Title

A Quest for Purpose: Investigating the Functional Implication of the Methylation of Proteins Involved in Translation

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A Quest for Purpose: Investigating the Functional Implication of Methylation of Proteins Involved in Translation

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biochemistry, Molecular and Structural Biology

by

Jonelle White

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ABSTRACT OF THE DISSERTATION

A Quest for Purpose: Investigating the Functional Implication of Methylation of Proteins Involved in Translation

by

Jonelle White

Doctor of Philosophy in Biochemistry, Molecular and Structural Biology

University of California, Los Angeles, 2019

Professor Steven G. Clarke, Chair

Methylation is an essential post translational modification that can be found on a variety of proteins in higher and lower species. The effects of these modifications are diverse and include targeting genes for activation or silencing and protein stability. The most extensively characterized methylation reaction is that of histone tails. In the past decade researchers became interested in identifying non-histone protein substrates and the enzymes responsible for methylating them (methyltransferases). In the yeast *Saccharomyces cerevisiae,* a substantial number of methyltransferases have been identified for modifying mitochondrial proteins, translational release factors, ribosomal proteins and translational elongation factors.

When I started my doctoral journey the newest methyltransferase of the yeast translational apparatus was found to modify elongation factor 1 alpha (EF1A), the protein responsible for bringing the aminoacyl-tRNA to the ribosome. This enzyme, Efm7, was the fifth

elongation factor methyltransferase (Efm) responsible for methylating EF1A! EF1A methylation by five methyltransferases is unique as other elongation factors, EF2 and EF3, only have two methyltransferases that modify them both. Additionally, each of these five EF1A methyltransferases appears to target specific residues on EF1A. This has not been seen in any other protein of the translational apparatus that is methylated. Thus I was captivated by this uniqueness of EF1A methylation.

Although it is known that EF1A and other translational proteins are methylated and the enzymes responsible for its methylation, we still don't know why it is methylated. GTP-bound EF1A functions as the courier of aminoacylated tRNA to the ribosome. When a correct codon-anticodon match is made, a conformational change in the ribosome results in the hydrolysis of GTP to GDP and the release of EF1A. Additionally, GDP bound-EF1A functions outside of the ribosome including being able to bind and bundle filamentous actin. This dissertation focused on identifying whether or not there was a direct link between methylation and functionality of EF1A. In a separate study, I examined the functional role of ribosomal protein methyltransferases.

My studies lead to the development and characterization of the first quintuplet knockout yeast strain for the enzymes responsible for methylating EF1A. These strains have been confirmed to be methylation deficient at the respective EF1A lysine sites, using mass spectrometry. I found that EF1A methylation appears to be necessary for helping yeast to survive and adapt to differences in its cellular environment but may not be necessary for the integrity of the actin cytoskeleton as well as EF1A induced actin binding.

The dissertation of Jonelle White is approved.

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DEDICATION

To Kareem- Thank you for being my rock, practice audience, and cheer squad these past 5 years. Your love gave me fuel when I felt I was running on empty.

To my parents- The research bug would not have bitten me if you guys had not nurtured my inquisitiveness as a child. Thank you for believing in me and encouraging me to pursue whatever my heart desired.

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My entire project would not have been possible without the strains generated by the Guillaume Chanfreau lab by Dr. Kevin Roy, Dr. Jason Gabunilas and Charles Wang. These strains were used in all the chapters of this dissertation. My collaboration with Kate Liu in the Joseph Loo laboratory was integral in confirming the EF1A methylation deficiencies of our mutant strains in chapter 4. Lastly, my venture into the world of the actin cytoskeleton was only possible due to guidance from Dr. Will Silkworth and Alex Bradley from the Margot Quinlan laboratory and Dr. Elena Grintsevich from the Emil Reisler laboratory. I am grateful to all of these people for their collaboration.

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J. T. White, T. Cato, N. Deramchi, J. Gabunilas, K. R. Roy, C. Wang, G. F. Chanfreau & S. Clarke. Protein methylation and translation: Role of lysine modification on the function of yeast elongation factor 1. *Manuscript in preparation*.

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J.White, N. Deramchi, T. Cato, J. Gabunilas, G. Chanfreau, and S.G. Clarke. Protein methylation and translation: Role of lysine modification on the function of yeast elongation factor 1 alpha. Presented at the American Society for Biochemistry and Molecular Biology Conference, San Diego, CA, 2018

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J. White, C. Theimer. Characterization of the *Saccharomyces cerevisiae* telomerase RNA pseudoknot. Presented at the McNair Research Conference, Niagara Falls, NY, 2013

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Chapter One

Plan of the Dissertation

Life as we know it is governed by the central dogma; DNA makes RNA, RNA makes protein. Our complex genetic information is encoded in DNA which is transcribed to a simpler format, RNA, that is then translated to proteins. Humans have about 20,000 protein-encoding genes but, there are more than 20,000 distinct proteins present in our cells (1). This discrepancy in protein count arises in part due to the complexity of RNA processing. Alternative splicing, polyadenylation, pre-RNA editing, and alternative translation initiation or transcription initiation sites give rise to a great variety of mRNA transcripts that are correlated with a great variety of proteins (2).

Proteins are the working force of cellular activity. However, due to the work demand, organisms developed a method to delegate who does what and when, through the addition of post-translational modifications that create even a wider variety of protein species. Enzymes are responsible for adding functional groups such as ubiquitin, phosphate or acetyl groups to modulate protein function and stability. One modification in particular, methylation, has emerged as a prevalent modification comparable to phosphorylation and acetylation as an essential regulator of many cellular processes (3–7).

Methylation is an enzymatic post-translational modification where the methyl (CH_3) group from the methyl donor *S*-adenosylmethionine is added to a wide variety of substrates, such as proteins, RNA, and DNA. The most well characterized type of protein methylation is for the modification of histone tails. These modifications are crucial regulators of transcriptional activity (8, 9). However a plethora of non-histone protein substrates have also been identified. In *Saccharomyces cerevisiae*, there are 166 methyltransferases found when methyltransferase genes are searched in the *Saccharomyces* genome database. About 70-80 are dependent on *S*-adenosylmethionine and have a known substrate with a majority being involved in some aspect

of protein synthesis (10, 11).

Protein synthesis can be summarized as having three steps. The first is initiation where the ribosome assembles with the help of ribosomal proteins and then binds to the mRNA with initiation factors. The second is elongation where the ribosome links the proper amino acids encoded in the mRNA forming the protein polypeptide chain. The last step of translation is termination where the ribosome is signaled to stop, allowing the newly made protein to be released.

Since the identification of translational methyltransferases and their substrates researchers have become more interested in investigating the relevance of these methylation reactions for their respective functions. Most recently in *Saccharomyces cerevisiae*, it has been seen that methylation of ribosomal protein Rpl3 by the histidine methyltransferase Hpm1 is needed for proper 60S ribosome subunit assembly and methylation of elongation factor 2 and 3 by Efm2 and Efm3 for fine tuning yeast growth under translation inhibition (12, 13). These phenotypes suggest that methylation of a protein involved in synthesis can act as a modulator of the cell's response in a change in environment as well as to maintain interactions responsible for stability/ functioning of the ribosome during translation.

Interestingly when my journey began in 2015, there were 9 out of 10 ribosomal methyltransferases in *Saccharomyces cerevisiae* whose purpose had yet to be elucidated. In chapter 2 of this dissertation the phenotypes associated with the individual loss of each ribosomal methyltransferases were assessed. This work was done in conjunction with a recent graduate at the time, Dr. Qais Al-Hadid. Dr. Al-Hadid measured differences in ribosome assembly and translation fidelity while I examined changes in yeast growth in response to the translational inhibitors that bind the ribosome. Ribosome assembly for this experiment was defined as

differences in the amount of small and large subunits found on a mRNA and translation fidelity defined as the ability to recognize errors that were programmed into a fusion plasmid. We found that like Hpm1-deficient cells, loss of four of the nine other ribosomal protein methyltransferases resulted in defects in ribosomal biogenesis. Yeast cells from all mutants tested were unable to recognize important translation termination signals whereas 7 out of 9 yeast strains incorrectly incorporated the wrong amino acid into its protein polypeptide. All of the mutant strains exhibited a growth resistance to the ribosome inhibitors anisomycin and/or cycloheximide in plate assays, but not in liquid culture.

Upon completion of the ribosomal project I explored the functional roles of the lysine methylation of the highly conserved and essential protein, elongation factor 1 alpha (EF1A). It is largely unknown as to why EF1A is methylated. Specifically, in *S. cerevisiae* only one publication from 1997 started to address this (14). Functional relevancy of EF1A methylation has also been investigated in *M. racemosus* (15), *E. coli* (16, 17), *P. aeruginosa* (18, 19), chicken (20) and humans (21–23). There are only 12 known yeast lysine methyltransferases involved in translation. Remarkably five of those methyltransferases (Efm 1, Efm4-7) appear to function solely to modify EF1A at five distinct sites. Compared to its comrades in elongation, EF1A is unique since EF2 and EF3 are both modified by Efm2 and Efm3.

Functionally, EF1A is mainly responsible for bringing the aminoacylated tRNA to the acceptor site of the large ribosomal subunit during protein synthesis. Once the correct tRNA/mRNA pairing is formed a conformational change in the ribosome is induced allowing for EF1A to leave the ribosome and be recycled (24). EF1A has also been implicated in having a direct interaction with ribosomal subunits and aiding in ribosome assembly (25). Additionally,

EF1A has non-canonical roles in apoptosis, actin bundling/polymerization, nuclear export, ubiquitin dependent degradation and viral functions (26–31). Structurally, three catalytically active domains characterize EF1A: A GTP binding domain, an aminoacyl-tRNA binding domain and an actin-binding domain. Three of the methylated lysine's (Lys-3, Lys-30, and Lys-79) are in the GTP-binding domain, one (Lys-316) in the aminoacyl-tRNA binding domain, and one site (Lys-390) in the actin-binding domain (31–33).

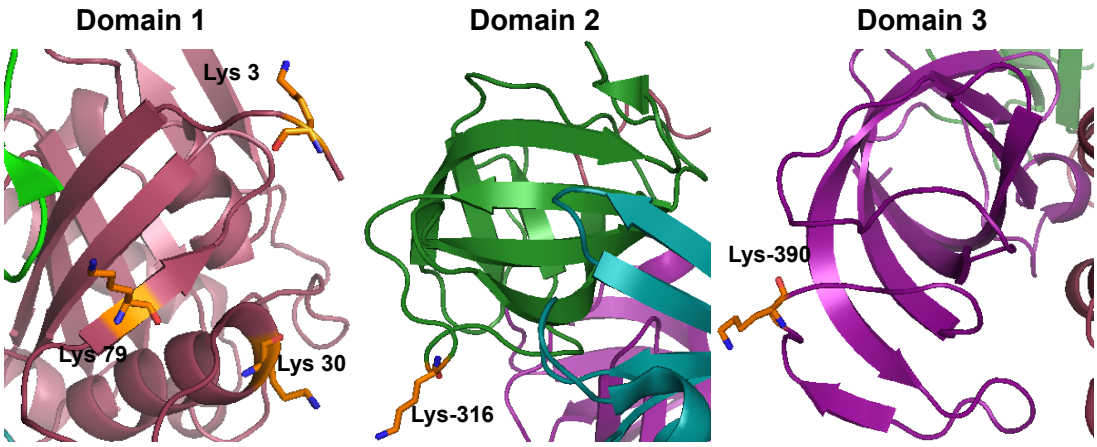

Figure 1-1: Functional Domains of *S. cerevisiae* EF1A. A Images were made from the EF1A crystal structure PDB file 1F60. Domain 1 (GTP binding, dark pink) is residues 2-243, domain 2 (aa-tRNA binding, green) is residues 244-333 and domain 3 (actin binding, purple) is residues 334-441. Lysine residues that are methylated are shown in orange.

In chapter 3, I examine the effect of EF1A methylation deficiency on its translation-associated roles when the methyltransferases for EF1A are knocked out or when the non N-terminal lysine residues are mutated. This work led to the generation of the first quintuplet knockout yeast strain for the enzymes responsible for methylating EF1A from a collaboration with Kevin Roy, Jason Gabunilas, Charles Wang, and Professor Guillaume Chanfreau. We found that the loss of these specific lysine residues appears to modulate yeast growth in response to

cellular stress since we observed decreased growth rates in the mutant strains under conditions such as osmotic and oxidative stress. Additionally, in the mutant strains we saw a significant decrease in the amount of EF1A protein detected by western blot, which surprisingly did not lead to a reduction in translation fidelity.

In collaboration with Kate Liu and Professor Joseph Loo, I was able to take a dive into EF1A's methyl proteome in chapter 4. We were able confirm the methylation deficiency of our mutant strains and identify potential novel methylated residues, specifically Lys 253 where an EF1A peptide containing this site has recently been shown to be methylated *in vitro* (34). We were able to consistently confirm the presence of the novel lysine methylation sites by bottom up analysis of enzyme digested peptides. However we were not able to confirm these sites with our preliminary heavy methyl SILAC labeling experiment.

The last chapter starts to delve into one of EF1A's non-canonical functions, its ability to bind and bundle actin. I investigated how this interaction is impacted with methylation-deficient EF1A. It still remains unclear how methylation-deficient EF1A interacts with the actin cytoskeleton but preliminary experiments suggest EF1A induced actin bundling but not binding may be altered.

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Chapter Two

Ribosomal Protein Methyltransferases in the Yeast *Saccharomyces cerevisiae*: Roles in Ribosome Biogenesis and Translation

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Ribosomal Protein Methyltransferases in the Yeast *Saccharomyces cerevisiae*: Roles in Ribosome Biogenesis and Translation

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Keywords: ribosomal protein, ribosome biogenesis, translation elongation, translation fidelity, aminoacyl-tRNA, peptidyl transferase

Abbreviations: Hpm1, Histidine protein methyltransferase 1; Ntm1, N-terminal methyltransferase 1; Rkm1, ribosomal lysine methyltransferase 1; Rkm2, ribosomal protein methyltransferase 2; Rkm3, ribosomal protein methyltransferase 3; Rkm4, ribosomal protein methyltransferase 4; Rkm5, ribosomal protein methyltransferase 5; Rmt1, arginine methyltransferase 1; Rmt2, arginine methyltransferase 2; Sfm1; spout family methyltransferase 1; A-site, aminoacyl site; P-site, peptidyl-tRNA site; E-site, exit site; PTC, peptidyl transferase center; -1 PRF, programmed -1 ribosomal frameshifting; AdoMet, *S*-adenosyl-L-methionine; [*methyl*-^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]-L-methionine.

ABSTRACT

A significant percentage of the methyltransferasome in *Saccharomyces cerevisiae* and higher eukaryotes is devoted to methylation of the translational machinery. Methylation of the RNA components of the translational machinery has been studied extensively and is important for structure stability, ribosome biogenesis, and translational fidelity. However, the functional effects of ribosomal protein methylation by their cognate methyltransferases lag far behind. Previous work has shown that the ribosomal protein Rpl3 methyltransferase, histidine protein methyltransferase 1 (Hpm1), is important for ribosome biogenesis and translation elongation fidelity. In this study, we aimed to uncover the functional roles of the nine other known ribosomal protein methyltransferases in *S. cerevisiae*. Yeast strains deficient in each of the ten ribosomal protein methyltransferases in *S. cerevisiae* were examined for potential defects in ribosome biogenesis and translation. Like Hpm1-deficient cells, loss of four of the nine other ribosomal protein methyltransferases resulted in defects in ribosomal subunit synthesis. All mutant strains exhibited elevated polysome levels and most were significantly resistant to the ribosome inhibitors, anisomycin and cycloheximide. Translational fidelity assays measuring stop codon readthrough, amino acid misincorporation, and programmed -1 ribosomal frameshifting, revealed that eight of the ten enzymes are important for translation elongation fidelity and the remaining two are necessary for translation termination efficiency. Altogether, these results demonstrate that ribosomal protein methyltransferases in *S. cerevisiae* are playing important roles in ribosome biogenesis and translation.

INTRODUCTION

Translational components are modified by the addition of one or more methyl groups, in all domains of life, by structurally related enzymes called methyltransferases. These

modifications occur on components of the translational machinery including: ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), translation factors, and ribosomal proteins (1–5). More than half of the known methyltransferases in the yeast *Saccharomyces cerevisiae* modify these ribosomal components, suggesting that methylation of translational components is important for cellular function (6). Methylation of the RNA components of the ribosome is important for ribosome synthesis, structure stability, and translational fidelity (3, 4, 7, 8). Similarly, methylation of elongation and release factors has been demonstrated to be important for translational fidelity and termination efficiency (5, 9). However, the functions of methyltransferases responsible for ribosomal protein methylation are not well characterized and little has been done to uncover their biological activities.

We previously showed that the yeast methyltransferase, Hpm1, plays an important role in ribosome biogenesis and translation (10). Cells deficient in Hpm1 exhibited defects in 60S large ribosomal subunit synthesis and decreased translation elongation fidelity (10). To determine if the nine other known ribosomal protein methyltransferases in *S. cerevisiae* are playing similar roles as Hpm1, we investigated the consequences of depleting each ribosomal protein methyltransferase on ribosome biogenesis and translation. We show that half of the enzymes are important for ribosome biogenesis as their loss resulted in altered levels of ribosomal subunits. Interestingly, all ribosomal protein methyltransferase-deficient strains exhibited higher levels of polysomes and almost all showed higher resistance to the ribosomal inhibitors anisomycin and cycloheximide. These results suggested that these strains may have translational defects and therefore, we tested for potential defects in translational accuracy. Loss of seven of the nine methyltransferases resulted in increased readthrough of stop codons and amino acid misincorporation, whereas, loss of the two other enzymes resulted only in increased stop codon

readthrough. Additionally, all mutant strains exhibited higher frequencies of programmed -1 ribosomal frameshifting (-1 PRF). This suggests that all the ribosomal protein methyltransferases in *S. cerevisiae* are necessary for accurate translation elongation or termination. Altogether, these data show that ribosomal protein methyltransferase in *S. cerevisiae* are important for ribosome biogenesis and translation.

MATERIALS AND METHODS

Strains and growth media – All strains used are in the BY4742 background (*MATα, his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) obtained from the Open Biosystems yeast knockout collection (Thermo Scientific). All strains contain a kanamycin resistance marker in place of the open reading frame of each ribosomal protein methyltransferase gene. Yeast strains were grown in 1% yeast extract, 2% peptone, and 2% dextrose (YPD, Difco) or minimal synthetic defined media lacking uracil (SD –ura) containing 0.17% yeast nitrogen base without ammonium sulfate and amino acids (BD Biosciences), 0.077% complete supplement mixture without uracil (MP Biomedicals; 114511212), 0.5% ammonium sulfate (Acros; 423400010), and 2% dextrose (Fisher; D16-1).

RESULTS

Loss of yeast ribosomal protein methyltransferases results in abnormal ribosomal subunit levels and increased polysomes.

In prior work, we showed that the protein histidine methyltransferase, Hpm1, in *S. cerevisiae*, is needed to promote normal ribosome biogenesis (10). We sought to determine if loss of the other yeast ribosomal protein methyltransferases results in defects in ribosomal subunit levels and/or translation by polysome profile analysis. Lysates were prepared from wild

type and each of the ten mutant strains and ribosomal components separated by sucrose density ultracentrifugation. Alterations in the levels of small (40S) and large (60S) ribosomal subunits, intact ribosomes (80S), and polyribosomes (polysomes) indicate defects in ribosome biogenesis and/or translation. Like Hpm1-deficient cells, loss of Rkm1, Ntm1, Rmt1, or Rmt2 resulted in a deficit of 60S subunits (Fig. 1A). This defect in 60S biogenesis is highlighted by a significant decrease in the free 60S/free 40S subunit ratio in these strains, compared to wild type (Fig. 1B). Ntm1 was previously shown to be important for 60S biogenesis (11). Loss of Rkm3 and Rkm4, on the other hand, resulted in slightly increased levels of 60S subunits and consequently, increased free subunit ratios (Fig. 1A, 1B). Loss of Rkm2, Rkm5, or Sfm1 had no impact on the levels of ribosomal components, suggesting they are not required for ribosomal subunit synthesis (Fig. 1A, 1B). These data suggest that in addition to Hpm1, the following methyltransferases are important for normal levels of ribosomal subunits: Rkm1, Rkm3, Rkm4, Ntm1, Rmt1, and Rmt2.

Interestingly, all ribosomal protein methyltransferase-deficient strains had an increased proportion of polysomes, compared to wild type (Fig. 1A). This increase in the polysome fraction is evident in the higher polysome/80S ratios in these strains (Fig. 1C). Polysomes are usually engaged in active translation and therefore increased polysomes should result in higher translational output and cellular proliferation. However, growth of these mutant strains on agar plates or liquid media (data not shown) was similar to wild type, suggesting that the increased polysome levels was not resulting in increased translational activity. Interestingly, studies have shown a correlation between increased levels of polysomes, ribosome transit time on mRNAs, and defects in translation (12, 13). Transit time corresponds to the time after translation initiation for the ribosome to complete polypeptide synthesis and peptide release. Defects in

translation elongation or termination are therefore expected to increased mRNA transit times and polysome levels. It is thus likely that the increased levels of polysomes in the ribosomal protein methyltransferase mutants are caused by defects in translation elongation or termination.

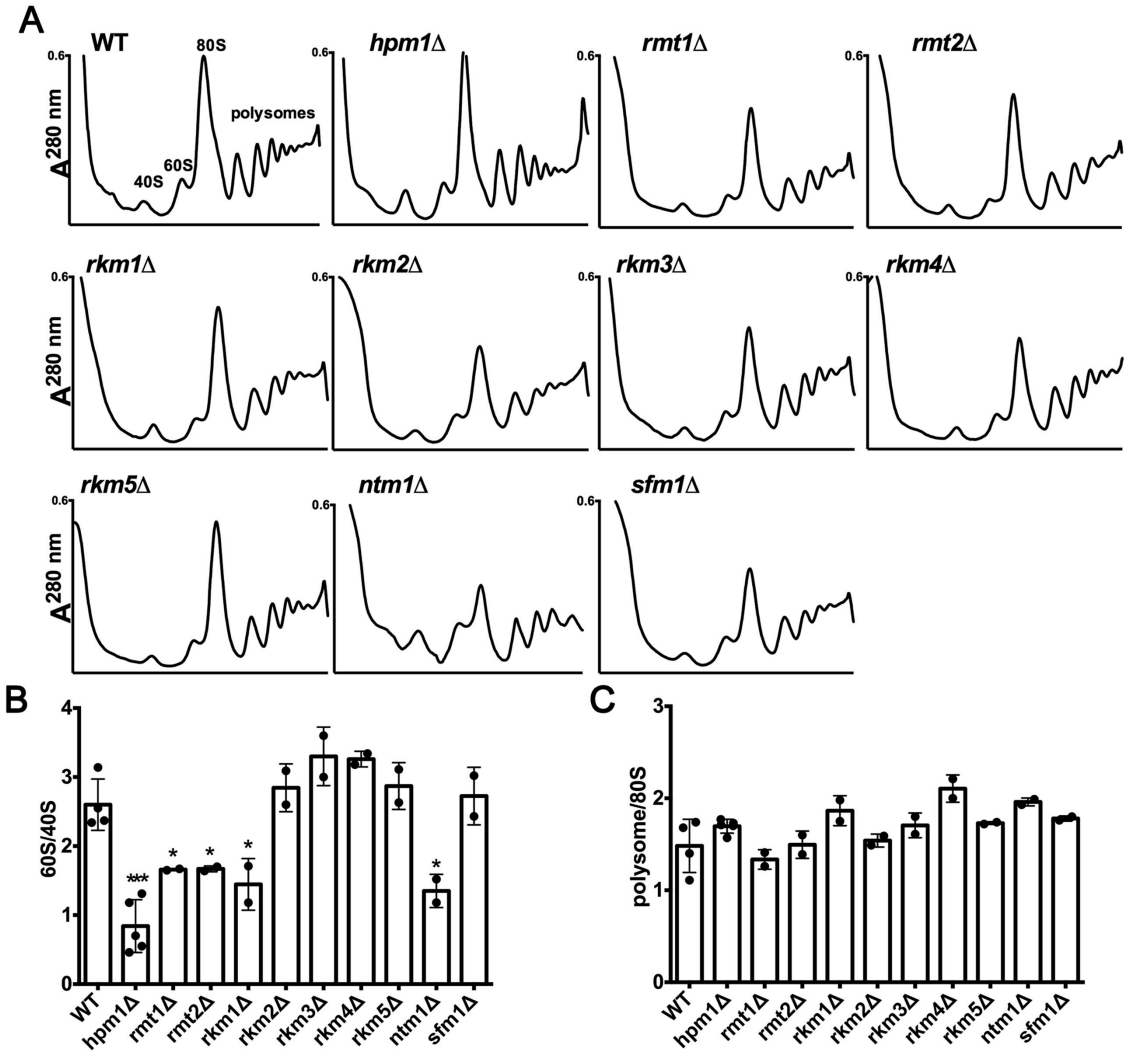

FIG. 2-1. Cells lacking ribosomal protein methyltransferases in S. cerevisiae have altered levels of ribosomal subunits. (A) Polysome profile analyses of wild type and cells deficient in each of the ribosomal protein methyltransferases in S. cerevisiae was done as described previously [12]. A total of four independent profiles were obtained for wild type cells, five profiles for hpm1Δ cells, and two profiles for each of the other strains. The profiles for wild type

and hpm1Δ cells were previously reported [13]. A representative profile for each strain is shown here. **(B)** Ribosomal subunit levels were determined by directly weighing cutouts of half peak areas (assuming Gaussian-like distribution of ribosome particles) from printer paper using an analytical balance. Error bars represent standard deviation of two or more independent profiles. Unpaired t-test two-tailed p-values were calculated to determine significant differences between wild type and each of the ribosomal protein methyltransferase mutants. hpm1Δ $p = 0.0002$, rmt1Δ $p = 0.028$, rmt2Δ $p = 0.0292$, rkm1Δ $p = 0.0232$, ntm1Δ $p = 0.0137$. **(C)** Levels of intact ribosomes (80S) was determined by directly weighing half the peak area, assuming Gaussian-like distribution, and polysome levels were determined by weighting the peaks after 80S. Error bars represent standard deviation of two or more independent profiles. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Cells deficient in ribosomal protein methyltransferases are resistant to the A-site and E-site ribosome-binding drugs, anisomycin and cycloheximide, respectively.

Next, we tested if the defects in ribosomal subunit biogenesis and/or the elevated levels of polysomes in the ribosomal protein methyltransferase mutants correlate with altered sensitivities to ribosome-binding drugs. Altered sensitivities to these drugs may indicate structural and/or functional distortions to the regions that these drugs bind. Drugs that bind to different functional centers of the ribosome were used including: anisomycin, paramomycin, and cycloheximide. Anisomycin binds to the A-site of the ribosome and acts as a competitive inhibitor of aminoacyl-tRNAs (14). Paramomycin binds to the decoding center of the small subunit and induces translational errors (15). Cycloheximide binds to the E-site of the large subunit and inhibits translation elongation (16). Previously, we showed that Hpm1-deficient

cells have enhanced resistance to cycloheximide in plate assays, suggesting alterations to the A and E-sites of the large ribosomal subunit of *hpm1*Δ cells (10). Remarkably, we were able to now show that all of the mutant strains demonstrated increased resistance to cycloheximide in plate assays with the exception of *rkm4*Δ (Fig. 2). Increased sensitivity of *rkm4*Δ strains to cycloheximide has previously been reported (17, 18). No differences in sensitivity of the mutants were seen on paramomycin-containing agar plates, compared to wild type (data not shown). In the presence of anisomycin, we also observed increased resistance in the ribosomal methyltransferase mutants with the exception of *hpm1*Δ cells. The latter result is in accord with a previous study (10). The similar resistance phenotype of many of these strains to anisomycin and cycloheximide indicates a defect at a common functional step, likely in translation elongation or termination, rather than a common structural distortion at the A-site and E-site of the large subunit. We also measured growth rates in liquid culture of wild type, *rkm4*Δ, and *hpm1*Δ cells (Fig. 3). Interestingly, we observed a slightly faster growth rate of the *hpm1*Δ cells in YPD compared to wild type cells. In the presence of cycloheximide, we detected no significant differences in growth rates, although both *rkm4*Δ,and *hpm1*Δ appeared to grow more slowly than wild type cells. Finally, we found highly variable growth rates in different experiments with anisomycin. The reason for this is not clear at present, although we do note that the four experiments with the most rapid growth all were with *rkm4*Δ cells.

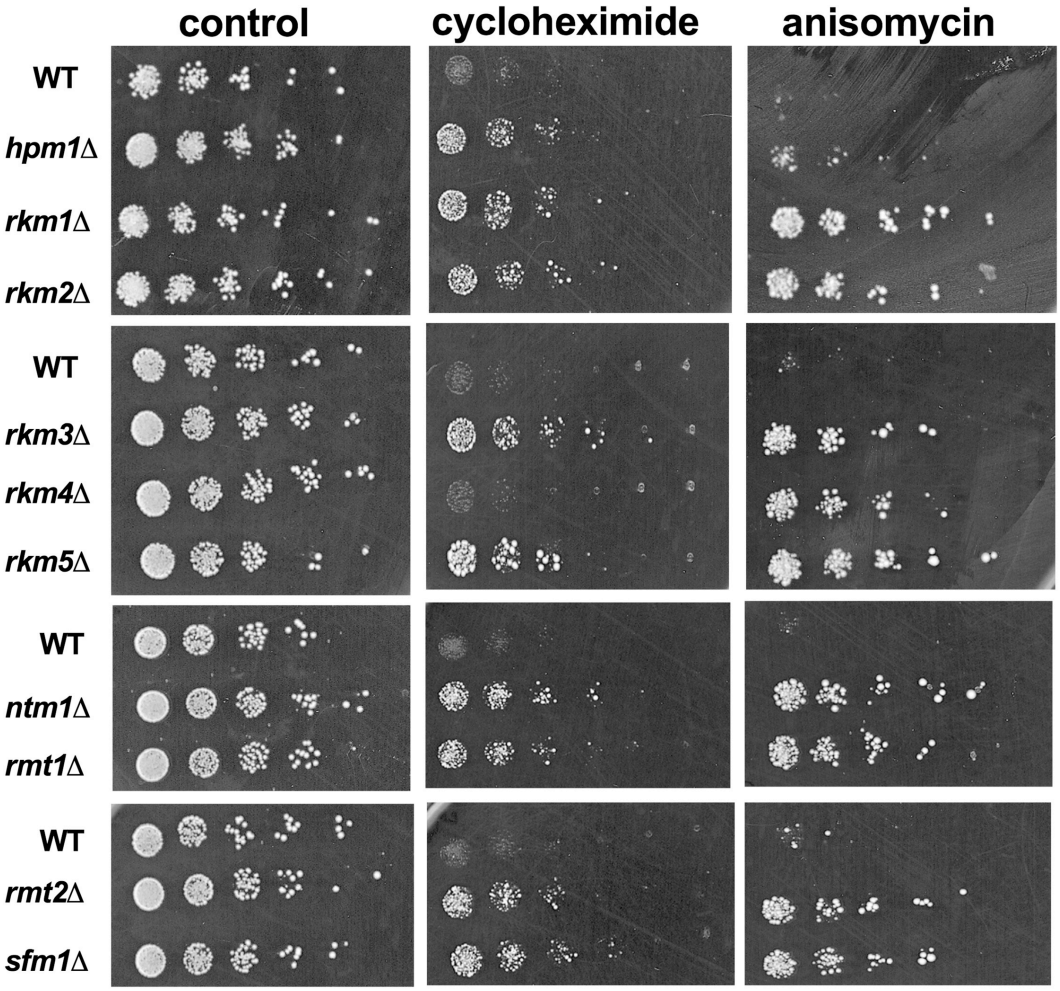

FIG. 2-2. Cells deficient in ribosomal protein methyltransferases show altered sensitivities to the ribosome-targeting drugs anisomycin and cycloheximide in plate assays. Exponentially growing wild type and ribosomal protein methyltransferase knockouts were inoculated overnight in 20 ml of YPD at 30 °C with shaking at 250 rpm at a dilution to ensure a starting OD600nm around 0.5 the following morning. Cells were pelleted by centrifugation for 5 min at 5000 × g and washed three times in sterile water. Cells were resuspended in water to a final OD600 nm of 0.5 and serially diluted in 5-fold steps with water. Three µl of each dilution starting at an OD of 0.02 was spotted on YPD agar plates in the presence or absence of anisomycin (10 µg/ml; CalBiochem #176880) and cycloheximide (500 ng/ml; Sigma #C7968).

Plates were then incubated for 4 days at 30 °C. Each panel shown is from a single plate of one of two biological replicates.

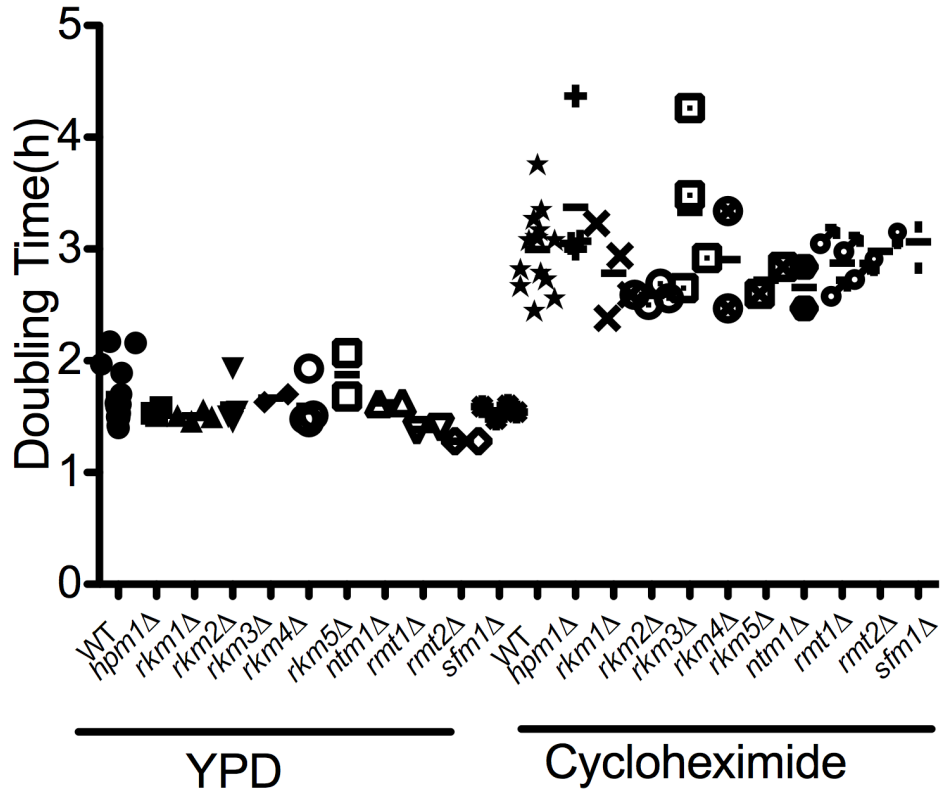

FIG. 2-3. Cells deficient in ribosomal protein methyltransferases show similar growth rates in liquid culture to wild type cells in the presence or absence of cycloheximide. Exponentially growing wild type and mutant strains were inoculated overnight in 25 ml of YPD at 30 °C with shaking at 250 rpm at a dilution to ensure a starting OD600nm around 0.1 the following morning. At that time, either no drug or cycloheximide (50 ng/ml final concentration) was added and cell growth at 30 °C was determined by the increase in OD600nm at 90 min intervals for 9 h. When needed, 5-fold or 10-fold dilutions were done to insure that the measured OD was below 1. Doubling times were calculated from the slope of a log OD versus time plot. The data shown are from five separate experiments done in duplicate.

Loss of ribosomal protein methyltransferases results in defects in the fidelity of translation elongation or termination.

Previously, we demonstrated that loss of Hpm1 results in reduced fidelity in translation elongation (10). To determine if the other ribosomal protein methyltransferases are important for translational fidelity, we performed assays measuring stop codon suppression, amino acid misincorporation, and programmed -1 ribosomal frameshifting (-1 PRF). These assays utilize dual-luciferase reporter genes: *Renilla* followed by firefly luciferase, separated by a linker region, under the control of a constitutive promoter. The amount of firefly luciferase luminescence correlates with translational errors in all three assays and the amount of *Renilla* luciferase luminescence is used to correct for differences in translation initiation and mRNA levels of the dual reporters. To measure stop codon suppression, vectors containing stop codons (UAA and UAG) in the linker region between the *Renilla* and firefly luciferase genes were used and the amount of reporter luminescence was measured. Increased readthrough of the stop codons, as a result of defects in elongation or termination, would result in increased firefly luciferase luminescence. Loss of each of the ten ribosomal protein methyltransferases resulted in increased readthrough of the UAA and UAG stop codons, compared to wild type (Fig. 4A, 4B). This result suggests that all ten ribosomal protein methyltransferases in *S. cerevisiae* are important for translation elongation or termination fidelity. To determine if the translational fidelity defects in all ten strains is occurring at the elongation or termination step, amino acid misincorporation was measured, which measures elongation fidelity defects. Amino acid misincorporation levels were determined using a dual-luciferase reporter vector with a point mutation in the firefly gene at a catalytically-important residue (K529) to a near-cognate asparagine residue (19). This mutation renders firefly luciferase catalytically-deficient. High

fidelity translation would result in incorporation of the asparagine residue at position 529 and synthesis of an inactive firefly luciferase. However, reduced translational accuracy would result in increased frequencies of near-cognate and non-cognate aminoacyl-tRNA accommodation and increases the chances of misincorporating the wild type lysine reside, resulting in the synthesis of an active firefly luciferase enzyme. Hence, reduced translation elongation accuracy would result in the production of more active firefly luciferase enzymes and as a consequence, greater firefly luciferase luminescence.

All ribosomal protein methyltransferase-deficient strains exhibited significantly higher frequencies of amino acid misincorporation (> 2-fold), except for *rkm2*Δ and *ntm1*Δ, which had similar levels of misincorporation as wild type (Fig. 3C). This suggests that most ribosomal protein methyltransferases are important for translation elongation fidelity, whereas Rkm2 and Ntm1 are important in translation termination efficiency. Finally, to corroborate that these strains have defects in translation, we measured the frequencies of programmed -1 ribosomal frameshifting (-1 PRF), which has previously been shown to positively correlate with translation elongation defects (20). To measure -1 PRF efficiency, a dual-luciferase reporter vector was used that contained a viral L-A direct -1 PRF signal between the *Renilla* and firefly genes (21). Firefly luciferase synthesis depends on the -1 PRF event as it is out of frame of the *Renilla* open reading frame. Defects in translation elongation or termination increases the transit times of ribosomes on mRNAs and consequently, increases the probability of a -1 PRF event occurring (12, 13, 22). Therefore, defects in translation elongation or termination should result in more firefly luciferase production. Loss of each of the ten ribosomal protein methyltransferases resulted in enhanced -1 PRF efficiency (Fig. 4D). Notably, loss of Rkm5 or Ntm1 resulted in > 2-fold increase in -1 PRF efficiency (Fig. 4D). These results suggest that ribosomal protein

methyltransferases in *S. cerevisiae* are important for translation elongation and/or termination fidelity.

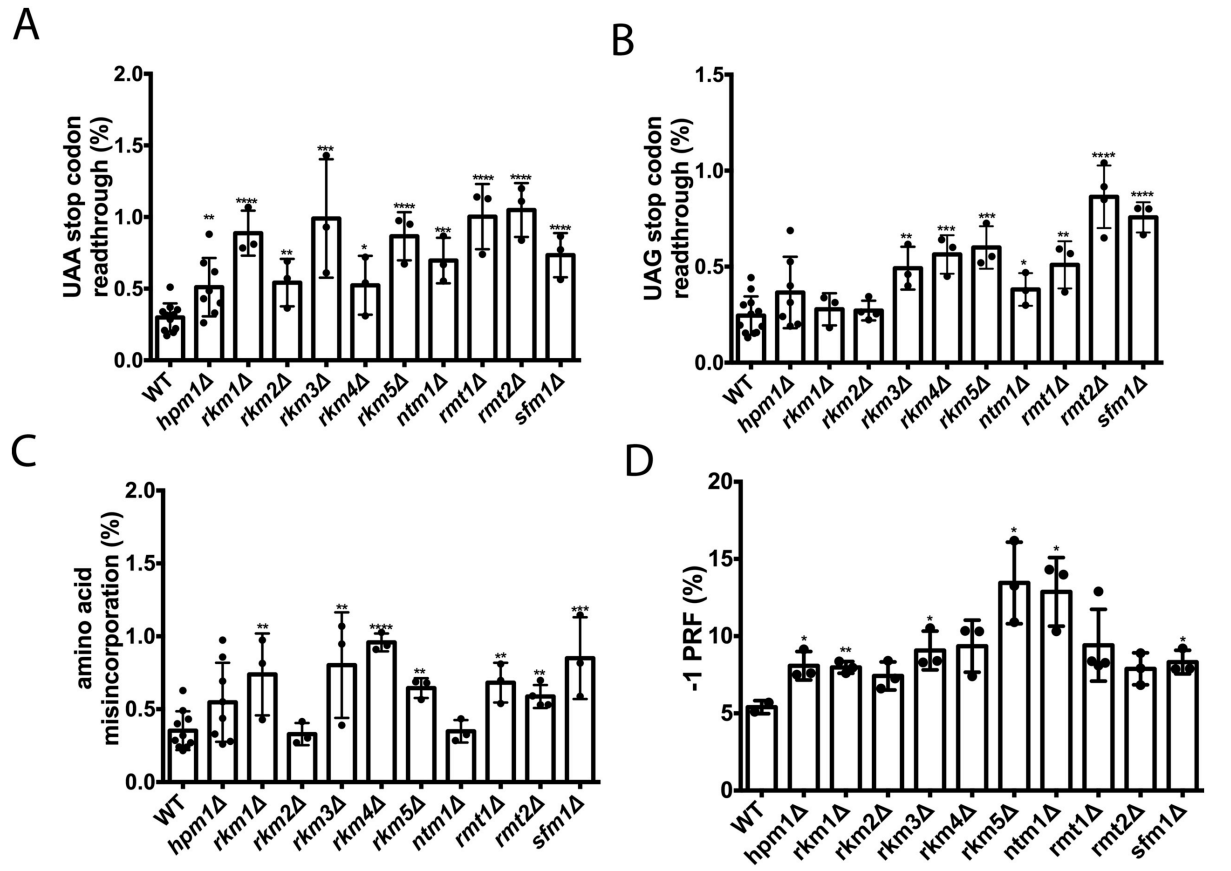

FIG. 2-4. Cells deficient in ribosomal protein methyltransferases have reduced translational fidelity. Translation elongation and termination accuracy were measured in cells lacking each of the ten ribosomal protein methyltransferases. A dual-luciferase reporter assay consisting of a Renilla luciferase gene fused C-terminally to a firefly luciferase gene separated by a linker region was used to measure stop codon readthrough, amino acid misincorporation, and programmed −1 ribosomal frameshifting (−1 PRF), as described previously [12], [22], [24]. (A) Percent readthrough was calculated by taking the firefly/Renilla luminescence ratio of the UAA-containing vector divided by the same ratio in the respective control. Error bars represent standard deviation. hpm1Δ $p = 0.0077$, rkm1Δ $p < 0.0001$, rkm2Δ $p = 0.006$, rkm3Δ $p = 0.0001$,

rkm4Δ p = 0.0158, rkm5Δ p < 0.0001, ntm1Δ p = 0.0001, rmt1Δ p < 0.0001, rmt2Δ p < 0.0001, sfm1Δ p < 0.0001. Wild type was assayed 11 independent times, hpm1Δ 8 times and mutants were assayed three independent times. Data for the wild type and hpm1Δ cells were previously reported [13]. (B) Same as in (A) except a UAG stop codon was used. Wild type was assayed for a total of 12 biological replicates, hpm1Δ was assayed 7 times, and mutants 3–4 times. Data for wild type and hpm1Δ cells were previously reported [13]. hpm1Δ p = 0.0811, rkm1Δ p = 0.6114, rkm2Δ p = 0.626, rkm3Δ p = 0.0024, rkm4Δ p = 0.0003, rkm5Δ p = 0.0001, ntm1Δ p = 0.05, rmt1Δ p = 0.0017, rmt2Δ p < 0.0001, sfm1Δ p < 0.0001. (C) Percent amino acid misincorporation was measured as in (A). Wild type was assayed 10 independent times, hpm1Δ was assayed 8 times, and mutants were assayed 3–4 independent time. Data for wild type and hpm1Δ cells were previously reported [13]. hpm1Δ p = 0.0621, rkm1Δ p = 0.0054, rkm2Δ p = 0.7818, rkm3Δ p = 0.0051, rkm4Δ p < 0.0001, rkm5Δ p = 0.0043, ntm1Δ p = 0.9623, rmt1Δ p = 0.0032, rmt2Δ p = 0.0069, sfm1Δ p = 0.001. (D) Percent −1 PRF was calculated by taking the firefly/Renilla luminescence ratio of cells containing pJD376 (L-A virus gag-pol frameshift signal) divided by the same ratio of cells containing the no frameshift control (pJD375). Error bars represent standard deviation of at least two independent experiments. Strains were assayed 2–4 independent times as indicated by the number of data points. hpm1Δ p = 0.03, rkm1Δ p = 0.006, rkm3Δ p = 0.03, rkm5Δ p = 0.03, ntm1Δ p = 0.02, sfm1Δ p = 0.02. Frameshift vectors were generously provided by Jonathan Dinman at the University of Maryland, MD and described [24]. p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), p < 0.0001 (****).

DISCUSSION

In this study, we showed that most ribosomal protein methyltransferases in *S. cerevisiae* are playing roles in ribosome biogenesis. These enzymes (except Ntm1) can localize or are predominantly localized in the nucleus (23, 24), where the bulk of ribosome assembly takes place (25–27). This suggests that these methyltransferases (Hpm1, Rkm1, Rkm3, Rkm4, Rmt1, and Rmt2) are likely active participants during the assembly process of the ribosome. Ntm1 may not be actively involved in the assembly process of the ribosome but instead methylation of its ribosomal protein substrate, Rpl12, may be important for ribosomal assembly, as Rpl12 is known to be imported into the nucleus and assemble with pre-ribosomes along with most of the ribosomal proteins (25, 27). Ntm1 may also have unknown substrates whose methylation is important for the assembly process. It is also interesting to note that the predominantly nuclear-localized methyltransferases, Rkm3 and Rkm4, are required for normal ribosome biogenesis, yet their ribosomal protein substrate, Rpl42, does not assemble with pre-ribosomes in the nucleus but rather, at a later step in the cytoplasm. This raises the question as to the spatial and temporal association between Rkm3 and Rkm4 with Rpl42 and suggests that the enzymes likely methylate free or ribosome-associated Rpl42 in the cytoplasm prior to their import into the nucleus. The latter possibility is unlikely because the Rpl42 methylation sites are embedded within the 25S rRNA of the mature ribosomes and are unlikely to be recognized by the methyltransferases (6, 28). Rkm3 and Rkm4 may also have additional unknown substrates in the nucleus whose methylation is important for ribosome biogenesis. To uncover these putative substrates, tandem-affinity purification of these methyltransferases, followed by *in vitro* methylation assays with interacting proteins need to be performed.

The ribosomal protein methyltransferase mutants exhibited elevated levels of polysomes and increased resistance to the ribosome-binding drugs anisomycin and cycloheximide, which

suggested a common translational defect in these strains. Several studies have shown a correlation between increased polysome levels and translation elongation defects (12, 13, 22). This prompted us to test if these methyltransferase-deficient strains have defects in translation elongation or termination. Translational fidelity assays measuring stop codon readthrough, amino acid misincorporation, and programmed -1 ribosomal frameshifting demonstrated that all of the ribosomal protein methyltransferases in *S. cerevisiae* are important for translational accuracy. Loss of Ntm1 or Rkm2 resulted in increased readthrough of stop codons but had no major effect on amino acid misincorporation, suggesting that these enzymes are important for translation termination but not necessary for elongation, unlike the other eight enzymes. Importantly, Ntm1 and Rkm2 methylate the same ribosomal substrate, Rpl12, at the N-terminus of the protein (17, 29) that is exposed to the cytoplasm and a component of the GTPase-associated center (GAC), which is known to interact with translation factors and couples GTP hydrolysis with translation elongation or termination (30–32). It is therefore possible that methylation of Rpl12 by Ntm1 and Rkm2 is important for recruitment of release factors to the GAC and/or coupling GTP hydrolysis to translation termination. Moreover, previous work has shown that these two enzymes may be functionally linked as cells deficient in Ntm1 not only lose methylation at the Ntm1 target site (P1) but also at the Rkm2 target site (K3) (29). The other eight mutant strains all had increased levels of amino acid misincorporation and stop codon suppression, suggesting these enzymes are important for elongation accuracy. The stop codon readthrough phenotype in these cells is likely a consequence of an elongation rather than a termination defect as has previously been reported (19). It is unclear if these translational fidelity phenotypes are dependent or independent of the ribosome biogenesis defects of cells lacking these enzymes. It is possible that aberrant ribosome biogenesis results in ribosomes with

altered structures and/or functionality that diminishes fidelity of protein synthesis. Alternatively, these enzymes may be multifunctional with separate roles in ribosome biogenesis and translation and methylation of their ribosomal protein substrates may be important for maintaining proper ribosome conformations during the decoding, peptidyl transfer, and translocation steps of elongation. To address this concern, similar analyses need to be done with ribosomes lacking methylation at each of the sites targeted by these ribosomal protein methyltransferases. Also rRNA structure, biochemical, and biophysical characterization of ribosomes isolated from each of these ribosomal protein methyltransferase-deficient strains needs to be done to get a clear understating as to how or if methylation of ribosomal proteins in *S. cerevisiae* promotes ribosome biogenesis and accurate protein production.

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Chapter 3

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

Manuscript prepared to submit to *Biochemistry*

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

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Keywords**:** translation elongation factor, yeast, protein methylation, post-translational modification (PTM), stress response, translation

ABSTRACT

To date, twelve protein lysine methyltransferases that modify translational elongation factors and ribosomal proteins (Efm1-7; Rkm 1-5) have been identified in the yeast *Saccharomyces cerevisiae*. Of these twelve, five (Efm1, 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. Here 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 with no evidence of acting on other substrates. This work starts to illuminate why one protein can need five different methyltransferases for its functions and highlights the resilience yeast have 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, 11–16). 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 316 being the most highly conserved (2, 17).

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 profiling, and dual luciferase assays to assess translation fidelity.

Here we provide phenotypes associated with the disruption of EF1A methylation. Our study shows that cellular growth can be slowed, perhaps due to lowered levels of EF1A, although translational fidelity is not affected. Our work demonstrates that methyl-deficient EF1A is still able to function in translation but may have some as yet unknown function in the TORC1 pathway. Finally, we provide evidence that the five EF1A methyltransferases appear to be

specific to EF1A and do not have additional cellular targets.

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 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 media 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 media 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 L and 20 g/L agar.

Strains used in this study are listed in Table 3-1 below. The *efm1456Δ* and *efm14567Δ* deletions strains were based on the *efm1*Δ strain obtained from Dharmacon online yeast knockout collection. Each successive deletion was created through homologous recombination following the protocol as described (18). Each primer contained either 40 base pairs upstream or downstream of the corresponding ORFs to be deleted. For the knockout using the *KIURA3* cassette, we used the *KIURA3* found in the CORE cassette as a template. The mutants were confirmed through 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,

210515). Sanger Sequencing of the *TEF1* open reading frame (ORF) was used to confirm the point mutations. Starting with wildtype 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.

Table 1: Yeast Strains Used in This Study

Strain	Genotype
BY4742	MATalpha *his3Δ1 leu2Δ0 lys2Δ ura3Δ0*
efm1Δefm4Δefm5Δefm6Δ	BY4742 background, *yhl039wΔ::hphMX, yil064wΔ::HIS3, ygr001cΔ::kanMX, ynl024cΔ::URA3*
efm1Δefm4Δefm5Δefm6Δefm 7Δ	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

Lysis Method 1. Yeast cells grown in YPD (7 $OD_{600\ nm}$) were washed 3 times with 1 ml of water and then resuspended in 0.2 mL of lysis buffer (0.2% SDS, 0.7 mM phenylmethylsulfonylfluoride (PMSF)). 0.2 g of baked glass beads (Biospec Products, 11079105) was added and the cells were lysed with 7 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 12,000 x g for 15 min.

Lysis Method 2. Yeast cells grown in YPD (7 $OD_{600\ nm}$) were washed once with 1 mL ice cold water, spun at 4000 x 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. (19) 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% (wt/vol) trichloroacetic acid was added and the mixture incubated on ice for another 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 water, 12.5 µL 2-mercaptoethanol, 25 µL of 1 M Tris base, and 100 µg of PMSF and heated for 3 min at 95°C. After the determination of protein concentration by Lowry analysis after trichloroacetic acid precipitation (20), a small amount of solid bromophenol blue was added and samples analyzed by SDS-PAGE.

SDS-PAGE. Cell lysates were fractionated on 4-12% Bis-Tris precast polyacrylamide gel (Genscript) with 1X 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 used to determine protein size. The gel was Coomassie stained (50% methanol, 10% acetic acid, 40% water, 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 Francisco et al. (21). A 50 mL overnight culture grown in YPD from wildtype or mutant strain was used to inoculate 2 flasks of 4 L of YPD and cells were grown to an $OD_{600\ nm}$ of ~ 2.5. The cells were centrifuged at 664 x g in pre-weighed centrifuge bottles and the weight of the pellet 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-Cl 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 passing through an emulsifier (EmulsiFlex-C3) four times at greater than 25,000 pounds per square inch pressure. Cell debris was removed by centrifugation at 11,300 x g for 30 min at 4 °C and then the supernatant clarified at 76,300 x 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-Cl 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 transferring to a 50 mL conical tube and centrifugation at 2,000 x g for 3 min. The supernatant was then incubated with 25 mL of sulphopropyl-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 2,000 x 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 step-wise salt gradient 100 mM KCl, 150 mM KCl, 200 mM KCl, 300 mM KCl, 350 mM KCl 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 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_{600nm} of ~0.7 was grown in *S*-adenosyl-[*methyl*-^{3}H]methionine, using the method described (22). Next the labeled cells were washed with water, resuspended in 1 mL binding buffer (20 mM Tris, 100 mM KCl, 10% glycerol, 1% Triton X-100, 200 µg/mL PMSF) and lysed with 0.2 g of baked glass beads using 7 rounds of 30 s vortexing followed by 30 s on ice. The radiolabeled lysates were collected and clarified at 5,000 x g for 5 min. Ten microliters were set aside as the input material. Protein A beads were prepared in binding buffer with three washes at 700 x g for 2 min and kept on ice

until needed. To start the immunoprecipitation, the labeled lysates (500 μg protein by 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 above, the protein-antibody-protein A bead complex was heated at 100 °C in 50 μL of 5X SDS- buffer (250mM Tris-Cl pH 6.8, 10% SDS, 30% glycerol, 0.5 M DTT, 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, then treated with En3hance (Perkin Elmer) 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 media at 30 °C to give an $OD_{600\ nm}$ of about 0.7 the following morning. The inhibitor chase was performed as described by Buchanan et al. (23) with the changes described below. Samples were collected at various time points and were 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 fractionated in duplicate using SDS-PAGE (described above). Protein sizes were determined using a Biorad broad range unstained molecular weight ladder and equal amounts of protein (by Lowry assay after precipitation with trichloroacetic acid) were loaded for each strain tested. One gel was stained and destained as above. A second gel was transferred to 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 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% Tween 20 (v/v, TBST) or 0.5%

BSA (w/v)/ 0.02% (w/v) SDS in phosphate-buffered saline with 0.1% Tween 20 (v/v, PBST). After blocking, the membranes were washed in 1X TBST or 1X PBST and incubated with primary antibodies (1:10000 rabbit anti-EF1A, Kerafast, ED7001) diluted into 1% dried nonfat milk in 1X TBST or 0.5% BSA/ 0.20% SDS in PBST, as indicated, for 1.5 h at 4 °C. After washing with the respective buffers, the membrane was incubated with anti-rabbit IgG-HRP (1:6666; Cell Signaling, 7074) secondary antibody in 1% dried nonfat milk or 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 HRP secondary antibody (Amersham Biosciences ECL Prime Western blotting, GE Healthcare, RPN2232) and LICOR Odyssey imager for the fluorescent probe. After probing, membranes were stained with Ponceau S or Coomassie to determine transfer efficiency.

Dual Luciferase (DLR) Assay. For amino acid misincorporation, the CTY775/luc CAAAFF K529N plasmid was used and for programmed frameshift, the pJD376 (L-A) termed PRF -1 and pJD377 (Ty1) PRF +1 plasmid was used. These plasmids were transformed into the wildtype and mutant strains using the lithium acetate-ssDNA-PEG method (24). 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 $OD_{600\,nm}$ of 0.5-0.8. Next 0.5 $OD_{600\,nm}$ units were harvested by centrifugation at 5,000 x 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 transferred to a white (Greiner bio-one, 82050-736) 96 well plate. 30 µL of LARII solution was added and immediately read using Sprectramax M5 microplate reader, giving firefly

luminescence; then 30 μL of Stop and Glo buffer immediately added to that same well and read to give Renilla luminescence. Spectra Max parameters were set as: read type - endpoint; read mode - luminescence with 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 elongation factor 1A (EF1A) N-terminal glycine residue 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 (*efm14567Δ*) or the four methyltransferases that methylate lysine residues 30, 79, 316, and 390 (*efm1456Δ*) through marker-based gene deletions. Secondly, 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 the "Experimental Procedures" section. The N-terminal modifications are still present in this strain (trimethyl Gly-2 and dimethyl Lys-3). The successful construction of the *efm14567Δ* 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 wildtype, *efm14567Δ*, and *TEF1* K(30,79,316,390)R strains labeled *in vivo* with *S*-adenosyl-[*methyl*-^{3}H]methionine. 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) (Fig. 1). In wildtype hydrolysates, all three lysine ^{3}H-methylated species were detected whereas in the *efm14567Δ* strain, no radioactivity was detected at the positions of TMK, DMK, and MMK, confirming biochemically the loss of the efm1, efm4, efm5, efm6, and efm7 methyltransferases. On the other hand, we observed reduced TMK and DMK/MMK methylation of *tef1 K(30,79,316,390)R* EF1A (Fig. 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.

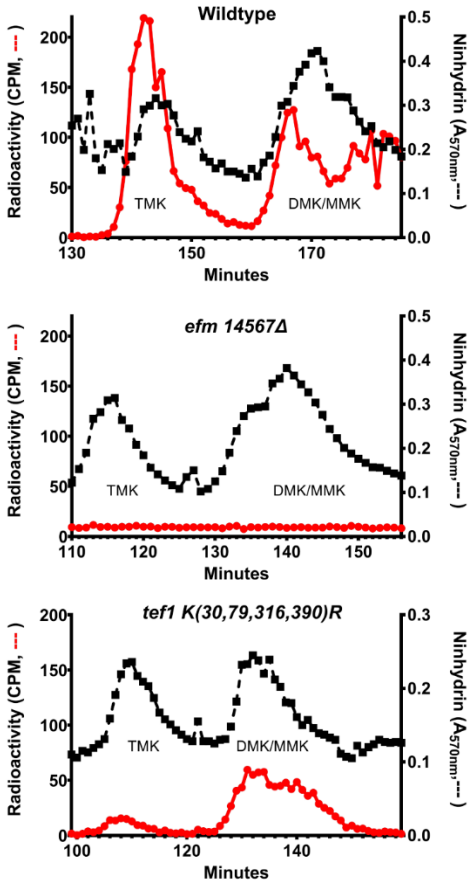

Figure 3-1: Loss of methylated lysine residues in EF1A from a strain lacking five Efm methyltransferases and a strain with lysine to arginine substitutions at positions 30, 79, 316, and 390 in EF1A. EF1A purified from yeast cells were labeled with *S*-adenosyl-[*methyl*-^{3}H]methionine, acid hydrolyzed, and the methylated amino acid derivatives separated by high resolution cation exchange chromatography using the method described (22) with the modifications shown below. Wildtype 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 ε-dimethyllysine (DMK) while *efm14567Δ* 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 ninhydrin assay in 25 µl aliquots (black squares and line; performed at 68 °C for 15 min). Data from the middle panel is from one experiment; the data in the upper and lower panels is from one experiment of two replicates.

To confirm the reduction or absence of methylation of EF1A in the mutant strains, we labeled intact 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 in long exposures, no radioactivity was detected at the 50 kDa position of EF1A in the *efm14567Δ* strain lacking all of the EF1A methyltransferases, and reduced methylation was observed in the *efm1456Δ* strain at shorter exposures (Fig. 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 (Fig. 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 (Fig. 2). Here we looked closely for evidence of methylated polypeptides on SDS-PAGE that were reduced or not found in the any of the three mutant strains. While we did observe a clear reduction of methylation in the *efm14567Δ* strain, confirming that the major methylated species at this polypeptide size was EF1A, we did not detect any other changes when the fluorographs were analyzed at a range of exposures (Fig. 2). These results did not provide any evidence that the EF1A methyltransferases could methylate polypeptides in addition to EF1A, although we would not detect the loss of minor methylated species.

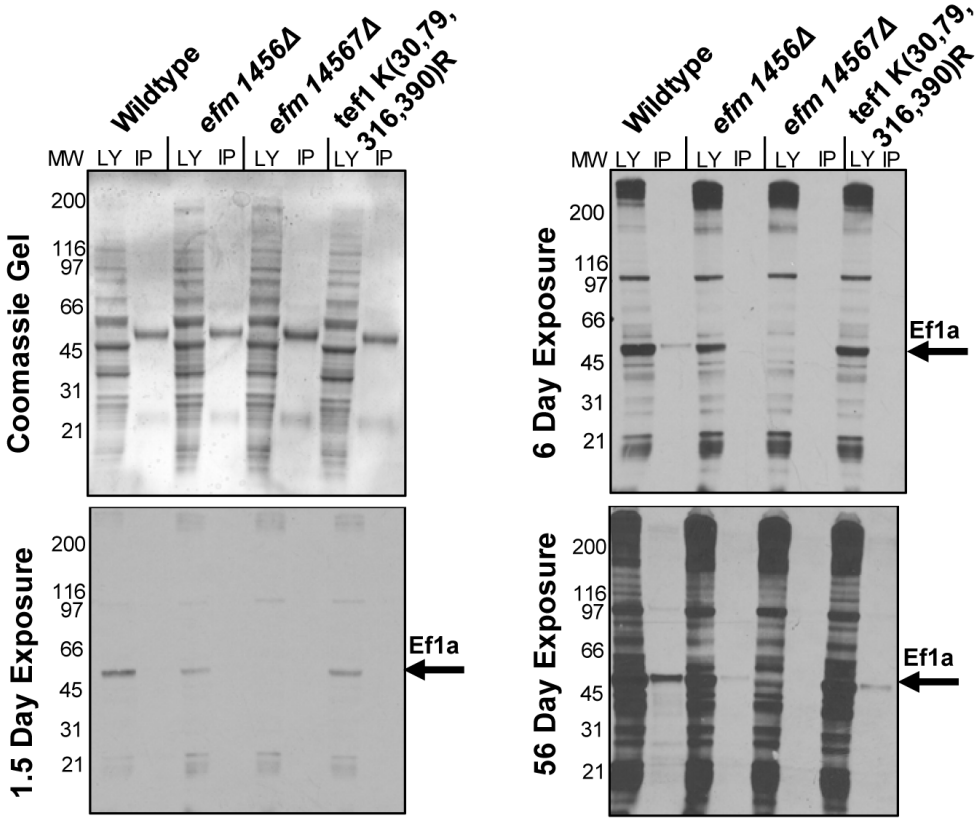

Figure 3-2: Immunoprecipitation of EF1A from methylation-deficient cells shows specificity of elongation factor methyltransferases. Yeast cells from wildtype and mutant strains that were labeled with *S*-adenosyl-[*methyl*-^{3}H] methionine, and immunoprecipitated with an anti-EF1A polyclonal antibody as described in "Experimental Procedures". 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 methylation 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. The figure shown is a representative from one out of two separate experiments.

Methylation deficient cells exhibit a slow growth phenotype and alter growth in response to cellular stress. We then assessed differences in the growth of the EF1A methylation-deficient strains. In Fig. 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 *efm1456Δ* strain as well as the *efm14567Δ* strain were much smaller than the wildtype colonies. These defects were confirmed and quantitated by observing slower growth in liquid YPD media as well. We found an increase in doubling times from about 1.7 h for the wildtype cells to 2.1 h for the *efm1456Δ* strain and 2.2 h for the *efm14567Δ* strain (Fig. 3B).

When similar experiments were performed for the *TEF1* K(30,79,316,390)R strain, somewhat reduced colony sizes were observed after 1 day of plate growth but not after 2 days (Fig. 3A). In liquid medium, we found a significantly increased doubling time of 2.1 h compared to 1.7 h for the wildtype (Fig. 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.

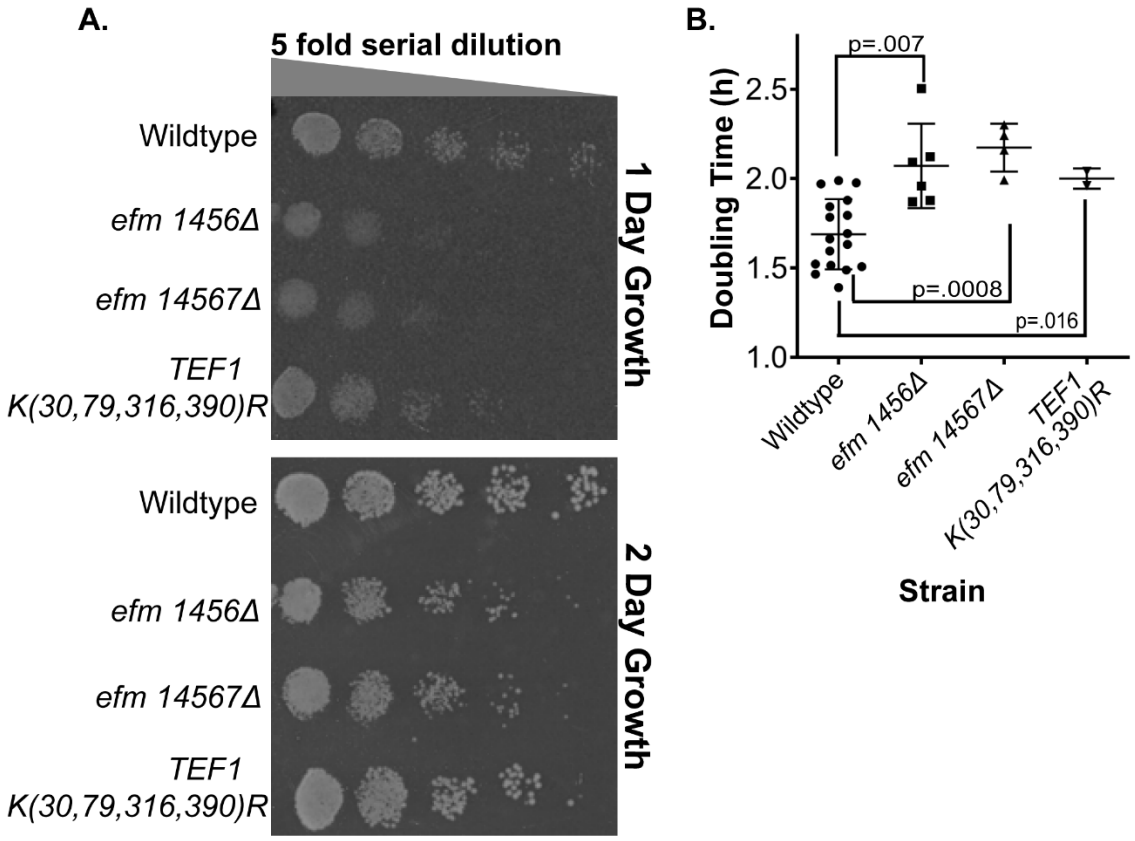

Figure 3-3: Loss of Efm methyltransferases results in slow growth in solid and liquid YPD growth media while EF1A with four lysine to arginine mutations shows slow growth in only liquid media. A) Yeast cells from wildtype and mutant strains grown at 30 °C in YPD to an $OD_{600\,nm}$ of about 0.5 and 3 µl of a cell suspension starting at 0.1 $OD_{600\,nm}$ 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 wildtype colonies in 16 out of 23 experiments; in the other 7 cases colonies were roughly the same size. Colonies for the efm14567Δ mutant were significantly smaller than wildtype colonies in 19 out of 21 replicate experiments; in the

other 2 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 the wildtype. **B)** Doubling times for growth in liquid YPD media at 30 °C were calculated from the linear portion of exponential growth measured by $OD_{600\ nm}$ over a 12 h time frame. Each point is a biological replicate. Error bars indicate standard deviation values and Student T- test p values (unpaired, two tails) are shown.

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 both the *efm1456Δ* and the *efm14567Δ* strains were markedly smaller than the wildtype strain (Fig. 4). We found that colonies of the *TEF1* K(30,79,316,390)R strain on the plates were somewhat smaller than wildtype colonies under osmotic and oxidative stress conditions (Fig. 4). The *TEF1* K(30,79,316,390)R colonies on YPG plates did not have any difference in size compared to wildtype. When cells were grown on lactate plates we observed no difference in the colony size of the mutants compared to wildtype (Fig 4). These results demonstrate the EF1A methylation deficient cells are less able to adapt to at least some stress conditions. However, it is unclear why these deficient cells are able to grow equally as well as wildtype cells with non-fermentable carbon sources. It is possible that reduced rates of translation in non-fermentative conditions allows the EF1A methylation deficient cells to grow at the same reduced rate as wildtype cells when EF1A function is not rate-limiting for growth.

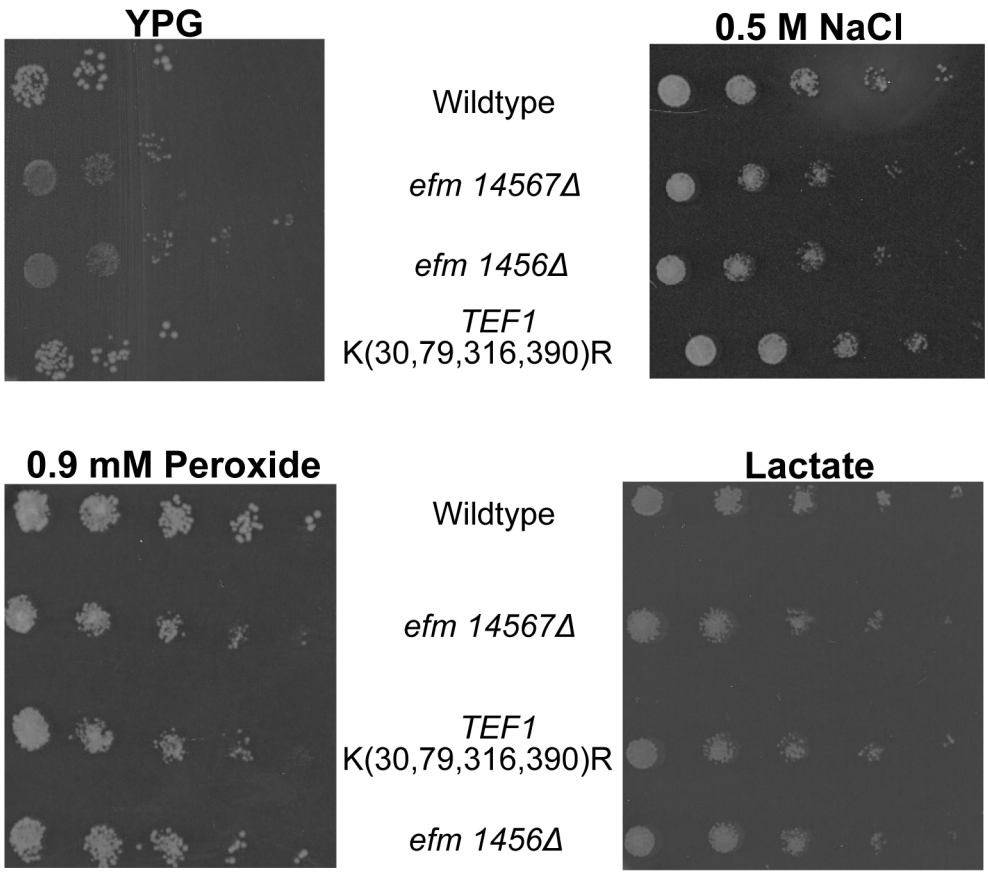

Figure 3-4: Loss of Efm methyltransferases causes sensitivity under different cellular stress conditions. Representative images showing yeast cells that were grown in YPD, serially diluted and then spotted on YPD agar containing 0.5 M NaCl, or 0.9 mM peroxide, or YPG, or lactate media at 30 °C as described in the Figure 3 legend. Colonies were imaged after 2 days. In YPG, colonies for the *efm1456Δ* and *efm14567Δ* mutant were significantly smaller than wildtype colonies in 2 out of 3 replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as wildtype in those replicates. Under oxidative stress, colonies for the *efm1456Δ* and *efm14567Δ* mutant were significantly smaller than wildtype colonies in 3 replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as wildtype in three replicates. In the presence of sodium chloride, mutant colonies were

smaller compared to wildtype in four replicate experiments. No difference in colony size was observed in lactate media for six replicates.

Lastly we assessed growth when the yeast cells were grown on YPD media containing caffeine or rapamycin (Fig. 5). The *efm1456Δ* and the *efm14567Δ* colonies were somewhat smaller compared to wildtype under rapamycin growth whereas they were significantly smaller compared to wildtype for caffeine growth (Fig. 5). The colonies of the *TEF1* K(30,79,316,390)R strain on the caffeine plates grew similarly to wildtype (Fig.5). Interestingly, we observed smaller colonies for the *TEF1* K(30,79,316,390)R strain in both rapamycin conditions tested (Fig. 5). Both rapamycin and caffeine affect protein synthesis and cellular growth through the TORC1 pathway (25, 26). Since growth under rapamycin stress was altered in the *efm1456Δ* and *TEF1* K(30,79,316,390)R strains, it suggests that there is some interaction between methylated EF1A and the TORC1 pathway that gets disrupted when EF1A is unmethylated.

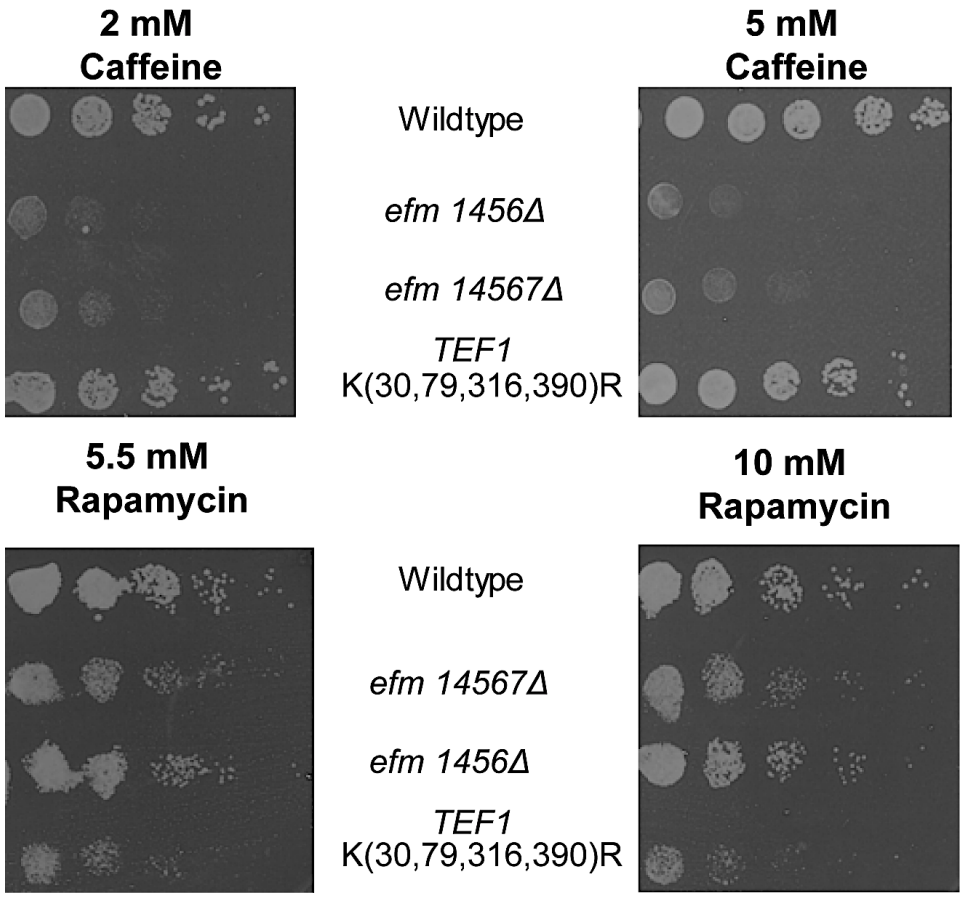

Figure 3-5: Methylation-deficient EF1A growth inhibited by caffeine and rapamycin. Representative images showing yeast cells that were grown in YPD, serially diluted and then spotted on YPD agar containing 2 mM or 5 mM caffeine and 5.5 nM or 10 nM rapamycin (diluted from a 50 mg/ml stock solution in ethanol) at 30 °C as described in the Figure 3 legend. Colonies were imaged after 2-4 days. In 2 mM caffeine, colonies for the *efm1456Δ* and *efm14567Δ* mutant were significantly smaller than wildtype colonies in all 4 replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as wildtype in those replicates. At 5 mM caffeine, colonies for the *efm1456Δ* and *efm14567Δ* mutant were significantly smaller than wildtype colonies in all 5 replicate experiments whereas the *TEF1* K(30,79,316,390)R mutant always grew relatively the same as wildtype in all

replicates. In the presence of 400 nM and 9 ng/mL rapamycin, all mutant colonies were smaller compared to wildtype 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 (Fig. 6). With puromycin, a drug that causes premature polypeptide chain release from the ribosome (27, 28), tunicamycin, a drug that activates the unfolded protein response and inhibits translation (29, 30), and anisomycin, a drug that interferes with the ribosomal acceptor site (31), we observed much smaller colonies of the *efm1456Δ* and the *efm14567Δ* strains compared to the wildtype strain. No decrease in cell size was seen with any of these inhibitors for the *TEF1* K(30,79,316,390)R strain (Fig. 6). Finally, we detected no decrease in colony size with cycloheximide, a drug that blocks translation elongation (32), 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.

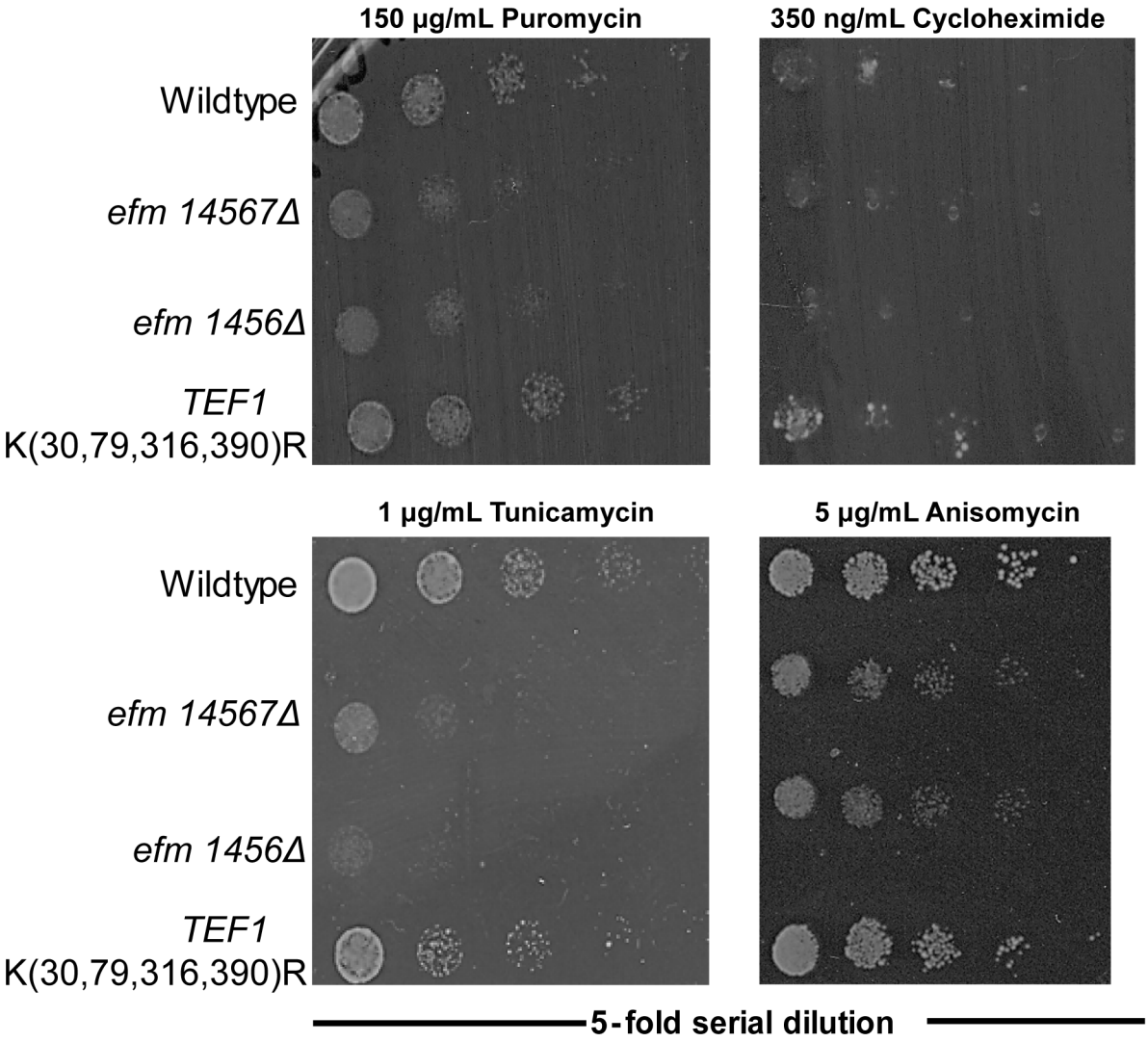

Figure 3-6: Loss of Efm methyltransferases and mutation of four lysines to arginines in EF1A results in differential responses to translational inhibitors. Representative image of yeast cells grown in YPD and then serially diluted onto agar plates as described in the Figure 3 legend but supplemented with either puromycin, cycloheximide, tunicamycin and anisomycin. In puromycin, 8 out of 10 replicates for *efm14567Δ*, and 10 out of 12 replicates for *efm1456Δ* strain had smaller colonies compared to wildtype colonies. Colonies for the *TEF1* K(30,79,316,390)R strain always had similar sized colonies compared to wildtype colonies in 12 replicates. Growth on cycloheximide displayed no difference in colony size compared to

wildtype colonies for the *efm14567Δ* mutant (four replicate experiments), *efm1456Δ* mutant (eight replicate experiments), and the *TEF1* K(30,79,316,390)R mutant (eight replicate experiments). Tunicamycin colony sizes were always smaller than wildtype for *efm1456Δ* (six replicate experiments) and *efm14567Δ* (two replicated experiments) but remained unchanged for *TEF1* K(30,79,316,390)R mutant. (six replicate experiments). On anisomycin plates, there were smaller colonies 4 out of 6 replicates for *efm14567Δ* and 5 out of 6 replicates for the *efm1456Δ* mutant compared to wildtype colonies. The *TEF1* K(30,79,316,390)R strain had similar sized colonies compared to wildtype colonies with the exception of 2 out of 6 replicates where the colony sizes were bigger.

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 wildtype and methylation deficient strains with a polyclonal antibody specific to the entire yeast EF1A protein (Fig. 7A). Quantitation of the immunoblot signal demonstrated that the deficient strains contained about half of the EF1A present in wildtype strains, although there was considerable variability (Fig. 7B). This may explain the slowed growth rates and responses to translation inhibitors observed previously.

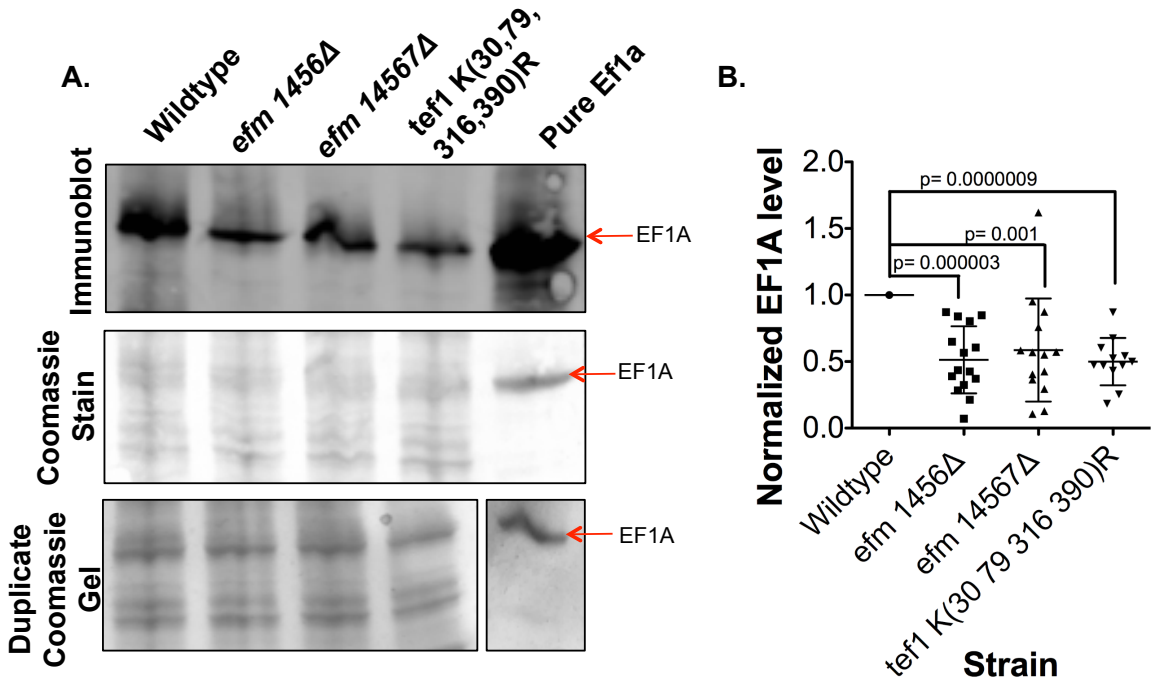

Figure 3-7: Loss of Efm methyltransferases and mutation of four lysines to arginines in EF1A affects protein abundance levels. Seven OD_{600nm} units of yeast cells were harvested after being grown in YPD media at 30 °C, then lysed using "method 2", fractionated using SDS-PAGE and immunoblotted for antibody detection of EF1A with the LICOR secondary antibody as described in "Experimental Procedures". **A)** A representative experiment showing a LICOR detected immunoblot of EF1A protein levels, the Coomassie-stained PVDF membrane and a duplicate Coomassie-stained gel of the lysates. **B)** EF1A protein expression levels determined from comparison of peak areas of immunoblots probed for EF1A. The densitometry for the mutant strains were normalized to wildtype and quantified using image J. Student T- test p values (unpaired, two tails) are shown.

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 Fig. 8, we show the levels of EF1A by immunoblotting over a 2 h time course. In both conditions, there is no evidence for more rapid degradation of methylation-deficient EF1A. While EF1A degradation is increased after two hours in puromycin, the rate of decay is similar in the mutant strains.

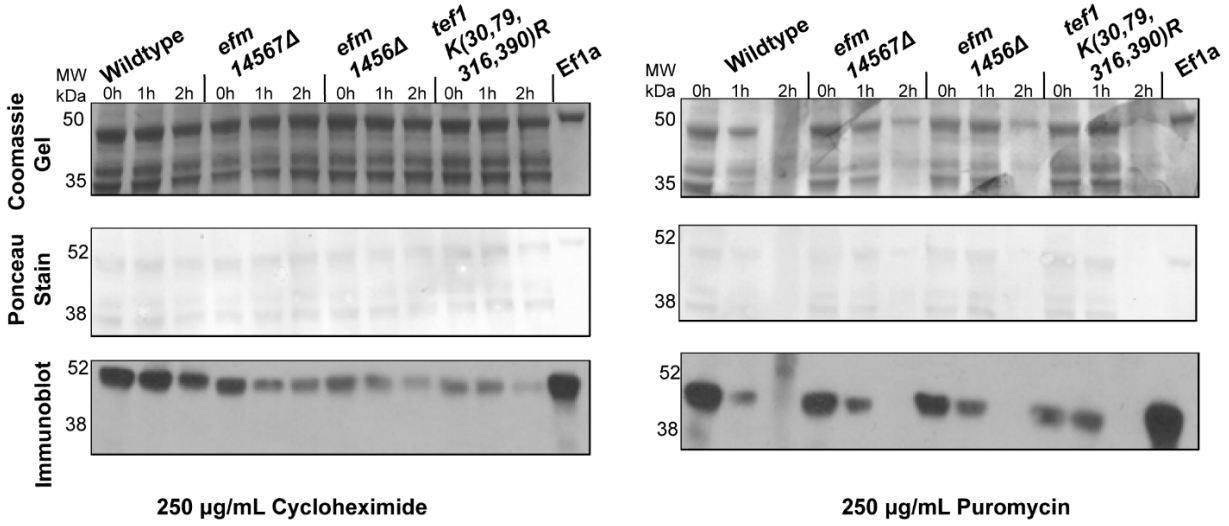

Figure 3-8: EF1A protein levels remain stable in presence of cycloheximide and puromycin in wildtype cells and in cells deficient in EF1A methylation. Yeast cells were grown to an OD of about 0.7 at 600 nm in YPD media at 30 °C. Cycloheximide or puromycin were then added to 7.5 OD_{600nm} units of cells to a final concentration of 250 µg/ml. Sample aliquots were collected at the indicated times, lysed (using method 2) and the proteins fractionated by SDS-PAGE as described in the "Experimental Procedures" section. 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 lower panel. This experiment was performed once for cycloheximide and twice for puromycin.

Ribosome assembly is unaffected by 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. Fig. 9A and B show a representative experiment of the separation of ribosomal subunits in the presence of cycloheximide for wildtype and *efm14567Δ* strains, respectively. Cycloheximide is used to stall translation to capture actively translating ribosomes on a transcript in order 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, 80S peaks and the polysomes peaks. We found that there are no discernible differences in the levels of large and small units and amount of polysomes detected. No significant differences were seen when the 60S:40S and 80S:polysome ratios were quantified (Fig. 9C and D, $p = 0.1$). Under these conditions at least, methylation of EF1A is not necessary for its ability to assemble ribosome subunits.

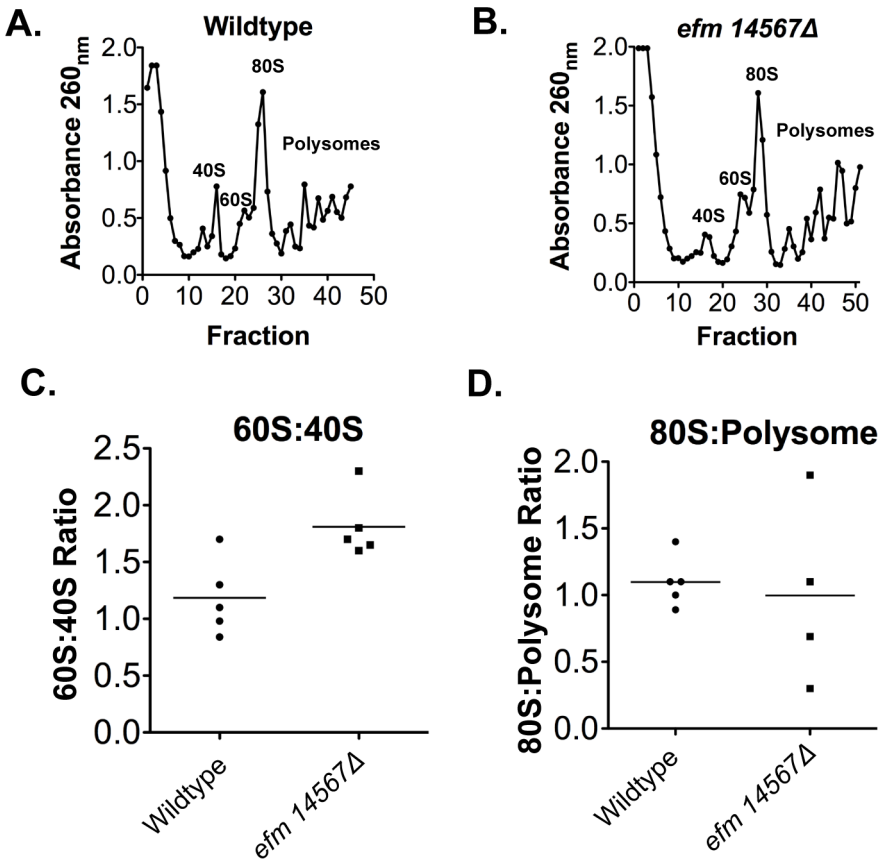

Figure 3-9: Deletion of EF1A methyltransferases Efm1, 4, 5, 6, and 7 does not affect ribosome assembly. Ribosomes were prepared from yeast cells grown to an OD_{600nm} of ~0.7 as described (31) and analyzed with the modifications described below. The top panels (A and B) show the fractionation of ribosomes by sucrose gradient centrifugation of 7 A_{260nm} units. In each case, 100 µL fractions were collected and the $A_{260\ nm}$ value of each fraction plotted. The absorbance of each of the peaks were summed to quantify the ratio of 80S:polysome ribosomal subunits (panel C) and the ratio of the 60S:40S subunits (Panel D) with the mean value indicated by the horizontal line.

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) (34–36), examining both amino acid misincorporation and programmed frameshift 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 frameshift plasmids a viral programmed frameshift is placed in the linker region and when it is bypassed firefly luminescence is detected (36). 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 activity (37). As shown in Fig. 10, we found no differences in the misincorporation or frameshift rate with the *efm1456Δ*, *efm14567Δ*, 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.

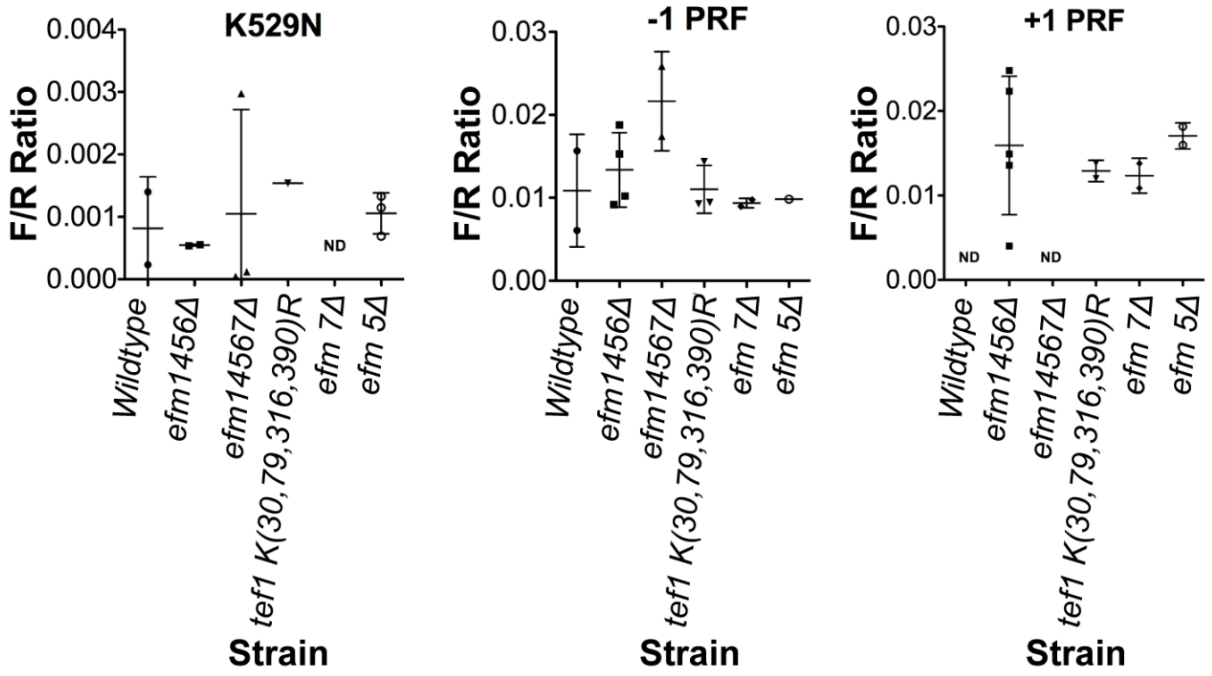

Figure 3-10: Loss of Efm methyltransferases and mutation of four lysines to arginines in EF1A does not affect translation fidelity. Yeast cells were prepared as described in "Experimental Procedures". Ratios of firefly and Renilla luciferase luminescence values are

shown with each point representing a biological replicate. There was no statistical difference in the ratios with any of the strains shown here. **ND- No data collected**

DISCUSSION

EF1A is extensively post-translationally modified across all organisms. It can be ubiquitinated at lysine residues (38), phosphorylated at serine and threonine residues (39, 40), acetylated (41), methyl esterified at its C-terminal lysine residue (42), methylated at multiple lysine residues and an N-terminal glycine residue (2, 3, 11, 12, 14–16) and glutaminylated at a glutamic acid residue (43). However, the functional relevance of these EF1A modifications are 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 (17) , chicken (44), brine shrimp (17), corn (17), thale cress (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 - di-methylation at Lys-57 and tri-methylation at Lys-5 respectively (47–50). Sequence analysis using BLASTp revealed no clear orthologs of yeast Efm1, 4, 5, 6, or 7 in the prokaryotic species or in *M. racemosus*. Using FungiDB, orthologs of Efm 1, 4, 6, and 7 are found for *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 (16, 57, 58). A similar point mutant strain was used in the *S. cerevisiae* study but was unavailable so

we constructed our own. They too observed that this strain is viable and had no *in vitro* difference in poly(U)-directed polyphenylalanine synthesis and 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 of about 8 lysine residues 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 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 additional substrates. Recently, evidence for 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 similar sequence motif as the Efm1 Lys-30 site. It is possible that methylation at Lys 253 could be contributing to the mono-methylation 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 wildtype strain when grown with glycerol as a carbon source, in the presence of caffeine or under oxidative and osmotic stress conditions. However, growth is also impaired in the mutant strains compared to wildtype in YPD media.

Therefore, these stress-induced phenotypes we are seeing may not be a specific result of respiratory growth or environmental stress but it is also possible that subtle growth differences are masked in this assay. For example, the *TEF1* K(30,79,316,390)R mutant did not appear to have reduced growth on solid media but the calculated doubling time revealed the slight reduction in growth. On the other hand, all of the EF1A methylation-deficient mutant strains had reduced growth in the presence of rapamycin which does appear to be a stress induced phenotype of unmethylated EF1A. This result suggests that methylated EF1A may have some role in the TORC pathway, the target of rapamycin (25).

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 since 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 of Ef-Tu (56). In yeast, it is unclear how the loss of EF1A methylation affects its protein abundance since 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 unimpaired. When amino acid incorporation, and programmed frameshift were measured by the dual luciferase translational fidelity assay system and ribosome assembly assessed using polysome analysis, the methylation deficient strains

performed similarly to the wildtype strain. There may be compensatory mechanisms in our mutant strains that allow translational functions with reduced EF1A levels.

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Chapter Four

Journey into the Methyl Proteome of Methylation-Deficient Elongation Factor 1A in Yeast

Introduction

The translational elongation factor that brings the aminoacyl-tRNA to the ribosome is a highly abundant protein that has been known to be methylated at Lys 30, 79, 316 and 390 in yeast since 1992 (1) but as early as 1979 for Lys 57 in *E. coli* (2). In the last 10 years, five methyltransferases, Efm1, Efm4, Efm5, Efm6 and Efm7 have been identified in yeast as being responsible for the methylation of the side chains of lysine residues 30, 79, 316, and 390, as well as at the N-terminus of Gly 2 and the side chain of Lys 3 (3). In my quest to understand the implication of these methylation reactions I manipulated the genes of *S. cerevisiae* in collaboration with Kevin Roy, Jason Gabunilas, Charles Wang, and Guillaume Chanfreau to develop mutant strains that would, in theory, leave EF1A unmethylated. The first approach was to mutate lysine 30, 79, 316 and 390 (the only known methylated lysine residues at that time of the construction) to arginine residues in order to preserve the bulkiness and basicity of a lysine residue at that position without the ability to be modified by lysine-specific protein methyltransferases resulting in the *TEF1 K(30,79,316,390)R* strain (Figure 4-1). This strain is not new to the literature as a similar one was constructed by Cavallius J. et al. 1997 (4). However, that strain was unavailable, so we constructed our own.

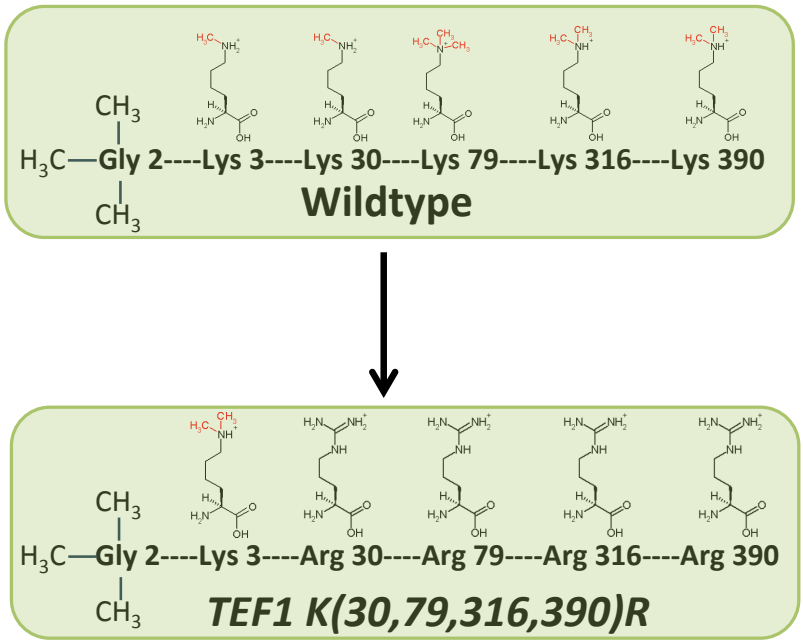


Figure 4-1: Point mutant strain constructed for this study. The predicted methylation state of the methylated residue in each strain is depicted.

The second approach was to knock out the genes associated with four enzymes responsible for methylation at those sites (*efm 1456Δ)* to serve as the compliment for the *TEF1 K(30,79,316,390)R* mutant strain (Figure 4-2). Upon learning of the discovery of the N-terminus modification (5) a mutant strain with all five methyltransferases knocked out was also generated (*efm 14567Δ*, Figure 4-2*)*.

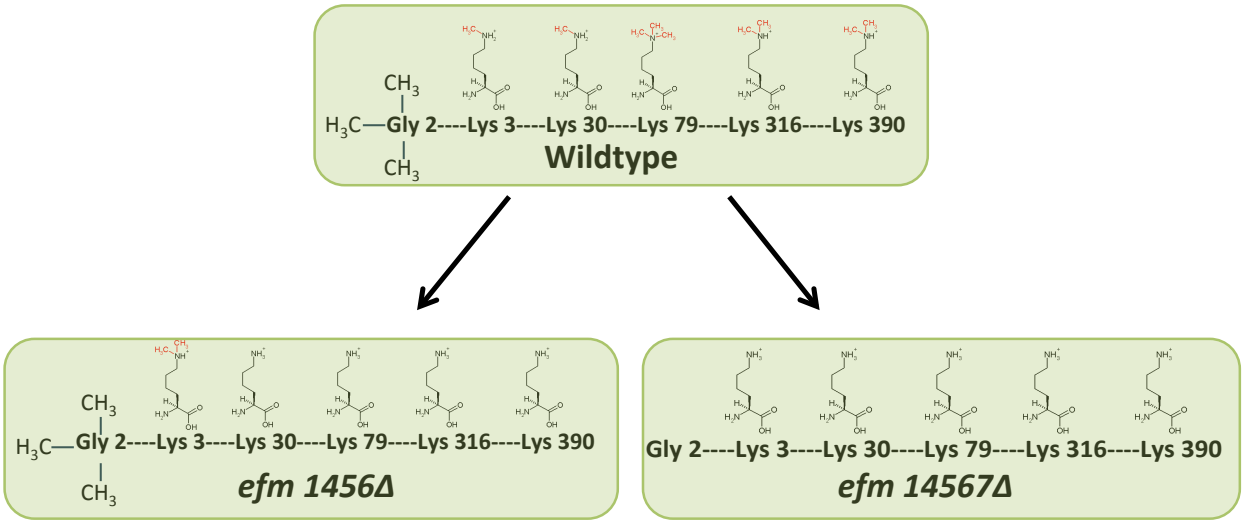


Figure 4-2: Methyltransferase mutant strains constructed for this study. The predicted methylation state of the methylated residue in each strain is depicted.

To confirm the methylation deficiency of these mutants, yeast cells were labeled *in vivo* with *S*-adenosyl-[*methyl*-^{3}H]-L-methionine, the 50 kDa band containing EF1A was excised after SDS-PAGE, and ^{3}H-methylated amino acids were analyzed by cation exchange chromatography after amino acid hydrolysis. In chapter 3, I observed that no ^{3}H-methylated lysine species were detected when all five of the elongation factor methyltransferases were knocked out. This result confirms that these five methyltransferases are the only (or the major) species responsible for EF1A lysine methylation.

However, the cation exchange studies revealed the possibility of alternative lysine residues being able to be methylated when the canonical sites were unavailable in the *TEF1 K(30,79,316,390)R* mutant (Figure 3-1). This method can identify the total population of ^{3}H mono, di-, and tri-methylated lysine species present in the protein analyzed. The canonical methylated lysine residues on wildtype EF1A contain one trimethyl (K79)(tmk), two dimethyl (K2, K316)(dmk), and two monomethyl (K30, K390)(mmk) lysine residues. Since I was unable to clearly resolve the mono- and dimethyllysine species, I would expect a ratio of tmk to

mmk/dmk of 3:6 or 0.5 in the wild type strain. In two experiments, I confirmed this ratio as 0.7 in one experiment and 0.8 in another experiment (Table 4-1). It is not clear why there appeared to be an excess of trimethyllysine species in the wild type cells. In the *TEF1 K(30,79,316,390)R* mutant I expected to see no trimethyllysine species and to see one-third of the amount of dmk/mmk species present compared to wildtype since in this strain dimethylation of lysine 3 is still present. I did observe a reduction of radiolabeled dmk/mmk species in the mutant; only 45% of dmk/mmk was found in the *TEF1 K(30,79,316,390)R* sample compared to the wildtype sample (Table 4-1). Surprisingly I also detected tri-methylated lysine when none was expected to exist (Table 4-1).

Radioactive Counts Per Minute			
	TMK	**DMK/MMK**	**Ratio**
Wildtype	1362	1957	0.6962
	1624	2006	0.8096
TEF1 K(30,79,316,390)R	122.7	865.7	0.1418
	126.2	883.0	0.1429

Table 4-1: Quantification of radioactivity found in methylated lysine peaks from cation exchange experiment in Figure 3-1. The radioactivity of two biological replicates was quantified.

Since there was a discrepancy in the expected versus observed amounts of trimethylated species in the cation exchange data of the wild type and *TEF1 K(30,79,316,390)R* mutant and because I was not able to clearly resolve the mono- and dimethylated lysine peaks, I turned to mass spectrometry to get a clearer picture of EF1A's post-translational modification status. Using LC-MS/MS in collaboration with Kate Liu and Joseph Loo, I was able to validate the methylation state of the EF1A methylation-deficient mutant strains. I was also able to demonstrate the importance of pairing conventional mass spectrometry analyses with mass spectrometry after heavy methyl SILAC labeling and the importance of validating these studies

with *in vitro* biochemical analyses. Conventional mass spectrometric identification of unlabeled peptides can be subject to the identification of false positive methylation sites (6).

Methods

Proteolytic cleavage and in-gel digestion

A single colony from the wildtype, *efm 14567Δ, or TEF1 K(30,79,316,390)R* strains described in Table 1 of chapter 3 was used to inoculate 50 mL of YPD (10 g/L yeast extract, 20 g/L tryptone, 20 g/L dextrose) growth media and grown overnight at 30 °C on a rotating shaker at 220 rpm. Next 25 mL of the overnight culture was added to two flasks containing 2L of YPD. The 2L cultures were grown under the same conditions to an OD_{600nm} of ~2 then harvested for purification. The ion-exchange purification procedure is described in chapter 3 methods. Purified EF1A (2 to 5 µg protein) from the wildtype or the mutant strains were incubated with 4 volumes of 5X SDS-PAGE sample buffer (250mM Tris-Cl pH 6.8, 10% SDS, 30% glycerol, 0.5 M DTT, 0.02% bromophenol blue) and fractionated on precast 4%-12% gradient polyacrylamide gel in MOPS running buffer (6.06 g/L Tris base, 10.46 g/L MOPS, 1 g/L SDS and 0.3 g/L EDTA, GenScript). The gel was run for 1 h at 140 V, stained with Coomassie brilliant blue and then destained with 15% methanol/ 10% acetic acid.

The Coomassie-stained gel slices from the 50-kDa region were excised (at least 3 lanes of protein per sample depending on concentration) and then washed with 3 mL of digestion buffer (50 mM ammonium bicarbonate for trypsin and 10 mM HEPES, 10 mM $CaCl_2$, pH 7.5 for lysargiNase) for 10 min and further washed with 3 mL of 50% digestion buffer and 50% acetonitrile for 2 hours, or until the gel slice became transparent. Acetonitrile (100%) was added to the transparent gel slices and the samples dried by vacuum centrifugation for 10 min. The

dried slice was then incubated in a minimal volume of 10 mM DTT in digestion buffer to cover the gel slices for 1 hr at 60 °C to reduce the disulfide bonds and then the proteins were alkylated by treatment in 50 mM iodoacetamide in digestion buffer for 45 min at 45 °C. Gel slices were then washed in a minimum volume to cover the gel slices by alternating 10-min incubations in digestion buffer and 100% acetonitrile. After the removal of the final acetonitrile wash, the gel slices were covered with a minimum volume of 20 ng/µl sequencing grade trypsin (Promega, V5111) or lysargiNase (Proteolysis Lab, IBMB-CSIC Barcelona Science Park, Barcelona) for 45 min and then the excess protease removed. The digest was performed for 16-20 h at 37 °C. The following day the peptides were eluted using 50% acetonitrile/1% trifluoroacetic acid in water and then dried by vacuum centrifugation. Peptides were stored at -20 ^{o}C until ready for analysis.

Mass Spectrometry analysis of peptides of wildtype and efm 14567Δ EF1A

The in-gel digested samples were analyzed by Top 10 data-dependent LC-MS/MS on a Q-Exactive mass spectrometer coupled to an Easy-nLC 1000. Peptides were identified using Proteome Discoverer 1.4 and searched with MASCOT with dynamic modifications for carbamidomethyl (C), oxidation (M), deamidation (N,Q), methyl (K), dimethyl (K), and trimethyl (K), trimethyl (protein N-term G), methyl (protein C-term). The precursor tolerance was 10 ppm and fragment mass tolerance was 0.02 Da. The enzyme specificity was either trypsin (C-term K/R) or lysargiNase (N-term K/R) with maximum 2 missed cleavages. The search results were filtered using fixed value PSM validator. The search result generated a list of hypothetical peptide modifications, which was manually examined and filtered based on mass accuracy of precursor and fragment ions, fragment ion coverage, precursor isotope distribution,

and retention time using Skyline by importing Mascot search result. Methylated peptides with high confidence were further confirmed with targeted mass spectrometry.

Peptides identified with known methylations (G2, K3, K30, K79, K316, K360) as well as peptides with putative methylations (K252, K375, K405) were inputted into the inclusion list for reanalysis with Parallel Reaction Monitoring mass spectrometry (PRM-MS). Both unmethylated and methylated versions were in the target list. Highly abundant peptides without detectable methylation were also included in the list as normalization standards. Two peptides were used for lysargiNase run normalization (RLPLQDVY and RVETGVI) and three for trypsin (FQEIVK[169], YAWVLDK[61], and IGGIGTVPVGR[263]. Triplicate PRM analysis was performed on both wildtype and *efm 14567Δ* EF1A, digested by either trypsin or lysargiNase. The resulting data set was analyzed in Skyline, where extracted chromatograms of all precursors were obtained. For each run, target peptide peak areas were normalized to the normalization peptides to ensure equal sample amount and minimize systematic variance from LC-MS/MS.

Mass Spectrometry analysis of TEF1 K(30,79,316,390)R EF1A

The trypsin digested sample was analyzed by Top 10 data-dependent LC-MS/MS on a Q-Exactive mass spectrometer coupled to an Easy-nLC 1000. Peptides were identified using Proteome Discoverer 2.2 and searched with MASCOT with dynamic modifications for carbamidomethyl (C), oxidation (M), deamidation (N,Q), methyl (K), di-methyl (K), and tri-methyl (K), methyl (R), di-methyl (R), and tri-methyl (R). The precursor tolerance was 10 ppm and fragment mass tolerance 0.02 Da. The enzyme specificity was trypsin with maximum 2 missed cleavages. The search results were filtered using fixed value PSM validator. The protein database was wildtype EF1A and *TEF1 K(30,79,316,390)R.*

Heavy Silac Labeling and Mass Spectrometry Analysis

Two yeast colonies from a *met6* deletion wildtype strain (GE Dharmacon, YSC3869-202332366) was first grown individually in 50 mL of synthetic growth media that contained L-methionine (CSM, MP Biomedicals) to an OD_{600nm} of 2. These cells are unable to synthesize methionine de novo and thus are dependent on methionine supplied in the culture media for protein synthesis and *S*-adenosylmethionine formation. The cells were then transferred separately to 2 L of synthetic growth media lacking L-methionine (0.75 g/L methionine amino acid dropout mix (CSM-Met, MP Biomedicals), 1.75 g/L yeast nitrogen base without amino acids or ammonium sulfate, 5 g/L ammonium sulfate and 5 g/L of dextrose supplemented with 20 mg/L of [*methyl*-D_3]-L-methionine for the "heavy" cultures (Cambridge Isotope Lab Inc., 13010-53-2) or 20 mg/L L-methionine for the "light" cultures (Calbiochem, 63-68-3). The 2 L cultures were grown at 30 °C at 250 rpm to an OD_{600nm} of ~2 and were harvested by centrifugation at 5000 x g for 5 min. The yeast pellet was stored at -80 °C until lysis could be performed. The frozen pellet was resuspended in ice cold lysis buffer (20 mM Tris-Cl pH 7.5, 20 mM MgOAc, 1 mM EDTA, 1.3% Triton X-100, and 2 mM beta-mercaptoethanol and lysed by passing through an emulsifier (EmulsiFlex-C3) six times at greater than 25,000 pounds per square inch pressure. Cell debris was removed by centrifugation at 30,000 x g for 10 min at 4 ^{o}C. 500 µg of both heavy and light lysates were mixed and then desalted (Protein desalting spin column, Thermo Scientific). The desalted heavy and light SILAC mixture was fractionated by SDS-PAGE and the 50 kDa bands containing EFlA were digested by trypsin and lysargiNase as described above and analyzed by LC-MS/MS on a Q-Exactive mass spectrometer. Data analysis was carried out in Thermo Proteome Discoverer 2.2. To identify methylated peptides, a

differential modification search was employed that considered variable mass shifts of 14.0157 (monomethylation), 28.0314 (dimethylation), and 42.0471 (tri-methylation) on lysine and arginine to identify unlabeled peptide. The heavy search considered a static mass shift of 3.0189 Da from L-[*methyl*-D_3]-methionine and variable mass shift of 17.0346 (monomethylation), 34.0692 (dimethylation), and 51.1038 Da (tri-methylation) on lysine or arginine. Database searches were conducted with trypsin and lysargiNase enzyme specificity. Peptide identifications were validated with Percolator with a 5% false discovery rate by target decoy strategy. The Skyline software package was used to manually evaluate the presence of heavy-light peptide doublet from extracted ion chromatograms generated for each peptide. PRM runs were performed to target these light-heavy peptide pairs.

Results and Discussion

Mass spectrometric analyses of EF1A from wildtype yeast

EF1A was purified from wildtype yeast grown in D-glucose media, as described in the "Methods" section and proteolytic peptides analyzed by a targeted parallel reaction monitoring (PRM) LC-MS/MS approach to detect methylated residues. Wildtype EF1A is known to have monomethylation at Lys 30 and 390, dimethylation at Lys 3 and 316, and trimethylation at the N-terminal residue Gly 2 and at Lys 79 (Figure 4-3).

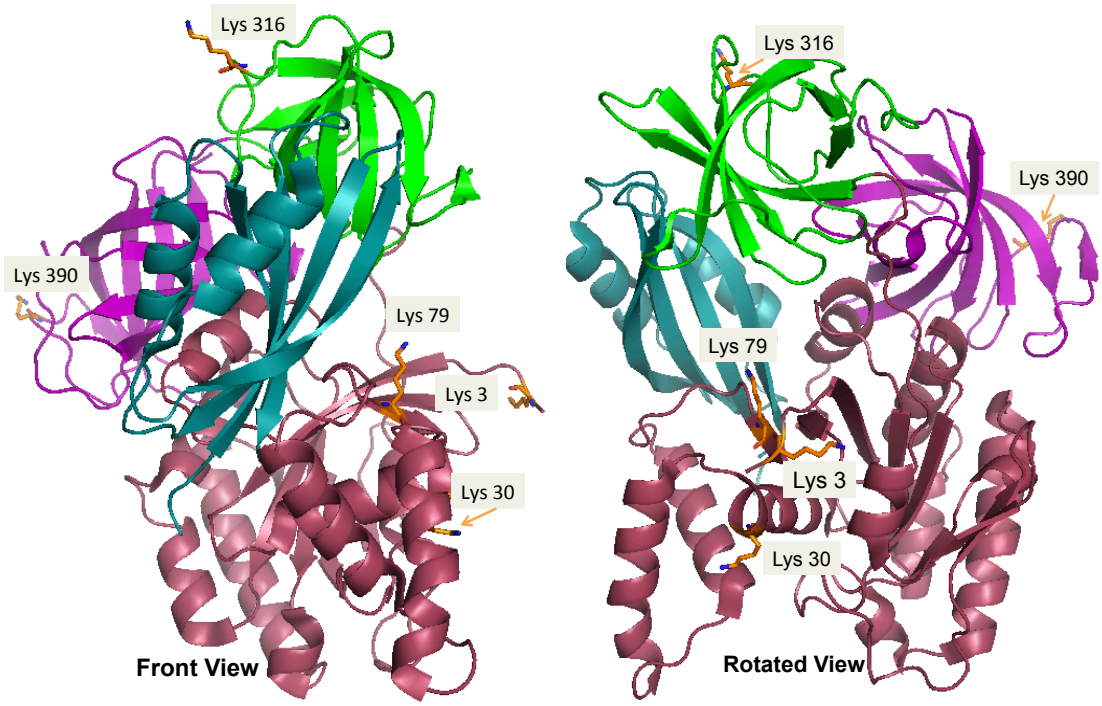


Figure 4-3: X-Ray crystallography image of yeast EF1A complexed with EF1B showing methylated lysine residues. Structure is from PDB 1F60 file. EF1B is colored in teal. The GTP binding domain of EF1A is shown in dark pink, aa-tRNA/EF1B binding domain green and the actin binding domain purple. The methylated lysine residues are shown in orange

For EFlA from wild type cells, we were able to detect peptides containing both unmodified and monomethylated Lys 30 when trypsin or lysargiNase was used to digest the protein (Figure 4-4). A negligible amount of unmodified Lys 30 was found in all three trypsin replicates while it was only detected in two out of three lysargiNase replicates. All three replicates from trypsin and lysargiNase show the presence of monomethylated Lys 30. Methylation at Lys 30 is very abundant and was best detected with trypsin since the lysargiNase peptides found were found in much lower abundance. Major b and y ions were found adjacent to the site of methylation, confirming its identification. Since the level of methylated Lys 30 was some ten-fold higher than the unmethylated form, it appears that methylation at this residue was

nearly complete under the growth conditions used.

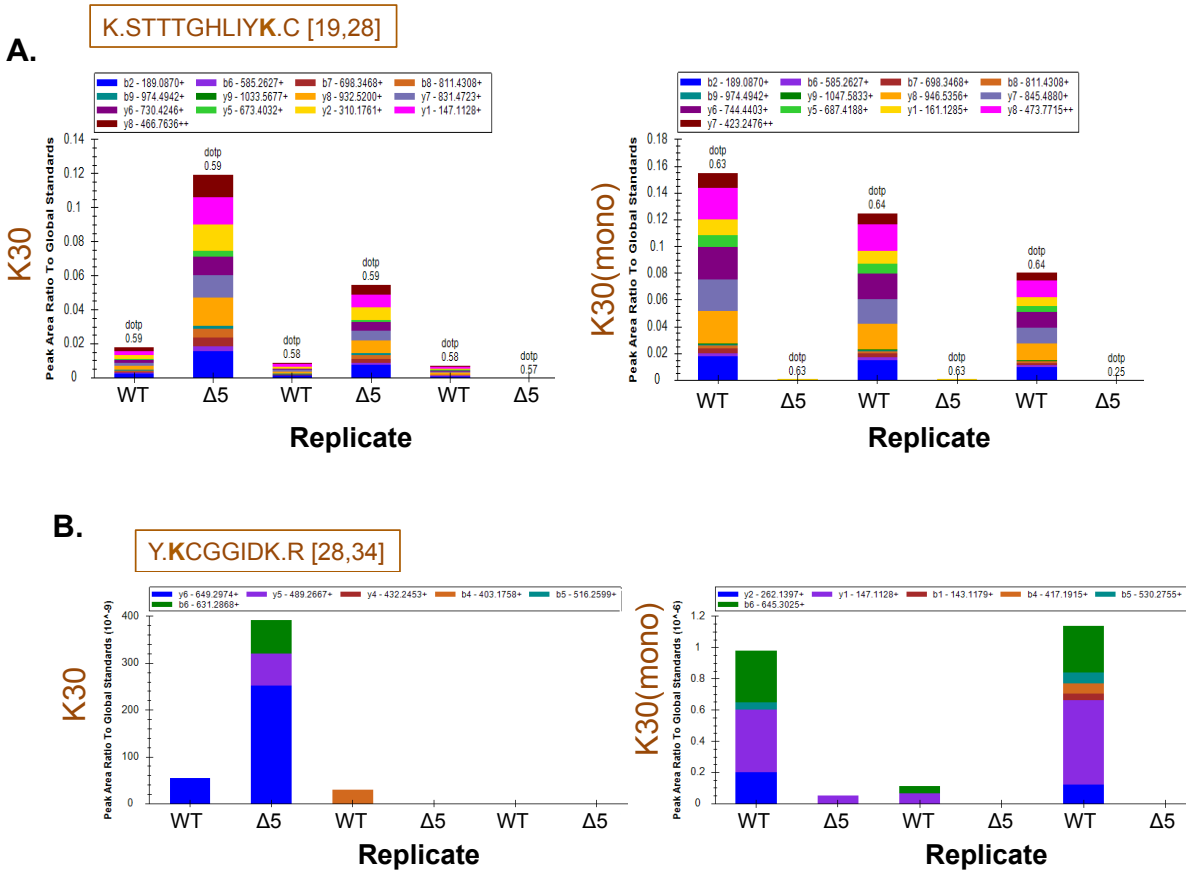


Figure 4-4: Stoichiometry of Lys 30 residue on EF1A when digested with trypsin or lysargiNase. Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain *efm 14567Δ* yeast cells were fractionated using SDS-PAGE and digested with the protease trypsin **(A)** or lysargiNase **(B)** The relative abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ*5) represents *efm 14567Δ* EF1A. This targeted PRM run was performed in triplicate. The full scan spectra for tryptic peptides are found in appendix figure 1.

Trimethylation at Lys 79 was abundantly found when wildtype EFlA was digested with both proteases in all repeat experiments (Figure 4-5). Significantly, we observed major b and y ions surrounding that modification site, giving confidence to its validity. No intermediate such as

mono- or dimethylation at Lys 79 was found. Using lysargiNase we were unable to detect any unmodified Lys 79 while only background levels of only a few ions were found in two out of three trypsin experiments suggesting Lys-79 is fully methylated in the cells.

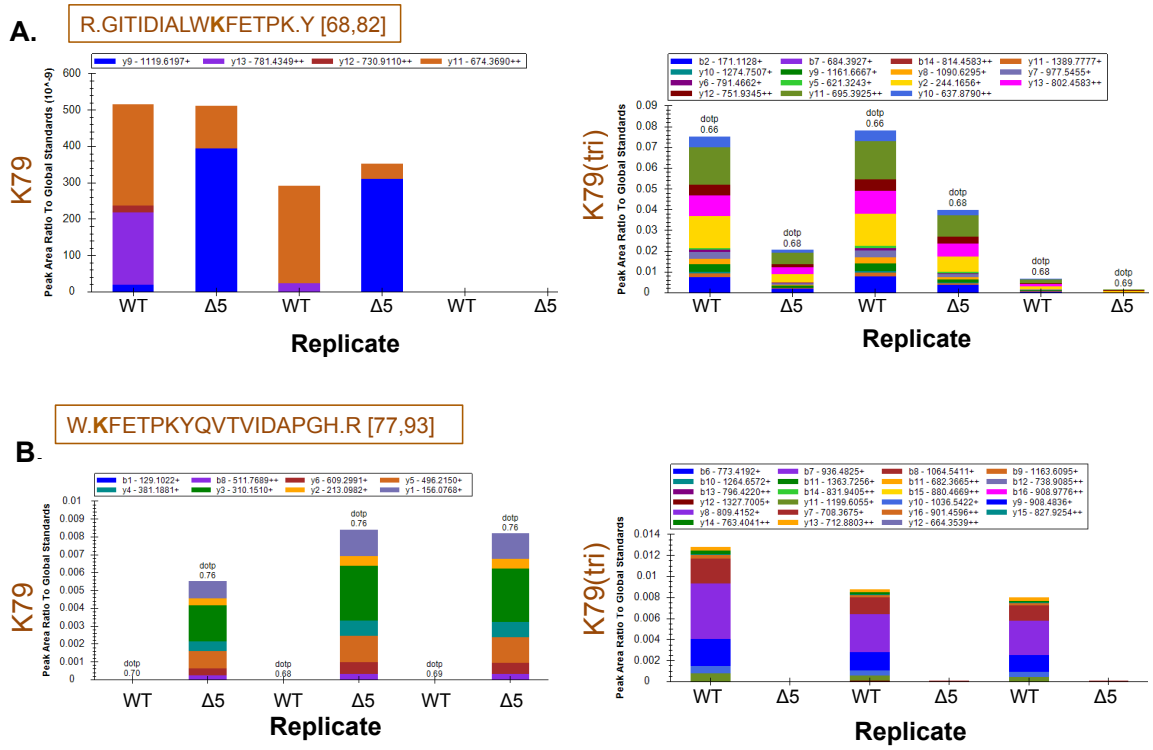


Figure 4-5: Stoichiometry of Lys 79 residue on EF1A when digested with trypsin or lysargiNase. Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain *efm 14567Δ* yeast cells were fractionated using SDS-PAGE and digested with the protease trypsin **(A)** or lysargiNase **(B)** The relative abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ*5) represents *efm 14567Δ* EF1A. This targeted PRM run was performed in triplicate.

Dimethylation at Lys 316 was found in all three replicate experiments for both enzyme conditions (Figure 4-6 A/B). Interestingly, we found in three replicates monomethylated Lys 316 for the trypsin digestion (Figure 4-6 A) but only one surrounding ion was present. We did not clearly identify any unmodified Lys 316 in lysariNase digests and only background levels of a

few b and y ions in the tryptic digests. We thus believe that dimethylation at this site is the major form since its abundance is much greater than what was observed for the other forms. Hart-Smith G. et al. 2014 previously reported both monomethylated and dimethylated Lys 316 (7).

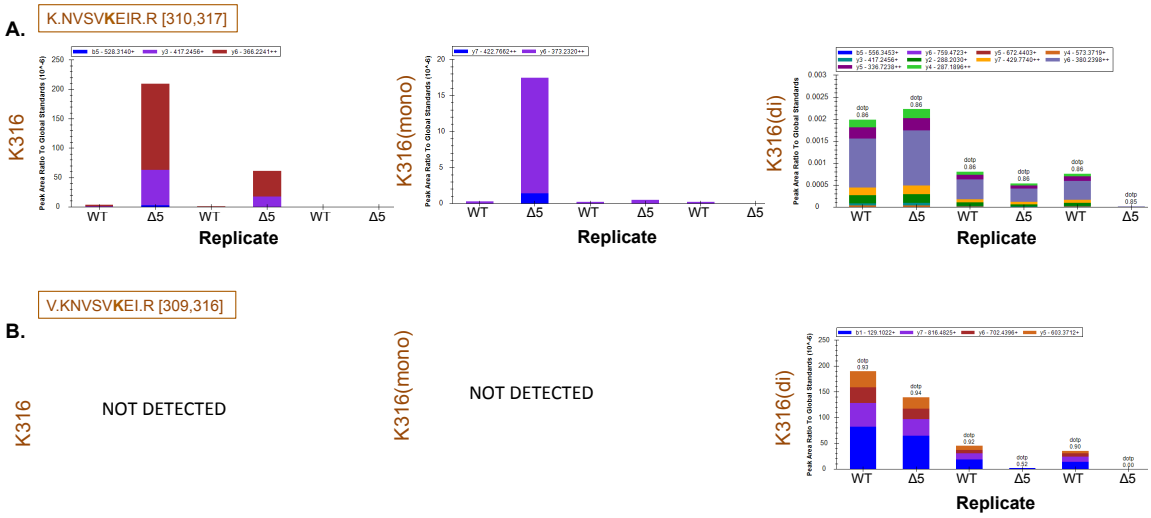


Figure 4-6: Stoichiometry of Lys 316 residue on EF1A when digested with trypsin or lysargiNase. Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain *efm 14567Δ* yeast cells were fractionated using SDS-PAGE and digested with the protease trypsin **(A)** or lysargiNase **(B)** The relative abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ*5) represents *efm 14567Δ* EF1A. The full scan spectrum for tryptic peptides is shown in appendix Figure 1. This targeted PRM run was performed in triplicate.

Only low levels of unmodified and methylated Lys 390 containing peptides were detected in the trypsin digestion; with a ratio of about 3:1 for unmodified:monomethylated (Figure 4-7 A). In the lysargiNase digestion, unmodified Lys 390 was about eight-fold more abundant than monomethylated Lys 390 (Figure 4-7 B). Overall, Lys 390 only appears to be partially methylated under our growth conditions. In previous work, it was found that monomethyl Lys

390 was most prevalent when EF1A was overexpressed (8). Additionally, only when EF1A is overexpressed could dimethylation at Lys 390 be detected (8).

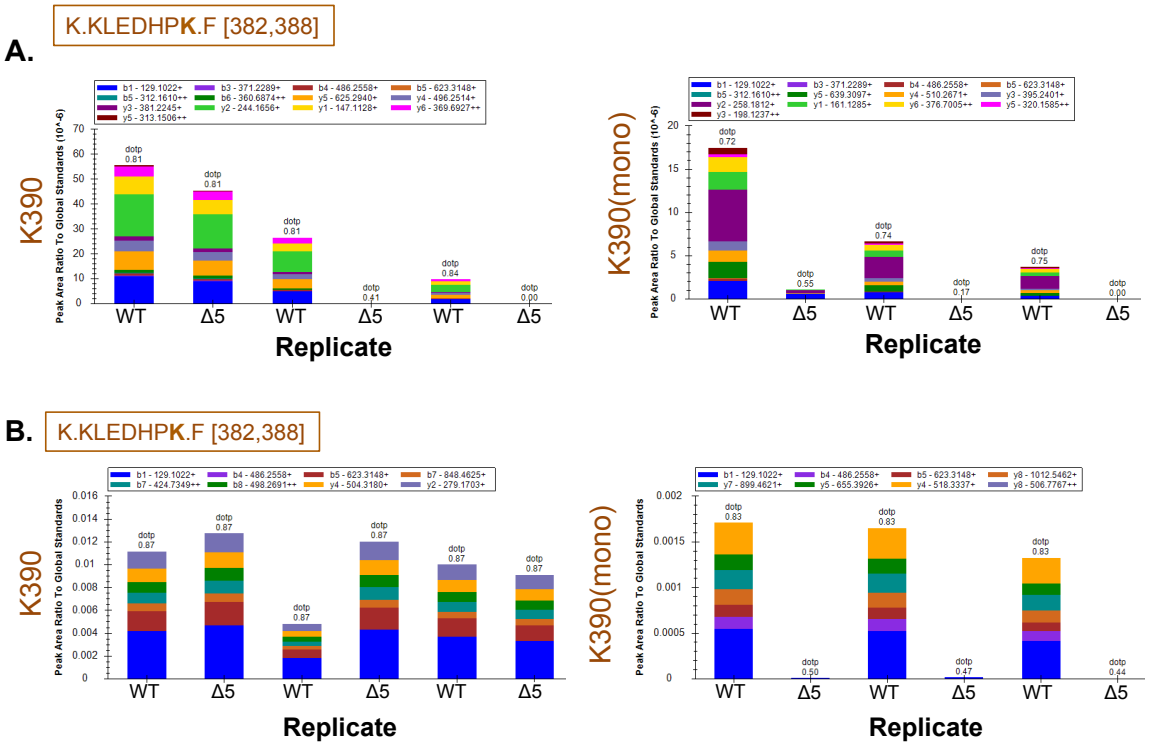


Figure 4-7: Stoichiometry of Lys 390 residue on EF1A when digested with trypsin or lysargiNase. Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain *efm 14567Δ* yeast cells were fractionated using SDS-PAGE and digested with the protease trypsin **(A)** or lysargiNase **(B)** The relative abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ*5) represents *efm 14567Δ* EF1A. The full scan spectrum for tryptic peptides is shown in appendix figure 1. This targeted PRM run was performed in triplicate.

Our mass spectrometry experiments were also able to detect the N-terminal trimethyl modification of EF1A at Gly 2 and variable levels of methylation of the adjoining Lys 3 residue in tryptic peptides (Figure 4-8). Gly 2 appears to be fully methylated in wildtype cells - no unmethylated or partially methylated peptides were detected. However, we detected unmodified,

monomethylated, and dimethylated Lys 3. Hamey et al. 2016 reported that Gly 2 and Lys 3 were trimethylated and dimethylated using Asp N protease digestion (5). These authors also detected the variants with only trimethylation at Gly 2 as well as trimethylation of Gly 2 and mono-methylation at Lys 3 (5).

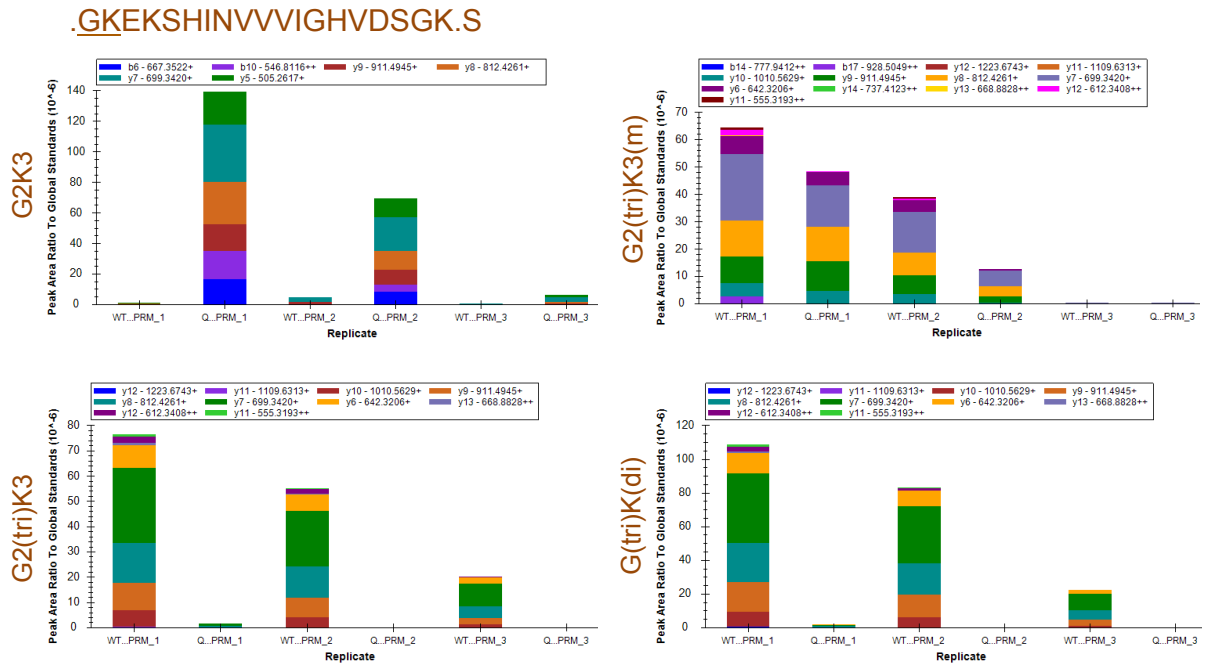


Figure 4-8: N-terminal modifications found on wildtype and methyltransferase deficient EF1A. Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain (*efm 14567Δ*) were fractionated using SDS-PAGE and digested with the protease trypsin which C-terminally cleaves lysine or arginine and analyzed using LC-MS/MS. The abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ*5) represents *efm 14567Δ* EF1A. This targeted PRM run was performed in triplicate.

Taken together these results demonstrate that our mass spectrometric methods are able to detect and confirm the methylation state of wildtype EF1A.

Putative novel methylation sites in EF1A

In the targeted PRM experiment described thus far, we also found evidence of potential new methylation sites on wildtype EF1A. Monomethylation of Lys 252, 375, and 405 were observed when digested with trypsin (Figure 4-9) and lysargiNase (Figure 4-10). Methylation at Lys 252 and 375 were detected at substoichiometric levels. The abundance of the unmethylated forms is more prevalent. Monomethylation at Lys 375 and 405 were detected in multiple experimental runs of trypsin/lysargiNase digestion as well as two runs with Asp-N digestion, which N-terminally cleaves at Asp and Cys residues (Table 4-2).

Data Analysis File Name	K252(m)	K375(m)	K400,405
021617_50kDa_Thermolysin			
032717_50kDa_Thermolysin			
032717_WT_Trypsin			
032717_WT_AspN			
052217_WT_Trypsin			
052217_WT_AspN			
052217_WT_Thermolysin			
052218_WT_Trypsin			
052218_Quint_Trypsin			
052218_WT_LysArg			
052218_Quint_LysArg			

Table 4-2: Putative methylation sites detected from each full scan LC-MS/MS experiment.

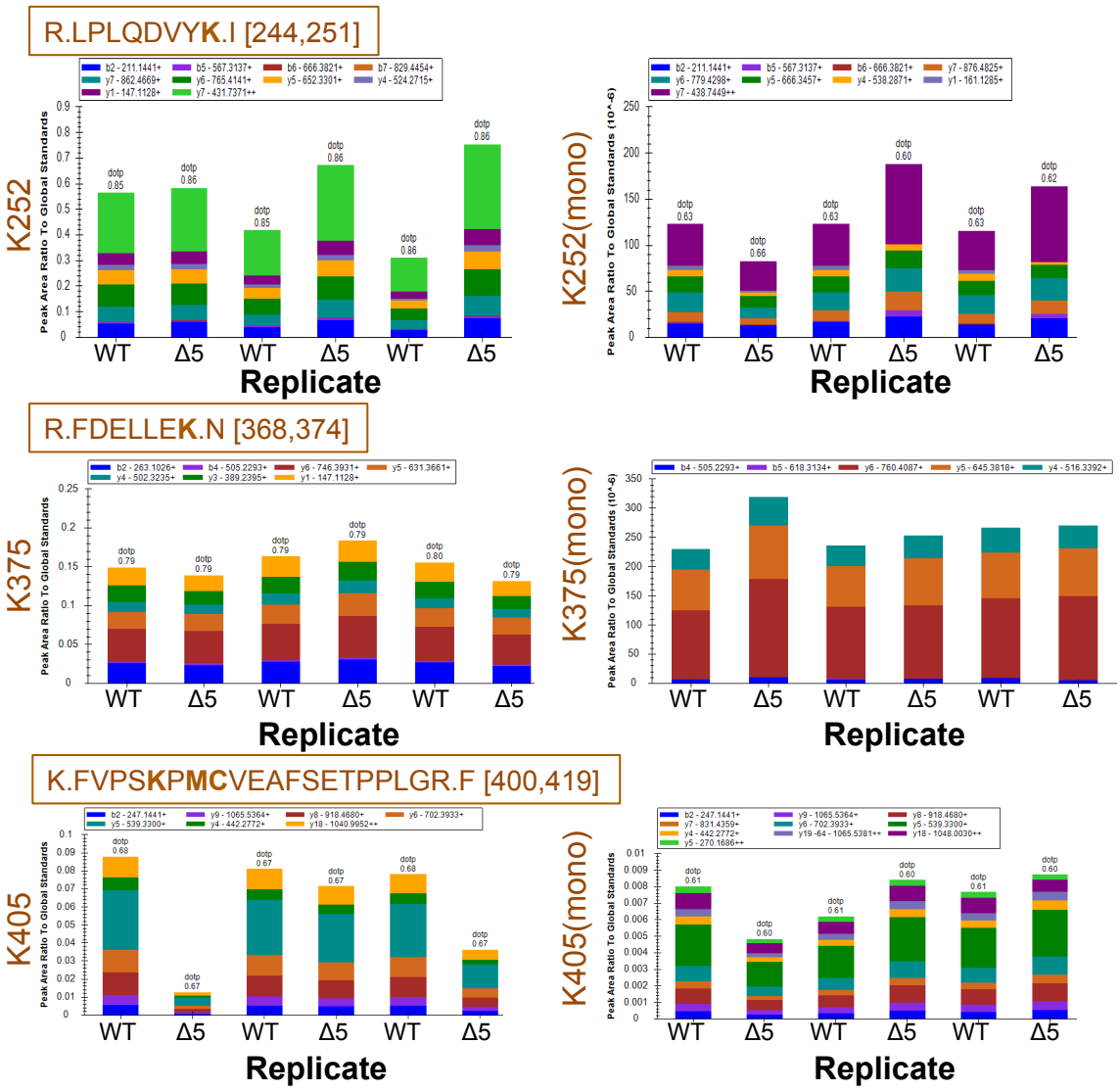


Figure 4-9: Trypsin detection of novel putative lysine methylation sites.

Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain (*efm 14567Δ*) were fractionated using SDS-PAGE and digested with the protease trypsin which C-terminally cleaves lysine or arginine and analyzed using LC-MS/MS. The abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ5*) represents *efm 14567Δ* EF1A. This targeted PRM run was performed in triplicate. The full scan spectrum is shown in appendix figure 2.

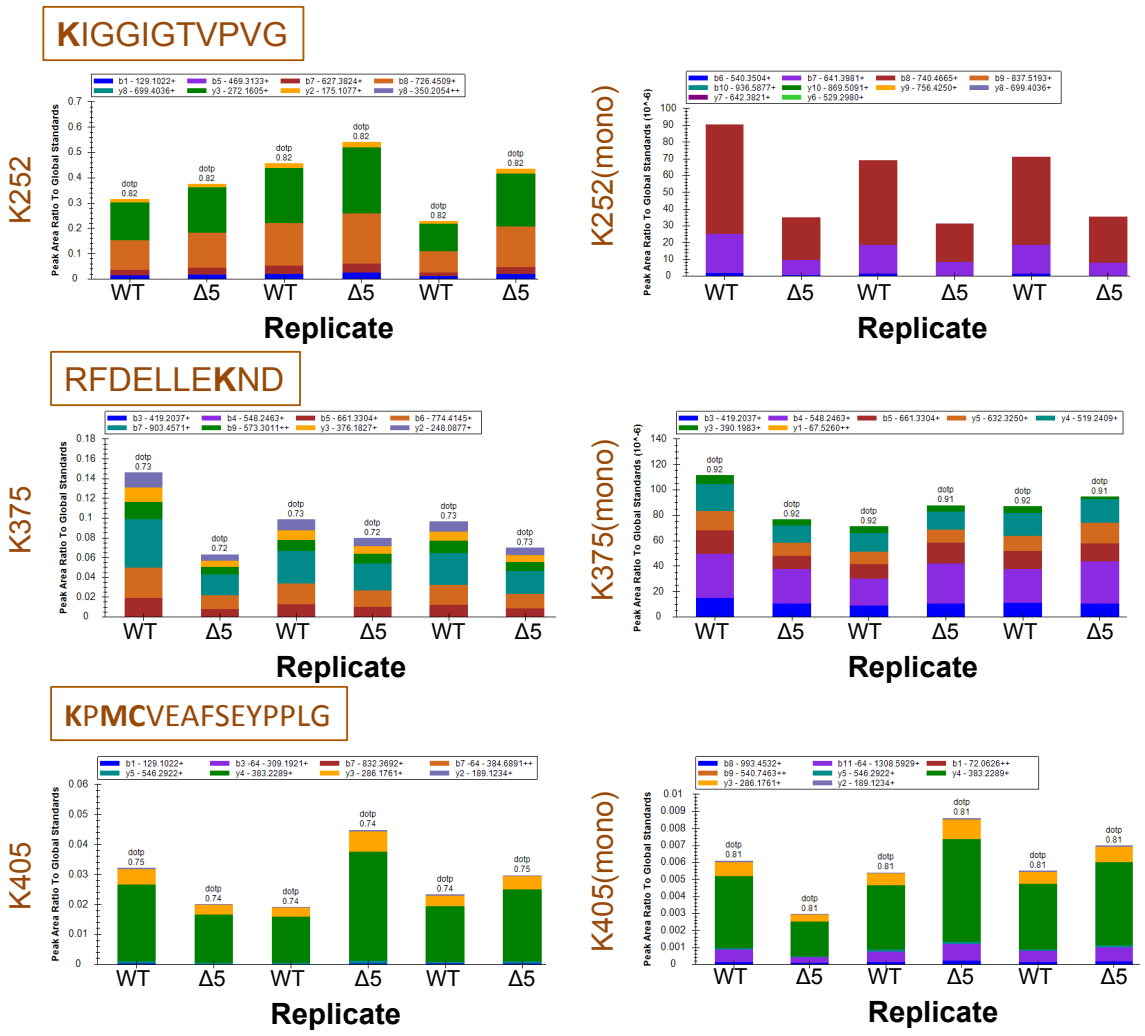


Figure 4-10: LysargiNase detection of novel putative lysine methylation sites.

Purified EF1A (described in chapter 3 methods) from wildtype and the quint methyltransferase mutant strain (*efm 14567Δ*) were fractionated using SDS-PAGE and digested with the protease lysargiNase which N-terminally cleaves lysine or arginine and analyzed using LC-MS/MS. The abundance of peptide fragments found for each modification is shown for each type of EF1A. In this image (*Δ*5) represents *efm 14567Δ* EF1A. This targeted PRM run was performed in triplicate.

Examination of wildtype EF1A structure when it is complexed with EF1B reveals that these putative methylation sites are solvent exposed and in close proximity to some of the

canonical lysine methylation sites (Figure 4-11). Lys 252 and 316 and Lys 376 and 390 are both 10.6 angstroms away from each other. This distance was determined using the Pymol measurement function.

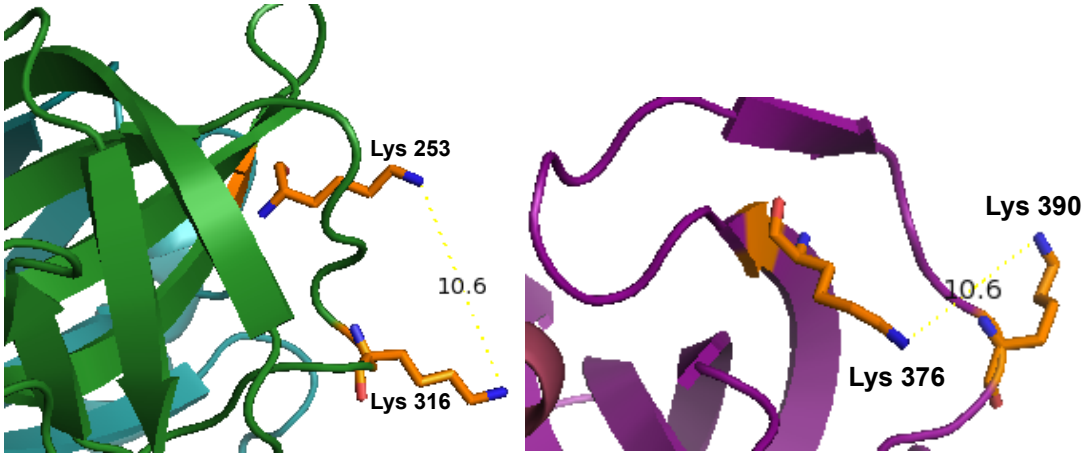


Figure 4-11: Zoomed in wildtype EF1A structure depicting the newly identified methylation sites. Distance between the known methylated site and the new site is depicted by the yellow dotted line. Pymol measurement function used to determine distance. PDB file 1F60.

Confirmation of wildtype methylation sites by SILAC analyses of yeast grown in normal L-methionine and in methyl-deuterated L-methionine.

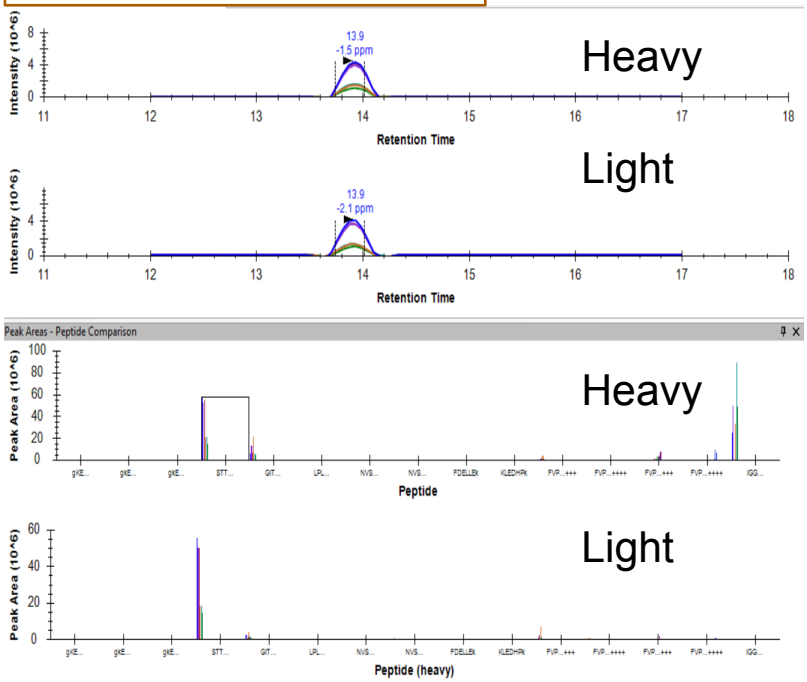
Identifying posttranslational modifications using a bottom-up approach as we have can be complicated. There can be a high false positive discovery rate, especially with low abundance peptides (6). Historically, wide-scale proteomic studies of post-translational modifications fall victim to these problems, which leads to minimal overlaps in the results of various proteomic studies to detect methylated residues in yeast proteins (9–11). Thus, we sought to verify our findings using heavy SILAC labeling. Heavy SILAC labeling is advantageous because methylation is a small modification that can cause a mass shift change of 14, 28, or 42 kDa, which can be non-ambiguous since other amino acid substitutions can have this same change.

For example, acetylation can also cause a mass shift of 42 kDa. Here, high-resolution mass spectrometry can detect the difference between acetylation and trimethylation but in other cases the masses would be precisely the same. Having a deuterated methyl group allows for comparison of the heavy and light version of these monomethylated lysine residues.

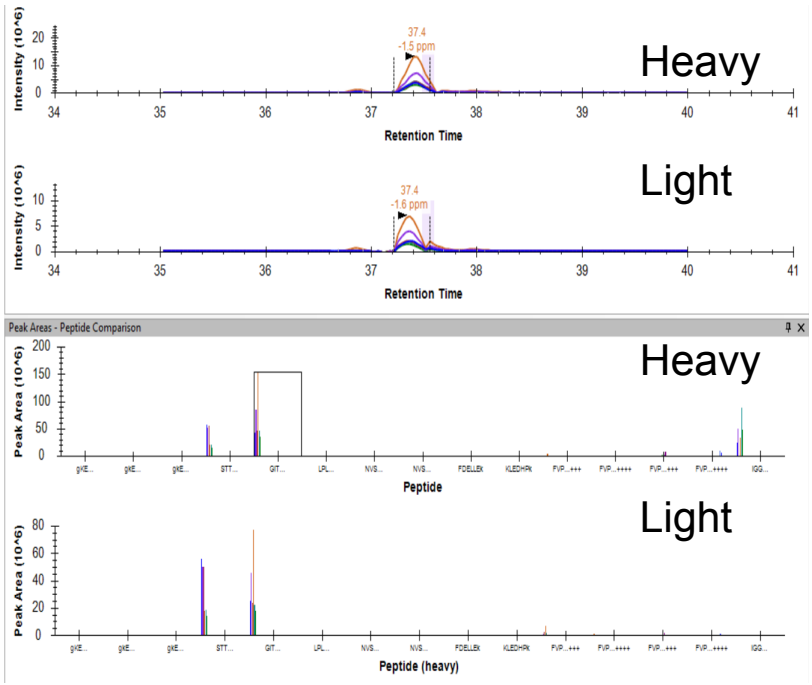
We examined the methylation status of lysates from a wildtype yeast strain that had a deletion of the MET6 gene (*met6*Δ) which is responsible for the biosynthesis of methionine (12, 13) that was grown with or without methyl-deuterated L-methionine as described in the methods section. We did not use our BY4742 wildtype strain because this strain can biosynthesize methionine and dilute out the deuterated label. Even with the BY4741 strain that is auxotrophic for methionine, recycling can dilute the label as described by Caslavka et al. (14). These authors demonstrated better heavy and light labeling for *met6*Δ cells compared to BY4741 cells (14). Maintaining the 1:1 ratio of heavy and light labeling is crucial for this method because if only the light form is found in the heavy sample that methylated modification cannot be confirmed.

First, we were able to detect heavy and light peptides for methylation at Lys 30, 79, 316 and 390 (Figure 4-12). We were unable to detect the N terminal modification in this experiment

K30m STTTGHLIY**K**



K79t GITIDIALW**K**FETPK



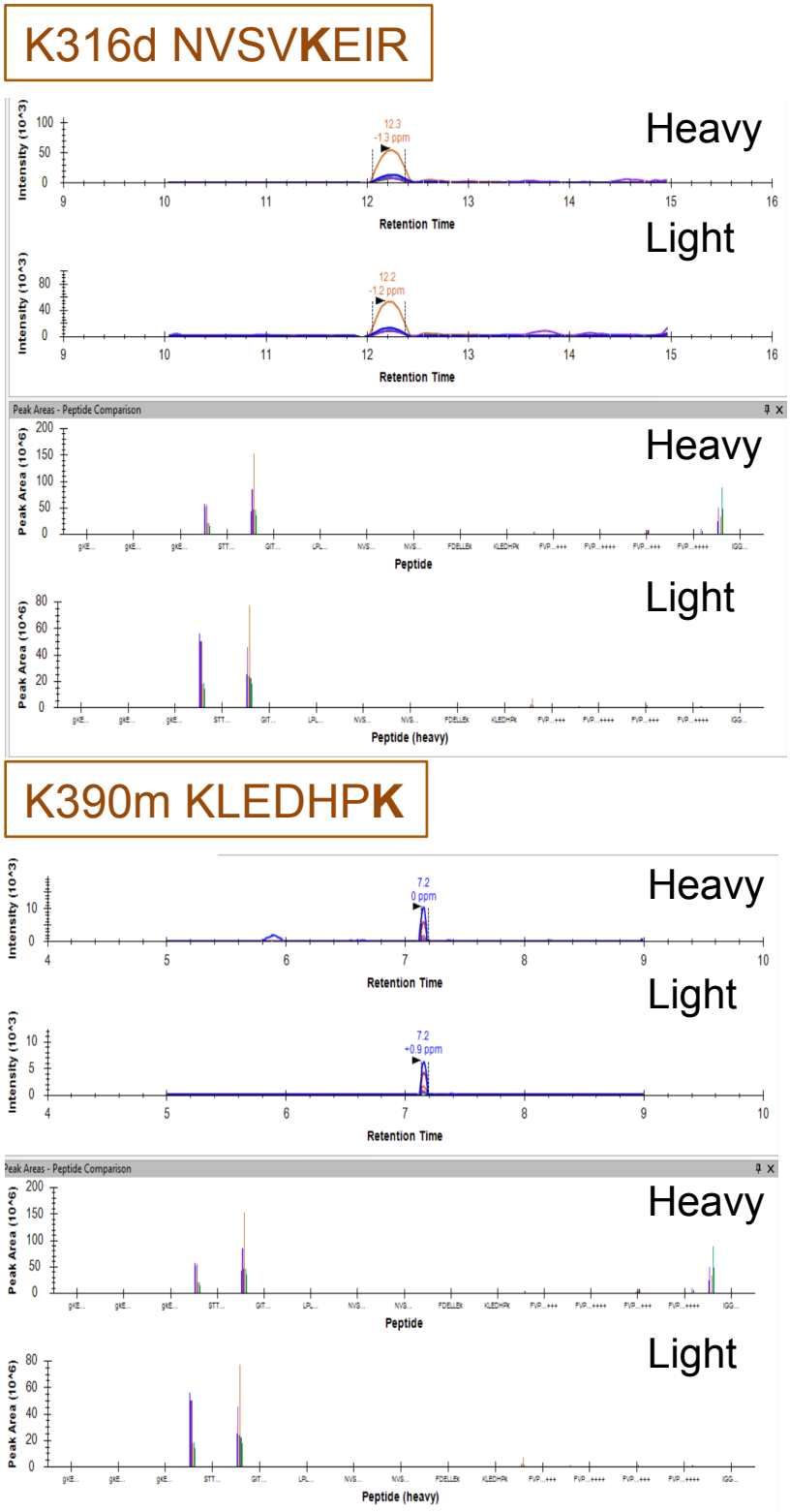


Figure 4-12: Peak areas and intensity plots of peptides from heavy and light wildtype peptides.

During our search to validate the authenticity of these sites Hamey et al. found that Efm 1 can recognize a distinct sequence motif YKXGG where Y can be any hydrophobic residue, K is the target lysine and X is any amino acid (15). They then found that a peptide with the K253 sequence, which contains this recognition motif (YKIGG), could be methylated *in vitro* (15). We both were able to detect methylation at Lys 252 *in vivo* (Figures 4-9 and 4-10). However, in our preliminary heavy SILAC labeling experiment, I was unable to further validate methylation at Lys 252, 375, and 405. Since the abundance of these sites are low for endogenous levels of EF1A, the overexpression EF1A should allow us to detect these sites better. I recently obtained an EF1A over expression plasmid from Charles Wang in the Chanfreau lab that can be transformed into the *met6*Δ strain for a repeat heavy SILAC labeling experiment. It is possible that the methylation at these sites is not dependent upon *S*-adenosylmethionine. For example, a bacterial flavin and methylenetetrahydrofolate-dependent methyltransferase has been described for a tRNA species (16).

Analysis of the quint methyltransferase mutant methylation

EF1A purified from the *efm 14567Δ* strain was also analyzed to confirm the methylation status of the known methylated sites and whether or not the putative methylation sites existed on it as well. No monomethylation at Lys 30 was detected in both conditions tested (Figure 4-4 A/B). Unmethylated Lys 30 was detected for the trypsin peptides in two out of three replicates (Figure 4-4 A). Only a few peptides were found for unmodified Lys 30 in one replicate when *efm 14567Δ* EF1A was digested with lysargiNase, therefore no conclusion can be made from that experiment (Figure 4-4 B).

Surprisingly *efm 14567Δ* EF1A was found to have eight-fold (replicate one), and 2-fold (replicate 2) less trimethylation at Lys 79 in tryptic peptides (Figure 4-5 A). In the third replicate Lys 79 trimethylation was undetectable (Figure 4-5 A). On the other hand, trimethylation at Lys 79 was undetectable in all replicates of lysargiNase peptides (Figure 4-5 B). The unmodified Lys 79 was not well detected for trypsin peptides since only two surrounding ions were found in two replicates and nothing in the third (Figure 4-5 A). Unmodified Lys 79 was clearly detected for lysargiNase peptides in all three replicates (Figure 4-5 B). Under these growth conditions, it appears Lys 79 is unmethylated on our *efm 14567Δ* EF1A.

We did not clearly detect unmodified and monomethylated Lys 316 for both protease conditions tested (Figure 4-6 A/B). A minimal amount of dimethylation of Lys 316 was found in tryptic peptides in two replicate experiments. In these two replicates; one replicate appeared to have four-fold more dimethylation than the other (Figure 4-6 A). A very low abundance of dimethylation at Lys 316 was detected in peptides digested with lysargiNase in one replicate experiment (Figure 4-6 B). Nothing was detected in the other two replicate experiments (Figure 4-6 B). The majority of this data suggests that methylation at Lys 316 would be unmethylated.

Only background levels of tryptic peptides of methylated and unmethylated Lys 390 were detected in one replicate each (Figure 4-6 A) where unmodified Lys 390 was 4- fold greater. Nothing was detected in the other replicate experiments (Figure 4-6 A). Unmethylated Lys 390 was more abundantly detected in lysargiNase digested peptides while the methylated form was not detected in all three replicates (Figure 4-6 B). Unmethylated Lys 390 appears to solely exist under these growth conditions.

At the N-terminus Gly 2 appears to be unmethylated since that was the most prevalent tryptic peptide found (Figure 4-8). Trimethylation at Gly 2 with monomethylation at Lys 3 was the only variant modification detected on *efm 14567Δ* EF1A. No tryptic peptides were found for the other variant modifications of the N-terminal Gly2 and the adjoining Lys3 residue.

We also examined whether these putative new methylation sites were present in this methyltransferase mutant yeast strain. We detected a very low abundance of monomethylation at Lys 252, 375, and 405 in tryptic (Figure 4-9) and lysargiNase peptide (Figure 4-10). The unmodified form of each of these sites was more abundant under both protease conditions (Figure 4-9/4-10). Methylation at Lys 252 and 375 was not convincingly demonstrated in this quint mutant (Figure 4-9/4-10) while methylation of Lys 405 was unaffected by the quint mutant suggesting methylation at this site is not dependent on the methyltransferases that are knocked out in this mutant strain (Fig. 4-9/4-10).

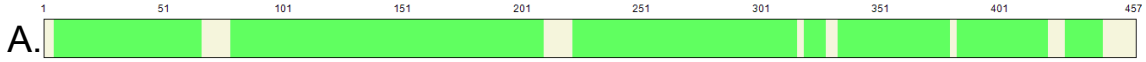
The finding of methylated sites at Lys 79 and Lys 316 in some of the EF1A tryptic digests of the quint mutant is unexpected since no radiolabeled methylated lysine species were detected in acid hydrolysates of yeast cells labeled *in vivo* (chapter 3). We note that no methylated species were seen in the lysargiNase digests. We have no good explanation for the tryptic results.

Analysis of the point mutant

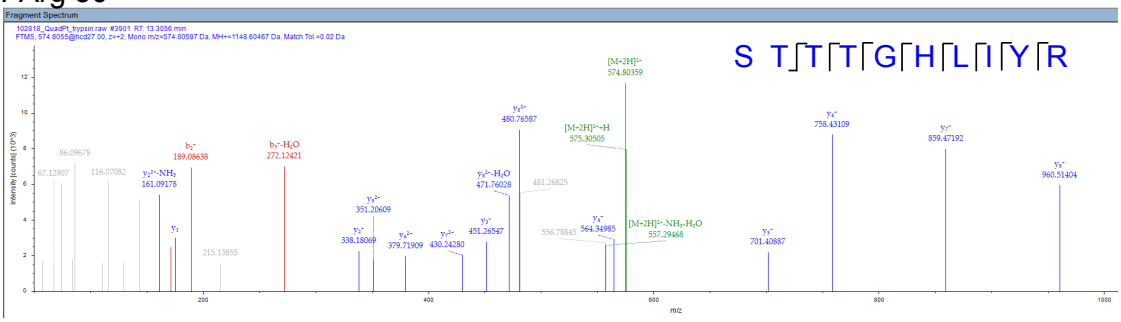
We were also able to confirm the presence of Arg 30, 79, 316 and 390 for the *TEF1 K(30,79,316,390)R* mutant strain. In the experiment depicted in figure 4-13, we were unable to get sequence coverage for Arg 79. (Figure 4-13 A) However, in a different replicate experiment

(Appendix Figure 3) this site was found. Figure 4-13 shows the fragment spectra for Arg 30, 316, and 390.

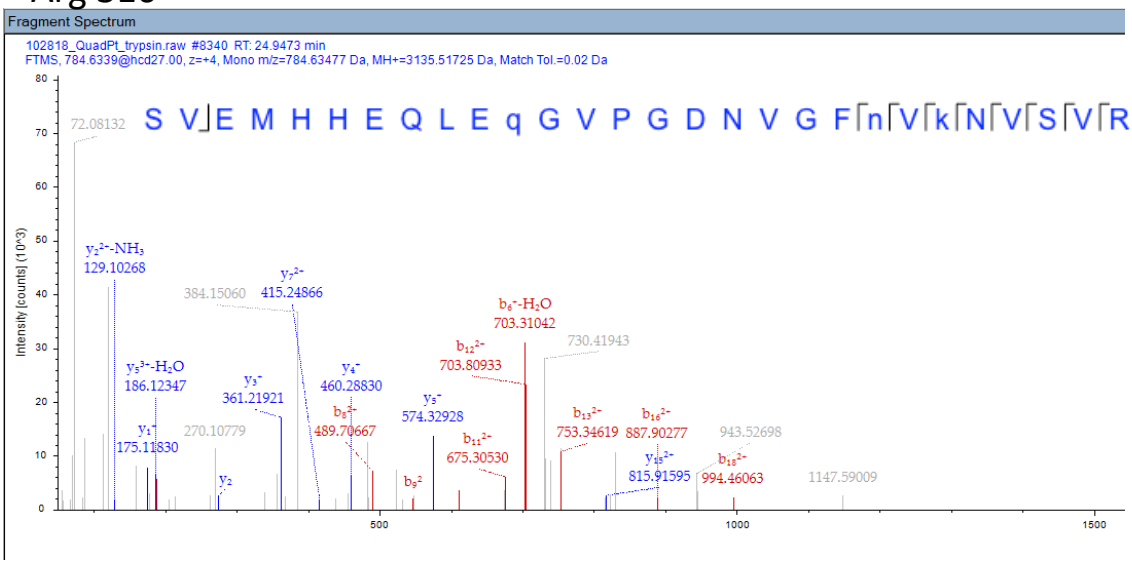
Interestingly the putative methylation sites we identified on wildtype and *efm 14567Δ* EF1A were also detected (Appendix Figure 4). Relative abundance of these sites using targeted PRM run was not performed.



B. Arg 30



Arg 316



Arg 390

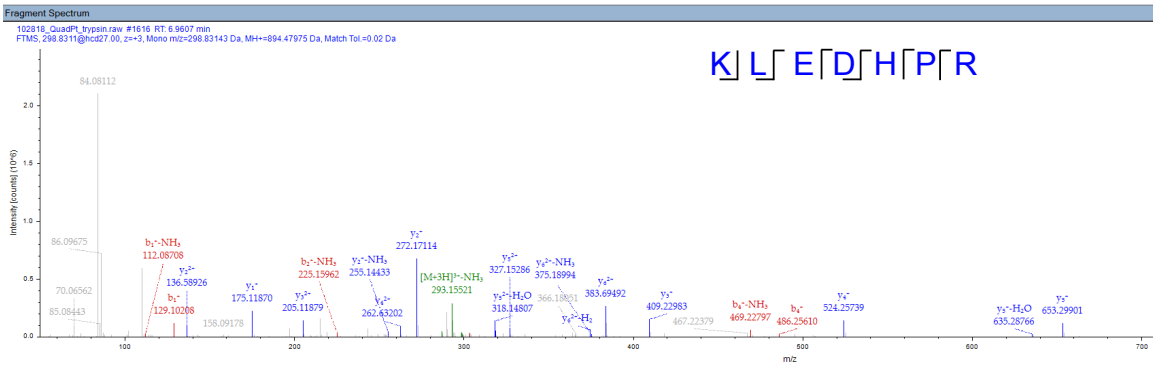


Figure 4-13: Fragment spectra of *TEF1 K(30,79,316,390)R* EF1A. EF1A from the mutant strain was trypsin digested and analyzed by LC-MS/MS. **A**) Sequence map showing amino acids found in green. No sequence coverage was available for Arg 79. B) Fragment spectra showing the b-ions found in red, y-ions in blue and the precursor peptide in green.

Possibility of a C-terminal methyl ester

In 2000 it was reported that the C-terminus of EF1A contains a carboxyl methyl esterification on a lysine residue (17). The specific lysine residue and methyltransferase responsible for this modification has yet to be elucidated. In our mass spectrometry analysis we probed for this modification as well. When purified wildtype EF1A from *S. cerevisiae* as described in chapter 3 methods was fractionated by SDS-PAGE and the 50 kDa region excised and digested with Asp-N protease, we were able to detect the entire C-terminus of EF1A (Figure 4-14). The most C-terminal peptide we found was DKTEKAAKVTKAAQKAAKK, corresponding to the terminal residues encoded by the open reading frame of the *TEF1/TEF2* genes, and we did not detect a modified form of any lysine residue in this region. The lack of this modification could be due to its volatile or reversible nature as well loss during the EF1A purification or the failure to detect the methylated form by mass spectrometry. Zobel-Thropp et al. purified EF1A with a one ion exchange chromatography step, whereas I used three to have a

purer preparation, which could have caused the modification to be lost. It will be worthwhile to use that one step purified EF1A, Asp-N digest it and subject it to a more sensitive targeted mass spectrometry run as opposed to Top 10 LC-MS/MS like we have shown here.

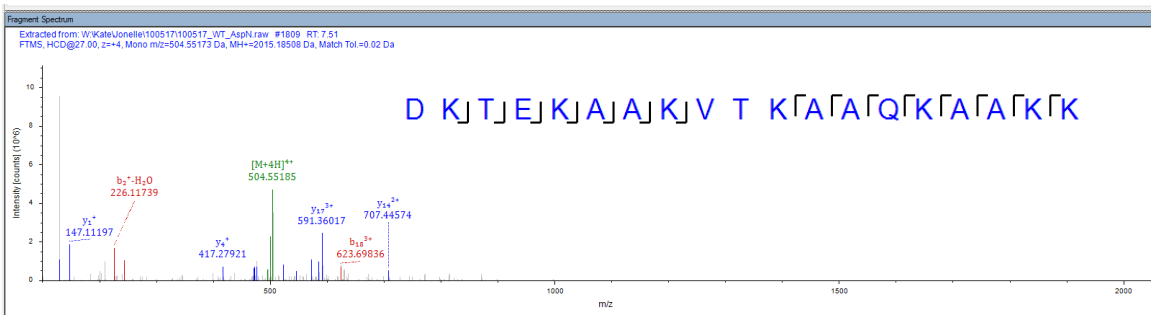


Figure 4-14: Fragment Spectra of Asp-N digested wildtype EF1A. LC-MS/MS was performed on Asp-N digested wildtype EF1A peptides. The b-ions found for this peptide are shown in red, y-ions in blue and the precursor peptide in green.

In conclusion, our mass spectrometry methods was able to detect similar wildtype methylation trends within the literature (7, 8), as a proof of validity of our methods. Our methods were able to detect a small abundance of 3 potentially novel lysine residues (Lys 252, 375, and 405) that are all monomethylated. We were able to elucidate the methylation status of *efm 14567Δ* mutant strains which agrees with the results from chapter 3 where I observed that no ^{3}H-methylated lysine species were detected when all five of the elongation factor methyltransferases were knocked out. This result confirms that these five methyltransferases are the only (or the major) species responsible for EF1A lysine methylation.

In the case of the putative methylation sites, we cannot unambiguously confirm their identification at this time. It will be worthwhile to repeat the heavy SILAC experiment once more optimizing for protein abundance to determine if those sites exist. I believe these sites might be real since it has continually been observed in different mutant EF1A yeast strains as

well as wildtype and with different proteases (Table 4-2). Detecting mono-methylation at Lys 252 with heavy SILAC labeling would ideally serve as a control since it is known that it can be methylated *in vitro* (15). However, even if they are found to be false positives, this study demonstrates the necessity for having alternative mass spectrometry validation or biochemical methods for post-translation modification identification.

Appendix

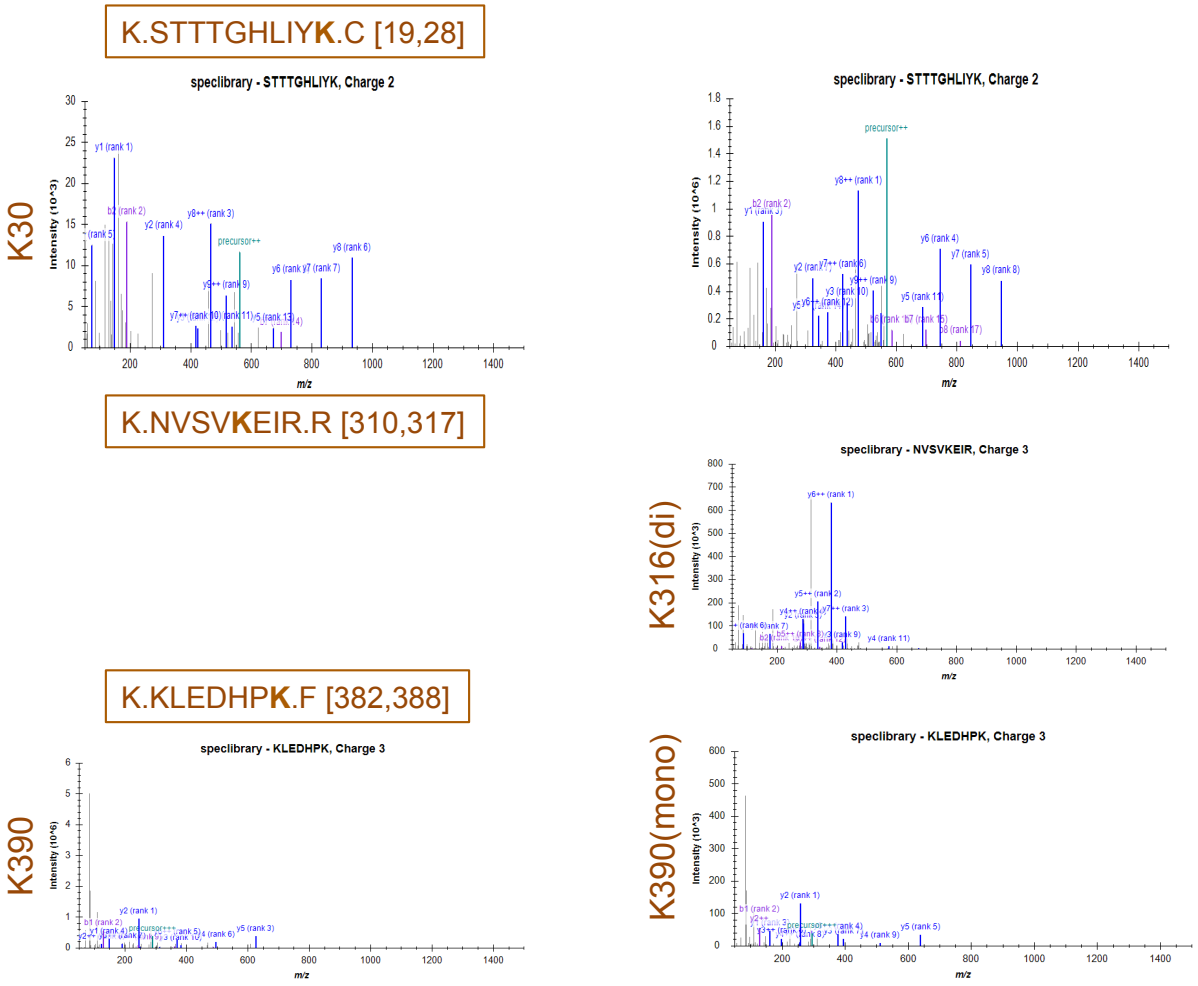


Figure 1: Full scan peptide detection in trypsin of canonical lysine methylation sites. Wildtype and *efm 14567Δ* EF1A was digested in trypsin and a LC-MS/MS scan performed. The ions found from both strains in all three replicates are shown. Peptide corresponding to Lys 79 was not detected in the full scan of this experiment.

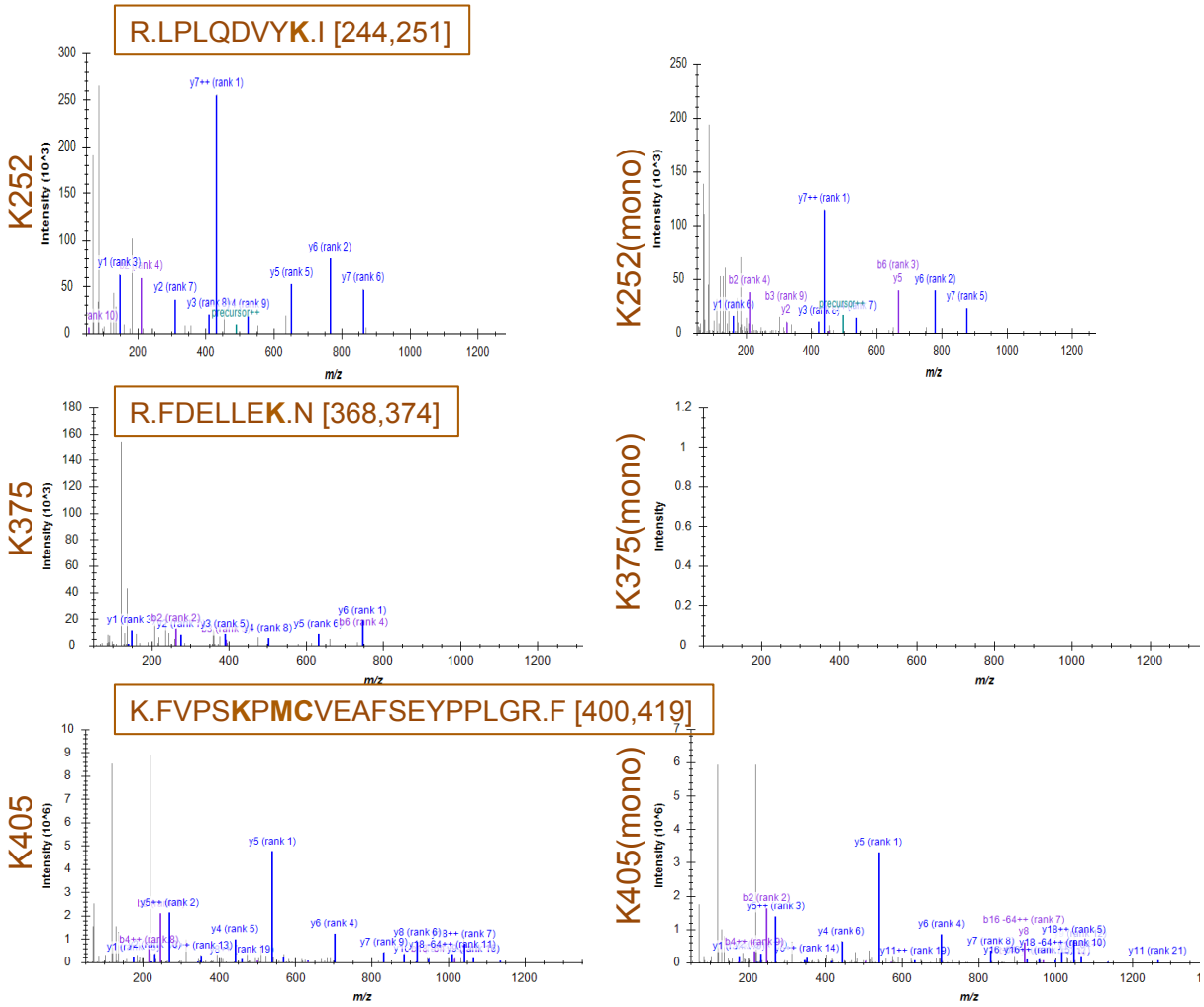
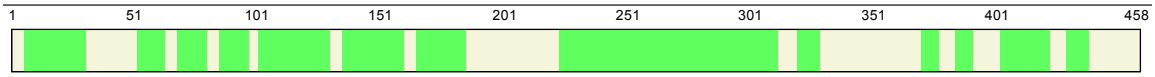


Figure 2: Full scan peptide detection spectra of putative methylation sites. Wildtype and *efm 14567Δ* EF1A was digested in trypsin and a full LC-MS/MS scan performed. The ions found from both strains in all three replicates are shown.



Arg 79 GITIDIALWR

Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b⁺	b²⁺	Seq.	y⁺	y²⁺	#2
1	58.02875	29.51801	G			10
2	171.11282	86.06005	I	1100.64631	550.82679	9
3	272.16050	136.58389	T	987.56224	494.28476	8
4	385.24457	193.12592	I	886.51456	443.76092	7
5	500.27152	250.63940	D	773.43049	387.21888	6
6	613.35559	307.18143	I	658.40354	329.70541	5
7	684.39271	342.69999	A	545.31947	273.16337	4
8	797.47678	399.24203	L	474.28235	237.64481	3
9	983.55610	492.28169	W	361.19828	181.10278	2
10			R	175.11896	88.06312	1

Figure 3: Sequence coverage and b/y ions found of *TEF1 K(30,79,316,390)R* EF1A digested with trypsin.

K252m

Peptide Summary

Sequence: LPLQDVYK. K8-Methyl (14.01565 Da)
Charge: +2, Monoisotopic m/z: 495.28595 Da (-1.01 mmu/-2.04 ppm), MH+: 989.56462 Da, RT: 21.81 min.
Identified with: Mascot (v1.30); IonScore:17, Exp Value:2.0E-002, Ions matched by search engine: 4/64
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

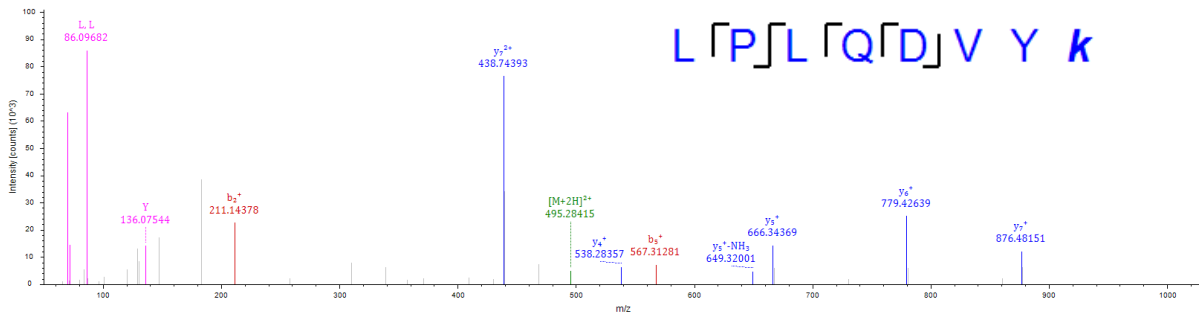
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b⁺	b²⁺	Seq.	y⁺	y²⁺	#2
1	114.09135	57.54931	L			8
2	211.14412	106.07570	P	876.48257	438.74492	7
3	324.22819	162.61773	L	779.42980	390.21854	6
4	452.28677	226.64702	Q	666.34573	333.67650	5
5	567.31372	284.16050	D	538.28715	269.64721	4
6	666.38214	333.69471	V	423.26020	212.13374	3
7	829.44546	415.22637	Y	324.19178	162.59953	2
8			K-Methyl	161.12846	81.06787	1

Fragment Spectrum

Extracted from: W:\Kate\Jonelle\1018 quad pt\102818_QuadPt_trypsin.raw #7132 RT: 21.81
FTMS, HCD@27.00, z=+2, Mono m/z=495.28595 Da, MH+=989.56462 Da, Match Tol.=0.02 Da



K375m

Peptide Summary

Sequence: FDELLEK, K7-Methyl (14.01565 Da)
Charge: +2. Monoisotopic m/z: 454.24191 Da (-0.31 mmu/-0.69 ppm). MH+: 907.47655 Da. RT: 23.38 min.
Identified with: Mascot (v1.30): IonScore:26, Exp Value:2.6E-003. Ions matched by search engine: 5/56
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Fragment Matches

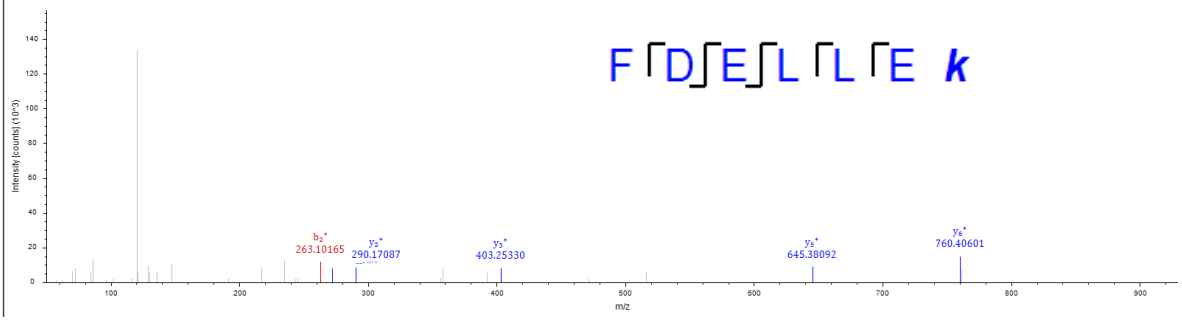
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	Seq.	y+	y2+	#2
1	148.07570	74.54149	F			7
2	263.10265	132.05496	D	760.40875	380.70801	6
3	392.14525	196.57626	E	645.38180	323.19454	5
4	505.22932	253.11830	L	516.33920	258.67324	4
5	618.31339	309.66033	L	403.25513	202.13120	3
6	747.35599	374.18163	E	290.17106	145.58917	2
7			K-Methyl	161.12846	81.06787	1

Fragment Spectrum

Extracted from: W:\Kate\Jonelle\1018 quad pt\102818_QuadPt_trypsin.raw #7728 RT: 23.38
FTMS, HCD@27.00, z=+2, Mono m/z=454.24191 Da, MH+=907.47655 Da, Match Tol.=0.02 Da



K405m

Peptide Summary

Sequence: FVPSKPMCVEAFSEYPPLGR, K5-Methyl (14.01565 Da), M7-Oxidation (15.99492 Da), C8-Carbamidomethyl (57.02146 Da)
Charge: +3. Monoisotopic m/z: 781.04926 Da (-0.81 mmu/-1.04 ppm). MH+: 2341.13321 Da. RT: 26.54 min.
Identified with: Mascot (v1.30): IonScore:27, Exp Value:1.8E-003. Ions matched by search engine: 5/202
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	b3+	Seq.	y+	y2+	y3+	#2
1	148.07570	74.54149	50.03008	F				20
2	247.14412	124.07570	83.05289	V	2194.06723	1097.53725	732.02726	19
3	344.19689	172.60208	115.40381	P	2094.99881	1048.00304	699.00445	18
4	431.22892	216.11810	144.41449	S	1997.94604	999.47666	666.65353	17
5	573.33954	287.17341	191.78470	K-Methyl	1910.91401	955.96064	637.64285	16
6	670.39231	335.69979	224.13562	P	1768.80339	884.90533	590.27265	15
7	817.42772	409.21750	273.14742	M-Oxidation	1671.75062	836.37895	557.92172	14
8	977.45838	489.23283	326.49098	C-Carbami...	1524.71521	762.86124	508.90992	13
9	1076.52680	538.76704	359.51378	V	1364.68455	682.84591	455.56637	12
10	1205.56940	603.28834	402.52798	E	1265.61613	633.31170	422.54356	11
11	1276.60652	638.80690	426.20702	A	1136.57353	568.79040	379.52936	10
12	1423.67494	712.34111	475.22983	F	1065.53641	533.27184	355.85032	9
13	1510.70697	755.85712	504.24051	S	918.46799	459.73763	306.82751	8
14	1639.74957	820.37842	547.25471	E	831.43596	416.22162	277.81684	7
15	1802.81289	901.91008	601.60915	Y	702.39336	351.70032	234.80264	6
16	1899.86566	950.43647	633.96007	P	539.32004	270.16566	180.44830	5

Fragment Spectrum

Extracted from: W:\Kate\Jonelle\1018 quad pt\102818_QuadPt_trypsin.raw #8958 RT: 26.54
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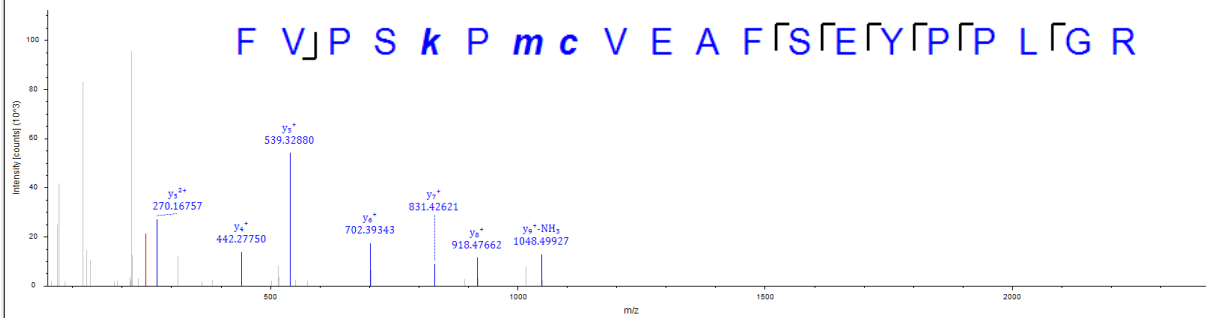

Figure 4: Fragment spectra of putative novel lysine residues found on *TEF1 K(30,79,316,390)R* EF1A. EF1A from the mutant strain was trypsin digested and analyzed by LC-MS/MS. Fragment spectra showing the b-ions found in red, y-ions in blue and the precursor peptide in green.

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Chapter Five

Does EF1A Methylation Direct its Interaction with the Actin Cytoskeleton?

Introduction

An additional role of elongation factor 1 as an actin binding protein was first discovered in 1990 in the amoebozoa *Dictyostelium discoideum* (1, 2). In this model organism, EF1A has been shown to bind F-actin with a Kd of 0.2 μM in a pH-dependent manner and can inhibit both polymerization and depolymerization of actin filaments (2–5). Later work in the yeast *Saccharomyces cerevisiae* showed that the GDP-bound form of EF1A could bind to and polymerize actin and result in actin bundling (6, 7). In yeast, it was demonstrated that the guanine nucleotide exchange factor, EF1B, modulates EF1A's ability to bundle actin (8). EF1B is responsible for reactivating EF1A by exchanging GDP for GTP. This exchange factor was structurally shown to bind in between domains 1 and 2 of EF1A (9). EF1B is also able to outcompete other EF1A binding partners that are known actin regulators. For example, EF1B inhibits EF1A binding to Rho1, which is a GTPase that activates the formin Bni1 that catalyzes the assembly of actin filaments with another actin binding protein profilin (10). This evidence supports the model that EF1A can be directed to protein synthesis as the GTP-bound form by the exchange of GDP for GTP by EF1B or to an interaction with the actin cytoskeleton as the GDP-bound form (4).

In the yeast EF1A-EF1B three-dimensional structure, methylation sites are present in each of its domains. EF1A's structure is divided into three functional domains, where domain 1 binds GTP or GDP, domain 2 binds the aminoacyl-tRNA and EF1B, and domain 3 binds actin (9, 11). Four of EF1A's methylated residues (Gly 2, Lys 3, Lys 30, and Lys 79) are in the GTP-binding domain, one (Lys 316) is in the aminoacyl-tRNA/EF1B binding domain, and one site (Lys 390) is in the actin-binding domain. Additionally, mutagenesis studies have identified

multiple residues within domains 1 and 2 that can inhibit actin binding, reduce actin bundling, and alter actin cytoskeletal organization of the yeast buds (6, 12, 13).

Since the methylated sites occur on the three domains that are involved in actin interactions, I investigated how EF1A methylation affects these interactions using a wildtype and two methylation-deficient mutant strains.

Methods

Cosedimentation Centrifugation

Centrifugation was performed as described in (6) with the following changes: HEPES instead of PIPES was used for the cosedimentation buffer and EF1A purified from yeast strains as described in chapter 3 was used. Once the centrifugation was over the pellet and supernatant fractions were solubilized with 3X SDS protein loading buffer (250 mM Tris-Cl pH 6.8, 10% SDS, 30% glycerol, 0.5 M DTT, 0.02% bromophenol blue) for 25 min at room temperature. All samples from the supernatant and pellet were fractionated on precast 4%-12% gradient polyacrylamide gel in MOPS running 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 140V, stained with Coomassie brilliant blue and then destained with 15% methanol/ 10% acetic acid. Rabbit skeletal actin or yeast actin (kind gifts from the laboratories of Margot Quinlan and Emil Reisler) was used as indicated.

Total Internal Reflection Microscopy (TIRF) of Actin Bundles formed In Vitro

Purified monomeric rabbit skeletal actin only (RSA, 5 μM final concentration), RSA (5 μM) and wildtype purified yeast EF1A (1 μM final concentration), or RSA (5 μM) and *efm 14567Δ* purified yeast EF1A (1 μM) were allowed to come to equilibrium overnight at 4^{o} C as

described below. Ten-fold less volume than the volume of the RSA stock of 500 mM $MgCl_2$, 2 mM EGTA was added. The reaction was then brought up to a final volume of 150 µL with 10 mM HEPES pH 7, 1 mM EGTA, 50 mM KCl, 1 mM $MgCl_2$ (KMEH buffer). The purification of EF1A is described in chapter 3 methods. The incubation mixtures were analyzed using a Leica DM16000 TIRF microscope as described below. The amount of actin in each sample was diluted from 5 µM to 2 µM with KMEH buffer and mixed with 2 µM Alexa Fluor 488 Phalloidin (ThermoFisher scientific) in a 1:1 volume ratio. A 50x dilution was then made for each sample and 10 µL of that sample was mounted on a glass cover slide with a poly-L-lysine coverslip. A cut pipette tip was used to avoid excessive shearing of the actin filaments.

Actin Staining and Confocal Microscopy of Intact Yeast Cells

Yeast cells were grown to an $OD_{600\ nm}$ of 0.5 at 30 ^{o}C in YPD. 37% formaldehyde (0.7 mL) was added to 5 mL of the actively growing culture and allowed to sit at room temperature for 1 h. Next, the cells were centrifuged at 3000 x g for 2 min and the pellet washed 3 times with 1 mL of PBS using the same spin time. After the last wash, the pellet was resuspended in 50 µL of PBS. A solution of 10 µL cells, 1 µL 488-phalloidin and 39 µL of PBS was incubated overnight in the dark. The next day the cells were centrifuged and washed three times with 100 µL of PBS. A 10x dilution of the overnight cells was mounted onto a regular microscope slide with mounting buffer (Prolong GOLD with DAPI). A poly-L-lysine coverslip was overlaid onto the solution for imaging.

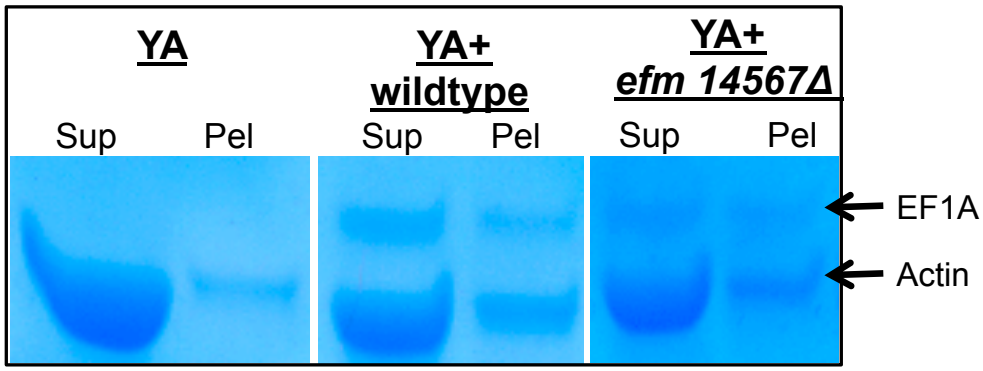
Each sample was imaged using a Leica SP6 confocal microscope. The actin structures were imaged using the 63x oil immersion lens. XYZ-planes slices starting at the top and bottom

of visible actin structures were taken and then merged to form a single image. The merging processing was done using FIJI- Z projection max intensity function.

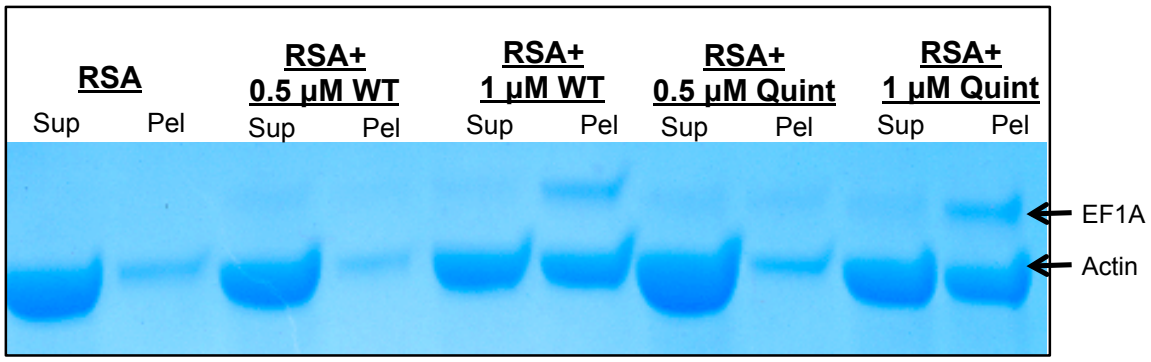
Results and Discussion

To perform *in vitro* studies, wildtype and methylation deficient EF1A (*efm 14567Δ)* was purified from *S. cerevisiae* using three steps of ion exchange chromatography described in chapter 3 "Experimental Methods". Using centrifugation, I first analyzed the bundling capabilities of purified wildtype compared to *efm 14567Δ* EF1A. At low centrifugal speeds actin is unable to be pelleted when alone but when it interacts with EF1A it becomes able to. In Figure 5-1 A, I show bundling for yeast actin. Here I detected no difference with the wildtype or mutant EFlA. I then focused my work on rabbit skeletal actin (RSA). In a similar bundling assay shown in Figure 5-1 B, I found more bundling of actin, but still no significant difference in the wildtype and mutant preparations of EF1A.

A.



B.



	Normalized Actin Peak Area Density		% Actin bundled	% Difference
	Supernatant	Pellet		
RSA Only	1	0.2	20.3	
0.5 µM Wildtype+RSA	0.91	0.16	17.0	
1 µM Wildtype+RSA	0.77	0.64	82.4	65.4
0.5 µM Efm 14567Δ +RSA	1.1	0.23	20.4	
1 µM Efm 14567Δ +RSA	0.75	0.51	68.4	48.0

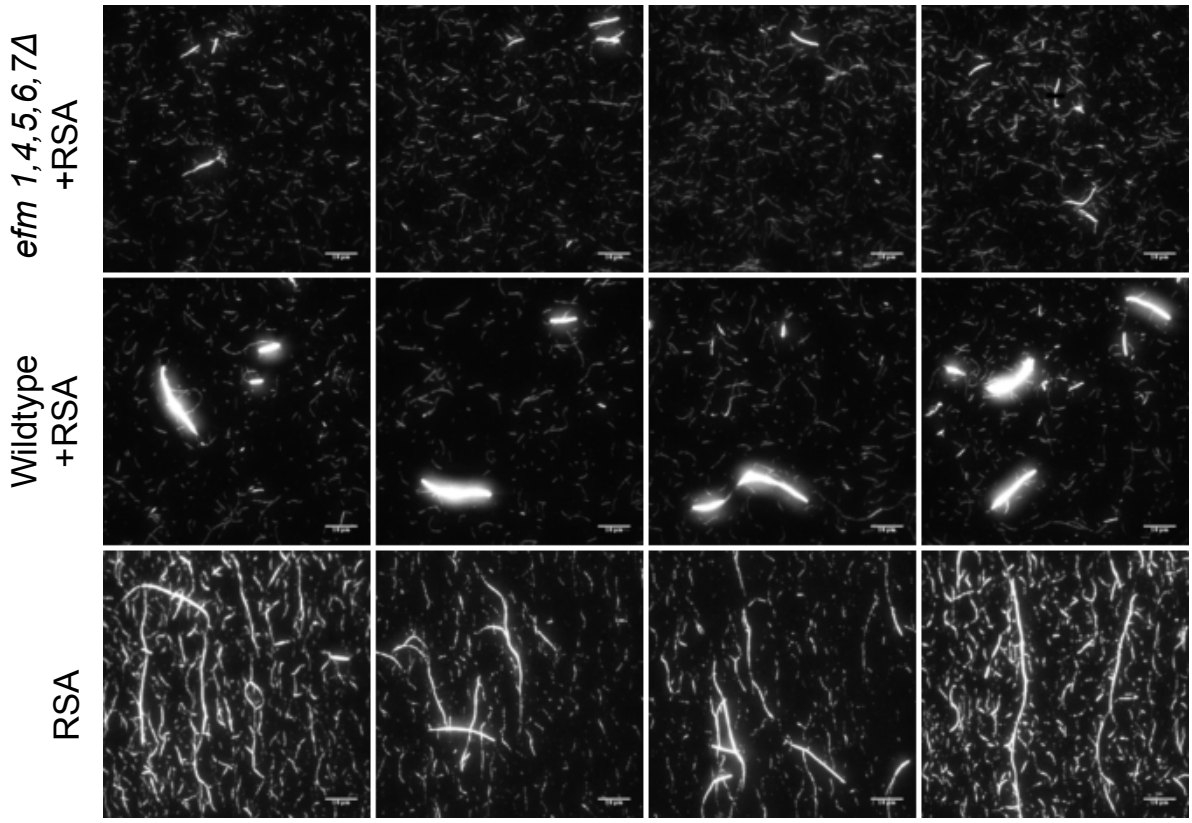
Figure 5-1: Rabbit skeletal muscle actin bundling by EF1A is concentration dependent and similar with wildtype EF1A and methylation-deficient EF1A. A) Representative cosedimentation performed using yeast actin and 1 µM wildtype or 1 µM mutant *efm 14567Δ* EF1A from one experiment. Other samples from the gel were omitted. This experiment was performed once. **B)** Cosedimentation analysis of varying wildtype or mutant EF1A concentrations prepared as described in "Experimental Methods" using rabbit skeletal muscle actin (RSA). One replicate was done. The table below shows the amount of actin present in the supernatant and pellet of the different samples normalized to the amount of actin from the actin only control. Values were determined by ImageJ densitometry.

To verify the extent of *efm 14567Δ* EF1A deficiency in actin bundling, I also looked at bundling *in vitro* using TIRF microscopy. I was able to visually look at the actin filaments stained with fluorescent phalloidin in the absence and presence of wildtype and mutant EF1A.

(Figure 5-2) With the methods used I was unable to assess filament length as some previous studies did but I was able to quantify the intensity (thickness) of the filament as a measure of bundling capability. Wildtype bundles were significantly thicker than those from the methyltransferase mutant EF1A in one replicate experiment. A Student's T-test analysis revealed a p-value of 8.06×10^{-8} (unpaired, two tails) when 44 bundles of each strain were measured (Table 5-1).

Filament Thickness (micron)					
Wildtype +RSA	*efm 14567Δ* + RSA	RSA only	Wildtype +RSA	*efm 14567Δ* + RSA	RSA only
2	1.2	1	3	3	1
2	2	1	8	2	1
6.5	2	1	6	1.5	1
8	2	1	4	3	1
8	1.6	1	5	2	1
2	2	1	5	2	1
3	1.5	1	11	2	1
6	1.8	1	4	2	1
8	2	1	11	2	1
8	2	1	15	1.5	1
8	2	1	4	2	1
8	1.4	1	2	2	1
10	3	1	12	2	1
25	1.6	1	3	2	1
7.5	1.6	1	3	2	1
10	1.5	1	4	2	1
2	2	1	15	2	1
4	2.2	1	2	1.5	1
2.5	2	1	2	3	1
5	2.6	1	5	2.5	1
4	3	1	8	2	1
10	2.5	1	2	2	1

Table 5-1: Filament thickness values of 44 individual actin bundles from one actin bundling experiment analyzed using FIJI



	Average Intensity	Filament Thickness (micron)
RSA	2591	1.0
Wildtype +RSA	1322	6.4
***efm 14567Δ* + RSA**	1208	2.0

Figure 5-2: Methyltransferase-deficient EF1A has reduced filamentous actin bundling capabilities. Four different fields of view are shown for each sample. The thickness of the actin filaments was quantified using FIJI software. These images are from one experiment performed.

I then turned to look at *in vitro* binding interactions of EFlA and rabbit skeletal actin. In these experiments, I centrifuged mixtures of these proteins at high speeds to separate soluble EFlA from EFlA bound to actin filaments. At high centrifugation forces, EF1A alone is found in the supernatant but its interaction with actin allows it to be pelleted. I found that there was no

visible difference in the actin-induced pelleting of *efm 14567Δ* EF1A when compared to wildtype EF1A (Figure 5-3). This was a surprising result based on the loss of actin bundling in the methylation-deficient EF1A seen in the previous experiment. If binding were disrupted due to the lack of EF1A methylation some amount of EF1A would remain in the supernatant fraction. This result may suggest that EF1A's ability to bind and bundle actin are separate since our methyltransferase mutant had decreased actin bundling potential. However more experiments are still needed.

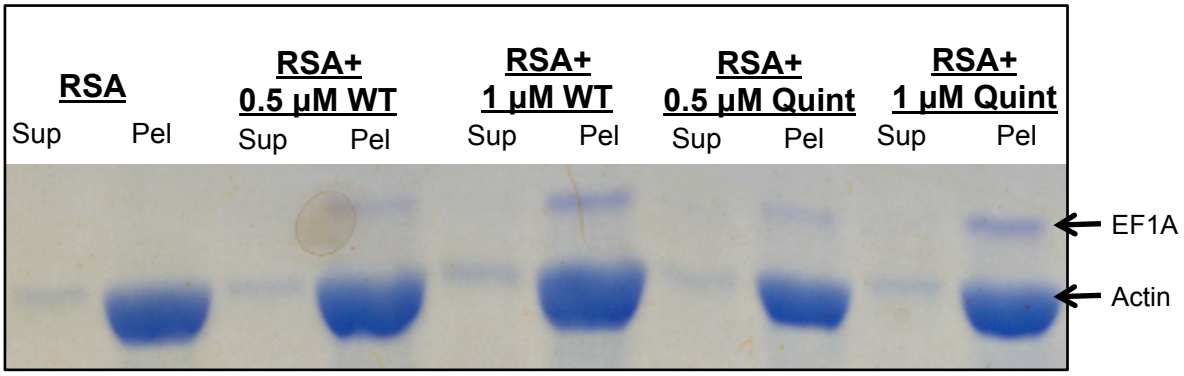


Figure 5-3: Methyltransferase-deficient EF1A is able to bind actin similar to Wildtype EF1A. Representative image of cosedimentation experiment with different concentrations of wildtype or mutant EF1A prepared as described in "Methods" using rabbit skeletal actin (RSA). One replicate was done with RSA and another with yeast actin. Quint is EF1A purified from the e*fm 14567Δ* yeast strain.

Finally, I examined the integrity of the actin cytoskeleton using confocal microscopy of wildtype, e*fm 14567Δ*, e*fm 1456Δ* and *TEF1 K(30,79,316,390)R* yeast cells that were fixed with formaldehyde. Previous studies have shown that when EF1A is overexpressed, yeast cells have morphological defects such as increased yeast bud size and a reduction in actin patch/cable formation (6). In chapter 3, I showed that the abundances of EF1A in the methylation deficient

strains (*Efm 1456Δ, Efm 14567Δ* and *TEF1 K(30,79,316,390)R*) were about two-fold less than wildtype EF1A protein abundance (chapter 3, Fig. 3-6). Since it is known that the overexpression of EF1A leads to bigger yeast buds, I expected the yeast cells in my experiments to be smaller or similar in size to wildtype yeast cells for these methylation-deficient yeast cells. I found no noticeable differences in cell morphology of all of the mutant yeast cells when compared to wildtype when the yeast cells were examined using the confocal microscope under visible light (Figures 5-4, 5-5, 5-6, 5-7, left panels).

I then examined cells under fluorescence to detect the phalloidin derivative bound to actin. In budding yeast the actin cytoskeleton is made up of actin cables, actin patches, and an actin ring (14, 15). In my experiments, actin patches were the most prominent actin structures present in each strain (Figures 5-4, 5-5, 5-6, 5-7, right panels). Actin patches were seen in mother buds as well as growing buds in each strain tested. I was unable to observe differences in the mutant yeast cells compared to wildtype (Figures 5-4, 5-5, 5-6, 5-7). If there were any changes, they were too subtle to observe using these methods.

I found it difficult to measure actin cables as they can vary in length depending on the stage of yeast growth (14). A more informative study would be to look at actin cable formation in live cells. Yang et al. fused GFP to Abp140 to visualize both bud-associated cables and randomly oriented cables over time (16). This experiment with GFP fused EF1A would also shed light on what EF1A is doing in the cell. Thus far it is only known that EF1A is found bound along actin filaments *in vitro* (7).

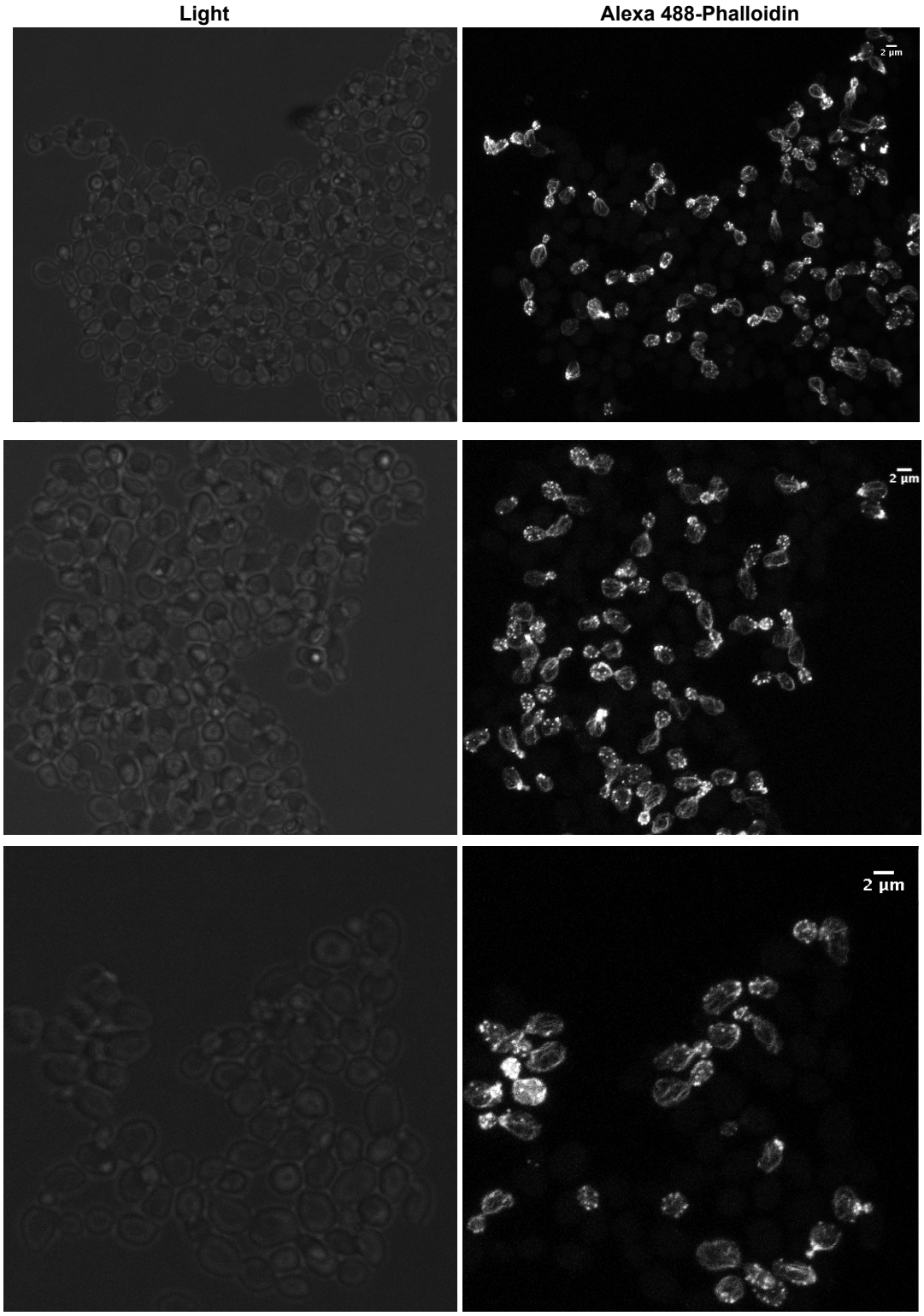


Figure 5-4: Phalloidin stained wildtype yeast cells visualized under confocal microscope.

Whole yeast cells were fixed with formaldehyde and actin structures stained with phalloidin and

imaged using a confocal microscope as described in the "Methods" section. Each panel pair represents a different field of view. This experiment was performed one time.

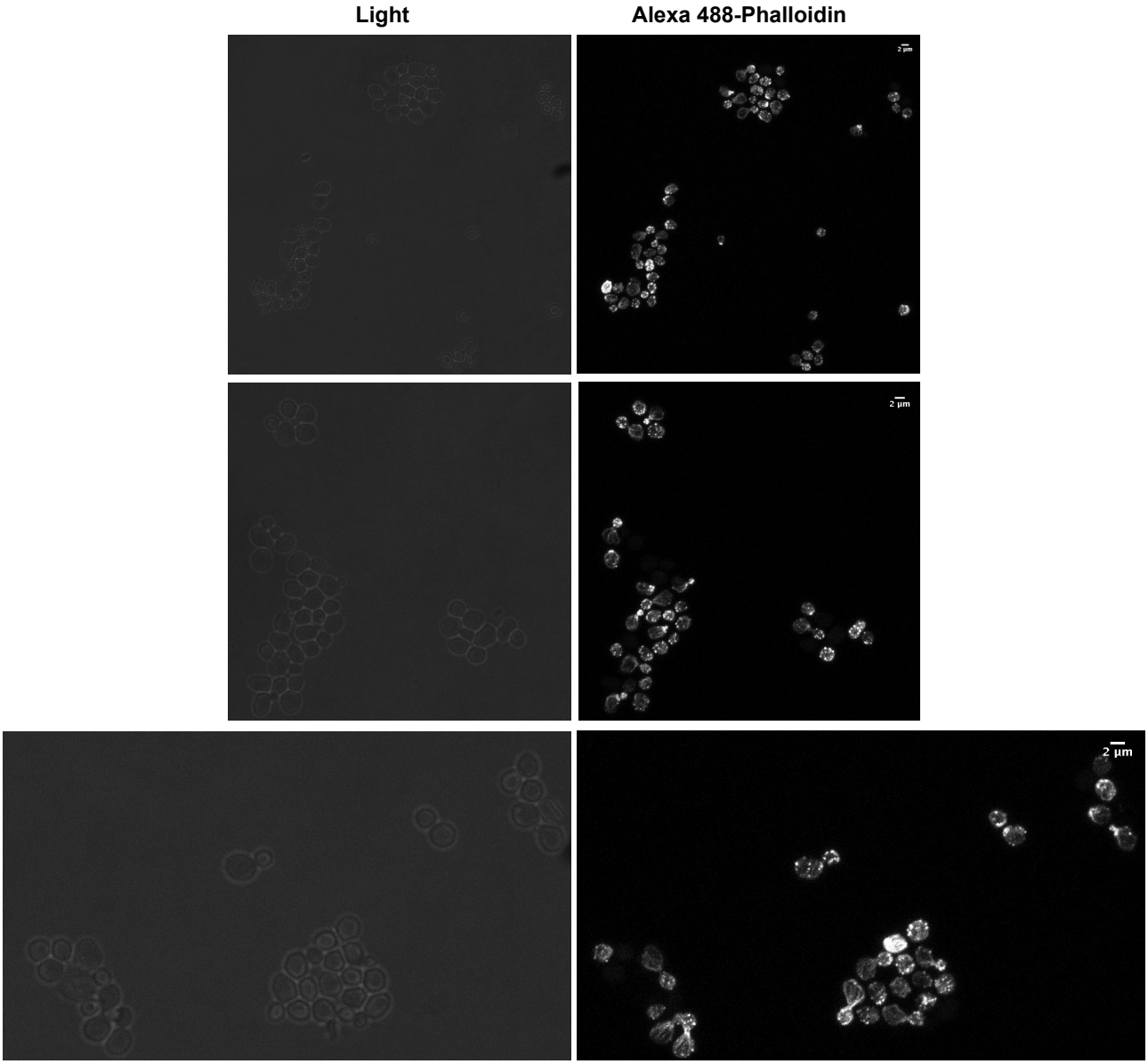


Figure 5-5: Phalloidin stained *efm 1456Δ* yeast cells visualized under confocal microscope. Whole yeast cells were fixed with formaldehyde and actin structures stained with phalloidin and imaged using a confocal microscope as described in the "Methods" section. Each panel pair represents a different field of view. This experiment was performed one time.

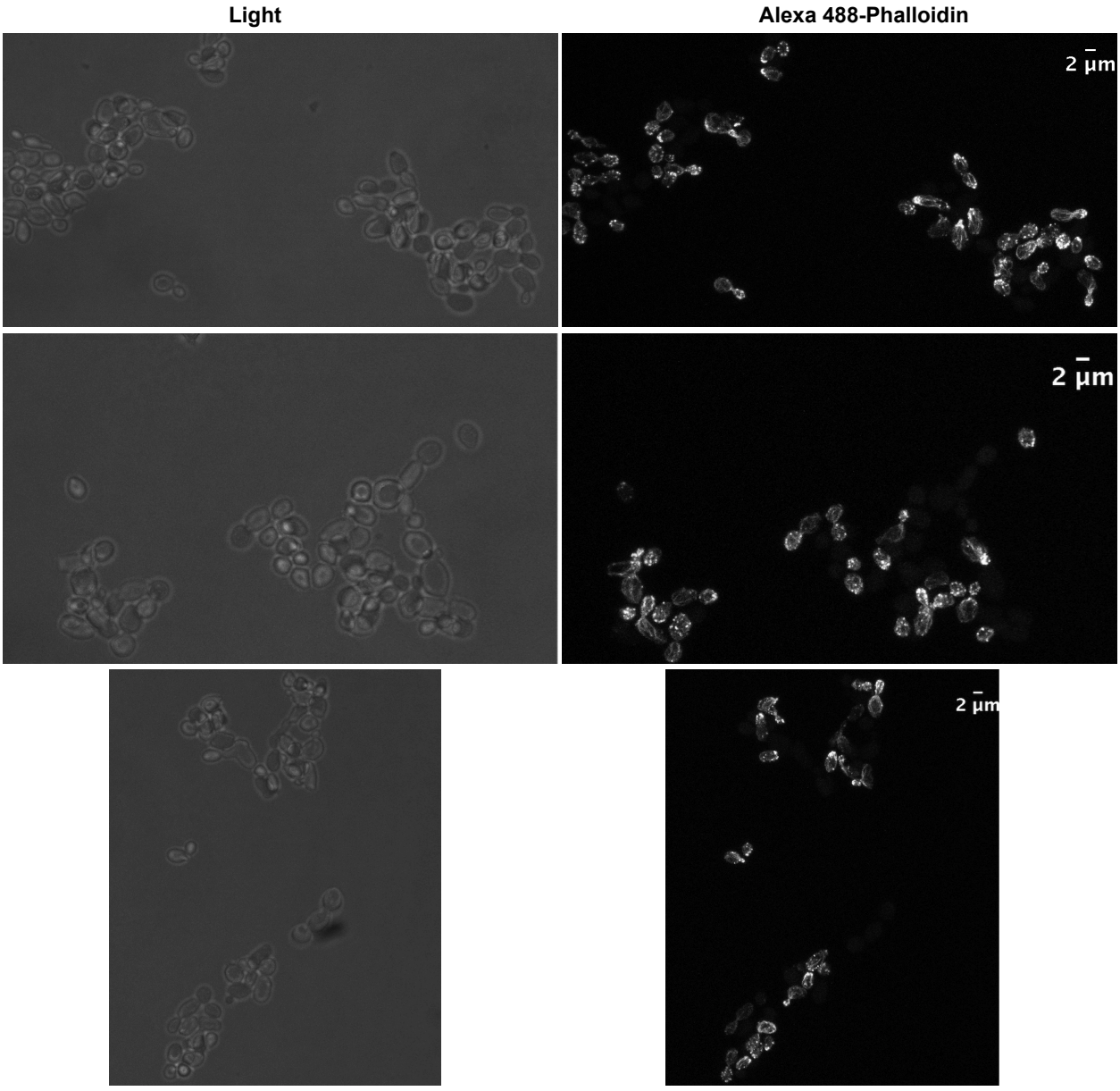


Figure 5-6: Phalloidin stained *TEF1* K(30,79,316,390)R yeast cells visualized under confocal microscope. Whole yeast cells were fixed with formaldehyde and actin structures stained with phalloidin and imaged using a confocal microscope as described in the "Methods" section. Each panel pair represents a different field of view. This experiment was performed one time.

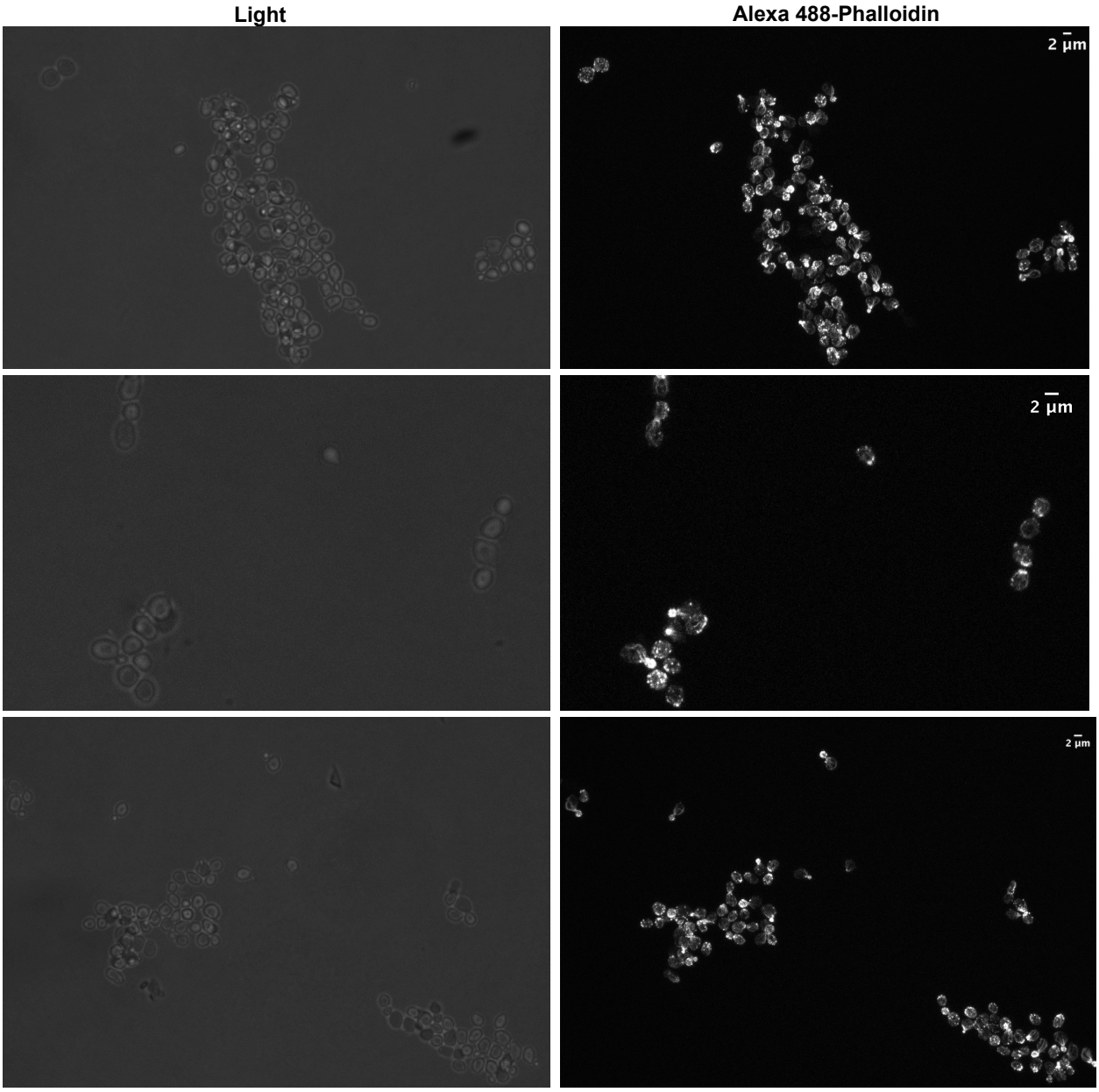


Figure 5-7: Phalloidin stained *efm 14567Δ* yeast cells visualized under confocal microscope. Whole yeast cells were fixed with formaldehyde and actin structures stained with phalloidin and imaged using a confocal microscope as described in the "Methods" section. Each panel pair represents a different field of view. This experiment was performed one time.

In summary, this work does not provide any firm evidence that the methylation state of EF1A can affect its interactions with actin. In *in vitro* low-speed centrifugation assays, no

evidence for bundling was seen (Fig. 5-1). However, when we used TIRF microscopy, it appeared at least in one experiment that methylation-deficient EF1A significantly reduced the thickness of the bundles (Fig. 5-2). In *in vitro* high-speed centrifugation assays, I found no effect of EFlA methylation state on actin binding (Fig. 5-3). Finally, in studies of intact yeast cells fixed in formaldehyde, I found no effect on actin organization (Fig. 5-4 to 5-7). Further work may help to confirm the TIRF results, or to see if there are more subtle changes induced by EF1A methylation. One problem in the TIRF assays is that bundles can be sheared which prevents the analysis of overall bundle length. The bundling assay used in this study was only able to visualize the bundles and quantifies filament thickness, which should not have been affected by shearing introduced by sample prep.

If methyl-deficient EF1A indeed affects actin bundling, it would be useful to look next at actin polymerization rates. A polymerization pyrene assay as used by the Quinlan laboratory can be performed (17). Another area for future research would be to examine the interactions of yeast EF1A with actin from various species, including yeast and amoeba.

I have previously attempted to use TIRF microscopy to visualize growing actin filaments. I tried doing a 1h incubation time for EF1A and yeast actin and no filaments were visualized. The polymerization of yeast actin appears to be too slow for this assay. The cosedimentation bundling assay normally requires an 18 h overnight polymerization step. However, rabbit actin has been shown to have detectable filaments with a 1h polymerization time (7). It appears that the polymerization of yeast actin by EF1A may be slow so I suggest doing further studies with rabbit skeletal muscle actin.

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Chapter Six

Conclusions and Future Perspectives

Methylation of proteins involved in translation has been a successful field thus far in the identification of enzymes and substrates involved in a plethora of organisms (1–5). This paved a way for the study of the functional implications of these methylation reactions to emerge, which were still largely unknown, specifically in yeast EF1A, prior to this work. Excitedly, the work presented in chapters two, three and five of this dissertation help to grow this field some more. I describe work studying growth phenotypes and functional consequences when the protein substrate can no longer be methylated.

Chapter two examines the role of the ten ribosomal protein methlytransferases in ribosome biogenesis and translation when it is genetically removed. Loss of methylation due to the lack of methyltransferase appears to be necessary for proper amino acid recognition while only two aided in translation termination. A follow up to this study would be to assess the biological implications when the substrate itself is altered to remove the methylated residue. The phenotypes we observed might be an affect from the lack of methyltransferase causing a cascade affect if they work on alternative substrates. Many of these methyltransferases have been shown to have both physical and genetic interactions with other proteins from affinity based pulled down assays. If this compliment study is performed and it is determined that the methyltransferase and not the methylation of the substrate is responsible for the phenotypes observed it may be worthwhile switching gears to examine what they are doing in the cell.

Chapter three and five both focus on the methylation of elongation factor 1 alpha and its role in translation (chapter three) and its cytoskeletal actin interaction (chapter five). This work allowed for the generation of a new methyltransferase mutant strain of EF1A, *efm14567Δ,* which has never been characterized before. The work in chapter 3 and five functionally characterizes this strain while chapter 4 elucidates its methylation profile using mass spectrometry. Thus far

we have showed that translation fidelity remains intact when EF1A is methylation deficient although the protein abundance of EF1A is drastically reduced in both mutant forms and that methylation of EF1A may help it adapt to changes in its cellular environment.

Chapter 4 is the first study to characterize the methylation proteome of EF1A in the *efm14567Δ* strain. Here we confirmed that Gly 2, and Lys 3, 30, 79, 316, and 390 on EF1A in our *efm14567Δ* strain are unmethylated. We also provide evidence of putative novel methylation sites that exists on wildtype, *efm14567Δ,* and *TEF1 K(30,79,316,390)R* EF1A under our specific growth conditions, which may not be *S*-adenosylmethionine dependent. We were also able to detect the C-terminal peptide of wildtype EF1A but this peptide did not contain the carboxyl methyl esterification that has previously been reported to exist there (6).

Preliminary evidence from one experiment in Chapter 5, suggests that when the methyltransferases are knocked out, methyl deficient EF1A loses its ability to bundle actin. Further verification will be needed to support this conclusion. This study should also be repeated with the compliment point mutation strain with all 6 methylation sites mutated to verify if this phenotype is true. I have started construction of the quint point strain with Dr. Kevin Roy. Additionally, I think it will be interesting to explore the mechanism of EF1A and actin interaction. The exact site(s) on EF1A necessary for binding actin has not been determined. Only a general region of EF1A is known to interact with actin. There is also evidence of EF1B being able to outcompete EF1A for actin binding suggesting they may share a similar binding site or region (7). Within EF1A domain 2 (EF1B binding) and 3 (actin binding) there are two methylated lysine residues (Lys 316, and 390) that would be of interest to assay for being a key player in actin binding.

Lastly, I am curious to know whether actin organization induced by EF1A is impaired when the yeast cells are treated with translation inhibitors. In the presence of translation inhibition I have shown that yeast growth is slowed while overall translation fidelity is not impaired when EF1A is rendered unmethylated due to the methyltransferase gene being deleted. It is known that EF1A's translational responsibilities and its interaction with the actin cytoskeleton is mutually exclusive but not the how. This experiment would hopefully shed light on if methylation of EF1A is a linker to this phenomenon (8).

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