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RESEARCH ARTICLE

Post-transcriptional regulation of miRNA biogenesis and functions

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Abstract MicroRNAs (miRNAs) are a highly conserved class of small (18–24 nucleotides) non-coding RNAs that regulate a broad spectrum of biological processes. Aberrations or corruptions of miRNA functions may lead to deregulated cell proliferation, tumorigenesis, and ultimately, cancer. Increasing evidences suggested that a large fraction of miRNAs is regulated at the post-transcriptional stage, which impacts on the level and function of miRNAs during cell development and human diseases. Recently, several distinct mechanisms are emerging to regulate the biogenesis, stability and function of miRNAs at post-transcriptional level, such as specific binding to terminal loops of miRNA precursors (pri-miRNAs or pre-miRNAs) by RNA-binding proteins and 3'-terminal modifications by particular enzymes. Signaling cascades and post-translational modifications of the core components of RNA machinery also take part in the post-transcriptional regulation of miRNAs.

Keywords miRNA biogenesis, post-transcriptional regulation, RNA-binding proteins, hua enhancer 1 (HEN1), non-canonical poly(A) polymerase, signal transduction, post-translational modification

1 Introduction

MicroRNAs (miRNAs) are a class of small, regulatory, noncoding RNAs (ncRNAs) that are 18–24 nucleotides (nts) in length, and are present in nearly all metazoans. More than 400 human miRNA genes have been cloned (Lagos-Quintana et al., 2001; Bentwich et al., 2005; Berezikov et al., 2005; Landgraf et al., 2007), and more than a thousand of miRNA genes are predicted in the human genome by bioinformatic computations (Lim et al., 2003; Berezikov et al., 2005; Berezikov et al., 2007). Functional studies indicate that miRNAs participate in the regulation of almost every cellular process through post-transcriptional silencing of specific target mRNA, and bioinformatic predictions indicate that approximately 30%–50% of protein-coding genes in humans are regulated by miRNAs (Lewis et al., 2003; Lewis et al., 2005; Miranda et al., 2006; Rajewsky, 2006; Grimson et al., 2007).

Studies in animal models have revealed that miRNAs play important roles in diverse biological processes during animal development (Bartel, 2004; Zamore and Haley, 2005). Deregulation of miRNAs has been found in many human diseases, including cancer (Calin and Croce, 2006; Hammond, 2006), and many miRNAs have been shown to be deregulated during tumorigenesis (Calin et al., 2004; Iorio et al., 2005; Volinia et al., 2006; Iorio et al., 2007; Dahiya et al., 2008). For example, the miR-17-92 cluster is amplified in human B-cell lymphomas, and enforced expression of this miRNA in mouse B-cell lymphoma models dramatically accelerated tumor development (Kurschat et al., 2005; Dews et al., 2006). In particular, the let-7 family miRNAs have been proposed to function as tumor suppressors, possibly by inhibiting the mRNAs of well-characterized oncogenes, such as Ras, HMGA2, and c-Myc (Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Sampson et al., 2007; Kumar et al., 2008). Reduced expression of let-7 family miRNAs is common in non-small cell lung cancer (NSCLC) (Kumar et al., 2008) and breast tumor-initiating cells (BT-IC) (Yu et al., 2007). Moreover, Yu et al. (2007) have demonstrated that self-renewal and maintenance of the undifferentiated state of cancer stem cells (CSCs) requires reduced levels of let-7, and the lack of let-7 expression can be used as a marker to prospectively isolate self-renewing progenitor cells (Ibarra et al., 2007).

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2 The miRNA biogenesis

Most animal miRNAs share common biogenesis and effector machineries. Mature miRNAs are produced from long primary transcripts (pri-miRNAs) through a series of endonucleolytic processing steps (Kim, 2005). Many primary miRNA transcripts are transcribed by RNA polymerase II (Pol II) (Cai et al., 2004; Lee et al., 2004) and contain 5' caps and 3' poly(A) tails, while others are transcribed by RNA polymerase III (Pol III) (Borchert et al., 2005). Some miRNA genes are clustered in the genome and may be co-transcribed and processed into multiple individual mature miRNAs (Altuvia et al., 2005), For example, the miR-17-92 cluster is a prototypical example of a polycistronic miRNA gene encoding six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92).

In the canonical pathway of miRNA biogenesis (Fig. 1), pri-miRNAs are processed into pre-miRNAs by a nucleus-specific microprocessor complex that contains the RNase III enzyme Drosha, and its partner DiGeorge syndrome critical region gene 8 (DGCR8) (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Han et al., 2006). Some intronic miRNA precursors, termed mitrons, are processed into pre-miRNAs by the RNA splicing machinery rather than the Drosha-DGCR8 complex (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). In either case, the about 70-nt pre-miRNA hairpin with a protruding 2-nt 3'-end overhang is specifically recognized by transport factor Exportin-5 (Exp-5) and transported into the cytoplasm in a Ras-related nuclear protein-guanosine triphosphate (RanGTP)-dependent manner (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004). In the cytoplasm, pre-miRNAs are further processed by the cytoplasmic RNase III enzyme Dicer and its partner TAR (transactivating response) RNA binding protein (TRBP) into about 22-nt miRNA duplexes with 5'-phospahte and 3' 2-nt overhangs (Bernstein et al., 2001; Knight et al., 2001; Chendrimada et al., 2005). The miRNA duplex is then asymmetrically loaded into the RNA-induced silencing complex (RISC) (Gregory et al., 2005; Förstemann et al., 2007), where the mature miRNA strand is preferentially retained and the complementary passenger strand (miRNA*) is selectively degraded. The major component of the active miRNA RISC (miRISC) consists of a single-stranded mature miRNA and a member of the Argonaute (AGO) proteins (Schwarz and Zamore, 2002). Both the 5'-phosphate and the 3'-terminal 2-nt overhang are specifically recognized by the Argonaute proteins (Song et al., 2003; Lingel et al., 2004; Ma et al., 2004; Ma et al., 2005; Parker et al., 2005). Following integration into miRISC, which often includes other proteins that probably function as the repressive effectors, such as the P-body protein GW182 (Peters and Meister, 2007), miRNAs inhibit the translation or guide the degradation of target mRNAs (Pillai et al., 2007; Filipowicz et al., 2008).

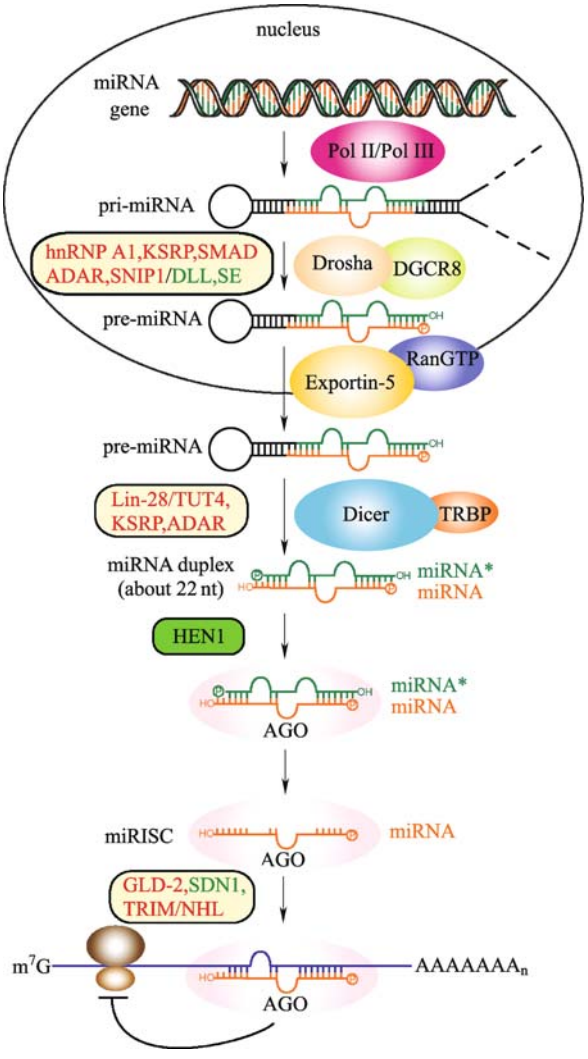

Fig. 1 The canonical pathway of miRNA biogenesis in animals and some known post-transcriptional regulators at cascaded steps. Factors in red are from animal, and factors in green are from plant.

3 Post-transcriptional regulation of the miRNA biogenesis

A multistep model has been proposed for the regulation of miRNA biogenesis (Obernosterer et al., 2006; Thomson et al., 2006; Wulczyn et al., 2007) consistent with sequential processing steps of miRNA maturation (Fig. 1). It has been shown that a large fraction of miRNA genes are post-transcriptionally regulated in embryonic stem (ES) cells, embryonal carcinoma (EC) cells, and primary tumors (Thomson et al., 2006). There are distinct mechanisms behind the post-transcriptional regulation of miRNA biogenesis. In mammals, certain pri-miRNAs are subject to RNA editing by adenosine deaminase (ADAR), which converts adenosine to inosine (A → I RNA editing)

(Luciano et al., 2004; Blow et al., 2006; Yang et al., 2006; Kawahara et al., 2007a; Kawahara et al., 2007b; Kawahara et al., 2008). The ADAR editing sites are frequently located on the double-stranded stem region of the pri-miRNA, and hence the A → I editing usually suppresses the processing of the pri-miRNA to pre-miRNA by the Drosha-DGCR8 complex (Yang et al., 2006) or of the pre-miRNA to mature miRNA by the Dicer-TRBP complex (Kawahara et al., 2007a).

Recently, several reports show that 3'-terminal modifications are more general mechanisms in the post-transcriptional regulation of miRNAs. In plants, miRNAs are modified by a small RNA methyltransferase Hua Enhancer 1 (HEN1) on the 2'-OH of their 3'-terminal ribose molecules (Yu et al., 2005; Yang et al., 2006b), and thus protected from degradation and uridylation at 3' end (Li et al., 2005). Recently, it has been demonstrated that 3'-ends of a subset of miRNAs undergo post-transcriptional adenylation or uridylation (Hagan et al., 2009; Heo et al., 2009; Katoh et al., 2009; Lehrbach et al., 2009) at different steps. For example, miR-122 is stabilized by GLD-2 through 3'-terminal adenylation after maturation (Katoh et al., 2009), while let-7 is destabilized by TUTase4 (TUT4) through 3'-uridylation before the process by Dicer in cytoplasm (Heo et al., 2009; Lehrbach et al., 2009).

Recently, increasing evidences showed that a subset of miRNAs is regulated by sequence-specific RNA-binding proteins at post-transcriptional level. For example, it has been shown that the RNA binding protein heterogeneous nuclear ribonucleoprotein (hnRNP) A1 binds specifically to human pri-miR-18a (one of miRNAs in miR-17-92 cluster) and facilitates its processing by the Drosha-DGCR8 complex (Guil and Caceres, 2007). Two RNA recognition motifs (RRMs) in the amino-terminal domain of hnRNP A1, also known as unwinding protein 1 (UP1) (Fig. 2A), exhibit the RNA binding specificity of full-length hnRNP A1. ADAR proteins have been shown to affect miRNA processing independently of catalytic RNA editing activity, and instead, function as RNA-binding proteins (Heale et al., 2009). The KH-type splicing regulatory protein (KSRP, also known as KHSRP), a key mediator of mRNA decay that specifically binds to single-stranded AU-rich-element-containing mRNAs, has been found as a component of both Drosha and Dicer complexes and regulates the biogenesis of a subset of miRNAs (Trabucchi et al., 2009). Lin-28, a developmentally regulated RNA-binding protein and a pluripotency factor (Yu et al., 2007), has been demonstrated to selectively

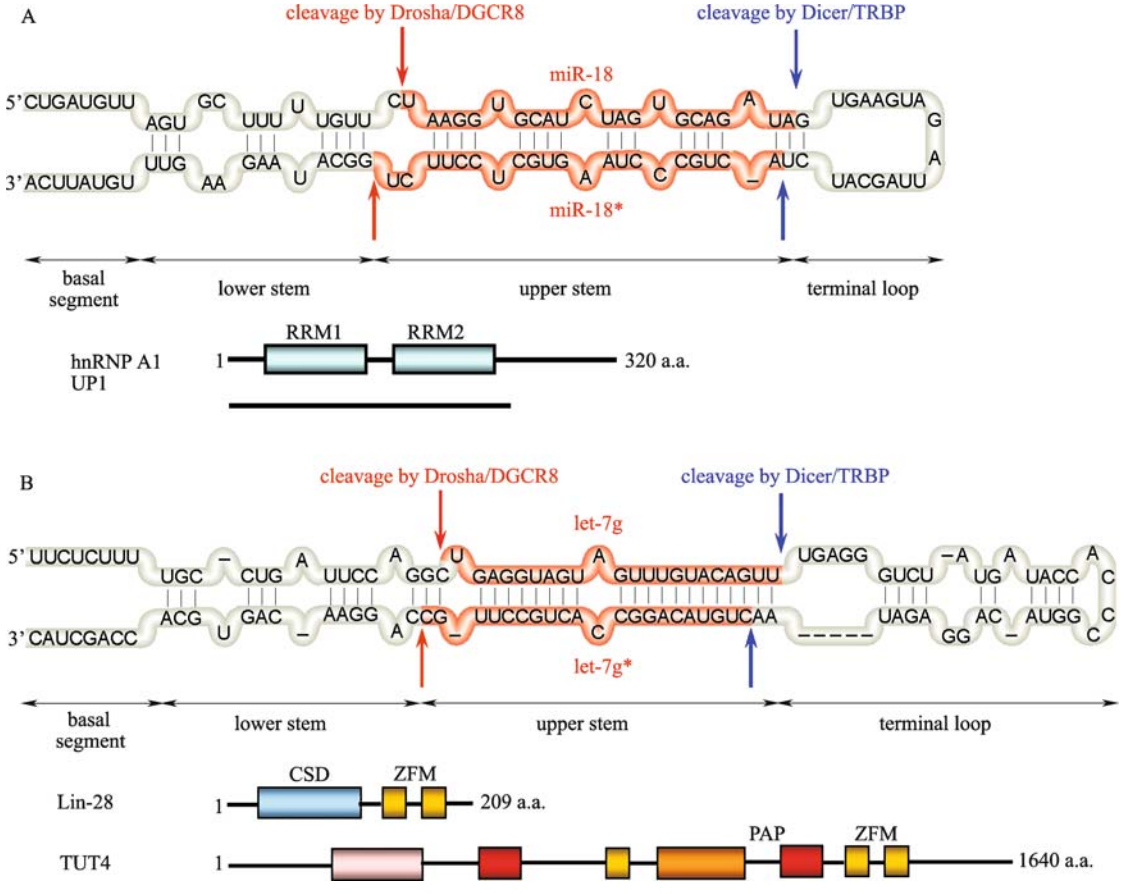

Fig. 2 Predicted hairpin structures of pri-miR-18a **(A)** and pri-let-7g **(B)**, and domain compositions of their regulators: hnRNP A1 **(A)**, Lin-28 and TUT4 **(B)**

block the processing of pri-let-7g by the Drosha-DGCR8 complex in EC cells (Viswanathan et al., 2008) and pre-let-7a-2 by the Dicer-TRBP complex in ES and EC cells (Heo et al., 2008; Rybak et al., 2008). Lin-28 contains a unique combination of three RNA-binding domains: a cold-shock domain (CSD) and a tandem retroviral-type CysCysHisCys (CCHC) zinc finger motif (ZFM) (Fig. 2B) (Balzer and Moss, 2007). Very recently, it has been shown that Lin-28 works with TUT4 to block Dicer processing through 3'-terminal uridylation (Chang et al., 2009; Hagan et al., 2009; Heo et al., 2009). In plant, a forehead-associated domain (FHA)-containing protein, DAWDLE (DDL) has been demonstrated as an RNA-binding protein that interacts with Dicer-like 1(DCL1) and acts in the biogenesis of miRNAs (Yu et al., 2008).

4 RNA-binding proteins in the post-transcriptional regulation of miRNA maturations

Little is known about the molecular mechanisms by which the RNA-binding proteins regulate the miRNA processing. In order to be recognized and processed precisely by the Drosha-DGCR8 complex (Han et al., 2006; Sohn et al., 2007), pri-miRNAs should possess a characteristic hairpin structure: a terminal loop ($\geqslant$10 nt), an upper stem (about 22 bp), a lower stem (about 11 bp), and basal segments (Fig. 2) (Zeng et al., 2005; Han et al., 2006). The terminal loops in the miRNA precursors have been demonstrated as the major sites recognized by various RNA-binding proteins (Michlewski et al., 2008; Piskounova et al., 2008; Heo et al., 2009). However, the molecular bases for the recognition and regulation of miRNAs by RNA-binding proteins are not fully understood.

It is expected that RNA-binding proteins behave as RNA chaperones through binding specific sites of miRNA precursors (pri-miRNAs or pre-miRNAs), i.e., to modulate their conformations and/or interactions with processing complexes (Drosha-DGCR8 complex or Dicer-TRBP complex), thus facilitating or blocking the processing of specific miRNAs. In fact, RNA chaperone activities of both hnRNP A1 and proteins containing the CSD (e.g. cold shock-like protein CspA) (Graumann and Marahiel, 1998) or the ZFM (e.g. human immunodeficiency virus-type 1 (HIV-1) nucleocapsid) (Rein et al., 1998) have been demonstrated. Due to intrinsic flexibilities of miRNA precursors originated from mismatches and internal loops, pri-miRNAs or pre-miRNAs may exhibit multiple conformations, such as structures (I) and (II) shown in Figs. 3A and B. Pri-miRNAs with structure (I) could be recognized by the Drosha-DGCR8 complex and correctly processed to pre-miRNAs, whereas pri-miRNAs with structures (II) could not be recognized or processed by the Drosha-DGCR8 complex. If an RNA-binding protein specifically binds and stabilizes the structure (I) of a

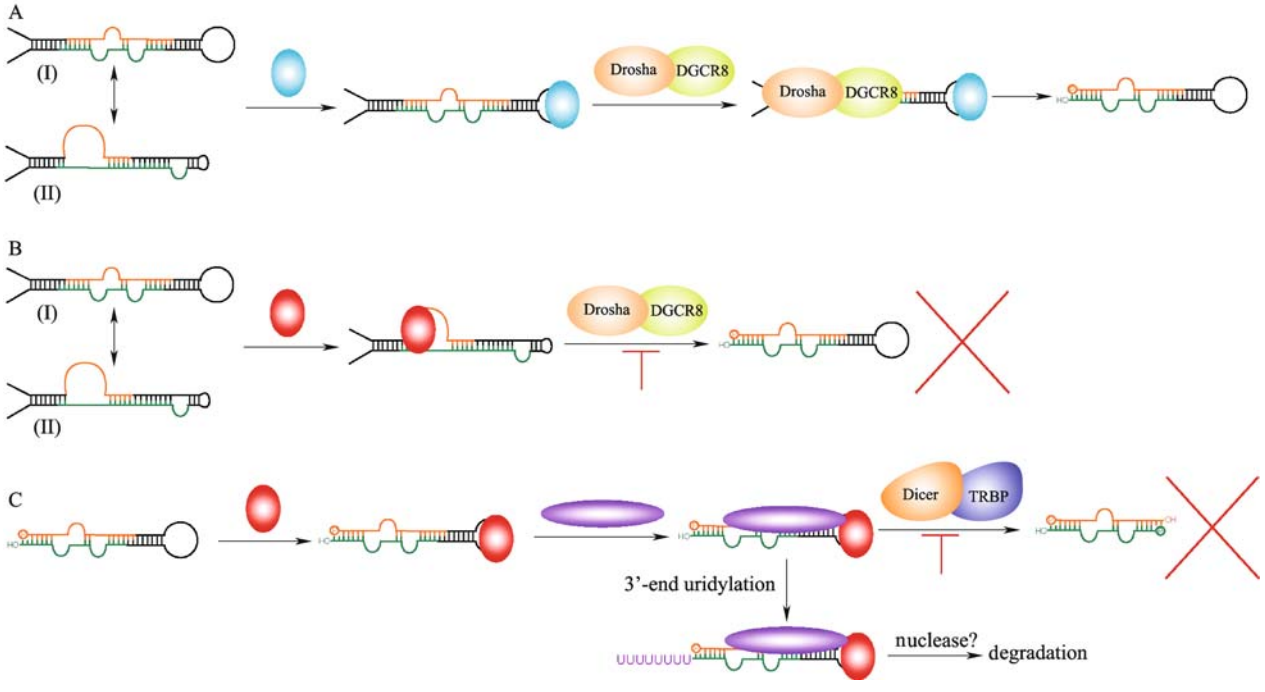

Fig. 3 Mechanisms of the RNA-binding proteins in the post-transcriptional regulation of miRNA maturation. **(A)** RNA-binding protein binds and stabilizes the pri-miRNA structure (I) that is recognized and processed by the Drosha-DGCR8 complex; **(B)** The RNA-binding protein binds and stabilizes the pri-miRNA structure (II) that cannot be recognized and processed by the Drosha-DGCR8 complex; **(C)** The RNA-binding protein binds the pre-miRNA and recruits another protein to block the access of the Dicer-TRBP complex to the pre-miRNA.

pri-miRNA, it will facilitate the processing of the pri-miRNA (Fig. 3A). On the other hand, if an RNA-binding protein specifically binds and stabilizes the structure (II) of a pri-miRNA, the pri-miRNA processing will be blocked (Fig. 3B). It is also possible that even an RNA-binding protein specifically binds the structure (I), but it can recruit another protein that prevents the access of the Dicer-TRBP complex to the pre-miRNA, and the pre-miRNA processing is blocked (Fig. 3C). If the recruited protein is a 3'-terminal modifying enzyme (such as TUT4), the pre-miRNA may be destabilized and degraded (Fig. 3C).

5 The 3'-terminal modifying enzymes in the post-transcriptional regulation of miRNA stabilities

The miRNA stability is regulated by a group of 3'-terminal modifying enzymes. In plant, HEN1 methylates both 3'-ends of miRNA duplex before loading into the RISC, which prevents the 3'-terminal oligo-uridylation and further degradation (Li et al., 2005; Yu et al., 2005). A recent structural study indicates that highly cooperative recognitions of the small RNA substrate by multiple RNA binding domains and the methyltransferase (MTase) domain in HEN1 measure the length of the RNA duplex and determine the substrate specificity (Fig. 4) (Huang et al., 2009). The metal ion coordination by both 2' and 3' hydroxyls on the 3'-terminal nucleotide and four invariant residues in the active site of the MTase domain suggests a novel Mg^{2+}-dependent 2'-O-methylation mechanism (Fig. 4). It has been shown that miRNA-specific exoribonuclease small RNA degrading nuclease 1 (SDN1) is also sensitive to the 2'-O-methyl modification on the 3'-terminal ribose of miRNAs (Ramachandran and Chen, 2008). In animals, a subset of miRNAs, such as miR-122, are selectively stabilized by 3'-terminal adenylation by a noncanonical poly(A) polymerase (PAP), GLD-2, after maturation in cytoplasm (Katoh et al., 2009). In *C. elegance*, it has been found that another noncanonical PAP, CDE-1, is required for the 3'-terminal uridylation of siRNAs bound CSR-1, and in the absence of CDE-1, siRNAs accumulate to inappropriate levels (van Wolfswinkel et al., 2009). CDE-1, a TUT4 homologue is also a noncanonical PAP required for 3'-terminal uridylation of pre-let-7. Similar to mRNAs, miRNAs and other small silencing RNAs are stabilized by 3'-terminal adenylation, but are destabilized by 3'-terminal uridylation. In addition, uridylation and adenylation may occur competitively, and a single-adenylation prevents further oligo-uridylation (Chen et al., 2000). Sequencing results have identified that many miRNAs from animals have 3'-terminal

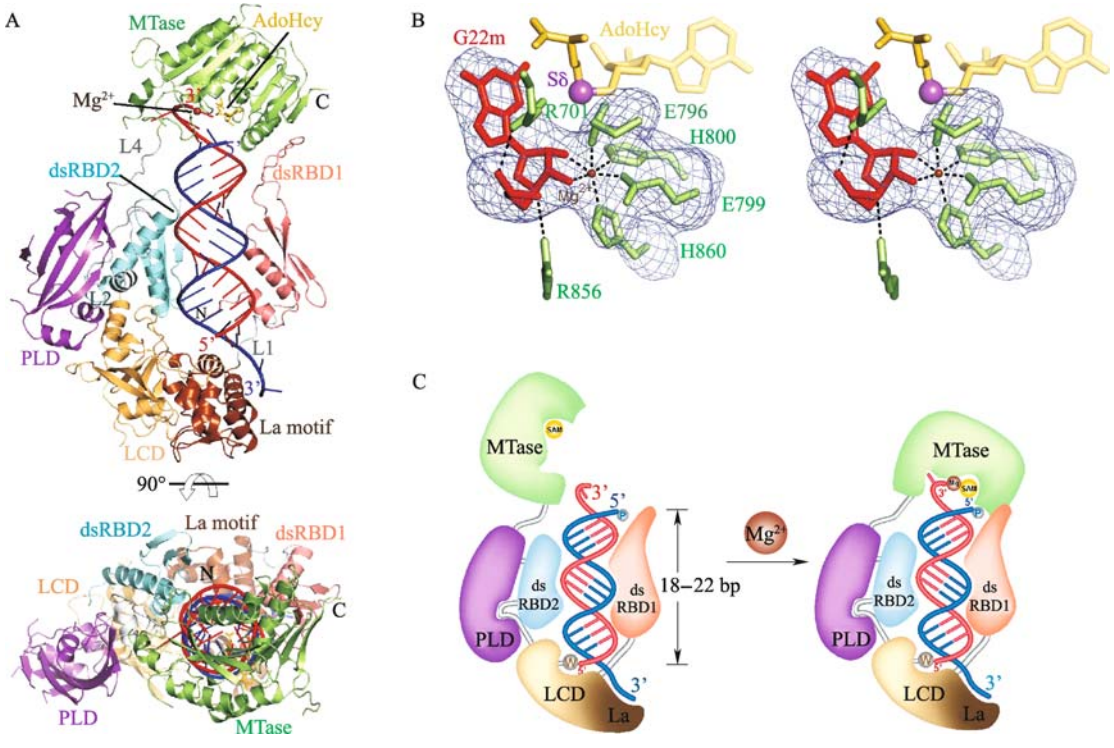

Fig. 4 Molecular mechanisms for the miRNA-specific 3'-terminal 2'-O-methyltransfease HEN1. **(A)** Structural overview of Arabidopsis hua enhancer 1 (HEN1) in a complex with an miRNA duplex and the cofactor product S-adenosyl-L-homocysteine (AdoHcy). (B) A stereoview of the Mg^{2+}-coordination by both 2' and 3' hydroxyls of the 3'-terminal nucleotide and four invariant residues in the MTase domain, E796, E799, H800 and H860. **(C)** Proposed model for the specific recognition of small RNA substrates by HEN1 and the Mg^{2+}-dependent 2'-O-methylation mechanism.

adenylation or uridylation (Ruby et al., 2006; Landgraf et al., 2007; Azuma-Mukai et al., 2008). These observations imply that 3'-terminal modifications are general mechanisms in the post-transcriptional regulation of miRNA stabilities.

6 The signal transduction regulation of miRNA machinery

There is increasing interest in the potential regulation of miRNA biogenesis by the signaling cascades. For example, the transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signaling pathways have been shown to positively regulate miR-21, which is processed by the Drosha-DGCR8 complex. This regulation will result in an induction of a contractile phenotype in human vascular smooth muscle cells (Davis et al., 2008). Work in the Kato Lab has shown that estradiol (E2) binds to estrogen receptor α (ERα), and further interact with the Drosha-DGCR8 complex (Yamagata et al., 2009). Importantly, both regulatory mechanisms require the presence of the microprocessor-associated RNA helicase p68 and p72, therefore, inhibit the Drosha-DGCR8 complex-mediated miRNA processing, and regulate miRNA production. TRBP, the critical partner of Dicer, was recently reported to be positively regulated by the mitogen-activated protein kinase (MARK) pathway (Paroo et al., 2009). Extracellular signal-regulated kinase (ERK), one of the mitogen-activated protein kinases (MARKs), plays an important role in cellular proliferation, differentiation, and survival. TRBP interacted with phosphorylated ERK *in vivo*, and was phosphorylated at four serine residues (142, 152, 283, and 286) under conditions where Erk was activated. Notably, a cell line expressing phospho-mimic TRBP showed an increasing level of Dicer-TRBP, consequently increasing the level of mature miRNA (Paroo et al., 2009). Another well-known RNA silencing factor, Argonaute (Ago) proteins, was demonstrated to have post-translational modifications in 2008. Human Ago2 was found to interact with the subunits of type I collagen prolyl-4-hydroxylase (C-P4H(I)), and hydroxylated at proline 700, which not only stabilized the Ago2 protein but also facilitated its localization to P bodies in the cytoplasm. P bodies are regions consisting of RNAs and enzymes involved in mRNA turnover (Qi et al., 2008). Ago2 protein was phosphorylated at serine 387 in the consequence of the activation of p38 MARK pathway, which facilitates the localization of Ago2 to P bodies. A similar mechanism has been found for Piwi-interacting RNA (piRNA) interacting proteins, the Piwi proteins (Kirino et al., 2009; Reuter et al., 2009; Vagin et al., 2009; Wang et al., 2009). The N-terminal fragment of several Piwi proteins has been shown to be symmetrically dimethylated at arginines at so-called symmetrically dimethylated arginines (sDMA) motifs (typically Gly-Arg-Gly) that are recognized by the Tudor-related (Tdrd) proteins (tudor domain containing proteins), which regulates the localization of the Piwi proteins in cells. The tripartite motif and Ncl-1, HT2A and Lin-41 domain (TRIM-NHL)-containing proteins, such Mei-P26, NHL-2, and TRIM32, have also recently been demonstrated to regulate certain miRNAs through interaction with Argonaute proteins (Neumuller et al., 2008; Hammell et al., 2009; Schwamborn et al., 2009).

7 Overall significance

Post-transcriptional regulations of miRNA maturations and stabilities are widely used to modulate the miRNA levels in cells. Several distinct mechanisms have been uncovered. However, much remains to be learned, such as molecular mechanisms for the conformational changes of miRNA precursors bound by RNA-binding proteins, substrate binding and molecular mechanisms of miRNA-specific 3'-terminal modifying enzymes, as well as the underlying mechanisms of the signaling transduction regulation of the key components within the miRNA pathway. Given that miRNAs are involved in broad biological processes and human diseases, the steady-state levels of miRNAs in the particular developmental stages are essential for their proper functions. It is expected that further studies on the mechanism of post-transcriptional regulation of miRNAs may shed light on miRNA-related pathogenesis and pave the way for developing innovative therapeutic strategies.

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