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POINT OF VIEW

Emerging roles of DROSHA beyond primary microRNA processing

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ABSTRACT

DROSHA is the catalytic subunit of the Microprocessor complex, which initiates microRNA (miRNA) maturation in the nucleus by recognizing and cleaving hairpin precursors embedded in primary transcripts. However, accumulating evidence suggests that not all hairpin substrates of DROSHA are associated with the generation of functional small RNAs. By targeting those hairpins, DROSHA regulates diverse aspects of RNA metabolism across the transcriptome, serves as a line of defense against the expression of potentially deleterious elements, and permits cell fate determination and differentiation. DROSHA is also versatile in the way that it executes these noncanonical functions, occasionally depending on its RNA-binding activity rather than its catalytic activity. Herein, we discuss the functional and mechanistic diversity of DROSHA beyond the miRNA biogenesis pathway in light of recent findings.

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Characterization of DROSHA as the initiator of microRNA maturation

MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides (nt), which recognize complementary sites within target mRNAs to direct their post-transcriptional repression [1]. In mammals, each miRNA downregulates hundreds of target mRNAs [2,3], and more than 60% of mRNAs have selectively maintained at least one miRNA target site [4]. Practically, this means that virtually every biological pathway is under the control of miRNAs. In this regard, it is not surprising that dysregulation of these tiny regulators is associated with various human diseases, including cancer [5,6].

Much is now known about how metazoan miRNAs are produced. In the canonical biogenesis pathway, miRNA genes are transcribed by RNA polymerase II (Pol II) into long primary transcripts, called primary miRNAs (pri-miRNAs), containing one or more characteristic hairpin structures [7]. These miRNA hairpins are recognized and cleaved by the nuclear Microprocessor complex, a heterotrimeric complex consisting of one molecule of DROSHA, an RNase III enzyme, and two molecules of DGCR8, a double-stranded RNA (dsRNA)-binding protein, to release ~60–80 nt precursor miRNAs (pre-miRNAs; Figs. 1, 2A & 3A) [8-13]. Pre-miRNAs are then exported to the cytoplasm with the aid of Exportin-5 (XPO5) [14-16], where they undergo further processing by a second RNase III, DICER (Fig. 3A) [17-19]. Of the resulting ~22 base-pair (bp) miRNA duplex, one strand is preferentially loaded onto an Argonaute (AGO) family protein to form a functional miRNA-induced silencing complex (miRISC; Fig. 3A) [20-24].

Human DROSHA contains proline-rich (P-rich) and arginine/serine-rich (RS-rich) stretches in the N-terminal region and tandem RNase III domains (RIIIDa and RIIIDb) followed by a dsRNA-binding domain (dsRBD) in the C-terminal region [25,26]. The N-terminal region of DROSHA has diverged among metazoan orthologues, and at least for human DROSHA, is dispensable for pri-miRNA processing activity *in vitro* [11]. However, diverse post-translational modifications within the N-terminal region of human DROSHA have been associated with subcellular localization or stability control of the protein [27-30], suggesting that this region may serve as a regulatory platform. The central and C-terminal regions of DROSHA are folded in the presence of two DGCR8 molecules to constitute the processing center of the Microprocessor [12,13], in which RIIIDa and RIIIDb dimerize intramolecularly to cleave the 3′ and 5′ strand of a pri-miRNA hairpin, respectively (Fig. 1) [11,13]. DROSHA plays a pivotal role in miRNA biogenesis, as demonstrated by the complete loss of canonical miRNA expression in *DROSHA* knockout cells [31].

Pri-miRNAs appear to be the canonical substrates of DROSHA. Inspection of *Caenorhabditis elegans, Drosophila*, and human pri-miRNAs revealed the typical structure of these molecules, in which an imperfect stem of ~3 helical turns is flanked by unpaired basal segments at the base and an apical loop at the top (Figs. 1 & 2A) [32,33]. DROSHA introduces staggered cuts ~1 helical turn away from the basal junction and ~2 helical turns away from the apical junction (Figs. 1 & 2A) [8,33-36]. In addition to these structural determinants, several primary sequence motifs in pri-miRNAs were found to promote DROSHA-mediated processing, including a UG motif at the basal junction, a CNNC motif ~17–18 nt downstream of the 3′ cleavage site, a mismatched GHG motif in the middle of the basal stem (where H is any nucleotide except G), and a UGU/GUG motif in the 5′ end of the apical loop (Fig. 1) [12,37-40]. More recently, N^6-methyladenosine (m^6A) RNA modifications emerged as an epigenetic determinant of pri-miRNA processing [41-43].

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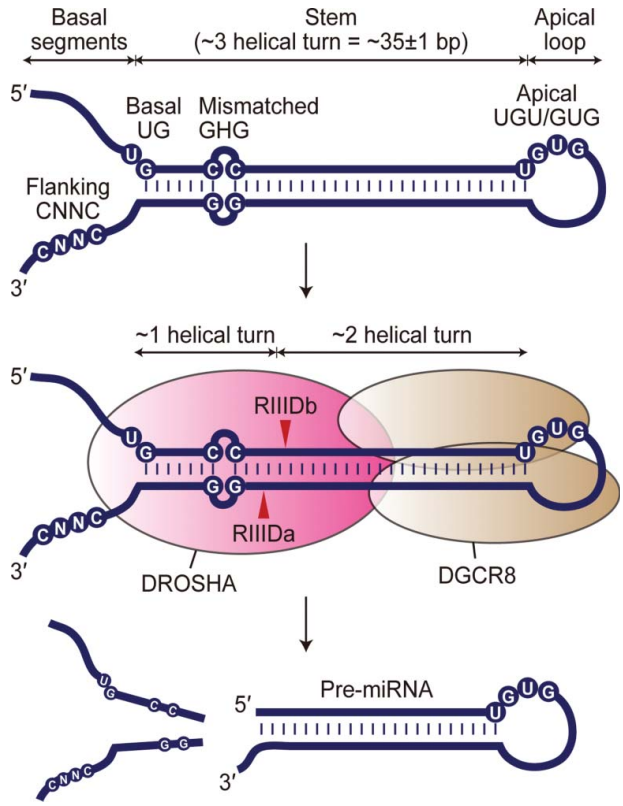

Figure 1. Recognition and processing of pri-miRNA by the Microprocessor complex. A representative pri-miRNA molecule is shown at the top. Pri-miRNAs are characterized by an imperfect stem of ~3 helical turns flanked by a stretch of unstructured nucleotides at both ends, termed the basal segment and apical loop. DROSHA introduces staggered cuts ~1 helical turn away from the basal junction and ~2 helical turns away from the apical junction. Primary sequence motifs that are known to enhance the processing efficiency of the Microprocessor are indicated, including a basal UG motif, a flanking CNNC motif, a mismatched GHG motif, and an apical UGU/GUG motif.

Noncanonical functions of DROSHA beyond nuclear pri-miRNA processing

In addition to understanding pri-miRNAs and their recognition and cleavage by DROSHA, several genome-wide approaches aimed at uncovering the cellular functions of DROSHA have revealed the existence of its non-miRNA substrates [44-46]. Given that DROSHA-mediated cleavage is accompanied by substantial destabilization of pri-miRNAs [8,10,11,47,48], transcriptomic changes upon DROSHA depletion were measured to identify any additional substrates [49-52]. mRNAs that are derepressed by DROSHA deficiency, but not DICER deficiency, tend to possess hairpin structures that closely resemble those of pri-miRNAs [49,51], suggesting that DROSHA-mediated destabilization of mRNAs involves their direct cleavage. Instead of measuring steady-state transcript abundance, cleavage products can be directly quantified using parallel analysis of RNA ends (PARE) or degradome sequencing, which aims to sequence the 5′ ends of RNA fragments bearing 5′ monophosphate termini [53,54]. Degradome sequencing in mammalian cells identified a number of DROSHA-dependent cleavage sites within mRNAs [55,56]. In addition, application of a crosslinking, immunoprecipitation, and sequencing (CLIP-seq) technique to the Microprocessor components demonstrated the presence of substrates that give rise to very little or no detectable small RNAs [57-59]. These findings raise the question of whether noncanonical targeting by DROSHA is widespread, and if so, what are its functions? Below, we describe the reported roles of DROSHA beyond nuclear pri-miRNA processing.

Ribosomal RNA biogenesis

The earliest characterization of DROSHA indicated a putative role in ribosomal RNA biogenesis [26], consistent with the previously reported functions of bacterial and yeast RNase III enzymes [60,61]. A significant fraction of nuclear DROSHA protein is translocated to the nucleolus during the S phase [26], and antisense inhibition of DROSHA leads to the accumulation of precursors of 5.8 S rRNA [26,62]. However, levels of mature rRNAs are not evidently changed following DROSHA depletion [26,62,63], suggesting that DROSHA may play a minor role, if any, in the maturation of rRNAs.

mRNA destabilization

The best-characterized function of DROSHA outside of the miRNA pathway is post-transcriptional destabilization of mRNAs through cleaving pri-miRNA-like hairpins embedded within them (Fig. 3B). The herald of this paradigm was the *DGCR8* mRNA, which contains two evolutionarily conserved hairpin structures in its 5′-untranslated region (5′-UTR) and coding region, respectively (Fig. 2B) [64]. These hairpins are cleaved by DROSHA *in vitro* and in cells, which imparts instability to the reporter construct in a DROSHA-dependent manner [50,65]. DROSHA-mediated destabilization of the *DGCR8* mRNA is proposed to serve as a means of maintaining constant Microprocessor activity, and its deep conservation in mammals and *Drosophila* may reflect its importance [49-51,56,65]. Notably, the *DGCR8* mRNA hairpins seem unlikely to serve as pri-miRNAs, because the corresponding small RNAs could only be detected, if at all, by deep sequencing [50,51]. For example, the ~60 nt pre-miRNA-like fragment generated from the 5′-UTR hairpin is not processed down to small RNAs in cells[50,65] despite its full potential to produce small RNAs *in vitro* [65], and this processing failure is attributed in part to its strict nuclear retention [50].

DROSHA-mediated cleavage also contributes to the clearance of a subset of mRNAs in progenitor cells, which appears to be essential for cell fate determination and differentiation [52,66-68]. In embryonic neural stem cells, DROSHA destabilizes the mRNA of the proneural factor Neurogenin2 (Ngn2) by cleaving pri-miRNA-like hairpins within its 3′-UTR (Fig. 2C) [66,69]. The loss of this repression largely explains the precocious differentiation of progenitors caused by DROSHA deficiency, because forced expression of Ngn2 leads to the same phenotype, and double depletion of DROSHA and Ngn2 restores normal progenitor status [66]. The *Tbr1* mRNA is another substrate for DROSHA in neuronal progenitors and its downregulation is critical for corticogenesis [68]. In adult hippocampal stem cells, DROSHA targets the *NF1B* mRNA to prevent oligodendrogenesis and to promote neurogenesis [67]. A similar mechanism operates in hematopoietic stem cells, where DROSHA-mediated destabilization of the *Myl9* and *Todr1*

mRNAs is necessary for dendritic cell development and myelopoiesis (Fig. 2D) [52]. It will be interesting to investigate whether this type of regulation is at play in other progenitor cells during their differentiation.

In addition to these biologically relevant examples, many other mRNAs are subject to DROSHA-mediated cleavage [49,51,56-59]. However, with the exception of the *DGCR8* mRNA, candidate mRNAs identified from one study tend to overlap poorly with those from another, probably due to differences in subjects and the experimental approaches employed. DROSHA-mediated cleavage of these mRNAs leads to their destabilization, but the extent to which they are derepressed upon DROSHA depletion is modest compared to pri-miRNAs [49,51,56,57,59]. Indeed, mRNA hairpins are cleaved less efficiently than pri-miRNAs *in vitro* [50-52,57,59,65], and are proposed to have suboptimal structures and sequences [59]. This may be because these hairpins have evolved to modulate the expression of their host transcripts, rather than to serve as a source for downstream effectors. Alternatively, but not mutually exclusively, this may represent a strategy that allows robust cleavage to occur only under certain conditions. For example, the *Ngn2* hairpin deviates significantly from optimal pri-miRNA hairpins in terms of its structure and sequence (Fig. 2C), but it is readily cleaved by DROSHA in neural stem cells, leading to degradation of more than 80% of the *Ngn2* mRNA [66]. TDP-43, a neural activity-responsive factor, was reported to associate with DROSHA[10,70] and to be required for DROSHA-mediated destabilization of the *Ngn2* mRNA [69]. This suggests that *trans*-acting factors may modulate DROSHA-mediated cleavage of mRNA hairpins in order to achieve spatiotemporally regulated destabilization of specific mRNAs.

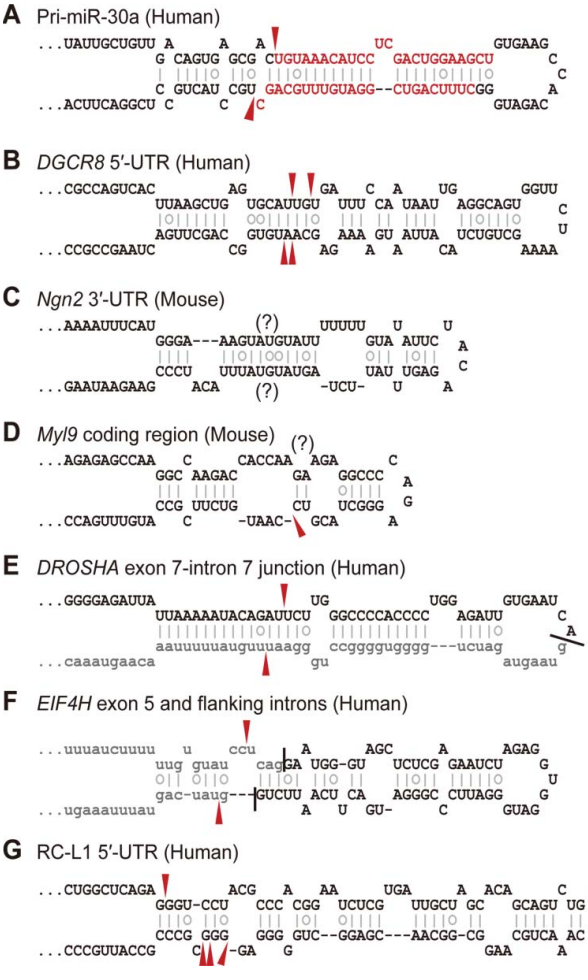

Figure 2. Canonical and noncanonical substrates of DROSHA. Predicted secondary structures of diverse DROSHA substrates are presented. Prediction was performed using the mfold RNA folding algorithm.[101] For pri-miR-30a, mature miRNA sequences are colored red. For the *DROSHA* and *EIF4H* hairpins, intronic sequences are shown as gray lowercase letters. The sites of DROSHA-mediated cleavage determined by characterization of *in vitro* or *in vivo* processing products are indicated by red arrowheads.

Regulation of transcription and RNA processing

Microprocessor-mediated processing of pri-miRNAs occurs co-transcriptionally, as evidenced by the chromatin association of DROSHA [71,72]. Interestingly, genome-wide analysis of its chromatin footprints found that DROSHA binds not only miRNA genes but also the 5′ ends of many other genes [73]. This binding is reportedly mediated by short hairpins within promoter-associated transcripts that are structurally distinct from pri-miRNAs or pri-miRNA-like hairpins found in mRNAs. Surprisingly, the consequence of this binding is neither cleavage nor destabilization of the corresponding transcripts, but rather transcriptional activation. Furthermore, the function of DROSHA as a transcriptional activator is independent of its catalytic activity and is mediated through its N-terminal region, which appears to interact with Pol II and CBP80 (Fig. 3C).

Consistent with the notion that nuclear RNA processing events are closely coupled to one another [74], it has been demonstrated that DROSHA can play dual roles in both pri-miRNA (or pri-miRNA-like hairpin) cleavage and other RNA processing. DGCR8 CLIP-seq revealed hundreds of cassette exons bound by DGCR8, and depletion of DROSHA or DGCR8 causes the accumulation of splice isoforms containing these exons [57]. Bioinformatics analysis identified dozens of loci in which pri-miRNA hairpins are juxtaposed with exon-intron junctions such that the Microprocessor and splicing machinery functionally antagonize each other [75,76]. Splicing regulation by DROSHA was recently corroborated by the discovery of a pri-miRNA-like hairpin located across a specific exon-intron junction of the human *DROSHA* gene itself (Fig. 2E) [59,77,78]. The *DROSHA* hairpin, likely derived from transposable elements, is structurally conserved among placental mammals but seems to be functionally divergent. For example, the human *DROSHA* hairpin is cleaved by DROSHA and tilts splicing of the adjacent exon toward skipping in a DROSHA-dependent manner, while the mouse *DROSHA* hairpin lacks these activities [77]. Interestingly, the suppressive effect of DROSHA on splicing of its own transcript does not require the catalytic activity of DROSHA, but it involves sterically hindering the splicing machinery from recognizing its cognate splice site, thereby expanding the repertoire of its mode of action (Fig. 3D) [77]. DROSHA can also act as a positive regulator of alternative splicing independently of its cleavage function (Figs. 2F & 3E) [79]. Although the underlying mechanism is poorly understood, documented interactions between the Microprocessor and spliceosome components may provide a clue [10,80,81].

Transcription termination and 3′-end processing is another process subject to DROSHA-mediated regulation (Fig. 3F). In the canonical pathway of Pol II transcriptional termination, endonucleolytic cleavage near a polyadenylation signal is followed by degradation of downstream cleavage products by the 5′-3′ exonuclease XRN2, and Pol II is released from the template when XRN2 catches up to it [82]. The very same exonuclease is recruited to the sites of DROSHA-mediated cleavage [71,83,84], suggesting that a similar 'torpedo-like' mechanism may be operational downstream of pri-miRNA processing. Indeed, abrogating pri-miRNA processing results in increased Pol II density at the downstream region and extensive transcriptional readthrough [83,85]. In this alternative pathway, DROSHA appears not only to provide an entry site for XRN2, but also to directly define the 3′ end of the transcript [85]. Interestingly, genome-wide analysis suggested that transcriptional termination by DROSHA is restricted to long non-coding RNAs (lncRNAs) that serve as pri-miRNAs [85]. It remains to be elucidated how miRNA hairpins within lncRNAs are distinguished from those embedded in protein-coding transcripts and are specifically destined for the transcription termination signals.

Maintenance of genome integrity and antiviral defense

The DNA-damage response (DDR) is a signaling pathway that has evolved to cope with various genotoxic stresses confronted by cells. In addition to a multitude of protein components comprising the cascade, a class of small RNAs distinct from miRNAs have been implicated in the DDR pathway in various organisms [86-89]. In mammals, proper formation of DDR foci following exogenous or oncogene-induced genotoxic insults requires small RNAs generated at sites of DNA damage in a DROSHA- and DICER-dependent manner, termed DNA-damage RNAs (DDRNAs) [87]. Similar to miRNAs, DDRNAs depend on AGO proteins to elicit their functions in DNA repair [88]. However, DDRNAs are distinguished from miRNAs by being independent of GW182-like proteins, the downstream effectors of miRNA-mediated post-transcriptional repression, and in their probable nuclear localization [87]. The mechanistic details of their biogenesis and functions await further investigation, and in particular, how DNA lesions occurring randomly throughout the genome are capable of providing adequate substrates for DROSHA remains to be established.

Another threat to the maintenance of genome stability is transposable elements. Although most transposable elements found in the human genome are currently inactive, a small fraction of retrotransposons still retain their activity and contribute to evolution and human diseases [90]. To prevent uncontrolled transposition of these elements, which would likely be detrimental to cells, several restriction mechanisms have evolved at multiple levels of gene expression. For example, a class of small RNAs, called PIWI-interacting RNAs (piRNAs), mediate transcriptional silencing of transposons in the germlines of mammals and insects [91]. The role for the Microprocessor as a post-transcriptional repressor of retrotransposons was hinted at by DGCR8 CLIP-seq, which revealed that around one third of identified binding sites correspond to repetitive elements, mainly LINE-1 and SINE (including Alu) retrotransposons [57]. Indeed, DROSHA negatively regulates LINE-1 and Alu retrotransposition by cleaving pri-miRNA-like hairpin structures embedded in their cognate transcripts (Figs. 2G & 3B) [92].

Accumulating evidence suggests that the protective functions of DROSHA extend to antiviral defense. DROSHA targets two pri-miRNA hairpins within the 3′-UTR of the mRNA for Kaposin B (KapB), a protein encoded by Kaposi's sarcoma-associated herpesvirus (KSHV), and decreased KapB expression mediated by DROSHA is associated with latent replication of the virus (Fig. 3B) [93]. Interestingly, DROSHA expression is reduced during lytic infection with concomitant derepression of KapB, suggesting that the virus may have evolved to utilize Microprocessor activity as a means of regulating its own life cycle. On the other hand, diverse RNA viruses including Sindbis virus (SINV) trigger translocation of DROSHA into the cytoplasm, where it cleaves viral genomic RNA [94,95]. Surprisingly, a recent study demonstrated that cytoplasmic DROSHA exerts its antiviral activity primarily through clamping viral RNA hairpins and conferring steric hindrance on the viral RNA-dependent RNA polymerases (RdRPs), rather than cleaving them (Fig. 3G) [96].

Concluding remarks

Extensive efforts over the past decade have greatly expanded the functional repertoire of DROSHA beyond nuclear pri-miRNA processing (Fig. 3A). From a molecular standpoint, DROSHA regulates diverse aspects of RNA metabolism across the transcriptome, ranging from post-transcriptional control of RNA stability (Fig. 3B) [49-52,57,59,65-68,92] to transcriptional activation (Fig. 3C) [73], alternative splicing (Fig. 3D & E) [57,75-77,79], and 3′-end processing and transcriptional termination (Fig. 3F) [83-85]. Meanwhile, at the cellular level, DROSHA defends against genotoxic stresses[87] and the expression of potentially deleterious elements such as retrotransposons (Fig. 3B) [92] and viruses (Fig. 3G) [93-96]. From a physiological stance, DROSHA ensures appropriate cell fate decisions in progenitor cells and allows their timely differentiation (Fig. 3B) [52,66-68]. Notably, multiple types of cancer exhibit aberrant expression of the Microprocessor components, and the resulting perturbation of the miRNA pool has been thought to contribute to tumorigenesis [6]. Further studies are needed to reveal whether dysregulation of other biological processes controlled by DROSHA plays a role in human diseases, and if so, to what extent.

The mechanistic repertoire of DROSHA has also been expanded, as illustrated by its recently-discovered cleavage-independent functions (Figs. 3C-E, G) [73,77,79,96]. Interestingly, cleavage-independent functions are not the exclusive property of DROSHA, and may represent a common regulatory paradigm for RNase III enzymes. The *Escherichia coli* RNase III has been reported to promote the translation of phage genes independently of its cleavage function [97]. Transcriptome-wide mapping of DICER-binding sites in human cells and *C. elegans* found that more than three quarters of identified loci are not associated with apparent small RNA production, and these 'passive sites' instead confer stability and granular localization on target RNAs [98]. Although the

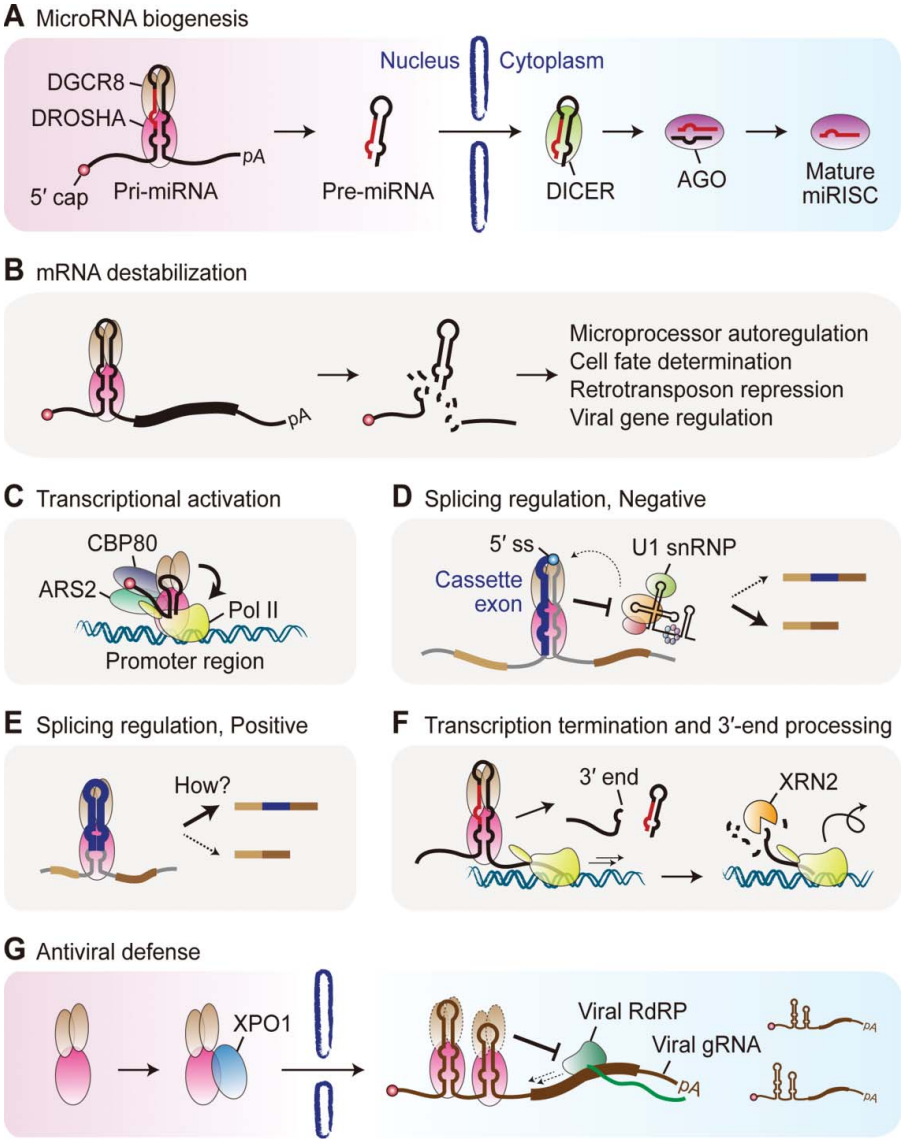

Figure 3. Emerging roles of DROSHA in RNA metabolism. (A) DROSHA functions as the initiator of miRNA biogenesis by cleaving pri-miRNA hairpins in the nucleus. The resulting pre-miRNAs are exported to the cytoplasm and further processed by DICER to produce mature miRNAs. (B) DROSHA destabilizes a subset of mRNAs by cleaving pri-miRNA-like hairpins, which is associated with various molecular and cellular outcomes including the homeostatic maintenance of Microprocessor activity, cell fate determination in progenitor cells, suppression of retrotransposons and viral gene regulation. (C) DROSHA is known to promote transcription of a subset of human genes, plausibly by binding to short hairpins within promoter-associated transcripts and interacting with RNA polymerase II (Pol II), CBP80 and ARS2. (D) DROSHA negatively regulates alternative splicing of its own transcript and other nascent transcripts whose splice sites overlap with pri-miRNA hairpins. For the *DROSHA* transcript, alternative splicing regulation by DROSHA primarily relies on its binding to a pri-miRNA-like hairpin spanning the 5′ splice site (5′ ss) and sterically blocking U1 small nuclear ribonucleoprotein (U1 snRNP). (E) DROSHA promotes splicing of a cassette exon of the *EIF4H* nascent transcript, which adopts a pri-miRNA-like hairpin structure, through an as-yet-uncharacterized, cleavage-independent mechanism. (F) DROSHA is involved in transcription termination and 3′-end processing of a subset of lncRNAs that serve as pri-miRNAs. DROSHA directly defines the 3′ ends of these transcripts and provides an entry site for XRN2, which is proposed to facilitate Pol II release from the template by a torpedo-like mechanism. (G) DROSHA serves as an antiviral effector against diverse positive-stranded RNA viruses by recognizing pri-miRNA-like hairpins in their genomic RNAs and conferring steric hindrance on the viral RNA-dependent RNA polymerase (RdRP). The antiviral activity of DROSHA is localized to the cytoplasm and this depends on XPO1-mediated nuclear export of DROSHA upon viral infection. It is not clear whether DGCR8 is co-exported and functions together with DROSHA in antiviral defense.

mechanistic details underlying cleavage-independent functions remain to be elucidated, DROSHA appears to employ at least two strategies: first, DROSHA may recruit positive regulators, possibly through its N-terminal region, to stimulate biological processes of interest such as transcription (Fig. 3C) [73] and splicing (Fig. 3E) [79] and second, DROSHA may bind to its substrates and catalyze little or no cleavage activity, but rather impede the access of other proteins to exert its inhibitory roles (Fig. 3D, G) [77,96].

The cytoplasmic functions of DROSHA are another important subject for future studies. Antiviral activity of DROSHA toward RNA viruses is localized to the cytoplasm [94-96], and this relies on Exportin-1 (XPO1)-mediated nuclear export of DROSHA upon viral infection (Fig. 3G) [95] Interestingly, a mutant version of DROSHA that fails to associate with DGCR8 suppresses viral replication as efficiently as wild-type DROSHA, suggesting the possibility of an alternative DROSHA complex in the cytoplasm [96]. In addition to viral entry, heat shock and oxidative stress are also known to cause cytoplasmic re-localization of DROSHA [30]. In fact, a small fraction of DROSHA protein is present in the cytoplasm even under normal conditions, due to alternative splicing [77,99,100], although

its biological role is largely unknown. It will be interesting to investigate whether cytoplasmic DROSHA has any distinct substrates, interacting partners, and functions.

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