

Mechanisms of microRNA-mediated gene regulation in animal cells

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MicroRNAs are a large family of regulatory molecules found in all multicellular organisms. Even though their functions are only beginning to be understood, it is evident that microRNAs have important roles in a wide range of biological processes, including developmental timing, growth control, and differentiation. Indeed, recent bioinformatic and experimental evidence suggests that a remarkably large proportion of genes (>30%) are subject to microRNA-mediated regulation. Although it is clear that microRNAs function by suppressing protein production from targeted mRNAs, there is, at present, no consensus about how such downregulation is accomplished. In this review, I describe the evidence that there are multiple mechanisms of microRNA-mediated repression and discuss the possible connections between these mechanisms.

Introduction

MicroRNAs (miRNAs) – single-stranded RNA molecules of ~21–23 nucleotides – were originally discovered in *Caenorhabditis elegans* as post-transcriptional regulators of genes involved in developmental timing and are now known to have pervasive effects on gene expression in all multicellular eukaryotes [1,2]. In animals, examples of documented miRNA functions include regulation of signaling pathways, apoptosis, metabolism, cardiogenesis and brain development (reviewed in Ref. [3]). In addition, misregulation of miRNA expression has been linked to many types of cancer (reviewed in Ref. [3]). In general, miRNAs function post-transcriptionally by reducing protein yield from specific target mRNAs. Here, I discuss the mechanisms by which this repression is achieved. For additional perspectives, see other recent reviews on the mechanistic aspects of miRNA function [4,5].

Several hundred miRNAs have been characterized, and each miRNA is thought to have several hundred mRNA targets. In animals, there is compelling evidence that miRNAs recognize their targets mainly through limited base-pairing interactions between the 5'-end of the miRNA (i.e. nucleotides 2–8, the seed region) and complementary sequences in the 3' untranslated regions (3'-UTRs) of the target mRNAs [6–8]. It is known that miRNAs do not function as naked RNAs but, instead, as components of ribonucleoprotein complexes (RNPs); a common constituent of all miRNA-containing RNPs (miRNPs) is a member

of the Argonaute protein family [1]. Given the apparent uniformity of miRNPs and the 'simple' way in which they engage their targets, it would seem probable that all miRNPs function by a common mechanism. Surprisingly, however, this expectation has not been borne out experimentally. There is now evidence for multiple modes of miRNA-mediated regulation, including translational inhibition, increased mRNA de-adenylation and degradation, and/or mRNA sequestration. How, or whether, these apparently diverse mechanisms are interrelated is not yet clear.

Translational inhibition

The first mechanistic analyses of miRNA function were carried out using *C. elegans*: it was found that the abundance of miRNA-regulated mRNAs was not substantially changed, but the abundance of proteins encoded by those mRNAs was markedly reduced [9,10]. Furthermore, the regulated mRNAs seemed to be present in polysomes [9,10] and engaged with ribosomes capable of subsequent elongation *in vitro* [10] (Figure 1). These striking observations suggested that miRNA-mediated suppression of protein production occurred by a mechanism that operated after the initiation of protein synthesis (Figure 2). Although subsequent studies have shown that miRNAs can exert their effects by a variety of mechanisms, there are now numerous examples in which miRNA-mediated reduction of protein production is not accompanied by corresponding changes in mRNA abundance (e.g. see Refs [11–15]). In most of these cases, the translational status of regulated mRNAs (i.e. whether they are associated with polysomes) has not been determined. Nevertheless, three recent reports provide evidence for repression of protein production after protein synthesis has been initiated [16–18]. In the first study, an miRNA-regulated mRNA in HeLa cells was found exclusively in polysomes and associated with elongationally competent ribosomes [16]. In the second study, a reporter mRNA responsive to an endogenous miRNA was also present in polysomes [17]. Several lines of evidence showed that these polysomes were actively translating; however, nascent polypeptides (Figure 1) could not be detected by immunoprecipitation, suggesting that repression of protein accumulation resulted from cotranslational protein degradation (i.e. protein degradation concomitant with translation) [17] (Figure 2). In the third study, a reporter construct containing a 3'-UTR with designed target sites for a synthetic miRNA that has extensive but imperfect complementarity to those sites

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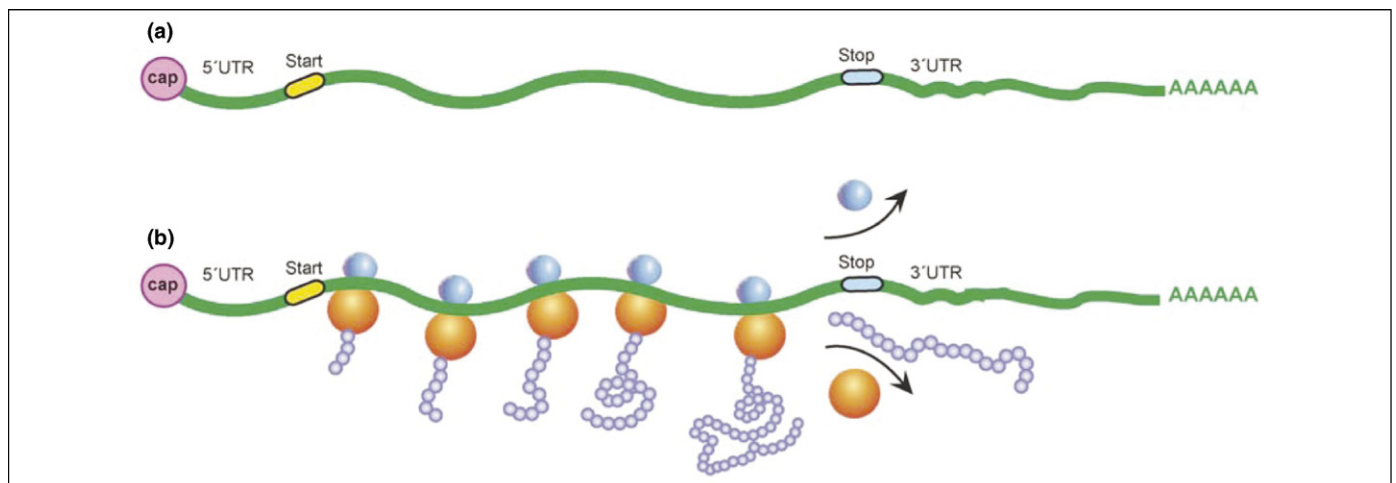


Figure 1. The process of translation. (a) A typical eukaryotic mRNA is shown, with start (initiation) and stop (termination) codons indicated. Protein synthesis occurs in three steps: initiation, elongation and termination. Initiation involves recognition of the 5'-cap by proteins called initiation factors, which recruit the small ribosomal subunit. After this subunit identifies the start codon, the large ribosomal subunit associates, and translation begins. (b) An mRNA occupied by several actively elongating ribosomes (which together constitute a polysome) is shown. As translation proceeds, newly synthesized proteins (nascent polypeptides) emerge from the large ribosomal subunit. Termination occurs when an elongating ribosome encounters the stop codon; the ribosome dissociates from the mRNA, and the completed protein is released.

was shown to be translationally repressed and associated with polysomes [18]. In this case, evidence suggested that the deficit in protein production resulted from premature termination of translation (premature ribosome drop off) [18] (Figure 2).

By contrast, two other reports have demonstrated miRNA-mediated inhibition at the level of initiation of protein synthesis [19,20] (Figure 2). In one study, an endogenous miRNA was shown to reduce polysome association of a reporter construct with appropriate target sites [19]. In the other study, complete repression of translation required both the 5'-cap and the 3'-poly(A)⁺ tail, whereas internal ribosome entry site (IRES)-directed translation was insensitive to inhibition [20]. Although the results of these two studies differ in the details [e.g. the role of the poly(A)⁺ tail], they both present persuasive evidence that initiation of protein synthesis is impaired by miRNAs at the level of cap recognition.

Collectively, the studies of translational inhibition have revealed distinct mechanisms by which cap-dependent translation can be repressed. Interestingly, differences have also been observed in the ability of miRNAs to suppress translation promoted by IRESs: two groups found that IRES-directed protein synthesis was unaffected by miRNAs, whereas another group showed that it was impaired [18–20]. The most straightforward interpretation of the results obtained so far is that miRNA-mediated inhibition of translation can occur in an mRNA-specific manner at distinct steps in the process of translation. Nevertheless, the features of individual mRNAs and/or mRNA–miRNA combinations that dictate the mechanism of repression remain to be elucidated.

mRNA de-adenylation and degradation

As discussed in the previous section, it was originally thought that miRNA-mediated regulation was exerted mainly at the level of translation and not at the level of mRNA degradation. It is now clear that this idea is only partially correct. There are numerous examples of miRNAs destabilizing their target mRNAs. Indeed, as detailed in

this section, the mechanism by which miRNAs increase mRNA turnover is perhaps better understood than the mechanism of translational repression.

Early evidence for miRNA-mediated destabilization came from studies in which an miRNA was transfected into cells that normally did not express this miRNA. The presence of such an miRNA was found to reduce the abundance of many mRNAs, most of which contained target sites for the miRNA [21]. In addition, examination of several miRNA targets in *C. elegans* revealed that these also were reduced in abundance in an miRNA-dependent manner [22].

The realization that miRNAs can promote mRNA degradation has enabled the use of mRNA microarray analysis for the identification of miRNA targets [21,23–25]. Although this approach has been fruitful, the proportion of the total number of targets that is identified by these types of analysis remains to be determined. There is no analogous high throughput method to identify targets that are regulated strictly at the translational level.

It now seems clear that miRNA-mediated destabilization is a consequence of de-adenylation followed by decapping (i.e. removal of the 5'-cap). Four studies – in zebrafish embryos, human cells and *Drosophila melanogaster* cells – have shown miRNA-dependent de-adenylation [24,26–28]. In *D. melanogaster* cells, de-adenylation is carried out by the de-adenylase complex CCR4–NOT [24]. Interestingly, although de-adenylation seems to be a prerequisite for mRNA decapping and degradation, it does not necessarily lead to these outcomes. Specifically, in the study in *D. melanogaster*, three reporter mRNAs were analyzed [24]. Although all three were completely de-adenylated, one remained stable, a second was only partially destabilized, and turnover of a third was markedly increased. Importantly, the level of regulation of these reporter mRNAs did not correlate with their stability in all cases: that is, the stable de-adenylated RNA was still strongly repressed at the translational level [24]. Although the translational repression observed could have resulted from the loss of the poly(A)⁺ tail, maintaining the poly(A)⁺ tail (by knock-down of the de-adenylase) did not relieve the inhibition [24].

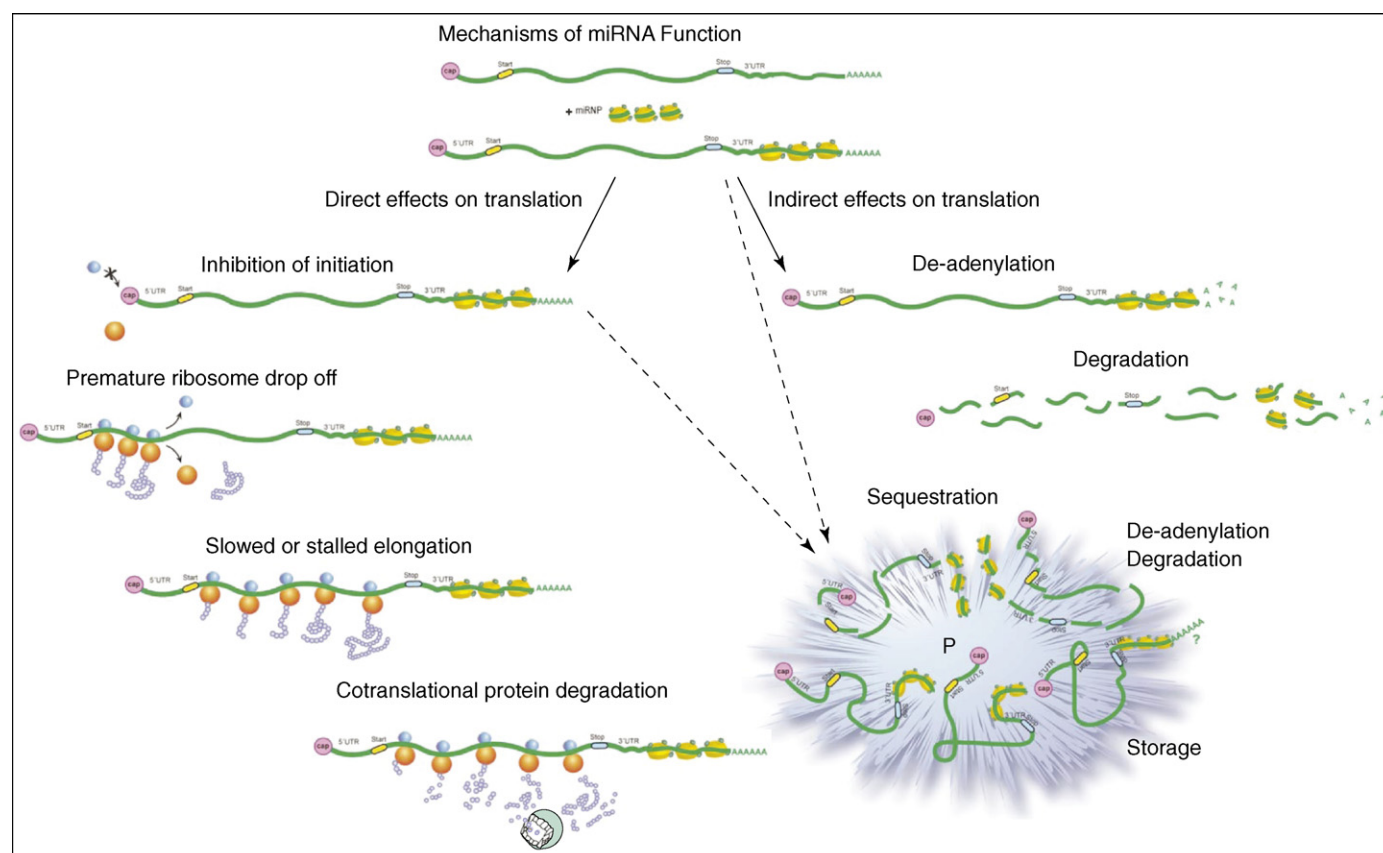


Figure 2. Mechanisms of miRNA-mediated repression. Target mRNAs are recognized by miRNAs in the form of ribonucleoprotein complexes (miRNPs), through sequence complementarity, usually between the miRNA and sequences in the 3'-UTR of the mRNA. The interaction between the miRNP and the mRNA can have several consequences. These include direct and indirect effects on translation. Direct effects occur either through inhibition of initiation of translation, which results in prevention of ribosome association with the target mRNA, or through inhibition of translation post-initiation. In the case of post-initiation repression – which includes premature ribosome drop off, slowed or stalled elongation, and cotranslational protein degradation – the repressed mRNA seems to be present in polysomes: i.e. associated with multiple ribosomes. In addition to direct effects on translation (or protein accumulation), miRNPs can have other effects on targeted mRNAs, including promoting de-adenylation, which might result in degradation (increased turnover). Both de-adenylation and degradation might take place in P bodies (denoted by P), which are cytoplasmic foci enriched for factors involved in mRNA degradation. It is possible that miRNA-targeted mRNAs could be sequestered from the translational machinery and degraded or stored for subsequent use. Alternatively, targeted mRNAs might be sequestered as a consequence of inhibition of initiation of translation. It is unknown whether stored mRNAs are de-adenylated. For discussion of the evidence in support of these mechanisms, see the main text.

Results from human cells, showing miRNA-dependent translational inhibition of a non-polyadenylated mRNA, are also inconsistent with de-adenylation being the sole cause of repression of protein synthesis [27]. Furthermore, repression of protein synthesis is not responsible for de-adenylation: two groups have shown that mRNAs that cannot be translated, because of either a strong stem-loop in the 5'-UTR or a defective cap, are still subject to de-adenylation [27,28].

These results from the study of several species and many distinct mRNAs suggest that increased de-adenylation might be a general consequence of miRNA-mRNA interactions. However, no change in poly(A)⁺ tail length was observed for an miRNA-regulated mRNA in *C. elegans*, although in this case the tail was short under all conditions examined [9]. It will be of considerable interest to determine whether other miRNA-regulated mRNAs are de-adenylated, particularly those that seem to be repressed by distinct mechanisms and those for which miRNA-mediated repression is reversible [29].

mRNA sequestration

It is clear that miRNA-mediated repression can be manifested by inhibition of translation and/or by increased

mRNA degradation. A variety of approaches, both cell biological and biochemical, have converged to indicate a central role for cytoplasmic foci known as processing (P) bodies (also called GW bodies) in both of these processes. P bodies, which were originally described in budding yeast, have been shown to contain colocalizing concentrations of a wide range of enzymes involved in mRNA turnover, including decapping enzymes, de-adenylases and exonucleases [30,31]. In animal cells, P bodies also contain the protein GW182, which was named for its extensive glycine and tryptophan repeats [32,33]. Although many other proteins have been shown to localize to P bodies [31] (for specific examples, see Refs [34–37]), the composition of P bodies has not been determined, because P bodies have been refractory to biochemical purification so far.

Notably, several groups have shown that Argonaute proteins – which bind to miRNAs – localize to P bodies [19,37–39] (reviewed in Ref. [31]). Furthermore, co-immunoprecipitation analyses indicate that Argonaute proteins interact in an RNA-independent way with the known P-body components DCPIA and GW182 [24,36,39], and, in *D. melanogaster*, there is evidence that Argonaute-1 and GW182 (also known as CG31992) interact directly [24]. Other proteins implicated in miRNA function also are

found to be concentrated in P bodies, and three studies have demonstrated that targeted mRNAs localize to P bodies in an miRNA-dependent way [19,29,39]. Finally, several lines of evidence, including knockdown and tethering of GW182 proteins, have established a direct role for this protein in miRNA-mediated downregulation of protein production from targeted mRNAs [24,36,37,39].

Collectively, these data provide the basis for a straightforward and attractive model for the mechanism by which miRNAs function [24,30,31]. In its simplest form, an miRNA bound to an Argonaute protein recognizes its mRNA targets by base pairing; the Argonaute protein, in turn, interacts with GW182, and the mRNA–miRNA–Argonaute complex is delivered to P bodies. When it reaches the P body, the targeted mRNA is de-adenylated by resident de-adenylases, then either decapped and degraded or held in stasis (i.e. spatially removed from the translational machinery, because P bodies are devoid of ribosomes) [30,31]. The latter outcome (stasis) is suggested by experiments in budding yeast showing that P bodies can be a repository for untranslated mRNAs [40] and is supported by an example from mammalian cells in which a specific mRNA seems to be sequestered from ribosomes in an miRNA-dependent manner [29]. As discussed extensively in Refs [30] and [31], this model has the advantage of rationalizing much of the current literature regarding the mechanisms of miRNA function, including the disparate fates of specific regulated mRNAs.

Although the P-body hypothesis is appealing and is supported by multiple lines of evidence, several observations seem to be incompatible with the idea that P-body-dependent processes account for all miRNA-mediated regulation. Specifically, a quantitative microscopic analysis of Argonaute protein localization has shown that only a small proportion (<2%) of Argonaute proteins are found in P bodies [41]. Furthermore, several groups have shown that miRNAs are associated with polysomes [16,17,42–46], and a recent report demonstrated that most miRNAs are associated with mRNAs undergoing translation [16]. Finally, knockdown of a P-body component was found to cause the disappearance of visible foci but did not compromise miRNA function [37].

The findings that only a minority of Argonaute proteins and miRNAs localize to P bodies [16,41] seem to indicate that the bulk of regulation takes place elsewhere. However, this might not be the case, because regulated mRNAs could exit translation and be ‘delivered’ to P bodies by a process that involves the removal of the miRNA–Argonaute complex. In this case, no marked accumulation of miRNAs or Argonaute proteins would be expected, and the apparent localization of these factors would be the result of transitory interactions. The demonstration that P-body structural integrity was not required for miRNA-mediated repression [37] is also not definitive, because it is possible that, although microscopically visible P bodies were absent, submicroscopic foci remained.

Examples of miRNA-mediated regulation in which repressed mRNAs are present in polysomes [16–18,42] are, however, more difficult to rationalize with the P-body hypothesis because P bodies lack ribosomes. Furthermore, any model of miRNA function must account for different

magnitudes of regulation. In this regard, the absolute level of repression of most miRNA-regulated mRNAs seems to be relatively mild (e.g. about twofold [7,21]) [47]. In these cases, by definition, regulated mRNAs must be translated. If regulation is mediated exclusively by accelerated mRNA degradation, then a decrease in steady-state mRNA levels would result, and this would account for decreased protein production; the intact mRNAs would seem to be normally translated. However, if regulation occurs without mRNA destabilization, then it is predicted by the P-body hypothesis that regulated mRNAs would partition between nontranslating (sequestered) and translating states proportional to the degree of regulation. Thus, at steady state, assuming twofold repression, half of a specific regulated mRNA would be expected to be associated with P bodies, and the other half would be expected to be in the translating pool. At present, there are two examples in which this prediction is fulfilled [19,29] and several in which it is not [9,10,16–18]. It will be of considerable interest to determine the subcellular distribution (whether ribosome associated) of additional mRNAs that are regulated in the absence of increased degradation. If most regulation occurs by sequestration, then biochemical purification of P bodies (assuming the development of appropriate techniques) would provide a powerful means both for identifying miRNA targets and for assessing levels of regulation.

Multiple mechanisms

Multiple factors

Because miRNA-mediated repression can be accomplished by several mechanisms and occurs over a wide range of magnitudes (less than twofold to more than tenfold repression), there must be specific interactions between distinct mRNPs (mRNA-containing RNPs) and the regulatory machinery. In principle, such specificity could result from features of the mRNP itself (e.g. secondary structure characteristics or the presence of bound proteins), from the composition of the miRNP itself (e.g. the presence of distinct auxiliary proteins), or from a combination of both (e.g. the composition of an mRNP could dictate which distinct miRNP can associate).

At present, it is clear that all miRNAs are bound to an Argonaute protein and that this protein is required for miRNA function. Similarly, it is generally agreed that GW182 (or GW182-like proteins) have an essential role in miRNA-mediated regulation regardless of the mechanism [24,36,48–50]. So these two proteins could be considered to be the ‘core’ of the miRNA machinery, and there are many candidates for additional factors. Several groups have used either biochemical approaches or co-immunoprecipitation to identify Argonaute-associated proteins. In addition to GW182, these include decapping enzymes, fragile X mental retardation protein (FMR), vasa intronic gene (VIG), the staphylococcal nuclease Tudor-SN, gem-associated protein 3 (GEMIN3) and GEMIN4, the arginine methyltransferase, PRMT5, and the DEXD box helicases MOV10 and RCK (also known as p54 and DDX6) [36,37,45,51–53]. In addition, Dicer and TRBP (also known as TARBP2) have been shown to interact with Argonaute [54–56]. The main function of these two proteins is thought to be to load mature miRNAs into Argonaute complexes

[54,56], but the possibility that these proteins have roles in subsequent steps in the pathway (e.g. target recognition) has not been excluded.

Many of the Argonaute-interacting proteins described here have been implicated in miRNA function, by using various techniques, including immunoprecipitation of mature miRNAs and/or small interfering RNA-mediated knockdown; however, the specific functions of these proteins are not yet known. It seems highly unlikely that all of these proteins constitute a common complex. Thus, it is possible that there is a range of miRNPs with distinct compositions. Whether such distinct complexes could confer specificity in terms of mechanism or magnitude of regulation is a matter of speculation, and it is worth noting that, so far, there is no evidence to support miRNA-specific effects for any identified factor.

mRNA context

Perhaps the most puzzling and interesting aspect of miRNA-mediated gene regulation is the role of mRNA context in determining the extent of regulation and the mechanism of regulation. Extensive bioinformatic analyses, together with experimental evidence, have indicated that many properties that contribute to context – including extent of miRNA pairing to complementary target sites (other than the seed region), secondary structure of mRNA and protein-binding sites on mRNA – often do not have essential roles in determining the ability of miRNAs to exert repressive effects [7]. The finding that engineered target sites in artificial or natural UTRs are responsive to natural or artificial miRNAs also suggests that context does not have a crucial role in determining miRNA function.

Although context might not always be important, there are now several clear examples in which it determines either the extent or the mechanism of repression [24,28,29,57–59]. In terms of mechanism, when three different miRNA-responsive 3'-UTRs were examined [24], the levels of repression were similar, but the mechanisms by which repression was achieved were different: the first mRNA was translationally repressed and stable; the second was destabilized; and the third was repressed by a combination of destabilization and translational repression. The most straightforward interpretation of these results is that each 3'-UTR, because of structure and/or bound proteins, interacts in a distinct way with a common miRNP or perhaps can recruit miRNPs of distinct composition (as discussed earlier). It will be of considerable interest to determine the *cis*-acting sequences and *trans*-acting factors that confer specificity.

There are also several examples in which mRNA context determines the extent of regulation [28,29,57–59]. In one case, recruitment of a factor (HuR; also known as ELAVL1) to a 3'-UTR under specific physiological conditions alleviates miRNA-mediated repression [29]. Although the mechanism by which HuR modulates miRNA activity remains to be determined, these observations indicate that regulation can be unexpectedly dynamic. In another example of negative modulation of miRNA function, an as-yet-unidentified regulatory factor(s) exerts its effect in a cell-type-specific manner [28]. In a further example, context does not negatively modulate miRNA function but, instead, is

necessary for the miRNA to exert its repressive effects [59]. Collectively, the observations that context can affect both the mechanism and the level of miRNA-mediated regulation (both positively and negatively) indicate that such regulation is more complex than was anticipated. Unraveling the complexity of this process awaits detailed analysis of many more specific examples.

Parallels to other regulatory pathways

Post-transcriptional downregulation of gene expression is not carried out only by miRNAs, and all of the mechanisms discussed here have numerous precedents. The similarities in regulation mediated by miRNAs and PUF proteins (a family of proteins named for its founding members *D. melanogaster* Pumilio and *C. elegans* FBF) are particularly striking. PUF proteins bind to 3'-UTRs and mediate repression either by increasing de-adenylation and promoting mRNA degradation or by inhibiting translation [60]. As discussed extensively in Ref. [60], PUF proteins have multiple targets, and their activities can be modulated by multiple binding partners. Although the mechanism(s) by which PUF proteins exert their many effects is not fully understood, the parallels between the end points of their regulation and miRNA-mediated regulation suggest that common factors are involved in these pathways. Therefore, it would be interesting to determine whether GW182 is important for PUF protein activity.

An additional potentially important connection between the miRNA-mediated regulation pathway and other translational control pathways comes from the finding that RCK is involved in miRNA-mediated translational repression [37]. The *D. melanogaster* homolog of RCK, ME31B, is required for suppression of *oskar* mRNA translation [61], and the budding yeast homolog of RCK, Dhh1p, is involved in general translational repression [62].

Finally, there are numerous other well-documented examples of post-transcriptional gene regulation at both the level of mRNA stability and the level of translation (at initiation and subsequent steps) that do not involve miRNAs (reviewed in Refs [63] and [64]). It would be surprising if these phenomena and miRNA-mediated repression were not linked by common mechanisms and factors.

Concluding remarks and perspectives

Despite rapid progress and a wealth of information, the study of the mechanisms by which miRNAs repress gene expression is in its infancy. The data reviewed here support the existence of several distinct, but perhaps overlapping, mechanisms. However, it is not clear whether all mechanisms have been uncovered, because relatively few regulated mRNAs have been studied in detail. It will be important to define which subsets of mRNAs are subject to different mechanisms, because such knowledge should enable studies that could uncover common features. Nevertheless, categorizing mRNAs will be difficult, because, at present, there is no straightforward way to identify mRNAs that are regulated solely or in part at the translational level.

Equally challenging or even more challenging will be the identification and characterization of *trans*-acting factors that promote or suppress miRNA-mediated function in

specific contexts. In this regard, there are clear cases of physiological conditions or cell type affecting miRNA activity. Whether miRNA-mediated regulation can be modulated by other factors, such as signal transduction pathways or cell growth status, remains to be determined. Future experiments will no doubt answer these questions and illuminate our general understanding of post-transcriptional regulatory mechanisms.

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