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The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC

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Since their discovery almost two decades ago, microRNAs (miRNAs) have been shown to function by post-transcriptionally regulating protein accumulation. Understanding how miRNAs silence targeted mRNAs has been the focus of intensive research. Multiple models have been proposed, with few mechanistic details having been worked out. However, the past few years have witnessed a quantum leap forward in our understanding of the molecular mechanics of miRNA-mediated gene silencing. In this review we describe recent discoveries, with an emphasis on how miRISC post-transcriptionally controls gene expression by inhibiting translation and/or initiating mRNA decay, and how *trans*-acting factors control miRNA action.

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MicroRNAs (miRNAs) are evolutionarily conserved, small (~21 nucleotides) noncoding RNAs that are encoded within the genomes of almost all eukaryotes, from plants to mammals. Organisms express hundreds of miRNAs, which are integral to almost all known biological processes. In general, miRNAs, especially in animals, post-transcriptionally regulate protein synthesis by base pairing to partially complementary sequences in the 3′ untranslated regions (UTRs) of target mRNAs[1–3]. miRNAs mediate mRNA repression by recruiting the miRNA-induced silencing complex (miRISC), a ribonucleoprotein complex, to target mRNAs (**Fig. 1a**). The core of the miRISC contains a miRNA-loaded Argonaute protein and a glycine-tryptophan repeat–containing protein of 182 kDa (GW182) (**Fig. 1a**,**b**). All nuclear transcribed eukaryotic mRNAs contain a 5′ m^7GpppN structure (where N is any nucleotide) termed the 5′ cap, which interacts with the eukaryotic translation initiation factor eIF4E (reviewed in ref. 4). eIF4E, along with other eIFs, in turn recruits ribosomes to initiate mRNA translation. The miRISC has been reported to inhibit cap-dependent translation at both initiation (by interfering with ribosome recruitment)[5–9] and post-initiation steps[10–13] (**Fig. 1a**, top). However, the molecular details of these mechanisms are unclear. The miRISC also engenders deadenylation and subsequent decapping and decay of target mRNAs (**Fig. 1a**, bottom)[14–18]. Exactly how the miRISC elicits these events, the order in which they operate and the proteins that the miRISC recruits remain controversial topics. Can the miRISC inhibit translation in the absence of mRNA decay, or is mRNA destabilization always the end result of gene silencing? Moreover, does miRNA-mediated mRNA deadenylation precede translational repression or vice versa, or are these two events mechanistically coupled? Furthermore, what proteins does the miRISC recruit to effect silencing? In this review, we address these topics and highlight important new advances and controversies in understanding the mechanisms of miRNA-mediated gene silencing.

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The miRNA-induced silencing complex

miRNAs act as the nucleic acid core of miRISC, which silences target mRNAs[19]. Thus, although the miRNA sequence dictates which mRNAs it potentially interacts with within a given transcriptome, it is the protein components of the miRISC that execute the silencing of target mRNAs. The core components of the mammalian miRISC consists of one of four Argonaute proteins (AGO1–4) and Argonaute-bound GW182 (**Fig. 1a**,**b**).

AGO proteins display a bilobal architecture, which consists of four evolutionarily conserved domains: the N-terminal and Piwi-Argonaute-Zwilli (PAZ) domains in one lobe and the MID and PIWI domains in the second[20] (**Fig. 1b**). AGOs are essential for miRNA-mediated gene silencing, inasmuch as depletion of AGOs from mammalian and insect cells impairs miRNA-mediated silencing[17,21]. Strikingly, AGO proteins repress protein synthesis when artificially tethered to reporter mRNA 3′ UTRs lacking miRNA-target sites[22]. Thus, miRNA-mRNA annealing is dispensable for the activity of miRNA-loaded AGO proteins but determines which endogenous mRNAs AGO proteins interact with. Several lines of evidence suggest that without their interacting partner GW182, AGO proteins fail to silence target mRNAs. Depleting GW182 proteins from human and *Drosophila melanogaster* cells in culture results in reduced miRNA-mediated silencing of reporter mRNAs to which AGO proteins are artificially tethered[23,24]. Blocking GW182-AGO interaction also impairs miRNA-mediated silencing *in vivo* and *in vitro*[17,24–27]. Mammals encode three GW182 paralogs, termed trinucleotide repeat-containing protein (TNRC) 6A, B and C, whereas insects possess a single GW182 protein (for example, dGW182 or Gawky in *D. melanogaster*), and the nematode *Caenorhabditis elegans* contains two GW182 proteins, AIN-1 and AIN-2 (reviewed in ref. 28). The N-terminal segment of animal and insect GW182 proteins contains a multitude of GW, WG or GWG repeats, many of which function as binding platforms that interact with AGO proteins (**Fig. 1b**)[17,24,27,29,30]. Additional GW repeats in the N-terminal half of dGW182 are also implicated in repression, via recruitment of the CCR4–NOT deadenylase complex (see below)[31,32]. The N-terminal GW repeat–containing region is followed by a putative ubiquitin-associated (UBA) domain and a glutamine-rich (Q-rich) domain,

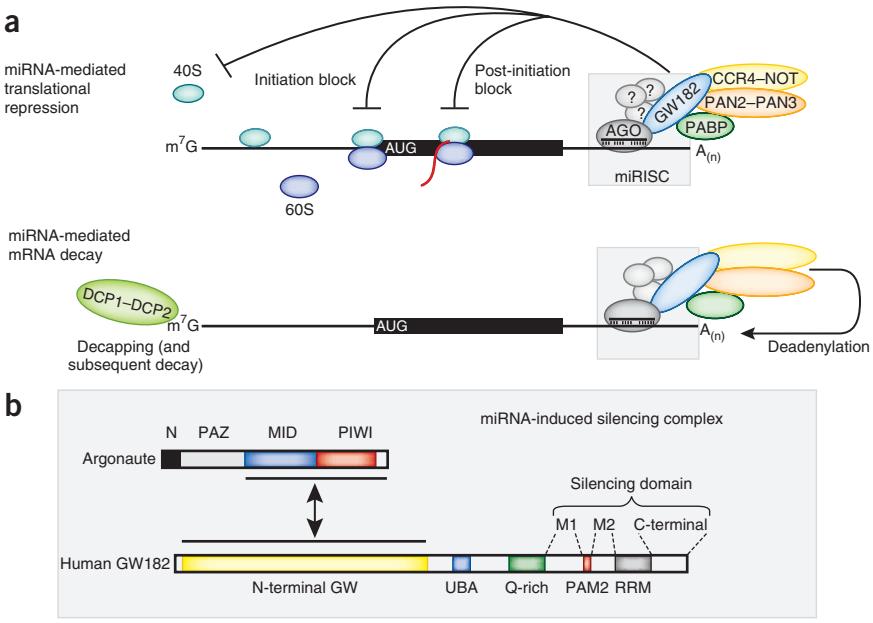

Figure 1 miRISC-mediated gene silencing. (**a**) Schematic diagram of miRNA-mediated translational repression and mRNA decay. Translational repression: the miRISC inhibits translation initiation by interfering with eIF4E-cap recognition and 40S small ribosomal subunit recruitment or by antagonizing 60S subunit joining and preventing 80S ribosomal complex formation. The miRISC might inhibit translation at post-initiation steps by inhibiting ribosome elongation. mRNA decay: the miRISC interacts with the CCR4–NOT and PAN2–PAN3 deadenylase complexes to facilitate deadenylation of the poly(A) tail (indicated by $A_{(n)}$). Following deadenylation, the 5-terminal cap (m^7G) is removed by the decapping the DCP1–DCP2 complex, and mRNA decay is effected by the Xrn1 5′–3′ exonuclease. (**b**) Domain structures of miRISC components Argonaute and GW182. AGO proteins contain N-terminal (N), PAZ, MID and PIWI domains. Human and *D. melanogaster* GW182 proteins share similar domain organizations. The N-terminal region of GW182, containing GW repeats, interacts with AGO proteins. This region, including GW-rich, UBA and Q-rich domains, is responsible for targeting GW182 proteins to P bodies. The C-terminal part of mammalian and *D. melanogaster* proteins contains a major effector domain called the silencing domain, comprised of M1 and M2 regions, PAM2 and an RRM, and the C-terminal domain.

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which is responsible for localizing GW182 to cytoplasmic foci called processing (P) bodies or GW bodies[23].

The GW182 C-terminal region, termed the 'silencing domain', engenders robust repression (**Fig. 1b**). The silencing domain is a bipartite region, which is predicted to be unstructured. It is divided into middle (Mid) and C-terminal subdomains that flank an RNA-recognition motif (RRM), which is predicted not to bind RNA, owing to the presence of an additional C-terminal alpha helix[33] (**Figs. 1b** and **2a**). The Mid domain is further subdivided into the M1 and M2 regions that flank a poly(A) binding protein (PABP)-interacting motif 2 (PAM2) (**Fig. 1b**). The GW182 protein counterparts in *C. elegans*, AIN-1 and AIN-2, also contain GW repeats that bind AGOs but lack identifiable PAM2 and RRM domains[17]. Human, *D. melanogaster* and zebrafish GW182 silencing domains confer strong repression *in vivo* when artificially tethered to reporter mRNAs[34–37]. Moreover, complementation assays in HeLa and *D. melanogaster* Schneider 2 (S2) cells, wherein cells were depleted of endogenous GW182 proteins via RNA interference and rescued with wild-type or mutant GW182 proteins, demonstrated that deleting the silencing domain from full-length GW182 proteins resulted in severely impaired repression of miRNA-targeted mRNAs[38,39]. Furthermore, studies carried out using a mammalian *in vitro* reconstituted system that recapitulates miRNA-mediated silencing showed that the tethered TNRC6C silencing domain alone promoted efficient deadenylation of reporter mRNAs[40,41].

In addition to its C-terminal silencing domain, dGW182 contains an N-terminal effector domain (NED), adjacent to the dGW182 AGO-binding region (**Fig. 2b**)[31,32,35]. The dGW182 NED silences mRNA in *D. melanogaster* S2 cells when artificially tethered to reporter mRNAs[31,32]. Also, an N-terminal fragment of dGW182, which binds AGO and contains the NED, silenced miRNA-targeted mRNAs in S2 cells[32]. In contrast to these results, another study found that dGW182 lacking the C-terminal silencing domain failed to repress miRNA-targeted mRNAs in S2 cells, despite the presence of the NED[38]. Furthermore, overexpressing an N-terminal fragment of dGW182, which contains both the AGO-interacting domain and the NED, suppressed miRNA-mediated silencing *in vivo*[23,32]. The dGW182 NED can interact with the CCR4–NOT deadenylase complex, which could account for its repression of reporter mRNAs[31] (see below for further details). Thus, it is conceivable that the context of the NED (that is, in a truncated GW182 fragment or full-length protein) dictates its role in repressing target mRNAs. However, it remains to be established whether the dGW182 NED interacts with the CCR4–NOT complex in the context of full-length GW182 protein.

GW182-interacting proteins and miRNA-mediated silencing

GW182 proteins were first linked to miRNA-mediated gene silencing several years ago[18,42–44]. However, molecular insight into how GW182 proteins facilitate miRNA repression has only come to light in the past few years. In addition to directly interacting with AGO proteins, animal and insect GW182 proteins serve as molecular platforms that bind a multitude of silencing effectors[31,38–41,45,46].

PABP. The PABP (**Fig. 2a**) binds the mRNA 3′ poly(A) tail and a variety of proteins that control both mRNA translation and mRNA metabolism (reviewed in ref. 47). PABP interactions with eIF4G and the PABP-interacting protein 1 (PAIP1) stimulate translation initiation through mRNA circularization, whereas PABP association with PAIP2 inhibits translation by displacing PABP from the mRNA poly(A) tail. PABP also interacts with the eukaryotic release factor 3 (eRF3), which functions in translation termination. PABP binding to the transducer of ERBB2 (Tob) or the poly(A) nuclease (PAN) complex helps regulate mRNA deadenylation and decay[48,49].

More recently, GW182 proteins have been shown to directly bind PABP via a stretch of amino acids within its C-terminal silencing domain, originally called domain of unknown function (DUF) and more recently renamed as a bona fide PAM2 (ref. 26) (**Fig. 2a**). The crystal structure of human TNRC6C PAM2 peptide in complex with a peptide from the PABP C-terminal (PABC) domain (also known as MLLE, which refers to the conserved KITGMLLE binding sequence[50])

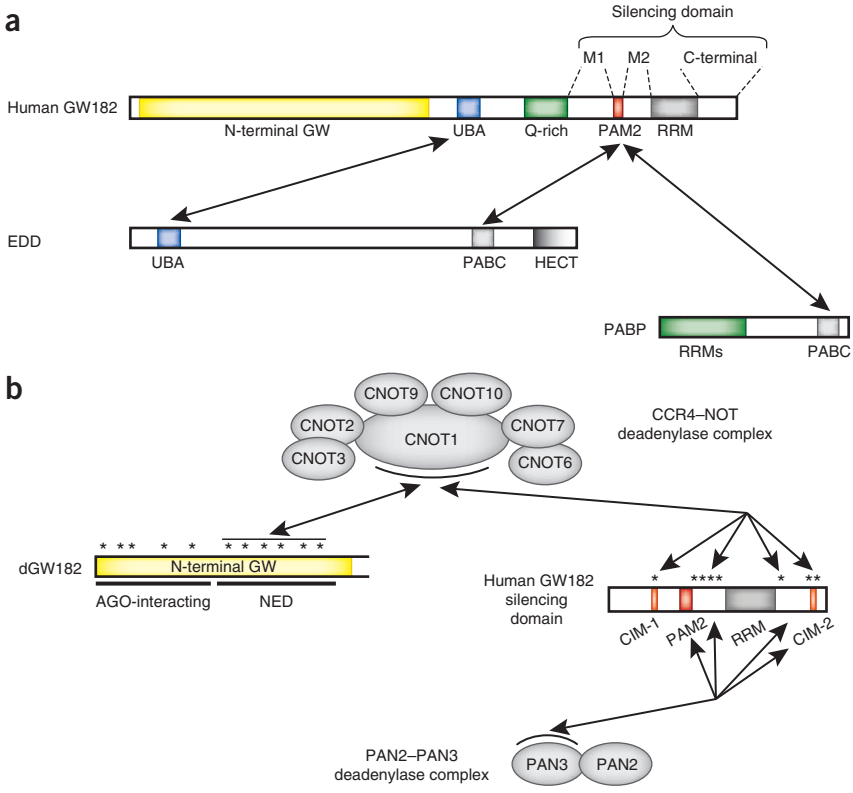

Figure 2 Schematic diagram of GW182 and its interaction partners. (**a**) Argonaute proteins interact with the N termini of GW182 proteins (see **Fig. 1b** for details). Mammalian and *D. melanogaster* PABP binds directly to the silencing domain of human GW182 proteins via PABC. Mammalian EDD interacts with the human GW182 PAM2 motif via its PABC domain and through EDD–GW182 UBA domain dimerization. (**b**) The CNOT1 subunit of the CCR4–NOT complex binds directly to the silencing domain of human and *D. melanogaster* GW182 proteins. CIM-1 and CIM-2, as well as multiple tryptophan residues (denoted by asterisks) in the silencing domain M2 and C-terminal regions are reported to interact with the CCR4–NOT complex. Evidence exists that the N-terminal domain of dGW182 also induces silencing that is mediated by GW repeats (denoted by asterisks) present in its NED, which interact with the CCR4–NOT complex. The PAN3 subunit of the PAN2–PAN3 complex has been reported to directly bind the M2 region and C-terminal domain of the GW182 silencing domain, as well as to the PABP. HECT, homologous to E6-AP C terminus.

demonstrates that mammalian GW182 proteins interact with PABP in a manner similar to that of other PABP-interacting proteins, such as PAIP1, PAIP2, eRF3 and the deadenylase-associated protein Tob[41,51]. The GW182 PAM2 motif is evolutionarily conserved in animals and insects, and dGW182 also directly binds PABP via its PAM2 motif[38]. However, in contrast to human GW182 proteins, dGW182 preferentially interacts, albeit indirectly, via the M2 and C-terminal sequences in the dGW182 silencing domain with the PABP N-terminal RRMs[38,39]. Interestingly, the *C. elegans* PABP PAB-1 interacts with the GW182 family protein AIN-1, even though AIN-1 lacks an identifiable PAM2 motif[46]. Using a heterologous system, in which tagged *C. elegans* proteins were overexpressed in *D. melanogaster* S2 cells and subsequently immunoprecipitated, Kuzuoglu-Ozturk and colleagues found that the PAB-1 N-terminal RRMs are responsible for interacting with AIN-1 (ref. 46). However, in contrast to an earlier report that showed PAB-1 immunoprecipitating GFP-tagged AIN-2 (ref. 52), they were unable to detect an interaction between PAB-1 and AIN-2 (ref. 46).

How could the GW182 interaction with PABP silence miRNA-targeted mRNAs? One proposed model posits that GW182 binding to PABP inhibits mRNA translation by interfering with PABP-eIF4G association and mRNA circularization[26,39]. In addition, the GW182-PABP interaction could enhance miRNA-mediated deadenylation by juxtaposing the miRISC-recruited deadenylation machineries (see below) next to the poly(A) tail[26]. Nevertheless, although PABP can directly interact with GW182, a role for this protein-protein interaction in miRNA-mediated gene silencing has been challenged. In one study, deleting the PAM2 motif from TNRC6B failed to rescue silencing in both HeLa cells and *D. melanogaster* S2 cells in complementation assays[38]. However, another study using complementation assays reported that mutating the PAM2 motif in dGW182 and human TNRC6C had little or no detectable impact on silencing in *D. melanogaster* S2 cells (both dGW182 and TNRC6C) and in HeLa cells (TNRC6C)[31]. Also, deleting the PAM2 motif in a tethered C-terminal silencing domain fragment had little impact on silencing in cell cultures[34].

Whether PABP plays a role in miRNA-mediated silencing independent of its contact with GW182 is currently unclear. Modulating PABP levels has been reported to affect miRNA-mediated silencing in HEK-293 cells, as PABP overexpression led to partial derepression of miRNA silencing[53], in part through antagonizing deadenylation of target mRNAs. Furthermore, overexpressing PAIP2, which should decrease the amount of available PABP, was found to increase miRNA-mediated silencing[53]. Depleting PABP from zebrafish embryos using antisense morpholino oligomers directed against PABP mRNA had no detectable impact on miRNA-mediated silencing of miR-430–targeted mRNAs, although optimal silencing in zebrafish embryos requires a GW182 PAM2 motif[37].

Mammalian and *D. melanogaster* cell-free systems have also generated contradictory results vis-à-vis the role of PABP in miRNA-mediated silencing. Depleting PABP from mouse Krebs extract abrogated miRNA-mediated deadenylation[26]. PABP depletion was accomplished by using recombinant glutathione *S*-transferase (GST)-PAIP2 to pull down almost 90% of endogenous PABP from Krebs extract[26]. Importantly, this defect can be easily rescued with the addition of recombinant PABP to depleted extracts[26]. In contrast, another study reported that PABP is not required for miRNA-mediated translational repression or deadenylation in a *D. melanogaster* S2 *in vitro* system[54]. Partially depleting PABP, using GST-PAIP2 and/or blocking its contact with dGW182 via the addition of recombinant PAIP2 had little impact on let-7 miRNA repression or tethered GW182 repression of respective reporter RNAs[54]. It is plausible that PABP-GW182 contact stimulates miRNA-mediated silencing in certain systems but not in others.

CCR4–NOT and PAN2–PAN3 deadenylase complexes. miRNAs effect deadenylation of target mRNAs in human and insect cells and in zebrafish embryos, as well as in a variety of cell-free extracts that recapitulate miRNA repression[14,16,17,26,55,56]. miRNA-mediated

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deadenylation requires the CCR4–NOT deadenylation complex and, to a lesser extent, the PAN2–PAN3 deadenylation complex[15,17,26,57]. Recently, several concurrent studies demonstrated that GW182 proteins act as a docking platform for both the CCR4–NOT and the PAN2–PAN3 deadenylase complexes[26,31,40,45,46] (**Fig. 2b**). These interactions drive GW182-mediated deadenylation *in vitro* in mouse Krebs-2 extracts and miRNA-mediated silencing in human and *D. melanogaster* cells[31,40,45]. Both deadenylation complexes interact with the C-terminal silencing domain of human and *D. melanogaster* GW182 proteins. Human GW182 proteins directly bind the CNOT1 subunit of the CCR4–NOT complex[40,45], whereas the PAN2–PAN3 complex has been reported to bind GW182 either directly through the PAN3 kinase-like domain[31,45] and/or indirectly through PABP[31,40]. *C. elegans* AIN-1 protein shows less than 12% sequence identity with human and *D. melanogaster* GW182 proteins. However, it is able to coimmunoprecipitate *C. elegans* CNOT1, CNOT2 and PAN3 proteins when they are overexpressed in *D. melanogaster* S2 cells, demonstrating that GW182 interaction with the deadenylation machineries is highly evolutionarily conserved[46].

As mentioned previously, AGO proteins interact with specific N-terminal tryptophan-containing sequences (GW, WG or GWG) in GW182 proteins[17,24,27,29]. Interestingly, several recent studies point to additional evolutionarily conserved tryptophan-containing sequences in GW182 proteins being responsible for recruiting the CCR4–NOT complex[31,40]. Specifically, tryptophans in the dGW182 NED and in the C-terminal silencing domain of both human and *D. melanogaster* GW182 interact with the CCR4–NOT complex (**Fig. 2b**). One report on GW182-mediated deadenylation in Krebs extract, using a tethered recombinant TNRC6C silencing domain, found that the CCR4–NOT complex interacts with two tryptophan-containing evolutionarily conserved motifs in the M1 region and C-terminal domain of the GW182 silencing domain termed CCR4-NOT interaction motifs 1 and 2 (CIM-1 and CIM-2), respectively[40]. Both motifs are conserved in mammals, but CIM-2 is not found in flies[40]. The CCR4–NOT complex also interacts with the M2 and C-terminal regions of the human GW182 silencing domain through seven tryptophan-containing motifs (WG, WS or WT), two of which reside in CIM-2 (ref. 31). Although it is clear that GW182 recruits the CCR4–NOT complex, exactly how the CCR4–NOT complex contacts GW182 proteins through these tryptophan-containing motifs (that is, do all tryptophans make contact with the CCR4–NOT complex or are some important for GW182 folding) is not known. Interestingly, although several tryptophan-containing motifs in both human and *D. melanogaster* GW182 silencing domains help recruit the PAN2–PAN3 complex, similar motifs in the *D. melanogaster* GW182 NED cannot[31]. Thus, the context of tryptophan-containing motifs may dictate whether they can recruit the PAN2–PAN3 complex.

In addition to its function as a platform for deadenylation machineries, an *in vitro* study suggests that GW182 may also act as a deadenylation coactivator[40]. GW182 silencing domain fragments containing either CIM-1 or CIM-2 are sufficient to bind the CCR4–NOT complex; however, only CIM-2 promotes processive deadenylation when artificially tethered to reporter RNA *in vitro*. A silencing domain fragment containing only CIM-1 initiates deadenylation but stalls after removing ~15 As, indicative of a defect in poly(A) tail processing. Thus, the manner by which the human GW182 silencing domain binds the CCR4–NOT complex may be critical for robust poly(A) tail removal from target mRNAs.

EDD. PABP is not the only protein to directly interact with GW182 via a PABC domain. An E3 ubiquitin ligase called 'E3 ubiquitin ligase identified by differential display' (EDD), which also contains a PABC domain, was recently identified as a novel GW182-interacting protein by using a genetic screen in mouse embryonic stem cells (ESCs) to search for novel effectors of miRNA silencing[58] (**Fig. 2a**). EDD depletion leads to impaired repression of miRNA-targeted reporters without affecting miRNA biogenesis pathways, suggesting that EDD is mechanistically linked to miRNA-mediated silencing in ESCs. GW182 directly binds EDD through a PAM2-PABC contact, and via an additional interface through dimerization of GW182 and EDD UBA domains. Curiously, EDD impact on miRNA-mediated silencing is independent of its E3 ubiquitin ligase activity, as inactivating it using gene-targeting strategies failed to affect repression[58]. In contrast, removing the PABC domain from EDD derepressed the endogenous miRNA target *Bim* (also known as *BCL2L11*), which codes for a proapoptotic protein in mouse ESCs, suggesting that the PABC domain is integral to the function of EDD in miRNA-mediated silencing.

EDD associates with other silencing effectors, such as the miRISC-associated protein DEAD box helicase RCK/p54, which enhances mRNA decapping and has been shown to repress cap-dependent translation[58–60]. Moreover, knocking down EDD in mouse embryonic fibroblasts diminished the AGO-RCK/p54 interaction[58]. Thus, EDD may serve as an additional module that assists GW182 to recruit miRNA effectors such as RCK/p54 to target RNAs. PABP and EDD bind the GW182 PAM2 motif, suggesting that these interactions are mutually exclusive. Whether GW182 can interact with EDD and the CCR4–NOT and PAN2–PAN3 deadenylase complexes simultaneously remains to be established. One intriguing model proposes that the GW182–EDD complex leads to deadenylation-independent translational repression and subsequent mRNA destabilization, whereas the GW182–CCR4–NOT complex causes deadenylation and subsequent mRNA decay.

A temporal order of miRNA mechanism of action?

Seminal studies on miRNA action in *C. elegans* suggested that miRNAs post-transcriptionally control protein abundance either by repressing target mRNA translation with no discernible impact on mRNA stability or by initiating mRNA degradation[61–63]. The first documented miRNA, lin-4, was reported to inhibit translation of the *lin-14* mRNA in the absence of mRNA destabilization[61]. Since this initial report, several other studies using *C. elegans* and mammalian cell cultures have observed both endogenous miRNA targets and miRNA-targeted reporters shifting to lighter polysomal fractions in sucrose density gradients, which is indicative of a defect in translation initiation due to impaired ribosome recruitment[9,64,65]. Moreover, many of these studies failed to detect substantial degradation of miRNA-targeted mRNAs. In contrast, others reported miRNA-mediated deadenylation, decapping and decay of target mRNAs[14,16–18,66]. Thus, it was important to determine how miRNA-mediated translational repression and mRNA destabilization relate to each other.

Several groups investigated the global effects of miRNA action on mRNA and protein levels by using genome-wide approaches. Initial attempts used stable isotope labeling of amino acids in cell culture (SILAC) coupled with MS to identify changes in protein levels upon overexpression of miRNAs in mammalian cell cultures[67,68]. Studies from the Bartel and Rajewsky labs found significant correlations between mRNA and protein levels of miRNA targets, indicative of widespread mRNA destabilization. However, Rajewsky also identified hundreds of miRNA targets for which protein levels were decreased far more than mRNA levels, indicative of translational repression, a conclusion reached by a subsequent SILAC-based study in pancreatic cancer cells[69]. Techniques that analyze ribosome occupancy

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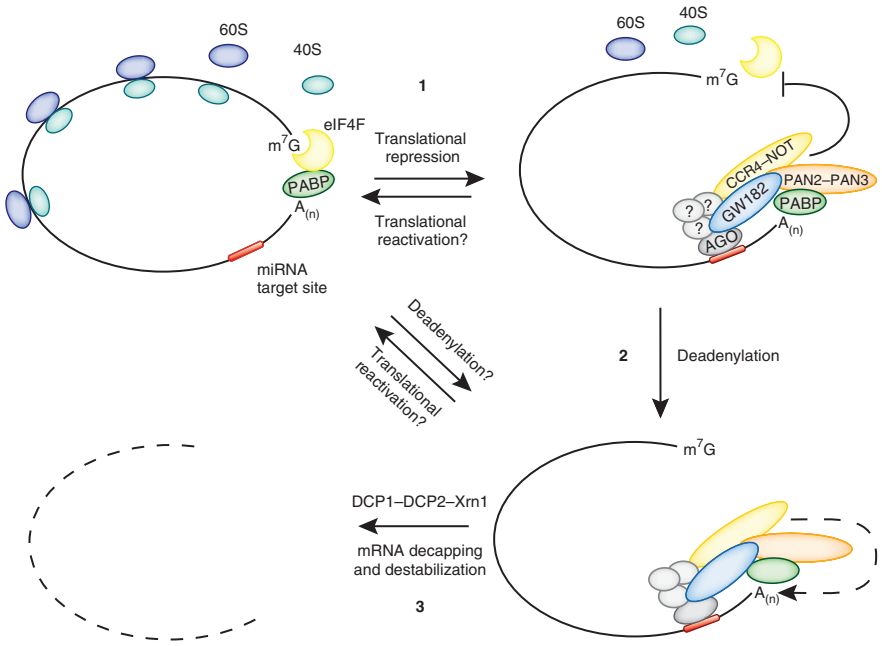

Figure 3 A temporal model of miRNA-mediated gene silencing. According to one model, the miRISC inhibits translation initiation by interfering with eIF4F-cap recognition through its eIF4E subunit and 40S ribosomal subunit recruitment[7,8,73]. GW182 protein interaction with PABP might interfere with the closed-loop formation mediated by the eIF4G-PABP interaction and thus contribute to the repression of translation initiation (1). The CCR4–NOT deadenylase complex, recruited by GW182, has also been reported to repress translation independently of its deadenylase activity. Deadenylation is mediated by the CCR4–NOT and PAN2–PAN3 deadenylation complexes (2). Following deadenylation, the 5′-terminal cap (m^7G) is removed by the DCP1–DCP2 decapping complex and the mRNA is degraded by the Xrn1 5′–3′ exonuclease (3).

(the fraction of a specific mRNA associated with ribosomes) and ribosome density (the number of ribosomes bound to unit length of coding sequence) of miRNA targets as readout of mRNA translation have also been used to address this question[70,71]. Polysome density gradient and ribosome profiling techniques coupled with microarray and mRNA-sequencing analyses, respectively, revealed that mammalian microRNAs have a major impact on mRNA stability, yet suggest an interplay between translation repression at the level of initiation and mRNA destabilization[70,71]. Nevertheless, upon subjecting cells to specific stress, miRNA-targeted mRNAs are translationally reactivated, demonstrating that mRNA destabilization is not always the end result of miRNA action[64,72].

Although both powerful and informative, the above-mentioned techniques are limited to investigating the steady-state effects of miRNAs and ignore early time points in miRNA-mediated silencing. In contrast, *in vitro* studies have been instrumental in dissecting both miRNA mechanisms of action and the order in which they operate. Studies using mouse and *D. melanogaster* cell-free extracts that recapitulate miRNA-mediated silencing show that translational repression and miRNA-mediated deadenylation, a precursor event to mRNA destabilization, can be uncoupled (that is, translational repression in the absence of mRNA deadenylation or destabilization)[26,73]. In addition, a time-course analysis of let-7–mediated repression in extract from Krebs-2 mouse ascites revealed translational repression before deadenylation. Two recent studies[74,75] that investigated the kinetics of miRNA action *in vivo* have bolstered the model for temporal order of miRNA-mediated events previously observed *in vitro*. In one study, a time-course analysis of miRNA silencing was achieved by transcriptionally pulsing a variety of metallothionein promoter–driven miRNA-targeted reporters in *D. melanogaster* S2 cells[74]. Transcription was induced for 90 min by incubating cells with copper(II) sulfate and shut off with a copper-specific chelator. Translational repression was consistently observed to precede mRNA deadenylation or destabilization for a variety of reporter mRNAs containing either artificial or natural 3′ UTRs.

A second study[75] took advantage of a natural developmental process in zebrafish embryos, in which the onset of zygotic transcription induces expression of miR-430, a miRNA previously shown to target and clear endogenous maternal mRNAs through deadenylation and subsequent destabilization[16]. Whether deadenylation-independent translational repression preceded deadenylation, however, was unknown. To address this question, miRNA repression was analyzed using a combination of ribosome profiling, mRNA-sequencing and a novel technique to resolve poly(A) tail lengths of miR-430 targets using capillary electrophoresis at early time points upon the onset of zygotic transcription. Importantly, the results demonstrated that miR-430 represses translation initiation before initiating deadenylation and decay of target mRNAs. In summary, persuasive data from several studies demonstrate that miRNAs can act through a two-step mode of repression, first inhibiting translation in a deadenylation-independent manner, then subsequently causing deadenylation, decapping and destabilization of targeted mRNAs (**Fig. 3**). That being said, it is quite possible that deadenylation proceeds at a faster rate in some biological systems. Moreover, it remains to be determined whether deadenylation can proceed before translational repression, or whether the two events, although additive in outcome, are mechanistically distinct.

Novel insights into translational repression by miRNAs

The past few years have witnessed considerable progress in the understanding of the molecular underpinnings and protein-protein contacts that drive miRNA-mediated deadenylation. How miRNAs repress translation in a deadenylation-independent manner remains far less clear. Several studies demonstrated that miRNAs repress cap-dependent translation, and mRNAs lacking functional 5′-cap structures are refractory to silencing[7,8,73,76]. This is supported by the observation that addition of the cap-binding complex eIF4F to mouse Krebs-2 cell-free extracts, which stimulates ribosome recruitment to the mRNA, antagonizes miRNA-mediated silencing in a dose-dependent manner[8]. Importantly, blocking miRNA-mediated deadenylation, by adding an additional 10–40 non-A nucleotides to the poly(A) tail, did not prohibit translational repression in zebrafish embryos or in *D. melanogaster* cell-free extracts[37,54,74]. However, miRNAs can still repress translation of nonadenylated mRNAs, suggesting that the poly(A) tail, PABP and/or PABP-poly(A) tail interactions may enhance, but are not a prerequisite for, such repression[23,31,35,45].

How would the miRISC inhibit mRNA translation before initiating deadenylation? The idea that miRNA-loaded AGO directly binds the 5′-cap structure of target mRNAs[77–79] is unlikely, as human AGO2 does not significantly interact with cap analogs, and structural studies

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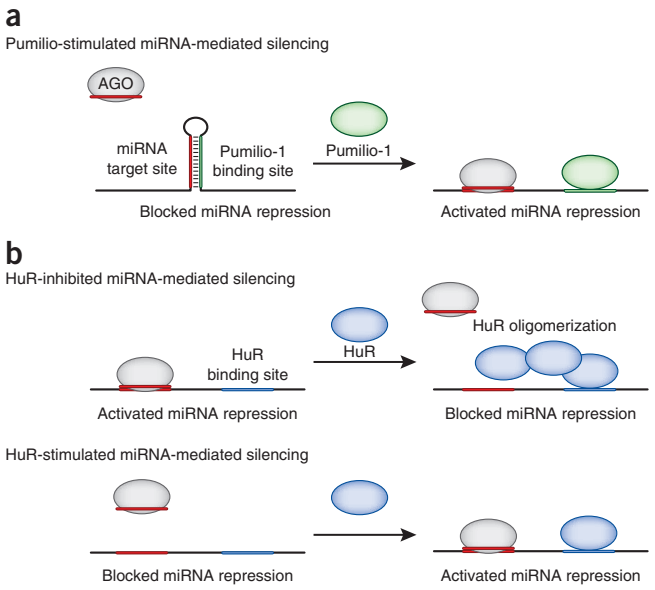

Figure 4 Schematic of 3′ UTR binding proteins modulating miRNA target site accessibility. (**a**) A miRNA target site resides within an RNA stem loop, rendering it inaccessible to a miRNA-loaded Argonaute. Pumilio binding remodels the RNA secondary structure and disrupts the RNA stem loop structure, thereby rendering the miRNA target site accessible and initiating miRNA-mediated repression. (**b**) A miRNA target site that recruits Argonaute is adjacent to an HuR protein-binding site. When HuR is bound, HuR proteins oligomerize and impede miRNA target site accessibility, thereby derepressing miRNA-targeted mRNAs[64,85]. In another model, a miRNA target site, which is positioned adjacent to an HuR protein-binding site, recruits Argonaute only when HuR is bound[84].

have not supported such an interaction[23,80,81]. Another model proposes that miRNAs repress translation through GW182 binding to PABP, thereby interfering with mRNA circularization[26,39], although this model remains controversial (see above). More recently, however, several studies suggest that the miRISC-recruited CCR4–NOT deadenylase complex may be required for deadenylation-independent translational repression. The CAF1-CNOT7 deadenylase subunit of the CCR4–NOT complex represses cap-dependent, but not cap-independent, translation in *Xenopus laevis* oocytes when artificially tethered to reporter mRNAs[82]. Importantly, this translational repression is independent of deadenylation, as inactivating CAF1-CNOT7 deadenylase activity does not abolish translational repression[82]. In addition, knocking down various components of the CCR4–NOT complex impairs GW182-mediated repression of nonadenylated target mRNAs in cell cultures[31,45]. Moreover, tethering CCR4–NOT complex components to nonadenylated reporters inhibits their expression without affecting their mRNA levels[31]. It is therefore likely that GW182 recruitment of the CCR4–NOT complex drives both translational repression and deadenylation of miRNA targets. Whether and how the CCR4–NOT complex interacts with the translation machinery to inhibit protein synthesis, however, is still unresolved.

Modulating miRNA target site accessibility

miRNAs and 3′ UTR binding proteins, such as HuR and PUF proteins, post-transcriptionally regulate gene expression by targeting the 3′ UTRs of specific mRNAs. Several mRNA subsets contain target sites for both types of *trans*-acting factors. Indeed, several studies have reported relationships between 3′ UTR binding proteins and miRNA target sites, with 3′ UTR binding proteins modulating miRNA silencing. Interestingly, 3′ UTR binding proteins have the potential to both agonize and antagonize miRNA-mediated silencing (**Fig. 4**)[64,83–92].

PUF proteins. PUF proteins activate miRNA-mediated silencing in multiple systems[91,92]. For example, miRNA-mediated repression of *hbl-1* mRNA in *C. elegans* requires its 3′ UTR to interact with both the let-7 miRNA and the pumilio homolog, *puf-9*, because let-7 is insufficient on its own to initiate silencing[91]. One model that may explain the cooperation between PUF proteins and miRNA silencing was postulated by Agami and colleagues in light of their studies of pumilio-1 (PUM1) protein's involvement in miRNA-mediated silencing of the *p27* mRNA (**Fig. 4a**)[92]. Both miR-221 and miR-222 are required to silence *p27* mRNA yet cannot do so in quiescent cells despite the presence of both miRNAs. Importantly, miRNA-mediated silencing of *p27* mRNA requires PUM1 binding to its 3′ UTR, which is activated via growth factor stimulation. Using FRET analysis, Agami and colleagues reported that PUM1 binding altered the local p27–3′ UTR secondary structure and increased miRNA target site accessibility in a manner reminiscent of bacterial riboswitches[92] (for reviews, see refs. 93,94). A comparative analysis of mRNA targets of PUF proteins in human cells showed an enrichment of PUF protein binding sites in the vicinity of predicted miRNA target sites, suggesting that PUF protein-miRNA target site networks may be rather widespread[95]. Interestingly, the human PUF protein PUM1 can interact with a subunit of the CCR4–NOT deadenylase complex[96]. However, whether PUF protein–mediated silencing augments miRNA-mediated silencing is currently unknown.

HuR proteins. The AU-rich element–binding protein HuR represents another class of RNA binding protein that modulates miRNA silencing (**Fig. 4b**). Similarly to PUF proteins, transcriptome-wide analyses of HuR-binding sites identified many as being proximal to, but not overlapping with, miRNA target sites[97,98]. HuR may also enhance miRNA target site accessibility via RNA remodeling in a manner similar to PUM1 and the p27 3′ UTR. Indeed, HuR is required for miRNA-mediated silencing of c-Myc and the Ras-related small GTPase *RhoB* mRNAs[82,83]. However, HuR binding has also been reported to negatively affect silencing of other miRNA-targeted mRNAs[64,87,88,99]. HuR-mediated antagonism of miRNA silencing was first observed in human hepatocarcinoma cells. Amino acid starvation led to HuR-binding and a relief of miR-122–mediated repression of the cationic amino acid transporter mRNA, CAT-1 (ref. 64). HuR binding impeded silencing *in vivo* and miRNA-mediated deadenylation *in vitro* of miRNA-targeted reporters, most likely via a mechanism whereby HuR oligomerization along the RNA leads to miRISC dissociation[85].

Dead end 1. Dead end 1 (Dnd1) represents another RNA binding protein that blocks miRNA-mediated silencing of mRNAs. Dnd1 impedes miR-221–mediated repression of the *p27* mRNA in mammalian cells and miR-430–mediated repression of nanos1 and TDRD7 mRNAs in zebrafish primordial germ cells[89,90]. In contrast to HuR binding sites, which are found in proximity to miRNA binding sites, experimentally validated Dnd1 binding sites overlap with miRNA binding sites, thereby interdicting miRNA target site accessibility.

Perspectives

Significant advances have been made to our understanding of miRNA-mediated silencing, but many outstanding questions remain unanswered. A critical issue is how the miRISC, in combination with the

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CCR4–NOT complex and/or other factors, represses translation at early time points in a deadenylation-independent manner. miRNA-mediated deadenylation does not require the target mRNA to be translated[26,55,73]. However, it remains to be established whether translation needs to be shut down before miRNA-mediated deadenylation can proceed on an actively translating mRNA, which could account for a temporal order of silencing mechanisms. GW182 binds AGO and serves as an interaction platform for PABP, EDD and multiple deadenylase machineries. However, GW182 also drives mRNA decapping through the Dcp1/2 decapping enzymes and mRNA destabilization through the Xrn1 5′–3′ exonuclease[15,18]. Are these factors actively recruited by the miRISC, or is miRNA-mediated decapping and decay of targets just a consequence of deadenylation? In addition, the PABP-interacting protein Ataxin-2 (which is responsible for spinocerebellar ataxia type 2 through the expansion of its polyglutamine domain[100]) and the DEAD-box helicase RCK/p54, represent two other proteins implicated in miRNA-mediated silencing[60,101]. They associate with AGO, but their molecular contacts with miRISC and mechanistic roles in miRNA-mediated silencing are not known.

Finally, understanding how specific miRNA-repressed targets remain stable such that they can be derepressed at a later time, as is the case for the miR-122 target CAT-1 and neuronal miRNA targets that become reactivated at post-synaptic densities, is of critical importance[64,72]. Are these targets repressed in the absence of deadenylation, or are they deadenylated and subsequently readenylated? These and other questions remain at the forefront of miRNA biology.

Note added in proof: A study detailing an additional role of PABP and the poly(A) tail in miRNA-mediated silencing has been published while this review was in press. This article[102] reports that PABP and the poly(A) tail augment miRNA-mediated silencing, at least in part, by stimulating miRISC association with target mRNAs.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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