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## Rmt1 catalyzes zinc-finger independent arginine methylation of the small ribosomal protein Rps2 in *Saccharomyces cerevisiae*

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### Abstract

Rps2/rpS2 is a well conserved protein of the eukaryotic ribosomal small subunit. Rps2 has previously been shown to contain asymmetric dimethylarginine residues, the addition of which is catalyzed by zinc-finger-containing arginine methyltransferase 3 (Rmt3) in the fission yeast *Schizosaccharomyces pombe* and protein arginine methyltransferase 3 (PRMT3) in mammalian cells. Here we demonstrate that despite the lack of a zinc-finger-containing homolog of Rmt3/PRMT3 in the budding yeast *Saccharomyces cerevisiae*, Rps2 is partially modified to generate asymmetric dimethylarginine and monomethylarginine residues. We find that this modification of Rps2 is dependent upon the major arginine methyltransferase 1 (Rmt1) in *S. cerevisiae*. These results are suggestive of a role for Rmt1 in modifying the function of Rps2 in a manner distinct from that occurring in *S. pombe* and mammalian cells.

### Keywords

ribosomes; protein arginine methylation; posttranslational modification of proteins; methyltransferases; ribosomal assembly; asymmetric dimethylarginine

### Introduction

The functional roles of many post-translational modifications are still being discovered. Protein arginine methyltransferases (PRMTs) have been found to act on many cellular proteins and tend to be well conserved between organisms [1-4]. In humans, there appear to be at least nine members of the PRMT family; fewer members are found in fungi, and no examples have been found to date in prokaryotes [1,2,5]. Enzyme family members can be distinguished by the number and configuration of methyl groups that they transfer to arginine residues, as well as by the presence of additional binding domains. The functional roles of this modification are varied and include regulation of transcription, splicing, DNA repair, protein translocation, and signaling [1-4].

We have been interested in the modification of ribosomal proteins by arginine methylation. Two sites of such modification have been described to date [6]. The large ribosomal protein Rpl12 is modified at an internal guanidino nitrogen atom in an arginine residue in fungal species

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by a unique enzyme Rmt2 [5,7,8]. The small ribosomal protein Rps2/rpS2 is modified at the terminal guanidine nitrogen atoms of one or more arginine residues to generate asymmetric dimethylated derivatives in both fungal and mammalian species [9-11].

The mammalian PRMT3 enzyme has been shown to catalyze the methylation of rpS2 [9]; its homolog Rmt3 in the fission yeast *S. pombe* has been shown to catalyze methylation of the ribosomal protein homolog Rps2 [10]. Notably, PRMT3 and Rmt3 have been found to physically associate with rpS2/Rps2 in mammalian cells and *S. pombe* and it appears that this ribosomal protein may be a major substrate of these enzymes [9-14]. Both of these enzymes contain an N-terminal addition of a zinc-finger domain that is required for the binding of enzyme and Rps2/rpS2 and for most or all of their methyltransferase activity with these substrates [9,11]. Interestingly, it appears that at least one role of the physical association of enzyme and substrate is independent of methyltransferase activity since catalytically inactive forms of Rmt3 can rescue the ribosomal subunit ratio defect found in *S. pombe* cells lacking the enzyme [11].

However, the budding yeast *S. cerevisiae* does not have a homolog to PRMT3. Despite this, we have found one or more arginine residues in Rps2 are methylated in these cells. We now show that the zinc-fingerless PRMT1 homolog Rmt1 is required for the methylation of Rps2 in *S. cerevisiae*. We suggest that protein arginine methylation of Rps2 may play a distinct role in organisms lacking homologs of PRMT3/Rmt3 [2,5].

## Materials and methods

### Yeast Strains

*S. cerevisiae* strains were obtained from the Saccharomyces Genome Deletion Project and included the parent strain BY4742 and the *RMT1*, *RMT2*, and *HSL7* gene deletion strains in this background. Cells were grown at 30° C in YPD media (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) to an optical density of 0.5 – 1.0 at 600 nm. The cells were subsequently harvested by centrifugation at 4° C for 5 min at 5,000 × g.

### Isolation of ribosomal small subunit proteins

Cells were *in vivo* labeled with *S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine ([<sup>3</sup>H]AdoMet), ribosomes were prepared from extracts, and ribosomal subunits were separated by centrifugation on a high salt sucrose gradient according to the protocol described in [15], except that buffer A contained proteinase inhibitors from the Roche Proteinase Inhibitor Cocktail Tablet and buffer B contained 750 mM KCl. Briefly, the ribosomal fraction re-suspended in buffer B was layered on top of a 10%-38% sucrose gradient made in the presence of buffer B and centrifuged for 60,000 × g for 18.5 h at 4° C (unless otherwise specified) using a Beckman type SW41 rotor. Selected fractions were combined and then ethanol and acetic acid extracted as described in [15] to precipitate protein and remove RNA. The post-dialysis sample was lyophilized and re-suspended in 100 μl water. Protein concentration was determined by precipitating an aliquot using 10% trichloroacetic acid by the Lowry method using bovine serum albumin as a standard. For SDS gel electrophoresis, samples were combined with 2× dye, heated at 2 min at 100° C, and loaded onto a 12.6% SDS-PAGE in the Lammeli buffer system [16]. The gels were rocked overnight submerged in Coomassie Brilliant Blue dye, and then de-stained to enable visualization of protein bands.

### Mass Spectrometry

Analysis by mass spectrometry was performed on an Applied Biosystems Q-Star Elite instrument in an approach similar to that used previously [17]. Briefly, the small subunit proteins were injected into a PLRP-S polymeric column with pore size of 300Å, bead size of

5  $\mu\text{m}$ , and dimensions of 150  $\times$  1.0 mm (Polymer Laboratories, Amherst, MA) which was maintained at 50° C for the duration of the run. The column was initially equilibrated with 5% B (0.05% trifluoroacetic acid in acetonitrile) and 95% A (0.05% trifluoroacetic acid in water), and the proteins were eluted at 50  $\mu\text{l}/\text{min}$  with 5 min at starting conditions, followed by a 25 min gradient from 5% to 30% B, then a 45 min gradient from 30% to 60% B, and finally a 15 min gradient from 60% to 100% B. The Q-Star mass spectrometer was calibrated using Glufib and run at a mass window of 300-2200, accumulation time of 1.5, source position 13;+5, and a DP of 140.

## Results

### Rps2 from *S. cerevisiae* contains asymmetric dimethylarginine

Previous studies have indicated that homologs of the Baker's yeast small subunit ribosomal protein 2 are asymmetrically dimethylated at arginine residues in *S. pombe* and in mammalian cells [9,10]. To determine whether Rps2 of *S. cerevisiae* was also modified in this manner, we purified the wild type polypeptide by isolating ribosomes, preparing small subunit proteins, and separating the polypeptides by SDS-PAGE from cells labeled *in vivo* with [<sup>3</sup>H]AdoMet as a methyl donor. [<sup>3</sup>H]labeled Rps2 was excised from the gel, acid hydrolyzed, and the resulting amino acids analyzed by high resolution cation exchange chromatography (Fig. 1). We found that radioactivity eluted in the positions expected for asymmetric dimethylarginine (ADMA) and monomethylarginine (MMA); no radioactivity eluted in the position of methylated lysine residues (Fig. 1). This result is consistent with product formation of type I protein arginine methyltransferases [1].

In *S. pombe* and mammalian cells, Rps2 homologs are substrates of a specific type I protein arginine methyltransferase (Rmt3 and PRMT3) that is characterized by an N-terminal domain including a zinc finger motif [9,10,18]. However, *S. cerevisiae* does not have such a protein arginine methyltransferase; the closest homolog is the Rmt1 species that is most similar to Rmt1 in *S. pombe* and PRMT1 in mammals (Fig. 2A). In spite of this difference, all of the Rps2 proteins of these organisms are characterized by N-terminal domains containing the canonical GRGG, RXR, and RGG recognition sequences for type I protein arginine methyltransferases, a group that includes both PRMT1 and PRMT3 (Fig. 2B).

### Loss of methylation of yeast Rps2 upon deletion of the RMT1 gene

Although the *S. cerevisiae* genome does not encode a PRMT3 homolog, there are three known protein arginine methyltransferases in *S. cerevisiae*: Rmt1 (also known as Hmt1), Rmt2, and Hsl7. Of these enzymes, only Rmt1 has been shown to catalyze the formation of ADMA [8, 19,20]. While, like PRMT3, these proteins all contain the conserved 7- $\beta$  sheet AdoMet binding domain of Class I methyltransferases, none contain the zinc finger domain that typifies PRMT3 homologs and has been shown to be required for PRMT3's enzymatic activity [9,11,18]. We began by testing knockout strains of all three *S. cerevisiae* arginine methyltransferases and screening by intact mass spectrometry to determine if any showed a loss of methylation for Rps2 (Fig. 3).

For Rps2 from wild type cells, the major peak at 27,362.2 Da corresponds to that of the unmodified protein lacking the initiator methionine residue and acetylated on the serine-2 residue as expected [21]. The calculated average mass is 27362.9; our experimental value is within 26 ppm, well within the expected error of 100 ppm. We also detected secondary peaks with masses that are about 13.4 and 31.3 Da larger that could correspond to the presence of substoichiometric levels of mono and dimethylated species (Fig. 3, top panel). The deconvoluted mass profile for Rps2 from cells lacking either the Rmt2 or the Hsl7 protein arginine methyltransferases was not different from that of the wild type cells shown in Fig. 3 (data not

shown). However, for Rps2 purified from cells lacking the Rmt1 methyltransferase, we see a reduction in both of the secondary peaks (Fig. 3, bottom panel). These results suggest that Rmt1 catalyzes the methylation of Rps2; the presence of a species from the protein lacking Rmt1 at 16 Da greater than the N-acetylated serine form suggests the presence of an oxidized species. Some of the signal in the secondary species in the wild type sample may also reflect the presence of some oxidized material.

To confirm the identification of Rmt1 as the methyltransferase responsible for the modification of Rps2, we *in vivo* labeled both wild type and  $\Delta rmt1$  strains to test whether Rmt1 was necessary for [ $^3\text{H}$ ]methyl addition to Rps2. Ribosomes were isolated by centrifugation, subunits separated by high salt sucrose gradient sedimentation, and the small subunit proteins pooled for each sample and further separated by SDS-PAGE. A protein band corresponding to Rps2 can be seen in both wild type and  $\Delta rmt1$  samples (Fig. 4, top panel). However, the radioautograph clearly shows the loss of [ $^3\text{H}$ ]methylation of Rps2 in the  $\Delta rmt1$  strain (Fig. 4, lower panel). This provides further evidence that Rps2 methylation is dependent upon the methyltransferase Rmt1 in *S. cerevisiae*.

## Discussion

Protein arginine methylation of Rps2 in the fission yeast *S. pombe* and mammalian cells has been closely linked to a zinc-finger containing enzymes designated Rmt3 and PRMT3 [9-14]. The zinc-finger of the methyltransferase appears to be essential for the modification of Rps2 in mammalian cells [9]. We were thus surprised to see Rps2 protein arginine methylation in the budding yeast *S. cerevisiae* that lacks this member of the protein arginine methyltransferase family. We have shown here that the methylation of Rps2 in *S. cerevisiae* is catalyzed by the major Rmt1 enzyme which does not contain a zinc-finger.

Like many other ribosomal proteins, Rps2 has been implicated as playing roles in the ribosomal biosynthesis [22,23]. Recent studies in *S. pombe* have indicated that Rps2 is essential for the proper processing of the A2 cleavage event that leads to the 18S rRNA [22]. In the absence of Rps2, synthesis of the 40S subunit is abolished [22]. Evidence has been presented that interaction of Rps2 in *S. pombe* with the Rmt3 protein arginine methyltransferase modulates the efficiency of 40S synthesis, as indicated by the altered 40S:60S ratio seen in cells lacking Rmt3 [10-12].

Studies of the role of Rps2 in rRNA processing in *S. cerevisiae* have shown that a depletion of Rps2 leads to export defects of the 20S rRNA [23]. This is interesting in light of the fact that Rmt1 has been shown to play a role in facilitating export for a number of its previously determined substrates, such as the mRNA-binding protein Npl3 [24,25]. Thus methylation of Rps2 in *S. cerevisiae* may play a role in the export to the cytosol of ribosomal RNA species. Significantly, we have found that the methylation of Rps2 at arginine residues is substoichiometric; the bulk of the protein is unmodified. Previous results have suggested that RNA binding may impede the ability of Rmt1 to methylate its substrates [26,27]. We have observed varying levels of methylation of Rps2 dependent upon growth conditions (unpublished data), which may support a functional regulatory role for this modification. Due to the nuclear localization of at least a fraction of Rmt1 [28], it seems possible that Rps2 methylation occurs in the nucleus. This is distinct from the situation with *S. pombe* and mammalian cells, where the zinc-finger containing enzymes Rmt3 and PRMT3 appear to be wholly cytosolic. In these organisms, the modification of Rps2 may occur in the cytosol before it is imported into the nucleolus for its role in ribosomal assembly. The degree of modification of rps2 in mammalian cells appears to be stoichiometric because protein purified from wild type cells is not a substrate for methylation by recombinant PRMT3 [13]. Additionally, mass

spectrometric analysis suggests that the formation of eight dimethylarginine residues in Rps2 of *S. pombe* [11].

These results provide evidence that protein arginine methylation of Rps2 in budding yeast may have a distinct function than that in fission yeast or mammalian cells [22]. In mammalian cells, it has been suggested that PRMT3 and Rps2 form a complex together that leads to resistance to ubiquitination and degradation [14]. This does not appear to be the case in *S. cerevisiae*, since the protein is largely unmodified. In *S. pombe*, it has been suggested that there is a methylation-independent function of Rmt3 in regulating levels of 40S [11].

Rmt1 has been most extensively studied in terms of its role in transcription, rRNA processing, and mRNA export from the nucleus. The arginine-3 residue of histone H4 is methylated by Rmt1 leading to transcription repression [29,30]. However, Rmt1 is also recruited to areas of high transcriptional activity for methylation of mRNA proteins involved in mRNA processing and export [31]. Rmt1 has also been demonstrated to methylate the nucleolar proteins Gar1, Nop1, and Nsr1 [32]. Although the functions of the modifications are unknown, these three proteins have previously been connected to rRNA processing. A number of the target proteins of Rmt1, including Npl3, Hrp1, and Nab2, function in shuttling mRNA to the cytosol. The methylation of these proteins by Rmt1 appears to promote their dissociation from nuclear factors and facilitate export from the nucleus [24,25,33]. Like with other Rmt1 substrates, it will be interesting to explore in the future whether methylation of Rps2 affects the protein-protein interactions that facilitate import and/or export of this RNA binding protein.

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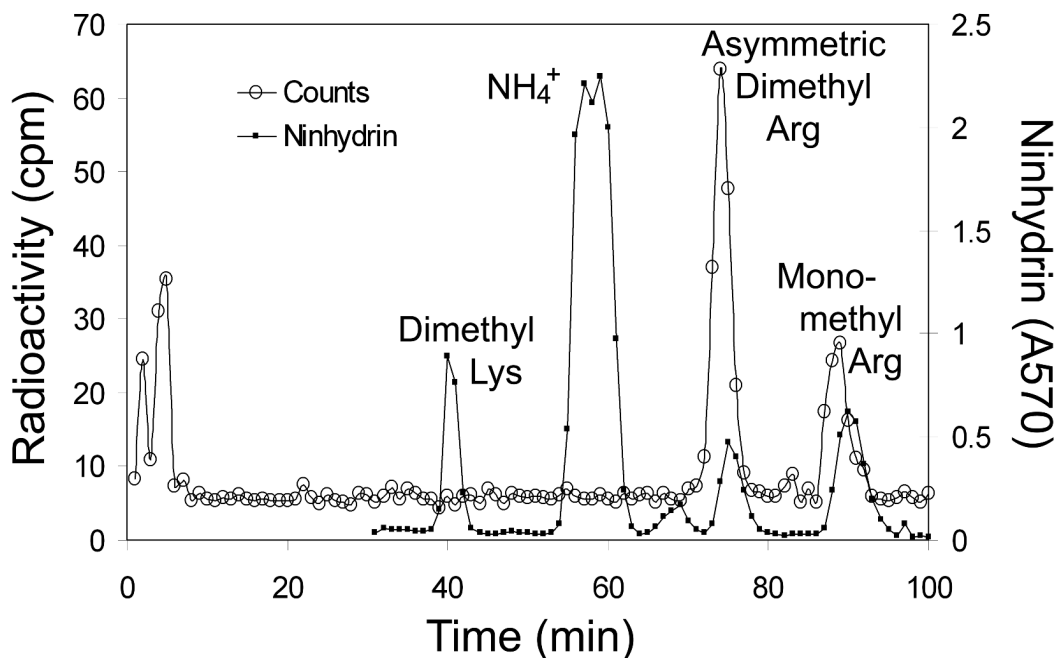
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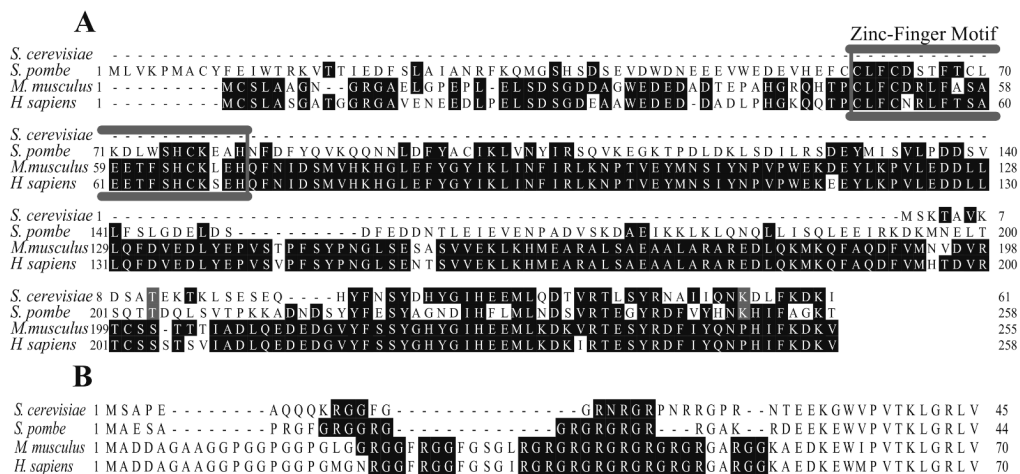
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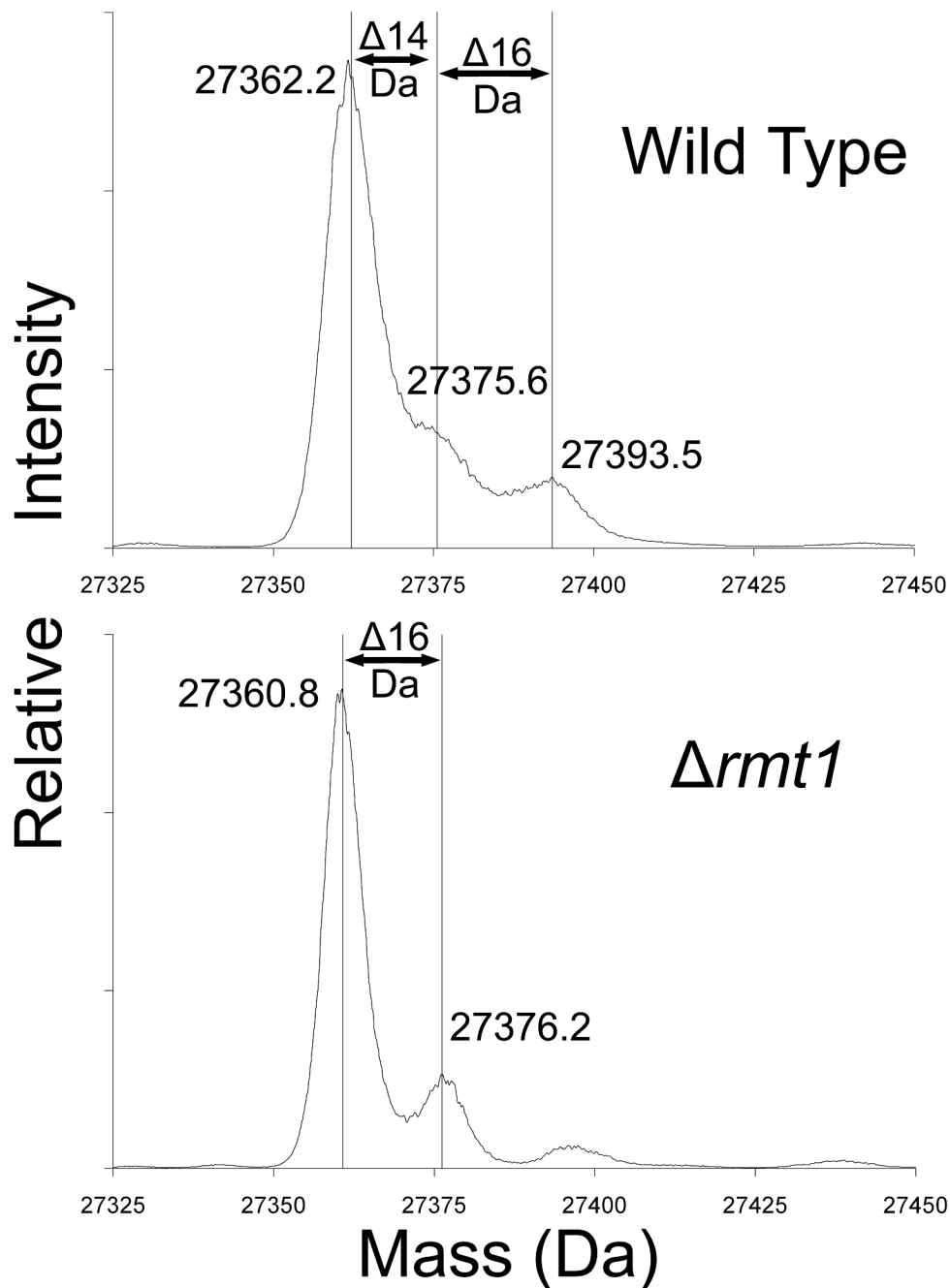
**Fig. 1.**

Detection of asymmetric dimethylarginine and monomethylarginine residues in yeast Rps2. Amino acid analysis of the [ $^3\text{H}$ ]methylated  $\sim 27$  kDa polypeptide (Rps2) of the *S. cerevisiae* small ribosomal subunit. Cells (BY4742, *RMT1+*) were labeled *in vivo* with [ $^3\text{H}$ ]AdoMet and small ribosomal subunit proteins were isolated as described in Materials and methods. These proteins were fractionated by SDS gel electrophoresis as described in Materials and methods for 35 mA for 2.5 h, and then at 20 mA for 5 h. After Coomassie-staining, the band under the 31 kDa marker was excised, minced, and placed in a  $6 \times 50$  mm flint glass tube. Acid hydrolysis and amino acid fractionation by ion exchange chromatography were performed as described in [16] with added standards of 1  $\mu\text{mol}$  each of epsilon-dimethyllysine,  $N^G, N^G$ -dimethylarginine, and  $N^G$ -monomethylarginine. The *open circles* show radioactivity from 200  $\mu\text{l}$  of 1 min (1 ml) fractions collected. The *closed circles* show the absorbance at 570 nm of ninhydrin assays of 100  $\mu\text{l}$  of the fractions for the added methylated amino acid standards. In this high-resolution chromatography, the tritiated methylated derivatives elute slightly before the non-isotopically labeled standards [19].



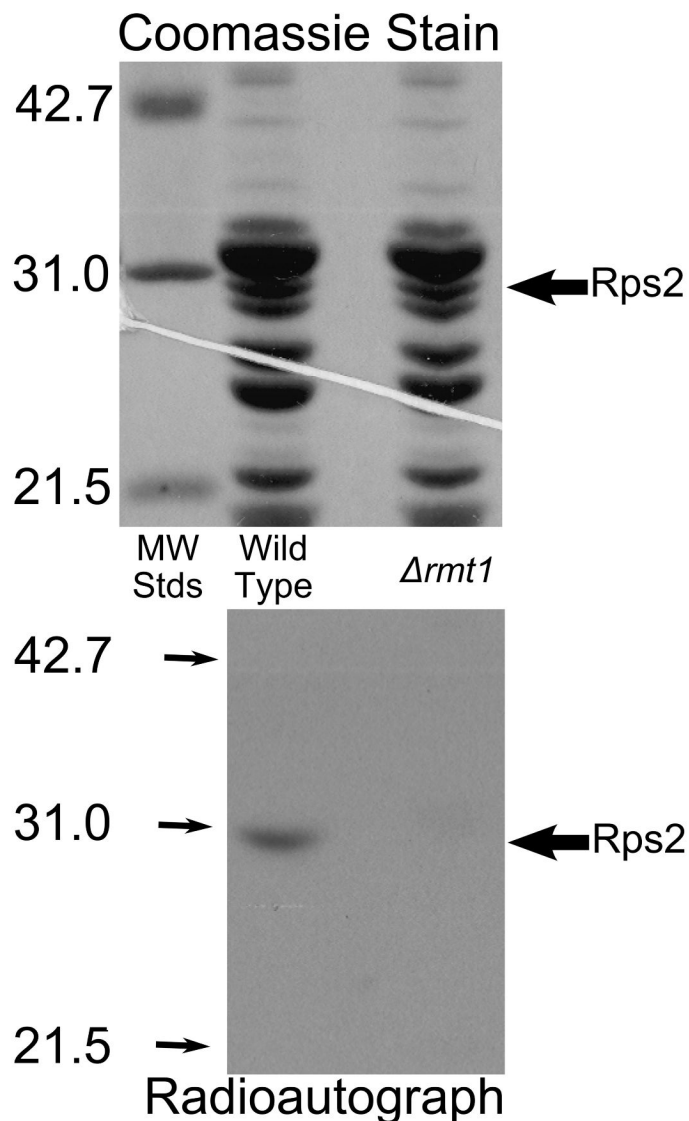


**Fig. 2.** Amino acid sequence conservation of Rmt1/Rmt3/PRMT3 and Rps2. (A) Aligned sequences for the N-terminal region of Rmt1 (*S. cerevisiae*, NP\_009590), Rmt3 (*S. pombe*, NP\_595572) and PRMT3 (*M. musculus*, NP\_598501; *H. sapiens*, NP\_005779). Identical residues are shaded, and the N-terminal zinc-finger motif is boxed in grey. (B) Aligned sequences for the N-terminus of Rps2 homologs, showing the RG-rich region (*S. cerevisiae*, NP\_011392; *S. pombe*, NP\_588435; *M. musculus*, NP\_032529; *H. sapiens*, NP\_002943). Protein arginine methyltransferase recognition motifs are shaded, including GRGG, RGG, and RXR [4,34, 35].

**Fig. 3.**

Loss of methylation of Rps2 in yeast cells lacking the Rmt1 protein arginine methyltransferase. Small subunits were isolated from the ribosomal fraction of *RMT1*<sup>+</sup> cells (wild type) and of  $\Delta rmt1$ <sup>-</sup> cells prepared as described in Methods and materials but in 1 l of growth media without the [<sup>3</sup>H]AdoMet incubation. Due to greater cell pellet volumes, 4.5 ml buffer A was used to lyse each cell pellet and ribosomal pellets were individually re-suspended in 1 ml buffer B. An abbreviated gradient of 7%-27% sucrose made in buffer B was centrifuged for 16 h under the same conditions noted in Materials and methods. Small subunit proteins were prepared as described in Materials and methods except that dialysis was not performed and the sample supernatants after ethanol and acetic acid treatment were directly concentrated by vacuum

centrifugation for about 35 min to bring the total volumes to approximately 100  $\mu$ l. A final centrifugation of  $20,000 \times g$  for 5 min removed any particulate matter from solution, and 30  $\mu$ l of each concentrated sample was analyzed by liquid chromatography/mass spectrometry using an Applied Biosystems Q-Star Elite instrument as described in Materials and methods. Data for the peak at 51 min containing Rps2 were de-convoluted using Analyst software and the resulting average masses of significant peaks are shown. Mass changes are indicated for 14 Da (methylation) as well as 16 Da (oxidation).



**Fig. 4.**

Loss of methylation of Rps2 in  $\Delta rmt1$  mutant cells. Intact cells were labeled with [ $^3\text{H}$ ]AdoMet as described in Materials and methods except that cells were grown initially in 1 l of medium and labeled with 2.0 nmol of [ $^3\text{H}$ ]AdoMet (Perkin Elmer Life Sciences, 83.3 Ci/mmol) in 40 ml of fresh YPD and the resulting cell pellets were lysed individually using 2 g beads and 4.5 ml buffer A for a total of ten cycles. Small ribosomal subunits were prepared by sucrose gradient centrifugation and the protein fraction isolated by ethanol/acetic acid treatment as described in Materials and methods. 50  $\mu\text{g}$  of small subunit proteins were separated by SDS gel electrophoresis for 35 mA for 40 min and then 25 mA for 5 h with molecular weight standards (Bio-Rad Low Molecular Weight; hen ovalbumin, bovine carbonic anhydrase, and soybean trypsin inhibitor) in an adjoining lane. After protein staining (*top panel*), the gel was treated in EN $^3$ HANCE (Perkin Elmer Life Sciences) for 1 h, followed by a 20 min wash in water before being dried under vacuum for 2 h at 80 $^\circ\text{C}$  followed by 1 h without heat. The gel was placed on Kodak BIOMAX XAR film for 3 months at -80 $^\circ\text{C}$  (*bottom panel*). Large arrows indicate the position of the Rps2 band; small arrows indicate the position of the standards in the radioautograph.