miRNAs on the move: miRNA biogenesis and the RNAi machinery

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Recent advances have led to a more detailed understanding of RNA interference and its role in microRNA biogenesis and function. Primary microRNA transcripts are processed by the RNaseIII nuclease, Drosha, and are exported from the nucleus by Exportin-5. Dicer cleaves microRNAs into their mature forms, which can be incorporated into effector complexes that mediate gene silencing activities. The 3′ two-nucleotide overhang structure, a signature of RNaseIII cleavage, has been identified as a critical specificity determinant in targeting and maintaining small RNAs in the RNA interference pathway. MicroRNA functional analyses and genetic and biochemical interrogation of components of the pathway are starting to provide a glimpse at the range of biological processes and phenomena regulated by RNA interference.

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Abbreviations

dFMR1	*Drosophila* fragile X mental retardation protein
dsRNA	double-stranded RNA
ES	embryonic stem
FMRP	fragile X mental retardation protein
miRNA	micro-RNA
nt	nucleotide
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
RISC	RNA-induced silencing complex
RITS	RNA-induced initiation of gene transcriptional gene silencing
RNAi	RNA interference
siRNA	small interfering RNA
SMN	survival of motor neuron protein
VIG	Vasa intronic gene

Introduction

RNA interference (RNAi) is a gene regulatory pathway triggered in response to double-stranded RNA (dsRNA) [1]. Since it was first formally described in *Caenorhabditis elegans*, RNAi has been found to exist in many eukaryotic organisms, and to be involved in an extraordinary number of gene-silencing phenomena [1,2]. The RNAi machinery consists of a conserved core of factors with roles in recognizing, processing and effecting responses to dsRNA. Although a great variety of dsRNA triggers and responses have been described, the characteristic hallmark of the process is the cleavage of dsRNA into ~22nt dsRNAs by the nuclease Dicer, and the subsequent incorporation of these small dsRNAs into a complex containing members of the Argonaute family of proteins.

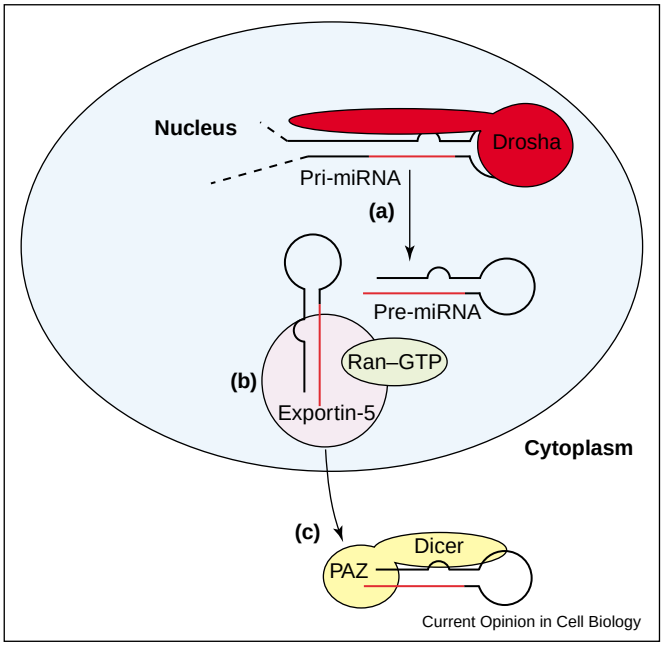
MicroRNAs (miRNAs) are a growing family of small non-protein-coding regulatory genes found in many eukaryotic organisms. miRNAs are processed via the RNAi machinery, and some have been shown to regulate the expression levels of homologous target-gene transcripts. miRNAs were first described in *C. elegans*; these are lin-4 and let-7, both of which target the 3′ untranslated regions (3′ UTRs) of developmental transcription factors and suppress their translation [3–6]. As more miRNA:target pairs were described, it became apparent that regulation of development might be a common theme in miRNA biology; miRNAs and their targets have been found to affect diverse processes, including flowering time and leaf patterning in *Arabidopsis*, neuronal asymmetry in *C. elegans*, and developmentally regulated cell proliferation in *Drosophila* [7–11]. Recently a number of algorithms have been described that aim to predict miRNA targets [12•–15•], and in mammals miRNAs are predicted to control a surprisingly wide variety of genes, thus impacting many aspects of biology [12•]. This review will focus on recent advances in our understanding of RNAi pathways and miRNA biogenesis.

miRNA production and trafficking

miRNA genes are often located in clusters that may be transcribed as polycistrons [16]. Although neither miRNA promoters nor the RNA polymerase responsible for miRNA transcription have been characterized, it is clear that miRNA genes are often under strict developmental-stage- and tissue-specific control [17•,18–21]. Many primary miRNA transcripts (pri-miRNAs) are predicted by computer algorithms to undergo folding into elaborate stem-loop structures [22–24,25••]. Cleavage of the stem-loops by the RNase III enzyme Drosha liberates ~70-nt precursor miRNAs (pre-miRNAs) [26••] (Figure 1a).

Drosha is one of three characterized RNaseIII family enzymes in the human and mouse genomes [27]. Initially described in *Drosophila*, Drosha mapped to a region containing microdeletions co-segregating with embryonic lethality [28]. The enzyme contains a proline-rich region, an arginine–serine-rich domain and two canonical C-terminal RNaseIII domains [29]. The RNaseIII domain structure of Drosha is similar to Dicer, but, unlike

Figure 1



A model for microRNA biogenesis, trafficking and assembly into RISC. **(a)** miRNAs are transcribed in the nucleus and pri-miRNAs are processed by Drosha into miRNA precursors, which have the two-nucleotide 3′ overhang characteristic of RNaseIII cleavage. **(b)** The two-nucleotide 3′ overhang end structure of the miRNA precursor is recognized by Exportin-5, a Ran–GTP-dependent nuclear export factor. The miRNA is transported into the cytoplasm. **(c)** The miRNA precursor is cleaved by Dicer, which probably uses the PAZ domain to specifically recognize and bind the two-nucleotide 3′ overhang. Dicer cleavage of the miRNA precursor liberates a ~22 nt mature miRNA. Having been processed by two RNaseIII enzymes, the miRNA now has symmetrical end structures.

Drosha, Dicer's second RNase III domain is thought to be partly inactive as it contains substitutions at critical active-site residues [30]. Drosha is found almost exclusively in the nucleus of HeLa cells, and appears to be concentrated in the nucleolus during S-phase. Initial functional characterization of Drosha suggested that the enzyme is required for preribosomal RNA processing, as Drosha knockdown using antisense RNA led to a substantial loss of HeLa cell viability with an accumulation of 12S and 32S precursor rRNAs [29]. Drosha emerged as a principle player in miRNA biogenesis when it was discovered that Drosha could cleave pri-miRNAs to pre-miRNAs in S2 cell extracts, and Drosha small interfering RNA (siRNA)-mediated deficiency in HeLa cells led to accumulation of pri-miRNA precursors [26••].

The mechanism by which Drosha establishes specificity for primary miRNA transcripts remains a subject for speculation at present, but it seems likely that, given the exquisite secondary structures predicted to be formed by miRNA precursors, Drosha may have some affinity for hairpin loops. The ribosomal precursors processed by Drosha also have characteristic secondary structures. Alternatively, Drosha may be recruited to miRNA transcripts via intermediate factors that specifically recognize pri-miRNA.

Drosha homologs can be found in the mouse, human, *C. elegans* and *Drosophila* genomes [27]; however, the notable absence of Drosha homologs from several RNAi-competent organisms, including *Schizosaccharomyces pombe* and *Arabidopsis*, suggests that Drosha may be a more recent addition to the RNAi pathway. In *Arabidopsis*, this is particularly puzzling, as pri-miRNAs must be processed to pre-miRNAs, presumably in the nucleus. However, it is possible that in plants Drosha's role is filled by one of the several Dicer homologs, some of which are nuclear [31]. The existence of miRNAs in *S. pombe* remains unproven, although a sequence motif has been described that defines a group of genes that are upregulated in RNAi mutants [32]. In this latter case, the absence of a Drosha enzyme could simply reflect the absence of an miRNA pathway altogether.

Pre-miRNAs of ~70nt that are produced by Drosha have the characteristic two-nucleotide 3′ overhang end structure left by the staggered cut of RNase III enzymes. Recent studies have shown that Exportin-5 mediates the nuclear export of pre-miRNAs in a Ran–GTP dependent manner [33•–35•]. Interestingly, Exportin-5-mediated nuclear export of another cargo RNA, the adenovirus VA1 noncoding RNA, also requires a two-to-three-nucleotide 3′ overhang [36], suggesting that the structural

determinates of Exportin-5 recognition may be implicit in Drosha processing (Figure 1b).

Dicer

Entry into the cytoplasm brings pre-miRNAs into contact with Dicer, a predominantly cytoplasmic enzyme [37–39]. Dicer cleaves pre-miRNAs into mature ~22mer miRNAs (Figure 1c); it can also cleave dsRNA into ~22mer siRNAs [3,40–43]. Dicer is a modular enzyme composed of two RNaseIII domains, a DExH/DEAH box RNA helicase domain and a PAZ domain as well as a domain of unknown function (DUF283) and a double-stranded RNA-binding motif [40].

The DExH/DEAH box family of ATP-dependent RNA helicases has a central and essential role in the RNAi machinery. The Dicer helicase domain itself is highly conserved in all Dicer enzymes [44], even though its ATP-binding domain has been found to be dispensable for the activity of recombinant mammalian Dicer *in vitro* [39,45]. Additionally, several accessory RNA helicases have been shown to be involved in RNAi in several organisms. For example, a conserved helicase has been found to interact with RDE-4, a double-stranded-RNA-binding protein essential for RNAi in *C. elegans* [46], and another worm RNA helicase, mut-14, is required for gene silencing induced by antisense oligos [47]. The *Drosophila* RNA helicase Spindle-E (homeless) is required for RNAi activation upon oocyte maturation [48]. Spindle-E is also essential for silencing retrotransposons, targeting heterochromatin to transgene arrays, and for silencing the stellate repeat locus in the testes [49–51]. In mammalian cells, Gemin3, an RNA helicase and component of 'gems', nuclear bodies enriched in SMN (survival of motor neuron protein), also copurifies with EIF2C2/Ago2, a human Argonaute homolog [25••]. In *Arabidopsis*, a putative helicase, SDE3, is required for long-range transport of RNA-mediated silencing triggers [52]. It has been suggested that the action of an RNA helicase may be two-fold: the classical view of a helicase is that it has unwinding activity and, in addition, it has been suggested that helicases could act as 'RNPases', remodeling the interactions between RNA and proteins [53]. The many processing steps that have been defined for miRNAs and other RNAs entering the RNAi pathway probably necessitate multiple reorganizations of ribonucleoprotein particles.

The PAZ domain is a highly conserved domain that is unique to Dicer enzymes and Argonaute proteins [54]. The structure of the PAZ domain was recently solved by X-ray crystallography and NMR, revealing a deviant OB-fold structure with a weak but consistent affinity for nucleic acids [55•–57•]. The PAZ domain has a particular specificity for single-stranded 3′ RNA ends, including the two-nucleotide 3′ overhang structure characteristic of RNaseIII processing. This suggests that a function for PAZ in Dicer could be to choose substrates that have been pre-processed by Drosha, thus allowing selection for pre-miRNAs [55•]. Interestingly, *S. pombe* Dicer does not have a PAZ domain — perhaps indicating that the *S. pombe* RNAi machinery uses a different mechanism for substrate specificity in the absence of miRNAs.

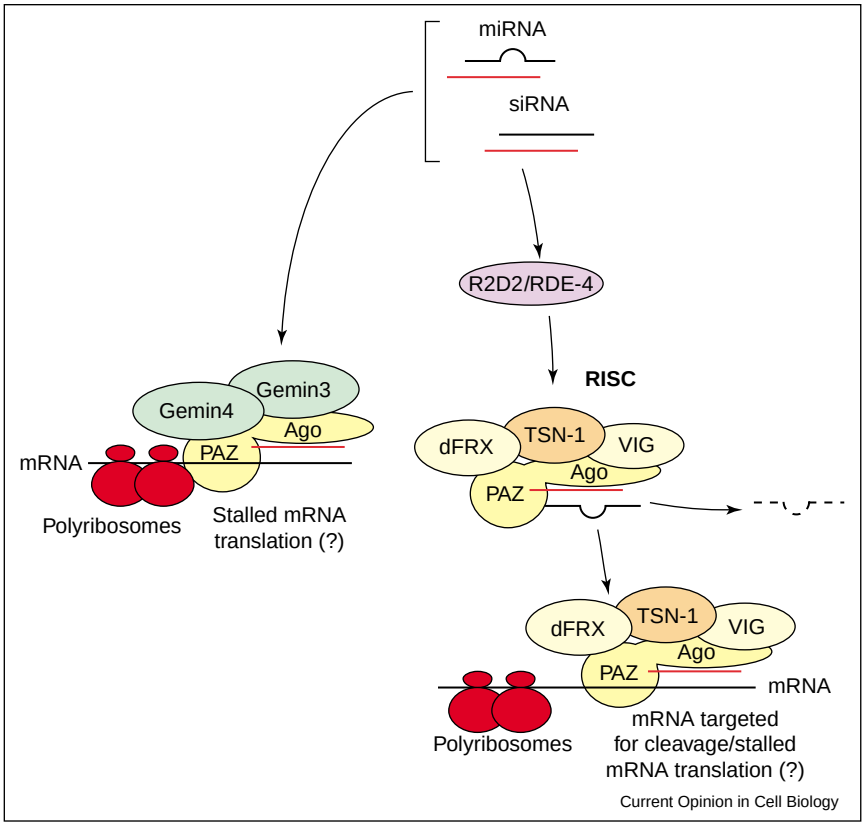
Effector complexes

Dicer cleavage must be followed by release of the mature miRNA or siRNA, and its incorporation into a RISC (RNA-induced silencing complex) or RITS (RNA-induced initiation of gene transcriptional gene silencing) effector complex whose diverse functions can include mRNA cleavage, translation suppression, transcriptional silencing and heterochromatin formation. The *C. elegans* double-stranded-RNA-binding protein RDE-4 and its *Drosophila* homolog R2D2 facilitate the transfer of siRNAs to RISC [46,58•] (Figure 2). There are also likely to be a number of additional components that aid in RISC assembly [59], particularly those that unwind siRNAs or miRNAs and through this action determine which strand of these small RNA duplexes becomes active for silencing [60••].

RISCs are ribonucleoprotein complexes that contain members of the PAZ–Piwi-domain Argonaute family of proteins, siRNAs or miRNAs, and miRNA/siRNA-complementary mRNAs (Figure 2). In addition, RISCs contain a number of accessory factors, some of which have activities necessary for effector function. Most organisms have a number of Argonaute family homologs — *Drosophila* has five, mouse has eight and worms have at least 24 [54]. It seems likely that these proteins give specificity to RISC and perhaps along with the other proteins and enzymes present in RISC determine the specific response to a particular miRNA or siRNA. The PAZ domain of Argonaute proteins is likely to directly engage siRNAs/miRNAs generated or released by Dicer [55•]; the PIWI domain was recently shown to interact directly with and inhibit the RNaseIII/dsRNA-binding domain region of Dicer [61•,62]. This was interpreted as a substrate transfer interaction, and it was speculated that PIWI interactions with Dicer may stimulate miRNA/siRNA release; furthermore, this interaction was found to take place in both the cytosolic and the membrane-associated fractions of 293T cells, consistent with previous reports that human Dicer colocalizes with calreticulin at the endoplasmic reticulum [39,61•].

A number of other factors have been found to associate with Argonaute proteins in RISC complexes. In *Drosophila* S2 cells, RISCs additionally contain Vasa intronic gene (VIG), a possible endonuclease known as TSN-1, and dFXR, a *Drosophila* homolog of fragile X mental retardation protein (FMRP) [63,64••,65]. A similar complex, containing Argonaute, TSN-1 and VIG homologs along with siRNAs, was detected in both *C. elegans*

Figure 2



RISC assembly. A 22 nt miRNA or siRNA is recognized by the PAZ domain of an Ago protein, and incorporated into RISC; R2D2/RDE-4 facilitates transfer of miRNAs or siRNAs into RISC. RISC components identified in the *Drosophila* S2 cell system include, besides Ago, TSN-1, VIG and dFRX. An additional complex has been described in mammalian cells, which contains miRNAs, Ago2 and Gemin3 and Gemin4. An early step in RISC maturation is the unwinding of the miRNA duplex into a single-stranded form. Depending upon its specific components, RISC may target homologous mRNA for cleavage, stall mRNA translation, perhaps in complex with polyribosomes, or induce chromatin modification and transcriptional gene silencing (this activity has only been directly observed in *S. pombe*).

extracts and mammalian cells [64••]. Mammalian Argonaute-containing complexes have been found to co-immunoprecipitate with SMN-interacting proteins Gemin3 and Gemin4 [25••] (Figure 2). A recent report has shown that human FMRP co-immunoprecipitates with EIF2C2/Ago2 and miRNAs in transformed human B-cells and that *Drosophila* Ago1 is required for FMRP function in neural development and synaptogenesis *in vivo* [66••]. This suggests that miRNAs may be required for FMRP function, and implicates RNAi in fragile X mental retardation syndrome.

The precise biochemical mechanisms whereby RISCs carry out their functions are unknown. Two well-characterized RISC activities are mRNA cleavage and translational suppression. The 'Slicer' activity that mediates messenger cleavage has not yet been identified, while a clue to the translational inhibition response may come from the tight association often found between RISC and polyribosomes [65,67,68]. It is tempting to speculate that RISC binds the polyribosome, forming a stable complex to stall further translation; however, in reality, the mechanism is likely to be much more subtle and intricate.

In *S. pombe*, Ago1, the only Argonaute homolog, is assembled into a RITS complex. RITS also contains Chp1, a chromodomain-containing centromere-binding protein required for methylation of histone H3 lysine 9 at the centromeric repeats, and Tas3, a protein of unknown function with a region of similarity to mouse protein OTT (ovaries and testes transcribed) [69••]. The RITS uses siRNAs derived from centromeric repeat transcripts to guide its localization at the centromeric repeats, where it induces heterochromatin formation [69••].

In *Drosophila*, the RNAi mutants Aubergine and Piwi (both members of the Argonaute family) and spindle-E (an RNA helicase) are required for heterochromatin formation at transgene arrays [51]. However, there is as yet no evidence for a RITS-like complex in *Drosophila*. Additionally, there have yet to emerge observations supporting the existence of a chromatin-regulatory arm of the

RNAi pathway in mammals. Although both strands of the centromeric satellite repeats in mouse are transcribed [70,71], no siRNAs derived from satellite repeats have been cloned from mammalian cells despite extensive efforts [17•,18,19,68,72]. It remains to be seen whether this is due to low abundance or an absence of centromeric siRNAs.

Turnover and transport

RISC is a multiple-turnover enzyme complex and, once incorporated, an siRNA or miRNA can direct multiple rounds of target cleavage [73], but whether RISC can act in a similarly catalytic manner in mediating translational suppression is not clear. One strand of the miRNA or siRNA duplex is preferentially incorporated into RISC in a manner which is dependent upon the thermodynamics of the duplex [60••,74]. It is therefore unlikely that siRNAs and miRNAs are recycled if released from RISC. Thus miRNP or siRNP stability defines a limiting step in the persistence of the RNAi response. Recently, a potential siRNA turnover enzyme was isolated in a genetic screen for enhancers of RNAi in worms [75••]. Called eri-1, this enzyme is related to the RnaseT family of 3′–5′ exonucleases, having specificity for 3′ overhangs [75••]. This led the authors to suggest that eri-1 destabilizes siRNAs by removing their 3′ overhangs, perhaps rendering them 'invisible' to RISC.

It is clear that siRNAs and miRNAs are not confined by the cell membrane. It has long been known that systemic RNAi in *C. elegans* and plants leads to an organismal silencing effect by intercellular transport of siRNA and dsRNA, and recent work has described the *C. elegans* transporter, SID-1, which specifically transports dsRNA into cells [76•]. Interestingly, one recent report has raised the possibility that a miRNA may act as a movable signal, reminiscent of a morphogen or hormone, to specify leaf cell identity in maize [77••]. It will be interesting to learn if such role has been conserved in other systems.

Conclusions

Recent advances in the areas of miRNA biogenesis and the identification of new interactions and players in the core RNAi machinery have broadened our mechanistic and biological understanding of the RNAi pathway. Future work will highlight the scope and function of miRNA and RNAi biology by confirming more miRNA:-target pairs, identifying more players and mechanistic details, and determining the scope and limitations of RNAi in different model systems.

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