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The intricate balance between microRNA-induced mRNA decay and translational repression

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Abstract:

Post-transcriptional regulation of messenger RNAs (mRNAs) (i.e., mechanisms that control translation, stability, and localisation) is a critical focal point in spatio-temporal regulation of gene expression in response to changes in environmental conditions. The human genome encodes...
~2,000 microRNAs (miRNAs), each of which could control the expression of hundreds of protein-coding mRNAs by inducing translational repression and/or promoting mRNA decay. While mRNA degradation is a terminal event, translational repression is reversible and can be employed for rapid response to internal or external cues. Recent years have seen significant progress in our understanding of how miRNAs induce degradation or translational repression of the target mRNAs. Here, we review the recent findings that illustrate the cellular machinery that contributes to miRNA-induced silencing, with a focus on the factors that could influence translational repression vs. decay.

**Introduction**

MicroRNAs (miRNAs) are a group of small, ~22 nucleotide, non-coding RNAs [1, 2]. Each miRNA can target multiple mRNAs that contain the cognate miRNA-binding site [3]. Similarly, each mRNA can be regulated by multiple miRNAs. It is estimated that more than 60% of human protein-coding genes have at least one conserved miRNA-binding site [4]. Hence, the expression and activity of miRNAs are interlinked with various cellular processes including development, maintenance of homeostasis, and diseases such as cancer and metabolic disorders [5-7]. It has been suggested that miRNAs often have a significant effect on mRNA stability, however their impact on translation alone could also be responsible for a large portion of the phenotypic effects downstream of miRNA dysregulation. Recent evidence suggest that most miRNAs use a successive process of translational repression followed by removal of the 3′ poly(A) tail (deadenylation) and 5′ cap structure (decapping) of the mRNA, leading to the exposure of the mRNA to exonuclease-mediated degradation [8-10] (Fig. 1). However, numerous examples have demonstrated deviations from this conventional model and significant variations in the extent of translational repression vs. decay have been described.

Here, we highlight the machinery that controls both translational repression and mRNA decay induced by miRNAs and discuss the interplay between the mRNA decay and translation processes that determine the outcome of miRNA-induced silencing. This review predominantly focusses on mechanisms of miRNA-induced silencing in human and, where relevant, highlights the differences in factors involved in miRNA-induced silencing in other organisms.

**miRNAs biogenesis**

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miRNAs originate from hairpin-containing primary transcripts (pri-miRNAs) that are mainly transcribed by RNA polymerase II [11]. In mammalian cells, the vast majority of pri-miRNAs are processed within the nucleus by the Microprocessor complex, composed of Drosha, an RNase III enzyme and two DGCR8 proteins [12]. This produces the precursor miRNA (pre-miRNA), a 60-70 nucleotide (nt) stem-loop RNA with a 2 nt overhang at its 3’end, which is then exported to the cytoplasm via the Exportin/Ran-GTPase transport system. Once in the cytosol, the ribonuclease Dicer, in cooperation with the RNA-binding protein TRBP, cleaves the loop structure in pre-miRNA and produces mature miRNAs in the form of a ~22 nt RNA duplex with a 5’ phosphate and a 2 nt 3’ overhang on each strand [13]. In a process known as RISC-loading, Dicer, TRBP, and the chaperone proteins HSC70/HSP90 load the miRNA duplex onto an Argonaute (AGO) protein which is an integral part of the miRNA-Induced Silencing Complex (miRISC) [14]. Once handed over, the ‘guide strand’, which is nearly always the one with the lower stability (pU or pA) at the 5´ terminus [15, 16] acts as a core component for the formation of the miRISC and directs the complex to the target mRNA, whereas the ‘passenger strand’ is either cleaved or released intact if the two strands are mismatched [17, 18]. Notably, while the biogenesis of the vast majority of miRNAs follows this “canonical” processing route, several “non-canonical” miRNA biogenesis pathways have also been characterised in plants, C. elegans, D. melanogaster, as well as mammalian cells (reviewed in [19]).

Key components of the miRNA-induced silencing machinery

miRISC

Biochemical isolation of RISC revealed complexes with different sizes ranging from ~150 kDa up to ~3 MDa, which demonstrates its variable composition [20]. The minimum requirements for a functional miRISC complex are the miRNA and AGO proteins [21]. AGO proteins can bind to any guide strand regardless of nucleotide sequence, however certain AGOs prefer specific bases on the 5´-end. For instance, human AGO2 shows a clear preference for uridine in the 5´ position [22]. AGOs recruit additional factors to the complex (Table1), such as Dicer, TRBP, PACT and TNRC6A-C (GW182) proteins [23].

The glycine-tryptophan (GW)-rich 182KD protein, GW182, is required for miRNA-induced silencing and is suggested to act as a scaffold for the assembly of miRISC. GW182 interacts with AGOs [24],
PAN3 [25], CNOT1, and CNOT9 [26], subunits of the PAN2/PAN3 and CCR4-NOT complexes, further discussed below. The three GW182 paralogues in vertebrates are known as trinucleotide repeat-containing 6A (TNRC6A), TNRC6B, and TNRC6C [27]. Each AGO protein has a single GW182 binding site, however each GW182 can potentially bind to 3 AGOs. As such, a single GW182 protein can enable cooperativity in miRNA-mediated gene silencing by up to 3 independent miRNA/AGO complexes [28]. GW182 can also translationally repress target mRNAs independent of AGO proteins, indicating that while miRNA/AGO complex is required for recruitment of GW182 to the target mRNA, the downstream effect on the target mRNA is independent of the miRNA/AGO [29].

**Poly(A)-binding proteins (PABPs)**

PABPs comprise a family of highly conserved RNA-binding proteins (RBPs) with key roles in regulation of mRNA nuclear export [30], translation initiation [31], termination [32], and regulation of the length of poly(A) tails and mRNA decay [33]. The typical mammalian mRNA has an approximately 200-250 nt poly(A) tail [34]. Transcriptome-wide measurement of the poly(A) tail length across eukaryotes revealed that most highly expressed and translated mRNAs contain relatively short poly(A) tails that accommodate a minimal number of PABPs [35]. However, in certain contexts [e.g., in pre-gastrulation Zebrafish (Danio rerio) embryos but not in non-embryonic tissues] the length of poly(A) tail directly correlates with the translation efficiency of the mRNA and deadenylation leads to robust translational repression [36, 37]. PABPs physically protect the poly(A) tail against deadenylase, evidenced by the observation that poly(A) tails are often shortened in a periodicity of ~30 nt, a signature of PABP footprint [38]. Removal of PABP or its inhibition with excess poly(A) competitors result in destabilisation of the mRNA [33]. Direct interaction of poly(A)-bound PABP with eIF4G enhances eIF4F-mediated cap-dependent translation via promotion of the interaction between eIF4E and the cap [39], and augmentation of the RNA helicase activity of eIF4A [40].

**PAN2/PAN3 (PAN2/3) complex**

The poly(A)-specific ribonuclease (PAN) was the first identified deadenylating enzyme, the mutation of which leads to alterations in mRNA poly(A) tail lengths [41]. The two subunits of the PAN complex,
PAN2 and PAN3, interact in a 1:2 stoichiometry [42, 43]. PAN2 is a Mg\(^{2+}\)-dependent exoribonuclease, while PAN3 lacks catalytic activity but directly binds to poly(A) through an N-terminal zinc finger and its pseudokinase/C-terminal domain. PAN3 recruits PAN2 to mRNA [42] and stimulates the intrinsic deadenylating activity of PAN2 [44]. The interacting partners of PAN3 could determine the level of specificity of the deadenylating activity of PAN2/3; interaction with the cytoplasmic poly(A)-binding protein (PABPC) results in bulk poly(A) mRNA deadenylation [45], whereas interaction with GW182 [46] results in mRNA-specific deadenylation.

**CCR4-NOT complex**

The multi-subunit carbon catabolite repression 4 (CCR4)-negative on TATA-less (NOT) (CCR4-NOT) complex (Table 1) plays a canonical role in miRNA-induced translational repression. CCR4-NOT is generally thought to consist of two major modules: i) the catalytic module comprised of the deadenylase proteins CNOT6, CNOT6L, CNOT7 and CNOT8 [47-50] and ii) the NOT module, minimally consisting of CNOT1, CNOT2, and CNOT3 [51]. The non-catalytic subunits substantially increase the deadenylase activity and sequence-specificity of the catalytic subunits [52], likely by providing binding sites for other factors that recruit CCR4-NOT to their target mRNAs. The modular structure allows for binding of the catalytic module to CNOT1 independent of the NOT module and vice-versa [47], thus allowing for the independent regulation of the downstream module-specific functions. CNOT1, the scaffolding subunit of this complex, provides docking sites for other subunits (Fig. 1).

**Multifaceted impacts of miRNAs on target mRNAs**

**Deadenylation and 3’→ 5’ decay**

PAN2/3 and the CCR4-NOT complexes are critical for miRNA-induced deadenylation. These complexes cooperate in a ‘bi-phasic’ deadenylation process, in which mRNA deadenylation is initiated by PAN2/3 without significantly affecting mRNA stability [53]. The remaining poly(A) stretch, which has low capacity for PABP binding is susceptible to rapid deadenylation by CCR4-NOT [53, 54]. The CCR4-NOT-mediated deadenylation is terminated once a stretch of more than two non-
adenosine residues is encountered [52], thus preventing the degradation of the 3’ UTR by CCR4-NOT. However, the resulting deadenylated mRNA is prone to 3’→5’ degradation, which is mainly achieved by the activity of the RNA exosome (reviewed in [55]).

A given CCR4-NOT complex contains either CNOT6 or 6L and either CNOT7 or 8, owing to their mutually exclusive interaction with the same binding sites on CNOT7/8 and CNOT1, respectively [56]. Overexpression of CNOT7/8 and to a lesser extent CNOT6/6L significantly enhances Let-7-induced deadenylation and mRNA decay. Conversely, depletion of CNOT7/8, but not CNOT6/6L, slows Let-7-induced deadenylation in HeLa cells [57]. However, overexpression or depletion of PAN2/PAN3 does not have any effect on Let-7-induced deadenylation [57], indicating the predominant role of CCR4-NOT in this process. Surprisingly, while deadenylation of the target mRNAs is a cardinal effect of miRNAs, the presence of a poly(A) tail promotes miRNA-induced repression and mRNAs that lack a poly(A) tail are relatively resistant to miRNA-induced silencing [58, 59]. However, translational repression by miRNAs does not strictly require a poly(A) tail as depletion of CNOT1 alleviates translational repression of poly(A)-less mRNAs induced by tethered GW182 [26] and replacement of poly(A) with a 3’ histone stem-loop effectively restores miRNA-induced translational repression [60].

Translational repression

All eukaryotic mRNAs contain a 5’-cap structure, m7GpppN, where m is a methyl group, and N is any nucleotide. In the cytoplasm, the eukaryotic translation initiation factor 4E (eIF4E) binds to the cap as well as to eIF4G, which functions as a scaffold and in turn interacts with eIF4A RNA helicase. eIF4G also directly interacts with the eIF3 complex, which recruits the 43S pre-initiation complex (PIC), an assembly of the 40S ribosomal subunit and the ternary complex (TC), consisting of eIF2, initiator methionyl-transfer RNA (tRNA^{Met}) and GTP. This results in the formation of the 48S PIC that scans the 5’ UTR in 5’→3’ direction until a start codon is recognised [61]. Upon detection of the start codon by the PIC, the 60S ribosomal subunit joins to form the 80S ribosome and initiate the “elongation phase”.

Translational repression of the target mRNAs was noticed at the dawn of the discovery of miRNAs [62]. However, the exact mechanism and the stage at which the mRNA translation process is most
susceptible to miRNA-induced repression were not clear. It was initially suggested that miRNAs could repress mRNA translation by abrogating the formation of the 80S complex [63] or by blocking the elongation phase [64-66]. However, latest evidence suggests cap-dependent initiation as the main stage at which miRNAs interfere with the translation machinery. Accordingly, mRNAs that lack the 5’ cap but possess an Internal Ribosome Entry Site (IRES), are refractory to miRNA-induced silencing [58, 67, 68]. Reporter mRNAs capable of various types of IRES-mediated translation initiation revealed that translation driven solely by encephalomyocarditis virus (EMCV) IRES is completely resistant to miRNA repression. The EMCV IRES requires the eIF4G subunit of eIF4F complex, without employing eIF4E [58]. Experiments with mouse Krebs-2 cell extracts demonstrated that miRNA-induced silencing was suppressed with increasing concentrations of purified eIF4F [68]. These observations indicated that miRNAs interrupt eIF4F-mediated cap-dependent initiation. However, contesting mechanisms have been proposed to describe how eIF4F function is disrupted by miRNAs as discussed below.

Interrupting ribosome scanning

RNA helicase eIF4A is critical for efficient scanning of the 5’ UTR by 48S PIC. The human genome encodes three different eIF4A paralogs (eIF4A1-3). eIF4A1 and 2 participate in the formation of the eIF4F complex. However, while eIF4A1 is essential for efficient translation, the absence of eIF4A2 does not affect global translation [69]. It was suggested that in HeLa cells eIF4A2, but not eIF4A1, is recruited to the target mRNA via direct binding to the MIF4G domain of CNOT1, thus blocking the scanning of the 5’ UTR by 48S PIC. According to this model (Fig. 2A), the presence of upstream open-reading frames (uORFs) [70], or secondary structure within the 5’ UTR are the major determinants of the sensitivity of mRNAs to miRNA-induced translational repression, and mRNAs with unstructured 5’ UTRs are refractory to miRNA-induced repression [71, 72]. Notably, this model suggests that the competition between eIF4A2 and RNase helicase DDX6 (discussed in more detail below) for binding to the MIF4G domain of CNOT1 determines the fate of target mRNA. Unlike DDX6, eIF4A2 interferes with the deadenylase activity of CNOT7, which also interacts with the MIF4G domain of CNOT1. Thus, mRNAs bound to eIF4A2 have longer poly(A) tails than mRNAs bound to DDX6 [73]. In line with these findings, bioinformatic analyses showed that local structures in 5’ UTRs near the cap and start codon contribute to the miRNA-induced translational repression [74]
and analysis of the miR-17-92 targets in mouse revealed that sensitivity of target mRNAs to miR-17-92 is determined by the CG content in their 5’ UTRs [75].

In a cell-free system derived from HEK293T cells, it was shown that AGO2-RISC could induce dissociation of both eIF4A1 and eIF4A2 from mRNA without affecting eIF4G or recognition of the 5´-cap by eIF4E [76]. Consistent with this observation, treatment with Silvestrol, a pharmacological inhibitor that locks eIF4A onto mRNA, antagonised miRISC-mediated translational repression. This suggests that CCR4-NOT displaces eIF4A1 and 2 from the target mRNA, rather than inhibiting initial recruitment of eIF4A to the mRNA [76]. Notably, the Drosophila genome only encodes one protein homologous to eIF4A, the absence of which in Drosophila S2 cells was shown to attenuate miRNA-induced translational repression [77]. However, while Drosophila AGO1 promoted eIF4A displacement independently of GW182, the Drosophila GW182 displaced both eIF4A and eIF4E [77]. Furthermore, CRISPR/Cas9-mediated knockout as well as shRNA-mediated knockdown of eIF4A2 in human (HeLa) and mouse (NIH3T3) cells revealed that eIF4A2 is dispensable for miRNA-induced silencing [69]. Reporter mRNAs that are translated in a scanning-independent mechanism via the translation initiator of short 5´ UTR (TISU) sequences were efficiently repressed by miRNAs [78]. In addition, the length or secondary structures of the 5´ UTRs of the reporter mRNAs were shown to have no linear correlation with miRNA-induced translational repression [71, 78].

**Competition with eIF4E for binding to the cap**

While eIF4E binds to the cap very efficiently, this binding is subject to competition by other cap-binding proteins such as the eIF4E paralogue proteins, eIF4E2 (also known as 4E-Homologous Protein; 4EHP) [79] and eIF4E3 [80]. Recent data have revealed that the 4E-Transporter (4E-T) protein plays a key role in miRNA-induced silencing (Fig. 2B). Besides regulating the nucleocytoplasmic shuttling of eIF4E, 4E-T also prevents eIF4E interaction with eIF4G [81], thus repressing translation initiation [82-84]. 4E-T is predominantly localised to P-bodies along with eIF4E, CNOT6/6L, and decapping factors [84, 85]. Depletion of 4E-T impairs miRNA and CCR4-NOT-induced silencing [83, 86, 87] and tethering of 4E-T to reporter mRNAs induces deadenylation and translationally repression in the absence of decapping, resulting in accumulation of deadenylated and translationally repressed mRNAs [84]. Similar to human 4E-T, CUP, the 4E-T homologue in
Drosophila, also promotes deadenylation and translational repression in the absence of decapping [88].

4E-T binds to eIF4E and 4EHP through the eIF4E-binding motif (YX₄LΦ). This interaction bridges the 3´UTR decay machinery to the 5´ cap that is necessary for decapping [85]. Although a mutant form of 4E-T that is not able to interact with eIF4E and 4EHP can enhance mRNA decapping and 5´→ 3´ decay, indicating that 4E-T can promote degradation of target mRNA even in the absence of interaction with eIF4E and 4EHP [84]. 4E-T also interacts with LSM14, Patl1, DDX6, and CNOT1 [84]. This modular interaction with different binding partners provides the opportunity for simultaneous regulation of multiple aspects of mRNA metabolism. Accordingly, while numerous studies suggest that 4E-T affects miRNA and CCR4-NOT-induced translational repression [84, 86-89], it has also been observed that mRNAs targeted by miRNAs and CCR4-NOT have increased stability in 4E-T-depleted cells [82, 85], indicating a potential role for 4E-T in determining the fate of target mRNAs (i.e., promoting translational repression or decay). Nevertheless, the precise mechanism by which 4E-T influences the cap-dependent translation initiation and subsequent decapping/decay remains unclear.

In addition to its direct binding to the C-terminus of CNOT1 [84], 4E-T is also recruited to the CCR4-NOT complex via the helicase DDX6, which in turn binds the MI4G motif of CNOT1 [90, 91]. Experiments conducted in both HeLa and Xenopus cells have shown that tethering of DDX6 in HeLa cells [92] or its Xenopus homologue, Xp54 [93] to the 3´ UTR of a luciferase reporter mRNA results in strong translational repression and depletion of DDX6 results in reduction of miRNA- and GW182-induced translational silencing [91, 94, 95]. Importantly, in contrast to 4E-T [84] [88], depletion of DDX6 results in deadenylation of miRNA targets in the absence of decapping and accumulation of translationally repressed, deadenylated mRNAs [96]. In fact, several studies have suggested that DDX6 plays a role in decoupling translational repression and decay, wherein absence of DDX6 leads to upregulated mRNA translation without concurrent changes in mRNA stability [97-99]. This could occur due to abrogation of recruitment of 4E-T and/or GIGYF2 protein and 4EHP (as described below) to the target mRNA in the absence of DDX6.

The eIF4E parologue 4EHP is ubiquitously expressed in all cell types [100] but shows 30-fold less affinity for the 5´ cap compared to eIF4E [101]. Unlike eIF4E, 4EHP does not bind to eIF4G and is unable to form a translation promoting complex [102], yet it extensively interacts with components of miRISC, CCR4-NOT and their associated factors such as 4E-T, DDX6, and GIGYF2 [86].
Furthermore, compared with eIF4E, 4EHP has greater efficiency for binding to 4E-T [86] and interaction with 4E-T significantly increases the affinity of 4EHP, but not eIF4E, for the cap [86]. Thus, recruitment of 4EHP to the CCR4-NOT complex through 4E-T could displace eIF4E from the cap and repress mRNA translation, while avoiding exposure of the cap to the decapping and mRNA decay machinery (Fig. 2B). Consistent with this model, depletion of 4EHP in human [86, 103] and C.elegans [104] abrogates miRNA, GW182, and CNOT1-induced translational silencing. miRNA-induced translation inhibition through 4EHP plays a key role in cellular response to viral infection. 4EHP suppresses production of IFN-β, a critical cytokine in the host defence against viral infection, by effecting the miR-34a-induced translational silencing of Ifnb1 mRNA [105]. Translational repression of target mRNAs by the miRNA/CCR4-NOT/4E-T/4EHP axis also leads to maintenance of the MAPK/ERK pathway’s activity by mediating the miR-145-induced translational repression of Dusp6 mRNA, which encodes the ERK-specific dual phosphatase DUSP6, thus maintaining cell proliferation and survival [106].

The GRB10-interacting GYF (glycine-tyrosine-phenylalanine motif) proteins GIGYF1 and GIGYF2 also play key roles in miRNA-induced silencing. GIGYF proteins compete with each other and with 4E-T for binding to 4EHP [104, 107, 108]. However, despite the presence of the canonical 4E-binding motif (YX₄LΦ), neither GIGYF1 nor GIGYF2 interacts with eIF4E in human cells. GIGYF2 also interacts with miRISC, through interaction of the GYF motif of GIGYF2 with the PPGL motif of GW182 [107-109] (Fig. 2C), presenting an alternative 4E-T independent mechanism of recruitment of 4EHP to miRNA-targeted mRNAs. Accordingly, while GW182 recruits GIGYF2 via its GYF motif, interaction of the N-terminal domain of GIGYF2 with 4EHP results in translational repression in the absence of decay. Alternatively, instead of 4EHP, GIGYF2 can interact with CNOT1 and promote translational repression accompanied by deadenylation [110]. This notion is consistent with a prior observation that while depletion of NOT1 in Drosophila completely abolishes the mRNA decay induced by tethered GW182, it only partially restores translation of the reporter mRNA [111]. It is worth noting that GIGYF2 does not interact with GW182 in Drosophila, which could partly explain the differences and likely complicates the comparison of observations in human cells and Drosophila [112]. GIGYF2 also directly interacts with DDX6 [113], thus provides an additional mechanism by which CNOT1/DDX6 could recruit GIGYF2 and 4EHP to the target mRNA. Importantly, the mechanism which dictates the recruitment of 4EHP to either 4E-T or GIGYF2 upon miRNA-induced silencing is not clear. Notably, the C.elegans GYF-1 protein (ortholog of GIGYF2) was recently shown to interact with IFE-4 (ortholog of 4EHP) in order to repress the translation of miRNA-targeted
mRNAs without inducing mRNA deadenylation and decay [104]. Interruption of this mechanism via deletion of GYF-1 leads to defects in early embryonic development [104].

Decapping and 5´→3´ decay

Decapping is an essential step in 5´→3´ mRNA decay (Fig. 3). Decapping exposes the 5´ end of the mRNA to degradation by the conserved 5´→3´ cytoplasmic exonuclease XRN1, which is recruited to the target mRNA through direct interaction with Decapping protein 1 (DCP1), ensuring the rapid removal of decapped mRNAs [114]. DCP2 is the best characterised decapping enzyme [115]. Association of DCP2 with the regulatory subunit DCP1 and several decapping enhancers including PatL1, DDX6, Edc1-4, the Lsm1-7 complex, and regulatory factors such as LIMD1, modulates its activity and efficiency [116-121]. Earlier studies in Drosophila revealed that mRNA decay induced by miRNAs or tethered GW182 requires the DCP1:DCP2 complex and co-depletion of DCP1 and DCP2 resulted in accumulation of shorter and deadenylated forms of mRNAs that are translationally repressed [111]. Interaction with DCP1 induces a conformational change in DCP2, resulting in rotation of the DCP2 catalytic domain and enhanced binding to the cap [122]. The enhancers of decapping proteins, Edc3 and Edc4, in turn augment DCP2 decapping activity by stabilising its interaction with DCP1 [123]. Members of the Lsm1-7 complex and PatL1 also play essential roles in linking mRNA deadenylation and decapping by distinguishing between oligoadenylated and polyadenylated mRNAs, thus recruiting DCP2 to the oligoadenylated mRNAs [124].

4E-T plays a nuanced role in regulation of miRNA-induced silencing by simultaneous promotion of deadenylation and repression of decapping, thus storing the mRNA in a translationally repressed but deadenylated state [84]. While the precise mechanism by which 4E-T blocks decapping of the translationally repressed mRNA remains unclear, this ability is likely dependent on its interaction with the cap-binding proteins 4EHP/eIF4E [125], which protect the cap against the decapping enzyme (Fig. 2B). This notion is consistent with previous observations that cap-binding proteins block the removal of the cap by DCP2 [126]. However, the biological significance of the storage of deadenylated mRNAs, the reversibility of this process, and the mechanism that could trigger the recovery of their adenylated state for efficient mRNA translation are not understood.
A microRNA conundrum: to degrade or keep a translationally repressed target mRNA?

In contrast to the irreversibility of mRNA decay, reversible translational repression is an agile ‘rheostat’ for maintenance of homeostasis, enabling the cell to rapidly resume protein synthesis in response to environmental stimuli such as stress. Therefore, attaining balance between inducing decay vs translational repression by miRNAs is critical in processes that require rapid modulations of protein expression, such as regulation of the antiviral immune response by cytokines.

While high-throughput analyses have shown that the bulk of miRNA-induced repression is due to mRNA decay [127, 128], temporal dissections have demonstrated that there is translational inhibition of various degrees preceding mRNA deadenylation [8-10, 127, 129-131] and that miRNA-induced decay can only occur after this translational repression [8, 132, 133]. Other studies have also revealed translational repression of target mRNAs in the absence of detectable mRNA decay or deadenylation [75, 106, 134-137]. A meta-analysis of the relative contributions of mRNA decay and translational repression to miRNA-induced silencing in primary cells or tissues from mutant mice with genetic alteration of individual miRNAs (total of 159 target genes from 77 miRNAs) revealed that 48% of target genes are predominantly regulated by translational repression, 29% are regulated mainly by decay, and 23% are regulated by both [138]. The variability in the outcome of miRNA-induced decay and translational repression may thus be attributed to the intrinsic features of the target mRNAs and extrinsic factors that influence the assembly of the miRNA-induced machinery.

Intrinsic mRNA features affect the outcome of miRNA-induced silencing

Besides the length and nucleotide composition of the UTRs (reviewed in [139]), the degree of complementarity between the miRNA and target mRNA sequences and the location of the miRNA-binding site in relation to the ORF and UTRs can also influence the rate of miRNA-induced decay and/or translational repression. Indeed, full complementarity between the sequences of miRNA and target mRNA normally results in degradation of both molecules by a process termed target-directed miRNA degradation (TDMD) that induces their decay via the slicing activity of AGO2 [140]. Only a relatively small fraction (<6%) of all miRNAs in mammalian cells are susceptible to this mechanism [141].

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While the majority of reported miRNA-binding sites are within the 3’ UTRs, the presence of a considerable number of miRNA-binding sites within ORFs were also reported [142-146], although the complementary seed motifs may differ from those that bind to the 3’ UTRs, (e.g. extensive base-pairing at the 3’ sites rather than the 5’ sites) [146]. Importantly, miRNA-binding sites residing in the ORFs more potently inhibit translation compared with miRNA-binding sites located in the 3’ UTRs, which are more efficient at triggering decay [147]. While the precise mechanisms by which the ORF miRNA-binding sites repress translation is not clear, recent evidence suggests an AGO-dependent but GW182-independent mechanism that induces transient ribosome stalling [146]. Formation of an alternative miRISC that contains AGO3, instead of the canonical AGO2, may also contribute to the distinct effects of ORF-targeting miRNAs [148].

Overall, less efficiently translated mRNAs, due to lower initiation or elongation rates, are more susceptible to degradation [149, 150]. Codon composition affects both polyadenylation status and translation efficiency in various organisms [149]. Although different studies demonstrated variations in the extent of the effect of high vs. low optimal codon composition on miRNA-induced silencing, recent evidence suggests that highly translated mRNAs are more resistant to miRNA-induced silencing [151, 152]. However, the exact mechanism by which codon optimality and translation efficiency influence miRNA-induced silencing is not clear. In yeast, which does not possess any miRNA, Dhh1 (the yeast homologue of DDX6) functions as a sensor of codon optimality that preferentially associates with mRNAs with suboptimal codon choice, leading to activation of decay [153]. Considering the critical role of DDX6 in miRNA-induced silencing as well as in decoupling translational repression and decay [97-99], it would be interesting to investigate the role of DDX6 in linking codon optimality to differential translational repression and decay induced by miRNAs.

The Fragile X syndrome related RBP, FMRP, also selectively affects the stability of mRNAs based on codon optimality in neural cells, without affecting translation efficiency of these mRNAs [154]. Given the strong connection between the CCR4-NOT complex, DDX6, and FRMP [155, 156] with the miRNA-induced silencing machinery, the possibility that these proteins also influence the extent of miRNA-induced mRNA decay and translational repression in response to the rate of translation remains to be examined.

**Composition of the miRNA-induced silencing machinery**
Rather than static assemblies, miRISC and CCR4-NOT are dynamic complexes, the composition of which can vary and thereby affect the outcome of miRNA-induced silencing. As described above, the modularity of CNOT1 and 4E-T proteins allows the formation of distinct complexes with various core subunits and associated proteins that influence translational repression or decay. For instance, direct binding of the RING finger protein 219 (RNF219) to CNOT9 inhibits the deadenylase activity of CCR4-NOT but enhances its capacity to repress translation of a targeted mRNA[157].

Similarly, miRISC exists in multiple, distinct conformations containing alternative subunits, with likely different impacts on miRNA induced decay and translational repression. Biochemical and genetic studies in C. elegans embryos indicate that miRISC can be present as a continuum of species which evolves from a free miRISC to a mRNA-bound form that contains additional subunits and recruits effector components of miRNA-mediated silencing [158]. As such, AIN-1 (the C. elegans homologue of GW182) interacts only with a subset of ALG-1 (homologue of AGO2)-containing RISCs [159]. GW182-independent miRNA-induced silencing was also observed in C. elegans embryos [160]. Further studies revealed a GW182-independent silencing mechanism in C. elegans germline, which translationally represses miRNA-target mRNAs in the absence of decay [161]. In Drosophila, absence of GW182 has a solely negative impact on miRNA-induced deadenylation but not translational repression [137]. Furthermore, miRNA-induced silencing in Drosophila occurs via distinct mechanisms by miRISC complexes that involve either AGO1 or AGO2. AGO1-containing miRISC represses translation primarily by deadenylation and by blocking the activity of the fully formed eIF4F on the mRNA cap. In contrast, AGO2-containing miRISC competitively blocks binding of eIF4G to eIF4E and inhibits the formation of eIF4F in the absence of deadenylation [162]. Thus, it appears that naturally occurring miRISC complexes with distinct components could differentially affect miRNA-induced decay and translational repression.

Post-translational modifications of key components of miRISC and CCR4-NOT also control the composition and function of these complexes. Phosphorylation of human AGO2 at the Ser387 residue by Akt3 facilitates its interaction with GW182 and translational repression in the absence of decay [163]. Notably, physiological stresses such as hypoxia could potentially influence AGO2–mRNA interactions resulting in altered mRNA translation rates [164], due to phosphorylation of Ser387, which is induced in stress conditions through the action of p38 MAPK [165]. Ser387 phosphorylation may also contribute to remodelling of the miRISC complex by influencing the binding of AGO2 with LIM-domain protein LIMD1, which in turn facilitates interaction of AGOs with TNRC6A

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and DDX6 [166]. Poly-ADP-ribosylation of AGO1-4 and other components of miRISC has also been observed under stress conditions, which reduces their activity [167]. This mechanism is critical for activation of translation of interferon mRNAs by antiviral signalling pathways upon detection of a viral infection [168]. Notably, SUMOylation of Lys400 on mRNA-loaded human AGOs by RanBP2 inhibits the ubiquitination of AGO protein, leading to stabilisation of miRISC and more efficient translational repression of the target mRNAs, such as interleukin 6 (IL6) mRNA [169]. Human AGO2 can also be S-nitrosylated on the highly conserved Cys691 upon treatment with nitric oxide. This modification blocks AGO interaction with GW182 and reduces the Let-7-induced silencing, without affecting the ability of AGO2 to interact with either miRNA or mRNA [170]. Considering the key role of GW182 in induction of both deadenylation and translational repression by miRNAs, it would be interesting to investigate the potentially divergent effects of this AGO S-nitrosylation on mRNA decay and translational repression.

GW182 was initially identified as a phospho-protein [171] and phosphorylation of TNRC6C within its PAM2 domain reduces its interaction with PABPC1 [172]. Phosphorylation of TNRC6A at Ser1332/Ser1346, located within the C-terminal region, reduces its interaction with CCR4-NOT complex [173], likely decreasing their ability to cause miRNA-induced silencing. However, the exact contribution of these post-translational modifications to the divergent control of miRNA-induced decay and translational repression remains to be understood.

Concluding remarks

Despite their tiny sizes, the over 1500 miRNAs that modulate the expression of more than 60% of human protein coding genes have a mighty impact on patho-physiological processes. Therefore, defining the mechanism(s) by which miRNAs function could have a profound impact on our understanding of homeostasis and disease and provide promising opportunities to intervene. The last two decades have witnessed an amazing level of progress in understanding the various aspects of the miRNA-induced silencing machinery, including the myriad of proteins and complexes involved in this process. This review aimed to summarise the most important findings in this field with particular emphasis on the key factors that determine the extent to which a cognate miRNA:mRNA pair will undergo transient translational repression vs. permanent decay. While we are only able to present a snapshot of the wealth of knowledge in this field, we have highlighted multiple and at times

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contradictory proposed mechanisms, which are not easily reconciled in light of our present understanding. It is to be expected that these contradictions will be addressed through further investigation, but we recognise that there may not be a single universally conserved mechanism responsible for miRNA-induced silencing in different organisms and developmental stages. The mode of miRNA-induced silencing could be highly influenced by factors such as developmental stage and environmental conditions. For instance, in early embryos, miRNAs predominantly induce translational repression without decay, whereas in other developmental contexts mRNA decay is the predominant mode of miRNA-induced silencing. This difference has been attributed to the observation that while in embryonic stages the length of the poly(A) tail is linked to the mRNA translation efficiency, this link is removed in post-embryonic systems [174, 175]. However, this general proposition does not explain the numerous examples of miRNA-induced translational repression in the absence of decay in post-embryonic cells [62, 75, 106, 134-137, 176]. Another critical issue is the considerable variation in mechanisms observed with different model organisms that further complicates the interpretation of existing data. A conspicuous example is the stark difference in the number of AGO genes, with both redundant and unique functions, among different species (e.g., 8 in mammals compared with 27 in C. elegans [177, 178]). This level of heterogeneity among various model organisms may reflect the presence of diverse mechanisms in different species and tissues.

Finally, dysregulated expression or function of various factors involved in miRNA-induced translational repression and/or mRNA decay has been linked to multiple diseases such as neurological [179], metabolic [180], and developmental disorders [181, 182], cancer [183], and altered innate immune system [105]. A better understanding of the mechanistic details of this mechanism, particularly the criteria that determine whether a target mRNA is degraded or translationally repressed by a cognate miRNA could ultimately benefit research into these pathophysiological processes, wherein rapid modulation of gene expression mediated by miRNAs is widespread.

Author contributions:

PN and SMJ wrote the manuscript with input from TW, LA, and APH.
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**Table 1. Main subunits of the mammalian miRISC and CCR4-NOT complexes and factors involved in miRNA-induced silencing.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Complex</th>
<th>Main Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drosha</td>
<td>Microprocessor</td>
<td>Type III RNase, miRNA biogenesis in the nucleus</td>
</tr>
<tr>
<td>DGCR8</td>
<td>Microprocessor</td>
<td>RNA- and heme-binding protein</td>
</tr>
<tr>
<td>Dicer</td>
<td>Microprocessor</td>
<td>Type III RNase, miRNA biogenesis, RISC-loading</td>
</tr>
<tr>
<td>AGO1-4</td>
<td>RISC</td>
<td>Endonuclease, strand selection, interaction with GW182 and other proteins</td>
</tr>
<tr>
<td>TRBP</td>
<td>RISC</td>
<td>Double-strand RNA binding protein</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td><strong>PACT</strong></td>
<td><strong>RISC</strong></td>
<td>Double-strand RNA binding protein</td>
</tr>
<tr>
<td><strong>TNRC6A-C</strong></td>
<td><strong>RISC</strong></td>
<td>Scaffold protein, repressing translation and enhancing mRNA decay</td>
</tr>
<tr>
<td><strong>PABC</strong></td>
<td></td>
<td>poly(A) binding protein</td>
</tr>
<tr>
<td><strong>PAN2</strong></td>
<td><strong>PAN2/3</strong></td>
<td>Exoribonuclease, mRNA deadenylation</td>
</tr>
<tr>
<td><strong>PAN3</strong></td>
<td><strong>PAN2/3</strong></td>
<td>poly(A) binding protein, recruitment of PAN2 to the mRNA, stimulation of the intrinsic deadenylating activity of PAN2</td>
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<td><strong>CNOT1</strong></td>
<td><strong>CCR4-NOT</strong></td>
<td>Scaffold</td>
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<td><strong>CCR4-NOT</strong></td>
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<td><strong>CCR4-NOT</strong></td>
<td>Promoting mRNA decay, interaction with ribosome</td>
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<td><strong>CCR4-NOT</strong></td>
<td>E3 ligase</td>
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<td><strong>CNOT6</strong></td>
<td><strong>CCR4-NOT</strong></td>
<td>Deadenylase</td>
</tr>
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<td><strong>CCR4-NOT</strong></td>
<td>Deadenylase</td>
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<tr>
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<td><strong>CCR4-NOT</strong></td>
<td>Binding site for TNRC6/GW182</td>
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<td><strong>CCR4-NOT</strong></td>
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<tr>
<td><strong>CNOT11</strong></td>
<td><strong>CCR4-NOT</strong></td>
<td>Unknown, contributing to complex stabilization through creating CNOT10-CNOT11 module</td>
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<td><strong>DDX6</strong></td>
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<td>Putative RNA helicase, repression of translation initiation, promotion of decapping</td>
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<table>
<thead>
<tr>
<th>Protein</th>
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<td>Cap-binding protein, repression of translation initiation</td>
</tr>
<tr>
<td>4E-T</td>
<td>Repression of translation initiation</td>
</tr>
<tr>
<td>GIGYF2</td>
<td>Repression of translation initiation, promotion of mRNA decay</td>
</tr>
<tr>
<td>DCP1</td>
<td>Decapping, DCP2 activator</td>
</tr>
<tr>
<td>DCP2</td>
<td>Decapping enzyme</td>
</tr>
<tr>
<td>PatL1</td>
<td>Decapping enhancer</td>
</tr>
<tr>
<td>Edc3</td>
<td>Stabilising DCP1:DCP2 complex, enhancing DCP2 enzymatic activity</td>
</tr>
<tr>
<td>Edc4</td>
<td>Stabilising DCP1:DCP2 complex, enhancing DCP2 enzymatic activity</td>
</tr>
<tr>
<td>XRN1</td>
<td>Cytoplasmic 5´→ 3´ exoribonuclease, mRNA decay</td>
</tr>
</tbody>
</table>

**Figure legends**

**Figure 1. miRNA-induced silencing pathway.** miRNAs bind to the mRNA targets in association with an AGO protein that forms the core of the miRISC complex. AGO protein binds a GW182 (TNRC6) protein, which in turn recruits the CCR4-NOT complex through direct interaction with CNOT1 and CNOT9 subunits. Interaction of GW182 with PAN3 results in recruitment of the PAN2/PAN3 deadenylase complex, leading to mRNA deadenylation through concerted actions of the PAN2/PAN3 complex and the deadenylase module of CCR4-NOT composed of the CNOT7/8 and CNOT6/6L subunits. The MIF4G domain of CNOT1 plays a key role in recruitment of the deadenylase module and decapping factors. MIF4G domain also promotes translational repression through interaction with factors involved in translational repression of the target mRNA (See Fig. 2 for
more detail). The dotted lines show the presence of the indicated domain in CNOT1 protein; Huntington, Elongation Factor 3, PR65/A, TOR (HEAT), Middle of Initiation Factor 4G (MIF4G), CNOT9-Binding Domain (CN9BD), Superfamily Homology Domain (SHD).

**Figure 2. Alternative models for miRNA-induced repression of translation initiation.**

A) eIF4A2 interaction with CNOT1 through MIF4G domain displaces it from eIF4F complex and blocks the ribosomal scanning of the 5´ UTR. eIF4A displaces DDX6 from CNOT1 through competition for binding to the MIF4G domain and thus interrupts the recruitment of the deadenylase module to CNOT1 and blocks mRNA decay. B) Upon binding to the MIF4G domain in CNOT1, DDX6 interacts with 4E-T, which in turn recruits the cap-binding protein and translation inhibitor 4EHP and inhibits the eIF4F complex by displacing eIF4E from the cap. C) Interaction of the GYF motif of GIGYF2 with the PPGL motif on GW182 enables its recruitment to miRISC. GIGYF2 regulates translational repression and/or decay through distinct and direct interactions with CNOT1, CNOT7 and 4EHP.

**Figure 3. miRNA-induced decapping and 5´→3´ decay.** Recruitment of the DCP2 decapping enzyme and its associated protein DCP1 by CNOT1 is essential for miRNA-induced 5´→3´ mRNA decay. DDX6 protein serves as a hub for interaction with several other proteins such as EDC3/4 and PATL1 that enhance the decapping activity of DCP1/2. The 5´→3´ exonuclease XRN1 degrades the mRNA once the cap is removed.
Figure 1

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Figure 2

(A) ORF miRNA binding site

m^G

4E-T

DDX6

Decapping

Deadenylation

(B) ORF miRNA binding site

m^G

4E-T

DDX6

Decapping

Deadenylation

(C) ORF miRNA binding site

m^G

DDX6

Decapping

Deadenylation

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Figure 3

miRNA binding site

ORF

Decapping

XRN1

DDX6

PATL1

EDC3

EDC4

Decapping

Deadenylation

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