The widespread regulation of microRNA biogenesis, function and decay

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Abstract | MicroRNAs (miRNAs) are a large family of post-transcriptional regulators of gene expression that are ~21 nucleotides in length and control many developmental and cellular processes in eukaryotic organisms. Research during the past decade has identified major factors participating in miRNA biogenesis and has established basic principles of miRNA function. More recently, it has become apparent that miRNA regulators themselves are subject to sophisticated control. Many reports over the past few years have reported the regulation of miRNA metabolism and function by a range of mechanisms involving numerous protein–protein and protein–RNA interactions. Such regulation has an important role in the context-specific functions of miRNAs.

Deadenylation
The removal of the poly(A) tail from the mRNA 3′ end. Deadenylation is the first step in mRNA decay, and is generally followed by removal of the m^7G cap (the 7-methyl-guanosine-triphosphate structure at the 5′ end of mRNAs, which promotes their translation and protects them from degradation) and exonucleolytic 5′ to 3′ degradation of mRNA. Deadenylation is mainly mediated by the CAF1–CCR4 deadenylase complex.

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MicroRNAs (miRNAs) comprise a large family of ~21-nucleotide-long RNAs that have emerged as key post-transcriptional regulators of gene expression in metazoans and plants, and have revolutionized our comprehension of the post-transcriptional regulation of gene expression[1,2]. In mammals, miRNAs are predicted to control the activity of ~50% of all protein-coding genes. Functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated so far and that changes in their expression are associated with many human pathologies.

During the past decade we have learned much about the basic mechanisms of miRNA biogenesis and function[1–5]. However, more recently it has become apparent that miRNAs themselves are subject to sophisticated control, which takes place at the levels of both miRNA metabolism and function. The numbers of individual miRNAs expressed in different organisms (for example, ~800 in humans) are comparable to those of transcription factors or RNA-binding proteins (RBPs), and many are expressed in a tissue-specific or developmental-stage-specific manner, thereby greatly contributing to cell-type-specific profiles of protein expression. The nature of miRNA interactions with their mRNA targets, which involve short sequence signatures, makes them well suited for combinatorial effects with other miRNAs or RBPs that associate with the same mRNA. With the potential to target dozens or even hundreds of different mRNAs, individual miRNAs can coordinate or fine-tune the expression of proteins in a cell. These considerations call for a tight and dynamic regulation of miRNA levels and activity, particularly during rapid developmental transitions or changes in cellular environment.

Here, we provide an overview of the regulation of miRNA metabolism and function, and also discuss steps in the miRNA pathway that are likely targets of additional control. The Review is primarily focused on reactions in metazoans, but examples of miRNA regulation operating in plants are also described. Control of the miRNA pathway in plants[6] and the mechanistic aspects of miRNA biogenesis and function[1–5] have been described in other reviews.

Overview of miRNA biogenesis and function

miRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes (BOX 1). The pri-miRNAs fold into hairpins, which act as substrates for two members of the RNase III family of enzymes, Drosha and Dicer. The product of Drosha cleavage, an ~70-nucleotide pre-miRNA, is exported to the cytoplasm where Dicer processes it to an ~20-bp miRNA/miRNA* duplex. One strand of this duplex, representing a mature miRNA, is then incorporated into the miRNA-induced silencing complex (miRISC). As part of miRISC, miRNAs base-pair to target mRNAs and induce their translational repression or deadenylation and degradation (BOX 2). Argonaute (AGO) proteins, which directly interact with

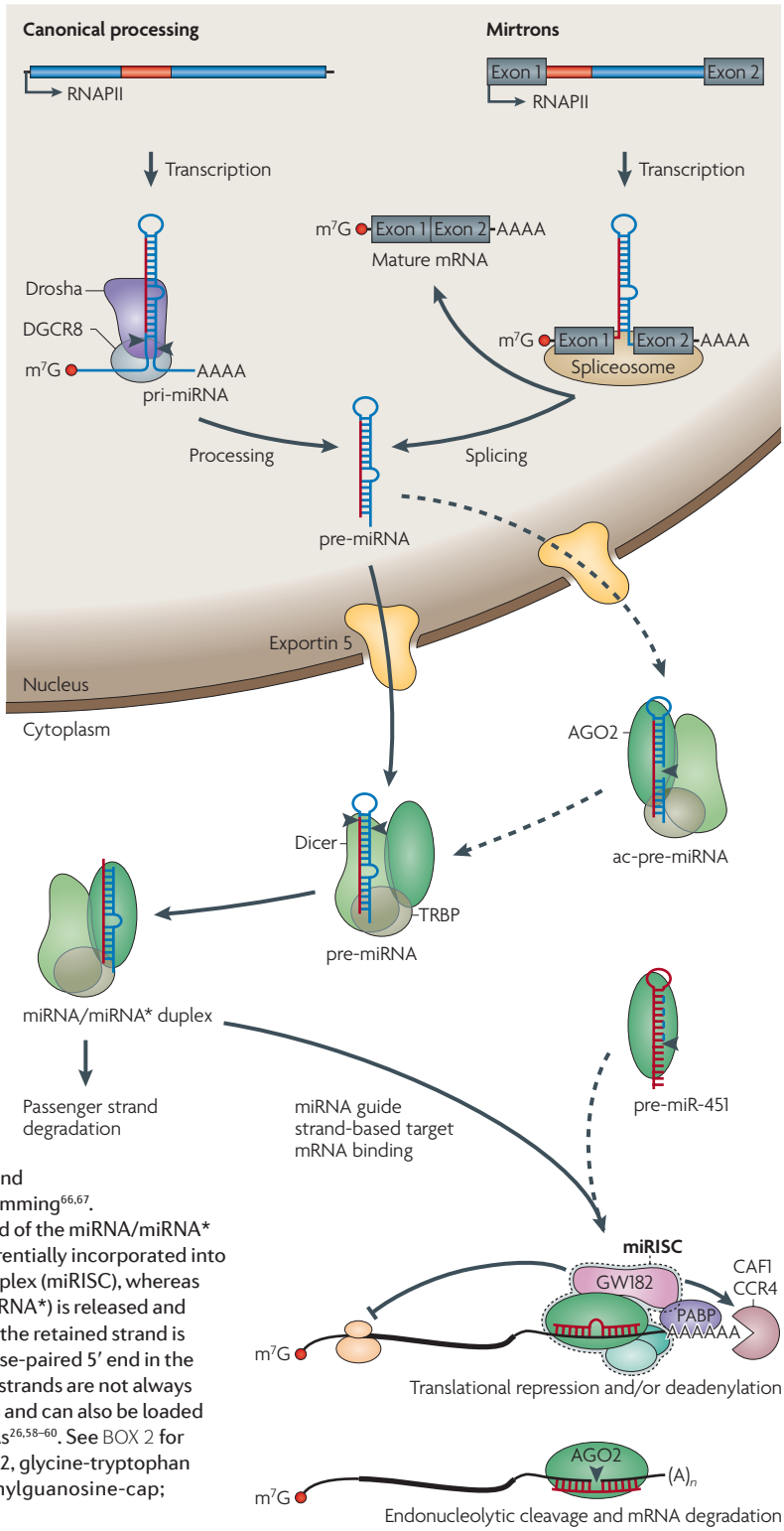
Box 1 | **MicroRNA biogenesis**

MicroRNAs (miRNAs) are processed from RNA polymerase II (RNAPII)-specific transcripts of independent genes or from introns of protein-coding genes[2,5]. In the canonical pathway, primary precursor (pri-miRNA) processing occurs in two steps, catalysed by two members of the RNase III family of enzymes, Drosha and Dicer, operating in complexes with dsRNA-binding proteins (dsRBPs), for example DGCR8 and transactivation-responsive (TAR) RNA-binding protein (TRBP) in mammals.

In the first nuclear step, the Drosha–DGCR8 complex processes pri-miRNA into an ~70-nucleotide precursor hairpin (pre-miRNA), which is exported to the cytoplasm. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing the Drosha–DGCR8 step. In either case, cleavage by Dicer, assisted by TRBP, in the cytoplasm yields an ~20-bp miRNA/miRNA* duplex.

In mammals, argonaute 2 (AGO2), which has robust RNaseH-like endonuclease activity, can support Dicer processing by cleaving the 3′ arm of some pre-miRNAs, thus forming an additional processing intermediate called AGO2-cleaved precursor miRNA (ac-pre-miRNA)[70]. Processing of pre-miR-451 also requires cleavage by AGO2, but is independent of Dicer and the 3′ end is generated by exonucleolytic trimming[66,67].

Following processing, one strand of the miRNA/miRNA* duplex (the guide strand) is preferentially incorporated into an miRNA-induced silencing complex (miRISC), whereas the other strand (passenger or miRNA*) is released and degraded (not shown). Generally, the retained strand is the one that has the less stably base-paired 5′ end in the miRNA/miRNA* duplex. miRNA* strands are not always by-products of miRNA biogenesis and can also be loaded into miRISC to function as miRNAs[26,58–60]. See BOX 2 for details of miRISC function. GW182, glycine-tryptophan protein of 182 kDa; m^{7}G, 7-methylguanosine-cap; PABP, poly(A) binding protein.



miRNAs, and glycine-tryptophan protein of 182 kDa (GW182) proteins, which act as downstream effectors in the repression, are key factors in the assembly and function of miRISCs. In their role in miRNA maturation both Drosha and Dicer are assisted by a number of cofactors or accessory proteins, with some playing an important regulatory function (see Supplementary information S1 (table)). Likewise, the formation of the miRISC and the execution of its activity involve many additional factors (BOX 1; Supplementary information S1 (table)).

Box 2 | **MicroRNA function**

Most animal microRNAs (miRNAs) imperfectly base-pair with sequences in the 3′-UTR of target mRNAs, and inhibit protein synthesis by either repressing translation or promoting mRNA deadenylation and decay (see the figure in BOX 1). Efficient mRNA targeting requires continuous base-pairing of miRNA nucleotides 2 to 8 (the seed region)[1]. Argonaute (AGO) proteins, which are directly associated with miRNAs, are core components of the miRNA-induced silencing complex (miRISC); most species express multiple AGO homologues: AGO1–AGO4 in mammals; dAGO1 and dAGO2 in flies; ALG-1 (argonaute-like gene) and ALG-2 in *Caenorhabditis elegans*. Mechanistic details of miRNA-mediated translational repression are not well understood, which is in contrast to mRNA deadenylation[3,4,82]. Deadenylation of mRNAs is mediated by glycine-tryptophan protein of 182 kDa (GW182) proteins, the other core components of miRISCs, which interact with AGOs and act downstream of them. While the amino-terminal part of GW182 interacts (through its GW repeats) with AGO, the carboxy-terminal part of mammalian and *Drosophila melanogaster* GW182 proteins interacts with the poly(A) binding protein (PABP) and recruits the deadenylases CCR4 and CAF1 (REFS 4,82). Interestingly, the PABP regions that are targeted by GW182 are also recognized by many other translational factors, suggesting that these interactions may be subject to sophisticated regulation[4]. In addition to the C terminus, *D. melanogaster* GW182 contains two additional regions that function in translational repression; the three repressive domains may be differentially regulated or may target distinct sets of mRNAs[151].

When miRISC containing AGO2 in mammals or dAGO2 in flies encounters mRNAs bearing sites nearly perfectly complementary to miRNA, these mRNAs are cleaved endonucleolytically and degraded[1–4]. Although rare in animals, this is a common mode of miRNA action in plants. However, miRNAs in plants may also imperfectly base-pair to mRNAs and repress translation[6].

Regulation of miRNA gene transcription

Transcription of miRNA genes is regulated in a similar manner to that of protein-coding genes, and is a major level of control responsible for tissue-specific or development-specific expression of miRNAs. Some examples of transcriptional control are summarized in BOX 3 and are also discussed in recent reviews[7,8]. Below, we only discuss a few aspects that are specifically related to miRNAs.

Control of gene expression by autoregulatory feedback loops is a common regulatory mechanism that is particularly important during cell fate determination and development. miRNAs are uniquely suited to participate in feedback circuits owing to their potential to directly base-pair with and repress mRNAs that encode factors involved in the biogenesis or function of the same miRNAs. Indeed, many examples have been described of miRNAs regulating their own transcription through single-negative or double-negative (or positive) feedback loops with specific transcription factors. For instance, the PITX3 transcription factor and miR-133b form a negative autoregulatory loop that controls dopaminergic neuron differentiation. PITX3 stimulates transcription of miR-133b, which in turn suppresses PITX3 expression[9]. More sophisticated regulation is provided by double-negative feedback loops like the one involving miRNAs lys-6 and miR-273, and transcription factors DIE-1 and COG-1 in *Caenorhabditis elegans* (BOX 3). This loop is instrumental in determining cell fate decisions between two alternative types of chemosensory neurons[10]. By fine-tuning miRNA expression and adjusting it to physiologically optimal levels, the circuits described above have a strong impact on the precise spatiotemporal expression of miRNA targets.

Seed sequence
Nucleotide positions 2–8 from the 5′ end of the microRNA, which generally perfectly base-pair with target mRNA, and are important for defining the target repertoire of a microRNA.

Interestingly, in both flies and mammals, some miRNAs are convergently transcribed from both DNA strands of a single locus, giving rise to two miRNAs with distinct seed sequences[11,12]. In *Drosophila melanogaster*, sense and antisense transcripts of miR-iab-4 are expressed in non-overlapping embryonic segments, and processed miRNAs regulate development by targeting homeotic Hox genes expressed in specific embryonic domains[11,12].

Regulation of miRNA processing

Control of miRNA processing has emerged as another important mechanism in defining the spatiotemporal pattern of miRNA expression.

Regulation of Drosha, Dicer and their double-stranded RBP partners. Drosha and Dicer generally operate in complexes with double-stranded RBP partners, such as DGCR8 and transactivation-responsive (TAR) RBP (TRBP) in mammals. Both the levels and activity of all of these proteins are subject to regulation that affects the accumulation of miRNAs. For example, DGCR8 has a stabilizing effect on Drosha through the interaction with its middle domain, whereas Drosha controls DGCR8 levels by cleaving hairpins present in the *DGCR8* mRNA, thereby inducing its degradation[13,14]. Keeping the Drosha to DGCR8 ratio in check may be important, as a threefold excess of DGCR8 dramatically inhibits Drosha processing activity *in vitro*[15]. Interestingly, haem binding to DGCR8 promotes its dimerization and facilitates pri-miRNA processing[16].

Accumulation of Dicer is dependent on its partner TRBP, and a decrease in TRBP leads to Dicer destabilization and pre-miRNA processing defects[17–19]. In human carcinomas, mutations causing diminished TRPB expression impair Dicer function[18]. TRBP itself is stabilized through serine phosphorylation, catalysed by mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK)[19]. Cell growth and survival are elevated on TRBP phosphorylation, possibly as a consequence of upregulation of growth-stimulatory miRNAs and a decrease in let-7, a known suppressor of proliferation[19]. Notably, let-7 can target *Dicer* mRNA[20], forming a negative feedback loop with the potential to broadly influence miRNA biogenesis, both physiologically and in cancer; other mechanisms can also contribute to the reported changes in Dicer and also Drosha levels in tumours[21].

Dicer is a large (~200 kDa) multi-domain protein, which is not only involved in the cleavage of pre-miRNAs but also participates in loading miRNAs into miRISC[2]. Dicer's amino-terminal helicase domain may have an autoregulatory function as its removal stimulates catalytic activity of human Dicer, similarly as does the addition of TRBP[22]. There is also evidence to suggest that proteolysis may play a role in modulating Dicer activity[23,24] or even changing its specificity towards DNA[25].

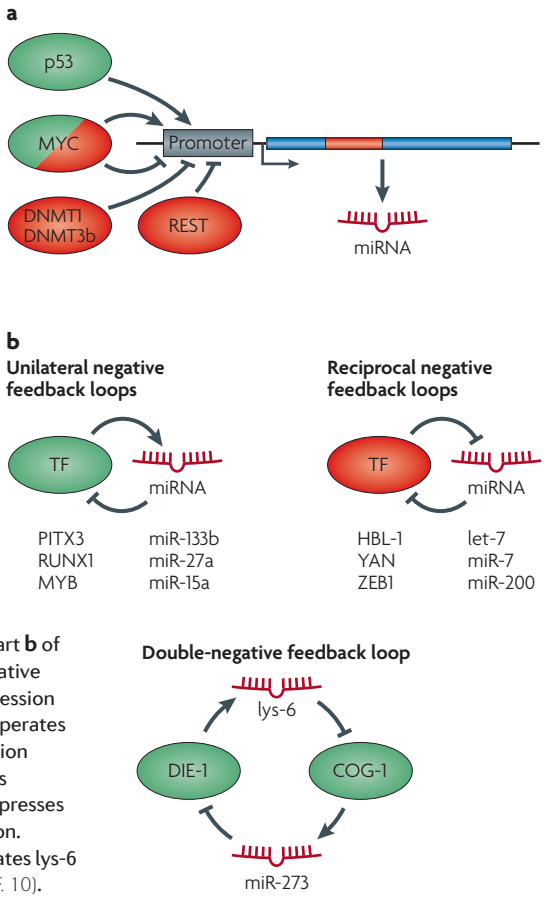
The position of Drosha and Dicer cleavage determines the identity of 5′-terminal and/or 3′-terminal miRNA nucleotides[2]. Notably, processing of some precursors by these enzymes is not uniform and generates

Box 3 | Regulation of microRNA gene transcription

The promoter regions of autonomously expressed microRNA (miRNA) genes are highly similar to those of protein-coding genes[152,153]. The presence of CpG islands, TATA box sequences, initiation elements and certain histone modifications indicate that the promoters of miRNA genes are controlled by transcription factors (TFs), enhancers, silencing elements and chromatin modifications, which is similar to protein-coding genes.

Activators and repressors of miRNA transcription

Many TFs regulate miRNA expression positively or negatively in a tissue-specific or developmental-specific manner (see part **a** in the figure; transcriptional activators or repressors are shown in green and red, respectively). For example, MYC and MYCN both stimulate expression of the miR-17-92 oncogenic cluster in lymphoma cells[154] and miR-9 in neuroblastoma cells[155], but inhibit expression of several tumour suppressor miRNAs (for example, miR-15a), which promote MYC-mediated tumorgenesis[156]. p53 stimulates the expression of miR-34 and miR-107 families, which enhances cell cycle arrest and apoptosis[157]. The RE1 silencing transcription factor (REST) recruits histone deacetylases and methyl CpG binding protein MeCP2 to the *mir-124* gene promoter, preventing its transcription in neuronal progenitors and non-neuronal cells[158]. REST is downregulated upon differentiation, allowing for high miR-124 expression in post-mitotic neurons. Transcription of miR-148a, miR-34b/c, miR-9 and let-7 is dependent on their gene promoter methylation status, which is regulated by the DNMT1 and DNMT3b DNA methyltransferases[159].



Regulatory networks of miRNA expression

miRNAs frequently act in regulatory networks with TFs, which can drive or repress the expression of the miRNAs. A few examples of autoregulatory feedback loops are shown in part **b** of the figure, with examples of specific miRNAs and TFs indicated. Unilateral or reciprocal-negative feedback loops (single or double loops) result in oscillatory or stable mutually exclusive expression of the TF and miRNA components. The double-negative feedback loop shown in the figure operates in the chemosensory neurons of *Caenorhabditis elegans*. Here, proper transcriptional activation and/or inactivation is accomplished by spatially controlled miRNA expression, and facilitates establishment of the left–right asymmetry of 'ASE' chemosensory neurons. The COG-1 TF represses the left ASE (ASEL) cell fate in the right ASE (ASER) neuron and stimulates miR-273 expression. miR-273 targets the DIE-1 transcription factor in ASER but not in ASEL, in which DIE-1 activates lys-6 expression and promotes the ASEL-specific cell fate. In ASEL, COG-1 is blocked by lys-6 (REF. 10).

miRNA isoforms with different termini. Heterogeneity at the 5′ end in particular can have important functional consequences, as it affects the seed register of miRNAs and, consequently, changes the identity of targeted mRNAs[26]. The thermodynamic stability of the miRNA duplex ends determines which strand, miRNA or miRNA*, is preferentially loaded into miRISC (BOX 1). Hence, cleavage heterogeneity can affect the end stability and alter strand selection. In addition, identity of the 5′-terminal nucleotide may also directly affect the efficiency of miRNA loading into miRISC, independent of the duplex end stability[27]. Generally, most miRNA genes produce one dominant miRNA species. However, the ratio of miRNA to miRNA* can vary in different tissues or developmental stages, which probably depends on specific properties of the pre-miRNA or miRNA duplex, or on the activity of different accessory processing factors[26,28,29]. Moreover, the ratio might be modulated by the availability of mRNA targets as a result of enhanced destabilization of either miRNA or miRNA* occurring in the absence of respective complementary mRNAs (REF. 146 and see below).

Role of accessory proteins. Recent work has identified a battery of proteins that regulate processing (either positively or negatively) either by interacting with Drosha or Dicer or by binding to miRNA precursors[30] (FIG. 1; Supplementary information S1 (table)). We discuss a few examples below. Although the activity of some of the regulators is restricted to specific miRNA families, most affect the processing of a broader range of miRNA precursors, suggesting that their activity can affect the expression of entire gene networks.

The best-studied negative regulator of miRNA biogenesis is LIN-28, which can act at different levels[31] (FIG. 1). Mature let-7 does not accumulate in undifferentiated embryonic stem cells (ESCs) and other progenitor cells, despite the high expression of the pri-let-7 transcripts[32]. The processing failure is due to LIN-28 binding to the terminal loop of pri-let-7, which interferes with cleavage by Drosha[31]. Binding of LIN-28 to pre-let-7 can also block its processing by Dicer. In the latter case, LIN-28 induces the 3′-terminal polyuridylation of pre-let-7 by attracting the TUT4 (also known as Zcchc11 or PUP-2 in worms) terminal poly(U) polymerase[33–37]. Uridylation prevents Dicer processing and targets pre-let-7 for degradation by an as yet unknown RNase[35] (FIG. 2). Repression of LIN-28 is highly specific and affects only members of the let-7 family[35,38].

The LIN-28–let-7 regulatory system is highly conserved in evolution and plays an important role in maintaining the pluripotency of ESCs, and also in

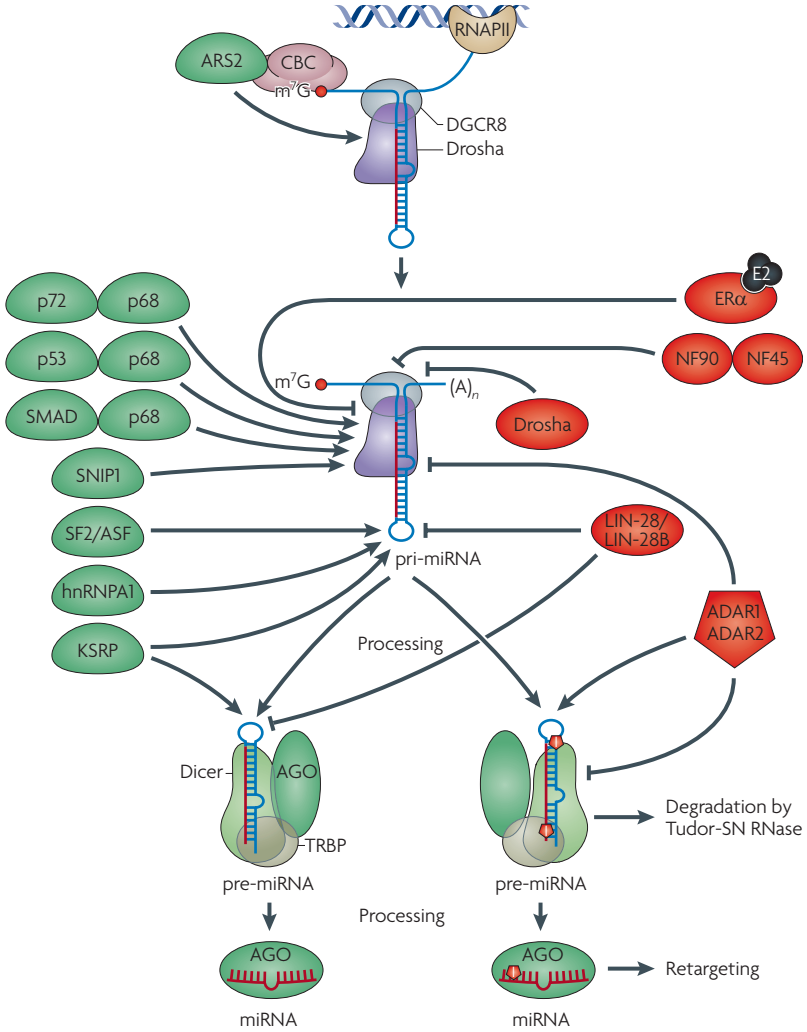


Figure 1 | **Regulators of microRNA processing.** Several activators and repressors regulate microRNA (miRNA) biogenesis through either protein–protein or protein–RNA interactions. Arsenite-resistance protein 2 (ARS2) supports Drosha processing of pri-miR-21, pri-miR-155 or pri-let-7, providing functional coupling of pri-miRNA transcription and processing[47,48]. The p68 and p72 helicases, identified as components of the Drosha Microprocessor complex, are thought to stimulate processing of one-third of murine pri-miRNAs[154]. p68 and p72 interact with various proteins and possibly act as a scaffold that recruits other factors. The SMAD–p68 complex, or a SMAD nuclear interacting protein 1 (SNIP1), enhances processing of pri-miRNAs, as well as the accumulation of mature miRNAs, like pri-miR-21 (REFS 43,44). Splicing factor SF2/ASF promotes Drosha-mediated processing of pri-miR-7 (REF. 50). Operating by direct protein–RNA interactions, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) binds to the loop regions in pri-miR-18a and facilitates its Drosha-mediated processing, possibly by inducing a re-arrangement of RNA structure[51]. The splicing regulatory protein KSRP binds to a subset of pri-miRNAs that have GGG triplet motifs in their terminal loops and enhances processing by Drosha. KSRP also promotes Dicer-mediated processing of some pre-miRNAs in the cytoplasm[52]. While the LIN-28 repressor effect seems to be restricted to let-7 family members, the nuclear factor NF90–NF45 heterodimer blocks maturation of a broader range of pre-miRNAs[175]. NF90–NF45 interacts with the stem of pri-miRNAs in a sequence-independent way and prevents DGCR8 binding[175]. The estrogen receptor α (ERα) interacting with p68 and p72 helicases[176] and Drosha[177] affects the Drosha complex formation and represses processing of several pri-miRNAs[177]. Drosha can also negatively regulate miRNA processing by decreasing DCGR8 levels. Editing of pri-miRNAs or pre-miRNAs by adenosine deaminases that act on RNA (ADAR1 and ADAR2) affects accumulation of mature miRNAs, and might also influence miRNA target specificity[54–57]. AGO, argonaute; CBC, cap-binding complex; m^{7}G, 7-methylguanosine-cap; RNAPII, RNA polymerase II; TRBP, transactivation-responsive (TAR) RNA-binding protein.

development and oncogenesis. Inhibition of let-7 maturation by LIN-28 is essential for maintaining self-renewal of ESCs and for blocking their differentiation; during differentiation LIN-28 levels decrease and let-7 miRNAs accumulate. Let-7 functions as a tumour suppressor by targeting several oncogenes, including *MYC*, *KRAS* and cyclin D1 (*CCND1*)[39]. By repressing maturation of let-7 miRNAs, LIN-28 acts as an oncogene; indeed, activation of LIN-28 is found in many human tumours[40]. Interestingly, LIN-28 itself is targeted by let-7, indicating that LIN-28 and let-7 control the levels of each other following differentiation[32].

The p68 and p72 helicases, identified as components of the Drosha Microprocessor complex, are thought to stimulate processing of one-third of murine pri-miRNAs[41]. In *p68* or *p72* knockout cells, levels of pre-miRNAs, but not pri-miRNAs, are significantly reduced as a consequence of attenuated Drosha binding and pri-miRNA processing[41]. p68 and p72 interact with a range of proteins, possibly acting as a scaffold that recruits factors to the Drosha complex and promote pri-miRNA processing. The p68-mediated interaction of the Drosha complex with the tumour suppressor p53 has a stimulatory effect on pri-miR-16-1, pri-miR-143 and pri-miR-145 processing in response to DNA damage in cancer cells[42].

The signal transducers of the transforming growth factor-β (TGFβ) and bone morphogenetic protein (BMP) signalling cascade, SMADs, regulate gene expression at the level of transcription, but also control Drosha-mediated miRNA processing (FIG. 1). SMADs are present, together with Drosha and p68, in a complex interacting with pri-miR-21. Upregulation of miR-21, induced by TGFβ and BMP4, facilitates differentiation of vascular smooth muscle cells into contractile cells[43]. It is unclear how SMADs control miRNA biogenesis. One possibility is that they interact with Drosha–pri-miRNA complexes through protein cofactors; alternatively, they might recognize consensus sites in pri-miRNAs. SMAD nuclear interacting protein 1 (SNIP1), a SMAD partner, is also found in Drosha complexes[44], and depletion of SNIP1 reduces expression of some miRNAs, including miR-21 (REF. 44). DAWDLE, a homologue of SNIP1 in *Arabidopsis thaliana*, promotes efficient pri-miRNA processing, probably by stimulating Dicer-like-1, a functional homologue of Drosha[44]. The SNIP1 complex regulates co-transcriptional splicing and stability of *CCND1* transcripts, and associates with both the *CCND1* gene and mRNA[45]. SNIP1 might also be involved in the coupling of transcription and the processing of pri-miRNAs.

Much evidence exists to suggest that, as in the case of mRNA processing, pri-miRNA processing by the Drosha complex also occurs co-transcriptionally, and that the excision of pre-miRNAs from introns may even precede splicing[46]. Pri-miRNA processing has been also linked to the 5′-terminal capping of transcripts. Arsenite-resistance protein 2 (ARS2), a component of the nuclear cap-binding complex, interacts with Drosha and is required for pri-miRNA stability and processing in flies and mammals[47,48] (FIG. 1). SERRATE, the ARS2 counterpart in

A. thaliana, plays a similar role[49]. ARS2 seems to influence the processing of a subset of pri-miRNAs[47], arguing for a regulatory rather than constitutive function.

Several splicing factors also function as miRNA processing regulators, independently from their role in splicing. SF2/ASF binds to pri-miR-7 and promotes its cleavage by Drosha[50]. Interestingly, this interaction is a subject of autoregulatory feedback, as mature miR-7 targets *SF2/ASF* mRNA[50]. Heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and KSRP (also known as KHSRP), which are known alternative splicing factors, also serve as auxiliary proteins in the biogenesis of miRNAs, sometimes acting at both the Drosha and Dicer cleavage steps[51,52] (FIG. 1).

RNA editing of miRNAs

Adenosine deaminases that act on RNA (ADARs) catalyse the conversion of adenosine to inosine in dsRNA segments, altering the base-pairing and structural properties of transcripts. Many pri-miRNAs and pre-miRNAs are targeted by ADARs at different stages in their processing, and the modifications can affect both Drosha-mediated and Dicer-mediated cleavage, and also prevent the export of pre-miRNAs (FIG. 1).

Selective editing inhibits cleavage of human pri-miR-142 by Drosha and contributes to its degradation by the nuclease Tudor-SN, which has affinity for dsRNA containing inosine-uracil pairs[53]. Editing of pre-miR-151 prevents Dicer processing, resulting in accumulation of pre-miR-151[54]. However, editing can also enhance Drosha processing[55]. These differential editing effects may underlie tissue-specific expression of some miRNAs. Interestingly, pri-miR-376a-2 processing in *D. melanogaster* is inhibited even by catalytically inactive ADAR2, which binds pri-miRNA and inhibits Drosha activity[56]. As ADARs interact with a range of pri-miRNAs, they may influence the accumulation of many miRNAs in an editing-independent way.

Editing within miRNA seed sequences can have an important impact on the target specificity of edited miRNAs[56,57] (FIG. 1). A single adenosine-to-inosine conversion in the seed sequence of miR-376-5p retargets it to the phosphoribosyl pyrophosphate synthetase 1 (*PRPS1*) mRNA, which is not repressed by unedited miRNA[57]. Deep sequencing has identified 16 edited mouse mature miRNAs, 8 of them in the seed; all originated from the brain, a tissue with the highest ADAR expression levels[26]. Hence, editing events can increase the number of miRNA targets.

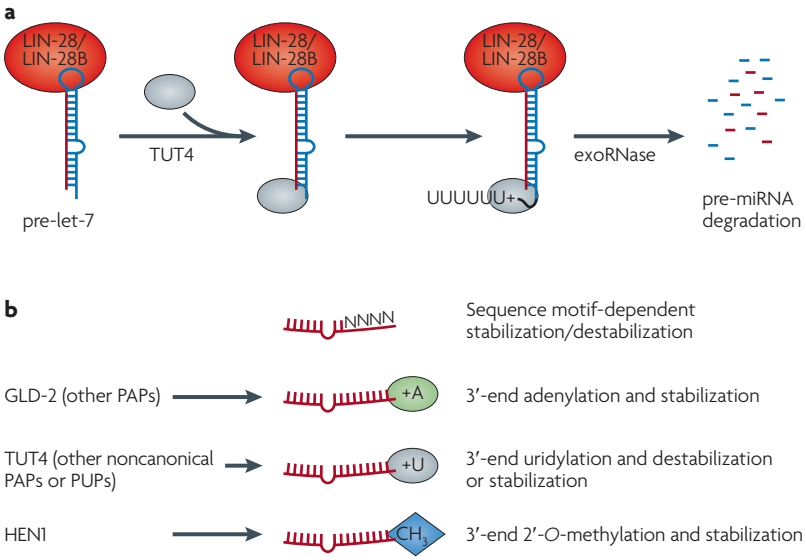


Figure 2 | **Modification at the 3′ end of microRNAs regulates stability.** The post-transcriptional addition of non-genome-encoded nucleotides to the 3′ end of either pre-microRNA (miRNA) or mature miRNA affects miRNA stability or abundance. **a** | The RNA-binding protein LIN-28 promotes uridylation of pre-let-7 in *Caenorhabditis elegans* and mammalian cells by recruiting the poly(U) polymerase (PUP) TUT4 (also known as Zcchc11 or PUP-2 in worms), which adds multiple uracil resides to the 3′ end of RNA substrates. Polyuridylation of pre-let-7 prevents Dicer processing and induces precursor degradation by an unknown nuclease. **b** | RNA stability is influenced by the 3′ end sequence motif or modifications (adenylation by poly(A) polymerase (PAP), uridylation by PUP or methylation) that mark miRNAs for degradation or protect them against exonucleolytic activity, depending on the specific miRNAs and the tissue. In liver cells, a single adenine residue added to the 3′ end of miR-122 prevents trimming and protects the miRNA against exonucleolytic degradation[142]. miRNA methylation at the 3′ end by HEN1 methyltransferase prevents uridylation and degradation in plants[144]. In *Drosophila melanogaster*, miRNAs that are sorted into Argonaute 2 (AGO2) instead of AGO1 are, like small interfering RNAs, modified at the 3′ end by methylation[58–60]. This modification is likely to increase their stability.

Regulation of miRNA function

The miRNA pathway is also extensively controlled at steps downstream of miRNA biogenesis. In addition to the miRISC core components — AGO and GW182 proteins — which represent the most obvious targets for regulation, dozens of other proteins have been identified which are implicated in positive or negative control of miRNA effects. Here, we summarize established examples of regulation that occur at effector steps of repression by miRNAs and discuss other potential targets of regulatory events.

Regulation at the level of AGO proteins. In many organisms, two or more different AGO or GW182 proteins operate in the miRNA pathway. Do different AGO or GW182 proteins have preferences for specific sets of miRNAs? Can association with a particular AGO or GW182 paralogue have an effect on the potency or even mechanism of miRNA repression? Recent data suggest that the control of miRNA function may occur at the level of AGO selection; so far no evidence exists supporting specific functions of individual GW182 paralogues.

Of the two AGO proteins in *D. melanogaster* dAGO1 is primarily dedicated to the miRNA pathway whereas dAGO2, which has a more potent endonuclease activity, functions in RNAi. Interestingly, some miRNAs, particularly those originating from hairpins with nearly perfect stems or those representing miRNA* strands, are selectively incorporated into dAGO2 complexes[58–60]. The functional consequences of sorting miRNAs into dAGO2 are not entirely clear[60]. The dAGO2 miRISC would be expected to primarily function in cleaving perfectly complementary targets, but such targets are rare. Notably, a recent report has indicated that dAGO2 can also act as a translational repressor, although it inhibits translation

by a mechanism that is independent of GW182 and distinct from that used by dAGO1 (REF. 61).

In contrast to *D. melanogaster*, all four vertebrate AGO proteins — including AGO2, which is able to endonucleolytically cleave perfectly complementary RNA targets — seem to have largely overlapping functions in miRNA repression[62], with no or only weak miRNA sorting preferences[63–65]. Nevertheless, recent findings point to some distinct functions of individual AGOs in vertebrates. AGO2, but not Dicer, functions in processing of miR-451 and remains specifically associated with this miRNA[66,67]. Although the role of AGO2 in miR-451 maturation requires its endonucleolytic activity, the protein also seems to have a specific but cleavage-independent role in haematopoesis[68]. Differential effects of mammalian AGOs are also supported by the observation that individual AGOs differ in their potency to repress protein synthesis when tethered to mRNA. Hence, differences in the relative abundance of individual AGO proteins may affect the strength of miRNA repression in particular cells or tissues[69]. Overexpression of each individual AGO family member enhances the abundance of mature miRNAs in HEK293 cells[70], which suggests that in these cells concentration of AGO proteins is a limiting factor in the formation of the miRISC. As different miRNAs may differ in their intrinsic ability to be loaded into the miRISC, changes in cellular concentration of AGO proteins might have not only quantitative but also qualitative effects on a range of miRNAs associated with miRISCs.

Significantly, several mechanisms have been described that regulate AGO2 levels in mammalian cells, including stabilization of AGO2 by the chaperone heat shock protein 90 (HSP90)[71,72], and modulating effects of protein modifications[73–75]. The TRIM-NHL protein TRIM71 promotes AGO2 polyubiquitylation and subsequent proteasomal degradation, resulting in impaired miRNA-mediated silencing[74]. Hydroxylation of the AGO2 Pro700 by type I collagen prolyl-4-hydroxylase C-P4H(I) stabilizes AGO2 and increases its localization to processing bodies (P-bodies)[73]. Increased recruitment of AGO2 to P-bodies was also observed on Ser387 phosphorylation, which occurs in response to stress and mediated by the MAPK/p38 kinase signalling pathway[75]. As cellular stress can modulate the degree of miRNA repression[76,77], it will be interesting to determine whether this effect is associated with modification of miRISC proteins.

Djuranovic *et al*. have proposed that AGO proteins that function in miRNA-mediated repression are subject to allosteric regulation through the binding of the miRNA and the mRNA 5′ cap to distinct sites in the AGO MID domain[78]. According to the authors, binding of miRNA to the protein would increase its affinity for the capped mRNA. The possibility that AGO proteins inhibit translation by directly contacting the 5′ cap has been reported[79], but has subsequently been challenged by demonstrations that mutations that abolish the proposed cap interaction are also defective in the recruitment of GW182, a protein essential for repression[80]. Unfortunately, the allosteric model suffers from a similar difficulty, as mutations that abolish allosteric effects also affect the binding of GW182. Despite these reservations, AGO allostery remains an interesting model, and the identification of mutations in AGO that uncouple allosteric effects from GW182 binding will be important. Notably, structural studies have demonstrated that prokaryotic AGO proteins undergo profound conformational changes in the course of target recognition[81].

Regulation at the level of GW182 proteins. A role for GW182 proteins as effectors of the repressive function of miRISCs has only been recognized recently and, consequently, little is known about their regulation. There are three GW182 paralogues in mammals (trinucleotide repeat-containing proteins TNRC6A, TNRC6B and TNRC6C) and a single protein in *D. melanogaster*. The *C. elegans* functional counterparts, AIN-1 and AIN-2, contain GW repeats but lack other domains that are characteristic of vertebrate and insect GW182 proteins[82]. In mammals, multiple transcription start or splice variants of GW182s are expressed[83].

TNRC6A was originally identified as a highly phosphorylated protein[84], yet the consequences of this phosphorylation and the identity of the signals mediating it are unknown. TNRC6A levels fluctuate during the cell cycle, which correlates with the number and size of P-bodies[85]. As cell cycle progression is associated with major phosphorylation and dephosphorylation events, it will be important to determine whether TNRC6A modification is linked to the cell cycle. Inhibition of HSP90 activity diminishes TNRC6A levels[71], and there is some evidence that TNRC6A also undergoes ubiquitylation[86]. Some GW182 proteins contain a putative ubiquitin-associated domain. All these observations raise questions about a possible role of protein modifications in the function and stability of GW182s, and their interaction with other proteins.

Role of proteins other than AGO and GW182. The core miRISC components, AGO and GW182, interact with many additional factors that are either required for miRNA function or for its modulation. Some of these factors have well-documented roles in translational repression or mRNA decay and some represent components of P-bodies. So far, the mode of action for most of the accessory proteins remains unknown. All known proteins of this category are listed in Supplementary information S1 (table); we discuss the properties of a few of them below, grouping them together based on their structural or enzymatic properties.

Many miRISC-interacting proteins belong to the family of DExD/H RNA helicases (for example, MOV10/Armitage, RHA and RCK/p54; see Supplementary information S1 (table)), which, generally catalyse ATP-dependent unwinding of RNA duplexes or remodelling of RNA or RNP structures[87]. Such activities are of potential importance for miRISC assembly, binding to and dissociation from mRNA targets, or miRISC disassembly and turnover. For example, human RHA, which binds AGO2, Dicer and TRBP, and the *D. melanogaster* Armitage, facilitate loading of small RNAs into the RISC[88,89].

Allosteric regulation
A mechanism by which an event at one region in a protein causes an effect at another site.

RNP
(Ribonucleoprotein). A complex of RNA and proteins. In the case of mRNPs the complex assembles on mRNA; in the case of miRNPs (also known as microRNA-induced silencing complexes), this involves microRNAs instead.

Another group of factors attenuating or enhancing the effect of miRNAs is represented by RBPs such as RBM4, HuR, fragile X mental retardation protein (FMRP) or DND1. Their association with miRISC components is often RNA-dependent, suggesting that they do not directly interact with miRISCs, but rather are recruited to the same targeted mRNA. The interplay between miRISCs and RBPs at the 3′-UTR of target mRNAs is discussed further below.

Several ubiquitin E3 ligases of the TRIM-NHL family act as positive or negative modulators of miRNA function[74,90–92]. Although all TRIM-NHL proteins share similar domain architecture and associate with AGO proteins, they seem to affect miRNA regulation in different ways. While *D. melanogaster* Mei-P26 functions as a negative regulator that decreases levels of mature miRNAs[91], mammalian TRIM32 and *C. elegans* NHL-2 enhance miRNA activity without changing miRNA levels. The strong interaction between NHL-2 and the worm homologue of RCK/p54 further suggests an involvement of NHL-2 in a step downstream of miRNA biogenesis[90]. Interestingly, TRIM32 and NHL-2 seem to enhance activity of only some miRNAs[90,92]. It is unknown how TRIM32 or NHL-2 enhance miRISC activity and whether they are able to bind RNA, either directly or through additional proteins. Specificity of these proteins for only selected miRNAs could possibly arise from the recognition of unique features of the miRNA/mRNA duplex or selective enrichment of TRIM-NHL binding sites in the vicinity of sites recognized by a specific miRNA. Recently, mammalian TRIM71 was shown to attenuate repression by miRNAs by promoting polyubiquitylation and proteasomal degradation of AGO2 (REF. 74). TRIM32 does not catalyse ubiquitylation of AGO and, consequently, its E3 ligase domain is dispensable for enhancing miRNA repression[92]. Whether Mei-P26 exerts its effect by ubiquitylating miRISC pathway components is unknown.

Several other proteins that interact with the miRISC have also been implicated in modulating miRNA function. One unanticipated miRISC cofactor is the nuclear import receptor importin 8 (IMP8). IMP8 associates with all four human AGO proteins independently of RNA, and localizes to the nucleus and P-bodies. IMP8 is required for efficient binding of AGO2 to a large set of target mRNAs[93]; possibly, it acts as a chaperone facilitating the binding of miRISC to target mRNAs.

One of the first AGO2-interacting proteins to be identified was the chaperone HSP90 (REF. 72), which stabilizes newly synthesized, unloaded AGO2 (REF. 71) and affects AGO2 localization and, possibly, function[71,72,94]. Inhibition of HSP90 activity decreases AGO and GW182 protein levels[71,72], and results in a loss of microscopically visible P-bodies in mammalian cells. These observations were recently complemented by *in vitro* studies showing that HSP90 facilitates loading of small RNA duplexes into AGO proteins of mammals, flies and plants[95,96].

The interplay between miRISCs and RBPs. The effects of miRNAs can be modulated by RBPs binding to the same mRNA (FIG. 3). Several examples of RBPs that counteract (for example, DND1, HuR and apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3G)) or facilitate (for example, FMRP, PUF and HuR) miRNA-mediated repression have been described. HuR, a member of the embryonic lethal abnormal vision (ELAV) family of proteins, translocates from the nucleus to the cytoplasm in response to stress. Upon binding to AU-rich elements in the *CAT-1* mRNA 3′-UTR, it relieves miR-122-mediated repression of this mRNA in Huh7 cells. The mRNA is released from P-bodies and recruited to polysomes for active translation[76]. Another RBP, DND1, antagonizes miR-430 repression of *NANOS1* and *TDRD7* mRNAs by binding to sequences that overlap with miRNA sites. As DND1 is expressed in primordial germ cells, but not somatic cells, it makes the miR-430 repression effectively cell specific[97,98].

The PUF proteins represent RBPs that collaborate with miRISCs. In *C. elegans*, PUF-9 synergizes with let-7 to regulate the repression of a shared target mRNA[99] and a systematic study of human PUFs has shown that miRNA sites are enriched in the vicinity of PUF elements[100]. The same RBP can, depending on the mRNA or cellular context, either prevent or activate miRISC repression. For example, in contrast to the CAT-1/miR-122 situation, HuR synergizes with let-7 to repress *MYC* mRNA translation[101]. Likewise, the interplay between FMRP and miRISCs is probably quite complex (see below).

Taking into account that the numbers of different RBPs and miRNAs expressed in metazoans reach several hundreds, the interplay between them at mRNA 3′-UTRs might be a general regulatory mechanism. Not only could RBPs modulate miRISC effects, but miRISCs could also activate or repress RBP function by either competing for binding sites or blocking RBP activity by binding to them (FIG. 3). In support of the latter possibility, miR-328 can relieve inhibition of *C/EBPA* mRNA by sequestering the negative translational regulator hnRNP protein E2 (REF. 102). As miR-328 can also act as a genuine miRNA, it will be interesting to see what governs its partitioning between AGO and E2 proteins.

Translational activation by miRNAs. Several reports indicate that miRNAs not only act as repressors but can also act as activators of translation. Under conditions of serum starvation (or general growth arrest, or at the G0 stage) the AGO2–miRISC complex has been shown to switch from a translational repressor to an activator. The switch required fragile X-related protein 1 (FXR1), a paralogue of FMRP[103,104]. Two other examples of activation include stimulation of 5′ TOP mRNAs translation (this class encompasses most mRNAs that encode ribosomal proteins) by miR-10a[105] and hepatitis virus C by miR-122 (REF. 106). It will be interesting to find out whether the change in AGO2 function is associated with its post-translational modification or its ability to interact with GW182.

Importance of intracellular localization

The appropriate subcellular localization of a protein or an RNP is essential to their function and regulation. Compartmentalization can control access to binding

3′-UTR
The 3′-UTR controls many aspects of mRNA metabolism, such as transport, localization, efficiency of translation and stability. 3′-UTRs can extend over several kilobases and generally contain binding sites for various regulatory proteins and microRNAs allowing dynamic and combinatorial regulation.

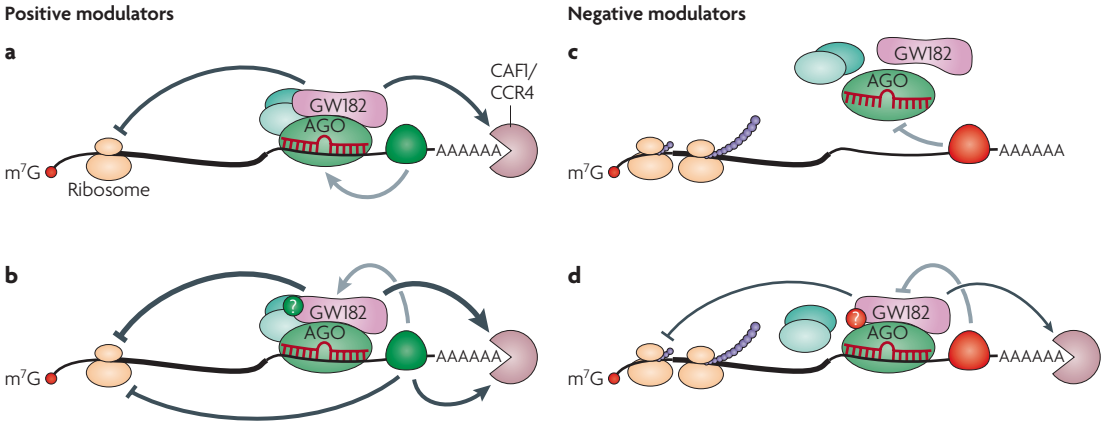


Figure 3 | **Interplay between RBPs and miRISCs at the mRNA 3′-UTR.** The binding of RNA-binding proteins (RBPs) to mRNAs can either facilitate or counteract microRNA (miRNA)-induced silencing complex (miRISC) activity. **a** | RBPs that enhance repression by miRNAs (depicted in dark green; for example, fragile X mental retardation protein (FMRP) or PUF)[99,100,130] could facilitate or stabilize miRISC binding. This could be by altering mRNA structure and thereby facilitating miRISC binding to mRNA; by direct interaction with miRISC components; or by directly interacting with the miRNA/mRNA duplex. **b** | Enhancement of silencing could also occur by strengthening interactions between miRISC components and the downstream effectors responsible for executing translational repression or deadenylation. This could possibly involve post-translational modifications of protein components (small green circle with question mark). Recruitment of translational repressors or deadenylation factors by RBPs independent of miRISC would also increase target repression. The exact mechanism by which positive modulators such as FMRP or PUF proteins synergize with miRISC to suppress shared mRNA targets needs to be determined. **c** | RBPs counteracting miRISC function (depicted in red) can act by either preventing miRISC binding or displacing miRISC from mRNA as exemplified by DND1 and HuR[76,97]. **d** | Other proteins could interfere with the interaction between miRISC components and downstream effectors involved in translational repression and deadenylation. Alternatively, they might promote post-translational modification of miRISC components (small red circle with question mark). AGO, argonaute; GW182, glycine-tryptophan protein of 182 kDa; m^{7}G, 7-methylguanosine-cap.

partners, concentrate factors that act together or temporarily segregate pathway components away from the rest of the cellular environment. Considerable effort has been put into establishing whether components of miRISCs are associated with particular cellular structures and whether miRNA repression can be regulated at this level. P-bodies and stress granules (BOX 4) have emerged as being potentially relevant to miRNA repression, as have multivesicular bodies (MVBs).

Role of MVBs in miRNA repression and miRNA secretion. Recent studies in *D. melanogaster* and mammalian cells have identified MVBs, specialized late-endosomal compartments, as organelles contributing to miRNA function[86,107]. In their lumen, MVBs accumulate vesicles, which they deliver to lysosomes for degradation or release extracellularly as exosomes. Blocking MVB formation by depleting endosomal sorting complex required for transport (ESCRT) factors inhibits miRNA silencing, whereas blocking MVB turnover by inactivation of the Hermansky–Pudlak syndrome 4 (*HSP4*) gene stimulates repression by miRNAs. Moreover, the depletion of some ESCRT factors leads to an increase in GW182 protein levels[86] and an impaired loading of miRISC with small RNAs[107], suggesting that selective removal of GW182 from the miRISC, and its transit to MVBs, is important for the efficient formation of new miRISCs. Components of the miRISC loading complex, namely Dicer and AGO2, had been found to be associated with membranous fractions in previous reports[72,108,109], and mammalian AGO2 was initially characterized as a Golgi-associated or endoplasmic reticulum-associated protein[108].

The association of miRISCs with MVBs raises many interesting questions. Does this interaction represent a major miRISC turnover pathway? Is the selective targeting of GW182 for degradation or exosomal secretion essential for the efficient formation of new miRISCs? Understanding the biological role of miRNA secretion is equally important. Purified exosomes contain miRNAs and are enriched in GW182 (REF. 86). Do miRNAs act as intercellular communication signals? In some instances, the uptake of miRNA-containing exosomes by other cells has been documented, but evidence that miRNA internalization is physiologically relevant for target cells is still missing. However, there are examples of non-cell-autonomous miRNA action[110,111], even though the short-distance cell-to-cell movements have been observed most probably do not involve exosomes. In the root of *A. thaliana*, the endodermally produced miR-165/6 moves to peripheral cells to target specific mRNA and to communicate positional information important for tissue patterning[110]. In mammals, miRNAs are transferred between B-lymphocytes and T-lymphocytes through immune synapses and can repress target genes in recipient cells[111].

Nucleo-cytoplasmic partitioning. miRNA-mediated repression is considered to be a cytoplasmic event, yet substantial amounts of AGO2 and miRNAs have been found in the nuclei of different mammalian cell lines[112–115], and human GW182 protein TNRC6B has been shown to shuttle between the cytoplasm and the nucleus[116]. It is currently unclear whether the nucleo-cytoplasmic shuttling of miRISC components is important for their cytoplasmic functions, or if it indicates that miRISCs have nuclear targets or perform nuclear function or functions unrelated to the established roles of miRNAs. At least in plants, a nuclear role has been described for miRNAs in transcriptional silencing[117–119]; and there is some evidence that miRNAs may also play a role in transcriptional silencing in mammalian cells[120].

Regulation of miRNA repression in neurons

Some of the most interesting examples of miRNA activity regulation are coming from neurons. Local mRNA translation at dendritic spines is important to ensure compartmentalized protein expression required for synaptic plasticity and long-term memory. Selected miRNAs are enriched at distal sites in dendrites and much evidence exists to suggest that synaptic stimulation is accompanied by reactivation of mRNAs targeted by miRNAs[121–124]. Schratt *et al.* found that miR-134 inhibits *LIMK1* mRNA translation at the synapses of hippocampal neurons. Exposure to brain-derived neurotrophic factor (BDNF) relieves the repression, contributing to synapse remodelling[123]. Another miRNA, miR-138, regulates translation of the depalmioylating enzyme APT1 at dendritic spines[124]. In both *D. melanogaster* olfactory and mammalian hippocampal neurons, stimulation induces rapid proteolysis of the miRISC assembly factor Armitage/MOV10, thus leading to the local relief of miRNA-mediated repression of several proteins involved in synaptic plasticity or memory formation[121,122].

In addition to miRNAs, AGO and GW182 proteins are also present in dendrites, consistent with a role of miRNAs in modulating synaptic plasticity[23,125–127]. Moreover, miRNA precursors and Dicer have been reported to be present in synaptosomes[23,128], raising speculations that maturation of some miRNAs can even occur in dendrites[128]. Interestingly, neuronal stimulation seems to induce the dispersal of dendritically localized P-body-like structures[127] or their remodelling, as evidenced by relocalization to more distant sites and decreased association with AGO2 (REF. 126).

Another player in the regulation of local translation in response to synaptic stimulation is FMRP, a protein generally implicated in translational repression. As FMRP associates with mature miRNAs and AGO, as well as with pre-miRNAs and Dicer, it has been implicated in the regulation of both miRNA biogenesis and miRISC function[129]. Recently, Erdbauer *et al.* demonstrated that the inhibitory effect of FMRP on translation of the mRNA, which encodes the NMDA (*N*-methyl-D-aspartate) receptor subunit NR2A, is reinforced by miR-125b[130]. The effect of miR-125b overexpression on spine morphology can be reversed by depleting FMRP, further supporting the idea of an FMRP–miRISC synergy[130] (FIG. 3). This model is consistent with earlier studies in *D. melanogaster*, which showed that the regulation of synaptic plasticity by dFMR, a homologue of FMRP, is partially dependent on dAGO1 (REF. 131).

Regulation of miRNA decay

In contrast to miRNA biogenesis, turnover of miRNAs has received only limited attention to date. It is generally thought that miRNAs represent highly stable molecules and, indeed, experimentation using RNA polymerase II inhibitors or depletion of miRNA processing enzymes, have indicated that the half-lives of miRNAs in cell lines or in organs such as liver or heart correspond to many hours or even days[132–134]. However, such slow turnover is unlikely to be a universal feature of miRNAs as they often play a role in developmental transitions or act as on and off switches, conditions that require more active metabolism.

Several examples of accelerated or regulated miRNA turnover are now known. miR-29b decays faster in cycling mammalian cells than in cells arrested in

Box 4 | The role of P-bodies and stress granules in miRNA function

Translationally repressed mRNAs can accumulate in discrete cytoplasmic foci known as processing bodies (P-bodies) or glycine-tryptophan bodies (GW-bodies)[160–162]. P-bodies function in both storage and decay of repressed mRNAs. Consequently, they are enriched in proteins involved in translational repression, and in mRNA deadenylation, decapping and degradation[160–162]. P-bodies are dynamic structures, with proteins and mRNAs continuously moving in and out of them[160–162], and the number and size of P-bodies varies depending on the translational activity of the cell[85].

Argonaute (AGO) and GW182 proteins, mature miRNAs and repressed mRNAs are all enriched in P-bodies[160], and the inhibition of microRNA (miRNA) biogenesis or depletion of GW182 proteins causes the disappearance of P-bodies[85,163–165]. Moreover, a positive correlation exists between miRNA-mediated repression and the accumulation of target mRNAs in P-bodies[76,166–168]. For example, cationic amino acid transporter-1 (*CAT-1*) mRNA, a target of miR-122 in hepatoma Huh7 cells, localizes to P-bodies when it is translationally repressed, but exits P-bodies upon stress, when its repression is relieved[76]. Similarly, miR-29a interacts with the 3′-UTR of the HIV-1 mRNA and targets it to P-bodies in human T-lymphocytes, and the artificial disruption of P-bodies enhances HIV-1 infection[169]. Although these examples focus on the fate of specific mRNAs, other studies link P-body status to miRNA function in a more general way. In mature mouse oocytes and early embryos, miRNA function is globally suppressed even though miRNAs are abundant[170,171]; this coincides with the loss of P-bodies[172,173]. P-bodies disappear in developing oocytes and reappear around the blastocyst stage[172], paralleled by a dispersal of AGO and the relocalization of GW182 to the cell cortex, a mechanism that possibly uncouples miRNA-induced silencing complexes (miRISCs) from translational repression. Dispersal of P-body-like structures[127], or the loss of AGO2 from them[126], has also been observed upon neuronal stimulation, a condition that can cause relief of miRNA-mediated silencing (see main text). Although all these examples clearly link P-bodies to miRNA silencing, other findings indicate that microscopically visible P-bodies are not essential for miRNA function, and that the formation of P-bodies is rather a consequence than the cause of miRNA-mediated repression[163–165].

AGO proteins, artificial miRNA mimics and repressed reporter mRNAs also accumulate in stress granules, another class of mRNA-containing cytoplasmic aggregates. Stress granules form on global repression of translation initiation in response to stress. They share some protein components with P-bodies, and stress granules and P-bodies are frequently located adjacent to each other, possibly exchanging their cargo material[174]. However, it remains to be established whether stress granules indeed play a role in miRNA silencing or if enrichment of miRISCs in stress granules just reflects a passive dragging of mRNA-associated miRISCs to these structures under conditions of general translation repression.

mitosis[135]. The accelerated decay depends on sequences present at the miR-29b 3′ end, and applies to miR-29b but not to the co-transcribed paralogue miR-29a. In contrast to most other miRNAs, miR-29b is predominantly localized in the nucleus. This localization is mediated by a sequence motif that is also present at the 3′ end, although the destabilization does not seem to be a function of nuclear import[135]. miRNA stability can be modulated by viral infection. In mouse cells, the level of miR-27a, but not of co-transcribed miRNAs, decreases after infection with the murine cytomegalovirus[136]. miR-27a demonstrates antiviral activity by interfering with virus replication, and murine cytomegalovirus-encoded or host-encoded factors may neutralize miR-27a by inducing its degradation, although the mechanism is unknown[136].

Rapid and regulated decay of many miRNAs occurs in different types of neurons. Characterization of several miRNAs downregulated in response to dark adaptation in mouse retina (miR-204 and miR-211, and miRNAs of the 183/96/182 cluster) has revealed that a decrease in their level is due to rapid decay. Surprisingly, the rapid turnover (half life of ~1 hour) may apply to many, if not all miRNAs, expressed in retinal neurons but not those expressed in glial cells. A similar high turnover of miRNAs (for example, miR-124, miR-128, miR-134 or miR-138) also occurs in primary dissociated rodent neurons and neurons differentiated from mouse ESCs[134], and in human primary neural cultured cells and post-mortem brain tissues[137]. Notably, miRNA turnover in neurons seems to be activity dependent. Blocking action potentials or glutamate receptors prevents miRNA decay, suggesting that active miRNA metabolism may be important for neuronal function[134]. In *Aplysia* spp., treatment with serotonin also rapidly decreased mature but not precursor levels of miR-124 and miR-184 by a mechanism depending on MAPK signalling[138]. Nevertheless, activity-dependent decay does not apply to all neuronal miRNAs. For example, blocking glutamate receptors did not inhibit the turnover of miR-219 (REFS 139,140) or miR-132 (REF. 134) in rodent brain or neuronal culture, but actually accelerated it.

The turnover-mediated decrease of miR-182, miR-183 and miR-96 in retina is physiologically relevant, as it results in upregulated expression of a specific glutamate transporter in photoreceptors, which helps to scavenge glutamate from the synaptic cleft in conditions of low light[134]. However, the significance of rapid and activity-dependent turnover of many other neuronal miRNAs remains unknown. miRNAs are implicated in the regulation of local translation at dendritic spines in response to synaptic stimulation, which is perhaps associated with rapid turnover of miRNAs. The observation that only ~50% of each miRNA decays rapidly[134] supports the possibility that turnover occurs only in one compartment of neurons, either processes or soma. Alternatively, the rapid turnover of miRNAs and, consequently, a continuous supply of *de novo*-produced miRNAs, might be required for regulation of the newly synthesized mRNAs that are known to be expressed in neurons in response to their activation[141].

The stability of mature miRNAs may be regulated by the untemplated addition of adenosine or uracil residues to the RNA 3′ end (FIG. 2). Deep sequencing has identified an abundance of such modifications in miRNAs. In liver cells, a single adenosine addition to the 3′ end of miR-122 by GLD-2 poly(A) polymerase protects it against exonucleolytic degradation[142], and in the plant *Populus trichocarpa* attachment of adenosines attenuates degradation of the ptc-MIR397 and ptc-MIR1447 families of miRNAs[143]. In human cells, miR-26a is uridylated by Zcchc11 nucleotidyltransferase, and this abrogates its repressive function[37]. In plants, 2′-*O*-methylation of the miRNA 3′-terminal nucleotide is a common modification; it prevents miRNA uridylation and degradation[144].

Recently, some progress has been made in identifying the enzymes involved in miRNA turnover. In *A. thaliana*, degradation of mature miRNAs is mediated by a family of 3′ to 5′ exoribonucleases, small RNA degrading nuclease 1 (SDN1), SDN2 and SDN3 (REF. 145). Inactivation of SDN genes, resulting in stabilization of several miRNAs, is associated with developmental phenotypes. In *C. elegans*, an enzyme with a polarity opposite to that of plants, the 5′ to 3′ exonuclease XRN-2, catalyses the degradation of mature miRNAs[146]. The degradation requires miRNAs to be released from the miRISC to make the miRNA 5′ end accessible to the enzyme. Importantly, the susceptibility of miRNAs to XRN-2 depends on target availability, as the miRISC association with mRNA prevents miRNA degradation[146]. Hence, in the absence of its complementary targets, the miRNA could be specifically released from miRISC and degraded, making AGO proteins available for loading with new miRNAs. In summary, turnover is likely to be an important step in the regulation of miRNA function, in a similar way to that established for mRNAs.

Future perspectives

Considering the fundamental role of miRNAs in organismal development, cellular differentiation and metabolism, viral infection, and oncogenesis, we can anticipate many more sophisticated mechanisms for the regulation of their biogenesis, function and catabolism to emerge in coming years. A few examples of post-translational modifications of miRISC proteins and factors controlling miRNA biogenesis are already known and it will be important to determine how these, and other modifications that might be discovered, affect miRISC function and what signalling pathways are responsible for them. Such information will be particularly important for the understanding of the basis of miRNA dysregulation known to occur in human pathologies.

Another important area of research will be understanding control at the level of the 3′-UTRs of mRNAs. Functionally, the mRNA 3′-UTR can be considered as a post-transcriptional equivalent of the gene promoter at which most transcription-related decisions are made. With hundreds of RBPs being expressed in eukaryotic cells, it is clear that the interplay between miRISC and RBPs, which both bind along kilobases of the 3′-UTR, will be of great importance for fine-tuning protein

CLIP
(Crosslinking immunoprecipitation). CLIP technology facilitates the identification and sequencing of short RNA regions associating with RNA-binding proteins or with microRNA-induced silencing complexes in intact cells.

synthesis. New technologies such as CLIP, which allows miRISC and RBP binding site mapping on a global scale, promise rapid progress in this area[65,147]. Most mammalian genes use alternative polyadenylation sites to form mRNA isoforms that differ in the 3′-UTR length. mRNAs with short 3′-UTRs, generally formed in highly proliferating or transformed cells, are more stable and more efficiently translated[148,149]. Is the escape from miRNA targeting the main purpose of this global 3′-UTR length switch? Were development-specific or tissue-specific poly(A) sites selected during evolution, allowing an increasingly important role of miRNAs in shaping tissue identity and sharpening developmental transitions?

Perhaps the most interesting findings concerning miRNA pathway regulation will emerge from research on neurons. Local miRNA-regulated translation in neuronal processes, transport of miRNAs to distal sites in dendrites, association of miRNA regulation with synaptic activation, and, finally, reported examples of activity-regulated miRNAs and their rapid turnover in neurons[121–124,134,138,150], all suggest elaborate mechanisms for controlling miRNA metabolism and function in these cells. It will be important to know how factors such as BDNF lead to the relief of miRNA-mediated repression in dendritic spines and what makes some components of miRISCs sensitive to proteolytic degradation. Other key questions that need to be answered include which RNA features are responsible for the selection of specific miRNAs to be delivered to dendrites, and how miRNAs are transported. These are just a few areas of research that are likely to keep miRNA biologists busy for years ahead.

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Competing interests statement
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