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Histidine methylation of yeast ribosomal protein Rpl3p is required for proper 60S subunit assembly

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Running title: Rpl3p histidine methylation promotes 60S assembly

Abbreviations: YPD, yeast peptone dextrose medium containing 1% yeast extract, 2% peptone, and 2% D-glucose (wt/vol); [^{3}H]AdoMet, S-adenosyl-[*methyl*-^{3}H]-L-methionine; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride.

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ABSTRACT

Histidine protein methylation is an unusual posttranslational modification. In the yeast *Saccharomyces cerevisiae*, the large ribosomal subunit protein Rpl3p is methylated at histidine-243, a residue that contacts the 25S rRNA near the P-site. Rpl3p methylation is dependent upon the presence of Hpm1p, a candidate seven beta strand methyltransferase. In this study, we elucidated the biological activities of Hpm1p *in vitro* and *in vivo*. Amino acid analyses reveal that Hpm1p is responsible for all detectable protein histidine methylation in yeast. The modification is found on a polypeptide corresponding to the size of Rpl3p in ribosomes and in a nuclei-containing organelle fraction, but was not detected in proteins of the ribosome-free cytosol fraction. *In vitro* assays demonstrate that Hpm1p has methyltransferase activity on ribosome-associated, but not free Rpl3p, suggesting that its activity depends on interactions with ribosomal components. *hpm1* null cells are defective in early rRNA processing resulting in a deficiency of 60S subunits and translation initiation defects that are exacerbated in minimal media. Cells lacking Hpm1p are resistant to cycloheximide and verrucarin A and have decreased translational fidelity. We propose that Hpm1p plays a role in orchestrating the early assembly of the large ribosomal subunit and in faithful protein production.

INTRODUCTION

Components of the translational apparatus are extensively posttransciptionally and posttranslationally modified in all three domains of life. One of the most common modifications is the addition of methyl groups to rRNA, tRNA, mRNA, snRNA, translation factors, and ribosomal proteins (1-4). These reactions are catalyzed by the members of several families of structurally-related methyltransferases that utilize *S*-adenosylmethionine as a methyl donor; some one percent of all genes in a variety of organisms encode such enzymes (5). In the yeast *Saccharomyces cerevisiae*, over half of the 64 known methyltransferases are devoted to the modification of the RNA and protein components of the translational machinery (6-8). The majority of rRNA modifications are found clustered in functional centers of the ribosome and increase in complexity in higher organisms. They are found in the peptidyl transferase center (PTC), the A/P/E sites, the polypeptide exit channel and the interface between the large and small ribosomal subunits, suggesting that these modifications help maintain the proper structures of these functional centers to ensure accurate and efficient translation (9). Methylation of tRNA is conserved in most organisms and can affect structural stability and translational fidelity (10,11). Methylation of translation release factors has been implicated in translation termination and fidelity (12-13). To date, relatively little is known about the roles of ribosomal protein methylation.

Previous mass spectrometric analyses of intact small and large ribosomal subunit proteins in *S. cerevisiae* have revealed eight stochiometrically methylated ribosomal proteins (Rps3p, Rps25p, Rps27p and Rpl1p, Rpl3p, Rpl12p, Rpl23p, Rpl42p) and one substoichiometrically methylated protein (Rps2p) (14-22). Unlike rRNA methylation, the sites of protein methylation are dispersed throughout the ribosome. The methylation sites on Rpl12p, Rps25p and Rps27p are exposed to the cytoplasm, whereas the methylated sites in Rpl3p, Rpl23p, Rpl42p, and Rps3p are in close proximity to rRNA. Although it is unknown what the precise roles of these modifications are, it is likely that protein modifications in close

63 contact with rRNA are playing structural or assembly roles, whereas protein modifications exposed to
64 the cytoplasm may be acting as binding platforms for translation factors or involved in signaling.
65
66 We previously reported that the large subunit protein Rpl3p is methylated in an unusual
67 reaction that results in the formation of 3-methylhistidine at position 243 (16). Although a few animal
68 proteins have been found with 3-methyl- and 1-methylhistidine, including actin and myosin, this
69 modification was novel to yeast and no enzymes catalyzing the modification in any organism had
70 previously been described (16). Methylation of His-243 is dependent upon the presence of a putative
71 methyltransferase that we designated Hpm1p (histidine protein methyltransferase 1) (16). His-243 lies
72 on the tryptophan finger domain of Rpl3p that extends deeply into large ribosomal subunit core, making
73 extensive contacts with the 25S rRNA (23). The tryptophan finger is involved in the accommodation of
74 aminoacyl-tRNAs into the ribosomal A site and in the activation of the peptidyltransferase center (24).
75 Interestingly, the *Escherichia coli* ortholog of yeast Rpl3p is also methylated (25). The ribosomal protein
76 L3 is methylated at a glutamine residue that aligns 12 residues from His-243 of *S. cerevisiae* Rpl3p. *E.*
77 *coli* cells lacking the PrmB methyltransferase, responsible for L3 methylation, demonstrated a defect in
78 the biogenesis of the small and large ribosomal subunits, indicating that L3 methylation might play a role
79 in the early steps of ribosome biogenesis (25). It is not known whether mammalian Rpl3 is methylated,
80 although the human C1orf156 protein (homolog of Hpm1p) has been found in a complex with human
81 Rpl3 (26).
82
83 In this paper, we have elucidated the biological roles of Hpm1p by analyzing its activities *in vivo*
84 and *in vitro*. Hpm1p is responsible for all detectable protein histidine methylation in *S. cerevisiae* and its
85 substrates are present in the ribosome and nuclei-containing organelles. We demonstrate that loss of
86 Hpm1p leads to a large subunit biogenesis defect that stems from a defect in early rRNA processing.

87 Hpm1p-deficient cells are significantly more resistant to the ribosome-targeting drugs cycloheximide
88 and verrucarin A, indicating an altered ribosomal structure. Additionally, Hpm1p-deficient cells exhibit
89 defects in translational fidelity shown by an increase in amino acid misincorporation and stop codon
90 readthrough. We propose that Hpm1p acts as an assembly factor during early ribosome biogenesis and
91 methylation of Rpl3p is needed for proper ribosome assembly and faithful protein production.
92

MATERIALS AND METHODS

Strains and growth media

All strains in this study are of the *BY4742* background (*MATα*; *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) and were obtained from the Open Biosystems yeast knockout collection (Thermo Scientific), containing a kanamycin resistance marker that replaces the open reading frame of the gene. Yeast strains were grown in 1% yeast extract, 2% peptone, and 2% dextrose (YPD, Difco) or minimal synthetic defined media lacking uracil and methionine (SD –ura –met). This media contains 0.67% yeast nitrogen base with ammonium sulfate without amino acids (BD Biosciences), 0.2% amino acids mix without uracil and methionine (Fisher), and 2% synthetic dextrose anhydrous (EMD Millipore).

Plasmids and expression of recombinant proteins. The *HPM1* rescue plasmid was made by amplifying its open reading frame from genomic DNA of the wild type *BY4742* strain. The inclusion of terminal SpeI and ClaI sites allowed its insertion into the pUG35 expression vector. Similarly, the human C1orf156 rescue plasmid was prepared by amplifying DNA from its open reading frame from pCMVSPORT6 (Open Biosystems) and cloned into the pUG35 expression vector using SpeI and ClaI restriction sites. The VEIG:AAAA and EIGCG:KIVCE substitutions in the *S*-adenosylmethionine binding motif 1 of Hpm1p were constructed using the Stratagene QuikChange Lightning kit. All sequences were confirmed by Sanger dideoxy sequencing (Laragen, Inc.) The following primers were used: HPM1 F SpeI- GACTGACT ACTAGTATGTCATTTTCCTTCGGCTTTAC, HPM1 R ClaI- GACTGACT ATCGATCTATCGGATAGCTTTATTTGTTTC, HPM1 (VEIG:AAAA) F-AACGATATCGACGCGGTTGCCGCAGCAGCCTGTGGTACGGCACTACCC, HPM1 (VEIG:AAAA) R-GGGTAGTGCCGTACCACAGGCTGCTGCGGCAACCGCGTCGATATCGTT, HPM1 (EIGCG:KIVCE) F-GATATCGACGCGGTTGTCAAAATAGTCTGTGAGACGGCACTACCCTCAG, HPM1 (EIGCG:KIVCE) R-CTCTGAGGGTAGTGCCGTCTCACAGACTATTTTGACAACCGCGTCGATATC, C1orf156 F SpeI-

GACTGACTACTAGTATGACCTTTCAGTTTAATTTCAC, and C1orf156 R ClaI-GACTGACTATCGATTTAACCAGGAAACTTAAAAGTTATTTC.

To prepare recombinant His-tagged proteins, the open reading frames of *HPM1*, *RPL3* and C1orf156 were cloned into the pET100/D-TOPO *Escherichia coli* expression vector as instructed (Invitrogen). The ORF of *HPM1* was amplified from a BG1805 plasmid containing the gene (Open Biosystems) by PCR using the forward primer CACCATGTCATTTTCCTTCG and the reverse primer CTATCGGATAGCTTTATTTG. The *RPL3* ORF was amplified from *BY4742* WT genomic DNA using the forward primer CACCATGTCTCACAGAAAG and the reverse primer TTACAAGTCCTTCTTCAAAGTA. C1orf156 ORF was amplified from a pCMVSPORT6 plasmid containing the gene (Open Biosystems) using the forward primer CACCATGACCTTTCAGTTTA and the reverse primer TTAACCAGGAAACTTAAAAG. Proper insertion into the pET100/D-TOPO vector was verified by DNA sequencing using T7 forward and reverse primers (GENEWIZ). The vector was transformed into *E. coli* BL21 (DE3) cells (Invitrogen). The recombinant N-terminal His-tagged protein was overexpressed by growing 2 L of the cells at 37°C in LB medium (Difco) with 100 µg/ml carbenicillin to an OD_{600} of 0.6 and then adding isopropyl β-D-thiogalactopyranoside (Anatrace, Maumee, OH; catalog number I1003) to a final concentration of 1 mM and growing cells for 6 h before harvesting. Cells were then washed, resuspended in lysis buffer (50 mM sodium phosphate, 500 mM NaCl, 5% glycerol, protease inhibitor cocktail (Roche applied science, IN, 11836145001), 5 mM beta-mercaptoethanol, pH 8.0) and lysed by an Avestin EmulsiFlex-C3 emulsifier (C315270) set to 18,000 psi. Lysates were centrifuged at 20,000 *x* g for 20 min and the resulting supernatant was loaded onto a 5-ml His-Trap HP nickel affinity column (GE Healthcare part number 17-5248-1), and the recombinant protein eluted using a gradient of 20 - 500 mM imidazole. Recombinant proteins were then buffer exchanged (50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, 5% glycerol, 5 mM beta-mercaptoethanol, pH 8.0) and stored at -80°C. Expression of the recombinant

proteins was verified by Western Blot analysis using a mouse anti-6XHis antibody (Abcam) and by bottom-up MS/MS using ESI-FTICR (Thermo Scientific).

***In vivo* radiolabeling with [^{3}H]AdoMet and subcellular fractionation.** Overnight cultures of wild type (BY4742) and *hpm1Δ* were diluted into 200 ml YPD media to an OD_{600} of 0.0001 and incubated on a rotary shaker at 30°C until the OD reached 0.6. At that time, cells from 23 ml of the culture (14 OD units) were harvested by centrifugation at 5000 *x* g for 5 min at room temperature; the remainder of the culture was placed at 4°C. The pelleted cells were washed with 1 ml water 3 times and then resuspended in 1696 µl of YPD and 304 µl of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine ([^{3}H]AdoMet; Perkin Elmer; 83.3 Ci/mmol; 0.55 mCi/ml in 10 mM H_2SO_4:ethanol 9:1). Cells were labeled for 30 min at 30°C on a rotatory shaker and then harvested at 5,000 *x* g for 5 min at 4°C. Radiolabeled cells were washed with water twice and then resuspended in 1 ml of YPD and remixed with the remaining 177 ml of the original culture. These cells were harvested at 5000 *x* g for 5 min, washed with water twice and stored at -80°C. Cells in the thawed pellet were then lysed by resuspension in 5 ml of buffer A (20 mM Tris base, 15 mM magnesium acetate, 60 mM KCl, 1 mM DTT, 1 mM PMSF, Roche protease inhibitor cocktail, adjusted to pH 7.5 with HCl) and 10 cycles of vortexing for 1 min with 1.5 g baked glass beads (Biospec Products, Bartlesville, OK) followed by cooling on ice for 1 min. The crude lysates were centrifuged at 800 *x* g for 5 min at 4°C to pellet unlysed cells and the supernatant volume was measured (this fraction is referred to as "lysate"). The lysates were then centrifuged at 12,000 *x* g for 5 min at 4°C in a JA17 rotor (Beckman). The pellet ("subcellular organelles") was resuspended in 500 µl of buffer A, the volume measured again, and the suspension frozen at -80°C. The supernatant was centrifuged at 20,000 x g for 15 min at 4°C and the pellet was discarded. The resulting supernatant was transferred into a fresh tube and centrifuged at 159,000 *x* g for 2 h at 4°C using a Ti65 rotor (Beckman). The pellet ("ribosome" fraction) was resuspended in 500 µl of buffer A at 0°C and the final volume measured and

the supernatant ("cytosol" fraction) volume was measured as well. Samples were stored at -80°C. The protein concentration of each fraction was determined by the Lowry method after protein precipitation with 10% trichloroacetic acid.

Amino acid analysis using high-resolution cation-exchange chromatography. Wild type and *hpm1Δ* subcellular fractions ("lysate", "subcellular organelles", "ribosome", and "cytosol") were acid hydrolyzed as follows: 400 µg protein of each sample was transferred to a 6 x 50-mm glass vial and precipitated with equal volume of 25% trichloroacetic acid for 30 min at room temperature and centrifuged at 4,000 *x* g for 30 min at room temperature. The pellets were washed with 100 µl of cold acetone and centrifuged again for 30 min. 50 µl of 6M HCl was added to each glass vial and placed in a reaction chamber (Eldex Labs, catalog #1163) containing 200 µl of 6M HCl. The vials were heated for 20 h *in vacuo* at 109°C using a Pico-Tag Vapor-Phase apparatus (Waters). Residual HCl was removed by vacuum centrifugation. Samples were resuspended in 50 µl of water and 100 µl of 0.2 M sodium citrate, pH 2.2. Each sample was added and spiked with 2 µmoles of a 3-methyl-L-histidine standard (Fisher #50-750-2805). Samples were loaded onto a cation-exchange column (Beckman AA-15 sulfonated polystyrene beads; 0.9-cm inner diameter by 12-cm column height) and equilibrated with sodium citrate buffer (0.2 M Na^+, pH 5.84 at 28 °C). Amino acids were eluted in the same buffer at 1 ml/min. One min fractions were collected from 50-105 minutes corresponding to the approximate elution time of 3-methyl-L-histidine standard. 500 µl of each fraction was added to 5 ml of scintillation fluor (Safety Solve; Research Products international) and counted. The 3-methyl-L-histidine standard was detected using the ninhydrin method (Gary and Clarke, 1995), with the following modifications. 50 µl of each fraction was added to a well in a flat bottom 96 well plate (Fisher #12565501) and mixed with 100 µl of ninhydrin reagent (2% (w/v) ninhydrin and 3 mg/ml hydrindantin in a solvent of 75% (v/v) DMSO and

188 25% (v/v) 4 M lithium acetate at pH 4.2). The plate was heated at 100°C for 15 min, and the absorbance
189 measured at 570 nm using a SpectraMax M5 microplate reader.
190
191 **Isolation of ribosomes.** Overnight cultures of wild type (BY4742) and *hpm1Δ* cells were diluted
192 into 500 - 1000 ml YPD media to an optical density at 600 nm of 0.1 and grown at 30 °C until cells
193 reached late log-phase at optical densities of 1-2. The cells were harvested at 5000 *x* g for 5 min,
194 washed with water twice and stored at -80°C. To lyse, cells were resuspended in 5 ml of buffer A and
195 lysed by vortexing in glass beads as described above. The crude lysate was centrifuged for 5 min at
196 12,000 *x* g at 4°C. The supernatant was transferred to a new tube and centrifuged at 20,000 *x* g for 15
197 min at 4°C. The supernatant was again transferred into a fresh tube and centrifuged at 159,000 *x* g for 2
198 h at 4°C using a Ti65 Beckman rotor. The resulting ribosomal pellet was resuspended in buffer A and
199 stored at -80°C. The protein concentration was determined using the Lowry method after precipitation
200 with trichloroacetic acid.
201
202 **SDS-PAGE/fluorography.** Polypeptides from cell lysates and sub-cellular fractions were
203 fractionated by SDS-PAGE. Samples were loaded onto a 15 x 17 X 0.2 cm Tris-glycine polyacrylamide gel
204 and resolved by applying 80 V until the bromphenol blue dye reached the interface of the stacking (4%)
205 and resolving (12%) gel followed by 180 V until the dye reached the bottom of the resolving gel. Gels
206 were Coomassie-stained and destained overnight (10% acetic acid, 5% methanol). After imaging, gels
207 were washed with water and then treated with EN3HANCE (PerkinElmer Life Sciences) solution for 1 h,
208 followed by a 30 min wash with water. The gel was then dried onto 3 mm Whatman paper under
209 vacuum for 2 h at 80°C, followed by 1 h without heat. The gel was placed on Kodak BIOMAX XAR film at
210 -80°C.
211

Polysome profile analysis and ribosomal subunit quantification. For polysome profile analysis, overnight cultures of yeast cells were diluted into 100-200 ml of the appropriate medium to an optical density at 600 nm of 0.1 (0.01 for cells growing in SD-ura-met). Growth was monitored until cells reached an optical density of 0.8-1.0. Cycloheximide was added to a final concentration of 100 µg/ml and cells were immediately placed in an ice-bath for 10 min. Cells were then pelleted by centrifugation at 3,000 *x* g at 4°C for 5 min and washed once with 20 ml of ice-cold lysis buffer (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$, 100 µg/ml cycloheximide, 200 µg/ml heparin, 0.1% diethylpyrocarbonate) and transferred to 15 ml polypropylene conical tubes. Cell pellets were stored at -80°C. For ribosomal subunit dissociation, yeast cells were grown and harvested similarly, except cells were not treated with cycloheximide and washed with buffer C (50 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1 mM DTT). To lyse, the pellet was resuspended in 1 ml of lysis buffer (buffer C for subunit dissociation) and a quarter volume of glass beads were added and the cells were vortexed for 30 s, followed by 30 s on ice, for a total of 12 cycles. The crude lysate was centrifuged at 5,000 *x* g for 5 min at 4°C. The supernatant was then transferred to pre-chilled microfuge tubes and centrifuged again at 12,000 *x* g for 8 min at 4 °C. The supernatant was transferred again to fresh pre-chilled tubes and absorbance at 260 nm was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific). 10 A260 units of lysate was layered onto 11-ml of a 8%-48% (wt/vol) sucrose gradient buffered with 50 mM Tris-Cl, pH 7.0, 50 mM NH_4Cl, 12 mM $MgCl_2$, 1 mM DTT (freshly prepared from solid), using Beckman Ultra-Clear centrifuge tubes (344059). For subunit dissociation, 2 A260 units of lysate was layered onto 11-ml of a 8%-48% (wt/vol) sucrose gradient buffered with buffer C. Tubes were centrifuged at 37,000 rpm (234,116.4 *x* g) at 4°C for 3.5 h using a Beckman SW41 Ti rotor. Continuous absorbance of the sucrose gradient at 280 nm was done using an ISCO gradient fractionator (Model 185). The flow rate was set to 1.5 ml/min using fluorinert FC-40 as a displacing agent. The absorbance detector used was an ISCO Model UA-5 Absorbance monitor; sensitivity was set to 0.5 absorbance units per volt. For digitizing the absorbance

236 readings, an RS-232 equipped voltmeter (Tekpower model TP4000ZC) was connected to the external
237 chart recorder outputs and recorded DC voltage at 1 s intervals using the included software (DMM
238 version 2.0) and then imported into Microsoft Excel for further data processing.
239
240 **Northern blot analysis.** Cells were grown in YPD to mid-log phase to an optical density at 600
241 nm of 0.4. Cells were harvested by centrifugation at 4000 *x* g for 2 min at room temperature. Cell
242 pellets were washed with 1 ml of ice-cold water, transferred to 1.5 ml tubes, spun down, and the
243 resulting pellets frozen in dry ice. Total RNA was extracted according to the hot phenol/acid-washed
244 glass beads protocol (27). 5 µg of RNA was denatured by glyoxal (28), and loaded onto 1.2% agarose / 1X
245 BPTE gels. The separated RNAs were passively transferred to Hybond N^+ nylon membranes (GE
246 Healthcare) in 10X SSPE. Membranes were cross-linked with 254 nm light using the UV Stratalinker 2400
247 as instructed by the manufacturer. Membranes were hybridized in standard Church's buffer (1% BSA, 1
248 mM EDTA, 0.5 M sodium phosphate pH 7.2, 7% SDS) using oligonucleotide probes labeled at the 5' end
249 with [γ-^{32}P]-ATP and T4 polynucleotide kinase. The following oligonucleotides were used: 18S-rRNA: 5'-
250 CATGGCTTAATCTTTGAGAC-3'; 25S-rRNA: 5'-CTCCGCTTATTGATATGC-3'; E-C2 region of pre-rRNA for
251 detection of 7S pre-rRNA and total 27S pre-rRNA: 5'-GGCCAGCAATTTCAAGTTA-3'; 5' ETS region
252 upstream of A0 for detection of 35S and 23S pre-rRNA: 5'-CGCTGCTCACCAATGG-3'; D-A2 region for
253 detection of 20S pre-rRNA: 5'-CGGTTTTAATTGTCCTA-3'. PDR5 mRNA was detected with a riboprobe
254 antisense to the PDR5 ORF. The 25S and 18S Northern blot signals were quantified using the Quantity
255 One software from the BioRad FX Plus Phosphorimaging System.
256
257 **Pulse-chase labeling of rRNA.** The pulse-chase protocol was modified from Tollervey *et al.*
258 (1991) (29). Cells were first transformed with an empty pUG35 plasmid (URA^+) to allow growth in the
259 absence of uracil, according to the LiOAc/ssDNA/PEG method (30). The transformed cells were then

grown at 25°C in 10 ml synthetic dextrose medium lacking uracil (SD-URA) to mid-log phase (optical density at 600 nm of 0.4). For the pulse, 400 µCi of [5,6-^{3}H]-uracil (Amersham) was added directly to the 10 ml culture. After two minutes of pulse, an excess of cold uracil (1 ml of 2.5 mg/ml uracil dissolved in SD-URA) was added directly to the labeled cultures. At the designated time points, 1 ml of culture was added to 10 ml of 100% ethanol pre-chilled on dry ice in 15 ml tubes. The tubes were then warmed to room temperature, and spun at 3000 *x* g for 3 min. The pellets were washed with 1 ml of ice-cold water to remove precipitate from the pellet, and then frozen on dry ice in 1.5 ml tubes. Total RNA was extracted according to the hot phenol / acid-washed glass beads protocol (27). The resulting RNA pellet was resuspended in 15 µl nuclease-free water (Ambion), and 1 µL was denatured in glyoxal buffer and loaded onto 1.2% agarose / 1X BPTE gels (Sambrook and Russell, 2001). The separated RNAs were then transferred to Hybon N^{+} nylon membranes (GE Healthcare), and the membrane was dried and exposed to BioMax MS film with a BioMax transcreen LE (Kodak) at -80°C for 5 days.

Dual-luciferase assay for measuring translational fidelity. The dual-luciferase systems described in Ref. 31 and 32 were used here. Luciferase reporters and control vectors were generously provided by Dr. Bedwell and Dr. Ming Du at the University of Alabama in Birmingham, AL. The following vectors were used: CTY775/luc CAAA FF K529 (AAA to AAT) and the control CTY775/luc CAAA vector for amino acid misincorporation. CTY775/luc UAAC, CTY775/luc UAGC, CTY775/luc UGAC and their respective control vectors CTY775/luc CAAC, CTY775/luc CAGC, and CTY775/luc CGAC. The control vectors are used to adjust for differences in protein expression between wild type and *hpm1Δ* cells. All eight vectors were transformed into wild type and *hpm1Δ* cells using the LiOAc/ssDNA/PEG method (30). The dual-luciferase assay was done as described in (31,32) using the Dual-Luciferase Reporter Assay system (Promega), with a few modifications: Lysates from each strain were diluted 10-fold using 1X Passive Lysis Buffer (Promega), to ensure measurements were in the linear range, and spotted into

each well of a white 96 well plate (Costar, 3912). Firefly and *Renilla* luciferase luminescence was measured at room temperature using a SpectraMax M5 microplate set to the following parameters: readtype = endpoint, readmode = luminescence with 1000 ms integration time, wavelength = all. Remaining parameters were set to default.

RESULTS

Hpm1p is the major histidine methyltransferase in *S. cerevisiae* with a primary target corresponding to ribosomal protein Rpl3p. We previously reported that the *S. cerevisiae* ribosomal protein Rpl3p contains a 3-methylhistidine modification on residue 243 using mass spectrometry and high-resolution cation-exchange chromatography (16). This modification is dependent on the presence of the seven-beta strand putative methyltransferase product of the *HPM1* gene (16). To determine other potential substrates of Hpm1p, we radioactively labeled all methylated products in both wild type and *hpm1* null yeast cells by incubating log phase cells with [^{3}H] AdoMet. Cell lysates were acid hydrolyzed and amino acids resolved by high resolution cation-exchange chromatography. Fractions containing 3-methylhistidine were collected and counted for radioactivity. Wild type lysate showed a radioactive peak co-eluting with a 3-methylhistidine standard (Fig. 1A). The radioactive species elutes one minute before the 3-methylhistidine non-isotopically labeled standard due to a known isotope effect (16, 33). Strikingly, the lysate from Hpm1p-deficient cells had complete loss of radioactivity in the 3-methylhistidine fractions. This result indicates that Hpm1p is responsible for all detectable histidine methylation in *S. cerevisiae* under these growth conditions.

To determine the subcellular distribution of Hpm1p-dependent methylation, ribosomes, nuclei-containing organelles and cytosolic fractions were isolated from the lysates of [^{3}H]AdoMet-labeled wild type and *hpm1* null cells and acid hydrolyzed for amino acid analysis as above. The majority of radioactive 3-methylhistidine was present in wild type ribosomes and absent from the *hpm1* mutant (Fig. 1A). A small amount of the modification was present in wild type organelles and again absent from *hpm1Δ* cells. Importantly, no radiolabeled 3-methylhistidine was found in the ribosome-depleted cytosol fraction of wild type and *hpm1Δ* cells. These results suggest that ribosomes are the major target of Hpm1p-dependent methylation.

315

316 To obtain a profile of methylated ribosomal proteins in wild type and *hpm1Δ* strains,
317 radiolabeled ribosomes prepared from [^{3}H]AdoMet-labeled cells, as described above, were analyzed by
318 SDS-PAGE/fluorography. The absence of Hpm1p resulted in the loss of only one radiolabeled
319 polypeptide that corresponds to the size of Rpl3p, suggesting that it is the only ribosomal protein
320 substrate of Hpm1p (Fig. 1B, arrow). To unambiguously confirm that this polypeptide is present on the
321 large ribosomal subunit, radiolabeled lysates were resolved by sucrose density ultracentrifugation to
322 separate the small and large ribosomal subunits. Peak fractions were analyzed by SDS-
323 PAGE/fluorography and showed one methylated band beneath the 45 kDa marker in only the 60S but
324 not the 40S or cytosol fractions (Fig. 1C). This band completely disappears in *hpm1Δ* cells.

325

326 Interestingly, a band corresponding to the size of the small ribosomal subunit proteins Rps2 and
327 Rps3 showed hypermethylation in the absence of Hpm1p (Fig. 1B, asterisk). Mass spectrometry analysis
328 of intact ribosomal proteins confirmed that Rps2 is hyper-methylated and that the content of fully-
329 methylated Rps3 is increased in the absence of Hpm1p (data not shown). It appears that the
330 methylation of other proteins can be affected in the absence of Hpm1p indicating a possible
331 compensatory mechanism. This was also seen in the parallel analysis of the *in vivo* [^{3}H]AdoMet-labeled
332 organelle fraction. This analysis also revealed a radiolabeled methylated polypeptide corresponding to
333 size of Rpl3p from wild type but not from *hpm1Δ* cells as well as alterations in the methylation patterns
334 of other proteins (Fig. 1B). At this point, it is unclear whether this represents methylation of the protein
335 in membrane-bound ribosomes of the endoplasmic reticulum or in an assembly intermediate of the
336 nucleus. Altogether, these data suggest that the bulk of 3-methylhistidine modification resides in the
337 ribosome on Rpl3p and that a small portion of this modification may be found in subcellular organelles.

338

Hpm1p is a bona fide methyltransferase that modifies ribosome-associated but not free Rpl3p. Since Rpl3p methylation is dependent on Hpm1p, we next investigated whether purified recombinant Hpm1p is in fact a methyltransferase that can directly methylate Rpl3p *in vitro*. Ribosomes from wild type and *hpm1Δ* strains were incubated with recombinant Hpm1p in the presence of [^{3}H]AdoMet. Proteins were then separated by SDS-PAGE and visualized by fluorography. A radioactive 43 kDa polypeptide band corresponding to Rpl3p was present in *hpm1Δ* ribosomes but not wild type ribosomes (Fig. 2A). This result is consistent with ribosomal proteins in wild type cells being stoichiometrically methylated, and therefore unable to be further methylated *in vitro.* However, *hpm1Δ* cells would contain unmodified Rpl3p and any other potential Hpm1p substrates, which would be competent for methylation *in vitro*. To verify that this radiolabeled protein is the substrate of Hpm1p, amino acid analyses of these radiolabeled ribosomes were performed. A radioactive species co-migrating with the 3-methylhistidine standard was only found when recombinant Hpm1p was incubated with ribosomes from the *hpm1Δ* strain but not wild type, confirming that Hpm1p catalyzes a 3-methylhistidine modification on a ribosomal protein corresponding to the size of Rpl3p (Fig. 2B). We also tested the ability of recombinant Hpm1p to methylate Rpl3p in the organelle fraction. This experiment showed that Hpm1p can also methylate a 43 kDa protein only in the organelle fraction from *hpm1Δ* cells (data not shown).

We next investigated the ability of recombinant Hpm1p to recognize and methylate a synthetic 16 amino acid-long peptide corresponding to the methylated region on Rpl3p. This peptide was incubated with recombinant Hpm1p and non-radioactive AdoMet and analyzed by LC-MS. Methylation of the peptide should result in a mass increase of 14 Da. However, addition of Hpm1p to the peptide resulted in no mass change, indicating that Hpm1p cannot detectably methylate the peptide (Fig. 2C). This result suggests that Hpm1p may require secondary or tertiary structures present in full-length Rpl3p

363 needed for recognition and subsequent methylation. We therefore tested the ability of recombinant
364 Hpm1p to methylate full-length recombinant Rpl3p when incubated with [^{3}H]AdoMet. No radiolabeled
365 polypeptide band corresponding to the size of recombinant Rpl3p was detected (Fig. 2A). These data
366 suggest that Hpm1p may require other factors associated with the ribosome to efficiently methylate
367 Rpl3p.
368
369 **Loss of Hpm1p results in reduced levels of 60S ribosomal subunits.** Since Rpl3p is currently the
370 only known substrate of Hpm1p and many of the ribosome modifications occur during ribosome
371 assembly, we next investigated the potential role of this enzyme in ribosome biogenesis and/or
372 translation. Growth studies of wild type and *hpm1Δ* cells in rich medium (YPD) at 15°C, 30°C, 38°C, or in
373 the presence of 250 mM NaCl showed no observed difference in growth rates (data not shown). To
374 dissect any abnormalities in ribosome biogenesis and translation, we performed sucrose density
375 centrifugation analysis to separate the small (40S) and large (60S) ribosomal subunits from intact
376 monosome ribosomes (80S) and polysomes. Loss of Hpm1p resulted in abnormal polysome profiles,
377 most strikingly seen with increased levels of 40S subunits and decreased levels of 60S subunits and 80S
378 monosomes when the background is taken into account (Fig. 3A). Additionally, *hpm1Δ* cells exhibited
379 increased ribosome runoff as noted by decreased levels of polysomes at the end of the profile. This
380 phenotype was accentuated under cold stress conditions (Fig. 3B). These changes are reflected in the
381 60S/40S ratio where, in *hpm1Δ* cells, the ratio decreased by over 50% (Fig. 3D, left). To confirm this,
382 yeast lysates from wild type and *hpm1Δ* were prepared using a buffer that results in complete
383 dissociation of the subunits (no cycloheximide and Mg^{2+}). Dissociation of the monosomes and
384 polysomes revealed a clear reduction of the large ribosomal subunit and no observable increase in the
385 small subunit (Fig. 3C), which is also reflected in a ~10% decrease in the 60S/40S ratio (Fig. 3D, right).

Since monosomes and polysomes contain stoichiometric amounts of large and small subunits, a decrease in the number of free 60S subunits would result in increased levels of free 40S subunits.

Loss of Hpm1p results in early processing defects and delayed kinetics of pre-rRNA maturation. To examine the basis for the large subunit biogenesis defect, we probed for alterations in the rRNA processing pathway. The 35S pre-rRNA transcript, which encodes for both the small and large subunit rRNAs, undergoes a series of sequential modifications and endo- and exonucleolytic cleavages to produce the mature 18S of the small subunit and 5.8S and 25S rRNAs of the large subunit (Fig. 4A) (34,35). The predominant processing pathway of the 35S rRNA transcript involves initial cleavages at sites A_0, A_1, and A_2. Defects in various aspects of ribosome biogenesis can result in impaired cleavages at sites A_0, A_1, and A_2, causing a shift towards more precursors undergoing the first cleavage at site A_3, and leading to a greater steady-state accumulation of 23S and 21S intermediate transcripts (Fig. 4A, right side) (36). We performed Northern blot analyses, using radiolabeled probes that bind to different regions of the pre-rRNA transcript, to determine steady-state levels of pre-rRNAs (Fig. 4B). A probe hybridizing upstream of the A_0 site revealed a substantial accumulation of the 35S precursor in *hpm1Δ* strain relative to wild type (Fig. 4B, top panel). There was also an accumulation of the 23S pre-RNA species in the *hpm1Δ* strain, suggesting defects in A_0, A_1, and A_2 cleavage in cells lacking Hpm1p. However, the levels of the large subunit pre-rRNAs, 7S and 27S, and the small subunit pre-rRNA, 20S, were relatively unchanged. This suggests that loss of Hpm1p results in an rRNA processing defect early on in the processing pathway. By contrast, other ribosomal protein methyltransferase mutant strains did not exhibit this defect, highlighting the specificity of the phenotype detected in the *hpm1Δ* strain. Comparison of the rRNA processing defect of *hpm1Δ* with a mutant deleted for *RNT1*, which processes the 3'ETS of pre-rRNAs, showed comparable accumulation of 35S and 23S species (Fig. 4C).

To further dissect the processing kinetics of rRNA precursors, a pulse-chase assay was performed using ^{3}H-uracil. Radiolabeled rRNA precursors, intermediates and mature products were fractionated on an agarose gel, transferred to a nylon membrane and analyzed by autoradiography. *hpm1Δ* cells exhibited a considerable delay in the processing of pre-rRNAs (Fig. 4D). A fraction of the unprocessed 35S pre-rRNA particle accumulated for over 20 min in the *hpm1Δ* strain and was slowly converted to 32S over the course of the chase, whereas in wild type, these precursors did not accumulate (Fig. 4D). In addition, *hpm1Δ* cells showed a delayed appearance of 20S and 27SA pre-rRNAs. To determine steady-state levels of mature rRNAs, the same membrane was probed for 18S and 25S rRNAs. It showed a reduced ratio of 25S to 18S rRNA, for cells lacking Hpm1p, throughout the entire chase (Fig. 4D). These data indicate that in addition to an overall delay in kinetics, a detectable fraction of the 35S precursor undergoes very slow processing, indicating that this fraction has not assembled properly. The delay in processing kinetics and increased steady-state levels of 35S and 23S pre-rRNAs are hallmarks of defects in early ribosome biogenesis (37).

Hpm1p methyltransferase activity is required for proper large subunit biogenesis and translation. To see if ectopic Hpm1p could rescue the defects in large subunit biogenesis and translational initiation associated with *hpm1Δ*, wild type and *hpm1Δ* cells were transformed with an inducible pUG35 vector containing the wild type *HPM1* open reading frame. Expression of the genes was induced by omitting methionine from the minimal growth media. Cell lysates were prepared and layered onto sucrose gradients and ribosomal particles were separated by ultracentrifugation. Consistent with the results shown in Fig. 3, *hpm1Δ* cells containing an empty pUG35 vector exhibited a 60S biogenesis defect with an accumulation of the small 40S subunit and decreased amounts of the large 60S subunit (Fig. 5A). Significantly fewer ribosomes were associated with translation-active polysomes in *hpm1Δ* compared to wild type, indicative of translation initiation defects. Reintroducing Hpm1p into *hpm1Δ* cells eliminated the 60S biogenesis defect phenotype and resulted in significantly

more ribosomes involved in active translation, showing that Hpm1p can rescue the defects in large ribosomal subunit biogenesis and translation associated with Hpm1p-deficient cells (Fig. 5A). These rescued cells had similar levels of 3-methylhistidine as the wild type cells (Fig. 5B).

To determine if the loss of Hpm1p methyltransferase activity is sufficient to cause the subunit imbalance, two mutant sequences that alter the AdoMet-binding motif were expressed in *hpm1Δ* cells. The mutation of EIGCG in Motif I to KIVCE (*hpm1-EK*) reduced the presence of 3-methylhistidine by at least ~80% and mutation of VEIG in Motif I to AAAA (*hpm1-VA*) resulted in the near absence of the modification as determined by amino acid analysis (Fig. 5B). Neither of these *hpm1* mutant plasmids was able to rescue *hpm1Δ* cells, as can be seen with the retention of the subunit reversal phenotype and diminished amounts of ribosomes engaged in active translation (Fig. 5A). The human homolog of Hpm1p, C1orf156, was also unable to rescue the diminished large subunit levels and translation defects. Currently, there is no evidence that human Rpl3 is methylated. Hence, the inability of C1orf156 to rescue Hpm1p-deficient cells may be due to C1orf156 not being an ortholog of Hpm1p, not being able to recognize the yeast protein, or that it is non-functional when expressed in yeast cells. Subunit ratios (60S/40S) were quantified and showed that *HPM1* partially restored the levels of 60S subunits to that of wild type, whereas *hpm1-EK* and *hpm1-VA* were unable to do so (Fig. 5C, left). Translational fitness of the various strains was quantified using the polysome/monosome ratio; a higher ratio indicates more ribosomes are engaged in active translation. Translational fitness is reduced in *hpm1Δ* cells and was only rescued by the introduction of wild type Hpm1p (Fig. 5C, right).

Cells lacking Hpm1p exhibit a hyper-resistance to the ribosome-targeting drugs cycloheximide and verrucarin A. We next examined the sensitivity of wild type and *hpm1Δ* cells to various ribosome-binding drugs to determine any potential consequences of a lack of Hpm1p on ribosome structure and

function. Growth rates of log-phase wild type and *hpm1Δ* cells were compared on YPD agar plates containing puromycin, anisomycin, paramomycin, cycloheximide, and verrucarin A. Each of these drugs interferes with different aspects of translation by binding to different regions of the ribosome. No changes in sensitivity of *hpm1Δ* were seen for puromycin, anisomycin, and paramomycin (Fig. 6A). However, *hpm1Δ* cells were significantly more resistant than wild type to cycloheximide and verrucarin A (Fig. 6A). The dramatic increase in resistance to these drugs indicates structural alterations to their binding sites (see Discussion).

Northern blot analyses detected similar levels of the multidrug transporter PDR5, suggesting that the resistance phenotypes are not a consequence of difference levels of the PDR5 exporter in wild type and *hpm1Δ* cells (Fig. 6B). Altogether, these results suggest that *hpm1Δ* cells have an altered ribosome structure that is different from wild type ribosomes, causing changes in ribosome-targeting drug sensitivity.

Cells deficient of Hpm1p have reduced translational fidelity. We next investigated the effect of Hpm1-depletion on translational fidelity by utilizing a dual-luciferase reporter assay to measure amino acid misincorporation and stop codon suppression. This system utilizes a plasmid with a *Renilla* luciferase gene upstream of a firefly luciferase gene (31, 32). The amount of *Renilla* enzyme luminescence is used to normalize differences in mRNA abundance and translation initiation efficiency. To measure amino acid misincorporation, the firefly gene contains a near-cognate K529N mutation that renders firefly luciferase catalytically inactive (32). Misincorporation of the wild type lysine residue through the recognition of the near-cognate lysyl-tRNA^{Lys} would restore enzymatic activity of the firefly enzyme and result in increased amounts of firefly luciferase luminescence. Stop codon readthrough reporter plasmids contain each of the three stop codons in between the *Renilla* and firefly genes (32). Suppression of these stop codons would again result in increased firefly luciferase activity. Hpm1p-

482 deficient cells displayed a significant increase in amino acid misincorporation that was more than 2-fold
483 higher than wild type cells (Fig. 7A). Additionally, there was increased readthrough of the UAA and UGA
484 stop codons but not the UAG stop codon. These findings indicate that *hpm1Δ* ribosomes have a reduced
485 ability to discriminate between cognate and near-cognate aminoacyl-tRNAs in the A-site and in the
486 recognition of translation terminating stop codons.
487
488

DISCUSSION

The large ribosomal subunit protein Rpl3p of *S. cerevisiae* contains an unusual 3-methylhistidine modification that makes extensive contacts with the 25S rRNA near the peptidyl transferase center (16, 23, 24). In this work, we show that the loss of Rpl3p methylation in *hpm1* null cells correlates with a deficit of large ribosomal subunits, decreased translational fitness, ribosomal structure alterations, and decreased fidelity in protein synthesis. These defects in large subunit biogenesis and translation likely stem from delayed and aberrant processing of pre-rRNA transcripts, as demonstrated by the accumulation of the 23S precursor, delayed appearance of the 27S and 20S precursors to the 25S and 18S mature rRNAs, respectively, and the detection of a fraction of 35S species that undergoes very slow A_0/A_1 cleavage to the 32S in *hpm1Δ* cells. Similar results have been reported for cells depleted of Rpl3p, which also demonstrated the subunit reversal phenotype and abnormal rRNA processing, as well as an accumulation of half-mer polysomes, indicative of initiation defects (38). Although we did not observe half-mers in the *hpm1Δ* cells, there was increased ribosome runoff, another manifestation of initiation defects. Furthermore, it has been reported that the loss of yeast Rrb1p, which physically interacts with Rpl3p, also results in a 60S biogenesis defect (39, 40). Rrb1p is proposed to target Rpl3p to the 35S pre-rRNA transcript. Notably, the human homolog of Hpm1p, C1orf156, co-purifies with both human RPL3 and GRWD1 (human homolog of Rrb1p) (26). These reports are consistent with our hypothesis that Hpm1p is involved in early 60S subunit assembly.

Our ability to rescue the phenotypes of *hpm1* null cells with a plasmid containing the wild type but not mutant *HPM1* suggests that Hpm1p's role in early large subunit assembly is dependent on its methyltransferase activity. This is unlike what has been reported for the Bud23p 18S rRNA methyltransferase in *S. cerevisiae* or the Rmt3p protein arginine methyltransferase in *Schizosaccharomyces pombe*; in both these cases, the methyltransferase activities are dispensable for their roles in ribosome biogenesis (41, 42). While Rmt3p is implicated in 40S small subunit biogenesis

513 (46), to our knowledge, Hpm1p is the only ribosomal protein methyltransferase shown to be involved in
514 large subunit biogenesis.

515 Using *in vitro* methylation assays, we now show that Rpl3p can be directly methylated by
516 Hpm1p, confirming that this protein is an active methyltransferase and not simply a required accessory
517 factor in the modification of Rpl3p at His-243. Hpm1p is capable of catalyzing the methylation of Rpl3p
518 when it is in complex with mature ribosomes, but not as a purified, recombinant protein. Additionally,
519 we find no evidence that Hpm1p can methylate a synthetic peptide containing the His-243 site. These
520 results suggest that Hpm1p requires specific interactions with ribosomal components to efficiently
521 methylate Rpl3p. This situation is similar to that observed with the *E. coli* PrmB L3 glutamine
522 methyltransferase where unfolded 70S ribosomes or a reconstituted mix of extracted ribosomal
523 proteins and rRNA is required for efficient methylation (25). The ability of Hpm1p to methylate
524 ribosome-associated Rpl3p *in vitro* seems counterintuitive, since the methylation site on mature
525 ribosomes is buried deep within the core of the 25S rRNA. This location makes it unlikely that Hpm1p
526 can locate its target site on mature ribosomes *in vivo*. GFP localization studies have shown that Hpm1p
527 is present in both the nucleus and cytoplasm, with higher levels in the nucleus (43). Hpm1p has also
528 been reported to co-purify with the Mlp2p nuclear protein (44) and the Rpa135p subunit of nucleolar
529 RNA polymerase I (45). Coupled with the rRNA processing defects that we observed and the appearance
530 of the 3-methylhistidine modification inside nuclei-containing subcellular organelle fractions, it is more
531 plausible that methylation occurs on partially assembled pre-ribosomes in the nucleus.
532
533 To examine the possibility that the alterations in ribosome biogenesis could affect the function
534 of the mature ribosome, Hpm1p –deficient cells were probed for changes in sensitivity to several
535 ribosome-binding drugs. Altered sensitivities may indicate structural abnormalities to the functional
536 centers that these drugs bind to. Puromycin and anisomycin act as competitive inhibitors of aminoacyl-

tRNAs by binding to a hydrophobic crevice in the A-site of the PTC (47) that is approximately 20 Å away from the methylated H243 site. Paramomycin binds to the decoding center of the small 40S subunit greater than 80 Å away from the H243 site; changes in sensitivity to this drug have been linked to alterations in the decoding center (48). Our findings that *hpm1* null cells had similar responses to sub-lethal levels of puromycin, anisomycin, and paramomycin as wild type cells suggests that the structural integrity of the A-site and the decoding center is largely unchanged. Cycloheximide binds to the E-site of the eukaryotic ribosome and inhibits the peptidyl transferase activity of the large subunit (49). *hpm1* null cells displayed a significantly increased resistance to cycloheximide. Considering that the cycloheximide-binding site is more than 80 Å away from the methylated H243 site, loss of Hpm1p appears to have a long-ranging effect on the structural integrity of the ribosome. The precise binding site of verrucarin A is unclear; however, it is known to inhibit peptide chain initiation resulting in ribosome runoff (50). It is therefore possible that this drug binds near the A-site of the PTC or in the aa-tRNA accommodation corridor. The resistance of *hpm1Δ* to verrucarin A might reflect an alteration to these two functional centers that could compromise the ability of *hpm1Δ* ribosomes to discriminate between cognate and near-cognate aa-tRNAs. Chemical probing for rRNA structural changes will be needed to confirm these hypothesis.

To test the possibility that ribosome function is altered in Hpm1p-null cells, we analyzed translational fidelity *in vivo* by measuring the extent of amino acid misincorporation and stop codon readthrough. There was more than a 2-fold increase in the misincorporation of near-cognate aminoacyl tRNAs in *hpm1* null cells compared to wild type. Hpm1p-deficient cells also displayed increased stop codon suppression of the UAA and UGA codons. This suggests that ribosomes with unmodified Rpl3p have difficulty in discerning between cognate and near-cognate tRNAs, as well as a decrease in translation termination efficiency.

In yeast, histidine 243 is located in close proximity (<20 Å) to three 2'-*O*- methylated nucleotides (U1888, C2337, U2980) and one pseudouridine (U2880)(23, 51), ; the clustering of these modifications may be functionally relevant. H243 lies on the tryptophan finger domain of Rpl3p at the core of the 25S rRNA, more specifically on a positively-charged "basic thumb" that protrudes perpendicularly to the finger (52). The basic thumb has recently been shown to play a role in coordinating the processes occurring in the PTC, aminoacyl-tRNA binding region, and the elongation factor binding site to promote unidirectional translation (52). Mutations of various amino acids in the basic thumb result in growth defects, decreased translation fidelity and peptidyl transfer rates, decreased binding of aminoacyl-tRNAs and the elongation factor eEF2 (52). Interestingly, H243 flanking residues, R240 and R247, displayed more severe phenotypes than the other basic thumb mutants examined (52). It is thus possible that methylation of H243 could be playing a role in the functionality of this basic thumb.

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FIGURE LEGENDS

FIG 1. Yeast protein 3-methylhistidine is found primarily on a ribosomal protein corresponding to Rpl3p and is dependent on *HPM1*. (A) Total lysate, ribosomes, sub-cellular organelles, and cytosol were prepared from wild type (BY4742) and *hpm1Δ* cells (BY4742) for amino acid analysis as described in "Materials and Methods". The radioactivity shown has been normalized by background subtraction. Solid lines with the square symbol represent radioactivity in counts per minute (CPM)). Dashed lines with the x symbol represent ninhydrin absorbance at 570 nm to detect the 3-methylhistidine standard. (B) *In vivo* [^{3}H] radiolabeled ribosomes and sub-cellular organelles from wild type and *hpm1Δ* (40 µg of protein) were pretreated with 2 units of benzonase (Novagen 70746-4) for 30 min at 37°C and resolved by SDS-PAGE. ^{3}H-methylated proteins were detected by fluorography for 13 weeks at -80°C as described in "Materials and Methods". The arrow shows the methylated polypeptide corresponding to the molecular weight of Rpl3p and the asterisk shows the positions of Rps2 and Rps3, whose levels of methylation are altered in the absence of *HPM1.* Radiolabeled bands correlating to known methylated ribosomal proteins are indicated on the right hand side (14-22). (C) 7 OD units of wild type and *hpm1Δ* cells were radiolabeled with [^{3}H]AdoMet and the small 40S and large 60S ribosomal subunits were dissociated as described in "Materials and Methods". 100 µl of the cytosol, 40S, and 60S peak fractions were resolved by SDS-PAGE as described in "Materials and Methods", except a 12% Bis-Tris gel was prepared and run using MOPS running buffer. ^{3}H-methylated proteins were detected by fluorography for 10 weeks at -80°C as described in "Materials and Methods". The three panels represent portions of lanes from the same gel.

FIG 2. Hpm1p can methylate Rpl3p on intact ribosomes but not free Rpl3p. (A) 46 µg of crude ribosome protein isolated as described in "Materials and Methods" from wild type and *hpm1Δ* cells or 46 µg of recombinant Rpl3p was incubated with or without recombinant histidine-tagged Hpm1p (His-

Hpm1p; 12 µg) in the presence of [^{3}H]AdoMet (1 µM) and 100 mM sodium chloride, 100 mM sodium phosphate, pH 7.5 (methylation buffer) for 5 h at 30°C. Reactions were terminated by the addition of equal volume of 2X Laemmli sample buffer and proteins analyzed by SDS-PAGE/fluorography as described in "Materials and Methods". Film was incubated with the dried gel for 7 weeks at -80°C. The arrow indicates approximate position of recombinant His-Rpl3p. The radiolabeled band at about 20 kDa appears to reflect a bacterial contaminant in the His-Rpl3p preparation. Vertical lines show where non-relevant lanes were removed from the single gel. (B) 37 µg of crude ribosome protein from wild type and *hpm1Δ* were incubated with or without His-Hpm1p, as above. Proteins were trichloroacetic acid precipitated and acid hydrolyzed for amino acid analysis as described in "Materials and Methods" except that fractions eluting at 60-72 ml were collected and 900 µl of each fraction was counted for [^{3}H] radioactivity. Solid lines with symbols represent [^{3}H] radioactivity (counts per minute) and the dashed line with the "x" symbol represents ninhydrin absorbance of the 3-methylhistidine standard. (C) The synthetic peptide WGTKKLPRKTHRGLRK (Biosynthesis, Lewisville, TX) corresponding to the methylated region on Rpl3p was incubated with or without His-Hpm1p (30 µg) in the presence of 200 µM S-adenosyl-L-methionine p-toluenesulfonate (Sigma) and methylation buffer for 16 h at 30°C. Reactions were terminated with trifluoroacetic acid to a final concentration of 1% and fractionated by HPLC using a PLRPS reverse-phase column (Polymer Laboratories, Amherst, MA, pore size 300 Å, bead size 5 µm, 120 x 2 mm). The column was maintained at 50°C and initially equilibrated in 95% solvent A (0.1% TFA in water) and 5% solvent B (0.1% trifluoroacetic acid in acetonitrile) with a flow rate of 0.5 ml/min. The following program was used: 10 min 5% B, 25 min gradient to 60% B, 1 min gradient to 100% B, 5 min at 100%, 1 min gradient to 5% B, 8 min at 5% B. The column effluent was directed to the electrospray ion source of a QSTAR Elite (Applied Biosystems) mass spectrometer running in MS-only mode and was calibrated using external peptide standards. The left y-axis represents the counts in the absence of His-Hpm1p and the right y-axis is for the counts in the presence of His-Hpm1p.

FIG 3. Lack of *HPM1* results in deficiencies in large-subunit biogenesis and translation initiation. (A) Polysome profile analyses of wild type and *hpm1Δ* cells were done as described in "Materials and Methods". The solid lines are two independent profiles of wild type and the dashed lines are two independent profiles of *hpm1Δ*. (B) Polysome profile analysis of cold-stressed (15°C) cells was done as in panel A. (C) Total subunit analysis of wild type and *hpm1Δ* was done as in panel A above except that cells were not pretreated with cycloheximide, the lysis buffer was replaced with buffer C (50 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1 mM DTT) and 2 A260 units of extract was loaded. (D) Ribosomal subunit ratio quantification of large to small subunit for both the polysome profiles (Fig.3A) and total subunits (Fig. 3C). Peak areas were determined using Graphical Analysis 3.8.4 software. Error bars represent standard deviation of four independent experiments. Unpaired t-test two-tailed p-values for the differences in the subunit ratios were 0.004 for the polysome ratio and 0.057 for the dissociated subunit ratio.

FIG 4. Loss of *HPM1* results in early ribosomal RNA processing defects and delayed kinetics of pre-rRNA maturation. (A) The rRNA precursor processing pathway in *S. cerevisiae.* The 35S pre-rRNA encodes the 18S rRNA of the small ribosomal subunit and the 5.8S and 25S rRNAs of the large subunit. The predominant pathway for the processing of the 35S pre-rRNA transcript is shown on the left side and the abnormal pathway is shown on the right (dashed arrow). First, the 5' end of the 35S pre-rRNA is cleaved at sites A_0 and A_1, generating the mature 5' end of the 18S rRNA. Cleavage at the A_2 site separates the 20S and $27SA_2$ precursors. The 20S precursor is cleaved at site D in the cytoplasm to yield the mature 18S transcript. The 5' end of the $27SA_2$ precursor is cleaved at site A_3, followed by 5' to 3' exonuclease trimming to generate the mature 5' end of the 5.8S rRNA. C_2 cleavage separates the 5.8S and 25S precursors, which are then trimmed by exonucleases to yield the mature 5.8S and 25S rRNAs. (B) Northern blot analysis of rRNA precursor steady-state levels in WT and *hpm1Δ* (lanes 1 and 2). Lanes

3-8 represent deletions of various ribosomal protein methyltransferases. The location of the probes within each rRNA species is indicated to the right of each panel. The 25S and 18S panels are from the ethidium bromide fluorescence of the gel prior to transfer. (C) Top panel: Northern blot analysis of rRNA precursor steady-state levels using the 35S probe in WT, *hpm1Δ* and *rnt1Δ*. Bottom panel: 25S and 18S rRNA detected by ethidium bromide fluorescence of the gel prior to transfer. (D) Top panel: Pulse-chase with ^{3}H-uracil for analysis of pre-rRNA processing kinetics in WT and *hpm1Δ* cells. Cultures in early exponential growth phase were pulsed with tritiated uracil for 2 minutes, and then chased with an excess of non-isotopically labeled uracil. Bottom panel: The same membrane from the pulse-chase was analyzed by Northern blot analysis of 25S and 18S rRNA. The ratios of the 25S to 18S rRNA are normalized to the 1 min chase lane of WT.

FIG 5. Plasmids containing wild-type *HPM1* but not the human homolog *C1orf156* can rescue *hpm1Δ* defects in large subunit biogenesis and translational initiation. (A) *hpm1Δ* cells were transformed with the pUG35 plasmid containing the *HPM1* gene, its human homolog *C1orf156*, or active-site mutants of *HPM1* under the control of the Met25 promoter. As controls, wild type and *hpm1Δ* were transformed with empty pUG35 expressing only GFP. *hpm1(EK)* and *hpm1(VA)* genes have mutations in Motif 1 of the MT domain, involved in AdoMet binding; EIGCG to KIVCE (EK) and VEIG to AAAA (VA). Cells were grown overnight in SD (-ura-met) at 30°C in a rotary shaker. Cells were then diluted in 100 ml of fresh SD (-ura-met) to an OD_{600} of 0.01 and grown overnight at 30°C until cells reached an OD_{600} of 0.8-1.0. Polysome profile analysis was done as described in "Materials and Methods". *HPM1*, *C1orf156*, *hpm1(EK)* and *hpm1(VA)* genes were cloned into pUG35 with their stop codons to express the genes without the GFP tag. (B) Wild type and *hpm1Δ* cells containing the plasmids described in panel A were *in vivo* labeled with [^{3}H]AdoMet as described in "Materials and Methods" except that 3.5 OD_{600} units of cells were labeled for 1 h. Total lysates were prepared in buffer C and acid hydrolyzed for amino acid

analysis as described in "Materials and Methods" except that the column was kept at 35°C and that 900 µl of each fraction was taken for radioactivity determination. Solid lines with symbols represent [^{3}H] radioactivity (counts per minute) and the dashed line with the "x" symbol represents ninhydrin absorbance of the 3-methylhistidine standard. (C) Quantification of polysome profiles. Ratios were calculated by determining the areas of the 40S, 60S, monosome (80S) and polysome peaks using Graphical Analysis 3.8.4 software. Error bars represent standard deviation of two independent experiments. The differences seen in the subunit ratio and translational fitness were not statistically significant at the $p < 0.05$ level.

FIG 6. Lack of *HPM1* results in a greatly increased resistance to the ribosome-targeting drugs cycloheximide and verrucarin A. (A) Dilution spot assays were done on mid-log phase yeast cells (OD_{600} of 0.5) that were grown in YPD media at 30°C in a rotary shaker for 2 generations from an overnight culture grown in YPD at 30°C. Cells were serially diluted five-fold on YPD agar plates in the presence or absence of ribosome-targeting antibiotics: puromycin (50 µg/ml), cycloheximide (500 ng/ml), paramomycin (50 µg/ml), anisomycin (5 µg/ml), and verrucarin A (2 µg/ml) and incubated at 30°C for 2 days (control, puromycin, anisomycin, and paramomycin), 3 days (verrucarin A) or 5 days (cycloheximide). WT and *hpm1Δ* cells containing the empty pUG35 vector were also spotted on synthetic dextrose (SD) media lacking uracil and methionine for 3 days. (B) Northern blot analysis of mRNA expressed from the *PDR5* gene encoding the multidrug exporter *PDR5*. The 25S and 18S panels are from the ethidium bromide fluorescence of the gel prior to transfer.

FIG 7. Cells deficient of Hpm1p have increased misincorporation of amino acids and stop codon readthrough. (A) The dual-luciferase assay was done as described in "Materials and Methods". % amino acid misincorporation and stop codon readthrough were calculated by taking the (firefly/*Renilla*)

luminescence ratio divided by the same ratio of the respective control vector. Error bars represent the standard deviation of three independent experiments. The unpaired t-test two-tailed p-value is shown for the amino acid misincorporation panel. Differences in stop codon readthrough between wild type and *hpm1Δ* were not statistically significant at the $p < 0.05$ level.