HHS Public Access

Author manuscript

Curr Opin Cell Biol. Author manuscript; available in PMC 2015 May 13.

Published in final edited form as:

Curr Opin Cell Biol. 2013 April ; 25(2): 233–240. doi:10.1016/j.ceb.2012.12.005.

Regulation of miRNA biogenesis as an integrated component of growth factor signaling

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Abstract

Transcriptional control of microRNAs (miRNA) by cell signaling pathways, especially in the context of growth factor regulation, is a widely recognized phenomenon with broad-reaching implications. However, several recent studies indicate that not just transcription, but also processing of miRNAs is subject to regulation as part of an integrated physiological response to various stimuli and environmental changes. The canonical miRNA biogenesis pathway; sequential steps of nucleolytic cleavage by the RNase III enzymes Drosha and Dicer, are emerging regulatory hubs for the modulation of miRNA expression as part of both physiological and pathological responses. In this article we use well-characterized growth-factor signaling pathways such as transforming growth factor-β (TGF-β), Protein Kinase B (PKB, also known as Akt) and extracellular-signal-regulated kinase (ERK) to illustrate how basic cell signaling pathways modulate the activities of these components of the miRNA biogenesis pathway to achieve optimal miRNA expression patterns.

Introduction: growth factors and miRNA maturation

One emerging paradigm in the study of cell signaling pathways is the role of post-transcriptional RNA regulation in modulating gene expression. A primary player in this regulation is microRNA (miRNA); small ~22 nucleotide (nt) RNA molecules that bind target mRNAs, usually in the 3′ untranslated regions (UTR), and inhibit their expression. Since their original description as mediators of development in *C. elegans* [1] miRNAs have proven integral components of nearly every aspect of biology. Perhaps nowhere is this importance illustrated as clearly as in the modulation of growth factor signaling pathways. Driven by the quest to discover novel targets for cancer therapeutics, knowledge of miRNA activity in response to growth factors has grown nearly exponentially.

Detailed molecular analyses have revealed a highly structured process of miRNA biogenesis involving sequential processing of long mRNA-like transcripts down into the ~22 nt single

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stranded RNA (ssRNA) effector molecule that is the mature miRNA. As the details of this processing pathway have emerged, it has become increasingly apparent that for each step in the miRNA biogenesis pathway, there exist alternative strategies through which they may be regulated or circumvented by cell signaling. Here we describe how growth factor signaling pathways utilize both the canonical and non-canonical miRNA biogenesis pathway to achieve the intricate balance necessary to respond appropriately to growth factor signaling pathways. We further speculate that the emerging interest in miRNA precursor stability (box 1) will eventually also be tied into growth factor signaling biology. In this article we do not discuss how individual miRNAs contribute to their respective pathways, as those subjects are extensively reviewed elsewhere [2, 3].

Box 1

New avenues for control - primary and precursor stability

In addition to altering the activity of miRNA-processing enzymes, miRNA biogenesis can be modulated by altering the stability of both pri-miRNAs and pre-miRNAs. By limiting the availability of pri-miRNAs or pre-miRNAs for processing, the downstream effect of miRNA-mediated gene regulation is affected. Pri-miRNA levels can be altered by RNA editing. The adenosine deaminase acting on RNA (ADAR) proteins are capable of deaminating adenosines on single-stranded RNA molecules [49]. Upon editing of pri-miR-142 by ADAR, pri-miR-142 is degraded by Tudor-SN, a component of RISC and also a ribonuclease [49].

Alternatively, pre-miRNAs can be destabilized. Expression of the RNA-binding protein is often inversely correlated with expression of members of the let-7 family of miRNAs. This is because Lin28 can bind the stem-loop of pre-let-7 and recruit the uridylatransferase Zcchc11 (TUT4) [50, 51]. Uridylation of pre-let-7 by Zcchc11 destabilizes pre-let-7, which results in degradation of the precursor and thus lower levels of mature let-7. To date neither pri-miRNA nor pre-miRNA destabilizing mechanisms have been linked to growth factor signaling pathways. However, expression of Zcchc11 can promote proliferation in different transformed cell lines [52], while ADAR has been shown to promote proliferation in astrocytomas [53]. Therefore, it is likely that these mechanisms of miRNA regulation will soon be placed in the wider context of growth factor signaling pathways under either physiological or pathological conditions.

Background: the miRNA biogenesis pipeline

A distinct set of rules governed by a specific series of proteins mediates the development of miRNAs from long, mRNA-like, primary (pri-miRNA) transcripts into the short [20–22 base pairs (bp)] single-stranded mature miRNA molecules that are physiologically relevant (Figure 1A, Reviewed in [4]). Briefly, this process involves sequential processing of the transcript by protein complexes primarily identified by the inclusion of a specific type III RNase enzyme. The biogenesis of miRNA begins with a long primary transcript known as pri-miRNA which bears a 7-methylguanosine cap and a poly-(A) tail. Pri-miRNA contains one or more stable stem-loop structures which encode mature miRNA(s). In the first processing step, the RNAse III enzyme Drosha, in complex with DeGiorgio Critical Region

8 (DGCR8 also known as Pasha) together with several other cofactors binds and cleaves the stem-loop region of pri-miRNA to generate a precursor miRNA (pre-miRNA) [4–6]. Pre-miRNA, usually <100 bp long, contains only the stem-loop portion of pri-miRNA and encodes what will become the mature miRNA in either its 5′ or 3′ arm extending out from the stem loop along with a second, nearly complementary, sequence on the opposing arm (Figure 1B). This structure is highly conserved among all miRNAs and is the basis for its recognition and export from the nucleus by Exportin 5 (EXP5) [7].

When in complex, Drosha and DGCR8 comprise the, “microprocessor complex” and can variably interact with two DEAD-box helicase proteins p68 (also called DDX5) and p72 (also called DDX17) whose expression is required for the biogenesis of some, though not all, miRNAs [8]. What role these RNA helicases play in miRNA biogenesis is presently unknown. Interestingly, some subset of miRNAs that require p72 for maturation, do not require p68; suggesting there exists heterogeneity in the composition of this important structure [8].

Once in the cytoplasm, the pre-miRNA stem-loop is processed into a dsRNA duplex containing only the 5′ (5p) and 3′ (3p) miRNA molecules by a second RNAse III enzyme named Dicer [9, 10]. After processing, one strand of the miRNA gets loaded into one of four argonaute (Ago) proteins [11]. These proteins mediate the effector function of the miRNAs either through inhibiting translation of the target mRNA or, in the case of Ago2, by directly degrading the mRNA transcript [11]. The remaining miRNA strand, termed the star or passenger strand and denoted by a *, is degraded.

Despite the highly ordered nature of this maturation process, multiple components of the developmental pathway are open to manipulation or deviation. The dissection of these deviations has proved extremely important in studying both the function and significance of miRNAs under physiological conditions. Growth factor signaling pathways utilize this typified biogenesis pathway to exert various biological effects.

Control of miRNA biogenesis by DNA binding proteins: Smads at the Drosha microprocessor

Perhaps the best understood, if not the most highly regulated, step of miRNA biogenesis is at the point of pri-to-pre-miRNA processing by the Drosha microprocessor complex. Canonically, increases in mature and pre-miRNA levels stem from higher pri-miRNA expression, usually as a result of increased transcription. However, this is not always the case. In 2009, it was reported that when pulmonary artery smooth muscle cells (PASMCs) are stimulated with ligands of the transforming growth factor β (TGF-β) family, such as TGF-β and bone morphogenetic protein 4 (BMP4), pri-miR-21 levels are unchanged while mature miR-21 expression increases significantly [12]. This result suggested a transcription-independent mechanism of miR-21 regulation which was confirmed by the induction of miR-21 even in the presence of the transcriptional inhibitor α-amanatin [12]. Biochemical analysis showed that the increase in mature miR-21 is caused by faster turnover in the biogenesis of miR-21 mediated by the nuclear translocation of Smad proteins [12, 13].

Smads represent the primary signal-transduction molecules of the TGF-β family of growth factors. Receptor activation by TGF-β family ligands induces phosphorylation of several Smad proteins collectively called the Receptor-specific Smads (R-Smads) [14]. Phosphorylated R-Smads form a complex with the common-Smad (co-Smad), Smad4, and translocate to the nucleus where they act as transcriptional regulators to promote or inhibit gene expression [14]. R-Smad/co-Smad hetero-dimerization is required for the transcriptional regulation mediated by TGF-β signaling. Surprisingly, Davis, et al. [12] found that Smad4 is dispensable for TGF-β induced induction of miR-21. Furthermore, stimulation with TGF-β ligands induced an association between Smads and the RNA helicase p68, indicating a direct recruitment of R-Smads to the Drosha microprocessor complex [12].

More recent miRNA expression profiling experiments revealed that stimulation of PASMCs with either TGF-β or the related BMP4 induces the expression of approximately 20 miRNAs [13]. Following stimulation, many of these miRNAs are bound directly by Smad proteins through a 5-base motif that closely resembles the Smad DNA binding element (SBE; 5′-CAGAC-3′) in a region overlapping the encoded mature miRNA [13]. Thus, R-Smads associate with pri-miRNA in a sequence specific manner and facilitate the Drosha microprocessor activity to enhance mature miRNA expression (Figure 2A). Interestingly, *in silico* analyses have found that pre-miRNA sequences encode a higher number of transcription factor biding sites than would be predicted by chance [15]. These results suggest that the mechanism for Smad-dependent regulation of pri-miRNAs may represent a widespread, though underappreciated, strategy for controlling miRNA expression.

A similar mechanism has been described for miRNA processing regulation by the tumor suppressor p53 [16]. Following DNA damage, p53 translocates to the nucleus where it binds p68 along with various pri-miRNAs to facilitate their biogenesis. For both the Smad and p53 pathways, knockdown of p68 by siRNA is sufficient to inhibit miRNA maturation [12, 16], suggesting that p68 is an essential factor for pri-miRNA processing. Whereas Smad proteins bind pri-miRNAs by a distinct sequence-specific mechanism [13], it is unclear how p53 specifically recognizes a set of pri-miRNAs.

Protein recruitment to the Drosha microprocessor complex does not always facilitate miRNA processing. Following stimulation with estradiol (E2), estrogen receptor α (ERα) also associates with p68 and the Drosha microprocessor [17]. However, unlike the previous examples, ERα inhibits pri-miRNA processing by Drosha [17]. Following E2 stimulation, nearly all miRNAs are affected, suggesting that ER-α act as a general regulator of miRNA expression unlike the more specific induction achieved by p53 and Smads. It is also unclear whether p72 (DDX17) plays a similar role to p68 in the induction of pri-miRNA processing by Smads, p53, or ERα.

Control of the Drosha microprocessor by RNA binding proteins: KSRP activation by Akt

Like the DNA binding proteins described above, RNA binding proteins are also known to facilitate pri-to-pre-miRNA processing. The singe-stranded RNA binding KH-type splicing

regulatory protein (KSRP) performs various cellular activities, such as facilitating alternative splicing and binding to the 3′ UTR of mRNAs to recruit the exosome and expedite their degradation [18]. In addition, KSRP binds the terminal loop of a subset of pri-miRNAs and facilitates their processing. Though the microprocessor activity of KSRP was originally attributed to direct interaction with a GGG motif in the terminal loop of let-7a [19], KSRP activity has since been found to modulate a broader repertoire of miRNAs include miR-155 [20], miR-16 [21] and miR-206 [22], suggesting that KSRP acts to enhance the catalytic activity of the Drosha microprocessor through a sequence-independent mechanism.

The activity of KSRP in regulating the Drosha microprocessor is closely tied to growth factor signaling. Mutational analyses of KSRP have revealed that phosphorylation of KSRP at various Serine residues including Ser193 [22] as well as Ser274 and Ser670 can enhance its pri-miRNA binding affinity and promote pri-miRNA processing [21]. During differentiation of myoblasts, Briata, et al. [22] found that phosphorylation of Ser193 mediates a switch of KSRP affinity from mRNA to pri-miRNA *in vitro*. They further found that this phosphorylation is Akt-dependent; constitutive activation of Akt2 was sufficient to enhance the production of myogenic miRNAs in a KSRP-dependent manner [22]. Conversely, Akt2-deficient cells grown in differentiation media displayed increased expression of pri-miRNAs, but not of the mature miRNAs, suggesting a block of pri-to pre-miRNA processing as a result of blocked KSRP phosphorylation by Akt2 [22]. Consistent with this idea, miRNA expression was significantly altered in the muscles of KSRP null mice following cardiac injury by administration of cardiotoxin [22]. These studies identify KSRP phosphorylation as an integral part of the Akt pathway (Figure 2B) and suggest that Akt signaling may activate a miRNA-processing cascade and mediate cell type-specific outcomes. KSRP-mediated miRNA biogenesis can also be regulated by other, non-growth factor, pathways as indicated by the finding that DNA-damage induced phosphorylation at Ser274 and Ser670 by ATM can also promote maturation of specific miRNAs [21].

The positive effect of KSRP on the biogenesis of let-7a-1 can be counteracted by the splicing factor heteronuclear ribonucleoprotein A1 (hnRNPA1) which binds the same region of pre-let-7a stem-loop and displaces KSRP [23]. Interestingly, hnRNPA1 can also promote the expression of a number of miRNAs [24] raising the intriguing possibility that the Drosha microprocessor components can alternate between agonistic and antagonistic roles in the context of specific miRNA biogenesis.

mirtrons

As described above, pri-to-pre-miRNA processing by the Drosha microprocessor complex is a critical regulatory step in the life of miRNAs. However, some miRNAs avoid this step by utilizing alternative processing pathways. In both flies [25] and humans [26] mirtrons, pre-miRNAs generated directly from the spliced introns of mRNA transcripts, have been identified by deep-sequencing and validated as functional. Because they are derived directly by splicing as a fully formed pre-miRNA-like stem-loop structure these sequences are able to bypass the microprocessor [25, 26]. To our knowledge no studies have yet placed mirtron development and/or activity into a physiological context. Therefore, how these miRNAs

contribute to cellular functions remains unknown. However, this may be changing, as the development of Drosha/DGCR8-deficient embryonic stem (ES) cell lines will make the analysis of such non-canonical miRNAs more straightforward [27, 28]. Furthermore, one recent study identified 237 mouse and 240 human splicing-derived mirtrons indicating the potential importance of this understudied class of miRNAs [29].

Control at the Dicer complex: regulation of TRBP by MAPK/ERK signaling

The pre-to-mature miRNA processing complex, of which the catalytic core is Dicer, can also serve as an important site for regulation of miRNA biogenesis. In fact, KSRP was originally described as an interacting protein of the Dicer complex where it acts through a sequence-specific association with the pre-miRNA stem-loop [20] TO promote maturation [19, 20]. It is unclear, however, whether Akt-mediated phosphorylation of KSRP plays a role in controlling Dicer processing.

Similarly, The human immunodeficiency virus (HIV)-1 transactivating response RNA binding protein (TRBP) is an important component of the Dicer complex [30, 31]. However, the activity of TRBP in this process is not constitutive. Rather, a recent publication identified four Serine residues, of which phosphorylation was required to promote this interaction [32]. miRNA microarray analysis comparing HEK293 cells expressing either a non-phosphorylatable TRBP with a phospho-mimic TRBP identified a variety of growth-associated miRNAs, including miR-17, miR-20a and miR-92a, that were induced upon phosphorylation. Conversely, the constitutively active protein uniformly decreased expression of various let-7 species, a family of miRNA with well-documented growth-suppressive functions [33]. The kinases responsible for phosphorylating TRBP were identified as the extracellular signal-regulated kinase, ERK1/2 [32]. Similar to the Akt pathway, the mitogen-activated kinase (MAPK)/ERK pathway is a central modulator of numerous cellular pathways and is commonly mutated in cancers [34]. Thus, TRBP recruitment to the Dicer complex may act as a regulatory switch activated by MAPK/ERK signaling to promote expression of growth-promoting miRNAs (Figure 2C) [32].

Another component of the Dicer complex, the protein kinase R activating protein (PACT) also acts as an important activator of pre-miRNA processing. Although PACT is not essential for miRNA maturation, inhibition of PACT decreases expression levels of multiple mature miRNAs [35]. It remains unclear whether this interaction can be controlled by growth factor signaling pathways. Interestingly, knocking down any components of the Dicer complex, (i.e. Dicer, PACT, or TRBP) inhibits the levels of both pre-miRNAs and mature miRNAs [36]. This suggests the existence of some positive feedback loop by which mature miRNAs modulate the levels of their pri- and/or pre-miRNAs. One such mechanism was recently described for let-7 expression in *C. elegans* where the Ago2 homolog, ALG-1 can bind a conserved region in the 3′ end of pri-let-7 to promote its processing [37]. This process is dependent on expression of mature let-7, and thus the mature miRNA acts through a positive feedback loop to promote its own biogenesis.

Dicer-independent maturation of pre-miRNA

As with the Drosha microprocessor complex, the Dicer complex may also be bypassed in miRNA biogenesis. Deep sequencing of both zebra fish [38] and mice [39] deficient in Dicer showed an enrichment of one specific mature miRNA: miR-451. Pre-miR-451 has a unique, highly conserved structure that allows it to associate with Ago2, which contains RNase activity. Loading of pre-miR-451 into Ago2 results in slicing of pre-miR-451 and generates a 30 nt intermediate miR-451, which is then further trimmed to become a mature miRNA [38, 39].

Pathological significance of growth factor regulated miRNAs

Aberrant expression of some miRNAs controlled by growth factor signaling pathways have been reported and implicated in the pathogenesis of disorders such as cancer and cardiovascular disease,. For example, miR-21 is known as an “onco-miR” because it is robustly upregulated in nearly all tumor samples [40] and it can propagate oncogenic activities by targeting tumor suppressors such as programmed cell death protein 4 (PDCD4) [41], tropomycin1 (TPM1) [42] and phosphatase and tensin homolog (PTEN) [43]. As activation of TGF-β signaling is often associated with various tumor types, it is intriguing to speculate that increased expression of miR-21 is, in part, due to aberrant activation of TGF-β signal and warrants anti-TGF-β therapies [44]. Similar to tumor samples, upregulatoin of miR-21 can be initiated by cardiac stress and may lead to cardiac hypertrophy or cardiac fibrosis ina MAPK/ERK pathway-dependent manner [40].

Other miRNAs may act as tumor suppressors. The let-7 family of miRNAs targets several oncogenes including c-Myc [45], numerous members of the Ras (N-Ras, K-Ras, and H-Ras) family of oncogenes [46] and high mobility group A2 (HMGA2) [47]. Biogenesis of let-7 is inhibited by ERK1/2-mediated phosphorylation of TRBP and unlike miR-21, let-7 expression is often lower in tumor samples than in normal tissues [33]. In some tumors, the Let-7 target c-Myc promotes the expression of Lin28b, a negative regulator of let-7, therefore, the c-Myc-let-7 axis forms a positive feedback loop to activate multiple oncogenic pathways. We speculate that future studies will uncover many more examples of human diseases that are initiated, and/or driven by aberrant miRNA expression as a result of misregulation of growth factor controlled miRNA biogenesis pathways.

Conclusion

As summarized in this article, control of miRNA biogenesis is an integral component of cell biological activities (Figure 3). Aberrations in miRNA biogenesis result in abnormal expression of multiple miRNAs, which can contribute to developmental defects and human diseases. Recent findings illustrate the importance of understanding not only transcriptional regulation but also post-transcriptional regulation of miRNA biogenesis. The existence of potential regulatory mechanisms was originally suggested in a 2006 study by Thompson, et al., who identified a substantial correlation between the primary and mature miRNA species expressed in normal cells but not in tumor samples [48]. It is clear that the ability to balance

miRNA expression is an important part of normal growth factor signaling and that maintaining this balance can be achieved at every step of the miRNA biogenesis pathway.

The examples discussed here represent the first insights into how miRNA biogenesis is integrated into growth factor signaling pathways. Future studies will likely identify additional pathways affecting these important signal transduction networks. Furthermore, the mechanisms underlying the specificity of regulated miRNAs by different signaling pathways remain to be elucidated. We anticipate that in the future these directions will provide insights into the pathogenesis of various human disorders and present novel therapeutic targets.

Acknowledgments

We apologize to those colleagues whose references we have omitted from this discussion; due to space restrictions and our focus we were unable to include all articles on this interesting and diverse subject matter. We wish to express our thanks to the members of the Hata/Lagna lab for their suggestions and animated discussion. This work was supported by grants from the National Institute of Health: HL093154 and HL108317, the American Heart Association: 0940095N and the LeDucq foundation Transatlantic network grant to A.H.

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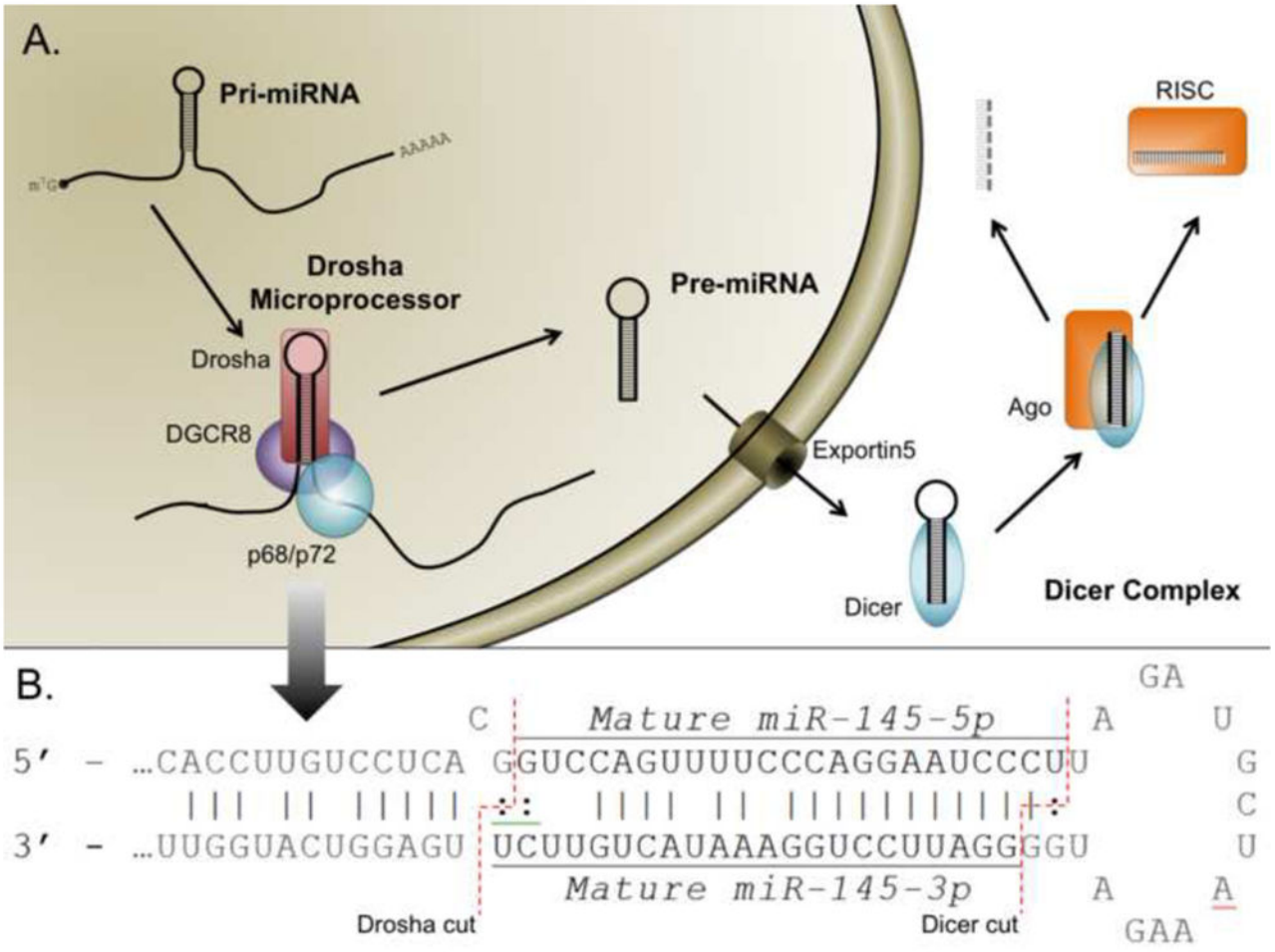

Figure 1. Basic components of the miRNA biogenesis pathway

miRNA begins as a long primary transcript (pri-miRNA) with a structure resembling mRNA. Pri-miRNAs contain a 5′ cap (m7-G) and a polyA tail at the 3′ end (AAAA). Pri-miRNA undergoes two sequential processing steps. First, the Drosha microprocessor complex which is composed of Drosha, DGCR8 and DEAD-Box RNA helicases (p68 or p72) cleaves pri-miRNA to generate precursor miRNA (pre-miRNA). Following cleavage by the Drosha microprocessor, the resulting pre-miRNA precursor maintains a stereotypical stem-loop structure. Following the export of pre-miRNA from the nucleus by exportin 5 (EXP5), Dicer processes the pre-miRNA into double stranded mature miRNA. Mature miRNA will be loaded into Argonaute proteins (Ago), which separates mature miRNA into two single stranded miRNAs. **(B)** Future miRNAs adopt a stereotypical stem-loop structure within the pri-miRNA sequence (shown here for miR-145). The mature sequence is encoded on one side of the loop and the degraded * strand on the other. More recent nomenclature has moved to calling each strand 5p and 3p according to which side of the strand they occupy. Here, the dotted lines indicate the site of future cleavage by Dicer and Drosha.

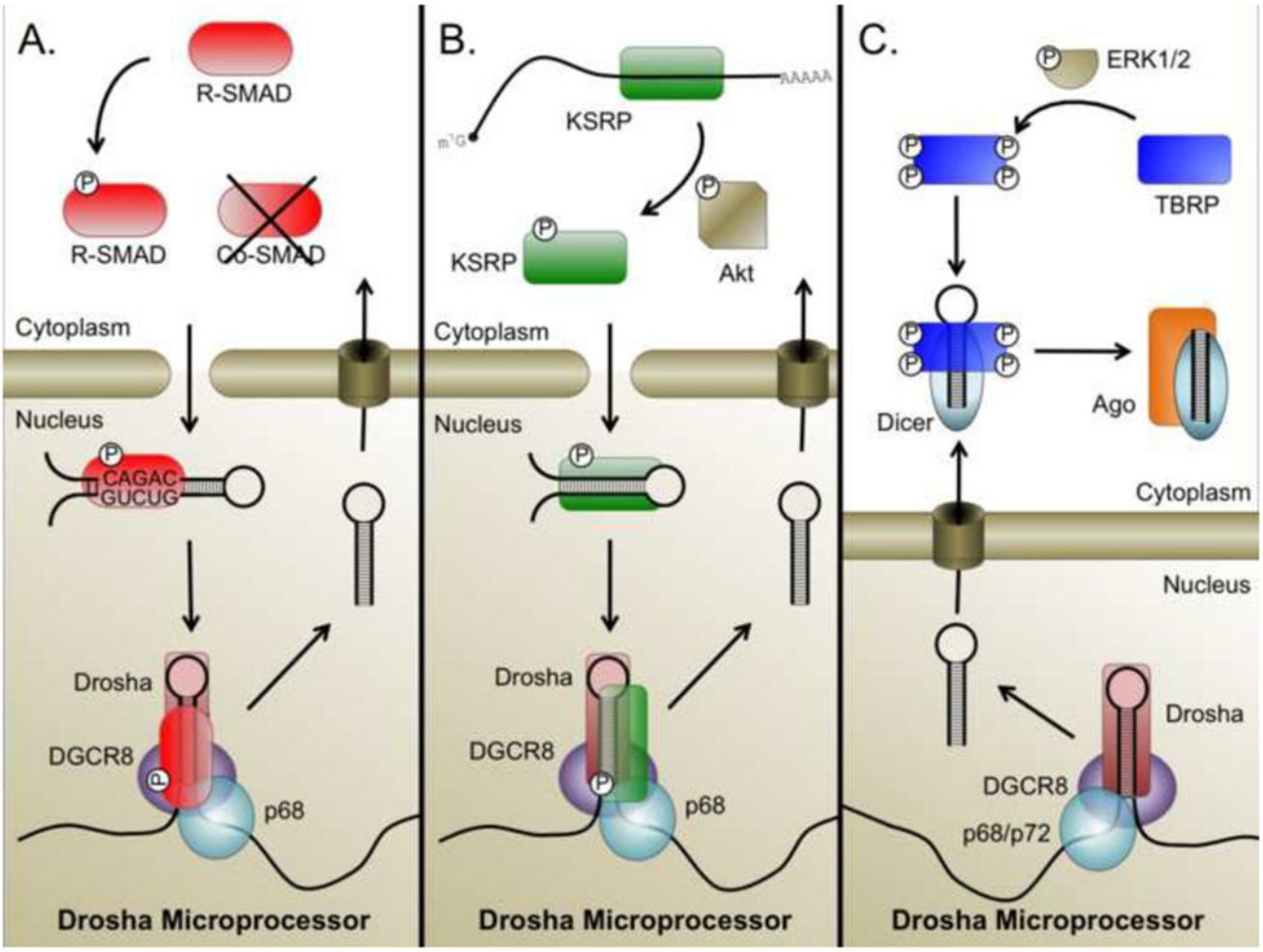

Figure 2. Integration of miRNA into cell growth factor signaling pathways

(A) Activation of receptor Smads by TGF-β family ligands leads to their translocation into the nucleus. In the nucleus, R-Smads bind, independent of the transcriptionally necessary co-Smad, to a conserved sequence in pri-miRNAs which they recruit to the Drosha microprocessor complex and facilitate pri- to pre-miRNA maturation. **(B)** PI3K-mediated activation of Akt induces the phosphorylation of KSRP. Phosphorylated KSRP is dissociated from mRNA and associates with targeted pri-miRNA sequence to recruit the Drosha microprocessor and facilitate pri- to pre-miRNA processing. **(C)** Activation of MAPK/ERK induces phophorylation of TRBP, which is subsequently recruited to Dicer and promotes the processing of miRNAs associated with cell proliferation.

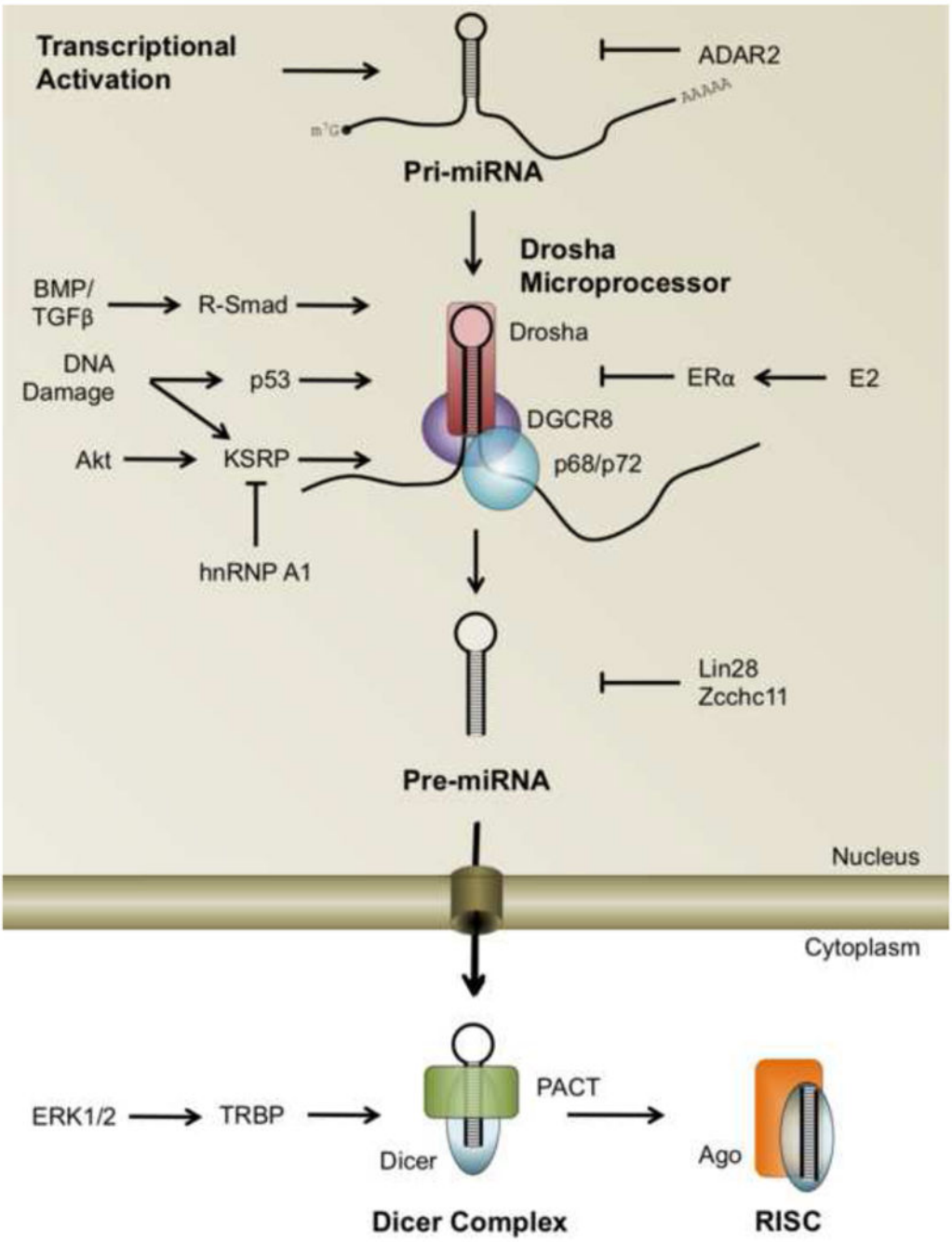

Figure 3. Integration of biological signaling pathways at the sites of miRNA biogenesis

miRNA biogenesis pathways are emerging as critical components of gene regulatory mechanism controlled by growth factor signaling (and other) pathways. Activation or inactivation of the Drosha microprocessor by DNA binding proteins (Smads, p53, and ERα), RNA binding proteins (KSRP) or TBRP has been well documented. However, mechanisms of regulation of many other proteins in the miRNA biogenesis pathway by different signaling pathways remain to be uncovered.