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Brian D. Young
David Geffen School of Medicine at UCLA

David I. Weiss
David Geffen School of Medicine at UCLA

Cecilia I. Zurita-Lopez
David Geffen School of Medicine at UCLA

Kristofor J. Webb
David Geffen School of Medicine at UCLA

Steven G. Clarke
David Geffen School of Medicine at UCLA

See next page for additional authors

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Identification of Methylated Proteins in the Yeast Small Ribosomal Subunit: A Role for SPOUT Methyltransferases in Protein Arginine Methylation

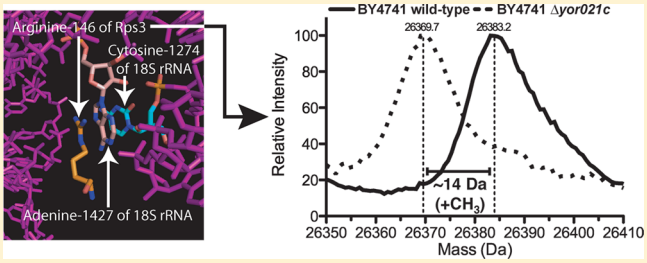
Brian D. Young,[§] David I. Weiss,[§] Cecilia I. Zurita-Lopez,[§] Kristofor J. Webb,[§] Steven G. Clarke,*[,§] and Anne E. McBride*[,§,‡]

[§]Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095, United States

[‡]Department of Biology, Bowdoin College, Brunswick, Maine 04011, United States

Supporting Information

ABSTRACT: We have characterized the posttranslational methylation of Rps2, Rps3, and Rps27a, three small ribosomal subunit proteins in the yeast *Saccharomyces cerevisiae*, using mass spectrometry and amino acid analysis. We found that Rps2 is substoichiometrically modified at arginine-10 by the Rmt1 methyltransferase. We demonstrated that Rps3 is stoichiometrically modified by ω-monomethylation at arginine-146 by mass spectrometric and site-directed mutagenic analyses. Substitution of alanine for arginine at position 146 is associated with slow cell growth, suggesting that the amino acid identity at this site may influence ribosomal function and/or biogenesis. Analysis of the three-dimensional structure of Rps3 in *S. cerevisiae* shows that arginine-146 makes contacts with the small subunit rRNA. Screening of deletion mutants encoding potential yeast methyltransferases revealed that the loss of the *YOR021C* gene results in the absence of methylation of Rps3. We demonstrated that recombinant Yor021c catalyzes ω-monomethylarginine formation when incubated with *S*-adenosylmethionine and hypomethylated ribosomes prepared from a *YOR021C* deletion strain. Interestingly, Yor021c belongs to the family of SPOUT methyltransferases that, to date, have only been shown to modify RNA substrates. Our findings suggest a wider role for SPOUT methyltransferases in nature. Finally, we have demonstrated the presence of a stoichiometrically methylated cysteine residue at position 39 of Rps27a in a zinc-cysteine cluster. The discovery of these three novel sites of protein modification within the small ribosomal subunit will now allow for an analysis of their functional roles in translation and possibly other cellular processes.



In nature, the library of stereochemical possibilities for macromolecules is vastly expanded by covalent modifications. Modifications of nucleic acids facilitate changes in gene expression and guide rRNA processing.[1,2] Protein function is also modulated by the posttranslational covalent modifications of amino acid residues.[3] These stereochemical changes can alter physical interactions within the protein and with other proteins, affecting signaling,[4,5] enzyme activity,[6] turnover,[7] and localization.[8] Families of enzymes have evolved to catalyze the formation and removal of these modifications, allowing precise control of a wide array of biochemical pathways. For proteins, methylation reactions, along with phosphorylation and acetylation reactions, represent major modification pathways.[9] Many amino acid side chains are known to be methylated in eukaryotes, including those of lysine,[10] arginine,[11,12] histidine,[13] glutamic acid,[14] and glutamine[15] residues. In addition, there can be methylation of the N- and C-termini of proteins.[16−18] Methylation of proteins is integral for epigenetics,[19] cellular signaling,[4,20] and other processes, including translation.[21]

We have been interested in exploring the methylation of proteins involved in translation and identifying novel enzymes catalyzing these modifications. In *Saccharomyces cerevisiae*, nearly 90 methyltransferases have been identified and predicted.[22,23] Nearly half of them modify components of the translational apparatus — rRNA, tRNAs, ribosomal proteins, and other proteins involved in translation. The majority of these enzymes belong to the seven β-strand methyltransferase family characterized by a series of four well-conserved motifs.[24] Different members of this family catalyze methylation of a wide range of substrates, including nucleic acids, proteins, small molecules, and lipids.[22,23] The other major subgroups of methyltransferases are the SET[25,26] and SPOUT[27,28] families, which, to date, have been shown to methylate proteins at lysine residues and RNAs, respectively. Our group has previously used bioinformatics to identify new putative members of these

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methyltransferase families.[22] Using a variety of proteomics approaches and amino acid analysis, we have been able to identify novel sites of protein methylation in ribosomes and have screened a library of single-gene deletion mutants lacking putative and uncharacterized protein methyltransferases to identify the enzymes responsible for these covalent modifications.

Our efforts have led to the identification of numerous modifications of ribosomal proteins in yeast and clarified the function of several previously uncharacterized methyltransferases. Mass spectrometric approaches allowed us to localize residues targeted by seven-β-strand family members Rkm5, Hpm1, and Ntm1 to lysine-46 in Rpl1ab,[29] histidine-243 in Rpl3, [13] and the N-terminal proline in Rpl12ab,[18] respectively. SET methyltransferases Rkm2, Rkm1, and Rkm3 and Rkm4 methylate specific lysines in Rpl12ab,[31] Rpl23ab,[30] and Rpl42ab,[31] respectively. In addition, a third methyltransferase, seven-β-strand family member Rmt2, modifies Rpl12ab at arginine-66.[32] Conversely, a single methyltransferase can modify multiple ribosomal targets: Ntm1 also methylates the N-terminal proline in the small ribosomal subunit protein Rps25ab.[18] A single member of each of these two methyltransferase families modifies translation elongation factor eEF1a, underscoring the ubiquity of translational machinery methylation.[33]

Here, we expand our understanding of protein methylation in the small ribosomal subunit, localizing several sites of methylation and identifying a previously uncharacterized SPOUT domain methyltransferase required for the methylation of a ribosomal protein. Using intact protein mass spectrometry and amino acid analysis, we observed the presence of ω-monomethylarginine at position 146 in Rps3, a ribosomal protein that may interact with the small ribosomal subunit export machinery.[34,35] We have explored the physiological relevance of this modification and have found that the amino acid identity of residue 146 affects cell growth. Analysis of this residue in a high-resolution ribosomal crystal structure has revealed that it contacts the 18S RNA, suggesting that its methylation might influence protein−rRNA interactions. In addition, through screening single-gene deletion mutants of known and putative methyltransferases, we showed that the *YOR021C* gene product is necessary for this modification. This finding is particularly noteworthy because Yor021c is a candidate SPOUT methyltransferase. To date, other methyltransferases in the SPOUT family have only been shown to methylate RNAs.[27,28,36−39] Our results suggest that SPOUT methyltransferases have a wider role than previously imagined and that other uncharacterized SPOUT enzymes might also be important for modification of proteins. In addition, our proteomics approaches have yielded the site of arginine methylation within Rps2 and the location of a previously uncharacterized methylcysteine residue in Rps27a. Coupled with the recent high-resolution structure of the yeast ribosome,[40] our studies identifying and localizing sites of posttranslational modifications in the small ribosomal subunit enhance our understanding of how protein methylation and ribosome structure and function are related.

EXPERIMENTAL PROCEDURES

Strains and Construction of RPS3 Mutants. Wild-type and methyltransferase deletion strains (in BY4741 and BY4742 backgrounds) were obtained from Open Biosystems (Huntsville, AL) and cultured in 1% yeast extract, 2% peptone, and 2% dextrose (YPD medium). Strains expressing Rps3 with various amino acid substitutions at arginine-143 and arginine-146 were constructed by site-directed mutagenesis. Plasmid K612 (*pGal RPS3 LEU2 CEN*[41]) was mutagenized using the PAGE-purified oligonucleotides (ValueGene, San Diego, CA) listed in Supplemental Table 1, Supporting Information by QuikChange II XL Site-Directed Mutagenesis (Stratagene) according to the manufacturer's protocol with an 8-min extension at 68 °C. The open reading frame was sequenced (GENEWIZ, La Jolla, CA) with oligonucleotides RPS3 (forward) and RPS3 (reverse) (ValueGene), confirming the presence of the expected mutations and no additional mutations. Wild-type and mutant plasmids were transformed into Y268 (*rps3Δ::kanMX4 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0*+Ycplac33-*RPS3*[41]) and selected on synthetic complete medium with 2% glucose lacking leucine and uracil (SCGlu-Leu-Ura). Transformants were grown for 5.5 h in SCGlu-Leu-Ura and then diluted to an optical density at 600 nm of 0.05 in synthetic complete medium with 2% raffinose and 1% galactose lacking leucine (SCGalRaf-Leu) to allow loss of the *URA3*-containing *Ycplac33-RPS3* plasmid. After overnight growth, cells were plated on SCGalRaf-Leu with 5-fluoroorotic acid to select against the *Ycplac33-RPS3* plasmid. Resulting strains bearing the wild-type and mutant *pGal-RPS3* plasmids as the sole copy of *RPS3* were cultured in rich medium containing 1% yeast extract, 2% peptone, 1% galactose, and 2% raffinose (YPGalRaf). To assess the effect of these mutations on growth, overnight cultures were grown in YPGalRaf, cells were counted, and serial dilutions were plated on YPGalRaf agar plates and grown for 2 days at 30 °C.

Isolation of Ribosomes and Ribosomal Proteins from *S. cerevisiae*. Yeast strains were cultured to midlog phase in either 1 L YPD medium or YPGalRaf growth medium at 30 °C before centrifugation at 5000*g* for 5 min at 4 °C. Cells were resuspended in 8 mL of 20 mM Tris-HCl, 15 mM magnesium acetate, 60 mM potassium chloride, 1 mM dithiothreitol, pH 7.4, containing one Complete protease inhibitor cocktail tablet (Roche #11836145001) per 50 mL. After addition of 3 g of 0.5-mm glass beads, the cells were lysed at 4 °C with 3 cycles of 3 min of rigorous vortexing followed by 3 min on ice. After centrifugation for 5 min at 12000*g* at 4 °C, the supernatant was spun again at 4 °C for 25 min at 20000*g*. Finally, ribosomes were isolated by ultracentrifugation at 103000*g* for 2 h at 4 °C.

Ribosomal proteins were isolated using an adaptation of the procedure described in Porras-Yakushi et al.[30] The precipitated ribosomes were resuspended in 900 μL of 50 mM Tris-HCl, 5 mM magnesium acetate, 750 mM potassium chloride, 1 mM dithiothreitol, pH 7.4, containing one Roche Complete protease inhibitor tablet per 50 mL (separation buffer). The samples were then loaded over 11-layer sucrose gradients, ranging from 7 to 27% sucrose (w/v) in separation buffer, and spun at 49400*g* for 16 h at 4 °C in a Beckman SW41 swinging bucket rotor. Gradient fractions were analyzed for absorbance at 260 nm to identify the large and small ribosomal subunits. The ribosomal proteins were then extracted by adding 507 μL 37 mM magnesium acetate in ethanol to 750 μL of fractions and freezing the mixture for 24 h at −20 °C. After centrifugation at 20800*g* at 4 °C for 20 min, the pellet was resuspended in 100 μL of H_2O, and 200 μL of glacial acetic acid and 10 μL of 1 M magnesium chloride were added. After vortexing at −20 °C for 45 min, the samples were spun at 20800*g* at 4 °C for 10 min, and the supernatants were concentrated by vacuum centrifugation.

Expression and Purification of Recombinant *YOR021C* Gene Product. Genomic DNA from *S. cerevisiae* was extracted from a 10-mL culture grown to saturation. The *YOR021C* open reading frame was amplified by PCR with a forward primer 5′-CACCATGAAGTACATTATTGAGCATATGG and a reverse primer 5′-CTACATCAACAGATCGTCCAAAC. The products were fractionated on a 1.1% agarose gel at 120 V for 60 min. The band containing the *YOR021C* open reading frame was cut from the gel and the DNA was purified using a QIAquick Gel Extraction Kit (QIAGEN, catalog no. 28704). Using the Champion pET100 Directional TOPO Expression Kit (Invitrogen), the DNA was inserted into a pET100/D-TOPO vector encoding a His-tagged N-terminal linker sequence MRGSHHHHHHGMASMTGGQQMGRDLYD-DDDKDHPFT that is followed by the complete sequence of the *YOR021C* open reading frame, including the initiator methionine residue, that is regulated by the T7 promoter. TOP10 chemically competent *Escherichia coli* cells were then transformed with the plasmid and plated onto LB plates containing 100 μg/mL ampicillin to select for colonies containing the plasmid. Plasmid DNA was sequenced on both strands to ensure proper insertion of the *YOR021C* gene without mutation (GENEWIZ, La Jolla, CA). Per the protocol of the Champion pET100 Directional TOPO Expression kit, the plasmid was used to transform competent BL21 Star (DE3) *E. coli* cells. These cells were then grown at 37 °C to an optical density of 0.5 at 600 nm in 1 L of LB containing 100 μg/mL ampicillin. At this time, isopropyl-ß-D-thiogalactopyranoside was added to give a concentration of 0.4 mM and the incubation continued for 18 h at 18 °C. Cells were pelleted for 5 min at 5000*g* at 4 °C, washed in 10 mL of water, and then resuspended in 30 mL of 50 mM sodium phosphate, pH 8.0, 500 mM sodium chloride, and 5% glycerol along with 1 Complete protease inhibitor tablet (Roche Diagnostics) and 11 μL of β-mercaptoethanol. Using an Emulsiflex C-3 emulsifier (Avestin), the cells were lysed and the supernatant was recovered after centrifugation at 20000*g* for 15 min at 4 °C. The supernatant was applied onto a 5-mL HisTrap HP nickel affinity column (GE Healthcare, catalog no. 17-5248-01) and the recombinant Yor021c protein was recovered using a gradient of 5−500 mM imidazole. The final protein preparation (2.6 mg/mL), in a buffer of 50 mM sodium phosphate, 260 mM imidazole chloride, 300 mM sodium chloride, 5% glycerol, pH 8.0, was stored at −20 °C.

In Vivo Labeling of Ribosomal Proteins from *S. cerevisiae* and Analysis by High-Resolution Cation-Exchange Chromatography. BY4742 wild-type ribosomes were labeled in vivo by incubating intact cells with *S*-adenosyl-L-[*methyl*-^{3}H]-L-methionine. Cells were grown in 500 mL of YPD medium to an optical density at 600 nm of 0.6, pelleted at 5000*g* for 5 min, and washed twice in water. Cells were then resuspended in 304 μL of *S*-adenosyl-L-[*methyl*-^{3}H]-L-methionine (PerkinElmer, 75−85 Ci/mmol, 0.55 mCi/mL in 10 mM H_2SO_4/ethanol (9:1, v/v)) and incubated in 40 mL of YPD medium in a rotary shaker for 30 min at 30 °C. Labeled cells were pelleted, washed, and stored at −80 °C until lysis and isolation of the protein fraction of the ribosomal small subunit as described above. Rps3 was purified by HPLC and acid hydrolyzed as described previously.[13] Purified Rps3 was placed in a 6 × 50-mm glass vial and dried by vacuum centrifugation. The protein was acid hydrolyzed by addition of 50 μL of 6 M HCl to the vial and 200 μL of 6 M HCl into the reaction chamber (Eldex Laboratories, catalog no. 1163). The vial was heated for 20 h in vacuo at 110 °C using a Pico-Tag Vapor-Phase apparatus (Waters). After 20 h, residual HCl was removed by vacuum centrifugation. The hydrolyzed amino acids were resuspended in 50 μL of H_2O and 500 μL of 0.2 M sodium citrate buffer, pH 2.2. This material was loaded onto a cation-exchange chromatography column (0.9-cm inner diameter × 10-cm column length; PA-35 sulfonated polystyrene beads; 6−12 μm, Benson Co.) along with 1.0 μmol of the following standards: *N*-ε-monomethyllysine hydrochloride (MMK, Bachem, #E-2155); 1-methyl-L-histidine (1-Me-His, Sigma, #M-9005); asymmetric N^G,N^G-dimethylarginine hydrochloride (ADMA, Sigma, #D4268); symmetric N^G, $N^{G\prime}$-dimethyl-L-arginine di(p-hydroxyazobenzene-*p*′-sulfonate (SDMA, Sigma, #D0390) and N^G-methyl-L-arginine acetate salt (MMA, Sigma, #M7033). The amino acids were eluted at 55 °C with 0.35 M sodium citrate buffer, pH 5.27 at 1 mL/min and 1-min fractions were collected. The column was regenerated with 0.2 N NaOH for 25 min and equilibrated with 0.35 M sodium citrate buffer, pH 5.27 for 25 min prior to each run. The eluted standards were identified via a ninhydrin assay. Briefly, 30 μL of each column fraction was mixed with 200 μL of water and 100 μL of a solution of 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 in a 96-well clear flat bottom plate. The plates were then heated in a 100 °C oven for 10 min, and the absorbance at 570 nm was read in a Molecular Devices SpectraMax M5 plate reader (0.9-cm path length). Radioactivity was measured by mixing 970 μL of sample with 400 μL of H_2O and 5 mL of fluor (Safety Solve, Research Products International, catalog no. 111177). Samples were counted on a Beckman LS6500 instrument three times for 5 min.

Localization of the Rps3 Methylation Site by Mass Spectrometry Analysis of Cyanogen Bromide Cleavage Products. Purified ribosomal proteins of the small subunit were isolated from wild-type BY4742, as detailed above, and Rps3 was purified by HPLC and digested with cyanogen bromide as described previously.[13] Briefly, 20 mg of cyanogen bromide was added to purified protein in 70 μL of H_2O/acetonitrile/trifluoroacetic acid (1:1:0.05) such that the molar ratio of cyanogen bromide to methionine residues was approximately 1000:1. After an overnight incubation in the absence of light, the digest was infused into a hybrid linear ion trap/FTICR mass spectrometer (LTQ FT Ultra, Thermo Scientific, San Jose, CA) operating in MS-only mode. The Xtract algorithm in the Xcalibur software suite (Thermo Scientific) was used to deconvolute the resulting spectra and calculate the uncharged monoisotopic masses.

Intact Protein Mass Spectrometry Analysis of Isolated Ribosomal Proteins. The extracted ribosomal proteins from the small ribosomal subunit were analyzed by liquid chromatography−mass spectrometry using an electrospray QSTAR Elite time-of-flight mass spectrometer (Applied Biosystems) calibrated with peptide standards. For the wild-type and mutant BY4742 strains, the chromatography and mass spectrometry method used has been described previously.[13] For the other strains, a slightly modified chromatography method was used. Briefly, 10 μL of the samples was mixed with 30 μL of H_2O and injected onto a PLRP-S column (150 mm × 1 mm, 5-μm particle size, 300-Å pore size) (Polymer Laboratories, Amherst, MA). Mobile phase A contained 2% acetonitrile and 0.1% formic acid while mobile phase B contained 98% acetonitrile and 0.1% formic acid. The following gradient was

used with a flow rate of 50 μL/min: t = 0−3 min, 5% B; t = 13 min, 25% B; t = 38 min, 62.5% B; t = 38.1−49 min, 95% B; t = 49.1−60 min; 5% B. Spectra were reconstructed using MagTran 1.03.[42]

Sample Preparation of In Vitro Methylation Reactions for Cation-Exchange Chromatography. Reaction mixtures (300 μL) were transferred to 6 × 50-mm glass vials and proteins were precipitated by incubation with an equal volume of 25% (wt/vol) trichloroacetic acid for 1 h at room temperature. The protein pellet from centrifugation at 4000g for 1 h at room temperature was washed with 200 μL of acetone at 0 °C. Acid hydrolysis of the pellet was performed as described above.

Localization of the Rps27a Methylation Site by Top-Down Mass Spectrometry using Collisionally Activated Dissociation and Electron-Capture Dissociation. Rps27a protein isolated and purified by HPLC from wild-type BY4742 was infused into the hybrid linear ion trap/FTICR mass spectrometer and fragmented using collisionally activated dissociation and electron-capture dissociation as described previously.[13] The spectra were deconvoluted, as described above, with the Xtract algorithm. The resulting uncharged monoisotopic masses were analyzed with the ProSight PTM online tool using the Rps27a and Rps27b protein sequences and a mass error range of 5.5 ppm.

Localization of the Rps2 Methylation Site by Bottom-Up Mass Spectrometry. Rps2 protein isolated and purified by HPLC from wild-type strain BY4742 was digested with chymotrypsin (Roche) and analyzed by liquid chromatography-tandem mass spectrometry as described previously.[13] Briefly, the protein was loaded onto a Biobasic C18 column (3.5 cm × 100 μm, 5-μm particle size, 300-Å pore size) (Microtech Scientific, Fontana, CA). The peptides were loaded at 5 μL/min and were eluted at 300 nL/min with the following gradient: t = 0, 2% B; t = 40 min, 60% B; t = 50 min, 80% B. Mobile phase A contained 1% acetonitrile and 0.1% formic acid while mobile phase B contained 100% acetonitrile and 0.1% formic acid. The eluted peptides were directed into the hybrid linear ion trap/FTICR mass spectrometer, which was operated in data-dependent mode with a full high-resolution scan (100 000 resolution at m/z= 400) followed by six MS/MS experiments using the low-resolution linear trap and collisionally activated dissociation. For the MS/MS experiments, the intensity threshold was 5000 and the m/z range was 300−2000. The data were processed using Mascot (Matrix Science, UK).

RESULTS

Rps3 Is Monomethylated at Arginine-146. Rps3 is an essential protein in *S. cerevisiae* with roles in ribosome biogenesis in the transport of the small subunit into the cytoplasm[41] and in translational fidelity.[43] In addition, physical interactions implicate Rps3 in translational initiation,[44−46] and recombinant Rps3 demonstrates endonuclease activity against abasic DNA.[47] Rps3 has been described as a target of methylation reactions in yeast[48] and higher organisms.[49] To better understand the functional role and nature of this posttranslational modification in *S. cerevisiae*, we labeled wild-type yeast cells with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. After isolation of ribosomes and extraction of ribosomal proteins of the small subunit, we acid hydrolyzed HPLC-purified Rps3 and separated the free amino acids by high-resolution cation-exchange chromatography alongside standards of methylated amino acids (Figure 1A). The radioactivity in the purified

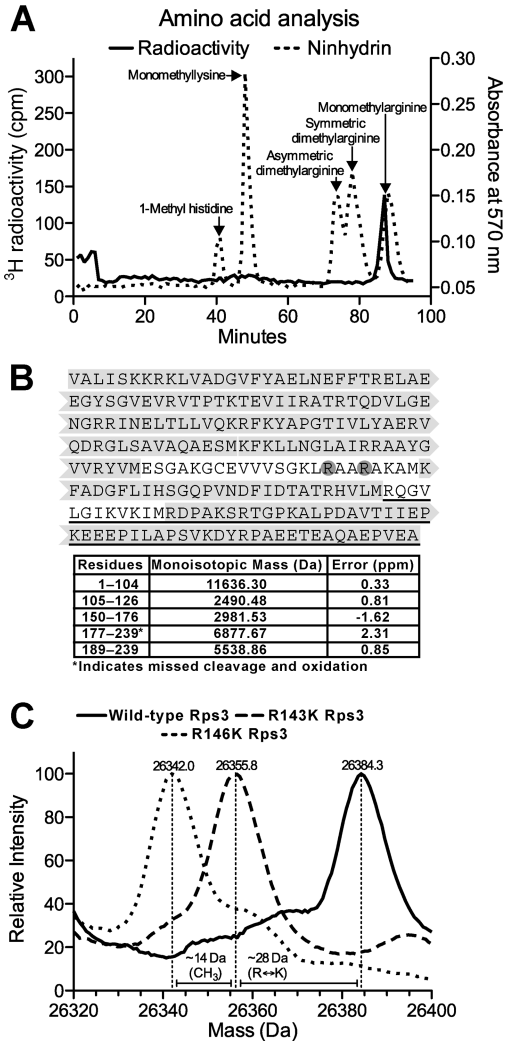

Figure 1. Rps3 is monomethylated at arginine-146. (A) HPLC-purified Rps3 from wild-type BY4742 cells grown in the presence of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine was acid hydrolyzed and the free amino acids were mixed with methylated standards and fractionated using high-resolution cation-exchange chromatography as described in the Experimental Procedures section. The ^{3}H-radioactivity in the resulting fractions was quantified with a liquid scintillation counter and is marked by a solid line; the position of the added methylated amino acid standards detected with a ninhydrin assay are shown by the dotted line. ^{3}H-radioactivity in Rps3 coelutes just prior to the standard of ω-monomethylarginine, as expected for the tritium-labeled species.[79] (B) Unlabeled HPLC-purified Rps3 was isolated from wild-type BY4742 cells and digested with cyanogen bromide prior to analysis of the resulting cleavage products with a hybrid linear ion trap/FTICR mass spectrometer. The spectra were deconvoluted and the resulting monoisotopic masses were searched against those predicted for Rps3 cyanogen bromide cleavage products. Matching unmodified peptides are boxed in light gray, while matching peptides containing one oxidation and one missed cleavage are underlined. Peptide matches are shown with the error. Matching unmethylated cleavage products were identified for all regions of Rps3 except between residues 127 and 149. The region spanning residues 127 and 149 contains the two arginine residues, arginine-143 and arginine-146, marked with gray circles. A detailed description of our mass spectrometry methods is given in the Experimental Procedures section. (C) Ribosomal proteins of the small subunit were isolated and analyzed by liquid chromatography−mass spectrometry from yeast strains expressing wild-type Rps3 and Rps3 containing arginine to lysine substitutions at residues 143 and 146, labeled as R143K Rps3 and R146K Rps3, respectively. Reconstructed

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Figure 1. continued

spectra of Rps3 in each of these strains are shown. The R143K Rps3 strain has a mass for Rps3 that is 28 Da lower than the wild-type, which is consistent with an arginine to lysine substitution. The R146K Rps3 strain, however, has a mass for Rps3 that is 42 Da lower than the wild-type, indicating an arginine to lysine substitution and a loss of methylation. The generation of these strains is detailed in the Experimental Procedures section.

protein eluted in the expected position of ω-monomethylarginine and had a retention profile distinct from 1-methyl histidine, monomethyllysine, asymmetric dimethylarginine, and symmetric dimethylarginine. These results revealed that Rps3 in *S. cerevisiae* contains an ω-monomethylated arginine residue.

To identify the site of arginine methylation within the protein, we analyzed cyanogen bromide cleavage products of HPLC-purified Rps3 by high-resolution mass spectrometry. We were able to detect all of the unmodified cyanogen bromide cleavage products of Rps3 with the exception of the peptides spanning residues 127−149 and 177−188 (Figure 1B). When we expanded our search to include peptides containing missed cleavages and oxidations, we found an unmethylated singly oxidized peptide containing residues 177−239. Taken together, these results suggested that the site of arginine methylation is between positions 127 and 149.

To further localize the site of monomethylation in Rps3, we used site-directed mutagenesis, constructing strains expressing wild-type and mutant Rps3 with arginine to lysine amino acid substitutions at arginine-143 and arginine-146, the two arginine residues present in the polypeptide between residues 127 and 149. We isolated ribosomes from these strains, purified proteins of the small subunit by sucrose density centrifugation, and analyzed the extracted proteins by liquid chromatography−intact protein mass spectrometry (Figure 1C). In the strain expressing wild-type Rps3 under a *GAL* promoter, we identified a 26384.3-Da species, consistent with the mass of monomethylated Rps3 after the previously described loss of the initiator methionine residue (error = 45 ppm).[50,51] Significantly, the unmethylated protein is not observed; we detected no ions corresponding to a mass of 26370 Da in two independent experiments. In the strain expressing Rps3 with an arginine to lysine substitution at position 143, we identified a 26355.8-Da species, which is consistent with the mass expected for monomethylated Rps3 with an arginine to lysine amino acid substitution (error = 64 ppm), indicating that arginine-143 is not the site of methylation. For a similar mutant strain with the arginine to lysine substitution at position 146, however, a 26342.0-Da species was observed. This mass is consistent with that of unmethylated Rps3 with the arginine to lysine substitution (error = 56 ppm), revealing that arginine-146 is the site of monomethylation in *S. cerevisiae*. In two replicate intact mass determinations, we did not detect ions corresponding to a methylated species of 26356 Da. Collectively, our amino acid analysis of Rps3, mass spectrometry analyses of cyanogen bromide digests, and mass spectrometry of intact wild-type and mutant Rps3 indicate that Rps3 is stoichiometrically ω-monomethylated at arginine-146, although minor amounts of unmethylated Rps3 species may have escaped detection.

Arginine-146 in Rps3 Interacts with the 18S rRNA in Crystal Structures of the Small Ribosomal Subunit. Knowing the site of methylation in Rps3, we wanted to understand the physiological significance of this posttranslational modification. Accordingly, we explored the high-resolution crystal structure of the yeast ribosome (PDB IDs: 3U5B, 3U5C, 3U5F, 3U5G).[40] Rps3 arginine-146 is on the exterior of the protein and its side chain does not interact with other amino acids within Rps3 (Figure 2A). In addition, this arginine residue does not form any close contacts with other ribosomal proteins (Figure 2B). This arginine residue, however, does interact with adenine-1427 in the 18S rRNA, which appears to also form contacts with cytosine-1274 (Figure 2C,D). The nitrogen atoms on the side chain of arginine-146 are within 4 Å of several of the nitrogen atoms of adenine-1427, indicating that methylation of the arginine residue might influence this protein−rRNA interaction. In addition, adenine-1427 also forms close contacts with cytosine-1274, suggesting the possibility that methylation of arginine-146 in *S. cerevisiae* might affect both intra-rRNA interactions as well as protein−rRNA interactions.

The Amino Acid Identity of Rps3 at Position 146 Affects Optimal Growth. Our analysis of high-resolution crystal structures of the small ribosomal subunit suggested that arginine-146 in Rps3 in *S. cerevisiae* might be involved in forming contacts with the 18S rRNA and thereby guiding interactions within the rRNA to optimize ribosome function. To evaluate this possibility, we constructed additional mutant strains expressing Rps3 under a *GAL* promoter with amino acid substitutions at positions 143 and 146. After growth in liquid medium, serial dilutions of equal numbers of cells were plated on YPGalRaf agar plates. After 2 days of growth at 30 °C, the strains expressing wild-type Rps3 and all mutant strains, except the mutant expressing Rps3 with an arginine to alanine substitution at position 146, grew equally well (Figure 3). Lysine substitution at positions 143 and 146 did not severely affect growth, although the expression of Rps3 from a strong galactose promoter in this assay might mask subtle growth effects caused by the small stereochemical changes that accompany the conservative arginine to lysine mutation. When the arginine residues were mutated to alanine residues, however, the strain with the mutation at residue 146 had a marked reduction in growth, while the mutation at residue 143 did not have an effect compared to the wild-type. Taken together, these results indicate that the amino acid identity at position 146 in Rps3 influences growth and fitness.

Arginine Methylation of Rps3 Is Not Mediated by Known Protein Arginine Methyltransferases. Given the role of the arginine residue at position 146 in fitness, we wanted to identify the enzyme catalyzing its modification. In *S. cerevisiae*, there are three enzymes known to methylate arginine residues: Rmt1, Rmt2, and Hsl7.[22,23] While Rmt1 methylates Rps2[52] and Rmt2 methylates arginine-66 in Rpl12ab[32] in *S. cerevisiae*, PRMT1, the mammalian homologue of Rmt1, methylates human Rps3 at multiple arginine residues within the KH RNA-binding domain.[49] To determine whether one of the three known arginine methyltransferases is involved in the posttranslational processing of yeast Rps3, we extracted small ribosomal proteins from single-gene deletion mutants of these enzymes and analyzed them by liquid chromatography-intact protein mass spectrometry (Figure 4A). In each of these deletion mutants and the wild-type strain, Rps3 appeared to be fully modified, indicating that Rps3 was not a substrate of any these enzymes.

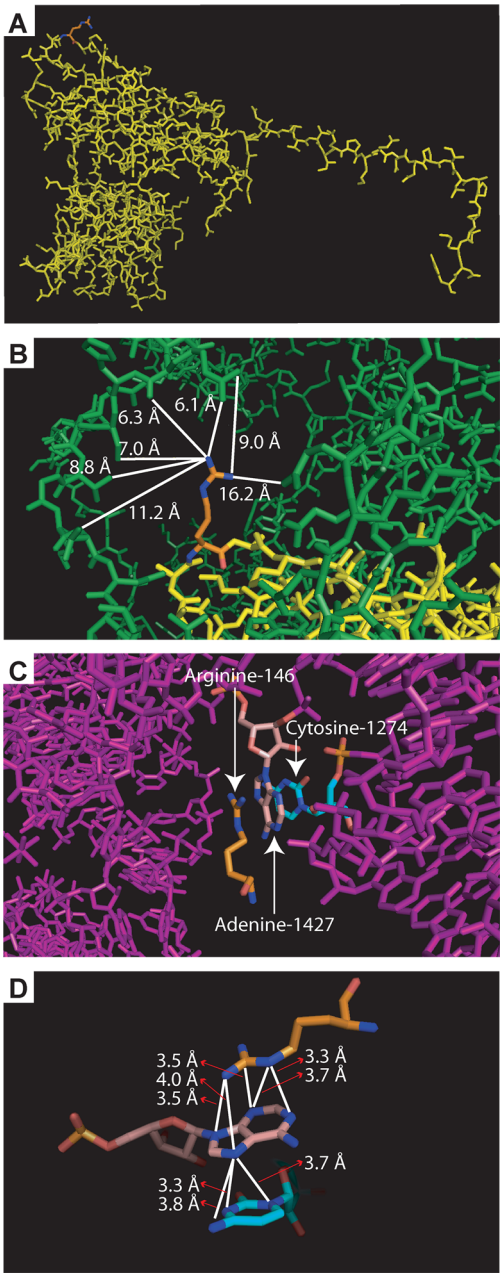

Figure 2. Arginine-146 in Rps3 likely forms close contacts with the 18S rRNA. The crystal structure of the small ribosomal subunit of *S. cerevisiae* (PDB IDs: 3U5B, 3U5C, 3U5F, 3U5G) [40] is explored to better understand the role of arginine-146 methylation in Rps3 from *S. cerevisiae*. (A) The structure of Rps3 alone is shown in yellow and arginine-146 is marked in orange for carbon atoms, red for oxygen atoms, and blue for nitrogen atoms. (B) The structure of Rps3 and arginine-146 (colored as in panel A) in the presence of other small ribosomal proteins (colored in green). Distances from arginine-146 in Rps3 to other nearby ribosomal proteins are shown. (C) Arginine-146 from Rps3 (colored as in panel A) and the 18S rRNA (purple) are shown. Adenine-1427, which has close interactions with arginine-146, is shown with carbon atoms in pink and nitrogen atoms in blue, and cytosine-1274, which interacts closely with adenine-1427, is shown with carbon atoms in teal, oxygen atoms in red, and nitrogen atoms in blue. (D) The distances between these bases and arginine-146 are indicated.

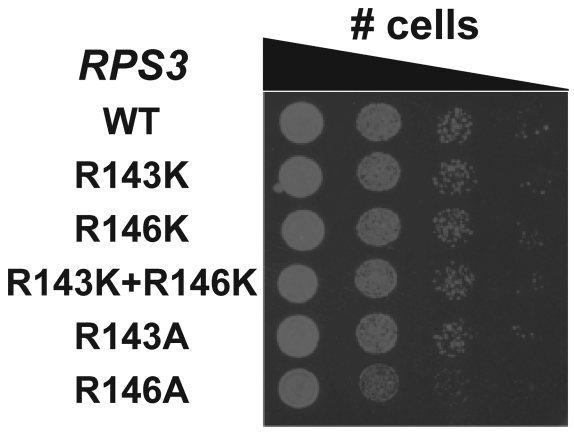

Figure 3. The amino acid identity at residue 146 in Rps3 is critical for optimal growth. Yeast strains expressing wild-type Rps3 and Rps3 containing amino acid substitutions at residues 143 and 146 were assayed for growth fitness. Serial dilutions of equal numbers of cells were plated and grown at 30 °C as described in the Experimental Procedures section. R143K, arginine to lysine substitution at position 143; R146K, arginine to lysine substitution at position 146; R143K + R146K, arginine to lysine substitutions at positions 143 and 146; R143A, arginine to alanine substitution at position 143; and R146A, arginine to alanine substitution at position 146.

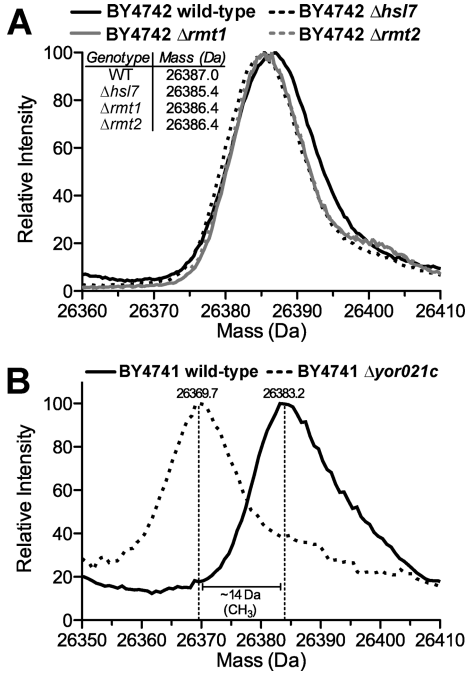

Figure 4. Rps3 methylation is dependent on a SPOUT methyltransferase. Ribosomal proteins of the small subunit were isolated from wild-type and single-gene deletion strains and analyzed by liquid chromatography–mass spectrometry as described in the Experimental Procedures section. (A) Reconstructed spectra of Rps3 in BY4742 wild-type yeast and BY4742 yeast strains lacking Rmt1, Rmt2, and Hsl7 (labeled as BY4742 Δ*rmt1*, BY4742 Δ*rmt2*, BY4742 Δ*hsl7*, respectively) are shown. (B) Reconstructed spectra of Rps3 in BY4741 wild-type yeast and a BY4741 strain lacking Yor021c (BY4741 Δ*yor021c*) are shown. Rps3 in BY4741 Δ*yor021c* has a mass that is approximately 14 Da lower than the wild-type strain, indicating a loss of methylation.

Arginine Methylation of Rps3 Is Catalyzed by the Yor021c SPOUT Methyltransferase. To identify the methyltransferase modifying Rps3, we screened the collection of single-gene deletion mutants of seven β-strand methyl-

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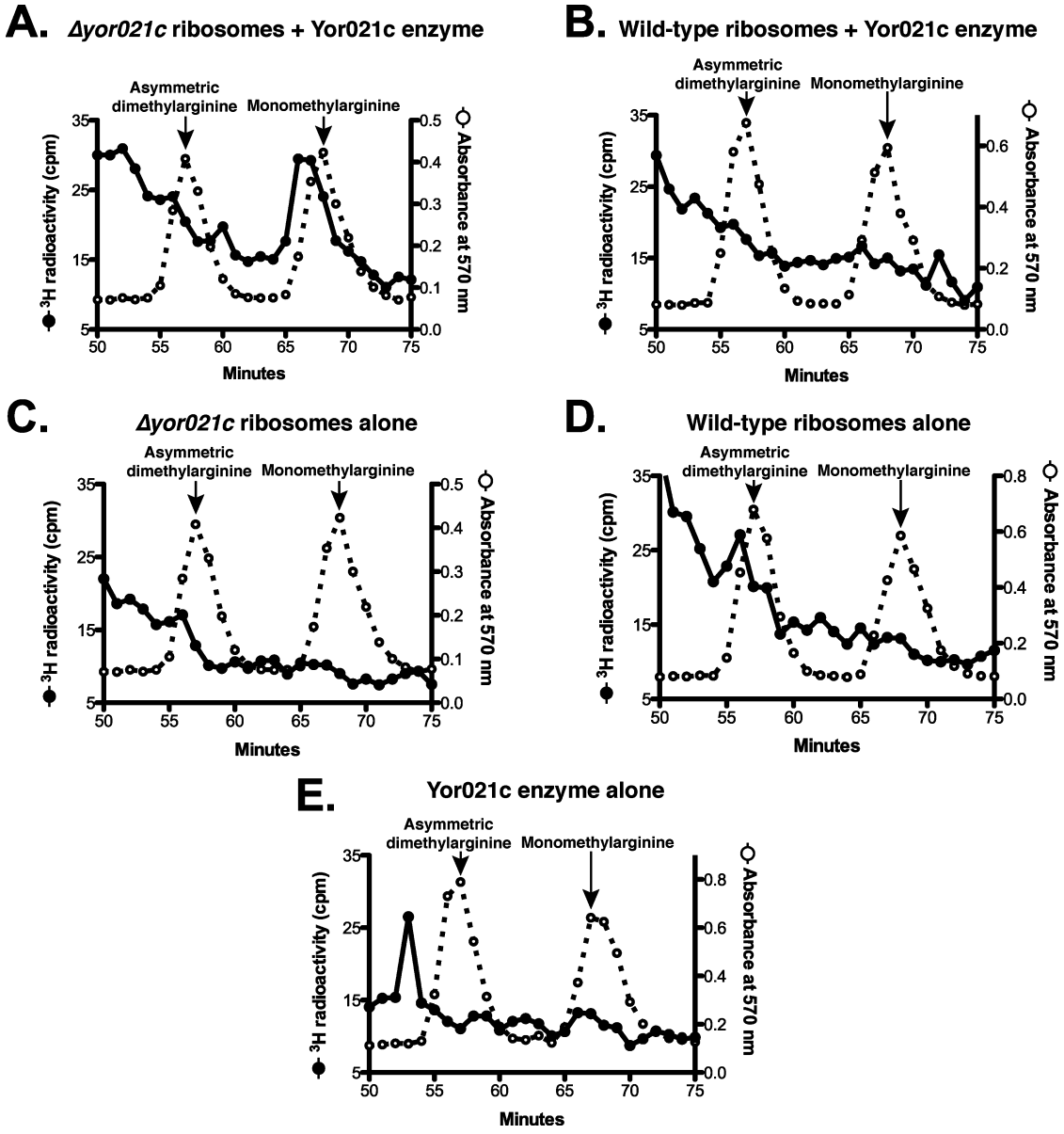

Figure 5. In vitro methyltransferase activity of recombinant Yor021c protein. (A) Yor021c was expressed as a His-tagged enzyme in *E. coli* as described in the Experimental Procedures section. This protein (40 μg) was incubated with ribosomes prepared from a yeast Δ*yor021c* strain in a BY4742 background (200 μg protein) with 1 μM *S*-adenosyl-L-[*methyl*-^{3}H]-L-methionine (75−85 Ci/mmol, from the stock described in the Experimental Procedures section) for 20 h at 30 °C in a buffer of 100 mM sodium phosphate, 100 mM NaCl, pH 7.0 in a final volume of 300 μL. After protein precipitation and acid hydrolysis as described in Experimental Procedures, 1 μmol of asymmetric N^G,N^G-dimethylarginine and 1 μmol of N^G-monomethylarginine were added as standards and high-resolution cation-exchange chromatography was performed as described in Experimental Procedures but with a 8-cm column. Radioactivity (closed circles, solid lines) was measured in 500 μL of each column fraction with 5 mL of Safety Solve fluor. Amino acid standards were detected by mixing 50 μL of each fraction with 100 μL of ninhydrin reagent as described in the Experimental Procedures section (path length = 0.4 cm) (open circles, dashed lines). Control reactions (B−E) were prepared and analyzed in a similar manner. (B) Wild-type ribosomes (strain BY4742) were substituted for the Δ*yor021c* ribosomes. (C) Recombinant Yor021c was absent from the reaction mixture. (D) Wild-type ribosomes (strain BY4742) were substituted for the Δ*yor021c* ribosomes and Yor021c was absent from the reaction mixture. (E) No ribosomes were included in the reaction mixture with the Yor021c enzyme. In panel (A), a peak of radioactivity elutes just prior to the standard of ω-monomethylarginine in the position expected for the tritium-labeled species.[79] This peak is absent in all of the controls (panels B−E).

transferases described previously[13] for loss of Rps3 methylation. None of the deletions tested appeared to affect the modification state of Rps3 (data not shown). A previous study showed that Yar1, an ankyrin-repeat protein, interacts physically with Rps3 and Yor021c through a two-hybrid screen.[35] Yor021c has been identified as a putative SPOUT family methyltransferase through bioinformatics analysis.[22,28] We isolated ribosomal proteins of the small ribosomal subunit from a deletion strain lacking this enzyme and wild-type strains (both strains in the BY4741 and BY4742 backgrounds, although the latter is not shown) (Figure 4B). Significantly, we detected no Rps3 methylation in the absence of Yor021c. We also tested deletion strains of two other uncharacterized putative SPOUT methyltransferases, *YGR283C* and *YMR310C*. Neither of them, however, was required for Rps3 modification (data not

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Table 1. SPOUT Methyltransferases in *S. cerevisiae* and Other Organisms

ORF	name	substrate[a]	methylation product	orthologs[a]					
YOR201C	Mrm1/Pet56	mitochondrial 21S rRNA[39]	2′-*O*-methylguanosine	M	F	P	OE	B	
YDL112W	Trm3	tRNA[36]	2′-*O*-methylguanosine-18	M	F	P	OE	B	
YLR186W	Emg1/Nep1	18S rRNA[38]	N^1-methyl pseudouridine-1191	M	F	P	OE		A
YOL093W	Trm10	tRNA[37]	N^1-methyl guanosine-9	M	F	P	OE		A
YOR021C		Rps3 (this work)	ω-monomethylarginine-146		F		OE		A
YGR283C		unknown	unknown	M	F	P	OE		A
YMR310C		unknown	unknown	M	F	P	OE		A

[a]M = metazoa, F = fungi, P = plants, OE = other eukaryotes, A = archea, B = bacteria.[28]

shown). These results indicate that Yor021c likely catalyzes the monomethylation of arginine-146 in Rps3.

The catalytic activity of recombinant Yor021c purified from *E. coli* was tested with ribosomes isolated from the *YOR021C* deletion strain that lack methylation of Rps3. As shown in Figure 5A, incubation of the recombinant protein with such hypomethylated ribosomes and *S*-adenosyl-[*methyl*-^{3}H]-L-methionine results in the formation of ^{3}H-ω-monomethylarginine. Radiolabeled ω-monomethylarginine was not detected in experiments using wild-type ribosomes, where the Rps3 protein was already methylated, as a methyl-accepting substrate (Figure 5B). We also did not detect the formation of ^{3}H-ω-monomethylarginine in control experiments lacking the Yor021c enzyme or ribosomes (Figure 5C–E). These experiments provide evidence that Rps3 is a substrate for the SPOUT methyltransferase Yor021c.

The methylation of Rps3 by Yor021c suggests that SPOUT methyltransferases can have a wider range of substrates than previously imagined, modifying both RNA and proteins (Table 1). Interestingly, homologues of *YOR021C* are present in other organisms but are absent in metazoa and plants, indicating that Rps3 might be similarly modified by SPOUT methyltransferases in lower eukaryotes and archaea (Table 1).

Rps27a Is Methylated at Cysteine-39. In addition to exploring the methylation of Rps3, we also identified other ribosomal proteins in the small subunit that were methylated. Our liquid chromatography–intact protein mass spectrometry analysis revealed an 8762.2-Da species, corresponding to either monomethylated Rps27a or dimethylated Rps27b with the loss of the initiator methionine residues (error = 10 ppm) (Figure 6A). To determine whether we were observing Rps27a or Rps27b, which only differs at position 62 (isoleucine and valine, respectively), we purified the protein and analyzed it by top-down mass spectrometry. Using ProSight PTM with a mass error range of 5.5 ppm, we searched the uncharged monoisotopic masses against the Rps27a and Rps27b protein sequences. When we placed one or two methyl groups on the N-terminus for Rps27a (Figure 6B) and Rps27b (Figure 6C), respectively, there were 63 matches for Rps27a and 25 matches for Rps27b. Among these, three of the fragments were exclusive to Rps27b and 42 were exclusive to Rps27a. When we placed one or two methyl groups on the C-terminus for Rps27a (Figure 6D) and Rps27b (Figure 6E), respectively, there were 53 matches for Rps27b and 53 matches for Rps27a. None of the fragments were exclusive to Rps27b or Rps27a. Taken together, these results indicate that we had isolated monomethylated Rps27a, not dimethylated Rps27b. Besides making this distinction, the fragmentation data also allowed us to localize the site of methylation within Rps27a. When the methyl group was placed on the N-terminus (Figure 6B), we observed the b_{39} ion (error = 1.8 ppm) and the c_{39} ion (error = 1.0 ppm), supporting a methyl group between positions 1–39 of Rps27a with its cleaved initiator methionine residue. When the methyl group was placed on the C-terminus (Figure 6D), we observed the c_{38} ion (error = −0.7 ppm), indicating an absence of methylation from positions 1–38. Taken together, these results support the methylation of Rps27a at cysteine-39.

Cysteine-39 in Rps27a Is Associated with a Cysteine-Zinc Cluster. In the crystal structure of the small ribosomal subunit from *Tetrahymena thermophila* (PBD ID: 2XZM) the homologous cysteine residue to yeast Rps27a cysteine-39 is a ligand in a typical four-cysteine zinc cluster.[53] However, in the crystal structure of the yeast ribosome (PDB IDs: 3U5B, 3U5C, 3U5F, 3U5G), cysteine-39 is found to be displaced from the zinc ion that is liganded to three other cysteine residues in an approximately planar configuration (Figure 7A).[40] These results suggest that the presence of the methyl group on cysteine-39 disrupts the four-cysteine zinc cluster in the yeast protein.

The four-cysteine zinc cluster in yeast Rps27a is remarkably similar to a four-cysteine zinc cluster in the *E. coli* Ada protein, which also contains a methylated cysteine residue (PBD ID: 1WPK)[54−56] (Figure 7A,B). In both cases, there are three cysteine ligands that form an approximate trigonal planar structure with the zinc atom, and one methylated cysteine residue. In the Ada structure, the sulfur atom of the methylated cysteine residue is liganded to the zinc atom. In the structure reported for the yeast Rps27a protein, it appears that the sulfur atom of the methylated cysteine residue is displaced from the zinc. However, since the presence of the methyl group was not known to the authors of the yeast ribosome structure,[40] it is possible that the electron density of the methyl group was attributed to the sulfur atom, resulting in an inaccurate zinc–sulfur distance. Ada scavenges alkyl groups from DNA to repair alkylating damage and has two functional domains. The N-terminal domain, which contains the 4-cysteine zinc cluster, repairs phosphotriester methylation damage in DNA by irreversibly transferring methyl groups from phosphates in DNA to cysteine-38.[57−59] Cysteine-321 in the C-terminal domain, not in a cysteine zinc cluster, removes alkyl damage from guanine residues to form various alkylated cysteine derivatives.[60−62] Whereas the C-terminal domain is present in eukaryotes as the *O*-6-methylguanine DNA methyltransferase, the N-terminal domain is not conserved. Given that Rps27 has been shown to be important in response to methyl methane sulfate, an alkylating agent, and UV–C exposure in plants,[63] this nonessential ribosomal protein might have a functional role in the repair of alkyl damage to the phosphodiester backbone of DNA in eukaryotic organisms. It remains to be tested whether yeast Rps27a is methylated spontaneously from alkylated nucleic acids or whether it is methylated in a *S*-adenosylmethionine-dependent enzymatic transmethylation reaction.[64]

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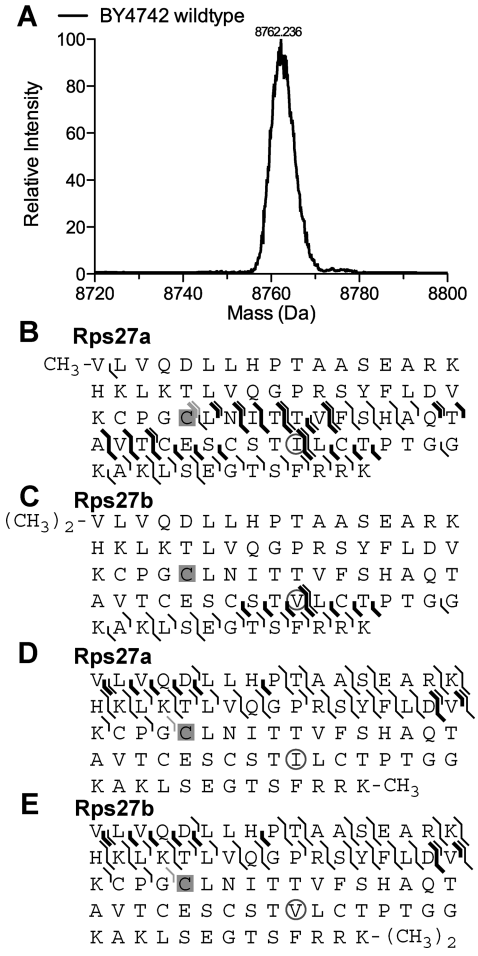

Figure 6. Rps27a is monomethylated at cysteine-39. (A) Ribosomal proteins of the small subunit were isolated from BY4742 wild-type yeast and analyzed by liquid chromatography−mass spectrometry, as described in the Experimental Procedures section. The reconstructed spectrum of Rps27a from wild-type yeast is shown. (B−E) HPLC-purified Rps27a from BY4742 wild-type yeast was fragmented and analyzed by top-down mass spectrometry. The resulting spectra were deconvoluted and the monoisotopic masses were searched with a 5.5-ppm error threshold against Rps27a with a theoretical N-terminal methyl group (B), Rps27b with two theoretical N-terminal methyl groups (C), Rps27a with a theoretical C-terminal methyl group (D), and Rps27b with two theoretical C-terminal methyl groups (E). Thick lines indicate matched fragments from electron capture dissociation fragmentation, while thin lines mark matched fragments from collisionally activated dissociation fragmentation. Informative fragments indicating methylation at cysteine-39 are shown with gray lines. In (B), the c_{39} and b_{39} ions indicate methylation between residues 1−39. In (D−E), the c_{38} ion supports an absence of methylation from residues 1−38, suggesting that cysteine-39 is the site of methylation. The sole residue where Rps27a and Rps27b differ is encircled in gray, and cysteine-39, the putative methylation site, is boxed in gray.

Rps2 Is Methylated at Arginine-10. We have previously explored the arginine methylation of Rps2 by Rmt1, which facilitates the formation of a single monomethylarginine or asymmetric dimethylarginine residue.[52] Although we identified the modifications in Rps2 and the associated methyltransferase, we wanted to localize the site of methylation so that we could better understand its biological role. Accordingly, we used a bottom-up mass spectrometry approach to pinpoint the site of the modification. Using MASCOT, chymotrypsin cleavage products of purified Rps2 were identified, including a peptide

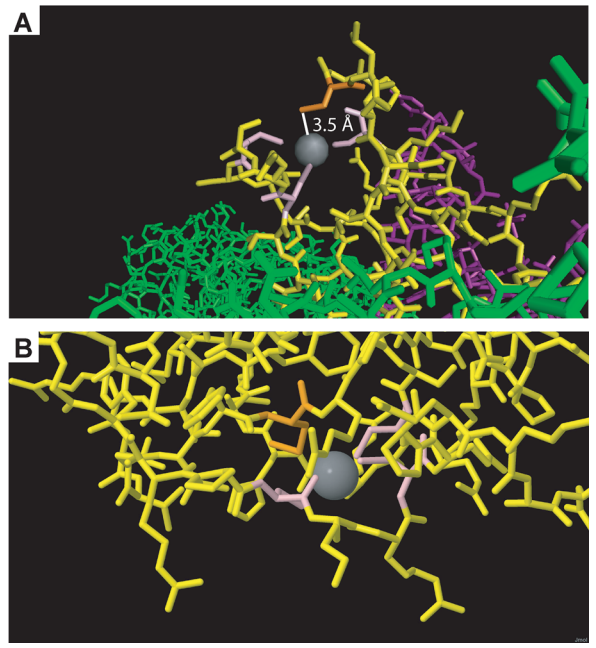

Figure 7. Cysteine-39 in Rps27a is likely in a disrupted four-cysteine zinc cluster. (A) The crystal structure of the small ribosomal subunit of *S. cerevisiae* (PDB IDs: 3U5B, 3U5C, 3U5F, 3U5G)[40] is explored to better understand the role of cysteine-39 methylation in Rps27a from *S. cerevisiae*. The structures of Rps27a, other ribosomal proteins, and the 18S rRNA are shown in yellow, green, and purple, respectively. Cysteine-39 in Rps27a is marked in orange, while the cysteine residues at positions 36, 55, and 58 are colored pink. The latter cysteine residues form a roughly planar trigonal three-cysteine zinc cluster (zinc is shown as a gray sphere), while cysteine-39 appears to be displaced from the cluster (see text). (B) The structure of the N-terminal domain of Ada, an *E. coli* protein that repairs phosphotriester damage in DNA by transferring methyl groups to a cysteine residue (PDB ID: 1WPK) is shown in yellow. Cysteine-38, which is methylated, is shown in orange, while the other cysteine residues forming the four-cysteine zinc cluster are colored pink. Zinc is represented by a gray sphere.

spanning residues 1−13 of acetylated Rps2 lacking its initiator methionine residue (Figure 8A). This peptide was observed in its unmodified, monomethylated, and dimethylated forms. The fragmentation of this peptide strongly supports methylation at arginine-10 (Figure 8B−D). We were hoping to use the available crystal structures to better understand this modification. However, the N-terminal residues of Rps2 and its Rps5 homologue in *T. thermophila* appear to be unstructured; the first 23 residues of Rps5 (including the corresponding arginine-8 residue) are missing from the *T. thermophila* structure[53] and the first 33 residues of Rps2 in yeast are missing from the *S. cerevisiae* structure.[40] These results suggest that this arginine residue is exposed and potentially available for other interactions.

DISCUSSION

Eukaryotic ribosome biogenesis is a complex process that involves modification and assembly of many ribosomal proteins and RNAs. A large set of proteins and RNAs modulates this process, many of which are not ultimately structural components of the ribosome, such as protein and RNA methyltransferases. The recent modeling of a high-resolution crystal structure of the yeast 80S ribosome[40] has highlighted eukaryotic-specific features that enhance our knowledge of

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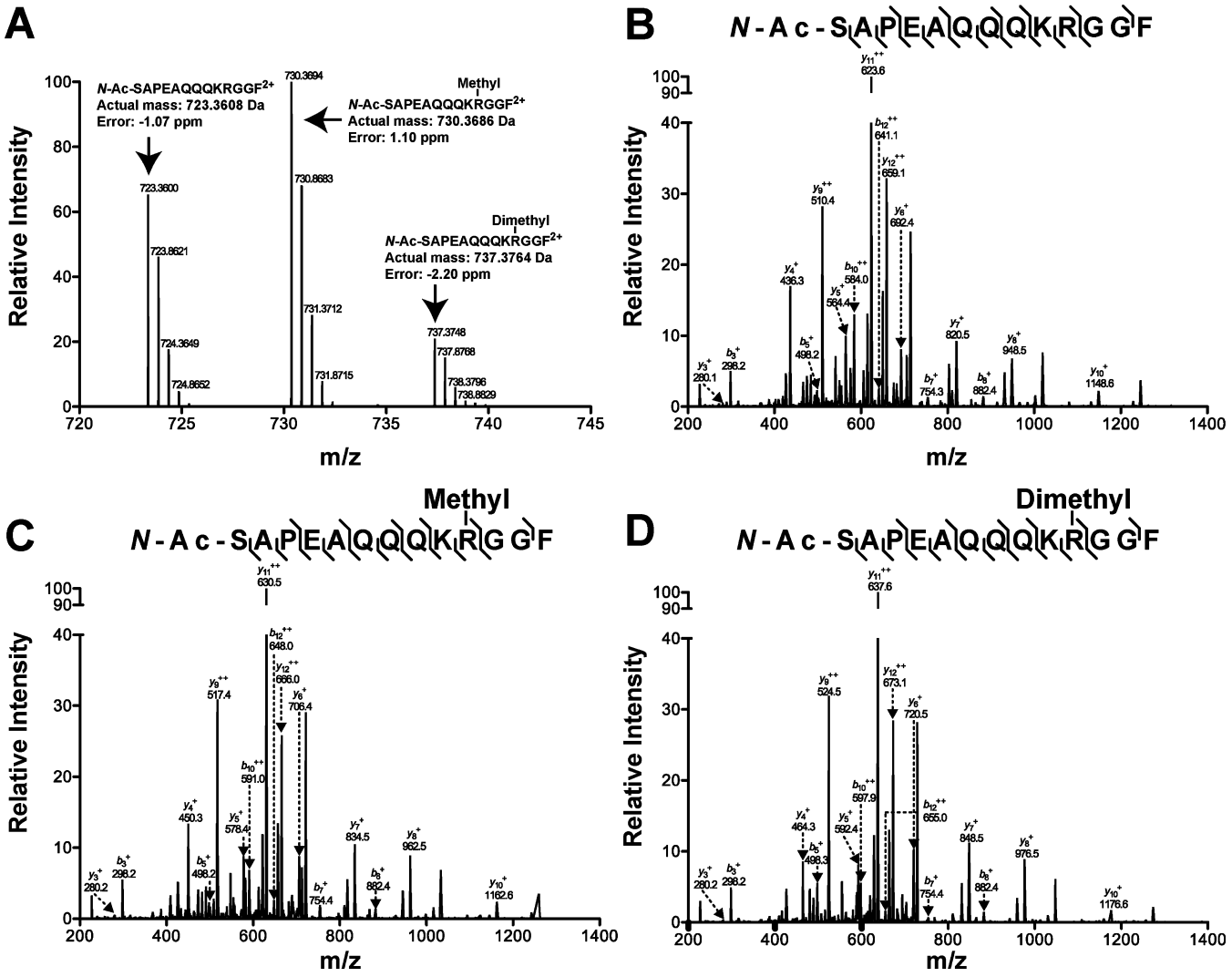

Figure 8. Rps2 is mono- and dimethylated at arginine-10. HPLC-purified Rps2 isolated from BY4742 wild-type yeast cells was digested with chymotrypsin and analyzed by bottom-up mass spectrometry as detailed in the Experimental Procedures section. (A) Averaged full-scan spectra of Rps2, indicating the presence of unmethylated, monomethylated, and dimethylated peptides spanning residues 1−13 of Rps2. (B−D) Fragmentation spectra of the unmethylated (B), monomethylated (C), and dimethylated (D) N-terminal peptides of Rps2. Prominent fragments with <500-ppm mass errors are indicated.

ribosome function; yet, a complete understanding of ribosome structure and its connection to function requires the identification of all modification sites within this complex ribonucleoprotein machine. Whereas some modifications are essential for proper ribosome assembly or function, others are not. For example, deletion of many genes that encode snoRNAs, which guide methylation of rRNA at specific sites, causes no growth defect on rich media.[65] Such posttranscriptional and posttranslational modifications of ribosomal components may cooperate in optimizing ribosome structure and function.

Rps3 Function and Methylation. In *S. cerevisiae*, Rps3 is an essential protein of the small ribosomal subunit.[66] Others have described a role for Rps3 in the export of the 40S subunit to the cytoplasm. Depletion of Rps3 results in decreased export of the 20S precursor rRNA and impairs its conversion to the 18S rRNA.[41] We were curious whether methylation of arginine-146 might affect the localization of GFP-tagged Rps3, but identified no differences in the localization pattern in a strain lacking Yor021c (data not shown). Rps3-interacting proteins Yar1 and Ltv1 are required for proper ribosome biogenesis and yeast cells lacking either protein are hypersensitive to some inhibitors of translation elongation, including paromomycin and anisomycin, but not cycloheximide.[34] In contrast, deletion of *YOR021c* did not affect sensitivity to any of these inhibitors (data not shown). These results suggest that the methylation of Arg-146 in Rps3 has more subtle effects than the removal of known factors in ribosome biogenesis.

Arginine methylation of mammalian Rps3 has been suggested to affect ribosome biogenesis, since simultaneous substitution of alanine for arginine at residues 64, 65, and 67 within the KH RNA-binding domain eliminated its in vitro methylation by PRMT1 as well as its incorporation into the ribosome.[49] In Rps3 from *S. cerevisiae*, the homologous residues are arginine-63, arginine-64, and asparagine-66. Interestingly, we did not detect methylation of either arginine-63 or arginine-64 by mass spectrometry (Figure 1B). In *S. cerevisiae*, the strain lacking the Yor021c methyltransferase does not exhibit decreased fitness[67] and the arginine-to-alanine substitution at 146 causes a slight growth defect, indicating that arginine

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methylation of Rps3 might have a distinct function from its mammalian homologue. In mammals, Rps3 has a wide range of extraribosomal roles,[57] and it is possible that some of these might be modulated by arginine methylation in *S. cerevisiae*. For example, Rps3 has been described to have an endonuclease activity for abasic DNA in both *S. cerevisiae* and mammals,[47,68] raising the possibility that methylation of arginine-146 in Rps3 might regulate this repair process. In addition, the ability of Rps3 to autoregulate the stability of its mRNA[43] suggests that methylation of arginine-146 might influence its interaction with *RPS3* mRNA and mRNA turnover.

The wild-type growth of Δ*yor021c* cells and of strains that expressed Rps3 with an arginine to lysine substitution at residue 146 under standard growth conditions, suggests that, rather than being crucial for basic ribosome function, methylation may cooperate with other modifications or factors to optimize the interactions between arginine-146 and the 18S rRNA. Our analysis of the high-resolution structure of the yeast ribosome[40] indicates that that arginine-146 closely interacts with adenine-1427, which is within 4 Å of cytosine-1274. Interestingly, one of the nonessential snoRNAs, snR56, directs 2′-*O*-methylation at guanosine-1425, two nucleotides 5′ to adenine-1427,[65] and the essential 40S ribosomal assembly factor Enp1 binds two nucleotides 3′ to adenine-1427, suggesting the significance of molecular interactions in this region. Strains expressing Rps3 with an arginine to alanine amino acid substitution at residue 146 exhibited a growth defect, indicating the importance of the amino acid identity at this position for optimal cellular growth. These interactions may become important for enhancing ribosome function or assembly under certain environmental conditions or in the absence of other factors or modifications.

SPOUT Methyltransferases and Translational Machinery. A striking observation in this work was the dependence of Rps3 methylation on the Yor021c SPOUT candidate methyltransferase. The many enzymes that facilitate ribosome biogenesis include ribonucleases that cleave precursor rRNAs, kinases that modulate ribosome function, and methyltransferases that modify rRNA and ribosomal proteins. Analyses of transcriptional profiles across multiple growth conditions demonstrated that many of these genes are coregulated with each other and with rRNA and ribosomal protein genes, forming an rRNA and ribosome biosynthesis (RRB) regulon.[69] On the basis of multiple genome- and proteome-wide data sets, a protein-function prediction algorithm predicted GO terms related to ribosome biogenesis or function for 16 of the previously uncharacterized RRB regulon genes.[70] Interestingly, five of these 16 genes are predicted to encode methyltransferases: *YBR271W*, *YNL022C*, *YGR283C*, *YMR310C*, and *YOR021C*.[22] Ybr271w and Ynl022c belong to the large family of seven β-strand methyltransferase genes, which includes all of the identified arginine methyltransferases in the eukaryotic domain.[22,23] The shared seven β-strand family structure allows modification of a variety of substrates; enzymes in this family also methylate proteins on lysine, histidine, and carboxyl groups, as well as RNA, DNA, and small molecules.[22,23] Ygr283c, Ymr310c, and Yor021c, however, are members of the SPOUT methyltransferase family.[22,28] Although there is less primary sequence similarity among the SPOUT family members than within the seven β-strand family, conserved structural features have allowed identification of SPOUT methyltransferases in all three domains of life.[28]

All SPOUT family members analyzed previously methylate RNAs (Table 1). The largest SPOUT cluster of orthologous groups (COG) includes 2′-*O*-ribose methyltransferases, represented by Mrm1 and Trm3 in yeast.[28] Ygr283c and Ymr310c belong to a distinct COG, which has no members of known function. The proteins within the COG containing Yor021c also have no known function, although this COG is closely related to the COG containing yeast Trm10, a guanine tRNA methyltransferase that modifies the N1 site. The association of Yor021c with monomethylation of arginine-146 of Rps3 indicates that SPOUT methyltransferases may have a broader range of substrates than previously imagined. Indeed, the nitrogen atom at position 1 in the guanine base that is methylated by Trm10 is structurally similar to the terminal nitrogen atom of the guanidino group of an arginine residue, suggesting the SPOUT enzymes might methylate both guanine and arginine. Such substrate variability is also seen among members of the HemK family of seven-beta strand methyltransferases, which include enzymes that methylate the chemically similar nitrogen atoms of the 6-amino group of adenine bases and the side chain amide of glutamine residues.[71]

Proteins in the Yor021c COG have a C-terminal extension beyond the SPOUT domain, in contrast to an N-terminal extension in the Trm10 COG.[28] Crystal structures of SPOUT methyltransferases have revealed the presence of flexible linkers between the SPOUT and terminal domains, as well as the roles of these domains in substrate binding.[72,73] The unique C-terminal domain of these Yor021c-related proteins may therefore guide methylation of the non-RNA substrate Rps3. In light of our direct demonstration of the methyltransferase activity of Yor021c on Rps3, we suggest the gene designation *SFM1* (SPOUT family methyltransferase 1) for the *S. cerevisiae* *YOR021C* open reading frame.

Rps27a and Rps2 Methylation. Our studies also led to the identification of a methylcysteine residue at position 39 in Rps27a. Rps27a is a nonessential ribosomal protein and has been suggested to be important for the synthesis of the 18S rRNA.[74] Our analysis of the crystal structure of Rps27a in the yeast small ribosomal subunit suggests that a four cysteine zinc cluster is present that may be similar to the one found in the N-terminal domain of the *E. coli* Ada protein that allows the repair of DNA phosphotriester damage by accepting methyl groups.[54] Eukaryotes lack the N-terminal region of Ada, and we wonder whether Rps27a might function in repairing these deleterious DNA lesions[57] in eukaryotes because its absence causes increased sensitivity to UV light and alkylating agents in *A. thaliana*.[63] Such a role, however, would require a population of unmethylated Rps27a. We have not observed such a species in ribosomes, although it might be present in the nucleus or cytosol.

In addition, we have identified the site of arginine methylation in Rps2 by bottom-up mass spectrometry. The short arginine-glycine-rich region of *S. cerevisiae* Rps2 is variably mono- or asymmetrically dimethylated by Rmt1 on arginine-10 within an RGGF sequence, a common substrate recognition motif for Rmt1-catalyzed methylation in *S. cerevisiae*.[75] In contrast, *S. pombe* Rps2 is asymmetrically dimethylated in vivo on eight arginines in its RG-rich N-terminus by PRMT3.[76] In vitro methylation of mammalian Rps2 by PRMT3 also occurs primarily on a stretch of eight RG dipeptides downstream of two RGGF peptides.[77] Similar to our results with *S. cerevisiae* Rps3, *S. pombe* Rps2 with arginine-to-lysine substitutions can replace wild-type Rps2 whereas alanine substitutions decrease Rps2 function,[76] indicating the greater importance of amino acid identity rather than methylation. Our previous mass

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spectrometry analysis of intact *S. cerevisiae* Rps2 revealed the presence of only one or two methyl groups per molecule, suggesting that the two downstream RG peptides are not methylated in this organism that lacks a PRMT3 homologue.[78] The physiological role of arginine-10 and the functional significance of RG region length in Rps2 remain to be determined; to date, the N-terminus of Rps2 has not been resolved in eukaryotic ribosome structures, suggesting its flexibility and the potential for interactions with other factors that might influence ribosome function.

In this paper, we enhance our understanding of methylation of proteins in the small ribosomal subunit in *S. cerevisiae*. We identified the sites of methylation modifications in Rps3, Rps27a, and Rps2 by a variety of analytical methods. Our analysis of available crystal structures has enabled us to suggest functional roles for these modifications, including methylated arginine-146 in Rps3 as a mediator of 18S rRNA interactions and cysteine-39 in Rps27a as a potential acceptor for alkylating damage of DNA. Finally, we have demonstrated that the Yor021c SPOUT methyltransferase is a protein arginine methyltransferase that methylates Rps3. To date, SPOUT methyltransferases have been shown to only modify RNA and our observations indicate that these enzymes are also capable of methylating ribosomal proteins. Our findings, which complement other efforts to identify sites of ribosomal protein modification and the enzymes that catalyze these modifications, are crucial for interpreting new high-resolution ribosome crystal structures and will help build our understanding of ribosomal structure and function.

■ ASSOCIATED CONTENT

Supporting Information

A list of oligonucleotides used to generate and verify Rps3 mutants is provided as Supplemental Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(S.G.C.) Address: Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, 607 Charles E. Young Drive East, Los Angeles, California 90095, United States. Tel: 310 825-8754. Fax: 310 825-1968. E-mail: clarke@mbi.ucla.edu. (A.E.M.) Address: Department of Biology, Bowdoin College, 6500 College Station, Brunswick, Maine 04011, United States. Tel: 207 798-7109. Fax: 207 725-3405. E-mail: amcbride@bowdoin.edu.

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Notes

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