NON-CODING RNA

MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship

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Abstract | MicroRNAs (miRNAs) have emerged as key gene regulators in diverse biological pathways. These small non-coding RNAs bind to target sequences in mRNAs, typically resulting in repressed gene expression. Several methods are now available for identifying miRNA target sites, but the mere presence of an miRNA-binding site is insufficient for predicting target regulation. Regulation of targets by miRNAs is subject to various levels of control, and recent developments have presented a new twist; targets can reciprocally control the level and function of miRNAs. This mutual regulation of miRNAs and target genes is challenging our understanding of the gene-regulatory role of miRNAs *in vivo* and has important implications for the use of these RNAs in therapeutic settings.

MicroRNAs (miRNAs) exemplify the emerging view that non-coding RNAs (ncRNAs) may rival proteins in regulatory importance. Since the initial discovery of miRNAs as essential regulators of development in the nematode *Caenorhabditis elegans*, thousands of miRNA genes have been identified in animal and plant genomes[1]. Well over half of the human transcriptome is predicted to be under miRNA regulation, embedding this post-transcriptional control pathway within nearly every major gene cascade[2,3]. Given this far-reaching role, it is not surprising that disruption of miRNA function contributes to many human diseases, including cancer, heart ailments and neurological dysfunctions[4]. Additionally, miRNAs are being developed as both targets and therapeutics for a growing industry hoping to harness the power of RNA-guided gene regulation to combat disease and infection[5].

miRNAs function as 21–24-nucleotide (nt) guides that regulate the expression of mRNAs containing complementary sequences. FIGURE 1 illustrates the general miRNA biogenesis pathway, and this topic has been comprehensively reviewed elsewhere[6]. Ultimately, the mature miRNA joins specific Argonaute proteins in what is referred to as an miRNA-induced silencing complex (miRISC). In animals, partial pairing between an miRNA and an mRNA target site usually results in reduced protein expression through a variety of mechanisms that involve mRNA degradation and translational repression[7]. Originally, miRNA function seemed to be distinct in plants, in which miRNAs could perfectly base-pair with targets and induce their cleavage and degradation. However, recent studies indicate that plants also use translational control as a mechanism for silencing their targets, and it remains to be determined how many plant mRNAs with sites that are partially complementary to miRNAs are subject to regulation[7]. Furthermore, in any organism, complete complementarity between an miRNA and a target site can only lead to cleavage if a catalytically active Argonaute is bound[7].

This Review focuses on the complexity of identifying endogenous miRNA targets and how they are regulated. Newly developed genome-wide approaches, such as ultraviolet crosslinking and immunoprecipitation (CLIP), are now facilitating the identification of endogenous miRNA-binding sites. Furthermore, techniques such as ribosome profiling are enabling the mechanism of gene regulation by miRNAs to be dissected. These cutting-edge methods have revealed new features of miRNA targeting on a global scale, and they provide rich data sets for deeper exploration of the rules used by miRNAs for the recognition and regulation of targets *in vivo*. However, predicting whether an mRNA will be subject to miRNA regulation in the endogenous context is a separate challenge, as there are now many examples of factors that control the ability of an miRISC to bind and repress specific targets. The newly appreciated reciprocal nature of miRNA and target regulation adds a level of complexity that must also be understood before miRNA–target relationships are fully resolved.

miRNA-induced silencing complex
(miRISC). This is composed of the miRNA guide and the effector proteins, which minimally include Argonaute proteins. The complex recruits additional proteins to regulate the stability or translation of targeted mRNAs.

Crosslinking immunoprecipitation
(CLIP). A method used to isolate and identify sequences that are bound by specific RNA-binding proteins. CLIP of microRNA complex proteins, such as Argonaute, has been used to detect microRNA-induced silencing complex (miRISC)-binding sites on genome-wide scales.

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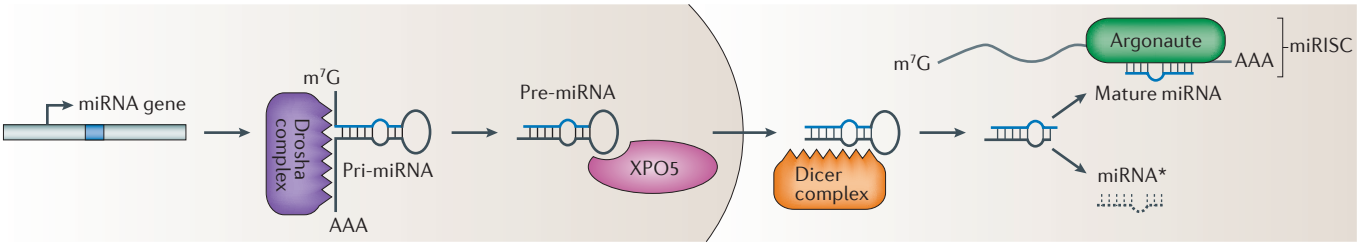


Figure 1 | **miRNA biogenesis.** In animals, microRNA (miRNA) genes are typically transcribed into primary miRNA (pri-miRNA) transcripts that undergo processing by Drosha-containing complexes[6]. The resulting hairpin precursor miRNAs (pre-miRNAs) are transported to the cytoplasm by exportin 5 (XPO5). The Dicer complex removes the loop region from pre-miRNAs, and one strand of the resulting duplex is bound by Argonaute to form an miRNA-induced silencing complex (miRISC), which targets mRNAs for regulation. The other strand, which is often called the star strand (miRNA*), is degraded. This pathway is similar in plants except that Dicer-like (DCL) proteins carry out both cleavage steps in the nucleus, and the resulting mature miRNA is bound by Argonaute proteins and transported to the cytoplasm.

In this Review, the identification of miRNA target sites and endogenous targets will be discussed first. Next, the mechanisms used in target regulation will be presented along with methods for pinpointing these target genes. The regulation of miRNA function will then be covered, finishing off with the emerging mutual regulatory relationship between miRNAs and their targets.

miRNA target sites

An outstanding problem in the miRNA field is how miRNAs recognize specific sequences of partial complementarity, complicating the prediction of target sites[2]. Given the challenge of matching miRNAs to specific target sequences, several approaches have been adopted for identifying functional interactions.

Characteristics of miRNA target sites. The small size of miRNAs provides a limited amount of sequence information for specificity. Furthermore, as partial pairing between an miRNA and a target site is often sufficient, a wide net can be cast for genes that are subject to regulation. This property not only means that a single miRNA can regulate multiple mRNAs but also that predicting those targets is not straightforward. In plants, target sites are present in coding exons as well as 3′ untranslated regions (UTRs), and the efficiency of regulation has not been linked to target site location[7]. Plant miRNAs may either form near-perfect duplexes with their targets, enabling endonucleolytic cleavage of the mRNA[8] (FIG. 2a), or they may regulate their targets through mechanisms that do not involve cleavage, obviating the need for strict complementary sites[9]. Cleavage products can be cloned and identified to validate direct miRNA–target interactions[10–12]. This has been a productive approach for discovering genuine miRNA target sites in plants. Targets that lack substantial miRNA-pairing capacity and, thus, would not be subject to cleavage are yet to be explored in plants[13].

In animals, it is clear that the vast majority of miRNAs only form partial duplexes with their targets, and most target mRNAs studied to date are regulated through 3′UTR interactions[2,3]. There are rare examples of near-perfect complementarity between an miRNA and a target site that enables cleavage of the mRNA, such as in the case of miR-196 and a sequence in the *HOXB8* mRNA[14]. Most cases, however, involve duplexes that contain mismatches and multiple nucleotide bulges. The most common motif is perfect pairing between nucleotides 2 and 7 at the 5′ end of the miRNA, which is called the 'seed' region, and the target site[2] (FIG. 2b). In some experimental contexts, seed pairing has been shown to be both necessary and sufficient for regulation by the miRNA pathway[15]. Imperfect pairing of the 5′ end of the miRNA to a target is sometimes compensated for by extensive 3′ end interactions, which seems to be the case for a lethal 7 (let-7) miRNA target site in the *abnormal cell lineage 41* (*lin-41*) 3′UTR in *C. elegans*[16–18] (FIG. 2c). More recently, 'centred sites' have been described, at which the middle region of the miRNA makes contiguous base pairs with a target sequence[19] (FIG. 2d). There are also numerous examples of functional miRNA target sites that do not readily fit any of the previously described patterns[3,20] (FIG. 2e). The apparent flexibility in animal miRNA-targeting rules suggests that factors beyond just pairing capacity mediate functional target interactions *in vivo*.

Methods for identifying target genes and their miRNA-binding sites. The perplexing problem of identifying miRNA targets and their regulatory sequences has been tackled by multiple complementary approaches (BOX 1; see REF. 21 for more details). The first miRNA target genes were discovered as genetic suppressors of miRNA loss-of-function phenotypes (BOX 1a). Based on the original evidence that miRNAs regulate mRNAs through base-pairing interactions, numerous computational approaches were developed to predict miRNA target sites *in silico*. Certain features, such as conserved seed pairing in unstructured and AU-rich regions, have emerged as good indicators of potential miRNA target sites[2] (BOX 1b). Nonetheless, most algorithms produce widely divergent predictions of partially complementary miRNA target sites with degrees of false positives and false negatives that are difficult to assess[22].

One of the many challenges of predicting functional miRNA target sites *in silico* is the limited availability of miRNA–target interactions that have been validated in the endogenous context. Often, target predictions are tested by fusing sequences that contain the target site

to a reporter gene and assaying for regulation in the presence or absence of the cognate miRNA. Here, the natural regulatory context is lost, both at the level of the target sequence within its mRNA as well as at the level of cellular context. However, it is now possible to identify endogenous target sites by sequencing those that co-immunoprecipitate with miRISC factors using techniques such as CLIP coupled with high-throughput sequencing (CLIP–seq) or high-throughput sequencing together with CLIP (HITS–CLIP)[23–26] (BOX 1c). These studies have provided extensive data supporting seed pairing, conservation and structural accessibility as common features of miRNA target sites. Nonetheless, they also point to new considerations, such as greater interaction of the miRISC with coding exons than had previously been thought, as well as to the existence of many binding sites that apparently deviate from known miRNA target prediction rules. A limitation of CLIP is that the functionality of the identified binding sites is indefinite without additional experiments. Comparison of CLIP data with results from assays that directly analyse changes in mRNA or protein levels is needed to judge whether association of the miRISC with a target affects its expression. This can be accomplished on a genome-wide scale through transcriptome profiling using microarrays or RNA sequencing (RNA-seq) and through quantitative proteomic platforms, such as stable isotope labelling with amino acids in culture (SILAC)[27–29].

Target regulation by miRNAs

The mechanism used by miRNAs to regulate target gene expression has been a controversial subject, as there is evidence for target mRNA destabilization, translational repression and even activation of gene expression[7]. In both plants and animals, miRNAs can silence targets through RNA degradation as well as translational repression pathways (reviewed in detail in REF. 7). Perfect pairing of an miRNA with its target site supports endonucleolytic cleavage of the mRNA by Argonaute[12,14] (FIG. 3a). This is a common mechanism in plants but is much rarer in animals. Nonetheless, destabilization of the target mRNA through other mechanisms is a common outcome of miRNA regulation in animals. Binding of the miRISC, which includes GW182 proteins, to 3′UTR target sequences can result in the recruitment of deadenylation factors that remove the poly(A) tail and make the mRNA susceptible to exonucleolytic degradation[30–35] (FIG. 3b). There are also cases in plants and animals in which miRNAs cause reduced protein (but not mRNA) levels, suggesting that translational repression is directed by miRISC. The actual mechanism that blocks protein production is not clear and there is evidence for inhibition of translational initiation or elongation, as well as for directed proteolysis of the peptide that is being synthesized from the targeted mRNA[36–42] (FIG. 3c,d). Recent work has shown that the CCR4–NOT complex that is recruited by GW182 to deadenylate target mRNAs also acts to repress translation initiation[31–33] (FIG. 3c). To make matters more complicated, stimulated translation of targets by miRNAs under certain circumstances

GW182 proteins
Glycine–tryptophan repeat-containing proteins that interact with the microRNA-induced silencing complex (miRISC) to recruit proteins that mediate degradation or translational repression of target mRNAs. Also known as TNRC6A, TNRC6B and TNRC6C in vertebrates and AIN-1 and AIN-2 in *Caenorhabditis elegans*.

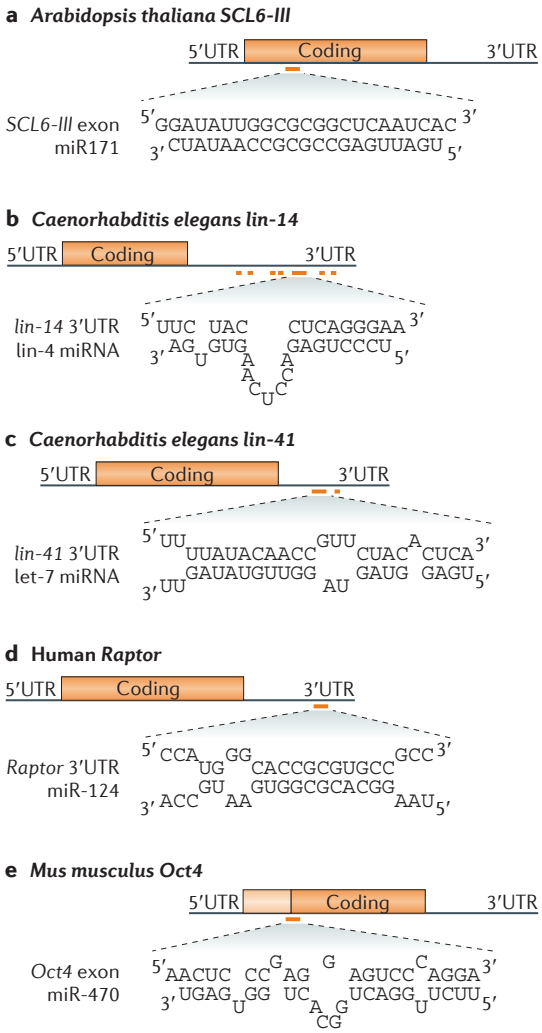
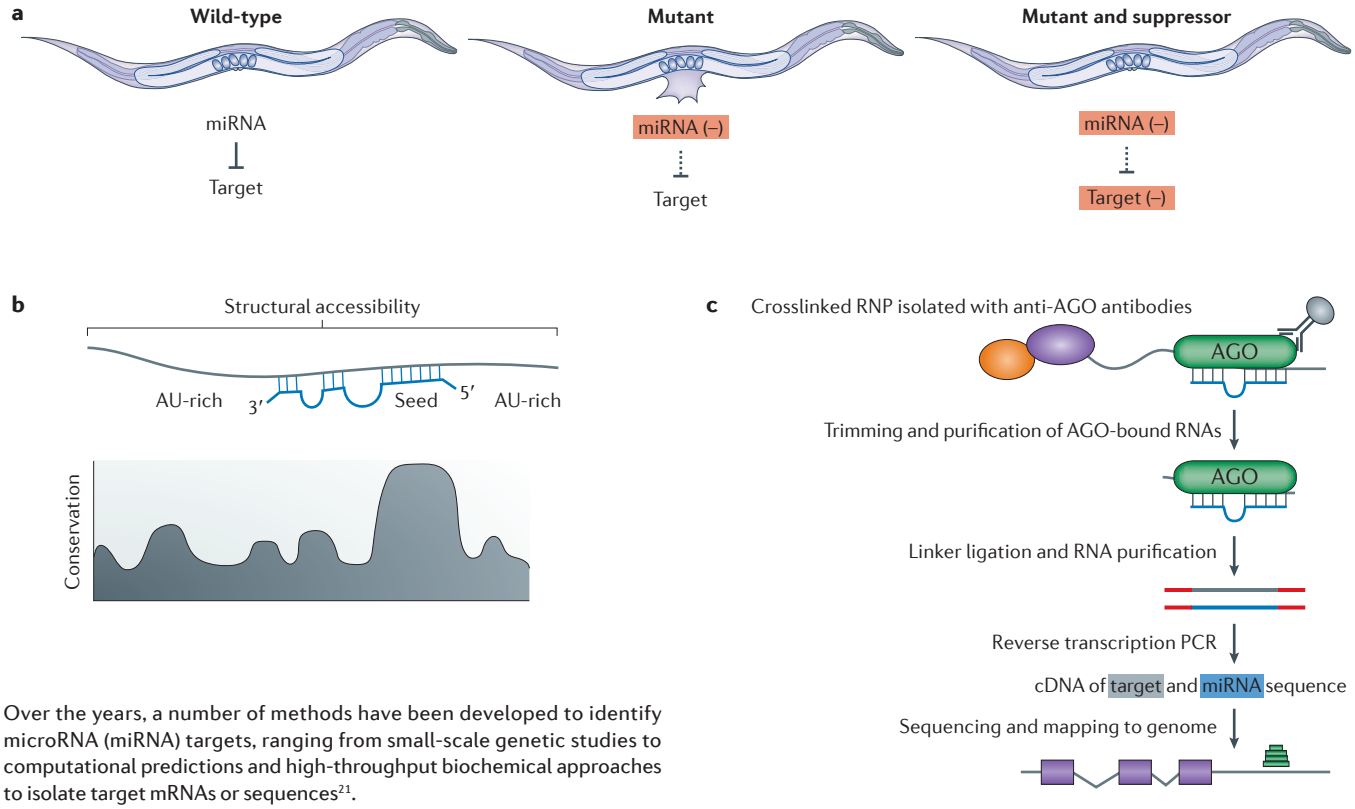


Figure 2 | **Examples of functional miRNA target sites.** Different degrees of base pairing mediate target recognition by microRNAs (miRNAs). **a** | In plants, miRNAs usually pair nearly perfectly with target sites. For example, miR171 (also known as miR39) regulates Scarecrow-like 6 III (*SCL6-III*) mRNA through a fully complementary site in its coding region in *Arabidopsis thaliana*[12]. **b** | In animals, partial pairing between miRNAs and their target sites is typical. Perfect pairing between a target and nucleotides 2–7 of the miRNA, which is called the seed region, is a common motif, as exemplified by a site in the 3′ untranslated region (UTR) of the *abnormal cell lineage 14* (*lin-14*) mRNA that is recognized by lin-4 miRNA in *Caenorhabditis elegans*[102,110]. The additional bars below the *lin-14* 3′UTR indicate other lin-4 target sites, not all of which exhibit seed pairing. **c** | Sometimes, the absence of perfect seed pairing is compensated for by the extensive pairing of the 3′ end of the miRNA to the target sequence, as is the case for a site in the *lin-41* 3′UTR that is paired to lethal 7 (let-7) miRNA in *C. elegans*; an additional let-7 target site is immediately downstream of the depicted duplex[16,17]. **d** | Pairing of miRNA middle sequences with target sites also mediates regulation for genes such as human *Raptor* and miR-124 (REF. 19). **e** | Regulation of animal mRNAs through coding regions has also been documented and, in some cases, the target site straddles an exon junction, as is the case for mouse *Oct4* (also known as *Pou5f1*) paired to miR-470 (REF. 20).

Box 1 | **Methods for identifying miRNA targets**



Over the years, a number of methods have been developed to identify microRNA (miRNA) targets, ranging from small-scale genetic studies to computational predictions and high-throughput biochemical approaches to isolate target mRNAs or sequences[21].

Genetic methods

This approach identifies miRNA targets through phenotypic suppression tests. Typically, screens are performed to search for candidate genes that rescue an miRNA loss-of-function phenotype. For example, the bursting vulva phenotype of *lethal 7* (*let-7*) mutants in *Caenorhabditis elegans* was found to be rescued by a mutation in *abnormal cell lineage 41* (*lin-41*), indicating that *lin-41* was a target of the let-7 miRNA (panel **a** of the figure)[16]. The miRNA mutant strain can be subjected to traditional mutagenesis or RNAi and screened for suppression. In the miRNA mutant background, target genes are usually upregulated. Thus, mutation or knockdown of a gene leading to a partial or complete rescue of the phenotype indicates that the gene is a target of the mutant miRNA. An important advantage of this approach is that the genetically identified target is a physiologically relevant gene that is regulated by the miRNA. Caveats of these genetic analyses include the inability to distinguish direct and indirect targets of miRNAs and the potential difficulty in detecting individual suppressors if many targets contribute to the phenotype.

Computational methods

Computational methods rely on algorithms that incorporate diverse criteria for the identification of candidate miRNA targets. Most of them, however, use a set of common experimentally derived conclusions that appear to reduce false-positive predictions (panel **b** of the figure)[22]. These include a requirement for perfect Watson–Crick pairing between the 5′ region for the miRNA and the mRNA target sequence, especially for the nucleotides 2–7, termed the miRNA 'seed'. The list of predicted targets can be further refined with the use of conservation criteria. The accessibility of the target site in the secondary structure of the 3′ untranslated region (UTR), as defined by the AU content in the vicinity of the site, is commonly used as a criterion for miRNA target predictions. Although these general guidelines have successfully predicted many miRNA target sites, exceptions are common in which seed pairing, conservation or AU context are not used for functional targeting[2,3]. Additionally, computational predictions are often tested for regulatory ability in the context of heterologous reporter genes fused to the target 3′UTR, leaving open the question of whether the predicted site functions in its natural genic and cellular setting.

Biochemical methods

Certain advantages are offered by biochemical methods, which are often coupled to bioinformatics analyses, when used to identify miRNA targets. These include increased sensitivity and the ability to identify endogenous target mRNA transcripts or even the target sequence in the mRNA on a large scale. Earlier approaches relied on the immunopurification of miRNA ribonucleoprotein (miRNP) complexes, isolation of the associated mRNAs, followed by identification of the targeted transcript with microarrays[102–107]. More recent methods, such as ultraviolet crosslinking and immunoprecipitation coupled to deep sequencing (CLIP–seq) or high-throughput sequencing coupled with ultraviolet crosslinking and immunoprecipitation (HITS–CLIP), aim at isolating the sequences in endogenous RNAs that are targeted by miRNAs and that take advantage of newly developed next-generation sequencing platforms, providing nucleotide-level resolution of the targeted sequences (panel **c** of the figure)[23–26]. Intact animals, tissues or cell lysates are irradiated with ultraviolet light, which crosslinks and stabilizes protein–RNA interactions. Immunopurification of miRNPs is followed by an RNase-treatment step to degrade RNA fragments that are not protected by miRNP components; the protected RNA fragments, including the associated miRNAs, are isolated, converted to cDNA and subjected to deep sequencing and bioinformatics analyses. A variation of this CLIP approach includes the addition of a photo-activatable ribonucleoside analogue (such as 4-thiouridine) in the culturing conditions (PAR-CLIP), which can lead to a single-nucleotide mutation at the site of the crosslink; this mutation can be used as a location marker of successful crosslinking[24]. These biochemical approaches yield genome-wide data sets of endogenous miRNA targets and potentially their direct binding sites. However, the detection of an mRNA bound by miRISC alone does not guarantee that it is actually being regulated, nor does it reveal the potential mechanism of control. AGO, Argonaute.

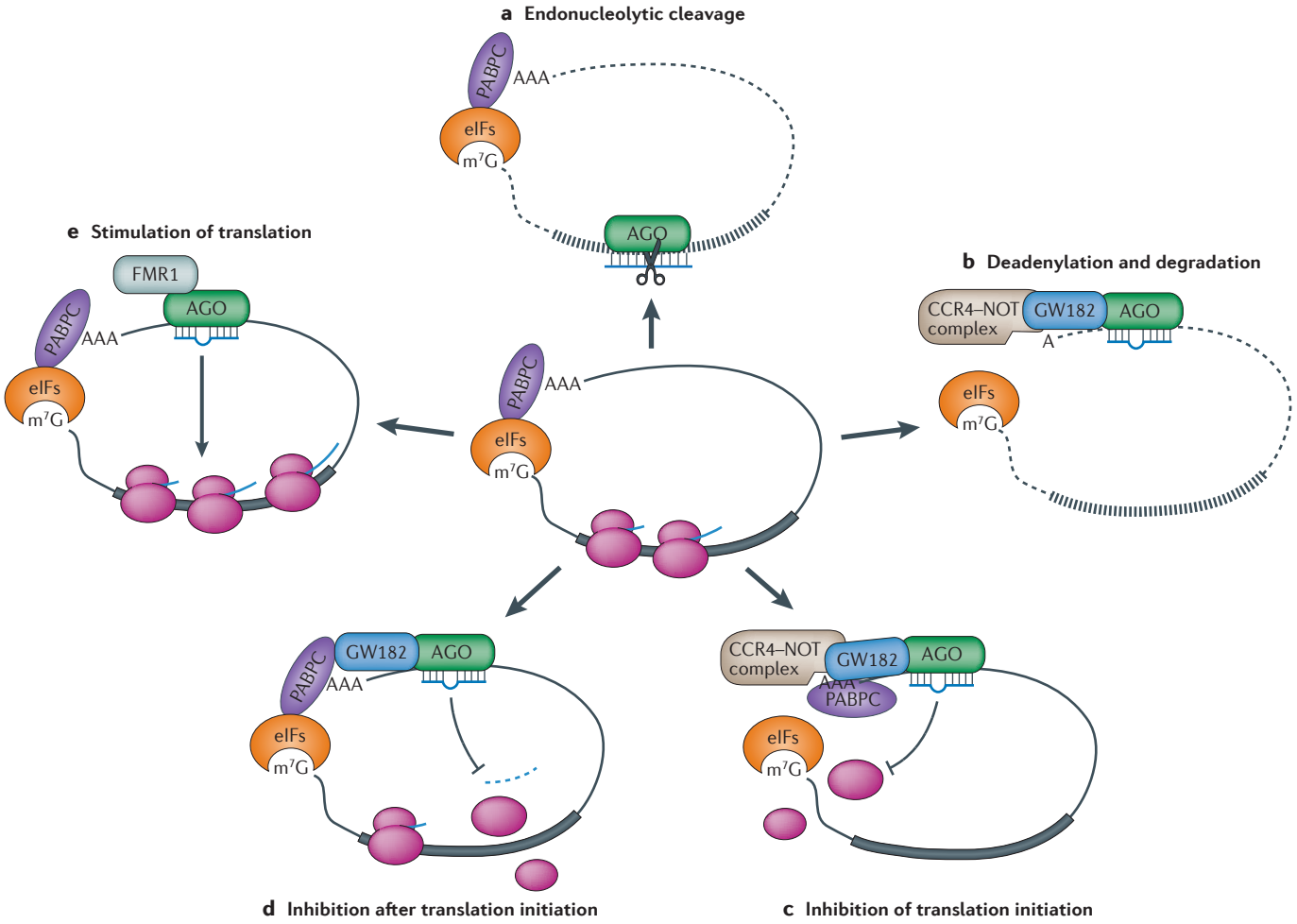


Figure 3 | **Mechanisms of target regulation by miRNAs.** MicroRNAs (miRNAs) regulate gene expression through multiple pathways[7]. A complex of eukaryotic initiation factors (eIFs) binds the 5′ cap and the cytoplasmic poly(A)-binding protein (PABPC), connecting the 5′ and 3′ ends of mRNAs and stimulating their translation[111] by the ribosome (shown in pink). **a** | Perfect pairing between an miRNA and its target site induces endonucleolytic cleavage by Argonaute (AGO), leading to rapid degradation of the mRNA. **b** | Partial pairing of the miRNA complex to target 3′ untranslated region (UTR) sites can result in deadenylation of the mRNA through recruitment of the CCR4–NOT complex by the miRNA-induced silencing complex (miRISC)-associated GW182 proteins. Loss of the poly(A) tail causes dissociation of PABPC and leads to degradation of the mRNA. **c** | The miRISC can also induce translational repression by blocking initiation via recruitment of CCR4–NOT by GW182. **d** | Translational repression can also be induced by the miRISC by inhibiting a step after initiation, such as promoting ribosome drop-off or stimulating proteolysis of the nascent peptide. **e** | miRNAs have also been shown to upregulate target expression under certain conditions through a mechanism that involves Argonaute and fragile X mental retardation protein 1 (FMR1).

has also been reported[43,44]. For example, miR-16 targets the mRNA for *myt1* kinase and, in conjunction with Argonaute and fragile X mental retardation syndrome-related protein 1 (fxr1), activates its expression in *Xenopus laevis* oocytes[45] (FIG. 3e). Overall, miRNAs typically repress gene expression, and it remains to be seen whether positive regulation of targets extends beyond the limited cases that have been uncovered so far.

Methods for discerning these different mechanisms of target regulation are summarized in BOX 2 (further details in REF. 21). Briefly, methods have traditionally focused on assaying mRNA levels. However, it is also important to investigate poly(A) tail length to identify cases in which deadenylation is induced by an miRNA, but the target mRNA is not degraded[46]. The first large-scale attempts to analyse miRNA-dependent changes in protein expression or association of transcripts with polysomes indicated that regulation is largely associated with mRNA destabilization[27,29,47]. The recent development of ribosome profiling (BOX 2b) has provided a sensitive method for examining the role of RNA degradation versus translational inhibition[48]. Ribosome profiling of mammalian cells in culture added further evidence that regulation by miRNAs is largely through mRNA destabilization, as opposed to translational repression[49]. It remains to be shown whether this is the case in other cellular contexts or whether mRNA degradation is a cause or a consequence of halted translation[50].

Fragile X mental retardation syndrome-related protein 1 (FXR1). An RNA-binding protein that can regulate the translation or stability of bound transcripts.

P bodies
Processing bodies, or P bodies, are cytoplasmic foci enriched for proteins that are involved in RNA degradation but that are devoid of translation factors. MicroRNAs, Argonaute and GW182 proteins also localize to P bodies, and silencing of some targets may occur in these structures.

Regulation of targeting efficiency

In the endogenous context, many factors influence the ability of the miRNA complex to bind and regulate specific targets.

Target site context. With the limited sequence specificity that is provided by miRNA guides, other factors must influence target site selection *in vivo*. The position of a target site within an mRNA has been associated with how well it is recognized and regulated by the miRISC. A feature that correlates with targeting efficacy in 3′UTRs is the location of the target site. AU-richness and generally unstructured areas seem to enhance accessibility to the miRNA complex[2]. Additionally, target sites tend to avoid sequences immediately after the translational stop codon that would fall into the ribosome shadow — the region covered by ~15 nt downstream from the stop codon that is bound by the ribosome before it dissociates from the mRNA[51]. This feature is consistent with studies showing that ribosomes can interfere with miRNA targeting of translated sequences[52] (FIG. 4a). Nonetheless, computational and biochemical experiments indicate that a substantial fraction of miRNA target sites exist in open reading frame (ORF) sequences[2,3]. For example, nearly half of the Argonaute-bound sites that were identified by CLIP assays in mammalian cells and worm extracts reside in coding-exon sequences[23–26]. Whether these sites are effective at inducing repression is yet to be shown. In one study, ORF target sites were generally found to be less effective on their own, although they could enhance the regulation of mRNAs that also contained 3′UTR target sites[53]. Thus, another consideration is how the number of miRNA target sites within an mRNA influences regulation. Generally, increasing the number of target sites for the same or different miRNAs improves the efficacy of regulation. However, when two target sites are nearly overlapping, the effect can be unpredictable, as instances of synergy as well as antagonism have been reported[51,54,55]. Context features may help to explain the variability in miRNA binding to identical target sites in different mRNAs[56]. Thus, all of these *cis*-acting features are important considerations when using reporter constructs to determine how well a target is recognized and regulated by the miRNA pathway.

RNA-binding proteins. RNA-binding proteins (RBPs) that associate with target mRNAs can interfere with miRISC binding or function. HuR (also known as ELAV1) is a broadly expressed RBP that binds AU-rich sequences in mRNAs, typically protecting them from degradation[57]. The effect of HuR on the repression of *CAT1* (also known as *SLC7A1*) mRNA by miR-122 is one of the first examples of an RBP directly antagonizing miRNA target regulation[58]. This study showed that under normal conditions in human hepatoma cells, miR-122 represses the translation of *CAT1* mRNA by binding to sites in its 3′UTR and sequestering the mRNA in P bodies. Under conditions of stress, such as amino acid starvation, the *CAT1* mRNA moves from P bodies back into the translating pool, which is accompanied by increased protein expression. This relief of miRNA-induced repression is mediated by HuR. This RBP is mostly nuclear, but certain stress conditions induce relocalization of HuR to the cytoplasm, where it can bind AU-rich sequences in the *CAT1* 3′UTR. The HuR-binding site is hundreds of nucleotides downstream of three miR-122 target sequences in the *CAT1* 3′UTR. Nonetheless, association of HuR with *CAT1* abolishes miRNA-mediated silencing of the mRNA through a mechanism that is yet to be determined. This effect of HuR is not restricted to miR-122 regulation, as reporters containing other miRNA target sites also responded to HuR as long as they contained the AU-rich sequences that are bound by the RBP. One possibility is that 3′UTR structure could bring HuR in the vicinity of the miRNA target sites, allowing for contacts that perturb either miRISC binding or its ability to regulate the mRNA.

The effect of HuR on miRNA-targeting efficacy may be widespread. Two recent CLIP studies found hundreds of HuR-binding sites that were adjacent to or overlapping with miRNA target sites in unstressed culture cells[59,60]. Overall, greater than 75% of 3′UTRs with Argonaute-binding sequences also contain HuR interaction sites[60]. Interestingly, only HuR sites that overlapped with miRNA target sequences seemed to antagonize miRISC regulation of mRNA levels[60]. The lack of a functional outcome for genes with HuR sites that are distant from miRNA target sequences could mean that *CAT1* is a rare example of HuR working remotely. Alternatively, the effect of such sites could be underestimated if some targets are more strongly regulated at the translational

Box 2 | Methods for studying target regulation by miRNAs

RNA-expression analyses

Because microRNAs (miRNAs) often promote destabilization of target mRNAs[7], decreased mRNA levels could be a result of direct miRNA regulation. Northern blotting or quantitative PCR can be used to analyse specific mRNAs. For genome-wide studies, microarrays and RNA sequencing are used to compare changes in mRNA abundance in the presence and absence of specific miRNAs[28]. Deadenylation is often a characteristic of miRNA-mediated target regulation, and this can be assessed by poly(A) tail length analysis using RNase H cleavage of mRNA 3′ ends followed by northern blotting or PCR-based methods for comparing adenylation states[108]. These approaches can show that a predicted target is subject to regulation at the mRNA level. However, failure to detect a change in mRNA abundance does not rule it out as a target, as the miRNA complex can also use mechanisms to block protein expression that do not involve mRNA destabilization.

Protein-expression analyses

Usually, the final outcome of target regulation by miRNAs is reduced protein expression. Western blotting is the most straightforward method for measuring changes in protein levels for specific targets. Larger-scale analysis of protein abundance is possible through mass spectrometry[27,29,109]. This method directly quantifies hundreds of proteins at once, but it is currently not sensitive enough to detect lower abundance proteins that may also be under miRNA control. An indirect method for assaying protein production is through ribosome profiling, where the association of mRNAs with ribosomes indicates their translation state. In ribosome profiling, the mRNA fragment that is associated with individual ribosomes is identified[48]. Not only can a change in ribosome association be detected by ribosome profiling, but the actual positions at which ribosomes are associated with the mRNA are revealed. Thus, an accumulation of ribosomes at the 5′ end of an mRNA can indicate stalled translational initiation. Because miRNAs have been reported to regulate translation at both initiation and post-initiation steps, ribosome profiling is a powerful method for determining the mechanism of regulation for individual mRNAs.

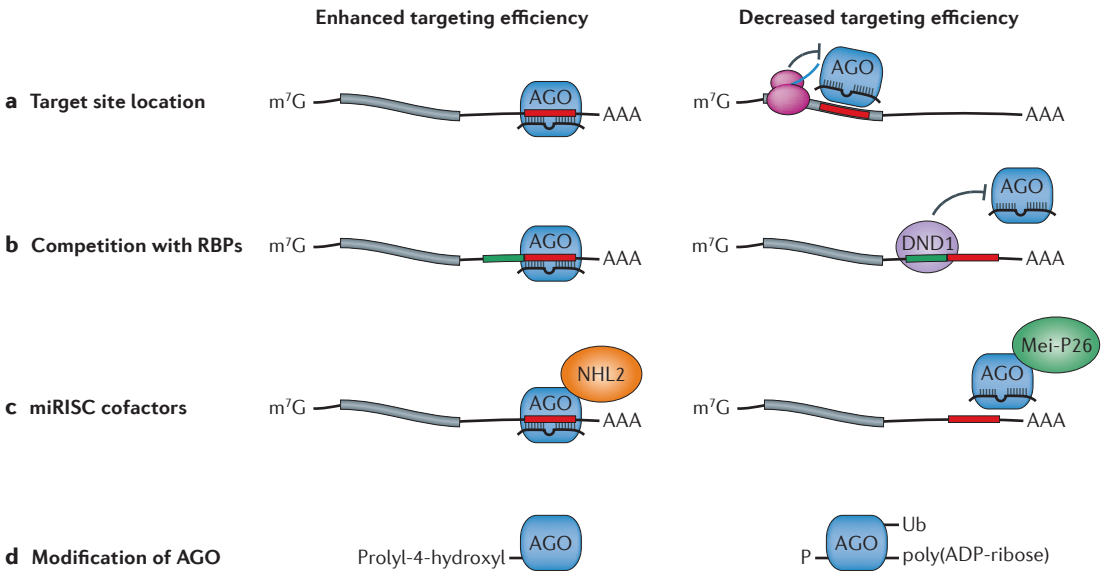


Figure 4 | **Regulation of miRNA-targeting efficiency.** The ability of the microRNA (miRNA) complex to recognize and regulate target mRNAs can be influenced by several factors. **a** | Target sites in 3′UTRs can be more efficiently bound and regulated by the miRNA-induced silencing complex (miRISC) than those in coding regions, where translating ribosomes may destabilize complexes containing partially base-paired miRNAs[52]. **b** | RNA-binding proteins (RBPs), such as deadend 1 (DND1), can shield miRNA target sites from miRISC binding[62]. **c** | Factors that associate with the miRISC can positively or negatively affect target binding and regulation (NHL2 or meiotic P26 (Mei-P26), respectively)[69,70]. **d** | Modification of Argonaute proteins can enhance targeting efficiency by stabilizing the proteins (prolyl-4-hydroxylation) or reduce Argonaute activity by decreasing the ability to bind small RNAs (P, phosphorylation), promoting destabilization (Ub, ubiquitylation) or interfering with target accessibility (poly(ADP-ribosylation))[112].

level and if HuR disrupts this mechanism. This possibility is supported by the finding that repression of *CAT1* by miR-122 is primarily through inhibition of translation initiation[58]. Hence, HuR might interfere with different levels of miRISC function, depending on the context of binding sites in target mRNAs.

HuR appears to have a dual role in regulating the ability of miRNAs to repress specific targets. In contrast to its antagonistic effect on miRNA-mediated inhibition of *CAT1* (REF. 58), binding of HuR to the *MYC* 3′UTR is necessary for repression of this target by let-7b and let-7c miRNA (referred to jointly as let-7b/c in this section) in HeLa cells[61]. HuR binds an AU-rich sequence that is adjacent to the let-7b/c target site, and this interaction seems to be necessary for the recruitment of let-7b/c and AGO2 to *MYC* mRNA. The implication is that HuR helps to unmask the let-7b/c target site or stabilizes miRISC association with the *MYC* 3′UTR. Given this cooperative example, the extensive list of genes with coexisting HuR- and miRNA-binding sites could include additional targets at which HuR positively affects regulation by the miRNA pathway[59,60].

Deadend 1 (DND1) is an RBP that provides preferential relief from miRNA-mediated repression of specific targets. DND1 recognizes sequences in endogenous target genes — such as *nanos* and *tdrd7* in zebrafish — in the vicinity of miR-430-binding sites and appears to reduce the accessibility of targets to the miRISC[62] (FIG. 4b). DND1 is naturally expressed in vertebrate primordial germ cells (PGCs), so it provided a possible explanation for how certain transcripts with miR-430 sites escaped repression by this miRNA in PGCs, but not in somatic cells, during early development in zebrafish[63].

miRISC regulation. Several members of the TRIM–NHL family of proteins directly associate with Argonaute and regulate the ability of miRISC to repress target gene expression. These proteins contain a tripartite motif (which consists of RING type, B box zinc fingers and a coiled-coil domain) coupled to an NHL domain (which is named for the founding protein members: NCL1 (also known as Calpain 3), HT2A (also known as TRIM32) and LIN41 (also known as TRIM71))[64], and many of them are known to contain E3 ubiquitin ligase activity[65]. The discovery that a mouse TRIM–NHL protein, LIN41, targets Argonaute for ubiquitylation and proteasome-mediated degradation shows that the miRNA complex itself is under the control of LIN41 (REF. 66). Reduced Argonaute levels result in compromised regulation of miRNA targets and decreased miRNA levels as association with RISC promotes miRNA stability[67,68]. Because LIN41 is itself regulated by the let-7 miRNAs, it is part of a complex regulatory circuit whereby LIN41 antagonizes the stability of Argonaute, and let-7 miRNAs directly repress the expression of LIN41.

Meiotic P26 (Mei-P26) is another TRIM–NHL protein that counteracts the miRNA pathway. Originally named for its role in meiosis, Mei-P26 is required for proper cellular proliferation and differentiation in the female germline of *Drosophila melanogaster*[69]. This

phenotype is at least partly due to hyperactivity of the miRNA pathway in *mei-p26* mutants. Additionally, Mei-P26 co-immunoprecipitates with AGO1, which is the Argonaute that is primarily responsible for miRNA function in *D. melanogaster* (FIG. 4c). It remains to be shown whether Mei-P26 acts as an E3 ubiquitin ligase for Argonaute or whether it works through another mechanism to reduce the levels and function of miRNAs generally.

In contrast to LIN41 and Mei-P26, other TRIM–NHL family proteins positively regulate miRISC function (FIG. 4c). Genetic and biochemical evidence points to a role for NHL-2 in stimulating the efficacy of miRNA target regulation in *C. elegans*[70]. Loss of *nhl-2* weakens the repression of certain miRNA targets without affecting miRNA expression levels. In addition to interacting with core miRISC proteins, including Argonaute (ALG-1 and ALG-2 in *C. elegans*), NHL-2 binds the helicase CGH-1 (which is known as RCK and p54 in humans, ME31 in *D. melanogaster* and Dhh1 in *Saccharomyces cerevisiae*). Previous work implicated homologues of CGH-1 in the miRNA pathway[71,72], raising the possibility that NHL-2 stimulates the function of this protein in miRNA target repression. The TRIM–NHL protein TRIM32 is another positive regulator of miRISC function. In mouse neural progenitor cells, TRIM32 promotes differentiation in part through activation of let-7 miRNA[73]. TRIM32 co-precipitates with AGO1 and an enriched set of miRNAs, including let-7. Through a mechanism that is yet to be defined, TRIM32 enhances the repression mediated by let-7. TRIM32 possesses E3 ligase activity and ubiquitylates MYC protein, promoting its degradation. Because MYC indirectly represses the expression of let-7 through LIN28 (REF. 74), TRIM32 enhances let-7 activity at multiple levels. Currently, it is unclear whether the effect of TRIM32 or NHL2 on the repressive function on miRISC requires E3 ligase activity.

In addition to ubiquitylation, Argonaute is subject to several other types of regulatory modifications (FIG. 4d). The stability of Argonaute can be regulated by prolyl-4-hydroxylation. In human cells, type I collagen prolyl-4-hydroxylase (C-P4H(I)) modifies proline 700 in AGO2 (REF. 75). Under hypoxia conditions, the expression of C-P4H(I) increases, resulting in modification of AGO2 and stabilization of the protein[76]. Other post-translational modifications of Argonaute regulate the function of the miRISC. Human AGO2 can be phosphorylated at multiple positions, although the factors that catalyse these modifications are yet to be determined[77]. One phosphorylation site maps to a conserved tyrosine in the region of AGO2 that serves as a binding pocket for the 5′ end of small RNAs. Phosphorylation is likely to result in a reduced ability to bind miRNAs[77], implying that the loading of Argonaute proteins with small RNAs can be regulated by phosphorylation. Another type of modification that can impede Argonaute function is poly(ADP-ribosylation)[78]. In mammalian cell culture, various stress conditions stimulate the modification of AGO1–4 by specific poly(ADP-ribose) polymerases (PARPs). How poly(ADP-ribosylation) interferes with the ability of AGO to repress target gene expression is, at present, a mystery[78].

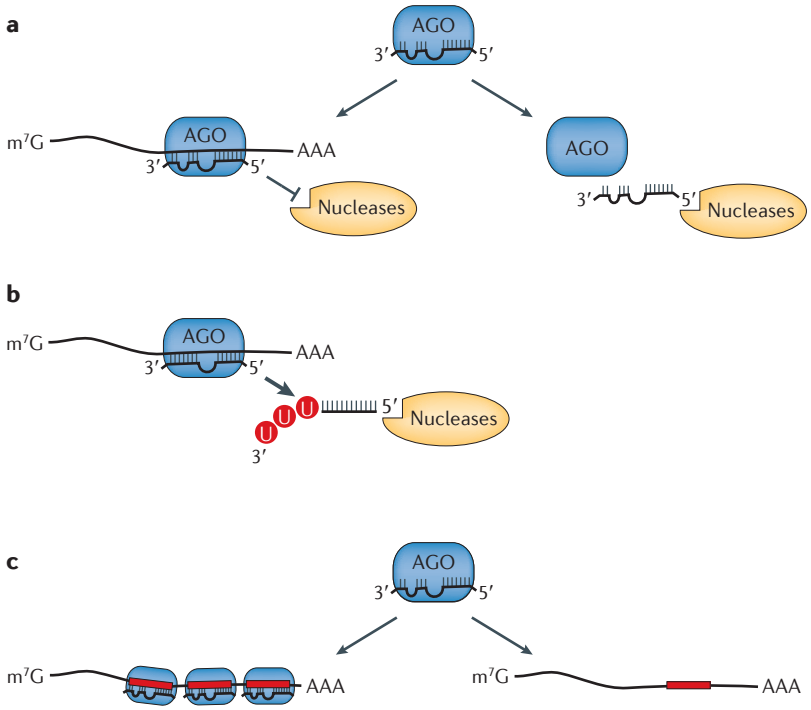


Figure 5 | **Regulation of miRNA stability and function by target interactions.** The presence and type of target site can affect the steady-state levels of specific microRNAs (miRNAs) and can create competition between targets for limiting miRNA complexes. **a** | In some cases, association of miRNAs with Argonaute proteins is stabilized through target interactions, preventing release and exonucleolytic degradation of miRNAs[67,80]. **b** | In other cases, substantial pairing of miRNAs with target sequences leads to 3′ end trimming and tailing of the miRNA, usually with uridines, marking it for degradation[81,84,113]. **c** | Transcripts with target sites for a common miRNA can compete for recognition. Titration of the miRNA complex by certain RNAs can release others from repression[106].

Regulation of miRNAs by target interactions

Recent work has revealed that regulation in the miRNA pathway is a two-way street. Not only can base pairing between an miRNA and its target result in repressed target expression, but these interactions can also have an impact on the levels of the miRNA.

Target effects on miRNA stability. Argonaute appears to be a limiting factor for miRNA stability. Increased expression of any of the four AGO proteins in human cells results in a general upregulation of mature miRNA levels[68]. Likewise, loss of the Argonaute that is dedicated to miRNA function in *C. elegans*, ALG-1, results in reduced miRNA abundance[79]. This effect is at least partly explained by the ability of ALG-1 to protect bound miRNAs from the 5′-to-3′ exonucleases XRN-1 and XRN-2 (REFS 67,80) (FIG. 5a). Moreover, this protective function depends on the availability of target sites for the miRNA. In *C. elegans*, the introduction of reporters with complementary sites to normally lowly expressed miRNAs boosted their accumulation[80]. This target-mediated miRNA protection (TMMP) implies that the level of some miRNAs may be positively correlated with the number of target sites that are available for base pairing[80].

Curiously, target–sequence interactions can also stimulate miRNA degradation. It has been shown in *D. melanogaster* and in human cells that extensive pairing between an miRNA and its target site can induce 3′ end trimming of the miRNA, which is often accompanied by the addition of non-templated uridines[81] (FIG. 5b). Multiple proteins have been implicated in the addition of uridines to the 3′ ends of miRNAs[82], but the specific enzyme that is responsible for modifying miRNAs that are base-paired to target sites is yet to be identified. Uridine addition to the 3′ end of miRNAs sometimes, but not always, leads to their destabilization[83]. More recent kinetic studies show that the introduction of a reporter with either perfectly paired or two-nucleotide-bulged target sites accelerated the decay of the cognate miRNA in human cells, and in both cases this was associated with the 3′ addition of a single uridine to the miRNA[84]. A faster decay rate was measured for the target that is capable of perfectly base pairing with the miRNA, supporting a correlation between the degree of complementarity and the effect of the target on miRNA stability.

The ability to regulate miRNA stability through base-pairing interactions has been usurped by viruses. Herpesvirus saimiri encodes seven U-rich ncRNAs — called HSURs — that contain regions of complementarity to three miRNAs in primate T cells[85]. All three miRNAs associate with the viral RNAs *in vivo*, but only miR-27a is destabilized through its interaction with HSUR1. Although downregulation of miR-27a in herpes simplex virus (HSV)-infected cells correlates with increased levels of some known miR-27a targets, how this benefits the virus is yet to be elucidated[85]. The specific degradation of miR-27a may be related to the higher degree of complementarity between miR-27a and the viral RNA, in comparison to the sites that are recognized by the other two miRNAs. Even so, pairing of miR-27a with HSUR1 is predicted to form only a partial duplex. Flexibility in the base-pairing requirements for target sites to induce miRNA degradation is also evident from *in vitro* experiments in *D. melanogaster* extracts, in which as many as seven mismatches could still induce trimming of the partially complementary miRNA[81]. How often miRNA destabilization results from pairing to endogenous target sites — which typically includes several mismatches and bulged nucleotides — is yet to be determined.

Target effects on miRNA function. Just as the expression of miRNA targets can be repressed through mechanisms other than mRNA destabilization, the activity of miRNAs can be repressed by target interactions that do not necessarily induce changes in miRNA levels (FIG. 5c). The first example of a natural target affecting the ability of an miRNA to regulate another target was discovered in the phosphate starvation response pathway in *Arabidopsis thaliana*[86]. When plants are deprived of inorganic phosphate, miR399 levels increase, resulting in cleavage of the putative ubiquitin-conjugating enzyme E2 24 (*PHO2*) target mRNA, which contains multiple near-perfect miR399 complementary sites. Another RNA that is induced in response to phosphate starvation is the ncRNA induced by phosphate starvation 1 (*IPS1*). As levels of *IPS1* rise, degradation of *PHO2* attenuates because miR399 activity is redirected to a target site in *IPS1*. The duplex between *IPS1* RNA and miR399 includes a mismatched region in the centre that prevents cleavage. This type of target site allows *IPS1* to persist and to sequester the miRNA from other targets. Since this discovery, 'target mimicry' has been exploited as a powerful tool for inhibiting the activity of specific miRNAs *in vivo*. In plants, RNAs that have been engineered to contain non-cleavable miRNA target sites can titrate the function of multiple miRNAs of similar sequence, effectively depleting families of miRNAs to test their biological roles[86–88]. For example, overexpression of an RNA with a non-cleavable target site for the *A. thaliana* miR156 family, which includes ten highly related miRNAs, resulted in plants with less leaves, a phenotype that was the opposite of plants overexpressing miR156 (REF. 86).

The idea that competition between targets for common miRNAs may widely influence the potency of miRNA regulation of individual targets has been substantiated by a flurry of recent studies in animal cells[89–95]. In mammalian cells, the tumour suppressor function of phosphatase and tensin (*PTEN*) is exquisitely sensitive to dosage. A computational search for RNAs that could act as miRNA decoys of *PTEN*, and thus could influence its expression levels, led to a candidate list of more than 100 protein-coding mRNAs that share multiple miRNA target sites with *PTEN*[95]. By presenting target sites for the same miRNAs, these competing endogenous RNAs (ceRNAs) mutually regulate the expression of each other. Because the *PTEN* ceRNAs titrate repressive miRNAs from this tumour suppressor gene, they represent new candidates in the cancer gene network. Zinc finger E-box-binding homeobox 2 (*ZEB2*) was validated as one such ceRNA for *PTEN*[90]. Previous studies indicated that *ZEB2* is a transcription factor that represses genes that are required for the differentiated state in epithelial cells and may have an oncogenic role in some cancers[96]. Thus, the mRNA and protein of *ZEB2* can have conflicting biological functions under certain circumstances.

Large-scale analysis of protein-coding and miRNA-expression profiles in human gliomas led to the conclusion that thousands of transcripts may be acting as target decoys[94]. Several cancer-relevant genes, including *PTEN*, are embedded in the network of mRNAs that can cross-regulate each other by titrating the activity of common miRNAs. Thus, the combinatorial effect of multiple ceRNAs can have a profound impact on gene expression and cellular phenotype.

An implication of the previously described studies is that any RNA with miRNA target sites can potentially function as a ceRNA. Thus, long ncRNAs (lncRNAs) are prime candidates for functioning as sinks of miRNA activity. lncRNAs are broadly defined as expressed RNAs of more than 200 nt in length that lack obvious protein-coding capacity[97]. Specific lncRNAs have been shown to regulate transcription through chromatin interactions and post-transcriptional events, such as mRNA processing and degradation by interacting with splicing factors

Competing endogenous RNAs
(ceRNAs). Bind miRNA complexes, titrating them from other target RNAs. Long non-coding RNAs, pseudogene RNAs and mRNAs can compete with other RNAs for access to specific miRNA complexes, effectively diluting their activity.

and 3′UTR elements, respectively. miRNA decoy can now be added to the growing list of lncRNA functions. The lncRNA linc-MD1 regulates muscle differentiation by sequestering miRNAs that target protein-coding genes needed to activate the differentiation programme[89]. Furthermore, in mammalian cells, the pseudogene *PTEN1* contains miRNA-binding sites that are common with those in *PTEN* in its 3′UTR[91]. These sites engender mutual regulation between *PTEN* and *PTEN1* in another case of target mimicry. A puzzling aspect of this cross-regulation is that the *PTEN1* transcript is expressed at much lower levels than *PTEN* in most of the studied cell types, raising the question of how the pseudogene RNA can effectively titrate miRNAs from *PTEN*. Overall, pseudogenes and lncRNAs that act as ceRNAs support the idea that regulation of miRNA activity is tuned by many types of target interactions.

Even before the vast potential of natural miRNA target decoys was realized, transcripts that could sequester specific miRNAs were developed as experimental tools in plant and animal cells[86,98]. Given their ability to soak up miRNAs, miRNA 'sponges' have been expressed in a variety of cell systems to study the functions of specific miRNAs or groups of related miRNAs[99]. Typically, miRNA sponges consist of multiple sites of partial complementarity to the miRNA of interest. Because seed pairing is sometimes sufficient for miRNA–target interactions, sponges can be designed to titrate an entire family of miRNAs that share the same 5′ end sequence. Although sponge RNAs are engineered to be highly expressed and to sequester specific miRNAs, the discovery of widespread regulation of miRNA targeting by natural ceRNAs raises concern over the potential effects of any ectopic RNA on the network of genes under miRNA regulation.

Conclusion

The recent discovery that miRNAs can both regulate and be regulated by target interactions has profound implications for understanding their roles in gene regulation. This intertwined relationship raises many questions about how miRNA targeting functions *in vivo*. An original and remaining challenge in the field is the ability to predict miRNA target sites with high confidence. New high-throughput methods have accelerated the discovery of sequences that are bound by the miRNA complex *in vivo*[23–26]. These data sets provide an experimental framework for training computational algorithms to predict the likelihood of a sequence being recognized by specific miRNAs in a biologically relevant context. The discovery of ceRNAs redefines what it means to be an miRNA target, and so it will be important for target-prediction programs to consider any expressed RNA as a possible partner for miRNA pairing.

Several proteins have been shown to regulate the ability of the miRISC to bind or to regulate specific targets. Conversely, most of these factors themselves are targets of miRNA regulation. For example, the let-7 miRNA and LIN-41 constitute an auto-regulatory feedback loop[100]. Core factors in the miRNA pathway are also subject to targeting by the miRNA complex. CLIP studies in *C. elegans* revealed that genes that are important for the expression and function of miRNAs are highly enriched for Argonaute-binding sites[26]. Auto-regulation of miRNA pathway genes could be an important mechanism for stabilizing miRNA function.

Although miRNAs typically repress target gene expression, the reciprocal effect of targets on miRNAs is not entirely clear. In some cases, target interactions offer a protective influence on miRNA stability, whereas in others the outcome is miRNA degradation[67,80,81,84,85]. So, what determines whether a target interaction increases or decreases the stability of the miRNA? Thus far, it seems that the degree of pairing between an miRNA and its target site determines the fate of the miRNA, and it seems that extensive complementarity favours miRNA destruction[81,84]. This is an important consideration for the design of small RNAs to regulate gene expression experimentally or therapeutically. RNAs that can pair perfectly with sites in their target genes will induce cleavage and may initially have a stronger repressive effect than small RNAs that form partial duplexes. However, long-term silencing may be better achieved by RNAs that are designed to pair with mRNA target sites partially, as they might be less prone to destabilization.

The recent appreciation that the activity of an miRNA is also at the mercy of the targets that it regulates in addition to other RNAs further complicates the network of genes that are involved in the miRNA pathway. In fact, what is the extent of target interactions that sponge miRNA function versus those that are important for specific gene regulation? It could be argued that any expressed RNA can function as a ceRNA by presenting sequences that can engage the miRNA complex, releasing it from other targets. Considering that 3′UTRs are often rich in miRNA target sites, the recent finding that these regions of mRNA can be independently transcribed suggests that this could be a prime source of RNAs acting as natural sponges[101]. Because target degradation is a common outcome of miRNA regulation, some RNAs may be better suited to function as decoys than others. There are likely to be *cis*-acting elements that enable some ceRNAs to be very effective sponges that sequester miRNA complexes without eliciting their own destabilization. The demonstration that miRNA-mediated repression of some cancer-related genes, such as *PTEN*, is exquisitely sensitive to the presence of ceRNAs suggests that genes with polymorphisms that alter miRNA recognition could have consequences that reach far beyond their own regulation. The discovery of ceRNAs also implies a dual function for many mRNAs as templates for protein expression as well as riboregulators of miRNA function. Thus, partially functional gene products are possible with genetic mutations that prevent protein but not mRNA expression. The concept of targets serving as miRNA decoys to control the stability and function of the very miRNAs that regulate them calls to mind the quotation by Sir Walter Scott, "Oh what a tangled web we weave, when first we practice to deceive!". Future studies will reveal the extent of this entanglement and the rules that guide the regulatory relationships between miRNAs and targets *in vivo*.

Pseudogene
Relatives of protein-coding genes that can no longer be translated into functional gene products. In some cases, pseudogene RNAs are still transcribed but are then improperly processed or translated.

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