POST-TRANSCRIPTIONAL CONTROL — OPINION

Revisiting the principles of microRNA target recognition and mode of action

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Abstract | MicroRNAs (miRNAs) are fundamental regulatory elements of animal and plant gene expression. Although rapid progress in our understanding of miRNA biogenesis has been achieved by experimentation, computational approaches have also been influential in determining the general principles that are thought to govern miRNA target recognition and mode of action. We discuss how these principles are being progressively challenged by genetic and biochemical studies. In addition, we discuss the role of target-site-specific endonucleolytic cleavage, which is the hallmark of experimental RNA interference and a mechanism that is used by plant miRNAs and a few animal miRNAs. Generally thought to be merely a degradation mechanism, we propose that this might also be a biogenesis mechanism for biologically functional, non-coding RNA fragments.

MicroRNAs (miRNAs) are 20–24 nucleotide (nt) RNAs that regulate eukaryotic gene expression post-transcriptionally. miRNAs use base-pairing to guide RNA-induced silencing complexes (RISCs) to specific messages with fully or partly complementary sequences. The repression of targeted messages is a common outcome of RISC recruitment and might occur through translational inhibition, accelerated exonucleolytic mRNA decay or site-specific endonucleolytic cleavage (slicing) in miRNA–mRNA pairs[1]. Examples of cell-cycle-dependent miRNA-mediated translational activation have also recently been described, although the underlying mechanism is unknown[2]. Animals and plants express hundreds of miRNAs that are thought, at least in animals, to regulate a large part of the protein-coding transcriptome. In both kingdoms, miRNAs have vital roles in development, stress adaptation and hormone signalling[3,4].

In the current molecular framework for miRNA biogenesis, primary (pri)-miRNA transcripts with stem-loop regions are usually produced by RNA polymerase II, but occasionally by RNA polymerase III[5,6]. The stem-loop precursor (pre)-miRNA is released by a cleavage event, which is catalysed by the nuclear Microprocessor complex that contains the RNase III Drosha. A distinct RNase III, Dicer, subsequently produces a ~22 base-pair duplex RNA that is composed of the eventual mature miRNA, base-paired to the so-called miRNA* strand[7]. Plants do not have Drosha, and both the pri- and pre-miRNA processing steps are apparently mediated by Dicer-like 1 (DCL1), one of several plant DCL paralogues[8]. Many animal miRNAs reside in introns, and, in some cases, splicing bypasses the Microprocessor requirement and directly produces pre-miRNAs[9].

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In miRNA duplexes, the strand with the weakest 5′-end base pairing is selected as the mature miRNA and loaded onto an Argonaute (Ago) protein, whereas the miRNA* is degraded[10]. Agos are ubiquitous RISC components and provide the endonucleolytic RNase H activity of slicer-competent RISCs[11]. Three fundamental questions then arise regarding the activity of Ago-loaded miRNAs. First, how do miRNAs select their targets? Second, following a specific miRNA–mRNA interaction, what mechanism of regulation will then be used? Third, what molecular mechanisms underlie miRNA-mediated translational inhibition, accelerated exonucleolysis and slicing?

The molecular mechanisms of miRNA action remain intensely debated, and will not be discussed here. This article focuses on attempts to answer the first two questions, which have led to the formulation of a now widely accepted but largely empirical framework that can be summarized as follows.

Animal miRNAs target transcripts through imperfect base-pairing to multiple sites in 3′ untranslated regions (UTRs). Watson–Crick base-pairing to the 5′ end of miRNAs, especially to the so-called ‘seed’ that comprises nucleotides 2–7, is crucial for targeting. Although less important, 3′-end pairing might contribute to target recognition, particularly when sites have weaker miRNA seed matches. Imperfect miRNA–mRNA hybrids with central bulges (nucleotides 9–12) enable translational inhibition or exonucleolytic mRNA decay, although the factors that govern the prevalence of one specific mechanism remain unknown. Highly complementary target sites with central pairing — which are rarely documented in animals — result in target regulation through slicing. Target site multiplicity is thought to enhance the degree of repression by animal miRNAs, each of which might have hundreds of target transcripts, owing to their relaxed base-pairing requirements.

Plant miRNAs regulate transcripts with single, highly complementary target sites primarily by slicing and rarely by translational inhibition. Target sites are predominantly found in coding regions, but can be located in UTRs. Seed and central matches are particularly important for target recognition, and each miRNA is thought to have only a limited number of mRNA targets. Slicing is thought to provide an efficient means of RNA degradation or ‘clearance’ in plants.

Below, we systematically re-evaluate these notions in the light of recent findings in plant and animal systems.

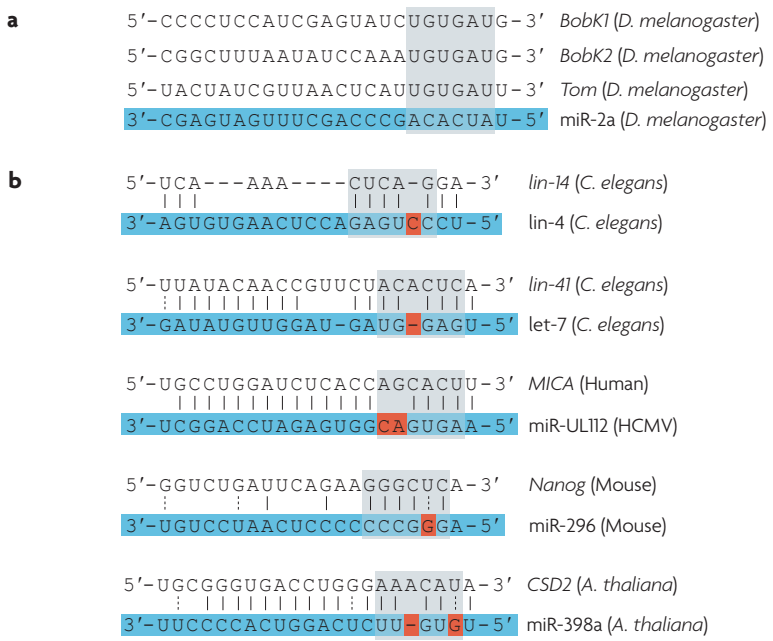


Figure 1 | **Seed and non-seed matches.** mRNA target sequences are shown in black and miRNAs are highlighted in blue. Connecting solid lines indicate a Watson–Crick base pair, and connecting interrupted lines indicate a GU wobble pair. The seed region is shaded in grey and positions that violate the seed rule are shown in red. **a** | Several Notch target genes in *Drosophila melanogaster* are regulated by microRNAs (miRNAs) through target sites that have perfect seed matches but minimal complementarity elsewhere. The seed complements shown (so-called K-boxes) had already been described as important *cis* elements for post-transcriptional control before the discovery of miRNAs[85]. **b** | Examples of non-seed matches. *Caenorhabditis elegans lin-14*–lin-4 and *lin-41*–let-7 contain non-seed target sites with single bulges in the seed match region (shown in red). The human cytomegalovirus (HCMV) miR-UL112 targets human *MICA* despite the occurrence of a double mismatch in the seed region, and several miR-296 sites in murine *Nanog* contain GU wobbles in the seed region. Similarly, the *Arabidopsis thaliana CSD2*–miR398a interaction contains both a bulge and GU wobble in the seed region. Figure part **a** is modified, with permission, from *Nature Genetics* REF. 27 © (2002) Macmillan Publishers Ltd. All rights reserved.

Animal miRNAs target 3′ UTRs

Two arguments underlie the notion that animal miRNAs target mRNAs in their 3′ UTRs. The first rests on experimental evidence: *Caenorhabditis elegans* lin-4 and let-7, the first two miRNAs to be discovered, have complementary sites in the 3′ UTRs of their genetically identified target mRNAs[12,13]. The second is best characterized as an *in silico* convenience. Many miRNA target site search algorithms rely on evolutionary conservation of putative target sites to filter 'signal' (functional miRNA target sites) from 'noise' (non-target sites that have miRNA sequence complementarity by chance). As conserved miRNA target sites might be masked by codon-imposed evolutionary conservation of open reading frames (ORFs) — particularly when a limited number of genomes is available — most miRNA target search algorithms were simply designed to focus on 3′ UTRs, supported by lin-4 and let-7 as precedents[14,15]. These algorithms were used successfully to identify a wealth of authentic miRNA target sites in 3′ UTRs[16], but they have left the possibility that miRNAs target other regions underinvestigated.

In microarray studies that involved miRNA transfection in human cells, transcripts with potential miRNA target sites in ORFs were noticed in repressed gene sets, although they were not pursued in depth because they were thought to be rare, weak or of uncertain importance *in vivo*[17,18]. In fact, experiments with artificial sensor constructs in human cells showed that miRNA-guided repression is equally efficient when sites are located in the 5′ or 3′ UTRs of reporter transcripts, thereby demonstrating that no mechanistic requirement confines miRNA action to 3′ UTRs[19]. Accordingly, two recent studies in human cells have identified functional miRNA target sites in the ORFs of DNA methyltransferase 3b (*DNMT3B*) and of the cell-cycle inhibitor *p16*^{INK4A} (also known as *CDKN2A*)[20,21]. Although bioinformatics search tools were used to identify precise sites in these messages[15,22,23], their in-depth characterization was pursued because experimental evidence suggested an important contribution of post-transcriptional regulation (miR-24–*p16*^{INK4A}) or because of an exceptionally high degree of target–miRNA complementarity (miR-148–*DNMT3B*).

Computational analysis of 12 *Drosophila melanogaster* genomes also revealed that the conservation of motifs with miRNA complementarity in ORFs is commonplace[24]. A similar search for conserved motifs in coding regions in 17 vertebrate genomes identified several high-scoring, potential miRNA sites and led to the interesting finding that let-7 directly represses human Dicer mRNA through three sites in the ORF[25]. Finally, using a miRNA target-prediction algorithm that does not impose a 3′ UTR bias[22], several experimentally validated miRNA target sites were recently found in the ORFs of mouse transcripts that encode the pluripotency factors nanog, OCT4 (also known as POU5F1) and SOX2 (REF. 26). Thus, the fact that validated 3′ UTR target sites currently outnumber those in coding regions reflects a potentially flawed bias of most bioinformatics tools towards 3′ UTRs. In fact, it is increasingly apparent that not only plant, but also animal, miRNAs frequently use coding region complementary sites to regulate biologically important targets. This indicates that no mechanistic differences in miRNA target recognition between the two kingdoms systematically dictate different target site positions in mRNAs.

The seed 'rule'

Early observations noted that ~6-nt *cis* elements that are required for post-transcriptional repression of *D. melanogaster* Notch targets are perfectly complementary to the 5′ ends of specific miRNAs[27] (FIG. 1a). Subsequently, systematic miRNA target site mutagenesis in human cells, *D. melanogaster* and *Arabidopsis thaliana* has shown that base-pairing at the 5′ end of miRNAs is important for target recognition[28–30], and the introduction of mismatches into the seed region of a presumptive miRNA–mRNA duplex has become standard practice in target site validation.

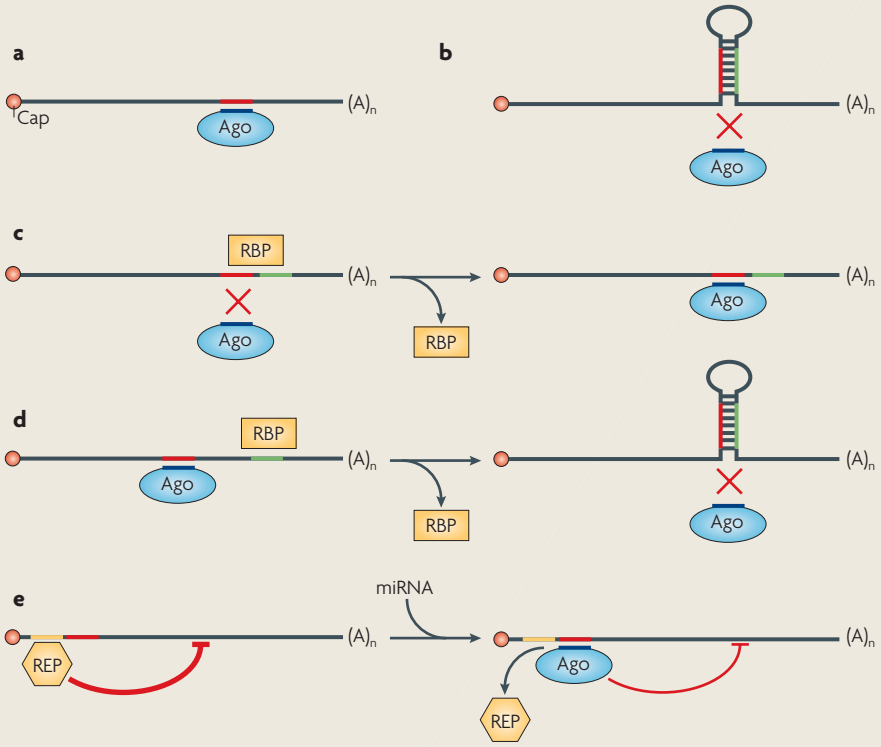
The seed 'rule' states that contiguous Watson–Crick base-pairing to the 5′ miRNA nucleotides 2–7 is required for activity in ways that are predictive of bona fide miRNA target sites[23] (FIG. 1). Initially, this idea was introduced only on the basis of evolutionary evidence, because the seed was the only contiguous region of miRNAs that retrieved more evolutionarily conserved target sites

than expected by chance in bioinformatics searches[14]. Nonetheless, there was and still is no evidence that any miRNA hexamer must be contiguously base-paired for a target site to be functional, nor that target sites must be evolutionarily conserved. Many seed-based target predictions have now been validated as functional sites *in vivo*, and large-scale transcriptomics and proteomics studies have recovered gene sets that are enriched in seed matches, following miRNA overexpression or inactivation in human cells[18,31,32]. Therefore, several miRNA–mRNA interactions obey the seed rule, and its introduction has helped to characterize biologically important miRNA functions.

Yet, can we be confident that seed pairing is mandatory for target recognition, or that most target recognition events follow the seed rule? It is noteworthy that the seed complement is the only over-represented 6-nt motif in downregulated genes following miRNA transfection, and could be identified without prior knowledge of its sequence[18]. If, however, seed-match regions that contain single mismatches, GU wobbles, insertions or deletions were also functional sites, none of those motifs would have been over-represented. Collectively, however, their number might rival that of perfect seed complements. There is now considerable evidence that many such 'non-seed' target sites of high biological relevance exist.

When miRNA target identification follows from tests of specific hypotheses assisted by biological context, or from completely unbiased genetic or biochemical approaches, miRNA–target site interactions often violate the seed rule. For example, well before the advent of the seed concept, it had been genetically shown that *C. elegans* lin-4 and let-7 use many functional non-seed sites in their *lin-41* and *lin-14* target mRNAs, in addition to seed-type target sites[13,33] (FIG. 1b). In human cells, cytomegalovirus produces a miRNA that uses a non-seed target site to repress a major histocompatibility complex-related gene, thereby avoiding the destruction of infected cells by the host immune system[34]. Also in human cells, crosslinking of biotinylated, photoactivatable miR-10a showed that it targets ribosomal protein transcripts via non-seed sites in the 5′ UTR[35]. In mice, computational predictions that do not rely on either the seed rule or the 3′ UTR bias[22] have identified several functional non-seed miRNA target sites in the coding regions of the key pluripotency factors that are discussed above[26]. Likewise, microarray hybridization of mRNAs associated with immunoprecipitated AGO1–miRNA complexes in *D. melanogaster* has uncovered many mRNAs that do not have seed-match enrichment, although it is unknown what fraction of these transcripts are bona fide miRNA targets[36]. Finally, although the *C. elegans* neuronal miRNA lsy-6 regulates its target *cog-1* through seed sites, an elegant use of *in vivo* lsy-6 sensors has shown that GU wobble pairs in the seed region are often tolerated, and that the occurrence of seed matches was in fact a poor predictor of lsy-6-mediated repression[37,38].

Box 1 | **Influence of secondary structure and RNA-binding proteins on target sites**



It is clear that the secondary structure of RNA and its association with RNA-binding proteins (RBPs) could influence target site accessibility, and thereby modulate regulation by small RNAs. For example, plant viroids — autonomously replicating, infectious, non-coding RNA molecules — produce small interfering RNAs in their plant host, but escape targeting by RNA-induced silencing complexes (RISCs) owing to their very stable secondary structure[76]. Similarly, a microRNA (miRNA) target site might be concealed by its secondary structure to avoid regulation. The interplay of miRNA target sites, RBP-binding sites and secondary structure can also generate more complex outcomes (see the figure). Black lines are mRNAs, red segments are miRNA target sites, green segments are mRNA regions with complementarity to the miRNA target site, yellow segments are other binding sites for RBPs and blue segments are miRNAs.

The accessible mRNA target site can be regulated by miRNA-loaded Argonaute (Ago) protein (collectively forming a RISC; see the figure, part **a**). Alternatively, regulation can be avoided, because the target site participates in a stem-loop structure (see the figure, part **b**). Several examples of engineered miRNA target sites embedded in secondary structures have been shown to behave in this way[48]. The target site can overlap with a binding site for an RBP, the association of which with mRNA inhibits miRNA-guided mRNA regulation (see the figure, part **c**). The inhibition of miRNA-mediated repression of zebrafish *nanos* and human *CAT1* repression by the RBPs Dnd1 and HUR, respectively, might provide examples of this type of interaction[77,78]. A segment that is complementary to a miRNA target site can overlap with a binding site for an RBP (see the figure, part **d**). If RBP associates with the mRNA, secondary-structure formation that involves the target site is precluded, and RISC can regulate the mRNA through the accessible miRNA target site. Apparent translational activation by miRNAs results from RISC competition with a strong translational repressor (REP) (see the figure, part **e**). If the miRNA target site is close to the REP-binding site, RISC might compete with REP for access to the mRNA and lead to an apparent translational activation if REP is a stronger repressor than RISC. Such a model might explain the miR-10a-dependent translational activation of ribosomal protein transcripts that contain a repressive 5′ terminal oligopyrimidine tract in the vicinity of the miR-10a site[35]. $(A)_n$, polyadenine.

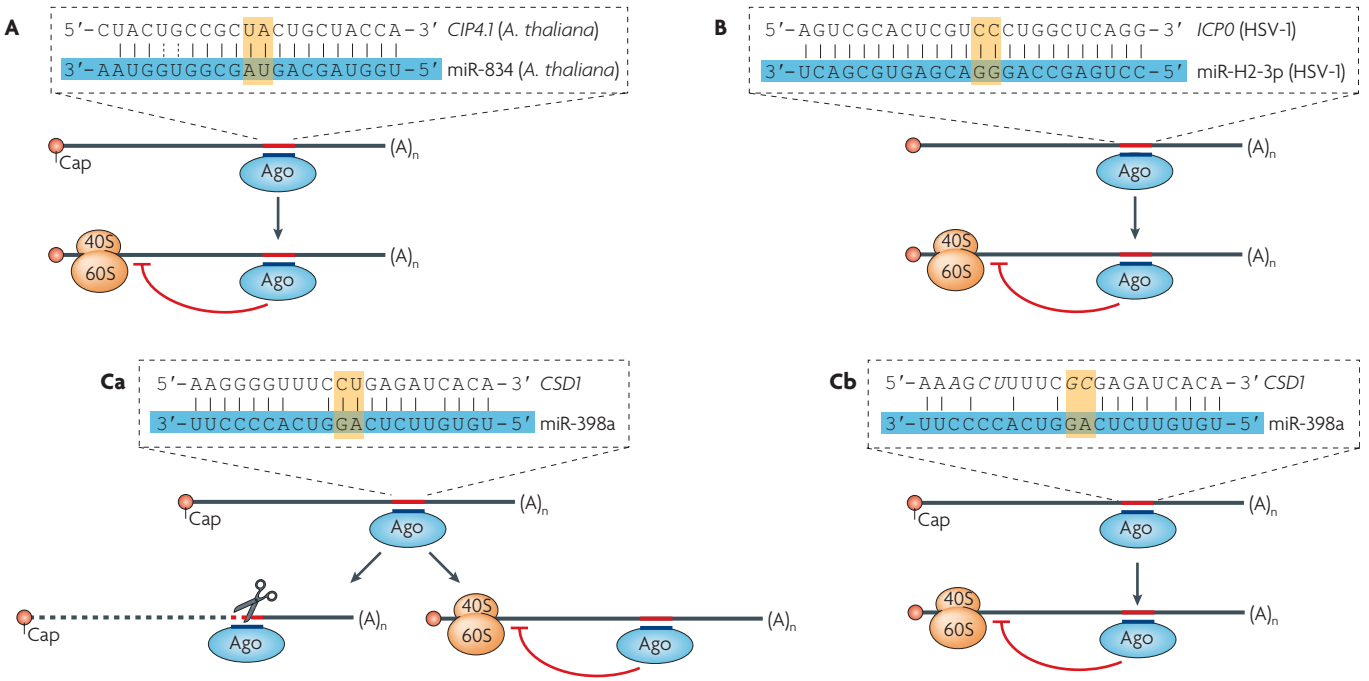


Figure 2 | **microRNA–target complementarity does not necessarily predict the regulatory output of interactions. A**,**B** | Examples of perfectly or near-perfectly matched target sites that nonetheless lead to target regulation predominantly, if not exclusively, by translational inhibition. The tenth and eleventh nucleotides in microRNA (miRNA)–target duplexes, in which slicing normally occurs, are highlighted in yellow. **A** | *Arabidopsis thaliana CIP4.1* targeted by miR-834. **B** | Herpes simplex virus 1 (HSV-1) *ICP0* targeted by the viral miR-H2-3p. **C** | An example of a functional, but highly mismatched, miRNA–target interaction in *A. thaliana*. Wild-type *CSD1*, which is highly matched to miR-398a despite containing a seed mismatch, is regulated both through slicing and translational inhibition (**Ca**). Regulation still occurs following the introduction of five mismatches (shown in italics), including the central nucleotides 10–11, but only through translational inhibition (**Cb**). $(A)_n$, polyadenine; Ago, Argonaute. Image is adapted from REF. 64.

Target site and miRNA mutagenesis studies have also highlighted the seed region as important for miRNA targeting in plants, as are the central nucleotides, the pairing of which is necessary for efficient slicing (see below)[28,39,40]. As well as confirming these findings, forward-genetic screens have revealed that single-nucleotide changes at positions that are normally paired to miRNA nucleotides 16 and 19 can release targets from miRNA control, thereby demonstrating that factors beyond seed and central pairing can be important *in planta*[41–43]. Moreover, the *A. thaliana* miR-398–*CSD2* seed match contains both a bulge and a GU wobble (FIG. 1b), and several other examples of plant miRNA–mRNA interactions with poor seed matches exist[44].

Somewhat disappointingly, therefore, we do not have a sufficiently refined understanding of mRNA targeting by miRNAs *in vivo* to explain why some experimentally discovered sites are recognized by miRNAs, whereas other regions that show similar or higher complementarity are not. This is exemplified by the surprising finding that a target site that is engineered to perfectly complement lsy-6 in its *cog-1* target is completely non-functional[45]. Clearly, focusing on the seed, even with additional helper parameters, such as compensatory 3′ pairing and AU-rich sequence occurrence around target sites[17], is not sufficient, and a better appreciation of the physical chemistry that underlies target selection is needed. The seed itself is likely to function as a nucleation site for miRNA–mRNA hybridization, as was suggested early on[14]. For example, purified small interfering RNA (siRNA)-loaded human RISC was inhibited by complementary oligonucleotides at greater than tenfold lower concentration when the oligonucleotide inhibitor was complementary to the seed compared with when it was complementary to the 3′ end[46]. This argues that the seed is particularly accessible to base-pairing, and the structure of an archaeal Ago protein bound to a 21-nt DNA guide strand indeed shows the Watson–Crick edges of seed nucleotides to be solvent exposed[47]. Nucleotides 12–17 were not visible in the structure, however, precluding direct comparisons of accessibility between the seed and the 3′ end of the guide strand. Another important factor is target site accessibility, which can be influenced by stable secondary structures and by association with RNA-binding proteins in, or near, target sites[46,48] (BOX 1). A third key parameter is the relative *in vivo* concentrations of miRNA and target mRNA[30] — a serious concern for those target site tests that rely exclusively on transfected miRNAs and reporter constructs. Last, the requirement for sequence 'context features', observed for at least *C. elegans* let-7 and lsy-6 targets[45,49], needs to be understood. These context features are elements of ill-defined function located outside of the target sites themselves.

Complementarity and multiplicity

The degree of complementarity of target sites is often presented as a decisive feature that determines whether slicing or translational repression and mRNA decay follows from RISC recruitment. Site multiplicity is frequently viewed as necessary for efficient translational inhibition. Both features have also been repeatedly used to contrast the mode of action of plant and animal miRNAs. However, several recent studies suggest that these notions require amendment.

Centrally matched pairs result in slicing. The idea that perfectly complementary miRNA–target pairs promote slicing, whereas imperfectly matched pairs with central bulges lead to translational

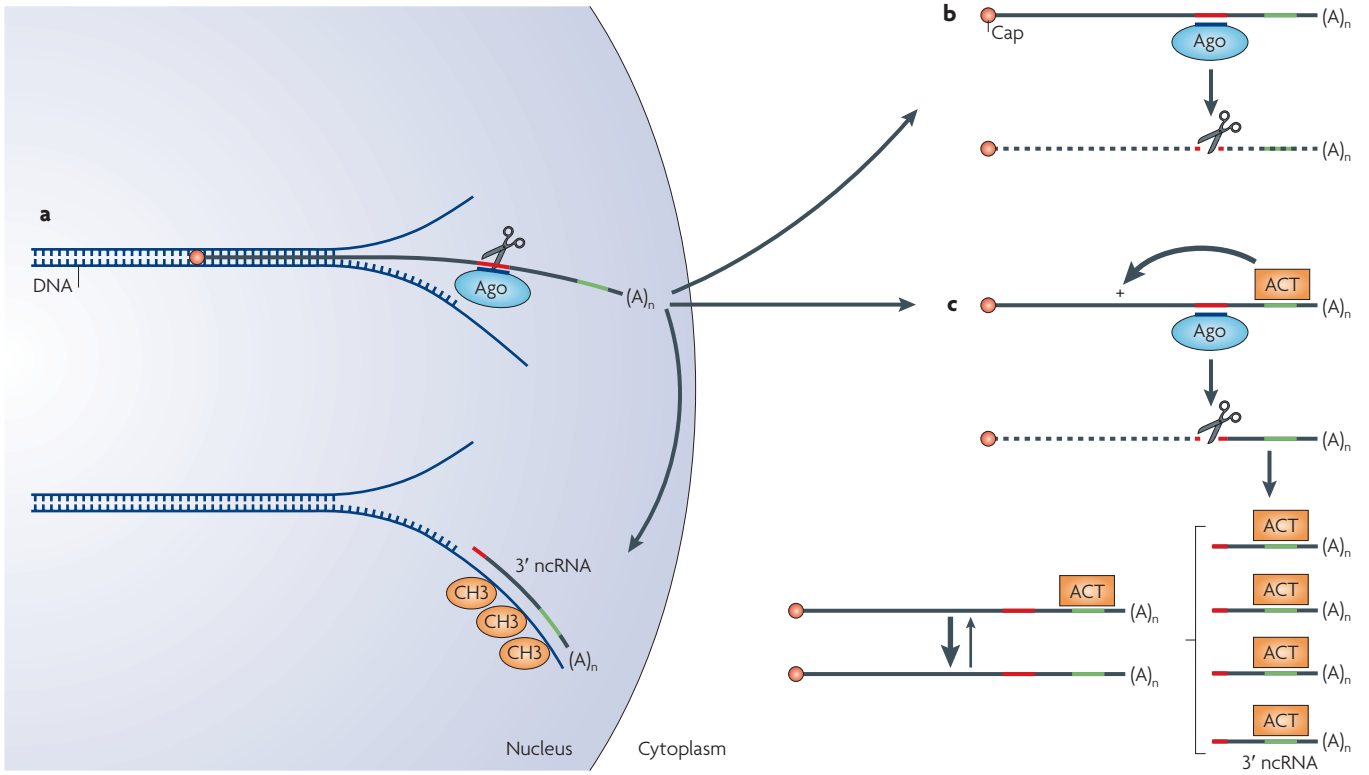


Figure 3 | **Possible functions of slicing and the resulting mRNA cleavage fragments. a** | In the nucleus, 3′ cleavage fragments might regulate chromatin, as do some other long non-coding RNAs (ncRNAs; BOX 2). The depicted example is inspired by the miR-165-dependent methylation of *PHABULOSA* (*PHB*) and *PHV* loci (see the main text). Co-transcriptional slicing could give rise to a 3′-end ncRNA that guides methylation (CH3) at template DNA strands. **b** | Slicing might function as a mere degradation mechanism, in which the two cleavage fragments are rapidly degraded by exonucleolysis. Although possible, this scenario does not seem to be common, because levels of 3′ cleavage fragments in *Arabidopsis thaliana* are often substantial. **c** | The stable 3′ cleavage fragment might act as a decoy for messenger ribonucleoprotein particle (mRNP) components, thereby depriving the full-length mRNA of association with, for instance, a 3′ untranslated region-binding activator protein (ACT). This scenario requires the 3′ fragment to accumulate to high levels — a frequently observed situation that might arise when the full-length transcript has rapid synthesis and slicing rates, and when the cleavage fragment undergoes slow degradation. $(A)_n$, polyadenine.

inhibition or mRNA decay, is based on several observations. Cleavage activity from siRNA-loaded human RISCs is strongly reduced by single central mismatches[50], and let-7 is made cleavage-competent simply by engineering its target to be centrally matched[51]. In addition, many highly matched plant targets undergo slicing[52], and only the very few highly matched animal targets (in contrast to all of the centrally mismatched ones) are sliced[53,54]. It seems safe, therefore, to conclude that central mismatches prevent slicing, which is consistent with structural models that suggest that the mRNA faces the Ago RNase H active site ~10 nt from the beginning of the miRNA–mRNA duplex[11]. It is incorrect, however, to infer that near-perfect target interactions lead to target regulation exclusively, or even predominantly, by slicing.

Early studies in *A. thaliana* showed that the transcription factor AP2 is regulated primarily at the translational level, despite slicing of *AP2* mRNA by the highly complementary miR-172 (REFS 55,56). Moreover, a genetic study in *A. thaliana* identified mutants in which silencing by several distinct miRNAs with highly matched targets is defective at the protein level, despite normal repression of their endogenous mRNA targets[57]. The same was also found with a hairpin-derived population of siRNAs that target an endogenous transcript[57]. With highly matched target sites, therefore, translational inhibition is commonly superimposed on slicing and may, under some circumstances, even dominate the outcome of miRNA–target interactions. For instance, evolutionarily young *A. thaliana*-specific miRNAs were initially suspected to be inactive because they tend not to affect the abundance of highly complementary mRNAs[58]. Investigation of such a miRNA, miR-834, and its target protein levels instead revealed that it exerts its effect primarily at the translation level[57] (FIG. 2A). Similarly, miR-H2-3p, which is produced by latent herpes simplex virus 1 in human cells, strongly represses the viral transcriptional activator ICP0 exclusively at the protein level, despite the existence of a perfectly complementary miR-H2-3p target site in the *ICP0* mRNA[59] (FIG. 2B).

If sequence parameters alone are insufficient to determine the regulatory output of miRNA–mRNA interactions, what other factors might then influence this process? One obvious factor might be the type of RISC or Ago protein onto which small RNAs are loaded. For example, some human Ago proteins that lack endonucleolytic activity contribute to target repression through translational inhibition during RNAi[60]. Another key aspect probably lies in the protein composition of the target mRNA–protein particle (or messenger ribonucleoprotein particle (mRNP)). mRNP biogenesis involves several nuclear events, including transcription, splicing and 3′-end formation[61], which suggests that such processes could ultimately influence the ways in which mRNAs are regulated by

Box 2 | **Non-coding RNAs in chromatin regulation**

In higher eukaryotes, most of the genome is transcribed despite the fact that protein-coding regions account for only ~30% of many plant genomes and as little as ~2% in humans[79]. It is now becoming increasingly clear that these many non-coding transcripts have important regulatory functions that are, in several cases, coupled to chromatin regulation[80]. A prominent example occurs in early mammalian embryogenesis, during which one of the two female X chromosomes is transcriptionally silenced (inactivated) to compensate for the double dose of X-linked genes compared with males. This inactivation is governed by several long non-coding RNAs (ncRNAs), including X-inactive-specific transcript (*Xist*)[81], the transgenic expression of which on an autosome is sufficient to inactivate the autosome[82]. X inactivation is initiated when *Xist* levels exceed a threshold, and *Xist* starts to coat the X chromosome from which it was transcribed, which eventually leads to the establishment of silent chromatin, characterized by repressive histone modifications and, ultimately, DNA methylation[80]. The precise mechanism of *Xist* action is unclear, although it is thought to recruit silencing factors to the X chromosome.

Transcription of long ncRNA is also generally associated with parental imprinting, in which one of the two parental alleles is silent (imprinted). In several cases, the ncRNAs themselves, not just their transcription, has been shown to be necessary for imprinting. For example, the paternal allele of mouse insulin-like growth factor 2 receptor (*Igf2r*) is silent because of transcription of the ~100 kb *Air* ncRNA from the same locus[83]. *Air* surrounds the paternal locus, and the mechanism of transcriptional silencing of genes in the vicinity of *Igf2r* includes recruitment of the histone H3 Lys9 methyltransferase G9a to their promoters[84].

miRNAs. Indeed, a recent study in human cells showed that the promoter that drives a miRNA target transcript determines the mechanism to be used for its translational inhibition in the cytoplasm[62]. Similar factors could influence whether mRNA decay by exonucleolysis or translational inhibition results from RISC recruitment.

Plant miRNAs use single, highly matched target sites. The idea that plant miRNAs only use highly complementary sites came from the computational demonstration that such sites exist for most, if not all, cloned *A. thaliana* miRNAs[63]. These sites might only represent an elite of sliceable targets, however, because the possibility that larger numbers of mRNAs are targeted for translational inhibition through imperfect sites has not been tested. Such a possibility is not unlikely: small RNA-mediated translational inhibition appears to be widespread in plants[57], even in perfectly paired small RNA–target interactions, and a mutational study of the *A. thaliana* miR-398–*CSD1* or *CSD2* interactions suggested that highly mismatched target sites have the potential to confer translational inhibition[64] (FIG. 2C).

Target site multiplicity favours efficient translational inhibition. Imperfectly paired target sites in animal transcripts often occur multiple times in the same mRNA. Cooperative action of multiple sites has been observed, and in some cases, individual inactivation of single sites disrupts miRNA-mediated regulation, thereby demonstrating that site multiplicity can indeed be an important factor for translational inhibition[26,65]. Site multiplicity is not a general requirement, however, as shown by the translational repression of *ICP0* by miR-H2-3p (REF. 59) (FIG. 2B), and by the numerous cases of translational inhibition of miRNA targets with single highly complementary sites in *A. thaliana*[57] (FIG. 2C).

The role of slicing

The observations of target repression through perfectly complementary sites in the absence of slicing raises the question of whether slicing has a special biological function. A standard explanation is that it is an efficient RNA degradation mechanism that could promote rapid clearance of unwanted transcripts. Nonetheless, without slicing, zebrafish miR-430 fulfils the clearance of maternal transcripts in the zygote by accelerating target mRNA deadenylation and decay[66]. Likewise, *A. thaliana* miR-164 accumulation overlaps with that of its transcription factor targets, and miR-164 seems to modulate their levels, rather than to clear them[67]. The fraction of target degradation that is mediated by slicing has not been addressed in any system, and it remains formally possible that the bulk of miRNA-mediated mRNA degradation occurs through accelerated exonucleolysis, even in plants. Could slicing of miRNA targets have another function beyond RNA degradation?

Work in plants and animals has shown that slicing can be used as a step in the biogenesis of small RNAs. For example, a few plant miRNAs use slicing of non-coding (nc) transcripts to generate cleavage fragments that are sources of biologically active *trans*-acting siRNAs (tasiRNAs) following their conversion to dsRNA by cellular RNA-dependent RNA polymerases[68]. The 5′ ends of a separate class of small RNAs in animals, the Piwi-interacting RNAs (piRNAs), are also formed by slicing[69,70]. The 3′ fragments that are generated by miRNA-guided slicing of canonical mRNA targets in *A. thaliana* and in human cells are stable[52–54], raising the possibility that these ncRNAs are also functional. If so, slicing could be generally seen as a biogenesis mechanism for ncRNAs in addition to being an mRNA degradation mechanism (FIG. 3). It is unlikely, however, that such ncRNAs would function as siRNA sources as in the tasiRNA example, because such siRNAs are extremely rare[71]. Intriguingly, long ncRNA fragments of protein-coding transcripts can have biologically important functions. For example, *D. melanogaster* oogenesis defects that are caused by a lack of *oskar* mRNA could be complemented not only by nonsense *oskar* alleles, but also by transgenic expression of the *oskar* 3′ UTR, although the molecular mechanisms that underlie the function of *oskar* ncRNA are elusive[72].

Regulation of chromatin in *cis* is a well-established function of long ncRNAs[73] (BOX 2). In this regard, it is interesting that *A. thaliana PHABULOSA* (*PHB*) undergoes miR-165-dependent DNA methylation. The methylation occurs in *cis*, because *PHB* heterozygotes with only one allele carrying an intact miR-165 target site are methylated exclusively on that allele[74]. Moreover, the DNA methylation is unlikely to be guided directly by miR-165, as its target site spans an intron, and is situated far upstream of the methylated DNA region. This region is, however, within the interval that is complementary to the *PHB* 3′-cleavage ncRNA fragment[74], which raises the possibility that this ncRNA is implicated in guiding DNA methylation (FIG. 3a). Two human transcripts, *HOXB8* and *RTL1*, known to be sliced and to generate detectable 3′-cleavage fragments might fit into this picture[53,54]: the *HOX* loci use several ncRNAs for their complex epigenetic regulation[75], whereas *RTL1* is an imprinted locus, and such loci are almost invariably associated with ncRNAs[73] (BOX 2). Sliced fragments might also modulate the degree of silencing of their precursor mRNAs, particularly if the mRNA is stabilized or translationally activated through 3′ UTR binding of activator proteins. The sliced ncRNA could, in this case, act as a decoy molecule to titrate such activators, resulting in exacerbated mRNA silencing (FIG. 3c).

Concluding remarks

Numerous studies have addressed the basic questions of how miRNAs select their targets, and what mechanism of regulation is used by RISC following mRNA binding. Although much progress has been made, the answers that have emerged can only explain a fraction of the available observations. Bioinformatic target search algorithms have helped to identify targets of miRNAs, but most of these are based on often unwarranted assumptions regarding the structure and evolutionary conservation of miRNA target sites. On the one hand, therefore, they might have retrieved only certain types of miRNA targets; on the other hand, these algorithms might overestimate the number of *in vivo* targets, because not all of the requirements for targeting *in vivo* might have been taken into account. Indeed, a key shortcoming of these studies is the recurrent failure to address the requirements for miRNA targeting under true *in vivo* conditions; that is, in cells in which the authentic miRNA–mRNA interaction takes place, and at physiological levels of these molecules. Such analyses will be a prerequisite to refining our understanding of target selection and regulation by miRNAs.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
AP2 | *cog-1* | *CSD1* | *CSD2* | *DNMT3B* | *p16*^{INK4A} | *PHB*
The miRNA Registry: http://microrna.sanger.ac.uk/sequences
let-7 | lin-4 | lsy-6 | miR-10a | miR-24 | miR-148 | miR-164 | miR-165 | miR-172 | miR-398 | miR-430 | miR-834 | miR-H2-3p
UniProtKB: http://www.uniprot.org
Dicer | Drosha | ICP0 | nanog | OCT4 | SOX2

FURTHER INFORMATION
Olivier Voinnet's homepage: http://ibmp.u-strasbg.fr/index.php?id=33&L=1&tx_labstructdef_pi1[showUid]=9&tx_labstructdef_pi1[viewType]=member&cHash=67efd20d58

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OPINION

Mechanisms of regulated unconventional protein secretion

Walter Nickel and Catherine Rabouille

Abstract | Most eukaryotic proteins are secreted through the conventional endoplasmic reticulum (ER)–Golgi secretory pathway. However, cytoplasmic, nuclear and signal-peptide-containing proteins have been shown to reach the cell surface by non-conventional transport pathways. The mechanisms and molecular components of unconventional protein secretion are beginning to emerge, including a role for caspase 1 and for the peripheral Golgi protein GRASP, which could function as a plasma membrane tether for membrane compartments during specific stages of development.

Most secretory proteins contain amino-terminal or internal signal peptides that direct their sorting to the endoplasmic reticulum (ER). From the ER, proteins are transported to the extracellular space or the plasma membrane through the ER–Golgi secretory pathway[1,2] (BOX 1).

Although the ER–Golgi system is an extremely efficient and accurate molecular machine of protein export[3], two types of non-conventional protein transport to the cell surface of eukaryotic cells have been discovered: these processes are known as unconventional protein secretion[4]. On the one hand, signal-peptide-containing proteins, such as yeast heat-shock protein 150 (Hsp150)[5], the cystic fibrosis transmembrane conductance regulator (CFTR)[6], CD45 (REF. 7), the yeast protein Ist2 (REF. 8) and the *Drosophila melanogaster* α integrin subunit[9], are inserted into the ER but reach the cell surface in a coat protein complex II (COPII) machinery- and/or Golgi-independent manner. On the other hand, cytoplasmic and nuclear proteins that lack an ER-signal peptide have been shown to exit cells through ER- and Golgi-independent pathways. Such proteins include fibroblast growth factor 2 (FGF2)[10–13], β-galactoside-specific lectins, galectin 1, galectin 3 (REFS 12,14–17), certain members of the interleukin family[12,18,19], the nuclear proteins HMGB1 (REFS 20–22) and engrailed homeoprotein[23–26], as well as the recently discovered *Dictyostelium discoideum* acyl-coenzyme A-binding protein (AcbA)[27]. We refer to these proteins as cytoplasmic/nuclear secretory proteins. In the extracellular environment, these macromolecules are crucial regulators of the immune response, cell growth, differentiation and angiogenesis.

Here, we begin by discussing the possible mechanisms that underlie these two types of unconventional protein transport. We then address the emerging role of caspase 1 in the unconventional secretion of cytoplasmic cytokines. Finally, we highlight possible roles for GRASP, a Golgi-associated peripheral membrane protein[28,29] that is involved in both types of unconventional secretion.

Signal-peptide-containing proteins

Some signal-peptide-containing proteins have been shown to traffic unconventionally; either their exit from the ER does not seem to involve COPII vesicles or their transport from the ER to the plasma membrane bypasses the Golgi apparatus.

Bypassing COPII vesicles. Although most of the signal-peptide-containing proteins use COPII-coated vesicles to exit the ER (BOX 1), a number of proteins have been shown to behave differently. For example, the yeast protein Hsp150 does not seem to depend on the COPII coat proteins Sec24 and Sec13 (REFS 5,30,31) to exit the ER *en route* to the plasma membrane. Overexpression of dominant-negative Sar1 mutants (Sar1 is one of the core yeast COPII proteins) does not block ER exit of the voltage-sensitive potassium channel (Kv4 K^+) when associated with its interacting proteins (KChIPs). Therefore, ER exit of this protein does not seem to be mediated by COPII-coated vesicles[32]. Lastly, ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1), which is a crucial regulator of ER-associated degradation (ERAD), does not accumulate in the ER under normal conditions because it is removed from the ER lumen by specific sequestration into ER-derived LC3-I-coated