

A Novel 3-Methylhistidine Modification of Yeast Ribosomal Protein Rpl3 Is Dependent upon the YIL110W Methyltransferase^{*[5]}

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We have shown that Rpl3, a protein of the large ribosomal subunit from baker's yeast (*Saccharomyces cerevisiae*), is stoichiometrically monomethylated at position 243, producing a 3-methylhistidine residue. This conclusion is supported by top-down and bottom-up mass spectrometry of Rpl3, as well as by biochemical analysis of Rpl3 radiolabeled *in vivo* with *S*-adenosyl-L-[methyl-³H]methionine. The results show that a +14-Da modification occurs within the GTKKLPRKTHRGLRKVAC sequence of Rpl3. Using high-resolution cation-exchange chromatography and thin layer chromatography, we demonstrate that neither lysine nor arginine residues are methylated and that a 3-methylhistidine residue is present. Analysis of 37 deletion strains of known and putative methyltransferases revealed that only the deletion of the YIL110W gene, encoding a seven β -strand methyltransferase, results in the loss of the +14-Da modification of Rpl3. We suggest that YIL110W encodes a protein histidine methyltransferase responsible for the modification of Rpl3 and potentially other yeast proteins, and now designate it Hpm1 (Histidine protein methyltransferase 1). Deletion of the YIL110W/*HPM1* gene results in numerous phenotypes including some that may result from abnormal interactions between Rpl3 and the 25 S ribosomal RNA. This is the first report of a methylated histidine residue in yeast cells, and the first example of a gene required for protein histidine methylation in nature.

The addition of methyl groups to proteins from the methyl donor *S*-adenosylmethionine is one of the most common post-translational modifications, resulting in an expansion of the physico-chemical characteristics of amino acids and the potential to modulate protein function (1). Major sites of protein methylation are at lysine and arginine residues (2, 3), and less major sites include glutamate, glutamine, and histidine residues, as well as N-terminal amino and C-terminal carboxyl groups (4–6). The extensive role of histone methylation in

transcriptional control highlights the biological significance of this modification (7–10). Protein methylation is also important in the translational machinery. Indeed, many proteins involved in translation, including ribosomal proteins and various elongation and release factors, are subject to methylation in both prokaryotes and eukaryotes (11).

Saccharomyces cerevisiae is an ideal organism to investigate the methylation of ribosomal proteins; its genome is well annotated and single open reading frame gene deletion mutants are available. High-resolution intact mass spectrometry suggested that six proteins of the large ribosomal subunit may be methylated: Rpl1ab, Rpl3, Rpl12ab, Rpl23ab, Rpl42ab, and Rpl43ab (12).² This study, however, did not identify the sites of methylation in these proteins nor did it identify the corresponding methyltransferases. In our laboratory, we have been interested in characterizing these modifications and identifying the methyltransferases involved in an effort to understand their physiological significance in translation. We have used mass spectrometry to screen for loss of methylation in the intact ribosomal proteins of yeast strains lacking a single known or putative methyltransferase. Using this approach, SET-domain methyltransferases responsible for modifying Rpl12ab, Rpl23ab, Rpl42ab, and eEF1a at specific lysine residues have been identified (13–17). In addition to screening the SET-domain methyltransferase gene knock-out strains, we also screened the seven β -strand methyltransferase gene family for loss of methylation in gene deletions. We found one seven β -strand methyltransferase involved in modifying eEF1a, presumably at a lysine residue (13), and one involved in methylating the small ribosomal protein Rps2 at one or more arginine residues (18). Finally, a seven β -strand methyltransferase has been shown to modify Rpl12ab and Rps25a/Rps25b at their N-terminal proline residues (6).

Protein methylation at histidine residues has been previously established for a small number of proteins in nature, but has not been observed previously in *S. cerevisiae* (Table 1). Several proteins in mammalian cells and the methyl-coenzyme M reductase of archaeal prokaryotes have been shown to contain 1-methylhistidine. Both actin and type I myosin heavy chains in

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2 and Figs. S1–S3.

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² Yeast ribosomal proteins are identified based on the gene designation. In cases where two genes encode identical protein products, the protein is designated "ab." For example, the Rpl1ab protein is the identical translation product of the *RPL1A* and *RPL1B* genes.

TABLE 1
Proteins methylated *in vivo* at histidine residues

Protein name	Sequence surrounding methylated histidine	Source	Modification (numbering including the initiator methionine residue)
Large subunit ribosomal protein Rpl3	KKLPRKTHRGLRKVA	<i>S. cerevisiae</i>	3-Methylhistidine-243 (this study)
Actin	TLKYPTEHGIITNWD	Rabbit skeletal muscle	3-Methylhistidine-75 (19)
Myosin heavy chain Type I fiber	LGSIDVDHTQYKFGH	Rabbit skeletal muscle	3-Methylhistidine-756 (20)
Myosin heavy chain Type I fiber	LGSIDVDHTQYRFGH	Chicken skeletal muscle	3-Methylhistidine-756 (21)
Myosin heavy chain Type I fiber	LASLDIDHNQYRFGH	Chicken cardiac muscle	3-Methylhistidine-754 (22)
Myosin light chain kinase	RGSPAFLHSPSCPAI	Rabbit skeletal muscle	1-Methylhistidine-158 (23)
14 kDa Migration-inhibitory-factor-related protein (MRP14, S100)	PRGHGHSHGKGCCK	Mouse spleen	1-Methylhistidine-107 (24)
Methyl-Coenzyme M reductase- α chain	DLSFAAKHAALVSMG	<i>Methanosarcina barkeri</i>	1-Methylhistidine-271 (25)
Methyl-Coenzyme M reductase- α chain	DFAYAAKHAIEVIHMG	<i>Methanobacterium thermoautotrophicum</i>	1-Methylhistidine-257 (26)

a number of species contain 3-methylhistidine. Actin histidine-73 methylation is well conserved in nature, although the modification is notably absent in two protozoan species (27, 28) and in the yeasts *Candida albicans* and *S. cerevisiae* (29). Evidence has been presented for functional roles of methylation in actin polymerization and ATP hydrolysis (30) and in actin structure (4). The enzyme(s) responsible for the formation of 3-methylhistidine in actin and/or the myosin heavy chain has been partially purified (31, 32), but no genes encoding protein histidine methyltransferases have been identified to date in any organism.

Here, we describe a member of the yeast seven β -strand methyltransferase family (33) required for the methylation of the ribosomal protein Rpl3. The mass of the Rpl3 protein from yeast strains lacking the YIL110W gene encoding this methyltransferase is reduced by 14 Da, corresponding to the loss of a single methyl group. We demonstrate that Rpl3 contains a single 3-methylhistidine residue and suggest that the YIL110W gene, previously designated *MNII*, now be designated *HPM1*³ for histidine protein methyltransferase 1. This work represents the first report of a gene product required for protein histidine methylation in nature, the first observation of a methylated histidine residue in a ribosomal protein, and the first identification of a methylated histidine residue in yeast.

EXPERIMENTAL PROCEDURES

Isolation of Ribosomal Proteins from *S. cerevisiae* Wild Type and Mutant Strains—The wild-type parent BY4742 and the YIL110W deletion strain in the BY4742 background, as well as the additional deletion strains listed in supplemental Table S1, were obtained from the *Saccharomyces* Genome Deletion Project (Open Biosystems, Huntsville, AL). The $\Delta set1$ gene deletion strain was a gift from Drs. Renee Chosed and Sharon Dent at the M. D. Anderson Cancer Center (Houston, TX). Large ribosomal subunits were isolated from the wild type and deletion strains as described previously (18).

Liquid Chromatography and Electrospray Ionization Mass Spectrometry of Intact Ribosomal Proteins—Proteins from the large ribosomal subunit were prepared after RNA extraction and were fractionated using reverse-phase liquid chromatography as described previously (14). The column effluent was

directed to the electrospray ion source of a QSTAR Elite (Applied Biosystems/MDX SCIEX) mass spectrometer running in MS-only mode. The instrument was calibrated using external peptide standards to yield a mass accuracy of 30 ppm or better.

Localization of Rpl3 Methylation Sites by Top-Down Mass Spectrometry—Large ribosomal proteins from the $\Delta YIL110W$ deletion and the parent wild-type BY4742 strain were separated by HPLC as described above. Fractions containing Rpl3 were then directly infused on a 7-T hybrid linear ion-trap/FTICR mass spectrometer (LTQ FT Ultra, Thermo Scientific, San Jose, CA) and fragmented using collisionally activated dissociation as previously described (14) with the exception that the source fragmentation was set to 10 V. The resulting spectra were processed using ProSightPC software (Thermo Scientific) in single protein mode with a 15 ppm mass accuracy threshold. The root mean square deviations for assigned fragments were less than 5 ppm. The reference data base for the ProSightPC software included the *S. cerevisiae* proteome from Swiss-Prot. Intact mass measurements were obtained using the manual Xtract program, version 1.516 (Thermo Scientific).

Mass Spectrometry of Cyanogen Bromide (CNBr) Fragments of Rpl3—HPLC-purified Rpl3 from wild-type *S. cerevisiae* was prepared as described above. CNBr (~20 mg) was dissolved in 70 μ l of water/acetonitrile/trifluoroacetic acid (1:1:0.05) prior to mixing with the protein solution. The molar ratio of CNBr to protein methionine residues was ~1,000:1. After incubation overnight in the dark at room temperature, the resulting solution was directly infused into the hybrid linear ion-trap/FTICR mass spectrometer, as described above, to determine the intact masses of the resulting fragments.

Nano-liquid Chromatography with Tandem Mass Spectrometry (nLC-MSMS)—HPLC-purified Rpl3 prepared as described above was digested at a protein:protease ratio of 20:1 overnight at room temperature with each of the following enzymes: sequencing grade trypsin (Promega), chymotrypsin (Roche Diagnostics), Glu-C (Roche Diagnostics), or Lys-C (Thermo Scientific). nLC-MSMS with collisionally activated dissociation was performed on the hybrid linear ion-trap/FTICR mass spectrometer integrated with an Eksigent nano-LC. A prepacked reverse-phase column (Biobasic C18, 5- μ m particle size, 300- \AA pore size, 100 μ m inner diameter, 3.5 cm long; Microtech Scientific, Fontana, CA) equilibrated in eluent A (water/acetonitrile/formic acid, 99:1:0.1, v/v/v) was used to fractionate the

³ The abbreviations used are: HPM, histidine protein methyltransferase; [³H]AdoMet, S-adenosyl-L-[methyl-³H]methionine; nLC-MSMS, nano-liquid chromatography with tandem mass spectrometry.

3-Methylhistidine, a Novel Yeast Post-translational Modification

peptide digest for mass spectrometry. Peptides were loaded at a flow rate of 5 μ l/min and eluted at 300 nl/min with increasing amounts of eluent B (acetonitrile/formic acid, 99.9:1, v/v) using the following gradient: 2–60% eluent B over 40 min, then increased to 80% eluent B over 10 min. The effluent was directed to the nano-spray ionization source (Thermo Scientific) at a capillary temperature of 220 $^{\circ}$ C, a tube lens voltage of 110 V, and a spray voltage of 2.3 kV connected to the hybrid linear ion-trap/FTICR mass spectrometer. The instrument was operated in data-dependent mode with a full precursor scan at high resolution (100,000 at m/z 400) and up to six MSMS experiments per cycle at low resolution on the linear trap. For the collisionally activated dissociation fragmentation, the intensity threshold was set to 1000 with a m/z range of 180–1800. Spectra were analyzed with Mascot software (Matrix Science, UK) using a significance threshold of $p < 0.05$.

High Resolution Cation Exchange Chromatography and Thin Layer Chromatography—Wild-type (BY4742) ribosomes were labeled *in vivo* with *S*-adenosyl-L-[methyl- 3 H]methionine (3 H]AdoMet) at a concentration of 84 μ Ci/ml (PerkinElmer, 75–85 Ci/mmol, 0.55 mCi/ml in 10 mM H_2SO_4 /ethanol (9:1, v/v)) as described previously (15). 3 H]methyl-labeled Rpl3 was then purified from extracted large ribosomal subunit proteins by SDS-12.6% polyacrylamide gel electrophoresis as described (15) or by reverse-phase HPLC as described (14). Diced gel slices containing the 43-kDa polypeptide corresponding to Rpl3, or the HPLC fractions containing Rpl3 were placed in a 6 \times 50-mm glass vial and dried by vacuum centrifugation. The samples were acid hydrolyzed by addition of 50 μ l of 6 M HCl to the 6 \times 50-mm glass vial and 200 μ l of 6 M HCl into the surrounding vacuum reaction chamber (Eldex Labs, catalogue 1163). The vial was heated for 20 h *in vacuo* at 110 $^{\circ}$ C with a Water Pico-Tag Vapor-Phase apparatus and residual HCl was removed by vacuum centrifugation. The samples were then resuspended in water and spiked with amino acid standards prior to analysis. Standards included 1-methyl-L-histidine (π -methyl-L-histidine; Sigma, No. M-9005) and 3-methyl-L-histidine (τ -methyl-L-histidine, Vega Biochemicals 12608). High-resolution cation-exchange chromatography was performed as indicated in the figure legends. Ascending thin layer chromatographic separation was performed using a 20 \times 20-cm silica-coated plate (Whatman, PE SIL G/UV) after preincubating the chamber with the mobile phase *n*-butanol/pyridine/water (1:1:1) (34). The solvent was allowed to migrate to near the top of the sheet and the plate was dried. Standards were detected by spraying with a ninhydrin solution consisting of 1 g of ninhydrin, 2.5 g of cadmium acetate, 10 ml of acetic acid, and 500 ml of ethanol. The plate was heated in an oven to 100 $^{\circ}$ C for 20 min until the purple stain developed. To detect radioactivity, the lane was cut into \sim 3-mm slices. Each slice was placed into a microcentrifuge tube with 300 μ l of water and incubated overnight with shaking at room temperature. The silica gel was removed by centrifugation, and the supernatant was counted for radioactivity with 5 ml of fluor (Safety Solve, Research Products International) in three 5-min counting cycles on a Beckman LS6500 instrument with an efficiency of 39% for a tritium standard.

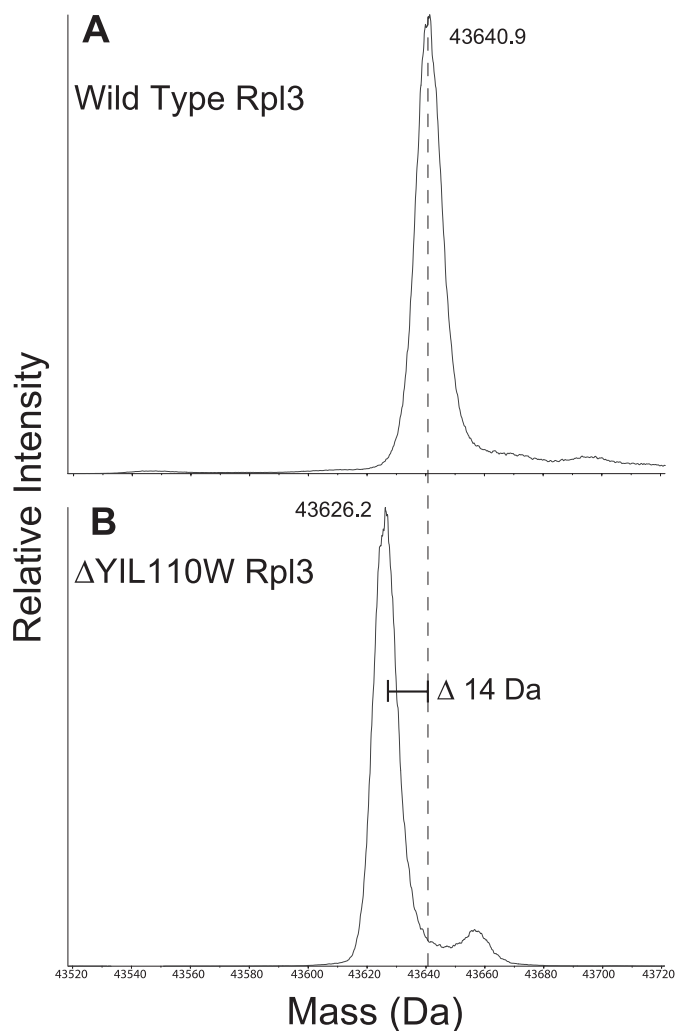


FIGURE 1. Intact mass reconstruction of Rpl3 derived from wild type (BY4742) and Δ YIL110W deletion strains. Proteins from the large ribosomal subunit were isolated from wild type (BY4742; panel A) and the Δ YIL110W/*HPM1* deletion (panel B) strains as described under “Experimental Procedures.” These proteins were fractionated using reverse-phase HPLC and the resulting effluent was introduced to an electrospray mass spectrometer (QSTAR Elite) to determine the intact mass of each protein. The MS spectra of the RPL3 protein from each strain were deconvoluted, and their measured average masses are shown. The loss of 14 Da in Rpl3 from the deletion strain, corresponding to the loss of a methyl group, is indicated.

RESULTS

YIL110W/HPM1 Is Required for the Methylation of the Ribosomal Protein Rpl3 in S. cerevisiae—Ribosomes from wild-type yeast strains and from 37 yeast deletion strains (supplemental Table S1) of known or putative methyltransferases were isolated using differential centrifugation. The large and small ribosomal subunits were separated using high-salt sucrose gradients as described previously (14, 18). Extracted proteins of the large subunit were fractionated using reverse-phase HPLC and the mass of the eluting intact proteins was determined by electrospray mass spectrometry as described under “Experimental Procedures.” Previous work has shown that Rpl3 is modified by the removal of the initiator methionine residue (35) and by a +14-Da modification corresponding to the mass of a single methyl group (12). In wild type and 36 of the deletion strains, the intact mass of Rpl3 was determined to be about 43641 Da,

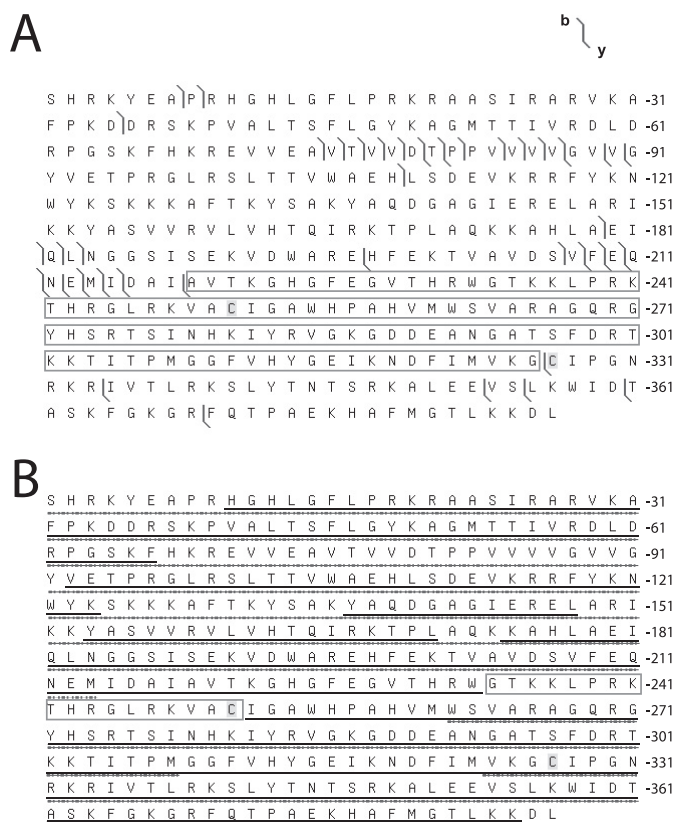


FIGURE 2. Intact fragmentation pattern and peptide coverage map of wild type ribosomal protein Rpl3 analyzed by mass spectrometry. Wild-type Rpl3 was purified using reverse-phase HPLC and used to determine the position of the +14-Da modification. *Panel A*, intact top-down mass spectrometric analysis of Rpl3 was performed using a LTQ-FT Ultra mass spectrometer with collisionally activated dissociation. All of the N-terminal b ion fragments (indicated by upper hash marks) up to the position of Asp-216 are unmodified; y ion fragments (indicated by lower hash marks) detected before Ala-219 each contain an additional 14 Da while y ion fragments detected after Gly-326 are unmodified. These results localize the +14 modification to a region between Ala-219 and Gly-326, which is boxed in *panel A*. *Panel B*, Rpl3 protein was also analyzed by a middle-down approach. CNBr-cleaved Rpl3 peptides were directly infused on the LTQ-FT Ultra instrument to measure their masses (supplemental Table S2). Detected masses corresponding to Rpl3 CNBr fragments are shown with a dashed line. Additionally, purified Rpl3 was digested in separate experiments with trypsin, chymotrypsin, Lys-C, and V8 protease. The resulting mixtures were analyzed using nLC-MSMS with collisionally activated dissociation. The identified fragments with an expected value of 0.05 or less are marked by a solid underline. Although no methylated fragments were detected, no fragments were observed between Gly-234 and Cys-251, suggesting that the +14-Da modification occurs in this region.

consistent with the loss of methionine and the addition of one methyl group (Fig. 1, *panel A*; data not shown). However, in the YIL110W/HPM1 deletion strain, we observed a mass of 43626.2 Da at the same retention time as the wild type protein and found no evidence for a 43641-Da species (Fig. 1, *panel B*). Knowing that YIL110W contains the signature sequence motifs of seven β -strand methyltransferases (33, 36–38), this ~14-Da decrease in the mass of Rpl3 suggests that the YIL110W gene product may encode the methyltransferase responsible for modifying the Rpl3 protein.

Site of Modification in the Rpl3 Protein—To localize the site of modification in Rpl3 we initially used an intact mass top-down fragmentation approach. Wild type large ribosomal subunits were HPLC purified, and fractions containing Rpl3 were collected. The purified intact protein was directly infused into a

hybrid linear ion trap/Fourier transform ion cyclotron resonance (FTICR) mass spectrometer as described in “Experimental Procedures.” Single charge states of intact Rpl3 species were selectively isolated and fragmented using collisionally activated dissociation to localize the site of methylation. Multiple MSMS spectra on different parent ions of the fragments were collected and deconvoluted to produce the fragmentation map for Rpl3 shown Fig. 2, *panel A*. Using this approach, we were able to localize the site of the +14-Da covalent modification in Rpl3 to the region between Ala-219 and Gly-326.

To narrow down the site of methylation, we cleaved the isolated Rpl3 protein at methionine residues with CNBr. The products were directly infused into the hybrid mass spectrometer and the fragments were analyzed. The detected species, charge state(s), and corresponding mass errors are listed in supplemental Table S2 and are highlighted in dashed lines in Fig. 2, *panel B*. All of the fragments detected were unmodified. However, we did not find peptide fragments in the region from Ile-215 to Met-261. This information, coupled with the knowledge that the wild-type Rpl3 protein is stoichiometrically methylated (Fig. 1), suggests that the location of the modification is between residues 219 and 261.

To further locate the methylated residue in Rpl3, we used a bottom-up mass spectrometry approach. Purified Rpl3 was digested with either trypsin, chymotrypsin, Glu-C, or Lys-C, and was analyzed by nLC-MSMS as described in “Experimental Procedures.” The fragment coverage of the Rpl3 protein is underlined with solid lines in Fig. 2, *panel B*. Although we were unable to detect a methylated peptide, we were able to pinpoint the site of the +14-Da modification to the region between Gly-234 and Cys-251 (GTKKLPRKTHRGLRKVAC). The MSMS spectra of the proteolytic fragments surrounding this region were manually inspected to verify their identity and ensure the proper localization of the modification. Significantly, we confirmed that the chymotryptic peptide IGAWHPAHVMW (residues 252–262) was unmodified (supplemental Fig. S1). The absence of sequenced peptides from residues 234 to 251 may reflect the presence of multiple cleavage sites for trypsin, Lys-C, and possibly chymotrypsin (39) that give rise to small fragments not retained by the HPLC column and thus not detected in our analysis. The region from residues 234–251 contains numerous lysine and arginine residues that are possible sites of methylation.

To determine whether an arginine or lysine residue is methylated in Rpl3, we labeled yeast cells with [³H]AdoMet, isolated large ribosomal subunits, and separated the polypeptides by SDS gel electrophoresis. We isolated the radioactive band corresponding to Rpl3, acid hydrolyzed the protein, and analyzed the methylated amino acids by high-resolution cation exchange chromatography. We detected no radioactivity co-eluting with the monomethyllysine or monomethylarginine standards (Fig. 3). However, a radioactive peak eluting at 43–44 min was found between the peaks of the monomethyllysine standard and the ammonium ion resulting from the hydrolysis of the polyacrylamide gel matrix. In this chromatography system, methylated neutral amino acid derivatives such as S-methylcysteine and O-methylthreonine are known to elute at about 4 min (data not shown). These results suggest that the Rpl3 modification is at a

3-Methylhistidine, a Novel Yeast Post-translational Modification

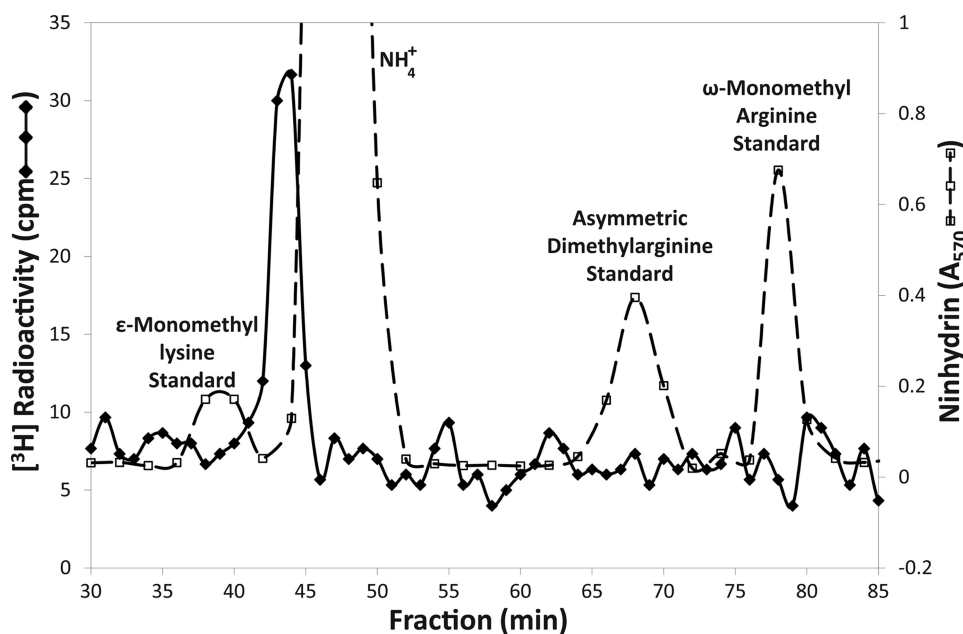


FIGURE 3. Rpl3 lacks a monomethylarginine or monomethyllysine residue. Amino acid analysis was performed on the ^3H -labeled ~ 43 -kDa polypeptide from the yeast large ribosomal subunit corresponding to Rpl3. Wild-type (BY4742) cells were labeled *in vivo* with ^3H AdoMet and the large ribosomal subunit proteins were isolated as described in "Experimental Procedures." The proteins were then separated on a 12.6% SDS-polyacrylamide gel, Coomassie stained, and the band below the ovalbumin marker was excised, diced, and acid hydrolyzed as described in "Experimental Procedures." 1 μmol of each of the standards (ϵ -monomethyllysine, N^G, N^G -dimethylarginine, and N^G -monomethylarginine) were mixed with the sample, and amino acid analysis was performed as previously described (Miranda *et al.*, 40). Closed diamonds show radioactivity from 200 μl of the 1-min (1 ml) fractions collected in 5 ml of scintillation fluor (Safety Solve, Research Products International). Samples were counted for three 5-min periods on a Beckman LS6500 instrument with an efficiency of 39% for a tritium standard. Standard amino acids were detected by ninhydrin reactivity (open squares). A 100- μl aliquot from each fraction was mixed with 600 μl of water and 300 μl of freshly prepared ninhydrin reagent (20 mg/ml ninhydrin and 3 mg/ml hydrindantin in a solvent of 75% (v/v) dimethyl sulfoxide and 25% (v/v) 4 M lithium acetate, pH 4.2), heated at 100 $^\circ\text{C}$ for 15 min, and the absorbance was measured at 570 nm. In this chromatography system, 1-methylhistidine and 3-methylhistidine elute shortly after monomethyllysine and 5 min before monomethylamine (40).

site distinct the nucleophilic side chains of lysine, arginine, cysteine, and threonine residues.

With this information, we considered the possibility that the modification was on the histidine residue at position 243. Two methylated derivatives of histidine have been found to date in a small number of proteins; these include 3-methylhistidine (or τ -methylhistidine) found in mammalian actin and myosin and 1-methylhistidine (or π -methyl-L-histidine) found in mammalian and archaeal proteins (Table 1). Both 3-methyl and 1-methylhistidine have been found to elute near the position of the ammonium ion between the methylated lysine and arginine derivatives under similar chromatographic conditions to those used here (40, 41); this is the region where we detected radiolabeled methyl groups from the hydrolysate of Rpl3 (Fig. 3).

With this knowledge, we performed additional analysis of acid-hydrolyzed Rpl3 to determine whether it contained a methylated histidine residue. We used a high-resolution cation-exchange chromatography system optimized for the separation of 1-methylhistidine and 3-methylhistidine (41). To avoid the generation of ammonium ions that can mask the presence of methylhistidine standards, we analyzed HPLC-purified ^3H methyl-labeled Rpl3 instead of gel-purified Rpl3. With MALDI mass spectrometry analysis, we noted that our HPLC fraction contained both Rpl3 and Rpl23ab. The ^3H -methylated Rpl3 and Rpl23ab proteins were then subjected to acid hydro-

lysis and analyzed by high resolution cation exchange chromatography (Fig. 4). The bulk of the ^3H -radiolabel eluted in two peaks at 95 and 120 min. The first peak eluted about 1 min prior to the standard of ϵ -dimethyllysine and reflects the presence of the two known dimethyllysine residues in Rpl23ab (15). The second peak eluted about 1.7 min before the standard of 3-methylhistidine. It has been shown that high-resolution cation exchange columns can partially resolve ^3H -labeled and unlabeled (^1H) amino acids. This has been shown for tritiated methyl forms of glutamate γ -methyl ester (42), aspartate β -methyl ester (43), N -methylated arginine derivatives (44), and other methylated amines and amino acids (45, 46). In each of these cases, the ^3H -methylated species elutes slightly before the non-isotopically labeled forms. Our results therefore suggest that the first radiolabeled peak represents ϵ - ^3H dimethyllysine from Rpl23ab and the second radiolabeled peak represents 3- ^3H methylhistidine from Rpl3.

In addition to modifications producing 3-methylhistidine, there

have been several reports of 1-methylhistidine in proteins (Table 1). To be confident that Rpl3 contains a 3-methylhistidine residue rather than a 1-methylhistidine residue, the high-resolution cation exchange chromatography was repeated with a 1-methylhistidine standard. In this case, we found that the second radiolabeled peak eluted significantly earlier from the standard of 1-methylhistidine (supplemental Fig. S2).

To further confirm the presence of 3-methylhistidine in Rpl3, we repeated the labeling of yeast cells with ^3H AdoMet, purified the Rpl3 protein by HPLC, and used thin layer chromatography to resolve the methylated species. The fraction containing ^3H -methylated Rpl3 and Rpl23ab was acid hydrolyzed, and the sample was analyzed on a silica gel plate using conditions developed previously to resolve methylhistidine isomers (Fig. 5; Ref. 34). Similar to the results shown in Fig. 4, we detect radioactivity migrating with both the dimethyllysine standard (Rpl23ab) and the 3-methylhistidine standard (Rpl3) but not with the 1-methylhistidine standard (Fig. 5).

Our results provide clear evidence for a 3-methylhistidine residue in Rpl3 at position 243, the only histidine residue in the region where we expected to find the modification (residues 234–251). This conclusion is consistent with the observed +14-Da modification to the intact protein (Fig. 1), our mass spectrometry-guided localization of the modification (Fig. 2),

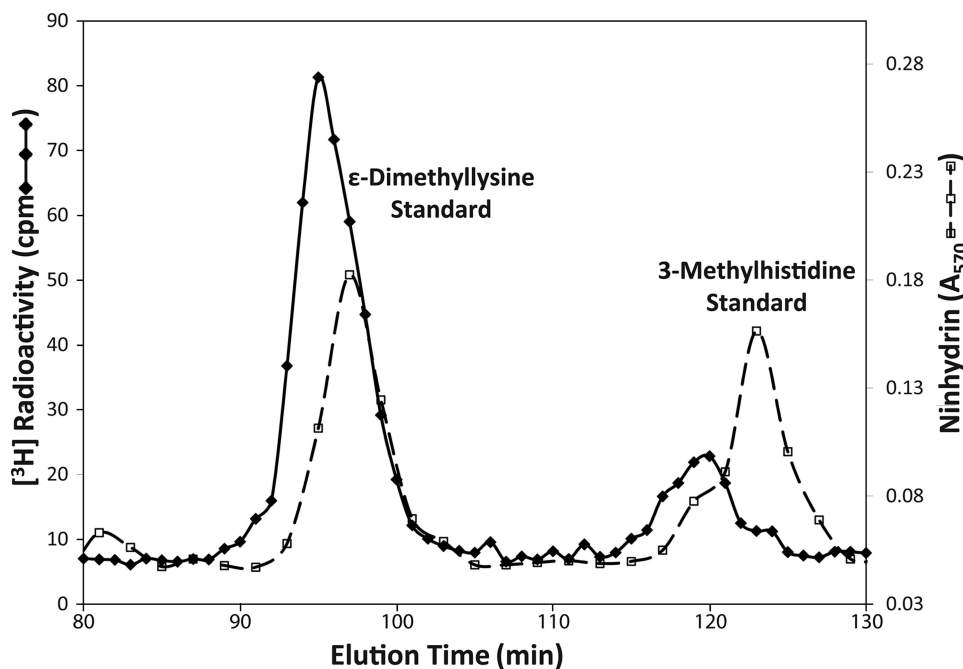


FIGURE 4. The methylated amino acid from Rpl3 co-elutes with 3-methylhistidine in high-resolution cation exchange chromatography. ^3H -methylated large ribosomal subunits were isolated from [^3H]AdoMet-labeled intact BY4742 wild-type cells and reverse-phase HPLC was used to isolate a fraction containing a mixture of Rpl3 and Rpl23ab as described in "Experimental Procedures." The latter protein is a known co-eluting species known to contain two dimethyllysine residues (17). This mixture was acid hydrolyzed and 1 μmol each of ϵ -dimethyllysine and 3-methyl-L-histidine (τ -methyl-L-histidine, Vega Biochemicals 12608) standards were added. A high-resolution cation exchange column (6–12 μm PA-35 sulfonated polystyrene beads, Benson Co., Reno, NV; 0.9 cm inner diameter \times 13 cm column height) was equilibrated and eluted with 0.35 M sodium citrate buffer (pH 5.84) at 28 $^{\circ}\text{C}$ at a flow rate of 0.5 ml/min. 1-min fractions were collected, and 20 μl of each fraction was added to 120 μl of water and 60 μl of the ninhydrin reagent described in the Fig. 3 legend. The mixture was then heated at 100 $^{\circ}\text{C}$ for 15 min and the absorbance was measured at 570 nm in a 96-well plate reader (open boxes). Radioactivity was measured by mixing 450 μl of the column fractions with 400 μl of water and 10 ml of fluor as in the Fig. 3 legend (closed diamonds). For methylated amino acids, a tritium isotope effect results in a slightly but consistently earlier elution of the radiolabeled species relative to the unlabeled standard (see text).

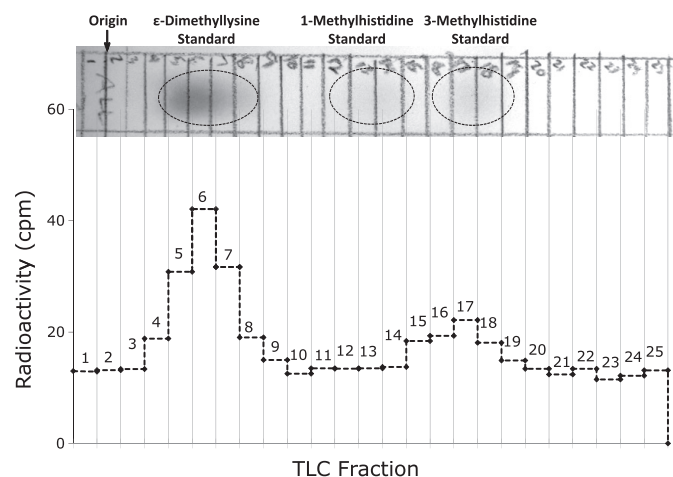


FIGURE 5. Confirmation of 3-methylhistidine in Rpl3 by TLC. ^3H -methylated Rpl3 and Rpl23ab from *in vivo* [^3H]AdoMet-labeled yeast cells were prepared as described in the Fig. 4 legend. As described in the "Experimental Procedures," the proteins were acid hydrolyzed, dried, and resuspended in water. The sample was mixed with standards (5 nmol each) of ϵ -dimethyllysine, 1-methyl-L-histidine, and 3-methyl-L-histidine, and chromatographed on a silica plate. Standards were detected with a ninhydrin spray reagent and their migration positions (determined by their migration as single species in adjacent lanes) are indicated by circles. To quantify radioactivity, the lane on the TLC plate was divided and cut into ~ 3 mm slices, as displayed as vertical lines, and each slice was counted as described in "Experimental Procedures." The origin is between fractions 1 and 2; the solvent front would correspond to fraction 60.

and the absence of methylated lysine and arginine derivatives in the SDS-purified Rpl3 (Fig. 3). Rpl3 is the first non-mammalian protein reported to contain a 3-methylhistidine modification (Table 1), and YIL110W/Hpm1 is the first and only gene that has been associated with a protein histidine methyltransferase in nature.

YIL110W/Hpm1 Homologs Are Present in Other Eukaryotes—Previously, the YIL110W open reading frame was predicted to encode a seven β -strand methyltransferase containing three of the four conserved motifs (Fig. 6 and Refs. 33, 36–38). A BLAST search revealed homologs in multiple eukaryotes, but not prokaryotes. Notably, one human homolog was found (UniProt O95568) with an expect value of 7×10^{-13} . There is high similarity between Hpm1 and the human homolog in the regions before motif I and after motif II. These regions are known to be important in substrate recognition in other seven β -strand methyltransferases. For example, residues preceding motif I are involved in peptide substrate recognition in the L-isoaspartyl protein repair methyltransferase (47) and residues following motif II in

protein arginine methyltransferases are involved in binding to the guanidino group of the substrate arginine residue (48, 49).

Structural Significance of Rpl3 and the 3-Methylhistidine Residue in the Large Ribosomal Subunit—The amino acid sequence of the Rpl3 protein is highly conserved. Crystal structures are available for the prokaryotic *Escherichia coli* L3 ortholog (50) and the *Haloarcula marismortui* L3 ortholog (51), both in complex with the 23 S ribosomal RNA. It is known that the proteins in the large ribosomal subunit stabilize interdomain interactions that are necessary for ribosomal biogenesis and stability (51). In *H. marismortui*, the L3 protein has two extensions, one on the N terminus and another designated the tryptophan finger, extending from residues 206 to 260 (residues 220–272 in *S. cerevisiae*) (supplemental Fig. S3). This tryptophan finger contains the 3-methylhistidine residue at position 243 in *S. cerevisiae*. Both extensions of the *H. marismortui* L3 protein reach deep into the core of the 23 S rRNA, making multiple hydrogen bonding contacts with the rRNA (51). Structure-function studies of the yeast Rpl3 protein identified multiple point mutations leading to effects on killer virus maintenance, programmed-1 ribosomal frame shifting, resistance to anisomycin, and the ability to translate reporter mRNA lacking the 5' mGppp cap and poly(A) tail (52). Interestingly, the majority of the yeast point mutations displaying phenotypes

3-Methylhistidine, a Novel Yeast Post-translational Modification

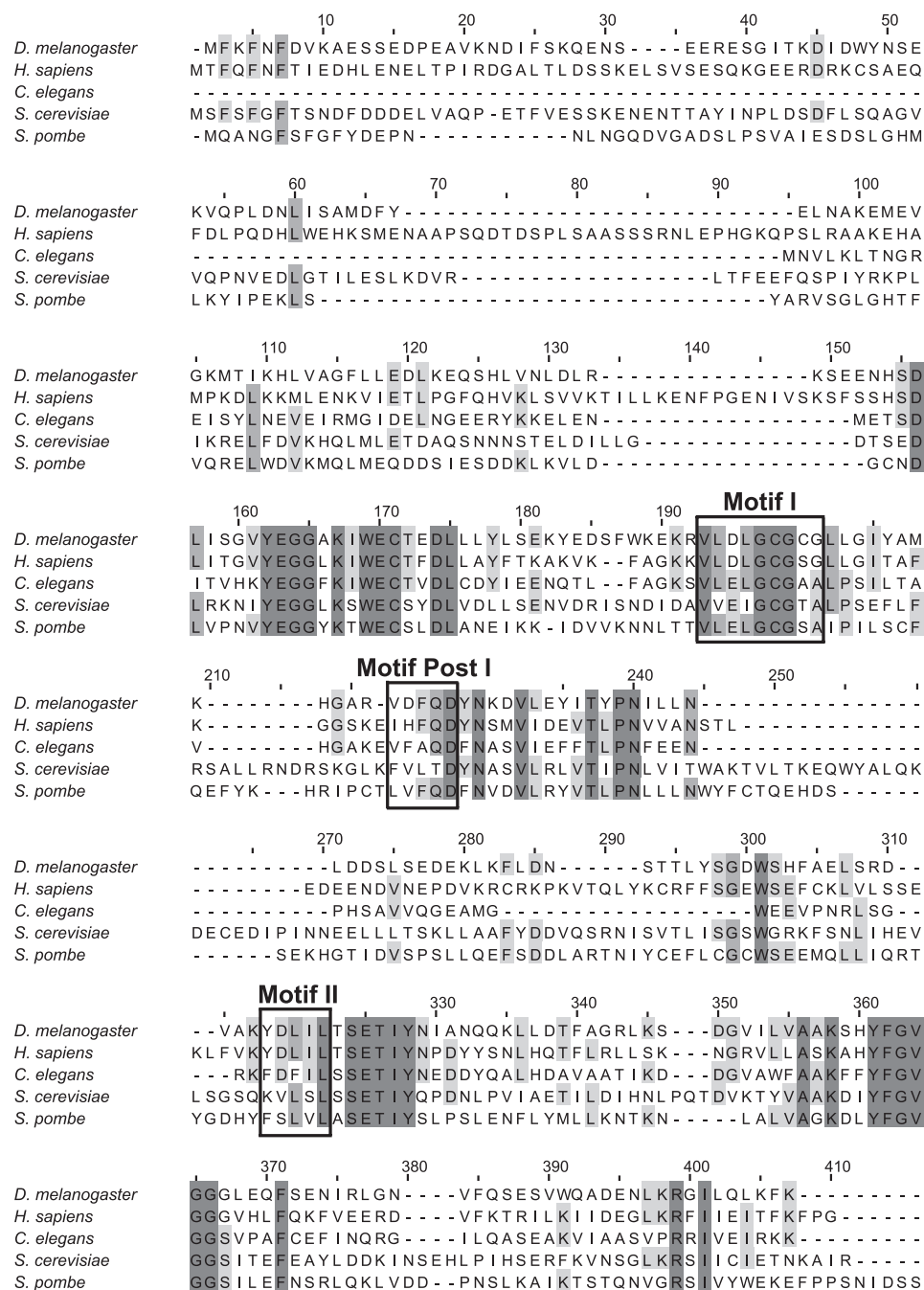


FIGURE 6. Yeast YIL110W/Hpm1 homologs are present in other eukaryotes. BLAST searches were performed against the yeast YIL110W/Hpm1 amino acid sequence to identify homologs in other organisms. ClustalW was used to align sequences. Signature methyltransferase motifs common to all class I protein methyltransferases (38) are boxed. The UniProt accession number and BLAST expect value for each sequence identified are: *Drosophila melanogaster* (Q9VQK8, expect value 8×10^{-13}), *Homo sapiens* (O95568, expect value 7×10^{-13}), *C. elegans* (Q21069, expect value 3×10^{-13}), *S. cerevisiae* (YIL110W/Hpm1, P40481), and *Schizosaccharomyces pombe* (Q9UTQ8, expect value 1×10^{-28}). Light gray shading indicates sites where three residues are identical, medium gray shading indicates sites where four residues are identical, and dark gray shading indicates sites where all residues are identical.

were at positions corresponding to those in direct rRNA contact in the L3 protein of *H. marismortui* (52). A reciprocal relationship was demonstrated between ribosomal affinity for the eEF1A aa-tRNA GTP complex and for eEF2, suggesting that the tryptophan finger and N-terminal extensions of Rpl3 coordinate the opening and closing of the corridor where the aa-tRNA moves during the accommodation process (52). The Rpl3 pro-

tein has been designated as the gatekeeper to the ribosomal A site, functioning as a rocker switch coordinating an allosteric signaling pathway between elongation factor binding sites and the peptidyltransferase center (53, 54).

These observations suggest the importance of physical contacts between the 25 S rRNA and the Rpl3 protein in yeast, and how the methylation of a single histidine residue in the tryptophan finger might affect ribosomal function. Sequence alignment between the *H. marismortui* L3 protein and the yeast Rpl3 protein can give insight into the local environment of the methylation site at His-243 (Fig. 7; supplemental Fig. S3). Two residues flanking the alanine residue that aligns to His-243 have close contacts with the 23 S rRNA of *H. marismortui*. In the *E. coli* L3 structure, the corresponding residues flanking the serine residue corresponding to yeast Rpl3 His-243 are also in close contact to the *E. coli* 23 S rRNA. Interestingly, methylation of the *E. coli* L3 protein has been shown to occur on the side chain of Gln-150 in a reaction catalyzed by the PrmB methyltransferase (55, 56). The methylation site at Gln-150 in the *E. coli* L3 protein aligns 12 residues from the *S. cerevisiae* Rpl3 methylation site at His-243 (Fig. 7), suggesting that both types of protein methylation in the tryptophan finger extension can help modulate the local structure to help optimal interactions with the rRNA (50).

DISCUSSION

Our observation of a 3-methylhistidine residue at position 243 in Rpl3, a protein in the large ribosomal subunit of *S. cerevisiae*, represents the first example of this protein modification in yeast and the first instance of 3-methylhistidine in a protein other than actin and myosin heavy chains. We show that Rpl3 is stoichiometrically modified at this site and we find no evidence for unmodified protein. Structural studies suggest that histidine 243 exists in a loop of Rpl3 that contacts the 25 S rRNA near the peptidyltransferase site (53, 54), although the importance of the methylation modification in Rpl3-rRNA interactions has yet to be

3-Methylhistidine, a Novel Yeast Post-translational Modification

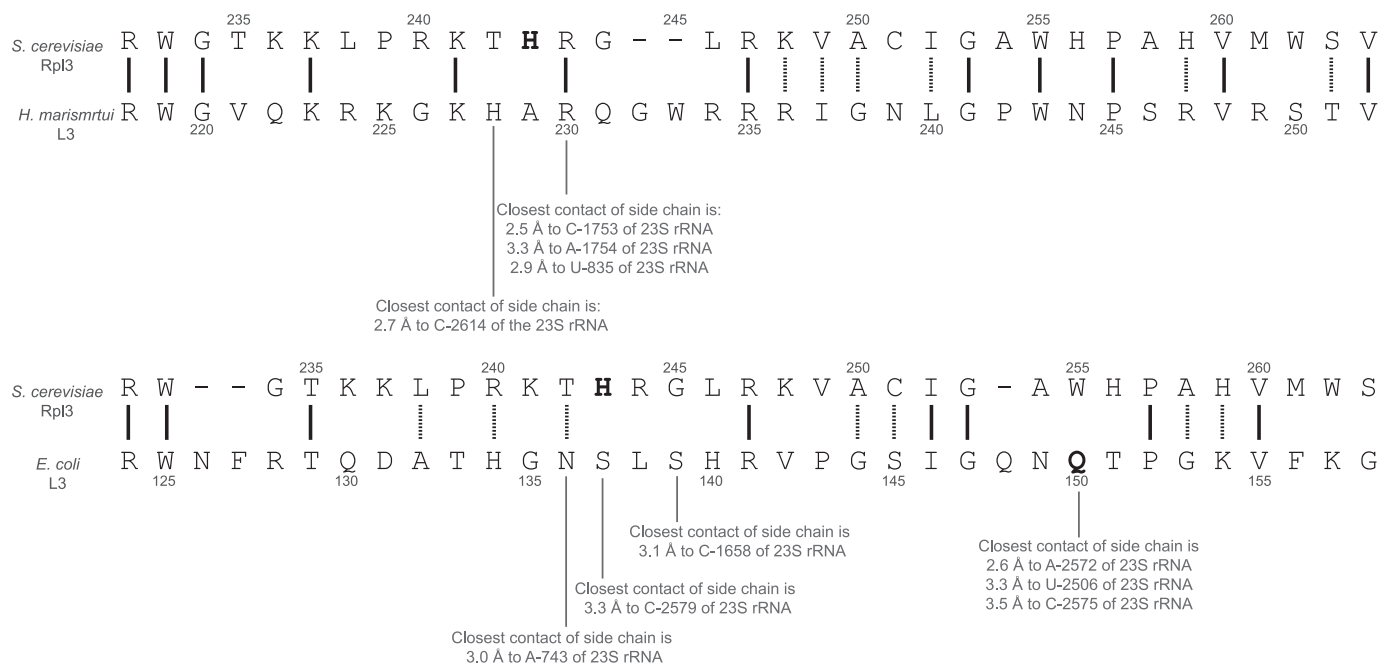


FIGURE 7. Methylation site alignment of His-243 in Rpl3 with prokaryotic homologs; predicted interaction with the yeast 25 S rRNA. *Top*, amino acid residues 232–264 of Rpl3 from *S. cerevisiae* are aligned with residues 218–252 of L3 from the archaeobacterium *H. marismortui*. The hydrogen-bonded 23 S rRNA contacts of the *H. marismortui* residues in the sequence corresponding to yeast residues 242–244 flanking the 3-methylhistidine site are shown as determined from the x-ray crystal structure (PDB file 1572). *Bottom*, amino acid residues 232–263 of Rpl3 from *S. cerevisiae* are aligned with residues 124–158 of L3 from *E. coli*. Contacts to the *E. coli* 23 S rRNA are shown for the *E. coli* residues from the x-ray crystal structure (PDB file 2AW4). ClustalW was used to align the sequences.

established. The addition of a 3-methyl group to histidine raises its pK_a from 6.01 to 6.47 (57) increasing the fraction of the side chain in the protonated form at neutral pH. Protonation of the nitrogen-1 atom in the imidazole ring may lead to enhanced hydrogen bond/ionic bond formation with rRNA. Alternatively, the presence of the methyl group on the nitrogen-3 atom may prevent a deleterious H-bond from forming. Additionally, the added bulk of the methyl group may play a role in enhancing some interactions and preventing others. In actin, mutants lacking 3-methylhistidine had an increased ATP exchange rate and showed instability, indicating that the loss of methylation alters the local structure of the nucleotide binding domain (30).

We present evidence that the yeast YIL110W gene product is the enzyme that catalyzes the modification of His-243 of Rpl3 and designate it Hpm1 for histidine protein methyltransferase 1. The YIL110W/Hpm1 protein is a member of the seven β -strand methyltransferase family (33, 36–38), and Rpl3 purified from strains with a deletion in YIL110W is completely unmodified at His-243. We have attempted, so far without success, to directly demonstrate this methyltransferase activity *in vitro* by incubating [3 H]AdoMet with purified recombinant YIL110W/Hpm1 protein with hypomethylated ribosomal proteins from a YIL110W mutant strain or with the synthetic peptide WGTKKLPKTRHGLRK that corresponds to residues 233–248 of Rpl3 (data not shown). *In vitro* activity analysis of ribosomal protein methyltransferases can be difficult because their substrates may not be the fully assembled ribosome but rather intermediates in ribosome biogenesis. Indeed, activity is readily observed in some cases (6) but not in others (16).

Yeast cells lacking the YIL110W/Hpm1 methyltransferase have a variety of phenotypes. These include those that may be

attributed to altered ribosomal function. For example, in comparison to wild type cells, mutants show reduced reproductive fitness in minimal media (relative growth rate of 0.76; Ref. 58), have reduced cell volume (32 fl compared with 41 fl; Ref. 59), and display a 3.5-fold increase in resistance to 0.2 mM selenomethionine (60). However, other mutant phenotypes appear to be unrelated to ribosomal function. Notably, YIL110W/Hpm1 mutant cells demonstrate enhanced growth on xylose media (61). YIL110W/Hpm1 mutants also have enhanced resistance to caspofungin, an inhibitor of β -1,3-glycan assembly (62), and are synthetically lethal in combination with a mutant of the *CHS1* gene for chitin synthase I, suggesting a role of the protein in cell wall biogenesis (63). Taken together, these results suggest that YIL110W/Hpm1 may have additional protein substrates other than Rpl3 in yeast.

Significantly, no genes have been described for histidine methyltransferases prior to this study. The apparent mammalian ortholog of the YIL110W/Hpm1 protein (the product of the C1orf156 gene in humans; UniProt O95568) might catalyze the methylation of actin or myosin. However, for actin, it seems unlikely that this is the case. Actin in *S. cerevisiae* and other fungal species is unmethylated *in vivo* (29) even though these species share a nearly identical primary sequence with mammalian actin (88% identity of the *S. cerevisiae* species with the human species), particularly in the region surrounding His-73, the site of 3-methylhistidine formation in mammals. Because the amino acid sequence surrounding the methylated histidine residue is distinct in actin and myosin (Table 1), it is possible that the mammalian ortholog of YIL110W is the myosin histidine methyltransferase. We note, however, that there is only weak sequence similarity in the amino acids surrounding the

3-Methylhistidine, a Novel Yeast Post-translational Modification

modified histidine site in Rpl3 and myosin. Another possibility is that the mammalian YIL110W/Hpm1 ortholog may modify its own Rpl3 ortholog as the amino acid sequence is highly conserved around the histidine 243 residue in yeast to mammals. Finally, it is possible that the mammalian ortholog may be the enzyme that converts the dipeptide carnosine (β -Ala-His) to anserine (β -Ala-1-methyl-His) (32, 64). Our studies now open the door to exploring protein histidine methylation in other yeast proteins and in identifying histidine protein methyltransferases in other organisms.

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