RNA PROCESSING AND MODIFICATIONS

Regulation of microRNA function in animals

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Abstract | Since their serendipitous discovery in nematodes, microRNAs (miRNAs) have emerged as key regulators of biological processes in animals. These small RNAs form complex networks that regulate cell differentiation, development and homeostasis. Deregulation of miRNA function is associated with an increasing number of human diseases, particularly cancer. Recent discoveries have expanded our understanding of the control of miRNA function. Here, we review the mechanisms that modulate miRNA activity, stability and cellular localization through alternative processing and maturation, sequence editing, post-translational modifications of Argonaute proteins, viral factors, transport from the cytoplasm and regulation of miRNA–target interactions. We conclude by discussing intriguing, unresolved research questions.

Isomirs
Variant forms of a canonical miRNA, generated by alternative cleavage during biogenesis, RNA editing or non-templated nucleotide addition.

MicroRNAs (miRNAs) are short non-coding RNAs (ncRNAs) of ~22 nucleotides that mediate gene silencing by guiding Argonaute (AGO) proteins to target sites in the 3′ untranslated region (UTR) of mRNAs. AGOs constitute a large family of proteins that use single-stranded small nucleic acids as guides to complementary sequences in RNA or DNA targeted for silencing[1]. The miRNA-loaded AGO forms the targeting module of the miRNA-induced silencing complex (miRISC), which promotes translation repression and degradation of targeted mRNAs[2]. The first miRNA was discovered in *Caenorhabditis elegans* as a short RNA produced by the *lin-4* gene, which post-transcriptionally represses the *lin-14* mRNA[3,4]. Such small RNAs were widely thought to be unique to nematodes, until they were shown to be abundant in diverse animal phyla[5]. This new class of regulators was subsequently named microRNAs[6–8].

miRNAs are involved in virtually every cellular process and are essential for animal development, cell differentiation and homeostasis; deletions of the fundamental miRNA biogenesis factors Dicer[9] and Drosha[10] are lethal in mouse embryos. Although the importance of miRNAs for embryonic development is well established[11], Drosha and Dicer are involved in other nuclear processes, such as pre-mRNA splicing, which may also contribute to their deletion phenotypes[12]. The miRNA repository miRBase currently lists 1,917 precursor miRNAs (pre-miRNAs) and 2,654 mature miRNAs in *Homo sapiens*[13], and more than 60% of human protein-coding genes harbour predicted miRNA target sites[14]. Deregulation of miRNA function is associated with numerous diseases[15], particularly cancer[16,17]: miRNAs can be both oncogenes (called oncomirs)[18] and tumour suppressors[19], although overall downregulation of miRNA expression is a hallmark of cancer[20]. Some miRNAs are prognostic markers[21,22] or potential targets for novel cancer therapies[23]. Plant miRNAs[24,25], which are not discussed here, differ considerably from animal miRNAs in their evolution[26], biogenesis[27] and function[28] (reviewed in REFS[29,30]).

In this Review, we first provide an overview of miRNA function and regulation. We then discuss in detail the regulation of miRNA function through the formation of isomirs, the addition of non-templated nucleotides, post-translational modification (PTM) of miRNA-binding proteins, miRNA sequestration, modulation by viral factors, transport from the cytoplasm and the regulation of interactions between miRNAs and their target mRNAs (miRNA–target interactions).

Overview of miRNA function

miRNA genes are transcribed into primary miRNA (pri-miRNA) transcripts and undergo multistep biogenesis, in which they are processed first into pre-miRNAs and finally into mature miRNAs (BOX 1). miRNAs exhibit tissue-specific expression patterns[31], which are primarily regulated transcriptionally[32]. They are transcribed mainly by RNA polymerase II[33–35] and can be derived from introns or from long non-coding RNAs (lncRNAs). Pri-miRNAs can consist of a single mature miRNA or of clusters of often related miRNAs[6,36]. miRNAs are grouped into families[37] on the basis of the similarity of their seed sequences; the seed comprises nucleotides 2–8 (counting from the 5′ end) and is primarily responsible for miRNA targeting of mRNAs[38].

The mature miRNA functions within an AGO protein (FIG. 1a), which comprises a single polypeptide chain composed of four characteristic domains: the amino (N)-terminal domain, the Piwi–Argonaute–Zwille (PAZ) domain, the middle (MID) domain and

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Box 1 | **miRNA biogenesis**

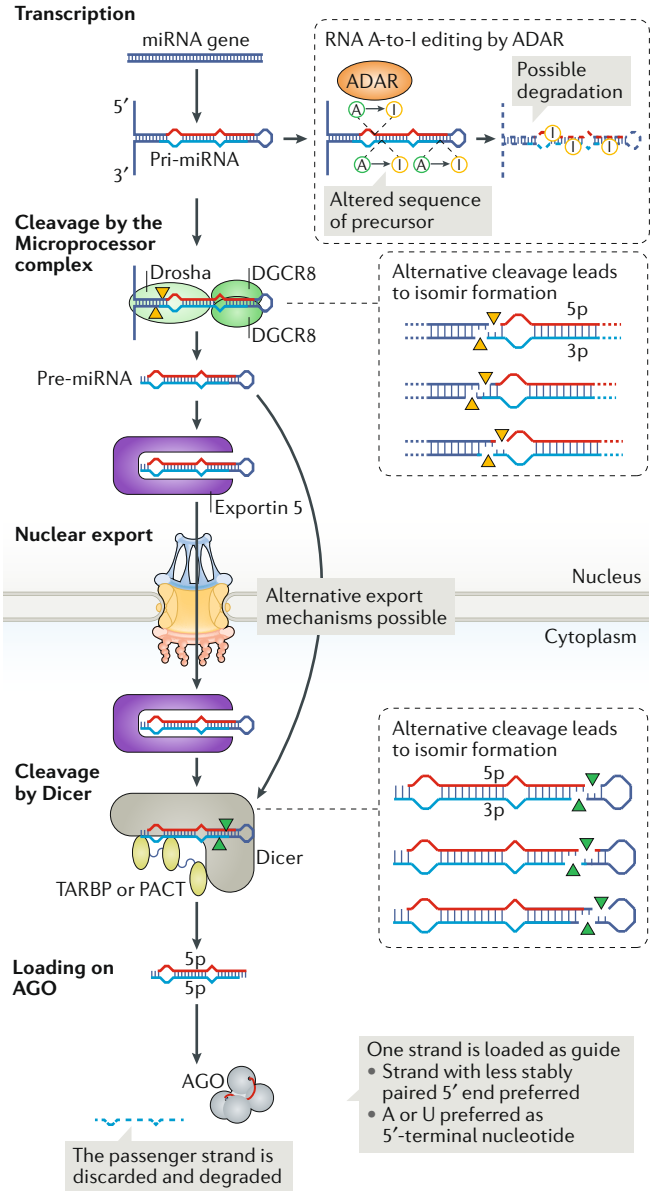
The biogenesis of microRNAs (miRNAs) is a multistep process. They are transcribed mainly by RNA polymerase II[33–35] as structured primary miRNAs (pri-miRNAs) and processed into precursor miRNAs (pre-miRNAs) and finally into mature-miRNA duplexes (see the figure). The mature miRNA comprises a 5p strand (arising from the 5′ arm of the pre-miRNA hairpin) and a 3p strand. The sequence of pri-miRNAs can be altered by A-to-I editing by double-stranded RNA-specific adenosine deaminase (ADAR) proteins, which may affect further biogenesis and the sequence of the mature miRNA or promote degradation of the pri-miRNA[99].

The pri-miRNA hairpin is excised in the nucleus by the Microprocessor complex, comprising the RNase III enzyme Drosha[252] and the protein DiGeorge syndrome critical region 8 (DGCR8)[253,254]. Drosha recognizes the double-strand RNA–single-strand RNA junction at the hairpin base, whereas two DGCR8 proteins bind the stem and ensure correct cleavage[255,256]. Alternative cleavage by Drosha leads to the production of isomirs[97,98] (FIG. 2; Supplementary Table 1).

Pre-miRNAs are hairpins of ~70 nucleotides[6–8]. The hairpin end features a 2-nucleotide overhang at the 3′ end, a 5′ phosphate and a 3′ hydroxyl, which are typical of RNase III products[257]. Exportin 5 recognizes the overhang and transports the pre-miRNA to the cytoplasm[258]. *XPO5* knockout in a human cell line reduced but did not eliminate nuclear export of pre-miRNAs, which suggested that alternative modes of pre-miRNA nuclear export exist[262].

In the cytoplasm, the RNase III enzyme Dicer[259–261] binds the pre-miRNA by recognizing the 5′ phosphate, 3′ overhang and loop structure[263–266]. Dicer is a 'molecular ruler'[263,267] that cleaves pre-miRNAs at a species-specific length[268] and yields a mature-miRNA duplex with another typical 2-nucleotide 3′ overhang[257,269]. Alternative cleavage by Dicer can also lead to the production of isomirs[98] (FIG. 2; Supplementary Table 1). In vertebrates, cleavage by Dicer is modulated by TAR RNA-binding protein (TARBP) and by protein activator of the interferon-induced protein kinase (PACT); in flies, it is modulated by Loquacious proteins[103–107].

One strand of the mature miRNA (the 'guide' strand) is loaded into Argonaute (AGO) whereas the other strand ('passenger strand') is discarded[270–272]. Loading preference is given to the strand possessing the less stably paired 5′ end[273–275]; Ago2 was also reported to prefer an A or U as the 5′-terminal nucleotide[276].



the P-element induced wimpy testes (PIWI) domain. Two linker domains (L1 and L2) connect the N-terminal and PAZ domains (L1) and the PAZ and MID domains (L2). The N-terminal and PAZ domains form one lobe of AGO, and the MID and PIWI domains form the second lobe. The MID and PIWI domains hold the 5′ end of the miRNA, whereas the PAZ domain binds its 3′ nucleotide[39–42]. The mammalian genome encodes four AGO proteins (Ago1–4). Ago2 is the most highly expressed and the only AGO protein able to cleave a target that is fully complementary to the guide strand of the miRNA[43,44], which is a feature important for the biogenesis of miR-451 (REFS[45,46]) and for regulation of a subset of miRNAs that are extensively paired with their targets[47,48]. Although some human miRNAs are preferentially loaded into specific AGO proteins, many associate with all AGO proteins[49–52].

miRNA target sites are generally located in the 3′ UTR of mRNAs; they possess strong complementarity to the seed region[38], which is the main criterion for target-site prediction[53–55]. The strongest canonical (seed-matching) target sites are those that complement miRNA

Guide strand
The strand in the mature miRNA duplex that is loaded into an Argonaute protein and used to identify complementary sites in target mRNAs.

Metastable
A stable state in a system that is not the state of least energy.

nucleotides 2–8 and have an adenine opposite miRNA nucleotide 1 (known as 't1A'), followed by those complementing nucleotides 2–8 without a t1A and nucleotides 2–7 with a t1A[54]. t1A is not recognized by the miRNA guide strand, but by a binding pocket within AGO[56,57] (FIG. 1a). Target sites with complementarity to nucleotides 2–7 or 3–8 of the miRNA are much weaker but still considered canonical[54]. Structural and single-molecule studies suggest that target recognition is achieved by a two-step mechanism in which first nucleotides 2–6 of the seed are pre-organized by the MID and PIWI domains in a stacked, helical conformation, with nucleotides 2–4 exposed to the solvent[41]. This conformation allows for rapid initial binding, which is weak and metastable and becomes long-lived only if the site presents complementarity to nucleotides 7–8 of the guide strand. Otherwise, AGO disengages the site and either laterally diffuses along the mRNA or dissociates from it completely[58]. Thus, the mRNA-binding behaviour of an miRNA is similar to that of an RNA-binding protein (RBP)[58–60].

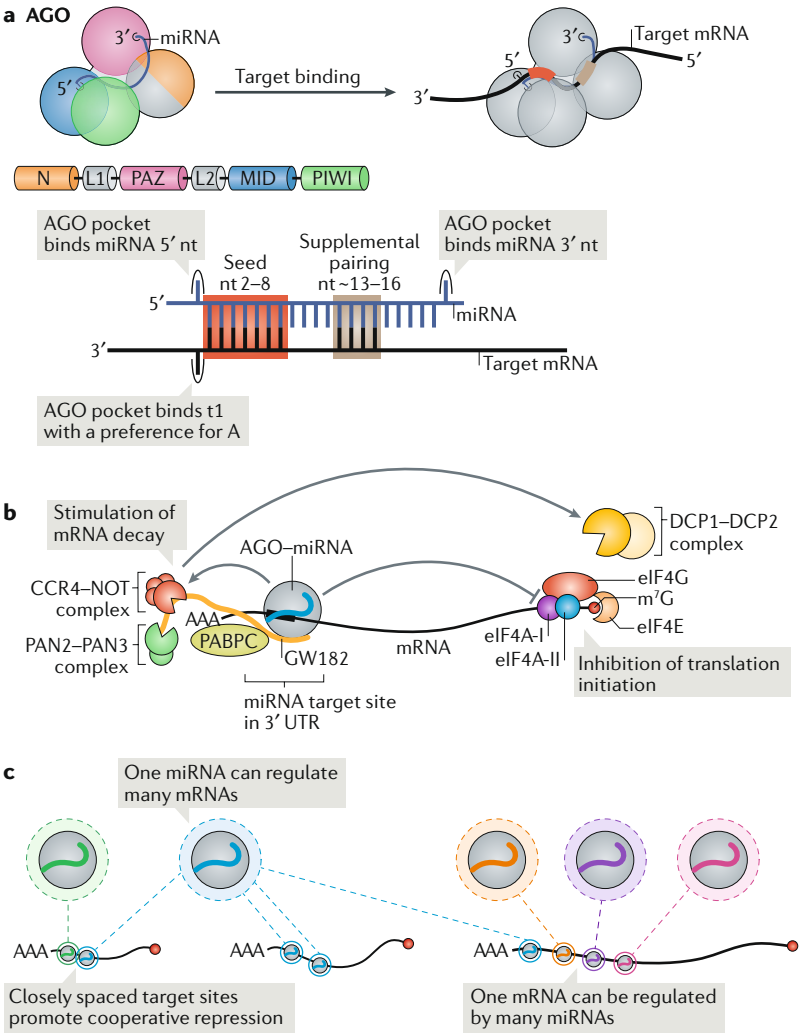


Fig. 1 | **Overview of miRNA function and its regulation. a** | Mature microRNAs (miRNAs) operate as functional units that include an Argonaute (AGO) protein. AGO proteins have four domains, the amino-terminal domain (N), the Piwi–Argonaute–Zwille (PAZ) domain, the middle (MID) domain and the P-element induced wimpy testes (PIWI) domain, and two linker regions (L1 and L2). The MID and PIWI domain hold the 5′ end of the miRNA and arrange it in a helical conformation; the MID domain has a binding pocket for the 5′-terminal nucleotide. Another binding pocket, in the PAZ domain, holds the 3′-terminal nucleotide[39–41]. Nucleotides 2–8 from the 5′ end of the miRNA form the seed, which is crucial for target mRNA recognition[38]. Seed interactions involve nucleotides 2–8, 2–7 and 2–6 (REF.[54]) and can be supplemented by the binding to the MID domain of an adenine in the target mRNA opposite miRNA nucleotide 1 (t1)[54,56,57], or through additional base pairing to nucleotides ~13–16 of the miRNA (the 'supplemental region')[65]. **b** | miRNAs silence gene expression by inhibiting translation at the initiation step, likely through release of eukaryotic initiation factor 4 A-I (eIF4A-I) and eIF4A-II (REFS[79–81]), and by mediating mRNA decay[2] through interactions with glycine-tryptophan protein of 182 kDa (GW182) proteins[68–71]. GW182 binds polyadenylate-binding protein (PABPC) and the deadenylation complexes poly(A)-nuclease deadenylation complex subunit 2 (PAN2)–PAN3 and carbon catabolite repressor protein 4 (CCR4)–NOT[72–76]. Deadenylation is followed by decapping by the complex mRNA-decapping enzyme subunit 1 (DCP1)–DCP2 (REF.[73]) and 5′–3′ mRNA degradation (not shown)[77]. **c** | miRNAs form complex networks of interactions, as one miRNA can target many different mRNAs[86], and one mRNA can be regulated by many different miRNAs[87], with cooperative repression achieved by binding closely spaced target sites[65,89,90]. m^{7}G, 7-methylguanylate cap; nt, nucleotide.

AGO–target mRNA crosslinking has enabled the identification of non-canonical (not seed-matching) binding sites[61–63], and a recent large-scale microarray-based survey in HeLa cells reported sites with minimal seed-pairing[64]. However, the biological function of non-canonical sites, when considered as a whole, has been disputed, as these sites imparted no detectable repression in meta-analyses of miRNA and small RNA transfection data sets[54]. In addition to the seed, the 3′ half of the miRNA can also contribute to target recognition (particularly nucleotides 13–16, which are termed the 'supplemental region') in a subset of sites[54,65] and can direct miRNA family members with the same seed to different target sites, as recently shown in *C. elegans*[66] and in mouse brains[63].

AGO–miRNA binding to the 3′ UTR leads to gene silencing through translation repression and mRNA decay[2,67] (FIG. 1b). The latter involves recruitment by AGO of a member of the glycine-tryptophan protein of 182 kDa (GW182) protein family (in humans, trinucleotide repeat-containing gene 6 A protein (TNRC6A), TNRC6B and TNRC6C)[68–71]. GW182 interacts with polyadenylate-binding protein (PABPC), thereby promoting mRNA deadenylation by recruiting the poly(A)-nuclease deadenylation complex subunit 2 (PAN2)–PAN3 and carbon catabolite repressor protein 4 (CCR4)–NOT complexes[72–76]. Deadenylation promotes decapping by the mRNA-decapping enzyme subunit 1 (DCP1)–DCP2 complex[73], thereby making the mRNA susceptible to rapid degradation by 5′–3′ exoribonuclease 1 (XRN1)[77]. GW182-mediated recruitment of CCR4–NOT also leads to translation repression through recruitment of the probable ATP-dependent RNA helicase DDX6 (REFS[2,78]).

Inhibition of translation initiation is also caused by interfering with the function of eukaryotic initiation factor 4 A-I (eIF4A-I) and eIF4A-II (REFS[2,79–81]). In *Drosophila melanogaster* S2 cell lysates, this activity was independent of GW182 (REF.[80]), suggesting the existence of multiple mechanisms of translation inhibition. There is disagreement on the mechanism of interference with eIF4A-I and eIF4A-II function[79–81], but most data indicate that the miRISC induces their dissociation from target mRNAs and thereby inhibits ribosome scanning and assembly of the eIF4F translation initiation complex. A recent investigation in human and *D. melanogaster* cells reported that the AGO Trp-binding pockets that mediate

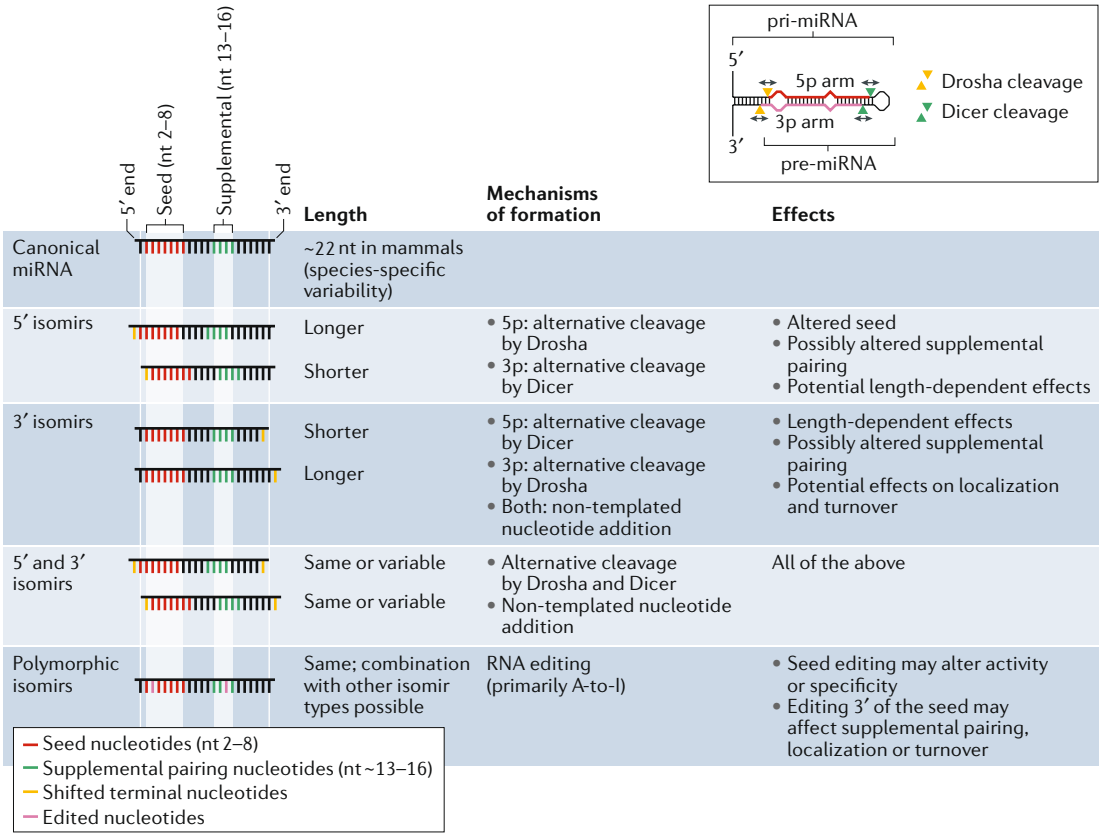


Fig. 2 | **Isomirs differ in length and sequence and expand the functional repertoire of miRNAs.** Isomirs are classified as 5′, 3′ or polymorphic, with combinations possible. Depending on the arm of the microRNA (miRNA) precursors (5p (arising from the 5′ arm of the precursor miRNA (pre-miRNA) hairpin) or 3p; see inset) used to produce the mature miRNA, cleavage by either Drosha or Dicer can result in the formation of the isomir[98]. 5′ isomirs have shifted seeds and can thereby target a different set of genes[109]. The functions of 3′ isomirs are less clear, but there is increasing evidence for their differential activity[113,114]. Polymorphic isomirs are generated by RNA editing, mainly by adenosine deaminase acting on RNA (ADAR). The editing can affect miRNA biogenesis, either by preventing it or by leading to the formation of 5′ isomirs or 3′ isomirs; if editing alters the seed, it could retarget an miRNA to other mRNAs[98,99]. nt, nucleotide; pri-miRNA, primary miRNA.

mRNA decay
Controlled mRNA degradation, usually starting with deadenylation, through either 3′–5′ exonucleolytic processing or decapping and 5′–3′ exonucleolytic processing.

Trp-binding pockets
AGO possesses three pockets located in the PIWI domain, which bind tryptophan and mediate the interaction with GW182.

miRNA clusters
Multiple miRNAs located in close proximity on the genome and transcribed as a single primary miRNA.

Multivalent protein interactions
Protein–protein interactions mediated by multiple, often fairly weak binding events or points of contact.

GW182 binding[41,82] are required for translation inhibition[83]. Thus, miRISC-mediated translation inhibition is still incompletely understood.

Both modes of miRISC-mediated gene silencing are thought to be interconnected[2], and ribosome profiling assays revealed that mRNA decay is generally responsible for 66–90% of silencing[67,84]. The observation that translation inhibition can be rescued[85] but mRNA degradation is irreversible raises the possibility that the regulated pauses, or blocks, in the molecular cascade leading to mRNA degradation could allow translation repression without mRNA decay.

One miRNA can silence hundreds of genes, although the effect on each gene is generally mild[86], and multiple miRNAs can regulate the same gene[87] (FIG. 1c). Furthermore, entire cellular pathways can be regulated by individual miRNAs[87] or miRNA clusters[88]. miRNA binding of neighbouring target sites on a target mRNA can result in cooperative repression[65,89,90], which might explain why the function of non-canonical sites can depend on the occupancy of neighbouring canonical sites[91]. Cooperativity is explained, in part, by the formation of multivalent protein interactions of GW182 with AGO proteins[82]. miRNAs can either switch off or fine-tune protein expression[92] and thereby buffer against fluctuations ('noise') in the levels of gene expression[93].

Modification of miRNA sequence

The interaction of miRNAs with their targets is largely based on their seed sequence[38,54], and miRNA biogenesis is affected by RNA secondary structures that mediate interactions with RBPs[94]. Cellular processes that alter the sequence of an miRNA or of its precursor therefore can affect miRNA biogenesis, activity and turnover.

Isomir formation is a regulated process that determines miRNA activity. Isomirs are mature-miRNA variants that differ in length, sequence or both[31,95–97]. The maturation, stability and turnover, activity or targetome of isomirs can vary (FIG. 2). Isomirs are classified into 5′, 3′ or polymorphic (internal) isomirs, depending on the site of variation, and can be derived from alternative Drosha or Dicer processing, RNA editing or non-templated nucleotide addition (NTA)[98,99]. A recent study in a human breast cancer cell line reported that many miRNAs had no isoforms whereas miR-21-5p had 43 isoforms[100]. Isomir expression patterns have been found useful in distinguishing different types of cancer and represent potential biomarkers[101].

The primary determinant of miRNA length and sequence is cleavage by Drosha and Dicer[32] (Supplementary Table 1). Alternative processing by Drosha was first reported for miR-142 and miR-342 in mouse CD8 T cells[102] and shown to be widespread in human cells[97]. Cleavage by Dicer has been more extensively studied; it is modulated by TAR RNA-binding protein (TARBP) and protein activator of the interferon-induced protein kinase (PACT; also known as PRKRA) in vertebrates (BOX 1) and by Loquacious in flies[103–107]. Variation in the Dicer cleavage site directly modulates the seed sequence of 3p miRNA strands (arising from the 3′ arm of the pre-miRNA hairpin) and can alter guide-strand selection[103,107] (FIG. 2; Supplementary Table 1). Dicer binds TARBP and PACT by the same domain, as revealed by a recent crystal structure of the binding interface, suggesting that Dicer is regulated by a mechanism based on binding competition between TARBP and PACT, which produce specific isomirs for some miRNAs[107].

Alternative cleavage by Drosha or Dicer can shift the position of the seed and is the primary mechanism of 5′ isomir production (BOX 1; FIG. 2), as nucleotide addition or removal at the 5′ end of mature miRNAs is rare[98]. Cleavage-directed 5′ end variation can retarget an miRNA, as observed in flies[104] and humans[108–111], and 5′ isomirs can target the same biological pathways together[109,112] (Supplementary Table 1). The abundance of 5′ isomirs can vary between cell types, with certain isomirs being predominant in specific cell types[96]. By contrast, 3′ isomirs mostly differ in their stability and turnover (see below), although recent findings point also to length-related effects on targeting and activity. For example, binding to the RNA of hepatitis C virus (HCV; see below) of a 21-mer 3′ isomir of miR-122 is weaker than binding of longer miR-122 isomirs[113], and longer 3′ isomirs of miR-222 have increased apoptotic activity[114].

Editing the sequence of miRNA precursors. RNA editing can change the miRNA sequence, generating isomirs, and can also affect biogenesis, leading to 5′ or 3′ variation[98,99] (BOX 1; FIG. 2). Deamination is the most commonly observed type of miRNA precursor editing. Adenosine deaminase acting on RNA (ADAR; also known as DRADA) converts adenosine into inosine[99], which is read as guanosine during splicing and translation. Similarly, cytidine deaminase acting on RNA (CDAR) proteins, better known as members of the apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) family, convert cytidine into uracil[115]. In vertebrates, ADAR1 and ADAR2 act on double-stranded RNA and edit miRNA precursors[99]. A-to-I editing sites appear widespread[116], and in the human brain ~16% of pri-miRNAs were predicted to be edited (on the basis of the analysis of 209 pri-miRNAs). However, editing levels at different miRNAs vary considerably[99,117].

A-to-I editing of miRNA precursors can interfere with cleavage by Drosha[117–121] or Dicer[117,122], or, less frequently, with AGO loading, and editing of the seed can redirect an miRNA to a new set of targets[120,123–126]. A-to-I editing of miRNAs is involved in miRNA deregulation in cancer[121,127,128]. For example, in melanoma, overexpression of cAMP-responsive element-binding protein (CREB) reduces ADAR1 expression, thereby reducing the editing of miR-455-5p; unedited miR-455-5p represses cytoplasmic polyadenylation element-binding protein 1 (CPEB1) and other tumour suppressors, thereby promoting tumour growth and metastasis[121]. Changes in seed sequences can also be mediated by the more elusive C-to-U editing[129]. miRNA A-to-I editing was first observed in pre-miR-22 in human and mouse tissues at species-specific positions and was predicted to alter miRNA targeting and activity[130]. In summary, RNA editing is a versatile regulatory mechanism that can control the abundance and target specificity of miRNAs, but in light of the relatively low frequency of editing[99], further research is required to characterize its biological importance.

Non-templated nucleotide addition. NTA was first suggested to be biologically regulated during *D. melanogaster* embryogenesis[131]. NTA predominantly involves adenylation or uridylation at 3′ ends and is miRNA-specific across tissue types, developmental stages, disease states and different species[132].

NTA is carried out by several enzymes and can modulate miRNA stability (FIG. 3a). The poly(A) RNA polymerase GLD2 (also known as PAPD4) mediates 3′-end monoadenylation that stabilizes miR-122 in the liver[133] and in mouse embryonic fibroblasts[134]. Poly(A)-specific ribonuclease PARN counteracts this as its depletion in a human liver cancer cell line leads to the appearance of miR-122 with 3′-oligo adenylation and to increased miR-122 stability. CUG triplet repeat RBP1 (CUGBP1; also known as CELF1) binds miR-122 and mediates its degradation by recruiting PARN (FIG. 3b). A similar mechanism regulates miR-93 and miR-652–3p[135]. By contrast, a recent analysis of miRNAs in the hippocampus of *Gld2*-knockout mice revealed reduced levels of 3′ adenylation but no effect on miRNA stability[136]; similarly, only about half of the investigated miRNAs were destabilized in human fibroblasts when GLD2-mediated adenylation was suppressed[137]. Moreover, 3′ adenylation by the poly(A) RNA polymerase PAP-associated domain-containing protein 5 (PAPD5) initiates degradation of miR-21 (REF.[138]).

Compared with 3′ adenylation, 3′ uridylation more consistently inhibits miRNA activity. Uridylation by terminal uridylyltransferase 4 (TUT4; also known as ZCCHC11) of miR-26b in a human adenocarcinoma cell line reduces target repression[139]. Moreover, TUT4-mediated uridylation primes numerous miRNAs for degradation during T cell activation[140] (FIG. 3a).

To date, the enzymes reported to mediate NTA include GLD2 (REFS[133,141]), PAPD5 (REF.[141]), poly(A) polymerase-γ and poly(A) RNA polymerase, mitochondrial[132] (adenylation) and TUT7, TUT4 (REF.[139]) and TUT1 (uridylation). Species-dependent differences in NTA have been observed, with uridylation being more common in *C. elegans* and adenylation more common in mouse and human[132]. In summary, NTA appears to influence, both positively and negatively, the stability of specific miRNAs in specific cell types. Recent findings demonstrate that interactions between the miRNA 3′ region and target RNAs can substantially affect miRNA modification and turnover (see below), thereby providing a potential mechanism for the cellular context dependence of NTA.

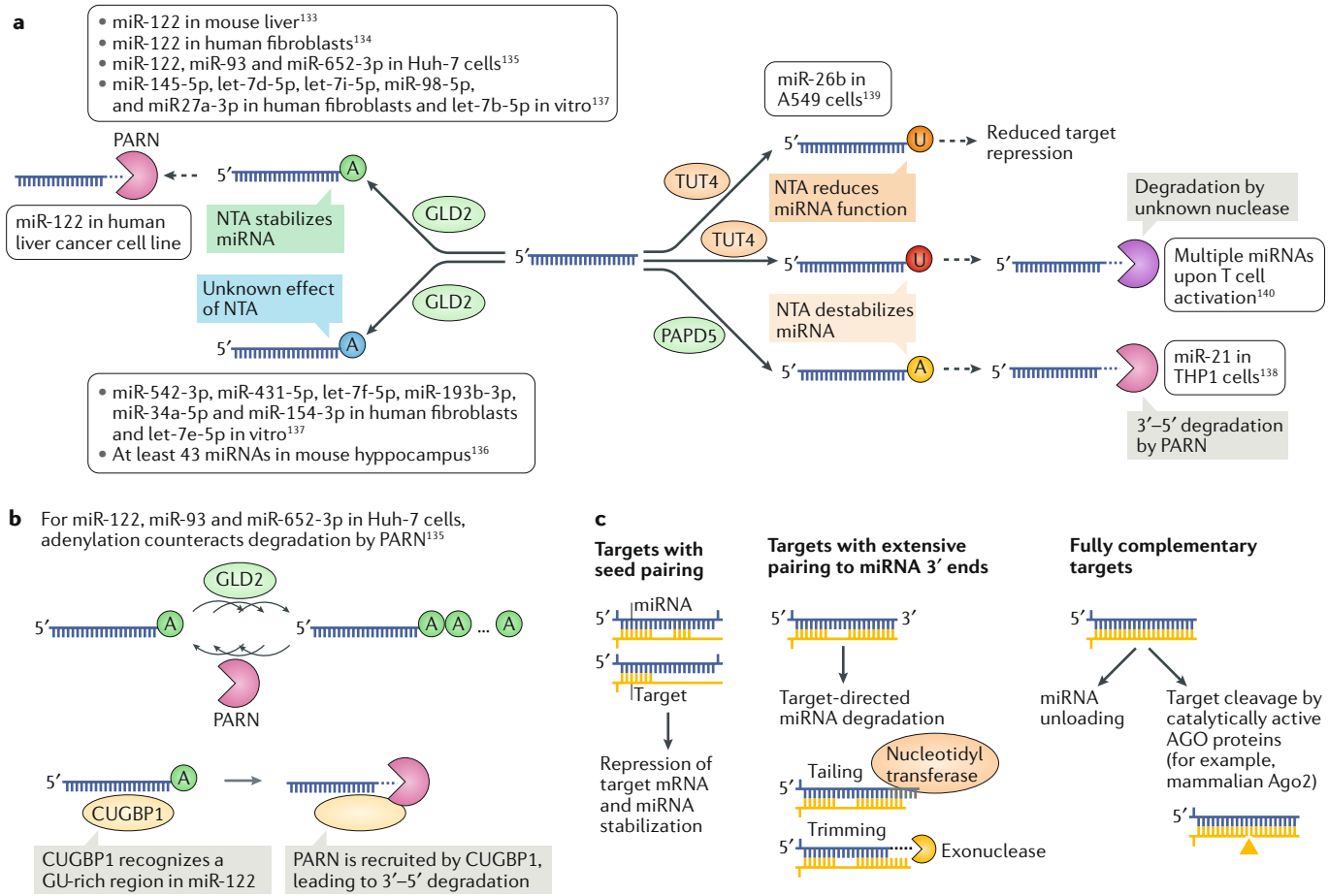


Fig. 3 | **Non-templated nucleotide addition and miRNA turnover.** **a** | Non-templated nucleotide addition (NTA) by poly(A) RNA polymerase GLD2 can stabilize some microRNAs (miRNAs) but has no effect on others. Terminal 3′ adenylation of miR-21 by PAP-associated domain-containing protein 5 (PAPD5) promotes exonucleolytic cleavage by poly(A)-specific ribonuclease PARN[138]. Terminal 3′ uridylation by terminal uridylyltransferase 4 (TUT4) can reduce the activity of miR-26b[139] or prime miRNAs for degradation following T cell activation[140]. **b** | Terminal 3′ adenylation stabilizes some miRNAs by counteracting 3′–5′ exonucleolytic activity by PARN. CUG triplet repeat RNA-binding protein 1 (CUGBP1) interacts with miR-122 and recruits PARN[135]. **c** | Interactions of an Argonaute (AGO)-bound miRNA with a target mRNA through the miRNA seed sequence result in translation repression and mRNA degradation. Targets with extensive pairing to miRNA 3′ ends promote tailing[144,151], trimming[148] and target-directed miRNA degradation[142,149,150]. A target mRNA that is fully complementary to a miRNA is cleaved when bound by a catalytic AGO, such as mammalian Ago2 (REFS[44,49]), but such pairing can also result in unloading of the miRNA from AGO[154].

miRNA turnover. miRNAs are generally thought to be stable in vivo, but turnover rates are regulated by multiple factors and across different miRNAs can range from minutes to days[142]. Turnover rates are miRNA-specific and isomir-specific and thus linked to miRNA sequence[143,144]; indeed, a 5′ guanine or cytosine is associated with faster turnover rates than a uracil in this position[143]. Stability can also be miRNA-specific. For example, miRNAs in mouse fibroblast 3T3 cells are generally stable, except for miR-16 family members, which are intrinsically unstable[145]. This instability allows miR-16 levels to vary with, and thereby help regulate, the progression of the cell cycle. Some miRNAs are stable unless specifically degraded in response to developmental cues; for example, miR-150 is rapidly lost when murine naive T cells differentiate into T helper 1 (T_H1) and T_H2 lymphocytes[146]. Turnover rates can also depend on tissue context: faster turnover rates are generally observed in neuronal miRNAs compared with miRNAs in other tissues[147].

One mechanism of miRNA destabilization is termed target RNA-directed miRNA degradation (TDMD)[148]. In TDMD, target RNAs with extensive complementarity to both 5′ and 3′ regions of an miRNA promote its turnover (FIG. 3c). TDMD is often associated with 3′ NTA, or 'tailing', and with 3′–5′ trimming of the degraded miRNA[142,149,150]. It has been proposed that 3′ tailing extends the AGO-loaded miRNA[144,151] until a 3′–5′ nuclease can bind and degrade it[148]. This hypothesis is consistent with the observation that 3′-remodelled isomirs generally coincide with TDMD. However, the hypothesis has recently been challenged by the observation that tailing of miR-7 by GLD2, which coincides with TDMD of miR-7 in mouse neurons, is not required for efficient miR-7 degradation[152]. Similarly, the 3′–5′ exoribonuclease DIS3-like exonuclease 2 (DIS3L2) was implicated in 3′ trimming associated with TDMD of miR-27a, but DIS3L2 depletion did not affect TDMD efficiency[151]. Thus, it is possible that TDMD and miRNA 3′ remodelling are distinct processes;

therefore, the molecular mechanism of TDMD remains poorly understood.

Although viral inducers of TDMD have been known for several years (see below), endogenous inducers of TDMD have only recently been reported. In zebrafish, miR-29b is selectively degraded in the cerebellar granule cell layer by a lncRNA and in mice by the 3′ UTR of a protein-coding gene[153]. Both transcripts show extensive sequence similarity around a highly complementary target site for miR-29b. Similarly, the lncRNA Cyrano can induce TDMD of miR-7, thereby affecting the stability of an miR-7-interacting circular RNA (circRNA) (see below)[152]. Furthermore, miR-503, which is inherently unstable in 3T3 cells, can be stabilized by mutating either its seed or 3′ regions[145], raising the possibility that its inherent instability is mediated by TDMD. It is possible that many inherently unstable miRNAs are subjected to TDMD through yet-to-be-identified target RNAs.

A biochemical screen using lysates of HEK239 cells (a human embryonic kidney cell line) revealed that miRNA degradation is promoted particularly by seedless targets with high complementarity to miRNA 3′ ends, with complementarity of the 3′-terminal nucleotide being crucial for degradation[150]. Combined with the observation that the sequences of both the 5′ end and the 3′ end influence turnover rates[143,144], this might partially explain the tissue-specific and miRNA-specific effects of NTA on stability. Alternatively, highly complementary targets can destabilize the AGO–miRNA interaction and promote release of the guide strand[154], although miRNAs appear to be stabilized by a high abundance of seed-matching targets[155].

In addition to exonucleolytic miRNA turnover, Tudor staphylococcal nuclease, which is responsible for degradation of A-to-I edited double-stranded RNA[99], was found to target miR-31-5p, miR-29b-3p and miR-125a-5p in HEK293T cells. Other tested miRNAs did not appear to be affected, suggesting that this is an miRNA-specific mechanism, although how selectivity is established remains to be determined[156]. Finally, phosphorylation-dependent regulation of miRNAs was recently discovered for the tumour suppressor miR-34. The nuclei of four human cancer cell lines were found to contain pools of inactive, mature, single-stranded miR-34 lacking a 5′ phosphate and not bound by AGO[157]. Irradiation of the cells led to 5′ phosphorylation, export to the cytoplasm and loading into AGO of miR-34. The serine-protein kinase ATM and the RNA kinase Clp1 were required for miR-34 phosphorylation. It is unclear how widespread this phenomenon is, but these findings suggest that miRNAs can be maintained in an inactive form and rapidly activated in response to stimuli.

Post-translational modification of AGO

AGO proteins undergo PTM on multiple residues (FIG. 4). Phosphorylation is the best characterized AGO PTM and it occurs at three main sites: at the L2 linker on Ser387 and Tyr393 of human Ago2; at the miRNA 5′-end binding region in the MID domain on Tyr529; and at a surface-exposed loop in the PIWI domain known as the eukaryotic insertion or the S824–S834 cluster, which includes Ser824, Ser828, Thr830, Ser831 and Ser834.

Several studies report phosphorylation of Ser387 in the L2 region of human Ago2 (REFS[158–160]) (FIG. 4a). Ser387 phosphorylation is stimulated by the p38 MAPK pathway-mediated stress response (for example, by MAP kinase-activated protein kinase 2 in vitro)[158]. The proto-oncogene RACγ serine/threonine-protein kinase (AKT3) also phosphorylates Ser387 in vitro and in HeLa cells, and its depletion or the expression of an S387A Ago2 mutant (which cannot be phosphorylated) led to derepression of a luciferase reporter and weakened the Ago2 interactions with TNRC6A[159]. Similarly, Ser387 phosphorylation is necessary for interactions between Ago2 and LIM domain-containing protein 1, which is a protein suggested to bridge Ago2 and GW182 (REF.[161]). Blocking Ser387 phosphorylation also reduced accumulation of Ago2 in cytoplasmic foci with GW182 (REFS[158,159]) and was suggested to reduce trafficking of Ago2–miRNA complexes to multivesicular endosomes and reduce the secretion of exosomes[162]. Notably however, a recent investigation found that S387A Ago2 did not reduce colocalization of GW182 in HeLa cells, indicating that additional factors may be involved in enabling the Ago2–GW182 interaction[163]. The combined data indicate that Ser387 phosphorylation promotes miRNA function by stimulating the assembly of the miRISC.

Tyr393 is adjacent to Ser387 in the L2 region, and its phosphorylation is also well documented[160,164,165] (FIG. 4a). It appears to be mediated by epidermal growth factor receptor (EGFR) and stimulated by hypoxic stress[164]. Overexpression of oncogenic RAS reversibly inhibits protein-tyrosine phosphatase 1B (also known as tyrosine-protein phosphatase non-receptor type 1), resulting in Ago2 hyperphosphorylation at Tyr393 (REF.[165]). Both studies reported diminished interactions between Ago2 and Dicer and reduced levels of Ago2-associated miRNAs following Tyr393 phosphorylation. Thus, in contrast to Ser387, Tyr393 phosphorylation appears to negatively regulate miRNA activity by inhibiting the loading of miRNAs into Ago2, thereby promoting tumorigenesis.

Tyr529 phosphorylation also potentially blocks miRNA loading[160] (FIG. 4a). Tyr529 binds the 5′ phosphate and first nucleotide of miRNAs loaded into AGO[41,166,167]. Tyr529 phosphorylation is therefore expected to preclude miRNA binding, as indeed was demonstrated in Tyr529 Ago2 mutants[160]. Tyr529 phosphorylation was also associated with decreased miRNA binding to Ago2 during macrophage activation[168].

A phosphorylation cycle of residues S824–S834 in Ago2 regulates the release of target mRNAs[169,170] (FIG. 4b). Target binding leads to phosphorylation of these residues by casein kinase I isoform-α (CSNK1A1), which reduces the affinity of Ago2 for mRNA and enables target release. The serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C (ANKRD52)–catalytic subunit (PPP6C) complex dephosphorylates the residues, which primes Ago2 for binding a new target. Interrupting this cycle strongly inhibited miRNA activity, and the cycle was suggested to prevent overly long association with mRNA targets[170]. Alternatively, the phosphorylation cycle may represent an AGO regulation mechanism that is mediated by mRNA-binding proteins.

Seedless targets
miRNA targets with considerably reduced complementarity to the miRNA seed.

Multivesicular endosomes
Type of late endosome that contains intraluminal vesicles formed by budding into the lumen of the endosome. Their content can be degraded by fusion with lysosomes or released into the extracellular space through fusion with the cell membrane.

Exosomes
Type of extracellular vesicle, ~50–150 nm in diameter, that contains proteins, lipids and RNA and can carry cargo to target cells.

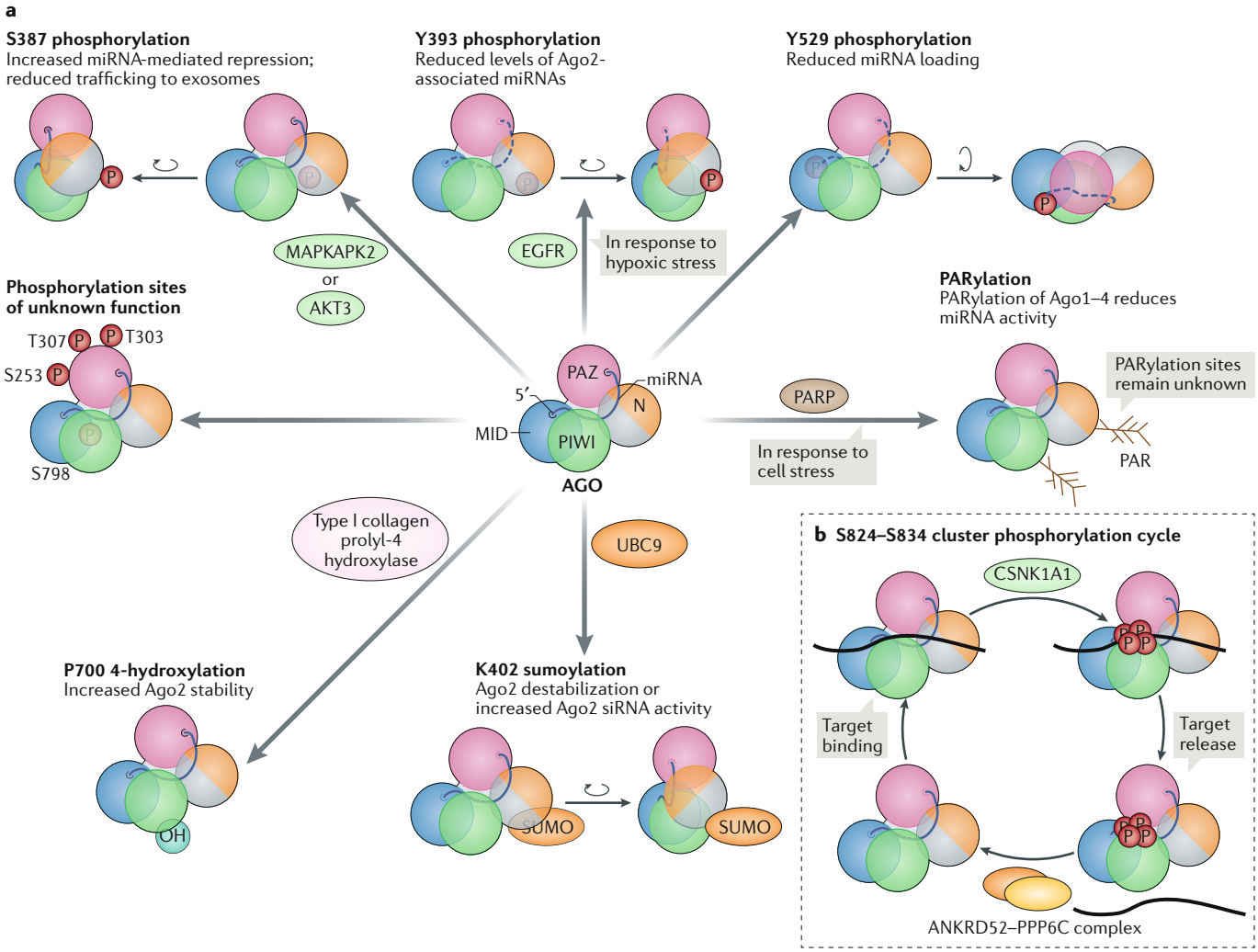


Fig. 4 | **The activity and the stability of miRNA-induced silencing complex is modulated by post-translational modifications of Argonaute proteins.** **a** | Phosphorylation of Ser387 (S387) in the linker 2 (L2) region of Argonaute (AGO) was found to be mediated by MAP kinase-activated protein kinase 2 (MAPKAPK2)[158] and RACγ serine/threonine-protein kinase (AKT3)[159] in vitro. Ser387 phosphorylation increases microRNA (miRNA) activity by stimulating the assembly of miRNA-induced silencing complexes and reduces translocation of Ago2 to multivesicular endosomes and secretion of exosomes[162]. Phosphorylation of the nearby Tyr393 (Y393), also in the L2 region, decreases the miRNA–Ago2 association, thereby reducing miRNA activity[164,165]. Tyr 529 (Y529) is located in the middle (MID) domain, near the miRNA 5′-nucleotide binding pocket, and its phosphorylation prevents miRNA loading[160]. No function has yet been assigned to phosphorylation sites in the Piwi–Argonaute–Zwille (PAZ) domain (S253, T303 and T307) and in the P-element induced wimpy testes (PIWI) domain (S798)[160]. Additional AGO post-translational modifications include Pro700 (P700) 4-hydroxylation, which increases Ago2 stability[171]; Lys402 (K402) sumoylation, which was reported to either destabilize Ago2 (REF.[177]) or be required for full small interfering RNA (siRNA) activity[178]; and poly(ADP-ribosylation) (PARylation), which inhibits miRNA activity[173,174], presumably by decreasing target accessibility. **b** | The S824–S834 cluster in the eukaryotic insertion region of human Ago2 undergoes a phosphorylation cycle, which regulates AGO–target interactions. Phosphorylation of the Ser residues in the cluster by casein kinase I isoform-α (CSNK1A1) favours target-mRNA release. Subsequent dephosphorylation by the serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C (ANKRD52)–catalytic subunit (PPP6C) complex primes Ago2 for the next round of target binding[169,170]. EGFR, epidermal growth factor receptor; N, amino-terminal domain; OH, hydroxyl group; P, phosphate; PAR, poly (ADP-ribose); PARP, poly(ADP-ribose) polymerase; SUMO, small ubiquitin-like modifier; UBC9, ubiquitin carrier protein 9.

The cluster is conserved in human AGO proteins, mouse Ago2, rat Ago2, zebrafish Ago2, the *C. elegans* argonaute protein (ALG-1) (REF.[169]) and in the fruitfly Ago1 (but not in the fruitfly Ago2, which is used for small interfering RNA (siRNA)-mediated target cleavage)[170].

Additional Ago2 phosphorylation sites have been reported in the PAZ domain (Ser253, Thr303 and Thr307) and the PIWI domain (Ser798), but their function is currently unknown[160] (FIG. 4a).

In addition to phosphorylation, other AGO PTMs are known (FIG. 4a). Hydroxylation of Ago2 Pro700 appears to increase the stability of Ago2 in mouse and human cells[171]. Interestingly, Pro700 is adjacent to a Trp-binding pocket in Ago2 that is used to interact with GW182 (REFS[41,82]); thus, its 4-hydroxylation could potentially alter the assembly of the miRISC, although this has not yet been demonstrated.

Ago1–4 undergo poly(ADP-ribosylation) (PARylation) in human cell lines[172]. Poly(ADP-ribose) (PAR) is a polymer of ADP-ribose units that can be enzymatically added to Asp, Glu and Lys residues. PARylation regulates the cellular stress response, particularly the formation of

cytoplasmic stress granules, which regulate mRNA translation and stability[172]. PARylation of Ago1–4 reduced translation repression and endonucleolytic cleavage and was hypothesized to impair target accessibility[173]. PARylation-induced downregulation of miRNA activity was observed in the antiviral response in HEK293 cells: cytotoxic interferon-stimulated genes are generally heavily regulated by miRNAs, and miRNA inhibition through AGO PARylation might enable a cytotoxic response to viral infection[174].

The ubiquitin–proteasome system reduces the abundance of the fruitfly Ago1 and mouse Ago2 (REF.[175]) and drives global miRNA downregulation during T cell activation[176]. Finally, two groups have reported Ago2 sumoylation at Lys402 with contradicting consequences: sumoylation might destabilize Ago2 (REF.[177]) or be required for full Ago2 siRNA-mediated activity[178].

Compared with AGO proteins, less is known about the regulation of other proteins involved in miRNA function. Tripartite motif-containing protein 65 ubiquitylates TNRC6 in HEK293 cells, leading to proteasomal degradation and derepression of miRNA targets[179]. TNRC6A has long been known to be phosphorylated in mammalian cells[180], and phosphorylation of the PAM2 motif of TNRC6C was suggested to reduce its interactions with PABPC1 (REF.[181]). A recent mass-spectrometry study of the TNRC6A interactome in HeLa cells revealed a large number of binding partners, but it is currently unknown whether any of these regulate TNRC6A[182].

Regulation of miRNAs by sequestration

The competing endogenous RNA (ceRNA) hypothesis[183] postulates that an increase in the cellular concentration of an miRNA target RNA would reduce the cytoplasmic availability of the specific miRNA by binding it, thereby derepressing other mRNAs that are targets of the same miRNA; this is similar to the proposed function of miRNA sponges[184]. Thus, according to the ceRNA model, gene expression would be shaped by the global competition of target RNAs for miRNAs[185] (FIG. 5a). The potential efficacy of ceRNAs is controversial, as the increased expression of any single ceRNA is expected to increase only slightly the total number of target sites of an miRNA and thus be unlikely to meaningfully affect miRNA activity[186–189]. Two studies measuring the cellular abundance of miRNAs and miRNA target sites proposed that the ability of a putative ceRNA to derepress the expression of other target mRNAs depends on miRNA abundance[190] and/or on target site abundance[186]. Both studies agreed that, in most cases, unphysiologically high levels of a ceRNA would generally be necessary to yield a biologically meaningful effect[186,190]. Mathematical modelling of miRNA distribution in the targetome indicates that a ceRNA must increase the cellular abundance of target sites at least twofold to be effective[188]. On the other hand, it was also noted that ceRNA efficacy could be increased if local concentrations of ceRNAs and/or miRNAs deviated strongly from the cytoplasmic average[188]. The discovery of the phosphorylation cycle regulating AGO activity also raises interesting new possibilities, as AGO–target binding might be modulated by mRNA-associated RBPs[170]. With respect to the ceRNA hypothesis, such a mechanism could alter the affinities of different target sites that otherwise seem equivalent on the basis of miRNA complementarity alone. Additionally, although this has yet to be demonstrated, ceRNAs that promote TDMD could function at lower concentrations by reducing miRNA abundance[189]. Thus, although the original ceRNA hypothesis may be generally implausible, ceRNA mechanisms may still have a biological role[188].

The controversy notwithstanding, the list of potential ceRNAs is growing and includes lncRNAs, pseudogenes, mRNAs (FIG. 5b) and specific circRNAs[187]. In mice, long intergenic non-protein coding RNA, muscle differentiation 1 (*Linc-md1*) is proposed to drive myoblast differentiation by sequestering miR-133 and miR-135, which target muscle-specific transcription factors[191]. Similarly, in human cells, the untranslated PTEN pseudogene 1 (*PTENP1*) derepresses *PTEN*[183]. This derepression was suggested to be a common function of pseudogenes[187], as they regularly share miRNA target sites with their parent genes. Recently, the overexpression of the *Braf* pseudogene was found to promote B cell lymphoma in mice through a possible ceRNA mechanism[192]. mRNAs have also been suggested to function as ceRNAs; for example, PTEN expression is regulated not just by pseudogenes but also by numerous protein-coding ceRNAs[193,194] (FIG. 5b). Different types of ceRNA were found to crosstalk in a network of various oncogenic pathways in glioblastoma[195].

circRNAs are enigmatic ncRNAs with dynamic and complex expression patterns; their function is still poorly understood[196]. The circRNA CDR1 antisense RNA (*CDR1as*) is expressed in human and mouse brains[197,198]. Because *CDR1as* contains many binding sites (63–74) for miR-7, it acts as an miR-7 sponge when overexpressed[197]. In support of the sponge mechanism, depletion of *CDR1as* in HEK293 cells led to downregulation of miR-7 target mRNAs[198]. However, knockout of *CDR1as* in mice led to a reduction in miR-7 levels and an increase in the levels of miR-7 target genes in the brain, suggesting that the sponge effect stabilizes miR-7, as none of its binding sites in *CDR1as* possesses the extensive 3′ complementarity required for TDMD[199]. The miR-7 molecules can be released from *CDR1as* through its slicing by miR-671, for which *CDR1as* contains a highly complementary target site. Thus, *CRD1as* may bind to miR-7 in order to help to localize it to specific subcellular compartments[197–199]. The lncRNA Cyrano was proposed to be responsible for the increased turnover of miR-7 upon *CDR1as* depletion[199] and has now been reported to induce TDMD of miR-7 (REF.[152]). Interestingly, Cyrano depletion increased miR-7 levels and led to a decrease in *CDR1as* levels, in part owing to an increased miR-671-mediated degradation of *CDR1as*[152].

Although thousands of circRNAs have been identified, only a handful stand out as harbouring large numbers of miRNA target sites[200]. In addition to *CDR1as*, ten different circRNAs derived from zinc-finger genes contain 7–24 target sites for the miR-23, miR-181 or miR-199 families[200]. Another proposed sponge, the testis-specific circRNA sex-determining region Y, possesses 16 sites for miR-138 in mice[197], but only 1 in humans[200]. Thus, although some circRNAs may

Stress granules
Following global translation shutdown during the cellular stress response, cytoplasmic granules form, which are composed of non-translating mRNAs, translation initiation factors and regulatory proteins.

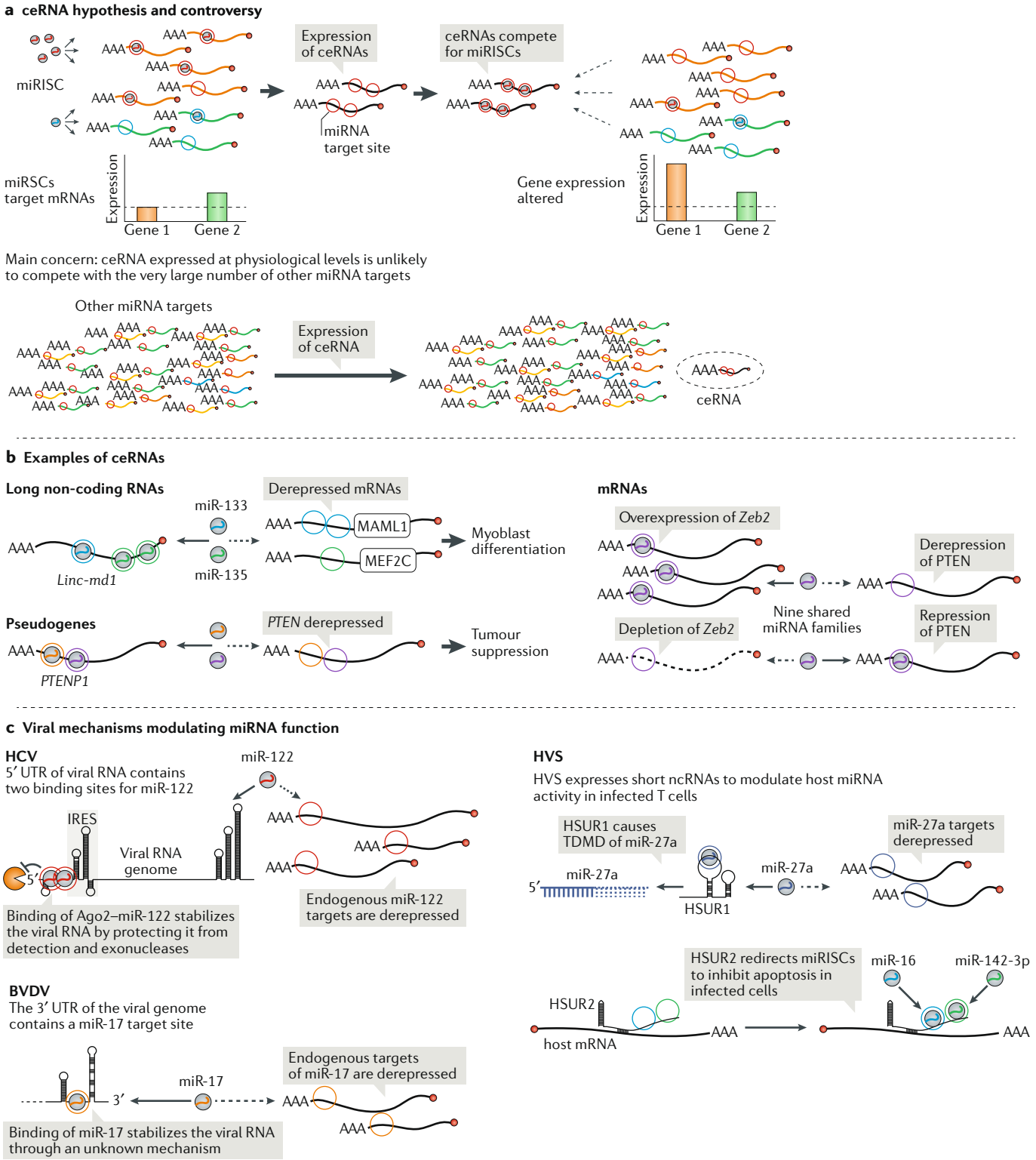
PAM2 motif
Poly(A) binding protein interacting motif 2 mediates the interaction between GW182 and PABP.

miRNA sponges
Transcripts that contain multiple target sites for a specific miRNA and bind miRNAs, thereby derepressing the miRNA target mRNAs.

interact with miRNAs, this does not appear to be a major function of most of them.

Viral modulation of miRNA activity

Viruses have evolved to repurpose or modulate host miRNAs for their replication, which often affects miRNA function (FIG. 5c). The best studied interaction is between the HCV and miR-122. HCV is a positive-sense single-stranded RNA virus of the *Flaviviridae* family that causes acute and chronic liver infection[201]. Its highly structured 5′ UTR contains an internal ribosomal entry site[202], and upstream of it, at the very 5′ end of the genomic RNA, two binding sites for the liver-abundant miR-122 (REF.[203]) (FIG. 5c). The binding sites recruit Ago2–miR-122 to the uncapped 5′ end of the viral RNA[204] to protect it from the cellular antiviral response



Small nuclear RNAs
Small non-coding RNAs in the nucleus that form complexes with proteins and are part of the splicing machinery.

and exonuclease activity[205,206]. This functionally sequesters cytoplasmic miR-122, which is a tumour suppressor miRNA responsible for hepatic maintenance and potentially explains the link between chronic HCV infection and an increased risk of developing hepatocellular carcinoma[207].

Another virus from the *Flaviviridae* family that binds host miRNAs is bovine viral diarrhoea virus, which is an economically important cattle virus. Binding of let-7 and miR-17 at a structured region of the viral RNA 3′ UTR promotes viral replication. miR-17 also stabilizes the viral RNA upon binding and increases its translation through an unknown mechanism. The binding of miR-17 to the viral RNA derepresses cellular targets of the miRNA, an effect also observed with classical swine fever virus[208]. The detection of similar interactions between miRNAs and viral genomic RNA of different members of the *Flaviviridae* family raises the question of whether this mechanism might be even more widespread in this family, which includes other health-relevant members such as dengue virus and zika virus.

Hepatitis B virus, which is a partially double-stranded DNA virus unrelated to HCV, directly downregulates miR-122 in liver cells, as this miRNA appears to inhibit viral infection[209]. A hybrid viral–human transcript[210] contains multiple miR-122 binding sites, one of which resembles a TDMD element that depletes cellular miR-122 levels and promotes loss of hepatic function and liver damage in mice[211].

Several members of the *Herpesviridae* family also modulate host miRNA function. *Herpesvirus saimiri* (HVS) expresses *H. saimiri* uracyl-rich RNAs (HSURs), which are short ncRNAs with structural similarity to small nuclear RNAs. Two HSURs harbour binding sites for host miRNAs: miR-16 (HSUR2), miR-27a (HSUR1) and miR-142-3p (both HSUR1 and HSUR2). HSUR1 reduces miR-27a levels in infected marmoset T cells through TDMD, thereby derepressing miR-27a cellular target mRNAs and promoting T cell activation[212]. TDMD of miR-27a is required for efficient HVS replication, as viral strains with HUSR1 bearing a mutated miR-27a binding site have reduced titres[213]. HSUR2 does not deplete the miRNAs it binds but instead acts as a tether that recruits AGO–miR-142-3p and AGO–miR-16 complexes to cellular mRNAs that encode pro-apoptosis factors, thereby inducing silencing of the tethered mRNAs and preventing apoptosis[214].

Analogously to HSUR1 function, TDMD and reduction in miR-27a levels were observed in mouse cell lines and primary macrophages upon murine cytomegalovirus infection[215], mediated by a transcript harbouring a miR-27a target site with substantial 3′-end complementarity[216]. Similarly, human cytomegalovirus (HCMV) targets miR-17 and miR-20a, two members of the miR-17-92 cluster. Degradation of these miRNAs, which is mediated by complementarity with sites in viral ncRNA, accelerates virus production during HCMV infection[217].

Fig. 5 | **miRNA sequestration by endogenous and viral RNAs. a** | The competing endogenous RNA (ceRNA) hypothesis postulates that a newly expressed RNA can compete with the already present microRNA (miRNA) targets for cytoplasmic miRNA-induced silencing complexes (miRISCs), potentially leading to derepression of certain genes[185]. However, a ceRNA is unlikely to lead to gene derepression when expressed at physiological levels[186–190]. **b** | Long non-coding RNAs, pseudogenes and mRNAs can have ceRNA activity. In mice, long intergenic non-protein coding RNA, muscle differentiation 1 (*Linc-md1*) contains one binding site for miR-133 (blue) and two for miR-135 (green). By sequestering these miRNAs, the muscle-specific transcription factors mastermind-like transcriptional co-activator 1 (MAML1) and myocyte enhancer factor 2C (MEF2C) are derepressed, thereby promoting myoblast differentiation[191]. The pseudogene PTEN pseudogene 1 (*PTENP1*) shares many miRNA target sites with the tumour suppressor *PTEN* mRNA and can derepress *PTEN* in human cells[183]. Similarly, the mouse zinc-finger E-box binding homeobox 2 (*Zeb2*) mRNA can derepress PTEN[193]. **c** | Different viral mechanisms affect miRNA function. Hepatitis C virus (HCV) harbours two binding sites for miR-122 at the very end of the 5′ untranslated region (UTR) of its RNA genome[203]. These recruit Argonaute 2 (Ago2)–miR-122 complexes[204] to protect the viral RNA from the cellular antiviral response and the activity of exonucleases[205] and functionally sequester miR-122 and derepress hepatic miR-122 target mRNAs[207]. Bovine viral diarrhoea virus (BVDV) is an RNA virus that contains a binding site for miR-17 in its 3′ UTR; miR-17 binding increases the stability of the viral RNA. The site functionally sequesters the miRNA and derepresses its cellular targets[208]. *Herpesvirus saimiri* (HVS) produces short non-coding RNAs (ncRNAs) termed HVS uracyl-rich RNAs (HSURs), two of which are known to modulate miRNA function. HSUR1 binds miR-27a and promotes its target RNA-directed miRNA degradation (TDMD), which leads to derepression of miR-27a cellular targets and promotes T cell activation[212]. HSUR2 binds miR-142-3p and miR-16 and tethers them to cellular target mRNAs, which prevents apoptosis[214]. IRES, internal ribosome entry site.

miRNA transport from the cytoplasm

As miRNAs function in the cytoplasm, their activity can be modulated by transferring them to the nucleus or to extracellular vesicles, where they potentially have location-specific functions[218].

Nuclear localization of miRNAs. miR-21 was the first miRNA to be found in the nucleus, as 20% of total miR-21 is present in nuclear extracts[49]; miR-29b was found to be predominantly localized to the nucleus owing to a 3′ hexanucleotide nuclear localization signal[219]. Ago2, Dicer, TARBP and GW182 are present in the nucleus and form complexes. In *C. elegans*, the AGO homologue ALG-1 uses mature let-7 to bind pri-let-7 transcripts in the nucleus and promote their biogenesis, thereby creating a positive feedback loop[220]. Recent single-molecule studies in mammalian cells indicated that nuclear miRNAs do not repress complementary targets. The importance of the hexanucleotide localization signal of miR-29b was also questioned, as miRNA nuclear localization was found to be primarily dependent on the nuclear presence of targets[155]. These findings suggest that our understanding of nuclear miRNA activity is still very partial.

Circulating miRNAs. Circulating miRNAs are potential cancer biomarkers[221]; their discovery in exosomes led to the hypothesis that they might contribute to intercellular signalling[222] (FIG. 6a). It remains unclear whether only a small fraction of circulating miRNAs travel within exosomes (~10% or less in plasma)[223,224] or whether exosomes contain the majority of circulating miRNAs (83–99% in serum)[225]. These apparently contrasting observations could be due to technical differences in exosome isolation or to differences between serum and plasma[225].

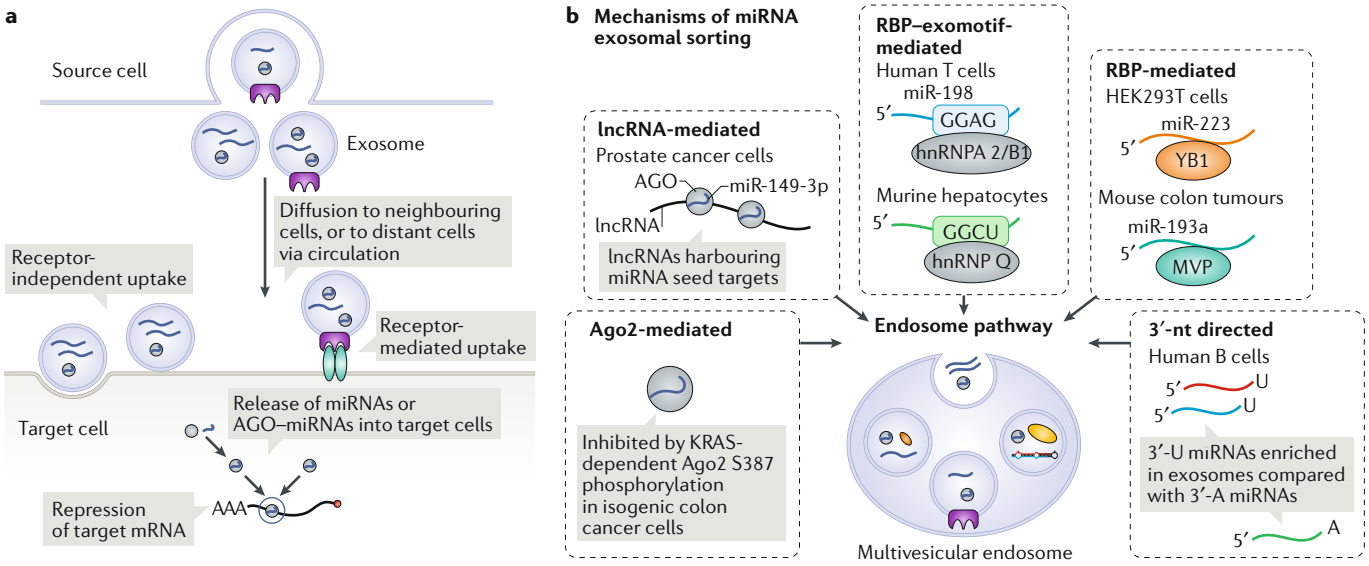


Fig. 6 | **Mechanisms of sorting miRNAs into exosomes. a** | MicroRNAs (miRNAs) can be packaged into exosomes and thus may contribute to intercellular signalling. Uptake of exosomes can be receptor-mediated or receptor-independent[277]. Upon entering a target cell, exosome-delivered miRNAs are thought to regulate target mRNAs[222]. **b** | Although the biological function of exosomal miRNAs is still incompletely understood, multiple mechanisms direct miRNAs into exosomes. Argonaute 2 (Ago2)-mediated sorting of specific miRNAs has been reported in isogenic colon cancer cells, and phosphorylation of Ago2 Ser387 inhibited loading of some miRNAs into exosomes[162]. Sorting based on sequence complementarity between miR-149-3p and exosomal long non-coding RNAs (lncRNAs) was shown in prostate cancer cells[232]. Exosomal RNA-binding proteins (RBPs) can direct miRNAs into exosomes by binding 'exomotifs' at miRNA 3′ ends. The exomotif GGAG promotes exosomal sorting of miR-198 by heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1) in human primary T cells[233], and the exomotif GGCU promotes hnRNP-Q-mediated exosomal sorting of miRNAs in murine hepatocytes[234]. No exomotifs are known for Y-box-binding protein 1 (YB1)-mediated sorting of miR-223 in HEK293T cells[235] and for major vault protein (MVP)-mediated sorting of miR-193a in mouse colon tumours[236]. Finally, in human B cells, 3′-adenylated miRNAs are depleted in exosomes whereas 3′-uridylated miRNAs are enriched in exosomes[237]. Exosomes have been reported to carry proteins, different RNAs and miRNA biogenesis components[226] but also Ago2–miRNA complexes[162] and AGO-free miRNAs[235].

Circulating miRNAs may have regulatory functions. Breast cancer cell-derived exosomes contain pre-miRNAs and the proteins required for cell-independent miRNA maturation, can induce cell proliferation in culture[226] and might have prometastatic properties in vivo[227,228]. A major concern with these studies is the technical challenge of clearly distinguishing between miRNA-mediated effects and changes caused by other exosomal components[229]. Moreover, exosomes from five different sources were found to have less than one miRNA molecule per exosome on average[230]. Nevertheless, a recent study showed that brown adipose tissue is a major source of exosomal miRNAs in humans and mice in support of a functional role of exosomal miRNAs[231]. Specifically, exosomes derived from a donor mouse expressing an exogenous miRNA in brown adipose tissue were found to repress a reporter gene in the liver of an acceptor mouse[231].

A biological function of exosomal miRNAs would require miRNA-specific mechanisms of sorting into exosomes (FIG. 6b). One proposed sorting mechanism is through exosomal lncRNAs and is based on the observed enrichment in exosomes of prostate cancer cell lines of miR-149-3p with lncRNAs harbouring its target sites[232]. Exosomal RBPs could also promote sorting by directly interacting with miRNA motifs termed 'exomotifs'. The exomotif GGAG located at the miRNA 3′ end was suggested to mediate binding of miR-198 and other exosomal miRNAs to the exosomal RBP heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1) in human primary T cells, thereby directing the miRNAs to exosomes[233]. Similarly, the exomotif GGCU, which is also preferentially located at miRNA 3′ ends, was suggested to recruit hnRNP Q (also known as SYNCRIP) in murine hepatocytes, yielding exosomal secretion of the bound miRNAs[234]. Other RBPs proposed to mediate miRNA sorting into exosomes, such as Y-box-binding protein 1 (also known as nuclease-sensitive element-binding protein 1) for miR-223 in HEK293T cells[235] and major vault protein (MVP) for miR-193a in colon tumour cells in mice[236], lack specific associated recognition motifs. The tumour suppressor miR-193a is an interesting case because the main reason for its sorting into exosomes appears to be its removal from the cytoplasm[236]. NTA at miRNA 3′ ends may also influence sorting, as reported for human B cells and B cell-derived exosomes, where adenylation was associated with miRNA depletion from and uridylation with miRNA enrichment in exosomes[237]. Finally, Ago2 Ser387 phosphorylation by the GTPase KRAS–MAPK pathway antagonizes exosomal sorting of Ago2 in colon cancer cells[162]. Although free, single-stranded miRNAs are rapidly degraded in the cell and thus are hardly functional[155], some reported sorting mechanisms[233,234] focus on miRNAs, with no mention of AGO proteins, or do not detect AGO proteins in exosomes[232]. Clearly, more studies are required to fully understand the mechanistic and functional aspects of exosomal miRNAs.

Circulating miRNAs
miRNAs present in circulation and found either as AGO–miRNA complexes or as cargo of vesicles (exosomes).

Regulation of miRNA target sites

The activity of miRNAs can also be modulated through changes in their target sites. RNA editing can alter target-site complementarity, and deregulation of target-site editing is associated with cancer[238,239]. Similarly, the t1A binding site on AGO does not recognize N^6-methyladenosine modification in RNA, raising the possibility that adenosine methylation of target mRNAs could subtly modulate their targeting by miRNAs, although this idea has yet to be tested in a cellular context[57]. Less subtly, formation of mRNA 3′-UTR isoforms can add or remove miRNA target sites, thereby altering mRNA susceptibility to miRNAs in a cell-type-specific or tissue-specific manner[240].

RBPs also can modulate miRNA–target interactions. Pumilio promotes cell-cycle re-entry of quiescent cells by binding the 3′ UTR of the mRNA encoding the tumour suppressor p27 (also known as CDKN1B) and remodelling it, thereby exposing target sites for miR-221 and miR-222 (REF.[241]). AU-rich element binding factor 1 (AUF1; also known as HNRNPD) facilitates interactions of Ago2 with mRNAs in HeLa cells[242]. The AU-rich element binding protein Hu-antigen R (HuR; also known as ELAVL1) derepresses the miR-122-targeted mRNA encoding high-affinity cationic amino acid transporter 1 (CAT1; also known as SLC7A1) by binding an AU-rich and U-rich region in the mRNA, thereby preventing miRNA function but apparently not miRNA binding[85]. Regulation by HuR appears to be widespread, as over 75% of human mRNAs that harbour miRNA-binding sites also possess HuR binding sites, often overlapping or in close proximity[243]. HuR prevents repression of p53 by miR-125b by binding to the mRNA and causing miRISC dissociation[244] and of programmed cell death protein 4 (PDCD4) by miR-21 by binding to targets in the mRNA and displacing the miRISC and by directly binding to miR-21 in MCF cells[245]. RBPs have also been suggested to regulate the Ago2 phosphorylation cycle, but no specific RBP has been discovered yet to do so[170].

Interestingly, in extracts of most adult mouse tissues, miRNAs are found in low-molecular-mass miRISCs of ~100 kDa, which is approximately the molecular mass of AGO with its miRNA guide and not bound to mRNA. By contrast, in cell lines, most miRNAs are found in high-molecular-mass miRISCs (up to 2 MDa) containing GW182, other proteins and target mRNAs. Consequently, in transformed cells, miRNA abundance may correlate more strongly with miRNA activity than in primary tissues, where additional regulation of AGO activity by the cell appears present[246]. Findings obtained in cell lines should therefore be considered with caution.

Future perspective

A number of interesting questions remain unanswered regarding the regulation of miRNA function. NTA at the 3′ end of miRNAs has been recognized as a widespread process[131,132]. However, neither the tissue-specific importance of the added nucleotides that promote miRNA stability or degradation nor the mechanism by which the cell selects which miRNAs to modify are known. Moreover, an extended miRNA 3′ end has been shown to improve interactions between miR-122 and HCV genomic RNA[113] and to alter the function of miR-222 to promote apoptosis in a breast cancer cell line[114], suggesting that 3′ isomir variation affects miRNA targeting in ways that remain to be understood. Thus, whereas altered seed-targeting has been explored in 5′ isomirs, very little is known about the functions and properties of 3′ isomirs.

The role of mRNA secondary and tertiary structure in miRNA–target recognition is also largely unexplored. Structures that render target sites inaccessible to the miRISC clearly inhibit silencing[247,248]. However, miRNA-binding sites have been identified within heavily structured segments of RNA viruses[203,208], raising the possibility that some structures may not be detrimental for targeting or might even enable recognition by miRNAs. Indeed, effective miR-159 target sites in *Arabidopsis thaliana* require an adjoining structural element composed of two stem loops[249]. The interactions between RNA structures and AGO and the degree to which they shape miRNA targeting remain unknown.

Although the activity of exosomal miRNAs and the presence of miRNA-selective exosomal sorting mechanisms suggest that exosomal miRNAs participate in intercellular communication, evidence in a physiological context remains elusive. The reported scarcity of miRNAs in exosomes[230] and the difficulty of disentangling miRNA-mediated effects from the effects of other exosomal cargoes add to this challenge[229]. Similarly, although exosomal Ago2-loaded miRNAs have been detected[162,226], so were single-stranded miRNAs, which would require loading into AGO in the target cells[235]. Thus, our understanding of exosomal miRNAs is far from complete.

Finally, the recent report of molecular condensation properties of AGO and TNRC6 (REF.[250]) connects miRNA regulation to the growing field of biological phase separation[251]. The data demonstrate that miRISCs can form large molecular condensates in vitro and in living cells, and it was hypothesized that the ability to form higher order complexes through molecular condensation may allow miRISCs to organize miRNA–target interactions within the cytoplasm and thereby modulate rates of mRNA translation and decay. This hypothesis raises the possibility that miRNA activity is regulated through the assembly of the miRISC itself by modulation of the biophysical properties of miRISC components.

In conclusion, although the first evidence of miRNAs was discovered over 25 years ago and major advances have been made since, many aspects of the complex mechanisms that govern the activity of these tiny regulators remain to be discovered and explored.

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