REVIEW

Many roads to maturity: microRNA biogenesis pathways and their regulation

Julia Winter[1,3], Stephanie Jung[1,3], Sarina Keller[1], Richard I. Gregory[2] and Sven Diederichs[1,4]

MicroRNAs are important regulators of gene expression that control both physiological and pathological processes such as development and cancer. Although their mode of action has attracted great attention, the principles governing their expression and activity are only beginning to emerge. Recent studies have introduced a paradigm shift in our understanding of the microRNA biogenesis pathway, which was previously believed to be universal to all microRNAs. Maturation steps specific to individual microRNAs have been uncovered, and these offer a plethora of regulatory options after transcription with multiple proteins affecting microRNA processing efficiency. Here we review the recent advances in knowledge of the microRNA biosynthesis pathways and discuss their impact on post-transcriptional microRNA regulation during tumour development.

MicroRNAs (miRNAs) are short (20–23-nucleotide), endogenous, single-stranded RNA molecules that regulate gene expression[1]. Mature miRNAs and Argonaute (Ago) proteins form the RNA-induced silencing complex (RISC), a ribonucleoprotein complex mediating post-transcriptional gene silencing[2–5]. Complementary base-pairing of the miRNA guides RISC to target messenger RNAs, which are degraded, destabilized or translationally inhibited by the Ago protein[6,7]. Proteomic studies have recently uncovered the broad impact of a single miRNA on hundreds of targets[8,9]. Many cellular pathways are affected by the regulatory function of miRNAs; the most prominent of these pathways control developmental and oncogenic processes[10–20]. Notably, miRNA processing defects also enhance tumorigenesis[21]. Although insights into the regulatory function of miRNAs are beginning to emerge, much less is known about the regulation of miRNA expression and activity. Recently, evidence for post-transcriptional control of miRNA activity has been accumulating[22–26].

In contrast to the linear miRNA processing pathway that was initially thought to be universal for the biogenesis of all mature miRNAs (Fig. 1), multiple discoveries led to the recognition of miRNA-specific differences that open a plethora of regulatory options to express and process individual miRNAs differentially. Here we review the recent progress made in elucidating the complexity of miRNA processing and post-transcriptional regulation. Although we focus predominantly on the mammalian system, related information obtained from other model systems including the fruitfly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and the plant *Arabidopsis thaliana* will also be presented where applicable.

Early steps: microRNA processing in the nucleus

Transcription of the pri-miRNA. miRNA genes are transcribed by either RNA polymerase II or RNA polymerase III into primary miRNA transcripts (pri-miRNA)[27–29]. Many pri-miRNAs are polyadenylated and capped — hallmarks of polymerase II transcription. Their transcription is sensitive to treatment with the polymerase II inhibitor α-amanitin, and polymerase II binds to promoter sequences upstream of the miR-23a/miR-27a/miR-24-2 cluster[27,28]. In contrast, miRNAs encoded by the largest human miRNA cluster, C19MC, are transcribed by polymerase III[29]. Both RNA polymerases are regulated differently and recognize specific promoter and terminator elements, facilitating a wide variety of regulatory options. Expression of selected miRNAs is under the control of transcription factors, for example c-Myc or p53 (refs 17, 19), or depends on the methylation of their promoter sequences[30–32]. In addition, it has been shown that each miRNA located in the same genomic cluster can be transcribed and regulated independently[33]. However, controls of miRNA transcription steps are not necessarily universal[34,35], and regulatory mechanisms at the transcriptional level are beyond the scope of this review.

microRNA editing. RNA editing of primary transcripts by ADARs (adenosine deaminases acting on RNA) modifies adenosine (A) into inosine (I). Because the base-pairing properties of inosine are similar to those of guanosine (G), A-to-I editing of miRNA precursors may change their sequence, base-pairing and structural properties and can influence their further processing as well as their target recognition abilities. Several examples of editing-mediated regulation of miRNA processing have been described (see Box 1).

[1]Helmholtz-University-Group 'Molecular RNA Biology & Cancer', German Cancer Research Center (DKFZ) and Institute of Pathology, University of Heidelberg, B150 INF 581, D-69120 Heidelberg, Germany. [2]Stem Cell Program, Children's Hospital Boston, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Harvard Stem Cell Institute, Boston, Massachusetts 02115, USA.
[3]These authors contributed equally to the work.
[4]Correspondence should be addressed to S.D. (e-mail: s.diederichs@dkfz.de)

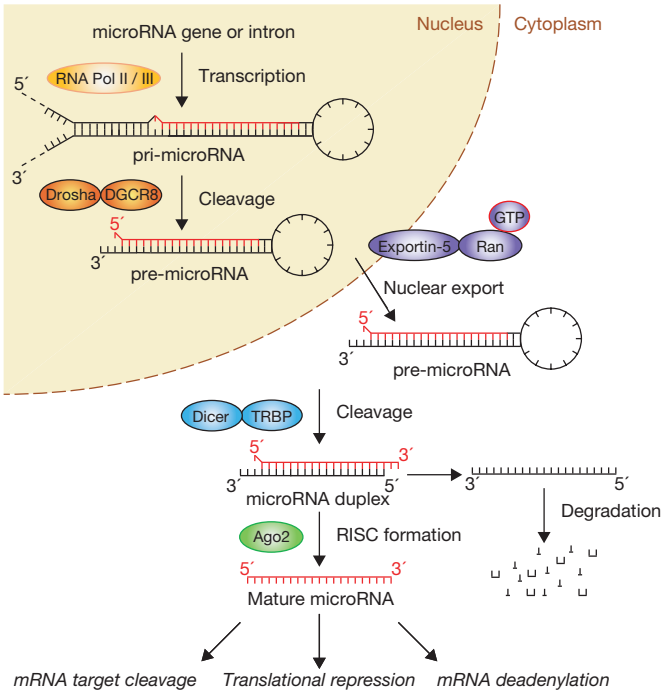

Figure 1 The 'linear' canonical pathway of microRNA processing. The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. In this review we discuss the many branches, crossroads and detours in miRNA processing that lead to the conclusion that many different ways exist to generate a mature miRNA.

pri-miRNA cleavage by the Drosha–DGCR8 microprocessor complex. The pri-miRNA is next endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha (RNASEN) and the DGCR8 (DiGeorge critical region 8) protein (also known as Pasha (Partner of Drosha) in *D. melanogaster* and *C. elegans*)[36] (Fig. 2a). DGCR8/Pasha contains two double-stranded RNA-binding domains and is essential for miRNA processing in all organisms tested[37–40]. An average human pri-miRNA contains a hairpin stem of 33 base-pairs, a terminal loop and two single-stranded flanking regions upstream and downstream of the hairpin. The double-stranded stem and the unpaired flanking regions are critical for DGCR8 binding and Drosha cleavage, but the loop region or the specific sequences are less important for this step[41–43]. A single nucleotide polymorphism in a miRNA precursor stem can block Drosha processing[44]. Nevertheless, many miRNA sequence aberrations observed in human tumours alter the secondary structure without affecting processing, and reveal the structural flexibility of the microprocessor[34].

The two RNase domains of Drosha cleave the 5′ and 3′ arms of the pri-miRNA hairpin[39], whereas DGCR8 directly and stably interacts with the pri-miRNA and functions as a molecular ruler to determine the precise cleavage site[41]. Drosha cleaves 11 base pairs away from the single-stranded RNA/double-stranded RNA junction at the base of the hairpin stem.

Drosha-mediated cleavage of the pri-miRNA occurs co-transcriptionally and precedes splicing of the protein-encoding or non-coding host RNA that contains the miRNAs. Splicing is not inhibited by Drosha-mediated cleavage, because a continuous intron is not required for splicing[45,46].

microRNA-specific regulation of the microprocessor complex. Drosha-mediated pri-miRNA processing was recently shown to be subject to regulation by miRNA-specific mechanisms. Drosha forms two different complexes, a small microprocessor complex that contains only Drosha and DGCR8 and processes many pri-miRNAs, and a larger complex that contains RNA helicases, double-stranded RNA binding proteins, heterogeneous nuclear ribonucleoproteins and Ewing's sarcoma proteins[38]. The RNA helicases p72 and p68 are part of the large Drosha complex and might act as specificity factors for the processing of a subset of pri-miRNAs (Fig. 2b). Expression levels of several miRNAs are reduced in homozygous $p68^{-/-}$ or $p72^{-/-}$ knockout mice, whereas other miRNAs remain unaffected[47].

Drosha-mediated cleavage can also be regulated for individual miRNAs: the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) binds specifically to pri-miR-18a and facilitates its processing. Loss of hnRNP A1 diminishes the abundance of mature miR-18a (Fig. 2c), but hnRNP A1 does not have any impact on other miRNAs that are located in the same miR-17 genomic cluster, demonstrating the extraordinary specificity of miR-18a biogenesis[48]. hnRNP A1 binds to the conserved loop of the pri-miR-18a and changes the hairpin conformation to create a more favourable cleavage site for Drosha[49]. About 14% of the human pri-miRNA loops are conserved between different species and could provide anchor points for similar regulatory mechanisms.

Transforming growth factor-β (TGF-β) and bone morphogenetic factors (BMPs) induce the maturation of miR-21 by regulating the microprocessor activity. TGF-β and BMP bring about the recruitment

BOX 1 microRNA editing

Editing is defined as a post-transcriptional change of RNA sequences by deamination of adenosine (A) to inosine (I), altering the base-pairing and structural properties of the transcript. Editing of miRNA transcripts by ADAR1 and ADAR2 was first described for miR-22 (ref. 116) followed by miR-151, miR-197, miR-223, miR-376a, miR-379 and miR-99a (ref. 117), as well as miR-142, miR-223, miR-1-1 and miR-143 (ref. 118). In pri-miR-142, A-to-I editing inhibits its cleavage by the endonuclease Drosha and results in its degradation by the ribonuclease Tudor-SN, which preferentially cleaves double-stranded RNA containing inosine–uracil pairs[118,119]. However, editing of other pri-miRNAs was shown to enhance their processing by Drosha[120]. Editing can also influence further downstream processing steps: pri-miR-151 editing abolishes its cleavage by Dicer in the cytoplasm. It remains to be established whether miRNA editing events are predominantly nuclear or cytoplasmic and whether they occur on the pri-miRNA or on the precursor miRNA (pre-miRNA)[121]. In addition to altering miRNA processing, miRNA editing can have an impact on miRNA target specificity. For example, a single A-to-I change in the miR-376 precursor redirects the mature miRNA to a new target, resulting in altered protein expression in mice[122]. In summary, miRNA editing can influence processing at multiple steps or can change the miRNA complementarity to target sequences, increasing the diversity of the cellular miRNA pool.

of ligand-specific signal transducers (the SMAD proteins) to the pri-miR-21 transcript in complex with the RNA helicase DDX5 (p68). As a consequence, Drosha-mediated processing of pri-miR-21 is strongly enhanced and the abundance of mature miR-21 increases, ultimately resulting in a contractile phenotype in vascular smooth muscle cells (Fig. 2d)[50].

Mirtrons: splicing replaces Drosha cleavage. Surprisingly, Drosha-mediated processing of pri-miRNAs into pre-miRNAs is not obligatory. Intron-derived miRNAs are released from their host transcripts after splicing (Fig. 2e). If the intron resulting from the action of the splicing machinery and the lariat debranching enzyme has the appropriate size to form a hairpin resembling a pre-miRNA, it bypasses Drosha cleavage and is further processed in the cytoplasm by Dicer[51,52]. These miRNAs, called mirtrons, have been discovered in several species including mammals, *D. melanogaster* and *C. elegans*[51–53].

Lin-28 regulates let-7 processing and precursor stability. Lin-28 is a stem-cell-specific regulator of let-7 processing that uses multiple mechanisms[54–58]. Lin-28 was found to be necessary and sufficient to block microprocessor-mediated cleavage of the pri-miRNA (Fig. 3a)[54]. Mature let-7g increases during embryonic stem cell differentiation but the pri-miRNA levels remain constant, indicating post-transcriptional regulation of maturation. Recombinant Lin-28 blocks pri-miRNA processing, and knockdown of *Lin-28* facilitates the expression of mature let-7 (ref. 54). The miRNA binding site of the Drosha competitor Lin-28 maps to conserved bases in the terminal loop of pri-let-7 (refs 56, 57). Intriguingly, although the loop region is considered dispensable for microprocessor action, many miRNAs have evolutionarily conserved loops potentially containing regulatory information[49].

Post-transcriptional self-regulation of the microprocessor complex. The miRNA processing factors are also regulated post-transcriptionally or post-translationally. For example, the two components of the microprocessor complex regulate each other. DGCR8 stabilizes Drosha through an interaction between its conserved carboxy-terminal domain with the middle domain of Drosha (Fig. 4a)[59]. In turn, Drosha cleaves two hairpin structures in the 5′ untranslated region and the coding sequence of the *Dgcr8* mRNA[60]. The *Dgcr8* mRNA is then degraded, resulting in a negative feedback loop reducing *Dgcr8* expression when sufficient microprocessor activity is available (Fig. 4b). The discovery that Drosha can directly cleave hairpin structures in mRNAs also points to the possibility that the two Drosha complexes in the cell regulate mRNAs independently of miRNAs.

Exportin-5–Ran-GTP mediate the export of the pre-miRNA. After nuclear processing, the pre-miRNA is exported into the cytoplasm by Exportin-5 (XPO5) in complex with Ran-GTP[61]. Knockdown of Exportin-5 leads to a decreased abundance of mature miRNAs but not to a nuclear accumulation of the pre-miRNA, indicating that Exportin-5 also protects pre-miRNAs against nuclear digestion[61–63]. Exportin-5 recognizes the pre-miRNA independently of its sequence or the loop structure. A defined length of the double-stranded stem and the 3′ overhangs are important for successful binding to Exportin-5, ensuring the export of only correctly processed pre-miRNAs[63–65].

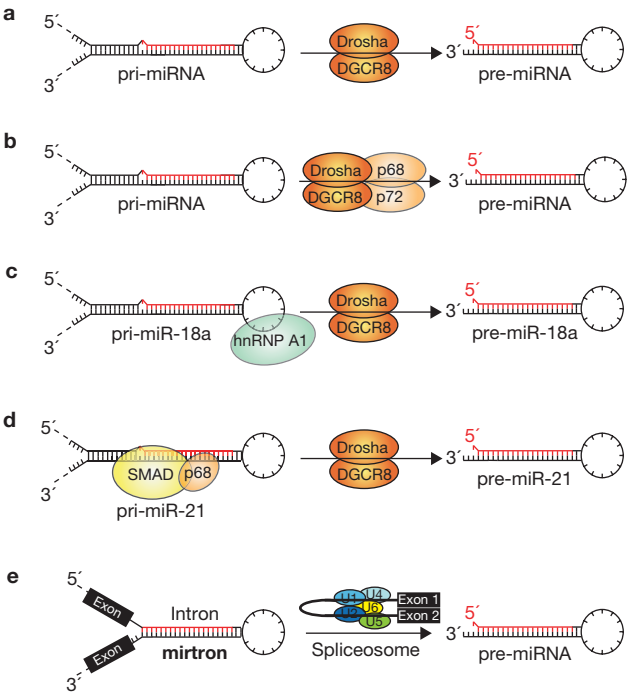

Figure 2 Regulation of pri-miRNA processing. **(a)** The microprocessor complex Drosha–DGCR8 cleaves the pri-miRNA, releasing the pre-miRNA. **(b)** Some miRNAs require additional specificity factors (for example p68 and p72) for efficient cleavage. **(c)** Interaction of pri-miR-18a with hnRNP A1 facilitates cleavage of this specific miRNA by Drosha. **(d)** TGF-β signalling induces SMAD binding to the miR-21 precursor and enhances its efficient processing by Drosha. **(e)** Splicing can replace Drosha processing if the released and debranched intron (mirtron) has the length and hairpin structure of a pre-miRNA.

Coming of age: microRNA maturation in the cytoplasm

The RISC loading complex (RLC): Dicer, TRBP and PACT join Ago2. RISC is the cytoplasmic effector machine of the miRNA pathway and contains a single-stranded miRNA guiding it to its target mRNAs. Cytoplasmic miRNA processing and RISC assembly are mediated by the RISC loading complex (RLC) (Fig. 5a). RLC is a multi-protein complex composed of the RNase Dicer, the double-stranded RNA-binding domain proteins TRBP (Tar RNA binding protein) and PACT (protein activator of PKR), and the core component Argonaute-2 (Ago2)[66–69], which also mediates RISC effects on mRNA targets.

TRBP and PACT are not essential for Dicer-mediated cleavage of the pre-miRNA (see below) but they facilitate it, and TRBP stabilizes Dicer[67,68,70]. Depletion of TRBP or PACT reduces the efficiency of post-transcriptional gene silencing, and both might have overlapping functions in the miRNA and small interfering RNA (siRNA) pathway. Although they both participate in the recruitment of Ago2 (refs 68, 70), the *in vitro* reconstitution of RISC loading and activation is achieved by Dicer, TRBP and Ago2 alone[69]. Formation of the human RLC complex is initiated independently of ATP hydrolysis by the assembly of Dicer, TRBP and Ago2, and the exported hairpin only joins the RLC after the formation of this ternary complex (Fig. 5a)[66,71].

Ago2-mediated pre-miRNA cleavage: the ac-pre-miRNA. For miRNAs that display a high degree of complementarity along the hairpin stem, an additional endonucleolytic cleavage step occurs before Dicer-mediated

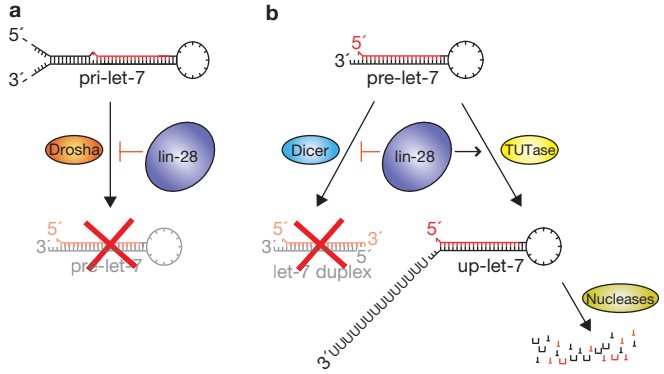

Figure 3 Lin-28 inhibits let-7 biogenesis. Different mechanisms suppress the maturation of let-7 by the RNA-binding protein Lin-28. **(a)** Lin-28 inhibits Drosha-mediated processing of pri-let-7. **(b)** Lin-28 inhibits Dicer-mediated cleavage of pre-let-7 and recruits a terminal uridylyl transferase (TUTase) to pre-let-7. The uridylated up-let-7 is not processed but is degraded by nucleases.

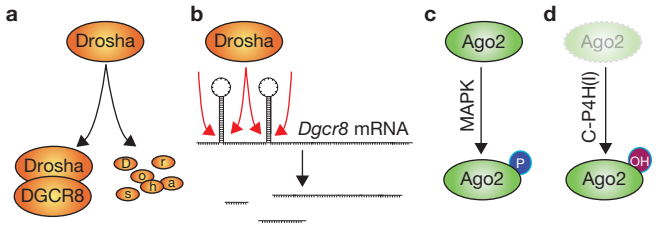

Figure 4 Regulation of microRNA processing factors. **(a)** DGCR8 enhances the protein stability of its partner Drosha. **(b)** Drosha cleaves two hairpin structures in the *Dgcr8* mRNA, which is subsequently degraded. **(c)** Serine phosphorylation of Ago2 regulates its localization to P-bodies. **(d)** Prolyl hydroxylation affects the stability of human Ago2.

cleavage: the slicer activity of Ago2 cleaves the 3′ arm of the hairpin — the prospective passenger strand — in the middle to generate a nicked hairpin, producing the Ago2-cleaved precursor miRNA or ac-pre-miRNA (Fig. 5b)[72]. Dicer can process this precursor as efficiently as the pre-miRNAs. The Ago2-mediated step most probably facilitates subsequent strand dissociation and RISC activation, in a similar manner to its function in the siRNA pathway[73–77]. Thus, in another example of miRNA-specific processing, pre-miRNAs undergo two different fates after nuclear export. This early function of Ago2 in miRNA processing might explain why it associates with the RLC before the pre-miRNA and corroborates earlier findings in other species that Ago proteins are active players in miRNA biogenesis[78,79].

Cleavage of the hairpin into a duplex by Dicer. The RNase III Dicer cleaves off the loop of the pre-miRNA or the nicked ac-pre-miRNA and generates a roughly 22-nucleotide miRNA duplex with two nucleotides protruding as overhangs at each 3′ end. This cleavage is essential for miRNA processing and has been described in many organisms, including *C. elegans*, *D. melanogaster* and mammals[80–83]. Deletion of Dicer decreases or abrogates the production of mature miRNAs[81,82]. In mice, deletion of this evolutionarily conserved endonuclease leads to lethality early in development[84], an effect that could be related to its crucial role in miRNA processing.

The number of genes encoding Dicer-like proteins varies from ten in *A. thaliana* to only one in vertebrates[80,85]. The single copy of Dicer in the mammalian genome might explain its essential role in miRNA biogenesis. Several modes of Dicer cleavage activity regulation have been described. The amino-terminal DExD/H-box helicase domain of human Dicer inhibits its cleavage activity; TRBP binds to Dicer in this region and activates Dicer through a conformational rearrangement[86]. Dicer is also regulated by its product let-7, which targets *Dicer* mRNA, creating a feedback loop[87]. Additional mechanisms to regulate Dicer activity may exist: pre-miR-138 is expressed ubiquitously but its mature form is restricted to certain cell types, indicating tissue-specific processing of this miRNA[22].

Lin-28 double act. Beyond its effect on nuclear microprocessor activity, Lin-28 also regulates pre-let-7 maturation in the cytoplasm. Notably, Lin-28 was shown to inhibit Dicer cleavage *in vitro* (Fig. 3b)[55]. Importantly, a third mode of action for Lin-28-mediated inhibition of let-7 maturation has been characterized in detail (Fig. 3b)[58]. Lin-28 associates with cytoplasmic pre-let-7 and induces its polyuridylation at the 3′ end by an unidentified terminal uridylyl transferase enzyme (TUTase), leading to its degradation by an unidentified nuclease activity. In *A. thaliana*, uridylation is known to accelerate the decay of mature miRNA, and miRNA methylation by Hen1 protects them against uridylation and degradation[88].

Only members of the let-7 family are subject to Lin-28-mediated processing inhibition or uridylation, whereas other human miRNAs are not affected, indicating the strong specificity of these effects[54–56,58]. Lin-28 could contribute post-transcriptionally to the regulation of *let-7* expression in development and cancer[23].

Unwinding the microRNA duplex into guide and passenger strand. After Dicer-mediated cleavage, Dicer and its interactors TRBP or PACT dissociate from the miRNA duplex. To form the active RISC that performs gene silencing, the double-stranded duplex needs to be separated into the functional guide strand, which is complementary to the target, and the passenger strand, which is subsequently degraded. Although multiple helicases have been linked to the miRNA pathway, a universal helicase responsible for duplex unwinding has not yet been identified. Helicases associated with RISC formation or activity include p68, p72, RNA helicase A (RHA), RCK/p54, TNRC6B, Gemin3/4 and human Mov10 or its *D. melanogaster* orthologue Armitage[89–93]. In mice, p68 is found complexed with let-7 and can unwind it[92]. Depletion of RCK/p54 leads to decreased miRNA-mediated RNA interference (RNAi) but not siRNA-mediated RNAi[91]. These findings indicate that specific helicases may regulate miRNAs differentially. However, the results of RISC loading and reconstitution experiments in the absence of ATP indicate that helicases might not be generally required[66,69,71]. For example, Ago2 facilitates duplex unwinding and RISC activation by cleaving the passenger strand of siRNAs or pre-miRNAs[72–76].

Guide strand selection, asymmetry and small RNA sorting. In principle, the miRNA duplex could give rise to two different mature miRNAs. However, in a similar manner to siRNA duplexes, only one strand is usually incorporated into RISC and guides the complex to target mRNAs; the other strand is degraded[94]. This functional asymmetry depends on the thermodynamic stability of the base pairs at the two ends of the duplex: the miRNA strand with the less stable base pair at its 5′ end in the duplex is loaded into RISC[95].

In *D. melanogaster*, miRNAs and siRNAs participate in a common sorting step that partitions them into effector complexes with different Ago proteins[96,97]; fully complementary duplex siRNAs are incorporated into an Ago2-RISC, whereas a distinct, unidentified mechanism incorporates

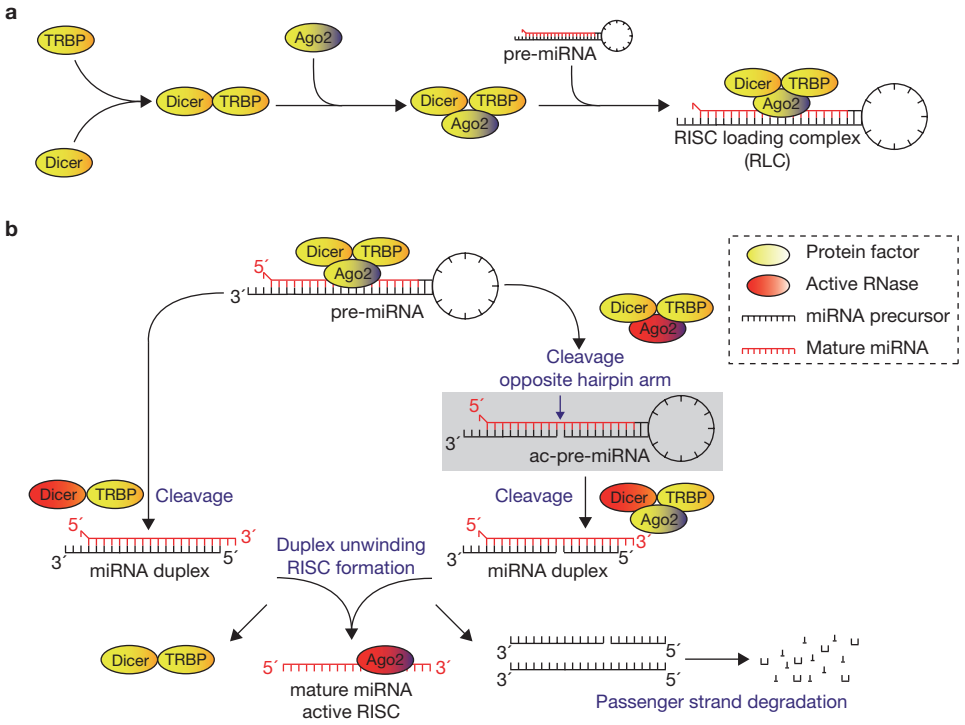

Figure 5 Ago2 generates an additional intermediate, the ac-pre-miRNA. **(a)** Dicer and TRBP interact before Ago2 is recruited to form a ternary complex that binds to the exported pre-miRNA constituting the RISC loading complex (RLC). **(b)** After pre-miRNA binding, Dicer releases the mature miRNA duplex. For some miRNAs, however, Ago2 cleaves first the prospective passenger strand, generating a nicked hairpin called ac-pre-miRNA or Ago2-cleaved pre-miRNA. Adapted from ref. 72, with permission.

partly complementary miRNAs into an Ago1-RISC. In flies, the precise length and position of the 5′ ends of guide and passenger strands increase after Ago2 loading, further ensuring the formation of the correct miRNA with the designated seed sequence[98]. Whereas sorting in *D. melanogaster* depends on duplex complementarity, the 5′ terminal nucleotide is the decisive point in *A. thaliana*[85]. It still remains to be explained how sorting is achieved in mammals.

Argonaute proteins: regulators and effectors. Ago proteins exert multiple functions in the miRNA pathway: they participate in miRNA processing by generating the ac-pre-miRNA[72], and they are the RISC effector proteins mediating the mRNA degradation, destabilization or translational inhibition[2–7]. In addition, Ago proteins regulate miRNA abundance post-transcriptionally, and loss of endogenous Ago2 diminishes the expression and activity of mature miRNA[72,99,100]. This particular function of Ago2 is independent of its slicer function and endonuclease activity. Most probably, the capacity of Ago proteins to bind to mature miRNAs stabilizes these short molecules. Hence, Ago2 is a prime candidate to coordinate the regulation of miRNAs, their biogenesis and their function.

Recent discoveries unravelled prolyl-4-hydroxylation and phosphorylation of Ago2 as regulatory mechanisms of Ago2 activity[101,102]. Human Ago2 is phosphorylated at residue Ser 387 by p38 MAP kinase under cellular stress conditions, aiding in the localization of Ago2 to processing bodies (Fig. 4c)[102]. P-bodies are sites of accumulation of untranslated mRNAs and of multiple enzymes involved in mRNA turnover and translational repression, including Ago proteins and miRNAs[103,104]. In addition, hydroxylation of Pro 700 on the Ago2 protein by the type I collagen prolyl-4-hydroxylase (C-P4H(I)) stabilizes it (Fig. 4d)[101].

Re-import of microRNA into the nucleus. In contrast to most other animal miRNAs, the mature human miR-29b is predominantly localized to the nucleus. It has a distinctive hexanucleotide terminal motif, a transferable nuclear localization element; this suggests that, despite their shortness, miRNAs might contain *cis*-acting regulatory motifs[105]. NRDE-3, a member of the extensive *C. elegans* Argonaute family, participates in nuclear import[106]. The nuclear localization of a fraction of the cellular Ago2 pool in human cells is affected by the RAN-GTP shuttle protein Importin-8, which is also required for miRNA-guided cytoplasmic regulation of a subset of mRNAs[107]. The re-import of miRNAs into the nucleus is especially relevant because evidence is accumulating that miRNAs could regulate gene expression in the nucleus at the transcriptional level[108,109].

Half-life and degradation of microRNA. In comparison with our increasing knowledge about miRNA processing, surprisingly little is known about the half-life and degradation of individual miRNAs. Only in *A. thaliana* has a family of exoribonucleases degrading miRNAs been identified[110]. Mature miRNAs are generally rather stable, as demonstrated by the long persistence of most miRNAs after RNAi-mediated depletion of processing enzymes[36,38]. Nevertheless, as yet unidentified mechanisms may control miRNA turnover. The marked decrease in miR-122 within 1 h after treatment of liver cells with interferon supports this notion[111]. In addition, miRNA activity could also be regulated after processing by blocking the miRNA binding sites on their target mRNA by RNA-binding proteins[112].

Conclusions and outlook: cellular effects of microRNA-specific processing and post-transcriptional regulation

In summary, miRNA biosynthesis can no longer be viewed as one general

pathway universal to all miRNAs. Many steps can be performed in multiple ways, omitted or replaced, and are affected by different mechanisms for individual miRNAs. Most importantly, these specific differences in miRNA processing suggest multiple opportunities for post-transcriptional regulation of miRNA expression. In addition, insights into the regulation of miRNA processing could be applied to enhance RNAi[100,113], which uses some of the same machinery. Because little is yet known about the stability and degradation of miRNAs, this is a promising area for the discovery of novel regulatory mechanisms. The identification of more interaction partners of individual precursors will further broaden the spectrum of control mechanisms. Ultimately, the characterization of miRNA–protein interactomes will be an invaluable tool with which to gain a full understanding of the complex circuitries governing miRNA activity.

Numerous studies have uncovered highly specific miRNA profiles during development or tumorigenesis. Their function as important regulators of differentiation, proliferation, apoptosis or metabolism is nowadays undisputed. The discovery of regulation of let-7 processing by Lin-28 during stem cell differentiation illustrates how insights into miRNA processing help elucidate the function of a miRNA and its regulator in the maintenance and differentiation of pluripotent stem cells[54,114].

Post-transcriptional regulation of miRNA processing also occurs in cancer cells[26] and might explain the aberrant miRNA expression patterns frequently observed in cancer[24,25] with a notable global reduction of mature miRNAs[15]. In addition, reduced expression of Dicer is associated with a poor prognosis in lung cancer[115]. The significance of miRNA processing regulation for tumorigenesis has recently been established experimentally: knockdown of *Drosha*, *Dgcr8* or *Dicer* was shown to promote transformation[21]. An appealing hypothesis to explain the general miRNA suppression observed in cancers is that it is linked to a deficit in miRNA processing. However, support from a primary tumour proving this causality is still lacking. Unravelling the mechanisms underlying miRNA regulation in cancer and other diseases is a central challenge for miRNA research in the coming years. Deepening our knowledge about miRNA maturation in pathological as well as physiological settings will enable us to gain a comprehensive understanding of their many roles in health and disease.

In the near future, therapeutic approaches will be developed that are based on small RNAs targeting genes with an established disease association, such as oncogenes. However, the small RNAs provide only the specificity component of the RNAi machinery, and they rely critically on the endogenous miRNA pathway to execute their function. Thus, understanding the regulatory mechanisms of the miRNA pathway is also a prerequisite for the development and successful application of all RNAi-based drugs.

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