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Review

Let Me Count the Ways: Mechanisms of Gene Regulation by miRNAs and siRNAs

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The downregulation of gene expression by miRNAs and siRNAs is a complex process involving both translational repression and accelerated mRNA turnover, each of which appears to occur by multiple mechanisms. Moreover, under certain conditions, miRNAs are also capable of activating translation. A variety of cellular proteins have been implicated in these regulatory mechanisms, yet their exact roles remain largely unresolved.

When discovered in the early 1990s, gene regulation by microRNAs (miRNAs) was initially treated as a curiosity of nematode biology (Lee et al., 1993; Wightman et al., 1993). Interest in this regulatory mechanism soared several years later when it was found to be widespread among eukaryotic organisms (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Reinhart et al., 2002), which often produce hundreds of different miRNAs that collectively control thousands of genes (Lim et al., 2003; Bentwich et al., 2005; Lewis et al., 2005). Over the past few years, significant progress has been made in delineating the influence of these small (~22 nucleotide) untranslated RNAs on many biological processes and human diseases and in devising technologies for manipulating gene expression with a related class of regulatory oligonucleotides known as small interfering RNAs (siRNAs). By contrast, progress toward elucidating the molecular mechanisms by which miRNAs act posttranscriptionally to alter gene expression has been considerably slower and more controversial due to the unexpected diversity of those mechanisms and the sometimes incongruous experimental findings from which they have been deduced. Fortunately, several recent reports have begun to suggest a molecular basis for understanding at least some of those mechanisms (Liu et al., 2004; Wu et al., 2006; Giraldez et al., 2006; Behm-Ansmant et al., 2006; Chendrimada et al., 2007; Kiriakidou et al., 2007; Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007; Vasudevan et al., 2007).

Studies in *Caenorhabditis elegans*, *Drosophila melanogaster*, and vertebrates have yielded most of what is known about metazoan miRNA and siRNA function, whose commonalities in those animal species are the focus of this review. Both miRNAs and siRNAs regulate gene expression by annealing to mRNA sequence elements that are partially or fully complementary. These two classes of small RNAs are distinguished from one another not by their size or function but rather by their origin, with miRNAs being processed from stem-loop structures in primary transcripts and siRNAs arising by cleavage of long double-stranded precursors or by chemical synthesis. Both have the same regulatory potential, as influenced by the complementarity of their mRNA targets (Hutvágner and Zamore, 2002; Doench et al., 2003; Zeng et al., 2003). In animals, that potential is manifested in multiple ways: by reductions, or sometimes increases, in translation efficiency and by diminished mRNA stability. Thus, acting in conjunction with a set of associated proteins, both miRNAs and siRNAs can inhibit the translation of messages to which they anneal with incomplete complementarity (Wightman et al., 1993; Doench et al., 2003; Zeng et al., 2003). Conversely, in growth-arrested cells, they can instead stimulate translation (Vasudevan et al., 2007). At first, miRNAs and siRNAs were thought to hasten mRNA decay only when paired with transcripts that are fully complementary. However, it is now clear that they have the capacity to trigger the decay of a variety of messages to which they bind (Lim et al., 2005; Bagga et al., 2005; Wu and Belasco, 2005), either by directing endonucleolytic cleavage within the target element in the case of perfectly (or almost perfectly) complementary mRNAs (Zamore et al., 2000; Elbashir et al., 2001; Llave et al., 2002; Yekta et al., 2004) or by accelerating poly(A) removal in the case of imperfectly complementary messages (Wu et al., 2006; Giraldez et al., 2006).

The overall degree of gene silencing by miRNAs and siRNAs is typically smaller for messages containing a single element that is only partially complementary: at most a factor of 2–3, versus a factor of up to 10–20 for messages that contain a fully complementary site (Zeng et al., 2002; Doench et al., 2003). Downregulation of genes that encode imperfectly complementary transcripts is generally most effective when mi/siRNA nucleotides #2–8 (the seed region) can form canonical base pairs with the target element and when mi/siRNAs have an opportunity to function additively by binding to multiple sites in the 3′ untranslated region (reviewed by Bartel, 2004).

Regulatory Mechanisms of mi/siRNAs

Translational Repression

Perhaps the most controversial aspect of mi/siRNA function is the mechanism by which they repress translation. The ability of

miRNAs to downregulate translation was originally deduced from the disparity between their effects on protein synthesis and mRNA concentration (Wightman et al., 1993). However, questions as basic as whether translation is inhibited at initiation or at a subsequent stage have remained in dispute for quite a while, raising the possibility that more than one mechanism might be involved.

The notion that miRNAs inhibit the initiation of translation rests on a number of observations. First, some studies involving sucrose density gradient centrifugation have revealed a shift of targeted mRNAs to translationally active polysomes of lower mass (i.e., fewer ribosomes per message), a telltale sign of impaired initiation (Pillai et al., 2005). Other studies utilizing in vitro-transcribed mRNAs introduced into cells by transfection have suggested that cap-independent translation under the control of an internal ribosome entry sites (IRES) may be insensitive to repression by miRNAs, a finding interpreted as evidence that miRNAs can only inhibit translation initiated by a cap-dependent mechanism (Humphreys et al., 2005; Pillai et al., 2005). Recent successes in reconstituting miRNA-dependent translational repression in cell-free systems have likewise led to evidence indicating that miRNAs cause an m^7G cap-dependent impediment to the recruitment of 80S ribosomes to mRNA (Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007). Finally, the discovery that translational repression is correlated with the cap-binding affinity of the miRNA-binding protein Ago has suggested a concrete mechanism by which miRNAs may inhibit translation initiation: concealment of the cap so that it cannot bind the initiation factor eIF4E (Figures 1A and 1B) (Kiriakidou et al., 2007). Other mechanisms for hindering initiation after cap recognition have also been proposed (Figure 1C) (Chendrimada et al., 2007).

Nevertheless, the mechanism of translational repression may not be so simple, as there is also persuasive evidence that miRNAs may be able to inhibit translation at a step after initiation. For example, unlike the results cited above, other studies have detected no change in the polysome profiles of messages targeted by miRNAs (Olsen and Ambros, 1999; Seggerson et al., 2002; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). Furthermore, despite reports to the contrary, there is ample evidence that miRNAs can indeed repress cap-independent translation directed by various IRES elements, including some that do not require any of the canonical translation initiation factors (Petersen et al., 2006; Lytle et al., 2007). These observations have led to proposals that miRNAs might cause retarded elongation by translating ribosomes, possibly coupled to premature termination (Figure 1D), or induce cotranslational degradation of nascent polypeptides (Figure 1E) (Olsen and Ambros, 1999; Seggerson et al., 2002; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006), though there is little precedent for the former mechanism in eukaryotic cells and the latter possibility seems at variance with the ability of miRNAs to repress the synthesis even of proteins that are exported cotranslationally to the endoplasmic reticulum (Pillai et al., 2005).

These conflicting findings are difficult to reconcile. Some of the discrepancies may be due to the disparate biological systems and methodologies used in various laboratories, reflecting either real differences between animal species, cell types, or growth conditions or artifactual differences between investigative techniques. Indeed, it has been observed that the same miRNA-sensitive reporter can yield different results depending on whether it is introduced into cells as DNA or mRNA and on the method of transfection (Lytle et al., 2007). When well-executed studies of posttranscriptional regulation produce contradictory results, greater credence should generally be given to those in which the mRNAs under investigation were transcribed, processed, and translated intracellularly, as studies utilizing cell-free systems or in vitro-transcribed mRNAs introduced into cells by transfection are more prone to artifacts.

On these grounds, neither a cap-dependent repression mechanism nor a cap-independent mechanism is easily dismissed. The evidence for each derives, in part, from analyses of reporter

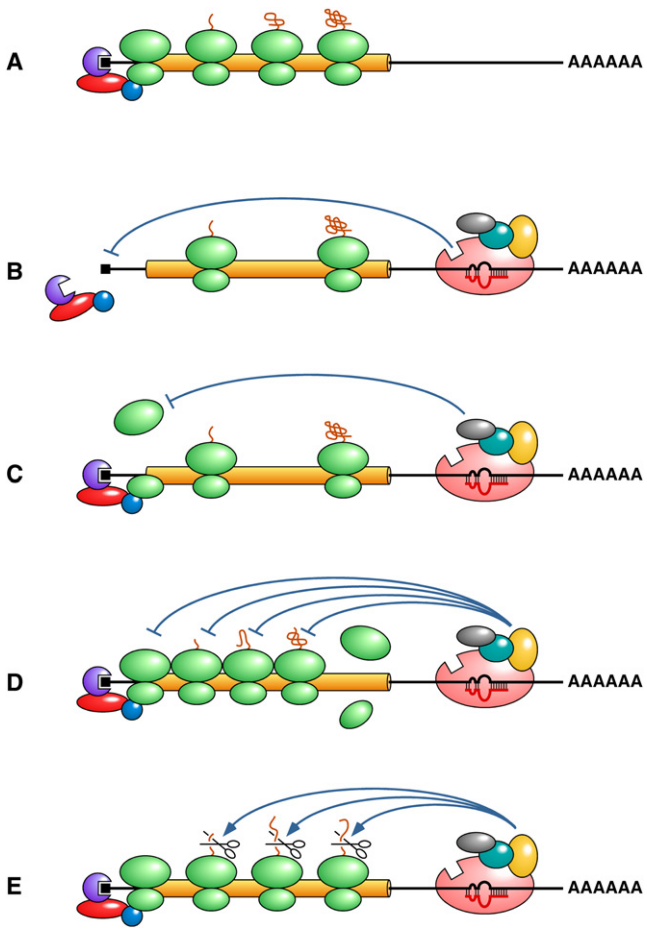

Figure 1. Hypothetical Mechanisms of Translational Repression by miRNAs

(A) mRNA undergoing translation in the absence of a bound miRNA. Black square, m^7G cap; amber cylinder, protein-coding region; and AAAAAA, poly(A) tail. Ribosomes are colored green, nascent polypeptides are brown, and the eIF4E subunit of the cap-binding complex is violet.

(B) Inhibition of translation initiation by competition between RISC and eIF4E for cap binding. RISC is depicted as a ribonucleoprotein complex comprising a microRNA (red), Ago (pink), and other protein subunits.

(C) Inhibition of translation initiation at a step after cap recognition, such as by impeding the association of the small and large ribosomal subunits.

(D) Inhibition of translation elongation coupled to premature termination.

(E) Cotranslational degradation of nascent polypeptides.

mRNAs produced and translated in cells, and in both cases, the key data (involving polysome profiles, Ago tethering [see below], and cap analogs for cap-dependent repression or IRES-containing intracellular transcripts for cap-independent repression) appear convincing. However, these two modes of regulation are not mutually exclusive, and some experiments designed to detect one may have obscured the other. Thus, on the basis of current evidence, it seems reasonable to conclude that miRNAs employ multiple mechanisms to repress the translation of targeted messages, including one that inhibits the earliest events of cap-dependent initiation and another that impedes a later, cap-independent event in translation.

Translation Activation

Although miRNAs have long been thought only to downregulate protein synthesis, new data indicate that they can also stimulate translation under certain conditions (Vasudevan et al., 2007). Thus, a miRNA that inhibits translation in proliferating mammalian cells can have the opposite effect after cell-cycle arrest caused by serum starvation or treatment with the replication inhibitor aphidicolin. Like translational repression, such activation requires base pairing of the targeted message with the seed region of the miRNA. The mechanism by which the efficiency of translation increases has not yet been determined, nor is it clear how changing the growth state of cells causes this striking reversal in the regulatory influence of miRNAs and the proteins with which they associate.

mRNA Degradation

siRNAs and miRNAs accelerate mRNA decay by two distinct mechanisms. Those that are fully complementary to their mRNA targets (or nearly so) direct endonucleolytic cleavage within the base-paired region (Figure 2A) (Zamore et al., 2000; Llave et al., 2002; Yekta et al., 2004). This event leads to rapid decay of the entire message by generating a pair of RNA fragments, each bearing an unprotected end that is susceptible to 5′- or 3′-exonuclease attack (Orban and Izaurralde, 2005). Although miRNAs share the potential to mediate internal mRNA cleavage, they rarely do so in animal cells due to the inadequate complementarity of nearly all mRNAs (Yekta et al., 2004). Instead, miRNAs accelerate mRNA turnover by directing removal of the 3′ poly(A) tail from messages to which they are partially complementary (Figure 2B) (Wu et al., 2006; Giraldez et al., 2006). Deadenylation and the consequent loss of poly(A)-binding protein trigger 5′ decapping, thereby exposing the message to exonucleolytic digestion from the 5′ end (Behm-Ansmant et al., 2006; Eulalio et al., 2007b).

The ability of miRNAs to hasten mRNA decay has two important consequences. First, by diminishing the concentration of targeted transcripts, it supplements the decreased efficiency with which each message is translated, resulting in a greater overall reduction in protein synthesis. Second, by inducing message degradation, miRNAs render irreversible their inhibitory influence on gene expression, an outcome not achievable by translational downregulation alone.

The relative contribution of accelerated mRNA decay to the overall influence of miRNAs varies widely from message to message (Behm-Ansmant et al., 2006). In some instances, it appears to be the principal mechanism of downregulation, whereas in others its effect can be quite modest compared to that of translational repression. Although the basis for such differences has not been determined, they may be related, at least in part, to the innate longevity of the targeted mRNAs. All else being equal, one would expect messages that are intrinsically long lived to be destabilized more than others that are inherently labile if miRNAs engaged in equivalent base-pairing interactions with distinct 3′ untranslated regions cause similar rates of deadenylation and decay.

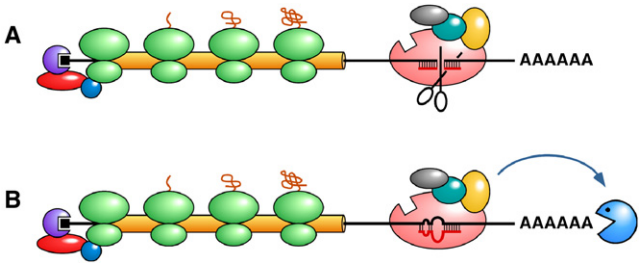

Figure 2. Mechanisms by which siRNAs and miRNAs Trigger mRNA Decay

(A) mRNA undergoing endonucleolytic cleavage by Ago2, as guided by a fully complementary siRNA or miRNA.

(B) mRNA undergoing poly(A) removal by the Ccr4/Not deadenylase (Pac-Man), as directed by a partially complementary miRNA.

Accelerated deadenylation appears to be neither a consequence nor a cause of impaired translation. Thus, blocking translation by other means (such as by introducing a large stem loop into the 5′ untranslated region) does not itself trigger poly(A) removal or impair the ability of miRNAs to do so (Wu et al., 2006; Giraldez et al., 2006; Mishima et al., 2006). Conversely, messages that cannot be deadenylated because they end in a histone mRNA stem loop rather than a poly(A) tail are nonetheless susceptible to the same degree of translational repression as otherwise identical mRNAs that are polyadenylated (Wu et al., 2006). Moreover, reductions in cellular deadenylase activity that diminish the influence of miRNAs on message stability do not impair their ability to repress translation (Behm-Ansmant et al., 2006). Although the failure of deadenylation to inhibit translation might at first seem surprising in view of previous evidence that poly(A)-binding protein stimulates translation by interacting with cap-bound initiation factors (Tarun and Sachs, 1996; Kahvejian et al., 2005), it is presumably a consequence of the marked instability of deadenylated mRNAs, whose prompt degradation ordinarily prevents them from lingering in a poorly translated state (Wu et al., 2006).

Proteins Important for mi/siRNA Function

A number of proteins help to mediate the influence of miRNAs and siRNAs on translation and mRNA decay, although the mechanisms by which they do so are, for the most part, unknown. Some of these proteins are thought to be integral components of the RNA-induced silencing complex (RISC) that delivers those small RNAs to complementary sites within mRNA, whereas others may associate with that complex only transiently if at all (Höck et al., 2007; Zhang et al., 2007).

Ago and TNRC6

The core subunit of RISC is the Argonaute protein Ago, which binds the mi/siRNA (reviewed by Peters and Meister, 2007). In

most species, this protein exists as multiple paralogs with distinct functions. At least one (e.g., Ago2 in humans, mice, and *Drosophila*) functions as an endonuclease able to cut mRNA within regions that are fully complementary to the mi/siRNA (Figure 2A) (Liu et al., 2004; Meister et al., 2004; Okamura et al., 2004). In addition, some Ago proteins (Ago1, Ago2, Ago3, and Ago4 in humans; Ago1 in *Drosophila*) appear capable of mediating both translational repression and accelerated deadenylation, as judged from the diminished regulatory potential of miRNAs and siRNAs when these proteins are depleted and from tethering experiments in which these proteins are bound to a reporter mRNA via a heterologous protein domain to which they are fused (Liu et al., 2004; Meister et al., 2004; Pillai et al., 2004; L.W. and J.G.B., unpublished data). Other studies of this kind have demonstrated a role for Ago2 in miRNA-mediated translation activation in growth-arrested cells (Vasudevan et al., 2007).

Recent evidence suggests that the repression of translation initiation by miRNAs may result, at least in part, from the ability of many Ago proteins to bind the m^7G cap at the 5′ end of mRNA and sequester it from the translation initiation factor eIF4E (Kiriakidou et al., 2007). Those experiments have shown that point mutations in Ago2 that reduce its cap-binding affinity also impair its ability to downregulate translation when tethered to mRNA. This discovery suggests a simple mechanism by which miRNAs could inhibit translation initiation on the messages they target via competition for the cap between the miRNA-associated Ago protein and eIF4E (Figure 1B). It would also explain an independent report that the efficacy of translational repression by miRNAs in a cell-free extract was diminished at higher concentrations of the translation initiation complex that contains eIF4E (Mathonnet et al., 2007).

As seductively uncomplicated as this model may be, it is unlikely to provide a complete explanation of translational repression by miRNAs. For one thing, results from a number of laboratories indicate that miRNAs can also repress cap-independent translation directed by an IRES (Petersen et al., 2006; Lytle et al., 2007). Moreover, although translational repression by *Drosophila* miRNAs normally requires Ago1, the only Ago protein in that species with a cap-binding motif, this requirement can be bypassed by tethering the RISC-associated protein GW182 (TNRC6A) directly to mRNA in cells depleted of Ago1 (Behm-Ansmant et al., 2006). Thus, GW182 (possibly in conjunction with other proteins) appears to be capable of inhibiting translation by an Ago-independent mechanism whose details are unknown. Consistent with this conclusion is the finding that depleting mammalian or *Drosophila* cells of GW182 or its paralog TNRC6B impairs the ability of mi/siRNAs to downregulate gene expression (Jakymiw et al., 2005; Liu et al., 2005a; Meister et al., 2005; Rehwinkel et al., 2005).

Translational Repression

Besides Ago and TNRC6, several additional proteins appear to contribute to translational repression by miRNAs. These include the RISC-associated proteins MOV10/Armitage and RCK/p54. The sequence of MOV10 suggests that it functions as an RNA helicase, whereas RCK is homologous to a yeast protein (Dhh1) previously implicated in miRNA-independent translational silencing in that species (Coller and Parker, 2005). Depletion of either from mammalian cells impairs gene silencing by miRNAs (Cook et al., 2004; Tomari et al., 2004; Meister et al., 2005; Chu and Rana, 2006). In addition, MOV10 (but not RCK) facilitates siRNA-guided Ago2 cleavage of messages that are perfectly complementary (Cook et al., 2004; Tomari et al., 2004; Meister et al., 2005; Chu and Rana, 2006). Exactly how these proteins influence RNA interference is not known.

Two other RISC components are Dicer and TRBP (Hammond et al., 2001; Sasaki et al., 2003; Chendrimada et al., 2005; Haase et al., 2005). Both are important for the production of miRNA and siRNA from precursor molecules, with Dicer functioning as the processing endonuclease and the RNA-binding protein TRBP serving as an ancillary factor (Bernstein et al., 2001; Chendrimada et al., 2005; Haase et al., 2005). However, only TRBP contributes to RISC function, as evidenced by the defect in RNA interference caused by depletion of that protein (but not Dicer) when synthetic, fully mature siRNAs are supplied by transfection (Martinez et al., 2002; Chendrimada et al., 2005; Haase et al., 2005; Kanellopoulou et al., 2005).

The translation factor eIF6 has also been implicated in gene silencing by miRNAs. Previous studies had shown that eIF6 can influence the biogenesis of 60S ribosomal subunits and inhibit their assembly with 40S subunits to form translationally active ribosomes (Russell and Spremulli, 1979; Ceci et al., 2003). This protein has recently been reported to copurify with RISC and to contribute to its function, as evidenced by the impaired capacity of miRNAs to downregulate targeted genes at the protein and mRNA levels when human or *C. elegans* cells are depleted of eIF6 (Chendrimada et al., 2007). The ability of eIF6 to inhibit ribosomal subunit joining suggests a possible cap-independent silencing mechanism (Figure 1C) that might account for translational repression by miRNAs even in the case of protein synthesis directed by IRES elements, such as those from cricket paralysis virus or hepatitis C virus, that do not require any of the canonical translation initiation factors (see above). Alternatively, the influence of eIF6 on miRNA function may be indirect.

Translation Activation

Presently, two proteins are known to be important for the ability of miRNAs to activate translation in growth-arrested mammalian cells. One is Ago2, as demonstrated by depletion and tethering experiments. The other is FXR1, an RNA-binding protein homologous to the fragile X mental retardation protein FMR1/FMRP (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). FXR1 associates with Ago2 and, by an unidentified mechanism, helps to mediate the positive influence of miRNAs on the translation of targeted messages in serum-starved cells. Interestingly, whereas the interaction of Ago2 with miRNA-responsive elements (miREs) in mRNA appears to occur in both growth-arrested and synchronously proliferating cells, FXR1 has been found to associate with miREs only in cells that are not growing (Vasudevan et al., 2007). Moreover, when tethered directly to mRNA, FXR1 is itself able to upregulate translation in both growth-arrested and proliferating cells. Conversely, the FXR1 paralog FMR1 has been reported to contribute to the downregulation of gene expression by RNA interference in mouse and *Drosophila* cells, possibly by helping siRNAs anneal to the messages they target (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004; Plante et al., 2006).

Deadenylation

Subunit depletion experiments have identified Ccr4-Not as the deadenylase that removes poly(A) from messages targeted by miRNAs in *Drosophila* (Figure 2B) (Behm-Ansmant et al., 2006). The deadenylated mRNAs that result then undergo 5′ cap removal by the Dcp2-Dcp1 decapping complex, which exposes them to 5′ → 3′ exonucleolytic degradation, presumably by Xrn1. The depletion of Ccr4-Not components important for miRNA-directed deadenylation stabilizes the targeted messages without impairing the ability of miRNAs to downregulate translation, a finding consistent with the aforementioned evidence that poly(A) removal is not the principal basis for translational repression by miRNAs.

P Bodies

A significant fraction of translationally silent mRNAs, including those repressed by miRNAs, are found concentrated in cytoplasmic foci known as P bodies (processing bodies) (Eystathioy et al., 2002; Liu et al., 2005b). These ribonucleoprotein aggregates, which are sufficiently large to be detected by immunofluorescence microscopy, also contain high concentrations of miRNAs, RISC-associated proteins (Ago1–4, GW182, etc.), and RNA degradative enzymes (Dcp1/2, Xrn1, Lsm1-7, etc.) (Ingelfinger et al., 2002; van Dijk et al., 2002; Liu et al., 2005b; Sen and Blau, 2005).

Three lines of evidence initially raised the possibility that P body localization might be important for silencing by miRNAs. First, P bodies are devoid of ribosomes (Teixeira et al., 2005). Second, miRNA-dependent localization of mRNA in P bodies requires both the miRNA and one or more miREs within the message (Liu et al., 2005b). Third, depleting cells of RISC-associated proteins such as GW182 or RCK disaggregates P bodies and impairs miRNA function (Jakymiw et al., 2005; Liu et al., 2005a; Chu and Rana, 2006). However, these findings did not prove that the ability of miRNAs to direct targeted messages to P bodies contributes to translational repression. Indeed, recent evidence suggests that P bodies likely play a less pivotal role either as graveyards where miRNA-associated messages that already have been translationally inactivated (and possibly also deadenylated) are sent to decompose or as depots where messages transiently repressed by miRNAs can be stored until needed (Bhattacharyya et al., 2006; Chu and Rana, 2006; Eulalio et al., 2007a). Thus, depletion of cellular Lsm1 or Lsm3 has been found to disaggregate P bodies, either completely or at least to a submicroscopic size, without impairing the ability of miRNAs to downregulate gene expression. These results suggest that it may be more informative to focus on the function of individual P body components than on their ability to aggregate.

Perspective

Taken together, current evidence indicates that miRNAs and siRNAs act in conjunction with a set of effector proteins to modulate gene expression by perturbing translation and by expediting mRNA decay and that each of those regulatory processes can occur by multiple mechanisms. Clues as to how mi/siRNAs repress or activate translation are only just beginning to emerge and remain controversial, whereas the contours of how they accelerate mRNA degradation are somewhat clearer. Very little is known about the functions of specific RISC-associated proteins in these regulatory events. The technique most often used to test the involvement of a protein in mi/siRNA-mediated regulation—reducing its cellular concentration by RNA interference—is of limited utility in this respect, as that approach generally cannot distinguish a direct functional role from an indirect or structural contribution, nor does it provide much insight into a protein's mechanism of action. Understanding the molecular mechanisms by which these proteins influence gene expression will require more incisive methods of investigation, such as in vivo studies with missense mutants defective in specific functions and in vitro experiments with purified components.

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