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Uridylation of mature miRNAs and siRNAs by the MUT68 nucleotidyltransferase promotes their degradation in Chlamydomonas

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Abstract: Regulation of gene expression by small RNAs (~20–30 nucleotides in length) plays an essential role in developmental pathways and defense responses against genomic parasites in eukaryotes. Micro-RNAs (miRNAs) and small interfering RNAs (siRNAs) are produced from long, near-perfect complementarity double-stranded RNAs (dsRNAs) of diverse origins, including the genome, physiological processes, or stress responses (1–3). siRNAs are then incorporated into the RNA-induced silencing complex (RISC) (1, 2). Argonaute proteins, as a guide to identify homologous RNAs, ultimately trigger the inactivation of cognate sequences, although siRNA may also participate in the activation of gene expression (1–3). At least two major classes of small RNAs have been identified in many eukaryotes: microRNAs and small interfering RNAs (1, 2). miRNAs originate from endogenous 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 (1–3). siRNAs are produced from long, near-perfect complementarity double-stranded RNAs (dsRNAs) of diverse origins, including the transcripts of long inverted repeats, the products of convergent transcription or RNA-dependent RNA polymerase activity, viral RNAs, or dsRNAs experimentally introduced into cells (1–5). These siRNAs play various roles in posttranscriptional regulation of gene expression, suppression of viruses and transposable elements, and/or heterochromatin formation (1–5). Hairpin and long dsRNAs are processed into siRNAs by an RNaseIII-like endonuclease named Dicer (1, 2). These small RNAs are then incorporated into multisubunit effector complexes, such as the RNA-induced silencing complex (RISC) (1, 2). 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 sRNA-guided endonucleases (1, 2, 6, 7). Recent evidence suggests that a sRNA duplex is first loaded into RISC and AGO cleaves one of the sRNA strands (the passenger strand) (1, 2). Ribonuclease, such as Trax or QIP, then promote RISC activation by removing the passenger strand cleavage products (8, 9). Activated RISC uses the remaining single-stranded siRNA as a guide to identify homologous RNAs, ultimately triggering transcript degradation and/or translation repression (1–3).

The biogenesis and the mode of action of sRNAs have attracted great attention (1–3, 10), but much less is known about mechanisms of miRNA/siRNA turnover and their role in small RNA function. Degradation of mature miRNAs in Caenorhabditis elegans, mediated by the 5′-to-3′ exoribonuclease XR2, has recently been shown to modulate miRNA accumulation in vivo (11). A conserved nuclease from C. elegans and Schizosaccharomyces pombe, Eri-1, degrades siRNA duplexes with 2-nucleotide 3′ overhangs in vitro and reduces the efficiency of RNAi in vivo (12, 13). Another family of 3′-to-5′ exoribonucleases, encoded by the SMALL RNA DEGRADING NUCLEASE (SDN) genes, has been implicated in mature miRNA turnover in A. thaliana (14). In C. elegans and mammalian cells, Lin-28 (a stem-cell-specific regulator) binds the precursor of the let-7 miRNA in the cytoplasm and promotes its 3′ end uridylation by a poly(U) polymerase (15–17). This leads to precursor RNA degradation and down-regulation of the let-7 miRNA. In contrast, terminal uridylation of mature miR-26 in mammalian cells appears to impart functional differences that attenuate miRNA-targeted repression without obvious changes in miRNA steady-state levels (18). In A. thaliana, 3′ end uridylation has been postulated, although not demonstrated yet, to stimulate the decay of small RNAs (19, 20). Indeed, the enzymes involved in untemplated nucleotide additions to the 3′ ends of mature sRNAs and the functional significance of these modifications remain largely uncharacterized in most eukaryotes. Moreover, plant miRNAs and siRNAs and animal siRNAs and PIWI-interacting RNAs (piRNAs) have a 2′-O-methyl group on their 3′ termini, introduced by the RNA methyltransferase HEN1, which seems to protect them against nucleotide additions and/or exonucleolytic shortening (19–22). However, the putative enzyme(s) involved in the proposed 3′-to-5′ degradation of unmethylated and uridylated sRNAs is not known because Arabidopsis SDN1 is inhibited by 3′ terminal uridylation (14).

We report here on the characterization of a C. reinhardtii mutant (Mut-68) that is defective in RNAi and provides further insight on the mechanisms of mature miRNA/siRNA degradation.
Mut-68, which lacks the MUT68 nucleotidyltransferase (23), displays elevated levels of miRNAs and siRNAs. The MUT68 enzyme is involved in the untemplated uridylation of the 3′ termini of small RNAs in vivo and in stimulating their degradation by the RRP6 exosome subunit in vitro. Moreover, RRP6 depletion by RNAi also results in the accumulation of miRNAs and siRNAs in vivo. We hypothesize that MUT68, in association with RRP6, is part of a quality control mechanism for the degradation of functionally defective small RNAs.

Results

Mut-68 Mutant Shows Enhanced Levels of Small RNAs and of the ARGONAUTE3 (AGO3) Protein. We have previously reported that MUT68 is required for the addition of untemplated nucleotides to the 5′ RNA fragments produced by RISC cleavage of target transcripts and for their efficient decay (23). Interestingly, a mutant strain, deleted for the cognate gene, also shows higher levels of several miRNAs (Figure 1A and Figure S1), relative to the wild type (CC-124) and the parental transgenic strain (Maa7-IR44). Likewise, Mut-68 displays enhanced accumulation of siRNAs produced from inverted repeat transgenes (Figure S2). These changes are paralleled by increased levels of AGO3 (Figure 1B), one of the three C. reinhardtii Argonaute proteins (4), and this effect is likely the result of translational or posttranslational regulation because transcripts for the three AGO genes are present at similar amounts in Mut-68 and the control strains (Figure S3A).

To define the step of small RNA biogenesis at which MUT68 may act to influence sRNA levels, we determined whether the amounts of the miRNA* [the strand complementary to a guide miRNA (1, 2)] were also affected by depletion of this enzyme. For two miRNA tested, miR912 and miR1157, the miRNA strand showed 70–90% higher accumulation in the mutant strain (relative to the controls) whereas the miRNA* was barely detectable (Figure 1C). In contrast, if the MUT68 deletion were to stabilize simultaneously and to the same degree both strands of a miRNA/miRNA* duplex, clearly detectable amounts of the miRNA* would be expected, given the observed enhancement in miRNA levels. An analysis of sRNAs by high-throughput sequencing is also consistent with a lack of an effect of MUT68 on miRNA’s (see below). Indeed, miR912 and miR1157 appear to be predominantly in a single-stranded conformation in both Mut-68 and its parental strain. When sRNAs were separated by non-denaturing polyacrylamide gel electrophoresis, with or without prior denaturation of the RNA samples, miRNA/miRNA* duplexes were undetectable (Figure 1D). This is in contrast to the accumulation of nicked duplex sRNAs caused by depletion of the QIP nuclease (9), which removes AGO-cleaved passenger strands in Neurospora crassa. Moreover, because no single-stranded guide sRNA seems to be produced before Argonaute

Figure 1. Mut-68 and strains depleted for the RRP6 exosome subunit show increased levels of miRNAs and of the AGO3 protein. (A) Northern blot analyses of sRNAs isolated from the indicated strains and detected with probes specific for Chlamydomonas miRNAs. Cad112, candidate miRNA 112 (31). The numbers below the blots indicate the relative abundance of the miRNAs. CC-124, wild-type strain; Maa7-IR44, CC-124 transformed with an IR transgene designed to induce RNAi of MAA7 (encoding tryptophan synthase β subunit); Mut-68, MUT68 deletion mutant; Rrp6-IR5 and Rrp6-IR11, Maa7-IR44 transformed with IR transgenes inducing RNAi of two distinct genes encoding the RRP6 exosome subunit; Csl4-IR2, strain transformed with an IR transgene triggering RNAi of CSL4 (encoding a core exosome subunit). (B) Immunoblot analysis of AGO3 protein levels. The specificity of the indicated AGO3 band was verified by peptide blocking assays. The asterisk shows a cross-reacting antigen. Coomassie-blue staining of an equivalent gel is shown as a control for similar loading of the lanes (Right). (C) Northern blot analysis of sRNAs isolated from the indicated strains and detected using probes specific for the guide strand (miRNA) or the passenger strand (miRNA*) of Chlamydomonas miR912 or miR1157. Control hybridizations to synthetic oligonucleotides are shown on the Right of each panel. (D) Northern blot analysis of sRNAs from the indicated strains separated by non-denaturing polyacrylamide gel electrophoresis. Isolated RNA was resuspended in a non-denaturing buffer (undenatured) or in formamide and denatured by heating (denatured) before loading the gel. The asterisks indicate uncharacterized RNA forms that are present in both Maa7-IR44 and Mut-68 although, due to reduced sample loading, they are less noticeable in the Mut-68 lanes. Because the intensity of these bands was not affected by denaturation, they do not appear to correspond to dsRNA. RNAi null, uncharacterized Chlamydomonas mutant lacking miRNAs. Synthetic oligoRNAs corresponding to miR912 and its miRNA* were used to demonstrate that an annealed duplex is stable under our extraction and electrophoretic conditions (Right). Annealed dsRNAs were resuspended in formamide and denatured by heating (single-stranded lane) or resuspended in non-denaturing buffer (duplex lane) before loading on the same gel as for the miR912 samples.
association and RISC activation (2, 21, 24), our observations imply that mature miRNAs and siRNAs appear to be stabilized by the MUT68 deletion. This interpretation is also consistent with the increase in AGO3 protein levels in Mut-68 (Figure 1B).

**Recombinant MUT68 Functions as a Terminal Nucleotidyltransferase on OligoRNA Substrates.** To begin addressing the role of MUT68 on sRNA stability, we tested whether the enzyme displayed terminal nucleotidyltransferase activity on oligoRNAs. Recombinant, His-tagged MUT68 was incubated with a synthetic C\textsubscript{15}'A\textsubscript{10} RNA, \textsuperscript{32}P-labeled at its 5' end, in the presence of different ribonucleotides or deoxyribonucleotides. His-MUT68 used preferentially ATP and UTP to add an untemplated oligonucleotide tail to the RNA primer (Figure 2A). In contrast, CTP, GTP, and deoxyribonucleotides were incorporated very poorly or not at all (Figure 2A). Two point mutations (D68A and D70Q) in the putative catalytic site resulted in a recombinant protein with abolished activity [Figure 2A, MUT68 (DADQ)]. His-MUT68 was also active on a single-stranded RNA corresponding in sequence to miR912 (Figure 2B). However, the enzyme was unable to use a 2'-O-methylated miR912 oligoRNA as a substrate (Figure 2B). Similarly to higher plant sRNAs (20), *Chlamydomonas* siRNAs and miRNAs appear to have a monophosphate group at their 5' ends (Figure S4A) and a 2'-O-methyl group at their 3' ends (25), as suggested by their resistance to periodate oxidation and \( \beta \) elimination (Figure S4B). Hence, MUT68 may only be able to modify in vivo newly processed or damaged siRNAs/miRNAs, lacking a terminal 2'-O-methyl group.

**MUT68 Promotes the Degradation of Small RNAs by the RRP6 Exosome Subunit.** In *Saccharomyces cerevisiae*, the Trf4/5-Air1/2-Mtr4 polyadenylation (TRAMP) complex has been implicated in stimulating the exosome, a 3'→5' multisubunit exonuclease, during the degradation of misfolded tRNAs and several noncoding RNAs (26–28). TRAMP is a nuclear complex that includes a nucleotidyltransferase (Trf4/5) weakly related to yeast Trf4 has been demonstrated to play a role in the turnover of certain transcripts that is independent of its polyadenylation activity (26, 27). In contrast, 2'-O-methylated miR912 was resistant to degradation under all of our in vitro conditions (Figure 2E). Thus, our results suggest that MUT68 and RRP6 cooperate in the degradation of unmethylated sRNAs.

**MUT68 Mutant Displays Reduced 3' Terminal Uridylation of miRNAs and siRNAs.** To gain further insight into the in vivo role of MUT68, we characterized the small RNA populations in two biological replicates of Mut-68 and Maa7-IR44 by high-throughput sequencing. During library construction each sample was given a unique 2-nt barcode: GA, UC, AG, and CU for the Mut-68–1, Mut-68–2, Maa7-IR44–1 and Maa7-IR44–2 libraries, respectively. To minimize any potential bias these barcodes might have on the sequenced small RNA populations, Mut-68–1 was compared to miR912, as a reducing terminal 2'-O-methyl group.  

![Figure 2. MUT68 acts as a terminal nucleotidyltransferase and promotes the degradation of oligoribonucleotides by the RRP6 exosome subunit in vitro. (A) Recombinant MUT68 was incubated with a \( \text{32}^\text{P} \)-labeled oligoRNA and the indicated nucleotide triphosphates for 5 or 20 min. Products were separated on a denaturing polyacrylamide/urea gel and analyzed by autoradiography. Controls included the omission of RNA, protein, or nucleotides as well as the substitution of MUT68 for the catalytically inactive MUT68(DADQ). (B) MUT68 activity, in the presence of UTP, on synthetic oligoRNA corresponding in sequence to miR912, either unmodified or 2'-O-methylated on the 3' terminal ribose. (C) 5'-end-labeled C\textsubscript{15}A\textsubscript{10} oligoRNA was incubated with affinity-purified RRP6 alone (buffer) or with the addition of MUT68 or the catalytically inactive MUT68(DADQ). Reactions were stopped at the indicated times, separated by denaturing PAGE, and analyzed by autoradiography. (D) Enzymatic activity of RRP6, RRP6/MUT68, or RRP6/MUT68(DADQ) on an unmodified miR912 oligoRNA. (E) Enzymatic activity of RRP6, RRP6/MUT68, or RRP6/MUT68(DADQ) on miR912 with a 3' terminal 2'-O-methyl group.

Maa7-IR44-1 and Mut-68-2 to Maa7-IR44-2. Only changes consistent between these two datasets are presented. As previously reported (25, 31), most sequenced sRNAs fell into the 20- to 22-nt size class (Figure 3A and Figure S8A). However, the size distribution was slightly shifted toward longer sRNAs in Mut-68 (Figure 3A and Figure S8A). The datasets also showed a decrease in the relative abundance of transposon siRNAs (76% for Mut-68–1 and 82% for Mut-68–2) and an increase in the relative abundance of miRNAs (50% for Mut-68–1 and 95% for Mut-68–2) in the mutant strain (Table S1 in Dataset S1).

Interestingly, ~7.3% of the sRNAs had 3′ untemplated nucleotides in Maa7-IR44 but this fraction was reduced to ~4.9% in Mut-68 (Table S2 in Dataset S1). Moreover, uridylation, the predominant addition to the 3′ termini of small RNAs, was markedly lower in the mutant (Figure 3B and Figure S9). 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, which is dominated by un-tailed sequences (Figure 3C and Figure S8B). Different classes of AGO-associated sRNAs (phased siRNAs, transposon siRNAs, miRNAs) followed these trends (Tables S3–S5 in Dataset S1). In contrast, putative RNA degradation products (including those matching rRNAs, tRNAs, and chloroplast and mitochondria RNAs) showed a fairly similar distribution and abundance of 3′ untemplated sequences in the two strains examined (Tables S3 and S4 in Dataset S1). Likewise, the miRNA population was not influenced by the MUT68 deletion (Tables S3 and S4 in Dataset S1). Overall, these observations are consistent with an in vivo role of MUT68 in the 3′ terminal uridylation and subsequent degradation of certain, but not all, miRNAs and siRNAs. However, uridylation of sRNAs was not entirely abolished in the deletion mutant, likely reflecting redundant activities from other nucleotidyltransferases encoded in the Chlamydomonas genome (32).

Discussion

Deletion of MUT68 in Chlamydomonas results in five defects linked to RNAi: (i) increased accumulation of miRNAs and siRNAs (Figure 1 and Figure S2), (ii) reduced 3′ untemplated uridylation of small RNAs (Figure 3B and Figure S9), (iii) lack of oligoadenylation of RISC generated 5′ RNA cleavage products (23), (iv) stabilization of partly degraded 5′ RNA fragments from target transcripts (23), and (v) enhanced levels of uncleaved, full-length target transcripts (ref. 23 and Figure S2B). The first four defects in the Mut-68 mutant can be explained by the function of MUT68 as a terminal nucleotidyltransferase (Figure 2 A and B) that promotes the exonucleolytic degradation of miRNAs/siRNAs and of the 5′ RNA fragments resulting from RISC cleavage. MUT68 appears to collaborate with the RRP6 exosome subunit in the decay of small RNAs (Figure 1 and Figure 2 C and D) and with the core exosome in the turnover of long RNAs (ref. 23 and Figure S6). Interestingly, in vivo, MUT68 seems to carry out preferentially uridylation of small RNAs (Figure 3B and Figure S9) and adenylation of RISC-cleaved transcripts (23). The basis for this differential specificity is presently unclear but nucleotidyltransferases with context-dependent nucleotide preferences have been previously described. For instance, human TUTase1 has been reported to adenylate mitochondrial miRNAs and to uridylate histone transcripts (33, 34). Moreover, recombinant S. pombe Cid1 can add either uridine or adenosine to RNA in vitro and its specificity is likely conferred by associated proteins because Cid1 complexes immunoprecipitated from cells only add uridine (35). In the context of siRNA degradation, 3′ terminal adenylation, unlike uridylation, has recently been proposed to lead to selective stabilization of mammalian miRNAs (36).

The last deficiency in Mut-68, increased levels of full-length siRNA-targeted transcripts (ref. 23 and Figure S2B), is suggestive of a defect in RISC activity. Indeed, this mutant was originally identified as being deficient in RNAi (23) and the Rrp6-IR strains also show diminished RNA interference triggered by another IR transgene. Because Mut-68 contains enhanced levels of sRNAs in single-stranded conformation, which correlate with higher amounts of AGO3, RISC assembly appears to occur normally. As already mentioned, 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, 37). Similarly, miRNA/miRNA* duplexes are loaded onto AGO and rapidly unwound by a poorly characterized mechanism (2, 37). No single-stranded siRNA or miRNA appears to be produced before this maturation step (2, 21, 24) and, thus, the single-stranded sRNAs detected in Chlamydomonas Mut-68 likely correspond to those associated with Argonautes. However, the function of a significant fraction of these RISC complexes may be compromised if the associated guide sRNAs are dysfunctional and/or damaged (see below), resulting in the sequestration of AGO proteins into inactive complexes. Although alternative explanations are possible, this interpretation for the diminished RNAi activity in Mut-68 (and in the Rrp6-IR strains) is consistent with a role for MUT68/RRP6 as a quality control mechanism for the removal of functionally defective sRNAs in Chlamydomonas. Moreover, this process may be operative in other eukaryotes because 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 (38). 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 CDE-1 absence these siRNAs accumulate to inappropriate levels, accompanied by defects in an RNAi pathway involved in chromosome segregation (39).
Untemplated nucleotide additions to sRNAs have been observed in animals, plants, and now algae (19, 36, 39–42). The role of these modifications is poorly understood but our findings and those of others (18, 36, 39) suggest that they may influence the stability and/or the function of mature small RNAs. MUT68 depletion resulted in a defect in 3′ terminal uridylation of certain small RNAs and an alteration in their steady-state levels. We hypothesize that the MUT68 nucleotidyltransferase activity may be required for the degradation of sRNAs that are dysfunctional in a RISC environment (Figure 4). Indeed, recent results suggest that RISC-bound sRNAs 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 miRNA (43). In A. thaliana, it has been proposed that HEN1 methylates sRNA duplexes before their loading onto RISC (1, 20). In contrast, the D. melanogaster HEN1 appears to methylate single-stranded piRNAs and siRNAs already associated with certain AGO-PiWI proteins (1, 21, 22). Thus, some sRNAs lacking 2′-O-methyl groups are loaded onto RISC in animals and, conceivably, this may also happen for at least a fraction of the small RNAs in Chlamydomonas. In these cases, the MUT68/RRP6 machinery may act as a quality control mechanism in competition with HEN1 (Figure 4A). Functional guide sRNAs (with respect to their interactions with AGO) may be protected by HEN1-mediated 3′ end methylation whereas subfunctional or dysfunctional sRNAs may be preferentially degraded by MUT68/RRP6. Kinetic competition with HEN1 may also result in the decay of a fraction of functional sRNAs. In the absence of MUT68, these small RNAs would be methylated by HEN1 and stabilized, consistent with the fact that miRNAs and siRNAs are resistant to periodate oxidation and β elimination in the mutant background (Figure 4B).

In degradative RNAi, RISC functions as a multiple turnover enzyme (2, 44) and a quality control mechanism(s) may also be necessary to assess the integrity of guide sRNAs after each round of target RNA cleavage (Figure 4B). 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 (6, 7, 37) (Figure 4B). After RISC-mediated endonucleolytic cleavage, the target RNA products are released and degraded by exonucleases (23, 45). At this step, the 3′ end of the guide siRNA may become accessible to the MUT68/RRP6 machinery before rebinding to the PAZ domain (Figure 4B). We speculate that MUT68/RRP6 may also operate here, as a quality control mechanism to degrade damaged sRNAs lacking 2′-O-methyl groups. However, addressing the molecular details of the proposed mechanism (Figure 4) will require further experimentation because the nature of the putative dysfunctional or damaged small RNAs is not clear.

Materials and Methods

Transgenic Strains, Mutants, and Culture Conditions. The isolation of Mut-68 has been reported (23) and transgenic strains containing inverted repeat constructs homologous to CSL4, RRP40, RRP6a, or RRP6b were generated as previously described (46). The Chlamydomonas genome contains two genes encoding RRP6 homologs: RRP6a (51,1862) and RRP6b (284930) (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). DNA fragments for building the IR constructs were generated by RT-PCR amplification with the following primers: for CSL4, Csl4-1 (5′-CGGATACATACGCTGGAG-3′) and Csl4-2 (5′-CAACCGCAACAATCCGCTG-3′); for RRP40, Rrp40-1 (5′-TGGCTTAAAAGGCCGGGTATTATA-3′) and Rrp40-2 (5′-GCCTAGCAAGAGTGAAC-3′); for RRP6a, Rrp6a-sca27-F1 (5′-ACATGGCGGACGACAAAGG-3′) and Rrp6a-sca27-R1 (5′-CTTCGCGGATAGGCTGAGG-3′); and for RRP6b, Rrp6b-sca11-F1 (5′-CCCG GACTACATCTGGACTG-3′) and Rrp6b-sca11-R1 (5′-CCCTGAGTCTTTGAGCCAG-3′). For all analyses, unless noted otherwise, C. reinhardtii cells were grown phototrophically in Tris-acetate-phosphate medium (4, 46).

RNA Analyses. Total cell RNA was purified with TRI reagent (Molecular Research Center). For Northern analysis, the isolated RNA was separated by agarose/formaldehyde gel electrophoresis, blotted onto nylon membranes, and hybridized with [32P]-labeled probes (4, 46, 47). Small RNAs, fractionated through Microcon YM-100 centrifugal devices (Millipore), were resolved on 15% polyacrylamide/7 M urea gels, and electrophoreted onto Hybond-XL membranes (GE Healthcare) (23, 46, 47). Blots were hybridized with [32P]-labeled DNA probes at 40 °C for 48 h using the High-Efficiency Hybridization System (Molecular Research Center). Specific miRNAs or miRNA* s were detected by hybridization with DNA oligonucleotides labeled at their 5′ termini with γ-[32P]ATP and T4 Polynucleotide Kinase (New England Biolabs). For the analysis of duplex RNA, total cell RNA was purified with TRI reagent, resuspended in TE buffer, and separated on a 16% native polyacrylamide gel (47). Parallel samples were resuspended in formamide and heated to 75 °C before loading onto the native gel. After electrophoresis, the gel was incubated for ~10 min in 50 mM NaOH and electrophoreted onto Hybond-XL membranes for Northern hybridization (46, 47). Control miR912/miR912* duplexes were generated by annealing synthetic oligoRNAs (miR912, UGGAUUGAUUCCACCGACGAC; miR912*, CCGUGGGAUCAAUGCAAG) in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.2 mM EDTA (47). The annealed duplexes were purified by the same protocol as total RNA, before gel loading, to ensure that our RNA extraction procedure preserves the integrity of small dsRNAs.

Figure 4. Proposed model for the role of MUT68 and RRP6 in the degradation of mature miRNAs and siRNAs (see text for details). (A) MUT68/RRP6 may eliminate dysfunctional or subfunctional guide small RNAs in kinetic competition with the methyltransferase HEN1 (after cleavage and dissociation of the passenger strand during RISC loading of siRNAs or perfectly complementary miRNA duplexes). (B) MUT68/RRP6 may also degrade damaged guide sRNAs during the multiple cycles of RISC activity.
Immunoblot Analysis. The Chlamydomonas AGO3 protein was immunodetected, following a standard protocol (46), by overnight incubation at 4 °C with a 1:10,000 dilution of a rabbit antibody raised against a C-terminal peptide (ASRSRGGAGAEGG) conjugated to KLH (GenScript).

Terminal Nucleotidyltransferase Assays. A DNA fragment corresponding to the coding sequence of MUT68 was subcloned into pET30a(+) and transformed into E. coli BL21(DE3), according to the manufacturer’s protocol (Novagen). To generate MUT68(DADQ), point mutations were introduced in the putative catalytic site (D68A and D70Q) of the wild-type sequence (23) by using the QuickChange XL mutagenesis kit (Stratagene). Recombinant proteins were purified under denaturing conditions on nickel-nitrilotriacetic acid agarose His-binding columns and refolded while attached to the solid matrix (48). These proteins were then used for pol(A) polymerase assays following a described protocol (26, 49). Briefly, reaction mixtures contained 20 mM Tris-HCl (pH 7.8), 50 mM KCl, 0.5 mM DTT, 0.7 mM MnCl2, 2.5 mM MgCl2, 200 μg/mL BSA, 0.3 μM synthetic 32P-labeled RNA substrates, and 0.5 mM of different nucleotide triphosphates. Reactions were incubated at 25 °C for 5–40 min, stopped by the addition of formamide/EDTA gel-loading buffer, and the products analyzed on 15% polyacrylamide/urea sequencing gels (47). Oligonucleotide substrates were labeled with γ-32P using T4 polynucleotide kinase (New England Biolabs) following the manufacturer’s instructions. Pol(A) polymerase was used to test the addition of poly(A) tails to the RNA substrates.

RPR6 Exosome Subunit Assays. Recombinant His-tagged RPR6 (PM/SC100 human) expressed in baculovirus-infected S. frugiperda cells, was obtained from ProSpec. For the exoribonuclease assay, an empty pGBKT7 plasmid (Clontech) or pGBKT7 plasmids encoding MUT68 or MUT68(DADQ) were included with unlabeled methionine in the TNT T7 Quick Coupled Transcription/ Translation System for 90 min at 30 °C, following the manufacturer’s directions (Promega). Recombinant RPR6 (~50 ng) and 5’-32P-labeled synthetic oligoRNAs were then added to the reactions and the buffer adjusted to 10 mM Tris-HCl (pH 8.0), 10 mM DTT, 50 mM KCl, 5 mM MgCl2, 0.5 mM UTP, and 1 μL/mL RNase inhibitor (Ambion). (50) Incubation was continued at 30 °C and 5-μL aliquots were removed at the indicated times (Figure 2 C–E and Figure S7). Samples were quenched with formamide/EDTA gel-loading buffer and resolved on 15% polyacrylamide/urea sequencing gels (47). These gels were dried on blotting paper and the radioactivity detected with a PhosphorImager (Molecular Dynamics).

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files were compared for each locus and only unique profiles are displayed (Table S5 in Dataset S1). To identify siRNAs with 1- or 2-nt untemplated tails, the first 18 nt of the small RNAs were matched to the genome. Sequences with a perfect match were then extended until a mismatch was found. To minimize including reads with sequencing errors as having 3' untemplated nucleotides, if the nucleotides after the first mismatch matched the genome, the siRNAs were not considered to be tailed. The deep sequencing libraries are deposited in NCBI GEO (GSE17815).

Analysis of the Chemical Nature of Small RNA Ends. Twenty micrograms of YM-100–enriched small RNAs were used for each reaction. Periodate oxidation and β-elimination of RNAs were carried out as described (9). Some reactions were spiked with an unmodified, 31-nt synthetic oligoRNA (2726 substrate, 5'-ACAUACACCCUGUCCCGUACCCUGAAAUCA-3'). To probe the siRNA 5' ends, samples were treated with 2 units terminator exonuclease, an enzyme that digests RNAs with a free 5' monophosphate, following the manufacturer’s instructions (Epicentre). Small RNAs were also incubated with 10 units antarctic phosphatase (New England Biolabs) to remove terminal phosphate groups (10). Some small RNA samples, subjected to dephosphorylation, were also resuspended in RNase-free water and then treated with T4 polynucleotide kinase (New England Biolabs).

Transient Expression and Analysis of MUT68:GUS Subcellular Localization in Onion Epidermal Cells. Because recombinant proteins are poorly expressed in Chlamydomonas (11), onion epidermal cells were used as a transient expression system, consisting of transparent cells that facilitate subcellular imaging. A fusion protein between MUT68 and E. coli β-glucuronidase, under the control of the cauliflower mosaic virus 35S promoter, was constructed as previously described (12). Cells in the epidermal layer of onion bulbs were transformed by microprojectile bombardment with DNA coated tungsten particles (13). After bombardment the epidermal peptides were incubated for 18–20 h at 23 °C under constant light of 50 μmol·m⁻²·s⁻¹ photosynthetically active radiation. Cell layers were stained for GUS activity as described (13), washed briefly in 100 mM sodium phosphate (pH 7.0), and mounted in the same solution containing 1 μg mL⁻¹ propidium iodide (PI) (12). The stained cells were inspected by bright-field microscopy for distribution of GUS activity and by epifluorescent microscopy for PI labeling of the nucleus (12).

References


Supporting Information

An additional supporting dataset (Excel files; .xls) is attached at the “Download” page for this article.

SI Materials and Methods

Reverse Transcriptase–PCR (RT-PCR) Analyses. Total RNA was isolated with TRI reagent and contaminant DNA was removed by DNase-I treatment (Ambion). First-strand cDNA synthesis and PCR reactions were performed as previously described (1, 2). PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining (2). The number of cycles showing a linear relationship between input RNA and the final product was determined in preliminary experiments. Controls included the use as template of reactions without RT and verification of PCR products by hybridization with specific probes (data not shown). The primer sequences were as follows: for AGO1, AGO1-F (5'-CTTCCTGTGGTGTCAGAAGTCGC-3') and AGO1-R (5'-CACCAGTACCCAGTGGTCG-3') for AGO2, AGO2-F (5'-GTCTAGGGAGAAGTGTCAGCG-3') and AGO2-R (5'-TGATGCCCTGTCGTCCACCC-3'); for AGO3, AGO3-F (5'-GGGACCTGTTGAGGAGAAGTCGC-3') and AGO3-R (5'-CGGAGTCGCTGGTCCAGAAGTCGC-3'); for RRP6a, Rrp6a-sca27-F (5'-ACTTCCGGTGCTATCTGCTGGTGA-3') and Rrp6a-sca27-R (5'-CCGGAGTCCTGTCGTCCACCC-3') and for RRP6b, Rrp6b-sca11-F (5'-CGCTAAGTCTGTCGTCCACCC-3') and Rrp6b-sca11-R (5'-CGTCAACCACCTCAGACGCG-3'); and for ACT1, ACT1-cod-F (5'-GACTCCCGAAGGACCTCTAC-3') and ACT-cod-R (5'-GATCCACATTGTCCTGGAAAGT-3').

Small RNA Library Construction and Data Analysis. The small RNA fraction was precipitated from 800 μg of total RNA and cDNA libraries were constructed as previously described (3). The 5’ RNA adapters contained unique 2-nt additional barcodes at their 3’ termini (GA, UC, AG, and CU for the Mut-68–1, Mut-68–2, Maa7-IR44–1, and Maa7-IR44–2 libraries, respectively). Equal amounts of the final DNA products from each library were pooled and sequenced by Illumina. After sequencing, the adapter sequences were removed and remaining sequence reads binned according to their barcodes. Sequences shorter than 18 nt or longer than 28 nt were discarded. Sequence reads were compared to the C. reinhardtii genome versions 3.1 and 4.0, ESTs, predicted transcripts, transposons, and bonus reads (http://genome.jgi-psf.org/Chlr4/Chlr4.download.ftp.html) (4). Sequences matching tRNAs, rRNAs, snRNAs, snoRNAs (5), the chloroplast or the mitochondrial genomes were removed as these were likely to be degradation products. The remaining sRNAs were normalized to transcripts per million (TPM) and grouped into different categories. Phased siRNAs were characterized as sRNAs matching previously described phased loci (6, 7). To identify potential miRNAs, the Chlamydomonas miRNAs in the miRNA database (http://microrna.sanger.ac.uk/sequences/) were matched to genome version 4.0. Because many Chlamydomonas miRNAs did not have reported miRNA* sequences in the Sanger database, the genomic regions were folded using mFOLD (8) and the miRNA* sequences retrieved. Sequence reads matching genome version 4.0 with 5’ ends +/- 1 nt relative to the 5’ end of a reported miRNA or miRNA* were considered miRNAs or miRNA*s, respectively. This was expanded to a 5-nt offset for the data reported in each miRNA cluster (Table S5 in Dataset S1). In cases where the miRNA or miRNA* resided in more than two loci in the genome, the small RNA pro-

**Figure S1.** Mut-68, a mutant defective in the nucleotidyltransferase MUT68, and strains depleted for the RRP6 exosome subunit show increased levels of miRNAs. The histograms display the relative abundance of small RNAs detected by Northern blotting with probes specific for the indicated *Chlamydomonas* miRNAs and normalized to the amount of the U6 small nuclear RNA. Cad112, candidate miRNA 112 (7). For illustration purposes, the miRNA levels in the wild-type strain (CC-124) were set to 1.0 and the remaining samples were then adjusted accordingly. The results shown are the average of four independent experiments ± the standard deviation. Samples marked with an asterisk are significantly different (*P < 0.05*) from the parental transgenic strain (Maa7-IR44) in a two-tailed Student’s *t* test. CC-124, wild-type strain; Maa7-IR44, CC-124 transformed with an IR transgene designed to induce RNAi of *MAA7* (encoding tryptophan synthase β subunit); Mut-68, MUT68 deletion mutant; Rrp6-IR5 and Rrp6-IR11, Maa7-IR44 transformed with IR transgenes inducing RNAi of two distinct genes encoding the RRP6 exosome subunit; Csl4-IR2, strain transformed with an IR transgene triggering RNAi of *CSL4* (encoding a core exosome subunit).
Figure S2. Mut-68 and strains depleted for the RRP6 exosome subunit show enhanced levels of small interfering RNAs (siRNAs). (A) Schematic representation of the RbcS2 transcript (encoding RUBISCO, an essential enzyme for photosynthetic CO2 fixation). The regions of homology to a dsRNA from an inverted repeat (IR) transgene, triggering RNAi, and to the probe used for Northern blot hybridization are indicated by black lines. (B) Northern blot analysis of total RNA isolated from the indicated strains. The same filter was sequentially hybridized with probes specific for the 5’ end of the RbcS2 transcript and the ACTIN1 3’-UTR sequence. CC-124, wild-type strain; RbcS-IR13, CC-124 transformed with an IR transgene designed to induce RNAi of RbcS2 (1); Mut-68, MUT68 deletion mutant (14); Mut-68(RbcS-IR13), Mut-68 containing the RbcS2 IR transgene introduced by crossing. (C) Detection of RbcS2 siRNAs by Northern blotting. A representative blot out of three replicates is shown. Hybridization to the U6 small nuclear RNA was used as a control for equivalent loading of the lanes. (D) Growth and survival of the indicated strains on Tris-acetate-phosphate (TAP) medium or on high salt (HS) minimal medium. Cells with RNAi-mediated suppression of RUBISCO are deficient in photosynthesis and grow very poorly on HS medium. (E) Northern blot analysis of siRNAs corresponding to the MAA7 gene (encoding tryptophan synthase β subunit). Maa7-IR44, CC-124 transformed with an IR transgene designed to induce RNAi of MAA7 (1); Mut-68 was isolated in the Maa7-IR44 background and already contains the MAA7 IR transgene (14); Rrp6-IR11(Maa7-IR44), Maa7-IR44 transformed with an IR transgene inducing RNAi-mediated depletion of the RRP6 exosome subunit. The numbers below the blot indicate the relative abundance of the siRNAs. A representative blot out of three replicates is shown.

Figure S3. Analysis of the steady-state mRNA levels corresponding to the ARGONAUTE and RRP6 genes by reverse transcriptase (RT)-PCR. (A) RT-PCR analysis of AGO1, AGO2, and AGO3 expression in the indicated strains. Amplification of ACT1 (encoding actin) transcripts is shown as an input control. The numbers below the panels indicate the relative abundance of the PCR products. Representative gels out of four replicates are shown. Reactions using RNA not treated with reverse transcriptase as the template were employed as a negative control (data not shown). CC-124, wild-type strain; Maa7-IR44, CC-124 transformed with an IR transgene designed to induce RNAi of MAA7 (encoding tryptophan synthase β subunit); Mut-68, MUT68 deletion mutant (14); Rrp6-IR5 and Rrp6-IR11, Maa7-IR44 transformed with IR transgenes inducing RNAi of two distinct genes encoding the RRP6 exosome subunit; Csl4-IR2, strain transformed with an IR transgene triggering RNAi of CSL4 (encoding a core exosome subunit). (B) RT-PCR analysis of RRP6a and RRP6b expression in the indicated strains. The numbers below the panels indicate the relative abundance of the PCR products. Representative gels out of four replicates are shown. The Chlamydomonas genome contains two genes coding for homologs of the RRP6 exosome subunit (including both 3’-to-5’ exonuclease and HRDC domains): RRP6a (511862; au5.g2165_t1) and RRP6b (284930; au.g3315.t1) [http://genome.jgi-psf.org/Chlre4/Chlre4.home.html]. The transgene introduced into Rrp6-IR5 was designed to suppress specifically RRP6a whereas the transgene introduced into Rrp6-IR11 was designed to knock down RRP6b. However, because of the sequence identity between these two RRP6 paralogs, each IR construct suppresses, to different degrees, the expression of both RRP6 genes.
**Figure S4.** Northern analysis of enzymatic and chemical probing of small RNA ends. (A) 5’ end analysis of small RNAs. Purified sRNAs were treated with terminator exonuclease (Ter Exo), alkaline phosphatase (Phos), or alkaline phosphatase followed by T4 polynucleotide kinase (PNK). After separation by denaturing PAGE, miR1157 was detected by Northern hybridization. The membrane was then stripped and reprobed for miR912. The susceptibility of the miRNAs to degradation by Terminator Exonuclease and the slight mobility shift caused by alkaline phosphatase (which was reversed by PNK) are consistent with miR1157 and miR912 having a 5’ monophosphate terminus. (B) 3’ end analysis of small RNAs. Purified sRNA samples, mixed with 0.05 pmol of an unmodified 3-mer oligoRNA partly homologous to miR1157, were subjected to periodate oxidation and β-elimination reactions. After separation by denaturing PAGE, small RNAs were electroblotted to a nylon membrane and detected by sequential hybridization with probes specific for miR1157, miR912, and MAA7 siRNAs. Representative blots out of three replicates are shown. Whereas most of the synthetic oligoRNA migrates faster following β-elimination, the miRNAs and siRNAs do not appear to be affected, indicating lack of a 2’,3’ hydroxy terminus. These results are consistent with methylation at the 2’ hydroxy position of the 3’ terminal nucleotide in *Chlamydomonas* small RNAs and with the existence of a functional HEN1 homolog, as previously reported (6).

**Figure S5.** Subcellular localization of GUS and MUT68-GUS fusion proteins in transiently transformed onion epidermal cells. (A) Diagram of GUS and of the MUT68-GUS fusion polypeptides. (B) The proteins expressed in onion epidermal cells were localized histochemically by the X-glucuronide assay. Transformed cells were classified as showing nuclear localization of GUS activity (empty bars, N) or not (solid bars, C). The results shown are the average of three independent experiments (n = 300 for each construct). (C) Representative cellular staining patterns in transiently transformed onion cells. Tissues were simultaneously analyzed by X-glucuronide staining (blue color, Left) and nuclei-specific propidium iodide staining (orange color, Right). The Upper and Middle panels correspond to cells transformed with the MUT68-GUS fusion protein whereas the bottom panel corresponds to a cell transformed with GUS alone. The Middle panel is shown as an example of the nuclear localization of MUT68-GUS although the frequency of these events was very low. The nuclei are indicated with arrowheads. The subcellular localization of the *Chlamydomonas* RRP6 exosome subunits was not determined but in *Arabidopsis thaliana*, which contains three RRP6-like proteins, one isoform is restricted to the cytoplasm and the other two show distinct intranuclear localizations (15).
Figure S7. Recombinant MUT68 promotes the in vitro degradation of oligoribonucleotides by the RRP6 exosome subunit. A C15A10 oligoRNA, 32P-labeled at its 5' end, was incubated with affinity-purified RRP6 alone (buffer) or with the addition of MUT68. Reactions were stopped at the indicated times, separated by denaturing PAGE, and analyzed by autoradiography. The gel was run for a short time to examine the accumulation of degradation products, which were run off the gels shown in Figure 2 C–E. Incubation with MUT68 alone for up to 180 min did not result in the accumulation of degradation products, indicating that recombinant MUT68 did not contain contaminating ribonuclease activity (data not shown).
Figure S8. Analysis of endogenous small RNA sizes in Mut-68 and the parental strain Maa7-IR44. (A) Size distribution of unique (distinct) small RNAs, after subtracting putative degradation products and normalization. Two independent libraries were examined for each strain. Unique sRNA sequences from Maa7-IR44 are represented by red (Maa7-IR44-1) or pink (Maa7-IR44-2) bars. Unique sRNA sequences from Mut-68 are represented by blue (Mut-68-1) or light blue (Mut-68-2) bars. The number following the strain names indicates the small cDNA library from which the data were obtained. (B) Size distribution of unique small RNAs with an untemplated 3' terminal U or UU (after removing the tails). Two independent libraries were examined for each strain and the bars are colored as indicated above (A).
Figure S9. Analysis of untemplated nucleotide additions to the 3’ termini of small RNAs in Mut-68 and the parental strain Maa7-IR44. (A) Frequency of the predominant untemplated nucleotides added to the 3' ends of small RNAs (relative to all sRNAs with 3’ nucleotide additions). Two independent libraries were examined for each strain and the number following the strain names indicates the small cDNA library from which the data were obtained. (B) Frequency of small RNAs (as a percentage of all sRNAs) with 3’ untemplated nucleotide additions in Mut-68 and the parental strain Maa7-IR44.