

# Control of translation and mRNA degradation by miRNAs and siRNAs

Marco Antonio Valencia-Sanchez,<sup>1,3</sup> Jidong Liu,<sup>2</sup> Gregory J. Hannon,<sup>2</sup> and Roy Parker<sup>1,4</sup>

<sup>1</sup>Department of Molecular and Cellular Biology and Howard Hughes Medical Institute, University of Arizona, Tucson, Arizona 85721, USA; <sup>2</sup>Cold Spring Harbor Laboratory, Watson School of Biological Sciences, Cold Spring Harbor, New York 11724, USA

**The control of translation and mRNA degradation is an important part of the regulation of gene expression. It is now clear that small RNA molecules are common and effective modulators of gene expression in many eukaryotic cells. These small RNAs that control gene expression can be either endogenous or exogenous micro RNAs (miRNAs) and short interfering RNAs (siRNAs) and can affect mRNA degradation and translation, as well as chromatin structure, thereby having impacts on transcription rates. In this review, we discuss possible mechanisms by which miRNAs control translation and mRNA degradation. An emerging theme is that miRNAs, and siRNAs to some extent, target mRNAs to the general eukaryotic machinery for mRNA degradation and translation control.**

**micro RNAs (miRNAs)/short interfering RNAs (siRNAs) are important regulators of eukaryotic mRNAs**

A key aspect of the regulation of eukaryotic gene expression is the cytoplasmic control of mRNA translation and degradation. Over the past decade, miRNAs and siRNAs have emerged as important regulators of translation and mRNA decay. The regulatory pathways mediated by these small RNAs are usually collectively referred to as RNA interference (RNAi) or RNA silencing. As discussed in more detail below, miRNAs and siRNAs can silence cytoplasmic mRNAs either by triggering an endonuclease cleavage, by promoting translation repression, or possibly by accelerating mRNA decapping. Originally described in *Caenorhabditis elegans*, hundreds of such molecules and their possible targets have now been discovered in the genomes of plants and animals (Bartel and Chen 2004). Strikingly, bioinformatics analyses suggest that up to 30% of human genes may be regulated by miRNAs (Lewis et al. 2005).

[Keywords: miRNA; siRNA; translation]

<sup>3</sup>Present address: Universidad de Sonora, Escuela de Medicina, Hermosillo, Sonora 83000, Mexico.

<sup>4</sup>Corresponding author.

E-MAIL rrparker@u.arizona.edu; FAX (520) 621-4524.

Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1399806>.

miRNAs and siRNAs are ~21–26-nucleotide (nt) RNA molecules. Although both types of molecules can be functionally equivalent, they are distinguished by their mode of biogenesis (Carmell and Hannon 2004; Kim 2005). miRNAs are produced from transcripts that form stem-loop structures. These are processed in the nucleus by a complex comprised of at least two components: the RNase III enzyme Drossha, and a protein called Pasha in *Drosophila* or DGCR8 in mammals (Lee et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). Initial cleavage is followed by exportin-5-mediated transport to the cytoplasm of a 65–75-nt pre-miRNA, which is further processed by the cytoplasmic RNase III endonuclease Dicer complex (Yi et al. 2003; Lund et al. 2004). Final processing by Dicer appears coupled to assembly of the miRNA into the RNA-induced silencing complex (RISC), which is the effector of RNAi (Gregory et al. 2005). In contrast, siRNAs are produced from long double-stranded RNA (dsRNA) precursors, which can be either endogenously produced or exogenously provided. Processing of siRNAs is also Dicer-dependent and their assembly into the RISC complex is facilitated by the Dicer enzyme complex, at least in some cases (Tomari et al. 2004).

The key component of the RISC complex is an Argonaute protein. Argonaute proteins are consistently found in RISC complexes from a variety of organisms (Carmell et al. 2002). The Argonaute protein family is diverse, with all members containing a PAZ domain, which is involved in miRNA/siRNA binding, and a PIWI domain, which is related to RNaseH endonucleases and functions in slicer activity (Lingel and Sattler 2005). Argonaute proteins directly interact with the miRNA/siRNA (Song et al. 2003; Ma et al. 2004, 2005). Most eukaryotes examined contain multiple Argonaute family members, with different Argonautes often specialized for distinct functions. For example, in *Drosophila*, Ago1 appears to primarily function in miRNA-mediated translation repression, while Ago2 acts in siRNA-catalyzed endonucleolytic cleavage (Okamura et al. 2004). Similarly, in humans, Ago2 is the only Argonaute capable of endonuclease cleavage (Liu et al. 2004; Meister et al. 2004). Additional proteins have been associated with the RISC complex—including the Vasa intronic gene (VIG) pro-

tein, the Tudor-SN protein, Fragile X-related protein, the putative RNA helicase Dmp68, and Gemin3 (Caudy et al. 2002, 2003; Ishizuka et al. 2002; Mourelatos et al. 2002)—although their generality or precise role in RNAi remains to be determined.

A key issue in miRNA/siRNA function is the specificity of their interactions with their target mRNAs and how each interaction leads to discrete downstream consequences. From a number of experiments, some key principles of this interaction have emerged. First, based on experimental manipulation, base-pairing between the 5' end of the miRNA (residues 2–7) and the mRNA target plays a primary role in establishing interactions, with the important feature being the thermal stability of the miRNA:mRNA interaction (Doench and Sharp 2004). Moreover, the 5' portion of related miRNAs is the most highly conserved. Second, the 3' portion of the miRNA can also contribute to efficient repression, and it has been suggested to work as a modulator of suppression (Doench and Sharp 2004; Kiriakidou et al. 2004; Kloosterman et al. 2004). Third, for efficient endonuclease cleavage, base-pairing is needed at the site of cleavage, between bases 10 and 11 (Elbashir et al. 2001; Haley and Zamore 2004; Martinez and Tuschl 2004). Fourth, while only one complementary site is generally sufficient to direct repression by cleavage, multiple sites are required for efficient translational repression, with a few exceptions (Doench et al. 2003; Zeng et al. 2003; Doench and Sharp 2004; Kiriakidou et al. 2004). Fifth, the interaction of miRNA and Argonaute with the mRNA may be influenced by other sequence-specific RNA-binding proteins, thus providing an additional level of specificity to miRNA:mRNA interactions. This possibility is suggested by the observations that an RNA-binding protein, GW182, interacts with Argonaute proteins and is required for efficient miRNA-mediated repression in animals (Ding et al. 2005; Jakymiw et al. 2005; Liu et al. 2005b; Rehwinkel et al. 2005), and that the RNA-binding protein TTP collaborates with a miRNA to affect the decay rate of some mRNAs (Jing et al. 2005). Surprisingly, in this latter case, the sequence within miRNA that is important for pairing with the target mRNAs is from nucleotide 11 to 18. This study suggests miRNAs could have more far reaching and general effects on gene regulation. Finally, because effective repression of the LIN-14 mRNA by the LIN-4 miRNA appears to require a bulge in the miRNA:mRNA duplex (Ha et al. 1996), the specific conformation of the miRNA:mRNA duplex may be important in function, perhaps to allow the recruitment of additional RNA-binding proteins in specific contexts.

### miRNAs and siRNAs can direct endonucleolytic cleavage of mRNAs

One manner in which miRNAs and siRNAs control post-transcriptional gene expression is by directing endonuclease cleavage of the target mRNA. Such endonuclease cleavage, referred to as “Slicer” activity, was first demonstrated in cell cultures with exogenously pro-

vided dsRNAs (Tuschl et al. 1999; Hammond et al. 2000). However, it is now appreciated that some endogenous miRNAs in both plants and metazoans direct endonucleolytic cleavage (Llave et al. 2002; Yekta et al. 2004; Allen et al. 2005). Endonucleolytic cleavage is generally favored by perfect base-pairing between the miRNA/siRNA and the mRNA, although some mismatches can be tolerated and still allow cleavage to occur (Mallory et al. 2004; Yekta et al. 2004; Guo et al. 2005). Interestingly, extensive base-pairing between the miRNA and the mRNA is not always sufficient to induce cleavage, suggesting that there can be additional requirements for a RISC complex to catalyze endonucleolytic cleavage (Chen 2004).

One additional requirement for slicer activity is that a specific Argonaute protein be present within RISC. For example, in mammalian cells, biochemical and genetic studies have identified Ago2 as the only one of the four mammalian Ago proteins capable of directing cleavage (Liu et al. 2004; Meister et al. 2004). Ago2 has an RNaseH-like domain and contains all of the critical active residues to carry out cleavage. Moreover, mutations in the RNaseH domain of Ago2 abrogate siRNA-mediated cleavage (Liu et al. 2004; Song et al. 2004). In vitro-specific cleavage activity is dependent on siRNA-Ago2 binding and it does not need the presence of any other factor (Rivas et al. 2005). These results define the minimal RISC composition needed for siRNA-directed cleavage in mammals as the miRNA/siRNA and the Ago2 protein. Some Argonaute proteins lack the catalytic residues and hence enzyme activity. However, there are also cases in which Argonaute proteins are inactive despite the presence of all known catalytic residues. The underlying cause of this deficit is currently unknown. The requirement for a specific Argonaute protein for endonuclease cleavage suggests that a specific miRNA that preferentially assembles with a particular Argonaute protein, perhaps due to its mode of biogenesis, might be unable to direct cleavage, even if the miRNA/mRNA base-pairing is perfect.

### mRNA fragments generated by RISC cleavage are directed to the general cellular mRNA degradation machinery

The products of RNAi-mediated cleavage appear to be degraded by the same enzymes that degrade bulk cellular mRNA. Eukaryotic cells contain two general and conserved pathways for the degradation of bulk mRNA, both of which require an initial removal of the 3' poly(A) tail in a process referred to as deadenylation (Parker and Song 2004). In one case, deadenylation is followed by 3'-to-5' exonucleolytic degradation by the exosome, a multimeric complex with 3'-to-5' exonuclease activity. Alternatively, after deadenylation, mRNAs can be decapped by the Dcp1/Dcp2 decapping enzymes and degraded 5'-to-3' by the abundant 5'-to-3' exoribonuclease, Xrn1p.

Evidence suggests that following mRNA cleavage triggered by siRNAs or miRNAs, the 3' fragment is degraded by major cellular 5'-to-3' exonucleases. For example, in

*Drosophila* S2 cells in culture, Xrn1p is required for degradation of the 3' cleavage product from RISC-mediated cleavage (Orban and Izaurralde 2005). Similar results are seen in plant cells, where loss of the *Arabidopsis* homolog of Xrn1 (Xrn4) leads to stabilization of some of the 3' products of miRNA induced cleavage (Souret et al. 2004).

The degradation mode of the 5' fragment from miRNA/siRNA-induced cleavage is less clear, and this fragment may be subject to two alternative fates. This 5' fragment can be a substrate for the exosome, since in S2 cells, knockdown of the exosome and/or the associated SKI complex leads to the accumulation of this 5' cleavage product (Orban and Izaurralde 2005). A second fate of the 5' product can be the addition of a 3' tail after the site of cleavage that includes predominantly Us, but can include As and Cs (Shen and Goodman 2004). This uridylation occurs in both plants and animals and could be a mechanism to enhance degradation for poor substrates for the exosome, which might require a 3' extension to activate the exosome. This would be similar to polyadenylation activating decay of structured RNAs in bacteria and of defective pre-RNAs in the eukaryotic nucleus (for review, see Jensen and Moore 2005). Alternatively, uridylation may compete with 3'-to-5' degradation, and substrates where uridylation occurs could end up being targeted for decapping and 5'-to-3' degradation. Consistent with this latter possibility, oligouridylation seems to correlate with shortening of the 5' end of the cleavage product (Shen and Goodman 2004).

#### miRNAs can target mRNAs for slicer-independent decay

Several observations now suggest the possibility that miRNAs also target mRNAs for increased decay by a slicer-independent mechanism. For example, introduction of specific miRNAs into HeLa cells by transfection decreases the levels of a population of transcripts that contain potential binding sites for the miRNA (Lim et al. 2005). Similarly, analyses in *C. elegans* indicate that the let-7 and lin-4 miRNAs reduce the levels of their target mRNAs (Bagga et al. 2005), although how these studies differ from those that previously led to different conclusions is unclear (Wightman et al. 1993). An additional example in which a miRNA can target mRNAs for decay comes from the analysis of mRNA decay stimulated by an AU-rich 3' UTR regulatory sequence (ARE). AREs are a common class of sequences that control mRNA decay rates and translation in eukaryotic cells (for review, see Espel 2005). Recent work suggests that the miR-16 miRNA functions with RISC and the sequence-specific RNA-binding protein TTP to target an ARE containing mRNA for degradation (Jing et al. 2005).

An unresolved issue is the mechanism by which these miRNAs are targeting mRNAs for degradation. In principle, these miRNAs could also be leading to activation of slicer activity. However, this possibility seems unlikely, because in many of these cases, the expected sites of cleavage are mismatched between the miRNAs and the mRNA target, and none of the expected mRNA de-

cay intermediates from slicer activity have been observed (Bagga et al. 2005; Jing et al. 2005). In the case of ARE-mediated degradation, experiments suggest the miRNA/RISC complex is not involved in exosome-mediated decay because RNAi inhibition of exosome function has no impact (Jing et al. 2005). These observations suggest that miRNAs, minimally in combination with Argonaute proteins, either target mRNAs to an unknown decay pathway, or might promote mRNA decapping and 5'-to-3' degradation.

Evidence that miRNAs might target mRNAs for decapping has come from comparing the subcellular distribution of Argonaute proteins with the decapping machinery. In a range of eukaryotic cells, including yeast and mammals, the decapping enzyme (consisting of Dcp1/Dcp2), Xrn1p, and several activators of decapping are concentrated in specific cytoplasmic foci known as cytoplasmic processing bodies (P-bodies, also referred to as GW-bodies), which can be sites of mRNA decapping and degradation (Sheth and Parker 2003; Cougot et al. 2004). In tissue culture cells, all four versions of the mammalian Argonaute proteins are concentrated in P-bodies and can coimmunoprecipitate with the decapping enzyme (Jakymiw et al. 2005; Liu et al. 2005b; Pillai et al. 2005; Sen and Blau 2005). Similarly, ALG-1, which is one of the Argonaute family members in *C. elegans*, can accumulate in P-bodies (Ding et al. 2005). The concentration of Argonautes in P-bodies in mammalian cells requires interaction with small RNAs, but is independent of catalytic activity (Liu et al. 2005a). The mRNA targets of miRNAs also accumulate within P-bodies in a miRNA-dependent manner (Liu et al. 2005a; Pillai et al. 2005). Quantitation of microscopic images suggests that at least 20% of the target mRNAs is concentrated in easily visualized P-bodies, and this fraction could be higher if there are additional P-bodies that are too small to be easily visualized in the light microscope (Pillai et al. 2005). Indeed, based on nonquantitative RT-PCR analysis, the majority of a mRNA repressed by the Let-7 miRNA, is found in a biochemical fraction containing P-bodies (Pillai et al. 2005). Based on these results, a strong prediction is that miRNAs target mRNAs to P-bodies, increasing their association with the decapping machinery and thereby potentially reducing their levels by decapping and 5'-to-3' degradation.

Several other observations are consistent with miRNAs and RISC increasing decapping rates. First, knockdown of Xrn1p in *C. elegans*, which would be required to degrade the mRNA body after decapping, was observed to attenuate the decrease in mRNAs levels in mRNA targets caused by let-7 and lin-4 miRNAs (Bagga et al. 2005). Second, partially degraded mRNAs were detected for the lin-41 mRNA in *C. elegans* that extended from the 5' side of the mRNA:miRNA duplex to the 3' end of the mRNA (Bagga et al. 2005). Such decay intermediates are consistent with 5'-to-3' exonucleolytic degradation of the mRNA, with stalling of the Xrn1p at the position of RISC on the target RNA. Previous results have also suggested that Xrn1p is required for efficient RNAi in *C. elegans*, possibly because Argonaute proteins fail to be



recycled if the transcript is not degraded (Newbury and Woollard 2004). Finally, knockdowns of Dcp1p and/or Dcp2p in *Drosophila* S2 cells, or mammalian cells, in culture led to an inhibition of miRNA-based repression of a reporter mRNA, although whether Dcp1/Dcp2 knockdown affected the reporter mRNA levels and/or mRNA decay rates in response to a miRNA was not examined (Liu et al. 2005b; Rehwinkel et al. 2005). The absence of this data leaves open to question whether miRNAs are driving mRNA decapping or translation repression, and Dcp1p/Dcp2p are required for efficient translation repression, as can be seen in yeast cells under some conditions (Holmes et al. 2004; Collier and Parker 2005).

In summary, the reduction in mRNA levels by miRNAs, the interaction and colocalization of miRNAs, Argonautes, and mRNA targets to P-bodies, and the functional interactions between miRNA-mediated repression and the decapping enzyme and Xrn1p, suggests the reasonable hypothesis that miRNAs will in some cases increase decapping rates. A direct test of this hypothesis will hopefully emerge and might include a direct demonstration that a miRNA can increase the decay rate of a mRNA in a manner dependent on the decapping enzyme both *in vivo* and in reconstituted systems *in vitro*. Moreover, because decapping generally occurs following translation repression and the mRNA exiting translation (for review, see Collier and Parker 2004), miRNAs might induce decapping as a downstream consequence of repressing translation.

### miRNAs can reduce translation

A third way that miRNAs silence mRNAs is by interfering with their translation. This was first suggested by the observation that the lin-4 miRNA reduced the amount of lin-14 protein, without reducing the amount of the lin-14 mRNA (Lee et al. 1993; Wightman et al. 1993). Although recent observations suggest that the lin-4 might also affect mRNA levels (Bagga et al. 2005), there are now multiple other examples where silencing by a miRNA is observed with either no change in the mRNA level, or with a significantly smaller decrease in mRNA levels than is observed for protein (e.g., Brennecke et al. 2003; Chen 2004; Poy et al. 2004; Cimmino et al. 2005). Similarly, several reporter mRNA systems have been constructed in mammalian cells, where silencing either by an endogenous miRNA, an exogenously provided miRNA, or tethering of the Argonaute to the mRNA fusion by a sequence-specific RNA-binding protein reduces protein production by a greater amount than mRNA levels (Doench et al. 2003; Saxena et al. 2003; Zeng et al. 2003; Pillai et al. 2005).

Translation repression by miRNAs can be generally distinguished from slicer activity by several features. First, while substantial bulges in the helix in the vicinity of the cleavage-site block slicer activity, they can still allow efficient translation repression. Second, the ability to repress translation is thought to be common to all members of the Argonaute family of proteins. For ex-

ample, tethering of either human Ago2 or Ago4 to a target mRNA can lead to translation repression (Pillai et al. 2004). Since Ago1, Ago3, and Ago4 also accumulate in P-bodies in mammalian cells (Liu et al. 2005b; Pillai et al. 2005), a reasonable assumption is that Ago1, Ago3, and Ago4 will also function in translation repression in human cells. In this regard, translational repression in response to miRNAs remains intact in Ago2-null cells (Liu et al. 2004). Third, efficient translation repression by miRNAs often utilizes multiple miRNA-binding sites. This was first suggested by the observation that the early identified mRNA targets of miRNAs contained multiple sites for miRNA binding, either the same miRNA or a combination of several different miRNAs (Bartel and Chen 2004). Moreover, this property has been experimentally reconstructed (Doench et al. 2003; Zeng et al. 2003; Kiriakidou et al. 2004). However, it should be noted that many predicted targets of miRNAs only contain a single miRNA-binding site in their 3' UTR (e.g., see Brennecke et al. 2005; Lewis et al. 2005), suggesting that such single sites may lead to fine "tuning" of mRNA function (Bartel and Chen 2004). Whether multiple sites are required for efficient repression to ensure occupancy of at least one site by a RISC complex, or because multiple RISC complexes act in an additive manner to repress translation remains unresolved.

### How do miRNAs repress translation?

Recent observations suggest that miRNA/RISC may decrease the rate of translation initiation. For example, Argonaute proteins, miRNAs, and mRNA targets of miRNAs accumulate in P-bodies in a miRNA-dependent manner (Jakymiw et al. 2005; Liu et al. 2005a; Pillai et al. 2005; Sen and Blau 2005). P-bodies are thought to contain pools of mRNAs not engaged in translation, because P-bodies show a reciprocal relationship with polysomes, do not contain the translation machinery, and contain and require mRNAs for assembly (Andrei et al. 2005; Brengues et al. 2005; Kedersha et al. 2005; Teixeira et al. 2005). Thus, the accumulation of mRNA targets of miRNAs and the Argonaute proteins in P-bodies argues that miRNAs are increasing the amount of ribosome-free mRNA. Moreover, in mammalian cells, translation repression by the Let-7 miRNA, or by tethered Argonaute proteins, can shift the mRNA target to lighter fractions in polysome gradients, which argues that miRNA-mediated repression can reduce translation initiation (Pillai et al. 2005). Additional evidence that miRNAs can affect translation initiation is that alterations in the translation initiation process can make an mRNA resistant to miRNA-induced translation repression (see below). For example, tethering of the translation factors eIF-4E or eIF-4G to an mRNA makes it resistant to miRNA-induced repression (Pillai et al. 2005). Note that if miRNAs affected nascent protein stability or a step after translation initiation, one would expect translation driven by tethered translation-initiation factors to still be repressed.

### Evidence for 'P-body formation' being important in RNA silencing

Evidence that the localization of Argonaute proteins in P-bodies is functionally important has come from the identification of a new and conserved Argonaute-binding protein. Specifically, the mammalian GW182 protein, a major component of P-bodies (Eystathiou et al. 2003), has been found to colocalize and coimmunoprecipitate with Argonaute proteins (Jakymiw et al. 2005; Liu et al. 2005a). Similarly, a homolog of GW182 in *C. elegans*, referred to as Ain-1, was found to coimmunoprecipitate, and in some cases, colocalize with a member of the Argonaute family in *C. elegans*, Alg-1 (Ding et al. 2005). Moreover, reconstitution of binding between recombinant Alg-1 and in vitro-translated Ain-1 suggests that the interaction between these GW182 family members and Argonaute proteins may be direct (Ding et al. 2005). Finally, both GW182 and Ain-1 coimmunoprecipitate with miRNAs (Ding et al. 2005; Jakymiw et al. 2005). These results identify the GW182 protein family as a conserved Argonaute-interacting protein.

Several experiments now indicate that the GW182 protein family is also functionally important in miRNA-mediated repression, in a manner that directly correlates with its ability to function in P-body assembly. First, siRNA knockdown of GW182 function in mammalian cells reduces P-body formation and inhibits miRNA-mediated translation repression, and may also affect slicer-dependent repression (Jakymiw et al. 2005; Liu et al. 2005b). Second, introduction of a dominant negative allele of GW182 in mammalian cells also reduces P-body formation and affects miRNA/siRNA silencing (Jakymiw et al. 2005). Third, mutations in Ain-1 have developmental phenotypes consistent with defects in miRNA-based repression (Ding et al. 2005). Fourth, siRNA knockdown of GW182 in *Drosophila* S2 cells inhibits miRNA-mediated repression (Rehwinkel et al. 2005), although whether P-bodies are affected in this case was not examined. The requirement for GW182 protein to form P-bodies and for miRNA-mediated repression argues that these two processes are linked.

Two experiments provide additional evidence that P-body formation and RNA silencing are linked. First, transfection of mammalian cells with a dominant negative fragment of Ago2 inhibits both RNA silencing and P-body formation (Jakymiw et al. 2005). Second, when the PAZ9 and PAZ10 mutant forms of the Ago2, which are unable to bind to miRNAs or accumulate in P-bodies, are tethered to reporter mRNAs, they are no longer able to repress translation (Liu et al. 2005a,b). However, the PAZ9 and PAZ10 Ago2 mutants still interact with Dcp1p, Dcp2p, and GW182, arguing that these proteins are not simply unfolded, but are defective in P-body localization and silencing per se (Liu et al. 2005a,b). These results suggest the possibility that translation repression and P-body targeting requires a transition in the RISC complex that could be dependent on Ago:miRNA interactions, miRNA:mRNA interactions, or possibly specific events in translation.

It remains to be clarified whether GW182 family members can also affect endonuclease cleavage triggered by siRNA/miRNAs. Two groups have only seen a small effect of GW182 knockdowns on slicer-mediated repression (Liu et al. 2005b; Rehwinkel et al. 2005), whereas another group saw a requirement for GW182 for what was anticipated to be a slicer-dependent mode of repression for the lamin A/C mRNA (Jakymiw et al. 2005). One simple explanation for these differences is that the siRNA used against lamin A/C represses by a combination of slicer-dependent and slicer-independent mechanisms.

In summary, the correlation between P-body formation and RNA silencing in multiple cases suggests that at least slicer-independent RNA silencing involves formation of a translationally repressed mRNP, which can then aggregate into P-bodies and might be subject to both translation repression and/or decapping. Whether the translation repression is sufficient once an individual mRNP has been formed or assembly into a larger P-body is required, is yet to be determined. In addition, a major issue to be addressed is whether RISC assembled on the mRNA interferes with a specific aspect of translation initiation and/or represses translation by promoting the assembly of the P-body mRNP.

### Translation initiation control mechanisms and their implications for the mechanism of miRNA-based repression

In order to discuss how miRNAs might repress translation initiation, it is helpful to review the process of translation initiation and how it is controlled on specific mRNAs. The process of translation initiation occurs by a series of key steps (for review, see Kapp and Lorsch 2004). For cap-dependent translation, which is the major mode of translation initiation, the 5' m<sup>7</sup>GpppG cap is recognized by the cap-binding protein, eIF-4E, part of the eIF-4F initiation complex. The eIF-4F complex then recruits a complex containing eIF3, the 40S subunit of the ribosome, and a ternary complex of eIF2, GTP, and the initiator tRNA. The 40S subunit is then thought to scan on the 5' UTR until an AUG is recognized, leading to joining of the 60S subunit to begin the elongation phase of translation. Initiation can also occur in cap-independent mechanisms, whereby internal ribosome entry sites (IRESs) recruit the translation machinery independent of the cap-binding protein in a variety of manners (see below).

There are two broad manners by which translation can be repressed. First, translation initiation can be regulated on specific mRNAs by affecting the ability of the mRNA to complete a step in the initiation process (for review, see Richter and Sonenberg 2005). For example, in *Drosophila* the *Oskar* mRNA assembles a tripartite complex wherein eIF-4E is bound to the cap, but is prevented from interaction with eIF-4G by the eIF-4E-binding protein *Cup*, which is delivered to the mRNA by an interaction with the sequence-specific binding of *Bruno* to the 3' UTR (Nakamura et al. 2004). Alternatively, recent re-

sults suggest that translation initiation rates can also be repressed by a competition between assembly of the translation initiation complex and a P-body mRNP, suggesting a model wherein cytoplasmic mRNAs are in equilibrium between translation complexes and P-body mRNPs, with the status of any individual mRNA being the summation and competition of interactions driving the assembly of these two biochemical states (Bregues et al. 2005; Collier and Parker 2005). Moreover, mRNA-specific repression complexes might feed into this general competition. For example, despite the eIF4E, Cup, Bruno repression complex, efficient translational repression of the *Oskar* mRNA during early development requires the *Drosophila* protein Me31b, whose yeast homolog functions in translation repression and P-body formation (Nakamura et al. 2001; Collier and Parker 2005).

These results suggest two possibly overlapping mechanisms by which miRNA and RISC might repress translation. In one model, a component of RISC, directly or through additional factors, inhibits the function of some translation initiation factor, thus leading to the mRNA exiting translation and accumulating in P-bodies. Alternatively, or in addition, RISC might contain or recruit proteins that promote the assembly of an mRNP that can accumulate within P-bodies and be sequestered from the translation machinery. It should be noted that because P-bodies are dynamic structures and, at least in yeast, mRNAs can cycle in and out of P-bodies (Bregues et al. 2005), the translation repression by RISC could be a kinetic effect on either increasing the rate of entry into P-bodies or decreasing the exit rate of mRNAs back into translation.



### Experimental alterations of translation initiation and its effect on miRNA-based translation repression

One manner to determine the mechanism by which miRNAs regulate translation is to alter aspects of the translation initiation process and then see whether miRNAs can still repress translation. For example, if miRNA/RISC represses translation by interfering with the cap-binding protein eIF-4E, then mRNAs without the cap structure would be expected to be resistant to miRNA-mediated repression. Several such experiments have recently been reported in mammalian cells examining how miRNAs repress translation when the mRNA is lacking a 5' cap structure, a poly(A) tail, or initiates translation through an IRES element, which bypasses the need for certain initiation factors. As summarized in Table 1, these experiments vary in design (e.g., some transfect DNA, some transfect mRNA directly), result, and interpretation. However, comparison across this set of experiments reveals some relatively clear results.

First, miRNAs can repress translation independent of the poly(A) tail. This is based on the observations that transfected capped, unadenylated mRNAs can be subject to miRNA-based repression (Humphreys et al. 2005; Pillai et al. 2005). Thus, miRNA-mediated repression does not work solely through the poly(A) tail of mRNAs.

Second, miRNAs can repress translation independent

**Table 1.** miRNA repression of mRNAs with altered translating initiation

<u>Structure of mRNA</u>	<u>Effect of miRNA</u>
<b>Transfected RNAs</b>	
m <sup>7</sup> G_____A <sup>+</sup>	6 - 10X repression <sup>1,2</sup>
m <sup>7</sup> G_____A <sup>-</sup>	2.5 - 10X repression <sup>*,1,2</sup>
Acap_____A <sup>+</sup>	2.5X repression <sup>†,2</sup>
Acap_____A <sup>-</sup>	No repression <sup>2</sup>
Acap_CrPV_____A <sup>-</sup>	No repression <sup>2</sup>
Acap_EMCV_____A <sup>-</sup>	No repression <sup>2</sup>
Acap_EMCV_____A <sup>+</sup>	2.5X repression <sup>†,2</sup>
m <sup>7</sup> G_HCV_____A <sup>+</sup>	No repression <sup>1</sup>
NoCap_EMCV_____A <sup>+</sup>	No repression <sup>1</sup>
<hr/>	
<b>in vivo Synthesized mRNAs</b>	
m <sup>7</sup> G_AUG UAA CrPV AUG UAA A <sup>+</sup>	miRNA repression of both cistron ORFs <sup>3</sup>
m <sup>7</sup> G_AUG UAA HCV AUG UAA A <sup>+</sup>	
m <sup>7</sup> G_AUG UAA  AUG UAA A <sup>+</sup>	miRNA repression of only upstream ORF <sup>1</sup>
m <sup>7</sup> G_AUG UAA  AUG UAA A <sup>+</sup>	

\* Evidence poly(A) tail not required for miRNA repression

† Evidence cap structure not required for miRNA repression

1. Pillai et al., 2005

2. Humphreys et al., 2005

3. Petersen et al., 2006

of the cap structure. This is based on the observation that transfected mRNA without an m<sup>7</sup>G cap, with or without an IRES, still are repressed by miRNAs (Humphreys et al. 2005). Thus, miRNA-mediated repression does not work solely through the 5' cap structure.

Third, some, but not all mRNAs containing IRES elements are subject to repression. For example, when a dual reporter mRNA is produced by in vivo transcription, translation from the HCV or CrPV IRES was still repressed by miRNAs (C.P. Petersen, M.E. Bordeleau, J. Pelletier, and P.A. Sharp, in prep.). Because the CrPV IRES initiates translation independent of all initiation factors, this would suggest that miRNA-mediated repression either affects a step in initiation involving the ribosome subunits, affects a step after translation initiation, or represses translation by sequestering the mRNA into a complex where it is not accessible to the ribosomes. However, when mRNAs with either the HCV or CrPV IRESs virus are transfected into cells, they escape repression by miRNAs (Humphreys et al. 2005; Pillai et al. 2005).

An unresolved question is why these experiments yield different results, although there are several possible factors. For example, transfected mRNAs may be differ-



ent from endogenously produced transcripts in terms of their associated proteins, which might affect their interaction with miRNAs or other aspects of cellular metabolism, which might indirectly affect miRNA control. Second, if miRNA-mediated translation repression affects one of two or more steps that can limit translation initiation rate, then one anticipates that only mRNAs that are limiting for the miRNA-affected step will be efficiently controlled by miRNAs. For example, since many mRNAs with IRES elements are relatively poorly translated, they may already be primarily in the P-body pool, and as such, may not be significantly affected by interactions promoting P-body targeting. This could explain why addition of a poly(A) tail to an mRNA where translation is IRES-dependent restores miRNA-mediated repression (Humphreys et al. 2005), since the poly(A) tail might promote the mRNA having a reduced concentration in P-bodies. Similarly, miRNAs may affect the balance between assembly of a translation complex and sequestration in a P-body, and it is the overall sum of the interactions dictating these competing assembly processes that determines whether or not an mRNA will be subject to miRNA repression. Interestingly, translation promoted by tethering of the cap-binding proteins, eIF4E or its binding partner eIF4G, upstream of an internal ORF is resistant to repression by the Let-7 miRNA (Pillai et al. 2005). This could be explained if the tethering of multiple copies of eIF-4E or eIF-4G produces robust translation initiation, which might then outcompete the assembly of a translation repression complex. Thus, it may be useful in future experiments to consider both the absolute and relative rates of translation when examining miRNA-based repression mechanisms.

### Can miRNAs repress translation by additional mechanisms?

Some observations suggest that miRNAs might repress translation by affecting a step in protein production after translation initiation (Olsen and Ambros 1999). This hypothesis was based on examining the control of the *lin-14* and *lin-28* mRNAs, which are developmentally regulated in *C. elegans* by the *lin-4* miRNA (Lee et al. 1993; Wightman et al. 1993). The crucial observation was that the polysomal distribution of both mRNAs did not change in response to repression (Olsen and Ambros 1999; Seggeron et al. 2002). More recently, similar observations have been made with a reporter mRNA in mammalian cells whose translation is repressed by the addition of an exogenous miRNA (C.P. Petersen, M.E. Bordeleau, J. Pelletier, and P.A. Sharp, in prep.). Two possible mechanisms to explain this observation were initially suggested (Olsen and Ambros 1999). These results could be explained if miRNA/RISC does not affect translation per se, but instead leads to the rapid destruction of the nascent polypeptide. However, because translation repression by miRNAs works on proteins targeted into the endoplasmic reticulum and is insensitive to proteasome inhibitors, such miRNA-dependent protein degradation would have to be proteasome independent and

work both in the cytosol and the endoplasmic reticulum (Pillai et al. 2005).

An alternative explanation for the similar polysome distribution of mRNAs with or without miRNA-based repression is that the miRNA/RISC might affect a combination of initiation, elongation, and termination rates, such that the average number of ribosomes remain constant but proteins were completed at a reduced rate. Note that in this latter model, multiple steps in translation would need to be affected, since only slowing elongation or termination rates would be expected to increase the average number of ribosomes per mRNA, while solely decreasing initiation rates would be expected to reduce the average number of ribosomes per mRNA.

It is difficult to reconcile the evidence that miRNAs can affect translation initiation with the failure in some cases to observe a change in polysome distribution. One possibility is that miRNAs and RISC can drive translation repression by multiple manners. Alternatively, it may be that the distribution of mRNAs in a polysome gradient is not solely a measure of mRNAs associated with ribosomes, and this overlap in biochemical fractionation is complicating the interpretation of polysome distributions. In either case, resolving the conflict between some polysome experiments and the evidence that miRNA/RISC may affect translation initiation is likely to provide new insights into miRNA function and/or polysome analysis.

### An integrated model for miRNAs/siRNAs and cytoplasmic mRNA metabolism

It is now possible to propose a working model for how RISC interacts with and affects cytoplasmic mRNAs. First, RISC interacts with specific mRNAs through base-pairing between the miRNA/siRNA and the mRNA. If this interaction is stable enough, RISC remains bound with each bound RISC contributing increased pressure for translation repression and eventual accumulation of the mRNA/RISC complex in P-bodies, although the molecular details of that effect remain unclear. One intriguing possibility is that a part of RISC-mediated translation repression will involve the sequestration of the cap structure in a complex with eIF4E, 4E-T, which binds to eIF4E and prevents it from recruiting eIF4G. This possibility is suggested by the presence of eIF4E and 4E-T in P-bodies in mammalian cells (Andrei et al. 2005; Ferraiuolo et al. 2005), and by the observation that knockdown of the 4E-T homolog in S2 cells did slightly reduce miRNA-mediated repression (Rehwinkel et al. 2005). If the base-pairing is sufficient and RISC contains a cleavage-competent Ago protein, the mRNA can be cleaved, before, during, or after accumulation of the mRNA:RISC complex in P-bodies. At what stage endonuclease cleavage would occur would simply be a function of the relative rates of cleavage versus translation repression and P-body accumulation.

In this model, whether an mRNA is subject to translation repression and/or decapping due to interaction

with a miRNA can be understood in terms of other properties of the specific mRNA. For example, when repressed by miRNAs, mRNAs with rapid decay rates may appear to be solely translationally repressed, since the mRNAs turnover is already fast. In contrast, long-lived mRNAs may be more susceptible to an increase in decay rates by miRNA repression. A subtler example may have to do with the relative rates of deadenylation. Specifically, because decapping usually requires prior deadenylation (Coller and Parker 2004), mRNAs with slow deadenylation rates, which thereby exist as adenylated mRNAs at steady state, might be expected to be translationally repressed, but not decapped following accumulation in a P-body biochemical state. In contrast, mRNAs that are generally deadenylated at steady state might be preferentially decapped due to accumulation in P-bodies. Finally, because translation and general mRNA decay can be differentially regulated in response to stresses or developmental stage (Zhang et al. 1999; Gowrishankar et al. 2005), the status of the cell may affect whether miRNAs trigger translation repression or decapping and degradation.

The hypothesis that miRNAs repress translation and/or enhance decapping by assembling a translationally repressed complex that accumulates in P-bodies predicts that miRNA-mediated repression will increase the deadenylation rate of the target mRNA. This prediction is based on the observations that decreases in translation initiation due to defects in translation factors, or the nonsense-mediated decay system in yeast, both target mRNAs to P-bodies and increase deadenylation rates (Schwartz and Parker 1999; Cao and Parker 2003; Teixeira et al. 2005). Because the poly(A) tail can enhance translation rates and inhibit mRNA decay, it should be noted that if miRNA/RISC increases deadenylation rates, this could provide an additional mechanism by which translation repression and mRNA decay could be stimulated.

### Future perspective and issues

The mechanisms by which miRNAs/siRNAs silence cytoplasmic mRNAs are becoming clarified with the mechanism of endonuclease cleavage the best understood at this time. A reasonable and testable hypothesis is that slicer-independent reductions in mRNA levels, and at least part of translation repression may be mechanistically similar and due to miRNAs/RISC assembling mRNAs into a translationally repressed mRNP that aggregates into P-bodies, although how this fits with the polysome experiments needs to be resolved. An additional important issue is how much the specificity and range of mRNA targets of miRNAs will be influenced by RNA-binding proteins that interact with the RISC complex. If this is a common phenomenon, then the range of mRNA repressed by miRNAs could be substantially broader than currently appreciated.

Finally, it should be considered that if miRNAs/RISC plays a role in targeting mRNAs to P-bodies, then we should anticipate that miRNAs/RISC will affect other

aspects of RNA metabolism that occur within P-bodies. For example, recent results argue that the Ty3 retrotransposon in yeast may assemble its virus-like particles in association with P-bodies (Beliakova-Bethell et al. 2006). This suggests that P-bodies may be important sites of specific steps in retrotransposon and viral life cycles that might then be modulated by miRNAs. Consistent with that possibility, the replication of the hepatitis C virus appears to be enhanced by the miR-122 miRNA (Jopling et al. 2005). Given this, there may still be additional roles for miRNAs and RISC that we do not yet appreciate.

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## Control of translation and mRNA degradation by miRNAs and siRNAs

Marco Antonio Valencia-Sanchez, Jidong Liu, Gregory J. Hannon, et al.

*Genes Dev.* 2006, **20**:

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