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A Novel Protein Arginine Methyltransferase Interacts with the RNA Induced Silencing Complex Component, MUT70, and Is Required For RNA Interference in *Chlamydomonas reinhardtii*

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RNA interference (RNAi) is an important gene regulatory mechanism. It involves 21-24 nucleotide small RNA molecules that downregulate complementary, or near complementary, mRNA sequences through transcript degradation or translation repression. The non-coding small RNA molecules act through an effector complex known as the RNA induced silencing complex (RISC). The components of RISC are evolutionarily conserved and consist of DICER and AGO proteins as well as several other polypeptides. These include, among others, MUT70, an RNA binding protein that has an RGG rich domain. Protein arginine methyltransferases typically methylate RGG rich regions, affecting protein stability, localization, and/or interactions. Here, we report that Protein Arginine Methyltransferase 2 (PRMT2) is required for RNAi in the green algae *Chlamydomonas reinhardtii*. Phylogenetic analyses indicate that the PRMT2 protein likely evolved in the plant/algal lineage. Interestingly, PRMT2 depletion resulted in a defect in RNAi-mediated translational repression. Moreover, *in vitro* analyses showed that MUT70 and PRMT2 interact directly and that PRMT2 can methylate MUT70. A point mutation in a highly conserved region of the methyltransferase domain disrupted methylation activity, but

increased the affinity of PRMT2 for the MUT70 substrate. Our results, taken together, indicate that PRMT2 is required for RNAi, presumably through its modification of the MUT70 RISC component. However, elucidating the precise mechanistic role of PRMT2 will require further exploration.

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Acknowledgements

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Literature Review

Chlamydomonas reinhardtii is a single celled, eukaryotic green alga. It is commonly found in the soil and can either assimilate CO_2 through photosynthesis or use certain organic compounds such as acetate as carbon sources ([1]). Active research areas involving *C. reinhardtii* include photosynthesis, flagellar proteins, gene regulation, and more recently, biofuels. The algal genome has been sequenced and annotated by the *Chlamydomonas* community ([2],[3]). This has led to the discovery of many genes that influence cell development and function. The genome of *C. reinhardtii* also includes a complex set of non-coding RNA genes, including endogenous microRNAs ([4]).

RNA Interference

RNA interference (RNAi) refers to the use of double stranded RNAs (dsRNA) to induce a silencing effect on complementary mRNA sequences through a variety of mechanisms, including mRNA degradation and translational repression. Small non-coding RNAs, which are between 21 and 24 nucleotides in length and derive from dsRNAs, function through the RNAi pathway to regulate gene expression. These small RNAs were first discovered in *Arabidopsis thaliana* but are found in most eukaryotes ([5]). Small non-coding RNAs have also been found to have regulatory roles in prokaryotes, although, they do not function in an RNA interference type pathway in these organisms ([6]).

There are several classes of small RNAs that act as guides for the RNAi pathway. The two most prominent classes are small interfering RNAs (siRNAs) and microRNAs (miRNAs). SiRNAs primarily originate from transposable element RNA, viral RNA, or exogenous double stranded RNA. MiRNAs originate from endogenous RNA transcripts and can be intronic or intergenic ([7],[8]). In animals, siRNAs function primarily through the endonucleolytic cleavage of homologous target transcripts, whereas miRNAs function through translational repression ([9],[10]). Several studies have provided considerable insights on the RNA mediated target cleavage and degradation process, while the mechanism of translational repression is poorly understood ([11]). Intriguingly, there is also some evidence that certain miRNAs can promote translation in animals ([12]). In plants, miRNAs, which are often perfectly complementary to target mRNA sequences, were originally shown to function through transcript degradation alone. However, new evidence has shown that miRNAs can also control gene expression via translational repression as in metazoans ([13]). Other classes of small RNAs related to the RNAi pathway include the PIWI-interacting RNAs (piRNAs), which are expressed in animal germ line cells and suppress transposable elements, and a newly discovered class of small RNAs in *Neurospora crassa,* which are induced by DNA damage ([14], [15],[16]).

The downregulation of genes by RNAi affects many biological processes. Plant miRNAs have been implicated in response to both biotic and abiotic stresses ([7],[17]). Animal miRNAs have been found to be involved in developmental regulation, a variety of diseases, and can be markers for certain cancers in humans ([19],[20]). SiRNAs often function as a defense mechanism against foreign RNA

transcripts, such as those derived from transposons and viruses. Interestingly, siRNAs can also originate from a virus to counter the host's defense systems ([18]).

Small RNA Biogenesis

Small interfering RNAs are produced from long double-stranded RNA (dsRNA) with perfect complementarity, whether they originate in the cell from transgenes or transposons or from outside the cell as viral RNAs or introduced dsRNA molecules. Long dsRNAs are processed by the Dicer protein, an RNase III enzyme, into ~21-25 nucleotide duplexes with 2-nucleotide overhangs at the 3' end ([8]). In contrast, miRNAs are transcribed from the genome into double stranded stem loop structures called primary miRNAs (pri-miRNAs) and are characterized by imperfect complementarity in the stem regions. In animals the pri-miRNAs are further processed in the nucleus by another RNase III – type enzyme, Drosha, which cleaves the pri-miRNA 11 base-pairs away from the base of the double-stranded stem loop structure to produce precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported to the cytoplasm via exportin 5, a nuclear transporter, and are further processed by Dicer in the cytosol to generate mature miRNAs ([21]).

The small RNA duplexes (miRNAs and siRNAs) are methylated on the 3' end in plants and algae, which is a modification that stabilizes the small RNAs by preventing uridylation and degradation ([22],[23]). In animals, only siRNAs and piRNAs have been found to be methylated by HEN1, the same methyltransferase used by plants and algae ([24]). One strand of each small RNA duplex (the guide

strand) is eventually loaded into the RNA-induced silencing complex (RISC), the effector RNAi complex. The other strand (called the passenger strand) is usually degraded ([25]).

RNA Induced Silencing Complex (RISC)

RISC incorporates Dicer-processed small RNAs as guides to target homologous mRNAs for their degradation or translational repression. Argonaute proteins (a class of proteins referred to as AGO) are core components of RISC. In humans, RISC loading involves Dicer, AGO, and a dsRNA binding protein, TRBP ([26]). However, there is no TRBP homolog in *Chlamydomonas reinhardtii*. Upon small RNA loading into RISC, the AGO proteins select the guide strand and then proceed to cleave the passenger strand, facilitating its removal. Yet the cleavage of the passenger strand is not necessary for functional RNAi, as not all AGO proteins contain a functional endoribonuclease domain ([27],[28]).

The AGO family of proteins consists of two members, the PIWI clade and the AGO clade. The PIWI proteins are limited to certain eukaryotes, whereas the AGO proteins are found in all cell types in animals and plants ([41]). AGO proteins contain two major domains, the PIWI and PAZ domains ([29]). The PAZ domain recognizes the 3' end of small RNAs, whereas the PIWI domain is homologous to RNaseH and cleaves target mRNAs (the slicer activity site) ([30]). While some AGO proteins have redundant functions, most recognize and function with specific types of small

RNAs ([31]). The nucleotides in the 2-8 positions from the 5' end of the guide strand play a major role in the identification of target mRNAs ([32]). The slicing of target

transcripts by AGO then occurs after the tenth nucleotide from the 5' end of the guide strand ([33],[34]).

The AGO proteins can also repress translation by a largely unknown mechanism. Translational repression in animals requires GW182, which is a P-body component ([35],[36]). Importin 8, a nuclear transport factor, has also been shown to interact with AGO proteins, localize to P-bodies, and be involved in the recruitment of RISC to mRNA targets ([37]). However, there is no evidence that translational repression by AGO actually requires P-bodies.

There are other proteins that associate with RISC, but whose function remains uncertain. The fragile X mental retardation protein 1 (FMRP), an RNA binding protein with RGG motifs, interacts with RISC and other ribosomal proteins in animals, though there is no corresponding homolog in *Chlamydomonas reinhardtii* ([38]). Another RNA binding protein that immunoprecipitated as a RISC component in *Drosophila* is the vasa intronic gene (VIG), which also has an RGG motif ([39],[40]). In *C. reinhardtii*, VIG (named MUT70) was found to be essential for RNAi (unpublished data). Recently, the protein arginine methyltransferase 5 (PRMT5) has been co-immunoprecipitated with PIWI-like proteins in animals. PRMT5, with its cofactor WDR77, interacts directly with PIWI and influences piRNA levels ([41],[42]).

Introduction

Post-translational modifications of proteins are prevalent in all organisms. The encephalitogenic protein from the human brain was the first protein to be described as having a methylated arginine as a posttranslational modification ([43]). Enzymes that methylate arginine residues, most often at conserved RGG or RGR rich regions, are collectively known as protein arginine methyltransferases or PRMTs and are evolutionarily conserved in eukaryotes ([44],[47]). Most organisms have multiple PRMTs: eleven in humans, nine in *Arabidopsis thaliana*, and three in *C. reinhardtii* ([45],[46]). PRMT activity has been found in both nuclear and cytoplasmic compartments, and its targets include transcription factors, histones, RNA binding and metabolic proteins, DNA damage repair proteins, and PIWI proteins ([42],[48],[49]). Arginine methylation has been implicated in affecting protein localization, stability, and protein-protein interactions ([50]).

There are three classes of PRMTs, but all of them use S-adenosyl-L-methionine (SAM) as a methyl donor. Class I PRMTs perform monomethylation and asymmetric dimethylation of ω-guanidine nitrogen atoms; class II PRMTs perform monomethylation and symmetric dimethylation of ω-guanidine nitrogen atoms; and class III are membrane bound PRMTs that perform only the monomethylation of δ-

guanidine nitrogen atoms of arginine amino acids ([46]). Human PRMTs have been the most intensely studied, and three of these enzymes are evolutionarily conserved in

eukaryotes, PRMT1, PRMT3, and PRMT5. The first two are class I PRMTs, while PRMT 5 is a class II PRMT ([50]).

Several components of RISC could be targets of arginine methylation by PRMTs. These include the FMRP protein and the vasa intronic gene protein, which are RNA binding polypeptides with RGG rich regions. In fact, FMRP in animals is methylated, and the methylation of arginines in the RGG box has a disruptive effect on RNA binding ([51]). It is not known whether the vasa intronic gene protein (named MUT70 in *Chlamydomonas*) may be a target of arginine methylation. Interestingly, PRMT5 has recently been pulled down in an immunoprecipitation assay using mammalian Ago4 ([37]). PRMT5 has also been demonstrated to be responsible for the methylation of PIWI proteins in animals, and the loss of PRMT5 activity results in lower levels of piRNAs, Ago3 and Aubergine in *Drosophila* ([41]).

In *C. reinhardtii*, RISC includes AGO and MUT70, among other proteins (unpublished data). Intriguingly, affinity purification experiments using MUT70 as the bait identified a protein arginine methyltransferase (named PRMT2) as a specific interactor. This finding prompted us to explore the role of PRMT2 in RNA interference. We show here that PRMT2, a class I PRMT, is essential for RNAi in a strain of *C. reinhardtii* that suppresses a transgenic inverted repeat targeting the *MAA7* gene (encoding the Tryptophan Synthase β subunit, TSβ). A phylogenetic analysis shows that PRMT2 is a plant specific PRMT and shares a conserved SAM

binding with all related proteins. We also demonstrate that PRMT2 interacts with MUT70 and methylates this protein *in vitro.* Our findings suggest that methylation of certain RISC components is necessary for optimal RNAi function in *C. reinhardtii*.

Materials and Methods

Culture Conditions, Genetic Transformations, and Generation of Transgenic Strains:

Unless otherwise noted, *C. reinhardtii* cells were grown on Tris-acetate-phosphate (TAP) medium ([1]). The wild type CC-124 and the transgenic Maa7-IR44 strains have been previously described ([1],[52]). Transformation with linearized plasmid DNA was carried out by the glass beads procedure ([53]). The PRMT-IRs15 strain, in which the PRMT mRNA is downregulated, was generated by transformation of the Maa7-IR44 strain with its construct of an inverted repeat designed to produce dsRNA homologous to the 3'UTR of the PRMT2 mRNA using previously described procedures ([52]). The production of the complement strains, PRMT-IRs15(PRMT)3, PRMT-IRs15(PRMT)7, and PRMT-IRs15(PRMT)12, were obtained by transformation of PRMT-IRs15 with a construct designed to contain the PRMT2 coding sequence with no flanking regions (3' and 5' UTRs) so as to avoid downregulation by the PRMT-IR.

To analyze the growth of the different strains on media containing 5-fuoroindole, the five strains, CC-124, Maa7-IR44, PRMT-IRs15, PRMT-IRs15(PRMT)3, and PRMT-IRs15(PRMT)7, were grown again for 2 days in liquid culture. A volume containing 3×10^6 cells was then pelleted via centrifugation at 5,000 × rpm for five minutes and then suspended in 150 μL of liquid TAP media for a total of approximately 1×10^5 cells. Four dilutions followed by taking 37.5 μL of the

original into 112.5 μL of liquid TAP media. The four dilutions had 2.5×10^4, 1.25×10^4, 3.125×10^3, and 781 cells respectively, assuming 1×10^5 cells in the original sample. Five μL of each dilution was plated in a row, with four rows total, one for each strain. The plates were grown under indirect light for seven to ten days.

Plasmid Construction

His-tagged PRMT2 was generated by amplifying the PRMT2 coding sequence from a cloned cDNA template with the primers PRMT-cod-F1 (ATCCATGGTAATGTCGTCGCCAAAGTCC), and PRMT-cod-R1 (TTGATATCTTACTCGATGTTCCAGCGGAACT) into a *Chlamydomonas* expression vector (unpublished data). The product was then cloned in forward orientation into the pET-30-C+ vector (Novagen, Madison, WI, USA) using *Nco*I and *Eco*RV restriction sites on both plasmids. The MUT70 coding sequence was previously cloned into the pET-30-C+ plasmid, and was then sub-cloned into the pGEX-6P-1 vector directly from the pET-30-C+ vector, using the *Kpn*I and *Xho*I restriction sites. To generate the point mutation of the 72nd residue from a glycine to and arginine in the conserved SAM binding motif, the coding sequence of PRMT2 was modified using the Quickchange Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the following primers: PRMT G72R antisense (5'-GATGCCGCTGCGGCCCACGT-3') and PRMT G72R (5'-ACGTGGGCACGCGCAGCGGCATC-3').

DNA Sequence and Phylogenic Analysis:

C. reinhardtii PRMT2 (XP_001702822.1) sequence was used to BLAST searches on the NCBI protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The highest scoring protein from each target organism (*Chlamydomonas reinhardtii*, *Ostreococcus tauri*, *Arabidopsis thaliana*, *Oryza sativa*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*) was used for phylogenetic analysis. The SMART database was used to identify the conserved methyltransferase domain present in the PRMT proteins obtained. The protein sequences were aligned with ClustalX ([54]) with manual corrections made using the GENEDOC program (http://www/psc.edu/biomed/genedoc). The phylogenetic relationships among related proteins were determined using the neighbor-joining (NJ) method ([55]), and trees were obtained using MEGA v4.0 ([56]), Poisson-corrected amino acid positions, and the bootstrap values for 1000 pseudoreplicates.

RNA Analysis:

Standard protocols were used for RNA extraction, fractionation by gel electrophoresis, and hybridization with ^{32}P labeled probes ([57],[58]). Total RNA was isolated with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's instructions. The isolated RNA was separated by agarose/formaldehyde gel electrophoresis, blotted onto a Hybond-NX nylon

membrane (GE Healthcare, Pittsburgh, PA, USA), all northern blots were chemically crosslinked using EDC ([59]), and hybridized with ^{32}P labeled probes corresponding to the *PRMT2*, *Maa7*, or *Actin* coding sequence ([58]). The *Actin* probe was used for equivalent loading of samples.

Small RNA Analysis

For detection of small RNA, tri-reagent isolated total RNA was resolved in 15% polyacrylamide/7M urea gels, electroblotted to Hybond-XL membranes, and chemically crosslinked using EDC ([58]). The small RNA blots were then probed using ^{32}P labeled antisense miRNA probes, miR1157 and miR912, and then a U6 probe was used for equivalent loading of samples. The hybridization was performed at 35° C for 48 hours using the High Efficiency Hybridization System (Molecular Research Center).

Protein Analysis

For *in vivo* analysis, approximately 5×10^6 cells, grown to logarithmic phase, were pelleted by centrifugation and resuspended in 50 μL of SDS-gel running buffer ([58]), 10 μL aliquots of boiled samples were separated by 12% SDS-PAGE and electroblotted to nitrocellulose membranes ([58]). The AcV5-tagged complemented PRMT2 proteins were immunodetected by overnight incubation at 4°C with a

1:50000 dilution of a mouse raised anti-AcV5 antibody in (AbCam, Cambridge, MA, USA), and after incubation, a rabbit anti-mouse secondary antibody, conjugated to horseradish peroxidase (HRP). A chemiluminescent substrate (Pierce, Rockford, IL, USA) was used for autoradiographic detection. For immunodetection of the tryptophan synthase β (TSβ) protein, an anti-TSβ antibody raised in polyclonal goat (kindly provided by Thomas McKnight) at a 1:4000 dilution was incubated at 4°C overnight. After incubation, an anti-rabbit secondary antibody conjugated to horseradish peroxidase was used for autoradiography. Coomassie Blue staining of the SDS-PAGE gel was used to adjust sample loading.

In vitro Protein Interactions

The recombinant proteins MUT70-GST, MUT70-His, PRMT2-G72R-His, and PRMT2-His were produced in BL21-DE3 *Escherichia coli* cells via 0.3 mM IPTG induction for three hours at room temperature. The cells were pelleted and then lysed and the proteins purified using GST or His purification kits (Novagen, Madison, WI, USA). For the GST pulldown, the glutathione beads were blocked for 2 hours with 1% bovine serum albumin at 4°C prior to adding lysate, and the MUT70-GST was immobilized on the glutathione beads ([48]). In concert, the PRMT2-His and PRMT2-G72R-His recombinant proteins were purified and eluted, followed by dialysis in 50 mM NaH_2PO_4 pH 7.2 overnight at 4°C. After dialysis, glycerol was added to a final concentration of 30% and stored at -20°C. 20 μL of the purified PRMT2-His or PRMT2-G72R-His solution was added to the MUT70-GST on

glutathione beads in 50 mM NaH_2PO_4 pH 7.2 with 0.1% Tween and incubated overnight at 4°C. After the incubation, it was centrifuged at 1000 rpm for 1 minute and the supernatant was discarded. The beads were then washed twice with 50 mM NaH_2PO_4 pH 7.2 and then resuspended in 50 μL of SDS gel loading buffer and examined by western blot ([58]). The primary antibody used was an S-tag antibody conjugated to HRP (Novagen, Madison, WI, USA) to analyze the bound proteins.

For the His pulldown, PRMT2-His and PRMT2-G72R-His recombinant proteins were purified and immobilized on the His-tag beads (Novagen Madison, WI, USA). At the same time, the recombinant MUT70-GST was purified, eluted (Novagen, Madison, WI, USA), and was subject to dialysis in 50 mM NaH_2PO_4 pH 7.2 overnight at 4°C and concentration of 30% glycerol was added and stored at -20°C. The incubation and washes were conducted in the same conditions as the GST pulldown. The bound proteins were analyzed by western blot using a GST primary antibody raised in a mouse (Applied Biological Materials Inc., Richmond, BC, Canada).

In vitro Methylation Assay:

The PRMT2-His and PRMT2-G72R-His proteins were purified, eluted (Novagen, Madison, WI, USA), and were subject to dialysis in 50 mM NaH_2PO_4 pH 7.2 overnight at 4°C, then brought to a 30% glycerol concentration. The MUT70-His recombinant protein was purified and immobilized on the His-tag beads (Novagen, Madison, WI, USA). The bound recombinant MUT70-His protein was incubated

with 10 μCi of (methyl-C^{14}) S-adenosyl-L-methione (SAM), 50 mM NaH_2PO_4 pH 7.2, either 5 μL of MUT70-His BL21 DE3 cell extract, 2 μL of PRMT2-His, or 2 μL of PRMT-G72R-His in a 40 μL reaction at room temperature for three hours. Following the reaction, 15 μL of 3× SDS gel loading buffer was added and subsequently boiled and loaded into a 10% SDS-PAGE gel. The gel was then stained with Coomassie Blue G-200 stain, dried onto filter paper, and then exposed to phosphorimager for 24 hours.

Results

PRMT2 Is Related to Protein Arginine Methyltransferases

Analysis of the predicted polypeptide encoded by the PRMT2 gene in *C. reinhardtii* indicated that it possesses a well-conserved protein methyltransferase domain. We were also able to identify a conserved SAM binding domain within the methyltransferase motif ([44]) (Figure 1A). Within this sub-domain, there is a highly conserved region that contains the following sequence: VLDVG_G (Figure 1B). A point mutation in the last G (to an R) has been shown to eliminate all methylation activity *in vitro* ([60],[61]). A phylogenetic analysis using the neighbor-joining method showed that PRMT2 is related to other class I asymmetric dimethyltransferases, including human PRMT3. However, PRMT2 clusters together with proteins from *Arabidopsis*, *Oryza*, and *Ostreococcus* in a well-supported group (Figure 1C). Although human PRMT3 is the more closely related mammalian homolog to *C. reinhardtii* PRMT2, it falls into a different clade of the tree together with proteins from *C. elegans*, *Drosophila*, *Arabidopsis* and *Oryza* (Figure 1C). This observation suggested that PRMT2 orthologs might have evolved specifically within the plant/algal lineage.

PRMT2 Is Involved in RNA Interference

Previous work has shown that MUT70, a component of RISC, co-immunoprecipitates with PRMT2 in *C. reinhardtii* (unpublished data). Thus, we decided to investigate the role of PRMT2 in RNAi. To this end, we used the Maa7-IR44 strain. This strain has an inverted repeat transgene targeting the *MAA7* mRNA, and, as a consequence, the protein produced by this gene, tryptophan synthase β subunit (TSβ), is downregulated. The TSβ protein converts 5-fluoroindole into a toxic tryptophan analog ([54]). However, downregulation of *MAA7* by RNAi allows the cells to grow in the presence of 5-fluoroindole. If PRMT2 is involved in RNAi, we predicted that the depletion of this protein would result in increased levels of *MAA7* RNA and/or the TSβ protein and inability of the cells to grow in the presence of 5-fluoroindole.

We generated an inverted repeat transgene intended to target the 3' UTR of the PRMT2 mRNA and, thus, downregulate the methyltransferase. The PRMT2-IR transgene was then introduced into the Maa7-IR44 strain. The obtained line, containing two inverted repeat transgenes targeting *MAA7* and PRMT2, respectively, was named PRMT-IRs15. The PRMT2 transcript levels were reduced about 19% in PRMT-IRs15, relative to the parental strain, as shown by a northern blot analysis of total RNA (Figures 2A and 2B). We also complemented the PRMT-IRs15 strain with an epitope (AcV5)-tagged PRMT2 expressed from a transgene with a different 3'UTR and, therefore, not targeted by the RNAi-triggering inverted repeat (Figure 2, strains PRMT-IRs15(PRMT)7 and PRMT-IRs15(PRMT)3). The complemented lines

expressed the AcV5-tagged PRMT2 as revealed by immunoblot analyses (Figure 2C). Moreover, in these strains, the PRMT2 mRNA levels were similar to those of the parental strain, Maa7-IR44 (Figure 2A and 2B)

The phenotype of PRMT-IRs15 resembled that of the wild type strain (CC-124), which expresses the TSβ protein and is sensitive to 5-fluoroindole (Figure 3A). In contrast, the complemented strains retained the ability to grow on medium containing 5-fluoroindole, strongly supporting the argument that the PRMT2 protein is necessary for RNAi. However, there was no difference in small RNA levels (miR1157 and miR912) among any of the strains (Figure S1), suggesting that PRMT2 is not required for the biogenesis of small RNAs.

Based on the lack of sensitivity to 5-fluoroindole, we expected that the *MAA7* mRNA levels in the Maa7-IR44 strain would be downregulated. In contrast, the PRMT-IRs15 line was anticipated to have restored *MAA7* mRNA levels and the complement strains were expected to have similar *MAA7* mRNA levels to Maa7-IR44. However, as shown in Figures 3B and 3C, this was not the case. All strains had slightly reduced *MAA7* mRNA levels relative to the wild type *Chlamydomonas* strain, CC-124. Interestingly, further analysis showed significant downregulation of the TSβ protein in the Maa7-IR44 strain whereas expression of this protein in PRMT-IRs15 was similar to the wild type strain (Figure 2D). These results suggested that TSβ is mainly regulated at the translational level in the Maa7-IR44 strain and that the PRMT2 protein appears to be required for this translational repression in *Chlamydomonas reinhardtii*.

PRMT2 Interacts with MUT70 *in vitro*

Given that PRMT2 is required for translational repression mediated by RNAi and has a conserved methyltransferase domain, we next set out to determine targets for methylation. One RISC component with RGG rich domains is MUT70 (Figure 4A). Moreover, as already mentioned, PRMT2 was shown to co-purify with epitope-tagged MUT70 (unpublished data). To determine if MUT70 and PRMT2 interact directly, PRMT2-His and MUT70-GST fusions were used in an *in vitro* pulldown assay. Another protein, PRMT2-G72R-His containing a point mutation at residue 72, that changes a glycine amino acid to an arginine and abolishes the methyltransferase activity ([61]), was also included in the assays (Figure 4B). All three tagged proteins were tested for interactions in two separate assays. First, the purified MUT70-GST fixed to glutathione beads was used to pulldown either PRMT2-His or PRMT2-G72R-His. Both PRMT2 proteins interacted with MUT70-GST, with PRMT2-G72R-His having stronger affinity for MUT70-GST (Figure 4B). Indeed, the mutation of the PRMT2 methyltransferase domain was expected to prolong substrate association because of the deficient catalytic activity and presumably delayed substrate release. However, the affinity of PRMT2 for MUT-70 was fairly weak since only a small amount of the input protein was retained in the MUT70-GST associated beads (Figure 4B). Similar results were obtained using PRMT2-His or PRMT2-G72R-His to pulldown MUT70-GST (Figure 4C). These results suggested that MUT70, which has two RGG rich regions, could interact directly, albeit weakly, with PRMT2.

PRMT2 Methylates MUT70 *in vitro*

The sequence analysis of PRMT2 indicated that it might have methyltransferase activity, and we have shown that it interacts *in vitro* with MUT70. We therefore tested if PRMT2 can methylate MUT70. Using purified recombinant proteins, we set out to see if PRMT2-His modifies MUT70-His when C^{14} labeled SAM was used as a methyl donor. By autoradiographic detection, MUT70-His was indeed found to be methylated by PRMT2-His in this *in vitro* assay (Figure 5). PRMT2-His methylated multiple proteins from an *E. coli* cell lysate, with the most pronounced band being the induced MUT70-His. Using purified MUT70-His as the substrate resulted in a stronger degree of methylation. In contrast, the PRMT2-G72R mutated protein showed no methylation activity, as expected, because of the point mutation in the SAM binding domain ([61]). Our results demonstrate that recombinant PRMT2 is functional as a protein methyltransferase and that MUT70 is a substrate for the PRMT2 activity at least *in vitro*.

Discussion and Conclusions

The machinery of the RNA interference pathway has been studied intensely, from components involved in the biogenesis of small RNAs to those responsible for the degradation of target mRNAs. However, there are still questions that need to be answered. For example, little is known about the regulation of gene expression by RNAi through translational repression ([62],[63]). The proteins associated with AGO and Dicer and their roles as a part of RISC are also poorly understood. Somewhat surprisingly and contrary to previous beliefs, it has recently been reported that complementary siRNAs with no mismatches can induce translational repression ([13]).

Protein arginine methyltransferases have been found to interact with the RNA interference machinery ([51], [37], unpublished data), and in animals have been shown to play a role in piRNA function through the methylation of Ago3 ([41], [42]). However, these findings involved symmetric arginine methylation catalyzed by PRMT5 homologs. Other studies reported the occurrence of asymmetric methylation of the RISC interactor, FMRP, but this modification has not been implicated in RNAi ([51]). Here we have shown for the first time that putative asymmetric arginine methylation may be required for RNAi translational repression mediated by RNAi in the model organism *Chlamydomonas*. PRMT2, a protein arginine methyltransferase related to enzymes that catalyze asymmetric arginine methylation, is necessary for

RNAi in *Chlamydomonas reinhardtii*. In fact, we have previously shown that PRMT2 co-purifies *in vivo* with MUT70 (unpublished data), which is a component RISC ([39]). MUT70 is an RNA binding protein that has two RGG rich regions, potential targets for arginine methylation. We have also demonstrated here that these proteins directly interact *in vitro*, and that recombinant PRMT2 is able to methylate recombinant MUT70.

The interaction between the recombinant PRMT2 and MUT70 proteins was relatively weak when compared to the amount of input proteins. We hypothesized that a mutation in the SAM binding domain of PRMT2 would allow PRMT2 and MUT70 to have a stronger interaction because of delayed substrate release by a catalytically inactive enzyme. Indeed, the mutant PRMT-G72R protein showed increased affinity for MUT70, but the interaction was still very weak compared to the input protein amounts. Future experiments will be needed to validate the interaction between PRMT2 and MUT70, perhaps by using affinity purification from *Chlamydomonas* cells, or by transforming the point-mutated PRMT2 into *C. reinhardtii* and using this tagged protein to identify interactors in pulldown assays.

Recent studies have identified arginine methylation as necessary for efficient RNA interference by piRNAs in metazoans. This modification stabilizes PIWI proteins in *Drosophila* ([41]). The murine PIWI proteins interact with and are methylated by PRMT5, along with its cofactor, WDR77. The PIWI associated RISC includes different interactors than the AGO associated RISC, but the mouse homolog of Vasa has been implicated as a PIWI interactor ([42]). Interestingly, the vasa intronic gene (called MUT70 in *Chlamydomonas*) is located within an intron of the

Vasa gene and is expressed at its highest levels in germline cells in *Drosophila*, although the two proteins share nothing else in common ([40]).

We have shown that PRMT2 interacts with and methylates MUT70. In the present study, we were unable to pulldown any RISC specific interactors while using epitope-tagged PRMT2 (data not shown). The same was seen when using an epitope-tagged PRMT5 in Drosophila germline cells, but its epitope-tagged cofactor, WDR77, did interact with the PIWI proteins. However, we have previously shown that MUT70 co-purifies with PRMT2 when an epitope-tagged MUT70 is used for affinity purification. This is similar to the findings that epitope-tagged PIWI proteins pulldown PRMT5 in metazoans. These observations raise the possibility that an accessory protein may be the keystone to an interaction between PRMT2 and RISC.

There are RGG regions at the N-terminal end of both the murine and Drosophila PIWI proteins that have been shown to be methylated by PRMT5 and are subsequently recognized and bound by Tudor-domain containing proteins ([64]). Tudor motifs are known to bind methylated arginines. This recognition and binding by Tudor proteins has been shown to be needed for proper subcellular localization of the polypeptides ([41], [42]). It seems reasonable, given this evidence, to look for Tudor proteins in *Chlamydomonas* in order to assess their role in RNAi.

New directions to examine the direct function of PRMT2 in RNA interference should also include *in vivo* methylation studies of MUT70 and AGO proteins. Further analysis of the methylation sites on MUT70 would likely give insight to the function of the methylation itself. The methylation of arginine residues neutralizes positive charges, and the methylation of multiple residues would alter the properties

of the protein more than at a single site. Therefore, the amount of methylation of the target protein should be identified. Arginine methylation also alters RNA binding on certain RNA binding proteins as is the case in FMRP ([51]). Thus, MUT70 methylation may alter specificity of the mRNA targets that are though to be scanned by this protein. Another possibility is that the methylation could affect localization of the MUT70 protein within the cell. The human Ki-1 antigen (which is related to MUT70) is methylated by PRMT1 and has both cytoplasmic and nuclear localization with higher concentrations in the cytoplasm. However, after treatment with methylation inhibitors, the Ki-1 antigen displays reduced concentration in the cytoplasm ([65]). Thus, the methylation on MUT70 could alter its subcellular localization, which would in turn affect the effectiveness of RNA interference, as seen for PIWI methylation and Tudor protein interactions.

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Figure 1

Refer to “A Novel Protein Arginine Methyltransferase.ppt”

Figure 1. Phylogenetic analysis of PRMT2. (A) Representation of the PRMT2 protein sequence showing a conserved methyltransferase domain, which contains a SAM binding motif. (B) Alignment of the SAM binding domain of related PRMT sequences using ClustalX. The asterisk denotes the conserved glycine that was changed to an arginine to produce a nonfunctional mutant. (C) A neighbor-joining tree showing the phylogenetic relationship among 15 protein arginine methyltransferases. Sequences aligned with the ClustalX program were used to draw a tree with the MEGA 4 program. The numbers on the branches indicate bootstrap values as a percentage based on 1000 pseudoreplicates (only values greater than 60% are shown). Abbreviations are as follows: Cr is *C. reinhardtii*, Ot is *O. tauri*, AT is *A. thaliana*, Os is *O. sativa*, Ce is *C. elegans*, Dmel is *D. melanogaster*, and Hs is *H. sapiens*. The accession numbers for the protein sequences used are as follows: Cr_PRMT2, XP_001702822.1; Ot07g00730, CAL54346.1; Os06g0142800, NP_001056772.1; OsJ_16622, EAZ32411.1; Os07g0671700, NP_001060600.1; AT1G04870, NP_563720.1; AT3G20020, NP_188637.2; AT4G29510, NP_194680.1; AT3G06930, NP_850528.1; AT5G49020, NP_974913.1; Dmel_CG6563, NP_731984.1; Dmel_CG5358, NP_649963.1; Ce_Y113G7B.17, NP_507909.1; Hs_PRMT3, NP_005779.1; and Hs_CARM1, NP_954592.1.

Figure 2

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Figure 2. RNAi mediated suppression of PRMT2 in *Chlamydomonas reinhardtii* and complementation of the epi-mutant with a PRMT2 transgene having an unrelated 3' UTR. (A) Northern blot Analysis of PRMT2 mRNA levels from the wild type strain (CC-124), Maa7-IR44, which has a transgene targeting the *MAA7* gene, PRMT-IRs15, which has a transgene targeting the PRMT2 gene introduced into the Maa7-IR44 strain, and the PRMT2 complemented strains, PRMT-IRs15(PRMT)3 and PRMT-IRs15(PRMT)7. (B) The graph illustrates PRMT2 mRNA levels normalized to the wild type strain. The results are the average of three independent experiments +/- the standard deviation. (C) Western blot analysis of ACV5-tagged PRMT2 protein expression levels. An AcV5-tagged MUT70 was used as a positive control.

Figure 3

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Figure 3. PRMT2 is required for RNAi in *Chlamydomonas*. (A) Growth and survival of the indicated strains on TAP or on TAP media containing 5-fluoroindole. The panel includes the wild type strain (CC-124), the transgenic parental strain (Maa7-IR44), the downregulated PRMT2 strain (PRMT-IRs15), and the PRMT2 complementary strains (PRMT-IRs15(PRMT)3 and PRMT-IRs15(PRMT)7). (B) Northern blot analysis of *MAA7* mRNA levels in the indicated strains. The *Actin* mRNA amount was used as a control for equivalent loading of the lanes. (C) The graph represents *MAA7* mRNA levels normalized to the wild type strain. The results are the average of three independent experiments +/- the standard deviation. (D) Western blot analysis of tryptophan synthase β subunit levels in the indicated strains. The same samples were run in parallel and stained with Coomassie Blue as a control for equivalent loading of the lanes.

Figure 4

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Figure 4. PRMT2 interacts with the RISC component MUT70 *in vitro*. (A) Diagram of the MUT70 protein showing RGG/RGR rich regions. (B) SDS-PAGE analysis of an *in vitro* pulldown assay, using glutathione beads bound to either GST or a MUT70-GST fusion protein. After incubation with PRMT2, PRMT2-G72R, or an ALFin-like protein a western blot analysis was performed using an S-tag antibody to detect bound PRMT2, PRMT2-G72R, and the ALFin-like protein (right panel). The left panel shows 5% of the input PRMT2, PRMT2 G72R, and the Zn finger protein that were incubated in the pulldown assay. Coomassie Blue stained gels of equivalent samples are shown at the bottom. (C) SDS-PAGE analysis of an *in vitro* pulldown, using His, PRMT2-His or PRMT2 G72R-His bound to Ni-NTA His beads to pulldown Mut70-GST. After incubation with MUT70-GST, a Western blot analysis using an anti-GST antibody was used to test for interaction. The membranes stained with Ponceau S are shown at the bottom.

Figure 5

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Figure 5. PRMT2 methylates MUT70 *in vitro*. SDS-PAGE analysis of a C^{14}-SAM methylation assay. The MUT70-His protein bound to Ni-NTA beads or an *E. coli* cell extract expressing MUT70-His were incubated with C^{14} labeled SAM and purified PRMT2-His, PRMT2-G72R-His, or nothing *in vitro.* Methylation activity was detected by autoradiography (top panels). The corresponding Coomassie Blue stained gels are shown at the bottom.

Supplemental Figure 1

Refer to “A Novel Protein Arginine Methyltransferase.ppt”

Supplemental Figure 1. PRMT2 depletion has no affect on miRNA levels. (A) A northern blot analysis of miRNAs miR1157 and miR912 in the wild type strain, the transgenic parental strain, the downregulated PRMT2 strain, and a complemented strain. Detection of U6 snRNA was used as a control for equivalent loading of the lanes. (B) The graph represents miRNA levels normalized to those of the wild type strain. The results are the average of two independent experiments.