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Review

The Functions of MicroRNAs: mRNA Decay and Translational Repression

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MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs, which regulate complementary mRNAs by inducing translational repression and mRNA decay. Although this dual repression system seems to operate in both animals and plants, genetic and biochemical studies suggest that the mechanism underlying the miRNA-mediated silencing is different in the two kingdoms. Here, we review the recent progress in our understanding of how miRNAs mediate translational repression and mRNA decay, and discuss the contributions of the two silencing modes to the overall silencing effect in both kingdoms.

miRNAs Mediate Two Modes of Silencing

miRNAs are endogenous, small noncoding RNAs approximately 20–22 nucleotides (nt) long that regulate gene expression by binding to their complementary target mRNAs. To date, over 2000 miRNAs have been identified in the human genome, while the model plant *Arabidopsis thaliana* has ~300 miRNAs [1]. They control a broad array of biological processes, including development, differentiation, proliferation, and stress responses [2–6].

miRNAs cannot work alone. To silence target mRNAs, they need to form a ribonucleoprotein complex, called RNA-induced silencing complex (RISC) [7,8]. The minimal RISC is composed of a small RNA and Argonaute protein (Ago) [9]. In mammals, all four Agos (Ago1–4) function in the miRNA pathway, while in *Drosophila* one of the two Agos (Ago1) is functionally specialized for miRNAs [10]. Via RISC, miRNAs mediate two modes of gene silencing: mRNA decay and translational repression [8]. mRNA decay can be induced by endonucleolytic cleavage by RISC. Indeed, Argonaute proteins have a domain homologous to RNase H, and when the small RNA is perfectly or nearly perfectly complementary to the target mRNA, RISC cleaves the target mRNA at the position facing nucleotides 10 and 11 of the small RNA [8]. The cleavage mode of RISC action is commonly seen in plants, in which most of miRNAs are nearly complementary to a single or a few target mRNAs [11]. In contrast to this, animal miRNAs recognize their target mRNAs through partial base pairing, especially within the 'seed' sequence at nucleotides 2–7 or 2–8 of the miRNA [8,12]. Such partial complementarity prevents the cleavage activity of RISC, but animal RISC can still silence target genes by recruiting additional effector proteins, which induce translational repression and/or mRNA decay in a manner independent of endonucleolytic cleavage.

In recent years, genetic, biochemical, and structural analyses have provided a detailed picture of the mechanism of miRNA-mediated mRNA decay. By contrast, it still remains unclear how animal miRNAs repress translation of target mRNAs, even though considerable progress was made in the past several years. Similar to animals, miRNAs also induce translational repression in plants, but the underlying mechanism has been poorly understood. In this review, we focus on the effector step of the miRNA-mediated gene silencing, that is, how miRNAs silence their target genes, in animals and plants. Specifically, we review recent progress on the molecular mechanisms of miRNA-mediated

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Animal miRNAs promote mRNA decay by recruiting deadenylases and decapping factors onto the target mRNAs through GW182/TNRC6.

Plant miRNAs do not promote deadenylation but cleave nearly perfectly complementary targets. The 3′ end of the 5′ fragment is uridylated, and both the 5′ and 3′ fragments are decayed by the 5′-to-3′ exoribonuclease.

Animal miRNAs repress translation initiation by promoting dissociation of eIF4F through GW182-mediated displacement of PABP, recruitment of translational inhibitors via GW182, and displacement of the ATP-dependent RNA helicase eIF4A from the translation initiation complex eIF4F.

Plant miRNAs repress translation via various organelle-bound factors. Although the mechanism is unclear, *in vitro* studies suggest that AGO1–RISC can block translation initiation and ribosome movement.

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mRNA decay and translational repression in animals and plants. Moreover, we discuss the relative contribution of mRNA decay and translational repression in miRNA-mediated gene silencing in both kingdoms.

Mechanism of miRNA-Mediated mRNA Decay in Animals

Although animal miRNAs were initially thought to repress translation of target mRNAs with little or no decrease in mRNA abundance [13,14], later studies revealed that miRNAs can also promote mRNA destabilization by recruiting deadenylases onto target mRNAs through GW182 protein (TNRC6A–C in mammals and GW182 or Gawky in *Drosophila*) [15–27]. GW182 protein plays key roles in the animal miRNA pathway through interaction with Ago. Biochemical and structural studies revealed that the tryptophan residues in the N-terminal glycine–tryptophan (GW) repeat domain of GW182 protein are recognized by the tandem tryptophan-binding pockets in the PIWI domain of Ago [20,28–36]. GW182 protein serves as a hub protein, and recruits several factors to the target mRNAs including poly(A)-binding protein (PABP) and two deadenylase complexes, CCR4–NOT and PAN2–PAN3 complexes (Box 1) [21–23,37–46].

Knockdown of CCR4–NOT components or overexpression of dominant negative form of CAF1, one of the two deadenylases in the CCR4–NOT complex, severely impeded miRNA-mediated deadenylation and mRNA decay [20,22,24–26,47]. By contrast, depletion of PAN3 or overexpression of catalytically inactive form of PAN2 had only a modest effect on poly(A) shortening [22,24,26]. These results indicate that CCR4–NOT complex, rather than PAN2–PAN3 complex, is the major trigger of miRNA-mediated deadenylation and mRNA decay (Figure 1) [20,22,24–26,47].

miRNAs can promote mRNA decay not just by recruiting deadenylases on the target mRNAs but also by increasing the accessibility of the poly(A) tail to deadenylases (Figure 1). Indeed, recent studies demonstrated that miRNA targeting or GW182 tethering promoted dissociation of PABP before deadenylation starts [48]. Furthermore, even when deadenylation was blocked by an internalized poly(A) tail, PABP was released from target mRNAs by the recruitment of the CCR4–NOT deadenylase complex via GW182 [49]. As such, dissociation of PABP as well as deadenylation via GW182 can be a cause of translational repression and subsequent mRNA decay (see later).

After deadenylation, target mRNAs undergo degradation in the 5′-to-3′ mRNA decay pathway [20,27,50–52]. Importantly, RISCs can directly promote decapping and the subsequent mRNA

Box 1. Interaction between GW182 Protein and Two Deadenylase Complexes

GW182 protein interacts with the two deadenylase complexes, CCR4–NOT and PAN2–PAN3, through its tryptophan (W) motifs located in the C-terminal silencing domain (the W motifs in the N-terminal domain of GW182 in *Drosophila* can recruit deadenylase complexes) [21–23,43,45,46]. Recent structural studies have provided important molecular insights into these interactions. The rod-shaped CN9BD (CAF40/CNOT9-binding domain) of CNOT1 (NOT1 ortholog in vertebrates) interacts with the ARM repeat domain of the CNOT9 (NOT9/CAF40/RQCD1/RCD1). This CNOT1–CNOT9 complex binds to TNRC6 through interaction between W motifs in TNRC6 and the two hydrophobic tryptophan-binding pockets located in the ARM repeat domain of the CNOT9 [45,46]. In addition to the CNOT9-mediated interaction, CNOT1 can interact with TNRC6 via the C-terminus region and the tristetraprolin-binding site of CNOT1 [45,46].

The crystal structure of PAN3 forms intertwined and asymmetric homodimers [43,140–142]. The crystal structure of the PAN2–PAN3 complex reveals that the catalytic subunit PAN2 and PAN3 interact with 1:2 stoichiometry [43,140–142]. The PAN3 dimerization interface harbors a W-binding pocket, which is required for the interaction with TNRC6 [43].

Interestingly, *Caenorhabditis elegans* GW182 homolog protein, AIN-1, has less than 12% sequence identity to *Drosophila* GW182 and human TNRC6, but still can interact with both *C. elegans* and *Drosophila* PAN3 and NOT1 [42]. Given that the AIN-1 mutant, in which all tryptophan residues were substituted with alanine residues, did not interact with NOT1 or PAN3, the W-motif-mediated recruitment of deadenylase complexes may be conserved among animal GW182 proteins. For more details on the structural bases for the interactions between GW182 and its interactors, see Jonas and Izaurralde [143].

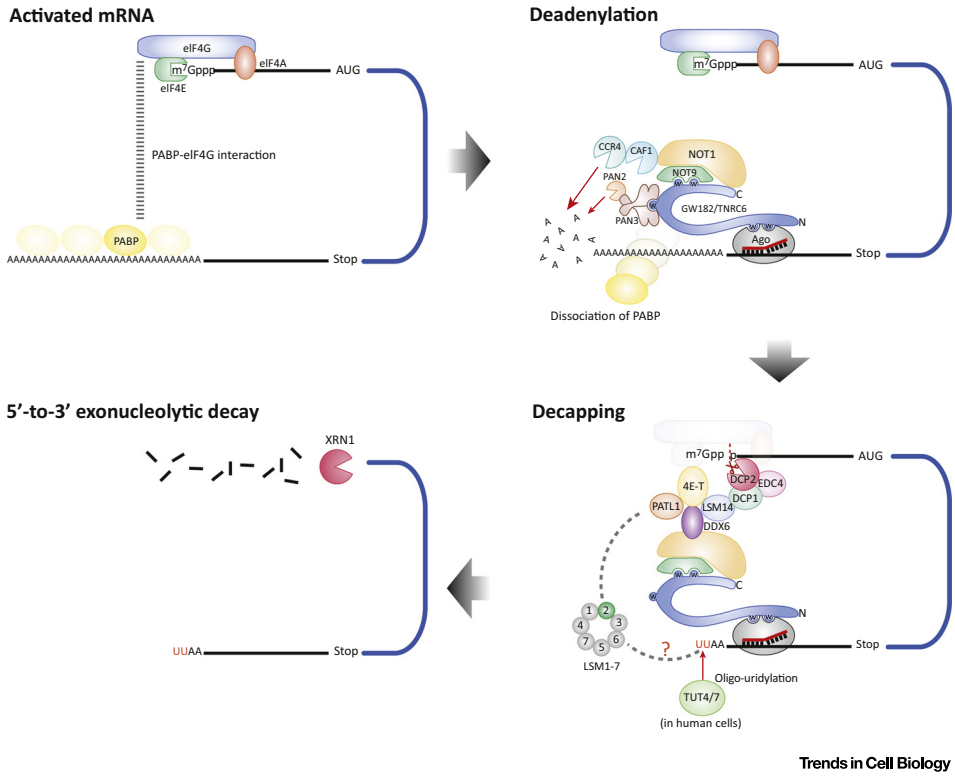

Figure 1. miRNA-Mediated mRNA Decay Pathway in Animals. There are three main steps in miRNA-mediated mRNA decay in animals. The first step is deadenylation. miRNAs induce poly(A) shortening by recruiting CCR4–NOT and PAN2–PAN3 deadenylase complexes to the target mRNAs via GW182 protein (Box 1). Deadenylated mRNAs are subjected to oligouridylation by TUT4/7, which then promotes general mRNA decay in mammals. In addition to deadenylase recruitment, GW182 can also promote dissociation of poly(A)-binding protein (PABP), raising the efficiency of deadenylation. The second step is decapping. Decapping activators, including DDX6, are recruited onto the CCR4–NOT complex, which promotes removing of the 5′ m^7G-cap structure via decapping enzyme DCP2. The third step is the 5′-to-3′ exonucleolytic mRNA decay by XRN1.

decay by recruiting decapping factors onto the target mRNAs [53–55]. In mammalian cells, the catalytic subunit of decapping complex (DCP2), and decapping activators DCP1, RCK/p54/DDX6 (also known as Dhh1 in yeast, Me31B in *Drosophila melanogaster*, and Xp54 in *Xenopus*) and EDC4 (also known as Ge-1 or Hedls) coimmunoprecipitated with Ago proteins [56–59]. Decapping factor HPat (PATL1 in humans) also coimmunoprecipitated with GW182 in *D. melanogaster* Schneider 2 cells (S2 cells) [60]. RNA coimmunoprecipitation experiments demonstrated that RISC recruits DCP1, Me31B, and HPat to the cap-less target mRNAs in *Drosophila* S2 cells [53]. Supporting these results, structural studies showed that CNOT1 directly interacts with DDX6 through the MIF4G domain [45,46,61]. A recent study demonstrated that in humans the eIF4E-binding protein 4E-T interacts with CNOT1 and a number of decapping activators, including PATL1, LSM14, DDX6, LSM2, and DCP1 by using a combination of coimmunoprecipitation and proximal biotinylation (Bio-ID) techniques [55]. They showed that 4E-T promotes miRNA-mediated mRNA decay through the eIF4E-binding domain, suggesting that 4E-T bridges the 3′-terminal mRNA decay complex to the 5′ m^7G-cap via binding to eIF4E [55]. Given that RISC can induce decapping and subsequent decay of target mRNAs with the internalized poly(A) tail or target mRNAs lacking poly(A) tail [53,54], decapping is not merely a consequence of miRNA-mediated deadenylation but it can be induced in a deadenylation-independent manner. However, deadenylation normally precedes mRNA decay in mammalian

cells and zebrafish embryos [62,63], and it remains to be investigated if miRNAs can directly promote decapping before deadenylation under physiological conditions.

Mechanism of miRNA-Mediated mRNA Decay in Plants

In contrast to animal miRNAs, plant miRNAs cannot promote deadenylation [64]; instead, they can direct target RNA cleavage (Figure 2) [8,65]. In the model plant *A. thaliana*, there are ten Ago proteins (AGO1–10) [66,67], of which miRNAs are mainly sorted into AGO1. AGO1 has a catalytic tetrad in its PIWI domain and cleaves target mRNAs with fully or nearly fully complementary sequence to the miRNA [65,68–70]. Although it is unknown how quickly miRNAs cleave their target mRNAs *in vivo*, *in vitro* kinetic studies demonstrated that plant AGO1–RISC cleaves targets with high efficiency [64,71], just like animal siRNAs.

After the small RNA-mediated endonucleolytic cleavage, the 3′ cleaved fragment is degraded by the 5′-to-3′ endoribonuclease XRN4 in *Arabidopsis* and by XRN1 in *Drosophila* [72,73]. The 5′ cleaved fragment can also be degraded by XRN4 in *Arabidopsis*, because the 5′ RNA fragment was accumulated in *xrn4* mutant plants but not in plants with mutations in the exosome components [74]. This is in stark contrast to the siRNA-mediated mRNA cleavage and decay pathway in *Drosophila* and *Chlamydomonas reinhardtii*, where the 5′ cleaved fragment is

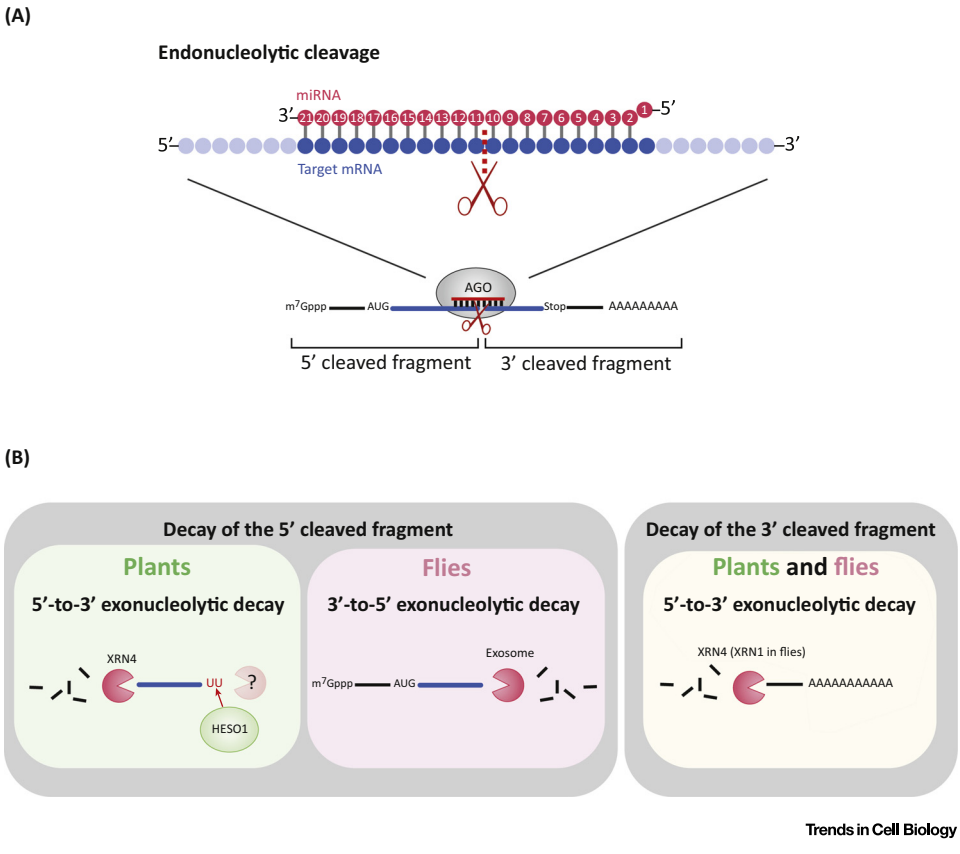

Figure 2. miRNA-Mediated mRNA Decay Pathway in Plants. (A) Plant miRNAs bind to nearly fully complementary target sites mainly located in the open reading frame (ORF) and induce endonucleolytic cleavage at the position facing nucleotides 10 and 11 of the miRNA. (B) The 5′ cleavage fragment is uridylated by HUA ENHANCER 1 (HEN1) suppressor 1 (HESO1) and degraded by XRN4 in the 5′-to-3′ direction in *Arabidopsis*. By contrast, the 5′ fragment generated by siRNA-mediated endonucleolytic cleavage is degraded by the 3′-to-5′ exoribonuclease complex, 'exosome' in *Drosophila*. The 3′ cleaved fragments are degraded by XRN4 or XRN1 in *Arabidopsis* or *Drosophila*, respectively, in the 5′-to-3′ direction.

degraded by the exosome in the 3′-to-5′ direction [73,75]. In *Arabidopsis*, degradation of the 5′ cleaved fragment can be accelerated by uridylation at the 3′ end by HUA ENHANCER 1 (HEN1) suppressor 1 (HESO1) [74].

miRNA-Mediated Translational Repression in Animals

To date, many studies using different organisms and methods have suggested that miRNAs inhibit the initiation step of translation (Box 2) [76–79]. Translational repression at the initiation step is also supported by recent genome-wide analyses of endogenous miRNA targets [80–82]. Although it is still unclear exactly how miRNAs repress translation, three major mechanisms have been proposed in the past few years, including (i) GW182-mediated PABP displacement [48,49], (ii) recruitment of the translational repressors through GW182 [45,46,55,61,83,84], and (iii) dissociation of eIF4A from the cap-binding complex eIF4F [85,86]. These mechanisms are not mutually exclusive; they may overlap, occur concurrently, or occur with different kinetics to augment the overall silencing effect (Figure 3).

GW182-Mediated PABP Displacement

Many studies have reported that GW182 protein induces not only deadenylation and subsequent mRNA decay but also translational repression [20–23,25,33–35,41,45–47,49,86–92]. GW182 protein directly interacts with the C-terminal MLLE domain of PABP through PAM2 motif located in the C-terminal silencing domain [37,38,40,93]. The high conservation of the GW182–PABP interaction suggests its crucial role in RNA silencing [42]. Indeed, several reports showed that the GW182–PABP interaction is important for miRNA-mediated silencing [37,39–41,49]. In addition, GW182 and eIF4G interact with PABP in a mutually exclusive manner in *Drosophila* [39]. These results raised the possibility that, via GW182, miRNAs inhibit the eIF4G–PABP interaction and compromise translation initiation. As mentioned earlier, miRNA promotes shortening of the poly(A) tail, which inevitably leads to dissociation of PABP from target mRNAs. However, it was recently demonstrated that miRNA promotes dissociation of PABP from target mRNAs even before deadenylation occurs in *Drosophila* embryo extract [48]. Supporting this, it was shown that GW182 displaces PABP from the target mRNA through interaction with the CCR4–NOT complex [49]. These results suggest that GW182-mediated displacement of PABP from the poly(A) tail breaks the 'closed-loop' structure formed by the interaction between eIF4G and PABP, thereby repressing translation initiation. By contrast, other papers demonstrated that mRNAs lacking the poly(A) tail, to which PABP does not specifically bind, are still repressed by miRNAs or GW182 tethering [25,35,49,56,91,92,94–97]. In S2 cell lysate, depletion of PABP or addition of Paip2 (PABP-binding protein 2), which inhibit both the eIF4G–PABP and PABP–poly(A) interactions, did not affect miRNA-mediated translational repression [89]. Furthermore, overexpression of Paip2 or depletion of PABP by injection with morpholino oligonucleotides against *pabpc1a* mRNA did not affect miRNA-mediated translational repression in zebrafish embryos [98]. Thus, although PABP is one of the targets for miRNAs, this inhibition can explain only a part of the overall miRNA-mediated translational repression.

Recruitment of the Translational Inhibitors through GW182

GW182 can also repress translation of the nonpolyadenylated target mRNAs through interaction with the CCR4–NOT complex [22,23]. Tethering of CAF1 induced translational repression of target mRNAs with or without poly(A) tail [22,23,90,99]. These results led to the model that deadenylases not only mediate poly(A) shorting but also play key roles in translational repression [90]. However, in *Drosophila* S2 cell lysate, CAF1 knockdown did not block translational repression by miRNAs [47]. Furthermore, tethering of a CAF1 mutant that has mutations in the catalytic domain as well as in CNOT1- and CCR4-binding sites did not inhibit translation of mRNAs with or without poly(A) tail. Another CAF1 mutant that has mutations only in the catalytic domain and CCR4-binding site repressed target translation, suggesting that CAF1 itself is not the translation inhibitor but instead recruits other inhibitory factors through CNOT1 [46].

Box 2. Mechanism of Translation Initiation in Eukaryotes

Translation proceeds in three steps: initiation, elongation, and termination. Among them the initiation step is the rate-limiting step in the eukaryotic canonical translation. The initiation step of translation is composed of serial reactions that place the P (peptidyl) site of the ribosome on the AUG start codon of an mRNA [144,145]. First, the 43S preinitiation complex (43S PIC), containing 40S small ribosomal subunit, eukaryotic initiation factor 1 (eIF1), eIF1A, eIF2–GTP initiator tRNA, eIF3, and eIF5 is recruited to the m^7G-cap structure at the 5′ end of mRNA through the interaction between eIF3 and the cap-binding complex eIF4F. eIF4F contains the cap-binding protein eIF4E, the large scaffold protein eIF4G, and the DEAD box RNA helicase eIF4A. PABP, which binds to the 3′ poly(A) tail of mRNAs, stimulates ribosome recruitment by stabilizing binding of eIF4F to the cap structure through interaction with eIF4G. The 43S PIC then scans the 5′ UTR of mRNA to the downstream AUG codon. eIF4A facilitates both the ribosomal recruitment and the scanning step in an ATP-dependent manner, supposedly by unwinding secondary structures in the 5′ UTR. After the initiator tRNA in the 48S complex recognizes the AUG codon, eIFs are released and the 60S ribosomal subunit joins the complex on the start codon to form the functional 80S complex (Figure I).

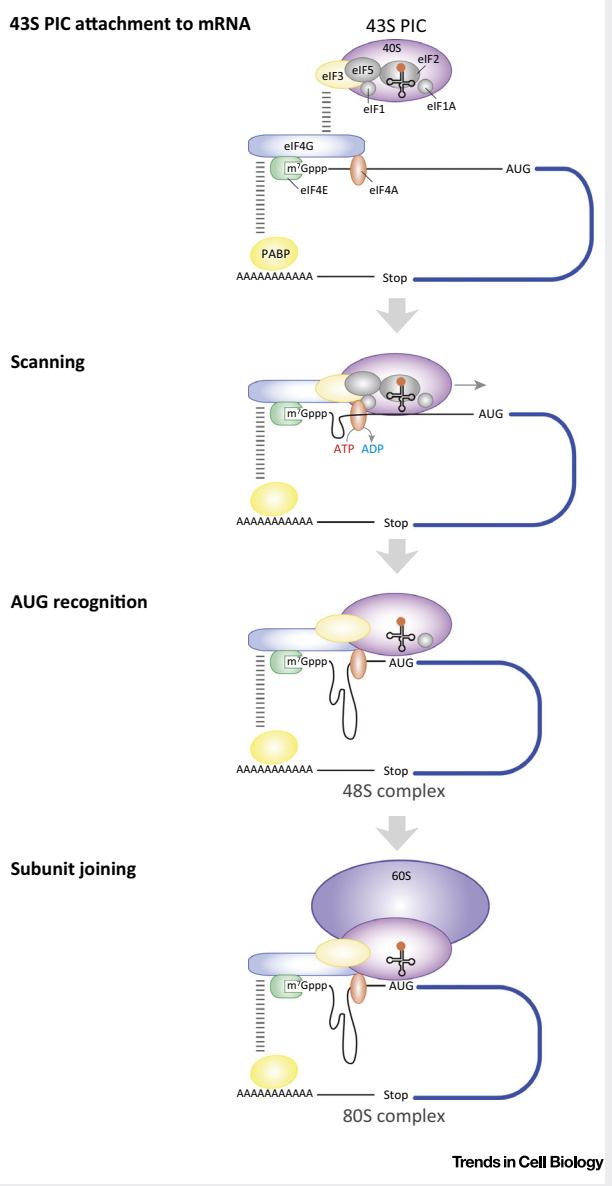

Figure I. A Model of Eukaryotic Translation Initiation.

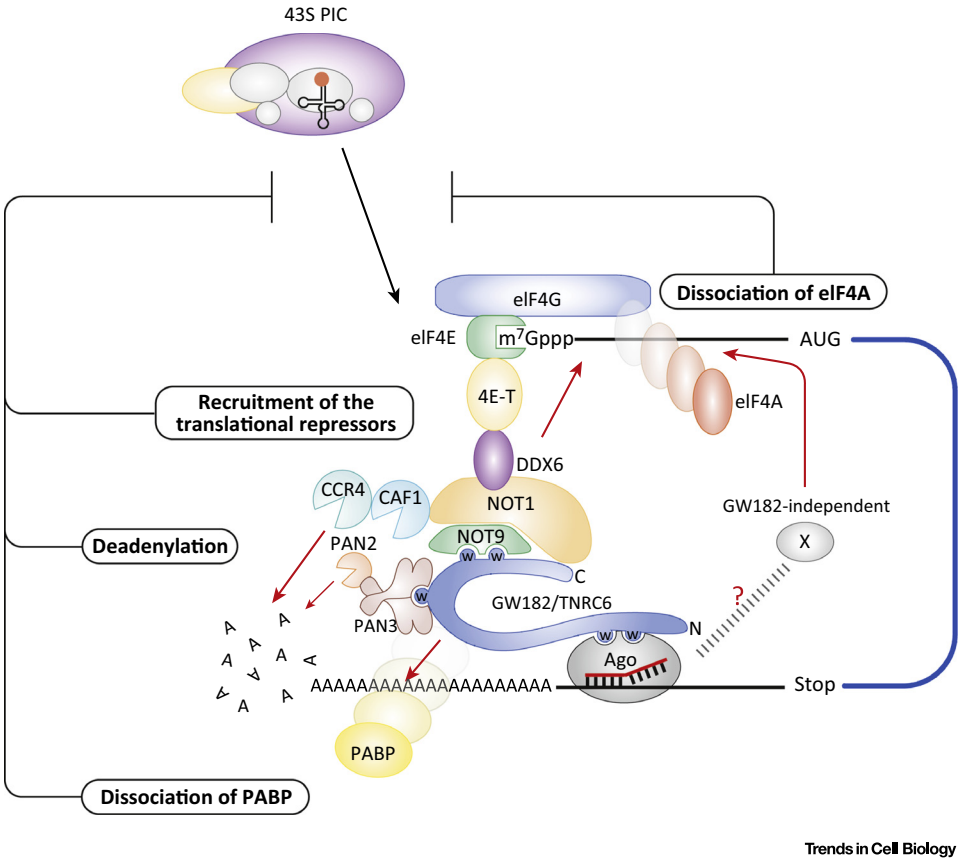

Figure 3. miRNA-Mediated Translational Repression in Animals. Through the CCR4–NOT complex, GW182 recruits downstream translational repressors (e.g., DDX6 or 4E-T) onto target mRNAs, but it remains unclear which step(s) in translation is blocked by them. miRNAs can also repress translation via displacement of the ATP-dependent RNA helicase eIF4A from the cap-binding complex eIF4F, which blocks the 43S PIC recruitment or ribosomal scanning *in vitro*. This mode of miRNA action does not require GW182 in *Drosophila*. Deadenylation as well as displacement of poly(A)-binding protein (PABP) through GW182 and CCR4–NOT also contribute to the overall miRNA-mediated translational repression.

It was proposed that, in mammalian cells, eIF4AII, but not eIF4AI, is specifically recruited onto target mRNAs through CNOT1–MIF4G domain (middle domain of eIF4G) and interferes with translation initiation mediated by eIF4G and eIF4AI [83]. However, this model was challenged by structural and biochemical studies, which showed that CNOT1–MIF4G domain does not interact with either eIF4AI or eIF4AII, but specifically interacts with the DEAD-box RNA helicase DDX6 [45,46,61]. DDX6 is known as a decapping activator but can also function in translational repression at the initiation step and/or at the elongation step [100,101]. In mouse embryonic stem cells, DDX6 was shown to be recruited onto miRNA targets through its interaction with the mammalian hyperplastic discs protein EDD, which binds to GW182 through the PABC domain [44]. Recent studies suggest that the eIF4E-binding protein 4E-T is recruited onto the CCR4–NOT complex through DDX6, PATL1, or LSM14 [55,84]. 4E-T represses general translation through the eIF4E-binding domain [84,102]. However, tethering of 4E-T repressed translation of the target mRNA in a manner independent of its 4E-binding domain in human cell lines and *Xenopus* oocyte [84,102]. In addition, a reporter assay using various internal ribosomal entry sites (IRESes) suggested that 4E-T targets eIF4F components (except for eIF4E) or eIF4B [84,102]. Given that depletion of this factor partially attenuates RNA silencing in HeLa cells and *Drosophila* S2 cells, 4E-T may promote miRNA-mediated silencing in diverse organisms [27,102].

It should be noted that mechanistic studies of GW182-dependent repression have often been conducted by direct tethering of GW182 or downstream components [20–23,25,33–35,46,47,49,86–91], which can potentially cause unnaturally high concentrations of the tethered protein(s) locally on the reporter mRNAs. Indeed, in *Drosophila* S2 cells, depletion of GW182 almost completely abolished deadenylation and subsequent mRNA decay, but only partially compromised silencing at the protein level [20,47,103]. Thus, although it is clear that GW182 is essential for deadenylation and mRNA decay by miRNAs, there are likely both GW182-dependent and GW182-independent mechanisms for translational repression.

miRNA-Mediated Dissociation of eIF4A

To date, many reports have shown that mRNAs lacking the 5′ m^7G-cap structure but instead bearing the nonphysiological A-cap structure or IRESes, which bypass the requirement for the 5′ m^7G-cap as well as one or more eIFs for translation initiation, are refractory to miRNA-mediated silencing [47,56,94,104–108]. Supplementation or depletion of initiation factors attenuated miRNA-mediated repression [83,85,86,106,109]. These results strongly suggest that eIF4F components are the molecular targets of miRNA-mediated translational repression. However, it had been unclear which eIF4F component is removed from the 5′ m^7G-cap structure by miRNA targeting. Recently, two different methods that can monitor the effect of miRNAs on the formation of initiation complex revealed that miRNAs displace eIF4A from the target mRNAs [85,86]. In one method, UV crosslinking with cap-labeled target mRNAs specifically detected eIF4E and eIF4A, which bind very close to the 5′ terminus of target mRNA [86]. Importantly, crosslinking of eIF4A was abolished in the presence of cognate miRNAs, whereas no change was observed in crosslinking of eIF4E. Furthermore, addition of recombinant eIF4A attenuated miRNA-mediated translational repression and eIF4A dissociation by miRNAs [86]. Neither miRNA-mediated translational repression nor the displacement of eIF4A from target mRNAs was impacted by depletion of GW182 in *Drosophila* S2 cell lysate, suggesting that in flies miRNAs can dissociate eIF4A from the cap-binding complex independently of GW182 [86]. In the other method, a pull-down assay of the mRNA–protein complex in a mammalian cell-free system demonstrated that both eIF4AI and eIF4AII were specifically released from the cap-binding complex eIF4F in the presence of miRNAs [85]. Taken together, these results suggest that animal miRNAs have the ability to dissociate eIF4A from the target mRNAs, which leads to inhibition of ribosome binding and/or scanning. Future studies are warranted to determine what factor mediates the dissociation of eIF4A from the target mRNA.

miRNA-Mediated Translational Repression in Plants

Evidence for Translational Silencing and Introduction of Factors Involved

Initially, plant miRNAs were thought to silence target genes only through endonucleolytic activity of Argonaute proteins. Contrary to animal miRNAs, however, which silence many partially complementary target mRNAs without cleavage, each plant miRNA has a few specific target mRNAs with fully or nearly fully complementary sequence [11]. Indeed, plant miRNA-mediated target cleavage has been validated with Northern blotting, 5′-Rapid Amplification of cDNA Ends (RACE) analyses and other assays in many reports [11,110,111]. Strikingly, plants lack GW182 homolog proteins, the core component for miRNA-mediated translational repression and mRNA decay in animals. However, a number of reports have shown that plant miRNAs can lead to a disproportional decrease in the protein accumulation versus mRNA accumulation of their targets, suggesting that plant miRNAs can induce translational repression in addition to target cleavage [112–116]. In 2008, the first genetic evidence for miRNA-mediated translational repression in plants was provided [117]. In a forward-genetic screen for *Arabidopsis* mutants defective in RNA silencing, Brodersen *et al.* found that the activity of the microtubule-severing enzyme KATANIN (KTN1) is required for translational repression. Brodersen *et al.* also demonstrated that the decapping activator Ge-1 homolog VARICOSE (VCS), AGO1, and AGO10 are

involved in translational repression [117]. Another factor implicated in translational repression in *Arabidopsis* is SUO, a large protein with N-terminal bromo-adjacent homology and transcription elongation factor S-II domains [118]. A mutation in *SUO* caused derepression of miR156/157 target reporters only at the protein level but not at the mRNA level. Although SUO possesses two GW repeats at the C-terminal region, it is unclear whether this protein directly binds to AGO proteins as the AGO–GW182 interaction in animals [118]. Recently, ALTERED MERISTEM PROGRAM1 (AMP1) and its homolog, LIKE AMP1 (LAMP1), were identified as new factors involved in miRNA-mediated translational repression in plants [119]. They are integral membrane proteins associated with endoplasmic reticulum (ER) and are coimmunoprecipitated with AGO1. In *amp1 lamp1* double mutant, miRNA target mRNAs were derepressed and exhibited enhanced association with the membrane-bound polysome but not with the total polysome, suggesting that translational repression occurs on the ER membrane [119]. Given that VCS and SUO localize to P-bodies [118,120], where mRNA degradation factors are concentrated, and KTN1 associates with microtubule [121], multiple dynamic cellular processes may participate in miRNA-mediated translational repression (Figure 4). However, it remains unclear what molecular roles these factors play and how they are interrelated in repression of protein synthesis in the plant miRNA pathway.

Proposed Mechanisms of miRNA-Mediated Translational Repression

How do plant miRNAs repress translation of target mRNAs? Given that the ribosome density on the target mRNAs is decreased by miRNAs specifically on the ER membrane, miRNAs may inhibit translation initiation by sequestering target mRNAs to the translationally inactive subcellular compartment [119]. By contrast, a significant amount of miRNAs is associated with polysome, implying that plant miRNAs function also at a post-initiation step [122].

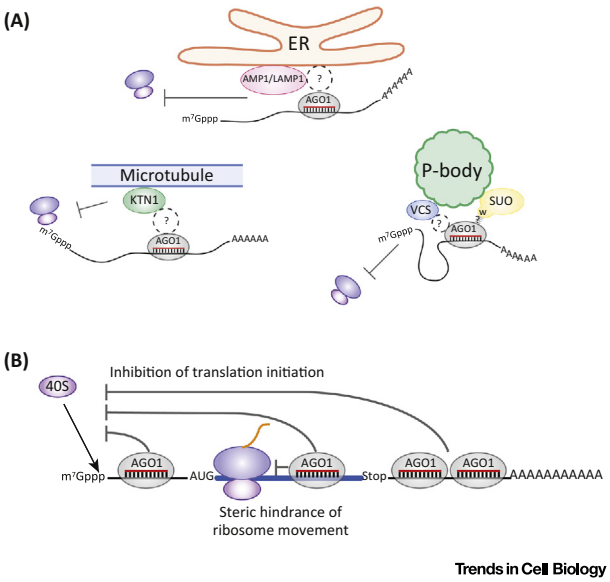

Figure 4. miRNA-Mediated Translational Repression in Plants. (A) Effector proteins for miRNA-mediated translational repression in plants. Mutations in *KTN1*, *VCS*, *SUO*, *AMP1/LAMP1*, or *AGO1* caused derepression of miRNA target genes at the protein level. KTN1 localizes with microtubules, VSC and SUO in P-bodies, and AMP1/LAMP1 on ER membranes. (B) Mechanisms of miRNA-mediated translational repression in plants. A plant cell-free system showed that catalytic mutant of *Arabidopsis* AGO1–RISC can repress translation initiation without inducing deadenylation and mRNA decay. When bound to the ORF, plant AGO1–RISC can block movement of ribosomes. Multiple target sites are needed for efficient translational repression from the 3′ UTR. Abbreviations: AMP1, ALTERED MERISTEM PROGRAM1; LAMP1, LIKE AMP1; RISC, RNA-induced silencing complex; AGO, Argonaute; ER, endoplasmic reticulum; ORF, open reading frame; UTR, untranslated region.

Cell-free assay systems are an invaluable tool for investigating molecular mechanisms of RNA silencing [123]. Many aspects of plant RNA silencing, for example, target cleavage and RNA-dependent RNA polymerization, have been recapitulated in lysates derived from *Arabidopsis* inflorescence tissue and cultured cells as well as in wheat germ extract [71,124]. Recently, a cell-free system for plant RISC assembly was developed using evacuolated tobacco protoplast [125]. In this system, *in vitro* translated plant AGOs can be programmed with synthetic miRNA duplexes to produce RISCs with the desired combination of an AGO protein and an miRNA guide sequence [125–128]. By using this system, the mechanism of miRNA-mediated translational repression was studied *in vitro* (Figure 4) [64]. The results showed that: (i) plant AGO1–RISC has the ability to inhibit translation initiation without inducing deadenylation and mRNA decay; (ii) when bound to the 5′ untranslated region (UTR) or open reading frame (ORF), catalytic mutant plant AGO1–RISC can block recruitment or movement of ribosomes; and (iii) plant AGO1–RISC requires more extensive base pairing to the target site for recognition and translational repression compared with animal RISCs [64]. These observations revealed that plant miRNAs can induce translational repression but the mode of action differs from animal miRNAs. The above *in vitro* study demonstrated that to repress translation of target mRNA bearing an miRNA-binding site within the ORF, plant miRNAs require near-perfect base pairing, which normally triggers rapid endonucleolytic target cleavage [64,71]. Currently, it remains unclear how translationally repressed target mRNAs evade endonucleolytic cleavage by AGO1–RISC *in vivo*.

Relative Contributions of Translational Repression and mRNA Decay in the Overall Silencing Effect

In Animals

Although genetic, biochemical, and structural analyses have uncovered the intricate mechanisms of miRNA-mediated silencing, the relative contributions of mRNA decay and translational repression to the overall silencing remain under debate. Of course the relative contributions of the two pathways should vary depending on the concentration of RISC components, the sequence of target mRNAs (e.g., number or position of miRNA-binding sites), or cell types, but recent genome-wide analyses have suggested that translational repression generally precedes mRNA decay, although mRNA decay contributes most substantially to overall silencing.

Comparing changes at the mRNA and protein levels of potential miRNA targets by using SILAC (Stable Isotope Labeling using Amino acids in cell Culture) or pulsed SILAC coupled with mass spectrometry after expression or deletion of individual mammalian miRNAs revealed that protein changes generally correlate with mRNA changes [129,130]. More sensitive methods, polysome profiling and ribosomal profiling, also found that mRNA decay can explain the majority of miRNA-mediated gene silencing [80,82,131]. However, the above studies were performed at the steady state and did not evaluate miRNA function at early time points upon miRNA expression. By contrast, *in vitro* studies demonstrated that translational repression preceded deadenylation [37,85]. Kinetic studies using reporter mRNAs were performed in *Drosophila* S2 cells and HeLa cells, and confirmed that translational repression occurs before deadenylation and mRNA decay [63,132]. In zebrafish embryos, miR-430, the expression of which peaks at 4 h post-fertilization (4 hpf), promotes deadenylation and mRNA decay of hundreds of target genes [18]. By monitoring the impact of miR-430 on ribosome occupancy of endogenous mRNAs, it was found that miR-430 reduces the number of ribosomes on target mRNAs in early embryos at 4 hpf before the completion of deadenylation and mRNA decay, supporting the model that miRNAs induce translational repression before mRNA decay *in vivo* [81].

Recently, a large-scale poly(A) tail length profiling method was developed [62,133], which was used for measuring the poly(A) tail length of endogenous RNAs isolated from various samples,

including mammalian cells and zebrafish embryos. The tail lengths correlated well with translational efficiency in the zebrafish early embryonic stage (2–4 hpf), but surprisingly no coupling was observed in the later stage (6 hpf) nor in other mammalian cells [62]. When injected into zebrafish embryos, miR-155 and miR-132, which are normally not expressed at the early embryonic stage, shortened poly(A) length of target mRNAs and simultaneously decreased translation efficiency at 4 hpf. In contrast to this, in gastrulating embryos collected at 6 hpf, no correlation was observed between the poly(A) length and translation efficiency. Rather, the target RNAs with shortened poly(A) tails tended to be degraded [62]. The authors concluded that a special condition in early zebrafish embryos, where poly(A) tail length and translation efficiency are strongly correlated and short poly(A) tailed mRNAs are stable, enables miRNA-mediated deadenylation to induce translational repression without mRNA decay [62]. Thus, the results obtained from the early embryonic stage may not provide general insights into the relative contributions of translational repression and mRNA decay in other animal cells.

To elucidate the relative contribution and dynamics of miRNA-mediated mRNA decay and translational repression for endogenous mRNAs in common cell types, ribosomal profiling was conducted with various mammalian cell types after expression or deletion of individual mammalian miRNAs [82]. They demonstrated that although translational repression occurs rapidly, its contribution to the overall silencing is relatively weak by the time substantial silencing ensues via mRNA decay [82]. A remaining question is whether or not the initial translational repression is required for efficient mRNA decay afterwards; it is well known that miRNAs can mediate deadenylation and mRNA decay in the absence of translation [15,18,24,25,37,97], but the kinetics of deadenylation and mRNA decay need to be directly compared between actively translated mRNAs and translationally repressed mRNAs in the future.

Decay is not always the fate of target mRNAs. Several reports suggest that under specific contexts, translationally repressed targets can be reactivated by degradation of a RISC component or dissociation of RISC from target mRNAs in mammals [134–136]. Future studies focused on the specific tissue and cells are needed to understand the physiological relevance of the miRNA-mediated translational repression.

In Plants

Because plant miRNAs do not promote deadenylation and directly mediate mRNA decay only through endonucleolytic activity of AGO1, mutations in the catalytic core of AGO1 enable investigation of the impact of the two modes of miRNA action – target cleavage and translational repression – on plant development. In *A. thaliana*, catalytic mutant of AGO1 cannot restore the morphological defects caused by *ago1* mutation [137]. This study clearly indicates that the endonucleolytic activity of AGO1 is essential for plant development. By contrast, the *amp1 lamp1* double mutant or the *suo* mutant, which compromise the miRNA-mediated translational repression but not the target cleavage activity, exhibited pleiotropic morphological defects [118,119], which are also seen by mutations in the miRNA biogenesis pathway. The above genetic experiments suggest that the two modes of silencing have important roles in plant development. However, it cannot be excluded that the morphological defects in the *amp1 lamp1* double mutant or *suo* mutant are independent of RNA silencing, because a mutation in KTN1, which is another mediator of miRNA-mediated translational repression in *Arabidopsis*, showed only mild developmental defects compared with that of the slicer deficient *ago1* mutant [117].

Sequencing the 5′ ends of noncapped polyadenylated mRNAs isolated from *A. thaliana* revealed that mRNAs with highly complementary target sites were cleaved by plant miRNAs at the position facing nucleotides 10 and 11 of miRNAs [110,138]. By contrast, ribosomal profiling in *Arabidopsis* showed that translation efficiency of miRNA targets was significantly lower than that of non-miRNA targets [139]. These results suggest that both endonucleolytic cleavage and

translational repression together augment the overall silencing efficiency. However, to precisely estimate the relative contribution of the two modes of miRNA-mediated repression for endogenous mRNAs, ribosomal profiling or polysome profiling after expression or depletion of individual miRNAs should be performed.

Concluding Remarks

Over the past years, much progress has been achieved in understanding the effector step of animal and plant miRNA pathways. Accordingly, our understanding of the mechanism of miRNA-mediated mRNA decay in animals and target cleavage in plants has significantly matured. In contrast to this, there are many unanswered questions regarding the mechanism of miRNA-mediated translational repression (see Outstanding Questions). In animals, it remains unclear at which step(s) of translation is blocked by GW182 and its associating factors (e.g., DDX6 and 4E-T). Moreover, it is also unknown what factors mediate dissociation of eIF4A from the cap-binding complex eIF4F. In plants, future research is needed to address how KTN1, SUO, AMP1/LAMP1, and VCS1 enhance miRNA-mediated translational repression. The most crucial uncertainty is how target mRNAs evade endonucleotytic cleavage by plant AGOs to be repressed at the translational level.

Large-scale analyses performed over the past decade have provided a macroscopic view in the understanding of the miRNA pathway in animals. These studies suggested that although translational repression generally occurs first, mRNA decay is the dominant effect of mammalian miRNAs. However, the physiological importance of the two modes of miRNA action is still unknown. To tackle this problem, it is needed to separate and/or modulate these two silencing modes *in vivo* and precise understanding of the molecular mechanism of miRNA-mediated translational repression should give us a hint. In plants, although it is clear that the endonucleolytic activity of AGO1 is essential for development, biological relevance of translational repression by miRNAs remains unclear. Future studies should determine why miRNAs mediate so many different molecular mechanisms to silence their target genes and why animals and plants have evolved to make use of miRNAs in distinct ways (see Outstanding Questions).

Outstanding Questions

How do GW182 and its associating factors repress translation? Although DDX6 and 4E-T are good candidates as mediators for translational repression downstream of GW182, their mode of action needs to be further characterized.

How is eIF4A dissociated from miRNA targets? In *Drosophila*, it was suggested that miRNAs can displace eIF4A from the cap-binding complex eIF4F independently of GW182, but what factor mediates eIF4A dissociation remains unknown.

How do target mRNAs evade endonucleolytic cleavage by plant miRNAs to be repressed at the translational level? Because target binding and translational repression require extensive miRNA-target complementarity, which usually promotes rapid degradation *in vitro*, unknown mechanisms must suppress the cleavage reaction.

Does translational repression promote miRNA-mediated mRNA decay? Although it is clear that deadenylation occurs independently of translation, it should be determined if miRNA-mediated translational repression facilitates decapping and subsequent mRNA decay.

What is the physiological role of miRNA-mediated translational repression? Although a few reports show that translationally repressed targets can be reactivated, the physiological importance of miRNA-mediated translational repression is still obscure in both animals and plants.

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