Sabine Guth,[2] Tyler Zarubin,[1] Andrea Motoyama,[1] Jianming Chen,[1] Franco Di Padova,[2] Sheng-Cai Lin,[3] Hermann Gram,[2] and Jiahuai Han[1,*]

[1]Department of Immunology
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, California 92037

[2]Novartis Institutes for Biomedical Research
CH-4002, Basel
Switzerland

[3]Department of Biochemistry
Hong Kong University of Science & Technology
Clear Water Bay, Kowloon, Hong Kong
China

Summary

AU-rich elements (AREs) in the 3′ untranslated region (UTR) of unstable mRNAs dictate their degradation. An RNAi-based screen performed in *Drosophila* S2 cells has revealed that Dicer1, Argonaute1 (Ago1) and Ago2, components involved in microRNA (miRNA) processing and function, are required for the rapid decay of mRNA containing AREs of tumor necrosis factor-α. The requirement for Dicer in the instability of ARE-containing mRNA (ARE-RNA) was confirmed in HeLa cells. We further observed that miR16, a human miRNA containing an UAAAUAUU sequence that is complementary to the ARE sequence, is required for ARE-RNA turnover. The role of miR16 in ARE-RNA decay is sequence-specific and requires the ARE binding protein tristetraprolin (TTP). TTP does not directly bind to miR16 but interacts through association with Ago/eiF2C family members to complex with miR16 and assists in the targeting of ARE. miRNA targeting of ARE, therefore, appears to be an essential step in ARE-mediated mRNA degradation.

Introduction

The expression of most genes is regulated by multiple mechanisms, and an important control of gene expression is exerted through mRNA stability. The stability of mRNAs is regulated by a variety of signals acting on specific sequences within the RNA. AU-rich elements (AREs), located in the 3′ untranslated region (UTR) of a variety of short-lived mRNAs such as cytokines and protooncogenes (Shaw and Kamen, 1986), are the most conspicuous among various *cis*-acting destabilizing elements identified so far. The destabilizing function of ARE is believed to be regulated by factors with specific ARE binding activities (Shyu and Wilkinson, 2000). Several ARE binding proteins have been identified, and their participation in regulating the stability of ARE-RNA is supported by experimental observation (Stoecklin et al., 2002; Zhang et al., 1993; Fan and Steitz, 1998; Laroia et al., 1999; Ma et al., 1996; Gueydan et al., 1999). These proteins either promote ARE-RNA degradation, as demonstrated for TTP (also known as Tis11) and BRF1 (Tis11B) (Carballo et al., 1998; Stoecklin et al., 2002), or increase their stability, which is the case for HuR (Fan and Steitz, 1998; Peng et al., 1998). In vitro studies have shown that the exosome can be recruited to AREs and execute the degradation of ARE-RNA (Chen et al., 2001; Mukherjee et al., 2002). However, despite the significant progress made, the mechanical basis of ARE-mediated mRNA instability remains nebulous.

miRNAs are short noncoding single-strand RNA species found in a wide variety of organisms. Binding of the miRNA to the mRNA results in translational repression of the protein-coding genes (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000) or cleavage of mRNA (Yekta et al., 2004) depending on the degree of complementarity (Hutvagner and Zamore, 2002; Doench et al., 2003; Saxena et al., 2003). With the exception of a few, very little is known about the targets of miRNAs. Although the mechanism of miRNA action remains elusive, their biogenesis is rapidly becoming clear. Primary miRNA transcripts are first processed in the nucleus to produce a hairpin RNA of ~70 nt (Lee et al., 2003). This pre-miRNA is then exported into the cytoplasm where Dicer cuts the hairpin (Bernstein et al., 2001; Hutvagner et al., 2001) and one of the two strands of the miRNA intermediate is incorporated into the RNA-induced silencing complex (RISC) (Martinez et al., 2002; Hutvagner and Zamore, 2002). The RISC complex in *Drosophila* cells contains argonaute 2, the vasa intronic gene product (VIG), the *Drosophila* homolog of the fragile X mental retardation protein dFXR, and the tudor staphylococcal nuclease (Tudor-SN) (Hammond et al., 2001; Caudy et al., 2003). A study using HeLa cells has revealed that a number of miRNAs reside in a 15S miRNP complex containing eiF2C2/hAgo2, Gemin3, and Gemin4 (Mourelatos et al., 2002). The involvement of mammalian eiF2C family members in posttranscriptional gene silencing also has been shown by other studies (Doi et al., 2003; Martinez et al., 2002). Whether the 15S miRNP and RISC are similar or distinct complexes is currently unclear. It should be noted that miRNA and RNAi share some cellular machinery in their maturation, and the mechanisms by which miRNA and RNAi execute their functions are similar, if not identical, since endogenous miRNAs can cleave mRNAs with perfect complementarity, and exogenously introduced siRNA can translationally repress mRNAs bearing imperfect complementary binding sites (Doench et al., 2003; Saxena et al., 2003).

To better understand the mechanisms of ARE-mediated mRNA instability, we used RNAi to screen a number of genes in S2 cells for their requirement in the degradation of ARE-RNA. We found that Dicer and argonaute, the key components of miRNA/RNAi system,

*Correspondence: jhan@scripps.edu
[4]Present address: Department of Cardiology, Changhai Hospital, Shanghai, China.

are required for the ARE-mediated mRNA decay in *Drosophila* cells. A requirement of Dicer in ARE-RNA degradation was further confirmed in HeLa cells. Sequence analysis revealed that a miRNA, miR16, carries a sequence of UAAAUAUU, which is complementary to ARE. We further provide evidence that mRNA decay, mediated by the ARE of TNF mRNA, is dependent on the presence of miR16. In the quest for the underlying mechanism, we found that TTP indirectly interacts with miR16. The association of TTP and miR16 in cells most likely occurs through interaction between TTP and eiF2C/Ago family members, which are components of RISC. The interaction between TTP and RISC may account for the TTP-enhanced sequence-specific pull-down of miR16 by ARE in our experiments. Our data suggest that two classes of molecules, ARE binding proteins and miRNA, are involved in recognition of the ARE sequence. The cooperation of miRNA and ARE binding proteins, like TTP, appears to be essential in ARE-mediated mRNA degradation.

Results

Double-Stranded RNAi in S2 Cells Can Be Used to Screen Genes that Are Required for the Degradation of ARE-RNA

To better understand the regulation of ARE-RNA degradation, we set out to identify genes that are required for ARE-RNA degradation. RNAi can specifically and effectively silence the expression of targeted genes in *Drosophila* S2 cells (Clemens et al., 2000), providing an opportunity to examine a large number of candidate genes regarding their function in ARE-RNA degradation. However, it is not intuitively clear whether S2 cells can be used to study ARE-mediated mRNA instability. Therefore, we first evaluated the possibility of using S2 cells to screen for genes involved in ARE-mediated mRNA degradation.

Destabilization of β-globin mRNA, by attaching ARE to its 3′UTR, has been widely used to assess the effect of ARE on mRNA stability (Shaw and Kamen, 1986). We applied the same strategy here in S2 cells. The rabbit β-globin gene was fused with DNA fragments encoding 3′UTR from several short-lived mRNAs of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-8 and was then subcloned into a *Drosophila* expression vector pRMHa-3, which utilizes a copper-inducible metallothionein promoter. As controls, we used inverted 3′UTR of TNF-α (inv-ARE^{TNF}) and ARE-deleted TNF-α 3′UTR (ΔARE). Next, these vectors were transfected into S2 cells to establish stable cell lines. The expression of β-globin mRNA was induced by adding $CuSO_4$. Transcription was inhibited by actinomycin D (ActD) (Figures 1A and 1B), or by inactivation of the metallothionein promoter through Cu^{2+} removal (Figure 1C). Real-time PCR was used to quantitate β-globin mRNA. The 3′UTR from these short-lived mRNA destabilized β-globin mRNA in S2 cells (Figures 1A and 1C). The half-life of the ARE-RNA reporter is about 1.2 hr in S2 cells, and its decay continues thereafter. In contrast, inverted 3′UTR of TNF-α and ARE-deleted TNF-α 3′UTR cannot destabilize β-globin mRNA (Figures 1B and 1C). These results demonstrate that ARE promotes mRNA decay in *Drosophila* S2 cells.

To determine whether the regulation of ARE-RNA stability in S2 cells is similar to that in mammalian cells, we examined whether *Drosophila* homologs of mammalian ARE binding protein, TTP and HuR, behave similarly to mammalian TTP and HuR in altering ARE-RNA stability. We used RNAi to suppress the expression of the *Drosophila* TTP (dTTP; gene name, *dTis11*) and HuR (dHuR; gene name, *Elav*). Our results show that the mRNA levels of the targeted genes were reduced more than 90% within 48 hr of dsRNA treatment (data not shown). To ensure reduction at the protein level, a second round of RNAi was performed on day 3, and RNA stability was analyzed on day 4. The reduction in the mRNA level of targeted genes on day 4 is shown in Figure 1D. As observed in mammalian cells, depletion of dTTP (*dTTP^d*) leads to stabilization of β-globin mRNA containing 3′UTR of TNF-α (Figure 1E), and depletion of dHuR (*dHuR^d*) did not change the half-life of short-lived ARE^{TNF}-RNA (Figure 1E). Decaying mRNAs without the ARE sequence were not affected by the depletion of *dTTP* or *dHuR* (data not shown). These data suggest that *Drosophila* and mammals possess a similar mechanism in controlling the decay of ARE-RNA.

Having shown the effectiveness of using RNAi to evaluate the requirement of ARE binding protein in ARE-mediated mRNA degradation, we next screened a number of genes for their involvement in ARE-RNA degradation.

Dicer Is Required for ARE-RNA Decay

Having screened more than thirty different genes using RNAi in S2 cells, we found that *Dicer1* is required for the rapid degradation of ARE-RNA (see Table S1 in the Supplemental Data available with this article online). As shown in Figures 2A and 2B, RNAi in S2 cells effectively reduced Dicer 1 mRNA, and reduction of *Dicer1* expression in S2 cells (*dDicer1^d*) caused stabilization of β-globin mRNA containing TNF-α ARE. The stability of ARE-RNA was not affected when the cells were treated with the RNAi of an unrelated gene, *Drosophila* peptidoglycan recognition protein LC (*dPgrp*). Dicer 1 is an RNase III family member, playing a key role in processing small RNAs in miRNA systems (Bernstein et al., 2001; Hutvagner et al., 2001). The requirement of Dicer1 in ARE-mediated RNA degradation suggests involvement of miRNA pathways in controlling the half-life of ARE-RNA. Therefore, we examined other core elements of the miRNA pathways for their involvement in ARE-RNA degradation. Proteins of the *RDE-1/AGO* family are known to function together with Dicer in processing miRNA precursors or double-strand RNA into small RNAs with lengths of ~21–26 nucleotides (Williams and Rubin, 2002; Hutvagner et al., 2001). Inhibition of *Drosophila Ago1* or *Ago2* also leads to the stabilization of ARE^{TNF}-RNA in S2 cells (Figures 2C and 2D).

Next, we examined whether the miRNA system has a role in ARE-mediated mRNA decay in mammalian cells. Silencing the human *Dicer* gene in HeLa cells by siRNA has been reported (Hutvagner et al., 2001) and was used here to diminish the expression of this gene. The reduction of Dicer protein in Dicer siRNA-expressing HeLa cells (*Dicer^d*) was confirmed by Western blots (Figure 2E). The levels of Let7 and miR16 in *Dicer^d*, and

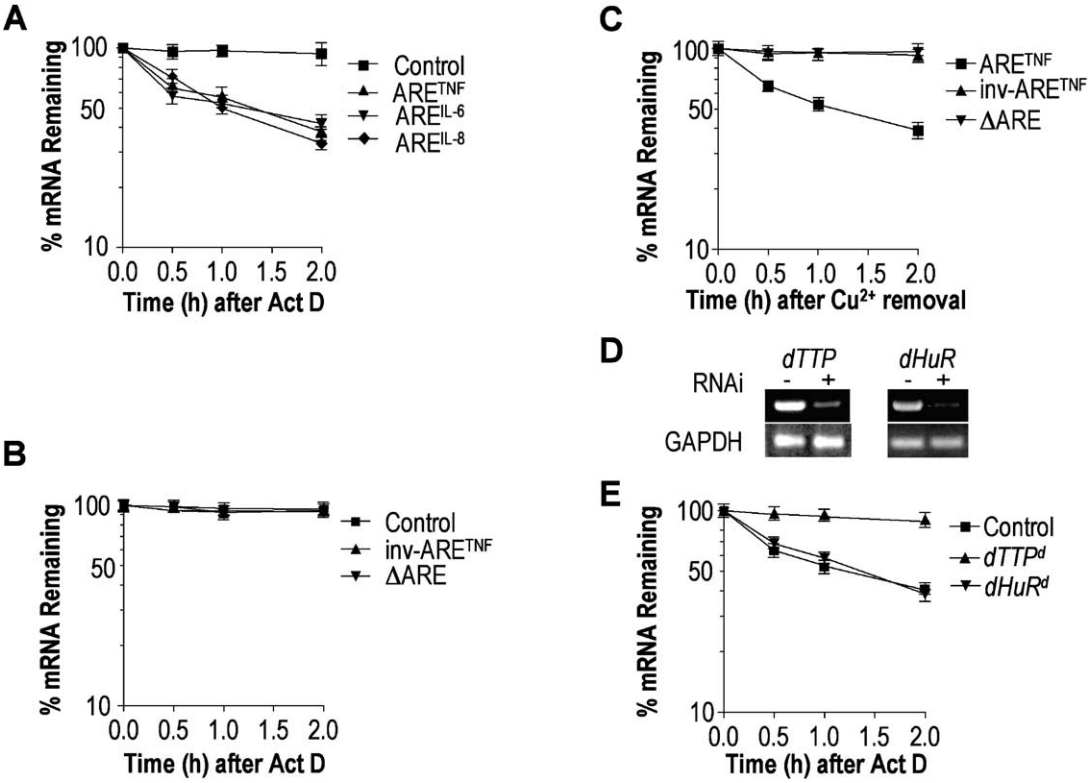

Figure 1. ARE-Mediated mRNA Instability in *Drosophila* S2 Cells

(A) S2 cells were stably transfected with a Cu^{2+}-inducible expression vector containing β-globin gene or β-globin gene fused with the 3′UTR of either TNF-α, IL-6, or IL-8. These cells were labeled as control, ARE^{TNF}, $ARE^{IL\text{-}6}$, and $ARE^{IL\text{-}8}$, respectively. Gene expression was induced by $CuSO_4$ (0.5 mM). ActD (10μg/ml) was added 10 hr after Cu^{2+} treatment, and total RNA was isolated at different time periods after ActD addition. The stability of these ARE-containing mRNAs was determined by measuring β-globin mRNA levels with real-time PCR.

(B) The same experiments were performed as in (A) except that the β-globin gene was fused with inverted 3′UTR of TNF-α (inv-ARE^{TNF}) or a TNF-α 3′UTR, in which ARE was deleted (ΔARE).

(C) The stability of β-globin mRNA containing ARE^{TNF}, inv-ARE^{TNF}, or ΔARE was determined as in (A) at different times after Cu^{2+} removal.

(D) S2 cells stably expressing RNA containing the ARE of TNF-α (ARE^{TNF}-RNA) were treated with two rounds of RNAi for dTTP or dHuR. The mRNA level of dTTP and dHuR in the RNAi-treated or -untreated cells were examined by RT-PCR.

(E) Stability of the ARE^{TNF}-RNA in the cells described in (D) was measured by real-time PCR. dTTP RNAi- and dHuR RNAi-treated cells are indicated as $dTTP^d$ and $dHuR^d$, respectively.

The data shown in this figure are representative of three to five independent experiments. The results show the means ± SE (n = 3).

control HeLa cells were determined using primer extension (Figure 2F). Let7 levels were decreased and miR16 was undetectable in *Dicer*^{d} cells. A serum-inducible *c-fos* promoter system has been used for a transient burst of mRNA synthesis in studies of ARE-mediated mRNA turnover in NIH3T3 cells (Chen and Shyu, 1994). We tested this method and found it can be used in HeLa cells. The pBBB vector-based constructs containing the β-globin gene fused with TNF-α 3′UTR, ARE-deleted TNF-α 3′UTR, or inverted TNF-α 3′UTR were transiently transfected into *Dicer*-deficient (*Dicer*^{d}) HeLa cells and control wild-type HeLa cells. The β-globin mRNA was induced by serum, and the decay of β-globin mRNA after the transcriptional pulse was determined as described (Chen and Shyu, 1994). The levels of β-globin mRNA were measured by real-time PCR (Figure 2G). Just as in the S2 cells, mRNA containing TNF-α 3′UTR (ARE^{TNF}) is unstable in wild-type HeLa cells but stable in *Dicer*^{d} HeLa cells (Figure 2G). The mRNA containing inverted TNF-α 3′UTR (inv-ARE^{TNF}) or ARE-deleted TNF-α 3′UTR (ΔARE) were stable in both wild-type and *Dicer*^{d} HeLa cells (Figure 2G). Thus, Dicer is required for ARE^{TNF}-RNA degradation in both S2 and HeLa cells.

miR16 Is Required for ARE^{TNF}-RNA Degradation

One possible reason for the requirement of Dicer and Ago in ARE-RNA degradation is that miRNA is necessary for ARE-RNA decay. Recent studies have identified hundreds of miRNAs from different species (Mourelatos et al., 2002; Lau et al., 2001; Lagos-Quintana et al., 2001). By analyzing the sequence of miRNA isolated from human cells, we found that miR16 contains eight bases (Figure 3A, in bold) that can pair with ARE in TNF-α mRNA and has no fortuitous match with TNF-α mRNA in other regions (Figure 3A). Two genes located in chromosomes 3 and 13 encode miR16 (Mourelatos et al., 2002). Predicted precursors of miR16 are shown in Figure 3B. To determine whether miR16 is involved in ARE-RNA degradation, we designed siRNA to selectively de-

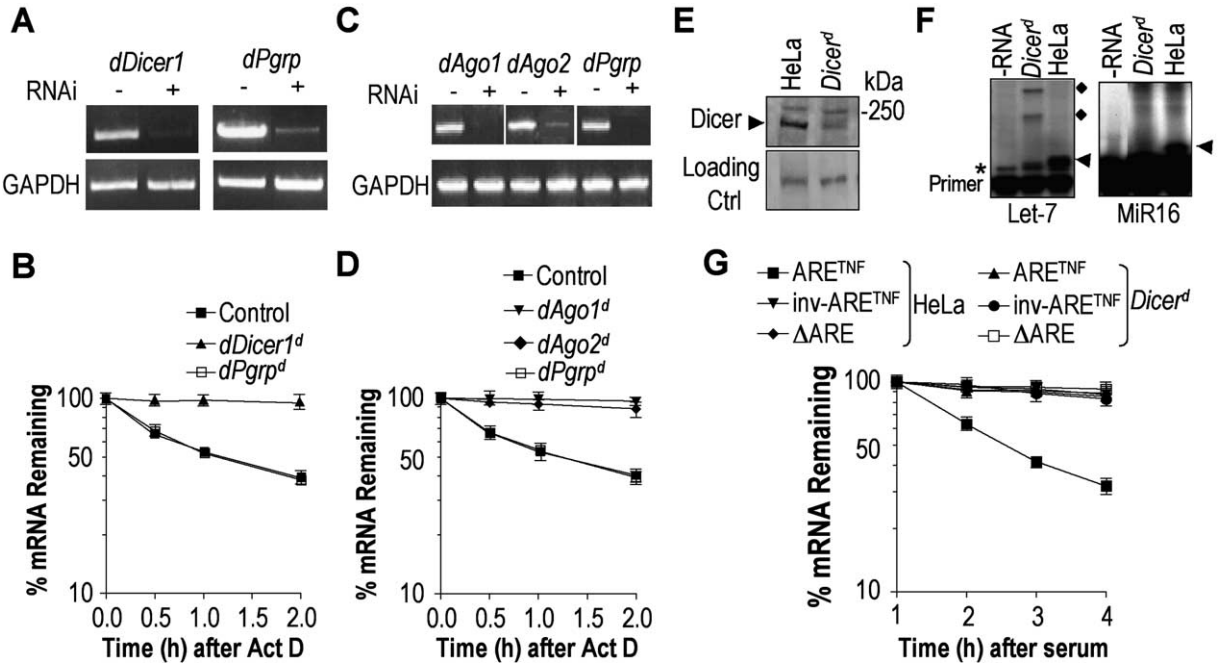

Figure 2. Core Elements of the miRNA System Are Required for ARE-Mediated mRNA Instability

(A) ARE^{TNF}-RNA-expressing S2 cells were treated with RNAi of *dDicer1* or an unrelated gene *dPgrp*. The levels of dDicer1 and dPgrp mRNA were determined by RT-PCR.

(B) Stability of ARE^{TNF}-RNA in dDicer1 RNAi-treated (*dDicer1^d*), dPgrp RNAi-treated (*dPgrp^d*), or untreated (control) cells was measured by real-time PCR.

(C) ARE^{TNF}-RNA expressing S2 cells were treated with RNAi of *dAgo1*, or *dAgo2*, or *dPgrp*. The mRNA level of dAgo1, dAgo2, and dPgrp were determined by RT-PCR.

(D) Stability of ARE^{TNF}-RNA in the cells treated with different RNAi in (C) was measured by real-time PCR. The cells in which the expression of *dAgo1* or *dAgo2* was suppressed by RNAi are indicated as *dAgo1^d* and *dAgo2^d*.

(E) Dicer protein levels in HeLa cells stably transfected with pSuper empty vector (HeLa) or Dicer siRNA expression vector (*Dicer^d*) were measured by Western blotting with anti-human Dicer antibody.

(F) Primer extensions using ^{32}P-labeled primer of Let7a or miR16 were performed with no RNA (–RNA) or total RNA from HeLa or *Dicer^d* HeLa cells. Arrows indicate the extension products. Star (*) points to an unrelated product generated from Let7a primer alone. Diamonds indicate possible precursors.

(G) ARE^{TNF}-RNA, inv-ARE^{TNF}-RNA, or ΔARE-RNA were transiently expressed in Dicer-deficient (*Dicer^d*) HeLa cells and control HeLa cells. mRNA decay was measured by real-time PCR 48 hr after transfection.

The results show the means ± SE (n = 3).

stroy the two predicted precursors, pre-miR16-1 and pre-miR16-2 (Figure 3C). After two rounds of siRNA treatment, miR16 levels were examined using primer extension and Northern blots. The siRNA of pre-miR16-1 (si-miR16-1) significantly reduced the miR16 level in HeLa cells while si-miR16-2 had no effect (Figures 3D and 3E), suggesting that miR16 in HeLa cells is primarily derived from pre-miR16-1. We then measured the stability of mRNA containing ARE of TNF-α. As shown in Figure 3F, si-miR16-1 treatment significantly increased the stability of ARE^{TNF}-RNA in contrast with the si-miR16-2, which had no effect. Pre-miR16-1 is encoded by a polycistronic gene that also encodes miR15. Since pre-miR16-1 and pre-miR15 are initially in the same transcript less than one hundred base pairs apart, they should be expressed simultaneously. We selected miR15 as a control since it does not pair with ARE, and found that inhibition of miR15 by siRNA has no effect on ARE^{TNF}-RNA stability (data not shown). Complementary 2′-O-methyl oligonucleotides have recently been shown to inhibit small RNA function (Meister et al., 2004). We used a 2′-O-methyl oligonucleotide that complements to miR16 (anti-miR16) and found that it inhibited ARE-RNA degradation while anti-miR21 had no influence on ARE-RNA decay (Figure 3G). Thus, we have determined that miR16 is required for ARE^{TNF}-RNA decay.

To determine how general miR16 functions in ARE-mediated RNA decay, we examined mRNA stability of β-globin mRNA containing the 3′UTR of cyclo-oxygenese 2 (Cox2) or urokinase plasminogen activator receptor (uPAR). Inhibition of miR16 expression increased the stability of mRNA containing Cox2 3′UTR (Figure 3H) but had almost no effect on the decay of uPAR 3′UTR containing mRNA (Figure 3I). Analysis of ARE from different mRNAs shows significant sequence diversity with the ARE of TNF, Cox2, and uPAR being classified into different groups of ARE sequences (Bakheet et al., 2001). Unlike in TNF and Cox2, ARE in uPAR is not a cluster and contains only one AUUUA motif (see Sup-

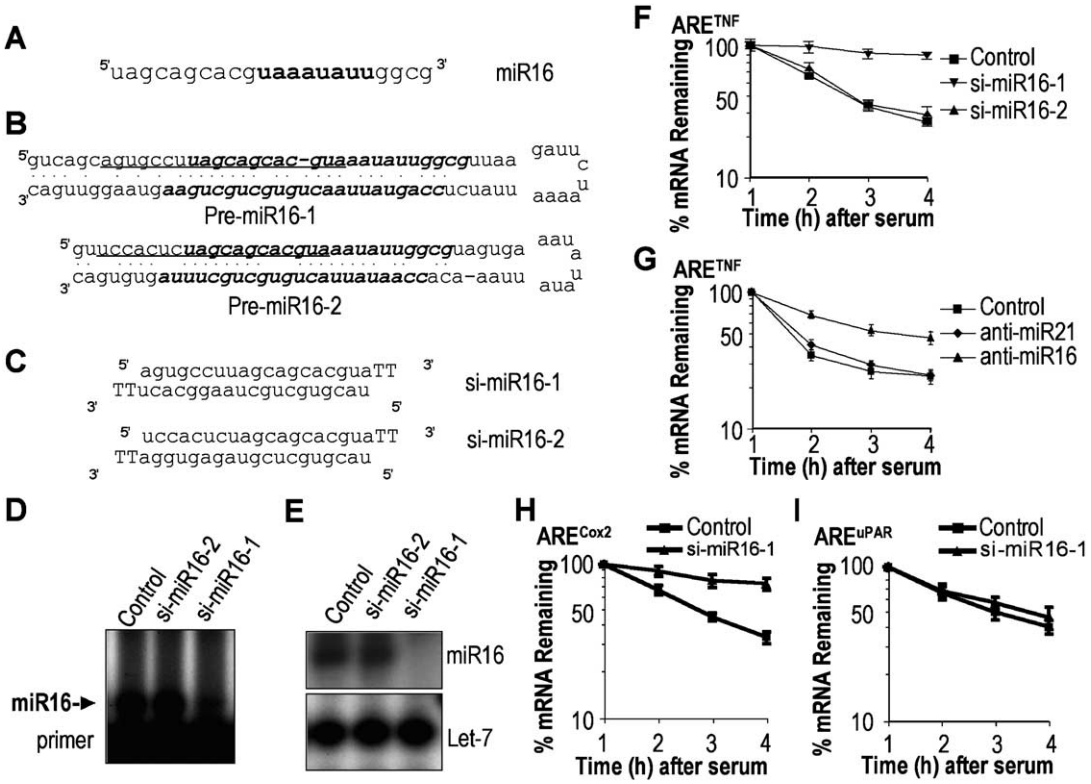

Figure 3. miR16 Is Required for ARE^{TNF}-RNA Turnover

(A) The sequence of miR16. The sequence in miR16 that complements ARE^{TNF} is shown in bold.

(B) The predicted precursors of miR16 encoded by two different genes. The double-strand intermediates of miR16 are shown in bold. The sequences that are targeted by siRNA in our experiments are underlined.

(C) The sequence of siRNA used to selectively target pre-miR16-1 and pre-miR16-2.

(D) HeLa cells were treated with two rounds of siRNA of pre-miR16-1 (si-miR16-1) or pre-miR16-2 (si-miR16-2). The levels of miR16 were measured by primer extension.

(E) Northern blot analysis of the samples described in (D) using probes for miR16 or Let7a.

(F) The expression vector of ARE^{TNF}-RNA was cotransfected at the second round of siRNA transfection. The stability of ARE^{TNF}-RNA was measured by real-time PCR 48 hr after transfection.

(G) The expression vector of ARE^{TNF}-RNA was cotransfected with anti-miR16 or anti-miR21 2′-O-methyl oligonucleotide (5 nM final concentration in medium). The stability of ARE^{TNF}-RNA was measured by real-time PCR 24 hr after transfection.

(H) The expression vector of ARE^{Cox2}-RNA or (I) ARE^{uPAR}-ARE was cotransfected at the second round of si-miR16-1 transfection. The stability of ARE^{Cox2}-RNA and ARE^{uPar}-RNA were measured by real-time PCR 48 hr after transfection.

The results show the means ± SE (n = 3).

plemental Data). Our results shown in Figures 3H and 3I suggest that miR16 is involved in the decay of some, but not all, types of ARE-RNA.

We next examined whether an increase in miR16 levels can enhance ARE^{TNF}-RNA decay. We transiently transfected a double-stranded miR16 intermediate (bolded sequence showed in the top panel of Figure 3B), together with the expression plasmid of β-globin fused with TNF-α 3′UTR (ARE^{TNF}; Figure 4A) or ARE-deleted TNF-α 3′UTR (ΔARE; Figure 4B). siRNA for green fluorescent protein (GFP) was used as a control for the miR16 intermediate. Increasing miR16 in HeLa cells increased the rate of decay of ARE-containing mRNA (Figure 4A) but did not change the decay rate for identical mRNA without the ARE sequence (Figure 4B). siRNA for GFP had no influence on ARE^{TNF}-RNA stability (Figure 4A).

To determine whether the effect of miR16 on ARE^{TNF}-RNA degradation is due to the sequence in miR16 that complements the ARE sequence, we constructed an expression vector of miR16 and two miR16 mutants: miR16G with three A to G and miR16C with three A to C substitutions (Figure 4C). Stable cell lines were established after transfection of HeLa cells with an empty vector (pSuper), miR16, miR16G, or miR16C expression vector. The expression of miR16, miR16G, and miR16C in the corresponding cell lines was determined by primer extension (Figure 4D). The level of miR16 in miR16-transfected cells is about 2-fold of that in parental or vector-transfected HeLa cells. The expression level of miR16G and miR16C were slightly higher than miR16 based on the radioactivity of extended primers (data not shown). The stability of ARE^{TNF}-RNA in vector-, miR16-, miR16G-, and miR16C-transfected cells

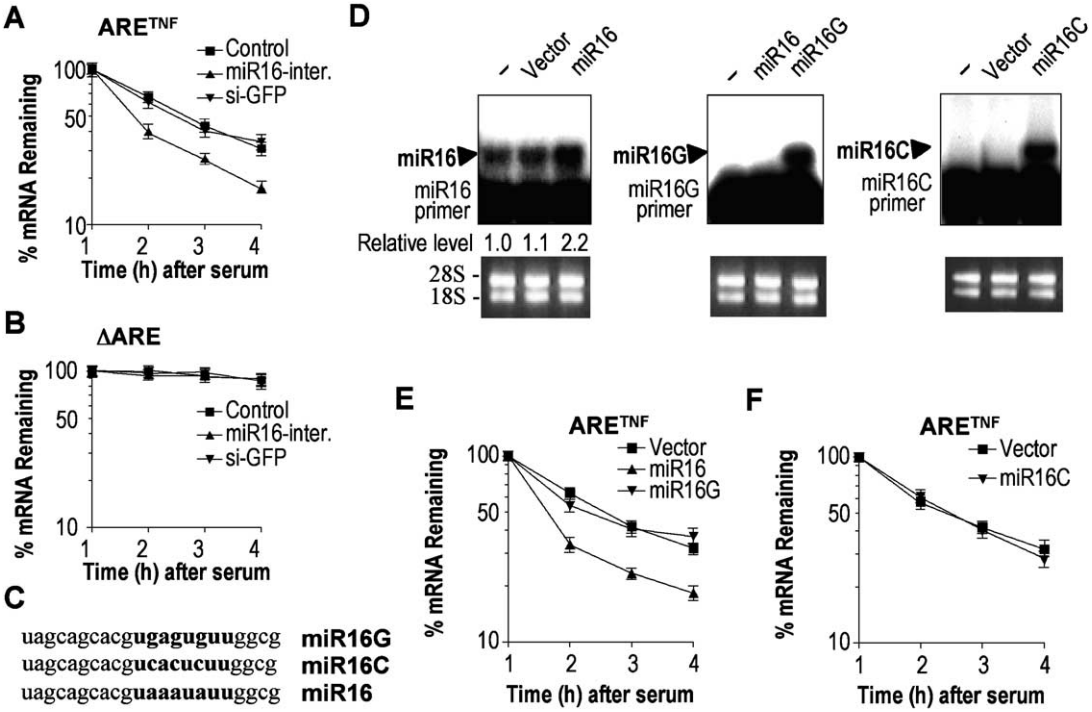

Figure 4. The Complementary Sequence of ARE in miR16 Is Required for miR16 Function in ARE-RNA Degradation

(A) Double-stranded miR16-1 intermediate (miR16-inter, bolded sequence in Figure 3B) or GFP siRNA (si-GFP) was cotransfected with ARE^{TNF}-RNA expression vector into HeLa cells. The stability of ARE^{TNF}-RNA was determined by real-time PCR.

(B) miR16-inter or si-GFP was cotransfected with ΔARE-RNA expression vector into HeLa cells. The stability of ΔARE-RNA was determined by real-time PCR.

(C) The sequence of miR16, miR16G, and miR16C.

(D) HeLa cells were stably transfected with pSuper empty vector, miR16, miR16G, or miR16C expression vector. The expression level of miR16, miR16G, and miR16C were determined by primer extension.

(E) ARE^{TNF}-RNA expression vector was transfected into miR16- or miR16G-expressing HeLa cells. The stability of ARE^{TNF}-RNA was measured by real-time PCR.

(F) ARE^{TNF}-RNA expression vector was transfected into miR16C-expressing HeLa cells. The stability of ARE^{TNF}-RNA was measured by real-time PCR.

The data shown in this figure are representative of two to three independent experiments. The results show the means ± SE (n = 3).

was measured (Figures 4E and 4F). miR16 expression reduced ARE^{TNF}-RNA stability, while miR16G and miR16C had no effect. Thus, the complementary sequence of ARE in miR16 is essential for miR16 function in destabilizing ARE^{TNF}-RNA.

miR16 and TTP Are Dependent on Each Other in the Destabilization of ARE-RNA

ARE binding proteins are known to play an important role in ARE-RNA decay. TTP is an ARE binding protein that binds to ARE sequences and destabilizes ARE-RNA (Carballo et al., 1998). To determine the relationship between miR16 and TTP in regulating ARE-RNA stability, we overexpressed miR16 in HeLa cells or HeLa cells in which TTP expression was inhibited by siRNA. Since TTP homologs can function similarly to TTP in destabilizing ARE-RNA (Stoecklin et al., 2002), the siRNA used here targets a common sequence in all three TTP family members. It should be noted that although the mRNA of TTP can be detected in HeLa cells, TTP protein is undetectable by Western blot analysis when cell lysates are directly used. We speculate that this undetectable signal is due to low protein level of TTP, and modifications of TTP in HeLa cells widen the TTP band on SDS-PAGE (Zhu et al., 2001). To detect TTP, we first immunoprecipitated TTP and then analyzed the TTP protein level by Western blot. As shown in Figure 5A, TTP appears as a smeared band and siRNA of TTP (si-TTP) diminishes TTP expression. As expected (Carballo et al., 1998; Stoecklin et al., 2002), diminishing TTP expression stabilized ARE^{TNF}-RNA (Figure 5B). As we showed earlier, the overexpression of miR16 destabilizes ARE^{TNF}-RNA (Figure 5B). Interestingly, miR16 cannot destabilize ARE^{TNF}-RNA when TTP expression is diminished (Figure 5B). It appears that miR16's effect on ARE^{TNF}-RNA stability requires the presence of TTP or TTP family members.

We then questioned if miR16 controls TTP's effect on ARE^{TNF}-RNA. We overexpressed TTP in the presence or absence of inhibiting miR16 by siRNA (si-miR16-1). The overexpressed TTP can be easily detected by Western blot in HeLa cell lysates (Figure 5C). As expected, the stability of ARE^{TNF}-RNA was reduced in TTP-overexpressed cells and increased in miR16-

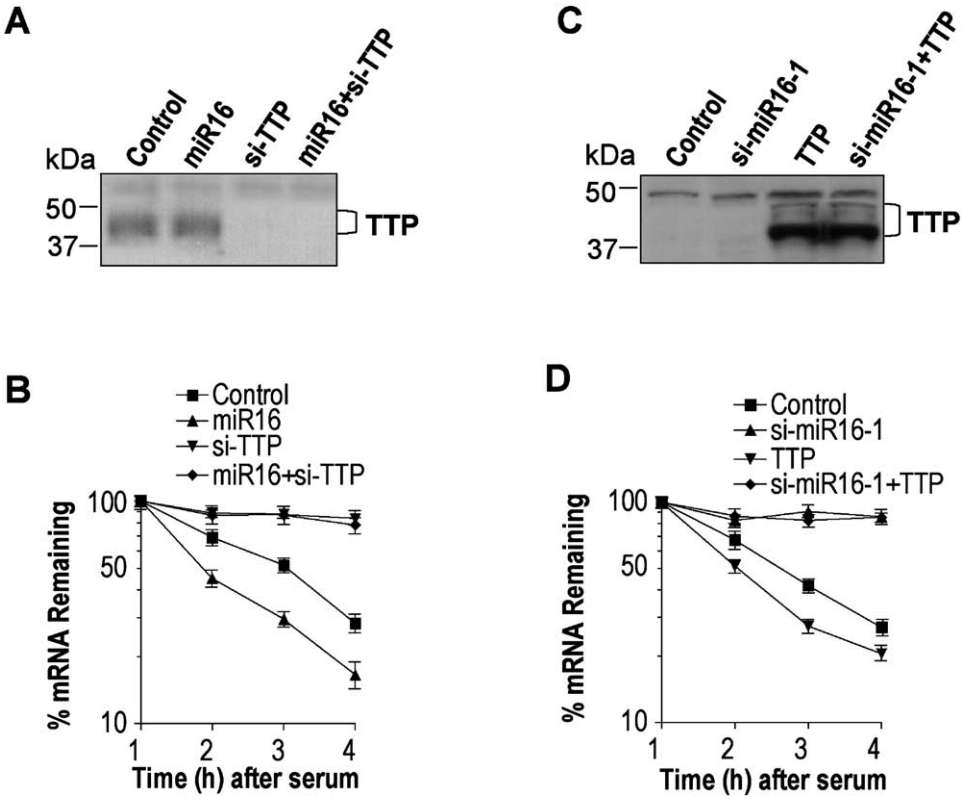

Figure 5. miR16 and TTP Are Dependent on Each Other in Destabilizing ARE^{TNF}-RNA

(A) HeLa cells and HeLa cells stably transfected with miR16 expression vector (miR16) were transfected with or without siRNA of TTP (si-TTP). The ARE^{TNF}-RNA expression vector was cotransfected at the second round of si-TTP transfection. The cells were lysed and TTP was immunoprecipitated with anti-TTP antibody immobilized to agarose beads. The TTP protein in the immunoprecipitates was analyzed by Western blot with anti-TTP antibody.

(B) The stability of ARE^{TNF}-RNA in the cells described in (A) was measured by real-time PCR.

(C) HeLa cells were transfected with or without si-miR16-1. ARE^{TNF}-RNA expression vector was cotransfected with or without TTP expression vector at the time of the second round of si-miR16-1 transfection. The expression of TTP was determined by Western blot with anti-TTP antibody.

(D) The stability of ARE^{TNF}-RNA in the cells described in (C) was measured by real-time PCR.

The results show the means ± SE (n = 3).

diminished cells (Figure 5D). The destabilization effect of TTP on ARE^{TNF}-RNA was not detected when miR16 was inhibited with si-miR16-1 (Figure 5D). Thus, TTP's effect on ARE-RNA stability also requires miR16.

TTP Does Not Directly Bind to miR16 but Interacts with Components of RISC and Assists miR16 in Targeting ARE

Because of the interdependency of miR16 and TTP in the destabilization of ARE^{TNF}-RNA, we set out to determine whether TTP can bind directly to miR16. ^{32}P-labeled miR16 was incubated with recombinant TTP in HeLa cell S-100 extracts. Samples were UV irradiated, digested with RNase and analyzed on SDS-PAGE. As a control for UV cross-linking, a ^{32}P-labeled ARE-RNA was used. As shown in Figure 6A, we did not detect any crosslinking of miR16 to TTP. In contrast, the cross-linking of the ARE-RNA to TTP was easily detected. Gel shift assays were also performed to evaluate the interaction between miR16 and TTP. In vitro incubation of miR16 or miR16G with TTP did not alter the mobility of these small RNAs (see Figure S1A). The interaction between TTP and Cox2 ARE, but not the ARE mutant, was detected (Figure S1B). The sequence of Cox2 ARE and ARE mutant are provided in the Supplemental Data.

Although TTP does not directly interact with miR16 in vitro, one cannot exclude the possibility that indirect interaction of these two molecules occurs inside cells. To assess this possibility, we immunoprecipitated TTP and extracted RNA from the immunocomplex. The presence of miR16 was determined by primer extension. As shown in Figure 6B, miR16, but not miR21 or miR23, was detected in the immunoprecipitates, suggesting that TTP and miR16 are in the same complex within the cells.

Since miR16 should be in a RISC/miRNP complex (Martinez et al., 2002; Mourelatos et al., 2002), we then determined if TTP interacts with this complex. It is known that Ago2 is a component of the RISC complex in *Drosophila*. The predicted human homolog of Ago2 is eiF2C2, which is found in RISC or 15S miRNP of HeLa

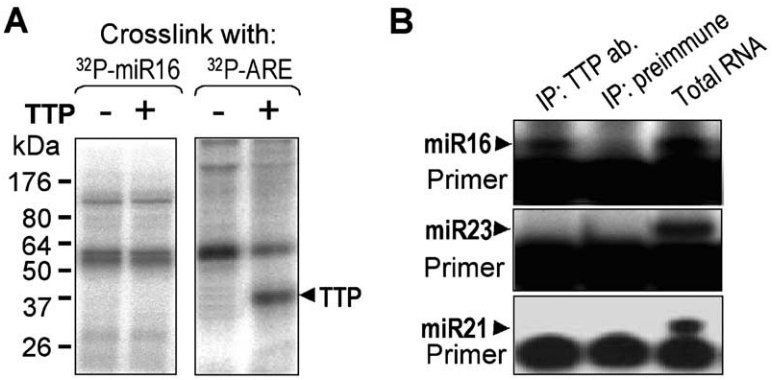

Figure 6. miR16 Does Not Directly Interact with TTP In Vitro but Can Be Coimmunoprecipitated with Cellular TTP

(A) In vitro transcribed ^{32}P-UTP-labeled miR16 and ARE-RNA were incubated with or without recombinant TTP (0.1 μg) in HeLa cell S-100 extracts at 30°C for 5 min before UV irradiation. The samples were digested with RNase and analyzed by SDS-PAGE.

(B) HeLa cells were lysed and immunoprecipitated with anti-TTP antibody or preimmune serum. RNA was extracted from the immunoprecipitates, and the presence of miR16, miR21, and miR23 was determined by primer extension. Arrows indicate the extension products.

The data shown in this figure are representative of two to three independent experiments.

cells (Mourelatos et al., 2002; Martinez et al., 2002). To determine whether TTP can interact with eiF2C2, we coexpressed HA-tagged TTP with myc-tagged eiF2C2 in 293 cells. As shown in Figure 7A, eiF2C2 coimmunoprecipitates with TTP. The interaction of eiF2C2 with TTP is selective because eiF2C2 cannot be detected in the immunoprecipitates of HA-PRAK (p38 regulated and activated kinase; Figure 7A). eiF2C4 is an eiF2C family member with ~80% sequence identical to eiF2C2. The coimmunoprecipitation of eiF2C4 and TTP is also observed when these two proteins are expressed in 293 cells (Figure 7A). Thus, TTP either directly or indirectly interacts with eiF2C/Ago family members inside cells.

To determine whether eiF2C2 can complex with the ARE binding protein that stabilizes ARE-RNA, we examined coimmunoprecipitation of eiF2C2 and HuR. Myc-eiF2C2 was coexpressed with HuR in 293 cells and immunoprecipitated with anti-myc antibody. We were unable to detect HuR in the immunocomplex (Figure 7B). Again, HA-TTP was detected in the immunoprecipitate of eiF2C2. These data indicate that the association of TTP to Ago family members is specific.

Since anti-eiF2C4 antibody is the only antibody currently available for us to detect an endogenous argonaute protein in human cell lysates, we examined whether endogenous TTP and eiF2C4 interact by coimmunoprecipitation. HeLa cell lysates were immunoprecipitated with anti-TTP antibody, preimmune serum, or anti-MKK3 (MAP kinase kinase 3) antibody. The immunoprecipitates were analyzed by Western blot and are shown in Figure 7C. We found that eiF2C4 is detected in the immunoprecipitate of TTP, but not in the controls.

We show that the sequence in miR16, which pairs with ARE, is required for miR16 to regulate ARE^{TNF}-RNA stability (Figure 4). However, studies suggest that short complementary sequences are not sufficient for guiding a miRNA to its target (Doench et al., 2003). Since TTP is required for miR16's effect on ARE^{TNF}-RNA stability (Figure 5), and TTP can interact with RISC complex, it is possible that TTP interacts with RISC to facilitate or stabilize miR16 targeting of ARE. To test this possibility, we transcribed RNA containing the ARE or ARE mutant (AREmut) sequence of Cox2 mRNA in vitro. Biotin-labeled adenine was added to the RNA by poly A polymerase. In order to have a miRNA containing compensatory mutations of the AREmut, miR16g2 was made by a three-base substitution in the UAAAUAUU region of miR16 (see Experimental Procedures for sequence). The biotin-labeled ARE-RNA (biotin-ARE) was incubated with ^{32}P-labeled miR16 or miR16g2, together with recombinant TTP or bovine serum albumin (BSA) in HeLa cell S-100 extracts. Since RISC is rapidly formed in HeLa cell cytoplasmic extracts after adding single-strand RNAs of length 19 to 29 nucleotides (Martinez et al., 2002), the miR16 in the above reaction mixture should enter RISC early in the reaction. The biotin-labeled RNA was pulled down by avidin-beads after incubation and the miRNA associated with biotin-labeled RNA was determined by ^{32}P counts. As shown in Figure 7D, miR16 was pulled down by ARE-RNA when TTP was present, supporting the idea that targeting ARE by miR16 is TTP dependent. The sequence-specific targeting is supported by the result that miR16g2 was not pulled down by ARE in the presence of TTP (Figure 7D). The interaction of TTP and ARE appears to be equally important since AREmut, which is not able to interact with TTP, cannot pull down miR16g2 in the presence of TTP.

Discussion

The ARE motif (AUUUA) is the most studied *cis*-acting element responsible for rapid turnover of unstable mRNAs in mammalian cells. In the quest for a genetic system that allows a comprehensive search for components involved in ARE-mediated decay of mRNA, we examined *Drosophila* S2 cells and found that the decay of ARE-containing RNA in S2 cells is regulated in a manner similar to that in mammalian cells (Figure 1). Inhibition of gene expression by RNAi is much easier and more cost effectively conducted in *Drosophila* S2 cells compared to mammalian cells (Clemens et al., 2000), which allowed us to investigate a large number of genes for their involvement in ARE-mediated RNA decay. Surprisingly, knockdown of *Drosophila Dicer1* gene expression led to stabilizing an ARE-RNA reporter (Figure 2B). Further studies revealed that *Drosophila Ago1* and *Ago2* are required for ARE-mediated RNA degradation (Figure 2D), suggesting involvement of the

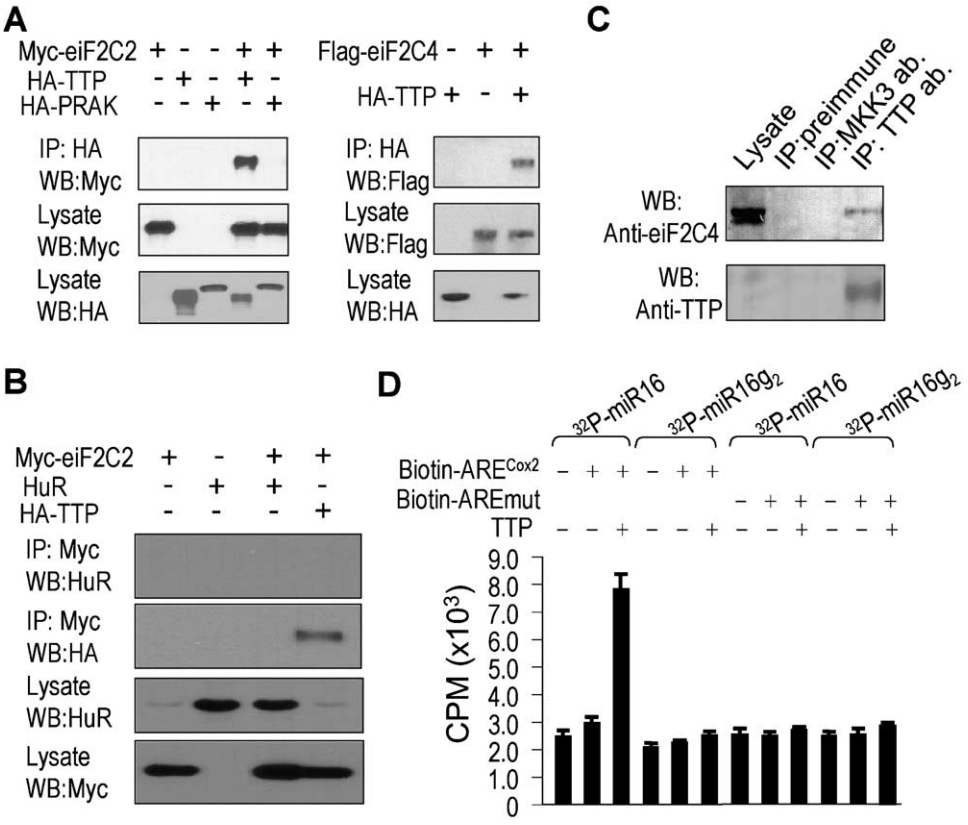

Figure 7. TTP Associates with Ago/eiF2C Family Members and Assists miR16 Target to ARE

(A) Myc-eiF2C2, flag-eiF2C4, and HA-TTP expression vectors were cotransfected into 293 cells in indicated combinations. HA-PRAK was used as a control for HA-TTP. Transfected cells were lysed and the HA-tagged proteins were pulled down with anti-HA antibody immobilized on agarose beads. The cell lysates and immunoprecipitates were analyzed by Western blots with anti-HA, anti-myc, and anti-flag antibodies.

(B) The expression vectors of myc-eiF2C2, HuR, and HA-TTP were cotransfected into 293 cells in different combinations as indicated. The cells were lysed and the myc-tagged proteins were pulled down with anti-myc antibody immobilized on agarose beads. The cell lysates and immunoprecipitates were analyzed by Western blots with anti-HuR, anti-HA, and anti-myc antibodies.

(C) HeLa cell lysates were immunoprecipitated with anti-TTP antibody. The preimmune serum and anti-MKK3 antibody were used as controls in the immunoprecipitation. The immunoprecipitates were analyzed by Western blotting with anti-eiF2C4 and anti-TTP antibodies.

(D) ARE^{Cox2}-RNA and AREmut was in vitro transcribed and labeled with biotin-N6-ATP by poly A polymerase. miR16 and miR16g2 were labeled with ^{32}P using T4 kinase. ^{32}P miR16 or miR16g2 was incubated with biotin-ARE^{Cox2}-RNA or biotin-AREmut-RNA in HeLa cell S-100 extracts in the presence or absence of recombinant TTP (1 μg) at 30°C for 10 min. Biotin-RNA was pulled down by NeutrAvidin beads. ^{32}P-labeled miRNAs that were pulled down with biotin-ARE-RNAs were quantified by scintillation counter after thoroughly washing the beads. The results show the means ± SE (n = 3).

miRNA system. We then confirmed that human *Dicer* is required in ARE-RNA degradation in HeLa cells, which implies that this underlying mechanism is conserved in the mammalian cells (Figure 2G). Given the involvement of *Dicer* in HeLa cells, we reasoned that miRNA(s) are involved in ARE-mediated RNA decay and conducted a search for miRNAs that possess a complementary sequence to the canonical AUUUA sequence of ARE. We identified miR16 as a potential candidate due to the presence of the sequence UAAAUAUU, and showed that downmodulation and overexpression of miR16 increased or decreased, respectively, the stability of a RNA reporter containing ARE of TNF or Cox2, but not uPAR (Figures 3 and 4). Furthermore, we determined that the regulation of ARE^{TNF}-RNA decay by miR16 is sequence specific (Figures 4E and 4F). Just as with Dicer, a function of Ago family members in ARE^{TNF}-RNA degradation is likely to be the processing of miR16. However, the interaction with the ARE binding protein TTP indicates that Ago/eiF2C family members also play a crucial role in the targeting of miR16 to ARE (Figures 6 and 7). Our data presented in this communication demonstrates the involvement of miR16 in controlling ARE-RNA turnover and suggests that cooperation of miRNA and ARE binding proteins is essential in the recognition of ARE and in triggering mRNA degradation.

Studies have shown that the ability of miRNA to target mRNA is directed by the pairing of miRNA to mRNA (Wightman et al., 1993; Lee et al., 1993; Yekta et al., 2004). We show here that the ARE-complementary sequence in miR16 is indeed required for miR16 function in destabilizing ARE-RNA (Figures 4E and 4F). However, pairing with no more than an eight-base ARE-sequence may not be sufficient for miR16 to target ARE-RNA. In addition, the pairing of miR16 to ARE is not in the 5′ region of miRNA, which is believed to be

more critical in causing gene repression than the 3′ region (Doench and Sharp, 2004). We speculate, then, that TTP is a factor that assists miR16 targeting to ARE sequences due to its ability to interact with the ARE and RISC complex. This explains why miR16-mediated ARE-RNA instability requires TTP (Figure 5B). In addition, the requirement of miR16 in TTP-mediated destabilization of ARE-RNA (Figure 5D) suggests that targeting of miR16 to ARE is a necessary step for RNA degradation.

ARE sequences from different mRNA can vary dramatically (Bakheet et al., 2001), with some containing multiple AU-rich elements that allow for simultaneous interaction with more than one miRNA. This could influence the ability of miRNA to promote RNA degradation because of the potential synergistic effect of miR16 to bind to multiple sites. This synergism has been demonstrated in a study that shows the addition of multiple binding sites of CXCR4 siRNA into 3′UTR of a reporter results in more translation inhibition than expected when summing up the individual effects of each binding site (Doench et al., 2003). The number of pairs that miR16 can form with different ARE sequences varies from five to eight, and the strength of interaction between miR16 and different AREs in a given mRNA may also vary. The number of miRNAs targeted to an mRNA and the strength of the interaction may both contribute to the quantitative control of mRNA turnover or translation. Perhaps since no more than six pairs can form between miR16 and ARE of uPAR and since uPAR has only one AUUUA motif in the 3′UTR, miR16 does not have a significant effect on the stability of mRNA containing uPAR 3′UTR (Figure 3I).

miR16 is conserved in mammals. Although a homolog of miR16 has not been found in *Drosophila*, miR289 contains UAAAUAUUUA, and four other known *Drosophila* miRNAs contain a UAAAU sequence (Figure S2A). Among them, at least miR277, miR289, and miR304 are expressed in S2 cells (data not shown). We used 2′-O-methyl oligonucleotides to test for *Drosophila* miRNA that could be involved in ARE-RNA degradation in S2 cells. The anti-miR289 oligo significantly stabilized mRNA containing TNF-α ARE, while the other four oligos had no or very modest effects on the stability of ARE^{TNF}-RNA (Figure S2B). miR289 had a similar effect on the stability of $ARE^{IL\text{-}6}$-RNA and $ARE^{IL\text{-}8}$-RNA (Figure S2C). Sequence comparisons showed that miR289 partially complements with ARE, but not the other regions of these 3′UTRs (Table S2). Thus, miR289 is likely to be a miRNA that has a role in regulating ARE-RNA in S2 cells.

Though we have shown the association of miR16 with ARE-RNA in the presence of TTP and S-100 in vitro, the exact mechanism of miRNA targeting of ARE and regulation of RNA degradation remains undetermined. Because of the similarity between siRNA and miRNA in regulating gene expression (Doench et al., 2003; Saxena et al., 2003), miR16-mediated ARE-RNA degradation could be similar to siRNA-mediated mRNA decay. It is theoretically possible that the targeting of ARE with miRNA leads to mRNA cleavage at the targeting site since RISC has been shown to be an RNA endonuclease in vitro (Martinez and Tuschl, 2004). However, translational suppression caused by miRNA or imperfect pairing of siRNA suggests that endonuclease activity is not always associated with RISC (Reinhart et al., 2000; Doench et al., 2003; Saxena et al., 2003). Since ARE-RNA degradation is believed to be initiated by deadenylation and subsequent targeting by the exosome pathway (Lai et al., 1999; Chen et al., 2001; Mukherjee et al., 2002), and since endocleaved ARE-RNA was not detected in our system (data not shown), we believe that the RISC involved in ARE-RNA decay is not associated with endonuclease activity. At the present, it is not clear if RISC can execute an exonuclease function, although an exonuclease, Tudor-SN, has been found in the RISC complex (Caudy et al., 2003). TTP has been shown to bind to extended ARE sequences by virtue of its zinc finger (Michel et al., 2003) and associates with components of exosomes (Chen et al., 2001); we have shown here that TTP is also associated with eiF2C/Ago family members. A recent study reported that an exosome associated DexH box helicase facilitates ARE-RNA deadenylation and decay in mammalian cells (Tran et al., 2004). Interestingly, a *C. elegens* homolog of this DexH box protein has been shown to interact with a protein complex containing Dicer, RDE-1, and RDE-4 (Tabara et al., 2002). It appears that ARE binding proteins, miRNA, deadenylase, and exosomes cooperate with each other in regulating mRNA degradation. Combining the evidence generated in our study with that reported in the literature, we favor a model in which TTP binds to an ARE and transiently interacts with the RISCs that scan mRNA. When a RISC containing miR16 encounters TTP, it stays with ARE and TTP due to base complementarity between miR16 and ARE. It is conceivable that RISC, in conjunction with TTP, serves to recruit proteins for deadenylation and/or exosomes for mRNA degradation.

Hundreds of miRNAs have been identified, but the targets of most miRNAs are unknown. Since perfectly or nearly perfectly paired sequences can only be found for a few miRNAs, computational as well as experimental approaches have been developed to identify potential miRNA targets that do not contain perfect complementary sequences (Lewis et al., 2003; Kiriakidou et al., 2004). Although these approaches have been shown to be very useful, ARE was not identified as the target of miR16 through currently available computer programs. Our data suggest that additional factors, such as sequence-specific RNA binding proteins, needs to be considered in studying the function of miRNA. As in the case of miR16, many miRNAs may require specific proteins in binding to their mRNA targets. The role of many miRNAs may need to be studied, not only in the context of miRNA-mRNA interaction, but also the interaction of miRNA complexes with other proteins.

Experimental Procedures

Plasmids

Reporter plasmids for S2 cells were constructed by subcloning the β-globin gene from the pBBB vector (Chen and Shyu, 1994) into pRMHα-3 vector using SacI and KpnI sites. The 3′UTR of TNF-α, IL-6, or IL-8 were inserted into the 3′UTR region of β-globin using

the BglII site. Deletion of AREs from TNF-α 3′UTR was accomplished by PCR recombination. The mutations of Cox2 ARE were made by replacement with synthetic oligonucleotides. The sequences of TNF UTR, TNF UTRΔARE, Cox2 ARE, Cox2 ARE mutant (AREmut), and uPAR UTR are shown in the Supplemental Data. pBBB vector was used to construct reporter plasmids for mammalian cells. The genes encoding pre-miR16 and pre-miR15 were amplified from human genomic DNA and subcloned into pSuper vector (Brummelkamp et al., 2002). The Dicer siRNA sequence has been reported (Hutvagner et al., 2001) and was subcloned into pSuper vector. The TTP, HA-TTP, and his-TTP expression vector has been described in our previous publication (Zhu et al., 2001). Flag-eiF2C4 was subcloned into pCMVFlag vector. Myc-eiF2C2 expression plasmid was kindly provided by Dr. K. Saigo (University of Tokyo).

RNAi and Antisense Inhibition

RNAi in S2 cells was performed using double-strand RNA, and siRNA treatment in HeLa cells was achieved using either synthetic oligonucleotides or stable expression by pSuper vector. Two rounds of RNAi or siRNA treatments were performed. The sequence of the functional strand of TTP siRNA is gttgtggatgaagtgg cagcg, which can also target Tis11B and Tis11D. Antisense 2′-O-methyl oligonucleotides were used to inhibit miRNAs as described (Meister et al., 2004). See Supplemental Data for more details.

Measurement of mRNA Stability

The β-globin reporter genes were stably transfected in S2 cells and transiently expressed in HeLa cells. β-globin mRNA in S2 cells was induced by addition of $CuSO_4$ (0.5 mM) for 10 hr. The transcription was stopped by actinomycin D (10 μg/ml) or the removal of $CuSO_4$. β-globin mRNA in HeLa cells was induced by 20% serum after serum-starvation (0.5% serum) for 24 hr. Real-time RT-PCR was performed on the ABI PRISM 7700 or 7900HT Sequence Detection System. Each sample was run in triplicate. The relative RNA amount was calculated with the ΔΔCt method and normalized with internal control glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

In Vitro Pull-Down Assay

RNA of Cox2 ARE and Cox2 AREmut were transcribed using T7 RNA polymerase for 2 hr at 37°C. The RNA was phenol/chloroform extracted and precipitated after the template had been digested by RNase-free DNase. Poly A polymerase (USB Corporation) was used to add an additional poly(A) tail to the RNA with 1:4 ratio of biotin-labeled ATP:ATP (ENZO Diagnostics). The biotin labeled RNA was repurified by phenol/chloroform extraction and ethanol precipitation. miR16 and miR16g2 (uagcagcacguacaaguuggcg) were labeled by ^{32}P at 5′ end with T4 kinase. Each of the labeled miRNA (5×10^6 cpm) was added to a 30 μl reaction mixture containing 15 μl HeLa cell S-100 extract (4C Biotech, Belgium), 1 mM ATP, 0.2 mM GTP, 40 U/ml RNasin, 5 mM EGTA, 30 μg/ml creatine kinase, 25 mM creatine phosphate, 200 pmol biotin-labeled RNA, and 1 μg of recombinant TTP. The reaction was carried out for 10 min at 30°C. The biotin-labeled RNA was pulled down by NeutrAvidin beads (Pierce) that had been preblocked with S-100 extracts. The amount of miRNA bound to RNA was determined by scintillation counter.

In Vitro Protein-RNA Crosslinking and RNA Electrophoretic Mobility Shift Assay

In vitro transcribed ^{32}P-labeled ARE-RNA and miR16 were used in a crosslinking assay. In vitro transcribed ARE-RNA and 5′ end ^{32}P-labeled mi16 or miR16G were used in gel-shift assay. See the Supplemental Data for details.

Immunoprecipitation, Western Blotting, Northern Blotting, and Primer Extension

Immunoprecipitations were performed using whole-cell extracts. Western blot was performed as described (Zhu et al., 2001). Primer extension was performed as described (Hutvagner et al., 2001). The Northern blot analysis of miRNA was performed as described (Sempere et al., 2002) except the oligo probes were ^{32}P labeled by T4 kinase. See the Supplemental Data for details.

Supplemental Data

Supplemental Data include two figures, two tables, Supplemental Discussion, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/120/5/623/DC1/.

Acknowledgments

We thank H. Holtmann for IL-6 and IL-8 cDNA, R. Agami for pSuper vector, A. Shyu for pBBB vector, K. Saigo and N. Doi for anti-human Dicer antibody and myc-eif2C2 expression vector, and S. Stanton for critical reading of this manuscript. This work was supported in part by NIH grants CA93926, AI41637, AI054796, and GM67101. Q.J. was supported in part by the National Basic Research Program of China (G20000056905).

Received: July 11, 2004
Revised: October 26, 2004
Accepted: December 23, 2004
Published: March 10, 2005

References

Bakheet, T., Frevel, M., Williams, B.R., Greer, W., and Khabar, K.S. (2001). ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res. *29*, 246–254.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science *296*, 550–553.

Carballo, E., Lai, W.S., and Blackshear, P.J. (1998). Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. Science *281*, 1001–1005.

Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M., Tops, B.B., Silva, J.M., Myers, M.M., Hannon, G.J., and Plasterk, R.H. (2003). A micrococcal nuclease homologue in RNAi effector complexes. Nature *425*, 411–414.

Chen, C.Y., and Shyu, A.B. (1994). Selective degradation of early-response-gene mRNAs: functional analyses of sequence features of the AU-rich elements. Mol. Cell. Biol. *14*, 8471–8482.

Chen, C.Y., Gherzi, R., Ong, S.E., Chan, E.L., Raijmakers, R., Pruijn, G.J., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001). AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell *107*, 451–464.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA *97*, 6499–6503.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev. *18*, 504–511.

Doench, J.G., Petersen, C.P., and Sharp, P.A. (2003). siRNAs can function as miRNAs. Genes Dev. *17*, 438–442.

Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K., and Saigo, K. (2003). Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr. Biol. *13*, 41–46.

Fan, X.C., and Steitz, J.A. (1998). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J. *17*, 3448–3460.

Gueydan, C., Droogmans, L., Chalon, P., Huez, G., Caput, D., and Kruys, V. (1999). Identification of TIAR as a protein binding to the

translational regulatory AU-rich element of tumor necrosis factor alpha mRNA. J. Biol. Chem. *274*, 2322–2326.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146–1150.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056–2060.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834–838.

Kiriakidou, M., Nelson, P.T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., and Hatzigeorgiou, A. (2004). A combined computational-experimental approach predicts human microRNA targets. Genes Dev. *18*, 1165–1178.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853–858.

Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S., and Blackshear, P.J. (1999). Evidence that Tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilisation of tumor necrosis factor-α mRNA. Mol. Cell. Biol. *19*, 4311–4323.

Laroia, G., Cuesta, R., Brewer, G., and Schneider, R.J. (1999). Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. Science *284*, 499–502.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858–862.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843–854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. (2003). Prediction of mammalian microRNA targets. Cell *115*, 787–798.

Ma, W.J., Cheng, S., Campbell, C., Wright, A., and Furneaux, H. (1996). Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J. Biol. Chem. *271*, 8144–8151.

Martinez, J., and Tuschl, T. (2004). RISC is a 5′ phosphomonoester-producing RNA endonuclease. Genes Dev. *18*, 975–980.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell *110*, 563–574.

Meister, G., Landthaler, M., Dorsett, Y., and Tuschl, T. (2004). Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA *10*, 544–550.

Michel, S.L., Guerrerio, A.L., and Berg, J.M. (2003). Selective RNA binding by a single CCCH zinc-binding domain from Nup475 (Tristetraprolin). Biochemistry *42*, 4626–4630.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. *16*, 720–728.

Mukherjee, D., Gao, M., O'Connor, J.P., Raijmakers, R., Pruijn, G., Lutz, C.S., and Wilusz, J. (2002). The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. EMBO J. *21*, 165–174.

Peng, S.S., Chen, C.Y., Xu, N., and Shyu, A.B. (1998). RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. *17*, 3461–3470.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901–906.

Saxena, S., Jonsson, Z.O., and Dutta, A. (2003). Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. J. Biol. Chem. *278*, 44312–44319.

Sempere, L.F., Dubrovsky, E.B., Dubrovskaya, V.A., Berger, E.M., and Ambros, V. (2002). The expression of the let-7 small regulatory RNA is controlled by ecdysone during metamorphosis in Drosophila melanogaster. Dev. Biol. *244*, 170–179.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46*, 659–667.

Shyu, A.B., and Wilkinson, M.F. (2000). The double lives of shuttling mRNA binding proteins. Cell *102*, 135–138.

Stoecklin, G., Colombi, M., Raineri, I., Leuenberger, S., Mallaun, M., Schmidlin, M., Gross, B., Lu, M., Kitamura, T., and Moroni, C. (2002). Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. EMBO J. *21*, 4709–4718.

Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. Cell *109*, 861–871.

Tran, H., Schilling, M., Wirbelauer, C., Hess, D., and Nagamine, Y. (2004). Facilitation of mRNA deadenylation and decay by the exosome-bound, DExH protein RHAU. Mol. Cell *13*, 101–111.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell *75*, 855–862.

Williams, R.W., and Rubin, G.M. (2002). ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos. Proc. Natl. Acad. Sci. USA *99*, 6889–6894.

Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304*, 594–596.

Zhang, W., Wagner, B.J., Ehrenman, K., Schaefer, A.W., DeMaria, C.T., Crater, D., DeHaven, K., Long, L., and Brewer, G. (1993). Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol. Cell. Biol. *13*, 7652–7665.

Zhu, W., Brauchle, M.A., di Padova, F., Gram, H., New, L., Ono, K., Downey, J.S., and Han, J. (2001). Gene suppression by tristetraprolin and release by the p38 pathway. Am. J. Physiol. *281*, L499–L508.