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Turnover of Mature miRNAs and siRNAs in Plants and Algae

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Abstract

microRNAs (miRNAs) and small interfering RNAs (siRNAs) play important roles in gene regulation and defense responses against transposons and viruses in eukaryotes. These small RNAs generally trigger the silencing of cognate sequences through a variety of mechanisms, including RNA degradation, translational inhibition, and transcriptional repression. In the past few years, the synthesis and the mode of action of miRNAs and siRNAs have attracted great attention. However, relatively little is known about mechanisms of quality control during small RNA biogenesis as well as those that regulate mature small RNA stability. Recent studies in *Arabidopsis thaliana* and *Caenorhabditis elegans* have implicated 3'-to-5' (SDNs) and 5'-to-3' (XRN-2) exoribonucleases in mature miRNA turnover and the modulation of small RNA levels and activity. In the green alga *Chlamydomonas reinhardtii*, a nucleotidyltransferase (MUT68) and an exosome subunit (RRP6) are involved in the 3' untemplated uridylation and the degradation of miRNAs and siRNAs. The latter enzymes appear to function as a quality control mechanism to eliminate putative dysfunctional or damaged small RNA molecules. Several post-transcriptional modifications of miRNAs and siRNAs, such as 3' terminal methylation and untemplated nucleotide additions, have also been reported to affect small RNA stability. These collective findings are beginning to uncover a new layer of regulatory control in the pathways involving small RNAs. We anticipate that understanding the mechanisms of mature miRNA and siRNA turnover will have direct implications for fundamental biology as well as for applications of RNA interference technology.

Introduction

RNA-mediated silencing is an evolutionarily conserved mechanism(s) by which small RNAs (sRNAs) induce the inactivation of cognate sequences.^{1–7} However, recent results indicate that these noncoding RNAs may also participate in transcriptional or translational activation.^{2,8} The regulation of gene expression by sRNAs, ~20–30 nucleotides in length,

plays an essential role in developmental pathways, metabolic processes and defense responses against viruses and transposons in many eukaryotes.¹⁻⁸ In plants and some algae, at least two major classes of small RNAs have been identified based on the molecules that trigger their production: microRNAs (miRNAs) and small interfering RNAs (siRNAs).^{3-7,9-12} miRNAs originate from single-stranded noncoding RNA transcripts or introns that fold into imperfect stem-loop structures and often modulate the expression of genes with roles in development, physiological processes, or stress responses.⁴⁻⁷ siRNAs are produced from long, near-perfect complementarity double-stranded RNAs (dsRNAs) of diverse origins, including transcripts of long inverted repeats, products of convergent transcription or RNA-dependent RNA polymerase activity, viral and transposon RNAs, or dsRNAs experimentally introduced into cells.⁴⁻⁷ In higher plants the siRNA population includes natural antisense transcript siRNAs (nat-siRNAs), trans-acting siRNAs (ta-siRNAs), heterochromatic siRNAs (hc-siRNAs), several other endogenous siRNAs (endo-siRNAs) as well as those derived from invading viral or transgene transcripts.^{5-7,13} These siRNAs play various roles in post-transcriptional regulation of gene expression, suppression of viruses and transposable elements, and/or DNA methylation and heterochromatin formation.^{3-7,13} However, there is a growing realization that, despite their differences, distinct small RNA pathways often interact, competing for and sharing substrates and effector proteins, and cross-regulating each other.

Hairpin and long dsRNAs are processed into small RNAs by an RNase III-like endonuclease named Dicer.^{1,2,5,6} The short RNA duplexes produced by Dicer are incorporated into multisubunit effector complexes, such as the RNA-induced silencing complex (RISC)^{1,2,5,6} Argonaute proteins, which include two main subfamilies of polypeptides named after *Arabidopsis thaliana* ARGONAUTE1 (AGO1) and *Drosophila melanogaster* PIWI, are core components of the RISC and some function as siRNA-guided endonucleases.^{1-6,14,15} Recent evidence suggests that an siRNA duplex is first loaded into RISC and then AGO cleaves one of the siRNA strands (the passenger strand) triggering its dissociation from the complex.^{1,2} Similarly, miRNA duplexes are loaded onto AGO and rapidly unwound by a poorly characterized mechanism.^{2,16} Activated RISC then uses the remaining single-stranded small RNA as a guide to identify homologous RNAs, ultimately triggering transcript degradation and/or translational repression.¹⁻⁶ sRNAs associated with certain AGOs can also direct cytosine DNA methylation and/or chromatin modifications^{4-7,13} and RISC complexes often contain auxiliary proteins that extend or modify their function(s).^{1,2,8}

The biogenesis and the mode of action of sRNAs have attracted great attention,^{1-8,17} but relatively little is known about mechanisms of mature miRNA/siRNA turnover and their role(s) in small RNA function. The accumulation of other cellular RNAs is dependent on the rates of transcription, processing, and also decay. For instance, messenger RNA degradation is now known to contribute significantly to the post-transcriptional regulation of gene expression and as a quality control mechanism to prevent the expression of inappropriate RNAs.^{18,19} By analogy, active small RNA turnover may conceivably modulate the levels of mature miRNAs/siRNAs and/or eliminate defective sRNA molecules. Here we examine the as yet relatively scant evidence on the mechanisms of small RNA degradation and their biological roles, with a specific focus on plants and algae. Along the way, we

briefly review the biogenesis of miRNAs/siRNAs and seek to delineate the current knowns and the many unknowns in the field of small RNA turnover.

Small RNA Processing

Most characterized eukaryotic miRNA genes correspond to RNA polymerase II transcription units, either in intergenic regions or embedded in introns of protein-coding genes that produce a primary miRNA transcript (pri-miRNA)^{1,2,5,7} This pri-miRNA typically forms an imperfect fold-back structure, which is processed into a short stem-loop precursor miRNA (pre-miRNA). In metazoans, this step is catalyzed by the nuclear microprocessor complex that includes as core components an RNase III enzyme (Drosha) and a double-stranded RNA-binding protein.^{1,2,5,6,17} Pre-miRNAs are then exported to the cytoplasm by the karyopherin Exportin 5²⁰ and further processed in the cytosol by Dicer to generate mature miRNAs.^{1,2,5,6,17} Dicer cleavage produces a short duplex containing two strands, named miRNA (equivalent to the guide strand) and miRNA* (the complementary, passenger strand).^{1,2,5,6,17} In plants, which lack Drosha-like enzymes, both pri-miRNA-to-pre-miRNA conversion and pre-miRNA-to-duplex-miRNA/miRNA* processing are carried out by Dicer-like proteins (Fig. 1).^{1,5,6,13} In *Arabidopsis* these steps are largely dependent on the activity of the nuclear DICER LIKE 1 (DCL1).^{5,6,21–23} However, higher plants and the green alga *Chlamydomonas reinhardtii* also have additional DCL proteins that are mostly responsible for the processing of a multitude of siRNAs from long dsRNAs, although they may also be involved in the making of some miRNAs.^{4–7,11}

In metazoans, the biogenesis of certain miRNAs is regulated at the level of microprocessor- and/or Dicer-mediated processing, as demonstrated by the identification of RNA-binding proteins such as Lin-28, hnRNP A1 and KSRP that can either prevent or promote the conversion of specific pri-/pre-miRNAs to mature miRNAs (see chapters by Lehrbach and Miska, Michlewski et al., and Trabucchi et al., respectively).^{17,24–26} Recently, the estrogen receptor α has also been implicated in inhibiting the processing of a subset of miRNAs that depend on the microprocessor-associated DEAD box helicases p68 and p72 for their biogenesis (see chapter by Fujiyama-Nakamura et al.).²⁷ In addition, *Caenorhabditis elegans* and mammalian Lin-28, besides its role in pri-miRNA processing, can also bind the precursor of the let-7 miRNA in the cytoplasm and stimulate its 3' end uridylation by a poly(U) polymerase, leading to precursor RNA degradation and downregulation of the mature let-7 miRNA levels (see chapter by Lehrbach and Miska).^{28–30} In contrast to this wealth of information, to our knowledge, there is as yet no experimental evidence supporting miRNA-specific regulation at the processing steps in plants or algae. However, discrepancies between pri-/pre-miRNA and mature miRNA levels in northern blot analyses of certain miRNAs suggest that post-transcriptional mechanisms affecting miRNA accumulation are also likely to exist in plants.^{5,31}

Small RNA Modification by 2'-O-Methylation

In plants, mature miRNAs and siRNAs are methylated at their 3' ends, a modification dependent on the RNA methyltransferase HUA ENHANCER 1 (HEN1).^{5,6,13,32} This is also

likely to occur in the alga *C. reinhardtii*, as suggested by the resistance of its small RNAs to periodate oxidation/ β elimination reactions.^{9,33} In vitro studies with recombinant HEN1 strongly suggest that the *Arabidopsis* protein prefers as substrates small RNA duplexes with 2-nt overhangs at their 3' ends, typical features of Dicer products.^{5,6,13,34,35} Thus, after DCL proteins catalyze the release of miRNA/miRNA* or siRNA duplexes from their precursors, it has been proposed that HEN1 methylates each strand of the duplex on the 2'-OH of their 3'-terminal ribose molecules.^{6,13,34} Interestingly, a HEN1-YFP fusion protein has been detected in both the nucleus and the cytosol in transgenic *Arabidopsis* lines²¹ and several viral RNA silencing suppressors, that appear to function in the cytoplasm, partly inhibit miRNA methylation.³⁶ Thus, it seems likely that HEN1-catalyzed reactions can occur in the nucleus as well as in the cytosol of plant cells (Fig. 1), although this has not been formally demonstrated.

Figure 1. General model of miRNA biogenesis and RISC loading in plants and some algae. Primary miRNA transcripts (pri-miRNAs), mostly generated by RNA polymerase II, are processed into hairpin precursor miRNAs (pre-miRNAs) by Dicer-like enzymes (DCL). These pre-miRNAs are further processed by DCL into short miRNA/miRNA* duplexes (miRNA duplexes). Mature miRNA duplexes are then methylated at the 3' end of each strand by HEN1. Some miRNA/miRNA* or methylated miRNA/miRNA* duplexes are likely exported to the cytoplasm by HASTY, the plant homolog of Exportin 5. Guide miRNA strands are eventually loaded, either in the nucleus or the cytosol, into effector complexes containing Argonautes (AGOs). Commonly accepted pathways in higher plants are indicated with solid lines whereas potential alternative pathways, currently lacking direct experimental evidence, are indicated with dashed lines (see text for details).

In metazoans, PIWI-interacting RNAs (piRNAs), a class of small RNAs specifically bound by PIWI proteins and absent in plants, as well as several siRNAs also have a 2'-O-methyl group on their 3' termini.³⁷⁻⁴⁰ In flies, miRNA*s associated with AGO2 have also been found to be 3'-modified.⁴¹⁻⁴³ In contrast, animal miRNAs do not appear to be methylated.^{1,37-40} Moreover, the animal homologs of HEN1 lack a dsRNA-binding domain and appear to act on single-stranded, mature small RNAs already associated with AGO or PIWI proteins.³⁸⁻⁴⁰ Indeed, the substrate specificity of HEN1 homologs in metazoans may reflect the fact that these proteins interact only with certain Argonaute polypeptides. Whether plant HEN1 could also methylate some single-stranded small RNAs already bound to AGOs is presently unknown (Fig. 1, dashed lines pathway). In both animals and plants, the methylation of small RNAs seems to protect them against untemplated nucleotide additions, such as uridylation and/or exonucleolytic shortening.^{6,13,38,39,44} Likewise, in the ciliated protozoan *Tetrahymena thermophila*, ~28-29 nt long sRNAs, which are expressed during sexual reproduction and required for DNA elimination, are selectively stabilized by 3'-terminal 2'-O-methylation.⁴⁵

Small RNA Loading and Activation of the RNA-Induced Silencing Complex

In metazoans, siRNAs seem to be loaded onto RISC as duplexes and then AGO cleaves the passenger strands triggering their dissociation from the complex and the concomitant maturation of RISC.^{1,2,17,46} Ribonucleases, such as C3PO (whose subunits Translin and Translin-associated factor X have homologs in plants), promote RISC activation by removing the passenger strand cleavage products.⁴⁶ Likewise, miRNA/miRNA* duplexes, which often contain mismatches or bulges, are loaded onto AGO, and the two strands are separated by a poorly defined "slicer-independent" mechanism.^{2,16} However, recent evidence suggests that AGO proteins themselves can function as RNA chaperones capable of unwinding small RNA duplexes.^{16,47} The dissociated miRNA* strands appear to be rapidly degraded, but the enzyme(s) involved in this process is presently unknown (Fig. 2). In either case, no single-stranded guide siRNA or miRNA appears to be produced prior to these RISC maturation steps.^{2,38,48}

In several metazoans, the relative thermodynamic stability of the 5' ends of the strands in a small duplex RNA, in some cases sensed by dsRNA-binding proteins partnering with Dicer in a RISC-loading complex, determines which strand is chosen as the guide siRNA or miRNA.^{2,16,41,49,50,51} In addition, in *Drosophila*, sorting of small RNA duplexes into specific AGO paralogs appears to be governed by the structure of the duplex, whether it is nearly perfectly double-stranded or contains central bulges and mismatches.^{16,41,43} As reported in plants, the identity of the first nucleotide of a small RNA may also play a role in this sorting process.^{41,43} However, the extent to which these factors weigh in the fate of specific small RNAs, in particular in different metazoans, is not clear as yet.^{52,53} Moreover, it has been commonly accepted that the passenger and miRNA* strands are simply byproducts of siRNA/miRNA biogenesis and RISC loading, destined to be degraded. Yet, recent evidence in flies suggests that certain miRNA/miRNA* duplexes could be bifunctional, with each

strand being independently sorted into different AGO proteins and most miRNA*s detected in cells appear to represent those associated with Argonaute proteins rather than undegraded discarded strands.^{41–43}

Figure 2. Proposed model for the turnover of mature miRNAs based on combined evidence from plants, algae, and metazoans. An miRNA/miRNA* duplex is loaded into AGO, and the two strands are separated by a poorly characterized mechanism. The unwound miRNA* strand is rapidly eliminated by an unknown enzyme(s). In *C. reinhardtii*, MUT68 (a terminal nucleotidyltransferase) and RRP6 (a 3'-to-5' exoribonuclease) may be part of a quality control mechanism to eliminate dysfunctional or damaged miRNAs that are loaded into Argonautes, in kinetic competition with the methyltransferase HEN1 (see text for details). Both *C. elegans* XRN-2 (a 5'-to-3' exoribonuclease) and *A. thaliana* SDN enzymes (3'-to-5' exoribonucleases) may contribute to the decay of mature overall RNAs dissociated from Argonautes (see text for details). For simplicity, the AGO-bound miRNA strand is shown methylated, as it occurs in plants and some algae, but *C. elegans* miRNAs would not undergo this modification.

Much less is known about RISC assembly in plants and algae, and elucidating this process is complicated by the existence of many Argonaute paralogs in a given organism^{4–7,11,13,54}. In *A. thaliana*, which contains ten AGOs, some heterochromatic and repetitive siRNAs are loaded into AGO4-containing complexes, likely in the nucleus.^{5,13,55–57} In contrast, most miRNAs appear to become associated with AGO1.^{5,6,54,56–58} At least part of the sorting into different *Arabidopsis* AGOs seems to be determined by the identity of the 5' nucleotide of the small RNAs.^{56,57,59} For instance, AGO1 predominantly associates with small RNAs with a uridine at the 5' terminus, which most miRNAs possess, whereas AGO4 prefers an adenine as the 5'-terminal nucleotide.^{5,56,57} Additionally, the asymmetric thermodynamic stability of the miRNA/miRNA* duplex termini also appears to play a role in miRNA strand selection in plants, but these rules do not seem to apply to at least some siRNAs.⁵¹

The subcellular location of miRNA loading into AGOs remains elusive in plants. HASTY (HST), the plant homolog of Exportin 5, is thought to transport miRNA/miRNA* or methylated miRNA/miRNA* duplexes to the cytoplasm^{5,6,60} for assembly into AGO complexes (Fig. 1). However, the role of HST is not as clear as that of Exportin 5 in animals since *Arabidopsis hasty* mutants show decreased accumulation of only a subset of miRNAs.^{5,60}

Moreover, in plants, miRNA abundance is higher than that of the corresponding miRNA* in both the cytoplasm and the nucleus, suggesting that mature, RISC-associated miRNAs are present in both compartments.⁶⁰ Interestingly, *Arabidopsis* HYPONASTIC LEAVES 1/DsRNA-BINDING PROTEIN 1 (HYL1/DRB1), a dsRNA-binding protein that cooperates with DCL1 in the processing of pri-/pre-miRNAs to mature miRNAs,⁶¹ influences miRNA strand selection, presumably in a similar way as related polypeptides in some metazoan RISC loading complexes.⁵¹ Since *Arabidopsis* HYL1/DRB1 is mainly localized in the nucleus^{21,22} and a YFP-AGO1 fusion protein is present in both the cytosol and the nucleus,²¹ at least a subset of AGO1 molecules may interact with HYL1/DRB1 and be loaded with miRNA/miRNA* or methylated miRNA/miRNA* duplexes in the nuclear compartment (Fig. 1). Recent findings in mammalian cells also indicate that Argonaute proteins and associated miRNAs can shuttle between the nucleus and the cytoplasm and that their transport depends on the import receptor Importin 8 and the karyopherin CRM1.^{62,63}

Another unresolved issue in plant RISC assembly is the exact role of the methyltransferase HEN1, proposed to act on short dsRNA substrates after DCL-mediated processing but prior to RISC loading.^{6,13,34} For instance, small RNA duplexes generated by DCL could be released, methylated by HEN1 and then rebound by metazoan-like RISC-loading complexes that associate with Argonautes.⁵¹ Alternatively, HEN1 could be an integral component of plant RISC-loading complexes and participate actively in the transfer of small RNAs to AGOs.

Mature Small RNA Degradation by Ribonucleases

Relatively little is known about the stability of endogenous small RNAs and the enzymes involved in their turnover in most eukaryotes. A conserved nuclease from *C. elegans* and *Schizosaccharomyces pombe*, ERI-1 (of which there are also six putative homologs in *Arabidopsis*),⁶⁴ degrades siRNA duplexes with 2-nucleotide 3'-overhangs in vitro and reduces the efficiency of RNAi in vivo.^{65,66} However, its role in small RNA turnover is not clear since, in nematodes, ERI-1 has recently been implicated in 5.8S rRNA processing and in the biogenesis of certain endo-siRNAs.^{67,68} In contrast, in *C. elegans*, the 5'-to-3' exoribonuclease XRN-2 (related to the yeast Rat1 enzyme) is involved in the degradation of mature, single-stranded miRNAs (Fig. 2) and has been shown to modulate miRNA accumulation in vivo (see chapter by Grosshans and Chatterjee).⁶⁹

In *Arabidopsis*, the existence of ribonucleases targeting siRNAs/miRNAs and the protective role of the 3'-terminal 2'-O-methyl group was recognized from analyses of small RNAs in mutants lacking sRNA methyltransferase activity.^{6,13,32,44} In *hen1* mutants, miRNAs and siRNAs fail to accumulate or their levels are considerably reduced.^{13,44} In addition, miRNA cloning and sequencing revealed the presence of 3' end-truncated miRNA molecules as well as others with untemplated 3'-terminal nucleotides, predominantly uridine residues.^{13,44} These observations indicated that methylation protects small RNAs from uridylation and degradation and, by analogy to the mechanism of decay of longer transcripts such as human histone mRNAs,^{70,71} led to the proposal that uridylation recruits and/or stimulates an exonuclease to degrade miRNAs.^{6,13} Interestingly, a family of 3'-to-5' exoribonucleases (re-

lated to the yeast Rex exonucleases) encoded by the *SMALL RNA DEGRADING NUCLEASE* (SDN) genes was recently implicated in the turnover of single-stranded, mature sRNA in *Arabidopsis*⁶⁴ (Fig. 2). However, the enzymes involved in untemplated nucleotide additions to the 3' ends of mature sRNAs and in the proposed 3'-to-5' degradation of unmethylated and uridylylated small RNAs remain unknown since SDN1 is inhibited by 3' terminal uridylation while it still acts, albeit with somewhat lower efficiency, on 2'-O-methylated sRNAs.⁶⁴

Recent studies with in vitro systems (either cell extracts or recombinant proteins) demonstrated that single-stranded, guide, small RNAs can be dissociated from Argonaute proteins.^{47,69} In *C. elegans* extracts, this process is partly dependent on XRN-2 and appears to be inhibited by interaction of the miRNA-AGO complex with a target RNA.⁶⁹ If confirmed in vivo, this mechanism could provide a way to recycle AGO proteins associated with sRNAs that lack a target transcript, allowing them to rebind to other guide siRNAs/miRNAs. Nevertheless, current evidence is most consistent with both *C. elegans* XRN-2⁶⁹ and *Arabidopsis* SDN enzymes⁶⁴ participating in the decay of mature small RNAs dissociated from Argonautes (Fig. 2). Moreover, since homologs of these proteins are widely distributed among eukaryotes,^{64,69,72} these pathways might be evolutionarily conserved; although partly redundant, multiple paralogs may complicate the detection of phenotypic defects in individual mutants or epi-mutants.^{64,69} Alternatively, the prevalence of 5'-to-3' versus 3'-to-5' degradation of dissociated mature small RNAs may vary in different organisms since *C. elegans* homologs of *Arabidopsis* SDN1 do not appear, individually, to be required for miRNA turnover⁶⁹ and the *Arabidopsis* XRN-2 homologs XRN2 and XRN3 seem to degrade the loop sequence of miRNA precursors without affecting mature miRNA levels.⁷³

A mutant in the green alga *Chlamydomonas reinhardtii* (Mut-68) also provided insight on the pathways of mature miRNA/siRNA degradation. Mut-68, which is deleted for a gene encoding a terminal nucleotidyltransferase named MUT68, was initially characterized as being deficient in the addition of untemplated nucleotides to the 5' RNA fragments produced by the RISC cleavage of target transcripts, a requirement for their efficient decay.⁷⁴ In addition, Mut-68 showed elevated levels of miRNAs and siRNAs and the MUT68 enzyme was found to play a role in the untemplated uridylation of the 3' termini of sRNAs in *Chlamydomonas*.³³ High throughput sequencing of small RNAs revealed that ~7.3% of the examined molecules had 3'-untemplated nucleotides in the wild type strain, but this fraction was reduced to ~4.9% in Mut-68. Moreover, sRNAs displayed markedly lower uridylation, the predominant addition to the 3' ends of miRNAs/siRNAs, in the mutant and, consistent with the possibility that U-tailed RNAs may be degradation intermediates, their average size was smaller than that of the sRNAs in the entire population.³³

The MUT68 activity stimulated in vitro the degradation of single-stranded small RNAs by RRP6,³³ a peripheral component of a 3'-to-5' multisubunit exoribonuclease, the exosome.^{75,76} Moreover, like the defect in MUT68, RNAi-mediated depletion of RRP6 in *Chlamydomonas* resulted in the accumulation of miRNAs and siRNAs in vivo.³³ RRP6, which is related to bacterial RNase D, is widely distributed in eukaryotes and acts as a distributive 3'-to-5' hydrolytic exonuclease that prefers unstructured substrates.^{75,77} As proposed before, it seems likely that, in *Chlamydomonas*, uridylation by MUT68 creates a short unstructured 3' end that facilitates small RNA degradation by the RRP6 enzyme (Fig. 2). Several

cycles of uridylation and truncation may be required for complete sRNA decay by this nonprocessive exoribonuclease. Interestingly, MUT68 appears to collaborate with RRP6 in the turnover of miRNAs/siRNAs³³ and with the core exosome in the degradation of longer RNAs generated by RISC cleavage.⁷⁴ Additionally, MUT68 seems to carry out preferentially uridylation of small RNAs³³ and adenylation of RISC-cleaved transcripts.⁷⁴ The basis for this differential specificity is presently unclear but nucleotidyltransferases with context-dependent nucleotide preferences have been previously described.^{70,78,79} Furthermore, in respect to sRNA degradation, 3'-terminal adenylation, unlike uridylation, has recently been proposed to lead to stabilization of miRNAs. The poly(A) polymerase GLD-2 adds a single adenine residue to the 3' end of mammalian miR-122, and this modification appears to stabilize selectively this particular miRNA in liver cells.⁸⁰ Untemplated adenylation of miRNAs has also been observed in plants and algae,^{33,44,81} and it also seems to protect small RNAs against degradation in an in vitro assay with *Populus trichocarpa* (black cottonwood) cell extracts.⁸¹

Both MUT68 and RRP6 are only active in vitro on small RNAs lacking a 3'-terminal 2'-O-methyl group.³³ Thus, homologs of MUT68 and RRP6 may conceivably be responsible for the observed uridylation and decay of small RNAs lacking 3' methylation in the *Arabidopsis hen1* mutants. However, defining the role(s) of MUT68 and RRP6 in *Arabidopsis* may be complicated by the fact that both proteins are encoded by small multigene families.^{74,82} More importantly, how these enzymes function in a wild-type background, where most miRNAs and siRNAs are methylated, is less obvious. As discussed in the next section, we have proposed³³ that, at least in *Chlamydomonas*, MUT68 and RRP6 may be part of a quality control mechanism to eliminate dysfunctional or damaged small RNAs associated with Argonautes (Fig. 3).

Quality Control of Mature Small RNAs

The *Chlamydomonas* Mut-68 mutant was originally identified as being deficient in RNAi,⁷⁴ and the RRP6-depleted strains also shows diminished RNAi activity. However, since Mut-68 contains enhanced levels of mature, single-stranded miRNAs and siRNAs, which correlate with higher amounts of an endogenous AGO protein, RISC assembly appears to occur normally.³³ As already mentioned, no single-stranded siRNA or miRNA appears to be produced prior to RISC maturation^{2,38,48} and, thus, the accumulated mature sRNAs detected in *Chlamydomonas* Mut-68 likely correspond to those associated with Argonautes. Yet, the function of a significant fraction of these RISC complexes may be compromised if the associated guide sRNAs are dysfunctional, inert, and/or damaged, resulting in the sequestration of AGO proteins into inactive complexes (Fig. 3). This interpretation for the diminished RNAi activity in Mut-68 (and in the RRP6-depleted strains) is consistent with a role for MUT68/RRP6 as a quality control mechanism for the removal of functionally defective sRNAs in *Chlamydomonas* (Fig. 3). Moreover, this process may be operative in other eukaryotes since a recent RNAi screen to identify genes involved in miRNA/siRNA pathways in *D. melanogaster* revealed that depletion of an RRP6 homolog resulted in an RNAi defect.⁸³ In addition, the *C. elegans* nucleotidyltransferase CDE-1 is required for the uridylation of siRNAs bound to a specific Argonaute protein (CSR-1) and in the absence of

CDE-1 these siRNAs accumulate to inappropriate levels, accompanied by defects in an RNAi pathway involved in chromosome segregation.⁸⁴

Figure 3. Proposed model for the role of MUT68 and RRP6 in the quality control of mature small RNAs in *Chlamydomonas reinhardtii*. Slight errors during Dicer-mediated processing and/or cleavage by alternative Dicer paralogs result in 5' nucleotide variants of at least some miRNAs and endo-siRNAs.³³ In the wild type strain, one sRNA isoform usually predominates and is presumably associated with an active RISC (1). However, in the Mut-68 mutant alternative, isoforms can also become quite noticeable,³³ suggesting that their decay depends on the MUT68 nucleotidyltransferase activity. Certain 5' nucleotide variants may be subfunctional or dysfunctional when associated with a particular Argonaute protein, for instance because of an unsuitable 5' terminal nucleotide (2) and/or a tendency to form intramolecular secondary structures (3) that will hinder the recognition of target RNAs. In the absence of the MUT68/RRP6 machinery, these small RNA variants can accumulate, conceivably sequestering AGO proteins into inactive RISC complexes. Additionally, some 5' nucleotide isoforms of miRNAs/siRNAs may be loaded into different AGO paralogs (4). In strains depleted for MUT68 and/or RRP6, these small RNAs may compete out those commonly associated with these Argonaute proteins, rendering the complexes functionally inert or leading to altered regulatory outcomes. Since small RNA processing isoforms have different seed sequences, if assembled into functional complexes, they could potentially affect the expression of distinct repertoires of target RNAs. For simplicity, MUT68 and RRP6 are shown degrading RISC-associated small RNAs, but this has not been directly demonstrated as yet.

Recent evidence suggests that RISC-bound small RNAs can be subfunctional. For instance, changing the 5' uracil residue of the let-7a miRNA did not affect the formation of a complex with human AGO2 but reduced significantly the association of this complex with a target mRNA.⁸⁵ In *Arabidopsis*, a uridine-to-adenosine change at the 5' end of engineered miRNAs resulted in an AGO1-to-AGO2 switch in sRNA loading and abolished their silencing activity.⁵⁶ In plants and some algae, slight errors during DCL processing and/or cleavage by alternative DCL paralogs may result in 5' nucleotide variants of miRNAs/siRNAs

that could be assembled into the wrong AGO isoform and have drastically altered regulatory outcomes,^{5,86} including rendering the miRNA/siRNA functionally inert and sequestering Argonaute proteins into ineffective complexes. Inaccuracies by RISC-loading complexes may also lead to the association of small RNAs with an incorrect AGO paralog. Thus, a quality control mechanism(s) may be required to eliminate AGO-bound dysfunctional or subfunctional small RNAs and MUT68 and RRP6 may participate in such a pathway (Fig. 3). We have not demonstrated directly that MUT68 and RRP6 act on Argonaute-associated small RNAs but, in *C. elegans*, uridylylated siRNAs are immunoprecipitated with the CSR-1 AGO.⁸⁴ In addition, both *Chlamydomonas* Mut-68 and CDE-1-defective *C. elegans* are deficient in RNA interference pathways, suggesting that, in these mutants, the accumulated small RNAs hinder RISC activity.^{33,84} In contrast this phenotype has not been reported upon depletion of *C. elegans* XRN-2 or *Arabidopsis* SDNs, implicated in the turnover of small RNAs dissociated from Argonautes.^{64,69} One expectation is that the populations of mature small RNAs accumulated in these sets of mutants would be different, including sRNAs with processing defects (and/or associated with incorrect AGOs) in the first case and predominantly correctly processed, functional miRNAs/siRNAs in the second.

Chlamydomonas MUT68/RRP6 may function in competition with HEN1 in a putative assessment of small RNA functionality. The *D. melanogaster* HEN1 homolog appears to methylate single-stranded piRNAs and siRNAs already associated with certain AGO/PIWI proteins.^{1,38,39} Thus, sRNAs lacking 2'-O-methyl groups are loaded into RISC in animals and, conceivably, this may also happen for at least a fraction of the small RNAs in *Chlamydomonas*. We proposed that, in these cases, the MUT68/RRP6 machinery may operate as a quality control mechanism in kinetic competition with HEN1 (Fig. 2)³³ Functional guide sRNAs (with respect to their interactions with a particular AGO isoform) may be protected by HEN1-mediated 3' end methylation, whereas subfunctional or dysfunctional sRNAs may be preferentially degraded by MUT68/RRP6 (Fig. 2). However, it is not clear whether a similar mechanism could also act in higher plants where HEN1 has been suggested to methylate small RNA duplexes prior to their loading into RISC.^{6,13,34} Additionally, dysfunctional or subfunctional small RNAs may be conceivably dissociated more easily from an Argonaute protein and XRN-2 and/or SDN homologs could also contribute to the degradation of some of these molecules.

In degradative RNAi, RISC functions as a multiple turnover enzyme^{2,87} and a quality control mechanism(s) may also be necessary to assess the integrity of guide siRNAs after each round of target RNA cleavage. In mature RISC, the 3' end of the guide siRNA is bound by the AGO PAZ domain but, when the siRNA forms an extensive duplex with a target RNA, its 3' terminus is released from the PAZ pocket.^{14,15,88} After RISC-mediated endonucleolytic cleavage, the target RNA products are released and degraded by exoribonucleases.^{74,89,90} At this step, the 3' end of the guide siRNA may become accessible to the MUT68/RRP6 machinery prior to rebinding to the PAZ domain. We speculated that MUT68/RRP6 may also operate here, as a quality control mechanism to degrade damaged sRNAs lacking 2'-O-methyl groups.³³ However, understanding the molecular details of the proposed quality control mechanism(s) will require addressing the nature of the putative dysfunctional or damaged small RNAs.

Conclusion

Small RNAs, both miRNAs and siRNAs, play important roles in the regulation of gene expression in eukaryotes. Yet, the complexity of small RNA biogenesis and function is just beginning to be understood. Recent studies have established that post-transcriptional sRNA modifications (such as 3' terminal methylation and untemplated nucleotide additions) and several exoribonucleases can affect the stability of mature, single-stranded miRNAs and siRNAs.^{33,64,69,91} Moreover, these factors can have profound effects on the homeostasis and the function of small RNAs in plants, algae, and metazoans.^{33,64,69,91} However, despite these advances, we still know relatively little about the molecular mechanisms of mature small RNA turnover and whether the discovered pathways are common to most eukaryotes. Additionally, many enzymes implicated in the modification and the degradation of miRNAs and/or siRNAs appear to be encoded by small multigene families, in plants, algae, and animals, and potential redundancy of function may complicate uncovering their significance in sRNA metabolism and developmental and physiological responses.

The biological role(s) of small RNA degradation also needs further exploration. Some pathways may operate as quality control mechanisms to eliminate AGO-associated dysfunctional or subfunctional small RNAs, resulting from errors in Argonaute loading and/or mistakes in the processing of miRNAs/siRNAs.^{5,33} Critical questions in this context are the nature of the postulated dysfunctional or subfunctional small RNAs and the way they are recognized by the degradation machinery. Other turnover mechanisms may modulate the overall levels of AGO-bound miRNAs.^{64,69} An intriguing possibility raised by work in *C. elegans* is that the accumulation of small RNAs may be linked to the availability of target RNAs,⁶⁹ provided that target binding maintains miRNAs in an Argonaute-associated state protected from exonuclease-mediated degradation. These pathways would potentially facilitate the recycling of AGO proteins in dysfunctional or inert complexes for re-binding to other small RNAs. Whether the levels of specific mature miRNAs could also be regulated by selective turnover is not clear as yet. In this case, factors that recognize certain miRNA sequences would presumably be needed to recruit ribonucleases to particular substrates. Interestingly, a 3' terminal hexanucleotide sequence in human miR-29b promotes its nuclear localization, suggesting that specific small RNA sequence motifs can direct distinct outputs, but the factors involved in this selective localization are not known.⁹²

Untemplated nucleotide additions appear to influence the stability of mature miRNAs and siRNAs, but the significance of small RNA modifications is not entirely obvious. For instance, 3'-terminal uridylation may create unstructured sRNA ends, facilitating their degradation by nonprocessive exoribonucleases.^{13,28,33,71} However, 3' end uridylation of mature miR-26 in mammalian cells appears to impart functional differences that attenuate miRNA-targeted repression without noticeable changes in miRNA steady-state levels.⁹³ Likewise, in *Arabidopsis* certain 3'-uridylated miRNAs are almost as abundant as the unmodified canonical forms in particular tissues, suggesting a specialized role for these modified small RNAs.⁸⁶ The 3'-terminal adenylation of some miRNAs appears to stabilize them,^{80,81} but this same modification promotes the degradation of the 5' RNA products of

RISC cleavage and a number of misprocessed and unstable RNAs.^{74–76,94–96} Thus, the consequences of untemplated nucleotide additions to small RNAs may be context-dependent. Conceivably, the effect of 3'-untemplated nucleotide additions may depend on how they alter the length and/or the 3'-terminal structure of a given miRNA or siRNA and, as a result, the sRNA interactions with AGO and susceptibility to ribonuclease activities.

Similarly, 3' end methylation of small RNAs seems to protect them directly from nucleotidyltransferases and exoribonucleases,^{13,38,39,44,45} but additional functions for this modification have not been explored. For instance, 3'-terminal methylation may affect the association of an sRNA with the PAZ domain of AGO, potentially influencing the kinetics of double-strand zippering with a target RNA. Indeed, a 2'-O-methyl group on the 3'-terminal nucleotide appears to decrease the siRNA-binding affinity by the PAZ domain of human AGO1.⁹⁷ In higher plants, 2'-O-methylation may also promote or decrease the ability of RNA-dependent RNA polymerases to use small RNAs as primers.¹³ Another outstanding question is why all siRNAs and miRNAs are methylated in plants (and likely in some algae), whereas miRNAs do not seem to undergo this modification in metazoans. Interestingly, it has been noted that all 2'-O-modified small RNAs identified thus far are associated with RISC complexes that have the capability to cleave efficiently their RNA targets.³⁸ This might reflect a requirement of 3' methylation of sRNAs (through its potential effect on AGO binding) for optimal duplex formation as an intermediate for target RNA cleavage and/or a role of this modification in preventing the unintended degradation of small RNAs by the exoribonucleases that participate in the decay of RISC-cleaved RNA products.

Finally, the subcellular localization of the pathways that affect small RNA stability remains to be evaluated in most eukaryotes. In *Chlamydomonas*, MUT68 appears to be located predominantly in the cytosol,³³ but it is becoming increasingly clear that distinct RISC complexes function in both the nucleus and the cytoplasm.^{1–8} Thus, certain pathways for small RNA degradation, for instance those associated with quality control, may be required to operate in both compartments. Conversely, selective subcellular localization of turnover processes might provide another layer of regulation for the degradation of specific miRNAs. We anticipate that deepening our knowledge about the mechanisms that regulate mature small RNA turnover will be relevant not only to the comprehensive understanding of how miRNA and siRNAs execute their function but also to the successful use of RNAi for practical applications.

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