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NONCODING RNAS

Dysregulation of microRNA biogenesis and gene silencing in cancer

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MicroRNAs (miRNAs) are small noncoding RNAs that suppress the abundance of partially complementary mRNAs and inhibit their translation. Each miRNA can regulate hundreds of mRNAs, sometimes strongly but often weakly, to mediate a diverse array of biological functions, including proliferation, cell signaling, differentiation, stress responses and DNA repair, cell adhesion and motility, inflammation, cell survival, senescence, and apoptosis, all intimately related to cancer initiation, treatment response, and metastasis. The expression and activity of miRNAs are spatially and temporally controlled. Global miRNA expression is reduced in many cancers. In addition, the expression and processing of cancer-related miRNAs that act as oncogenes ("oncomiRs") or tumor suppressors are often dysregulated in cancer. In this review, we summarize emerging knowledge about how miRNA biogenesis and gene silencing are altered to promote cancer.

Introduction

MicroRNAs (miRNAs) are ~22–nucleotide (nt)–long, noncoding RNAs (ncRNAs) that mediate the destabilization and translational suppression of target messenger RNAs (mRNAs) that bear partially complementary sequences (*1*, *2*). The biogenesis of miRNAs, which are encoded in the genome, is a stepwise process that is regulated on multiple levels (Fig. 1, A to G). miRNA biogenesis begins with the transcription of the miRNA-encoding gene by RNA polymerase II (RNA Pol II) into longer primary (pri-miRNA) transcripts (Fig.1A). miRNAs, which are embedded as short hairpins in pri-miRNAs, undergo stepwise processing. The cleavage of the pri-miRNA by the ribonuclease (RNase) III family enzyme Drosha and its cofactor DGCR8 (DiGeorge syndrome critical region gene 8), which constitute the microprocessor complex, takes place in the nucleus to generate a precursor miRNA ("pre-miRNA") hairpin. The pre-miRNA is transported to the cytoplasm and cleaved by another RNase III enzyme Dicer to generate a miRNA/miRNA* duplex. In addition to this canonical pathway, a small subset of miRNAs (less than ~1%) is generated by Drosha- or Dicer-independent mechanisms, such as through the splicing of miRNA-containing introns (*3*). The RNA duplex is loaded into an Argonaute (Ago) protein, which preferentially ejects the miRNA* strand and retains the mature miRNA (*4*). Ago proteins associate with cofactors of the GW182/TNRC6 family to form the RNA-induced silencing complex (RISC). miRNA/Ago complexes recognize target mRNAs by pairing to as few as ~7 nt, often in the 5′ end of the miRNA sequence (nucleotides 2 to 8), called the seed region. A majority of human mRNAs is regulated by evolutionarily conserved miRNAs, but even primate-specific miRNAs can be functionally important. A particular miRNA can potentially posttranscriptionally regulate hundreds of target genes, but the down-regulation of many of them is subtle. How many regulated genes typically contribute to miRNA function in any particular cell context is still uncertain (*5*). As one might expect for any important gene expression regulatory pathway, miRNA expression, processing, and functional activity are tightly controlled. These controls are critical for maintaining homeostasis under normal physiological conditions but also become important during the cell's response to changes in its environment, both to normal developmental or activating signals and to stress (*6*). During environmental stresses, such as hypoxia or DNA damage, cells adapt to a new environment by rapidly modulating gene expression. miRNAs are important mediators of these adaptive changes. Although under basal conditions, mature miRNAs bound to RISC are very stable in cells, in response to activation or stress, and miRNA transcription and processing and miRNA function are rapidly altered. The mechanisms that control miRNA abundance and function are only just beginning to be uncovered.

Role of miRNAs in Cancer-Associated Signaling

The first appreciation of the role of miRNAs in human disease came from studies of miRNA function in cancer cells (*7*–*17*). The earliest observation that provided a potential link between miRNA and tumor biology was the phenotype of *lin-4* and *let-7* loss-of-function mutations in *Caenorhabditis elegans*. These mutants reiterated larval stages and underwent extra cell divisions during the adult stage, implicating lin-4 and let-7 in the control of cell differentiation and proliferation (*18*, *19*). Subsequent studies on *bantam* and *miR-14* in *Drosophila* provided further evidence that miRNAs regulate cell proliferation and apoptotic cell death by modulating pathways relevant to tumorigenesis (*20*, *21*). The most direct evidence linking miRNAs to cancer came from the discovery by Calin and colleagues (*22*) of deletions of the *miR-15a/16-1* cluster in chromosome 13q14 in chronic lymphocytic leukemia cells. Since then, thousands of tumor miRNA expression profiling studies have generated an expansive list of miRNAs that are differentially expressed in tumors versus normal tissue. The miRNAs up-regulated in some cancers that promote oncogenesis are known as "oncomiRs," whereas the down-regulated ones often act as tumor suppressors and are known as "tumor suppressor miRs." miRNA expression signatures can be used to identify tumor subtypes and cancer prognosis. miRNA tumor profiles are more accurate than mRNA expression profiles at accomplishing this task (*23*)—a reflection of the importance of miRNAs in cancer biology. miRNA profiles can even be used to identify the primary origin of cancers, and profiling of serum miRNAs secreted by tumors within exosomes may eventually be useful for cancer diagnostics. Like protein-coding genes, miRNAs are transcribed by RNA Pol II and regulated by transcription factors that bind to promoters and enhancers in association with shared transcriptional coactivators and co-repressors. The same epigenetic marks that influence mRNA expression affect miRNA expression. About one-quarter of all miRNA genes are embedded in the

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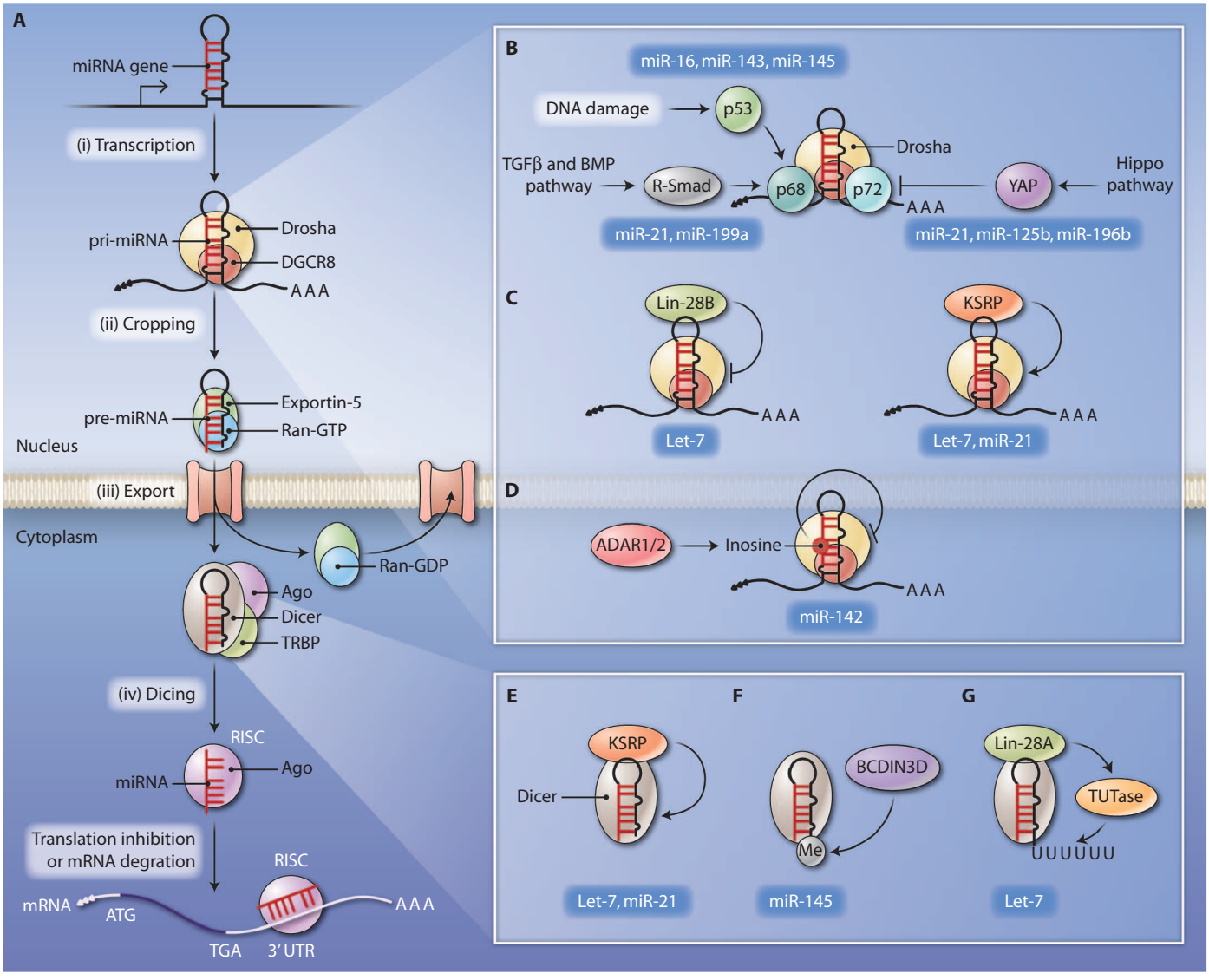

Fig. 1. Mechanisms that regulate miRNA biogenesis and RISC loading. (**A**) Biogenesis of a miRNA is a stepwise process involving (i) transcription of a primary transcript (pri-miRNA), (ii) processing ("cropping") by the Drosha/DGCR8 microprocessor complex to produce a pre-miRNA, (iii) export from the nucleus into the cytoplasm, mediated by the exportin XPO5 and the guanosine 5′-triphosphate (GTP)–binding protein Ran, and (iv) additional processing ("dicing") by a complex containing the RNase Dicer, a catalytic component, Argonaute (Ago), and the RNA-binding protein TRBP (transactivation response RNA binding protein) to produce the mature miRNA. The resulting complex of Ago and the mature, single-stranded miRNA is called the RISC. The miRNA binds complementary sequences in mRNAs; Ago either inhibits the translation of the mRNA or cleaves it to cause its degradation. (**B** to **D**) Every step of miRNA biogenesis and RISC assembly can be modified by posttranslational modifications (PTMs) (see also Table 1) or protein interactions, either affecting the activity of Drosha, Dicer, and other proteins involved in miRNA production or altering the stability of miRNAs themselves. For example, Drosha/DGCR8 complex–mediated processing of pri-miRNAs is regulated by (B) transcription factors that regulate the p68 and p72 RNA helicase subunits in the complex, (C) RNA binding proteins that associate with the terminal loop of specific pri-miRNAs, and (D) RNA-editing enzymes that mediate base replacements that impair pri-miRNA processing. (**E**) KSRP also promotes the function of Dicer in processing pre-miRNAs. (**F** and **G**) RNA modifications such as methylation (F) or uridylation (G) reduce pre-miRNA processing and stability, respectively. GDP, guanosine diphosphate.

introns of coding genes, and the host gene promoters and enhancers mostly, but not exclusively, regulate their expression (*24*). The regulatory regions that control transcription of extragenic miRNAs are not as well characterized. Gain- or loss-of-function studies of miRNAs differentially expressed in cancer have revealed their contribution to cellular transformation and tumorigenesis (*9*, *10*).

We first discuss examples of changes in miRNA abundance in response to environmental cues relevant to cancer, such as DNA damage (*12*) and hypoxia (*11*). Then, we discuss some important examples of oncomiRs and tumor suppressor miRs and the feedback regulatory networks in which they participate. Finally, we discuss how miRNA biogenesis and the function of miRNAs are regulated. Because of space limitations, modes

of miRNA control that are not directly relevant to cancer cells are not discussed in this review. We recommend various other reviews on those topics (*9*, *15*–*17*, *25*, *26*).

miRNAs in the DNA damage response

Genomic instability and increased resistance to DNA damage contribute to tumorigenesis. Mammalian cells are equipped with multiple pathways of DNA damage response (DDR) to protect the genome from double-strand breaks (DSBs) by either repairing DSBs or promoting apoptosis. Expression of DDR genes is orchestrated by the transcription factor p53, the "guardian" of the genome. In addition to inducing coding genes, p53 activates the transcription of a subset of miRNAs (*10*), of which the *miR-34* family is the most studied (*27*) (Fig. 2A). This family of miRNAs (miR-34a/b/c and miR449a/b/c) acts as tumor suppressors. They are induced either by p53 in response to DNA damage or oncogenic stress or independently of p53 in response to proliferative signals (*28*). Their expression is repressed by the transcription factor Myc. Reduced expression of *miR-34* in p53-mutant tumors derepresses oncoproteins, such as Myc itself, KRAS, the receptor kinases MET, and AXL, and thus is thought to contribute to tumorigenesis. miR-34a also targets cyclins and cyclin-dependent kinases to inhibit cell cycle progression at the G_1-S transition and inhibits a dense network of genes involved in cell signaling and proliferation in response to growth factors (*29*). In addition, miR-34 suppresses the expansion of the cancer stem cell compartment and metastasis. For example, in ovarian cancers with mutations in p53, suppression of the *miR-34* family increases the abundance of MET, which in turn promotes tumor cell proliferation, motility, and invasion (*30*). Conversely, in lung cancer, where the KRAS and p53 pathways are most frequently altered, exogenous expression of miR-34a can prevent tumor formation and progression in vivo by suppressing the abundance of KRAS (*31*). miR-34a also represses the abundance of various inhibitors of p53, including HDM4 [the human homolog of MDM4 (mouse double minute 4)], the deacetylases SIRT1 and HDAC1 (histone deacetylase 1), and the transcriptional regulators YY1 and MTA2, thus creating a positive feedback loop on the p53 pathway (*32*). miR-34 also curbs the epithelial-to-mesenchymal transition (EMT) program by targeting two EMT-associated transcription factors, SNAIL and ZNF281/ZBP-99 (*33*, *34*) (Fig. 2A). ZNF281 directly induces *SNAIL* expression, and SNAIL directly induces *ZNF281* expression and represses *miR-34*, which in turn derepresses *ZNF281*, indicating an intricate regulatory circuit among miR-34, SNAIL, and ZNF281 in the regulation of EMT and its contribution to motility and stemness of tumor cells (Fig. 2A). Despite the role of miR-34 in the regulation of key genes involved in cell proliferation, DDR, and cancer, the genetic ablation of all three isoforms of *miR-34* results in viable, fertile mice without an increase in spontaneous tumors or blunted response to DNA damage, unlike in the case of p53 deficiency. Ablation of both the *miR-34* and *miR-449* families unexpectedly leads to a defect in the generation of cilia, leading to early death rather than a cancer-related phenotype (*35*). Thus, miR-34a/b/c is not critical to the p53 response (*36*). Clinical trials are in progress to assess whether replacement therapy with miR-34a mimics will suppress tumors (*37*).

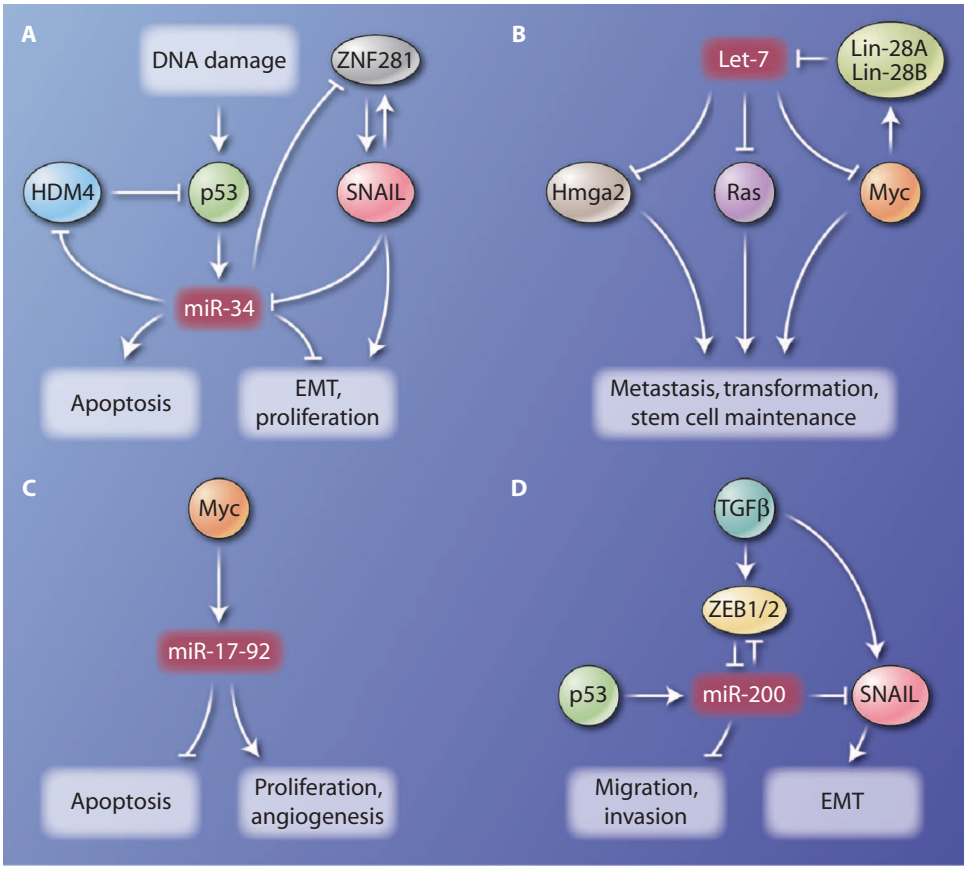

Fig. 2. Oncogenic and tumor suppressor roles of miRNAs. (A to D) Pathway schematics of signals and feedback mechanisms regulating some tumor-suppressive (A, B, and D) or tumorigenic (C) miRNAs. In cancer, miRNA-mediated regulatory feedback loops are disrupted, contributing to cell transformation; EMT, migration, and metastasis; stem cell maintenance and dedifferentiation; and the suppression of apoptosis.

Besides miR-34, a few other miRNAs modulate gene expression in the DDR (*38*). For example, miR-24 targets the transcript of histone H2A variant, H2AX, which marks DSBs and helps recruit DNA repair factors. miR-24–mediated suppression of H2AX renders hematopoietic cells hypersensitive to γ irradiation and genotoxic drugs, a circumstance that might contribute to the reduced DNA repair capacity of terminally differentiated hematopoietic cells, in which the abundance of miR-24 is increased (*39*). Moreover, pri-miRNA processing, nuclear export of pre-miRNA (*40*), and consequently, mature miRNA abundance of a subset of miRNAs are rapidly enhanced in an ataxia telangiectasia mutated (ATM)–dependent manner in response to DNA damage, as summarized below. A recent study identified a group of tumor suppressor miRNAs (*miR-1255b*, *miR-148b**, and *miR-193b**) that are deleted in ovarian cancer, and their deletion is linked to chromosomal instability (*41*). These miRNAs target genes are important for homologous recombination (HR). This upsets the balance between HR and DSB repair by nonhomologous end joining, which normally takes over during the G_1 phase of the cell cycle. The aberrant use

of HR during this phase of the cell cycle can lead to carcinogenic loss of heterozygosity.

p53 not only receives the DDR signal and transmits it to downstream miRNAs but also is itself under the control of miRNAs, such as miR-504 and miR-125b. miR-504 suppresses p53 protein abundance and thus p53-mediated apoptosis and cell cycle arrest and promotes tumorigenesis in vivo (*42*). Suppression of miR-125b, which also targets the transcript of p53 and transcripts of multiple p53-regulated genes, facilitates the DDR after irradiation by increasing the abundance of p53 and inducing apoptosis (*43*).

Thus, miRNA-mediated gene regulation is an integral component of the DDR. The lesson learned from *miR-34* family knockout mice, however, is that caution is in order when interpreting the functional importance of miRNA overexpression experiments. It is likely that research so far has only begun to describe the myriad effects that miRNAs have on the DDR. Modulating the expression of miRNAs that repress core proteins in the DDR might be an attractive strategy for cancer treatment to increase the sensitivity of cancer cells to radiation or chemotherapy.

miRNAs in hypoxia

Hypoxia is common in rapidly growing solid tumors, and the ability to survive hypoxia is critical for tumor growth. The response to hypoxic stress is orchestrated by hypoxia-inducible factor 1 (HIF1) transcription factors. The importance of miRNAs in hypoxia may be blunted by the global arrest of new protein translation that occurs in response to hypoxia and other cellular stresses and by reduced RISC activity caused by PTMs of Ago in response to hypoxia (discussed below). Hypoxia also inhibits the activity of Drosha and Dicer, thus interfering with miRNA maturation [also discussed further below (*44*, *45*)]. However, the abundance of a handful of miRNAs, some of which are transcriptionally regulated by HIF1 transcription factors, is increased by hypoxia (*11*). Among them, the best studied is miR-210, the abundance of which is strongly increased in response to hypoxia and in various solid tumors, where it is associated with adverse prognosis (*46*). miR-210 protects hypoxic tumor cells from apoptosis in part by targeting transcripts of the caspase-8–associated protein FLASH and the proapoptotic BCL-2 family member BNIP3; promotes tumor cell growth by repressing the Myc antagonist MNT; stimulates angiogenesis by targeting the transcript of EphA3; and suppresses mitochondrial respiration by targeting the transcripts of ISCU1/2 Fe-S cluster assembly proteins (*47*). The abundance of miR-210 is also induced by proinflammatory signals, such as through the activation of TLRs (Toll-like receptors), mediated in part through the activation of NF-κB (nuclear factor κB). miR-210 targets the transcripts of important inflammatory and oncogenic mediators (including potentially NF-κB1) in a negative feedback loop to limit proinflammatory cytokine production (*48*). Because miR-210 helps cells survive hypoxia, it may be a good target for antitumor therapy.

OncomiRs, Tumor Suppressor miRs, and Their Regulatory Networks

MiRNAs that modulate a diverse array of biological functions that are critical to either promote or suppress cancer initiation, progression, metastasis, and treatment response (*7*, *8*) are often mutated or aberrantly expressed in cancer cells. Although some miRNAs that suppress cancer development and progression [such as miR-200, which promotes the mesenchymal-to-epithelial transition, or let-7, which promotes cellular differentiation] may act as master regulators, others may serve the more modest function of stabilizing complex networks that maintain cellular homeostasis or the appropriate and measured response to changes in the cellular environment (*7*, *8*). Below, we describe a few well-characterized examples of miRNA regulatory networks and their relevance to human tumors.

Let-7

The *let-7* family of tumor suppressor miRNAs in humans has 12 homologs, encoded at multiple chromosomal sites, some of which are deleted in various human tumors. *Let-7* expression causes stem cells and stem-like cells to differentiate (*49*). Mature let-7 is barely detected in stem and progenitor cells, as well as in aggressive, poorly differentiated tumors. Members of the Lin-28 family, Lin-28A and Lin-28B, selectively inhibit the abundance of let-7 through different mechanisms (Fig. 2B). Whereas Lin-28B binds to the terminal loop of *pri-let-7* and blocks miRNA processing by Drosha in the nucleus (Fig. 1C), Lin-28A promotes uridylation of *pre-let-7* by the TUTase (terminal uridylyl transferase) Zcchc11 (also called TUT4) and blocks miRNA processing by Dicer primarily in the cytoplasm (*50*–*55*) (Fig. 1, A and G). Uridylation of the 3′ ends of *pre-let-7* (*51*–*53*) primes it for degradation by the Perlman syndrome–associated exonuclease Dis3L2 (*56*–*58*). *DIS3L2* is mutated in about a third of Wilms tumors, and its mutation in Perlman syndrome predisposes patients to cancer (*59*). Myc transactivates the expression of *Lin-28B* and, hence, suppresses the abundance of let-7 (*60*). Increased abundance of Lin-28 in tumors is associated with poor prognosis in patients (*61*), whereas *let-7* homologs are substantially decreased in lung tumors, and the degree of their reduction correlates with poor prognosis (*62*). Loss of *let-7* homologs results in constitutive overexpression of the let-7–repressed *RAS* family of oncogenes, which contributes to tumor pathogenesis (*63*). Conversely, overexpression of let-7 suppresses tumor development in breast and lung cancer models.

In some cancers, oncogenes escape repression by let-7 by preventing its interaction with target transcripts. For example, alternate polyadenylation shortens the 3′ untranslated region (3′UTR) of the development-associated transcript *HMGA2*, thereby removing let-7–binding sites, or MREs (miRNA recognition elements) (*64*). Alternatively, let-7–mediated repression is abrogated through the mutation of let-7 MREs in oncogenic transcripts, such as *KRAS* (*65*). An additional level of regulation is seen through target competition. For example, increased abundance of the let-7 target *HMGA2* in metastatic lung adenocarcinomas dominates the occupation of let-7, thereby decreasing its availability to repress other targets (*66*) (Fig. 2B). These studies highlight the complex interplay among miRNAs and mRNAs in the cell.

miR-17-92

The *miR-17-92* cluster consists of six miRNAs (*miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19-b-1*, and *miR-92a-1*), some of which exhibit oncogenic activities, whereas others act as tumor suppressors (*67*). The Myc oncogene activates expression of the cluster (*15*, *68*). Inactivation of p53 leads to increased expression of oncogenic miRNAs in this cluster, such as *miR-92a* and *miR-19a*, in tandem with decreased expression of the tumor-suppressive member *miR-17* (*69*) (Fig. 2C). Inactivation of *miR-17-92* suppresses retinoblastoma (RB) formation in mice. Silencing of both *miR-17/20a* and *p53* cooperatively decreases the viability of human RB cells, suggesting inhibiting these miRNAs as an approach to induce synthetic lethality in cancer cells (*70*). In a Burkitt lymphoma mouse model, miR-17-92 exhibits potent oncogenic activity through independent but ultimately cooperative functions. miR-92 increases the stability of Myc by inhibiting the abundance of the ubiquitin ligase FBW7 that marks Myc for proteasomal degradation (*71*). In turn, the apoptotic pathways that are induced by increased Myc abundance are repressed by another miRNA in the cluster, miR-19, which targets the transcript of proapoptotic BCL-2 family member BIM (*72*, *73*). It is likely that suppression of many additional target genes by the multiple miRNAs in this cluster contributes to the cluster's oncogenic function.

miR-200

The *miR-200* family of miRNAs, frequently down-regulated in human tumors, targets the ZEB transcription factors (ZEB1 and ZEB2), which

suppress the expression of epithelial genes and potently induce EMT (Fig. 2D). miR-200 thus suppresses migration and invasion (*74*). However, paradoxically, in some breast cancer models, ectopic miR-200 expression promotes metastasis by increasing mesenchymal-to-epithelial transition and colonization of distant tissues, the last and rate-limiting step for formation of clinically relevant macroscopic metastases (*75*, *76*). miR-200 is enriched in microvesicles in the serum of patients with metastatic cancer. A recent study showed that miR-200 secreted in microvesicles by metastatic human and mouse breast cancer tumors can transfer the ability to metastasize to poorly metastatic tumors at distant sites in mouse models (*77*). Recent work shows that, in addition to targeting *ZEB1/2*, miR-200 also targets *SNAIL* mRNA and additional transcripts encoding components of the ZEB and SNAIL repressor complexes (including the transcripts encoding EMT-promoting, TGFβ (transforming growth factor–β)–regulated transcription factors SMAD2 and SMAD5) to enhance its ability to promote epithelial gene expression (*78*). A recent study using HITS-CLIP (high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation) technology also identified a network of miR-200 target genes that control the actin cytoskeleton to inhibit cell migration (*79*). In negative regulatory loops, ZEB1 and ZEB2 bind directly to an E-box proximal promoter element to repress the transcription of *miR-200*. Moreover, the ratio of the isoforms of the kinase AKT (AKT1 and AKT2) plays a critical role in regulating the expression of the *miR-200* family, which in turn controls EMT in mesenchymal breast cancers (*80*). Thus, the miR-200 family acts as master regulators to promote epithelial gene expression and suppress mesenchymal features.

miR-195

In most contexts, miR-195 is a tumor suppressor miRNA that inhibits cell growth and enhances apoptosis after chemotherapy (*81*). Some of its targets that contribute to these effects encode cyclin D1, cyclin-dependent kinase CDK6, the transcription factor E2F3, and the proapoptotic protein BCL-2. In line with its tumor suppressor properties, *miR-195* expression is repressed by promoter hypermethylation in some cancers. A recent study in HCT116 colorectal carcinoma cells revealed a previously unknown mechanism regulating pri-miRNA processing mediated by the long noncoding RNA (lncRNA) *Uc.283+A* (*82*). *Uc.283+A* is an ultraconserved lncRNA that is partially complementary to the sequence of a lower stem region of *pri-miR-195*. Binding of *Uc.283+A* to the stem impairs *pri-miR-195* recognition and cleavage by Drosha and reduces the abundance of the mature miR-195 (*82*). Paradoxically, the expression of *Uc.283+A* is reduced as a result of promoter hypermethylation in a broad range of tumors (*83*), which should increase the abundance of mature miR-195. This suggests that *Uc.283+A* and *miR-195* may regulate a more complex network of cancer-related genes that is yet to be revealed.

Regulation of pre-miRNA and miRNA stability in cancer

Control of pre-miRNA and miRNA stability affects the abundance of mature miRNAs. As described above, uridylation of the 3′ ends of *pre-let-7* promotes its degradation mediated by Dis3L2 (*56*–*58*). Stability of pre-miRNAs, such as *pre-miR-146a* and *pre-miR-135b*, is negatively regulated by cleavage of the terminal loop by the endoribonuclease MCP-induced protein1 (MCPIP1, also known as ZC3H12A) (*84*). After endoplasmic reticulum stress, the Ser/Thr protein kinase and endoribonuclease IRE1α cleaves a subset of pre-miRNAs (in particular, *pre-miR-17*, *pre-miR-34a*, *pre-miR-96*, and *pre-miR-125b*) to cause their rapid decay. These miRNAs all target the transcript encoding caspase-2. Thus, IRE1α-mediated cleavage of these miRNAs increases the abundance of caspase-2 protein, thereby enhancing the mitochondrial apoptotic pathway (*85*). In some cases, mature miRNA stability is enhanced by the expression of target mRNAs (*86*, *87*). In melanoma cells, human polynucleotide phosphorylase (hPNPase) old-35 (also known as PNPT1), an interferon-inducible exonuclease, selectively degrades certain mature miRNAs, such as miR-221 (*88*). Details of the mechanism and physiological relevance of target mRNA–dependent miRNA stability control and its role in cancer biology remain to be explored.

Global Dysregulation of miRNAs During Tumorigenesis

miRNAs are globally down-regulated in tumors compared to normal tissues, especially in poorly differentiated tumors (*23*). Although we understand how this down-regulation occurs in some cancers, it is still unclear in most. Although most mature miRNAs are down-regulated, a small number of miRNAs, such as the *miR-290-295* cluster in mice and the *miR-302* and *miR-371/miR-372/miR-373* families and chromosome 19q13.42 cluster in humans, are highly expressed only in stem-like cells and some poorly differentiated tumors and contribute to maintaining pluripotency. One interpretation of the widespread underexpression of miRNAs in poorly differentiated cells and tumors is that many miRNAs function to define lineage-specific properties of differentiated cells, either by suppressing alternate lineages or actively promoting lineage-restricted functions and properties. Many miRNAs that are underexpressed in cancer also directly inhibit cell proliferation, which is mostly turned off in terminally differentiated cells. In support of an overall tendency for tumor suppression by miRNAs, global inhibition of miRNA biogenesis by knockdown or haploinsufficiency of *DICER1* promotes tumor formation and progression in several mouse models of spontaneous cancer, indicating an oncogenic role for the global down-regulation of miRNAs (*89*–*91*). A reduction of Dicer in human breast cancer as a result of up-regulation of the *miR-103/miR-107* family, which directly targets *DICER1* mRNA, fosters not only migration and metastasis of cancer cells but also induction of EMT through down-regulation of *miR-200* (*92*). Below, we discuss different mechanisms that regulate the expression and activity of mediators of miRNA biogenesis, the PTM of components of the RISC, and the loading of RNA duplexes into the RISC. An understanding of these mechanisms identifies accessory proteins that might be novel therapeutic targets for cancer (*92*–*100*).

Aberrant expression and mutations of genes encoding mediators of miRNA biosynthesis

Many mediators of miRNA biogenesis, including Drosha, Dicer, TRBP, and Xpo5 (exportin-5), act as oncogenes or tumor suppressors depending on the tumor type. Reduced *DICER1* or *DROSHA* mRNA correlates with a worse outcome in lung, breast, skin, endometrial, and ovarian cancers (*101*). Reduced expression of *DICER1* in some cancers may be induced by hypoxia through the epigenetic mechanism of promoter methylation. For example, the inhibition of oxygen-dependent histone demethylases KDM6A and KDM6B prevents their action on trimethylated histone H3 Lys^{27} (H3K27me3) on the *DICER1* promoter (*44*). Conversely, *DICER1* is overexpressed in metastatic lesions of prostate cancer, and *DROSHA* is overexpressed in advanced-stage cervical cancers and metastasis-prone esophageal cancers that are associated with poor patient survival (*101*). It is unclear whether in these tumors, the increased abundance of Dicer or Drosha results in an increase in miRNA abundance globally, and if so, how that contributes to carcinogenesis.

Recurrent somatic mutations of *DROSHA* and *DICER1* that affect miRNA processing and alter miRNA profiles have been reported in Wilms tumor patients (*102*, *103*) (Fig. 3, A and B). Furthermore, both somatic and germline *DICER1* mutations have been associated with human cancer syndromes, including familial pleuropulmonary blastoma, pituitary blastoma, and embryonal rhabdomyosarcoma (*104*–*106*) (Fig. 3B). Mutations in *XPO5*, whose protein product exports pre-miRNAs from the nucleus to the cytoplasm, occur in some human tumors with microsatellite instability (*107*) (Fig. 3C). Mutant forms of XPO5 are truncated in the C terminus and

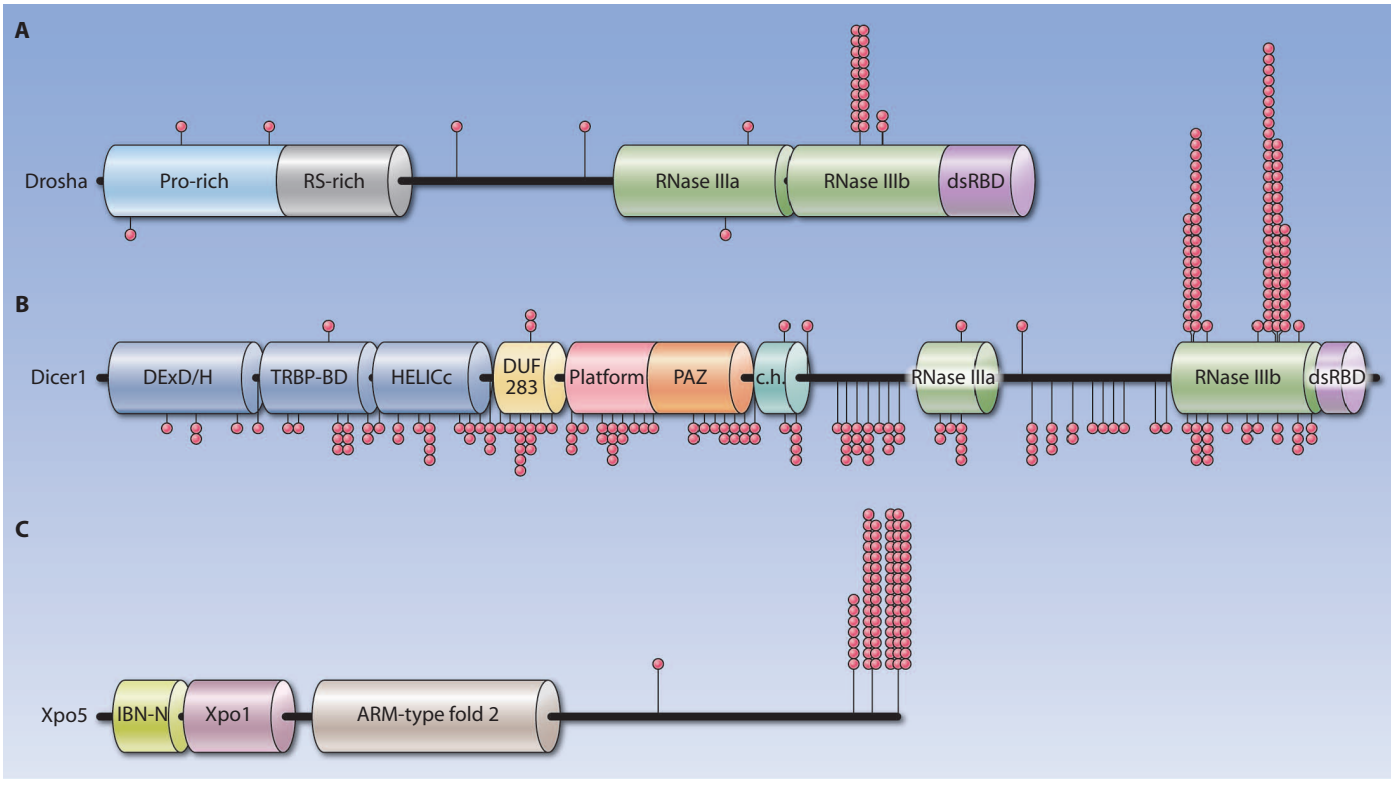

Fig. 3. Cancer-associated mutations in the miRNA biogenesis machinery. (**A** to **C**) Various mutations in key proteins in the miRNA biogenesis machinery—Drosha (A), Dicer (B), and XPO5 (C)—are found in patients with Wilms tumor, familial pleuropulmonary blastoma, pituitary blastoma, and embryonal rhabdomyosarcoma (A and B) or various cancers with microsatellite instability (C). Circles indicate the type (above, somatic; below, germline), position (relative to functional domains), and relative frequency of the mutations (*102*–*107*). RS, Arg/Ser; dsRBD, double-stranded RNA binding domain; DexD/H, DExD/H box helicase domain; TRBP-BD, transactivation response RNA binding protein–binding domain; HELICc, helicase-conserved C-terminal domain; PAZ, PIWI, Ago, and Zwille domain; IBN-N, importin-β–N; Xpo1, exportin-1/importin-β–like; ARM, armadillo; DUF283, domain of unknown function 283; c.h., connector helix.

are unable to export pre-miRNAs, causing down-regulation of mature miRNAs. Tumors with microsatellite instability also carry inactivating mutations of the *TARBP2* gene encoding TRBP, an essential cofactor of Dicer (*108*). Inactivation of TRBP destabilizes Dicer and impairs miRNA biogenesis. Thus, changes in overall abundance of mature miRNAs and the proteins that regulate their processing can have opposing oncogenic and tumor-suppressive roles in different types of tumors. Nonetheless, their recurrent links to cancer prognosis underscore the significance of miRNA-mediated gene silencing in the gene regulatory networks in cancer.

Regulation of microprocessors by DNA binding transcription factors

In addition to activating pri-miRNA transcription, some transcription factors also bind to related sequences in pri-miRNAs to regulate processing of key growth-related miRNAs. Posttranscriptional miRNA-dependent gene regulation is an important module of canonical growth factor signaling. Growth factors and their downstream signaling pathways sculpt the embryo and maintain the homeostasis of adult tissues; they also mediate adaptation to a new environment by responding to stress, such as nutrient starvation or hypoxia. The signal transducers downstream of membrane receptors activate gene transcription by binding to well-defined sequence elements in the enhancers and promoters of genes. The first example of growth factor signaling regulating a specific set of miRNAs was identified in vascular smooth muscle cells (VSMCs) stimulated with TGFβ and the related bone morphogenetic protein 4 (BMP4), which causes a transition to a contractile phenotype. The signal transducers of TGFβ and BMP4, R-Smad proteins, are bona fide transcription factors with DNA binding and transcription-activating domains (*109*). However, their activation also increases the abundance of *miR-21*, and about 20 other miRNAs posttranscriptionally. R-Smads are recruited by the RNA helicase p68 (also known as DDX5) to the Drosha microprocessor complex in the nucleus to promote *pri-miR-21* to *pre-miR-21* processing by Drosha (*110*) (Fig. 1B). *miR-21*, one of the most commonly up-regulated oncomiRs in nearly all tumors, down-regulates a long list of tumor suppressors, including *PTEN*, *PDCD4*, *TPM1*, *SPRY1/2*, and *TP53BP2* (*7*). In addition, it can drive tumorigenesis by inhibiting negative regulators of the RAS–MEK [mitogen-activated protein kinase (MAPK) kinase]–ERK (extracellular signal–regulated kinase) pathway (*111*). The specificity of R-Smad–mediated regulation of pri-miRNA processing is achieved by direct association of R-Smads with a sequence element in the stem region of *pri-miR-21* (*112*). Unlike transcriptional activation, regulation of Drosha processing by R-Smads does not require Smad4 (an essential cofactor of R-Smads for DNA binding and transcriptional regulation) (*112*). miRNA-mediated gene regulation is at least one plausible mechanism by which TGFβ can alter gene expression in pancreatic carcinoma cells harboring a deletion of *SMAD4*.

The p53 transcription factor not only induces the transcription of some pri-miRNAs (such as the *miR-34* family) but also posttranscriptionally enhances the abundance of a subset of tumor suppressor miRNAs (such as

miR-15/16, *miR-143*, *miR-145*, and *miR-203*) by acting as a cofactor of the Drosha microprocessor complex together with p68 to facilitate Drosha-mediated pri-miRNA processing, using a mechanism analogous to R-Smads (*113*) (Fig. 1B). Genotoxic stimuli, which induce acetylation of Lys^{120} in the DNA binding domain of p53, do not affect the transcriptional activity of p53 but facilitate its association with the Drosha microprocessor complex and thus increase the abundance of miR-203 to promote apoptosis instead of cell cycle arrest (*114*).

Regulation of microprocessors by RNA binding proteins

AKT phosphorylates and modulates the activity of the single-strand RNA binding factor KSRP (KH-type splicing regulatory protein) (*115*). KSRP binds the terminal loop of a subset of pri-miRNAs, including those of the *let-7* family, *miR-21* and *miR-125*, and promotes processing by Drosha by an unknown mechanism (*115*, *116*) (Fig. 1C). KSRP binding also promotes XPO5-mediated export of the pre-miRNA, and it binds to Dicer to promote pre-miRNA processing of the same subset of miRNAs (Fig. 1E). When the phosphatidylinositol 3-kinase (PI3K)–AKT signaling pathway is activated, KSRP is phosphorylated at Ser^{274} and Ser^{670}, which enhances its binding to pri-miRNAs and Drosha-dependent processing (*116*). KSRP-enhanced miRNA biogenesis is also activated by DNA damage. The DDR leads to phosphorylation of KSRP at the same residues in an ATM-dependent manner to promote maturation of specific miRNAs (*117*). KSRP is highly expressed in chronic myeloid leukemia in the acute/blast crisis phase compared with chronic phase disease. It remains to be investigated whether altered expression or function of KSRP contributes to leukemogenesis through the regulation of miRNA maturation (*118*).

A recent study links the global down-regulation of miRNAs in cancers with Hippo signaling, which regulates the Drosha processing step in a cell density–dependent manner (*119*). At low cell density, nuclear YAP (Yes-associated protein), the downstream target of the Hippo signaling pathway, binds the Drosha microprocessor-associated RNA helicase p72 (also known as DDX17) and sequesters it from the Drosha microprocessor complex, hence the suppression of p72-dependent miRNAs (*119*) (Fig. 1B). At high cell density, YAP is retained in the cytoplasm and facilitates Drosha processing (*119*). p72 binds to a 6-nt sequence in the 3′-flanking region of the pri-miRNAs that are regulated by p72. Some of the p72-dependent miRNAs are tumor suppressor miRNAs and target the oncogene *Myc*. Thus, constitutive activation of YAP or down-regulation of Hippo signaling in cancer could mediate generalized down-regulation of miRNAs and promote tumorigenesis via induction of MYC (*119*). It will be worthwhile to determine whether the subset of miRNAs highly expressed in stem cells and cancer lack a p72-binding motif, and how much this mechanism contributes to tumor formation in different types of cancer.

Regulation of microprocessors by RNA-editing enzymes

The adenosine deaminases acting on RNAs (ADARs), RNA-editing enzymes that convert adenosine to inosine in double-stranded RNAs (dsRNAs), also intersect with miRNA biogenesis. Adenosine-to-inosine conversion can change the sequence of the mature miRNA (including the critical seed sequence) to block target recognition and also change base pairing and hence can reduce dsRNA structure to interfere with pri-miRNA processing (Fig. 1D). RNA editing of *pri-miR-142* by ADAR1 and ADAR2 inhibits its processing by Drosha and also facilitates *pri-miR-142* degradation (*120*). More recently, another regulatory role of ADAR1 in the nucleus has been revealed (*121*). ADAR1 forms a complex with the Drosha cofactor DGCR8 and competes with its binding to Drosha. The exact role of the ADAR1/DGCR8 complex remains unclear, but it has been suggested that it contributes to the global dysregulation of pri-miRNA processing by Drosha. An ADAR dimer is required for RNA editing. Monomeric ADARs also complex with Dicer to increase the rate of pre-miRNA cleavage and facilitate miRNA loading onto RISC independently of their editing function. ADAR knockout embryos show a global reduction of mature miRNA abundance and gene silencing, suggesting that the role of ADARs in promoting pre-miRNA processing may dominate (*122*). ADAR1 is frequently reduced in metastatic melanoma, which results in dysregulation of more than 100 miRNAs (*121*).

Recently, a human RNA methyltransferase BCDIN3D was found to dimethylate the 5′ monophosphate of *pre-miR-145* and inhibit its processing by Dicer, leading to reduced mature *miR-145* (*123*). BCDIN3D mRNA is highly expressed in breast cancer stem cells, and its expression is linked to tumor cell invasiveness. Depletion of BCDIN3D in breast cancer cells increased mature miR-145 abundance and suppressed colony formation in soft agar and tumor cell invasivity in a miR-145b–dependent manner, indicating a critical contribution of BCDIN3D-mediated miRNA methylation in tumorigenesis. Further work is required to determine whether other pre-miRNAs are targets of BCDIN3D, the structural/sequence features of BCDIN3D target pre-miRNAs, whether BCDIN3D contributes to the global down-regulation of mature miRNAs in cancer, what the role of BCDIN3D is in normal cell differentiation, and whether changes in BCDIN3D expression or activity play a role in other cancers.

PTMs that control miRNA processing

PTMs of miRNA processing enzymes and their cofactors, which can affect miRNA processing, have also been identified (Table 1). Phosphorylation of serine residues by glycogen synthase kinase 3β (GSK3β) is required for the nuclear localization of Drosha (*124*, *125*). Acetylation of Drosha by the acetylases p300, CBP, and GCN5 prevents its ubiquitin-mediated degradation (*126*). Deacetylation of the Drosha cofactor DGCR8 by HDAC1 increases the affinity of DGCR8 for pri-miRNAs (*127*), whereas phosphorylation of DGCR8 by ERK stabilizes DGCR8 and facilitates miRNA production (*128*) (Table 1). It is not yet known whether aberrant miRNA production as a result of these microprocessor PTMs plays a role in cancer.

The MAPK-ERK pathway mediates phosphorylation of the Dicer cofactor TRBP, which enhances global miRNA biogenesis by increasing Dicer abundance through protein stabilization and facilitating Dicer/TRBP-mediated miRNA processing (*129*) (Table 1). Expression of phosphomimetic TRBP enhances growth-promoting miRNAs and increases cell proliferation (*129*).

Table 1. PTMs of the miRNA biogenesis machinery. Phosphorylation, acetylation, or deacetylation of Drosha or DGCR8 modulates Drosha microprocessor activity. Catalytic activity of Dicer and the silencing activity of RISC are regulated by phosphorylation of Ago and TRBP. In addition, Ago is known to undergo PTMs that affect protein stability, including hydroxylation, ubiquitylation, PARylation, and sumoylation.

Protein	PTM
Drosha	Phosphorylation by GSK3β Acetylation by p300, CBP, and GCN5
DGCR8	Phosphorylation by ERK Deacetylation by HDAC1
Ago	Phosphorylation by p38, AKT3, and EGFR Ubiquitylation, sumoylation; PARylation by poly(ADP-ribose) polymerase Hydroxylation by C-P4H(I)
TRBP	Phosphorylation by ERK

Control of nuclear-to-cytoplasmic export of pre-miRNA

Pre-miRNAs are exported into the cytoplasm after being processed by Drosha (Fig. 1A). XPO5 and GTP-bound Ran form a nuclear complex that transports and then releases pre-miRNAs into the cytoplasm (Fig. 1A). XPO5 is rapidly induced during cell cycle entry by a PI3K-dependent posttranscriptional mechanism, leading to an overall increase in mature miRNAs in proliferating cells (*130*). Nuclear export of pre-miRNAs also increases after DNA damage in an ATM-dependent manner. ATM activates the kinase AKT to phosphorylate the nucleopore component Nup153, leading to enhanced interaction between Nup153 and Xpo5 and more efficient nuclear export of pre-miRNAs (*40*). The PI3K-dependent mechanism underlying increased Xpo5 protein and pre-miRNA nuclear export has yet to be defined but could potentially also be mediated by AKT, which is activated by PI3K.

PTMs of Ago proteins and their cofactors

Ago proteins are the critical downstream effectors of miRNA-mediated gene silencing. They associate with the TNRC6 family proteins (TNRC6A, also known as GW182, and TNRC6B) in the RISC to guide miRNAs to target a specific set of transcripts and mediate gene silencing (*4*). Of the four human Ago proteins, Ago2 is the most abundant, then Ago1. In the absence of Ago2, miRNAs are unstable in cells with half-lives of ~8 hours, and global miRNA abundance is markedly reduced (*131*). Binding to Ago stabilizes a mature miRNA, possibly for weeks under basal conditions. Thus, the amount of Ago in a cell (estimated to be ~10^5 molecules, which is comparable to estimates of miRNA abundance) may be a key determinant of miRNA abundance and, hence, activity. Hence, PTMs of Ago proteins that change Ago stability or activity are potentially important mechanisms for globally and rapidly modulating miRNA abundance and activity (Table 1).

The first discovered Ago PTM was prolyl hydroxylation, which is mediated by type I collagen prolyl-4-hydroxylase I [C-P4H(I)] (*132*) (Table 1). C-P4H(I) hydroxylates multiple human Ago proteins, especially Ago2 at Pro^{700} (P700); this stabilizes Ago2 and augments small interfering RNA–mediated silencing. Hypoxia potently induces C-P4H(I) expression in VSMC, which increases Ago2 hydroxylation and leads to a rapid increase in Ago2 protein abundance and, hence, a rapid global increase in miRNAs and their mRNA-silencing activity (*133*).

Human Ago1 and Ago2 are phosphorylated in response to mitogens, including growth factors (Table 1). Phosphorylation of Ago2 at Ser^{387}, mediated by the p38 MAPK pathway and the kinase AKT3, promotes its association with TNRC6 cofactors and its localization to processing bodies (*134*), which enhances miRNA-mediated translational repression (*135*). The physiological significance of this PTM has not yet been demonstrated. Epidermal growth factor receptor (EGFR) signaling leads to the phosphorylation of Ago2 at Tyr^{393}, which reduces the association of Ago2 with Dicer and its cofactor TRBP (*136*). Hypoxia, which increases the abundance and activity of EGFR, consequently also enhances this phosphorylation of Ago2, which suppresses the maturation of a subset of growth-inhibiting pre-miRNAs that have long terminal loops and increases cell survival in response to hypoxia. Phosphorylated Ago2 is increased in hypoxic areas of human breast tumors. Moreover, increased abundance of phosphorylated Ago2 correlates with poor overall survival in breast cancer patients (*136*). Thus, mitogenic stimuli lead to phosphorylation of Ago and orchestrate a proliferation-promoting gene program, not only by increasing the abundance of growth-promoting miRNAs through the phosphorylation of TRBP (*129*) but also by reducing the abundance of tumor-suppressing miRNAs through the phosphorylation of Ago2 (*136*). The potential effects of the phosphorylation of Ago2 on the activity of the RISC, the loading of RNA duplexes into RISC, mRNA silencing by RISC, and miRNA stability and localization have yet to be revealed. Additionally, Ago proteins also undergo ubiquitination, poly(adenosine diphosphate–ribosyl)ation (PARylation), and sumoylation, all of which reduce the stability of Ago protein and, hence, RISC activity (*137*–*139*) (Table 1). However, the role of these PTMs in tumorigenesis or the status of these PTMs in cancer cells has not yet been reported.

A recent study found that the association of Ago proteins with their TNRC6 cofactors, especially TNRC6A/GW182, is greatly reduced in nondividing cells, compared to cancer cell lines or activated dividing primary cells (*140*). In most tissues, Ago is mostly found in nonfunctional low–molecular weight complexes that are not bound to target mRNAs or ribosomes (*140*). miRNA function and GW182 abundance increase after cells are activated in a PI3K–AKT–mTOR (mammalian target of rapamycin) pathway–dependent manner. Thus, miRNAs may be mobilized to function when cell division is stimulated to put the brakes on unrestricted cell proliferation. The authors attributed the change in Ago function to an increase in GW182 abundance (*140*), but they did not define a mechanism or investigate whether changes in Ago phosphorylation (discussed above) might also be important. In any event, this study suggests that measuring miRNA abundance may give an incorrect assessment of miRNA function.

Concluding Remarks

Cancer cells appear to be able to hijack every step of miRNA biogenesis to alter miRNA expression and activity to promote proliferation, survival, metastasis, and adaptation to new cellular environments. Although the basic mechanisms of miRNA biogenesis are now reasonably well understood, understanding the ways in which miRNA biogenesis and activity are regulated is still a work in progress. It is hard to predict the net effect of the opposing perturbations of miRNAs and mRNAs in cancer cells. Because the miRNA biogenesis machinery is often inhibited (Fig. 1, B to D), the abundance of miRNAs is generally reduced; however, because of other signals that are increased in cancer—either independently or because of the loss of miRNA-mediated repression (for example, through MYC, RAS, and TGFβ pathways), the expression and gene silencing activity of a subset of miRNAs are increased, leading to enhanced proliferative and migratory behavior in cancer cells (Fig. 2). These changes must be considered in the context of the general trend observed in dividing cells, whereby alternate polyadenylation yields mRNAs with shorter 3′ UTRs that lack many of their usual MREs and are therefore less able to be regulated by miRNAs.

Future studies will likely add many more mechanisms into the pool that regulates miRNA expression, processing, and function. These will likely include additional PTMs of the proteins that orchestrate miRNA biogenesis. Further studies are needed to understand the cell type and context specificity of these PTMs and understand how different PTMs influence each other and regulate cellular responses. With a clearer understanding of miRNA regulation and the role of PTMs, future cancer drugs might be developed to stimulate or antagonize specific mediators of miRNA biogenesis and gene silencing.

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