NON-CODING RNA

Towards a molecular understanding of microRNA-mediated gene silencing

Stefanie Jonas and Elisa Izaurralde

Abstract | MicroRNAs (miRNAs) are a conserved class of small non-coding RNAs that assemble with Argonaute proteins into miRNA-induced silencing complexes (miRISCs) to direct post-transcriptional silencing of complementary mRNA targets. Silencing is accomplished through a combination of translational repression and mRNA destabilization, with the latter contributing to most of the steady-state repression in animal cell cultures. Degradation of the mRNA target is initiated by deadenylation, which is followed by decapping and 5′-to-3′ exonucleolytic decay. Recent work has enhanced our understanding of the mechanisms of silencing, making it possible to describe in molecular terms a continuum of direct interactions from miRNA target recognition to mRNA deadenylation, decapping and 5′-to-3′ degradation. Furthermore, an intricate interplay between translational repression and mRNA degradation is emerging.

Deadenylation
Shortening of mRNA poly(A) tails. In eukaryotes, this process is catalysed by the consecutive but partially redundant action of two cytoplasmic deadenylase complexes: PAN2–PAN3 and CCR4–NOT.

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MicroRNAs (miRNAs) are conserved post-transcriptional regulators of gene expression that are integral to almost all known biological processes, including cell growth, proliferation and differentiation, as well as organismal metabolism and development[1]. The number of miRNAs encoded within the genomes of different organisms varies considerably, from a handful of miRNAs in sponges up to approximately 1,500 miRNAs in humans[1]. Computational predictions and genome-wide identification of miRNA targets estimate that each miRNA can bind to hundreds of different mRNAs, which collectively results in the regulation of more than half of the protein-coding genes in humans[2]. It is therefore not surprising that dysregulation of miRNA expression is linked to various human diseases, including cancer[3].

To exert their regulatory function, miRNAs associate with Argonaute (AGO) family proteins to form the basic core of miRNA-induced silencing complexes (miRISCs)[1]. These complexes mediate the post-transcriptional silencing of mRNAs containing sequences that are partially or fully complementary to the miRNA[1]. Perfectly complementary targets are cleaved by catalytically active AGOs[1,4]. However, in animals the mRNA targets are often only partially complementary to the miRNA, which precludes cleavage by AGO proteins[4]. Moreover, in humans, only AGO2 is catalytically active, whereas AGO1, AGO3 and AGO4 are not[4]. In cases in which cleavage is not possible, AGO proteins recruit additional protein partners to mediate silencing[5,6]. Silencing occurs through a combination of translational repression, deadenylation, decapping and 5′-to-3′ mRNA degradation[5,6] (FIG. 1). The GW182 proteins play a central part in this process and are among the most extensively studied AGO partners[5,6]. They function as flexible scaffolds to bridge the interaction between AGO proteins and downstream effector complexes, such as the cytoplasmic deadenylase complexes PAN2–PAN3 and CCR4–NOT[5,6] (FIG. 1).

Over the past several years, remarkable progress has been made in our understanding of the structural basis of miRNA target degradation. However, much less is known about the mechanisms by which animal miRNAs repress translation in the absence of target mRNA degradation. In this Review, we describe emerging models of the molecular mechanisms driving miRNA silencing in animals. Although the information available on miRNA biogenesis, target selection and function has considerably increased in recent years[1,7], we do not cover it in this Review. Rather, we focus on recent molecular and structural insights obtained with respect to the effector step of silencing and on what happens after a target is recognized by miRISCs.

Mechanisms of miRNA target degradation

miRNAs silence gene expression by repressing translation and accelerating target mRNA degradation[4–6]

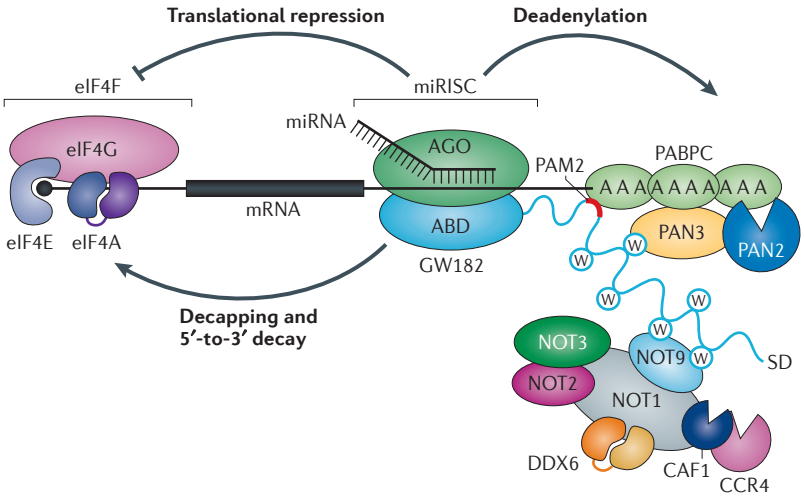


Figure 1 | **Overview of miRNA-mediated gene silencing in animals.** Animal microRNAs (miRNAs) bound to an Argonaute (AGO) protein in miRNA-induced silencing complexes (miRISCs) recognize their mRNA targets by base-pairing to partially complementary binding sites, which are predominantly located in the 3′ untranslated region of the mRNA. AGO proteins interact with a GW182 protein, which in turn interacts with cytoplasmic poly(A)-binding protein (PABPC) and with the cytoplasmic deadenylase complexes PAN2–PAN3 and CCR4–NOT. The PAN2–PAN3 and CCR4–NOT complexes catalyse the deadenylation of the mRNA target. The GW182 proteins consist of an amino-terminal AGO-binding domain (ABD) and a silencing domain (SD; see BOX 2 for additional information). In animal cell cultures, deadenylated mRNAs are decapped and rapidly degraded by 5′-to-3′ exoribonuclease 1 (XRN1; not shown) (BOX 1). In addition, miRNAs repress translation, but the precise molecular mechanism for this remains unclear. The emerging consensus is that miRNAs inhibit translation initiation by interfering with the activity and/or assembly of the eukaryotic initiation factor 4F (eIF4F) complex. The eIF4F complex consists of the cap-binding protein eIF4E, the adaptor protein eIF4G and the DEAD box RNA helicase eIF4A[99]. eIF4G serves as a scaffold for protein–protein interactions that are essential for the recruitment of the 43S pre-initiation complex and for translation initiation[99]. The cap structure is shown as a black circle. DDX6, DEAD box protein 6; PAM2, PABP-interacting motif 2.

(FIG. 1). Genome-wide measurements of the effects of miRNAs on protein and mRNA levels, combined with ribosome profiling experiments, have demonstrated that the degradation of miRNA targets is a widespread effect, which at steady state accounts for most (66–90%) of the miRNA-mediated repression observed in cultured mammalian cells[8–13].

Cumulative evidence has indicated that the degradation of miRNA targets is catalysed by enzymes involved in the cellular 5′-to-3′ mRNA decay pathway[14–30]. In this pathway, mRNAs are first deadenylated by the consecutive and partially redundant action of the PAN2–PAN3 and CCR4–NOT deadenylase complexes[31] (BOX 1). Deadenylated mRNAs are then decapped by decapping protein 2 (DCP2), which requires additional cofactors for full activity. In metazoans, these cofactors include DCP1, enhancer of decapping 3 (EDC3), EDC4, PATL1 and DEAD box protein 6 (DDX6; also known as Dhh1, RCK, p54 and Me31B in different species)[32]. Finally, deadenylated and decapped mRNAs are degraded by the major cytoplasmic nuclease 5′-to-3′ exoribonuclease 1 (XRN1) (BOX 1).

Decapping
Hydrolysis of the 5′ cap structure on the mRNA. A major decapping enzyme in eukaryotes is decapping protein 2 (DCP2), which hydrolyses the cap structure, releasing 7-methyl-GDP and a 5′ monophosphorylated mRNA. This 5′ monophosphorylated mRNA is a substrate for 5′-to-3′ exoribonuclease 1 (XRN1), which rapidly degrades decapped mRNA.

Consecutive steps in the 5′-to-3′ mRNA decay pathway are coupled through a network of direct interactions between subunits of the catalytic complexes involved. For example, in metazoans, the CCR4–NOT complex interacts with the decapping factors DDX6 and PATL1, which in turn interact with additional components of the decapping complex, providing a physical link that couples deadenylation to decapping[29,30,32]. Additionally, decapping factors (EDC4 in vertebrates and Dcp1 in *Drosophila melanogaster*) interact with XRN1 (REFS 32,33). These interactions ensure that XRN1 is recruited to the location where decapped mRNAs are produced. Consequently, mRNA degradation proceeds efficiently, and mRNA decay intermediates such as deadenylated but capped mRNAs do not accumulate in rapidly dividing cells[12,13,34], unless decapping is inhibited[16,19–22].

Coupling between steps in the mRNA decay pathway seems to be disrupted in oocytes, early embryos, cell-free extracts and probably neuronal cells. In these systems, miRNA targets typically accumulate in a deadenylated and translationally repressed form without undergoing further decay[12,35–41]. As a result, silencing is potentially reversible in oocytes, early embryos and neurons, as repressed mRNAs could eventually return to the translational pool after cytoplasmic polyadenylation[12,42,43].

miRISCs make use of these efficiently coupled decay machineries by direct recruitment via the adaptor GW182 proteins. Below, we introduce the GW182 proteins and describe how they interact with AGO proteins and deadenylase complexes to elicit deadenylation and the subsequent decay of miRNA targets.

The GW182 protein family

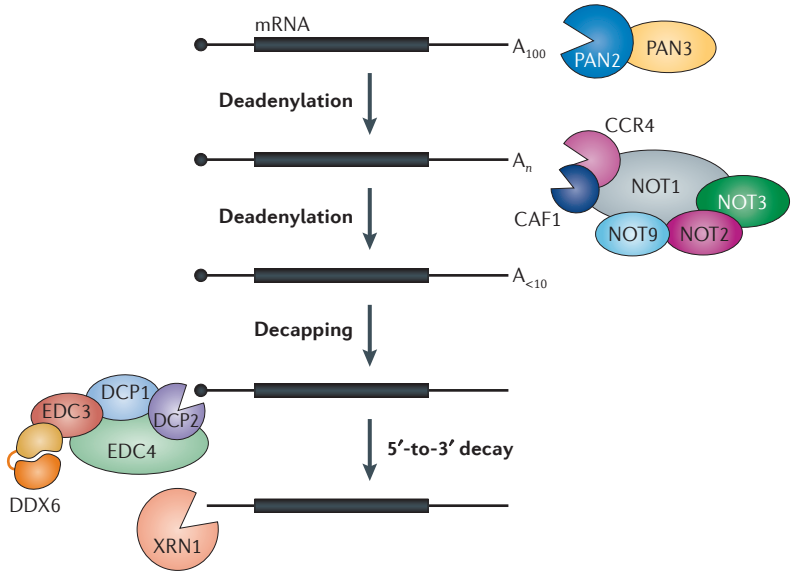
GW182 proteins co-purify with AGO family proteins and are required for miRNA-mediated gene silencing, as indicated by the observations that depletion of these proteins in mammalian and *D. melanogaster* cells and in *Caenorhabditis elegans* severely inhibits silencing[15,16,44–50].

The GW182 family of proteins is found in metazoans. In vertebrates and some insect species, the family comprises three paralogues, termed trinucleotide repeat-containing 6 (TNRC6) proteins: TNRC6A (also known as GW182), TNRC6B and TNRC6C. In *D. melanogaster*, there is one family member (GW182), and in *C. elegans* and most other nematodes, there are two highly divergent members, AIN-1 and AIN-2 (REFS 16,47,50) (BOX 2). To date, no orthologues have been identified in fungi or plants[16,50].

The three vertebrate GW182 paralogues and the single *D. melanogaster* orthologue share a similar domain organization, which is characterized by the presence of two functional domains: an amino-terminal AGO-binding domain (ABD) and a carboxy-terminal silencing domain (SD)[5,6,50–54] (BOX 2). These functional domains are predicted to be mainly unstructured and feature multiple tryptophan (W)-containing motifs, often flanked by glycine residues (GW, WG or GWG repeats), which provide the name for the protein family[5,6,50] (BOX 2).

Box 1 | **The cellular 5′-to-3′ mRNA decay pathway**

Bulk mRNA decay is initiated by deadenylation (see the figure). The PAN2–PAN3 complex is thought to catalyse the first phase of deadenylation, which is then continued by the CCR4–NOT complex[31,68]. Following deadenylation, mRNAs are decapped by the enzyme decapping protein 2 (DCP2). Multiple proteins — such as DCP1, enhancer of decapping 3 (EDC3), EDC4, DEAD box protein 6 (DDX6) and PATL1 (not shown) — have been shown to stimulate decapping using different mechanisms[32]. DDX6 also interacts with the CCR4–NOT complex (FIGS 4,5). Deadenylated and decapped mRNAs are degraded from the 5′ end by 5′-to-3′ exoribonuclease 1 (XRN1). Recent studies indicate that the average poly(A) tail in mammalian cells contains approximately 50–100 adenosines[12,34]. It is not known exactly when the CCR4–NOT complex takes over deadenylation, but it can catalyse deadenylation in the absence of the PAN2–PAN3 complex[31,68]. The decapping factors, along with XRN1, Argonaute proteins and GW182 proteins, localize to mRNA-processing bodies (also known as P-bodies), which are cytoplasmic foci where proteins involved in translational repression and mRNA decapping and decay accumulate[6,32]. The functional importance of this localization remains unclear, as GW182 proteins that fail to localize to P-bodies are active in complementation assays, and P-body integrity is not required for silencing[50,51,113,114]. The cap structure is shown as a black circle.



Ribosome profiling
A method that allows the determination of the position of ribosomes on cellular mRNAs with high sequence resolution. Briefly, cells are treated with cycloheximide to stabilize ribosomes on mRNAs, then lysed and treated with nucleases to degrade mRNA regions not protected by ribosomes. Translating ribosomes protect RNA fragments of about 30 nucleotides in length (known as ribosome-protected fragments) that can be sequenced, generating millions of mRNA sequence tags.

The W-containing motifs mediate binding to AGO proteins as well as to subunits of the deadenylase complexes, namely PAN3, NOT1 and NOT9 (also known as CNOT9, CAF40, RQCD1 and RQD1 (REFS 29,30))[24–30,48,50–58]. Over the past 5 years, a burst of three-dimensional structures have revealed general common principles for how GW182 proteins interact with their partners: the W residues are inserted into hydrophobic pockets exposed on the surface of AGO proteins, PAN3 and NOT9 (REFS 28–30,59). Molecular recognition is predominantly restricted to the W residues, although the flanking residues contribute to the affinity of the interactions[26,28–30,58–60]. As a consequence of this mode of recognition, multiple W residues can mediate binding in a redundant manner and contribute to the affinity of the interaction through cooperativity and/or avidity effects[26,28–30,51–60]. It is therefore likely that the stoichiometry of the complexes is not strictly defined and, to some extent, is determined by the relative affinities and concentrations of the interacting partners in the cell.

Interaction of GW182 proteins with AGO proteins. Structural and biochemical studies of archaeal, bacterial, yeast and human AGO proteins have revealed that they feature a bi-lobed architecture, in which one lobe contains the N-terminal and PIWI–AGO–ZWILLE (PAZ) domains, and the other contains the MID and PIWI domains[4] (FIG. 2a). The PAZ and the PIWI domains provide binding pockets for the 3′-hydroxyl group and the 5′ phosphate, respectively, of the small RNA (that is, the miRNA or small interfering RNA (siRNA)). The PIWI domain adopts an RNaseH fold and has endonucleolytic activity in some but not all AGO proteins[4]. The small RNA and the complementary RNA target bind in a cleft between the two lobes[4,59,61,62] (FIG. 2b).

Despite conservation of the individual domains, eukaryotic AGO proteins display specific structural features — such as extended loops, additional secondary structural elements and a distinct orientation of the lobes — that are not observed in prokaryotic AGO proteins[4,59,61,62]. Particularly relevant to the miRNA pathway is the presence of tandem W-binding pockets on the surface of the PIWI domain, opposite the miRNA-binding surface, in human AGO2 (REF. 59) (FIG. 2b,c), which represent binding sites for the GW182 proteins.

The residues lining the W-binding pockets are conserved in AGO proteins that play a part in the miRNA pathway, such as human AGO1, AGO2, AGO3 and AGO4, as well as *D. melanogaster* Ago1 (REF. 59). By contrast, these residues are less conserved in the PIWI clade of AGO proteins and in *D. melanogaster* Ago2, which do not interact with GW182 proteins[16,63].

The spatial arrangement of the AGO2 W-binding pockets is such that they can accommodate consecutive W residues as long as they are at least 8–10 residues apart (that is, 20–25 Å)[59] (FIG. 2c). This is a typical intervening distance between W residues in GW182 proteins, suggesting that concerted or consecutive binding of adjacent W residues contributes to the affinity of the interaction[59]. Accordingly, *in vitro* binding assays indicate that human AGO2 exhibits affinity for synthetic peptides containing at least two W residues separated by a minimal distance of 10 residues[60]. The residues flanking the W residues mediate weak interactions, which are most likely nonspecific, as different amino acids are tolerated at these positions as long as they have a small side chain. However, in the context of full-length AGO proteins, not all W residues contribute equally to the affinity of the interaction[51–58,60], indicating that the flanking regions influence binding in ways that are not completely understood.

Interaction of GW182 proteins with the PAN2–PAN3 complex. The PAN2–PAN3 deadenylase complex functions in both general and miRNA-mediated mRNA deadenylation[31]. In both cases, the catalytic subunit, PAN2, is recruited to target mRNAs through interactions mediated by the adaptor protein, PAN3. Indeed, PAN2 is recruited to bulk polyadenylated mRNA through direct interactions between PAN3 and cytoplasmic poly(A)-binding protein (PABPC) bound to the mRNA poly(A) tails[31,64]. Similarly, PAN2 is specifically recruited to miRNA

Cooperativity
The changes that occur when the binding of a ligand to a binding site on one molecule increases (or decreases) the affinity for binding to a second ligand on another binding site on the same molecule.

Avidity
The phenomenon by which individual binding events increase the likelihood of other interactions occurring, for example, by increasing the local concentration of each binding partner in proximity to the binding site.

targets through direct interactions between PAN3 and GW182 proteins[25,28]. In addition, PAN3 binds to RNA through an N-terminal zinc-finger domain[65].

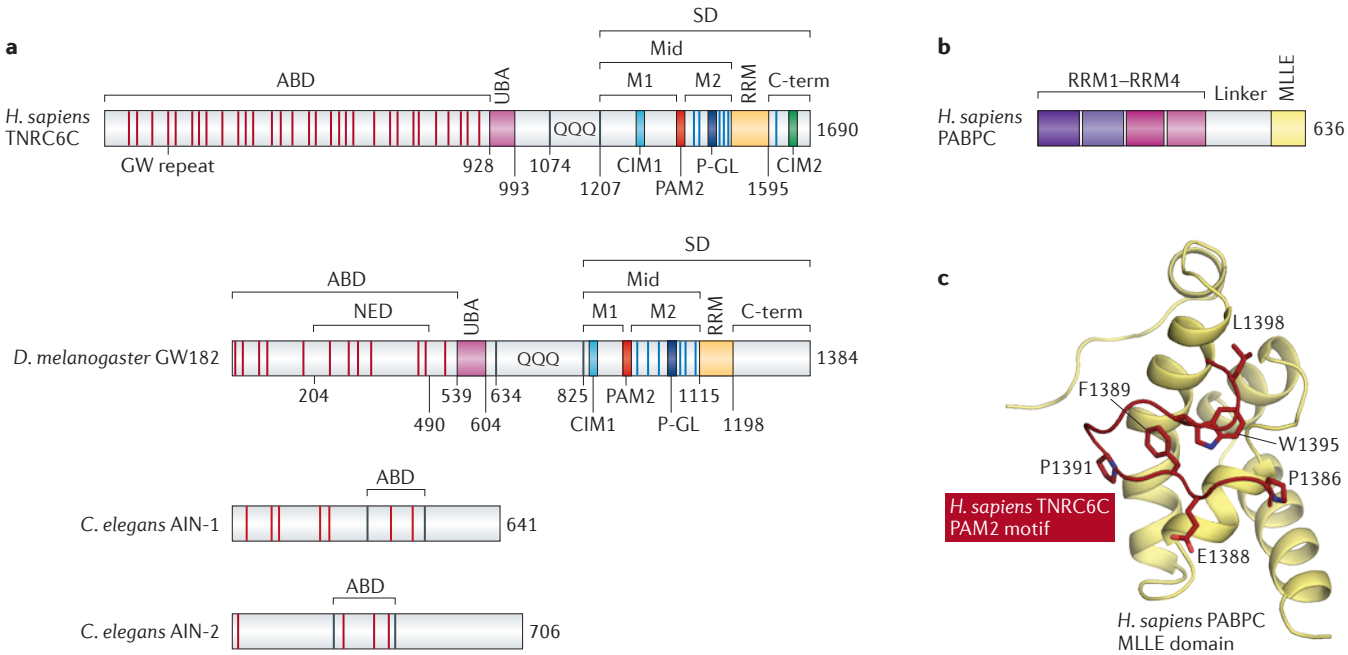
The structure of PAN3 bound to a W-containing peptide (derived from a neighbouring PAN3 molecule in the crystal lattice), together with structures of PAN3 bound to PAN2 (REFS 28,65–67), provides a detailed molecular model for the assembly of the PAN2–PAN3 complex and its recruitment to miRNA targets. The PAN3 protein homodimerizes through an extended coiled-coil that connects an N-terminal pseudokinase domain with a C-terminal globular domain (FIG. 3). This C-terminal domain provides a binding surface for the PAN2 deadenylase[28,66,67]. At the N-terminal end of the coiled-coil, metazoan PAN3 proteins harbour a W-binding pocket, which mediates binding to the GW182 proteins[28] (FIG. 3).

In contrast to the detailed structural understanding of the PAN2–PAN3 complex, much less has been learned about its exact contribution to silencing. This question has been complicated by the fact that the W residues in GW182 proteins mediate promiscuous binding to PAN3 and the CCR4–NOT complex, which precludes selective disruption of the interaction with only one complex[24]. Moreover, only a minor effect on silencing efficiency is observed when the PAN2–PAN3 complex is depleted or when catalytically inactive PAN2 mutants are overexpressed in *D. melanogaster* and human cells[20,25]. The CCR4–NOT complex is probably able to compensate and is sufficient for mRNA deadenylation in the absence of PAN2–PAN3 (REFS 31,68). This observation is consistent with current models of general mRNA turnover, which indicate that the PAN2–PAN3 complex is involved in an early phase of deadenylation,

Box 2 | The GW182 protein family and the interaction with PABPC

GW182 proteins consist of two functional domains: an Argonaute (AGO)-binding domain (ABD) and a silencing domain (SD) (see the figure, part **a**). The SD is bipartite and consists of a middle region (Mid; containing the M1 and M2 fragments) and a carboxy-terminal region (C-term). The ABD and the SD are predicted to be mainly unstructured and contain a variable number of tryptophan (W)-containing motifs (shown as vertical lines coloured red in the ABD and blue in the SD, and often flanked by glycine (G) residues). The ABD of *Drosophila melanogaster* GW182 contains an amino-terminal effector domain (NED), which also promotes silencing[52]. The SD contains several additional motifs, which include PABP-interacting motif 2 (PAM2)[23], CCR4-interacting motif 1 (CIM1), CIM2[27] and a proline-rich motif (P-GL)[82]. The PAM2 motif mediates binding to cytoplasmic poly(A)-binding protein (PABPC)[23,76–78], whereas the CIM1 and CIM2 motifs, together with the W motifs in the M2 and C-term regions, mediate binding to the deadenylase complexes[24–27,52,113,115]. Additionally, the vertebrate and insect proteins typically contain a region rich in glutamine (QQQ), a central ubiquitin-associated (UBA) domain and a C-terminal RNA recognition motif (RRM)[50,113,115]. Although the P-GL motif, the UBA domain and the RRM are highly conserved, their roles remain enigmatic because they can be mutated or deleted in cellular-based reporter assays without affecting the activity of the protein[24,50–54,113,115]. *Homo sapiens* trinucleotide repeat-containing 6C (TNRC6C) and *D. melanogaster* GW182 are shown as representative family members. The highly divergent *Caenorhabditis elegans* AIN-1 and AIN-2 proteins are included for comparison. These proteins contain fewer W motifs[47,83]. Nevertheless, they interact with AGO proteins through the ABD[83]. AIN-1 interacts with PABPC and the CCR4–NOT complex through motifs that have not yet been defined[83].

PABPC consists of four N-terminal RRMs (RRM1–RRM4), a proline-rich unstructured linker and a conserved C-terminal domain termed MLLE[77,79] (see the figure, part **b**). The RRMs interact with the PAM1 motif of PABP-interacting protein 1 (PAIP1) and PAIP2 and with the SDs of GW182 proteins[75,78,79]. The PABPC MLLE domain interacts with proteins containing PAM2, including PAIP1, PAIP2, PAN3 and the GW182 proteins[23,76–78]. Human PABPC1 is shown as a representative example. The structure of the human PABPC1 MLLE domain (yellow) bound to the PAM2 motif of TNRC6C (red; RCSB Protein Data Bank code: 2X04)[76] is shown in part **c** of the figure. TNRC6C residues involved in the interaction with PABPC are shown as sticks and are labelled.



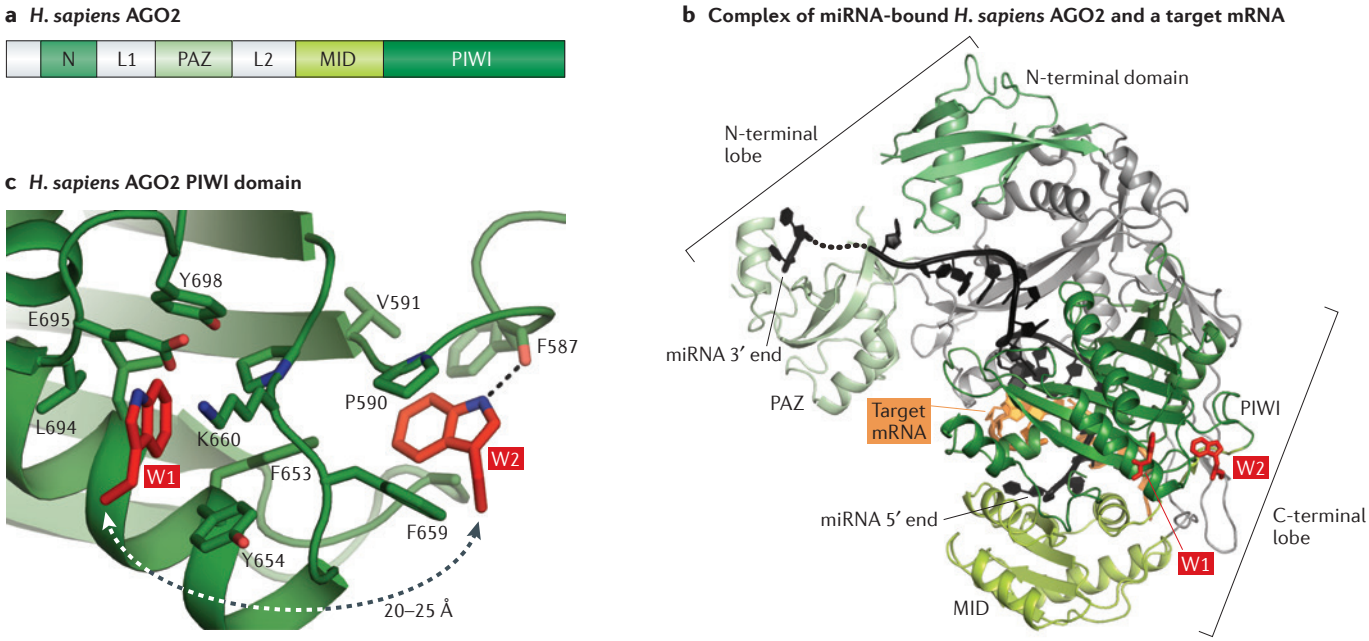


Figure 2 | **Structural insight into the interaction of AGO proteins with GW182 proteins.** **a** | Argonaute (AGO) proteins have four domains: the amino-terminal domain, the PIWI–AGO–ZWILLE (PAZ) domain, the MID domain and the PIWI domain. The PAZ domain is connected to the N-terminal and MID domains by linker regions called L1 and L2, respectively. *Homo sapiens* AGO2 is shown as a representative example of this family. **b** | The structure of *H. sapiens* AGO2 (green and grey) in complex with a microRNA (miRNA; black) and a short piece of target mRNA (orange) (RCSB Protein Data Bank code: 4W5O)[61] highlights the position of the tryptophan (W) residues (red) bound to pockets on the surface of the PIWI domain. **c** | Close-up view of the W-binding pockets (PDB code: 4OLB)[59] is shown. Residues lining the pockets are shown as sticks and partially labelled for orientation. The minimal distance between the two W residues is indicated. C-terminal, carboxy-terminal.

in which poly(A) tails are shortened in a distributive manner, without causing full mRNA degradation. Only when the poly(A) tails are shortened to a certain length does the CCR4–NOT complex take over and deadenylate the mRNA in a processive manner, committing it to full decay[31,68]. Consequently, the activity of the PAN2–PAN3 complex may not be detectable unless the CCR4–NOT complex is inhibited and the length of the mRNA poly(A) tail is accurately determined. Nevertheless, depletion of PAN3 exacerbates the effects of NOT1 depletion[25], indicating that PAN2–PAN3 participates in the degradation of miRNA reporters.

Interaction of GW182 proteins with the CCR4–NOT complex. The CCR4–NOT deadenylase complex has a central role in post-transcriptional mRNA regulation[31]. It catalyses the shortening of mRNA poly(A) tails and consequently causes or consolidates translational repression. As mentioned above, the complex couples deadenylation to decapping and 5′-to-3′ exonucleolytic degradation by XRN1; therefore, it can also lead to full degradation of the target mRNA in some cellular contexts[31]. In addition, the CCR4–NOT complex has the remarkable ability to repress translation in the absence of mRNA deadenylation and decay[26,30,69–71]. Accordingly, in the context of the miRNA pathway, the CCR4–NOT complex not only mediates deadenylation but is also involved in both the translational repression and the degradation of miRNA targets[16,21,24–27,29,30,70].

The CCR4–NOT complex consists of several independent modules that dock with NOT1, the central scaffold subunit (FIG. 4a). NOT1 features a modular domain organization (FIG. 4a) consisting of separate α-helical domains, which provide binding surfaces for the individual modules. A central domain of NOT1 — structurally related to the middle portion of eukaryotic translation initiation factor 4G (eIF4G) and thus termed the NOT1 MIF4G domain — is the docking site for the catalytic module, which comprises two deadenylases, namely CAF1 (or its paralogue POP2; also known as CNOT7 and CNOT8, respectively, in humans) and CCR4A (or its paralogue CCR4B, also known as CNOT6 and CNOT6L, respectively, in humans)[31,72,73] (FIG. 4a,b). The NOT1 MIF4G domain also serves as a binding platform for DDX6 (REFS 29,30) (FIG. 4c–e), which functions as a translational repressor in addition to interacting with decapping factors such as EDC3 (REFS 29,30,32,74). Thus, the NOT1 MIF4G domain coordinates the activities of the CCR4–NOT complex by providing binding sites for the factors that catalyse deadenylation, translational repression and decapping[29].

Immediately downstream of the MIF4G domain, NOT1 contains a CAF40/NOT9-binding domain (CN9BD), which forms a stoichiometric complex with the highly conserved NOT9 subunit[29,30,71]. This binary complex mediates binding to the GW182 proteins through tandem W-binding pockets present in the NOT9 subunit[29,30] (FIG. 4a,f,g). Similar to the structure of AGO2 (REF. 59), the

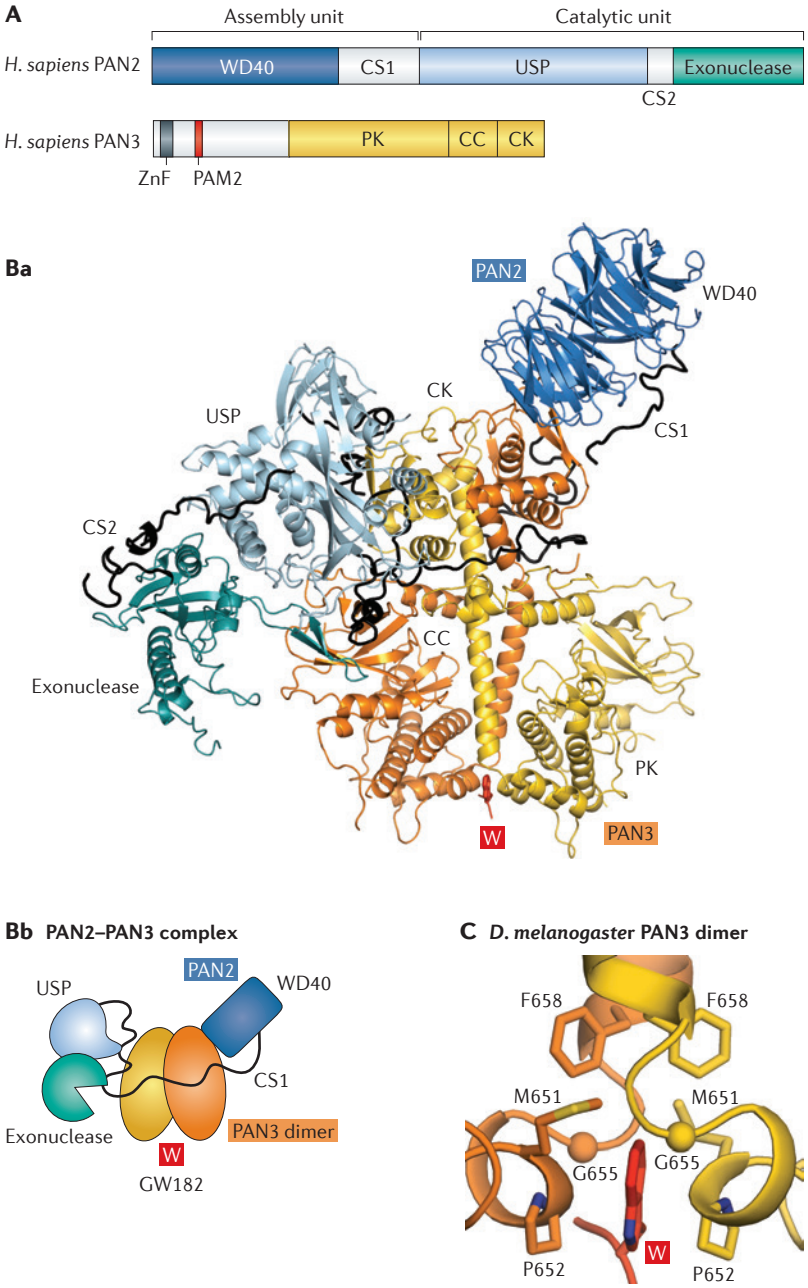


Figure 3 | **Assembly and interaction of the PAN2–PAN3 complex with GW182 proteins. A** | Domain organization of *Homo sapiens* PAN2 and PAN3 proteins is depicted. PAN2 contains three globular domains: an amino-terminal WD40 domain, a ubiquitin-specific protease (USP) domain and an exonuclease domain, which are linked by connector sequence 1 (CS1) and CS2[28,67]. PAN3 contains a zinc-finger (ZnF) domain, PABP-interacting motif 2 (PAM2), a pseudokinase (PK) domain, a coiled-coil (CC) and a carboxy-terminal knob (CK) domain[28,65–67]. The CC mediates PAN3 homodimerization and provides a tryptophan (W)-binding pocket at its base. PAN2 interacts with the PAN3 CK domain using an extensive interface contributed by the WD40 domain and CS1 (REFS 28,65–67). **B** | Structural model shows the assembly of the PAN2–PAN3 complex with a W-containing peptide (red) bound to PAN3. The model was generated by superposing the structures of the *Saccharomyces cerevisiae* and *Chaetomium thermophilum* PAN2–PAN3 complexes with the *Drosophila melanogaster* PAN3 structure (RCSB Protein Data Bank codes: 4D0K, 4XR7 (see also 4Q8J) and 4BWP, respectively)[28,66,67]. The crystal structures are shown in part **Ba**, and the schematic organization of the domains is shown in part **Bb**. **C** | Close-up view of the W-binding pocket at the *D. melanogaster* PAN3 dimer interface is shown. Residues lining the pocket are indicated as sticks and labelled.

distance between the two W-binding pockets in NOT9 is 20–25 Å, which could be spanned by a minimum of 8–10 residues in an extended conformation[29,30] (FIG. 4f,g). Thus, the molecular modes of interaction of GW182 proteins with AGO proteins and with the CN9BD–NOT9 complex are similar. In both cases, the main contacts are mediated by two W residues that are 8–10 residues apart[29,30,59], which raises the question of how binding specificity is achieved. As discussed above, the flanking sequences and the spatial arrangement of the W residues seem to confer specificity and contribute to the affinity of the interactions.

In addition to the W-binding pockets identified on NOT9, the CCR4–NOT complex contains other binding sites for GW182 proteins[29,30]. The precise location of these additional binding sites remains unclear, but they are most likely to be present in NOT1 because a NOT1 mutant that does not interact with NOT9 still retains the ability to bind to GW182 proteins[29,30]. Accordingly, various NOT1 fragments, including the C-terminal NOT1 superfamily homology domain (SHD), interact with TNRC6 proteins[24,30]. Thus, the CCR4–NOT complex probably features multiple and redundant W-binding pockets that synergize to mediate its recruitment to miRNA targets.

Interestingly, GW182 proteins may be more than just binding platforms for the CCR4–NOT complex. They can also activate the deadenylase activity of the complex *in vitro*[27], thereby functioning as deadenylation co-activators, although it is not yet clear how this activation is achieved.

Interaction of GW182 proteins with PABPC. In addition to their interaction with AGO proteins and deadenylase complexes, GW182 proteins bind to PABPC[23,24,75–78]. PABPC is a highly conserved eukaryotic protein that interacts with various translation factors and thereby stimulates translation[79]. PABPC consists of four N-terminal RNA recognition motifs (RRM1–RRM4), a proline-rich unstructured linker and a C-terminal domain termed MLLE (referring to a conserved signature motif KITGMLLE; also known as PABC)[79] (BOX 2). The MLLE domain recognizes PABP-interacting motif 2 (PAM2) sequences, which are present in several proteins involved in translation regulation or mRNA decay[79]. These include the translational regulators PABP-interacting protein 1 (PAIP1) and PAIP2, as well as PAN3 (REFS 64,79). PAIP1 and PAIP2 also contain a PAM1 sequence, which interacts with the PABPC RRMs[79].

Similarly to PAIP1 and PAIP2, GW182 proteins contain two binding sites for PABPC[78]. These binding sites are located in the SD. One binding site is a PAM2 motif, which interacts with the C-terminal MLLE domain in a manner similar to that of other PABP-interacting proteins[23,76–78] (BOX 2). The second, less-defined site is contributed by the M2 and C-terminal regions of the SD[75,78]. This second binding site mediates binding to PABPC RRMs[75,78] and is required for the GW182–Pabpc interaction in *D. melanogaster*. By contrast, the PAM2 motif of human TNRC6 proteins is sufficient for PABPC binding[23,75–78].

How does the GW182–PABPC interaction contribute to silencing? One model posits that PABPC may serve as an anchoring point for miRISCs to hook on to polyadenylated mRNAs, thereby enhancing miRISC affinity and specificity for functional mRNAs containing an intact poly(A) tail. Accordingly, polyadenylated mRNAs associate more efficiently with AGO proteins *in vitro*[41] and are more efficiently repressed in human and *D. melanogaster* cells[70,80,81]. Alternatively, GW182 binding to PABPC may interfere with its normal function in stimulating translation, thereby directly contributing to the repression[23,35,75,78,81].

Several observations in diverse systems are consistent with a role for PABPC in silencing. For example, PABPC depletion from cell-free extracts abolishes miRNA-mediated deadenylation[23]. Likewise, PABPC integrity is required for miRNA-mediated repression in rabbit reticulocyte lysates[40]. Moreover, mutations in the PAM2 motif impair the silencing activity of GW182 proteins in *D. melanogaster* and humans cells and exacerbate the inhibitory effect of mutations in the W motifs of the SD in complementation assays[24,78]. Although GW182 proteins carrying mutations in the PAM2 motif are functionally impaired in complementation assays, they are still active when artificially tethered to target mRNAs[24,70,78,82], suggesting a role for the GW182–PABPC interaction in the recruitment of miRISCs to target mRNAs.

Arguing against an essential role for Pabpc in silencing is the observation that its depletion does not inhibit silencing in *D. melanogaster* cell-free extracts or in zebrafish embryos[37,82]. More importantly, miRNAs can silence mRNA reporters lacking poly(A) tails in human and *D. melanogaster* cells[18,25–27,37,48,70,81,82], suggesting that the poly(A) tail and, consequently, PABPC may enhance silencing but are not prerequisites for miRISC activity, at least in some cellular contexts.

Nevertheless, the GW182–PABPC interaction has been maintained throughout evolution, suggesting that it has an important role in silencing. Indeed, the PAM2 motif is evolutionarily conserved and is a defining feature of GW182 proteins[23,50,54]. Even the highly divergent *C. elegans* AIN-1 protein, which does not contain an identifiable PAM2 motif, has retained the ability to interact with PABPC[83]. Interestingly, the genome of the parasitic nematode *Brugia malayi* encodes a GW182 protein that displays a similar domain organization to the vertebrate proteins and contains a PAM2 motif[54]. Thus, the GW182–PABPC interaction might be important for silencing, perhaps in cellular contexts in which translation efficiency and poly(A) tail length are correlated[13] and translation is susceptible to PABPC-mediated stimulation. Alternatively, the GW182–PABPC interaction may represent one of many redundant interactions that promote the association of miRISCs with target mRNAs.

miRNA-mediated translational repression

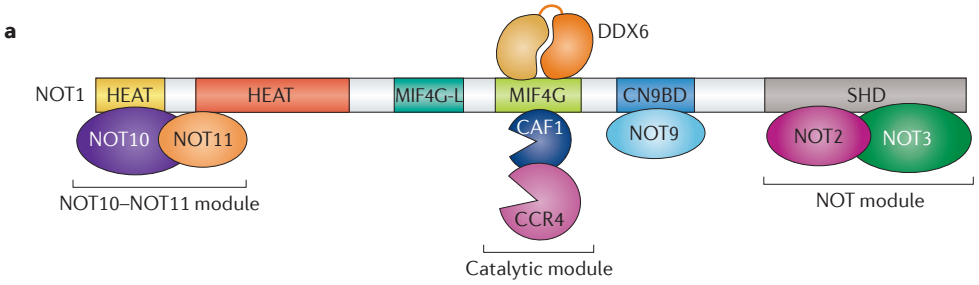
In addition to accelerating mRNA degradation, miRNAs also trigger translational repression. Translational repression is observed at early time points after miRNA expression, but the effects are generally weak, and by the time full repression is established, mRNA deadenylation or full destabilization is the dominant effect of miRNA-mediated silencing[13]. Nevertheless, global analysis of miRNA function indicates that 'pure' translational repression (that is, that which cannot be attributed to mRNA decay) accounts for 6–26% of the repression of each endogenous target in mammalian cells[13]. More importantly, translational repression can be uncoupled from mRNA destabilization effects through the use of various artificial mRNA reporters that lack poly(A) tails and are resistant to deadenylation. These reporters are efficiently silenced, indicating that miRISCs have the ability to repress translation in the absence of mRNA degradation[18,25,26,37,38,40,48,70,82,84–87].

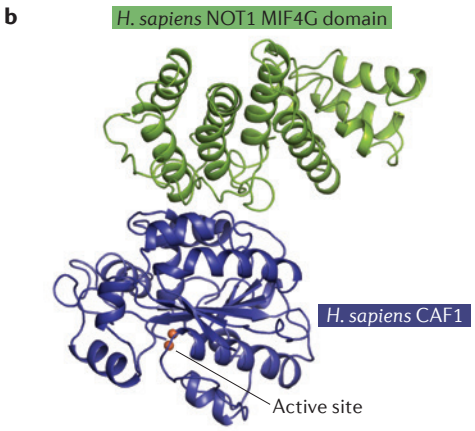
The question of how miRNAs repress translation has long been controversial, and models invoking inhibition at translation initiation and at post-initiation steps have been proposed[5,6]. This controversy has been largely resolved with the advent of the ribosome profiling method, which allows accurate measurements of translation efficiencies[5,11–13]. The use of this approach to untangle the effects of miRNAs on translation indicated that inhibitory mechanisms occurring post-initiation could be ruled out[5,11]. The emerging consensus in the field is that miRNAs inhibit cap-dependent translation at initiation[35,38,40,49,84–90], but the precise molecular mechanism for this remains to be resolved.

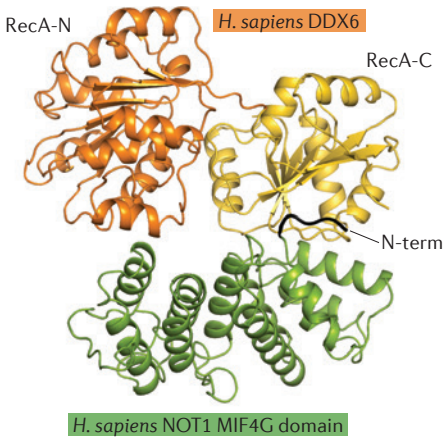
Interplay between translational repression and mRNA decay. In spite of the vast progress in our understanding of miRNA-mediated target degradation, the question of whether translation must be inhibited before deadenylation and decay can occur remains unresolved. This question is not specific to the miRNA pathway; instead, it applies generally to all cellular mRNAs. Kinetic studies aimed at dissecting the temporal effects of miRNAs on mRNA translation, deadenylation and decay have demonstrated that translational repression precedes mRNA degradation[8–13,39,91,92]. This order of events has been interpreted as evidence that mRNA decay is a consequence of the translational block[39,92]. However, this temporal order could simply reflect kinetic differences between translational repression and decay, rather than causality[12,13,91]. In agreement with this interpretation of non-causality, it has been shown that mRNAs with different poly(A) tail lengths are translated equally well and associate with polysomes (multiple ribosomes) in diverse cellular systems[12]. This observation argues against a requirement for translational inhibition before deadenylation can start and rather suggests that, in general, deadenylation is a slow process that occurs co-translationally until the mRNA is definitively degraded.

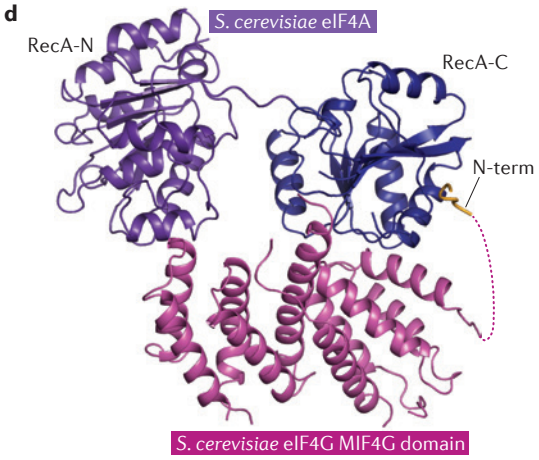
Furthermore, in the same way that translational repression can be uncoupled from mRNA degradation, mRNA degradation can occur when translation is blocked at different steps or even in the complete absence of translation. For example, miRNA targets are degraded in the presence of translation inhibitors (for example, puromycin, Torin 1 and cycloheximide), or

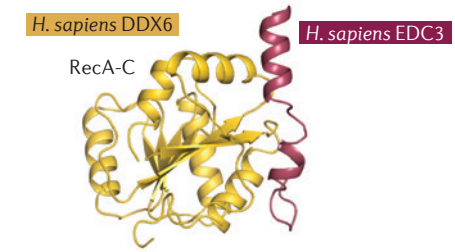
Cap-dependent translation
Initiation of translation that requires the ternary eukaryotic translation initiation factor 4F (eIF4F) complex, which consists of the cap-binding protein eIF4E, the adaptor protein eIF4G and the DEAD box RNA helicase eIF4A. This complex interacts with the cap structure at the 5′ end of an mRNA molecule and recruits the 43S pre-initiation complex.

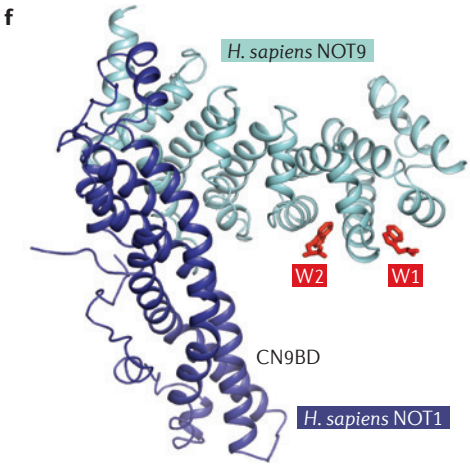












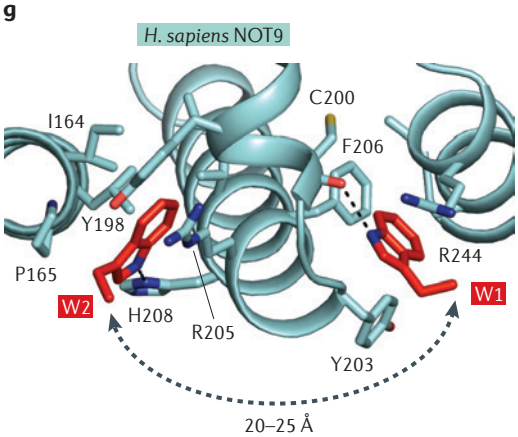


Figure 4 | **Assembly and interaction of the CCR4–NOT complex with GW182 proteins.** **a** | NOT1 consists of independently folded α-helical domains, most of which are structurally related to HEAT repeat domains. Two domains show additional homology with the middle domain of eukaryotic translation initiation factor 4G (eIF4G): the MIF4G-like (MIF4G-L) and the MIF4G domain. Furthermore, NOT1 contains a CAF40/NOT9-binding domain (CN9BD) and a carboxy-terminal NOT1 superfamily homology domain (SHD). The subunits that form independent modules and their binding sites on NOT1 are indicated. The catalytic module comprises the two deadenylases CAF1 and CCR4A (or its paralogue CCR4B)[72,73]. **b** | Structure of the *Homo sapiens* NOT1 MIF4G domain bound to the CAF1 deadenylase (RCSB Protein Data Bank code: 4GMJ) is shown. The enzyme active site contains two bound catalytic magnesium ions (orange)[72]. **c** | Crystal structure of the human NOT1 MIF4G domain bound to DEAD box protein 6 (DDX6; PDB code: 4CT4)[30] is depicted. Comparison of structures in part **b** and part **c** indicates that CAF1 and DDX6 could bind to NOT1 simultaneously. **d** | The *Saccharomyces cerevisiae* eIF4G MIF4G domain bound to eIF4A (PDB code: 2VSO)[105] shows structural similarity to the NOT1–DDX6 complex shown in part **c**. The eIF4A1 residues involved in binding to the eIF4G MIF4G domain are conserved in eIF4A2, and both proteins bind to eIF4G[29,30]. By contrast, DDX6 is specifically recognized by NOT1 (REFS 29,30,101). The binding specificities are mediated, in part, by the extended amino-termini of the MIF4G domains (N-term; shown in black and yellow for NOT1 and eIF4G, respectively), which read out additional parts of the helicase surface and increase binding affinity. **e** | Structure of the human DDX6 RecA-C domain bound to an enhancer of decapping 3 (EDC3) peptide[106] is shown. **f** | Crystal structure of CN9BD bound to the NOT9 subunit (PDB code: 4CRV)[29] shows that NOT9 contains two binding pockets for tryptophan (W). **g** | Close-up view of the W-binding pockets of NOT9 is shown. Residues lining the pockets are shown as sticks and labelled for orientation. The minimal distance between the two W residues is indicated.

when translation initiation is blocked owing to the lack of a natural cap structure or the insertion of strong secondary structures in the proximity of the 5′ cap structure[19,22,35,89]. Another observation that has been largely ignored is that miRNAs can trigger the deadenylation of short mRNA fragments containing miRNA-binding sites but lacking open reading frames[23]. These results clearly exclude silencing models that invoke ribosome scanning[93] or a reduction in the number of translating ribosomes as a prerequisite for mRNA destabilization, but they do not rule out that translation must be inhibited before actively translating mRNAs can be degraded. In yeast, mRNA degradation can occur co-translationally[94], so it would be of interest to determine whether this is also the case in metazoans for both bulk mRNA and miRNA targets.

CCR4–NOT links translational repression and mRNA decay. Although translational repression and mRNA destabilization can be uncoupled, the two processes could still be mechanistically linked and could therefore represent two consecutive outcomes of a single molecular mechanism that both interferes with translation and triggers deadenylation. One appealing possibility is that both effects are mediated by the CCR4–NOT complex and are thus intimately interconnected. This model is based on the following observations. First, in *D. melanogaster* and mammalian cells, GW182 proteins play a central part in miRNA-target repression and degradation through their interaction with the CCR4–NOT complex[24–27,29,30]. Second, the *C. elegans* GW182 protein AIN-1 has also retained the ability to interact with the CCR4–NOT complex, although using a different mode of interaction[83]. Last, and most importantly, similarly to miRISCs, the CCR4–NOT complex can repress translation independently of deadenylation[26,69–71]. Together, these observations indicate that the CCR4–NOT complex is a conserved and central downstream effector of silencing that can elicit both of the effects that miRISCs have on their targets.

GW182-independent silencing mechanisms. Although a central role for the GW182 proteins and the downstream CCR4–NOT effector complex in silencing is undisputed, several studies have provided evidence for alternative repressive mechanisms that act independently of GW182 proteins and CCR4–NOT, at least in *D. melanogaster* cells. For example, *D. melanogaster* Ago1 can mediate translational repression independently of the GW182 protein[37,38,95,96]. It is not known whether this is also the case for the human AGO proteins. Moreover, GW182 proteins can inhibit translation independently of the CCR4–NOT complex[38]. The molecular bases of these alternative pathways, their contribution to silencing *in vivo* and their conservation remain to be determined.

A role for DEAD box RNA helicases in silencing

A crucial unanswered question regarding the mode of action of the miRISCs (which by extension also applies to the CCR4–NOT complex) is the precise molecular mechanism by which translation initiation is inhibited. Current models are linked to RNA helicases: they involve either the initiation factor paralogues eIF4A1 and eIF4A2 (bona fide RNA helicases) or the translational repressor and decapping activator DDX6 (a putative RNA helicase). However, it is important to note that other proteins, including EDD[97] (also known as UBR5) and tripartite motif-containing protein 32 (TRIM32)[98], have been implicated in the regulation of translational repression by miRNAs through interactions with DDX6, but because even less is known about their mechanisms, we do not discuss them here.

eIF4A-dependent mechanisms. eIF4A RNA helicases are translation initiation factors that unwind secondary structures within mRNA 5′ untranslated regions (UTRs), allowing the 43S pre-initiation complexes to scan the 5′ UTR towards the start codon[99]. Several studies indicate that the miRISCs interfere with this process of ribosome scanning by targeting eIF4A[93,95,100]. However, individual studies diverge on how this interference is achieved, and a consensus of how eIF4A contributes to silencing is lacking. One study found that miRISCs specifically recruit eIF4A2 (but not its paralogue eIF4A1) through interactions with the CCR4–NOT complex in human cells[93]. Specifically, the MIF4G domain of NOT1 was postulated to interact with eIF4A2 in a manner analogous to the interaction of eIF4G with eIF4A[93] (FIG. 4d). The interaction with NOT1 was suggested to lock eIF4A2 onto the mRNA 5′ UTR, thereby setting up a roadblock for scanning by the 43S complexes and consequently repressing translation initiation[93].

Cap structure
Eukaryotic mRNA is modified by the addition of an $m^7G(5')ppp(5')N$ structure (7-methylguanosine attached, via its 5′ hydroxyl group, by a triphosphate group to the 5′ hydroxyl group of the first encoded nucleoside) at the 5′ terminus. Capping is essential for several important steps of gene expression, including mRNA stabilization, splicing, mRNA export from the nucleus and translation initiation.

Ribosome scanning
The 5′-to-3′ migration of the 43S pre-initiation complex towards the initiation codon. The 43S pre-initiation complex comprises a 40S ribosomal subunit, eukaryotic translation initiation factor 3 (eIF3), eIF1 and eIF1A, the ternary eIF2–GTP–Met-$tRNA^{Met}_i$ complex and most likely eIF5.

This model has been questioned in subsequent studies showing that the MIF4G domain of human NOT1 directly binds to the putative RNA helicase DDX6 but not eIF4A2 (see below)[29,30,101]. Furthermore, experiments in human and *D. melanogaster* cell-free extracts have indicated that miRNAs repress translation by releasing, rather than recruiting, eIF4A and that there is no distinction between the release of eIF4A1 and the release of eIF4A2 from the mRNA[95,100]. In *D. melanogaster* cell-free extracts, GW182 tethering to reporter mRNAs causes displacement of eIF4A as well as of the cap-binding protein eIF4E[95], although whether this dissociation requires interaction with the Ccr4–Not complex or other partners has not been determined. Similarly, whether the CCR4–NOT complex is required for the release of eIF4A1 or eIF4A2 in human cells has not been directly investigated[100]. Notably, in *D. melanogaster* cell-free extracts, Ago1 could also promote eIF4A displacement independently of GW182, providing further support for the idea that a GW182- and Ccr4–Not-independent repressive mechanism operates in insect cells[37,38,95,96].

Independently of whether eIF4A is recruited or released, an important outcome of the models involving eIF4A is that miRNAs can repress only mRNAs that are translated via a scanning- and eIF4A-dependent mechanism. Consistent with this model, an mRNA with an unstructured 5′ UTR, which does not require eIF4A activity for translation, has been shown to be impervious to silencing[93]. However, another study failed to detect a correlation between 5′ UTR secondary structure and the magnitude of miRNA repression[40]. Similarly, drugs that inhibit eIF4A activity, such as pateamine A, hippuristanol and silvestrol, have yielded contradictory results, most likely owing to the difficulty of finding appropriate normalization controls that are not affected by the treatment[93,100,102].

Perhaps the most compelling evidence that miRNAs interfere with ribosome scanning stems from the use of internal ribosomal entry sites (IRESs) to drive the translation of miRNA reporters. IRESs bypass specific steps in canonical cap-dependent translation initiation[99] and can therefore help to pinpoint the step of translation that is targeted by miRNAs. In particular, several studies have reported that miRNAs fail to repress translation initiated by IRESs that bypass scanning, such as those in hepatitis C virus, cricket paralysis virus (CrPV) and the *D. melanogaster reaper* gene[40,85,93,95,100], but miRNAs do repress translation driven by IRESs that require eIF4A activity, such as those in encephalomyocarditis virus (EMCV)[93,100] and poliovirus[40]. However, the results obtained with the EMCV IRES are controversial, as this IRES has been reported to confer both immunity[35,40,85–87] and sensitivity[93,100] to miRNA-mediated repression. Furthermore, numerous studies have reported that only cap-mediated, and not IRES-mediated, translation initiation is sensitive to miRNA-mediated repression[35,40,80,85–88,90]. Despite these inconsistencies, the picture that emerges from these and additional studies is that miRNAs interfere with the assembly and/or function of the eIF4F complex by targeting eIF4E[86,90], eIF4G[103] or eIF4A[93,95,100].

Internal ribosome entry sites
(IRESs). Structured RNA elements, usually present in the 5′ untranslated region, that allow cap-independent association of ribosomes with mRNAs.

A DDX6-dependent mechanism. An alternative, although not mutually exclusive, model for how the miRISCs and the CCR4–NOT complex can repress translation involves DDX6. This model is based on the observation that DDX6 depletion suppresses miRNA-mediated silencing, as well as the silencing caused by TNRC6 SDs tethered to reporter mRNAs in human cells[29,30,101,104]. Furthermore, DDX6 mutants that do not interact with NOT1 do not rescue the silencing of miRNA reporters in human cells depleted of endogenous DDX6 (REFS 29,30,101), indicating that DDX6 is recruited to miRNA targets through interactions with the CCR4–NOT complex and acts downstream of CCR4–NOT in this pathway.

Interaction of DDX6 with the CCR4–NOT complex. The complex formed by DDX6 and the NOT1 MIF4G domain is structurally related to the eIF4A–eIF4G complex, and the interfaces involve equivalent structural elements[29,30,101,105] (FIG. 4a,c,d). Similarly to all DEAD box proteins, DDX6 and eIF4A consist of tandem RecA-like domains. These domains interact with the MIF4G domains of NOT1 and eIF4G, respectively, in a manner similar to those described for other DEAD box proteins and MIF4G domains in a wide range of proteins[29,30,105]. Although binding is mediated by equivalent structural elements, specificity is imparted by differences in the amino acid sequences of the binding pairs.

The structural similarity between the eIF4G–eIF4A and NOT1–DDX6 complexes extends beyond the interactions mediated by the RecA and MIF4G domains. Indeed, in the eIF4G–eIF4A complex, the interface is extended by interactions mediated by an unstructured N-terminal tail of the MIF4G domain of eIF4G, which contacts the lateral surface of the C-terminal RecA domain (RecA-C) of eIF4A[105] (FIG. 4d). This extension contributes to the affinity of the interaction in the eIF4G–eIF4A complex[105]. An analogous interaction between an unstructured N-terminal tail of the NOT1 MIF4G domain and the DDX6 RecA-C domain is observed in the NOT1–DDX6 complex, and this is likely to contribute to the specificity of NOT1 for DDX6 (REF. 30) (FIG. 4c). Furthermore, upon binding, the NOT1 MIF4G domain induces conformational changes in DDX6 that stimulate its ATPase activity, similarly to the stimulation of eIF4A activity by eIF4G[30,105].

The structure of the DDX6–NOT1 complex has revealed a long-sought direct molecular link between the deadenylation and decapping machineries and, together with previous structures, enabled the connection of consecutive steps in the 5′-to-3′ mRNA decay pathway (FIG. 5). These previous structures include the following complexes: NOT1–CAF1 (REFS 72,73) (FIG. 4b), DDX6–EDC3 (REF. 106) (FIG. 4e), EDC3 bound to an α-helical leucine-rich motif (HLM) present in DCP1 in metazoans[107] and the yeast Dcp1–Dcp2 decapping complex[108]. It remains to be determined whether all of these structurally characterized molecular interactions occur simultaneously in the context of the full-length proteins and whether the assembly of these complexes on the mRNA is regulated.

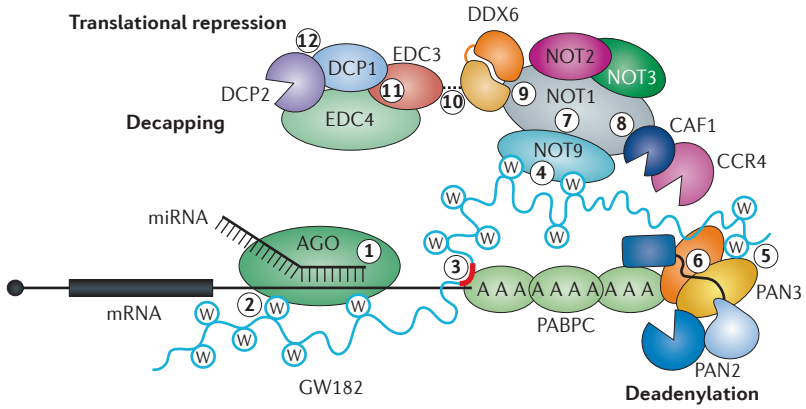


Figure 5 | **Structure-based model of miRNA-mediated silencing.** Structural studies allow us to describe in molecular terms a chain of direct interactions that connects microRNA (miRNA) target recognition to translational repression, deadenylation and decapping of the miRNA target. The numbers in white circles indicate the interactions supported by crystal or nuclear magnetic resonance (NMR) structures, including the structures of human Argonaute 2 (AGO2) bound to miRNA mimics[59,61,62] (interaction 1); human AGO2 bound to tryptophan (W) residues[59] (interaction 2); the cytoplasmic poly(A)-binding protein (PABPC) MLLE domain bound to PABP-interacting motif 2 (PAM2) of the GW182 family member trinucleotide repeat-containing 6C (TNRC6C)[76,77] (interaction 3); negative regulator of transcription 9 (NOT9) and PAN3 bound to W residues[28–30] (interactions 4 and 5, respectively); the PAN2–PAN3 complex[28,65–67] (interaction 6); the NOT1 CAF40/NOT9-binding domain (CN9BD) bound to NOT9 (REFS 29,30) (interaction 7); the NOT1 MIF4G domain bound to CAF1 (REFS 72,73) (interaction 8); the NOT1 MIF4G domain bound to DEAD box protein 6 (DDX6)[29,30] (interaction 9); DDX6 bound to an enhancer of decapping 3 (EDC3) peptide[106] (interaction 10; shown as a dashed line because it is not known whether DDX6 binds to EDC3 and NOT1 simultaneously); EDC3 bound to a helical leucine-rich motif (HLM) present in decapping protein 1 (DCP1) in metazoans[107] (interaction 11); and the *Schizosaccharomyces pombe* Dcp1–Dcp2 complex[108] (interaction 12). The cap structure is shown as a black circle.

Mechanisms of DDX6 function. Mechanistically, DDX6 has a dual function in post-transcriptional mRNA regulation. It can repress translation in the absence of mRNA degradation and can also stimulate decapping, thus committing deadenylated mRNAs to full degradation[74]. For example, in *D. melanogaster* cells, depletion of the DDX6 orthologue Me31B allows deadenylation but inhibits decapping and the subsequent degradation of polyadenylated miRNA targets. As a result, miRNA targets accumulate as deadenylated, repressed mRNA decay intermediates[19,21]. These and additional observations support a role for DDX6 in the activation of decapping[32,74], which could be achieved, at least in part, through the DDX6-mediated recruitment of decapping factors to mRNAs undergoing deadenylation.

A key remaining question is how DDX6 represses translation. DDX6 interacts with eIF4E transporter (4E-T; also known as EIF4ENIF1)[109], an eIF4E-binding protein that competes with eIF4G for binding to eIF4E and represses translation initiation. However, depletion of 4E-T only partially alleviates silencing[110]. This implies that DDX6, beyond its putative disruption of the eIF4E–eIF4G interaction, may use additional mechanisms to repress translation that are so far unknown. In this context, it is interesting to note that the ATPase activity of DDX6, which is stimulated by NOT1, is required for miRNA-mediated silencing[30].

Conclusions and future directions

Structural studies along with genome-wide measurements of miRNA effects on mRNA and protein levels have streamlined our understanding of miRNA-mediated silencing and simplified future studies. It is now well established that the miRISCs directly recruit the cellular mRNA decay machinery and that mRNA degradation is the dominant effect of miRNAs at steady state. Although there is still much to be learned about the mechanism of silencing, the structures of human AGO2 bound to miRNA mimics[59,61,62], of AGO2, PAN3 and NOT9 bound to W residues[28–30,59], of the NOT1–DDX6 complex[29,30] and of DDX6 bound to decapping factors[106] enable a detailed molecular-level understanding of consecutive steps in this pathway (FIG. 5) and provide a structural framework for future mechanistic studies.

Despite the wealth of molecular information, studying the mechanism of silencing remains challenging because of the combinatorial, redundant and promiscuous interactions between the factors involved. An extreme example is provided by the W motifs of GW182 proteins. Although structural studies have yielded initial clues for how these W motifs are recognized, we are still far from being able to explain how specificity and sufficiently high affinity are achieved and whether there is a hierarchy of binding. Furthermore, GW182 partners have been reported to interact with each other independently of GW182, adding redundancy and functional plasticity to the interaction network. For example, the PAN2–PAN3 complex interacts with PABPC and eventually with the CCR4–NOT complex[64,111]. DDX6 interacts with the CCR4–NOT complex and forms mutually exclusive interactions with multiple decapping factors[29,30,32,101]. In addition, DDX6 interacts with EDD and TRIM32, which have been implicated in miRNA-mediated silencing[97,98]. This redundancy offers alternative ways to assemble silencing complexes, which might differ in their functional outcomes. For example, the recruitment of decay factors through direct interactions with GW182 proteins might have a different effect on silencing than recruitment through indirect interactions, potentially resulting in distinct molecular mechanisms. These mechanisms might become dominant in a context-dependent manner or under specific experimental conditions, providing a potential explanation for the seemingly conflicting results reported in the literature.

It is also conceivable that additional complexity is introduced through the expression of alternative isoforms or post-translational modifications that alter the binding properties or affinities of the interacting partners. For example, phosphorylation of human AGO2 by AKT3 increases the interaction with GW182 proteins[112]. Similarly, mitogenic signalling in *D. melanogaster* cells regulates the association of Ago1 with GW182 (REF. 96). The available structural information can now be harnessed to investigate the regulatory role of these and additional post-translational modifications, which has not yet been fully explored.

An additional challenge for future studies involves understanding exactly how the miRISCs repress translation and the interplay between translational repression

and mRNA destabilization. In particular, it remains unclear whether the recruitment of CCR4–NOT fully explains silencing or whether parallel repressive mechanisms occur in a species-specific manner. In our view, the answers to these questions lie, in part, in the elucidation of the mechanisms by which eIF4A is dislodged from the mRNA target and by which CCR4–NOT and DDX6 repress translation. Bearing in mind that the CCR4–NOT complex is recruited by various RNA-binding proteins to a range of mRNA targets in addition to those targeted by miRNAs[29,31], there is no doubt that these studies will be worthwhile and will substantially contribute to our understanding of post-transcriptional mRNA regulation in eukaryotic cells.

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