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A Novel SET Domain Methyltransferase in Yeast

*Rkm2-DEPENDENT TRIMETHYLATION OF RIBOSOMAL PROTEIN L12ab AT LYSINE 10**

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The ribosomal protein L12ab (Rpl12ab) in *Saccharomyces cerevisiae* is modified by methylation at both arginine and lysine residues. Although the enzyme responsible for the modification reaction at arginine 66 has been identified (Rmt2), the enzyme(s) responsible for the lysine modification(s) has not been found, and the site(s) of methylation has not been determined. Here we demonstrate, using a combination of mass spectrometry and labeling assays, that the yeast gene *YDR198c* encodes the enzyme responsible for the predominant ε-trimethylation at lysine 10 in Rpl12ab. An additional site of predominant ε-dimethylation is observed at lysine 3; the enzyme catalyzing this modification is not known. The *YDR198c* gene encodes a SET domain similar to that of the Rkm1 enzyme responsible for modifying Rpl23ab, and we have now designated the *YDR198c* gene product as Rkm2 (ribosomal lysine methyltransferase 2). The effect of the loss of the enzyme on ribosomal complex stability was studied by polysomal fractionation. However, no difference was observed between the Δ*rkm2* deletion strain and its parent wild type strain. With the identification of this enzyme, it appears that the 12 SET domain family members in yeast can now be divided into two subfamilies based on function and amino acid sequence identity. One branch includes enzymes that modify histones, including Set1 and Set2; the other branch includes Rkm1, Rkm2, and Ctm1, the cytochrome *c* methyltransferase. These studies suggest that the remaining seven SET domain proteins may also be lysine methyltransferases.

Ribosomal proteins are highly modified by post-translational methylation reactions (1). The extent to which ribosomal proteins are modified and the enzymes responsible for the modifications have only begun to be understood. For example, in the yeast *Saccharomyces cerevisiae*, of the three most highly methylated large subunit proteins (Rpl1ab, Rpl12ab, and Rpl23ab) (1, 2), methyltransferases have only been recently identified for two of these species. The Rmt2 enzyme catalyzes the monomethylation of arginine 66 of Rpl12ab (3), and the Rkm1 enzyme catalyzes the dimethylation of two lysine residues in Rpl23ab (4). A mass spectral analysis identified other potentially methylated species within the large subunit of *S. cerevisiae* (5). This study confirmed the modifications of Rpl1ab, Rpl12ab, and Rpl23ab and also provided evidence for the modification of Rpl3, Rpl42ab, and Rpl43ab. Studies of the small subunit in yeast have been less extensive, with the identification of methylated Rps2, Rps3, Rps13, Rps21ab, Rps23a, and Rps25ab (1, 6, 7). The physiological role(s) of these modifications is not known.

In the fission yeast *Schizosaccharomyces pombe*, Prmt3 has been shown to be a ribosomal protein methyltransferase (8). This protein-arginine methyltransferase 3 homolog is responsible for asymmetrically dimethylating an arginine residue in the small ribosomal subunit protein S2 (8). The direct role this modification plays is not known. However, when the gene is absent, an accumulation of 60 S subunits is observed, implicating the modification in small subunit stability (8). In addition, the decreased level of small subunits in Prmt3-deficient cells results in increased ribosome biosynthesis (9).

In mammals, several studies have demonstrated the methylation of ribosomal proteins and the possible effect of the modifications, including modulation of the cell cycle (10–14, 15). A mass spectrometric analysis of 22 of the 32 human small subunit proteins demonstrated that the ribosomal proteins RS10, RS12, and RS25 are modified by methylation (12). Interestingly, the methylation state of RS25 changes when viral RNA binds to the small subunit. Currently, only one mammalian ribosomal methyltransferase (Prmt3) has been identified, and it was shown to modify the rpS2 protein (16). The methyltransferases responsible for modifying the remaining ribosomal proteins and the genes that encode them have yet to be identified.

Since it had previously been demonstrated that the ribosomal large subunit proteins Rpl12ab and Rpl23ab are highly modified at lysine residues in *S. cerevisiae* (1, 2), we decided to identify the methyltransferases that were responsible by analyzing putative methyltransferases that contain a SET domain. The SET domain is an *S*-adenosyl-L-methionine-binding fold, specifically observed in lysine methyltransferases, that does not resemble the canonical seven-β-strand *S*-adenosyl-L-methionine-binding fold observed in most methyltransferases whose structures are currently known (17, 18). SET stands for Su(var), Enhancer of Zeste, and Trithorax, the names of the three *Drosophila* genes in which it was first discovered (19) and which were later shown to encode histone lysine methyltransferases (20–22). The importance of the SET domain in regulating gene

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TABLE 1
Strains
These strains were prepared by the *Saccharomyces* Genome Deletion Project (available on the World Wide Web at www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) and obtained through Invitrogen.

Strain	Genotype
BY4741	MAT**a** *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0
Δ*ydr198c* (BY4741)	BY4741, Δ*ydr198c*::*Kan*^{r}
Δ*rmt2* (BY4741)	BY4741, Δ*ydr465c*::*Kan*^{r}
BY4742	MATα *his*3Δ1 *leu*2Δ0 *lys*2Δ0 *ura*3Δ0
Δ*ydr198c*	BY4742, Δ*ydr198c*::*Kan*^{r}
Δ*ydr465c*	BY4742, Δ*ydr465c*::*Kan*^{r}

expression through the post-translational modification of lysine residues on histones has been recently demonstrated in a variety of studies (23–26). The modification of side chain lysine residues in Rubisco is also catalyzed by a SET domain methyltransferase, although the function of this modification is still under investigation (27–29). However, less attention has been paid to the involvement of SET domain methyltransferases in the modulation of translational function through the modification of ribosomal proteins. Nevertheless, we have recently demonstrated that the SET domain-containing enzyme Rkm1 is responsible for methylating Rpl23ab in *S. cerevisiae* (4).

In this study, we analyzed yeast genes encoding SET domain methyltransferases with the highest sequence identity to Rkm1. We found that the *YDR198c* gene encodes a SET domain-containing lysine methyltransferase that is responsible for the trimethylation of Rpl12ab at lysine 10. With the identification of this second ribosomal lysine methyltransferase, we were able to re-evaluate the yeast SET domain family to show that it consists of two branches, one with the histone methyltransferases and one with the ribosomal and cytochrome *c* methyltransferases.

EXPERIMENTAL PROCEDURES

Purification of Yeast Ribosomal Proteins—Strains used are listed in Table 1. *S. cerevisiae* ribosomal proteins corresponding to the large subunit were purified as described by Porras-Yakushi *et al.* (4), with the modifications indicated below. One-liter cultures were grown to an optical density that ranged between 0.6 and 1.0 at 600 nm, and the cell pellets were lysed in 5 ml of baked zirconium beads and 3 ml of buffer A. After lysis, the beads were washed with an additional 3 ml of buffer A to maximize protein yield. Sucrose gradients (7–27%) were employed. The *in vivo* labeling step was omitted when the ribosomal proteins were only analyzed by mass spectrometry. Under these conditions, we observed a complete separation of the large and small subunits from each other and from contaminating soluble proteins.

Liquid Column Chromatography Coupled to Electrospray Ionization Mass Spectrometry (LC-MS+)[3]—Lyophilized ribosomal proteins purified as described above were resuspended in 100 μl of water and fractionated by reverse-phase liquid chromatography with the effluent split between direct injection into an electrospray ionization mass spectrometer or a fraction collector as described previously (30). A PLRP-S polymeric column was used with a pore size of 300 Å, a bead size of 5 μm, and 150 × 2.1-mm dimensions (Polymer Laboratories, Amherst, MA). The column was equilibrated at 95% solvent A (0.1% trifluoroacetic acid in water) and 5% solvent B (0.1% trifluoroacetic acid in acetonitrile), and the proteins were eluted using a program of 5 min at 5% B, followed by 25 min changing from 5 to 40% B and ending with a 120-min gradient that changed from 40 to 100% B, all at a flow rate of 100 μl/min. An API III+ mass spectrometer (PE Sciex) was tuned and calibrated as described previously (31) to yield a mass accuracy of 0.01% (±2.0 Da at 20 kDa). Approximately 50% of the column flow was directed toward a fraction collector using a "T" flow splitter for further analysis. The 50-μl fractions collected were stored at −20 °C for subsequent proteolytic digestion. Data were analyzed using BioMultiview 1.3.1 software (Applied Biosystems).

Digestion of Rpl12ab with ArgC Proteinase or Chymotrypsin—Half of the fraction (25 μl) collected during the LC-MS+ analysis that was shown to contain Rpl12ab was diluted with 45 μl of 0.5 M ammonium bicarbonate on ice. Following the dilution, 200 ng of ArgC (endoproteinase ArgC from mouse submaxillary gland, suitable for protein sequencing, lyophilized powder; Sigma catalog number P6056) dissolved in 5 μl of 0.5 M ammonium bicarbonate, was added to the reaction mixture. The reaction was then allowed to proceed for 3 h at 37 °C, after which time the reaction was quenched by lyophilization and stored at −20 °C. The other half of the fraction was digested with chymotrypsin (α-chymotrypsin from bovine pancreas, suitable for protein sequencing, salt-free, lyophilized powder; Sigma catalog number C6423) using the same method and concentrations. The peptide fragments obtained were then analyzed by μLC-MSMS mass spectrometry on a Q-Star instrument as described below.

Analysis of Methylated Peptides by Micro-liquid Chromatography Tandem Mass Spectrometry (μLC-MSMS)—Samples were analyzed by μLC-MSMS with data-dependent acquisition (Q STAR XL; Applied Biosystems, Foster City, CA) after dissolution in 10 μl of 0.1% formic acid, 5% acetonitrile (v/v). A reverse-phase column (200 μm × 10 cm; PLRP/S 5 μm, 300 Å; Michrom Biosciences, San Jose, CA) was equilibrated for 20 min at 2 μl/min with 100% A (A, 0.1% formic acid, 5% acetonitrile in water; B, 0.1% formic acid in acetonitrile) prior to sample injection (5 μl). A compound linear gradient was initiated 3 min after sample injection ramping to 80% A, 20% B at 8 min; 65% A, 35% B at 13 min; 25% A, 75% B at 23 min; and 90% A, 10% B at 23.1 min. The column flow was directed to a stainless steel nanoelectrospray emitter (ES301; Proxeon, Odense, Denmark) at 4.4 kV for ionization without nebulizer gas. The mass spectrometer was operated in information-dependent acquisition mode with a survey scan (m/z 400–1500), data-dependent MSMS on the two most abundant ions with exclusion after two MSMS experiments. Individual sequencing experiments were matched to a custom yeast ribosomal protein sequence data base downloaded from NCBI (available on the World Wide Web at www.ncbi.nlm.nih.gov) using Mascot software (Matrix Sciences, London, UK). The search was run under the "no enzyme" mode to identify all peptides, and variable modification of lysine to mono-, di-, and trimethyla-

[3] The abbreviations used are: LC-MS+, liquid chromatography with electrospray ionization mass spectrometry and fraction collection; [^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]L-methionine; MSMS, tandem mass spectrometry; μLC, micro-liquid chromatography; HPLC, high pressure liquid chromatography.

tion was included. The results of Mascot searches were carefully scrutinized, and in some cases MSMS spectra were interpreted further using Prosight PTM (available on the World Wide Web at prosightptm.scs.uiuc.edu/), and peak lists were extracted from the data using the Bayesian peptide reconstruct software (BioAnalyst; Applied Biosystems).

In Vitro Methylation of Rpl12ab by Tap-tagged Rkm2—Large subunit proteins were purified from the Δ*ydr198c* deletion strain as described above. Rpl12ab was further purified from this preparation by the LC-MS+ method described above. Tap-tagged Rkm2 was purified by the method described previously for Rkm1 (4). Fractions 2 and 3 from the last step of the purification were pooled and used as the enzyme source. The yeast strain containing tap-Rkm2 was purchased from Open Biosystems (Huntsville, AL).

For the *in vitro* reactions, 100 μl of purified enzyme was incubated with 10 μl of either purified large subunit proteins or purified Rpl12ab and 10 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; Amersham Biosciences; 1 mCi/ml, 70–81 Ci/mmol, in dilute HCl/ethanol (9:1, v/v), pH 2–2.5), for 2 h at 30 °C. The *in vitro* reaction was buffered by the addition of 10.9 μl of 600 mM Tris-HCl, 1.2 M KCl, 144 mM magnesium acetate, 12 mM phenylmethylsulfonyl fluoride, and 12 mM dithiothreitol. The reaction was precipitated by adding an equal volume of 25% trichloroacetic acid and 2 μl of a 10.4 mg/ml solution of bovine serum albumin, vortexing, and incubating at room temperature for 30 min. After centrifugation for 30 min at 1,000 × *g*, the pellet was washed with 100 μl of acetone at 0 °C. The pellet was dissolved in 100 μl of 6 N HCl and hydrolyzed *in vacuo* for 20 h at 108 °C using a Waters Pico-Tag apparatus. Residual HCl was removed by vacuum centrifugation. The free amino acids were resuspended in 100 μl of water and added to 500 μl of citrate sample buffer (0.2 M NaOH titrated to pH 2.2 with citric acid). 1 μmol of a N^{ϵ},N^{ϵ}-dimethyllysine (Bachem product E-1810) standard was added to the sample before chromatography on a high performance cation exchange column as described previously (4).

Polysomal Fractionation—Yeast strains carrying a single gene deletion in either the *ydr198c* gene (BY4742, Δ*ydr198c*::*Kan*) or the *rmt2* gene (BY4742, Δ*ydr465c*::*Kan*) were grown to an optical density of 0.5–0.8 at 600 nm along with their parent wild type strain BY4742. All strains were purchased from Invitrogen. Polysomal analysis was performed as described by Zanchin *et al.* (32) with a few modifications. Briefly, 300-ml cultures of the wild type, Δ*ydr198c*, and Δ*rmt2* strains were grown to an optical density of 0.5–0.8 at 600 nm and harvested by spinning at 5,000 × *g* for 5 min. The cell pellet was then washed with 10 ml of sterile water and again harvested by centrifugation at 5,000 × *g* for 5 min. The cell pellet was then resuspended in 6 ml of fresh YPD liquid medium and 60 μl of 10 mg/ml cycloheximide and allowed to incubate with shaking for 30 min at 30 °C. The cells were then again harvested and washed as before. The cell pellet obtained was resuspended in 0.5 ml of breaking buffer (20 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate, 100 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl, 100 μg/ml cyclohexamide) and ~0.5 ml of baked zirconium beads (Biospec Products, Bartlesville, OK). The cells were lysed by vortexing for 20 s followed by cooling on ice for 1 min, for a total of eight cycles. Unbroken cells and cell debris were then removed by centrifuging once at 8,000 × *g* for 5 min. A total of 20 units at an optical density of 254 nm were layered onto a 15–49% stepwise sucrose gradient prepared in polysomal buffer (10 mM Tris-HCl, pH 7.4, 70 mM ammonium acetate, 4 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride). The total volume of the gradient was 11.5 ml, including the volume of the sample layered on top. The gradients were then spun for 4 h at 40,000 rpm (274,355 × *g*) in a Beckman type SW41Ti rotor (153.1-mm maximal radius) and then fractionated using an ISCO UA6 apparatus with absorbance monitor attached.

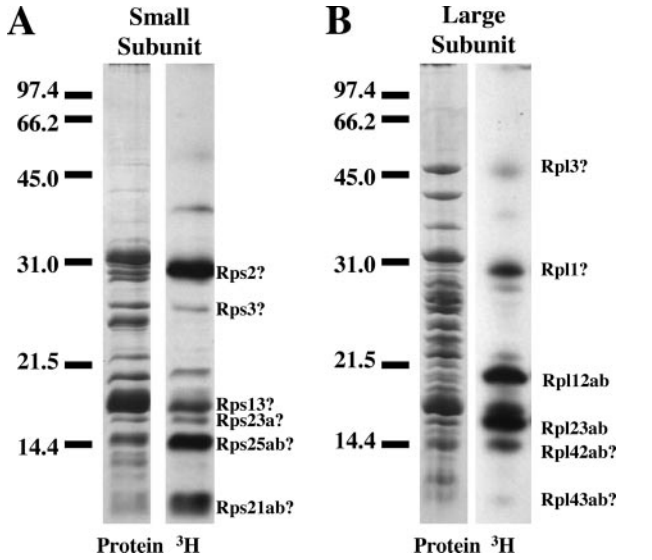
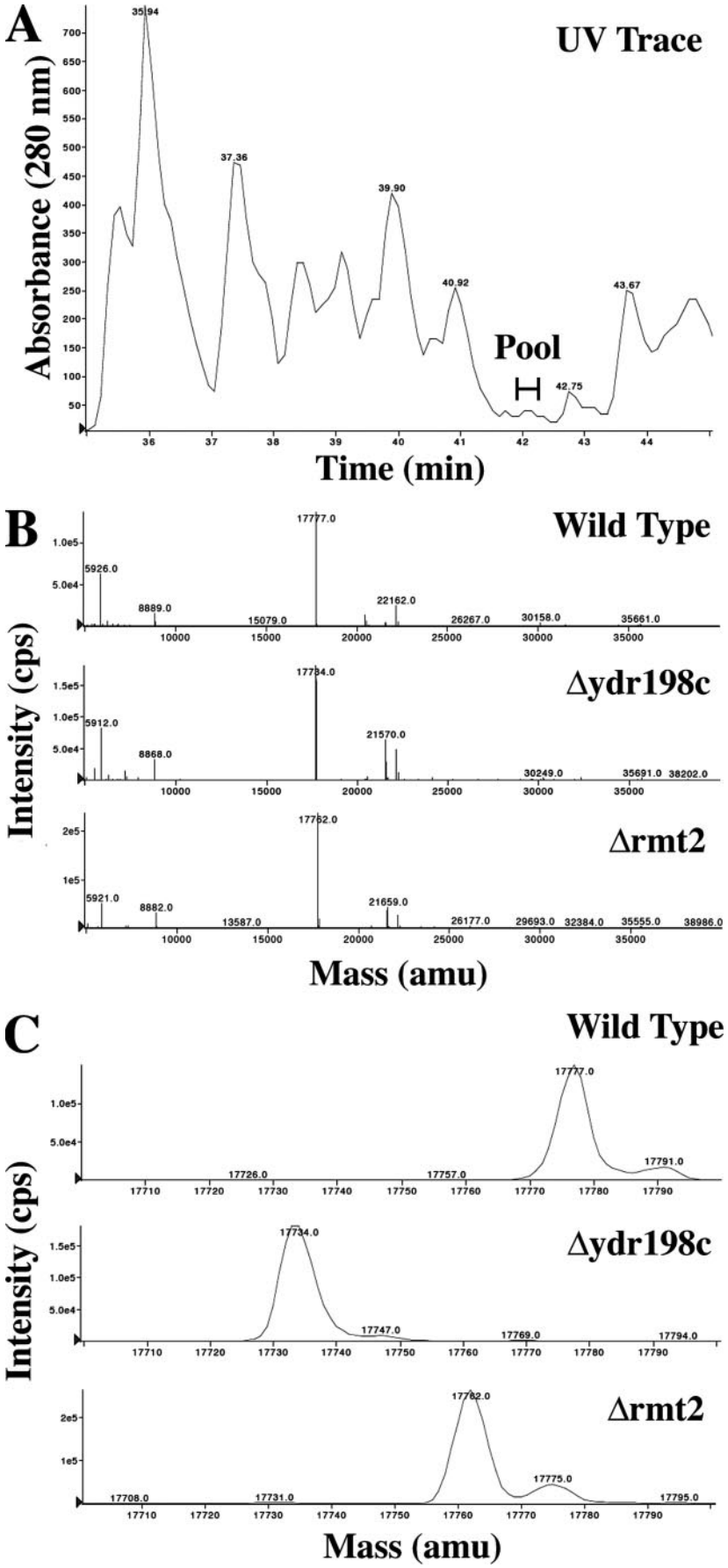


FIGURE 1. **Analysis of methylated small and large subunit ribosomal proteins in yeast.** *In vivo* [^{3}H]AdoMet-labeled small (*A*) and large (*B*) subunits were purified from wild type (BY4742) yeast cells and analyzed by SDS-PAGE (12.6% acrylamide monomer) as described previously (4). The Coomassie-stained gels were then treated with EN^{3}HANCE (PerkinElmer Life Sciences), dried, and exposed to film at −80 °C for 2 months. In each *panel*, the *first lane* corresponds to the Coomassie-stained gel, and the second lane corresponds to the autoradiograph. Protein assignments were made according to polypeptide molecular weight and the previous assignment of methylated species (1, 5–7). Species with a *question mark* include those for which no methyltransferase has been identified.

RESULTS AND DISCUSSION

Methylated Proteins in the Small and Large Subunit of the Yeast Ribosome—Intact cells were labeled *in vivo* with [^{3}H]AdoMet as described previously (4). Ribosomes were purified by differential centrifugation, and their subunits were separated using a high salt sucrose gradient as described under "Experimental Procedures." Proteins were fractionated by SDS-PAGE and analyzed by Coomassie staining and radioautography. As depicted in Fig. 1, a number of proteins are ^{3}H-methylated in both the small and the large subunit. Many of these radiolabeled species could be identified on the basis of their size and prior knowledge of their degree of methylation (1, 5–7). Additional ^{3}H-methylated species were also found that have not been identified to date. This experiment demonstrates that a significant percentage of ribosomal proteins are modified by methylation. However, the enzymes that modify these species largely remain to be identified. We decided to focus our atten-



tion on one of the three most highly modified proteins of the large subunit, Rpl12ab.

Identifying the Rpl12ab Lysine Methyltransferase by an LC-MS+ Screen—In 1984, Lhoest *et al.* (1) demonstrated that the yeast ribosomal protein Rpl12ab was modified at lysine residues to give both dimethylated and trimethylated species and represented one of the three major methylated species in the large subunit. Using a mass spectrometric approach, Lee *et al.* (5) determined that the ribosomal protein L12ab contained either six methyl groups or three methyl groups and an acetyl group. One of these sites represents a δ-monomethylarginine residue at position 66 that is the product of the Rmt2 protein arginine methyltransferase (3). However, the methyltransferase(s) responsible for the lysine residue modifications have not been identified.

We thus analyzed yeast strains containing deletion mutations in candidate genes for SET domain proteins that have been shown to encode protein lysine methyltransferases for histones and other proteins. Seven of the SET domain-encoding genes identified by the Pfam protein family data base (available on the World Wide Web at www.sanger.ac.uk/gi-bin/Pfam; version of 31 October 2002) were analyzed previously by SDS-PAGE of [^{3}H]AdoMet-labeled whole cell lysates from each of the deletion strains (4). No loss of ^{3}H methylation of the Rpl12ab protein was observed in any of the seven strains.

BLAST searches for homologues of Rkm1 in yeast (available on the World Wide Web at www.ncbi.nlm.nih.gov/BLAST) demonstrated the presence of an additional SET domain protein encoded by the *YDR198c* gene. SDS-PAGE analysis of a deletion strain for this gene showed a partial loss of methylation in the position of Rpl12ab (data not shown). Because of the possibility that multiple enzymes may modify this and other ribosomal proteins, a new mass spectrometric approach was then taken that would reveal individual enzyme contributions to the modification of the whole protein.

This approach involved developing a mass spectrometry screen to determine if deleting any of these SET domain-containing genes results in a change in the intact mass of any of the large ribosomal subunit proteins when compared with the masses observed in the wild type. Large ribosomal subunit proteins were isolated from the wild type and knock-out strains and fractionated using a reverse phase HPLC column. With the aid of a splitter, half of the eluent was collected for later analysis, whereas the other half was directed to an electrospray ionization mass spectrometer to determine the intact masses of the proteins as they eluted from the column. The ribosomal proteins that gave a strong reproducible signal were compared with the masses observed in the wild type parent strain and the other knock-out strains. The accuracy of this method allows us to distinguish small differences in mass, such as that resulting from the addition/loss of a single methyl group (14 Da).

TABLE 2
LC-MS+ analysis of Rpl12ab

Rpl12ab purified from strain	Observed mass[a]	Calculated mass[b]	Error[c]	Modifications observed[d]
	Da	*Da*	*ppm*	
Wild type	17,776 ± 1	17,775	56	Monomethyl group (Arg^{66}) Dimethyl group Trimethyl group
Δ*ydr198c*	17,734 ± 0	17,733	56	Monomethyl group (Arg^{66})
Δ*rmt2*	17,762 ± 1	17,761	56	Dimethyl group Trimethyl group

[a] Observed average isotopic mass is reported as an average ± S.D. of at least three masses observed in spectra obtained for Rpl12ab derived from each of the strains listed. In addition, for the calculation, masses obtained for Rpl12ab purified from the independently derived BY4741 strains were also used at least once for each strain.
[b] The calculated average isotopic mass listed was obtained using the ExPASy proteomic server (us.expasy.org/). The calculated mass for Rpl12ab completely unmodified minus the mass of the initiator methionine is 17,691 Da.
[c] Error is calculated ((theoretical − observed)/theoretical) × 1,000,000 and is reported as an absolute value. In these experiments, the error is estimated at about ±0.01% of the total weight of the protein, which corresponds to an error of ±1.8 Da for a protein of this size.
[d] The masses for each modification are the following: one monomethyl group is 14 Da, one dimethyl group is 28 Da, and one trimethyl group is 42 Da. Because error exists in the analysis, any mass difference approximately equal to these values is considered to be one of these modifications.

We focused our efforts here on Rpl12ab modifications, analyzing an HPLC-purified protein with the mass expected for this protein (5) (Fig. 2). In the wild type strain, we observed an intact mass of 17,777 Da, whereas in the Δ*ydr198c* strain, we observed an intact mass of 17,734 Da. In the Δ*ydr198c* strain we thus observed a loss of about 43 Da compared with the wild type strain, corresponding nearly to the mass of three methyl groups. Because Rpl12ab had previously been shown to be monomethylated by Rmt2, we decided to confirm the validity of this approach by analyzing a Δ*rmt2* deletion strain. For the Rpl12ab protein isolated from a Δ*rmt2* strain, we observed an intact mass of 17,762 Da, a loss of about 15 Da corresponding to the mass of one methyl group. This result is consistent with the previous finding of the monoarginine methylation of Rpl12ab by Rmt2 (3). In all three strains, a minor peak ~14 Da larger than the major Rpl12ab peak was observed (Fig. 2*C*), indicating the possibility that a small percentage of this protein is hypermodified by an additional methyl group. In Table 2, we summarize LC-MS+ data from this experiment and from two additional independent purifications of Rpl12ab. We included in these analyses at least one sample from each mutant in the independently derived BY4741 and BY4742 backgrounds (Table 1). These data confirm the loss of three methyl groups in

FIGURE 2. **LC-MS+ analysis of Rpl12ab derived from wild type (BY4742), Δ*ydr198c*, and Δ*rmt2* strains.** Large subunit proteins were isolated and separated by reverse phase HPLC as described under "Experimental Procedures." The column effluent was split; half was directed to a SCIEX electrospray ionization mass spectrometer to determine the intact masses of the proteins, whereas the other half was directed to a fraction collector, where 1-min (50 μl) fractions were collected as described under "Experimental Procedures." *Panel A*, the UV trace recorded for the elution of the large ribosomal subunit proteins from the wild type strain. *Pool*, the fractions containing Rpl12ab (see below). Similar elution profiles for the Δ*ydr198c* and Δ*rmt2* strains were obtained (data not shown). *Panel B*, the deconvoluted MS spectrum over the range of 0–40,000 Da for the proteins eluting in the "pool" region. In all three strains, the major species observed in this area corresponds to Rpl12ab. In the sample from the wild type strain, an average isotopic mass of 17,777 Da was obtained, whereas the major peak was 17,734 Da for the sample from the Δ*ydr198c* strain and 17,762 Da for the sample from the Δ*rmt2* strain. *Panel C*, an enlarged view of the region from 17,700 to 17,800 Da for each of the samples to demonstrate the presence of a minor species ~14 Da larger. All masses are given as an average mass based on natural isotopic abundance. *amu*, atomic mass units; cps, counts/s.

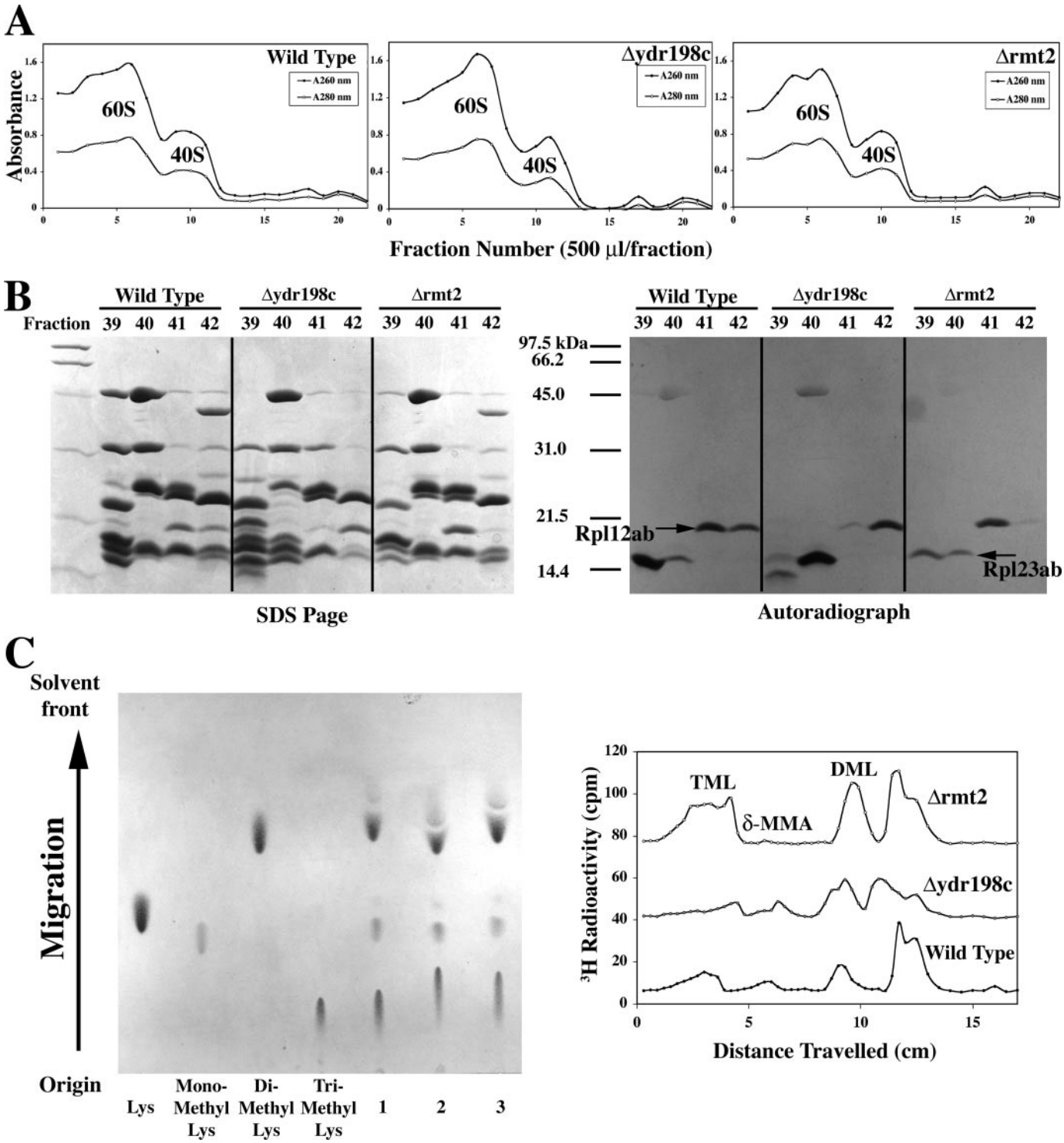


FIGURE 3. **Analysis of *in vivo* labeled HPLC-purified Rpl12ab.** *A*, the separation of the large and small ribosomal subunits using high salt sucrose gradients as described under "Experimental Procedures." Sucrose gradient fractions (500 μl) were collected from the bottom of the tube, 5 μl of each fraction was then diluted with 95 μl of water, and the absorbances at 260 and 280 nm were measured. Large ribosomal subunits in fractions 2–7 were pooled, and protein was extracted from RNA, dried down, resuspended in 100 μl of water, and separated on a reverse phase HPLC column as described in the legend to Fig. 2. *B*, the results of SDS-PAGE analysis followed by autoradiography for 45 days at −80 °C for selected HPLC fractions. The *arrows* denote the points of migration for the ^{3}H-methylated Rpl12ab and Rpl23ab proteins. In the wild type and the Δ*rmt2* strains, Rpl12ab primarily eluted in fraction 41, whereas in the Δ*ydr198c* strain, Rpl12ab was found to elute characteristically one fraction later. *C* shows the results of amino acid analysis of the modified residues. HPLC-purified Rpl12ab-containing fractions from each of the three strains were acid-hydrolyzed into their component amino acids and analyzed by thin layer chromatography as described previously (4). Standards of lysine and mono-, di-, and tri-ϵ-lysine (40 nmol each) were mixed with the hydrolyzed samples prior to chromatography and were also loaded individually in adjacent *lanes*. The wild type sample was loaded in *lane 1*, the Δ*ydr198c* sample was loaded in *lane 2*, and the Δ*rmt2* sample was loaded in *lane 3*. The migration of the radioactivity was quantified by cutting the sample lanes into 0.3-cm pieces, scraping the silica into 1 ml of H_2O, adding 5 ml of scintillation fluor, and counting each sample three times for 20 min. [^{3}H]trimethyllysine was found to migrate from 2 to 4.5 cm; [^{3}H]δ-monomethylarginine migrated from 5 to 7 cm, and [^{3}H]dimethyllysine migrated from 8.5 to 10 cm. The identity of the radiolabeled peak migrating from 10.5 to 13 cm is unknown. *δ-MMA*, δ-monomethylarginine; *DML*, di-*N*$^{\epsilon}$-methyllysine; TML, tri-*N*$^{\epsilon}$-methyllysine.

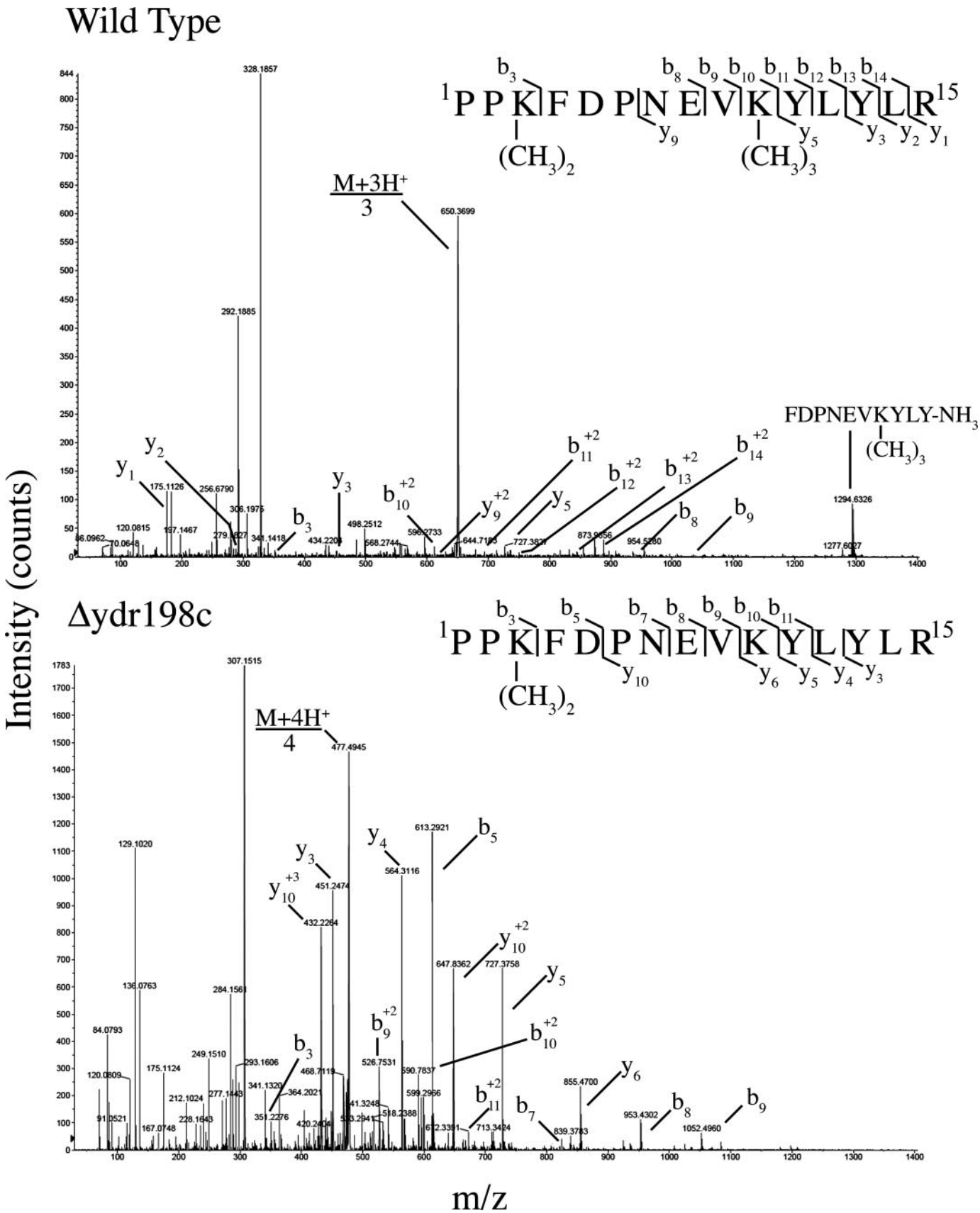


FIGURE 4. **MSMS spectrum of methyllysine-modified peptide in Rpl12ab.** Rpl12ab purified by LC-MS+ was digested with ArgC and analyzed using a Q-Star mass spectrometer. The MSMS spectrum of the peptide fragment spanning residues 1–15 is depicted for both the wild type and Δ*ydr198c* strain. The Δ*rmt2* strain gave similar results to what was observed for the wild type in this region (data not shown). The y and b ions observed are depicted for both strains as well as the fragmentation map.

TABLE 3
MSMS ions for Rpl12ab methylated peptide 1–15
All values are reported as monoisotopic uncharged masses.

Strain	Peptide	Ion	Observed mass	Theoretical mass	Mass error
			Da	*Da*[a]	*ppm*[b]
Wild type	PPKFDPNEVKYLYLR | | $(CH_3)_2$ $(CH_3)_3$	b3	350.233	350.232	4.9
		b8	952.429	952.465	−38.6
		b9	1051.518	1051.534	−15
		b10	1221.630	1221.676	−37.5
		b11	1384.736	1384.739	−2.2
		b12	1497.846	1497.823	15.1
		b13	1660.873	1660.886	−8.3
		b14	1773.940	1773.971	−17.1
		y1	174.105	174.114	−48.9
		y2	287.183	287.198	−50.7
		y3	450.241	450.261	−44.6
		y5	726.391	726.408	−23.5
		y9	1238.656	1238.704	−39.2
	PPKFDPNEVKYLYLR | | $(CH_3)_3$ $(CH_3)_2$	b3	364.278	364.247	84.1
		b4	511.352	511.316	71.4
		b7	837.488	837.439	58.6
		b8	966.521	966.481	41.2
		b9	1065.599	1065.550	46.2
		b10	1221.630	1221.676	−37.5
		b11	1384.736	1384.739	−2.2
		b12	1497.846	1497.823	15.1
		b13	1660.873	1660.886	−8.3
		b14	1773.940	1773.971	−17.1
		y1	174.105	174.114	−48.9
		y2	287.183	287.198	−50.7
		y3	450.241	450.261	−44.6
		y5	726.391	726.408	−23.5
		y8	1110.586	1110.646	−54.1
Δ*ydr198c*	PPKFDPNEVKYLYLR | $(CH_3)_2$	b3	350.220	350.232	−34.8
		b5	612.319	612.327	−12.8
		b7	823.396	823.423	−32.2
		b8	952.413	952.465	−54.7
		b9	1051.488	1051.534	−43.6
		b10	1179.551	1179.629	65.4
		b11	1342.672	1342.692	−14.6
		y3	450.239	450.261	−48.0
		y4	563.304	563.345	−73.6
		y5	726.390	726.408	−25.2
		y6	854.478	854.503	−30.1
		y10	1293.691	1293.71	−14.7

[a] The calculated mass listed was obtained using the Prosight proteomic server (available on the World Wide Web at prosightptm.scs.uiuc.edu/).
[b] Error was calculated using the Prosight server (available on the World Wide Web at prosightptm.scs.uiuc.edu/), taking into consideration the post-translational modification.

the Δ*ydr198c* strains and one methyl group in the Δ*rmt2* strains.

These results demonstrate that the protein is modified by the addition of six (and possibly seven) methyl groups, with one methyl residing on arginine 66 as previously established (3), three methyl groups residing on a different site or sites added by the protein encoded by *YDR198c*, and two (and sometimes three) methyl groups added to an additional site or sites by an as yet unidentified protein. These results are consistent with the earlier finding that Rpl12ab was modified either by acetylation/trimethylation or hexamethylation (5). The apparent lack of an acetylated N terminus in Rpl12ab is consistent with the results of Polevedo and Sherman (33). In several yeast proteins, they found that an N-terminal Met-Pro sequence, as found in Rpl12ab, results in the loss of the initiator methionine but not the N-acetylation of the proline residue (33).

Analysis of the Type of Methylation on Rpl12ab—To directly demonstrate that the difference of 42 Da in Rpl12ab observed in the Δ*ydr198c* strain was due to the loss of one or more specific protein modifications, [^{3}H]Rpl12ab was purified from cells labeled *in vivo* with [^{3}H]AdoMet and acid-hydrolyzed, and the resulting amino acids were analyzed by thin layer chromatography.

In Fig. 3*A*, we show the UV profiles of the fractions collected from the sucrose gradients involved in the large subunit purification. These profiles demonstrate the efficient separation of the large and small subunits and lack of soluble protein contaminants after the ribosomal pelleting step. The *in vivo* labeled large ribosomal proteins were then fractionated and analyzed by LC-MS+ as described above. Fractions corresponding to the times where Rpl12ab is observed to elute were analyzed by SDS-PAGE followed by autoradiography (Fig. 3*B*). These results show that we successfully isolated [^{3}H]Rpl12ab free of other methylated species. The fraction containing [^{3}H]Rpl12ab was then acid-hydrolyzed, the resulting modified amino acids were separated by thin layer chromatography, and slices of each lane were quantified for ^{3}H-radioactive species (Fig. 3*C*).

We were able to cleanly separate the ^{3}H-methylated species of δ-monomethylarginine, ϵ-dimethyllysine, and ϵ-trimethyllysine (Fig. 3*C*). In the wild type-derived fraction, we observed all of these species in a ratio similar to what would be expected for a protein containing one residue each of δ-monomethylarginine, ϵ-dimethyllysine, and ϵ-trimethyllysine. In the Δ*rmt2*-derived fraction, the radioactive peak corresponding to the δ-monomethylarginine was missing as expected according to previous results (3); the absence of radioactivity near its migra-

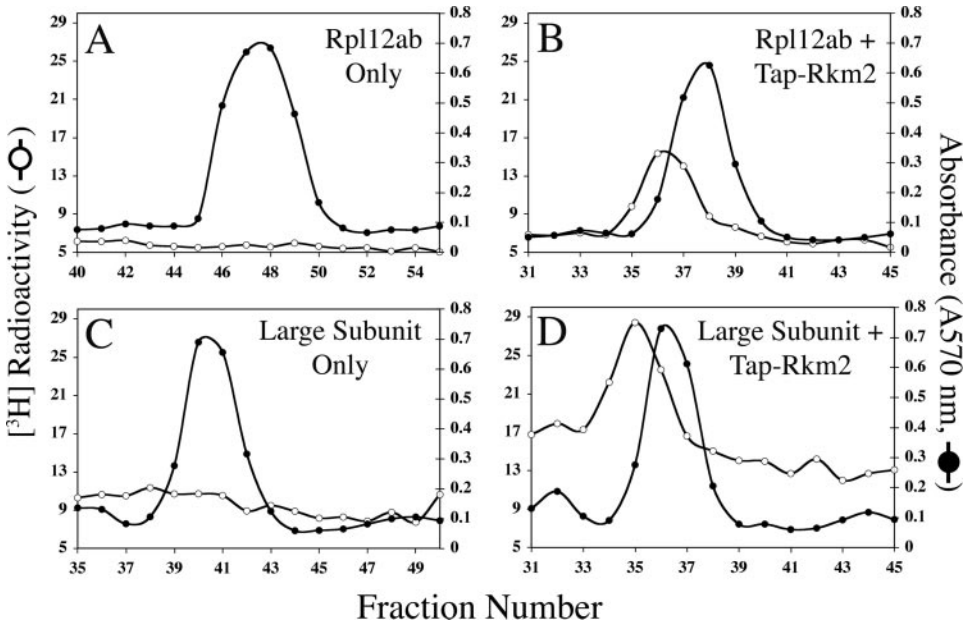


FIGURE 5. ***In vitro* methylation of Rpl12ab by Tap-Rkm2.** Hypomethylated Rpl12ab or large subunit proteins purified from the Δ*ydr198c* deletion strain were incubated either alone (*A* and *C*) or with purified tap-tagged Rkm2 (*B* and *D*) in the presence of [^{3}H]AdoMet, as described under "Experimental Procedures." After acid hydrolysis, ^{3}H-methylated amino acid derivatives were mixed with a standard of N^{ϵ},N^{ϵ}-dimethyllysine and fractionated by ion exchange chromatography as described previously (4). Of the 1-ml fractions collected, 700 μl was assayed for radioactivity (*open circles*) by diluting the sample with 400 μl of water and 5 ml of fluor and counting three times for 20 min each. The position of the dimethyllysine standard was determined by analyzing 100 μl of the fraction by ninhydrin assays (*closed circles*) (4). The radioactivity in *B* and *D* elutes in the position expected for N^{ϵ}-monomethyllysine.

tion position demonstrates the absence of ϵ-monomethyllysine in the protein. In this material, the ratio of ϵ-dimethyllysine to ϵ-trimethyllysine was found to be consistent with the presence of one residue of each form. When the fraction derived from the Δ*ydr198c* strain was analyzed, the ratio of radioactivity in δ-monomethylarginine and ϵ-dimethyllysine was found to be similar to that in the wild type sample, but the amount of ϵ-trimethyllysine was found to be greatly reduced. These results suggest that the Ydr198c protein catalyzes a major trimethylation reaction of a lysine residue. The small residual amount of trimethyllysine found in the mutant may be formed from the as yet unidentified enzyme that normally catalyzes the dimethylation reaction at the third site of methylation.

Determining the Sites of Methylation of Rpl12ab by MSMS—The sites of modification of Rpl12ab were directly identified by an MSMS analysis. Rpl12ab isolated using the LC-MS+ method was digested with either proteinase ArgC, which cleaves preferentially on the C-terminal side of arginine residues, or chymotrypsin, which cleaves primarily on the C-terminal side of aromatic amino acids but also cleaves at other residues, including leucine and glycine (34). Peptides were then analyzed by μLC-MSMS as described under "Experimental Procedures." For Rpl12ab peptides from all three strains, ions that correspond to peptides with unmodified lysine residues were observed with the exception of peptides that contained lysine 3 and lysine 10. In the wild type and Δ*rmt2*-derived ArgC peptides, a triply charged ion with an *m*/*z* of 650.37 was observed that corresponded to amino acids 1–15 (PPKFDPNEVKYLYLR) with five methyl groups. The MSMS spectrum of the peptide derived from the wild type strain is displayed in the *top panel* of Fig. 4. Analysis of the y and b ladder indicates that lysine 3 is dimethylated, whereas lysine 10 is trimethylated. However, we also found evidence for a minor species containing a trimethylated lysine 3 and a dimethylated lysine 10. Here, the intensities of the ions were much lower, suggesting that the predominant species is dimethylated at lysine 3 and trimethylated at lysine 10. Table 3 is a summary of the y and b ions observed during the analysis.

In the Δ*ydr198c* strain, we did not observe the *m*/*z* 650.37 ion corresponding to the pentamethylated 1–15 peptide. We did observe, however, a quadruply charged ion with an *m*/*z* of 477.51, which corresponded to the peptide of amino acids 1–15 with only two methyl groups. This ion was not found in either of the wild type or the Δ*rmt2*-derived peptides. When the *y* and *b* ladder of the 477.51 *m*/*z* species was analyzed, we found that lysine 3 was modified by dimethylation, whereas lysine 10 was not modified. The MSMS spectrum of this ion is shown in the *lower panel* of Fig. 4, and the y and b ions observed are summarized in Table 3. These results suggest that the *YDR198c* gene product is responsible for catalyzing the ϵ-trimethylation of Rpl12ab at lysine 10. We thus now designate the *YDR198c* gene product Rkm2 (for ribosomal lysine methyltransferase 2).

Recombinant Rkm2 Exhibits Lysine Methyltransferase Activity in Vitro but Does Not Recapitulate the Physiological Methylation Reaction—To demonstrate that the Rkm2 polypeptide catalyzes lysine side chain modification, *in vitro* methylation reactions were performed with [^{3}H]AdoMet using purified recombinant tap-tagged Rkm2. Hypomethylated Rpl12ab purified from the *rkm2* deletion strain was used as a methyl-accepting substrate. In addition, to determine whether other components of the large subunit are necessary for the enzymatic activity of Rkm2, large subunit proteins from the *rkm2* deletion strain were also used as potential methyl-accepting substrates. After the incubation, modified proteins were hydrolyzed in strong acid, and the ^{3}H-methylated amino acid derivatives were fractionated by column chromatography. When purified tap-Rkm2 is allowed to react with either purified Rpl12ab or purified large subunit proteins, we detect the production of a product that co-migrates with [^{3}H]N^{ϵ}-monomethyllysine (Fig. 5, *B* and *D*). However, no methylated products were found when tap-Rkm2 was not added (Fig. 5, *A* and *C*). Interestingly, the [^{3}H]monomethyllysine product was also seen when tap-Rkm2 was incubated by itself (data not shown). These results suggest that the Rkm2 polypeptide can catalyze lysine methylation but cannot recapitulate the specific modification of Rpl12ab to

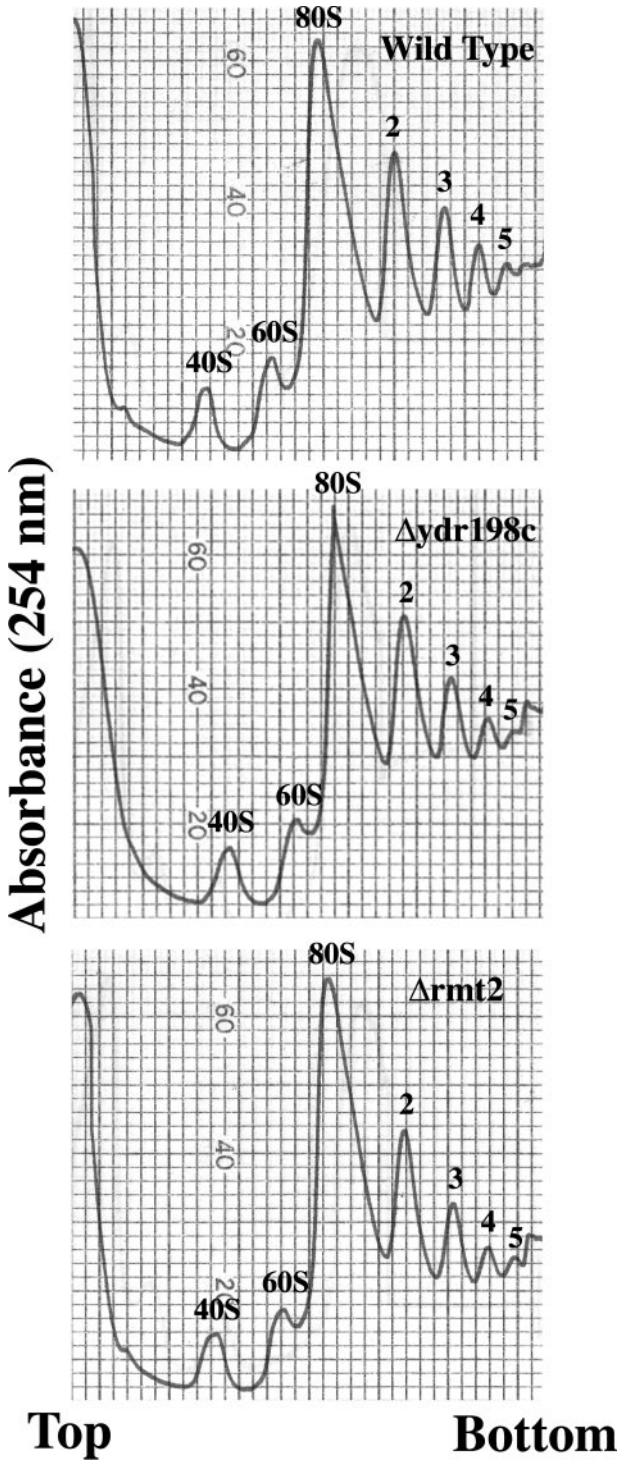


FIGURE 6. **Polysomal analysis of the wild type, Δ*rkm2*, and Δ*rmt2* strains.** Ribosomal complexes were studied using low salt sucrose gradients as previously described (14). The sucrose gradient was monitored by UV at 254 nm using an ISCO UA6 apparatus. In this case, the gradient was extruded by pumping a high density solution through the bottom of the tube. The positions of the 40 and 60 S small and large ribosomal subunits, the 80 S ribosomes, and the various polysomal complexes (2–5) are indicated.

form trimethyllyisne derivatives *in vitro*. It is possible that Rkm2 requires an unfolded form of Rpl12ab, such as the nascent polypeptide chain. It is also possible that additional polypeptides are required to direct the specific modification of Rpl12ab.

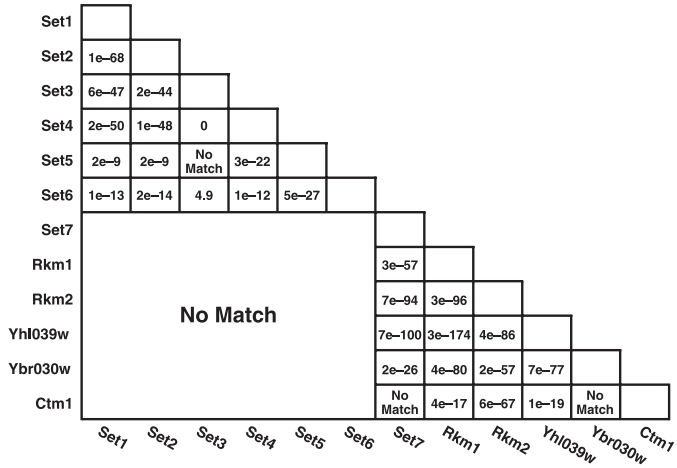


FIGURE 7. **Two subfamilies of SET domain proteins in *S. cerevisiae*.** Amino acid sequences of the SET domain family were Psi-Phi-blasted against all yeast proteins for five iterations, and the results of their matches are depicted. In addition, the expected values are also listed.

Physiological Roles of Rkm2 Methylation of Rpl12ab—The cellular roles of nonhistone protein lysine methylation are still poorly understood. In ribosomes, evidence has been presented that protein arginine methylation may be involved in the formation and stability of ribosome-mRNA complexes (8, 9). To ask if the methylation of Rpl12ab may play a similar role, polysomes were analyzed from wild type, Δ*rmt2*, and Δ*rkm2* strains as described in the legend to Fig. 6. We observed no difference between the wild type and the two mutant strains analyzed, suggesting that protein lysine methylation, at least of the lysine 10 residue of Rpl12ab, may not be involved in ribosomal stability. It is possible, however, that multiple modifications on one or more ribosomal proteins may act cooperatively, and the lack of one modification is not sufficient to produce an observable change. The identification of this second ribosomal protein lysine methyltransferase Rkm2 and the future identification of other modifying enzymes may then allow testing of this hypothesis.

Recent work with histone lysine methylation has indicated that modified residues can be recognized by a number of protein domains, including Tudor, MBI, chromo, and PHD domains (35, 36). It will be interesting to see if the lysine 3 and lysine 10 sites of Rpl12ab may be recognized by specific domains, since this may point to functional interactions of ribosomes with accessory proteins. Additionally, methylation of histone lysine residues is now known to be reversible by oxidative enzymes, including LSD1 and JHDM1 (37). We have no direct evidence to indicate that demethylation occurs in ribosomal proteins, but the presence of both the dimethyl and trimethyl derivatives at lysine 3 and lysine 10 of Rpl12ab suggests the possibility of such reactions.

The SET Domain Family of Methyltransferases in Yeast—With the identification of this second SET domain containing ribosomal lysine methyltransferase member Rkm2, we can reevaluate the SET domain family and group the SET proteins according to sequence similarity and function. The SET domain family in yeast is composed of five known SET methyltransferases: Set1 and Set2, which are histone lysine methyltransferases (24–26); Ctm1, which is the cytochrome *c* lysine

methyltransferase (38); and the Rkm1 and Rkm2 ribosomal lysine methyltransferases (4) (this work). In addition, the Pfam site on the World Wide Web (www.sanger.ac.uk/gi-bin/Pfam) identified seven additional SET domain-containing proteins, Set3 to -7, Yhl039w, and Ybr030w.

Fig. 7 illustrates the results of a Psi-Phi blast (available on the World Wide Web at www.ncbi.nlm.nih.gov/BLAST) of all of the SET and putative SET methyltransferase amino acid sequences. When Set1 to -6 were Psi-Phi-blasted for five iterations, the reports only identified each other and did not identify Rkm1, Rkm2, Set7, Yhl039w, Ybr030w, and Ctm1. However, when Rkm1, Rkm2, Set7, Yhl039w, Ybr030w, and Ctm1 were Psi-Phi-blasted, the report did not identify Set1 to -6. In the SET domain region of all of these proteins there is enough sequence similarity to group them all into the larger Set family, but it now appears that the group can be further subdivided into two families of proteins that have a stronger relationship among themselves then among the broader SET family designation. Here, the histone methyltransferases Set1 and Set2 define a group of six related proteins, whereas Rkm1, Rkm2, and Ctm1 define a second group. It will be interesting to identify the activities of the remaining seven family members.

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REFERENCES

1. Lhoest, J., Lobet, Y., Costers, E., and Colson, C. (1984) *Eur. J. Biochem.* **141,** 585–590
2. Lobet, Y., Lhoest, J., and Colson, C. (1989) *Biochim. Biophys. Acta* **997,** 224–231
3. Chern, M. K., Chang, K. N., Liu, L. F., Tam, T. C. S., Liu, Y. C., Liang, Y. L., and Tam, M. F. (2002) *J. Biol. Chem.* **277,** 15345–15353
4. Porras-Yakushi, T. R., Whitelegge, J. P., Miranda, T. B., and Clarke, S. (2005) *J. Biol. Chem.* **280,** 34590–34598
5. Lee, S. W., Berger, S. J., Martinovic, S., Pasa-Tolic, L., Anderson, G. A., Shen, Y., Zhao, R., and Smith, R. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 5942–5947
6. Kruiswijk, T., Kunst, A., Planta, R. J., and Mager, W. H. (1978) *Biochem. J.* **175,** 221–225
7. Meng, F., Du, Y., Miller, L. M., Patrie, S. M., Robinson, D. E., and Kelleher, N. L. (2004) *Anal. Chem.* **76,** 2852–2858
8. Bachand, F., and Silver, P. A. (2004) *EMBO J.* **23,** 2641–2650
9. Bachand, F., Lackner, D. H., Bähler, J., and Silver, P. A. (2006) *Mol. Cell Biol.* **26,** 1731–1742
10. Williamson, N. A., Raliegh, J., Morrice, N. A., and Wettenhall, R. E. H. (1997) *Eur. J. Biochem.* **246,** 786–793
11. Louie, D. F., Resing, K. A., Lewis, T. S., and Ahn, N. G. (1996) *J. Biol. Chem.* **271,** 28189–28198
12. Yu, Y., Ji, H., Doudna, J. A., and Leary, J. A. (2005) *Protein Sci.* **14,** 1438–1446
13. Chang, F. N., Navickas, I. J., Chang, C. N., and Dancis, B. M. (1976) *Arch. Biochem. Biophys.* **172,** 627–633
14. Scolnik, P. A., and Eliceiri, G. L. (1979) *Eur. J. Biochem.* **101,** 93–101
15. Chang, F. N., Navickas, I. J., Au, C., and Budzilowicz, C., (1978) *Biochim. Biophys. Acta* **518,** 89–94
16. Swiercz, R., Person, M. D., and Bedford, M. T. (2005) *Biochem. J.* **386,** 85–91
17. Yeates, T. O. (2002) *Cell* **111,** 5–7
18. Schubert, H. L., Blumenthal, R. M., and Cheng, X. (2003) *Trends Biochem. Sci.* **28,** 329–335
19. Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998) *Cell Mol. Life Sci.* **54,** 80–93
20. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting C. P., Allis, C. D., and Jenuwein, T. (2000) *Nature* **406,** 579–580
21. Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001) *J. Biol. Chem.* **276,** 25309–25317
22. Nishioka, K., Chuikov., S., Sarma, K., Erdjument-Bronmage, H., Allis, C. D., Tempst, P., and Reinberg, D. (2002) *Genes Dev.* **16,** 479–489
23. Shilatifard, A. (2006) *Annu. Rev. Biochem.* **75,** 243–269
24. Boa, S., Coert, C., and Patterton, H.-G. (2003) *Yeast* **20,** 827–835
25. Roguev, A., Schaft, D., Shevchenko, A., Pim Pijnappel, W. W. M., Wilm, M., Aasland, R., and Stewart, A. F. (2001) *EMBO J.* **20,** 7137–7148
26. Strahl, B. D., Grant, P. A., Briggs, S. D., Sun, Z.-W., Bone, J. R., Caldwell, J. A., Mollah, S., Cook, R. G., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2002) *Mol. Cell Biol.* **22,** 1298–1306
27. Houtz, R. L., Stults, J. T., Mulligan, R. M., and Tolbert, N. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1855–1859
28. Houtz, R. L., and Portis, A. R., Jr. (2003) *Arch. Biochem. Biophys.* **414,** 150–158
29. Dirk, L. M. A., Trievel, R. C., and Houtz, R. L. (2006) in *The Enzymes: Protein Methyltransferases* (Clarke, S.G., and Tamanoi, F., eds) pp. 179–228, Elsevier Inc., San Francisco
30. Whitelegge, J. P. (2004) *Methods Mol. Biol.* **251,** 323–339
31. Whitelegge, J. P., Gundersen, C. B., and Faull, K. F. (1998) *Protein Sci.* **7,** 1423–1430
32. Zanchin, N. I., Roberts, P., DeSilva, A., Sherman, F., and Goldfarb, D. S. (1997) *Mol. Cell. Biol.* **17,** 5001–5015
33. Polevoda, B., and Sherman, F. (2003) *J. Mol. Biol.* **325,** 595–622
34. Hirs, C. H. W. (1970) in *CRC Handbook of Biochemistry* (Sober, H. A., ed) p. C-125, The Chemical Rubber Co., Cleveland, OH
35. Kim, J., Daniel, J., Espejo, A., Lake, A., Krishna, M., Xia, L., Zhang, Y., and Bedford, M. T. (2006) *EMBO Rep.* **7,** 397–403
36. Wysocka, J., Swigut, T., Xiao, H., Milne, T. A., Kwon, S. Y., Landry, J., Kauer, M., Tackett, A. J., Chait, B. T., Badenhorst, P., Wu, C., and Allis, C. D. (2006) *Nature* **442,** 86–90
37. Whetstine, J.R., Nottke, A., Lan, F., Huarate, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacoo, M., and Shi, Y. (2006) *Cell* **125,** 467–481
38. Polevoda, B., Martzen, M. R., Das, B., Phizicky, E. M., and Sherman, F. (2000) *J. Biol. Chem.* **275,** 20508–20513