Ago-TNRC6 complex triggers microRNA-mediated mRNA decay by promoting biphasic deadenylation followed by decapping

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Abstract

MicroRNAs (miRNAs) silence the expression of their mRNA targets mainly through promoting mRNA decay. The mechanism, kinetics and participating enzymes for miRNA-mediated decay in mammalian cells remain largely unclear. Combining the approaches of transcriptional pulsing, RNA-tethering, over-expression of dominant-negative mutants, and siRNA-mediated gene knockdown, we show that let-7 miRNA-induced silencing complexes (miRISCs), which contain Argonaute (Ago) and TNRC6 (also known as GW182) proteins, trigger highly rapid mRNA decay by inducing accelerated biphasic deadenylation mediated via Pan2-Pan3 and Ccr4-Caf1 deadenylase complexes followed by Dcp1-Dcp2 complex-directed decapping in mammalian cells. When tethered to mRNAs, all four human Ago proteins and TNRC6C are each able to recapitulate the two deadenylation steps. Two conserved human Ago2 phenylalanines (F470 and F505) are critical for recruiting TNRC6 to promote deadenylation. These findings indicate that promoting biphasic deadenylation to trigger mRNA decay is an intrinsic property of miRISCs.

Keywords

mRNA turnover; microRNA; deadenylation; Caf1; Pan2; Argonaute protein; TNRC6

INTRODCTION

MicroRNAs (miRNAs) consist of a large family of evolutionarily conserved small regulatory RNAs. To accomplish their regulatory roles, one strand of the mature miRNA duplexes is incorporated into Argonaute (Ago) proteins to form miRNA-induced silencing complexes (miRISCs) in the cytoplasm, which repress the expression of partially or fully complementary target mRNAs (reviewed in refs 1–4,5). It has now become clear that many mRNAs targeted by miRISCs undergo rapid decay in the cytoplasm 6–9. The recognition of mRNAs by miRISCs through imperfect miRNA-mRNA base-pairing often results in rapid

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deadenylation of target mRNAs 10–13. However, if miRNA-mRNA base-pairing is perfectly complementary, endonucleolytic cleavage of targeted transcripts ensues 14–16.

Studies in *D. melanogaster* S2 cells show that miRNA-mediated decay (miRMD) involves several components: GW182, the Ccr4-Not deadenylase complex, the Dcp1-Dcp2 decapping complex, and Ago1. It was proposed that Ago1 recruits the Ccr4–Not deadenylase complex and/or the decapping complex via GW182 to miRNA target mRNAs to promote their decay 17–19. However, several key mechanistic issues of this model need to be directly addressed by kinetic studies. For example, is miRMD triggered by deadenylation or by decapping? Are deadenylation and decapping coordinated during miRMD? If so, how is this accomplished? Moreover, does miRISC directly recruit a deadenylase complex to promote deadenylation?

The mechanism of miRMD in mammalian cells is still somewhat unclear. In a cell-free system using HEK293F cell extracts, let-7 miRNA was shown to repress translation mainly by directing deadenylation 20. Similarly, an in vivo study using a c-*fos* inducible promoter system to monitor the kinetics of miRMD during the G0 to G1 transition showed that miRNAs modestly enhance deadenylation of target mRNAs 12. However, the cause-and-effect relationship between deadenylation and decay of the RNA body was not directly addressed nor were the participating poly(A) nucleases identified. In addition, it is unclear whether decapping also plays a role in mammalian miRNA-mediated decay.

There are three paralogs of GW182 in mammals (reviewed in refs. 17,21). The human members of this protein family are designated as trinucleotide repeat containing (TNRC)-6A, 6B, and 6C. All three TNRC6 proteins can interact with members of the Ago subfamily and are involved in miRNA-mediated mRNA repression in mammalian cells22–30 23,31. Mammalian Ago proteins have a modestly conserved "m7G-cap-binding protein" motif (also known as the MID domain) required for miRNA-mediated translation repression 32. When two conserved phenylalanines (F470 and F505) in the MID domain of human Ago2 were mutated to valines, the Ago2(F2V2) mutant protein was unable to repress translation 32. It was suggested that the human Ago2(F2V2) lost the ability to repress translation due to its incapability to bind the 5'-cap 32. However, the F2V2 mutant of Drosophila Ago1 (equivalent to Ago2 in mammals) was reported to retain the ability to bind the m7G–cap but could no longer interact with GW182 18. Thus, it remains controversial as to why the F2V2 mutation impairs the mRNA silencing function of miRNAs.

In this study, we used a transcriptional pulse-chase approach to monitor the effect of inactivating individual deadenylases, the decapping enzyme Dcp2, or a combination of these enzymes on the deadenylation and decay kinetics of an mRNA targeted by let-7 in mouse NIH3T3 fibroblasts. Moreover, we combined a λ-N peptide/boxB hairpin mediated RNA-tethering approach with transcriptional pulse-chase to further address the roles of Ago and GW182 proteins in miRMD. Our results provide crucial new insights into the mechanism of miRMD.

RESULTS

Deadenylation of let-7 target mRNA is biphasic

To decipher the mechanistic steps involved in miRNA-mediated mRNA decay (miRMD), we used the Tet-promoter driven transcriptional pulsing approach 33 to monitor the decay kinetics of β-globin mRNA carrying three let-7 recognition sites (Fig. 1a, BBB+let-7wt) in mouse NIH3T3 fibroblasts. The transcriptional pulse-chase approach made it possible to monitor the nearly synchronous degradation of mRNA molecules that were similar in age, thus unequivocally revealing precursor product relationships 34,35.

Our results show that insertion of let-7 recognition sites in the 3' UTR of β-globin mRNA (BBB+let-7wt) markedly accelerated the deadenylation and decay of the otherwise stable β-globin (BBB) mRNA (Fig. 1b, compare left panel with middle panel). In contrast, mutations in the let-7 recognition sites that prevent base-pairing to the seed-region of let-7 miRNAs (Fig. 1a, BBB+let-7mut) completely abolished the accelerated deadenylation and decay (Fig. 1b, right). The detection of two decay intermediates of BBB+let-7wt mRNA corresponding to RNA species with either a ~110-nt poly(A) tail or an oligo(A) tail respectively (Fig. 1b, middle, open and solid arrowheads) indicates that deadenylation of the BBB+let-7wt mRNA is biphasic.

Let-7 miRMD is triggered by deadenylation followed by decapping

Previously, we found that a major route of mammalian cytoplasmic mRNA decay begins with deadenylation and involves two consecutive phases. Pan2-Pan3 shortens the poly(A) tail to ~110 nt followed by a highly processive action of Ccr4-Caf1 to generate the oligo(A) intermediate 34. Since our data show that deadenylation of let-7 target mRNA is biphasic (Fig. 1b, middle), we next tested whether miRMD also utilizes the major mRNA decay pathway. We over-expressed dominant-negative mutants of deadenylases (Pan2 and Caf1) or a decapping enzyme (Dcp2) either individually or in combination 34,35 and determined the effects of these mutants on the decay of BBB+let-7wt mRNA (Fig. 1, c–e).

As shown in Fig. 1c (second panel from the right), over-expression of the Caf1 mutant completely blocked the second phase of deadenylation and markedly slowed the decay of BBB+let-7wt mRNA. In contrast, over-expression of the Pan2 mutant or the Dcp2 mutant had only a modest effect on mRNA decay. While the Pan2 mutant slightly slowed the first phase of deadenylation (Fig. 1c, compare the first two blots from the left), rapid deadenylation and decay of the BBB+let-7wt mRNA occurred after the 2-h time point, consistent with our previous finding that Ccr-4-Caf1 can take over and shorten the poly(A) tail in a highly processive manner 34. Dcp2 mutant over-expression slowed the decay of the intermediate with a ~110-nt poly(A) tail as well as the intermediate with an oligo(A) tail, particularly that of the latter (Fig. 1c, compare the first panel from the right with the first panel from the left), indicating that decapping occurs after both the first and the second phases of deadenylation 34. The results of our time-course/Northern blotting experiments combined with siRNA-mediated gene knockdown also show that deadenylation and decay of BBB+let-7wt mRNA are drastically slowed by Caf1 knockdown but are only modestly

affected by Pan2 or Dcp2 knockdown (Supplementary Fig. 1.). Taken together, we conclude that miRMD through let-7 is triggered by deadenylation and not by decapping.

When Pan2 and Dcp2 mutants were co-expressed, the stabilization effect was similar to that caused by over-expressing the Dcp2 mutant alone (compare Fig. 1d, left with Fig. 1c, right). This observation further substantiates our conclusion that Ccr4-Caf1 can initiate deadenylation when the function of Pan2 is impaired (see above and ref 34).–Similarly, Pan2 mutant and Caf1 mutant co-expression did not result in further stabilization beyond that caused by over-expressing the Caf1 mutant alone (compare Fig. 1d, second panel from the right with Fig. 1c, second panel from the right). On the other hand, co-expression of both the Caf1 mutant and Dcp2 mutant to block both the second phase of deadenylation and decapping markedly stabilized BBB+let-7wt mRNA as an intermediate with ~110 nt of poly(A) (Fig. 1d, second panel from the left). Moreover, co-expressing Pan2, Caf1 and Dcp2 mutants to impair both first and second phases of deadenylation as well as decapping nearly blocked the decay of BBB+let-7wt mRNA completely (Fig. 1d, right). Collectively, we conclude that let-7 routes target mRNAs for rapid degradation that is triggered by Pan2-Pan3 and Caf1- mediated deadenylation, followed by decapping.

Kinetics of siRNA-mediated mRNA decay

To compare RNA decay kinetics between the miRMD, which is induced through imperfect miRNA-mRNA base-pairing, and the decay directed by siRNAs that are perfectly complementary to their target mRNAs (siRMD), we introduced a let-7 recognition site that forms a perfect match with let-7 miRNA into the 3' UTR of the β-globin mRNA (Fig. 2a, BBB+let-7per). The results show that insertion of a perfect let-7 complementary site caused extremely rapid mRNA decay, which is much faster than that promoted by three miRNA recognition sites (compare Fig. 2b, left panel with Fig. 1b, middle panel). Moreover, as early as the 0-h time point, an intermediate lacking poly(A) and shorter than the full-length Poly(A)$^-$ RNA was detected (Fig. 2b, left). The size of this Poly(A)$^-$ intermediate is consistent with the prediction that a 5' endonucleolytic fragment was derived from cleavage within the perfect let-7 complementary site in the 3' UTR.

Closer examination of the decay kinetics of BBB+let-7per mRNA with finer time points revealed the presence of a sub-group of BBB+let-7per mRNA that exhibits deadenylation and decay kinetics similar to those of BBB+let-7wt mRNA (compare Fig. 2b, right panel with Fig. 1b, middle panel). When the Caf1 mutant was over-expressed, a small portion of BBB+let-7per mRNA was stabilized as a decay intermediate with ~110 nt of poly(A) tail (Fig. 2c, left, open arrowhead), a phenomenon also observed for BBB+let-7wt mRNA (Fig. 1c, second panel from the right). These observations indicate that some of the BBB+let-7per mRNA undergoes miRMD.

MiRMD plays an important role in let-7-mediated gene silencing

To see whether miRMD plays an important role in let-7-mediated gene silencing, we carried out the dual luciferase assay 36 using a reporter system consisting of a gene encoding the Renilla luciferase (RL) or its derivatives with either three let-7 target sites (RL+let-7wt), three mutated non-functional let-7 target sites (RL+let-7mut), or a single sequence that

matches perfectly with let-7a (RL+let-7per) that induces an RNAi effect, to test whether the luciferase activity derived from the let-7 target mRNA is elevated when miRMD is compromised by over-expressing the Caf1 mutant (Supplementary Fig. 2). The ability of let-7 miRNA to repress the expression of its target mRNA was demonstrated by observations that the luciferase activity derived from the RL+let-7wt transcript but not from the RL+let7-mut mRNA was much lower than that from RL transcript (Supplementary Fig. 2a). When miRMD was compromised by over-expressing the Caf1 mutant, the luciferase activity expressed from RL+let-7wt mRNA increased by ~1.8-fold (Supplementary Fig. 2a). In contrast, over-expression of the Caf1 mutant had relatively little effect on control Renilla luciferase transcripts lacking a wild-type (wt) let-7 target site (Supplementary Fig. 2a). These results indicate that let-7 directed gene silencing in NIH3T3 cells is accomplished appreciably at the level of mRNA turnover.

Tethering Ago proteins triggers biphasic deadenylation and decay

To elucidate the role of Ago proteins in miRMD, we employed a RNA-tethering assay to see whether Ago proteins can promote biphasic deadenylation and thus down-regulate the expression of mRNAs when directly tethered to them. This assay 37 involves co-expression of a reporter mRNA whose 3' UTR contains four boxB elements (BBB+boxB) and an Ago protein fused to a peptide derived from the N protein of bacteriophage-λ (λN-Ago), which binds with high affinity and specificity to the boxB elements (Fig. 3a). To facilitate detection of λN-Ago proteins, a hemagglutinin (HA)-epitope tag was fused immediately after the λN peptide to create λN-HA-Ago proteins 38. Our results (Supplementary Fig. 3a) showed that tethering any one of the Ago 1– 4 proteins greatly reduced the level of BBB +boxB mRNA to about 30% of that observed when tethered to the lacZ control protein. Moreover, the reduction in BBB+box mRNA level that resulted from tethering to Ago proteins was appreciably lessened by over-expressing the Caf1 mutant that blocks deadenylation but not by the Dcp2 mutant (Supplementary Fig. 3a). When both Dcp2 and Caf1 mutants were co-expressed, the increase in the level of BBB+boxB mRNA was similar to that resulted from over-expressing the Caf1 mutant alone (Supplementary Fig. 3a). Taken together, these results suggest that all four mammalian Ago proteins can promote deadenylation and decay when tethered to mRNAs.

To obtain direct evidence in support of the above conclusion and also to see whether tethering Ago proteins to mRNAs can trigger biphasic deadenylation, we carried out kinetic studies to determine whether tethering λN-HA-Ago2 to BBB+boxB mRNA is sufficient to recapitulate let-7 miRMD (Fig. 3b). Our results showed that BBB+boxB mRNA exhibited accelerated deadenylation and decay kinetics in the presence of λN-HA-Ago2 (Fig. 3b, compare the first two blots from the left). Intermediates with either a ~110-nt poly(A) tail or an oligo(A) tail both appeared at the 0-h time point (Fig. 3b, middle panel), indicating that the deadenylation is biphasic. In contrast, expression of the control λN-HA-lacZ did not have any enhancing effect on the decay of BBB+boxB mRNA (Fig. 3c, left). Moreover, the BBB mRNA without any boxB elements remains stable in the presence of λN-HA-Ago2 (Fig. 3c, right). When deadenylation was blocked by over-expression of the Caf1mutant, the BBB+boxB mRNA, even though co-expressed with λN-HA-Ago2, remained stable (Fig. 3b, right), demonstrating that the reporter mRNA was degraded through a deadenylation-

dependent pathway when tethered to the Ago2 protein. Taking all the observations (Fig. 3 and Supplementary Fig. 3) together, we conclude that tethering individual Ago proteins to mRNA is sufficient to recapitulate let-7 miRMD, a process that is triggered by biphasic deadenylation and followed by decapping.

It is worth noting that no discernable 5' endonucleolytic cleavage intermediates were readily detected when BBB+boxB mRNA was tethered to λN-HA-Ago2 (Fig. 3b, middle), suggesting that endonuclease activity is not necessary for Ago2 to promote miRMD. This notion is further substantiated by experiments showing that when tethered to the Ago2 (H634P) mutant lacking endonucleolytic activity 39, BBB+boxB mRNA exhibited an enhanced deadenylation and decay pattern that is similar to BBB+let-7wt mRNA (Fig. 3d). Collectively, these results indicate that as for miRISCs, promoting mRNA deadenylation and decay is an intrinsic property of the Ago proteins.

Ago2 and GW182 (TNRC-6) interaction is essential for miRMD

Previously, it was reported that the human Ago2 protein lost its ability to repress translation when two conserved phenylalanines (F470 and F505) in its MID-domain were mutated to valines (F2V2 mutation) 32. However, it remains controversial as to why the F2V2 mutation renders Ago2 non-functional 18,32. Using the RNA-tethering assay, we found that the F2V2 mutation effectively abrogates the ability of Ago2 to promote rapid deadenylation and decay of BBB+boxB mRNA (Fig. 4a). On the other hand, a conservative mutation F2W2, which changed the two phenylalanines to tryptophans and was reported to not affect the translation repression activity of Ago2 32, did not impede the ability of Ago2 to promote rapid mRNA deadenylation and decay (Fig. 4b, left). Like let-7 miRMD (Fig. 1c) and the mRNA tethered by wild-type Ago2 (Fig. 3b), the rapid deadenylation and decay of BBB+boxB tethered by Ago2(F2W2) was nearly completely impaired by over-expression of the Caf1 mutant alone (Fig. 4b, right). These observations further indicate the importance of deadenylation in Ago2-directed mRNA decay and suggest that F470 and F505 in the MID-domain of Ago2 play a critical role in miRMD.

To elucidate the mechanism underlying the negative effect of the F2V2 mutation on the ability of Ago2 to promote deadenylation and decay in mammalian cells, we carried out co-IP/Western blot analysis to determine if the interaction of human Ago2 with TNRC6 proteins is affected by the F2V2 mutation. Our results showed that ectopically expressed Flag-tagged TNRC6B and TNRC6C proteins can readily pull down either wild-type Ago2 or Ago2(F2W2) (Fig. 4d). In contrast, very little Ago2(F2V2) was pulled down. In a separate set of experiments, when ectopically expressed HA-tagged wild-type Ago2, Ago2(F2V2), and Ago2(F2W2) proteins were immunoprecipitated from NIH3T3 cell extracts, an appreciably higher amount of endogenous TNRC6B protein was pulled down by Ago2 and Ago2(F2W2) than by Ago2(F2V2) (Supplementary Fig. 4). It should be noted that in both sets of experiments, no endogenous Caf1 could be co-immunoprecipitated, even though it can be readily detected in the lysates. Consistently, we were not able to co-immunoprecipitate Ago2 or GW182 along with any of the ectopically expressed deadenylases (data not shown). Collectively, our data indicate that the interaction between Ago2 and TNRC6 is essential for miRISCs to promote rapid deadenylation and decay. It

appears that these two proteins do not directly interact with Pan2-Pan3 or the Ccr4-Caf1 deadenylase complex.

Tethering TNRC6 to mRNAs is sufficient to recapitulate miRMD

GW182 (or TNRC6) has been shown to be required for efficient miRNA-mediated mRNA silencing in *C. elegans*, *Drosophila* and mammalian cultured cells[10,18,21,28,30]. Although circumstantial evidence suggests the involvement of this protein in miRNA-mediated deadenylation [10,20,27], its role in miRMD has never been directly demonstrated by kinetic studies. We therefore carried out time-course experiments to see if tethering the λN-HA-TNRC-6C protein to BBB+boxB mRNA is sufficient to recapitulate let-7 miRMD. The results (Fig. 4e) directly paralleled those obtained by tethering Ago2, showing that TNRC-6C markedly accelerated biphasic deadenylation and decay of the otherwise stable BBB+boxB mRNA (Fig. 4e, upper panels). Moreover, over-expressing the Caf1mut to block deadenylation abrogates the rapid deadenylation promoted by TNRC6C (Fig. 4e, lower left). These results provide direct evidence that tethering TNRC6 protein is sufficient to induce rapid decay by triggering deadenylation of the target mRNA.

DISCUSSION

Combining a transcriptional pulsing approach that allows monitoring of mRNA decay kinetics with approaches for compromising specific endogenous decay enzymes [33,40], we were able to trap intermediates derived from miRNA-mediated mRNA decay (miRMD). Our data show that miRISC targets mRNA for rapid decay by triggering deadenylation that exhibits biphasic kinetics and involves Pan2-Pan3 and Ccr4-Caf1 deadenylase complexes followed by decapping involving the Dcp1-Dcp2 complex (Fig. 5). Moreover, β-globin mRNA bearing let-7 recognition sites exhibits much faster deadenylation and decay kinetics than β-globin mRNA carrying a nonsense-codon or the AU-rich element (ARE) did [41,42].

We also employed the transcriptional pulsing approach to monitor the decay kinetics of an siRNA target (Fig. 2, BBB+let-7per mRNA). Although siRNAs are believed to trigger rapid mRNA degradation via an Ago2-mediated endonucleolytic cleavage, in vivo kinetic studies have not yet been done to directly chase their target mRNAs into the decay intermediates to prove this point. Our data show that insertion of a single copy of the let-7 recognition site which forms a perfect match with let-7 miRNA into the 3' UTR of β-globin mRNA results in the most rapid mRNA decay among all the mammalian mRNA decay pathways tested thus far, e.g., miRMD (this study), ARE-mediated decay, nonsense-mediated decay (NMD), and the c-fos or c-myc coding-determinant mediated decay [41,43–45]. Moreover, our detection of the 5' fragment of BBB+let-7per mRNA (Fig. 2) may lend support to an emerging idea that siRNA-mediated endocleavage generates relatively stable long non-coding RNAs that have biological functions in gene regulation [46].

Although Ago proteins are the key effector in miRNA-mediated gene silencing, their role in miRISC-directed deadenylation and decay remains controversial. Our kinetic studies here demonstrate that all four human Ago paralogs as well as the Ago2(H634P) mutant, which lacks endocleavage activity, potently promote biphasic deadenylation and decay of their target mRNAs when tethered to the mRNAs. Moreover, tethering these proteins can no

longer exert such decay promoting effects when deadenylation activity is compromised by over-expressing the Caf1 mutant (Fig. 3 and Supplementary Fig. 3). These observations suggest that when bound to an mRNA, individual Ago proteins are sufficient to induce RISC-like complex formation to promote rapid deadenylation to trigger target mRNA decay. They further support the notion that promoting rapid deadenylation to trigger mRNA decay is an intrinsic property of miRISC, which is consistent with observations that animal miRNAs generally induce significant mRNA deadenylation or degradation[19,47,48]. Since miRNA targets normally contain a combination of different recognition sites, it will be interesting to determine the deadenylation and decay kinetics of endogenous mRNAs that are natural targets of miRNAs.

Two conserved phenylalanines (F470 and F505) in the MID domain of human Ago and Drosophila Ago1 proteins were found to be critical for miRISC-mediated gene silencing [18,32]. However, the underlying mechanism remains unclear. In this study, we show that these two phenylalanines are required for recruiting GW182 to promote rapid deadenylation and decay of the mRNA targeted by Ago2. Since these two phenylalanines are conserved among all four Ago proteins, we propose that they constitute an indispensable part of a binding site on human Ago proteins for interaction with GW182. This notion is furthered supported by our finding that tethering human GW182 protein TNRC6C to mRNAs is sufficient to trigger biphasic deadenylation and rapid decay and thus recapitulate miRMD (Fig. 4e).

In summary, our data indicate that promoting deadenylation to trigger target mRNA decay is an intrinsic function of miRISC in gene silencing effected through Ago and GW182 proteins. Given the lack of co-immunoprecipitation between deadenylases and Ago-GW182 complexes (Fig. 4d and Supplementary Fig. 4) and observations that several recent affinity purifications of animal Ago- or GW182-associated protein complexes failed to identify any deadenylase [24,26,49,50], our model does not favor a direct recruitment of a deadenylase or decapping complex by the miRISC to promote rapid mRNA decay as previously proposed [10]. Rather, it favors a model in which miRISCs render their mRNA targets more susceptible to degradation by the cytoplasmic decay machinery. In this regard, the identification of PABP in Ago- or GW182-associated complexes (Supplementary Fig. 5 and ref. 26) and the in vitro finding that PABP is involved in let-7 mediated deadenylation [51] suggest that the association between Ago2-GW182 and PABPs, either in an mRNA-dependent manner or via a transient protein-protein interaction, may occur to destabilize the 3' PABP-poly(A) tail complex or the close-loop formed between the 5 cap complex'-3' PABP-poly(A) tail complex, leading to accelerated deadenylation. To allow miRNAs to function as a simple translation repressor so that mRNA targets can re-enter the translation pool to resume gene expression, the mRNA decay promoting activity of miRISC complex must be altered to prevent the target mRNA from being degraded. This may depend on the context of the mRNA-protein complex where the miRNA recognition sites reside, the presence of other 3' UTR elements, the type of miRNAs, and/or the extent of base-pairing between a miRNA and its recognition site[3,52].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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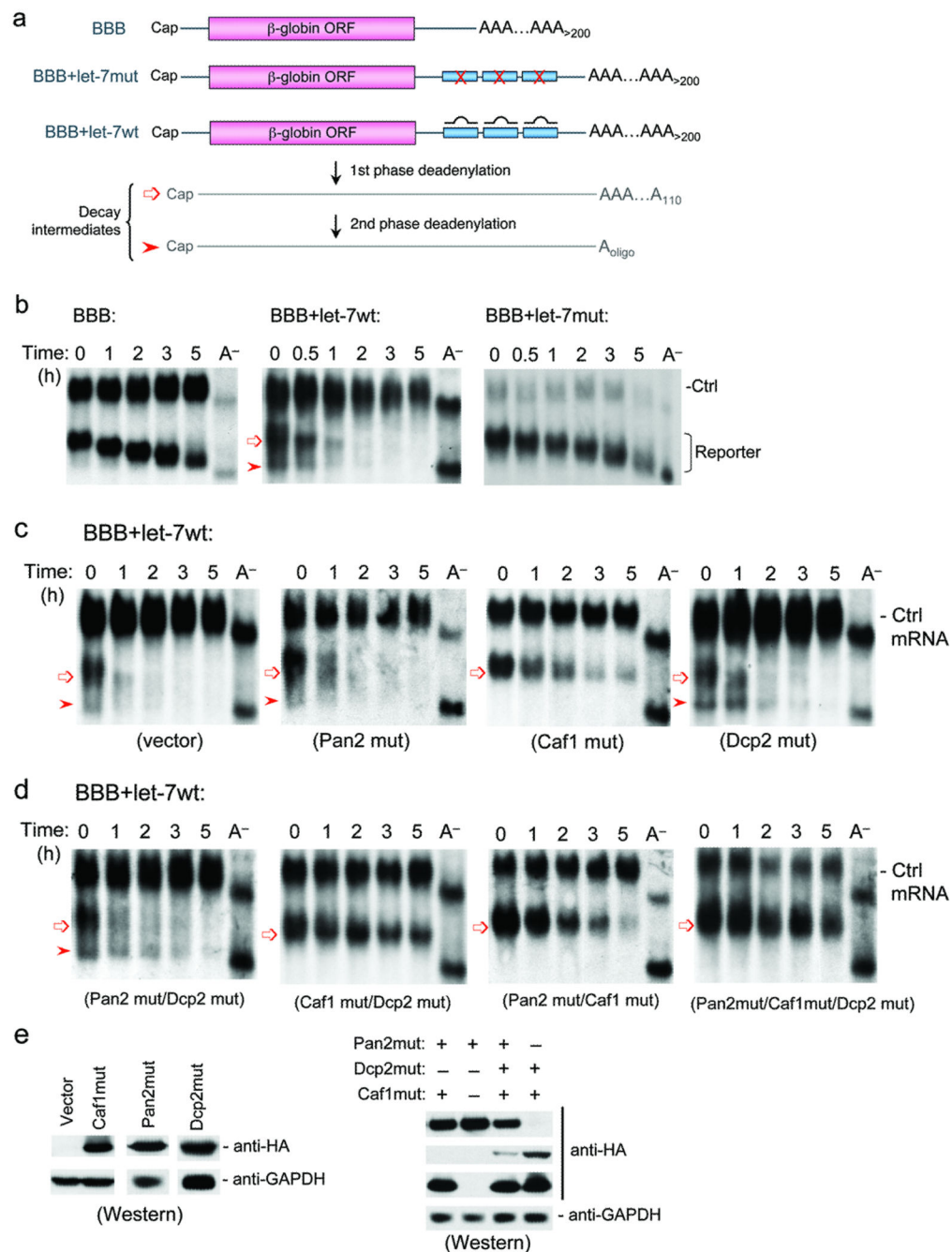
Figure 1. Let-7 miRMD is triggered by deadenylation followed by decapping
(a) Diagram showing β-globin mRNA itself (BBB) or with 3 copies of either mutated let-7 (BBB+let-7mut) or wild-type let-7 (BBB+let-7wt) target sites and the biphasic deadenylation kinetics of BBB+let-7wt mRNA. (b) Northern blots showing deadenylation and decay of BBB, BBB+let-7wt, or BBB+let-7mut mRNAs. (c) and (d) Northern blots showing mRNA decay of the BBB+let-7wt mRNA in the absence (vector) or presence of ectopically expressed mutant (mut) Pan2, Caf1, or Dcp2 either alone (a) or in combination (b) as indicated below each panel. Open arrows indicate decay intermediates with a ~110 nt

of poly(A) tail and solid arrowheads show decay intermediates with an oligo(A) tail. NIH3T3 B2A2 cells were transiently transfected with a Tet-promoter regulated plasmid encoding a reporter mRNA and the plasmids encoding Pan2mut, Caf1mut, and/or Dcp2mut as indicated. A plasmid encoding constitutively expressed α-globin/GAPDH mRNA was also co-transfected to provide an internal standard for transfection efficiency and sample handling (ctrl). The times given at the top correspond to hours (h) after Tetracycline addition. Poly(A)⁻ RNA (A⁻) was prepared in vitro by treating an RNA sample from an early time point with oligo(dT) and RNase H. (e) Western blots showing the expression levels of the ectopically expressed proteins. GAPDH served as a loading control.
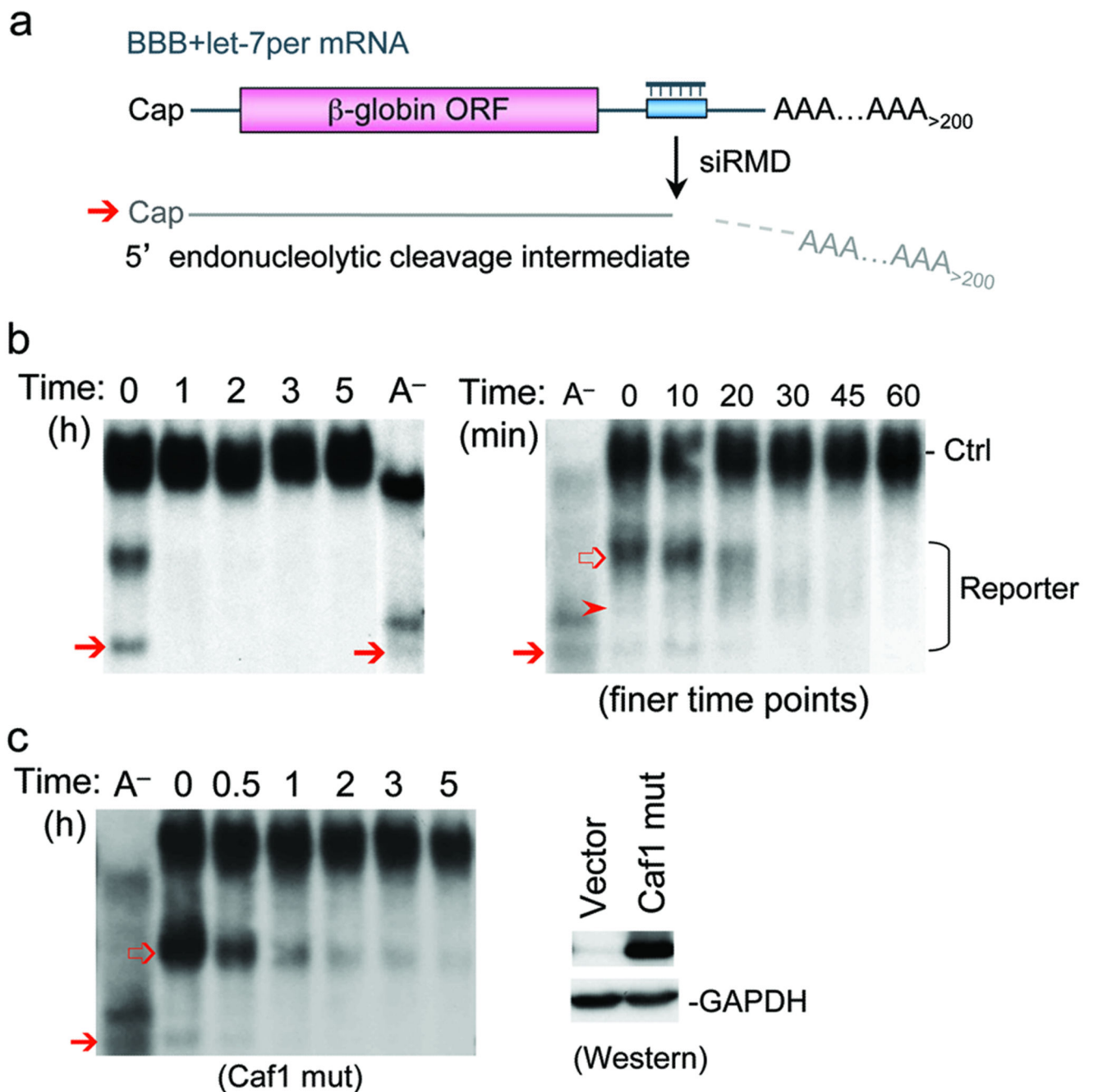
Figure 2. Decay kinetics of mRNA carrying a sequence that perfectly matches let-7 miRNA
(a) Diagram showing the physical map of β-globin mRNA carrying a sequence that perfectly matches let-7 miRNA (BBB+let-7per) and the 5' decay intermediate (red arrow) produced through siRNA-mediated endonucleolytic cleavage within the perfect let-7 complementary site. (b) Northern blots showing that the rapid decay of BBB+let-7per mRNA can be triggered by endonucleolytic cleavage through siRMD or by deadenylation through miRMD (see the finer time points in the right). Solid red arrows point out the 5' endonucleolytic products, open arrow points out the decay intermediates with a ~110 nt of poly(A) tail, and

the solid arrowhead points out the decay intermediates with an oligo(A) tail. (c) Over-expression of mutant Caf1 (Caf1mut) impairs deadenylation and stabilizes a small portion of BBB+let-7per mRNA as a decay intermediate with a ~110 nt of poly(A) tail (pointed out by an open arrow). NIH3T3 B2A2 cells were transiently co-transfected with a Tet-promoter regulated plasmid encoding BBB+let-7per mRNA and the plasmid encoding Caf1mut. The α-globin/GAPDH mRNA was expressed constitutively and served as an internal standard for transfection efficiency and sample handling (ctrl). The times given at the top correspond to hr (left panels of b and c) or minutes (right panel of b) after tetracycline addition. Poly(A)⁻ RNA (A⁻) was prepared as described in the legend to Figure 1. The expression level of the ectopically expressed Caf1mut was analyzed by Western blotting (right panel of c). GAPDH served as a loading control.
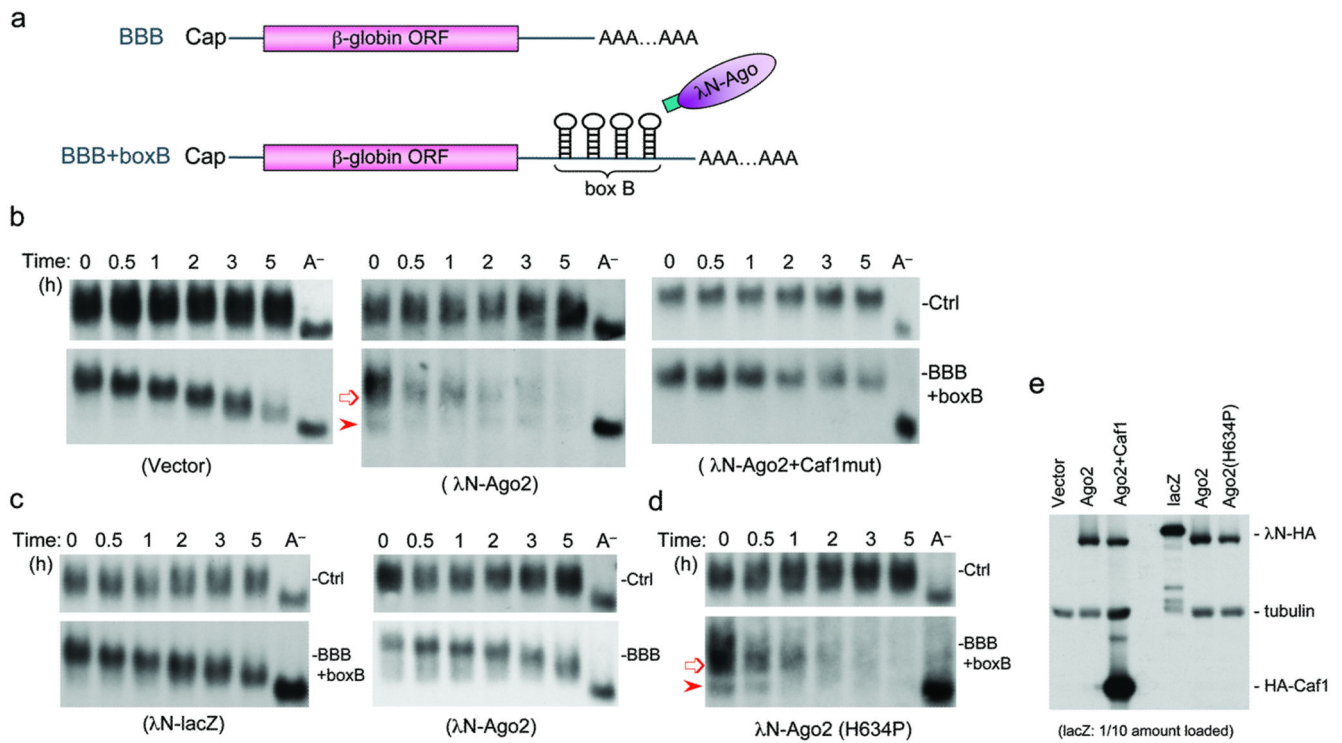
Figure 3. Tethering Ago2 to mRNAs triggers biphasic deadenylation and decay
(a) Diagram showing BBB and BBB+boxB mRNAs. The Ago protein N-terminally fused to a peptide derived from the N protein of bacteriophage-λ (λN-Ago) binds with high affinity to four boxB elements in the 3' UTR of BBB+boxB mRNA. (b) Northern blots showing that tethering Ago2 (middle) to the otherwise stable BBB+boxB mRNA triggers highly processive deadenylation and rapid decay, which is impaired by over-expression of Caf1mut (right). (c) Northern blots showing that expression of the control λN-lacZ did not enhance deadenylation or decay of BBB+boxB mRNA (left) and that deadenylation and decay of BBB mRNA lacking boxB was not accelerated in the presence of λN-Ago2 (right). (d) Northern blot showing that tethering Ago2 (H634P) mutant that lacks endonuclease activity enhances the deadenylation and decay of BBB+boxB mRNA. (e) Western blots showing the expression levels of the ectopically expressed λN-lacZ and λN-Ago2 or its mutant derivatives. α-tubulin served as a loading control. For panels b, c and d: times correspond to hr after tetracycline addition. The control mRNA (ctrl) and the preparation of Poly(A)⁻ RNA (A⁻) were as described in the legend to Figure 1.
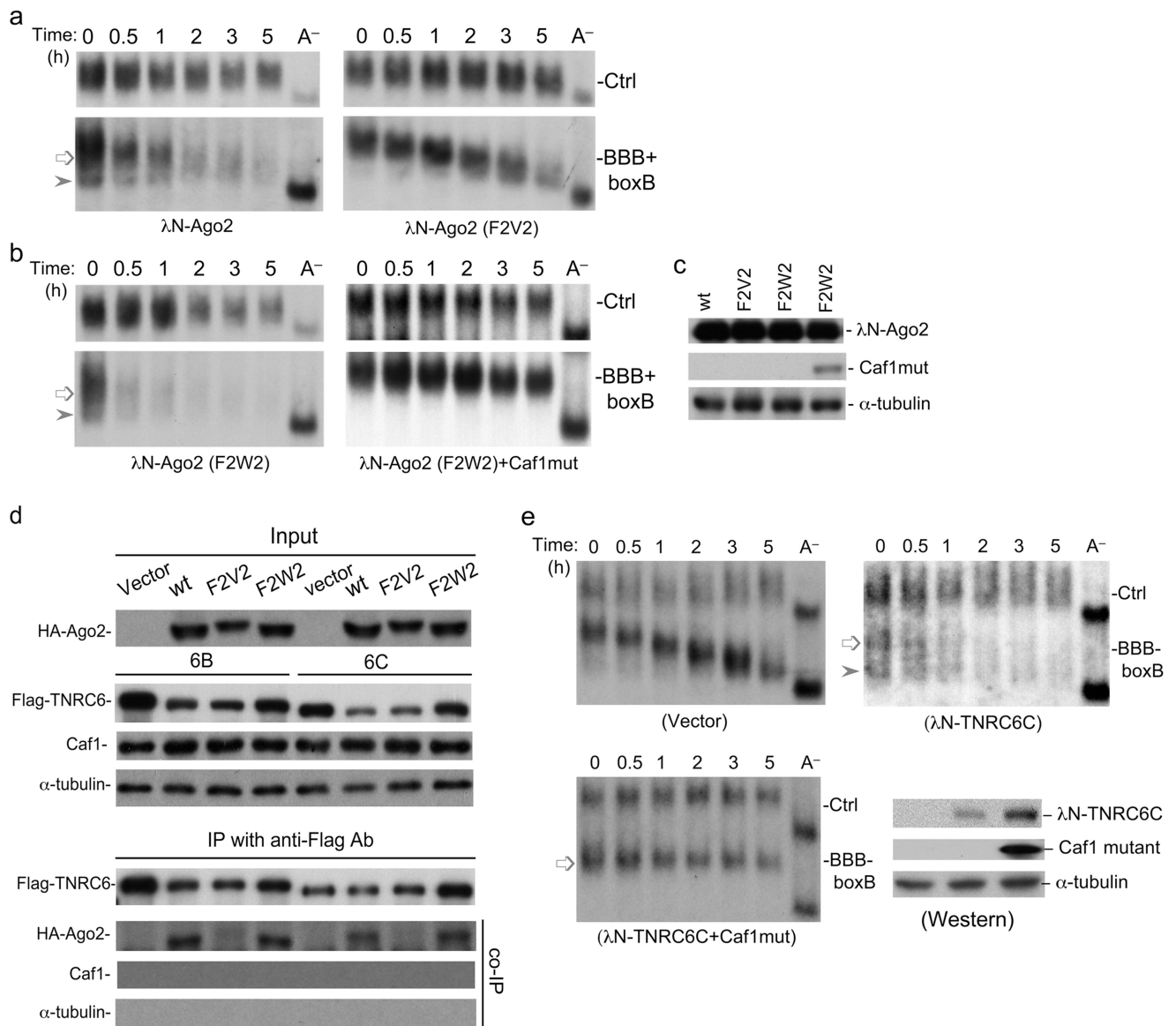
Figure 4. Tethering Ago2 or TNRC6C to mRNAs is sufficient to recapitulate miRMD
(a) Northern blots showing the effects of over-expressing λN-Ago2 (left) or λN-Ago2 (F2V2) (right) on deadenylation and decay of BBB+boxB. (b) Northern blots showing the effect of over-expressing λN-Ago2 (F2W2) (left) on deadenylation and decay of BBB+boxB and the effect of ectopically expressed Caf1 mutant (mut) on the decay of BBB+boxB tethered by λN-Ago2 (F2W2) (right). (c) Western blots showing the expression levels of the ectopically expressed proteins as indicated. α-tubulin served as a loading control. (d) Co-immunoprecipitation and Western blotting experiments showing that ectopically expressed Flag-TNRC6C and Flag-TNRC6B cannot effectively pull down ectopically expressed HA-Ago2(F2V2) or endogenous Caf1 deadenylase. Rabbit anti-Caf1 antibody was used at 1:4000 dilution. (e) Northern blots showing that tethering TNRC6C (upper right) to the otherwise stable BBB+boxB mRNA triggers highly processive deadenylation and rapid

decay, which is impaired by over-expression of Caf1mut (lower left). Western blots (lower right) showing the expression levels of the ectopically expressed λN-TNRC6C and Caf1 mutant. α-tubulin served as a loading control. NIH3T3 B2A2 cells were transiently co-transfected with a Tet-promoter regulated plasmid encoding BBB+boxB and the plasmids encoding the proteins as indicated. Times correspond to hours after Tetracycline addition. The control mRNA (ctrl) and the preparation of Poly(A)⁻ RNA (A⁻) were as described in the legend to Figure 1.
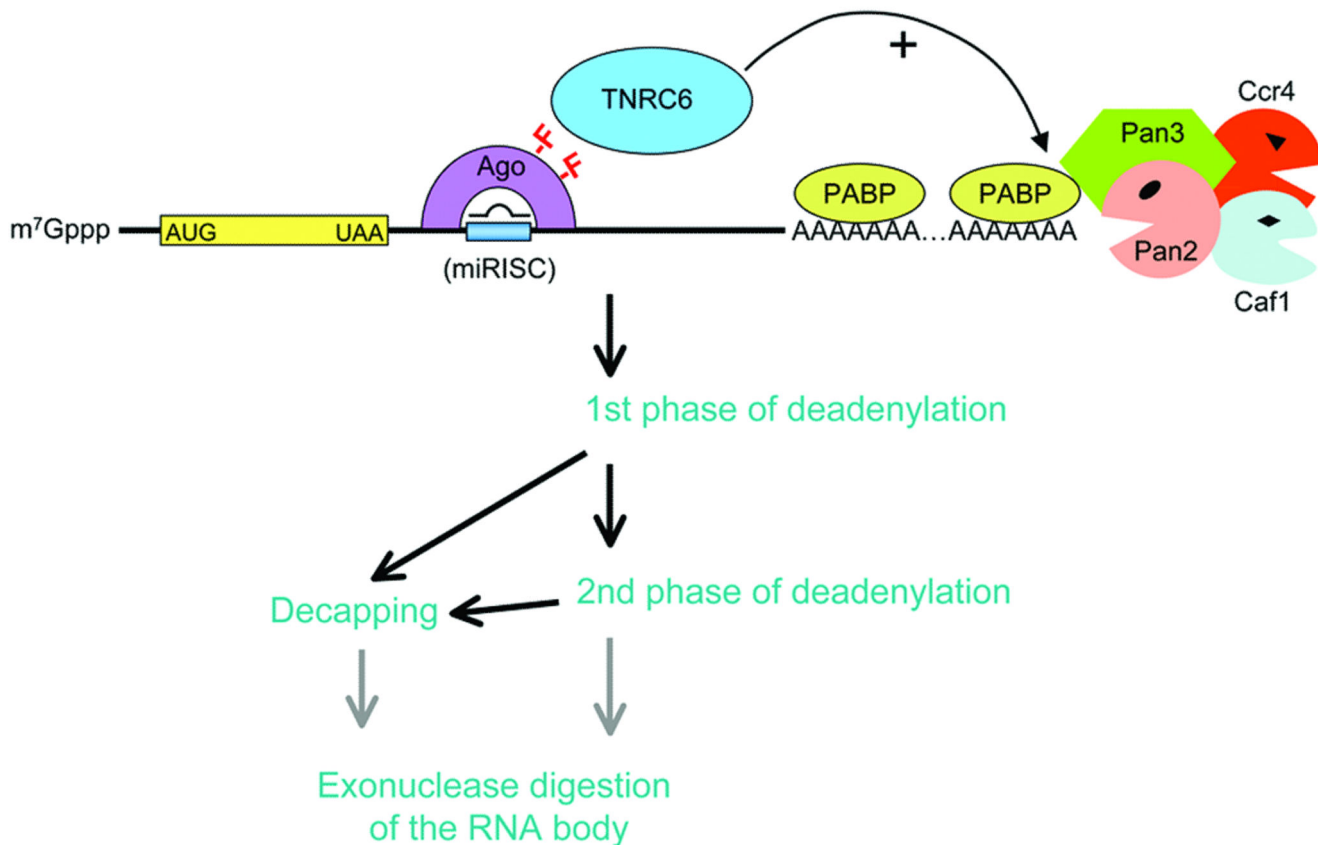
Figure 5. A model for miRMD in mammalian cells
See text for detailed discussion. miRNA-targeted mRNA decay is triggered by deadenylation followed by decapping. Pan2-Pan3 and Ccr4-Caf1 deadenylases form a super-complex that coordinates the two consecutive phases of deadenylation 35. For simplicity, cap-binding complex and its interaction with the 3' poly(A)-PABP complex are not depicted.