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Emerging complexity of microRNA generation cascades

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MicroRNA (miRNA) modules are built in genetic networks as a complex regulatory layer directing post-transcriptional gene regulation. miRNAs coordinate a broad spectra of gene expression programs mainly through modulation of mRNA metabolism. Perturbations of miRNA networks are linked to a wide variety of pathological processes, including cardiovascular diseases and cancer. While the mechanisms regulating miRNA biogenesis were previously poorly understood, recent findings have shed light on the regulatory mechanisms of miRNAs themselves, especially their biogenesis. Multiple steps of miRNA maturation could potentially provide a variety of regulatory options to generate mature miRNAs differentially and produce gradation in miRNA processing efficiency. Several studies have demonstrated that miRNA maturation pathways crosstalk with intracellular signalling molecules, including p53, Smad proteins and estrogen receptor. Other lines of evidence have demonstrated the involvement of multiple RNA binding proteins in biased processing of different miRNA species. This review summarizes accumulating evidence for the emerging complexity and dynamics of regulated miRNA processing. These findings will lead to better understanding of miRNA dynamics in various pathogenetic pathways and provide the molecular basis for diagnostic and therapeutic strategies based on small RNA biology.

Keywords: Dicer/Drosha/microRNA/p53/processing.

Abbreviations: ADAR, adenosine deaminase acting on RNA; Ago, Argonaute; ARE, AU-rich-element; ARS2, arsenite resistance protein 2; ATM, ataxia telangiectasia mutated; BMP, bone morphogenetic protein; CBC, cap binding complex; DGCR8, DiGeorge critical region 8; dsRBD, double-stranded RNA-binding domain; ERα, estrogen receptor α; hnRNP, heterogenous nuclear ribonucleoprotein; hp-RNA, hairpin RNA; KSRP, KH-type splicing regulatory protein; MAPK, mitogen-activated protein kinase; miR/miRNA, microRNA; NF90, nuclear factor 90; nt, nucleotide; PDCD4, programmed cell death 4; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; SF2/ASF, splicing factor 2/alternative splicing factor; SNIP1, smad nuclear interacting protein 1; snoRNA, small nucleolar RNA; TGF-β, transforming growth factor-β; TRBP, trans-activation response (TAR) RNA-binding protein; tsRNA, tRNA-derived small RNA; TUT4, terminal uridylyl transferase 4.

microRNAs (miRNAs) are small bits of RNA that coordinate a broad range of gene expression programs mainly through modulation of mRNA metabolism, one of the central aspects of gene regulation (*1*, *2*). Through complementary base pairing between individual miRNAs and their target mRNAs, miRNAs interfere with the transformation of transcriptome information into proteome output via RNA-induced gene silencing. Reflecting the multiplicity of relationships between miRNAs and target mRNAs, miRNAs regulate diverse cellular functions such as differentiation, proliferation and cell death in a highly context-dependent fashion. In addition, the essential roles of miRNAs in cellular pathways have expanded, to include their widespread commitment in many physiological and pathological processes such as development and tumourigenesis (*3*, *4*). Intense research is now increasing knowledge of the miRNA involvement in these processes. However, details of the metabolism of miRNAs themselves remain largely unknown. Recent advances have shed light on the understanding of regulated miRNA biogenesis. In this review, we summarize the recent advances in the understanding of miRNA biogenesis pathways, focusing in particular on interfaces with nuclear networks and the diversity of miRNA biogenesis pathways for distinct miRNA species.

General route of miRNA processing

The mammalian miRNA processing pathway is comprised of a series of several biochemical steps that convert primary miRNA transcripts (pri-miRNAs) into biologically active, mature miRNAs (Fig. 1A) (*5*). Similar to other protein-coding mRNAs, most miRNA genes are initially transcribed by RNA polymerase II to pri-miRNAs, which contain one or more stem–loop structures. RNA polymerase III has also been shown to be exceptionally responsible for the transcription of some pri-miRNAs (*6*). The canonical miRNA biogenesis pathway to funnel pri-miRNAs to mature miRNAs is characterized by two subsequent central steps utilizing ribonuclease reactions (*7*). In the nucleus, pri-miRNAs are recognized and cropped into hairpin-structured precursor miRNAs (pre-miRNAs) by the Drosha complex (also

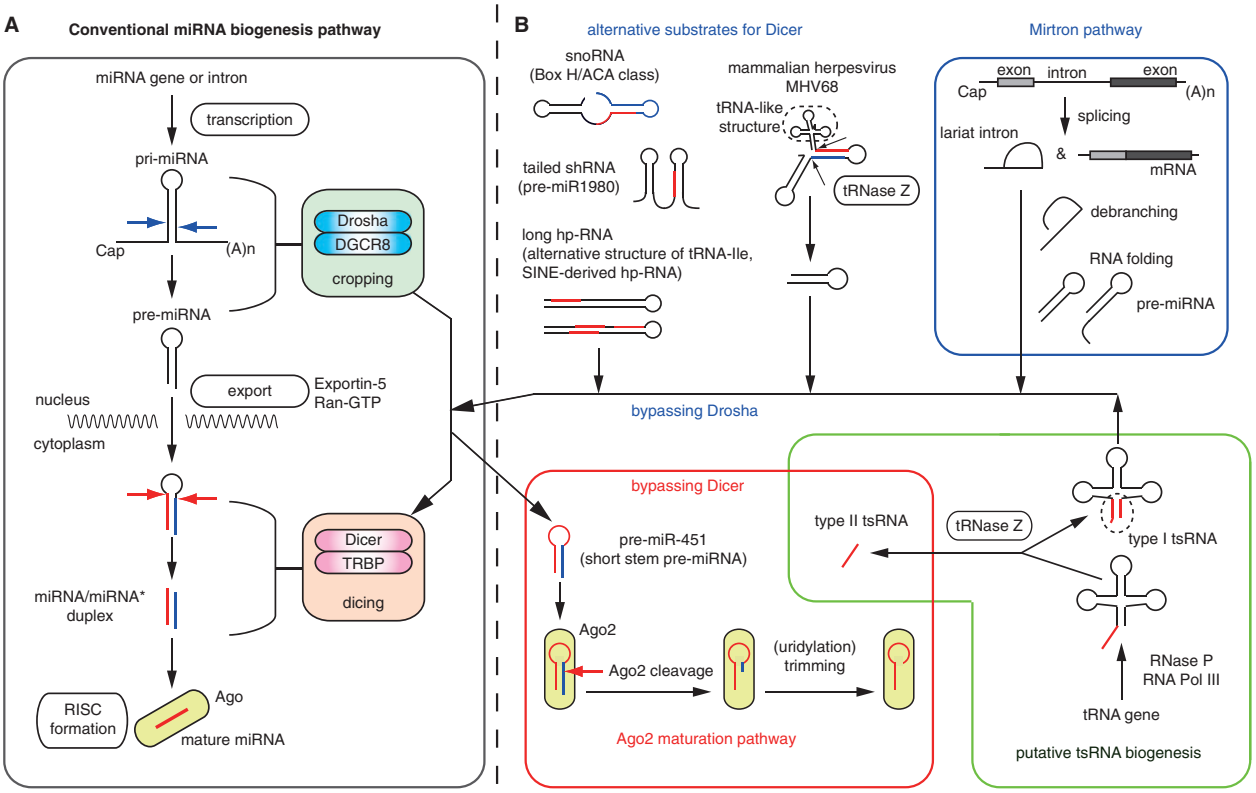

Fig. 1 Overview of small RNA biogenesis pathways. (A) Canonical miRNA biogenesis in animals is executed through sequential biochemical steps, including (i) transcription of pri-miRNA, (ii) Drosha-mediated cropping of pri-miRNA to pre-miRNA, (iii) nucleo-cytoplasmic transport of pre-miRNA, (iv) dicing of pre-miRNA to miRNA duplex by Dicer and (v) RISC formation. (B) Several pathways of generation of miRNAs and related small RNAs deviate from the conventional miRNA biogenesis pathway. In the mirtron pathway, spliced lariat introns become pre-miRNAs after linearization by the lariat debranching enzyme and RNA refolding. Besides mirtron, several RNA molecules such as snoRNA, tailed shRNA, long hairpin RNA (hp-RNA) and viral RNA (MHV68) could be alternative sources for Dicer cleavage. In the biosynthesis of tRNA-derived small RNAs (tsRNAs), type II tsRNAs are cleaved from tRNA precursors by tRNase Z. Type I tsRNAs are then produced by Dicer cleavage. Among the various pre-miRNAs, pre-miR-451, whose stem is shorter than those of other miRNAs, are matured by Ago2 cleavage and further trimming, independently of Dicer.

known as microprocessor complex). Drosha (RNASEN), an RNase III enzyme, and DGCR8 (DiGeorge critical region 8), a double-stranded RNA-binding domain (dsRBD) protein, are two essential components of the microprocessor complex. Drosha liberates the stem–loop pre-miRNAs from pri-miRNAs in cooperation with DGCR8-mediated recognition of the junctional region between the single-stranded and double-stranded portions of pri-miRNAs (*8*). The Drosha complex also contains several auxiliary factors such as EWSR1, FUS, numerous heterogeneous nuclear ribonucleoproteins (hnRNPs) and p68 (DDX5) and p72 (DDX17) DEAD-box helicases (*9*). While the roles of these accessory molecules in miRNA maturation are largely unknown, some hnRNPs and p68/p72 appear to promote the fidelity and activity of Drosha processing (*10*, *11*). miRNA biogenesis is homeostatically controlled by an auto-feedback loop between Drosha and DGCR8 (*12*), in which DGCR8 stabilizes Drosha protein level, while Drosha destabilizes DGCR8 mRNA through cleavage of the hairpin structures in the DGCR8 mRNA. The pre-miRNAs harbour a 2 nt 3′-overhang, which is recognized by exportin-5 to mediate nucleo-cytoplasmic transport of pre-miRNAs together with Ran-GTP (*13*).

In the cytoplasm, pre-miRNAs proceed to second-round ribonuclease reaction to generate mature miRNAs. This step is mediated by another important RNase III, Dicer. Dicer is associated with other dsRBD proteins, TRBP/PACT and dices the pre-miRNAs into a ~22 nt long miRNA duplex. The miRNA duplex is comprised of two miRNA strands, with one strand loaded onto the RNA-induced silencing complex (RISC), which contains the Argonaute (Ago) family protein as a core component. In these processes, another strand (miRNA* strand) is usually degraded, while miRNA* strands are retained and function in some cases (*14*). Mature miRNAs serve as guides directing RISC to target mRNAs, which are degraded, destabilized or translationally inhibited by the Ago proteins (*2*). Ago proteins play roles in miRNA maturation of several, but not all, miRNAs (*15*), in addition to their central roles in miRNA-mediated gene silencing. For some miRNAs that show a high degree of complementarity along the hairpin stem, Ago2 first nicks the prospective miRNA* strand (also designated as passenger strand) and generates an additional intermediate, Ago2-cleaved pre-miRNA (ac-pre-miRNA) before Dicer processing and stabilizes mature miRNAs (*15*). The principles of transcriptional regulation of numerous protein-coding

mRNAs are similarly applied to the transcription-dependent regulation of miRNAs, although the mechanisms of transcription of many miRNA genes are not fully investigated. In contrast, recent findings have shown that these sequential and multiple steps of miRNA processing could offer a plethora of regulatory options to differentially generate mature miRNAs.

Alternative pathways of miRNA maturation bypassing Drosha or Dicer

In addition to the complexity of regulated miRNA biogenesis, several studies have indicated that the pathways for maturation of certain miRNAs and related small RNAs deviate from the conventional miRNA biogenesis pathway and do not necessarily require Drosha or Dicer (Fig. 1B). In the mirtron pathway, pre-miRNA hairpins are generated by splicing and debranching of short hairpin introns, termed mirtrons, bypassing Drosha cleavage (*16*, *17*). After RNA refolding of short introns, these pre-miRNAs are subjected to exportin-5-mediated transport and Dicer cleavage. Several small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), tailed endogenous shRNA, long hairpin RNAs (hp-RNAs) derived from short interspersed nuclear elements (SINEs) and viral miRNA precursors (MHV68) are also Drosha-independent alternative sources of miRNA-like molecules (*18*–*21*).

In addition, several studies have shown that a large proportion of small RNAs similar to miRNAs in size are derived from tRNAs (*22*–*25*). It has been proposed that these are referred to as tRNA-derived RNA fragments (tRF) or tRNA-derived small RNAs (tsRNAs) (*24*, *25*). In the current model, the biogenesis of tsRNAs is mediated by tRNase Z and Dicer, utilizing the conventional tRNA processing system (*25*). In the biosynthesis of tsRNAs, type II tsRNAs are first cleaved from tRNA precursors by tRNase Z. Type I tsRNAs are then produced from mature tRNAs by Dicer cleavage.

Most recently, the processing of miR-451 involved in erythropoiesis has been pursued independently of Dicer (*26*, *27*). After Drosha cleavage, pre-miR-451, whose stem is shorter than those of canonical miRNAs, is cleaved by Ago2 slicer catalytic activity in a similar manner to ac-pre-miRNA production in the maturation of other miRNAs. Ago2-cleaved pre-miR-451 is uridylated and further trimmed independently of Dicer to generate functional miRNAs, though ac-pre-miRNAs of other typical miRNAs with usual hairpin stem length can serve as Dicer substrates. Since the sequence and secondary structure of miR-451 are highly conserved across vertebrates, this Ago2-mediated miRNA maturation pathway appears to be maintained through evolution.

Emergence of the importance of regulated miRNA biogenesis

While the abundance of mature miRNAs can be controlled by the regulation of pri-miRNA transcription, several early studies have shown that expression of mature miRNA does not always correlate with that of pri-miRNA in certain settings, including development and tumourigenesis, indicating post-transcriptional control of miRNAs (*28*–*30*). In one possible mechanism of this, miRNA sequence information could be modified by RNA editing, leading to alteration of miRNA processing efficiency and miRNA target recognition. RNA editing of several pri-miRNAs by adenosine deaminase acting on RNA (ADAR) prevents Drosha processing (*31*). Further, accumulating evidence has shown that miRNA biogenesis pathways are regulated in a complex manner and that each maturation step of miRNA biogenesis provides an array of regulatory options enabling differential generation of individual miRNAs (*32*). In this review, we summarized these advances as follows: (i) microprocessor regulation by intracellular signalling pathways; (ii) functional links between the microprocessor function and nuclear gene expression instruments; (iii) multiple roles of RNA binding proteins in miRNA biogenesis and (iv) modification of miRNA processing factors and RISC factors.

Post-transcriptional control of miRNA processing by tumour suppressor p53

First, a paradigm shift is occurring regarding the post-transcriptional modulation of microprocessor complex function. We and others have recently demonstrated that several intracellular networks can converge onto microprocessor activity via p68 and p72, important cofactors of the Drosha complex (*33*, *34*). While the roles of Drosha cofactors including EWSR1, FUS, numerous hnRNPs and p68/p72 have not been sufficiently examined, p68/p72 have been shown to be important regulators of processing for many miRNAs (*11*). p68 and p72 are well conserved RNA helicases implicated in diverse RNA processing activities, such as transcription, splicing, RNA degradation, RNA export and translation (*35*, *36*). In regulation of the transcriptional machinery, p68 has been shown to interact with core components of the transcriptional machinery such as RNA polymerase II and CBP/p300 and also to serve as a transcriptional co-regulator for several nuclear factors, including p53 and estrogen receptor α (ERα) (*35*, *37*, *38*). In addition, Fukuda *et al*. (*11*) have shown that these helicases enhance Drosha-mediated processing of pri-miRNAs for several, but not all, miRNAs including miR-16 and miR-143. p68/p72 function in the recruitment of the Drosha complex to some pri-miRNAs and the pri-miRNA cleavage regulated by Drosha, which depends on their helicase activity.

We recently found that the p53 tumour suppressor pathway utilizes the association between the Drosha complex and p68/p72 to modulate pri-miRNA processing (*33*). p53 responds to DNA damage, oncogene activation and other stress signals as the 'guardian of the genome' through induction of cell cycle arrest, senescence and apoptosis. Loss of p53 function occurs in most human tumours, either by mutation of p53 itself or perturbation of the p53 signalling pathway

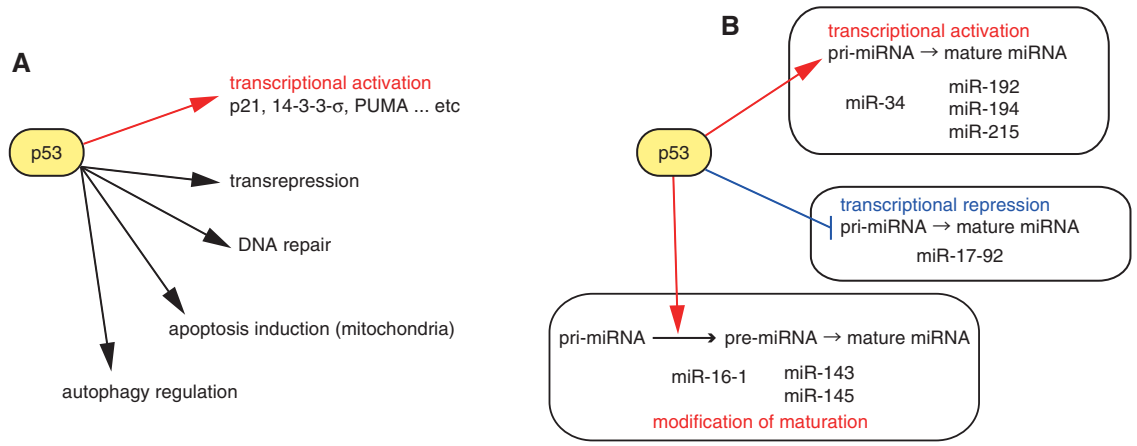

Fig. 2 Regulation of miRNA expression by p53. (A) Diverse cellular functions of p53. Besides transcriptional activation, p53 has several transcription-independent functions involved in many cellular activities. (B) Control of miRNA expression by p53. p53 regulates the expression of several miRNAs through transcriptional activation or repression of miRNA genes. Further, p53 directly modulates Drosha-mediated miRNA processing. The red line and blue line indicate upregulation and downregulation, respectively.

(*39*, *40*). The effects of p53 are conventionally recognized to rely on its ability to transactivate downstream effector genes such as p21, 14-3-3σ and PUMA (Fig. 2A). Although p53 is clearly a transcriptional activator, many studies have demonstrated that p53 also down-regulates the expression of specific genes in direct or indirect fashion (*41*). The emergence of extensive gene regulatory networks of miRNAs suggests the possibility of miRNA involvement in their regulation. In fact, p53 has previously been shown to transactivate the transcription of several miRNA genes, such as miR-34 family and miR-194-1/miR-215 cluster, in response to DNA damage (Fig. 2B) (*42*–*46*). These target miRNAs function as effectors and amplifiers of the p53 pathway (*43*, *45*, *47*). p53 has also been shown to suppress miR-17-92 via transcriptional repression (*48*). p53 also possesses certain transcriptional-independent and/or cytoplasmic activities (*49*). We observed that some miRNAs such as miR-143 and miR-16 were induced under DNA damage-inducing conditions. Interestingly, increase in these mature miRNA levels was accompanied by upregulation of pre-miRNAs, but not pri-miRNAs, suggesting the involvement of post-transcriptional mechanisms, particularly at the Drosha cleavage step. Further analyses revealed that p53 and p68/p72 mediated enhanced production of several miRNAs in response to DNA damage. Moreover, global miRNA profiling showed that p72-dependent miRNAs tended to be upregulated in response to DNA damage. Consistent with the previous report of interaction between p53 and p68/p72 (*37*), endogenous p53 accumulated and interacted with the Drosha/DGCR8-p68/p72 complex in response to DNA damage in HCT116 colon cancer cells and human diploid fibroblasts. The association between p53 and Drosha complex was sensitive to RNase treatment and depended on the presence of p68/p72 and the DNA binding domain of p53. An RNA chromatin-immunoprecipitation study showed that p53 enhanced the association between target pri-miRNAs and Drosha or p68. Drosha complex in association with p53 exhibited higher pri-miRNA processing activity in an *in vitro* cleavage analysis. In addition, a combination of immunoprecipitated p53 and Drosha complex augmented pri-miRNA cleavage. Taken together, these findings demonstrate that wild-type p53 enhances post-transcriptional maturation of several miRNAs through association with Drosha complex and enhancement of Drosha activity (Fig. 2B) (*33*). DNA damage-induced miRNAs such as miR-16 and miR-143 reduced cell proliferation and targeted important regulators of the cell cycle and cell proliferation, such as K-Ras and CDK6. Given the findings that these miRNAs are often reduced in various cancers (*50*–*54*), facilitation of miRNA maturation might support full-brown activation of the p53 pathway (*33*, *55*).

In contrast to the role of wild-type p53 in miRNA maturation, transcriptionally inactive cancer-derived p53 mutants have been shown to hinder the miRNA processing of certain miRNAs, such as miR-143 and miR-16. These effects may be mediated through negative titration of p68/p72 accessory factors from Drosha complex by mutant p53. The central DNA-binding domain of p53 plays an important role in the interaction with Drosha complex. Tumour-derived p53 mutations most frequently occur in the DNA-binding domain and might thus alter the functional properties of p53 and interfere with miRNA processing by perturbation of interactions between Drosha and auxiliary factors (*33*). Since some mutant p53 proteins exhibit several oncogenic activities such as inhibition of p53 family members and ataxia telangiectasia mutated (ATM) signalling pathways in addition to loss of wild-type p53 function (*56*, *57*), attenuation of miRNA maturation might be a novel oncogenic property of mutant p53.

While p53 plays an important role as a transcription activator, it also has certain transcriptional activation-independent and/or cytosolic activities, including modulation of DNA replication and homologous recombination, direct regulation of apoptosis on mitochondria and autophagy regulation (Fig. 2A)

(*58–61*). Adding to the spectrum of diverse cellular functions of p53, these observations have revealed a novel function of p53 as a modifier of miRNA biogenesis (Fig. 2B). The transcription-independent emergent production of certain miRNAs by p53 in response to DNA damage might complement the transcriptional response by p53 to yield a fully functional DNA damage response. Modulation of miRNA biogenesis by p53 might be somewhat non-stringent in contrast to sequence-specific p53 transcriptional responses and instead function as a safeguard system taking advantage of its broad effects when stringent transcriptional responses are dysregulated.

Integration of intracellular networks onto the microprocessor complex: roles of Smad, estrogen receptor and Nanog

The multimodal involvement of p68/p72 in miRNA biosynthesis has been demonstrated by other studies on microprocessor modulation by Smad, estrogen receptor-α (ERα), and Nanog (*62–64*). Davis *et al.* (*62*) revealed that induction of a contractile phenotype in vascular smooth muscle cells (VSMCs) by transforming growth factor-β (TGF-β) or bone morphogenetic proteins (BMPs) is dependent on the rapid induction of miR-21. miR-21 in turn suppressed programmed cell death 4 (PDCD4), a negative regulator of VSMC contractility. Both BMP and TGF-β increased the expression of mature miR-21 by enhancing the processing of pri-miR-21 into pre-miR-21 by the Drosha complex, independently of Smad4, a shared cofactor in TGF-β and BMP signalling (*62*). TGF-β and BMP-specific Smads (Smad2/3 and Smad1/5) have been shown to interact with p68 and to be recruited to pri-miR-21. This study showed that receptor-regulated Smads could function as a modifier of miRNA processing in transcription-independent fashion (*62*). Another study showed that Smad nuclear interacting protein 1 (SNIP1) interacts with Drosha and is involved in pri-miRNA processing of certain miRNAs including *let-7i*, miR-21, miR-22 and miR-23 as a Drosha cofactor (*65*).

In contrast to p53, the study by Yamagata *et al.* demonstrated an opposing functional link between the microprocessor and hormone receptor. ERα was previously shown to interact with p68 (*66*). Consistent with this, ERα was recruited to the Drosha complex in estrogen-dependent manner and hindered the pri-miRNA processing of several miRNAs, such as miR-16, miR-125a and miR-143 (*63*). The bridge between Drosha complex and ERα is mediated through the N-terminal domain of ERα and the C-terminal domain of Drosha, which contains the RNase domain and dsRBD. VEGF 3′-UTR is targeted by estrogen-repressed miRNAs and estrogen enhances the mRNA stability of VEGF by inhibiting miRNA biogenesis. Moreover, a stem cell pluripotency factor, Nanog, has also been demonstrated to interact with Drosha-p68 complex and to promote the production of miR-21, leading to chemotherapy resistance in breast cancer cells (*64*).

These findings have demonstrated that several nuclear factors modulate miRNA biogenesis in addition to their roles in transcriptional regulation (*34*). As described in the next section, several studies have shown that the transcription, splicing and processing of pri-miRNAs are closely coupled events (*67–69*). Drosha cleavage occurs during transcription acting on both independently transcribed and intron-encoded miRNAs, suggesting that miRNA processing machinery is intimately coupled with the transcriptional machinery in spatiotemporal fashion. Therefore, p68/p72 and other RNA-binding molecules might function as an interface integrating intracellular signalling pathways into the modification of miRNA processomes. Further studies will provide new insights into the mode of p68/p72-mediated microprocessor control.

Link between miRNA processing and nuclear gene expression instruments

In addition to p68/p72-bridged crosstalk with transcription factors and miRNA biogenesis machinery, several studies have revealed dynamic interplay between miRNA processing and several nuclear functional instruments executing transcription, 5′-end capping and splicing (Fig. 3) (*70*). In the nucleus, several events generating pri-miRNAs and mRNAs, such as transcription, splicing and processing, are spatiotemporally coupled. Many human miRNA loci are located in intronic regions. Kim and Kim (*67*) showed that Drosha may cleave intronic pri-miRNAs between the splicing commitment step and the excision step and not after the completion of splicing catalysis. Drosha cleavage did not affect the production of mature miRNAs. This might thus ensure both miRNA biosynthesis and protein production from a single host gene. The association between Drosha and spliceosome supports these findings (*71*). Other studies have shown that Drosha is recruited cotranscriptionally and cleaves pri-miRNAs during transcription, acting on both intergenic and intron-encoded miRNAs (*68*, *69*). These studies also showed that pri-miRNA processing might affect other transcription-dependent processes including transcription termination and splicing. It has been shown that both 5′–3′ and 3′–5′ exonucleases XRN2 and PMScl100 associate with the sites where Drosha cleavage occurs along with transcription (*68*, *69*). Subsequent exonucleolytic degradation of intronic sequences might enhance splicing efficiency by omitting extra sequences of introns for intronic miRNA genes (*68*). On the other hand, in the case of intergenic miRNAs, XRN2 might be involved in transcriptional termination through release of RNA PolII-associated transcripts from the template, while it does not affect miRNA cluster genes with short spacers (*68*). These observations suggested that the miRNA processing machinery is closely coupled with the transcriptional machinery. In this context, Drosha recruitment might be modulated by several factors independently of its association with Drosha. For instance, the complex comprised of nuclear factor 90 (NF90) and NF45 with dsRBDs interacted with

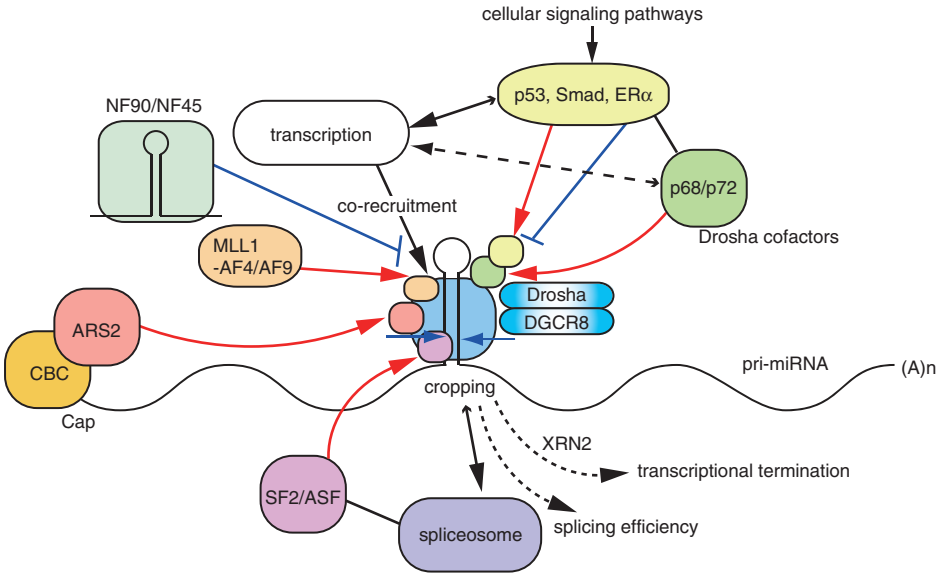

Fig. 3 Crosstalk between nuclear networks and miRNA processing. Recent findings have demonstrated dynamic interplay between miRNA processing and several elements of the nuclear functional apparatus including p68/p72-bridged crosstalk. Several nuclear factors such as Smad, p53, Nanog and ERα regulate pri-miRNA processing by Drosha. In the nucleus, the miRNA processing machinery is closely coupled with the transcription and splicing machinery. Pri-miRNA processing might also affect transcription-related processes including transcription termination and splicing. Some factors involved in 5′-end capping and splicing, such as ARS2 and SF2/ASF, have been shown to modulate Drosha processing. The red line and blue line indicate enhancement and suppression, respectively.

pri-miRNAs and suppressed pri-miRNA cleavage, while it did not associate with endogenous Drosha (*72*). NF90/NF45 might mask miRNA loci through binding to pri-miRNA, thereby inhibiting the recruitment of Drosha (*72*).

5′-end capping has recently been shown to be linked to pri-miRNA processing. ARS2 (arsenite resistance protein 2), a component of the cap binding complex (CBC), which plays an important role in maintenance of proliferation of mammalian cells, has an instrumental role in miRNA biogenesis (*73*, *74*). ARS2 interacts with both CBC and Drosha. It facilitates the recruitment of the microprocessor complex to nascent pri-miRNAs and the processing of pri-miRNAs for several miRNAs including miR-21 and *let-7* (*73*). In *Drosophila*, ARS2 interacts with Dcr-2 and is required for miRNA- and siRNA-mediated gene silencing (*74*). In addition, the splicing factor SF2/ASF has recently been shown to promote the processing of some pri-miRNAs including miR-7 through interaction with the stem region of pri-miRNAs, independently of its splicing function (*75*). Furthermore, the involvement of Mll/Af4 and Mll/Af9 fusion proteins in miRNA processing also suggests intimate crosstalk between miRNA biogenesis and nuclear networks. Mll/Af4 and Mll/Af9 fusion proteins, which are involved in a subset of acute lymphoblastic leukaemia and acute myeloblastic leukaemia, interact with Drosha complex and enhance the maturation of some miRNAs (*76*). A recent study revealed that Mll fusion partners such as Af4 and Af9 comprise a higher-order complex [AF4/ENL/P-TEFb complex (AEP complex)] containing P-TEFb transcription elongation factor (*77*). The Mll/AEP hybrid complex resulting from Mll fusion to AEP components constitutively activates Mll-target gene expression (*77*). Since Mll is an important histone methyltransferase, these findings suggest a possible additional link between chromatin remodelling and miRNA processing in the nuclear networks. Collectively, these studies demonstrate elaborate crosstalk between miRNA processing and the nuclear gene expression apparatus.

Multiple roles of RNA binding proteins in miRNA biogenesis: importance of the terminal loop

Additional levels of complexity in the regulation of miRNA processing have been revealed by a series of studies. A second highlighted issue demonstrates the important roles of multiple RNA binding proteins in miRNA biosynthesis through interaction with pri-miRNA or pre-miRNA, especially their terminal loops (Fig. 4). It has been demonstrated that several regulatory molecules, including hnRNP A1, KH-type splicing regulatory protein (KSRP), and Lin-28, modulate the miRNA processing by Drosha and/or Dicer (*10*, *72*, *76*, *78–83*).

These studies uncovered another aspect of RNA-binding proteins with multiple functions in RNA metabolism. hnRNP A1 has been well established as an essential regulator involved in many types of RNA processing and transport (*84*). hnRNP A1 interacts with specific nucleotides of the terminal loop of pri-miR-18a and enhances nuclear processing from pri-miR-18a selectively from the miR-17-92 cluster (*10*). Pri-miR-18a contains two regions similar to the consensus hnRNP A1-binding site, UAGGGA/U, within its stem and terminal loop (*10*). hnRNP A1 also

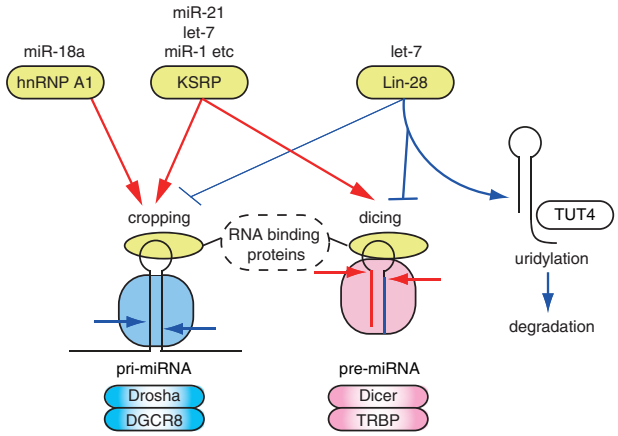

Fig. 4 Posttranscriptional modulation of miRNA maturation through interaction between RNA-binding proteins and miRNA precursors. Several RNA binding proteins such as hnRNP A1, KSRP and Lin-28 interact with the terminal loop of a subset of miRNA precursors, and promote or attenuate their processing, highlighting the importance of the terminal loops of miRNA precursors in their biogenesis.

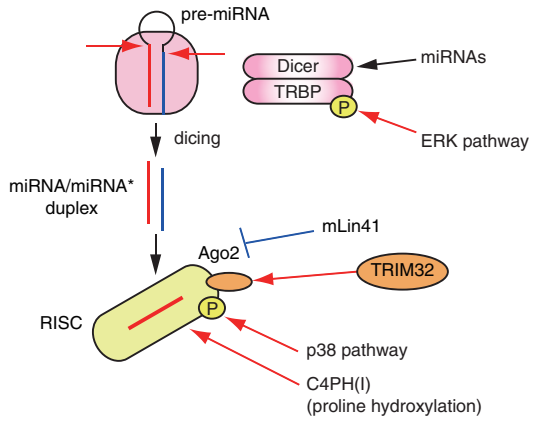

Fig. 5 Regulation of miRNA biogenesis components in the cytoplasm. The multiple steps in miRNA biosynthesis offer a plethora of regulatory options for differential production and functioning of individual miRNAs. In the cytoplasm, the processing factors and RISC components, such as Dicer, TRBP and Ago, are regulated by several mechanisms of protein modification including miRNA-mediated downregulation, phosphorylation, hydroxylation, ubiquitination and interaction with cofactors, leading to alterations of miRNA expression and/or function.

interacts with the apical regions of pri-let-7a-1 and pri-miR-101-1 (*78*). Their conserved terminal loops have also been shown to associate with hnRNP L and hnRNP I (PTB), respectively (*78*). KSRP also plays important roles in both mRNA and miRNA metabolism (*85*). KSRP interacts with single-strand AU-rich-element (ARE)-containing mRNAs and promotes their decay. In the setting of miRNA production, KSRP interacts with 5′-guanosine-rich portions, including GGG triplets, of the loop regions of various miRNA precursors, including pre-miR-21, pre-let-7, pre-miR-1 and pre-miR-15 (*79*). KSRP also serves as a cofactor for both Drosha and Dicer complex and promotes the processing of these miRNAs. This mechanism is involved in *let-7*-regulated cell proliferation.

While hnRNP A1 and KSRP positively modify miRNA maturation via the association with terminal loops of certain miRNA precursors (*10*, *78–82*), Lin-28 has been shown to inhibit Drosha and Dicer processing of *let-7* family precursors in specific fashion (*80–83*). *let-7* is an important regulator of cell differentiation and growth (*82*, *86*). Lin-28 interacts with the loop region of pre-let-7 and inhibits its maturation. This inhibition is accompanied by the polyuridylation of pre-let-7 (*83*). Recent studies have demonstrated that Lin-28 recognizes the GGAG motif in the terminal loop and induces its polyuridylation at the 3′-end by TUT4 (Zcchc11), a noncanonical poly(A) polymerase, leading to the suppression of Dicer processing and its degradation (*81*, *83*, *87*, *88*). Furthermore, Lin-28 plays an important role in stem cell maintenance and tumourigenesis by suppressing *let-7* (*81*, *86*, *89*). Lin-28b also potentially lies downstream of NF-κB signalling in tumour progression (*90*). In addition, TUT4 has been shown to uridylate miR-26 family members and repress miR-26a function (*91*).

These findings demonstrate the importance of the terminal loops of miRNA precursors in determining the processing efficiency of different miRNA species. Approximately 14% of human miRNAs contain highly conserved loop sequences (*78*), suggesting widespread commitment of the terminal loops in the control of the fate of miRNA precursors. Since the loop region is to a large extent not required for the basic activities of Drosha and Dicer, these loops of miRNA precursors might contain conserved regulatory information for RNA binding proteins. The terminal loops of several miRNAs including *let-7* could be targeted by multiple factors such as Lin-28 and KSRP, the balance of which might control the final output of miRNA biogenesis pathways.

Protein modification controls miRNA biogenesis pathways

Previous studies have demonstrated intimate crosstalk between the miRNA processing pathway and nuclear networks and dynamic interplay between mRNA metabolism and that of miRNA. The emerging complexity of miRNA biogenesis pathways could also be expanded to cytoplasmic regulation of miRNA maturation, in which the importance of protein modification has emerged in contrast to the nuclear networking of miRNA processing (Fig. 5).

Paroo *et al.* (*92*) showed that the mitogen-activated protein kinase (MAPK) pathway phosphorylates TRBP and positively regulates Dicer-TRBP complex. The phospho-mimic TRBP showed elevated TRBP protein levels and also an increase in Dicer protein. These effects are accompanied by a coordinated increase in levels of growth-promoting miRNA and reduced expression of *let-7* tumour suppressor miRNA, suggesting that the degree of stability of the Dicer-TRBP complex might affect recognition of preferential substrates for pre-miRNA processing. In tumourigenesis, these factors could also be targets of tumour-related events. Frameshift mutations of TRBP2, a Dicer interacting

partner, are observed in sporadic and hereditary carcinomas with microsatellite instability (*93*) and Dicer1 mutations are associated with familial pleuropulmonary blastoma (*94*). Accordingly, it has been reported that reduced expression of miRNA processing factors is associated with poor prognosis in lung cancer and ovarian cancer (*95*, *96*).

In addition, several studies have shown that a number of pathways regulate the stability of the Ago proteins, the core component of RISC. Ago2 has been shown to be controlled through phosphorylation by the p38 MAPK pathway and hydroxylation by type I collagen prolyl-4-hydroxylase (*97–99*). mLin41 (mouse homologue of *lin-41*), a *let-7* target gene, is a stem cell-specific E3 ubiquitin ligase antagonizing Ago2 (*100*). mLin-41 interacts with Dicer and Ago proteins and mediates ubiquitination of Ago2, leading to interference with *let-7*-mediated gene silencing. Other proteins containing the TRIM domain, such as TRIM32, have also been shown to regulate RISC loading and RISC activity (*101*, *102*).

Conclusion

Recent advances have revealed that the multiple stages of miRNA processing could serve a plethora of regulatory options in control of miRNA-dependent gene regulation. These discoveries have changed the conventional concepts of miRNA biogenesis pathway and have demonstrated an additional level of complexity in the miRNA network. As described earlier, we recently found that a cardinal tumour suppressor, p53, controls miRNA biogenesis via association with the microprocessor complex. Our findings suggest that posttranscriptional control of miRNA biogenesis is intrinsically equipped with a p53 tumour suppressive system. While numerous studies have demonstrated perturbation of miRNA expression and function during development and tumourigenesis, research on the regulation of miRNA processing is just beginning. In contrast to miRNA biogenesis, the mechanisms of modification, turnover, stabilization and reduction of miRNAs are largely unknown, though some studies have begun to deal with them and revealed some regulators such as GLD-2 (*103–105*). Further characterization of the regulatory elements of miRNA biosynthesis and function will provide new insights yielding comprehensive understanding of the complex gene-regulatory networks governed by miRNAs and the involvement of miRNAs in various pathological mechanisms. These findings will also offer a molecular basis for diagnostic and therapeutic strategies based on miRNA biology.

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Conflict of interest

None declared.

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