Regulation of microRNA biogenesis

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Abstract | MicroRNAs (miRNAs) are small non-coding RNAs that function as guide molecules in RNA silencing. Targeting most protein-coding transcripts, miRNAs are involved in nearly all developmental and pathological processes in animals. The biogenesis of miRNAs is under tight temporal and spatial control, and their dysregulation is associated with many human diseases, particularly cancer. In animals, miRNAs are ~22 nucleotides in length, and they are produced by two RNase III proteins — Drosha and Dicer. miRNA biogenesis is regulated at multiple levels, including at the level of miRNA transcription; its processing by Drosha and Dicer in the nucleus and cytoplasm, respectively; its modification by RNA editing, RNA methylation, uridylation and adenylation; Argonaute loading; and RNA decay. Non-canonical pathways for miRNA biogenesis, including those that are independent of Drosha or Dicer, are also emerging.

Argonaute family proteins (AGO family proteins). Proteins that associate with small RNAs and function as effectors in RNA silencing. AGO proteins carry two characteristic domains — PIWI (an endoribonuclease domain) and PAZ (PIWI–AGO–ZWILLE; the 3′ end-binding module).

PIWI-interacting RNA (piRNA). Small silencing RNAs (24–30 nucleotides long) that bind PIWI clade Argonaute proteins in animals and silence germline transposons. They are thought to derive from single-stranded RNA precursors and do not require RNase III enzymes for their maturation.

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Multiple types of small RNAs have evolved in eukaryotes to suppress unwanted genetic materials and transcripts[1,2]. Small RNAs are defined by their length (20–30 nucleotides) and their association with Argonaute family proteins (AGO family proteins), and they are classified into three classes in animals: microRNA (miRNA), siRNA and PIWI-interacting RNA (piRNA) (BOX 1). miRNAs constitute a dominating class of small RNAs in most somatic tissues. They are ~22 nucleotides in length and are produced by two RNase III proteins, Drosha and Dicer.

In RNA silencing, miRNA functions as a guide by base pairing with its target mRNAs, whereas AGO proteins function as effectors by recruiting factors that induce translational repression, mRNA deadenylation and mRNA decay[3]. miRNA-binding sites are usually located in the 3′ untranslated region (UTR) of mRNAs[4]. The domain at the 5′ end of miRNAs that spans from nucleotide position 2 to 7 is crucial for target recognition and has been termed the 'miRNA seed'. The downstream nucleotides of miRNA (particularly nucleotide 8 and less importantly nucleotides 13–16) also contribute to base pairing with the targets. More than 60% of human protein-coding genes contain at least one conserved miRNA-binding site, and, considering that numerous non-conserved sites also exist, most protein-coding genes may be under the control of miRNAs[5]. Thus, it is not surprising that the biogenesis and function of miRNAs themselves are tightly regulated, and their dysregulation is often associated with human diseases, including cancer[6] and neurodevelopmental disorders[7]. miRNA regulation takes place at multiple steps, including their transcription, their processing by Drosha and Dicer, their loading onto AGO proteins and miRNA turnover[8–10]. Various strategies are applied by the cell to interfere with or to facilitate each step, including the recruitment of transcription factors, RNA-binding proteins, protein-modifying enzymes, RNA-modifying enzymes, exoribonucleases and endoribonucleases.

This Review summarizes our current knowledge of how the biogenesis of animal miRNAs is regulated. As plant miRNAs are substantially different from animal miRNAs in terms of their sequence, precursor structure, evolutionary origin and biogenesis mechanism[11,12] (BOX 2), and the miRNA pathway in plants has been thoroughly reviewed elsewhere[11,13,14], we focus on miRNA biogenesis in animals.

miRNA transcription

miRNA genes are transcribed by RNA polymerase II (Pol II), and the long primary transcript has a local hairpin structure where miRNA sequences are embedded (FIG. 1).

miRNA gene families and nomenclature. miRNA genes constitute one of the most abundant gene families, and are widely distributed in animals, plants, protists and viruses[15]. The latest release of the miRNA database (miRBase) has catalogued 434 miRNAs in *Caenorhabditis elegans*, 466 miRNAs in *Drosophila melanogaster* and 2,588 miRNAs in humans, although the functional importance of many of these miRNA annotations remains to be determined[16,17] (BOX 3).

In many species, there are multiple miRNA loci with related sequences that arose mainly from gene duplication[18,19]. Classification rules have not yet been unified,

Box 1 | **Classification of small silencing RNAs in animals**

There are three major types of small silencing RNAs in animals: microRNAs (miRNAs), siRNAs and PIWI-interacting RNAs (piRNAs)[1,8]. miRNAs are generated from short hairpin RNAs by the sequential action of two RNase III-type proteins (Drosha and Dicer). siRNAs (~21 nucleotides long) are derived from long double-stranded RNAs or long stem–loop structures through Dicer processing[1]. They mediate the post-transcriptional suppression of transcripts and transposons, and contribute to antiviral defence[1]. piRNAs (24–30 nucleotides long) are not dependent on RNase III-type proteins and are produced from single-stranded precursors by an endonuclease called Zucchini (also known as mitochondrial cardiolipin hydrolase in humans) and as yet unidentified trimming enzymes[2,267–269]. The main function of piRNAs is to silence transposable elements in germline cells, although the roles of some piRNAs (for example, pachytene piRNAs and those produced outside of the germ line) are still enigmatic. Of the two subclades of Argonaute proteins (AGO and PIWI), miRNAs and siRNAs are associated with the AGO proteins, whereas piRNAs bind to PIWI proteins.

Features	miRNA	siRNA	piRNA
Length	~22 nucleotides	~21 nucleotides	24–30 nucleotides
Processing enzymes	Drosha and Dicer	Dicer	Zucchini and unknown trimming enzymes
AGO subclades	AGO	AGO	PIWI
Mechanism of action	• Translational repression • mRNA degradation	RNA cleavage	• Transcriptional or post-transcriptional repression of transposons • Multigenerational epigenetic phenomena in worms
Function	Regulation of protein-coding genes	• Regulation of protein-coding genes and transposons • Antiviral defence	• Pre-pachytene piRNA: transposon silencing • Pachytene piRNA: unknown • piRNA-like small RNA in soma: unknown

but it is generally considered that miRNAs with identical sequences at nucleotides 2–8 of the mature miRNA belong to the same 'miRNA family' (REF. 4). For instance, the human genome contains 14 paralogous loci (encoding 'miRNA sisters') that belong to the let-7 family. Thirty-four miRNA families are phylogenetically conserved from *C. elegans* to humans, and 196 miRNA families are conserved among mammals[16,20]. miRNA sisters generally act redundantly on target mRNAs, but distinct roles have also been suggested[21]. Some miRNAs share a common evolutionary origin but diverge in the miRNA seed. For instance, miR-141 and miR-200c belong to a deeply conserved miR-200 superfamily and differ by one nucleotide in their miRNA seeds. Deletion of each sister locus showed that the targets of miR-141 and miR-200c barely overlap, which illustrates the importance of the miRNA seed sequence in miRNA function and evolution[22].

The nomenclature of miRNA genes is somewhat inconsistent. The genes found in early genetic studies were named after their phenotypes (for example, *lin-4*, *let-7* and *lsy-6*), whereas most miRNAs found from cloning or sequencing received numerical names (for example, the *lin-4* homologues in other species are called *mir-125*). Genes encoding miRNA sisters are indicated with lettered suffixes (for example, *mir-125a* and *mir-125b*). If the same mature miRNA is generated from multiple separate loci, numeric suffixes are added at the end of the names of the miRNA loci (for example, *mir-125b-1* and *mir-125b-2*). Each locus produces two mature miRNAs: one from the 5′ strand and one from the 3′ strand of the precursor (for example, miR-125a-5p and miR-125a-3p). However, one arm (called the 'guide' strand) is usually much more prevalent (96–99% of the sum on average) and more biologically active than the other arm (the 'passenger' strand, which is known as miRNA*).

Transcriptional regulation. miRNA sequences are located within various genomic contexts. In humans, the majority of canonical miRNAs are encoded by introns of non-coding or coding transcripts, but some miRNAs are encoded by exonic regions. Often, several miRNA loci are in close proximity to each other, constituting a polycistronic transcription unit[23]. The miRNAs in the same cluster are generally co-transcribed, but the individual miRNAs can be additionally regulated at the post-transcriptional level. One of the most deeply conserved clusters is the *mir-100~let-7~mir-125* cluster, which has an important role in the development of bilaterian animals[24]. The let-7 miRNA, but not the other miRNAs, is suppressed post-transcriptionally in embryonic stem cells and in certain cancer cells in mammals.

Precise locations of the miRNA promoters have not yet been mapped for most miRNA genes but can be inferred from collective analysis of CpG islands, RNA sequencing data and ChIP–seq (chromatin immunoprecipitation followed by sequencing) data[25]. Some miRNA genes reside in the introns of protein-coding genes and, thus, share the promoter of the host gene. However, it has

Pachytene
The stage of meiotic prophase that immediately follows the zygotene. It is characterized by paired chromosomes that are condensed and visibly divided into chromatids, and by the occurrence of crossing-over.

Let-7 family
The *let-7* gene was initially discovered as an essential developmental gene in *Caenorhabditis elegans* and, later, as one of the first two microRNAs (miRNAs). The let-7 miRNA family is highly conserved throughout bilaterian animals, and it suppresses cell proliferation and promotes cell differentiation. It is also a tumour suppressor.

Box 2 | **miRNA biogenesis pathway in plants**

Like their animal counterparts, plant primary microRNAs (pri-miRNAs) are mostly transcribed by RNA polymerase II and their length is highly heterogeneous[11,13,14]. However, unlike animal miRNAs, plant miRNA processing is completed in the nucleus, and homologues of Drosha and DGCR8 are not found in plants. DICER-LIKE 1 (DCL1) processes most pri-miRNAs by sequential cleavage. The RNA-binding protein DAWDLE (DDL) interacts with DCL1 and stabilizes pri-miRNAs in nuclear foci called dicing bodies (D-bodies). The zinc-finger protein SERRATE (SE), the double-stranded RNA-binding protein HYPONASTIC LEAVES 1 (HYL1), DCL1 and the nuclear cap-binding complex form a complex and process pri-miRNAs. Following processing, the miRNA–'passenger strand' (miRNA*) duplex is 2′-*O*-methylated at the 3′ end by HUA ENHANCER 1 (HEN1), which blocks uridylation (by HEN1 SUPPRESSOR 1 (HESO1)) and decay of miRNAs (by 3′–5′ exoribonucleases SMALL-RNA-DEGRADING NUCLEASE 1 (SDN1), SDN2 and SDN3). pre-miRNAs or mature miRNAs are then exported to the cytoplasm by HASTY (HST), the plant homologue of exportin 5 (EXP5). An additional export pathway seems to be involved but remains unknown. In the cytoplasm, miRNAs are loaded onto cytoplasmic ARGONAUTE (AGO) proteins, of which AGO1 is the major player for the miRNA pathway.

ChIP–seq
(Chromatin immunoprecipitation followed by sequencing). A method used to analyse protein interactions with DNA. It combines ChIP with parallel DNA sequencing to identify the binding sites of DNA-associated proteins.

been noted that miRNA genes often have multiple transcription start sites[25] and that the promoters of intronic miRNAs are sometimes distinct from the promoters of their host genes[26].

miRNA transcription is carried out by RNA Pol II and is controlled by RNA Pol II-associated transcription factors and epigenetic regulators[27,28] (FIG. 1a,b). In addition, RNA Pol III has been shown to transcribe some viral miRNAs[29]. Some endogenous miRNA-like small RNAs are derived from tRNAs that are transcribed by RNA Pol III[30]. Transcription factors, such as p53, MYC, ZEB1 and ZEB2, and myoblast determination protein 1 (MYOD1) positively or negatively regulate miRNA expression[8,9] (FIG. 1b). Epigenetic control, such as DNA methylation and histone modifications also contribute to miRNA gene regulation[31].

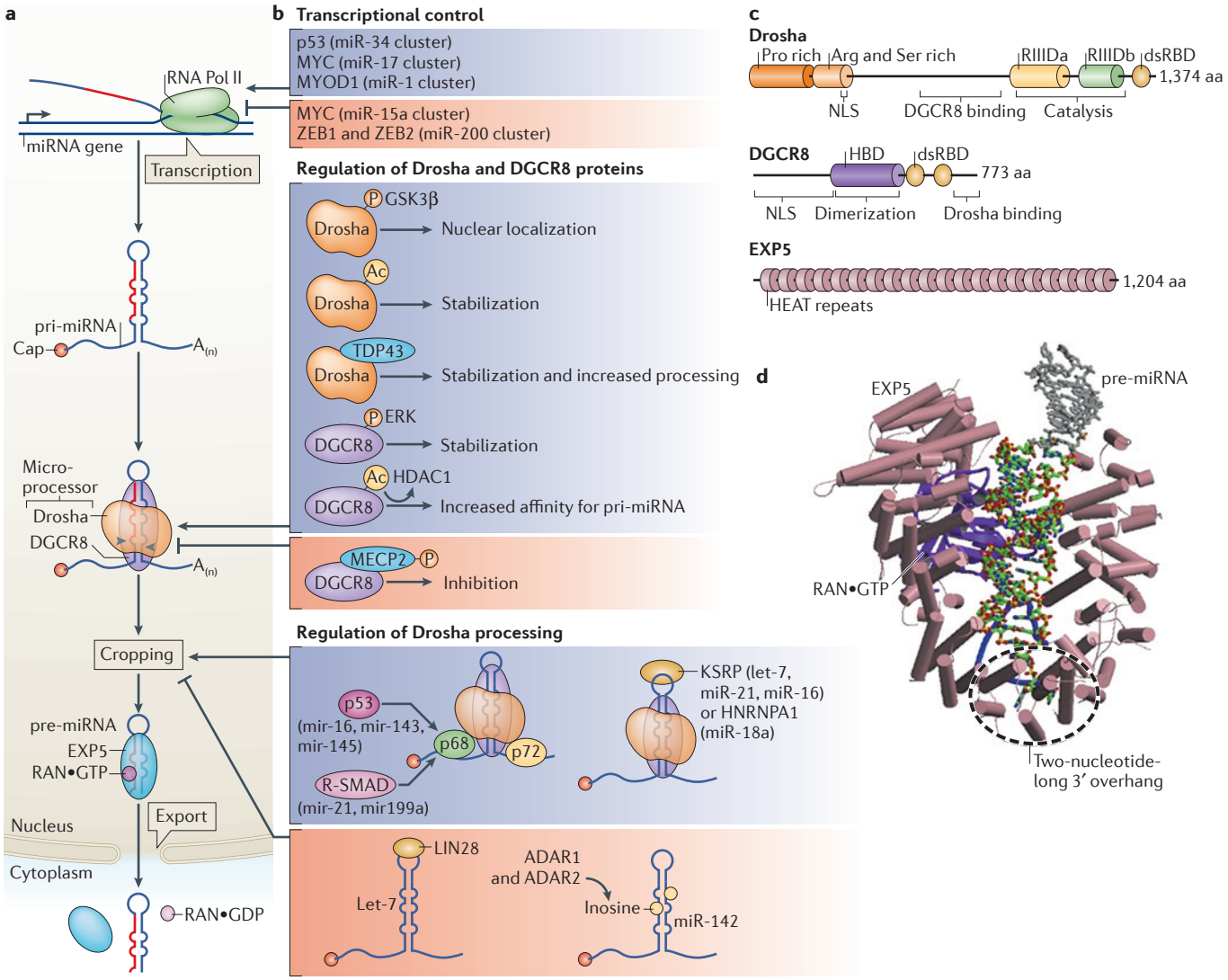


Figure 1 | **Nuclear events in the miRNA biogenesis pathway.** **a** | Schematic model of microRNA (miRNA) transcription by RNA polymerase II (Pol II), nuclear processing by the Microprocessor complex (comprising Drosha and DGCR8) and export by exportin 5 (EXP5) in complex with RAN•GTP. **b** | Examples of how miRNA transcription and primary miRNA (pri-miRNA) processing are regulated. p53, MYC and myoblast determination protein 1 (MYOD1) transactivate the miR-34, miR-17 and miR-1 clusters, respectively. MYC transcriptionally suppresses the miR-15a cluster, and ZEB1 and ZEB2 transcriptionally suppress the miR-200 cluster. Various post-translational modifications of Drosha and DGCR8 control the activity and/or localization of these proteins; the protein responsible for the modification, and the effect on Drosha or DGCR8, is shown in each case. The RNA-binding protein TAR DNA-binding protein 43 (TDP43) also interacts with Drosha. RNA-binding proteins, such as p68, p72, KH-type splicing regulatory protein (KSRP), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and LIN28, also regulate pri-miRNA processing. Finally, ADAR1 and ADAR2 mediate RNA editing from adenosine to inosine, which interferes with Drosha processing. **c** | Domain organization of Drosha, DGCR8 and EXP5 in humans. **d** | Structure of EXP5 (pink) complexed with RAN•GTP (violet) and pre-miRNA[83]. The position of the two-nucleotide-long 3′ overhang, which interacts with a basic tunnel-like structure on EXP5, is indicated with a circle. aa, amino acid; Ac, acetyl; dsRBD, double-stranded RNA-binding domain; GSK3β, glycogen synthase kinase 3β; HBD, haem-binding domain; HDAC1, histone deacetylase 1; HEAT, huntingtin, EF3, PP2A and TOR1; MECP2, methyl-CpG-binding protein 2; NLS, nuclear localization signal; P, phosphate; RIIID, RNase III domain; R-SMAD, receptor-activated SMAD. Part **d** from Okada, C. *et al.* A high-resolution structure of the pre-microRNA nuclear export machinery. *Science* 326, 1275–1279 (2009). Reprinted with permission from AAAS.

Box 3 | **To be, or not to be, a miRNA**

The current microRNA (miRNA) database (miRBase release 21) inevitably contains a substantial number of dubious annotations[17]. The majority of entries, particularly those since 2007, have been annotated on the basis of high-throughput sequencing, which is sensitive enough to detect decay intermediates of other RNA species. A sizeable proportion of miRNA entries are supported only by small numbers of sequencing reads, and their 5′ ends are highly heterogeneous, which casts doubts on their authenticity as miRNA. miRNA genes are under strong selective pressure to preserve the 5′ end of mature miRNAs, because the nucleotide position of the seed sequence is important for target recognition. A systematic effort to experimentally validate miRNAs in miRBase version 14 revealed that nearly one-third of the tested loci (173 of 564) lacked convincing evidence that they produce authentic miRNAs[16]. Although some of the misannotated miRNAs have been removed from the database, many more have since been added without validation, suggesting that current miRNA estimates might be substantially inflated. In an effort to distinguish authentic miRNAs from false annotations, miRBase has now implemented a poll to receive feedback from the users. The main criteria to consider in determining authentic miRNAs include: the 5′ homogeneity (although one should be cautious, because some authentic miRNA loci naturally produce alternatively processed isoforms); the abundance of sequencing reads; phylogenetic conservation; the evidence for RNase III-mediated processing (as inferred by the presence of a two-nucleotide-long 3′ overhang structure in the miRNA duplex or by the loss of miRNA following *Drosha* or *Dicer* knockdown); and evidence for the association with Argonaute proteins.

Nuclear processing

Following transcription, the primary miRNA (pri-miRNA) undergoes several steps of maturation[23] (FIG. 1a). pri-miRNA is long (typically over 1 kb) and contains a local stem–loop structure, in which mature miRNA sequences are embedded. A typical pri-miRNA consists of a stem of 33–35 bp, a terminal loop and single-stranded RNA segments at both the 5′ and 3′ sides (FIG. 2). The nuclear RNase III Drosha initiates the maturation process by cropping the stem–loop to release a small hairpin-shaped RNA of ~65 nucleotides in length (pre-miRNA)[32] (FIG. 2a). Together with its essential cofactor DGCR8 (also known as Pasha in *D. melanogaster* and as PASH-1 in *C. elegans*), Drosha forms a complex called Microprocessor[33–36]. Germline deficiency of Drosha causes lethality early in embryogenesis (by embryonic day 7.5 in mice)[37], which reflects the essential roles of miRNAs in development. Similarly, *Dgcr8*-knockout mouse embryos arrest early in development and the knockout embryonic stem cells show defects in proliferation and differentiation[38]. In humans, deletion of the genomic region including *DGCR8* is implicated in a genetic disorder called DiGeorge syndrome[39,40]. Drosha and DGCR8 are conserved in animals[41–43], whereas plants only use Dicer-like enzymes for miRNA processing (BOX 2).

The Microprocessor complex. Drosha is a nuclear protein of ~160 kDa, and Drosha, as well as Dicer, belongs to a family of RNase III-type endonucleases that act specifically on double-stranded RNA (dsRNA) (FIG. 1c) (see below). The amino-terminal part of Drosha is dispensable for pri-miRNA processing *in vitro*[35] but is necessary for nuclear localization in cells[44]. At its carboxyl terminus, Drosha has tandem RNase III domains (RIIIDs) and a dsRNA-binding domain (dsRBD). Two RIIIDs dimerize intramolecularly to form one processing centre at the interface between the RIIIDs[35]. The first RIIID (RIIIDa) cuts the 3′ strand of the stem of pri-miRNA, whereas the second RIIID (RIIIDb) cuts the 5′ strand to produce a staggered end with a two-nucleotide-long 3′ overhang[35,45,46]. The dsRBD of Drosha is necessary[35] but not sufficient for substrate interaction. Additional RNA-binding activity is provided by DGCR8, which is recruited through the middle region of Drosha[35].

DGCR8 is a protein of ~90 kDa (although it has an apparent mass of ~120 kDa on SDS–PAGE) that localizes to the nucleoplasm and the nucleolus[47,48] (FIG. 1c). The two dsRBDs of DGCR8 recognize pri-miRNA[49], while its conserved C terminus interacts with Drosha[48]. The crystal structure of a partial DGCR8 protein suggests that the two dsRBDs are tightly packed against the C-terminal helix[50]. The N-terminal region contains the nuclear localization signal[48]. The central region of DGCR8 binds to haem and mediates dimerization[48,51,52]. The haem-binding domain and its interaction with ferric ions is required for efficient pri-miRNA processing[53,54].

pri-miRNA processing. As Drosha cleavage defines the terminus of an miRNA and thereby determines its specificity, it is important that Microprocessor precisely recognizes and cleaves a pri-miRNA (FIG. 2a). Drosha cleaves the hairpin at approximately 11 bp away from the 'basal' junction between single-stranded RNA and dsRNA, and approximately 22 bp away from the 'apical' junction linked to the terminal loop[49,55] (FIG. 2a). The basal junction functions as the major reference point in determining the cleavage site[49,56], but the apical junction is also important for efficient and accurate processing[55,57]. It is still unclear how Drosha and DGCR8 specifically interact with the junctions and the stem. Thus, further studies, and particularly structural analyses, are necessary to understand the molecular basis of selective recognition of pri-miRNAs by the Microprocessor complex.

A recent report showed that additional sequence elements are involved in pri-miRNA processing[56]. The elements reside in the basal region (the UG motif and the CNNC motif) and terminal loop (the UGUG motif) of human pri-miRNAs (FIG. 2a). At least one of these three motifs is present in 79% of human miRNAs, suggesting that other determinants may also exist. SRp20 (also known as SRSF3), which is a splicing factor, binds the CNNC motif and increases the processing of human pri-miRNAs[56]. Recently, it has been reported that the CNNC motif is also required for DEAD-box RNA helicase p72 (also known as DDX17) binding, which increases processing by Drosha[58]. Thus, multiple auxiliary factors may contribute to pri-miRNA processing, and the mechanistic roles of these auxiliary factors need to be delineated.

Drosha-mediated processing of intronic miRNA does not affect splicing of the host pre-mRNA[59,270]. Cleavage of pri-miRNA is thought to occur co-transcriptionally before splicing catalysis[59]. Consistent with this idea, pri-miRNAs are enriched in the chromatin-associated nuclear fraction[60,61]. When an miRNA hairpin (or an miRNA-like hairpin) is located in the exonic region, Drosha-mediated cleavage leads to destabilization of

DiGeorge syndrome
An autosomal recessive genetic disorder caused by a deletion in chromosome 22 that is commonly associated with heart defects, poor immune system function, a cleft palate and behavioural disorders.

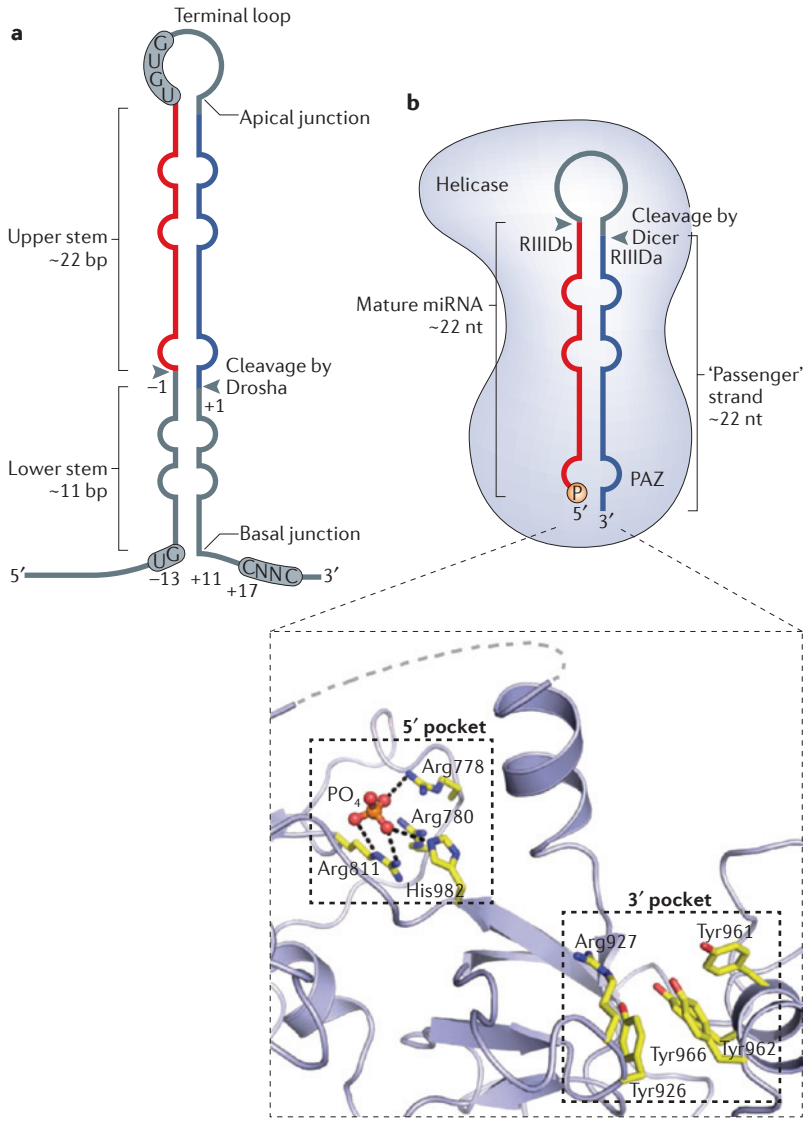


Figure 2 | **Substrate recognition of RNase III enzymes. a** | The Microprocessor complex (comprising Drosha and DGCR8) recognizes the single-stranded RNA tails, the stem of ~35 bp in length and a terminal loop of the primary microRNA (pri-miRNA). Microprocessor measures ~11 bp from the basal junction and ~22 bp from the apical junction, and Drosha cleaves the pri-miRNA at this position. **b** | Dicer recognizes pre-miRNA. The termini of pre-miRNA are recognized by the PAZ (PIWI–Argonaute (AGO)–ZWILLE) domain of human Dicer, which contains two basic pockets: one that interacts with the 5′-phosphorylated end of the pre-miRNA and one that interacts with the 3′ end[105,106]. The stem of the pre-mRNA is aligned along the axis of the protein in a way that Dicer can measure a set distance from both termini (like a 'molecular ruler'), because the catalytic domains of RNase III domain a (RIIIDa) and RIIIDb are placed ~22 nucleotides (nt) away from the termini. P, phosphate. Figure part **b** from REF. 105, Nature Publishing Group.

the host mRNA[62]. For example, Drosha can antagonize *FSTL1* (follistatin-like 1) expression by cleaving a miR-198 hairpin located at the 3′ UTR of the *FSTL1* mRNA[63]. KH-type splicing regulatory protein (KSRP; also known as FUBP2) binds to the terminal loop of miR-198 and facilitates this cleavage. Upon injury, transforming growth factor-β (TGFβ) signalling represses KSRP expression, which results in the upregulation of FSTL1 (REF. 63). It is of note that the miR-198 hairpin may only function as an mRNA destabilizing element rather than as an authentic pri-miRNA, as mature miR-198 is unusually low in abundance and highly heterogeneous at its 5′ end (BOX 3). There are other examples in which Drosha directly controls mRNA stability by cleaving miRNA-like hairpins on the exonic regions of the neurogenin 2 (*Neurog2*)[64] and *DGCR8* mRNAs (as discussed below).

Regulation of the Microprocessor. The efficiency of Drosha-mediated processing is crucial for determining miRNA abundance. Multiple mechanisms exist to control the expression level, activity and specificity of Drosha. One notable example is the autoregulation between Drosha and DGCR8 (REF. 62). DGCR8 stabilizes Drosha through protein–protein interactions[48,62], whereas Drosha destabilizes *DGCR8* mRNA by cleaving it at a hairpin in the second exon[37,62,65]. This cross-regulatory loop enables the homeostatic maintenance of Microprocessor activity and is deeply conserved throughout the animal kingdom. The mRNA fragment resulting from Drosha-mediated cleavage has been annotated as miR-1306 but is unlikely to function as an miRNA (BOX 3).

Post-translational modification can regulate the protein stability[66,69], nuclear localization[44,67] and processing activity of Microprocessor[68,70] (FIG. 1b). Phosphorylation of Drosha by glycogen synthase kinase 3β (GSK3β) is required for the nuclear localization of Drosha[44,67]. Acetylation of Drosha by an unidentified enzyme inhibits its degradation and stabilizes it[69]. DGCR8 can be deacetylated by histone deacetylase 1 (HDAC1), which increases the affinity of DGCR8 for pri-miRNAs[68]. DGCR8 is phosphorylated by ERK, which increases the stability of DGCR8[66]. A recent study showed that DGCR8 is sequestered by methyl-CpG-binding protein 2 (MECP2) when MECP2 is phosphorylated[70]. Following neuronal activity, MECP2 is rapidly dephosphorylated, releasing DGCR8, which in turn leads to miRNA production and dendritic growth.

Drosha-mediated processing is often controlled specifically by RNA-binding proteins that selectively interact with Drosha and/or certain pri-miRNAs (FIG. 1b). p68 (also known as DDX5) and p72 are required for the Drosha-mediated processing of a subset of miRNAs[71]. Receptor-activated SMAD proteins (R-SMADs) SMAD1–3 and SMAD5 and p53 promote Microprocessor activity through their interaction with p68 (REFS 72–74). For instance, when R-SMAD proteins are activated by bone morphogenetic protein (BMP) and TGFβ, they interact with p68 and the stem of pri-miRNAs to stimulate Drosha-mediated processing of *mir-21* and *mir-199a*[72,73]. The RNA-binding protein TAR DNA-binding protein 43 (TDP43) was reported to interact with and increase the stability of the Drosha protein[75] and to promote Drosha and Dicer processing[76] by as yet unknown mechanisms. Some proteins bind selectively to the terminal loop of pri-miRNA. For example, heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and KSRP bind to the terminal

loop of pri-mir-18a and pri-let-7, respectively, and facilitate Drosha-mediated processing[77–79]. LIN28A and its paralogue LIN28B also bind to pri-let-7 at the terminal loop, and suppress Drosha and Dicer-mediated processing (see below). Thus, terminal loops of miRNA precursors are enriched with *cis*-elements that recruit regulatory proteins.

Nuclear export

Following Drosha processing, pre-miRNA is exported into the cytoplasm, where maturation can be completed. The protein exportin 5 (EXP5; encoded by *XPO5*) forms a transport complex with GTP-binding nuclear protein RAN•GTP and a pre-miRNA[80–82] (FIG. 1a). Following translocation through the nuclear pore complex, GTP is hydrolysed, resulting in the disassembly of the complex and the release of the pre-miRNA into the cytosol. The crystal structure of this complex shows that EXP5–Ran•GTP forms a 'baseball mitt'-like structure into which the pre-miRNA stem is placed, enabling the interaction of the pre-miRNA stem with the positively charged inner surface[83] (FIG. 1d). There is a basic tunnel-like structure at the bottom of the mitt-like structure, which strongly interacts with the two-nucleotide-long 3′ overhang of the pre-miRNA[83]. This tunnel-like structure is consistent with the results from earlier biochemical analyses: EXP5 recognizes a dsRNA stem of >14 bp in length together with a short 3′ overhang (that is 1–8 nucleotides in length)[81,84,85]. Knockdown of *XPO5* results in the reduction of miRNA levels without an accumulation of nuclear pre-miRNA, which suggests that EXP5 may not only export pre-miRNA but may also protect it from nucleolytic attack in the nucleus[82].

EXP5 is ubiquitously expressed, but it has been shown that EXP5 is post-transcriptionally induced during cell cycle entry in a PI3K-mediated mechanism[86]. A recent report found that nuclear export of pre-miRNA increases upon DNA damage, in an ATM-dependent manner[87]. ATM activates AKT, which in turn phosphorylates the nucleopore protein NUP153. This leads to increased interactions between NUP153 and EXP5. In some tumours, *XPO5* is mutated and the resulting C-terminal-truncated EXP5 cannot transport its pre-miRNA cargo, which globally reduces the level of mature miRNAs[88]. Regulation of EXP5 has been less well investigated compared to that of other miRNA biogenesis factors. It will be interesting to examine whether there are any regulatory cofactors for EXP5 and whether other export factors also contribute to miRNA export. A recent study showed that EXP1 (also known as CRM1) transports some non-canonical pre-miRNAs (for example, pre-mir-320)[89] (see below).

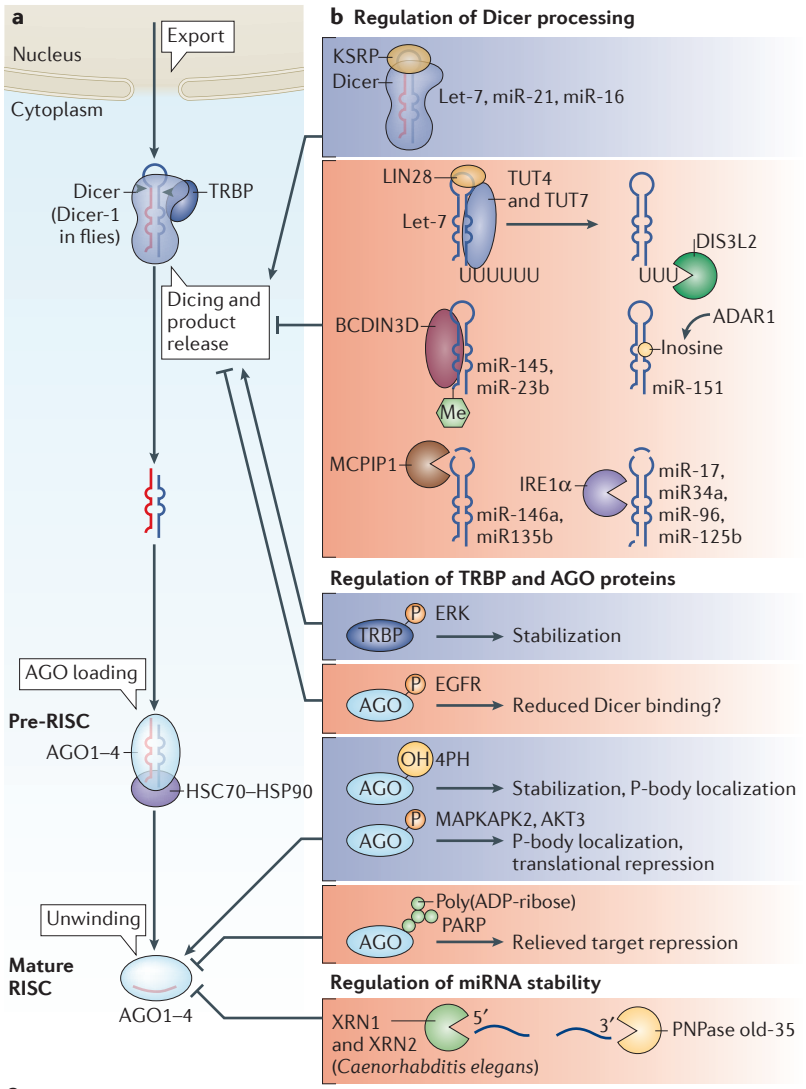
Cytoplasmic pre-miRNA processing

Upon export to the cytoplasm, pre-miRNA is cleaved by Dicer near the terminal loop, liberating a small RNA duplex[90–94] (FIG. 3a,b). Dicer homologues are widely distributed in eukaryotes, including fungi, plants and animals. Although most species, including nematodes and mammals, have a single *Dicer* gene, some species possess multiple *Dicer* homologues with different roles[95,96].

Figure 3 | **Cytoplasmic events in the miRNA biogenesis pathway.** **a** | Schematic model of Dicer-mediated processing and Argonaute (AGO) loading. Dicer interacts with a double-stranded RNA-binding domain (dsRBD) protein (TAR RNA-binding protein (TRBP) in humans and Loquacious (Loqs) in flies). Following Dicer processing, the RNA duplex is released and subsequently loaded onto human AGO1–4. A heat shock cognate 70 (HSC70)–heat shock protein 90 (HSP90) complex hydrolyses ATP to load the RNA duplex. The 'passenger' strand is discarded and the 'guide' (mature) microRNA (miRNA) remains in one of the AGO proteins. **b** | TRBP and AGO proteins are subject to post-translational modifications, which influence their ability to regulate Dicer processing, RNA-induced silencing complex (RISC) formation and miRNA activity. miRNA tailing by TUT4 and TUT7, RNA methylation by BCDIN3D, RNA editing by ADAR1, a decrease in RNA stability mediated by MCP-induced protein 1 (MCPIP1) and Ser/Thr protein kinase/endoribonuclease IRE1α also inhibit Dicer processing. A decrease in miRNA stability can downregulate miRNA activity. **c** | Domain organization of Dicer, TRBP and AGO2 in humans. **d** | Cryo-electron microscopic reconstruction of human Dicer[108]. **e** | Structure of human AGO2 and the guide RNA (red)[136]. The 5′ monophosphate of the guide RNA is tightly anchored to the 5′-phosphate-binding pocket at the interface between the MID (middle) and PIWI domain. 4PH, type I collagen prolyl 4-hydroxylase; aa, amino acid; EGFR, epidermal growth factor receptor; KSRP, KH-type splicing regulatory protein; L, linker; MAPKAPK2, MAPK-activated protein kinase 2; N, amino-terminal domain; OH, hydroxyl; P, phosphate; P-body, processing body; PARP, poly(ADP-ribose) polymerase; PAZ, PIWI–AGO–ZWILLE; RIIID, RNase III domain. Figure part **d** from REF. 108, Nature Publishing Group. Part **e** from Schirle, N. T. & MacRae, I. J. The crystal structure of human Argonaute2. *Science* 336, 1037–1040 (2012). Reprinted with permission from AAAS.

For instance, in *D. melanogaster*, Dicer-1 (Dcr-1) is required for miRNA biogenesis, whereas Dcr-2 is devoted to siRNA production[95]. Deletion of *Dicer1* in the mouse germ line results in early embryonic lethality (at approximately embryonic day 7.5)[97], and *Dicer1*-knockout embryonic stem cells show strong defects in cell proliferation and differentiation[98,99]. In addition, numerous defects have been reported in conditional knockouts in various tissues[100]. Furthermore, Dicer can function as a haploinsufficient tumour suppressor in mice[101].

Structure and functions of Dicer. Dicer is an RNase III-type endonuclease of ~200 kDa (FIG. 3c). The C-terminal tandem RNase III domains of Dicer form an intramolecular dimer to create a catalytic centre[46], similarly to those of Drosha (FIGS 1c,3c). The N-terminal helicase domain of Dicer facilitates pre-miRNA recognition by interacting with the terminal loop[102] and increases the processing of certain pre-miRNAs[103]. The PAZ (PIWI–AGO–ZWILLE) domain binds to the termini of pre-miRNA[104–106]. The crystal structure of apo-Dicer from *Giardia intestinalis* showed that the PAZ domain is separated from the catalytic centre by a positively charged connecting helix[104]. To explain how Dicer produces a discrete size of small RNAs, it has been proposed that



the region between the PAZ and RNase III domains functions as a 'molecular ruler' (REFS 46,104) (FIG. 2b). The structure of the Dicer helicase domain remains unknown, because Dicer of *G. intestinalis* lacks the domains upstream of the PAZ domain. However, on the basis of several electron microscopy studies, it has been suggested that human Dicer is an L-shaped molecule[107–109] (FIG. 3d). It will be important to solve the high-resolution structure of mammalian Dicer in complex with its natural substrate.

Dicer binds to pre-miRNA with a preference for a two-nucleotide-long 3′ overhang that was initially generated by Drosha[46] (FIG. 2b). In general, Dicer cleavage sites are located at a fixed distance from the 3′ end of the terminus of dsRNAs (the 3′-counting rule)[46,104,110–112]. This distance is typically 21–25 nucleotides in length and depends on the species and the type of Dicer. In mammals and flies, there is an additional mechanism to determine the cleavage site of pre-miRNA: Dicer binds to the 5′ phosphorylated end of the pre-miRNA and cleaves it 22 nucleotides away from the 5′ end (the 5′-counting rule)[105]. The 5′ end binding occurs when the end is thermodynamically unstable, but not when the end is strongly paired (such as through G·C base pairs). The structure of the PAZ domain of human Dicer shows two basic pockets that bind to the 5′ end and 3′ end of the pre-miRNA[105,106] (FIG. 2b). The pockets are spatially arranged in a way that they can be occupied simultaneously by the 5′ end and 3′ end of the pre-miRNA when the RNA has a two-nucleotide-long 3′ overhang. This structural arrangement may explain at least in part the preference of human Dicer for a two-nucleotide-long 3′ overhang structure.

Dicer regulation by its cofactors and products. RNase III proteins often interact with dsRBD proteins (FIG. 3a). *Drosophila* spp. Dcr-1 binds to one of the two isoforms of Loquacious (Loqs), Loqs-PA and Loqs-PB, each of which contains three dsRBDs[113–115]. Both isoforms are necessary for the production of most miRNAs, whereas a subset of miRNAs requires Loqs-PB specifically for their generation[116–118]. Loqs-PB can alter the Dcr-1-processing site to yield specific miRNA isoforms[116]. Similarly, human Dicer interacts with TAR RNA-binding protein (TRBP; encoded by *TARBP2*), a homologue of Loqs-PB with three dsRBDs[119–121] (FIG. 3c). The dsRBD cofactor PACT (also known as PRKRA) also associates with mammalian Dicer[121,122], but its exact role in the miRNA pathway remains to be determined. Like Loqs, TRBP modulates the processing efficiency of some pre-miRNAs and tunes the length of mature miRNAs[116,123,124]. Unlike Loqs, the mammalian dsRBD cofactors (TRBP or PACT) do not seem to be essential for Dicer-mediated pre-miRNA processing, as purified mammalian Dicer is comparable to the Dicer–dsRBD cofactor complex in the processing of pre-miRNAs[121]. Of note, TRBP and PACT were previously described as negative and positive regulators of dsRNA-dependent protein kinase R (PKR), respectively[125]. The relationship between the miRNA pathway and PKR signalling is unknown.

TRBP can be phosphorylated by the MAPK ERK, and this leads to the preferential upregulation of growth-promoting miRNAs and the downregulation of let-7 miRNAs, although the mechanism underlying the specificity remains unclear[126] (FIG. 3b). It was also reported that *TARBP2* is mutated in human cancers[127]. It is thought that the reduction of the TRBP protein leads to the destabilization of the Dicer protein and to the decrease of miRNA levels. However, the extent of its effect on Dicer seems to vary between different reports, calling for further investigation.

Human *DICER1* mRNA contains binding sites for let-7 miRNA, which results in a negative feedback loop between Dicer and its product let-7 (REFS 128,129). This regulation is thought to contribute to the homeostatic regulation of Dicer activity. RNA-binding proteins also participate in the control of pre-miRNA processing either positively or negatively (FIG. 3b). KSRP facilitates Dicer-mediated processing of various pre-miRNAs through its interaction with the terminal loop[79]. The LIN28 proteins bind to the terminal loop of pre-let-7, interfering with Dicer processing, and further block Dicer processing by inducing oligouridylation of pre-let-7 (see below). RNA editing and methylation also have a negative impact on the processing of a subset of pre-miRNAs (see below).

RNA-induced silencing complex formation

A small RNA duplex generated by Dicer is subsequently loaded onto an AGO protein to form an effector complex called RNA-induced silencing complex (RISC)[130–132] (FIG. 3a). RISC assembly involves two steps: the loading of the RNA duplex and its subsequent unwinding[133].

The AGO proteins. The AGO proteins are divided into three subclades: AGO, PIWI and worm-specific AGO proteins (WAGOs). Proteins of the AGO subclade are ubiquitously expressed and associate with miRNAs or siRNAs, whereas PIWI proteins are germ-cell-specific and interact with piRNAs (BOX 1).

The crystal structures of human and yeast AGO proteins in complex with a small RNA ('guide RNA') have been recently reported[134–136] (FIG. 3c,e). They revealed that eukaryotic AGO proteins are closely related to the prokaryotic AGO homologues, the crystal structures of which had previously been resolved [137–140]. AGO proteins adopt a bilobal architecture, composed of the N-terminal lobe with an N-terminal domain and a PAZ domain, and the C-terminal lobe with a middle (MID) domain and a PIWI domain (FIG. 3e). The 5′ monophosphate of the guide RNA is tightly anchored to the 5′-phosphate-binding pocket at the interface between the MID and PIWI domains[134–136,138,139]. The nucleotide specificity loop in the MID domain of human AGO2 contacts the 5′ nucleobase of the guide RNA with a preference for U or A binding[141]. The guide RNA threads along the basic channel of the AGO MID–PIWI lobe to reach the PAZ domain that binds to the 3′ end of the guide RNA[134–136,140,142,143]. The seed of guide miRNA is pre-arranged in an A-form helix conformation that facilitates efficient scanning of target mRNAs for complementary sequences.

The structure of the PIWI domain is similar to that of RNase H and contains an active site that enables cleaving ('slicing') of target mRNAs between nucleotide positions 10 and 11 (relative to the 5′ end of the guide RNA)[137,139,144]. Among the four AGO subclade proteins in humans (AGO1–4), only AGO2 can slice perfectly matched target mRNAs. All human AGO proteins are capable of inducing translational repression and decay of target mRNAs through interaction with the translation machinery and mRNA decay factors[3]. AGO2-deficient mice are embryonic lethal and show several developmental abnormalities in embryonic day 9.5 and 10.5 (REF. 144). Knock-in mice bearing the *Ago2*-slicer-deficient allele died shortly after birth with a prominent anaemia, and this phenotype can be at least partially explained by the loss of an erythropoietic miRNA, miR-451, which requires AGO2 for its maturation[145] (see below). Mouse embryonic stem cells that are deficient in AGO1–4 show defective miRNA silencing and undergo apoptosis[146]. Genetic inactivation of mouse *Ago4* revealed that it is required for spermatogenesis[147].

Selective miRNA loading. Following Dicer processing, RNA duplexes are preferentially loaded onto particular types of AGO proteins (FIG. 3a). In flies, miRNA duplexes and siRNA duplexes are sorted into AGO1 and AGO2, respectively, according to their intrinsic structural properties and independently of the type of Dicer that produced the duplex[148–150]. Central mismatches in the guide strand at nucleotide positions 9–10 direct the miRNA duplex to AGO1 and prevent it from entering AGO2 (REFS 150–154). Fly Dcr-2 (in complex with its dsRBD partner R2D2) can strongly bind to perfectly complementary small RNA duplexes and functions as a gatekeeper for RISC assembly involving AGO2. The identity of the 5′ nucleotide of the guide miRNA also contributes to sorting of small RNAs into AGO proteins, as AGO1 preferably binds to miRNAs with a 5′ U, whereas AGO2 favours siRNAs with a 5′ C[151,152,154]. Similar sorting mechanisms occur in *C. elegans*, in which miRNAs interact with ALG-1 and ALG-2 proteins, and siRNAs interact with RDE-1 proteins[155,156]. Like in flies, these differential interactions are guided by the structure of the RNA duplex[155,156].

By contrast, in humans, no strict small-RNA-sorting system exists, and the four AGO proteins (AGO1–4) are associated with almost indistinguishable sets of miRNAs[144,146,157–159]. All four human AGO proteins can incorporate both siRNA and miRNA duplexes, with a preference for small RNA duplexes with central mismatches (nucleotide positions 8–11)[144,158,160].

Control of RISC loading by Dicer complexes. In *D. melanogaster*, the RISC-loading complex (RLC), which consists of Dcr-2 and R2D2, is required for loading of an siRNA duplex onto AGO2 (REFS 161–163). R2D2 binds the more stable end of duplexes, whereas Dcr-2 is positioned at the less stable end of duplexes, thereby orienting small RNA duplexes for AGO2 loading.

By contrast, the role of the RLC with respect to loading on *D. melanogaster* AGO1 and human AGO1–4 is not clear. Earlier studies suggested that in mammals Dicer and TRBP (or PACT) (Dcr-1 and Loqs in flies) may

RNA editing
A molecular process through which some cells make discrete changes to specific nucleotide sequences within an RNA molecule after it has been generated by the RNA polymerase. Editing events may include the insertion, deletion and substitution of nucleotides within the edited RNA molecule.

RNA-induced silencing complex
(RISC). A ribonucleoprotein complex that consists of a small RNA guide strand bound to an Argonaute protein. RISC mediates all RNA-silencing pathways, and it can also include auxiliary proteins that extend or modify its function.

RISC-loading complex
(RLC). A protein complex containing Dicer, a double-stranded-RNA-binding protein, an Argonaute (AGO) protein and potentially other proteins that are required for loading of small RNAs onto the AGO protein.

have a role in RISC assembly that resembles the function of the RLC component Dcr-2 (REFS 119,164–167). A recombinant human Dicer–TRBP complex has been shown to bind to siRNA duplexes *in vitro*[168,169]. It has also been reported that the RLC has both pre-miRNA processing activity and target cleavage activity *in vitro*[170]. These findings support the idea that miRNA duplex loading may be coupled with Dicer-dependent pre-miRNA processing in humans (known as the 'Dicer-dependent AGO loading' model). However, *Dicer1*-knockout mouse embryonic stem cells are able to undergo siRNA-directed gene silencing[98,99], which strongly indicates that Dicer is not important for small RNA loading into AGO proteins. Moreover, in flies and mammals, Dicer has been reported to be dispensable for asymmetric RISC assembly *in vitro* and also in cells[133,153,160,171,172]. Thus, the RLC may not be essential for small RNA loading on *D. melanogaster* AGO1 and human AGO proteins, although it is important for loading onto *D. melanogaster* AGO2.

miRNA duplex unwinding. Following miRNA duplex loading, the pre-RISC (in which AGO proteins associate with RNA duplexes) quickly removes the passenger strand to generate a mature RISC (FIG. 3a). Slicing-competent AGO proteins (namely, AGO2 in flies and humans) can cleave the passenger strand if the duplex is matched at the centre[167,173–176]. Removal of the cleaved passenger strand is facilitated by the endonuclease C3PO, which is a multimeric complex of translin and translin-associated protein X (TRAX)[161,172]. However, this mechanism is rarely used in the miRNA pathway, as most miRNA duplexes have central mismatches (that prevent slicing) and human AGO1, AGO3 and AGO4 lack slicer activity[144,148,158]. Thus, miRNA duplex unwinding without cleavage is a more general process than passenger strand cleavage. Mismatches in the guide strand at nucleotide positions 2–8 and 12–15 promote unwinding of miRNA duplexes[150,153,160].

RISC loading of small RNA duplexes is an active process that requires ATP, whereas the release of the passenger strand is ATP-independent[153,160,162,163,177]. The heat shock cognate 70 (HSC70 (also known as HSPA8))–heat shock protein 90 (HSP90) chaperone complex uses ATP and mediates a conformational opening of AGO proteins, so that AGO proteins can bind to stiff dsRNA[178,179]. According to the 'rubber band' model, structural tension is introduced to the open conformation of AGO proteins (like a stretched rubber band)[133]. The release of this tension may drive the ATP-independent unwinding of the passenger strand.

Some miRNAs are longer than average (~23–24 nucleotides), owing to unusual bulge structures in their precursors, but these miRNAs can be trimmed down to ~22 nucleotides in length following RISC assembly by a 3′–5′ exonuclease called Nibbler (Nbr) in flies[180,181].

Rules for strand selection. The guide strand is determined during the AGO loading step, mainly on the basis of the relative thermodynamic stability of the two ends of the small RNA duplex[182,183] (FIG. 3a). The strand with a relatively unstable terminus at the 5′ side is typically selected as the guide strand. An additional determinant for strand choice is the first nucleotide sequence: AGO proteins select for guide strands with a U at nucleotide position 1 (REFS 151,152,154,184,185). The released passenger strand is degraded quickly, resulting in a strong bias towards the guide strand in the mature miRNA pool.

As strand selection is not completely strict, the strand that is not favoured can also be selected with varying frequency. The less abundant passenger strand (miRNA*) is also active in silencing, albeit usually less potently than the more abundant guide strand. Alternative strand selection ('arm switching') has been observed in studies comparing miRNA isoforms sequenced from multiple tissues[16]. miR-142-5p is a dominant isoform in ovaries, testes and the brain, whereas miR-142-3p was found more frequently in embryonic and newborn tissue samples. Such arm-switching events may be at least partly explained by alternative Drosha processing, which changes the relative thermodynamic stability of the miRNA duplex ends[186].

Regulation of the AGO proteins. AGO proteins can be modulated by numerous modifications (FIG. 3b). Prolyl 4-hydroxylation of human AGO2 by type I collagen prolyl 4-hydroxylase (4PH; also known as C-P4H(I)) increases the stability of AGO2 or localization within processing bodies (P-bodies)[187,188]. Phosphorylation at Ser387 of human AGO2 was reported to be mediated by MAPK-activated protein kinase 2 (MAPKAPK2)[189] or AKT3 (also known as RACγ Ser/Thr protein kinase)[190], resulting in its localization to P-bodies or translational repression, respectively. Under hypoxia, epidermal growth factor receptor (EGFR) phosphorylates human AGO2 (at Tyr393), which results in the dissociation of human AGO2 from Dicer and the reduction of pre-miRNA processing for some miRNAs[191]. Additionally, phosphorylation at Tyr529 of human AGO2 by an unknown kinase was proposed to reduce small RNA binding and target repression[192,193].

Upon stress or viral infection, human AGO proteins are subject to poly(ADP-ribosyl)ation, which inhibits their activity to repress targets[194,195]. AGO proteins can also be ubiquitylated and degraded by the proteasome in various contexts[196–199]. In studies using mouse embryonic stem cells, LIN41 (also known as TRIM71) was suggested to be an E3 ubiquitin ligase for AGO2 (REF. 196). However, it was also reported that mouse LIN41 directly represses target mRNAs[200,201] and does not affect the stability of AGO proteins[200–202]. Interestingly, AGO proteins are stabilized when they are occupied by miRNA, whereas empty AGO proteins are unstable[198,203]. Proteasome-mediated degradation[198] and autophagy[203] have been proposed to be responsible for the destabilization of AGO proteins.

miRNA intrinsic regulation

Alterations in RNA sequence and/or structure affect the maturation and turnover of miRNAs, and there are many different ways to introduce changes to RNA molecules.

Processing bodies
(P-bodies). Distinct foci within the cytoplasm of the eukaryotic cell that consist of many enzymes involved in mRNA turnover. They are thought to be sites for translational suppression and/or mRNA decay, and to be involved in RNA silencing. Some mRNAs can exit P-bodies and reinitiate translation.

Single nucleotide polymorphisms affecting miRNA biogenesis. Single nucleotide polymorphisms (SNPs) are found in miRNA genes and sometimes affect their biogenesis and/or alter their target specificity[204]. For example, a C to T SNP in the first C of the CNNC motif in *pri-miR-15a~16-1* reduces Drosha-mediated processing and thereby lowers miR-16 production[56]. This may be involved in the pathogenesis of chronic lymphocytic leukaemia[205]. Moreover, SNPs in the nucleotide sequence of mature miRNAs that resulted from the passenger strand, miR-196a-2* (miR-196a-2-3p)[206] and miR-146a* (miR-146a-3p)[207,208], have been associated with cancer. These SNPs change target specificity and may also affect the processing of these miRNAs.

Regulation through miRNA tailing. RNA tailing (untemplated nucleotidyl addition to the 3′ end of RNA) modifies pre-miRNA and mature miRNA[209] (FIG. 3b). Uridylation of the let-7 precursor (pre-let-7) has been most extensively studied. The let-7 family members are post-transcriptionally suppressed in embryonic stages[210,211]. LIN28A and its paralogue LIN28B bind to the terminal loop of pri-let-7 and pre-let-7 and interfere with Drosha and Dicer processing, respectively[212–219]. Moreover, the LIN28 proteins recruit terminal uridylyl transferases TUT4 (also known as ZCCHC11) and, to a lesser extent, TUT7 (also known as ZCCHC6) to induce oligouridylation of pre-let-7 (REFS 214,220,221). An oligo-U tail blocks Dicer processing and facilitates miRNA decay[214]. The 3′–5′ exonuclease that recognizes the U tail is DIS3L2 (REFS 222–223). In cells that do not express LIN28, terminal uridylyl transferases (mainly TUT7 but also TUT4 and TUT2) induce monouridylation at the 3′ end of group II pre-let-7 miRNAs and increase let-7 biogenesis (see below)[224]. Thus, LIN28 acts as a molecular switch that converts TUT4 and TUT7 from biogenesis factors into negative regulators of let-7 miRNAs.

Adenylation is another type of RNA tailing and occurs mainly after Dicer processing. A hepatic miRNA, miR-122, is frequently adenylated, which stabilizes the miRNA[225]. A poxvirus-encoded VP55 (also known as PAPL) catalyses adenylation of host miRNAs and facilitates miRNA decay[226]. Thus, the consequences of miRNA adenylation may depend on the context, and it is currently unclear what causes such differences.

RNA editing. Apart from RNA tailing, several types of RNA modifications affect miRNA biogenesis. RNA editing involves the conversion of adenosine to inosine, which is catalysed by adenosine deaminases (ADARs). RNA editing has been observed in a subset of pri-miRNAs, including pri-miR-142 (REF. 227) (FIG. 1b). The editing occurs in the stem region and makes pri-miR-142 poor substrates for Drosha. ADAR1 (also known as DRADA) edits pri-miR-151, and the product (pre-mir-151 with edited sites) cannot be efficiently processed by Dicer[228] (FIG. 3b).

RNA methylation. Recently, the human RNA methyltransferase BCDIN3D was shown to *O*-methylate the 5′ monophosphate of pre-mir-145 and pre-mir-23b[229] (FIG. 3b). As human Dicer interacts with the 5′-terminal phosphate[105], the modification interferes with Dicer-mediated processing[229]. Notably, BCDIN3D is involved in the tumorigenesis of breast cancer, whereas miR-145 and miR-23b can suppress tumorigenesis.

Regulation through miRNA stability. The abundance of some miRNAs is regulated at the RNA stability level[230] (FIG. 3b). Pre-miRNAs, including miR-146a and miR-135b, are cleaved at the terminal loop by the mammalian endoribonuclease MCP-induced protein 1 (MCPIP1; also known as ZC3H12A)[231]. More recently, Ser/Thr protein kinase/endoribonuclease IRE1α has been shown to be activated by endoplasmic reticulum stress and cleave select pre-miRNAs (pre-mir-17, pre-mir-34a, pre-mir-96 and pre-mir-125b), which leads to translational derepression of the pro-apoptotic caspase 2 (REF. 232).

The turnover of mature miRNA has been studied in various systems. Several nucleases have been proposed to cleave and degrade miRNAs, but it is unknown how they achieve substrate specificity and whether there is a conserved machinery for miRNA decay (FIG. 3b). Active degradation of miRNA was initially reported in *Arabidopsis thaliana*, in which a group of 3′–5′ exoribonucleases that are known as small-RNA-degrading nucleases removes miRNAs[233]. In *C. elegans*, the 5′–3′ exoribonucleases XRN-1 and XRN-2 were shown to degrade mature miRNAs[234,235]. In mammals, several neural miRNAs (miR-183, miR-96, miR-192, miR-204 and miR-211) rapidly turnover in the retina, although the nucleases involved in this turnover remain unknown[236]. In addition, human polynucleotide phosphorylase PNPase old-35 (also known as PNPT1), which is an interferon-inducible 3′–5′ exonuclease, degrades certain mature miRNAs (including miR-221, miR-222 and miR-106) in melanoma cells[237]. Loss of another 3′–5′ exonuclease, ERI1, was shown to increase the levels of many miRNAs in mouse immune cells, but it remains to be determined whether ERI1 degrades mature miRNAs directly[238].

Moreover, there are several reports that the target mRNA can modulate the stability of miRNAs[230,239]. Highly complementary targets induce miRNA degradation accompanied by tailing and trimming[240–242]. By contrast, it has been reported in *C. elegans* that miRNAs are stabilized by their targets[234]. Thus, further investigation is required to understand the mechanisms and physiological importance of target-mediated stability control.

Viruses sometimes use their own RNAs to destabilize host miRNAs through specific base pairing[230]. When T cells are infected with Herpesvirus saimiri, viral non-coding RNA HSUR binds to and destabilizes miR-27 specifically[243]. Mouse cytomegalovirus produces the m169 RNA that contains miR-27-binding sites in its 3′ UTR and induces the degradation of miR-27 (REF. 239). Furthermore, virulent strains of human cytomegalovirus express a ~240-nucleotide-long non-coding RNA (UL144-145) that discriminately binds to host miR-17 and miR-20a, and induces selective decay of these miRNAs[244]. The interactions between UL144-145 and these miRNAs are required for viral production during lytic infection, illustrating the importance of miRNA regulation in viral pathogenicity.

Terminal uridylyl transferases
Nucleotidyl transferases that covalently add one or more UMP moieties from UTP to the 3′-OH group of an RNA substrate in a template-independent manner.

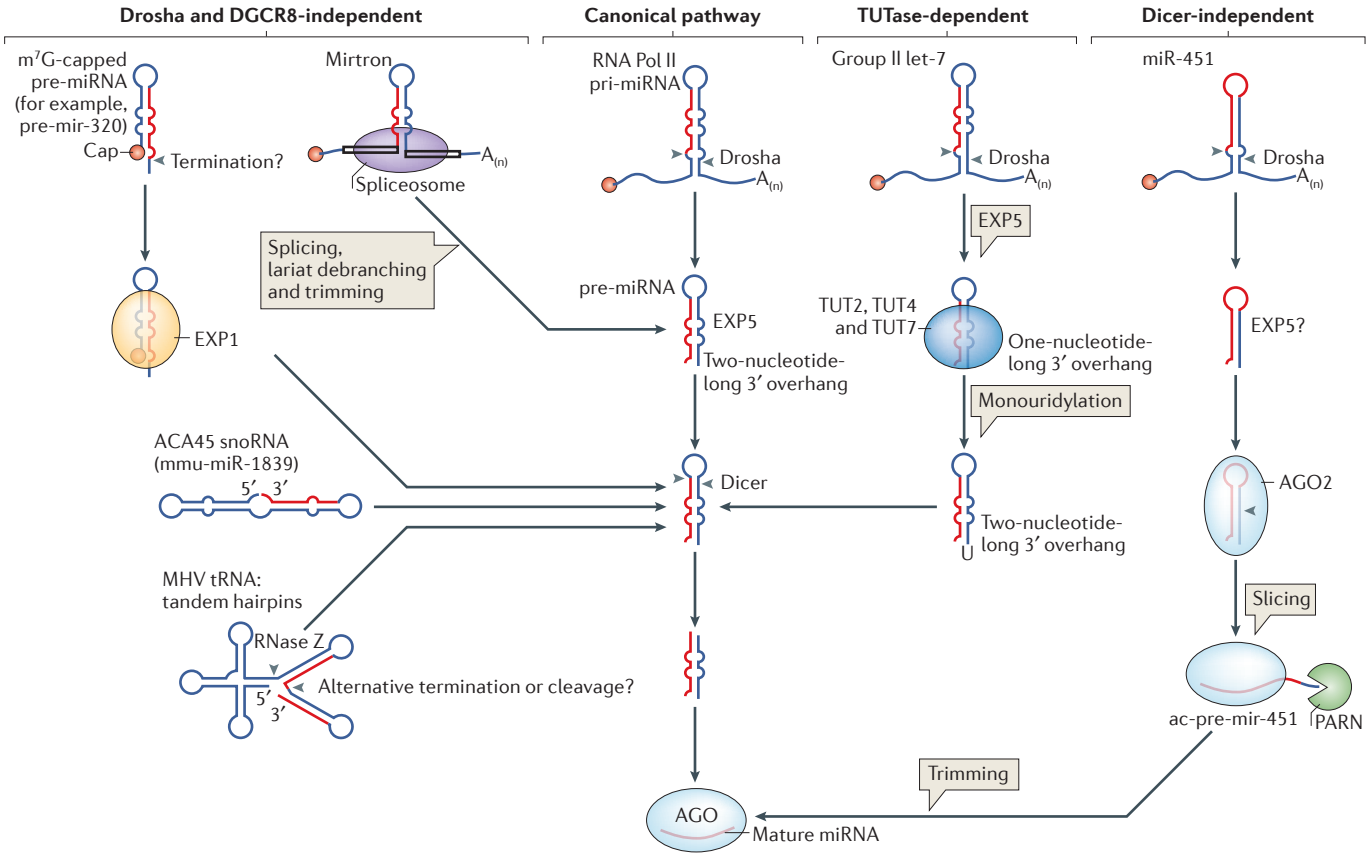


Figure 4 | **Non-canonical pathways of miRNA biogenesis.** The 7-methylguanosine (m^7G)-capped pre-mir-320 is directly generated through transcription, bypassing Drosha processing, and it is exported to the cytoplasm by exportin 1 (EXP1). Mirtron loci produce pre-miRNAs directly through splicing and debranching. Some mirtrons contain 5′ or 3′ single-stranded RNA tails that need to be trimmed before Dicer processing. Some small nucleolar RNAs (snoRNAs), such as ACA45, and tRNAs (or tRNA-like RNAs) may also be cleaved to produce pre-miRNAs. Terminal uridylyl transferase (TUTase)-dependent group II pri-miRNAs produce pre-miRNAs with a shorter 3′ overhang that is suboptimal for Dicer processing. This means that they need to be monouridylated for efficient Dicer processing. In a Dicer-independent pathway, a short pre-mir-451 is produced by Drosha, exported to the cytoplasm (possibly by EXP5) and loaded on Argonaute 2 (AGO2) without Dicer processing. AGO2 cleaves ('slices') the stem of pre-mir-451, generating AGO-cleaved pre-mir-451 (ac-pre-mir-451), which is further trimmed by the 3′–5′ exonuclease poly(A)-specific ribonuclease PARN. The question marks indicate places in which the depicted action has not be fully confirmed. MHV, murine γ-herpesvirus; mmu, *Mus musculus*; Pol II, polymerase II.

miRNAs are highly stable once they enter RISC, because both ends are protected by AGO proteins[134,136]. Therefore, for miRNA decay to occur, miRNAs may need to be unloaded first so that exonucleases can access their termini. It remains unclear if and how miRNA unloading takes place or what controls the specificity.

Non-canonical pathways

Apart from the canonical miRNA biogenesis pathways described above, various alternative mechanisms can generate miRNAs or miRNA-like small RNAs[100,245] (FIG. 4). Deep sequencing of small RNAs from cells deficient in *Dgcr8*, *Drosha* or *Dicer* uncovered unconventional miRNAs that can be produced in a Microprocessor-independent or Dicer-independent manner[30,37]. A non-canonical pathway was first described to take place during mirtron production[246–248], in which the Drosha-mediated processing step is bypassed and a small RNA precursor is instead generated through mRNA splicing. After splicing from host mRNAs, the lariat is debranched and refolds into a short stem–loop structure that resembles a pre-miRNA. Some mirtrons have extra sequences at the 5′ or 3′ end and need trimming by exonucleases[249].

Drosha-mediated processing is also bypassed in the cases of small RNAs derived from endogenous short hairpin RNAs, which are generated directly through transcription[30,37]. Although these endogenous short hairpin RNAs were initially thought to be transcribed by RNA Pol III, it has been recently shown that at least some of them (for example, *mir-320*) are transcribed by RNA Pol II[89]. The 7-methylguanosine-capped pre-mir-320 is recognized and exported by EXP1. Following Dicer processing, the 3p miRNA is exclusively selected, presumably because the 5p miRNA has a 7-methylguanosine cap at the 5′ end that may interfere with AGO loading.

In addition, some small RNAs may originate from other non-coding RNAs, such as tRNAs or tRNA-like precursors[30], small nucleolar RNAs (snoRNAs)[250] or small nuclear RNA-like viral RNAs[251], without

Short hairpin RNAs
Sequences of RNA that have a tight hairpin turn that can be processed by Dicer.

Small nucleolar RNAs
(snoRNAs). A class of small RNA molecules that primarily guide chemical modifications of other RNAs. This mainly includes rRNAs, tRNAs and small nuclear RNAs.

Drosha processing. Biogenesis of these small RNAs still depends on Dicer. For instance, in cells infected by murine γ-herpesvirus 68, viral pri-miRNAs are co-transcribed downstream of tRNAs[29]. The 5′ end of viral pre-miRNA is generated by the 3′ end processing of the tRNA, which is mediated by RNase Z and is independent of Drosha[252].

Although most alternative miRNA pathways depend on Dicer, biogenesis of miR-451 does not require Dicer and instead involves the catalytic activity of AGO2 (REFS 145,253,254). miR-451 is an erythropoietic miRNA conserved in vertebrates. Drosha-mediated cleavage of pri-miR-451 generates a short hairpin with a stem of ~18 bp that is too short to be processed by Dicer. Thus, pre-mir-451 is directly loaded onto AGO2, and is sliced in the middle of its 3′ strand, which yields a 30-nucleotide-long intermediate species (AGO-cleaved pre-mir-451 (ac-pre-mir-451)). Poly(A)-specific ribonuclease PARN trims down the 3′ end of ac-pre-mir-451 to produce the mature miR-451, which is ~23 nucleotides in length[255]. Notably, trimming of ac-pre-mir-451 is not essential for miRNA function: ac-pre-mir-451 (~30 nucleotides in length) is as active as the shorter miR-451 counterpart (~23 nucleotides in length), demonstrating that the 3′ heterogeneity is not crucial for miRNA activity.

Unlike prototypical pre-miRNAs that have a two-nucleotide-long 3′ overhang (group I pre-miRNAs), some pre-miRNAs (group II pre-miRNAs, including most members of the let-7 family in vertebrates) carry a shorter (1-nucleotide long) 3′ overhang, owing to their unusual pri-miRNA structure[224]. Group II pre-miRNAs are extended by 1 nucleotide through monouridylation mediated by terminal uridylyl transferases (including TUT2, TUT4 and TUT7). Monouridylation is required for efficient Dicer-mediated processing[224].

The existence of alternative pathways reflects the evolutionary flexibility of miRNA biogenesis. However, it is notable that the vast majority of functional miRNAs follow the canonical pathway for their biogenesis, and that only about 1% of conserved miRNAs (for example, miR-320 and miR-451) are produced independently of Dicer or Drosha in vertebrates. Most other non-canonical miRNAs are low in abundance and poorly conserved. So, the functional relevance of non-canonical miRNAs should be interpreted with caution.

Open questions

Although the basics of the miRNA biogenesis pathway have been established, there are still many unresolved questions. The structure of Drosha is yet to be solved, which will greatly advance our understanding of miRNA maturation. The crystal structure of the human Dicer–TRBP complex will also answer many questions regarding how Dicer interacts with its substrates and products during the pre-miRNA processing and loading steps. The precise roles of dsRBD proteins need to be investigated at the molecular and cell biological levels.

Further studies are needed to reveal whether a conserved pathway for miRNA decay exists and which nucleases are involved. In addition, although RNA tailing such as adenylation and uridylation is often associated with miRNA decay, the mechanisms linking tailing and decay are unknown. The nucleases that recognize the modified end should be identified to gain mechanistic insights into miRNA decay.

The boundaries between miRNAs and other small RNAs are becoming increasingly vague due to the discovery of non-canonical miRNAs. Additional alternative biogenesis pathways may be uncovered in the future. It will be interesting to uncover whether the use of the alternative pathways is differentially regulated under various cellular conditions.

Numerous auxiliary regulatory factors have been reported so far to be involved in miRNA biogenesis, and many more are expected to emerge in the near future, as miRNAs seem to be controlled at multiple levels in various developmental and pathological contexts. Moreover, as in some reported cases the mechanisms through which these regulatory factors control miRNA biogenesis are obscure, higher standards are needed to scrutinize if and exactly how these factors control miRNA maturation.

miRNAs are intricately connected to signalling pathways. Transcription factors and miRNA processing factors are under the control of cell signalling. It will be of interest to uncover the signalling molecules and their relationships upstream and downstream of miRNA biogenesis. Previous studies demonstrated that miRNAs are often engaged in feedback loops, thereby potentiating their regulatory power. A notable example is the bi-stable switch composed of LIN28 proteins (LIN28A and LIN28B in mammals) and let-7 in mammals. LIN28 proteins block let-7 maturation, while let-7 downregulates LIN28 proteins by binding to their 3′ UTR. Moreover, let-7 targets MYC, which activates the transcription of LIN28 proteins in mammals. It will be interesting to identify additional regulatory loops that involve miRNAs.

It has been proposed that miRNA biogenesis factors are also involved in other RNA pathways. Drosha cleaves and destabilizes several mRNAs[37,62,64,65,256–259] and retrotransposon transcripts[260]. DGCR8 has been reported to bind to snoRNAs in a Drosha-independent manner[261]. A recent genetic study in flies suggested that DGCR8 may have additional Drosha-independent functions[262]. Moreover, Dicer was reported to cleave various types of RNAs including Alu transcripts and mRNAs[263,264]. TRBP and PACT were initially shown to function as regulators of PKR signalling. AGO proteins[265] have been recently reported to function independently of miRNAs by binding to ribosomes and destabilizing stalled mRNAs during translation[266]. It is overwhelming to digest all these observations and come up with a unifying picture to understand the links between the miRNA pathway and the other RNA pathways. It awaits rigorous investigation to define physiologically relevant targets of the miRNA biogenesis factors, as many of the current tools are vulnerable to producing false-positive results. However, what seems clear at this point is that the miRNA biogenesis pathway will continue to be a rich source of exciting new discoveries.

Retrotransposon
A transposable element that replicates through an RNA intermediate, which is converted by a reverse transcriptase to cDNA. The cDNA can be inserted into genomic DNA, which increases the number of copies of the retrotransposon in the genome.

Alu
A short (~300 bp long) stretch of repetitive elements that are classified as short interspersed elements among the class of repetitive DNA elements. Alu elements of different kinds occur in large numbers in primate genomes, accounting for over 10% of the human genome.

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Competing interests statement

The authors declare no competing interests.

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