Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?

Witold Filipowicz, Suvendra N. Bhattacharyya* and Nahum Sonenberg‡*

Abstract | MicroRNAs constitute a large family of small, approximately 21-nucleotide-long, non-coding RNAs that have emerged as key post-transcriptional regulators of gene expression in metazoans and plants. In mammals, microRNAs are predicted to control the activity of approximately 30% of all protein-coding genes, and have been shown to participate in the regulation of almost every cellular process investigated so far. By base pairing to mRNAs, microRNAs mediate translational repression or mRNA degradation. This Review summarizes the current understanding of the mechanistic aspects of microRNA-induced repression of translation and discusses some of the controversies regarding different modes of microRNA function.

MicroRNAs (miRNAs), which are approximately 21-nucleotide-long RNA regulators of gene expression[1–3], have become a major focus of research in molecular biology. Although for a long time they were considered to be exclusive to multicellular organisms and possibly essential for the transition to a more complex organism design, the recent identification of miRNAs in the unicellular algae *Chlamydomonas reinhardtii*[4,5] indicates that miRNAs are probably evolutionarily older than originally thought. One to two hundred miRNAs are expressed in lower metazoans and plants, but at least a thousand are predicted to operate in humans. Functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated and that changes in their expression are observed in — and might underlie — human pathologies, including cancer[1,2,6–8]. These findings are perhaps not so surprising as bioinformatic predictions indicate that mammalian miRNAs can regulate ~30% of all protein-coding genes.

With just one possible exception noted so far[9], miRNAs control gene expression post-transcriptionally by regulating mRNA translation or stability in the cytoplasm[10–14]. However, further functions of miRNAs seem likely. For example, by virtue of base pairing to RNA, miRNAs could regulate pre-mRNA processing in the nucleus or act as chaperones that modify mRNA structure or modulate mRNA–protein interactions. Indications that mammalian miRNAs can be imported into the nucleus[15] or even secreted from the cell[16] will motivate searches for currently unidentified functions for this class of molecule. What is already certain is that the discovery of miRNAs has revealed an important new dimension in the complexity of post-transcriptional regulation of eukaryotic gene expression. We are beginning to understand why the 3′ UTRs of mRNA, with which the miRNAs and other factors interact, are often so long and so important for gene function.

The mechanistic details of the function of miRNAs in repressing protein synthesis are still poorly understood. miRNAs can affect both the translation and stability of mRNAs, but the results from studies conducted in different systems and different laboratories have often been contradictory: a comprehensive and lucid picture of the mechanism of miRNA-mediated repression is difficult to elaborate. In this Review, after briefly introducing miRNAs and their biogenesis, we summarize what is currently known about the mechanistic aspects of their function in controlling mRNA stability and translation, focusing primarily on animal cells. We also discuss the cellular localization and reversibility of miRNA-mediated repression. For further recent Reviews covering these topics, see REFS 11–14,17,18, and more general information about the biogenesis, diversity and function of miRNAs can be found in REFS 1–3,19–23.

miRNA and micro-ribonucleoprotein biogenesis

miRNA precursors fold into imperfect dsRNA-like hairpins, from which miRNAs are excised in two steps, both of which are catalyzed by Drosha (also known as

**Friedrich Miescher Institute for Biomedical Research, 4002 Basel, Switzerland.*
‡Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, Quebec, Canada, H3G 1Y6.
Correspondence to W.F. or N.S.
e-mails:
witold.filipowicz@fmi.ch;
nahum.sonenberg@mcgill.ca
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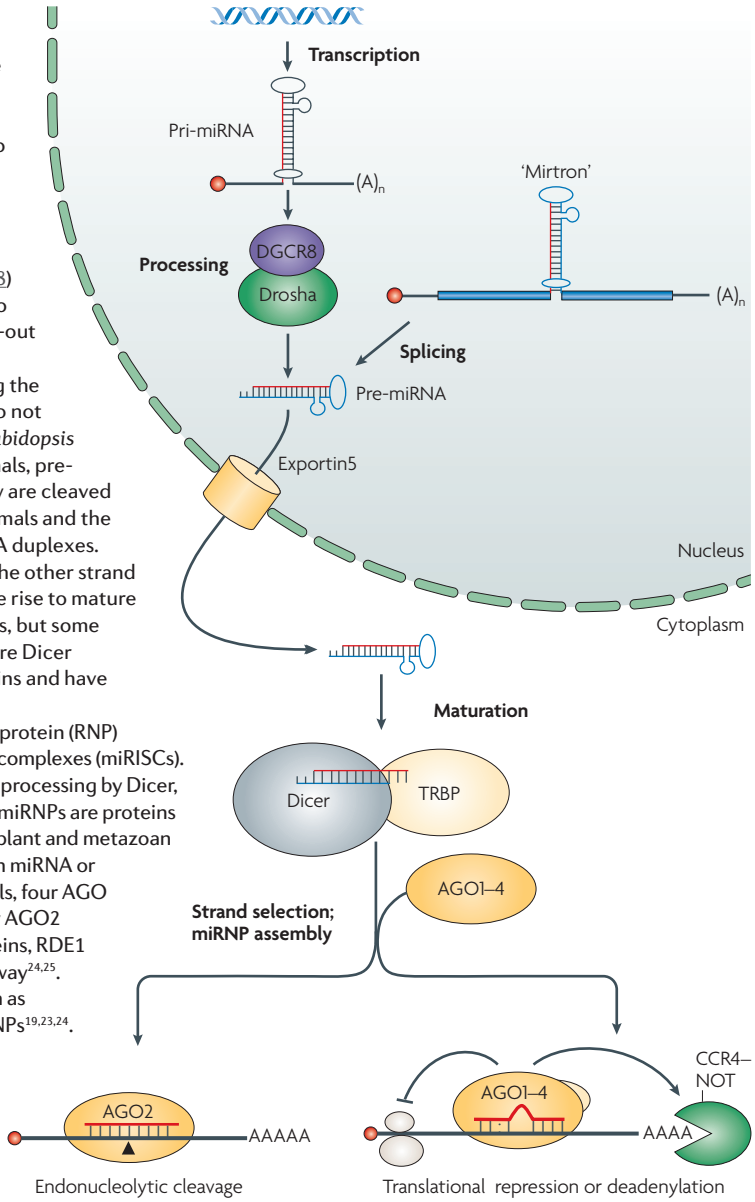
Box 1 | **Biogenesis of miroRNAs and their assembly into microribonucleoproteins**

microRNAs (miRNAs) are processed from precursor molecules (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase II transcripts. A single pri-miRNA often contains sequences for several different miRNAs. Pri-miRNAs fold into hairpin structures containing imperfectly base-paired stems and are processed in two steps, catalysed by the RNase III type endonucleases Drosha (also known as RN3) and Dicer. Both Drosha and Dicer function in complexes with proteins containing dsRNA-binding domains (dsRBDs). The Drosha partners are the *pasha* protein in *Drosophila melanogaster* or DiGeorge syndrome critical region gene 8 (DGCR8) in mammals. The Drosha–DGCR8 complex processes pri-miRNAs to ~70-nucleotide hairpins known as pre-miRNAs[1,3,21,24]. Some spliced-out introns in *Caenorhabditis elegans*, *D. melanogaster* and mammals correspond precisely to pre-miRNAs (mirtrons), thus circumventing the requirement for Drosha–DGCR8 (REFS 125–127). Plant genomes do not encode Drosha homologues, and all miRNA biogenesis steps in *Arabidopsis thaliana* are carried out by one of four Dicer-like proteins[29]. In animals, pre-miRNAs are transported to the cytoplasm by exportin5, where they are cleaved by Dicer (complexed with TAR RNA binding protein (TRBP) in mammals and the *loquacious* gene product in *D. melanogaster*) to yield ~20-bp miRNA duplexes. One strand is then selected to function as a mature miRNA, while the other strand is degraded. Occasionally, both arms of the pre-miRNA hairpin give rise to mature miRNAs[1,3,21,24]. Vertebrates and *C. elegans* contain single *dicer* genes, but some other organisms like *D. melanogaster* and plants express two or more Dicer proteins that function as heterodimers with different dsRBD proteins and have specialized functions[1,3,21,24].

Following their processing, miRNAs are assembled into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). The assembly is a dynamic process, usually coupled with pre-miRNA processing by Dicer, but its details are not well understood[1,3,21,24]. The key components of miRNPs are proteins of the Argonaute (AGO) family. Of the many paralogues encoded in plant and metazoan genomes, usually only some — known as AGO proteins — function in miRNA or both miRNA and small interfering RNA (siRNA) pathways. In mammals, four AGO proteins (AGO1 to AGO4) function in the miRNA repression but only AGO2 functions in RNAi. In *C. elegans*, which expresses 27 Argonaute proteins, RDE1 is involved in RNAi and ALG1 and ALG2 function in the miRNA pathway[24,25]. Apart from AGOs, miRNPs can contain further proteins that function as regulatory factors or effectors mediating inhibitory function of miRNPs[19,23,24]. Examples are the fragile X mental retardation protein, FMRP, and its *D. melanogaster* orthologue, dFXR, which are RNA-binding proteins known to act as modulators of translation, particularly in neurons (reviewed in REF. 128). Some P-body components such as GW182 and RCK/p54 (see BOX 4) interact with miRNP AGO proteins and are essential for inducing repression[78,92,104].



Dicer
An RNase III family endonuclease that processes dsRNA and pre-miRNAs into small interfering RNAs and microRNAs, respectively.

Small interfering RNAs
(siRNAs). Small RNAs that are similar in size to microRNAs but are derived from the progressive cleavage of long dsRNA by Dicer. Upon incorporation into an RISC, siRNAs guide the endonucleolytic cleavage of the target mRNA.

RN3) and the endoribonuclease Dicer — enzymes of the RNase III family (BOX 1). The final processing of the ~70-nucleotide pre-miRNA hairpin by Dicer yields ~21-bp miRNA duplexes with protruding 2-nucleotide 3′ ends, similar to small interfering RNAs (siRNAs) operating in RNA interference (RNAi). Generally, the strand with the 5′ terminus located at the thermodynamically less-stable end of the duplex is selected to function as a mature miRNA, and the other strand is degraded[3,19,21–23].

miRNAs function as components of ribonucleoprotein (RNP) complexes or RNA-induced silencing complexes (RISCs), referred to as either micro-ribonucleoproteins (miRNPs) or miRNA-induced silencing complexes (miRISCs) (BOX 1). The most important and best-characterized components of miRNPs are proteins of the Argonaute family[24,25]. Mammals contain four Argonaute (AGO) proteins, AGO1 to AGO4. Their function in miRNA repression is demonstrated by their association with similar sets of miRNAs and their ability to repress protein synthesis when artificially tethered to the mRNA 3′ UTR[26–28] (FIG. 1). AGO2 is the only AGO that functions in RNAi because its RNaseH-like P-element induced wimpy testis (PIWI) domain, but not those of the other AGOs, can cleave mRNA at the centre of the siRNA–mRNA duplex (BOX 1). In *Drosophila melanogaster*, Argonaute1 is dedicated to the miRNA pathway, and Argonaute2 mainly functions in RNAi[24,25]. Apart from the AGO proteins, miRNPs often include other proteins, which probably function as miRNP assembly or regulatory factors, or as effectors mediating the repressive miRNP functions[24].

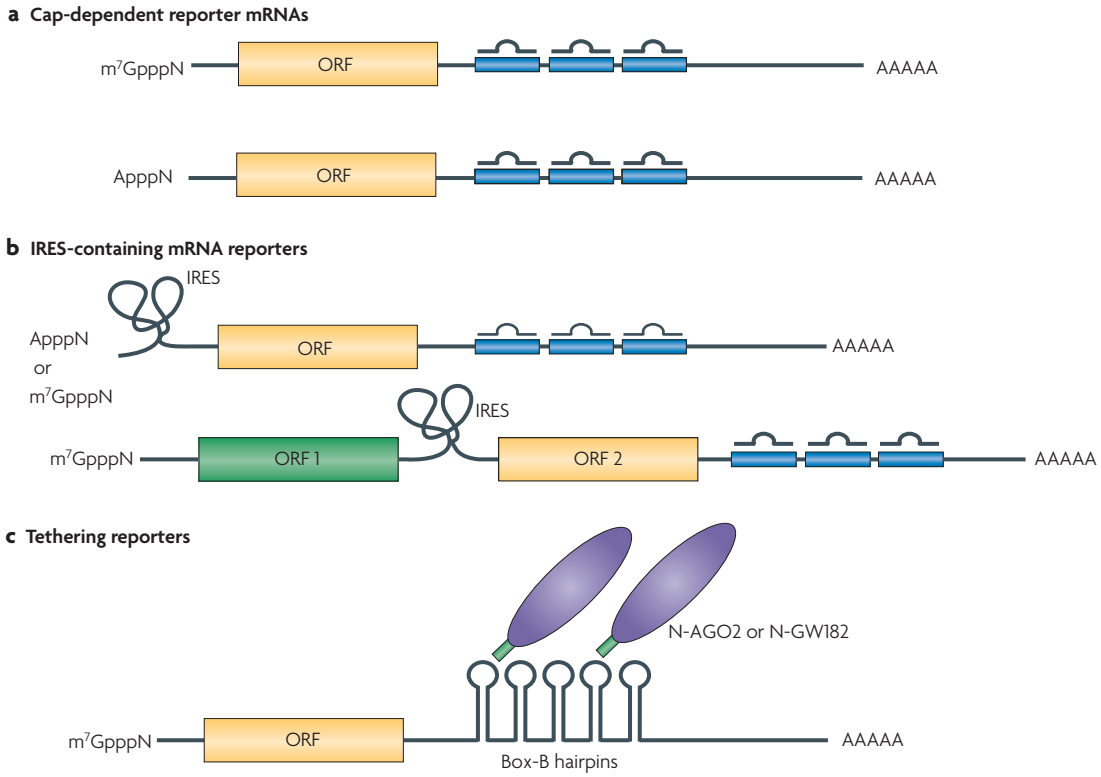


Figure 1 | **Examples of reporters used in studies of microRNA function. a** | Capped reporters containing multiple mRNA binding sites. mRNAs containing a non-functional ApppN cap (instead of the 7-methylguanosine (m^7G) cap) can be prepared by *in vitro* transcription with T7 phage RNA polymerase and either introduced into cells by transfection or used in studies in cell-free extracts. **b** | Mono-cistronic and bi-cistronic reporters containing a viral internal ribosomal entry sites (IRES). Reporters containing ApppN or pppN at the 5′ end can be prepared by *in vitro* transcription and then transfected into cells. **c** | Reporters used to study the effects of tethering to mRNA of Argonaute (AGO) proteins or GW182 on protein synthesis. The investigated proteins are expressed as fusions with a phage λN-peptide, which can bind the short Box-B hairpins that are inserted to the mRNA 3′ UTR[27,106]. The λN-peptide–Box-B system can also be used to tether initiation factors eIF4E or eIF4G to the intercistronic region of the bi-cistronic reporter[43]. Reporters that are generated *in vitro* and used for either RNA transfection experiments or studies in cell-free extracts can be prepared with or without the poly (A) tail[43,44,52,55]. Reporters can also differ in the number of miRNA binding sites that are present in the 3′ UTR. Reporters that are devoid of microRNA binding sites or that contain mutated sites are used as controls.

RNA interference
The dsRNA-induced, sequence-homology dependent gene-silencing mechanism. The dsRNA is processed to siRNAs, which, upon incorporation into an RISC, guide the endonucleolytic cleavage of the target mRNA.

RNA-induced silencing complex
(RISC). The ribonucleoprotein complex, consisting of small interfering RNA and an AGO protein, that harbours the 'slicer' activity, which cleaves an mRNA target in the middle of siRNA–mRNA complementarity.

micro-ribonucleoprotein
(miRNP). A ribonucleoprotein complex containing a miRNA and one of the AGO proteins. Depending on the identity of the associated AGO, it might harbour a 'slicer' activity, characteristic of an RISC.

m^7G cap
The 7-methylguanosine (m^7G) that is linked by a 5–5′ triphosphate bridge to the first transcribed nucleoside at the 5′ end of eukaryotic mRNAs.

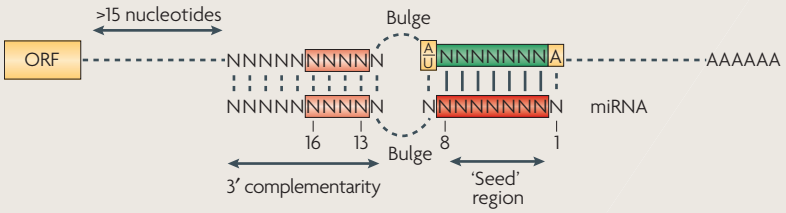
Principles of miRNA–mRNA interactions

In plants, miRNAs generally base pair to mRNAs with nearly perfect complementarity and trigger endonucleolytic mRNA cleavage by an RNAi-like mechanism[29]. In rare instances, a similar mechanism is used by vertebrate and viral miRNAs (see REF. 21 for examples). However, in most cases, metazoan miRNAs pair imperfectly with their targets, following a set of rules determined by experimental and bioinformatic analyses[30–34] (BOX 2). The most stringent requirement is a contiguous and perfect base pairing of the miRNA nucleotides 2–8, representing the 'seed' region, which nucleates the interaction. With few exceptions, miRNA-binding sites in metazoan mRNAs lie in the 3′ UTR and are usually present in multiple copies — this is required for effective repression of translation[30–34]. miRNAs also exert their repressive function when their binding sites are artificially placed in 5′ UTRs or coding regions[35,36], although the physiological effects of the coding-region sites might be only marginal[37].

Modes of translational repression

mRNA translation can be divided into three steps: initiation, elongation and termination. Initiation starts with the recognition of the mRNA 5′-terminal cap structure m^7GpppN (in which N is any nucleotide) by the eIF4E subunit of the eukaryotic translation initiation factor (eIF) eIF4F, which also contains eIF4G, an important scaffold for the assembly of the ribosome initiation complex (BOX 3). Interaction of eIF4G with another initiation factor, eIF3, facilitates the recruitment of the 40S ribosomal subunit[38,39]. eIF4G also interacts with the polyadenylate-binding protein 1 (PABP1). The ability of eIF4G to interact simultaneously with eIF4E and PABP1 brings the two ends of the mRNA in close proximity[40,41]. This 'circularization' stimulates translation initiation by increasing the affinity of eIF4E for m^7GpppN, and might facilitate ribosome recycling[41]. Some cellular and viral mRNAs initiate translation independently of the m^7G cap and eIF4E; in this case, 40S ribosomes are recruited to the mRNA through

Box 2 | **Principles of microRNA–mRNA interactions**



MicroRNAs (miRNAs) interact with their mRNA targets by base pairing. In plants, most miRNAs base pair to mRNAs with nearly perfect complementarity and induce mRNA degradation by an RNAi-like mechanism — the mRNA is cleaved endonucleolytically in the middle of the miRNA–mRNA duplex[29]. By contrast, with few exceptions, metazoan miRNAs base pair with their targets imperfectly, following a set of rules that have been identified by experimental and bioinformatics analyses[30–34].

- One rule for miRNA–target base paring is perfect and contiguous base pairing of miRNA nucleotides 2 to 8, representing the 'seed' region (shown in dark red and green), which nucleates the miRNA–mRNA association. GU pairs or mismatches and bulges in the seed region greatly affect repression. However, an A residue across position 1 of the miRNA, and an A or U across position 9 (shown in yellow), improve the site efficiency, although they do not need to base pair with miRNA nucleotides.
- Another rule is that bulges or mismatches must be present in the central region of the miRNA–mRNA duplex, precluding the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA.
- The third rule is that there must be reasonable complementarity to the miRNA 3′ half to stabilize the interaction. Mismatches and bulges are generally tolerated in this region, although good base pairing, particularly to residues 13–16 of the miRNA (shown in orange), becomes important when matching in the seed region is suboptimal[31,33].

Other factors that can improve site efficacy include an AU-rich neighbourhood and, for long 3′ UTRs, a position that is not too far away from the poly(A) tail or the termination codon; these factors can make the 3′ UTR regions less structured and hence more accessible to miRNP recognition[33,34,129]. Indeed, accessibility of binding sites might have an important effect on miRNA-mediated repression[130]. Some experimentally characterized sites deviate significantly from these rules and can, for example, even require a bulged nucleotide in the seed region pairing[131,132]. In addition, combinations of sites can require a specific configuration (for example, separation by a stretch of nucleotides of specific sequence and length) for efficient repression[131]. Usually, miRNA-binding sites in metazoan mRNAs lie in the 3′ UTR and are present in multiple copies. Importantly, multiple sites for the same or different miRNAs are generally required for effective repression[30–34]. When they are present close to each other (10–40 nucleotides apart) they tend to act cooperatively, that is, their effect exceeds that expected from the independent contributions of two single sites[30,33].

interaction with an internal ribosome entry site (IRES)[42]. Joining of the 60S subunit at the AUG codon precedes the elongation phase of translation.

Although it is now clear that the effects of miRNAs on protein synthesis can result from mRNA destabilization or translational repression, whether the latter occurs at the initiation or post-initiation step (or both) remains a matter of debate. Several recently published papers provide important mechanistic insights into the repression-at-the-initiation step, giving extra credence to this model.

Repression at the initiation step. Investigations were carried out using HeLa cells and reporter mRNAs that had multiple binding sites for either natural or synthetic miRNAs in their 3′ UTR. The investigations revealed that the translation of m^{7}G-capped mRNAs, but not of mRNAs containing an IRES or a non-functional ApppN cap, is repressed by miRNAs[43,44]. As in numerous subsequent studies, the specificity of repression was assessed using reporters containing mutated miRNA sites or by antisense oligonucleotides that specifically block the targeting miRNA. The conclusion that the m^{7}G cap is essential for translational repression was corroborated by experiments with bi-cistronic mRNAs. In these experiments, the activity of the first cap-dependent cistron, but not the second cistron, placed under the control of eIF4E or eIF4G artificially tethered to the mRNA, was repressed by the endogenous *let-7* miRNA[43] (FIG. 1). Polysome gradient analysis independently supports an effect on the initiation step: reporter mRNAs that either contained functional *let-7*-binding sites or that were repressed by AGO2 (artificially tethered to the 3′ UTR) showed a marked shift in sedimentation toward the top of the gradient, indicating reduced ribosome loading on the repressed mRNA[43]. Likewise, the amino-acid-starvation-induced release of endogenous cationic amino acid transporter 1 (CAT1) mRNA from repression that was mediated by the miRNA *miR-122* was accompanied by a more effective recruitment of CAT1 mRNA to polysomes in human hepatoma cells[45].

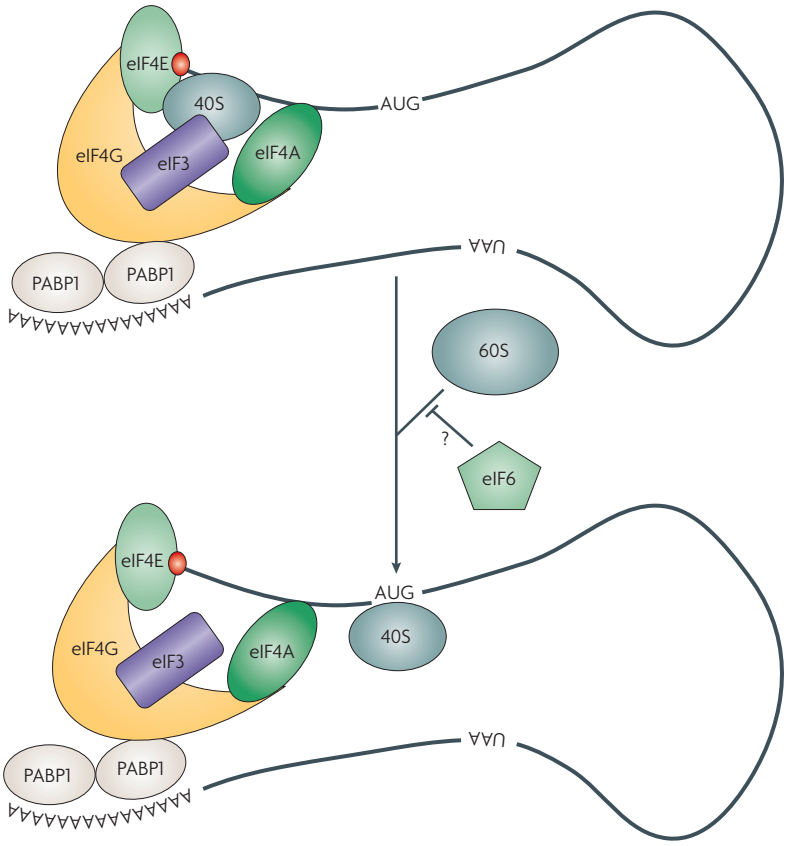
There is substantial evidence that factors bound at the 3′ UTR exert their inhibitory effect on translational initiation by recruiting proteins that either interfere with the eIF4E–eIF4G interaction or bind directly to the cap but, unlike eIF4E, are unable to associate with eIF4G and promote assembly of the 40S initiation complex[46–48]. Could miRNPs or tethered AGO proteins function in a similar manner? Kiriakidou *et al.*[49] recently reported that the central domain of AGO proteins contains limited sequence homology to the cap-binding region of eIF4E. Importantly, the similarity includes two aromatic residues (FIG. 2), which are crucial for cap binding in eIF4E and other cap-binding proteins[49,50]. Mutations of one or both aromatic amino acids in AGO2 to valine but, significantly, not to other aromatic amino acids, prevented the interaction with m^{7}GTP–Sepharose and abolished the ability of AGO2 to repress translation when tethered to the mRNA 3′ UTR. These data indicate that AGO2 and related proteins can compete with eIF4E for m^{7}G binding and thus prevent translation of capped, but not IRES-containing, mRNAs[49]. The data also provide a plausible explanation for the requirement of multiple miRNPs or tethered AGO molecules for robust repression[27,30,43,51]. Multiple copies of AGO, with an apparently lower affinity for m^{7}G than eIF4E[49], would increase the likelihood of AGO association with the cap. It will be important to determine whether the AGO aromatic residues are essential for miRNA-mediated repression in a physiological assay. Additional evidence, for example from cross-linking experiments, should be obtained in support of direct interaction of AGO with the mRNA m^{7}G cap structure.

Lessons from* in vitro *studies. Four different cell-free extracts that recapitulate many features of the miRNA-mediated *in vivo* effects have recently been described. In all of them, the presence of the m^{7}G cap was required for translational repression[52–55]; the mRNAs containing

Internal ribosomal entry site (IRES). An RNA element, usually present in the 5′ UTR, that allows m^{7}G-cap-independent association of ribosome with mRNA.

ApppN cap
An unmethylated cap analogue that is not bound by eIF4E. The mRNAs with an artificially introduced ApppN cap instead of a physiological m^{7}GpppN cap are translated inefficiently.

Box 3 | **Steps in eukaryotic translation**



Translation of mRNA consists of three steps: initiation, elongation and termination. Initiation is the most complex step and is subject to a large number of interventions, with the phosphorylation of initiation factors being the key regulator[133]. Translation requires the participation of at least 10 initiation factors, many of them multisubunit complexes[38,39]. Initiation of translation of most cellular mRNAs starts with the recognition of the mRNA 5′-terminal 7-methylguanosine (m^7G) cap (represented by the red circle in the figure) by the eukaryotic translation initiation factor (eIF) 4E subunit of the initiation factor eIF4F, which also contains eIF4A (an RNA helicase) and eIF4G (a large multidomain protein that functions as a scaffold for the assembly of the translation initiation complex). Interaction of eIF4G with another multi-subunit initiation factor, eIF3, facilitates the recruitment of the 40S subunit, which then begins scanning the mRNA 5′ UTR in search of the AUG (or in rare cases its cognate) initiation codon. Following the joining of the 60S ribosomal subunit the elongation phase ensues. The elongation step can also be regulated by phosphorylation of the elongation factor eEF2 (REF. 134). When the ribosome encounters a termination codon, translation release factors mediate the termination process, in which the ribosomal subunits dissociate from both the mRNA and from each other. An important function of eIF4G is its interaction with the poly(A)-binding protein 1, PABP1, which is associated with the poly(A) tail. This interaction brings about the circularization of the mRNA, which stimulates translation initiation and possibly recycling of ribosomes[40,41]. eIF6 is required for 60S subunit biogenesis, and might also act as an initiation factor that regulates subunit joining[58–61]. Some cellular and many viral mRNAs initiate translation independently of the m^7G cap and eIF4E, and sometimes also independently of other initiation factors. During this mode of translation, ribosomes are recruited to the mRNA through interaction with internal ribosome entry sites (IRES), which are usually highly structured regions in the 5′-UTR. The best-studied IRES are those of the encephalomyocarditis and polio viruses, hepatitis C virus and the insect cricket paralysis virus. Bi-cistronic constructs, in which translation of the upstream cistron requires the presence of the cap and eIF4E and that of the downstream cistron requires internal initiation, are widely used as one of the means to identify a putative IRES[38,42] (FIG. 1).

an IRES or an ApppN cap were not inhibited[53,54]. Extracts derived from *D. melanogaster* embryos and mouse Krebs2 ascites cells were used to define the repression step more precisely. In both systems, miRNAs inhibited the association of mRNA with either the 40S or the 80S ribosome, consistent with miRNAs targeting translation initiation, probably at the 40S–mRNA complex assembly step[53,54]. In agreement with the model that AGO proteins compete with eIF4E for cap binding[49], the addition of purified initiation factor eIF4F to the ascites extract rescued mRNA from the miRNA-mediated inhibition[54].

Extracts that were prepared from rabbit reticulocytes and from human HEK293 cells were also tested for the poly(A)-tail requirement[55] — translational repression occurred only when target mRNAs contained both an m^7G cap and a poly(A) tail. In the reticulocyte lysate, m^7G cap dependence could be partially relieved by the addition of poly(A) tails of non-physiological length — 0.8 kb or longer — implying that polyadenylation might have a role in miRNA-mediated repression[52]. Studies in HEK293 cell extracts showed that mRNAs containing miRNA-binding sites underwent deadenylation irrespective of whether they contained an m^7G cap (translationally repressed mRNAs) or an ApppN cap or IRES (non-repressed mRNAs). Thus, although the miRNA-mediated deadenylation had no apparent effect on the translation of IRES-containing or ApppN-containing mRNAs, it might have contributed to the repression of m^7G-capped mRNAs by disrupting the eIF4G-mediated mRNA circularization[55].

Taken together, the data support the notion that by targeting one of the two terminal mRNA structures, miRNAs prevent the synergy between the 5′ cap and 3′ poly(A) tail. Notably, HEK293 cell lysate was supplemented with an extract of cells overexpressing GW182 (REF. 55), a protein that recruits the CCR4–NOT deadenylation complex to the miRNA-bound mRNA (discussed below). Hence, in this system, miRNA-mediated repression might be biased towards deadenylation. Of the remaining three systems, the *D. melanogaster* and mouse ascites extracts originated from non-modified cells and responded to endogenous miRNPs[53,54]. By contrast, the repression in the reticulocyte lysate required pre-annealing of the synthetic miRNA to the template mRNA[52]. It is not known how effectively such a preformed miRNA–mRNA duplex associates with AGO proteins and thus to what extent this system recapitulates the physiological miRNA response. Interestingly, binding of miRNPs to the 3′ UTR in *D. melanogaster* extracts resulted in the formation of heavy aggregates, termed pseudo-polysomes, even in the absence of translation[53]. Whether they are related to P-bodies (discussed below) remains unknown.

Data on the requirement of a poly(A) tail for repression *in vitro* differ from some findings obtained in intact cells. Reporter RNA transcripts that were directly transfected to HeLa cells were repressed even in the absence of a poly(A) tail. In one study[44], its presence resulted in stronger repression, but this effect was not seen in a different study[43]. Although it is unlikely, the possibility that the poly(A)-free RNA becomes polyadenylated in

Polysome gradient analysis
A technique that involves the sedimentation of cell extracts through a gradient of sucrose or glycerol, thereby allowing the determination of the number of ribosomes that are associated with a specific mRNA. Repression of translational initiation, which results in the less efficient loading of ribosomes onto mRNA, is usually associated with a shift of mRNA towards the top of the gradient.

transfected cells was not excluded by these studies. With this caveat in mind, the data suggest that a poly(A) tail *per se* is not absolutely required for the repression, a conclusion supported by the observation that mRNA containing a 3′ histone stem-loop in place of a poly(A) tail also undergoes translational repression in HEK293 cells[56].

Repression by preventing 60S subunit joining. An alternative mechanism of miRNA action was recently proposed by Chendrimada *et al.*[57] The authors reported that eIF6 and 60S ribosomal subunit proteins co-immunoprecipitate with the AGO2–Dicer–TRBP complex. eIF6 was first described as a protein that binds the 60S subunit to prevent its precocious interaction with the 40S subunit[58], and was thought to act as an initiation factor. However, it was shown later that eIF6 is not involved in translation in yeast, but rather has a crucial function (both in yeast and mammals) in the biogenesis of the 60S subunit in the nucleolus, and accompanies the 60S subunit to the cytoplasm[59–61]. Chendrimada *et al.*[57] showed that partial depletion of eIF6 in either human cells or *Caenorhabditis elegans* rescues mRNA targets from miRNA inhibition, possibly by reducing eIF6-mediated impediment of 60S joining.

The involvement of eIF6 in ribosome biogenesis complicates the interpretation of the data that support its role in miRNA repression, and invites another possible scenario. Sachs and Davis[62,63] demonstrated that mutations in a ribosomal protein and a helicase involved in yeast 60S biogenesis could act as bypass suppressors of complete deletion of the gene encoding poly(A) binding protein (Pab1). As in metazoans, the yeast Pab1 is an essential protein, contributing to translation initiation through its function in mRNA circularization. The bypass suppressor mutations, which all resulted in a 60S ribosomal subunit deficit, allowed growth, albeit reduced, in the absence of Pab1. This rescue can be explained by an increase in the free 40S subunit pool (resulting from a partial depletion of 60S ribosomes), leading to an enhanced rate of their recruitment to mRNA. This would partially compensate for the lack of Pab1, which stimulates 40S recruitment, and would switch the rate-limiting step from the 40S subunit-loading step to the 60S joining step.

A similar switch, negating the advantages of the circularization of bulk mRNAs, could be caused by the knockdown of eIF6. The resulting limited 60S deficit[57] would bring some relief of the miRNA-mediated repression, because the target mRNAs could now compete with the bulk of mRNAs on a more equal footing. If this explanation is correct, the work of Chendrimada *et al.*[57] would be consistent with the idea that miRNA-mediated repression affects the initiation of translation by targeting the 5′ cap and poly(A) tail, although perhaps not because of a direct involvement of eIF6 in repression. Admittedly, this model does not explain why eIF6 co-purifies with the RISC.

Repression at post-initiation steps. Despite compelling *in vitro* and *in vivo* evidence, targeting of translation initiation is unlikely to be the only mechanism by which miRNAs bring about mRNA repression. Early studies in *C. elegans* showed that *lin-14* and *lin-28* mRNAs, which are targets of *lin-4* miRNA, remain associated with polysomes despite a strong reduction in their protein products at a specific stage of larval development[64,65]. Similar results, which are incompatible with the initiation model, were seen in mammalian cells. In two studies that used reporter mRNAs targeted by either synthetic

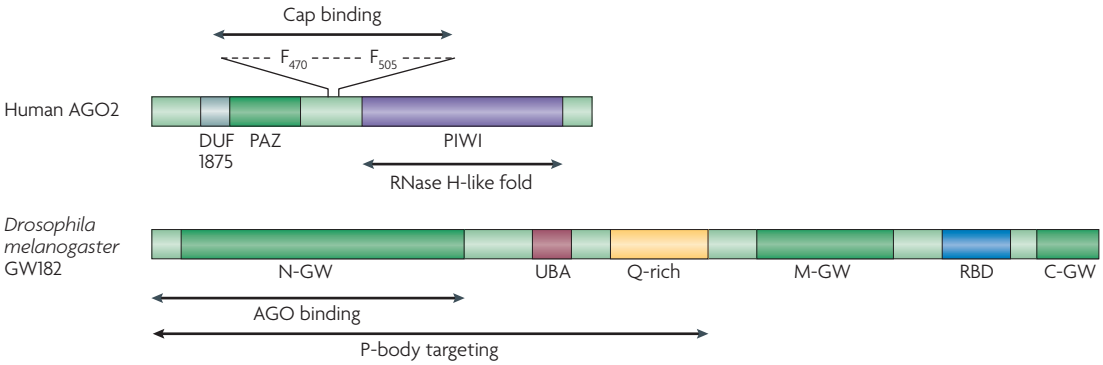


Figure 2 | **Domain organization of Argonaute and GW182 proteins.** The schemes represent human Argonaute2 (AGO2) and *Drosophila melanogaster* GW182, two proteins extensively characterized in mediating the microRNA (miRNA)-mediated repression. AGO2 is the only mammalian AGO protein that, in addition to miRNA repression, also functions in RNAi. Its RNAseH-like P-element induced wimpy testis (PIWI) domain is competent in endonucleolytically cleaving the mRNA. The region separating the PIWI Argonaute Zwille (PAZ) and PIWI domains of AGO2 contains two aromatic amino acids (phenylalanines F_{470} and F_{505}), mutation of which was reported to prevent both the repression of translation in the AGO2 tethering assay and the binding of AGO2 to 7-methylguanosine triphosphate (m^7GTP)-Sepharose[49]. *D. melanogaster* contains only one GW182 protein[78,136] but there are three GW182 paralogues in mammals (known as TNRC6A–C). One related protein, AIN1, is expressed in *Caenorhabditis elegans*[90]. N-GW, M-GW and C-GW are regions enriched in glycine (G)–tryptophan (W) dipeptides. The N-GW domain or shorter GW-repeats-containing peptides were shown to mediate interaction of GW182 proteins with the PIWI domain of AGO proteins[78,94]. The region extending from the N terminus to the glutamine-rich domain is responsible for targeting GW182 to P-bodies[78]. DUF, domain of unknown function; RBD, RNA binding domain; UBA, ubiquitin associated domain; Q; glutamine.

or endogenous miRNAs[66,67], the repressed mRNAs associated with active polysomes — as demonstrated by sensitivity of the polysomes to different conditions that inhibit translation. Moreover, Peterson *et al.*[66] found that, like the cap-dependent upstream ORF, IRES-mediated translation of the downstream ORF in the bi-cistronic reporter is sensitive to miRNAs. Drawing on additional data, the authors proposed a drop-off model, in which miRNAs render ribosomes prone to premature termination of translation. Lytle *et al.* also reported repression of IRES-containing reporters[35].

The observation that three endogenous miRNAs and *KRAS* mRNA, a known target of *let-7* miRNA, cosediment with polysomes led Maroney *et al.*[68] to conclude that repression occurs at a post-initiation step. Because puromycin or hypertonic conditions — factors causing general inhibition of translation — shifted polysome-associated miRNAs towards the top of the gradient during polysome gradient analyses, whereas the shift of *KRAS* mRNA was only partial, the authors proposed that miRNAs decelerate translation elongation. Cosedimentation of a significant fraction of cellular miRNAs or AGO proteins with polysomes has also been reported in other studies[69–71] and is often quoted in support of the post-initiation mechanism. However, it should be stressed that repression of mRNA targets by miRNAs is generally only partial, and binding of a single miRNP to mRNA frequently has no significant effect (see REFS 43,51 for examples). Hence, cosedimentation of miRNPs with polysomes is not necessarily diagnostic of post-initiation repression, but might simply reflect the association of miRNPs with mRNAs undergoing productive translation.

How miRNAs could modulate the elongation or termination process remains unclear. Apart from the proposed miRNA-mediated control, few other examples of regulation targeting post-initiation steps have been reported. Repression of *pal1* mRNA by GLD1 in *C. elegans* seems to involve the stalling or slowing down of elongating ribosomes[72], as does translational repression of unspliced *HAC1* mRNA in yeast[73]. Other examples include *nanos* and *oskar* mRNAs in *D. melanogaster* embryos[74,75], although the proposed mode of their regulation has recently been either reinterpreted or questioned[46,76]. Despite undeniable evidence that translational repression by miRNAs can occur by post-initiation mechanisms, the findings do not demonstrate unequivocally that the initiation and post-initiation mechanisms are mutually exclusive. It is possible that initiation is always inhibited, but when the elongation step is also repressed, ribosomes would queue on the mRNA, thereby masking the effect of an initiation block.

The association of repressed mRNAs with translationally competent polysomes has also fuelled speculations that proteins are continually synthesized from these mRNAs but do not accumulate because they are rapidly degraded by proteases recruited by miRNPs[64] (FIG. 3). This possibility has been experimentally addressed; in immunoprecipitation experiments, nascent polypeptides produced from the repressed reporter could not be detected[67]. Likewise, in pulse-labelling experiments, neither full-length nor nascent polypeptides could be identified when the reporter mRNA was repressed[66]. On the other hand, repression was not prevented when reporter proteins were targeted to the endoplasmic reticulum (ER). This excludes the possibility that nascent proteins are degraded in the cytosol[43]. In conclusion, the proteolysis proposal is at present based on negative rather than positive data. Proteasome inhibitors had no effect on miRNA-mediated repression[43,66,67] and other proteases have not been identified.

mRNA deadenylation and decay

Although initial studies suggested that the levels of miRNA-inhibited mRNAs remain mostly unchanged, more recent work has demonstrated that the repression of many miRNA targets is frequently associated with their destabilization[56,77–80] (FIG. 3). Likewise, microarray studies of transcript levels in cells and tissues in which the miRNA pathway was inhibited[78,79,81–83], or in which miRNA levels were experimentally altered[84–87], revealed marked changes in the abundance of dozens of validated or predicted miRNA targets, consistent with a role for miRNAs in mRNA destabilization.

In eukaryotes, mRNA degradation can follow two pathways, each of which is initiated by a gradual shortening of the mRNA poly(A) tail. The mRNA body can then be degraded by progressive 3′→5′ decay, which is catalysed by the exosome, or by the removal of the cap followed by 5′→3′ degradation, which is catalysed by the exonuclease XRN1 (REF. 88). Levels of mRNA are controlled by mRNPs through the recruitment of decay machinery components, leading to mRNA deadenylation and decapping. The degradation, or at least its final steps, is thought to occur in P-bodies — cellular structures that are enriched in mRNA-catabolizing enzymes and translational repressors[17,89] (BOX 4).

The mechanism of miRNA-mediated mRNA destabilization is best understood in *D. melanogaster*. Studies in *D. melanogaster* S2 cells demonstrated that the P-body protein GW182 (product of the *gawky* gene), which interacts with the miRNP Argonaute1 (the interaction also occurs between mammalian and worm orthologues[90–93]), is a key factor that marks mRNAs for decay[78]. The AGO PIWI domain and glycine–tryptophan (GW) dipeptide-containing domains or peptides of GW182 family proteins are important for this interaction[78,94] (FIG. 2). Consistent with its role in mediating mRNA degradation, GW182 depletion leads to an upregulation of many mRNA targets that are also upregulated in cells that are depleted of Argonaute1. Tethering of GW182 to the mRNA bypasses the Argonaute1 requirement for repression, further demonstrating that GW182 functions in the same pathway downstream of Argonaute1. Depletion of the components of the CCR4–NOT deadenylating complex prevents the decay-promoting activity of GW182, suggesting that it plays a part in recruiting CCR4–NOT to repressed mRNAs. Likewise, the knockdown of the decapping-complex proteins, DCP1 and DCP2, or different combinations of decapping activators, prevents miRNA-mediated degradation but leads to an accumulation of deadenylated mRNAs[78,83].

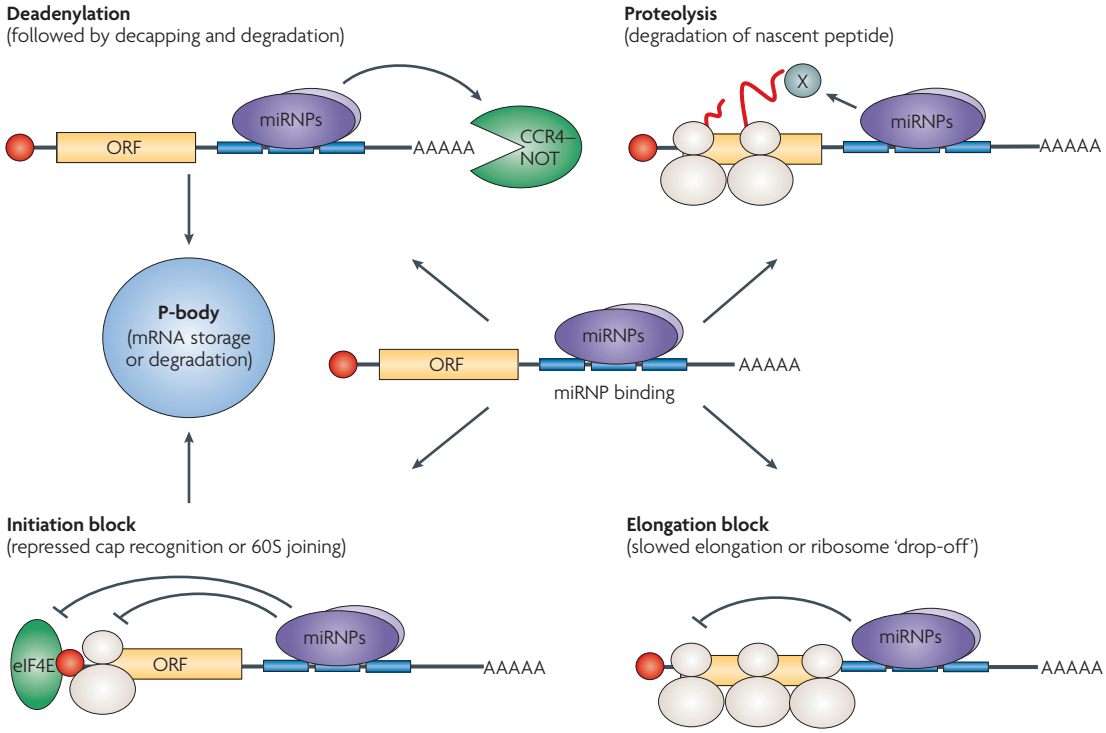


Figure 3 | **Possible mechanisms of the microRNA-mediated post-transcriptional gene repression in animal cells.** Binding of micro-ribonucleoproteins (miRNPs), possibly complexed with accessory factors, to mRNA 3′ UTR can induce deadenylation and decay of target mRNAs[56,78,79,83] (upper left). Alternatively, miRNPs can repress translation initiation at either the cap-recognition stage[43,44,53–55] or the 60S subunit joining stage[57] (bottom left). mRNAs repressed by deadenylation or at the translation-initiation stage are moved to P-bodies for either degradation or storage. The repression can also occur at post-initiation phases of translation[66–68], owing to either slowed elongation or ribosome 'drop-off' (bottom right). Proteolytic cleavage of nascent polypeptides was also proposed as a mechanism of the miRNA-induced repression of protein production[67] (upper-right). A protease (X) that might be involved in the process has not been identified. The 7-methylguanosine cap is represented by a red circle. eIF4E, eukaryotic initiation factor 4E.

Accelerated deadenylation also results in a reduced abundance of miRNA-repressed mRNAs in mammalian cells[56]. Moreover, knockdown experiments in *C. elegans*[77], and analysis of the decay intermediates originating from repressed mRNAs in worms[77] and mammalian cells[56,82], support the role of decapping and 5′→3′ exonucleolytic activities in these systems.

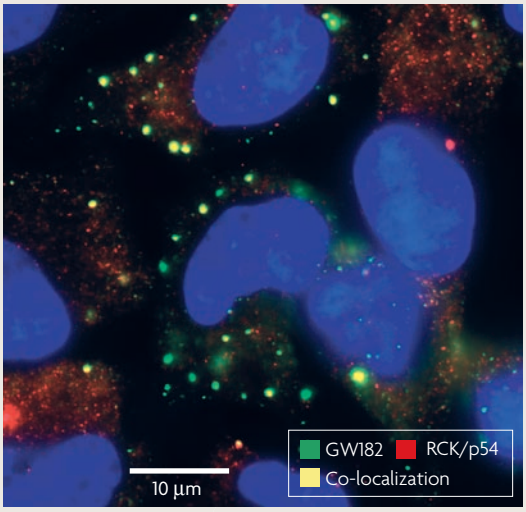
Widespread miRNA-mediated deadenylation of mRNAs occurs during zebrafish embryogenesis. The miRNA *miR-430* facilitates the removal of hundreds of maternal mRNAs by inducing their deadenylation and subsequent decay at the onset of zygotic transcription[79]. Interestingly, some *miR-430* targets, such as *nanos1* and tudor-like *tdrd7* mRNAs, are repressed by *miR-430* in somatic but not germ cells, indicating that target destabilization and/or repression can be tissue or cell specific[95]. Likewise, mRNA reporters targeted by *let-7* miRNA are destabilized to different degrees in different mammalian cell lines[82].

Although many of the mRNAs that are targeted by miRNAs undergo substantial destabilization, there are also numerous examples of repression at the translational level, with no or only a minimal effect on mRNA decay (Supplementary information S1 (table)). Studies using *D. melanogaster* S2 cells identified some endogenous or reporter miRNA targets, for which repression could be entirely accounted for by either mRNA degradation or translational repression, or by a combination of both processes[78,83]. It is not known what determines whether an mRNA follows the degradation or translational-repression pathway. Accessory proteins bound to the 3′ UTR might be involved, or structural subtleties of imperfect miRNA–mRNA duplexes, particularly of their central regions, could be important[82,96].

Whether the deadenylation and the ensuing decay are primary or secondary to the translational repression remains unknown. Clearly, the association of AGO instead of eIF4E with the m^7G cap would not only prevent effective recruitment of ribosomes, but would also disrupt the circularization of the mRNA, probably rendering the poly(A) tail more vulnerable to exonucleolytic degradation. Experiments that have been carried out to explore whether deadenylation is a primary or secondary event have not proved to be conclusive. Reporter mRNAs that are repressed by either oligonucleotides that are complementary to the AUG codon or the 5′ UTR hairpins do not undergo deadenylation unless they contain miRNA sites[79,80]. However, it is unlikely that mRNA circularization is disrupted by the oligonucleotide or the hairpin, both of which act at some distance from the cap. By contrast, the disruption could be effected by the miRNP AGO

interacting with the cap. Perhaps the strongest support for deadenylation as a primary event comes from the finding that the translationally inactive ApppN-capped mRNA (which does not interact with eIF4E and hence is unable to circularize) is deadenylated when injected into zebrafish embryos only when it contains *miR-430* sites in its 3′ UTR[95]. This, and other experiments[56,79,83], indicate that miRNA-dependent mRNA deadenylation and decay is not dependent on active translation, although examples of mRNA targets, decay of which requires ongoing translation, have also been reported[83].

Box 4 | **P-bodies and stress granules**



P-bodies (also known as GW-bodies) are discrete granules that are localized in the cytoplasm of eukaryotic cells. They are enriched in proteins that are involved in mRNA catabolism (deadenylation, decapping and mRNA degradation) and translational repression[17,89,97]. The core P-body components, conserved from budding yeast (which are devoid of RNA-silencing pathways) to mammals, include the decapping enzyme complex DCP1–DCP2, the decapping activators RCK/p54 (Dhh1 in yeast), Pat1 (or the *Drosophila melanogaster* orthologue CG5208, also known as HPat), RAP55 (Scd6 in yeast), and EDC3 (Edc3 in yeast) and the heptameric LSm1–7 complex. Metazoa contain yet another decapping activator, Ge-1 or Hedls. P-bodies also contain other mRNA decay enzymes: the deadenylase complex CAF1–CCR4–NOT and the 5′→3′ exonuclease XRN1 (REFS 17,89). Some proteins involved in nonsense-mediated mRNA decay (NMD) and other mRNA degradation pathways are also enriched in P-bodies. P-bodies lack ribosomes and all translation initiation factors with the exception of eukaryotic initiation factor (eIF) 4E. However, eIF4G and Pab1 accumulate in P-bodies under specific repressive conditions in yeast[99]. In metazoa, P-bodies are enriched in proteins participating in miRNA repression — Argonaute (AGO) proteins and GW182 — and miRNAs themselves. Consistent with their localization, AGO and GW182 proteins and miRNAs interact, directly or indirectly, with different P-body components[43,78,90,92,93,100,104,108] (see figure). The decapping activators RCK/p54 and Pat1, and another P-body-resident protein 4E-T, have the ability to repress translation, with some affecting the initiation step. These proteins can contribute to the repressive function of miRNAs[78,83,91–93,104,106,107,109].

P-bodies are highly dynamic structures, fluctuating in size and number during the cell cycle and in response to changes in the translational status of the cell. They require a continuous supply of repressed mRNAs, and a global translation-initiation block leads to an increase in P-body size in yeast and metazoa; inhibition of elongation by cycloheximide, which retains mRNAs on polysomes, results in their dispersion[17,89]. Likewise, depletion of some P-body components has a strong effect on their integrity, at least as visualized by microscopy. The mRNAs targeted to P-bodies either undergo degradation or are stored there for future use.

Stress granules (SGs)[97] are another type of mRNA-containing cytoplasmic aggregates, formed in response to global repression of translation initiation or to various stress conditions. Many proteins found in P-bodies are absent from SGs and vice versa. However, they share some proteins, and P-bodies and SGs are frequently located adjacent to each other, possibly exchanging their cargo material[115,135].

Compartmentalization of miRNA repression

Translationally inactive eukaryotic mRNAs generally assemble into repressive mRNPs that accumulate in discrete cytoplasmic foci known as P-bodies or GW-bodies[17,89]. Another type of aggregate that contains repressed mRNAs are stress granules (SGs), which accumulate in response to various stress conditions[97] (BOX 4). Originally considered as being primarily involved in mRNA degradation[17,89], P-bodies are now known to also be temporary sites of storage for repressed mRNAs in yeast and mammals[45,98,99]. The demonstration that AGO proteins, miRNAs and mRNAs repressed by miRNAs are all enriched in P-bodies[43,45,78,92,93,100,101] implicated P-bodies in miRNA repression and in the fate of repressed mRNAs. Relevant data are emerging, although their interpretation is sometimes difficult owing to the lack of a precise definition of P-bodies (microscopically-visible versus submicroscopic) and limited information on the distribution of miRNP components and other factors between P-bodies and the cytosol.

There is a good correlation between miRNA-mediated translational repression and accumulation of mRNAs in visible P-bodies[43,45,100,102]. Moreover, there is an inverse relationship between P-body localization and polysome association of target mRNAs in mammalian cells[43,45,102]. The endogenous *CAT1* mRNA, a target of *miR-122*, localizes to P-bodies when translation is repressed but not when it is reversed by stress. In addition, transfection of *miR-122* into cells that normally do not express it is sufficient to concentrate *CAT1* mRNA in P-bodies[45]. So far, quantitative data on the cytosolic distribution of P-bodies are available only for *let-7* miRNA and its reporter mRNA target, both of which are ectopically expressed in HeLa cells. Approximately 20% of each RNA was localized to visible P-bodies[43], indicating that the repression either involves submicroscopic P-bodies or occurs outside them. Note also that the knockdown of some P-body components (such as LSM1 or LSM3), which results in dispersion of microscopic P-bodies, has no effect on miRNA function[103,104]. Hence, the microscopically visible P-bodies are not essential for repression, and their formation is a consequence rather than the cause of silencing[43,103]. These data are consistent with the recent analysis of yeast cells that demonstrate that submicroscopic mRNPs, containing a set of core P-body components, are sufficient for basic control of translation repression and mRNA decay[105]. In contrast to knockdowns of LSM1 and LSM3, depletion of other P-body components such as DCP1 or DCP2, GW182, and various decapping activators, either individually (for example, RCK/p54) or in combinations, prevents efficient inhibition of target mRNAs in cultured cells[78,83,91–93,104,106,107].

Notwithstanding the above findings, a functional miRNA pathway is clearly essential for the formation of large P-body aggregates. Global inhibition of miRNA biogenesis or depletion of the proteins that are involved in miRNA repression, such as GW182 or Argonaute1, results in strong dispersal of visible P-bodies in mammalian and *D. melanogaster* S2 cells[103,108]. Interestingly, depletion of Dicer2 or Argonaute2, which are involved

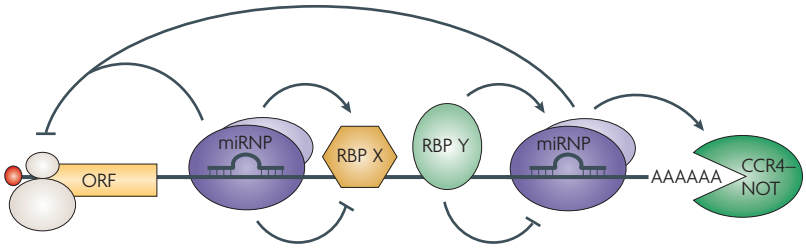


Figure 4 | **Possible interplay between RNA binding proteins and micro-ribonucleoproteins interacting with the mRNAs 3′ UTR.** A single mRNA can have several *cis*-acting motifs interacting with different RNA binding proteins (RBPs) and micro-ribonucleoproteins (miRNPs), which together will determine mRNA translatability or stability. The suppressive effect of the 3′ UTR-binding protein ELAV1 on the miRNA-mediated repression (not shown) has recently been documented[45]. However, it is possible that RBPs will also interact with miRNPs to augment their repressive function, and that miRNPs will have a positive or negative effect on the activity of RBPs bound at the 3′ UTR. The 7-methylguanosine cap is represented by a red circle in the figure.

in RNAi, also results in dispersion of large P-bodies in *D. melanogaster* cells, arguing for a role of both RNA-silencing pathways in P-body formation[103].

Most P-body components, including AGO proteins, are also found throughout the cytosol[17]. Hence, it is probable that repression by miRNPs is initiated in the cytosol (or at least outside P-bodies) and that the repressed mRNAs form P-body aggregates, either small or large, upon run-off from the ribosomes. **P-body-resident** proteins such as RCK/p54 (and the yeast orthologue Dhh1)[104,109–111], 4E-T[112,113], Pat1 (and the *D. melanogaster* orthologue HPat1)[103,109] and RAP55 (REF. 114) **have an** established inhibitory activity on translation, some at the initiation step. These proteins, as well as GW182 (**GW182 functions as a translational repressor in addition** to recruiting the **CAF1–CCR4–NOT deadenylase**[78]), can assist miRNPs in initiating the repression. Whereas RCK/p54 and GW182 can be enrolled directly through their interaction with AGO proteins[78,94,104], recruitment of others might occur through RCK/p54 or GW182.

Surprisingly, only ~1.3% of enhanced GFP (EGFP)-tagged AGO2 localized to P-bodies in HeLa cells[101]. Moreover, the P-body-associated EGFP–AGO2 exchanged with the cytoplasm at a much slower rate than DCP1–DCP2 or LSM6, the P-body components involved in decapping[101,113]; GW182 also exchanges slowly at P-bodies[115]. Rationalization of these observations is difficult at present. P-bodies could consist of compartments with differing component dynamics[43,116]. Alternatively, miRNPs and associated proteins, such as GW182, could be 'anchored' to some cytoplasmic structures and not be readily available for diffusion into the pre-existing photo-bleached P-bodies. In support of this model, most cellular AGO proteins fractionate with the ER or Golgi[117,118]. Moreover, following permeabilization of the plasma membrane, only a small fraction of AGO2 is readily extractable and is probably cytosolic[43]. However, the observation that the EGFP–AGO2 that accumulates in SGs following stress, or treatment with initiation inhibitors, exchanges rapidly with the cytosolic AGO2 pool[101] is at odds with the anchoring model. Association of AGO proteins with mRNAs stored in P-bodies, but not those undergoing degradation, could be another explanation for their low enrichment in these structures.

Leung *et al.*[101] found that, in addition to AGO proteins, miRNA mimics and the repressed reporter mRNA accumulate in SGs. Moreover, the localization of AGO proteins to SGs but not P-bodies was miRNA-dependant. Because SGs are now known to form not only in response to stress but also following general inhibition of translational initiation[18,119], SGs (like P-bodies) might have a role in the miRNA-mediated regulation of translation[18,101]. Alternatively, localization of miRNP components to SGs might reflect dragging of the mRNA-associated, but not necessarily inhibitory, miRNPs to SGs that are formed in response to general translational inhibition. This scenario could also explain why the localization of AGO to SGs, but not to P-bodies, is miRNA dependent: AGO proteins directly interact with other P-body components[78,94,104] but their localization to SGs might require assembly into miRNP to allow association with mRNA by base pairing.

Reversibility of miRNA-mediated repression

Recent findings indicate that under certain conditions, or in specific cells, miRNA-mediated repression can be effectively reversed or prevented[45,95,120,121], and miRNPs or their components can even act as translational activators[71]. The ability to disengage miRNPs from the repressed mRNA, or render them stimulatory, makes miRNA regulation much more wide-ranging and dynamic.

In human hepatoma cells, *CAT1* mRNA is translationally repressed by the liver-specific *miR-122* and accumulates in P-bodies. Following amino-acid starvation or other types of stress, *CAT1* mRNA is released from P-bodies and recruited to polysomes, in a process that depends on binding of ELAVL1 (also known as HuR), a member of the embryonic lethal abnormal vision (ELAV) protein family, to the *CAT1* 3′ UTR. APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G), also interferes with the miRNA action, possibly by altering the distribution of target messages between P-bodies and polysomes[102]. Other examples of the reversible action of miRNAs have been reported in neuronal cells. In neurons, some mRNAs are transported along the dendrites as repressed mRNPs to become translated at dendritic spines upon synaptic activation[122]. *miR-134* is implicated in the regulation of LIMK1, a protein kinase that is important for the development of the spine. In response to extracellular stimuli, *miR-134*-mediated repression of *Limk1* mRNA is partially relieved at dendritic spines of rat neurons[120]. In *D. melanogaster*, stimulation of olfactory neurons is associated with proteolysis of the *armitage* (armi) protein, which is essential for the assembly of miRNPs. Following armi degradation, mRNAs that are normally repressed by miRNAs become translated at the synapse[121]. Given that many miRNAs are specifically expressed in the brain[123], and that three of the four mammalian ELAV proteins — ELAV2, ELAV3 and ELAV4

(also known as HuB, HuC and HuD) — are restricted to neurons[124], reversible miRNA regulation might have a general role in brain development and function.

It is likely that RNA-binding proteins (RBPs) other than ELAV proteins act as modifiers of miRNA-mediated repression. *miR-430* repression of *nanos1* and *tdrd7* mRNAs in somatic but not germline cells can be attributed to a specific 3′ UTR-binding protein that prevents *miR-430* function in germline cells[95]. Intriguingly, together with the FMRP-related protein FXR1, AGO2 (possibly as a part of an miRNP) acts as an activator of translation when bound to the 3′ UTR of tumour necrosis factor-α mRNA in serum-starved human cells[71]. This finding possibly reveals one of many potential combinations of the interplay between the miRNPs and RBPs that are interacting with mRNA 3′ UTRs. Because RBPs such as ELAV1 can act as translational activators by interfering with the miRNP-mediated repression of translation, it is also possible that miRNPs might act as translational activators by either displacing or modulating inhibitory RBPs bound at the 3′ UTR. Likewise, in other circumstances, miRNPs and RBPs might act synergistically to either repress or activate mRNA translation (FIG. 4).

Conclusions and prospects

Perhaps the paramount open question is whether miRNAs inhibit protein synthesis by a primary single mechanism or by different mechanisms. In other words, is it possible that miRNAs trigger an initial event that is then amplified by different mechanisms? On the basis of the many lines of evidence, it is widely believed that miRNAs suppress protein synthesis by a bevy of mechanisms. Although this could be the case, it is too early to draw this conclusion with certainty. A simple, alternative mechanistic model posits that the earliest event in protein-synthesis repression is the inhibition of cap-dependent translation through the binding of AGO to the cap structure. Secondary effects of this inhibition could then be manifested at other steps, such as mRNA degradation or proteolysis of the nascent polypeptide chains. It is conceivable that the different outcomes of the miRNA repression experiments occur partially because of the different experimental systems and methodologies. Although the use of *in vitro* systems allows identification and biochemical characterization of early events during repression, the reporter mRNAs lack a nuclear history that could involve deposition of RBPs that modify the mRNA properties and affect the response to miRNAs. The same applies to the *in vitro* transcribed mRNAs that were transfected into cultured cells. Indeed, differences in the outcome of miRNA-mediated repression have been reported, depending on whether RNA or DNA was used for transfection, or even on the method of transfection[35]. Finally, it should be recognized that the steps that limit protein expression can differ among different transfected reporter genes or *in vitro* transcribed mRNAs[10].

It will be crucial to understand the regulation of miRNA function through modulation of the activity of RISC components and associated factors, possibly by phosphorylation and other protein modifications. Thus, the involvement of different signalling pathways in the control of miRNA function should be studied. It is also highly likely that the mechanisms that control translation initiation will have a significant impact on miRNA-regulated gene expression. It will also be important to determine the precise contributions of different cellular structures, such as P-bodies and SGs, to miRNA-mediated repression of translation. The fact that miRNA function can be recapitulated in cell-free extracts argues against a primary and essential role of P-bodies and SGs in miRNA repression, inasmuch as these microscopic structures are unlikely to exist in cell-free extracts. However, pseudo-polysomes that are formed in extracts from *D. melanogaster* embryos[53] might contain constituents of P-bodies, and it will be interesting to find out if this is indeed the case. The availability of cell-free systems to study miRNA function is a significant development. It is hoped that these systems will generate a detailed and precise mechanistic picture of the miRNA-mediated inhibition of protein synthesis, as has been accomplished for transcription, translation and splicing.

Finally, a complete and accurate understanding of the mechanism of miRNA function will require elucidation of three-dimensional structures of animal AGO proteins, their complexes with the miRNA and the cap structure, and ultimately the structure of miRNP bound to mRNA. Structural information would help validate or refute the current models for miRNA function.

Note added in proof

Two papers have recently appeared which add new information about the miRNA-mediated repression. Vasudevan *et al.*[137] showed that while miRNAs repress translation in proliferating mammalian cells, they induce translation upregulation of target mRNAs upon cell-cycle arrest. Kedde *et al.*[138] identified dead end 1 (Dnd1) as a protein that, by binding to the target mRNA 3′UTR, counteracts the function of miRNAs in human cells and zebrafish germ cells.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
HAC1 | *KRAS* | *let-7* | *lin-4* | *lin-14* | *lin-28* | *miR-430* | *pal1* | *tdrd7*

FlyBase: http://flybase.bio.indiana.edu
Argonaute1 | Argonaute2 | *armitage* | *gawky* | *nanos* | *oskar* | *loquacious* | *pasha*

UniProtKB: http://ca.expasy.org/sprot
AGO1 | AGO2 | AGO4 | APOBEC3G | CAT1 | DCP1 | DCP2 | DGCR8 | Dicer | ELAVL1 | ELAV2 | ELAV3 | ELAV4 | exportin5 | FXR1 | GLD1 | GW182 | LIMK1 | LSM1 | LSM3 | PABP1 | RN3 | TRBP | XRN1

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