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A Novel SET Domain Methyltransferase Modifies Ribosomal Protein Rpl23ab in Yeast*

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***In vivo* studies have shown that the ribosomal large subunit protein L23a (Rpl23ab) in *Saccharomyces cerevisiae* is methylated at lysine residues. However, the gene encoding the methyltransferase responsible for the modification has not been identified. We show here that the yeast *YPL208w* gene product, a member of the SET domain family of methyltransferases, catalyzes the reaction, and we have now designated it Rkm1 (ribosomal lysine (K) methyltransferase 1). Yeast strains with deletion mutations in candidate SET domain-containing genes were *in vivo* labeled with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. [^{3}H]Methyl radioactivity was determined after lysates were fractionated by SDS gel electrophoresis. When compared with the parent strain or other candidate deletion strains, a loss of a radiolabeled 15-kDa species was observed in the *rkm1* (Δ*ypl208w*) knock-out strain. Treatment of wild-type cell extracts with RNase or proteinase K demonstrated that the methyl-accepting substrate is a protein. Cellular lysates from parent and knock-out strains were fractionated using high salt sucrose gradients. Analysis of the gradient fractions by SDS gel electrophoresis demonstrated that the 15-kDa methyl-accepting substrate elutes with the large ribosomal subunit. *In vitro* methylation experiments using purified ribosomes confirmed that the methyl-accepting substrate is a ribosomal protein. Amino acid analysis of the *in vivo* labeled 15 kDa polypeptide showed that it contains ε-[^{3}H]dimethyllysine residues. Mass spectrometry of tryptic peptides of the 15 kDa polypeptide identified it as Rpl23ab. Analysis of the intact masses of the large ribosomal subunit proteins by electrospray mass spectrometry confirmed that the substrate is Rpl23ab and that it is specifically dimethylated at two distinct sites by Rkm1. These results show that SET domain methyltransferases can be involved in translational roles as well as in the previously described transcriptional roles.**

Methyltransferases containing the SET domain have been shown to post-translationally modify cytochrome *c*, Rubisco, and histones H3 and H4 at the ε-amino groups of lysine side chains (1). The SET domain was first identified in the *Drosophila* heterochromatin-associated proteins Su(var), Enhancer of zeste, and Trithorax, species later determined to be histone lysine methyltransferases (2–5). The SET domain is an *S*-adenosylmethionine (AdoMet)[3] binding domain that does not resemble the canonical seven β-strand AdoMet-binding fold seen in the majority of methyltransferases whose structures are presently known (6).

In the yeast *Saccharomyces cerevisiae*, three SET domain methyltransferases have been identified, two of which are involved in regulating transcription by methylating histone proteins in chromatin and one is the cytochrome *c* lysine methyltransferase Ctm1p (7–9, 10). The two SET domain methyltransferases involved in transcription are designated Set1 and Set2. Set1 is a histone H3 lysine-4 methyltransferase involved in transcriptional activation, while Set2 is a histone H3 lysine-36 methyltransferase involved in transcriptional repression (7–9). In higher organisms homologous SET domain protein histone methyltransferases have also been identified and their roles have also been linked to both the activation and repression of transcription (11). Ctm1p was found to specifically trimethylate lysine-72 of iso-1-cytochrome *c*. The functional role of this modification is not understood (10), although it has been suggested that the trimethyl group on lysine-72 may aid in abrogating the pro-apoptotic activity of cytochrome *c* (12). In contrast to the methylation of histones, the methylation of cytochrome *c* has only been found to occur in plants and fungi and not in higher animals (10, 13).

Other cellular processes may also be regulated by methylation, including translation. In a mass spectral analysis of the large ribosomal proteins of *S. cerevisiae*, it was found that six of the proteins are post-translationally modified by the addition of methyl groups including L1ab, L3, L12ab, L23ab, L42ab, and L43ab (14). The yeast ribosomal protein L23a or YL32 is encoded as identical amino acid sequences by the *RPL23a* and *RPL23b* genes and is designated here Rpl23ab. Rpl23ab was shown to be one of the three most highly methylated proteins in the large ribosomal subunit (15, 16), modified *in vivo* and *in vitro* by dimethylation at the side chain of one or more lysine residues (15). The small subunit of the ribosome is also modified by methylation (17). However, the physiological roles of ribosomal protein methylation are poorly understood (18).

Currently, only two genes have been identified that encode ribosomal protein methyltransferases. In *S. cerevisiae*, the *RMT2* gene encodes an enzyme that monomethylates the δ nitrogen of arginine 67 in the L12 protein of the large subunit (19). In the fission yeast *Schizosaccharomyces pombe*, the protein-arginine methyltransferase 3 homolog (Prmt3) asymmetrically dimethylates an arginine residue in the small ribosomal subunit protein S2 (20). Deleting the PRMT3 gene resulted in an accumulation of free 60 S subunits (20). The mammalian PRMT3 gene product can also specifically methylate the S2 protein (21). Biochemical efforts have been made to identify the methyltransferase responsible for modifying Rpl23ab. However, purification to homogeneity was not achieved due largely to the instability of the activity (16). Nevertheless, these efforts did lead to the estimation of the native molecular mass of the partially purified enzyme at 82 kDa by size exclusion chromatography and an isoelectric point of 4.45 by isoelectric focusing (16). The genes and proteins for the methyltransferases that modify the remain-

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[3] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; Rkm1, ribosomal lysine (K) methyltransferase 1.

ing ribosomal proteins have not yet been identified. Here we have determined that the gene encoding the Rpl23ab lysine-*N*-methyltransferase is *YPL208w*, which we now refer to as *RKM1* (ribosomal protein lysine (K) methyltransferase 1).

MATERIALS AND METHODS

In Vivo Labeling of Potential SET Methyltransferase Mutants with [^{3}H]AdoMet—Yeast strains were obtained from Invitrogen (Carlsbad, CA) in which the gene encoding a potential SET methyltransferase was deleted. Genotypes of strains used in this study are described in TABLE ONE. These strains were grown at 30 °C in YPD media (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) to an optical density of 0.7–0.9 at 600 nm. Once the cells reached the desired optical density, 7 $A_{600\text{ nm}}$ units of each culture were harvested by centrifugation at 5,000 × *g* for 5 min at 4 °C and washed twice with 1 ml of sterile water. The pellet was resuspended in 924 µl of fresh YPD and 76 µl of [^{3}H]AdoMet; Amersham Biosciences, 1 miCi/ml, 70–81 Ci/mmol, in dilute HCl/ethanol (9:1, v/v), pH 2 to 2.5). Cells were labeled for 30 min at 30 °C with shaking, pelleted at 5,000 × *g* for 5 min at 4 °C, washed twice with water, and lysed in 100 µl of 1% SDS and 0.7 mM phenylmethylsulfonyl fluoride. Lysis was performed by vortexing the cells for 1 min in the presence of 0.2 g of baked zirconium beads (Biospec Products; Bartlesville, OK), followed by cooling on ice for 1 min, for a total of 7 cycles. The lysate obtained for each mutant was then centrifuged for 15 min at 12,000 × *g* followed by a 10-min centrifugation at 17,000 × *g*, both at 4 °C. 10 µl of the resulting lysate was mixed with an equal volume of 2× SDS gel sample buffer (180 mM Tris/HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromphenol blue) and heated at 100 °C for 3 min. Samples were then electrophoresed at 30 mA for 5 h using a Laemmli buffer system (24) on a gel prepared with 12.6% acrylamide and 0.43% *N,N*-methylene-bisacrylamide (unless otherwise stated) (1.5-mm thick, 10.5-cm long resolving gel, 2-cm long stacking gel). Gels were stained with Coomassie Brilliant Blue R-250 for 1 h and destained in 10% methanol and 5% acetic acid overnight. For fluorography, the gels were treated with EN^{3}HANCE (PerkinElmer Life Sciences) for 1 h, followed by a 20-min wash in water. Gels were dried at 70 °C for 2 h *in vacuo* and allowed to cool for 1 h *in vacuo*. The gels were exposed to Kodak X-Omat AR scientific imaging film at −80 °C.

Cellular Fractionation by High Salt Sucrose Gradients—The procedure of Lhoest et al (15) and Hardy et al. (25) was used with a few modifications. Briefly, 500-ml cultures were grown in YPD to an optical density of 0.5–0.8 at 600 nm, harvested and washed as described above and *in vivo* labeled for 30 min at 30 °C in the presence of 152 µl of [^{3}H]AdoMet and 20 ml of fresh YPD medium. The amount of radioactivity used in this label is 20-fold less than the amount used in the *in vivo* label procedure. After labeling the cells were again harvested by centrifugation at 5,000 × *g* for 5 min, washed twice with water, lysed in 1.5 ml of buffer A (20 mM Tris/HCl, 15 mM magnesium acetate, 60 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and 1.5 g of baked zirconium beads using the method described above. After lysis, the beads were washed with an additional 1.5 ml of buffer to maximize the yield of lysate. The combined lysate was centrifuged twice at 12,000 × *g* for 5 min at 4 °C, followed by one centrifugation at 20,000 × *g* for 15 min at 4 °C. In each case the pellet was discarded. The resulting supernatant was then centrifuged at 100,000 × *g* for 2 h at 4 °C using a Beckman type Ti 65 rotor. The ribosomal pellet obtained was resuspended in 500 µl of buffer B (50 mM Tris-HCl 5 mM magnesium acetate, 500 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and layered onto a 7–25% sucrose gradient made in the presence of buffer B. The gradients were centrifuged at 60,000 × *g* for 16 h at 4 °C, using a Beckman type SW41 rotor. Fractions were then collected from

TABLE ONE

Strains used

Strain	Genotype	Source
BY4741	MAT**a** *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0	[a]
Δ*ypl208w* (BY4741)	BY4741, Δ*ypl208w*::*Kan*^{r}	[a]
BY4742	MATα his3D1 *leu*2Δ0 *lys*2Δ0 *ura*3Δ0	[a]
Δ*ybr030w*	BY4742, Δ*ybr030w*::*Kan*^{r}	[a]
Δ*ydr257c*	BY4742, Δ*ydr257c*::*Kan*^{r}	[a]
Δ*yhl039w*	BY4742, Δ*yhl039w*::*Kan*^{r}	[a]
Δ*yhr207c*	BY4742, Δ*yhr207c*::*Kan*^{r}	[a]
Δ*yjl105w*	BY4742, Δ*yjl105w*::*Kan*^{r}	[a]
Δ*ypl165c*	BY4742, Δ*ypl165c*::*Kan*^{r}	[a]
Δ*ypl208w* (BY4742)	BY4742, Δ*ypl208w*::*Kan*^{r}	[a]
YIT617(CB012)	MAT**a** *ade*2–1 *his*3–11,15 *leu*2–3,112 *trp*1–1 *ura*3–1 *pep*4Δ::*HIS3* *prc1*Δ::*hisG*	[b]
YIT613	YIT617 rpl25::LEU2 [p*RPL25-FH-URA3CEN*]	[b]

[a] Strains were purchased from the *Saccharomyces* Genome Deletion Project (www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html).
[b] The YIT617 and YIT613 strains were kindly provided by Dr. Toshifumi Inada (40).

TABLE TWO

SET domain proteins in *Saccharomyces cerevisiae*

Information on localization (42), amino acid length, calculated isoelectric point, and predicted transmembrane domain (TD) region was obtained from the *Saccharomyces* Genome Database, www.yeastgenome.org. Information on the location of the SET-domain and PHD domain was obtained from the Protein Family Database, www.sanger.ac.uk/Software/Pfam/. The PHD domain is an interleaved type of zinc finger fold that chelates two Zn^{2+} ions.

Gene name	ORF name	Function	Localization	Length of protein/pI	SET-domain residues	Other domains	Ref.
SET1	*YHR119w*	Histone H3-K4 MT[a]	Nuclear	1,080/9.68	932–1061		7, 8
SET2	*YJL168c*	Histone H3-K36 MT	Nuclear	733/8.56	114–243		9
SET3	*YKR029c*	NAD-dependent histone deacetylase	Nuclear	751/9.12	315–463	PHD: 119–166	31
SET4	*YJL105w*	Unknown	Unknown	560/8.76	340–482	PHD: 162–210	
SET5	*YHR207c*	Unknown	Cytoplasmic/Nuclear	526/6.43	106–140 & 364–409	TD: 236–253	
SET6	*YPL165c*	Unknown	Unknown	373/7.59	297–345	TD: 232–260	
SET7 (RMS1)	*YDR257c*	Unknown	Nuclear	494/4.80	19–271	TD: 166–189 & 344–372	
YBR030w	*YBR030w*	Unknown	Nuclear	552/4.37	10–341	TD: 74–92	
YHL039w	*YHL039w*	Unknown	Cytoplasmic	585/6.64	8–287	TD: 345–362 & 457–473	
YPL208w	*YPL208w*	Unknown	Cytoplasmic/Nuclear	583/4.86		TD: 196–224	
CTM1	*YHR109w*	Cytochrome *c* lysine MT	Cytoplasmic	585/4.72			10

[a] MT designates methyltransferase.

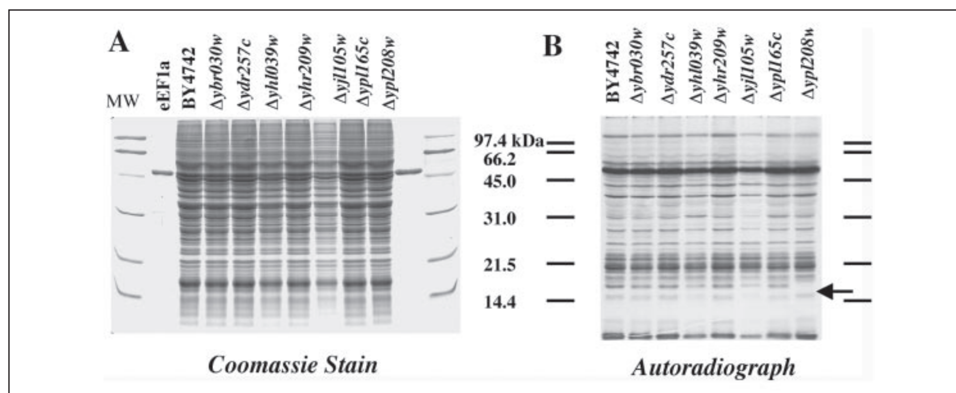

FIGURE 1. ***In vivo* labeling of putative SET methyltransferase mutants.** Yeast strains were obtained in which the gene encoding a potential SET methyltransferase was deleted. These strains were *in vivo* labeled with [^{3}H]AdoMet, and lysates of each mutant were fractionated by SDS gel electrophoresis on a 12.6% polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue (*A*), treated with EN^{3}HANCE, dried, and exposed to film for 76 h (*B*). The *arrow* indicates where loss of [^{3}H]methylation is observed in a 15-kDa band in the Δ*ypl208w* deletion strain. No obvious difference was observed in the other six putative SET methyltransferase knock-out strains when compared with the parent BY4742 strain.

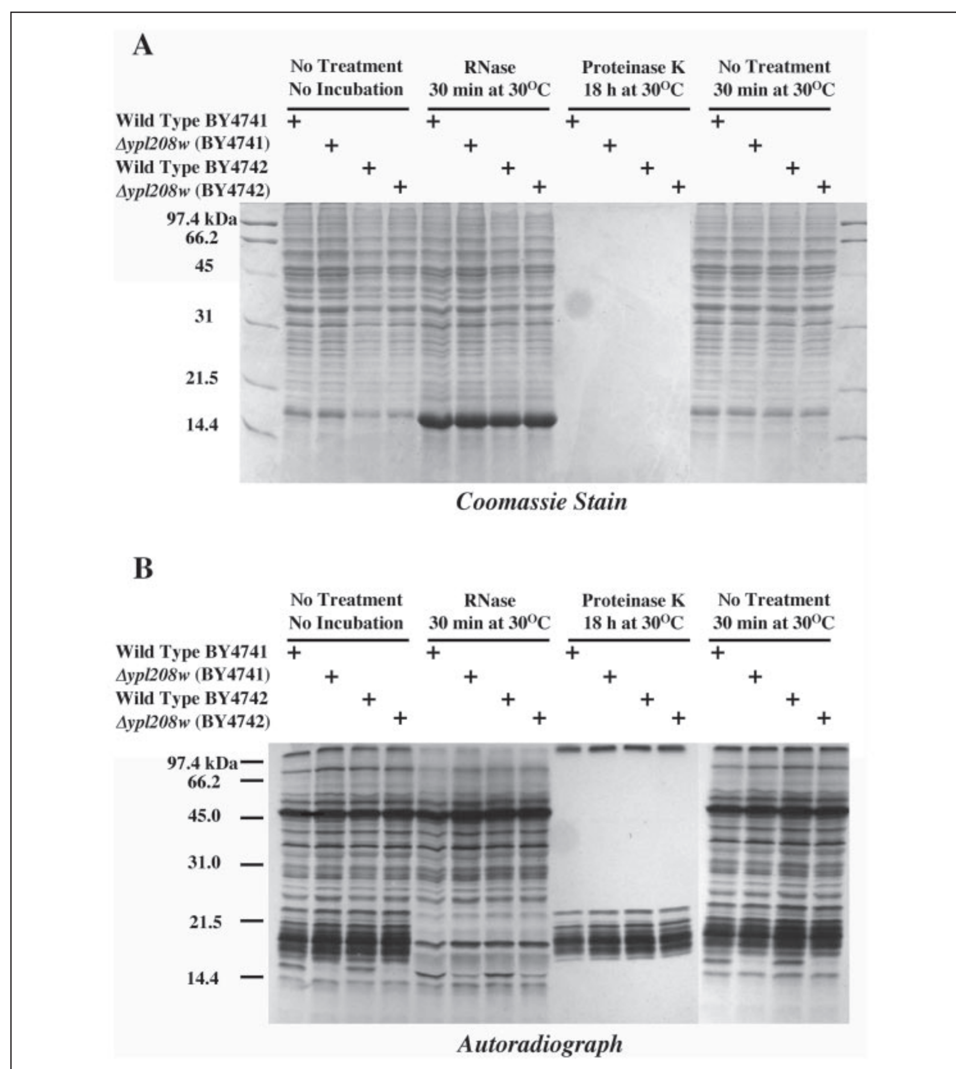

FIGURE 2. **Proteinase K and RNase treatment of [^{3}H]AdoMet-labeled cell extracts.** *In vivo* labeled lysate (from 0.35 $A_{600\ nm}$ units of cells) was incubated with either 2.4 mg/ml proteinase K for 18 h at 30 °C (*lanes 5–8*), 0.2 mg/ml RNase A for 30 min at 30 °C (*lanes 9–12*), or not treated, and incubated for 30 min at 30 °C (*lanes 13–16*). As a control, fresh lysate was loaded without incubation and without treatment (*lanes 1–4*). Lysates obtained from the wild-type BY4741 and BY4742 strains were analyzed in addition to their respective Δ*ypl208w* deletion strains. The reaction mixtures were adjusted to final concentrations of 9.6 mM Tris/HCl, 12 mM NaCl, and 0.24 mM EDTA, pH 7.5. The reactions were quenched by the addition of an equal volume of SDS gel sample buffer, electrophoresed, and analyzed by fluorography as described in the legend to Fig. 1 above with a film exposure of 4 days. The *arrow* indicates the migration position of the Ypl208w-methyl-accepting substrate.

the bottom and the $A_{260\ nm}$ and $A_{280\ nm}$ were measured. Fractions containing either the 60 S or 40 S ribosomal subunits, or cytosolic proteins, were individually pooled and ethanol precipitated by adding 0.7 volumes of cold ethanol in the presence of 15 mM magnesium acetate. The proteins were allowed to precipitate for 24 h at −20 °C. Proteins were pelleted by centrifugation at 12,000 × *g* for 20 min at 4 °C. The pelleted proteins were resuspended in 200 μl of water, and the RNA was extracted by the addition of 400 μl of glacial acetic acid and 20 μl of 1 M magnesium chloride, in rapid succession. The mixture was stirred for 45 min in an ice bath, after which the precipitated RNA was removed by spinning at 20,000 × *g* for 10 min at 4 °C. The supernatant was dialyzed against 2% acetic acid, and lyophilized. The presence of the ribosomal subunits was confirmed by phenol extracting the RNA and determining the content of the 25 S- and 18 S-rRNA species after agarose gel electrophoresis using methods described previously (26, 27).

Electrospray-Ionization Mass Spectrometry—Acetic acid-extracted lyophilized ribosomal proteins were dissolved in 90% formic acid and immediately injected for reverse-phase liquid chromatography with

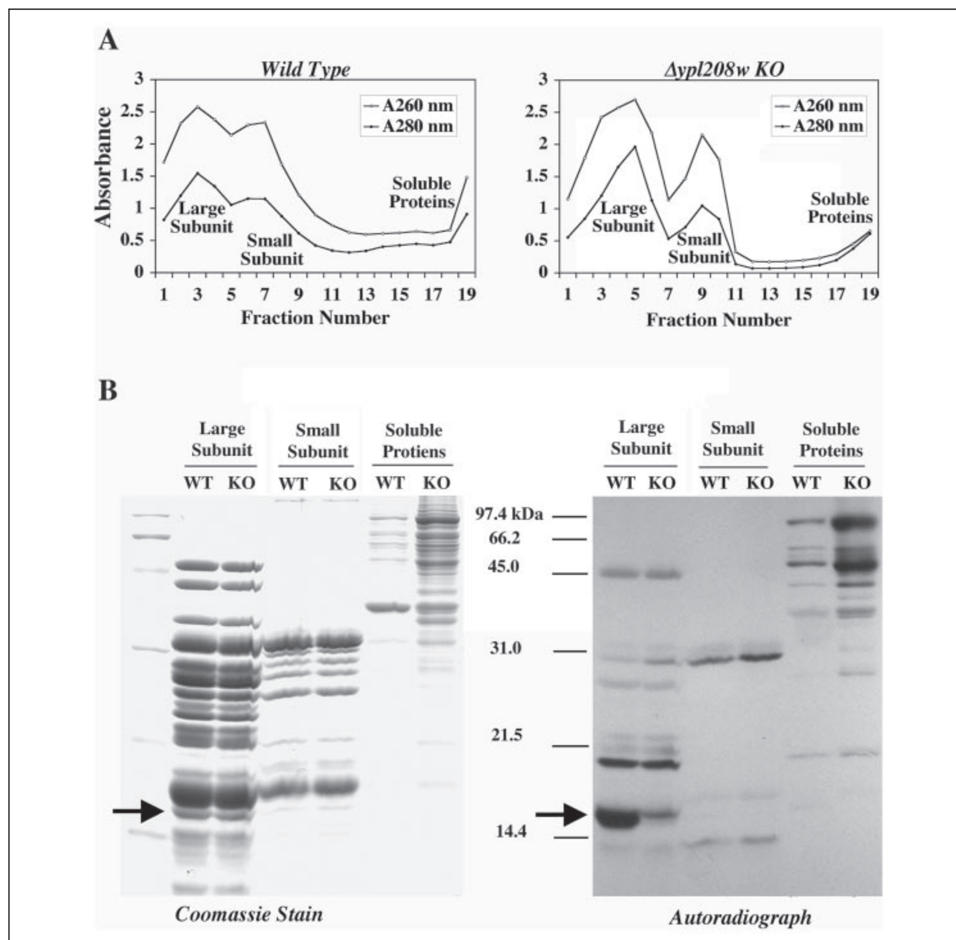

FIGURE 3. **Fractionation of BY4742 and Δ*ypl208w* extracts using high salt sucrose gradients.** *A*, fractions were collected from the bottom of the tube and the $A_{260\text{ nm}}$ and $A_{280\text{ nm}}$ were measured. Peaks observed represent the large 60 S or small 40 S ribosomal subunits, or free soluble protein. The identity of the ribosomal subunits was determined by extracting RNA from 100 μl of the pooled sample and demonstrating the presence of the 25 S rRNA in the large subunit and the 18 S rRNA in the small subunit by gel electrophoresis (data not shown). *B*, protein (100 μg) from the large ribosomal subunit, small ribosomal subunit, and soluble protein fraction was fractionated by SDS gel electrophoresis on a gel prepared with 15% acrylamide and 0.52% *N*,*N*-methylene-bisacrylamide, followed by fluorography for 2 months. A longer exposure was required than in the experiments shown in Figs. 1 and 2, because 20-fold less [^{3}H]AdoMet was used. The *arrow* indicates the position where loss of methylation is observed in the Δ*ypl208w* knock-out strain when compared with parent BY4742 strain.

electrospray-ionization mass spectrometry and fraction collection (LC-MS+). The procedure has been described in detail (28); briefly, the stationary phase used is polymeric (PLRP/S, Polymer Laboratories) and buffers A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile) are used for equilibration (95% A; 5% B) and an extended gradient. The mass spectrometer (API III+, PE Sciex) was tuned and calibrated as described (29) yielding mass accuracy of 0.01% (±1.5 Da at 15 kDa). Approximately 50% of the column eluent was directed to a fraction collector using a T flow splitter. Fractions were stored at −20 °C for further processing.

Purification of Tap-Tagged Rkm1—Tap-tagged Rkm1 (Open Biosystems; Huntsville, AL) was purified by slightly modifying the method used by Puig et al. (30). Briefly, 2 liters of tap-tagged Rkm1-containing cells were grown to 2 OD at an absorbance of 600 nm. The cells were harvested by centrifugation at 5,000 × *g* for 5 min and washed twice with 50 ml of water. The cell pellet was stored at −20 °C overnight. The next day the cells were thawed and lysed in 10 ml of Tap Buffer A (10 mM K-HEPES, 10 mM KCl, 1.5 mM magnesium chloride, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) and an equal volume of baked zirconium beads by seven vortexing/icing cycles. After lysis the KCl concentration was adjusted to 0.2 M by adding 1:9 volume of 2 M KCl. The lysate was centrifuged at 25,000 × *g* for 30 min and the supernatant was recovered. This material was dialyzed against Tap buffer D (20 mM K-HEPES, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) for 3 h at 4 °C. Purification with IgG affinity beads (Amersham Biosciences) and cleavage from beads with TEV protease (Invitrogen) was performed as described in Puig *et al.* (30). The TEV protease was then separated from the purified protein using calmodulin-Sepharose 4B chromatography (Amersham Biosciences) as described previously (30).

RESULTS AND DISCUSSION

As described in TABLE TWO, the genome of the yeast *S. cerevisiae* appears to encode at least eleven SET domain-containing proteins. Two of these genes (*SET1* and *SET2*) have been shown to encode histone protein lysine methyltransferases, one gene (*SET3*) encodes a histone deacetylase, and another gene (*CTM1*) encodes the cytochrome *c* lysine methyltransferase (7–10, 31). The remaining seven SET domain-encoding genes were identified by the Pfam protein family data base (www.sanger.ac.uk/gi-bin/Pfam; version of 31 October 2002) (TABLE TWO). The function of these seven gene products is unknown. In this work, we investigated the possible role of these species as protein methyltransferases.

We first compared the methylation of size-fractionated polypeptides derived from parent cells and cells with deletions in the seven SET domain-containing genes. In *S. cerevisiae*, AdoMet is readily taken up from the external media through a plasma membrane transporter (32, 33), and it is possible to directly label methylated species by incubating intact cells with [^{3}H]AdoMet. Here, [^{3}H]methyl groups are transferred by the endogenous yeast methyltransferases to their methyl-accepting substrates. When cell extracts were fractionated by SDS gel electrophoresis and analyzed for [^{3}H]methyl groups by autoradiography, a number of radiolabeled species were found (Fig. 1). We detected little or no difference between the parent BY4742 strain and six of the deletion mutants. However, when analyzing the Δ*ypl208w* deletion strain, we noted the loss of a 15-kDa radiolabeled species (Fig. 1). It does not

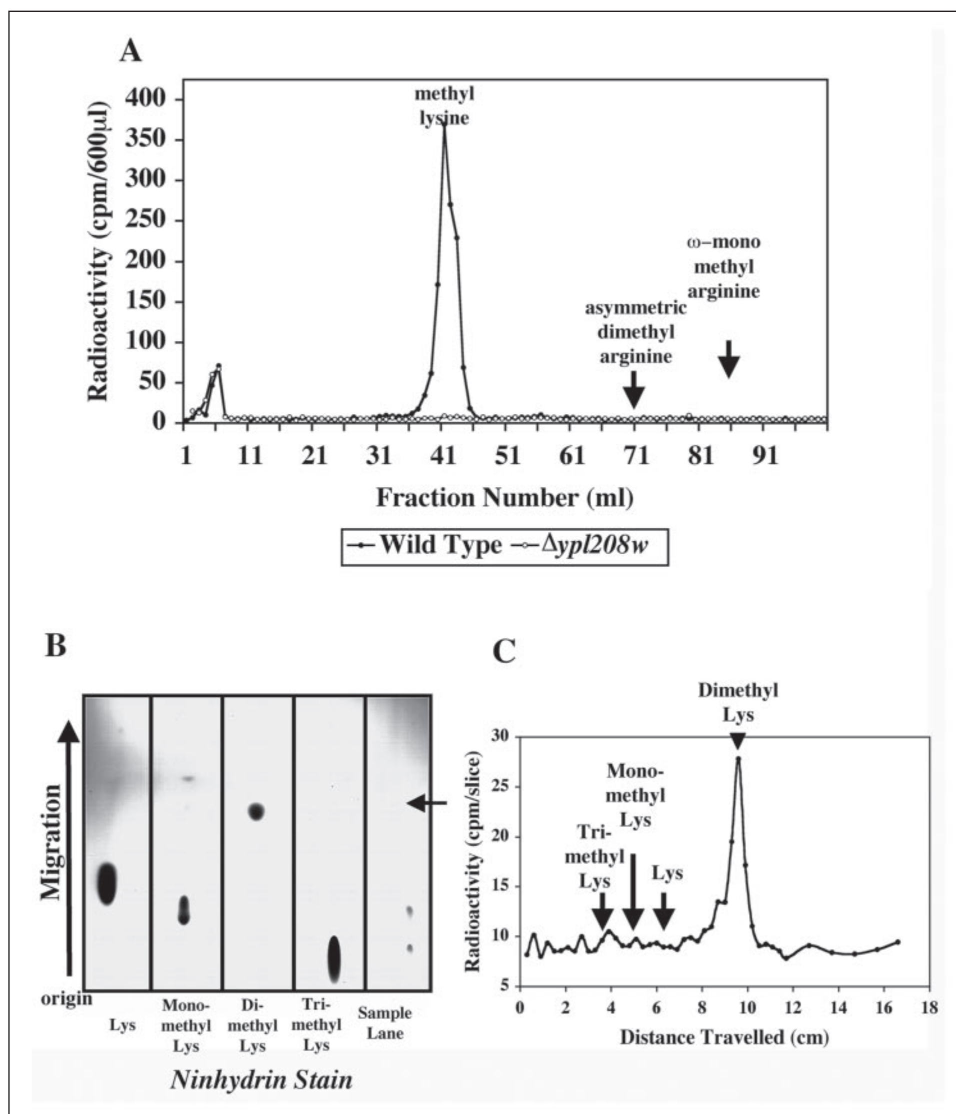

FIGURE 4. **Methylation occurs on lysine residue(s) of the 15-kDa substrate.** *A*, amino acid analysis was performed as described previously (39). Gel slices corresponding to the 15-kDa methylated substrate were excised from SDS gels of purified large ribosomal subunit proteins from both the BY4742 and Δ*ypl208w* strains. The gel slice was added to a 6 × 50-mm glass vial containing 100 μl of 6 N HCl and hydrolysis was carried out *in vacuo* for 24 h at 108 °C using a Waters Pico-Tag vapor-phase apparatus. Residual HCl was removed by vacuum centrifugation. The free amino acids were resuspended in 50 μl of water and then added to 500 μl of citrate dilution buffer (0.2 M NaOH titrated to pH 2.2 with citric acid). The hydrolyzed sample was then mixed with standards (1 μmol each of ω-N^G-monomethylarginine (Sigma product M7033; acetate salt), asymmetric ω-N^G,N^G-dimethylarginine (Sigma product D4268; hydrochloride), *N*ε, *N*ε,*N*ε-trimethyllysine (Sigma product T1660), and *N*ε-monomethyllysine (Sigma product M6004; hydrochloride) and loaded onto a high performance cation-exchange column (0.9-cm inner diameter × 11-cm column height, containing Beckman AA-15 sulfonated polystyrene beads). The column was equilibrated and eluted with sodium citrate buffer (0.35 M NaOH, titrated to pH 5.27 with citric acid) at a flow rate of 1 ml/min at 55 °C. Fractions were collected, and aliquots counted for radioactivity and assayed for amino acid content using a ninhydrin assay (39). *B*, radioactive fractions 39–44 from the wild-type substrate amino acid analysis were pooled and desalted using a Sephadex G-15 column (1.5 cm in diameter and 77 cm in length), equilibrated with 0.1 M acetic acid. Elution of the desalted sample was monitored by counting a fraction of each sample for the presence of radioactivity. The peak fractions collected from the desalting column were pooled and concentrated by vacuum centrifugation to 200 μl. One-tenth of this fraction was spotted onto a 20-cm silica-coated thin layer chromatography plate and developed using a mobile phase consisting of methanol and 14.8 N ammonium hydroxide (3:1, v/v). The sheet was heated at 37 °C to evaporate the solvent and then sprayed with 10 mg/ml of ninhydrin in acetone and incubated at 37 °C until the Ruheman's purple color appeared. 1 μmol of standard amino acids, L-lysine, *N*ε-monomethyllysine, *N*ε, *N*ε-dimethyllysine, and *N*ε,*N*ε,*N*ε-trimethyllysine were spotted in adjacent lanes. The solvent front is at the *top*. *C*, sample lane was divided into slices and radioactivity determined after scraping the silica into scintillation vials. The scraped silica was diluted with 500 μl of water in 5 ml of fluor and counted three times for 3 min each.

appear that the loss of methylation at 15 kDa is caused by the loss of the *YPL208w* gene product as a methyl-accepting protein because its encoded polypeptide has a mass of 67.2 kDa. We further confirmed these results by analyzing the independently-derived Δ*ypl208w* deletion strain obtained in the BY4741 background. Here, we also observed a similar loss of methylation at 15 kDa (Fig. 2). The Δ*ypl208w* deletion in both strains was confirmed by PCR analysis (data not shown). These results suggest that the *YPL208w* gene encodes a methyltransferase active on a 15-kDa species.

Because tRNA molecules are also modified by AdoMet-dependent methyltransferases and because they can migrate on an SDS gel in the position of 15–25 kDa polypeptides (34), we wanted to determine if the *YPL208w*-dependent 15-kDa labeled species represented a polypeptide or an RNA-methylated species. Lysates from *in vivo* labeled cells were treated with either RNase, which would be expected to degrade all of the RNA in the sample, or proteinase K, which would be expected to degrade all polypeptides. After SDS gel electrophoresis, we found that the samples treated with proteinase K did not contain the 15-kDa [^{3}H]methylated species, whereas it was present in the sample treated with RNase (Fig. 2). A slight shift in the mobility of the labeled 15-kDa species in the RNase-treated samples was observed, possibly because of the presence of the RNase protein itself at this position. These results indicate that the 15-kDa species is a protein and that the *YPL208w* gene product is a protein methyltransferase.

Because the previously identified yeast Set1 and Set2 proteins were shown to be histone lysine methyltransferases (TABLE TWO; Refs. 7–9), we first asked whether the 15-kDa labeled polypeptide might correspond to one or more histone species. However, we found that none of the yeast histones (purified according to Ref. 35) comigrated with the 15-kDa labeled species (data not shown). Because a number of methylated ribosomal proteins had been identified (14–16), we then asked if the [^{3}H]methylated 15-kDa species co-sedimented with ribosomes or with the soluble protein fraction. In preliminary experiments, we used sucrose gradient centrifugation to fractionate labeled cellular lysates under low salt conditions and found that the 15-kDa [^{3}H]methylated species in fact migrated with 80 S ribosomes (data not shown).

We then wanted to distinguish whether the 15-kDa species was associated with the large 60 S subunit or with the small 40 S subunit. *In vivo* labeled cellular components from both the BY4742 parent strain and the Δ*ypl208w* knock-out strain were thus fractionated using high salt

TABLE THREE

Mass spectral analysis of the Rkm1 substrate

Analysis was performed as described previously with modifications (43, 44). Protein bands were cut from a Coomassie gel of purified large ribosomal proteins. Gel slices were washed by repeated cycles of shaking for 10 min with 50 μl of 25 mM ammonium bicarbonate, pH 8.0:acetonitrile (1:1, v/v) (three times) followed by 50 μl of 25 mM ammonium bicarbonate, pH 8.0 (one time) until the stain was removed. The gel slices were then dried by vacuum centrifugation. Proteins in the gel were reduced and *S*-carboxyamidated by incubation for 1 h at 56 °C in the dark with 25 μl of 10 mM dithiothreitol, followed by incubation with 100 μl of 55 mM iodoacetamide for 1 h at room temperature in the dark, while shaking, after removal of excess dithiothreitol. The gel slice was then washed as described above for removing stain and dried by vacuum centrifugation. In gel trypsin, digestion was performed by adding 30 μl of 0.25 units/μl of trypsin and allowing the gel slices to rehydrate over ice for 15 min, followed by incubating the digest at 37 °C for 14–20 h. The digested peptides were eluted from the gel slices by incubating them with 50 μl of 50% acetonitrile, 5% formic acid in water for 10 min with shaking, followed by placing in a sonicator bath for 5 min. The process was repeated three times, and each time the supernatant was collected and pooled. The pooled supernatant, which contains the digested peptide fragments, was concentrated down to 20 μl by vacuum centrifugation, desalted using a 10-μl C18 column ("ZipTip"ZTC18S096, Fisher Scientific, Tustin, CA). This column was equilibrated by washing first with two 10-μl aliquots of methanol, then with three 10-μl aliquots of 0.1% trifluoroacetic acid/70%acetonitrile, and finally with five 10-μl aliquots of 0.1% trifluoroaceticacid/5% acetonitrile. The peptides were then loaded on the column, and the column was washed with six 10-μl aliquots of 0.1% trifluoroacetic acid/5% acetonitrile and then was eluted with 6 μl of a 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid (Sigma Product, C2020) in 0.1% trifluoroacetic acid/70% acetonitrile. The eluted material was then spotted on a plate and analyzed by MALDI on a Voyager-DE STR (Applied Biosystems) mass spectrometer in the positive ion mode using a laser intensity of ~1500–1900 and shooting the laser 800 times per spectrum. The data were analyzed using the Data Explorer program (Applied Biosystems).

Trypsin fragments	Residues in Rpl23ab	Observed mass[a]	Theoretical mass	Error[b]
		m/z	*m/z*	*ppm*
LNRLPAASLGDMVMATVK	46–63	1887.0266	1887.0196	3.7
GKPELR	65–70	699.4145	699.4153	1.1
VMPAIVVR	73–80	884.5412	884.5392	2.2
RRDGVFLYFEDNAGVIANPK[c]	87–106	2281.1796	2281.1729	2.9
RDGVFLYFEDNAGVIANPK[c]	88–106	2125.0823	2125.0718	5.0

[a] Observed mass is reported as an average of at least three masses observed in spectra obtained for the Rpl23ab protein excised from both the wild type and Δ*ypl208w* gel lane.
[b] Error is calculated ((theoretical − observed)/theoretical) × 1,000,000 and is reported as an absolute value. The observed mass used in the calculation is the calculated average observed mass.
[c] These peptide fragments were only observed in spectra obtained when analyzing the Rpl23ab protein obtained from the Δ*ypl208w*strain and were not observed when analyzing the Rpl23ab protein obtained from the parent strain BY4742.

sucrose gradients designed to dissociate the large and small ribosomal subunits. The majority of contaminating proteins were removed by differential centrifugation before loading the extracts onto the high salt sucrose gradient in order to better isolate the substrate. Upon SDS gel electrophoresis of individual gradient fractions, we observed that the [^{3}H]methylated 15-kDa species migrated with the large ribosomal subunit in the parent BY4742 strain. However, the radioactivity observed in that region was greatly reduced when the Δ*ypl208w* knock-out strain was analyzed, indicating that the bulk of the methylation seen was because of the activity of the Ypl208w gene product (Fig. 3). Two-dimensional gel electrophoresis was also performed on the *in vivo* labeled proteins of the large subunit and the 15-kDa labeled species was found to migrate in an elongated spot at a pH value consistent with its isoelectric point (data not shown).

To identify the amino acid modified by the Ypl208w-dependent methylation reaction, the 15-kDa [^{3}H]methylated species from the purified ribosomal large subunit was converted into its component amino acids by acid hydrolysis. Fractionation of the hydrolysate by high performance cation exchange chromatography demonstrated that the bulk of the radiolabel elutes with a standard mixture of ϵ-monomethyl and trimethyllysine with a small amount of label eluting in the early fractions (Fig. 4*A*). In this chromatography system, no separation of mono, di, and trimethyllysine is observed. No detectable radioactivity was seen at the position of the methylated arginine standards. The material in the region of the SDS gel corresponding to the 15-kDa substrate in the Δ*ypl208w* mutant strain was also acid hydrolyzed as a control. Significantly, no detectable radioactivity was observed in the methyllysine peak, but a similar amount of radioactivity was observed in the early fractions (Fig. 4*A*). Thus, the small amount of residual radioactivity seen in the autoradiograph of the mutant in Fig. 3*B* was not due to protein lysine methyltransferase activity. Taken together, these results demonstrate that Ypl208w is a protein lysine methyltransferase.

Lysine residues in proteins can be modified by mono, di, or trimethylation on the ϵ-side chain amino group; all three derivatives comigrate on the cation-exchange column shown in Fig. 4*A*. However, we were able to separate the mono-, di-, and trimethyl derivatives of lysine by thin layer chromatography (Fig. 4*B*). We pooled and desalted the radiolabeled fractions corresponding to the methyllysine peak shown in Fig. 4*A* and fractionated the methylated species by thin layer chromatography. Here, the radioactivity was found to migrate only with the dimethyllysine standard (Fig. 4*C*). These results suggest that the reaction catalyzed by the YPL208w gene product is a dimethyllysine modification of a protein of the large ribosomal subunit. We have thus designated the *YPL208w* gene *RKM1* for ribosomal protein lysine (K) methyltransferase 1.

Examination of the literature revealed a candidate for a large ribosomal protein corresponding to the 15-kDa methylated species. Lhoest *et al.* (15) had previously observed that the ribosomal protein L23a, with a polypeptide molecular mass of ~14.5 kDa, is dimethylated at lysine residues. Both the polypeptide size and the type of methylation match that seen here for the Rkm1-catalyzed reaction, although some monomethyllysine was also observed by Lhoest *et al.* (15). Furthermore, Lobet *et al.* (16) estimated the native molecular mass of the L23a methyltransferase to be about 82 kDa by gel filtration chromatography and the isoelectric point to be 4.45 by isoelectric focusing (16). We then compared these properties to those predicted for the Rkm1 methyltransferase. This polypeptide has an expected molecular mass of 67.2 kDa and a calculated isoelectric point of 4.86, values consistent with those reported for the L23a methyltransferase (16). The authors also found that the methyltransferase was very specific in modifying only Rpl23ab (16). Additional evidence for the possible identification of Rpl23ab as the 15-kDa substrate for Rkm1 comes from proteome-wide affinity precipitation assays that indicate a physical interaction between the Rkm1 protein and the Rpl23ab ribosomal protein (22, 23).

To determine whether the substrate for Rkm1 is indeed Rpl23ab, mass spectral analysis was performed. The band corresponding to the Rkm1 substrate was excised from a gel in which purified large ribosomal proteins had been separated. The band was trypsin digested and analyzed by MALDI-TOF. The trypsin fragments produced in the MALDI analysis were queried in the Protein Prospector data base (prospector.

ucsf.edu/). From these results, we were able to clearly identify several peptides from the large ribosomal protein Rpl23ab from both the BY4742 and Δ*ypl208w* strains (TABLE THREE). To confirm that the methylation sites were on Rpl23ab, we analyzed intact large ribosomal proteins purified from both the wild-type BY4742 strain and the Δ*ypl208w*(BY4742) strain by electrospray mass spectrometry. In the protein from the Δ*ypl208w* strain we observed a species with a mass of 14,384 Da, which corresponds to that of the Rpl23ab sequence with the loss of the initiator methionine and the addition of an acetyl group. However, this species was not detected in the protein prepared from the wild-type yeast, where a species of mass 14,440 was detected instead. The 56-dalton difference corresponds exactly to that expected for the presence of two dimethylated lysine residues. This result led us to conclude that the 15-kDa protein whose methylation is absent in the Δ*ypl208w* deletion strain is in fact Rpl23ab. Because we know that the substrate is exclusively dimethylated, as determined by thin layer chromatography (Fig. 4, *B* and *C*), we concluded that the Rpl23ab protein is modified at two distinct lysine residues by the Ypl208w protein. A yeast ribosomal species with a mass of 14,440 was also identified with Rpl23ab by Lee *et al.* (14). By MS/MS analysis of proteinase ArgC peptides, we have shown that the N terminus is indeed acetylated on Ser-2 and have been able to obtain preliminary evidence to narrow down possible sites of dimethylation to Lys-40, Lys-106, or Lys-110 (data not shown).

We then wanted to demonstrate that the Rkm1 protein encoded by *YPL208w* was directly responsible for modifying Rpl23ab. Therefore, we tested the *in vitro* activity of Rkm1. The yeast strain YIT613 contains a FLAG tag on the ribosomal protein L25 that allows rapid purification of the intact 80 S ribosomal complex. Purified FLAG-tagged ribosomes were *in vitro* methylated using wild-type and *rkm1* lysate as the methyltransferase source. We found that Rpl23ab was methylated in FLAG-tagged ribosomes incubated with the wild-type lysate but not with mutant *rkm1* lysate (Fig. 5*A*). Non-tagged ribosomes from the YIT617 isogenic parent strain of YIT613 were purified in parallel as a control, and no proteins were observed to non-specifically pull-down after Coomassie Blue staining. To confirm that the gene product of *YPL208w* is directly responsible for methylating Rpl23ab, FLAG-tagged ribosomes were *in vitro* methylated in the presence and the absence of purified Tap-tagged Rkm1. The isogenic BY4741 parent strain of the Tap-tagged Rkm1 strain was purified in parallel with the tagged strain as a control, but no 15-kDa labeling was observed (data not shown). When Tap-tagged Rkm1 is present the Rpl23ab protein is methylated. However, methylation is not observed when the Tap-tagged Rkm1 is not present in the reaction (Fig. 5*B*). The relatively low level of methylation in the *in vitro* experiments may be because of the instability of the methyltransferase. This is evident in the low yield of methyltransferase observed during the Tap tag purification; silver staining was required in order to visualize the enzyme. Lobet *et al.* (16) also encountered similar difficulties, which prevented them from purifying the methyltransferase to homogeneity and identifying the methyltransferase. The *in vitro* data demonstrates that Rkm1 is directly responsible for methylating Rpl23ab and that the interaction observed in the proteome-wide affinity precipitation assays is a real interaction between the Rkm1 methyltransferase and its substrate Rpl23ab (22, 23).

At this point, the physiological function of the Rkm1 methyltransferase is unknown. It is clear that yeast cells lacking the enzyme are viable. Affinity precipitation experiments demonstrate a physical interaction with its substrate protein Rpl23ab as well as with the *BCP1* (*YDR361c*) gene product (36). Currently, it is known that Bcp1 is required for the nuclear export of Mss4, a phosphatidylinositol 4-phosphate 5-kinase, and is involved in regulating the production of phosphatidylinositol 4,5-bisphosphate (37). However, we find it of interest that it appears to interact not only with our methyltransferase but also with the Rpl23ab substrate, two proteins that do not seem to be involved in either nuclear export or synthesis of phosphatidylinositides. In an attempt to identify a phenotype, we compared growth of the Δ*ypl208w* deletion strain to its parent strain under different conditions. We found no difference in growth on YPD solid medium between the *rkm1* knockout and its parent strain at 18 °C, 25 °C, 30 °C, and 37 °C, nor did we see

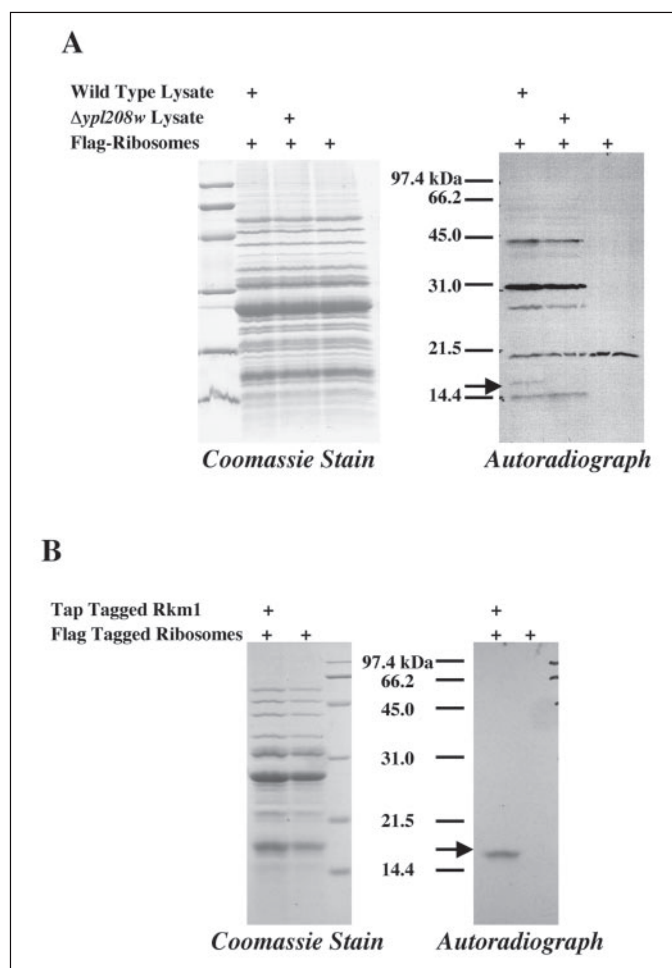

FIGURE 5. ***In vitro* methylation of Rpl23ab.** *A*, yeast strain YIT613 that carries a FLAG tag on the ribosomal protein L25 and permits the purification of intact ribosomes and its respective isogenic parent strain YIT617 were kindly provided by Dr. T. Inada (Nagoya University, Japan). FLAG-tagged ribosomes were purified according to the method of Inada *et al.* (40). Purified FLAG-tagged ribosomes still bound to anti-FLAG beads were incubated with wild-type lysate, Δ*ypl208w* lysate, or no lysate and 1 μM of [^{3}H]AdoMet for 2 h at 30 °C in the presence of lysis buffer (9.0 mM HEPES, 0.9 mM magnesium acetate, 45.2 mM potassium acetate, 45.2 μg/ml cycloheximide, 0.23 mM dithiothreitol, pH 7.4). The BY4742 and Δ*ypl208w* lysate was obtained as described under "Materials and Methods." After the incubation, the FLAG-tagged ribosomes were spun down by centrifugation at 1,000 × *g* and washed three times with IXA buffer (50 mM Tris/HCl, 100 mM KCl, 12 mM magnesium acetate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) to rid the ribosomes of the lysate and unused [^{3}H]AdoMet. The polypeptides of the ribosomes were analyzed by 12.6% SDS-PAGE. After electrophoresis the gel was enhanced for 1 h, dried and exposed to film for 2 months at −80 °C. The *arrow* indicates the 15-kDa substrate point of migration. *B*, to test the *in vitro* activity of the *YPL208w* gene product, 50 μl of Tap-tagged Rkm1 was incubated with 50 μl of FLAG-tagged ribosomes and 10 μl of 12 μM [^{3}H]AdoMet for 2 h at 30 °C (*lane 1*). The reaction was buffered by the addition of 10 μl of 12× buffer (600 mM Tris-HCl, 1.2 M KCl, 144 mM magnesium acetate, 12 mM phenylmethylsulfonyl fluoride, and 12 mM dithiothreitol). As a control the reaction the Tap-tagged Rkm1 was incubated with 50 μl of water instead of FLAG-tagged ribosomes (*lane 2*). The reaction was quenched by the addition of an equal volume of 2× SDS loading buffer. The degree of methylation was analyzed by loading the sample onto 15% SDS-PAGE as described above. After electrophoresis the gel was prepared for fluorography and exposed to film for 3 weeks at −80 °C. The *arrow* indicates the position of migration of the 15-kDa substrate.

TABLE FOUR
SET-domain methyltransferases

SET-domain methyltransferase	Substrate	Modification site	Species	Ref.
Ctm1 (*S. cerevisiae*)	Cytochrome *c*	Trimethyllysine-72	Plants/fungi	10
Rubisco LSMT (*Pisum sativum)*	Rubisco large subunit	Trimethyllysine-14	Plants	45
Set1 (*S. cerevisiae)*	Histone H3	Mono-,di-,trimethyllysine-4	Widespread	7,8
Set2 (*S. cerevisiae*)	Histone H3	Lysine-36, ND[a]	Widespread	9
Rkm1 (*S. cerevisiae*)	Rpl23ab	Dimethyllysine, ND	Plants/fungi	This study

[a] ND, not determined.

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197WYSFpAFLWShlIFISRAFP216   230VLlPIVDLLNHD241   268ELsNNYGgKgNEELLsgYGFV288   Ypl208w
198WnSFvAyLWSycIFnSRAFP217   235fLyPIVDLLNHk246   275ELfNNYGnisNEkcLlnYGFw295   Yhl039w
187sYSFdlemqdssvneneeee206   220smiPlaDmLNaD231   259qvyNiYGehpNsELLrrYGyV279   Ydr257c
209dvtqddFfWafgmlrSRAFt228   236VLiPlaDLaNHs247   285vLiqydlnKsNaELaldYGlt305   SSMT
201ritlddFiWafgIlkSRAFs220   228VLiPlaDLiNHn239   277vyiqydlnKsNaELaldYGFV297   LSMT
228ftSdelFslfvhvyfiinsr247   265tLvPyVDfmNHi276   219ELflNYGahsNdfLLneYGFV239   Ydr198c
228sdpdlkFvslydvcdkcgeP247   274metrviDedlik285   329EifNsYGelsNvfLLarYGFt349   Ybr030w
187vlnlsdikhlysaiISRcle206   221tLvPIVDfaNHe232   268vfisysptedlfsmLvtYGFt288   Ctm1p
317tvwekcyelfcgaFpkasee336   357qvyhwisfiNHD368   415ELrvNwGflcqcdrcqnelst435   Yhr207c
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FIGURE 6. **Amino acid sequence alignment of Ypl208w and other SET domain-containing proteins.** Protein sequences of known SET methyltransferases (Rubisco SSMT and LSMT and Ctm1p) and putative methyltransferases (Yhl039w, Set7, Ydr198c, Ybr030w, and SET5) were compared with the amino acid sequence of Ypl208w. Initial alignments were performed using PSI BLAST (41). These proteins were chosen because they had the highest sequence similarity to Ypl208w.

any difference in growth on YPD solid media at 30 °C with or without 0.4 M NaCl. No differences were seen for growth at 30 °C in liquid media. In addition, we detected no growth differences on solid media between the *rkm1* knock-out and its parent when grown on minimal media (38) supplemented with 2% D-glucose, ethanol, sodium lactate, glycerol, or potassium acetate. Finally we tested growth on various antibiotics including cycloheximide, gentamicin, hygromycin B, paromomycin, and tetracyclin and observed no difference in growth between the deletion strain and the wild-type strain on solid media. In an attempt to identify a role for the methylation of Rpl23ab we assessed ribosomal and polysomal assembly by comparing polysomal profiles between the wild-type BY4742 strain and the Δ*ypl208w* deletion strain. However, we were unable to detect a significant difference in the polysomal profile of the deletion strain as compared with the wild-type strain (data not shown). Further experimentation will be performed in an attempt to determine a role for the methylation of Rpl23ab.

The novel observation that SET domain-containing methyltransferases can also methylate ribosomal proteins in addition to histones or cytochrome *c*, opens up a new area of study because of their possible role in regulating translation. TABLE FOUR lists various SET domain-containing methyltransferases and the various processes in which they are currently known to be involved. From the sequence alignment analysis illustrated in Fig. 6, it is evident that Rkm1 shares much similarity to many of the putative SET methyltransferases but little or no sequence similarity to the known histone lysine methyltransferases Set1 and Set2. This opens up the possibility that the other putative SET domain-containing methyltransferases listed may also function as ribosomal lysine methyltransferases, like Rkm1. Currently, there are various ribosomal proteins known to be methylated (14), but their methyltransferases are still unknown.

BLAST searches of Rkm1 did not pull up any statistically significant protein matches in higher non-plant organisms. Rkm1 does share sequence similarity to the cytochrome *c* lysine methyltransferase, Ctm1, which also appears to be present only in plants and fungi, as well as the plant Rubisco lysine methyltransferase. It will be interesting to see which non-histone SET methyltransferases are more widely distributed to animal species.

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