PERSPECTIVE

MicroRNAs repress translation of m⁷Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation

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The past decade has witnessed a veritable explosion in research into identifying the microRNA (miRNA) repertoires of various organisms and predicting the mRNA targets of these numerous miRNAs. By contrast, until quite recently, relatively little work had been done on investigating the mechanisms by which miRNAs control gene expression, which is the topic of this Perspective. We will concentrate primarily on mechanisms in vertebrates, drawing on *Drosophila* and *Caenorhabditis elegans* data where relevant but largely omitting plant miRNAs, as these seem sufficiently different in terms of mechanisms of action to make extrapolation between plant and vertebrate systems problematic. Although the current controversies over the exact mechanism of miRNA-mediated regulation of gene expression have been discussed in a number of recent reviews (Jackson and Standart 2007; Nilsen 2007; Pillai et al. 2007), what makes this additional article particularly timely are three reports of successful recapitulation of repression in cell-free systems, notably the results of Wakiyama et al. (2007) published in the previous issue, which is arguably the system that is the closest parallel to what is believed to occur in intact cells. Before discussing this article, however, we will first provide some background and will review the current (rather controversial) state of knowledge derived from transfection assays and other work with intact cells.

What are microRNAs and how do they differ from other small RNAs?

Strictly speaking, miRNAs are defined and distinguished from other small RNAs mainly on the basis of their biogenesis pathway rather than the mechanism by which they regulate gene expression. This pathway, reviewed by Bushati and Cohen (2007), starts with the scission of a larger precursor RNA by the nuclear enzyme Drosha (a

member of the ribonuclease III family of enzymes), to generate a ~60-nucleotide (nt) pre-miRNA that has a fold-back hairpin structure (Fig. 1). On export to the cytoplasm, the pre-miRNA is cleaved by Dicer (another RNaseIII-like enzyme) that is present in a complex with many other polypeptides, including Argonaute (Ago) proteins, as well as a dsRNA-binding protein TRBP (Chen-drimada et al. 2005; Haase et al. 2005). This results in the formation of a staggered duplex RNA, typically of 19 bp plus 2-nt unpaired overhangs at each 3'-end and, most important, a 5'-phosphate on each strand, which is essential for biological activity. One of the two strands, generally the one with the lower stability base-pairing at its 5'-end, is then assimilated into a multiprotein complex, designated as an miRNP or a RISC (RNA-induced silencing complex), while the other ("passenger") strand is degraded. miRNPs have a complex protein composition, including Ago proteins and GW182, an RNA-binding protein (Jakymiw et al. 2005). As is the case in RNA interference studies, exogenous miRNA mimics are introduced either by RNA transfection of the staggered duplex intermediate or the ~60-nt pre-miRNA or, alternatively, as a DNA construct that will give rise to the pre-miRNA via transcription.

In contrast to plant miRNAs, mammalian miRNAs rarely have perfect complementarity to their mRNA target sites. In the few cases where they are perfectly complementary (or show near-perfect complementarity with no internal mismatches or bulges), the outcome is endonucleolytic cleavage of the target mRNA at the annealing site, provided the miRNP/RISC included Ago2, the only one of the four mammalian Ago proteins with endonuclease activity (Meister et al. 2004; Yekta et al. 2004). Following this scission, the two mRNA fragments are degraded by enzymes of the normal mRNA turnover pathway.

By far the most usual relationship between vertebrate miRNAs and their target sites is one of imperfect complementarity, with substantial internal mismatched bulges, particularly in the vicinity of where the endonucleolytic cleavage would occur in a perfectly comple-

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1591507>.

Figure 1. The pathway of miRNA biogenesis. Pri-miRNAs, which are generally synthesized by RNA polymerase II, are trimmed in the nucleus by Droscha to generate a ~60-nt pre-miRNA which is exported to the cytoplasm, where it is further processed by Dicer, in association with one of the four mammalian Argonaute proteins (Ago) and TBRP (human immunodeficiency virus trans-activating response RNA-binding protein). The miRNA in the resulting miRNP complex is loaded onto the target mRNA, where it may result in translational repression and/or deadenylation followed by decapping and exonuclease-mediated degradation if base-pairing is partially complementary or, in the case of perfect complementarity and provided the miRNP contains specifically Ago2, may result in endonucleolytic cleavage of the mRNA at the site where the miRNA is annealed.

mentary miRNA/mRNA pair. Consequently, there is no cleavage with these mismatched pairs, not even if the miRNP/RISC includes the catalytically active Ago2. Instead, the outcome is repression of target gene expression by mechanisms that will be discussed in the following section. Repression normally requires perfect contiguous base-pairing of the mRNA target site with miRNA residues 2–8, the “seed match” (Doench and Sharp 2004; Brennecke et al. 2005). Repression is reduced if this region includes G-U pairs, and even a single mismatch or bulged residue may abrogate repression. In contrast, base-pairing between the mRNA target site and the 3′-proximal part of the miRNA is much less critical, though good pairing here can rescue repression if the base-pairing of the seed is marginally suboptimal. In addition, recent publications suggest that the exact configuration of the mismatches, the mRNA sequences flanking the target sites, and the distance separating target sites may influence the efficiency of repression and/or the preferred mechanism of repression (Aleman et al. 2007; Grimson et al. 2007).

In all cases of validated target sites in vertebrates, flies, and nematodes, these sites are located in the 3′-untranslated region (3′-UTR), often in multiple copies, and most bioinformatic exercises in target site prediction examine just the 3′-UTR. However, there would seem to be no a priori reason why repression would not, or could not, function with target sites located in the coding region or

the 5′-UTR, and results to this effect have recently been reported (Lytle et al. 2007). The degree of repression is related to the number of 3′-UTR target sites, at least up to six such sites, the maximum tested so far (Doench et al. 2003; Petersen et al. 2006). Moreover, miRNAs can act in a combinatorial fashion, in the sense that a reporter with two 3′-UTR sites for each of two different miRNAs would be repressed to a similar degree as one with four sites for either one of the miRNAs (Doench and Sharp 2004). All four mammalian Ago proteins seem capable of promoting repression dependent on mismatched target site/miRNA interactions (Pillai et al. 2004), even though Ago1, 3, and 4 have no known enzymatic activity. By contrast, *Drosophila* have just two AGO proteins, with largely non-overlapping functions, as will be discussed in the final section of this article.

The current state of the controversy over mechanism(s) of miRNA-mediated repression

The mechanism(s) of miRNA-mediated repression remain elusive and controversial. Although publications dating from more than two years ago invariably suggested that miRNAs repress target mRNA translation at some ill-defined stage subsequent to the initiation step, without decreasing target mRNA abundance, both of these propositions have been recently questioned (for reviews, see Jackson and Standart 2007; Nilsen 2007; Pillai et al. 2007). First, it is now clear that with some target mRNA/miRNA pairs, there is a significant increase in mRNA degradation rates and, consequently, a reduction in mRNA abundance. This increased degradation is via the normal pathway of deadenylation followed by decapping and then degradation of the body of the mRNA, *not* via an initial endonucleolytic cleavage such as occurs in RNA interference with siRNAs that have perfect complementarity to the target mRNA (Behm-Ansmant et al. 2006). At present it is unclear why the quantitative significance of this miRNA-mediated degradation should differ between different target mRNA/miRNA pairs: whether it is an intrinsic feature of the mRNA sequence or of the particular miRNA or, as has recently been suggested (Aleman et al. 2007), the exact configuration of the mismatches in the miRNA–mRNA interaction.

Second, although the early investigations into miRNA-mediated repression of target mRNAs by *C. elegans* *lin-4* and *let-7* miRNAs have suggested that repression operated at some stage after the translation initiation step, some (but by no means all) recent investigations into miRNA action in mammalian cells unambiguously indicate that initiation is the susceptible step. The most commonly used criterion for judging this issue is whether sucrose density gradient centrifugation shows the target mRNA to be located either in very small polysomes and the free mRNP pool (indicative of inhibited initiation), or whether it is found in large polysomes of similar size as when the target mRNA is not

subject to repression by miRNAs. A secondary criterion is whether target mRNAs with a variety of viral IRESs (internal ribosome entry sites) are sensitive or resistant to miRNA-mediated repression. These viral IRESs either require only a subset of the canonical translation initiation factors, as is the case with the encephalomyocarditis virus (EMCV) and hepatitis C virus (HCV) IRESs, or like cricket paralysis virus (CrPV) IRES, they can function completely independently of any initiation factors (for review, see Jackson 2005).

In the first polysome profile studies, *lin-14* and *lin-28* mRNAs, targets of *lin-4* and *let-7* miRNAs, were found in large polysomes in late L2 or early L3 *C. elegans* larvae, where they are largely repressed (Olsen and Ambros 1999; Seggerson et al. 2002). The same was seen with target reporter mRNAs expressed in HeLa or 293T cells in transfection assays (Nottrott et al. 2006; Petersen et al. 2006), and these polysomes were judged to be dynamic (rather than “frozen”) by the criterion that they showed the typical decrease in size on incubation of the cells with puromycin for a relatively brief period of time. They also decreased in size on very brief incubation with a specific inhibitor of translation initiation (Petersen et al. 2006) or more prolonged incubation under conditions of iron starvation where only the target reporter mRNA would be subject to inhibition of initiation because it had an IRE (iron response element) in its 5′-UTR (Nottrott et al. 2006).

In addition, Petersen et al. (2006) observed that reporter target mRNAs driven by the HCV or CrPV IRESs were just as susceptible to repression as when the target mRNA would be translated via the conventional scanning mechanism. This implies that repression is independent of the exact mechanism of initiation, which would be entirely consistent with a repression that operates at some stage after the initiation step, in agreement with the polysome profile data. However, as the polysomes with the repressed target mRNAs appear to be dynamic, it remains a mystery why output of the encoded protein should be so markedly reduced. This dilemma has led to the suggestion, more by default and elimination rather than for any positive reason, that maybe the miRNA association with the mRNA results in a selective and specific cotranslational proteolytic degradation of the nascent polypeptide (Olsen and Ambros 1999; Nottrott et al. 2006).

If the repressed targets are indeed in polysomes, then miRNAs themselves should mostly cosediment with polysomes, and this was found to be the case in an analysis of HeLa cell extracts in which the integrity of the polysomes appeared to be exceptionally high (Maroney et al. 2006). Again, the cosedimentation of the miRNAs with polysomes was sensitive to puromycin. However, this cosedimentation of endogenous miRNAs with polysomes may be less decisive than appears at first sight, because although laboratory-generated reporters often have multiple target sites (typically four to six) for miRNA binding in order to maximize repression, many endogenous (or “natural”) mRNAs have only one or two imperfectly complementary target sites. Consequently,

the repression of these target mRNAs may be relatively weak, which in turn implies that both the target reporter mRNA and any associated miRNP would actually be expected to cosediment with polysomes, irrespective of whether the weak repression is exerted at initiation or at some post-initiation stage.

In contrast to all the above results, which are consistent with inhibition of a post-initiation step, Pillai et al. (2005) found the target reporter mRNA sedimenting mainly with small polysomes and free mRNPs in transfection assays of HeLa cells, indicative of an inhibition of initiation (Pillai et al. 2005). A similar distribution was noted for endogenous CAT-1 mRNA in Huh7 cells, showing that this distribution was not a peculiarity of an overexpressed target mRNA (Bhattacharyya et al. 2006). Moreover, a significant fraction of the repressed target mRNA was associated with P-bodies, cytoplasmic foci enriched in proteins involved in mRNA degradation and other RNA-binding proteins (including GW182) but lacking ribosomes and all translation initiation factors except eIF4E, the cap-binding factor (for reviews, see Eulalio et al. 2007; Parker and Sheth 2007).

Pillai et al. (2005) also found that transfected target mRNAs with an EMCV or HCV IRES were immune from repression, and similar results were reported by Humphreys et al. (2005) for the EMCV and CrPV IRESs, except that up to twofold repression was seen if the target mRNA with the EMCV IRES was polyadenylated. Taken together, these results suggest that miRNAs repress their target mRNAs by somehow interfering with either the function of the cap-binding initiation factor eIF4E (which is not required for any of the viral IRESs) or the recognition of the m⁷Gppp-cap. An alternative possibility, suggested by the influence of polyadenylation on repression of the target mRNA with the EMCV IRES, is that the miRNAs might interfere with the cap-poly(A) synergy (Fig. 2), the so-called “closed loop” configuration of the mRNA resulting from the interaction between poly(A) binding protein (PABP) bound to the 3′-poly(A) tail and the eIF4G subunit of the eIF4F complex bound to the 5′-end via its eIF4E subunit (Sachs et al. 1997; Imataka et al. 1998). A closed loop mRNA configuration greatly enhances translation efficiency, particularly under conditions of strong competition between different mRNAs (Proweller and Butler 1997), i.e., a relatively high ratio of mRNA to ribosomes and/or initiation factors.

Thus although the results of any one group are self-consistent, a consideration of all published work ends up with the two radically different conclusions that repression may be exerted either at the initiation step or at some stage subsequent to initiation. There is no clear-cut majority opinion in favor of a one or other conclusion, nor is there any very good reason to think that one answer is “right” and the other “wrong.” Thus a truly detached and unbiased standpoint would be that both mechanisms might be possible, but if this is the case, there must be at least one uncontrolled variable (or perhaps one should say “unrecognized variable”) that determines which route the system follows in different experiments in different laboratories.

Figure 2. (A) Schematic diagram of the “closed loop” mRNA configuration formed by the interaction between poly(A)-binding protein (PABP) bound to the 3′-poly(A) tail and the scaffolding initiation factor, eIF4G, which is associated with eIF4E, the cap-binding initiation factor. (B) The possible ways that the binding of a miRNA to a 3′-UTR target site may interfere with such a closed loop, as discussed in this Perspective. The presence of an Ago protein and GW182 in the miRNP complex may lead to deadenylation of the target mRNA (Wakiyama et al. 2007), or binding of Ago to the 5′- m^7 Gppp-cap may inhibit initiation by evicting eIF4E (Kiriakidou et al. 2007).

Recapitulation of miRNA-mediated repression in a cell-free system from 293 cells

There has been considerable interest in trying to recapitulate miRNA-mediated repression in cell-free extracts, which might allow a more direct biochemical analysis of the mechanism of repression than is possible with transfected cells, and might even help to resolve the controversy over which step in mRNA translation is inhibited. In the preceding issue, Wakiyama et al. (2007) describe such a successful recapitulation in a system that quite closely mirrors what is believed to happen in intact cells. These investigations used post-mitochondrial supernatant extracts prepared from 293F cells grown in suspension, and in most experiments, these preparations were supplemented with 10%–15% (by volume) of similar extracts made from 293F cells in which Ago2 or other proteins had been overexpressed. The extract was supplemented with a 60-nt *let-7* pre-miRNA, and preincubated for 60 min at 37°C, apparently without added ATP, GTP, or an energy generating system. Efficient processing of the pre-miRNA to mature *let-7* miRNA required enrichment of the extract with Ago2, whereas enrichment with Dicer, TRBP2, or the P-body component GW182 had no effect either individually or in any combination with or without supplementation with Ago2. Following this preincubation, a luciferase reporter mRNA with six target sites in the 3′-UTR was added, along with ATP, GTP, and creatine phosphate. The presence of *let-7* pre-miRNA and supplemental Ago2 resulted in an approximately twofold repression of luciferase output, which was increased to an approximately fourfold reduction with supplemental GW182 (in addition to extra Ago2).

This degree of repression was seen only if the target mRNA had both a m^7 Gppp-cap and a poly(A) tail. No repression at all was found if the reporter was m^7 Gppp-capped but had no poly(A) tail, or if it was Appp-capped/poly(A)+. Appp-capped reporters with the EMCV IRES were only 20% inhibited if they were polyadenylated, or 10% if they were poly(A) minus. Analysis of the polyadenylated reporter mRNAs recovered at the end of a 60-min translation assay showed that they had all been largely deadenylated, irrespective of whether they were strongly repressed (m^7 Gppp-capped) or not (Appp-capped mRNAs with or without the EMCV IRES), but there had been no significant degradation of the body of the mRNA.

Deadenylation of the target mRNA would almost certainly reduce the efficiency of its translation as a consequence of abrogation of the cap/poly(A) synergy (Fig. 2). To test this proposition, the investigators performed parallel time courses of the expression of luciferase and the deadenylation, with the m^7 Gppp-capped/polyadenylated reporter. Luciferase output was largely uninhibited by *let-7* in the first ~20 min of incubation but shut down once deadenylation was nearly complete, indicating that the decrease in luciferase expression is almost entirely the consequence of deadenylation, although as the mRNA retained a short poly(A) tail even after longer incubation times, there may be a minor direct effect on translation per se (Wakiyama et al. 2007). This question could be addressed more directly by testing whether a reporter RNA similar to that described by Wu et al. (2006) is subject to repression. This has a histone mRNA 3′-terminal stem-loop in place of a poly(A) tail and thus cannot be deadenylated, leaving the question of whether it is completely immune to any form of miRNA-mediated repression or still (slightly) sensitive.

As GW182 is a critical component of P-bodies, the supplemental GW182 in this system may have promoted recruitment and aggregation of other P-body components, including the Ccr4/Not1 deadenylase (and other enzymes involved in mRNA degradation), thereby directing the target mRNA down the deadenylation route to a greater extent than would happen in more physiologically relevant conditions. In this respect, it is regrettable that the degree to which the supplementation with GW182 had increased the concentration of this protein over the level found in standard unsupplemented extracts was not determined.

Other cell-free systems recapitulating miRNA mediated repression in vitro

The first successful recapitulation of miRNA-mediated repression in vitro was achieved in rabbit reticulocyte lysates (RRL) with an artificial miRNA mimic (a 21-nt RNA designed for RNA-interference knock-down of CXCR4 mRNA) and a luciferase mRNA with up to six bulged target sites, precisely the same reporter mRNA/miRNA pair as used by Petersen et al. (2006) and Humphreys et al. (2005) in their transfection assays. The essential trick was to preanneal the miRNA to the target

mRNA before addition to the nuclease-treated reticulocyte lysate (Wang et al. 2006). Although this requirement for preannealing means that the miRNP/mRNA complex is not assembled by the physiological route, nevertheless the outcome has all the hallmarks of miRNA-mediated regulation established *in vivo*, including increased repression with increasing number of target sites; a strict requirement for a 5'-phosphate on the miRNA; requirement for perfectly complementary contiguous base-pairing of the seed residues 2–8 of the miRNA, coupled with much less stringent requirement for contiguous base-pairing of the 3'-end; no repression if the 3'-end of the 21-mer miRNA was extended by 10 residues perfectly complementary to the target mRNA; and no repression with a 2'-O-methyl derivative of the 21-mer.

If the mRNA had a standard length poly(A) tail (200 A residues), repression was only seen if it also had a m⁷Gppp-cap, but uncapped mRNAs were subject to repression if the tail was extended to unphysiological lengths (2000 residues). Reporter RNAs were stable and apparently not deadenylated, though this remains to be rigorously tested. Unlike the results of Wakiyama et al. (2007), where the repression of luciferase output became more severe with longer incubation times, repression in this RRL system was maximal at the early stages and decreased with increasing incubation time, which may be due to the repression mechanism being rather labile. Although the time-course data do not allow an unambiguous identification of which step of translation is being inhibited, the requirement for a cap and a poly(A) tail strongly suggests that it is initiation rather than elongation that is affected.

The other successful recapitulation of miRNA-mediated repression was achieved with *Drosophila* embryo extracts, relying on endogenous miR-2 specifically repressing a luciferase reporter mRNA with six copies of the miR-2 target site present in the 3'-UTR of reaper mRNA but having no effect if these sites (21 nt) were replaced by a six-residue linker (Thermann and Hentze 2007). When this reporter mRNA was incubated for 60 min in the embryo extracts under translation conditions, no repression was observed, possibly because formation of the miRNP/mRNA complex is a slow process that is outcompeted by translation. In view of this possibility, the investigators devised a somewhat complex protocol, in which the reporter mRNA was preincubated in the absence of an energy regenerating system (creatine phosphate), for 3 h, with the addition of an equal volume of fresh extract every 60 min. After this extended preincubation, creatine phosphate was added to allow mRNA translation, and a 3.5-fold repression (dependent on the endogenous miR-2 and the appropriate target sites in the reporter mRNA) was observed over the ensuing 60 min. Repression was seen with m⁷Gppp-capped reporter, not with Appp-capped mRNA, but the question of whether a poly(A) tail is also needed remains open as all mRNAs were polyadenylated. Sucrose density gradient centrifugation analysis showed that initiation was decidedly the inhibited step, specifically the loading of 40S preinitiation complexes on to the mRNA.

An interesting feature of these sucrose gradient analyses is that some of the reporter mRNA was found to sediment quite rapidly (>150–200S) in structures that the investigators designated “pseudo-polysomes.” These pseudo-polysomes resembled conventional polysomes in that they were disrupted by EDTA but differed in that their sedimentation was unaffected by incubation with puromycin. Pseudo-polysome formation was dependent on the presence of both miR-2 and the target sites in the reporter mRNA but was not influenced by whether the mRNA was m⁷Gppp- or Appp-capped. Thus the pseudo-polysome state appears to be necessary but not sufficient for repression, and is thought to represent a complex between the target mRNA and the miRNP. It will be interesting to know whether pseudo-polysomes contain P-body proteins but not ribosomes.

Conclusions that can be drawn from the *in vitro* recapitulations of miRNA-dependent repression

The results, summarized in Table 1, of all three reports of recapitulation of miRNA-mediated repression *in vitro* are indicative of a mechanism that impacts, either directly or indirectly, on translation initiation. In the case of Wakiyama et al. (2007), this inhibition seems to be largely if not entirely a secondary consequence of the deadenylation, whereas this is unlikely to be the case with the RRL system of Wang et al. (2006), while the issue remains untested for the *Drosophila* embryo extract (Thermann and Hentze 2007). In all three cases, efficient repression was only seen if the target mRNA was m⁷Gppp-capped, suggesting that the mechanism of repression either impacts directly on the recognition of the 5'-cap by translation initiation factors or disrupts the poly(A)/cap synergy, perhaps by physically disrupting the closed loop configuration.

Significantly, there is no strong indication of inhibition at some stage after the initiation step. So, does this mean that the “right” answer must be that miRNA-mediated repression impacts exclusively on initiation, and that there must be some flaw or artifact in those transfected cell assays which suggested inhibition at a post-initiation stage? That would certainly be one interpretation, but probably not a valid one. As suggested previously, a detached view of the transfected cell and nematode larvae data is that miRNAs have the ability to repress translation either at the initiation step or at some post-initiation stage, according to the status of some not-yet-recognized variable. If this premise is accepted, then it could be argued that the *in vitro* systems developed so far have only succeeded in recapitulating the initiation lesion. It could be that the methods used to date have been unsuitable for revealing any repression at the post-initiation stage. Nevertheless, it does seem legitimate to conclude from the cell-free extract work that miRNAs *can* affect the initiation of translation of their target mRNAs, irrespective of whether they may also participate in an alternative or additional post-initiation regulatory mechanism.

Table 1. Summary of characteristics of cell-free systems recapitulating miRNA-mediated repression

	Wakiyama et al. (2007)	Wang et al. (2006)	Thermann and Hentze (2007)
Cell-free system	293F cells plus extra GW182 and Ago2	Rabbit reticulocyte lysate (nuclease treated)	<i>Drosophila</i> embryos
miRNAs	Exogenous pre- <i>let7</i> miRNA	Exogenous CXCR4 siRNA duplex precursor (used as a miRNA mimic)	Endogenous miR-2
Number of 3'-UTR target sites	6	6	6
Degree of repression	Fourfold at 60 min ^a Zero at 15 min	4.5-fold at 10 min ^b 1.7-fold at 60 min	3.5-fold (at 60 min)
Reporter mRNA requirements for repression	m ⁷ Gppp-cap + poly(A) tail	m ⁷ Gppp-cap ^c + poly(A) tail	m ⁷ Gppp-cap <i>poly(A) tail requirement not tested</i>
Primary mechanism of repression	Deadenylation of reporter mRNA	Probably inhibited initiation (see text)	Inhibition of initiation at the step of 40S subunit loading on to mRNA
Comments	Additional Ago2 needed for processing pre-miRNA Additional GW182 increases degree of repression	Melting and preannealing of miRNA to mRNA absolutely required when using the staggered duplex mi/siRNA precursor ^b	Repression requires preincubation of the target reporter mRNA in the cell-free extract in the absence of an energy regenerating system (creatine phosphate)

^aApproximately twofold repression if no supplementary GW182 present.

^bIn the absence of the melting and preannealing step, 1.4-fold repression seen if a single-stranded mi/siRNA was used, but no repression at all with the staggered duplex precursor.

^cUncapped mRNA was repressed if the poly(A) tail was 800 residues or longer; maximum threefold repression (at 10 min) with an A₂₀₀₀ tail.

The significance of interaction between Ago and the 5'-cap

The idea that miRNAs can inhibit initiation of translation of target mRNAs that are m⁷Gppp-capped and translated via a scanning mechanism (as opposed to Appp-capped, or uncapped, and/or possessing a viral IRES element) has recently received strong support from some most unexpected results reported by Kiriakidou et al. (2007). These investigators astutely observed that the central domain of some Ago proteins, including mammalian Ago2, shows similarities to the cap-binding translation initiation factor eIF4E, except that the two tryptophans (W56 and W102) of eIF4E that stack on the m⁷Gppp-cap are replaced by two phenylalanines (F470 and F505) in Ago2. Human Ago2 was shown to bind specifically to m⁷GTP-Sepharose but not if the two phenylalanines were mutated to valines. These mutations did not affect the ability of the Ago2 to be assimilated into an miRNP/RISC nor did they compromise its endonucleolytic cleavage activity, but they did abolish its ability to act as a translational repressor in a tethered function assay. In this system, the Ago protein is expressed as a fusion with bacteriophage λ N-peptide, and the 3'-UTR of the target reporter mRNA has multiple λ Box B sites (rather than any miRNA target site), so that the expressed Ago fusion protein will bind to the 3'-UTR even in the absence of any miRNA/mRNA interactions (Pillai et al. 2004). These findings carry the clear implication that Ago2 can inhibit translation initiation by in-

teracting with the m⁷Gppp-cap via the two critical phenylalanines. This conclusion is supported by the poly-some distribution of the target reporter mRNA and by the fact that, in RNA transfection assays, an uncapped reporter mRNA with an EMCV IRES and 5 Box B sites in its 3'-UTR was refractory to repression by a fusion of wild-type Ago2 with λ N-peptide (Kiriakidou et al. 2007).

What is striking about this unexpected development is that although the two critical phenylalanines are found in all four mammalian Ago proteins (all of which can promote repression of target mRNA translation), they are present only in ALG-1 and ALG-2 out of the >25 Ago proteins of *C. elegans*. In the case of *Drosophila*, where the two Ago proteins have largely non-overlapping functions, they are absent from AGO2, which is thought to be involved mainly, or perhaps exclusively, in the endonucleolytic cleavage pathway directed by siRNAs perfectly complementary to their mRNA target site, but they are present in AGO1, which is involved in target mRNA repression via miRNA-dependent nonendonucleolytic pathways (Behm-Ansmant et al. 2006). Moreover, these phenylalanines are absent from the Agos of organisms such as archaeobacteria and fission yeast, which do not have miRNAs (Kiriakidou et al. 2007).

Of course, the replacement of the two tryptophan residues of eIF4E with phenylalanines in Agos would be expected to result in Ago2 having a significantly lower affinity than eIF4E for the m⁷Gppp-cap (Altmann et al. 1988). Consequently, free Ago2 would be most unlikely to repress translation initiation in *trans*. Therefore effi-

cient repression is only likely to occur in *cis*, through the anchoring of the Ago to the target mRNA 3'-UTR, either via the normal miRNP/target mRNA interaction or via the laboratory-designed by-pass of the miRNA requirement using the λ N-peptide fusions and multiple Box B sites in the target mRNA. Moreover, the weaker stacking interactions of the phenylalanines offers an explanation for why repression of translation increases with increasing number of 3'-UTR target sites for miRNAs (for example, see Thermann and Hentze 2007), which would increase the number of Agos associated with the repressed mRNA and hence increase the fraction of time during which the 5'-cap would be associated with an Ago rather than productively bound to the eIF4E subunit of the eIF4F complex.

This seminal discovery not only gives very strong support to the notion that miRNAs *can* result in inhibition of initiation but further suggests that initiation *will invariably* be affected, as it is hard to see how the Ago/cap interactions could be prevented unless one were to invoke some regulatory protein that can interact with the Ago central domain to mask the cap-binding pocket. [This reasoning does not exclude the possibility of miRNA-mediated inhibition of some step *after* initiation, but any such post-initiation inhibition would have to be *in addition* to an inhibition of cap-dependent initiation, and *not* an alternative to it. It is worth bearing in mind that in a situation where initiation frequency was already decreased fivefold, and thus protein output would be fivefold reduced with the repressed mRNA located in very small polysomes, superimposing an approximately fourfold decrease in elongation rate would result in little additional reduction in reporter protein output, even though the target mRNA would move into larger polysomes. For a fuller discussion of the relationship between protein output rates, polysome size, initiation frequency, and elongation rate, under steady-state conditions likely to pertain in transfected cell assays, the reader is referred to Hunt et al. (1968, 1969).]

The conclusion that Ago can block initiation by sequestering the 5'-cap supports the belief that the results obtained so far with *in vitro* systems are indeed valid and physiologically relevant, and that further work with such systems would be very worthwhile.

Note added in proof

In a very recent publication, repression has been recapitulated in a Krebs-2 ascites cell-free extract using a m⁷Gppp-capped and polyadenylated luciferase reporter mRNA with six target sites for the endogenous *let-7* miRNA present in such extracts (Mathonnet et al. 2007). Following a 60-min incubation, the degree of repression averaged ~2.5-fold, almost entirely attributable to inhibition of translation rather than mRNA degradation. Although this repression is slightly weaker than in other *in vitro* systems, the repressed state was established more rapidly, within 20 min at 16°C (when little, if any, reporter mRNA translation would occur) or ~12 min at 30°C. In common with the other *in vitro* systems discussed here (Thermann and Hentze 2007; Wakiyama et al. 2007), no repression was seen if the reporter was Appp-capped, or if its translation was depen-

dent on an EMCV IRES but the requirement for a poly(A) tail was not tested. Sucrose density gradient centrifugation assays showed that initiation was inhibited by *let-7* miRNA, but these particular assays could not distinguish whether it was 40S subunit loading onto mRNA rather than some subsequent step that was affected. The significant novelty in this work is the demonstration that supplementary eIF4F could overcome the miRNA-mediated repression, consistent with the suggestion of Kiriakidou et al. (2007) that the mechanism of repression involves competition between Ago (anchored to the target mRNA as a component of the miRNP/RISC) and the eIF4E subunit of the eIF4F complex for binding to the 5'-m⁷Gppp cap.

Acknowledgments

We thank with Matthias Hentze, Wittek Filipowicz, and Elmar Wahle for discussions on aspects of this Perspective, and Catherine Jopling for a precursor version of Figure 1.

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Genes Dev. 2007, **21**:

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