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Protein Methylation and Translation: Role of Lysine Modification on the Function of Yeast Elongation Factor 1A

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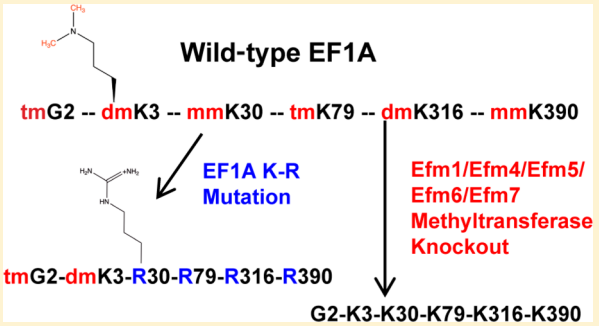
Supporting Information

ABSTRACT: To date, 12 protein lysine methyltransferases that modify translational elongation factors and ribosomal proteins (Efm1−7 and Rkm 1−5) have been identified in the yeast *Saccharomyces cerevisiae*. Of these 12, five (Efm1 and Efm4−7) appear to be specific to elongation factor 1A (EF1A), the protein responsible for bringing aminoacyl-tRNAs to the ribosome. In *S. cerevisiae*, the functional implications of lysine methylation in translation are mostly unknown. In this work, we assessed the physiological impact of disrupting EF1A methylation in a strain where four of the most conserved methylated lysine sites are mutated to arginine residues and in strains lacking either four or five of the Efm lysine methyltransferases specific to EF1A. We found that loss of EF1A methylation was not lethal but resulted in reduced growth rates, particularly under caffeine and rapamycin stress conditions, suggesting EF1A interacts with the TORC1 pathway, as well as altered sensitivities to ribosomal inhibitors. We also detected reduced cellular levels of the EF1A protein, which surprisingly was not reflected in its stability *in vivo*. We present evidence that these Efm methyltransferases appear to be largely devoted to the modification of EF1A, finding no evidence of the methylation of other substrates in the yeast cell. This work starts to illuminate why one protein can need five different methyltransferases for its functions and highlights the resilience of yeast to alterations in their posttranslational modifications.



Methylation of proteins of the translational apparatus, including ribosomal proteins and elongation factors, has been well-characterized in recent years.[1−6] One protein from *Saccharomyces cerevisiae*, elongation factor 1A (EF1A), stands out by the extensive methylation of its lysine residues. EF1A is primarily responsible for transporting the aminoacyl-tRNA to the ribosomal A site as a GTP complex and ensuring a correct codon−anticodon match.[7] Additionally, EF1A has been shown to have a role in the assembly of the ribosomal subunits,[8] the regulation of the actin cytoskeleton, and other cellular functions.[9,10] Five distinct enzymes methylate EF1A at Lys-3 (Efm7), Lys-30 (Efm1), Lys-79 (Efm5), Lys-316 (Efm4), and Lys-390 (Efm6).[2,3,11−15] Efm7 is also able to methylate the N-terminal amino group of Gly-2.[13] It is presently unknown whether these methyltransferases are specific for EF1A or whether they also modify other cellular proteins. Methylation of EF1A is conserved between different species, with methylation at Lys-79 and Lys-316 being the most highly conserved.[2,16]

Since the discovery of EF1A and its posttranslational modifications, the connection between EF1A function and its methylation has remained poorly characterized. To address the question of whether EF1A lysine methylation is necessary for EF1A's functional roles in the cell, we used EF1A methyl-deficient strains and assayed function using multiple biochemical approaches. These approaches included measuring yeast growth under different stress conditions, ribosome sedimentation, and dual luciferase assays to assess translation fidelity.

Here we provide phenotypes associated with the disruption of EF1A methylation, including slow growth and sensitivity to translational inhibitors as well as to rapamycin and caffeine. This work demonstrates that methyl-deficient EF1A can still function in translation and ribosomal assembly but may disrupt the TORC1 pathway. Finally, we provide evidence that the five EF1A methyltransferases appear to be specific to EF1A and do not have additional major cellular targets, although we cannot rule out the methylation of minor species.

MATERIALS AND METHODS

Yeast Strains and Growth Media. All yeast strains were grown in 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose (YPD, Fisher) at 30 °C. For spot test analyses, yeast strains were plated on 2% agar in YPD or on such plates

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Table 1. Yeast Strains Used in This Study

strain	genotype
BY4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
efm1Δefm4Δefm5Δefm6Δ	BY4742 background, *yhl039wΔ::hphMX, yil064wΔ::HIS3, ygr001cΔ::kanMX, ynl024cΔ::URA3*
efm1Δefm4Δefm5Δefm6Δefm7Δ	BY4742 background, *yhl039wΔ::hphMX, yil064wΔ::HIS3, ygr001cΔ::kanMX, ynl024cΔ::URA3, ylr285wΔ::LYS2*
EF1A K(30, 79, 316, 390)R	*tef1Δ::kanMX, tef2Δ::hphMX, TEF1* K(30, 79, 316, 390)R/pUG23

supplemented with hydrogen peroxide, NaCl, caffeine (Alfa Aesar, AA3921414), rapamycin (Alfa Aesar, AAJ62473MF), anisomycin (Millipore, 176880), cycloheximide (Sigma, C7698), tunicamycin (Sigma, T7765), and puromycin (Sigma-Aldrich, P8833) as described in the figure legends. Solid growth medium was also made as YPG with 10 g/L yeast extract, 20 g/L peptone, 3% glycerol, and 20 g/L agar or as lactate medium with 3 g/L yeast extract, 0.5 g/L dextrose, 0.5 g/L $CaCl_2$, 0.5 g/L NaCl, 0.6 g/L $MgCl_2$, 1 g/L NH_4Cl, 1 g/L KH_2PO_4, 8 g/L NaOH, 22 mL of 90% DL-lactic acid per liter, and 20 g/L agar.

Strains used in this study are listed in Table 1. The *efm1456Δ* and *efm14567Δ* deletion strains were based on the *efm1Δ* strain obtained from the Dharmacon online yeast knockout collection. Each successive deletion was created through homologous recombination following the protocol as described previously.[17] Each primer contained 40 bp upstream or downstream of the corresponding ORFs to be deleted. For the knockout using the *KIURA3* cassette, we used the *KIURA3* cassette found in the CORE cassette as a template. The mutants were confirmed through polymerase chain reaction (PCR) using primers upstream and downstream of the corresponding gene.

For introducing arginine substitutions at lysine codons in *TEF1*, the endogenous yeast *TEF1* gene was cloned into pUG23 (CEN/ARS HIS3 vector) under its native promoter and terminator using a standard cloning protocol. The point mutations were introduced via site-directed mutagenesis using QuikChange Lightning mutagenesis (Agilent 210518 and 210515). Sanger sequencing of the *TEF1* open reading frame (ORF) was used to confirm the point mutations. Starting with wild-type yeast cells, the *TEF1* ORF was first deleted with a kanMX cassette, and then the plasmid harboring the quadruple *TEF1* mutant was transformed into cells and selected for under growth in -HIS. *TEF2* was then deleted with the *hphMX* cassette, and the absence of both *TEF1* and *TEF2* was confirmed by PCR.

Lysis Method 1. Yeast cells grown in YPD (7 OD_{600} units) were washed three times with 1 mL of water and then resuspended in 0.2 mL of lysis buffer [0.2% sodium dodecyl sulfate (SDS) and 0.7 mM phenylmethanesulfonyl fluoride (PMSF)]; 0.2 g of baked glass beads (Biospec Products, 11079105) was added, and the cells were lysed with seven cycles of 1 min on vortex and 1 min on ice. Lysates were separated from beads using a gel loading tip and then clarified by centrifugation at 12000*g* for 15 min.

Lysis Method 2. Yeast cells grown in YPD (7 OD_{600} units) were washed once with 1 mL of ice-cold water, spun at 4000*g* for 4 min, and then washed again with 1 mL of ice-cold water supplemented with 100 μg/mL PMSF. Cells were lysed by the method of Yaffe et al.[18] with the following modifications. Washed cells were incubated for 10 min in 150 μL of ice-cold 1.85 M NaOH containing 2% 2-mercaptoethanol. After 10 min, ice-cold 50% (w/v) trichloroacetic acid was added and the mixture incubated on ice for an additional 10 min. The mixture was centrifuged for 2 min, and the resulting pellet washed with 1 mL of cold acetone and centrifuged again. The pellet was dried using vacuum centrifugation for 2 min. The pellet was then resuspended in 200 μL of sample buffer prepared from 500 μL of 0.2 M Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 500 μL of water, 12.5 μL of 2-mercaptoethanol, 25 μL of 1 M Tris base, and 100 μg of PMSF and heated for 3 min at 95 °C. After the protein concentration had been determined by Lowry analysis after trichloroacetic acid precipitation,[19] a small amount of solid bromophenol blue was added and samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

SDS−PAGE. Cell lysates were fractionated on a 4−12% Bis-Tris precast polyacrylamide gel (GenScript) with 1× MOPS buffer [6.06 g/L Tris base, 10.46 g/L MOPS, 1 g/L SDS, and 0.3 g/L EDTA (GenScript)] for 1 h at 140 V. An unstained protein marker ladder was used to determine protein size. The gel was Coomassie stained [50% methanol, 10% acetic acid, 40% water, and 0.2% Brilliant Blue R-250 (w/v)] for 1 h and destained in 10% acetic acid and 15% methanol until bands became visible.

EF1A Purification. The method of purification described below was adapted from ref 20. A 50 mL overnight culture grown in YPD from the wild-type or mutant strain was used to inoculate two flasks of 4 L of YPD, and cells were grown to an OD_{600} of ∼2.5. The cells were centrifuged at 664*g* in preweighed centrifuge bottles, and the weight of the pellet was recorded. Cells were stored at −80 °C until lysis could be performed. The pellet was resuspended in 2 mL/g of pellet in ice-cold lysis buffer [60 mM Tris-HCl (pH 7.5), 50 mM NH_4Cl, 5 mM $MgCl_2$, 0.1 mM EDTA (pH 8), 10% glycerol, 1 mM dithiothreitol (DTT), and 0.2 mM PMSF] and lysed by being passed through an emulsifier (EmulsiFlex-C3) four times at >25000 lb/in.^{2} of pressure. Cell debris was removed by centrifugation at 11300*g* for 30 min at 4 °C, and then the supernatant clarified at 76300*g* for 1.5 h at 4 °C. The supernatant was added to diethylaminoethyl cellulose resin (DE52, Whatman) that was pre-equilibrated with buffer 1 [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (pH 8), 25% glycerol, 1 mM DTT, and 0.2 mM PMSF] and 100 mM KCl for 1 h with light stirring at 4 °C.

Unbound EF1A was recovered by being transferred to a 50 mL conical tube and centrifugation at 2000*g* for 3 min. The supernatant was then incubated with 25 mL of sulfopropyl-Sepharose (fast flow, Sigma) also equilibrated with buffer 1 containing 100 mM KCl for 1 h with light stirring at 4 °C. Unbound material was removed by centrifugation as before, and then EF1A eluted by incubating the resin with 25 mL of buffer 1 containing 500 mM KCl for 1 h with light stirring at 4 °C. Eluted proteins were then recovered by centrifugation at 2000*g* for 3 min and dialyzed overnight in 3 L of buffer 1 with no salt. Lastly, the dialyzed protein was applied to 15 mL of carboxymethyl cellulose resin (CM52, Whatman) equilibrated with buffer 1 containing 50 mM KCl packed into a column and allowed to elute by gravity flow with a stepwise salt gradient of 100, 150, 200, 300, 350, and 500 mM KCl; 1.5 mL fractions were collected and analyzed by SDS−PAGE to determine where EF1A eluted. Fractions containing pure EF1A were

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pooled and dialyzed into buffer 1 containing 100 mM KCl overnight at 4 °C for storage at −80 °C.

Immunoprecipitation. Seven OD units of yeast cells grown to an OD_{600} of ∼0.7 was grown in *S*-adenosyl-[*methyl*-^{3}H]methionine, using the method described previously.[21] Next the labeled cells were washed with water, resuspended in 1 mL of binding buffer (20 mM Tris, 100 mM KCl, 10% glycerol, 1% Triton X-100, and 200 μg/mL PMSF), and lysed with 0.2 g of baked glass beads using seven rounds of 30 s vortexing followed by 30 s on ice. The radiolabeled lysates were collected and clarified at 5000*g* for 5 min. Ten microliters was set aside as the input material. Protein A beads were prepared in binding buffer with three washes at 700*g* for 2 min and kept on ice until needed. To start the immunoprecipitation, the labeled lysates (500 μg of protein as determined by the Lowry assay) were incubated with 5 μL of anti-EF1A antibody (Kerafast, ED7001) for 3.5 h and then with protein A beads for 2 h. Following centrifugation as described above, the protein−antibody−protein A bead complex was heated at 100 °C in 50 μL of 5× SDS buffer [250 mM Tris-HCl (pH 6.8), 10% SDS, 30% glycerol, 0.5 M DTT, and 0.02% bromophenol blue] for 8 min to release protein. Forty microliters of each sample and 5 μL of each input sample were analyzed by SDS−PAGE as described above. The destained gel was incubated in water overnight and then treated with En3hance (PerkinElmer) for 1 h followed by a 30 min water wash. The dried gel was then exposed to film at −80 °C.

Protein Stability Assay. Yeast cells were inoculated the night before in YPD medium at 30 °C to give an OD_{600} of ∼0.7 the following morning. The inhibitor chase was performed as described by Buchanan et al.[22] with the changes described below. Samples were collected at various time points and spun down and frozen at −20 °C until lysis. Puromycin or cycloheximide was used to perform the chase. Lysis was performed using method 2 described above, and the lysates were fractionated in duplicate using SDS−PAGE (described above). Protein sizes were determined using a Bio-Rad broad range unstained molecular weight ladder, and equal amounts of protein (as determined by the Lowry assay after precipitation with trichloroacetic acid) were loaded for each strain tested. One gel was stained and destained as described above. A second gel was transferred to a PVDF membrane for Western blot analysis with 7 μL of Amersham full range ECL rainbow ladder as described below.

Immunoblot Analysis. Proteins from lysates separated by SDS−PAGE were transferred to a PVDF membrane (Hybond-P) at 30 V for 1 h. The membrane was then blocked overnight at 4 °C in 5% dried nonfat milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST) or 0.5% (w/v) BSA/0.02% (w/v) SDS in phosphate-buffered saline with 0.1% (v/v) Tween 20 (PBST). After being blocked, the membranes were washed in 1× TBST or 1× PBST and incubated with primary antibodies (1:10000 rabbit anti-EF1A, Kerafast, ED7001) diluted into 1% dried nonfat milk in 1× TBST or 0.5% BSA/0.20% SDS in PBST, as indicated, for 1.5 h at 4 °C. After being washed with the respective buffers, the membrane was incubated with the anti-rabbit IgG-HRP (1:6666; Cell Signaling, 7074) secondary antibody in 1% dried nonfat milk or the LICOR anti-goat fluorescent antibody in 0.5% BSA/0.02% SDS in PBST for 1 h at room temperature. ECL was used to visualize bands probed with the HRP secondary antibody (Amersham Biosciences ECL Prime Western blotting, GE Healthcare, RPN2232) and the LICOR Odyssey imager for the fluorescent probe. After being probed, membranes were stained with Ponceau S or Coomassie to determine the transfer efficiency.

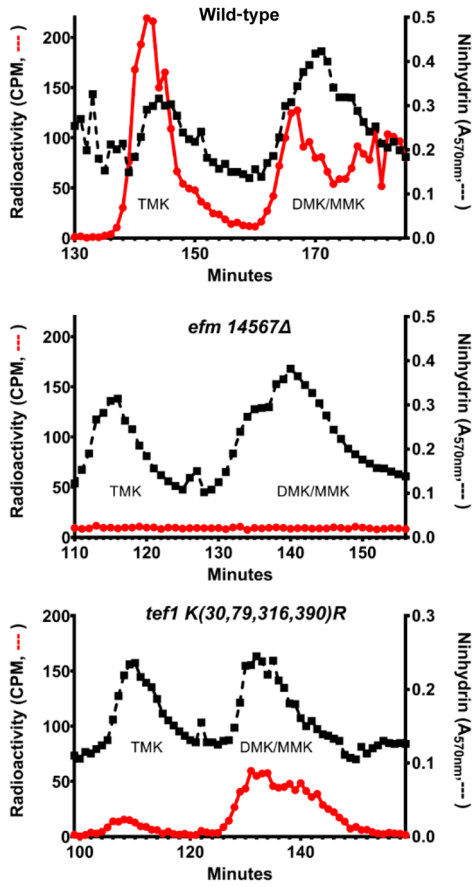

Figure 1. Loss of methylated lysine residues in EF1A from a strain lacking five Efm methyltransferases and a strain with lysine to arginine substitutions at positions 30, 79, 316, and 390 in EF1A. EF1A's purified from yeast cells that were labeled with *S*-adenosyl-[*methyl*-^{3}H]methionine and acid hydrolyzed, and the methylated amino acid derivatives separated by high-resolution cation exchange chromatography using the method described previously[21] with the modifications shown below. Wild-type and *tef1 K(30,79,316,390)R* hydrolysates were fractionated, mixed with standards of 2 μmol of ε-trimethyllysine (TMK) and 1.4 μmol of ε-dimethyllysine (DMK), while *efm14567*Δ was fractionated with the same amount of TMK and DMK with the addition of 0.6 μmol of ε-monomethyllysine (MMK). The column was eluted with a sodium citrate buffer (0.3 M Na^+) at pH 3.8. Radioactivity (red circles and line) was measured in 975 μL of the fractions eluting in the positions of the methylated lysine standards that were determined by a ninhydrin assay in 25 μL aliquots (black squares and line; performed at 68 °C for 15 min). Data from the middle panel are from one experiment; data in the top and bottom panels are from one experiment of two replicates.

Dual Luciferase (DLR) Assay. For amino acid misincorporation, the CTY775/luc CAAAFF K529N plasmid was used, and for programmed frame shift, the pJD376 (L-A) termed PRF −1 and pJD377 (Ty1) PRF + 1 plasmid was used. These plasmids were transformed into the wild-type and mutant strains using the lithium acetate−single-stranded DNA−PEG method.[23] Transformed strains were grown in SD −Ura [minimal synthetic defined medium lacking uracil; 0.07% (w/v) CSM-Ura powder, 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, and 2% (w/v) dextrose] to an OD_{600} of

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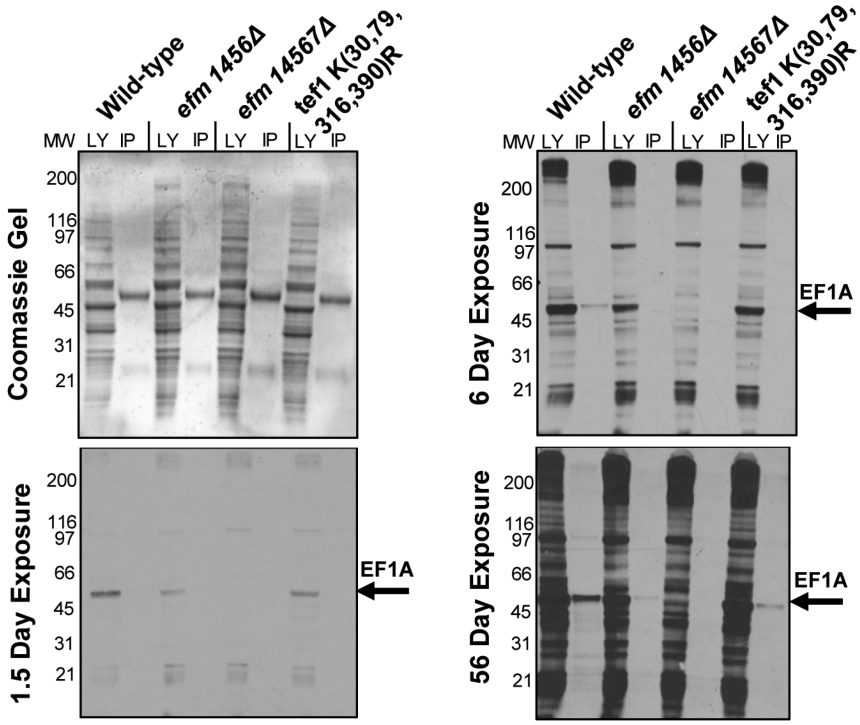

Figure 2. Immunoprecipitation of EF1A from methylation-deficient cells shows the specificity of elongation factor methyltransferases. Yeast cells from wild-type and mutant strains were labeled with *S*-adenosyl[*methyl*-^{3}H]methionine and immunoprecipitated with an anti-EF1A polyclonal antibody as described in Materials and Methods. The top left panel is a Coomassie-stained polyacrylamide gel, which serves as a protein loading control. The remaining panels show the detection of radioactive material in each sample at different time intervals. The longer exposure reveals that the level of methylated EF1A is decreased in the methyltransferase knockout mutants. The LY lane shows the total lysate before the immunoprecipitation, while the IP lanes show what was pulled down with the EF1A antibody. The figure is a representative from one of two separate experiments.

0.5−0.8. Next 0.5 OD_{600} unit was harvested by centrifugation at 5000*g* and stored on ice until ready for use. The DLR reagents, from Promega, were thawed to room temperature and diluted according to the assay manual. Harvested cells were individually lysed with 0.5 mL of passive lysis buffer, and then 6 μL was transferred to a white (Greiner bio-one, 82050-736) 96-well plate. Thirty microliters of a LARII solution was added and immediately read using a SpectraMax M5 microplate reader, giving firefly luminescence; then 30 μL of Stop and Glo buffer was immediately added to that same well and read to give Renilla luminescence. SpectraMax parameters were set as follows: read type, end point; read mode, luminescence with a 1500 ms integration time; wavelength, all; automix, off; autocal, on; setting time, off; autoread, off.

RESULTS

Generation of Yeast Strains Deficient in Multiple EF1A Methyltransferases or with Arginine Substitutions of EF1A Methyl-Accepting Lysine Residues. To assess the functional role of the methylation of the N-terminal glycine residue of EF1A and lysine residues 3, 30, 79, 316, and 390, two approaches were taken. First, we constructed yeast strains lacking the five methyltransferases responsible for methylation at all of these sites (*efm14567*Δ) or the four methyltransferases that methylate lysine residues 30, 79, 316, and 390 (*efm1456*Δ) through marker-based gene deletions. Second, we mutated a plasmid-borne *TEF1* gene encoding one copy of EF1A to replace lysine codons at positions 30, 79, 316, and 390 with arginine codons [Tef1 K(30,79,316,390)R] and then deleted both endogenous genes (*TEF1* and *TEF2*) encoding EF1A as described in Materials and Methods. The N-terminal modifications are still present in this strain (trimethyl Gly-2 and dimethyl Lys-3). The successful construction of the *efm14567*Δ mutant strain indicates that the loss of all five methyltransferase genes does not result in lethality.

We then analyzed the extent of lysine methylation in wild-type, *efm14567*Δ, and *TEF1* K(30,79,316,390)R strains labeled *in vivo* with *S*-adenosyl[*methyl*-^{3}H]methionine.[11] We performed acid hydrolysis on the 50 kDa polypeptides separated by SDS−PAGE that contain EF1A and analyzed the radiolabeled methylated lysine derivatives by high-resolution cation exchange chromatography. We were able to clearly resolve a peak of the ^{3}H-trimethylated species (TMK) and a poorly resolved peak that included both the ^{3}H-dimethylated and ^{3}H-monomethylated derivatives (DMK and MMK, respectively) (Figure 1). In wild-type hydrolysates, all three lysine ^{3}H-methylated species were detected, whereas in the *efm14567*Δ strain, no radioactivity was detected at the positions of TMK, DMK, and MMK, confirming biochemically the loss of the Efm1 and Efm4−Efm7 methyltransferases. On the other hand, we observed reduced levels of TMK and DMK/MMK methylation of *tef1 K(30,79,316,390)R* EF1A (Figure 1). Although we expected some [^{3}H]MMK and [^{3}H]DMK from the methylation at Lys-2, we were surprised to see the formation of a small amount of [^{3}H]TMK. These results suggest that alternative lysine residues may become available for methylation when lysines 30, 79, 316, and 390 are converted to arginine residues.

To confirm the reduction in the level or absence of methylation of EF1A in the mutant strains, we labeled intact

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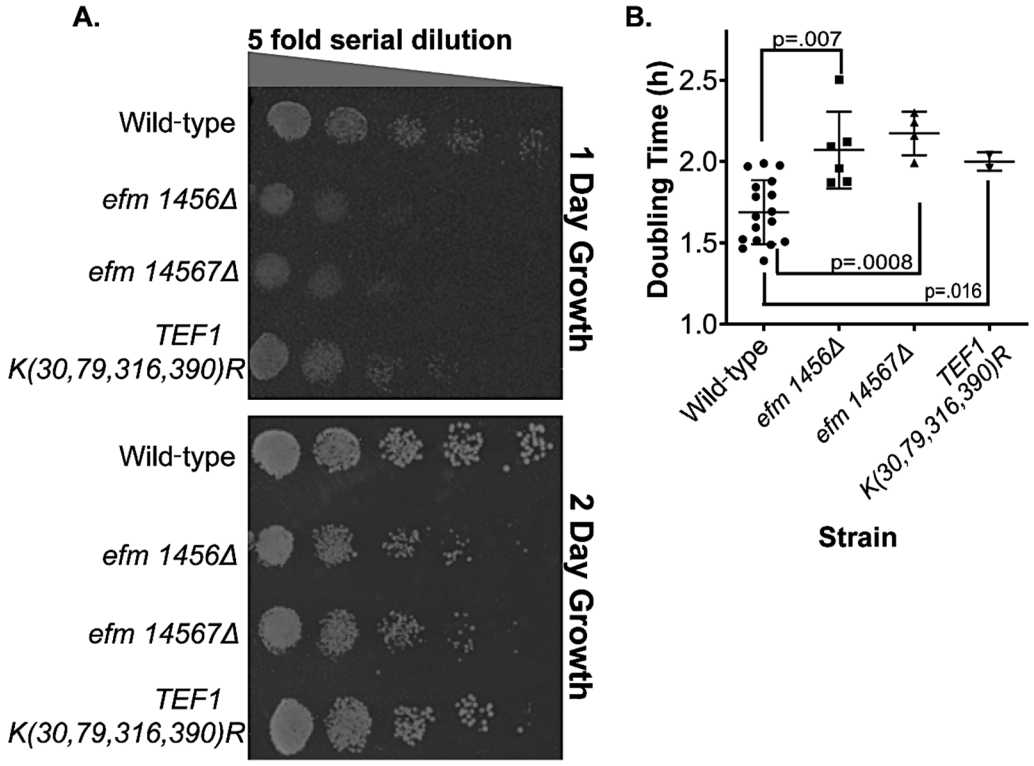

Figure 3. Loss of Efm methyltransferases results in slow growth in solid and liquid YPD growth media, while EF1A with four lysine to arginine mutations shows slow growth in only liquid media. (A) Yeast cells from wild-type and mutant strains grown at 30 °C in YPD to an OD_{600} of ∼0.5 and 3 μL of a cell suspension starting at 0.1 OD_{600} were then serially diluted and plated on YPD agar plates at 30 °C. Colonies were photographed for a representative experiment after 1 day or 2 days. In replicate experiments, we found that colonies for the efm1456Δ mutant were significantly smaller than wild-type colonies in 16 of 23 experiments; in the seven other cases, colonies were roughly the same size. Colonies for the efm14567Δ mutant were significantly smaller than wild-type colonies in 19 of 21 replicate experiments; in the two other cases, colonies were roughly the same size. In 23 replicate experiments, the colony sizes for the *TEF1* K(30,79,316,390)R mutants were indistinguishable from those of the wild type. (B) Doubling times for growth in liquid YPD media at 30 °C were calculated from the linear portion of exponential growth measured by OD_{600} over a 12 h time frame. Each point is a biological replicate. Error bars indicate the standard deviation values, and Student's *t* test *p* values (unpaired, two tails) are shown.

yeast cells with *S*-adenosyl[*methyl*-^{3}H]methionine and then analyzed ^{3}H-methylated polypeptides by SDS−PAGE before and after immunoprecipitation with antibodies to EFlA. Even with long exposures, no radioactivity was detected at the 50 kDa position of EF1A in the *efm14567*Δ strain lacking all of the EF1A methyltransferases, and a reduced level of methylation was observed in the *efm1456*Δ strain with shorter exposures (Figure 2). As shown for the amino acid analysis experiment described above, we found significant ^{3}H methylation in the 50 kDa immunoprecipitated EF1A in the K(30,79,316,390)R strain, again suggesting that alternate methylation sites may be used when these four lysine residues were unavailable (Figure 2).

To probe if the EF1A methyltransferases had alternative methylation substrates, we also analyzed the entire spectrum of methylated polypeptides in lysates of the intact cells labeled with *S*-adenosyl[*methyl*-^{3}H]methionine (Figure 2 and Figure S1). Here we looked closely for evidence of methylated polypeptides on SDS−PAGE that were reduced or not found in any of the three mutant strains on the fluorograph. The Coomassie-stained gel was a control for protein loading and to show the electrophoretic mobility of EF1A. The heavy and light chains of the EF1A antibody (∼60 and ∼25 kDa, respectively) can be seen on the Coomassie-stained gel bracketing the ∼50 kDa position of EF1A (Figure S1). In both the experiment shown in Figure 2 and the replicate experiment shown in Figure S1, we observed a complete loss of methylation in the *efm14567*Δ strain, confirming that the major methylated species at this polypeptide size was EF1A. However, we were unable to detect any reduction in the level of methylation of any other polypeptide band seen in the fluorographs (Figure 2 and Figure S1). These results suggest that none of the five EF1A methyltransferases catalyze the modification of non-EF1A polypeptides, although we would not be able to detect the loss of minor methylated species.

Methylation-Deficient Cells Exhibit a Slow Growth Phenotype and Alter the Growth in Response to Cellular Stress. We then assessed differences in the growth of the EF1A methylation-deficient strains. In Figure 3A, we show yeast growth on plates containing yeast extract, peptone, and dextrose (YPD). Serially diluted strains were spotted and allowed to grow for 1 day (early growth) and 2 days (later growth). At both stages, colonies of the *efm1456*Δ strain as well as the *efm14567*Δ strain were much smaller than the wild-type colonies. These defects were confirmed and quantitated by observing slower growth in liquid YPD medium, as well. We found an increase in doubling times from ∼1.7 h for the wild-type cells to 2.1 h for the *efm1456*Δ strain and 2.2 h for the *efm14567*Δ strain (Figure 3B).

When similar experiments were performed for the *TEF1* K(30,79,316,390)R strain, somewhat reduced colony sizes were observed after plate growth for 1 day but not for 2 days

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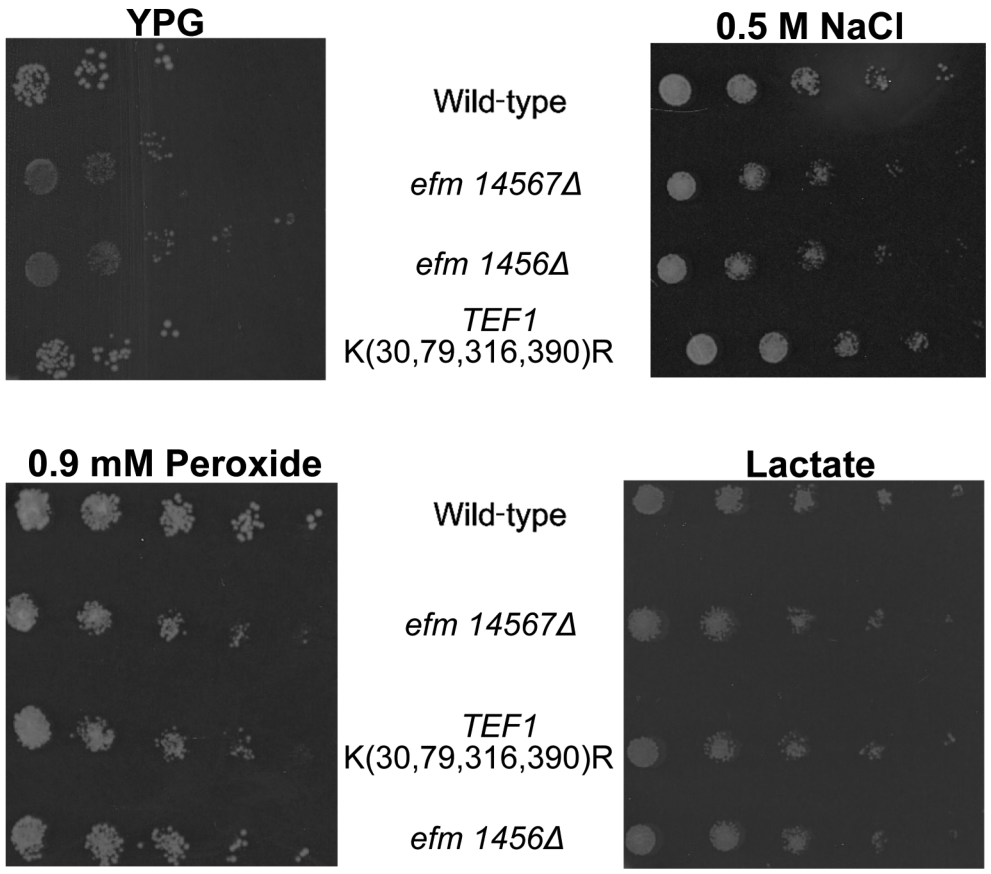

Figure 4. Loss of Efm methyltransferases causes sensitivity under different cellular stress conditions. Representative images showing yeast cells that were grown in YPD, serially diluted, and then spotted on YPD agar containing 0.5 M NaCl, 0.9 mM peroxide, YPG, or lactate medium at 30 °C as described in the legend of Figure 3. Colonies were imaged after 2 days. In YPG, colonies for the *efm1456*Δ and *efm14567*Δ mutants were significantly smaller than wild-type colonies in two of three replicate experiments, whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as the wild type in those replicates. Under oxidative stress, colonies for the *efm1456*Δ and *efm14567*Δ mutants were significantly smaller than wild-type colonies in three replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as the wild type in three replicates. In the presence of sodium chloride, mutant colonies were smaller than wild-type colonies in four replicate experiments. No difference in colony size was observed in lactate media for six replicates.

(Figure 3A). In liquid medium, we found a significantly increased doubling time of 2.1 h compared to 1.7 h for the wild type (Figure 3B). Thus, it is clear that the loss of either four or five of the EF1A methyltransferase genes, or the replacement of four of the methylated lysine residues on EF1A, results in significant decreases in the rate of growth.

We then tested the growth of the mutant strains under respiratory, osmotic, and oxidative stress conditions. When cells were grown on agar plates containing glycerol (YPG) as the carbon source or YPD plates containing 0.5 M NaCl or 0.9 mM hydrogen peroxide, the colonies of the *efm1456*Δ and *efm14567*Δ strains were markedly smaller than the wild-type strain (Figure 4). We found that colonies of the *TEF1* K(30,79,316,390)R strain on the plates were somewhat smaller than wild-type colonies under osmotic and oxidative stress conditions (Figure 4). The *TEF1* K(30,79,316,390)R colonies on YPG plates did not exhibit any difference in size compared to those of the wild type. When cells were grown on lactate plates, we observed no difference in the colony size of the mutants compared to that of the wild type (Figure 4). These results demonstrate the EF1A methylation-deficient cells are less able to adapt under at least some stress conditions. However, it is unclear why these deficient cells are able to grow as well as wild-type cells with nonfermentable carbon sources. It is possible that reduced rates of translation in nonfermentative conditions allow the EF1A methylation-deficient cells to grow at the same reduced rate as wild-type cells when EF1A function is not rate-limiting for growth.

Lastly, we assessed growth when the yeast cells were grown on YPD media containing caffeine or rapamycin (Figure 5). The *efm1456*Δ and *efm14567*Δ colonies were somewhat smaller than wild-type colonies under rapamycin growth, whereas they were significantly smaller than those of the wild type for caffeine growth (Figure 5). The colonies of the *TEF1* K(30,79,316,390)R strain on the caffeine plates grew like the wild type (Figure 5). Interestingly, we also observed smaller colonies for the *TEF1* K(30,79,316,390)R strain under both rapamycin conditions tested (Figure 5). Both rapamycin and caffeine affect protein synthesis and cellular growth through the TORC1 pathway.[24,25] Because growth under rapamycin stress was altered in the *efm1456*Δ and *TEF1* K(30,79,316,390)R strains, it suggests that there may be some interaction between methylated EF1A and the TORC1 pathway that is disrupted when EF1A is unmethylated. Alternatively, some or all of these methyltransferases may have additional methyl-accepting substrates (other than EF1A) in the TORC1 pathway.

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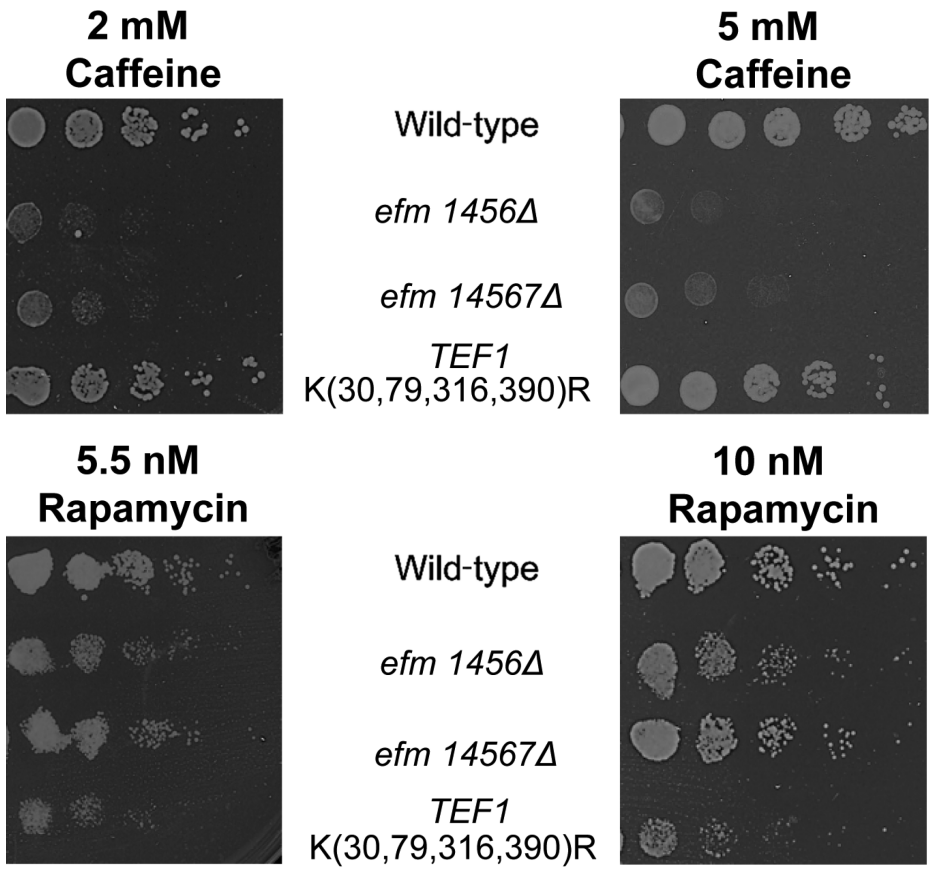

Figure 5. Methylation-deficient EF1A growth inhibited by caffeine and rapamycin. Representative images showing yeast cells that were grown in YPD, serially diluted, and then spotted on YPD agar containing 2 or 5 mM caffeine and 5.5 or 10 nM rapamycin (diluted from a 50 mg/mL stock solution in ethanol) at 30 °C as described in the legend of Figure 3. Colonies were imaged after 2−4 days. In 2 mM caffeine, colonies for the *efm1456*Δ and *efm14567*Δ mutant were significantly smaller than wild-type colonies in all four replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as the wild type in those replicates. At 5 mM caffeine, colonies for the *efm1456*Δ and *efm14567*Δ mutant were significantly smaller than wild-type colonies in all five replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as the wild type in all replicates. In the presence of 5.5 and 10 nM rapamycin, all mutant colonies were smaller than wild-type colonies in two replicate experiments each.

EF1A Methyltransferase-Deficient Cells Have Altered Sensitivity to Translation Inhibitors. A major cellular role of EF1A is bringing aminoacyl-tRNAs to the ribosomal A decoding site. To address whether this role was dependent or affected by its methylation, we first treated yeast cells with different translational inhibitors and assessed growth on YPD plates (Figure 6). With puromycin, a drug that causes premature release of the polypeptide chain from the ribosome,[26,27] tunicamycin, a drug that activates the unfolded protein response and inhibits translation,[28,29] and anisomycin, a drug that interferes with the ribosomal acceptor site,[30] we observed much smaller colonies of the *efm1456*Δ and the *efm14567*Δ strains compared to those of the wild-type strain. No decrease in cell size was seen with any of these inhibitors for the *TEF1* K(30,79,316,390)R strain (Figure 6). Finally, we detected no decrease in colony size with cycloheximide, a drug that blocks translation elongation,[31] in any of the EF1A methylation-deficient strains. These results indicate that changes in ribosomal architecture mediated by these inhibitors can affect translation more when EF1A is unmethylated, although the mechanisms for these effects are unknown.

Stability of EF1A in Methylation-Deficient Cells. We then asked if the phenotypes seen might result from changes in the level of the EF1A protein itself. We thus measured EF1A by immunoblotting whole cell lysates of wild-type and methylation-deficient strains with a polyclonal antibody specific to the entire yeast EF1A protein (Figure 7A). Quantitation of the immunoblot signal demonstrated that the deficient strains contained approximately half of the EF1A present in wild-type strains, although there was considerable variability (Figure 7B). This may explain the slowed growth rates and responses to translation inhibitors observed previously. Under these experimental conditions, it is also possible that the reduction of EF1A level in the *TEF1* K(30,79,316,390)R strain could be due to its plasmid expressing only one copy of the EF1A gene.

We considered the possibility that the absence of lysine methylation may enhance one or more ubiquitin-dependent proteolytic pathways. EF1A has been known to interact with ubiquitinated proteins to assist in ubiquitin-mediated degradation.[9] We thus examined the stability of EF1A in intact cells grown in YPD after the addition of puromycin and cycloheximide to prevent new protein synthesis. In Figure 8, we show the levels of EF1A by immunoblotting over a 2 h time course. In the puromycin chase experiment, we found that EF1A levels fell rapidly but in a similar fashion in the wild-type and mutant strains and that there was a similar loss of total protein as well as indicated by the Coomassie and Ponceau

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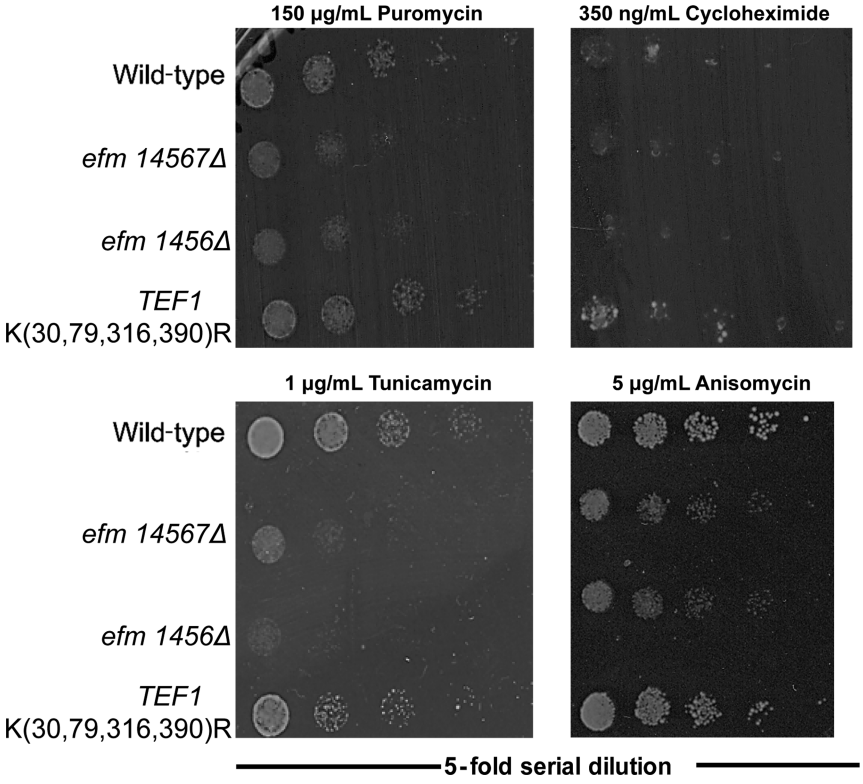

Figure 6. Loss of Efm methyltransferases and mutation of four lysine residues to arginine residues in EF1A result in differential responses to translational inhibitors. Representative image of yeast cells grown in YPD and then serially diluted onto agar plates as described in the legend of Figure 3 but supplemented with either puromycin, cycloheximide, tunicamycin, or anisomycin. In puromycin, 8 of 10 replicates for *efm14567*Δ and 10 of 12 replicates for *efm1456*Δ strain had colonies that were smaller than wild-type colonies. Colonies for the *TEF1* K(30,79,316,390)R strain always had similar sized colonies compared to wild-type colonies in 12 replicates. The growth on cycloheximide displayed no difference in colony size compared to wild-type colonies for the *efm14567*Δ mutant (four replicate experiments), the *efm1456*Δ mutant (eight replicate experiments), and the *TEF1* K(30,79,316,390)R mutant (eight replicate experiments). Tunicamycin colony sizes were always smaller than wild-type colony sizes for *efm1456*Δ (six replicate experiments) and *efm14567*Δ (two replicated experiments) but remained unchanged for the *TEF1* K(30,79,316,390)R mutant (six replicate experiments). On anisomycin plates, there were smaller colonies in four of six replicates for *efm14567*Δ and five of six replicates for the *efm1456*Δ mutant compared to wild-type colonies. The *TEF1* K(30,79,316,390)R strain had similar-sized colonies compared to wild-type colonies with the exception of two of six replicates where the colony sizes were larger.

staining. For the cycloheximide chase experiment, we also found little change in the relative loss of EF1A over 2 h in the wild-type and mutant strains, although the mutant strains had less EF1A at the zero time point and the total protein remained fairly constant. The similarity in the degradation rates of EF1A in the wild-type and mutant cells was quantitated by densitometry in replicate cycloheximide and puromycin chase experiments (Figure 8). From these data, we concluded that there was no large difference in the degradation of EF1A in the wild-type and mutant strains.

Ribosome Assembly Is Unaffected by the Loss of EF1A Methylation. Although EF1A is primarily responsible for the transport of aminoacylated tRNA to the ribosomal A site, it has been shown that it can also directly affect the assembly of the ribosomal subunits.[8] We then asked if methylation of EF1A influenced levels of ribosomal subunits, polyribosomes, or ribosomes. Panels A and B of Figure 9 show a representative experiment of the separation of ribosomal subunits in the presence of cycloheximide for wild-type and *efm14567*Δ strains, respectively. Cycloheximide is used to stall translation to capture actively translating ribosomes on a transcript to analyze the differences in the amount of small ribosome subunit (40S), large ribosome subunit (60S), single fully formed active ribosomes (80S), and polysomes (more than one active ribosome on transcript) found. We were able to clearly resolve the 40S, 60S, and 80S peaks and the polysome peaks. We found that the *efm14567*Δ cells had a reduction in the levels of 40S subunits because the 60S:40S ratio was ∼50% higher than that of wild-type cells. However, this change was not statistically significant as determined by a Student's *t* test (Figure 9C; $p = 0.16$). No significant differences were also seen when the 80S:polysome ratios were quantified (Figure 9D; $p = 0.79$). Under these conditions, it appears that methylation of EF1A is not necessary for the assembly of ribosome subunits, although we cannot rule out weak effects.

Protein Synthesis Fidelity Is Unaffected in Methylation-Deficient Cells. Lastly, we examined the translation fidelity of the methylation-deficient strains using the dual luciferase reporter system (DLR),[33−35] examining both amino acid misincorporation and programmed frame-shift errors. In these experiments, plasmids expressing fusion proteins of an N-terminal Renilla luciferase and a C-terminal firefly luciferase allow the expression of the firefly luciferase only when translational errors are made. For the frame-shift plasmids, a viral programmed frame shift is placed in the linker region, and when it is bypassed, firefly luminescence is detected.[35] Alternatively, the amino acid misincorporation plasmid has a mutation in the firefly gene itself that changes lysine 529 to an asparagine residue that results in the loss of luciferase

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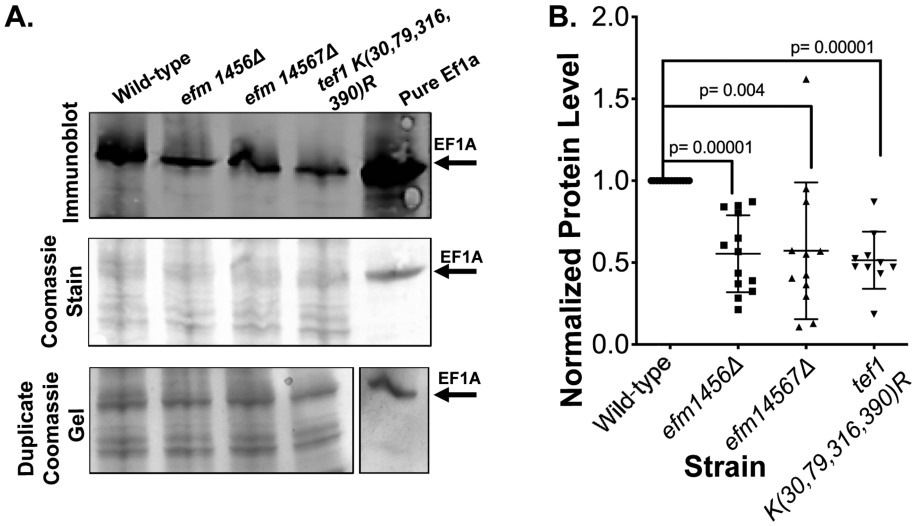

Figure 7. Loss of Efm methyltransferases and mutation of four lysine residues to arginine residues in EF1A affects protein abundance levels. Seven OD_{600} units of yeast cells was harvested after being grown in YPD medium at 30 °C, lysed using "method 2", fractionated using SDS−PAGE, and immunoblotted for antibody detection of EF1A with the LICOR secondary antibody as described in Materials and Methods. (A) Representative experiment showing a Coomassie-stained PVDF membrane, the LICOR-detected immunoblot showing EF1A protein levels, and a duplicate Coomassie-stained gel of the lysates. (B) EF1A protein expression levels determined from the comparison of peak areas of immunoblots probed for EF1A in yeast lysates. For each strain, each point represents a biological replicate. The densitometric signals for EF1A in the mutant strains were normalized to that of the wild-type strain in each experiment (making all wild-type values equal to 1) and quantified using ImageJ. Student's *t* test *p* values (unpaired, two tails) are shown.

activity.[36] As shown in Figure 10, we found no differences in the misincorporation or frame-shift rate with the *efm1456*Δ, *efm14567*Δ, or the *TEF1* K(30,79,316,390)R strains. These results suggest that the loss of methylation does not result in the loss of translational fidelity, at least in this system under normal growth conditions.

DISCUSSION

EF1A is extensively post-translationally modified across all organisms. It can be ubiquitinated at lysine residues,[37] phosphorylated at serine and threonine residues,[38,39] acetylated,[40] methyl esterified at its C-terminal lysine residue,[41] methylated at multiple lysine residues and an N-terminal glycine residue,[2,3,11,12,14,15,42] and glutaminylated at a glutamic acid residue.[43] However, the functional relevance of these EF1A modifications is largely unknown. In this study, we characterized two types of EF1A methylation-deficient yeast strains to elucidate the roles that lysine methylation of EF1A may have on its functions.

Extensive lysine methylation of EF1A is seen in a variety of eukaryotic species, including humans,[2] rabbits,[16] chickens,[44] brine shrimp,[16] corn,[16] *Arabidopsis*,[45] and the zygomycotan fungi *Mucor racemosus*[46] in addition to the ascomycotan yeast *S. cerevisiae*. However, lysine methylation of the corresponding EF-Tu protein in prokaryotes is not as extensive. *Escherichia coli* and *Pseudomonas aeruginosa* both only have one site of lysine methylation: dimethylation at Lys-57 and trimethylation at Lys-5, respectively.[47−50] Sequence analysis using BLASTp revealed no clear orthologs of yeast Efm1, Efm4, Efm5, Efm6, or Efm7 in the prokaryotic species or in *Mucor racemosus*. However, a FungiDB search revealed orthologs of Efm1, Efm4, Efm6, and Efm7 in *Mucor circinelloides*. On the other hand, there are clear orthologs for Efm4 and Efm5 in humans.[13,51]

Thus far, the functional relevance of EF1A methylation has been studied in *S. cerevisiae*,[52] *M. racemosus*,[53] *E. coli*,[54,55] *P. aeruginosa*,[48,56] chicken,[44] and humans.[42,57,58] A similar point mutant strain was used in the *S. cerevisiae* study but was unavailable, so we constructed our own. In that study, it was found that this strain was viable and had no *in vitro* difference in poly(U)-directed polyphenylalanine synthesis or GTP binding.[52] In *E. coli*, the methylation at Lys-57 was shown to affect the aa-tRNA-induced GTP hydrolysis *in vitro*.[55] Unmethylated EF1A did not affect EF1A's ability to bind GTP or the aa-tRNA in *M. racemosus*[53] or affect translation fidelity in *P. aeruginosa*.[56] Significantly, the extensive methylation characteristic to lysine residues on EF1A (approximately eight methyl groups) in *M. racemosus* was not found in the protein isolated from the spores of this organism. Additionally, *E. coli* EF-Tu was more methylated when cells were grown without nitrogen, phosphate, or carbon present.[54] These changes suggest some regulation of the prokaryotic methyltransferases under growth conditions.

This is the first study showing that the five known methyltransferases responsible for methylating EF1A in *S. cerevisiae* do not appear to have any major additional substrates. Recently, evidence of the *in vitro* methylation of an EF1A-derived peptide containing Lys-253 in *S. cerevisiae* by Efm1 was presented.[59] This lysine residue is found in a sequence motif similar to that of the Efm1 Lys-30 site. It is possible that methylation at Lys-253 could be contributing to the monomethylation peak observed in the cation exchange chromatography of the *TEF1* K(30,79,316,390)R mutant. However, whether this site is definitively methylated *in vivo* is not known.

We tested the ability of our *S. cerevisiae* strains to adapt to changing environments. As described above, the methylation of both *M. racemosus* and *E. coli* is dependent upon the stage of growth and nutritional status.[53,60] We did observe increased sensitivity of our yeast methyltransferase mutant strains compared to the wild-type strain when grown with glycerol as a carbon source, or under oxidative and osmotic stress conditions. However, growth is also impaired in the mutant

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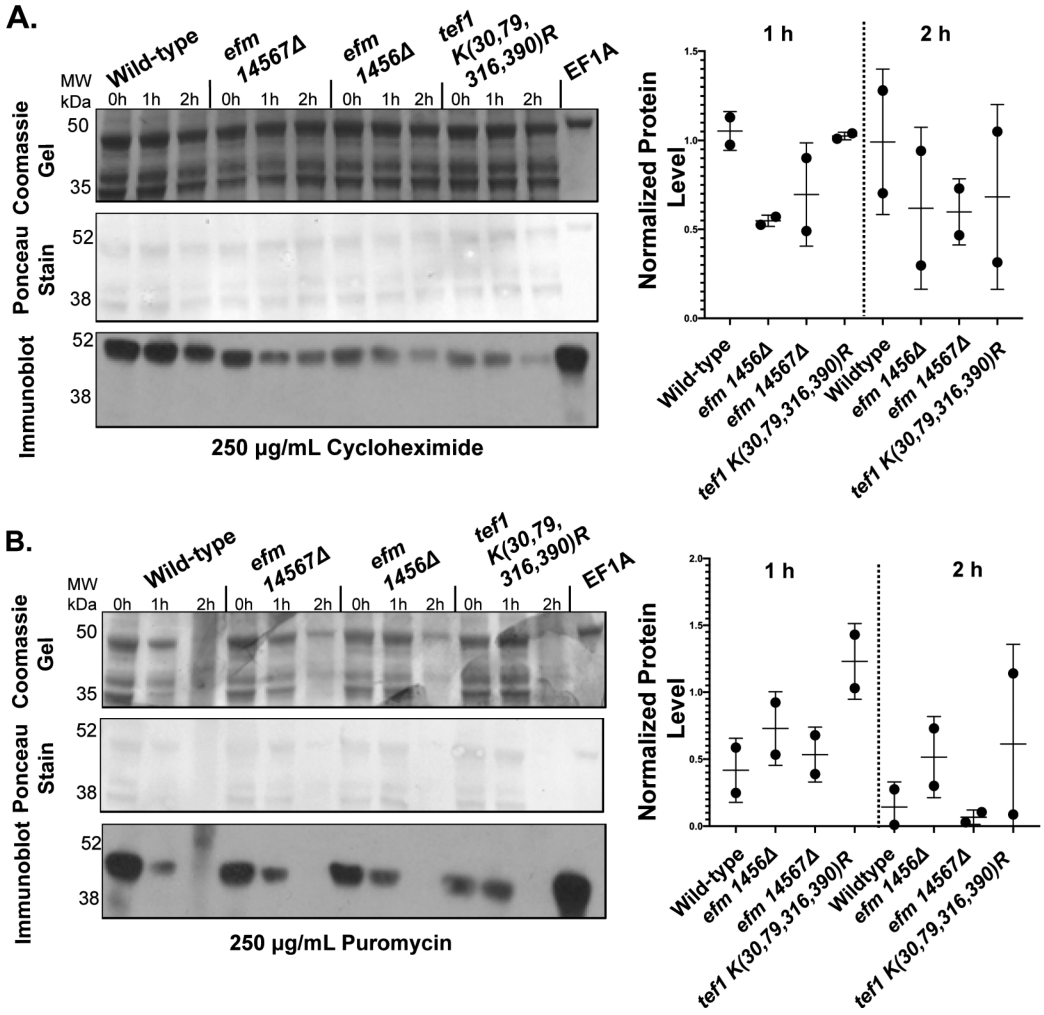

Figure 8. EF1A protein levels remain equally stable in the presence of cycloheximide and puromycin in wild-type cells and in cells deficient in EF1A methylation. Yeast cells were grown to an OD_{600} of ~0.7 in YPD medium at 30 °C. (A) Cycloheximide or (B) puromycin was then added individually to 3 mL of cells containing 7.5 OD_{600} units to a final concentration of 250 µg/mL. Aliquots (1 mL) of the 7.5 OD_{600} cells were collected at the indicated times per strain and drug condition and lysed (using method 2), and then the proteins were fractionated by SDS−PAGE as described in Materials and Methods. A representative gel and immunoblot are shown for both conditions. The top panel shows a Coomassie-stained gel. The middle panel is a Ponceau S-stained PVDF membrane from a duplicate gel. An immunoblot using antibodies to EF1A is shown in the bottom panel. This experiment was performed twice for cycloheximide and twice for puromycin. The relative EF1A protein levels in each wild-type or mutant strain at the 1 or 2 h time points compared to the EF1A level of the same strain at the zero time point were quantified using ImageJ densitometry and are shown to the right of its respective drug condition.

strains compared to the wild-type strain in YPD medium. Therefore, these stress-induced phenotypes we are seeing may not be a specific result of respiratory growth or environmental stress. We should point out that subtle growth differences may be masked in these assays. For example, the *TEF1* K-(30,79,316,390)R mutant did not appear to have a reduced level of growth on solid medium but a slight reduction in the level of growth was observed in liquid medium. On the other hand, all of the EF1A methylation-deficient mutant strains had a reduced level of growth in the presence of rapamycin and caffeine. These effects on growth may to be due to stress-induced phenotypes of unmethylated EF1A in the TORC1 pathway.[24] It is also possible that one or more of the Efm methyltransferases Efm1 and Efm4−7 can methylate non-EF1A substrates that affect TORC1 signaling. Further examination of the individual methyltransferase knockout strains may be useful in distinguishing these possibilities.

From our examination of EF1A protein levels, we found that the mutant strains had significantly less EF1A present. This protein expression phenotype appears to be an additive effect of methyltransferase loss because single-knockout methyltransferase mutations in yeast did not have alterations of EF1A protein levels (data not shown). In the prokaryote *P. aeruginosa*, loss of the single EftM methyltransferase does not result in the marked reduction in the level of Ef-Tu.[56] In yeast, it is unclear how the loss of EF1A methylation affects its protein abundance because we showed that the rate of degradation in the presence of cycloheximide or puromycin is unaltered in our methylation-deficient strains. On the other hand, the overexpression of EF1A also does not affect global translation efficiency.[61] Thus, it appears translation fidelity is independent of the amount of EF1A present.

Interestingly, although less EF1A protein is present in methylation-deficient strains, the translational function of EF1A remains apparently unimpaired. When amino acid incorporation and programmed frame shifts were measured by the dual luciferase translational fidelity assay system and ribosome assembly was assessed using polysome analysis, the

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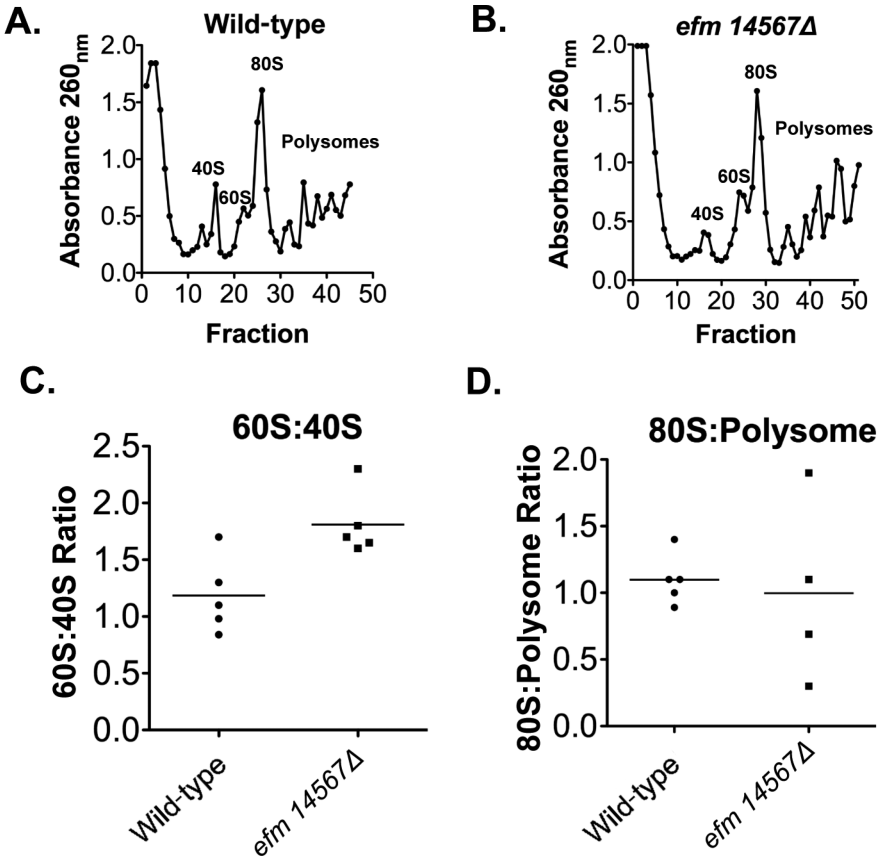

Figure 9. Deletion of EF1A methyltransferases Efm1 and Efm4−7 does not affect ribosome assembly. Ribosomes were prepared from yeast cells grown to an OD_{600} of ∼0.7 as described previously[32] and analyzed with the modifications described below. The top panels (A and B) show the fractionation of ribosomes by sucrose gradient centrifugation of 7 A_{260} units. In each case, 100 μL fractions were collected and the A_{260} value of each fraction was plotted. The absorbance of each of the peaks was summed to quantify the ratio of the 80S to polysome ribosomal subunits (C) and the ratio of the 60S to 40S subunits (D) with the mean value indicated by the horizontal line.

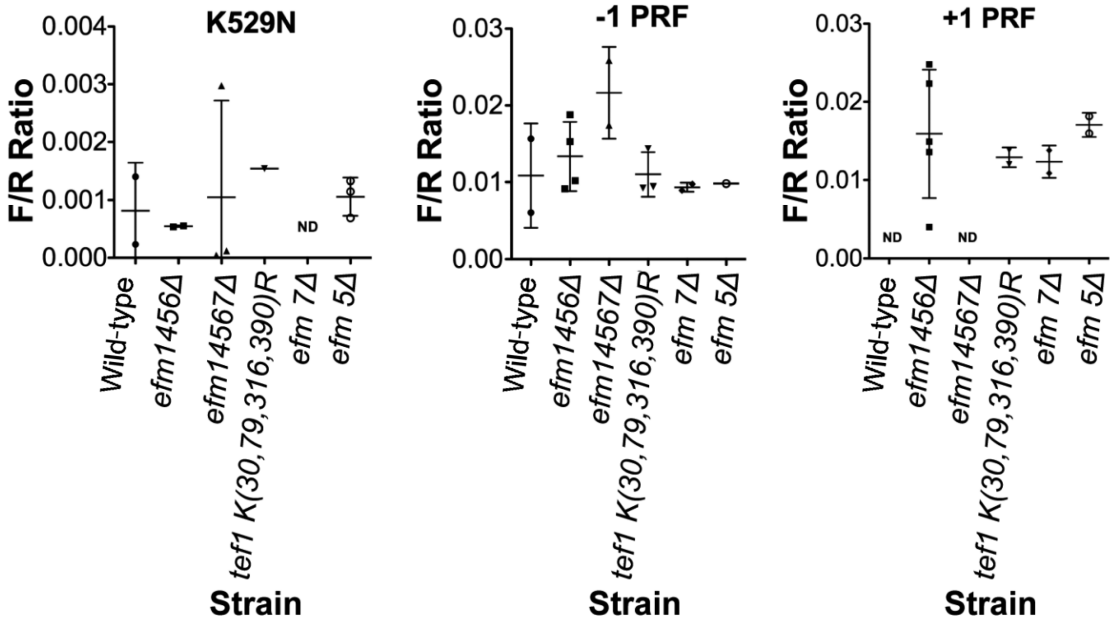

Figure 10. Loss of Efm methyltransferases and mutation of four lysine residues to arginine residues in EF1A does not affect translation fidelity. Yeast cells were prepared as described in Materials and Methods. Ratios of firefly and Renilla luciferase luminescence values are shown with each point representing a biological replicate. There was no statistical difference in the ratios with any of the strains shown here. ND, not done. The *efm5* and *efm7* mutants were used here to show there was no effect in single-deletion strains, as well.

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methylation-deficient strains performed like the wild-type strain. There may be compensatory mechanisms in our mutant strains that allow translational functions with reduced EF1A levels.

CONCLUSIONS

We have shown that the five protein lysine methyltransferases that modify elongation factor 1A in *S. cerevisiae* (Efm1 and Efm4−7) are not essential to the viability of yeast. However, their loss results in slow growth and a particular sensitivity to caffeine and rapamycin, inhibitors of the Tor1 protein kinase component of the TORC1 signaling complex. Further work will be required to establish the mechanism(s) of these effects. The loss of these methyltransferases did not affect the fidelity of translation or the assembly of ribosomal subunits. We present evidence that EF1A is the major if not the sole substrate for these five methyltransferases. It appears that the fine-tuning of EF1A function by modification at five distinct sites by five distinct methyltransferase enzymes optimizes cell physiology.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.9b00818.

Immunoprecipitation of EF1A from methylation-deficient cells (Figure S1) (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Protein methylation and translation: Role of lysine modification on the function of yeast elongation factor 1A

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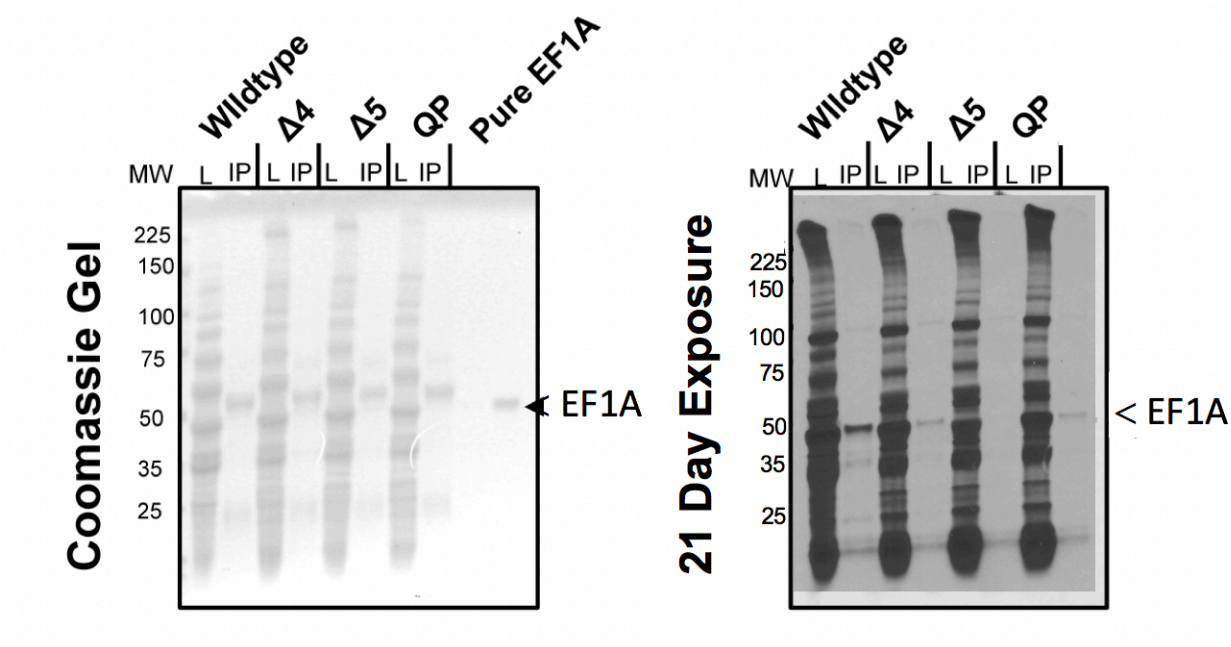

Figure S1: Immunoprecipitation of EF1A from methylation-deficient cells shows specificity of elongation factor methyltransferases. This is a replicate experiment of that shown in Figure 2. Yeast cells from wild-type and mutant strains were labeled with *S*-adenosyl-[*methyl*-^{3}H] methionine. This experiment was performed exactly as described in Figure 2 with the addition of purified unlabeled EF1A included on the gel as a control. Samples of total lysate (L), immunoprecipitated EF1A (IP), and pure EF1A were subjected to SDS-PAGE. The prominent Coomassie-stained band in the immunoprecipitated lanes migrates more slowly than EF1A (left panel), suggesting that it corresponds to the heavy chain of the antibody. The fluorograph is shown on the right panel. The symbols Δ4 and Δ5 represent lysates from the *efm1456Δ* and *efm14567Δ* strains respectively and QP represents lysates from the *tef1*K(30,79,316,390)R strain.