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Found in Translation:

The Search for Functional Roles of Translation Elongation Factor Methylation and
the Discovery of a Novel Type of Protein Methylation

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy in
Biochemistry and Molecular Biology

by

Maria Dzialo

2015

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ABSTRACT OF THE DISSERTATION

Found in Translation:

The Search for Functional Roles of Translation Elongation Factor Methylation and
the Discovery of a Novel Type of Protein Methylation

by

Maria Dzialo

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles, 2015

Professor Steven G. Clarke, Chair

Methylation has emerged as an essential modification of small molecules, lipids, nucleic acids, and proteins. Given the variety of potential substrates, the effects the addition of a small methyl group can have are expansive and diverse. Methylation of DNA can turn a gene off and methylation of a tRNA can affect its stability. Proteins offer even more variety of methylatable targets; methyl groups can be added to the side chains of lysine, arginine, glutamine, glutamate, asparagine, histidine, methionine, and cysteine as well as the N- and C-terminal amino and carboxyl groups of alanine, serine, proline, phenylalanine, and leucine. The most well-known function of protein methylation is arguably the modification of histone tails, part of the histone code that regulates gene expression. However, there are a number of non-histone methylations that are often less well understood in terms of functional relevance. Interestingly, in *Saccharomyces cerevisiae*, a large number of methylation reactions occur on translation-related proteins including ribosomal proteins, release factors, and elongation factors. This fact served as

the initial spark for a variety of my projects that centered on identifying the enzymes responsible for these reactions and delving into potential roles of the methylation reactions.

The elongation factors in *S. cerevisiae* are heavily methylated containing a total of ten methylated lysine residues. The original focus of this dissertation was to identify as many of the enzymes for these ten reactions as possible. Two had been identified at the onset of this project and this dissertation adds two more along with some evidence for a third. In my work, Efm3 was shown to trimethylate lysine 509 on elongation factor 2 and Efm5 was shown to catalyze the trimethylation of lysine 79 of elongation factor 1A. I also obtained preliminary evidence that YNL024C catalyzes the monomethylation of lysine 390 on EF1A. This identification was later confirmed by another group and the enzymes was designated Efm6. This second group also suggested that there is a relatively low occupancy of this modification, which explains the difficulty we had confirming the activity with our methods.

An interesting discovery was made during this search for elongation factor methyltransferases: a potentially new type of protein methylation that has yet to be described. It is possible that this methylation went undetected in previous screens and analyses due to its unusual characteristics and its behavior in our assays. I have been able to show that a radioactive product from acid hydrolysates of yeast cells labeled with S-adenosyl-[methyl-3H]-L-methionine is only formed in cells expressing the YLR285W gene. This gene encodes a putative methyltransferase but has not been associated directly with a substrate *in vivo*. This dissertation describes my attempts made to identify this new substrate and modification but as of yet, it remains elusive.

The dissertation of Maria Dzialo is approved.

Joseph A. Loo

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Steven G. Clarke, Committee Chair

University of California, Los Angeles

2015

Dedication

To my parents – I am eternally grateful for your support and encouragement through my long, twenty-two years of education. I love learning, something you ignited in me long ago.

To Dr. Brian D. Cohen – I cannot thank you enough for giving me the opportunity to perform research in your lab. You helped me realize my potential as a scientist and set me on my path.

To Mark – Thank you for helping me get through the past five years. You are my best friend, my support, my inspiration, my partner in life.

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I am forever indebted to my undergraduate researcher, Kyle J. Travaglini. I was exceptionally lucky to work with a student like Kyle. His eagerness to learn and his sharp scientific mind greatly enhanced my graduate career on a scientific, professional, and personal level. I am incredibly proud of what Kyle accomplished in such a short amount of time and cannot wait to see him succeed during graduate school as he “goes where no one has gone before”.

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Chapter Two of this dissertation is the manuscript published in the Journal of Biological Chemistry in 2014. Sean and Dr. Loo performed the mass spectrometry of my samples and Dr. Roy performed the Northern Blot analysis. Chapter Three contains the manuscript published in Biochemical and Biophysical Research Communications in 2014. Sean Shen and Dr. Joseph Loo also performed the mass spectrometry for this publication. An extra thank you goes to Dr. Roy and his lab mates Jason Gabunilas and Charles Wang for their timely and excellent construction of various knockout strains. These strains are described and utilized in Chapter Five. Also thank you to Dr. William Munroe for the use of the ISCO fractionator and his assistance running it. Thank you to Dr. Kym Faull for his assistance with the mass spectrometry analysis done in Chapter Six.

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Dzialo, M.C., Travaglini, K.J., Shen, S., Loo, J.A. and Clarke, S.G. *A New Type of Protein Lysine Methyltransferase Trimethylates Lys-79 on Elongation Factor 1A* Biochemical and Biophysical Research Communications. 2014 Dec 12; 455(3-4):382-389. doi: 10.1016/j.bbrc.2014.11.022. Epub 2014 Nov 15.

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Dzialo MC, Clarke SG. *Identifying novel protein methyltransferases involved in translation.* Poster presented at the Molecular Biology Institute Retreat at UCLA January 25-26, 2014

Dzialo MC, Clarke SG. *Identifying novel protein methyltransferases involved in translation.* Poster presented at the Cell Biology of Yeasts Conference at Cold Spring Harbor Laboratory November 5-9, 2013

Dzialo MC, Clarke SG. *Heavy Methyl: Using Radioactivity Assays to Characterize the Heavily Methylated eEF1A.* Poster presented at the Tri-Campus Symposium at USC April 19, 2013

Dzialo MC, Dias JA, Cohen BD. *Characterization of a putative caveolin interaction motif in the human follicle stimulating hormone receptor.* Poster presented at the Endocrine Society Annual Meeting and Expo in San Diego, CA June 19-22, 2010

Commonly Used Abbreviations in this Dissertation

mmK	ϵ -monomethyl lysine
dmK	ϵ -dimethyl lysine
tmK	ϵ -trimethyl lysine
AdoMet	<i>S</i> -adenosyl-L-methionine
[³ H]AdoMet	<i>S</i> -adenosyl- [<i>methyl</i> - ³ H]-L-methionine
SMM	<i>S</i> -methylmethionine
dmP	dimethyl proline
mH	3-methyl histidine
mC	<i>S</i> -methyl cysteine
TFA	trifluoroacetic acid
EF	elongation factor
EFM	elongation factor methyltransferase

Chapter One

Plan of the Dissertation

A PubMed search for “methylation” from the past year extracts over six thousand publications; more than double what was seen just ten years ago. Methylation has risen to stand alongside phosphorylation and acetylation as an essential component of regulating cellular functions (Walsh 2006). In *Saccharomyces cerevisiae*, a large proportion of methylated substrates have roles in protein translation including mRNA, tRNA, ribosomal proteins, release factors, and elongation factors (Clarke 2013). Histone methylation is relatively well understood in that the presence or absence of specific histone marks can activate or deactivate transcription of specific genes but the biological relevance of translation-related methylation is largely unexplored (Biggar and Li 2015; Lanouette et al. 2014; Clarke 2013).

In *Saccharomyces cerevisiae*, there are 86 known and putative methyltransferases (Petrossian and Clarke 2009). As of September 2015, 71 of those enzymes have confirmed substrates and over 50% of the known enzymes are involved in modifying some component of the translational apparatus (Appendix). The roles of some of these methylations have been characterized and demonstrate a wide variety of effects. Loss of rRNA methylation by Rcm1 results in reduced translational fidelity (Schosserer 2015) whereas loss of rRNA methylation by human Merm1 results in improper 18S processing (Haag et al. 2015). Methylation of ribosomal proteins can also play a role in translational fidelity as well as ribosome biogenesis (Al-Hadid et al. 2014). The translation release factor eRF1 is methylated on the highly conserved GGQ tripeptide that is vital to its activity (Heurgue-Hamard et al. 2005). These phenotypes indicate that methylation in translation may function to “fine-tune” various interactions between the large number of proteins and RNAs that are required to synthesize polypeptides.

The elongation factors are essential components of the translational cycle (Figure 1-1). Elongation factor 1A (EF1A) shuttles amino-acylated tRNAs to the A-site of the ribosome. Upon

a correct codon match, EF1A releases the aminoacyl-tRNA in a GTP-hydrolysis-dependent manner (Matayek et al. 2010). This now GDP-loaded EF1A leaves the ribosome and is recharged with a new GTP molecule with the assistance of its nucleotide exchange factor, EF1B (Anderson 2001). Elongation factor 2 (EF2) performs the translocation step, moving the tRNAs from A-site to P-site and P-site to E-site (Justice et al. 1998). Elongation factor 3 (EF3), is a fungal-specific A-protein that is thought to aid in the removal of empty tRNAs (Belfield et al. 1993 and Kurata et al. 2013).

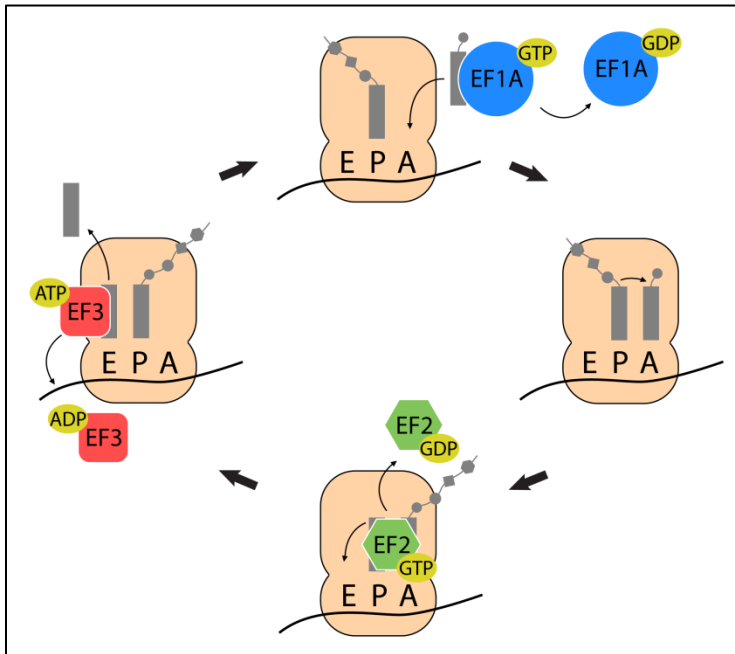


Figure 1-1. The elongation cycle in *Saccharomyces cerevisiae* In yeast, elongation factor 1A shuttles aminoacylated tRNAs to the A-site of the ribosome in a GTP-dependent manner. EF2 translocates the translational apparatus, also with energy from GTP. EF3 is an ATP-binding protein thought to aid in the exit of deacylated-tRNAs. This image was created by Kyle Travaglini and reproduced with his permission.

Methylation of yeast elongation factor 1A (EF1A) was first identified over twenty years ago utilizing chemical sequencing (Cavallius et al.1993). This method identified four methylated lysine residues: monomethyllysine 30 (mmK30), trimethyllysine 79 (tmK79), dimethyllysine 316 (dmK316), and monomethyllysine 390 (mmK390). Interestingly, these methylation sites are

only partially conserved in higher eukaryotes: tmK79 is found in rabbit and brine shrimp and was predicted to be in humans based on sequence similarity, K316 is trimethylated in higher eukaryotes and the monomethyl modifications are absent in many other organisms (Cavallius et al. 1993). Additionally, the unique glycerylphosphorylethanolamine modification is absent in yeast although it is widely found in eukaryotes. One other unique modification has been found on EF1A: a reversible C-terminal methyl ester was discovered through base lability screens of radiolabeled yeast lysates (Zobel-Thropp et al. 2000).

The first EF1A methyltransferases were found in 2010 (Lipson et al 2010). EF1A was purified from various deletion strains of putative methyltransferases and subjected to intact mass spectrometry. Only two of the thirty-seven deletion strains tested resulted in any mass changes of EF1A. Deletion of YHL039W resulted in a mass loss of 14 Da and deletion of YIL064W resulted in a 28 Da loss. At the time of publication, it was inferred that YHL039W was responsible for one of the monomethylations and was renamed Elongation Factor Methyltransferase 1 (Efm1). Loss of 28 Da implies loss of two methyls but it was not clear if YIL064W was responsible for the dimethylation or assisted with the monomethylations. At this time, YIL064W was annotated as See1 (Secretion and Early Endocytosis) based on endocytosis defects in the deletion strain (Martin-Granados et al. 2008). Later it was renamed as Efm4 to conform to the naming of elongation factor methyltransferases (see Chapter Three). Two years later, it was shown by mass spectrometry that Efm1 monomethylates K30 and Efm4 dimethylates K316 (Couttas et al. 2012).

In 2012, five new methylation sites were found on EF2 and EF3A (Couttas et al. 2012). Additionally, one of the methyltransferases was identified encoded by YBR271W (Efm2). However, the site Efm2 actually modified was not clear from these findings. We later confirmed

that Efm2 dimethylates EF2 at K613 and Efm3 trimethylates K509 (see Chapter Two). The EF3A sites remain unconfirmed.

The methylation of the elongation factors may be necessary given the extensive number of contacts required for various functions. While the canonical function of EF1A, EF2, and EF3 are to coordinate the movement of tRNAs through the elongation cycle, they each have been implicated in a variety of roles. EF1A has been implicated in tRNA export from the ribosome, proteasome function, apoptosis, actin bundling, and even viral replication (Mateyak and Kinzy 2010). All of these functions require extensive interactions and coordination with a variety of different proteins and RNAs. Given the “fine-tuning” function of methylation in translation, it is likely that the extensive modification of these three proteins aids in facilitating the numerous interactions they require.

The goal of this dissertation was to identify remaining elongation factor methyltransferases and elucidate possible roles of their modifications in the context of the cell. Since the onset of this project in 2010, many of the enzymes have been identified (Table 1-1). Chapter Two of this dissertation describes the identification and initial characterization of Efm3, which is responsible for trimethylation EF2 at K509. Chapter Three describes the identification of Efm5 which trimethylates K316 of EF1A. Chapter Four describes the various methods that were used in an attempt to identify the enzymes responsible for EF1A monomethyllysine 390 and the C-terminal methyl ester, respectively. Chapter Five describes the initial exploration of possible roles of EF1A methylation by analyzing phenotypes of various double and triple knockouts of the known EFMs. Chapter Six focuses on attempts to identify the substrate for the putative methyltransferase, YLR285W, which came into focus through screens described in

Chapter Four. This Chapter is in manuscript form as it is currently being formatted for submission. Chapter Seven provides goals and perspectives for future study of these topics.

TABLE 1-1

Known methylation sites of translational elongation factors in *Saccharomyces cerevisiae* and the associated methyltransferases

Substrate	Methylation type and site*	Methyltransferase (Gene)	Evidence
EF1A	mmK-30	Efm1 (YHL039W)	Lipson et al. 2010 Couttas et al. 2012
	tmK-79	Efm5 (YGR001C)	Dzialo et al. 2014b
	dmK-316	Efm4 (YIL064W)	Lipson et al. 2010 Couttas et al. 2012
	mmK-390	Efm6 (YNL024C)	Cavallius et al. 1993 Jakobsson et al. 2015
	C-terminal K-methyl ester		Zobel-Thropp et al. 2000
EF2	tmK-509	Efm3 (YJR129C)	Zhao et al. 2014 Dzialo et al. 2014a Davydova et al. 2014
	dmK-613	Efm2 (YBR271W)	Couttas et al. 2012
EF3A	tmK-187		Couttas et al. 2012
	tmK-196		Couttas et al. 2012
	tmK-789		Couttas et al. 2012

*mmK = monomethyllysine, dmK = dimethyllysine, tmK = trimethyllysine

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Chapter Two

Translational Roles of Elongation Factor 2 Protein Lysine Methylation

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Introduction to the Chapter

Elongation factors 1A and 2 from *S. cerevisiae* each contain a trimethylated lysine residue. However, the enzymes that catalyze these modification reactions were still unidentified at the onset of this project. Elongation factor 3 could contain up to three trimethylated lysine residues, although these modifications have never been fully confirmed. Since amino acid analysis of each Coomassie-stained protein band corresponding to EF1A and EF2 within each putative methyltransferase knockout strain would have been very time consuming, I looked for alternative approaches to searching for trimethylating enzymes.

The trimethyllysine on the bacterial homolog of EF1A, EF-Tu, was implicated in the initiation of *Pseudomonas aeruginosa* pneumonia (Barbier et al. 2013). Here, the trimethylated lysine residue mimics the phosphorylcholine moiety of platelet activating factor, a crucial epitope recognized by many human pathogens. Not only did this article describe an interesting role of elongation factor methylation, it also provided a possible way to detect loss of trimethylation on EF1A. Barbier et al. utilized several antibodies that would recognize trimethyllysine and phosphorylcholine with high specificity in *Pseudomonas*. Dr. Joanna Goldberg kindly sent us all of these antibodies along with the corresponding secondary antibodies to test on yeast lysates. Although my initial utilization of these antibodies demonstrated less specificity for yeast trimethyllysine and phosphorylcholine compared to that in *Pseudomonas*, a 50 kDa and 100 kDa band clearly immunoreacted with each antibody. I therefore utilized the one with the greatest specificity to EF1A to screen various knockout strains.

The most dramatic result obtained was the complete loss of immunoreactive signal at 100 kDa in a knockout of a putative methyltransferase, YJR129C. I pursued this as the potential trimethylating enzyme of EF2 and found that loss of this enzyme in fact results in loss of trimethylation of EF2.

Interestingly, two other research groups came to this same conclusion around the same time, resulting in three nearly concurrent publications on Efm3. The Wilkins group from the University of New South Wales (Australia) identified Efm3 in yeast and the Falnes group from the University of Oslo (Norway) demonstrated activity of the human homolog, FAM86A (Zhang et al. 2014; Davydova et al. 2014). Our publication specifically showed that the modification was indeed a trimethyllysine and probed at possible functional ramifications when the dimethyl and trimethylations are lost (Dzialo et al. 2014).

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Translational Roles of Elongation Factor 2 Protein Lysine Methylation*

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*Running title: *Translational Roles of Yeast EF2 Protein Lysine Methylation*

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Keywords: Protein methylation, translation elongation factor, protein synthesis, S-adenosylmethionine (SAM), lysine methylation, ribosome function.

Background: Translational elongation factors are extensively methylated but the roles of these modifications are not established.

Results: Loss of methylation on elongation factor 2 in *Saccharomyces cerevisiae* by deletion of *EFM3/YJR129C* or *EFM2* results in translational defects.

Conclusions: Elongation factor methylation is required for normal translational function.

Significance: Protein lysine methylation fine-tunes the translational apparatus.

SUMMARY

Methylation of various components of the translational machinery has been shown to globally affect protein synthesis. Little is currently known about the role of lysine methylation on elongation factors. Here we show that in *Saccharomyces cerevisiae*, the product of the EFM3/YJR129C gene is responsible for the trimethylation of lysine 509 on elongation factor 2. Deletion of EFM3 or of the previously described EFM2 increases sensitivity to antibiotics that target translation and decreases translational fidelity. Furthermore, the amino acid sequence of Efm3 and Efm2, as well as their respective methylation sites on EF2, are conserved in other eukaryotes. These results suggest the importance of lysine methylation modification of EF2 in fine-tuning the translational apparatus.

INTRODUCTION

Methylation of translational components has been shown to have a broad spectrum of functional consequences (1-6). Methylation of rRNA plays a role in ribosome biogenesis (6) and modifications to tRNAs increase their stability or affect translational fidelity (2). Protein modifications are also important and are found on various components including ribosomal proteins, release factors, and elongation factors (1,5,7). In a few cases, the functional consequences of these methylations have been established. For example, a 3-methyl histidine on ribosomal protein Rpl3 was recently shown to be involved in large ribosomal subunit biogenesis and translational fidelity (8). In prokaryotes and eukaryotes, release factor 1 is methylated on the conserved GGQ motif that enters the peptidyl transfer center. Loss of the methyltransferase in bacteria results in termination defects (1); in yeast, the loss of the release factor 1 methylation

site increases resistance to zymocin (9). However, in most cases, the functional relevance of protein methylation in translation is not known.

Methylation of elongation factors has been well established in *Saccharomyces cerevisiae* and many of the modification sites are conserved in higher eukaryotes (10,11). There are three protein elongation factors in budding yeast: the evolutionarily conserved EF1A and EF2 and the fungal specific EF3. These three proteins guide tRNAs through the various active sites of the ribosome (12-15). EF1A ensures that correct codon matches occur between the amino acyl-tRNA and the mRNA while EF2 and EF3 help facilitate the timely translocation of peptidyl-tRNAs and removal of deacylated tRNAs. These three proteins together contain ten methylated lysine residues (10,11). The methyltransferases responsible for catalyzing the modification of only three of these residues have been identified (11,16). Furthermore, the functional relevance of these modifications has been largely unexplored.

In yeast, EF1A is the most heavily methylated of the elongation factors, containing two monomethyllysines (K30 and K390), one dimethyllysine (K316), one trimethyllysine (K79), and a C-terminal lysine alpha-carboxyl methyl ester (10,17). EF2 contains trimethyl K509 and dimethyl K613 whereas EF3 has three trimethyllysines on K187, K196, and K789 (11). With the exception of the C-terminal methyl ester, there is no evidence that these modifications are reversible. While the functional role of these methylations during translation elongation is unclear, the locations of these modifications hint at their importance. Structural studies of EF2 and the 40S ribosome subunit indicate that the K509 site is in close contact with ribosomal protein Rps23b (18). K613 is in proximity to helix 33 of the 18S rRNA (18) and is on the same domain as the diphthamide modification at H699 that aids in maintaining proper transcript frame (19). The potential for enhancing contact with ribosomal components suggests that these

methylation sites could be crucial to maintain proper communication with the ribosome during translocation.

Three elongation factor methyltransferases (EFMs) have been identified in yeast. Efm1 monomethylated K30 of EF1A (11,16). See1, which we now refer to as Efm4, dimethylates K316 of EF1A (11,16). Efm2 has been shown to dimethylate K613 on EF2 and indirect evidence suggests it may trimethylate K196 of EF3 (11). This leaves five methylation events with no known responsible enzyme. As the majority of those sites are trimethylated, we sought to search for these enzymes through trimethyllysine immunoblot-based screens.

In this study, we identified Yjr129c as the enzyme responsible for the trimethylation at lysine 509 on elongation factor 2. While this work was being prepared for publication, this finding was reported by another group and the protein was designated Efm3 (20). In addition to mass spectrometric and immunoblot identification, we directly confirm the identity of this modification as a trimethyllysine by amino acid analysis. We then tested possible functions of elongation factor methylation including Efm2 catalyzed modification of EF2. Deletion of EFM2 or EFM3 increased sensitivity to translation inhibitors, indicating changes in the ability for EF2 to interact and communicate with ribosomal components. Additionally, we found that translational fidelity is reduced in *efm2Δ* indicating possible termination defects.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

S. cerevisiae strains used in this study are listed in Table 1. Growth media in this study include YPD (BD Difco 242810, 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose), SD-URA (minimal synthetic defined medium lacking uracil, 0.07% (w/v) CSM-URA

powder (MP Biomedicals, 114511212), 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) dextrose), SC (synthetic complete, 0.07% (w/v) CSM (MP Biomedicals 114500012), 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, with or without 2% (w/v) glucose).

Overnight 5 ml cultures were used to inoculate cultures to OD₆₀₀ 0.1 or 0.15 and grown to ODs needed for the specific experiment. Cultures were grown in flasks on a rotary shaker (250 rpm) at 30 °C.

Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Biological function	Source
BY4741	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Wild type	Open Biosystems
BY4742	MATalpha <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Wild type	Open Biosystems
<i>efm3Δ a</i>	BY4741 background	Putative/Elongation factor methyltransferase	Open Biosystems
<i>efm3Δ α</i>	BY4742 background	Putative/Elongation factor methyltransferase	Open Biosystems
GST-EFM3	BY4741 background	GST-tagged EFM3	Open Biosystems YSC1048
<i>efm2Δ</i>	BY4741 background	Elongation factor methyltransferase	Open Biosystems
<i>efm4Δ(see1Δ)</i>	BY4741 background	Elongation factor methyltransferase	Open Biosystems
<i>efm1Δ</i>	BY4741 background	Elongation factor methyltransferase	Open Biosystems
<i>rkm1Δ</i>	BY4741 background	Ribosomal protein lysine methyltransferase	Open Biosystems
<i>rkm2Δ</i>	BY4741 background	Ribosomal protein lysine methyltransferase	Open Biosystems
<i>yjr093cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>ykl162cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>ynl024cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>ymr209cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>yor021cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>ylr063cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>ymr310cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems
<i>ygr283cΔ</i>	BY4741 background	Putative methyltransferase	Open Biosystems

Immunoblotting

Strains of interest were grown in YPD to OD₆₀₀ 0.7 and cells from 14 ml of the culture were harvested and washed twice with water. Lysis was performed using 0.2 g of glass beads (Biospec Products 11079105) and 50 µl of lysis buffer (1% SDS, 0.7 mM PMSF). Samples were vortexed for 1 min, and then incubated on ice for 1 min, repeated ten times. Crude lysates were extracted and beads washed once with 50 µl of lysis buffer. Unbroken cells and membranes were pelleted by centrifugation at 12,000 x g for 15 min at 4°C. Protein concentrations were determined using the Lowry method (21). 50 µg of protein from each sample was loaded onto a 4-12% Bis-Tris gel (Life Technologies, NuPAGE Novex) and run at 200 V for 1 h with MOPS buffer. Rainbow full range molecular weight markers (GE Healthcare, RPN800E) were used as standards. Proteins were transferred to PVDF membrane (Hybond-P) at 30 V for 1 h. Membranes were blocked overnight at 4 °C in 5% dried non-fat milk in PBST (phosphate buffered saline with 0.1% Tween 20 (v/v)). Membranes were washed in PBST, incubated with primary antibodies diluted into 1% dried non-fat milk in PBST for 1.5 h at room temperature, and then with secondary antibodies diluted in the same solution for 1 h at room temperature. ECL was used to visualize bands (Amersham ECL Prime Western Blotting, GE Healthcare RPN2232). After probing, membranes were stained with Ponceau (1% Ponceau S (w/v), 0.1% acetic acid (v/v)) to determine loading equality.

Antibodies in this study include anti-trimethyllysine-HRP (1:5000, Immunechem ICP0602), anti-di/trimethyllysine (1:10,000, Upstate 07-756), anti-pan methyl lysine (1:10,000, Abcam ab7315), anti-rabbit IgG-HRP (1:6666, Cell Signaling 7074). The Immunechem and Upstate antibodies were kind gifts from Joanna Goldberg (Emory University). The Abcam "anti-pan methyl lysine" antibody was prepared against calf histone H1 containing dimethyllysine

residues. The Upstate antibody was raised against a synthetic peptide containing dimethyllysine at position 9 of human histone H3, and is listed by the manufacturer as an anti-di/trimethyllysine antibody. In the figures, we have emphasized which modified form the antibody prefers in the yeast elongation factors by bolding and underlining the respective degree of lysine methylation. Thus we describe the Abcam antibody as α -M/D/TMK and the Upstate antibody as α -D/TMK.

Mouse tissue cytosolic extracts were a kind gift of Dr. Jonathan Lowenson from the University of California, Los Angeles.

In Vivo Radiolabeling and Amino Acid Analysis

Cultures of wildtype and knockout cells were grown in YPD to OD₆₀₀ 0.7 and cells from 14 mL of culture were harvested by centrifugation at 5,000 x g for 5 min), resuspended in 1 mL of water and transferred to a microcentrifuge tube. After centrifugation, cells were resuspended in 900 μ L of YPD and 100 μ L of S-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet; 83.3 Ci/mmol; 0.55 mCi/mL in 10 mM H₂SO₄-ethanol (9:1); PerkinElmer). Cells were incubated for 30 min at 30 °C on a rotary shaker. Radiolabeled cells were washed twice with water and lysed using the glass bead method described above.

Lysates from each strain were loaded onto a 4% stacking, 12% resolving SDS/Tris-glycine polyacrylamide gel (15x17x0.2 cm) and run at 35 mA through the stacking and 45 mA through the resolving gels. Gels were Coomassie stained (50% methanol, 10% acetic acid, 40% water, 0.2% Brilliant Blue R-250 (w/v)) and destained overnight (10% methanol, 10% acetic acid, 80% water). The protein band running just above the 97 kDa marker was excised and placed into a 6 mm x 50 mm glass test tube. 100 μ L of 6N HCl was added to each slice and tubes placed in a reaction chamber (Eldex Labs, 1163) containing 500 μ L of 6N HCl. Chambers were

heated for 20 h in vacuo at 109 °C in a Pico-Tag vapor phase apparatus (Waters). Residual HCl was removed by vacuum centrifugation.

Dried gel slices were resuspended in 400 µl of cation exchange loading buffer (sodium citrate, 0.2 M Na⁺, pH 2.2). Half of the sample was spiked with 2 µmol of each methyllysine standard (Sigma, N ϵ -methyl-L-lysine hydrochloride 04685, N ϵ ,N ϵ -dimethyl-L-lysine monohydrochloride 19773, N ϵ ,N ϵ ,N ϵ -trimethyllysine hydrochloride T1660) and loaded onto a cation-exchange column (Beckman AA-15 sulfonated polystyrene resin, 0.9 cm inner diameter by 12 cm height) equilibrated with running buffer (sodium citrate, 0.3 M Na⁺) at 55 °C. For full separation of mono-, di- and tri-methyllysine, low pH buffer (pH 3.8) was used. To reduce run times when separation of mono- and dimethyllysine was not required, a pH 4.5 buffer was used. Buffer at pH 5.5 was used to analyze when the separation of mono-, di-, and trimethyllysine species was not needed. Amino acids were eluted in the equilibration buffer at 1 mL/min while collecting 1 min fractions at the expected elution position of the methyllysine standards. 50 µl of each fraction was added to a flat-bottom 96-well plate to detect standards by the ninhydrin method. Each well was mixed with 100 µl of ninhydrin reagent (2% ninhydrin (w/v), 0.3% hydrindantin (w/v), 75% dimethyl sulfoxide (v/v), 25% 4 M lithium acetate pH 4.2 (v/v)) and the plate heated at 100 °C for 15 min. Standards were detected by measuring absorbance at 570 nm using a SpectraMax M5 microplate reader. The remainder of each fraction was added to 5 ml of scintillation fluor (Safety Solve, Research Products International) in a 20 ml scintillation vial and counted for three five min cycles using a Beckman LS6500 instrument to detect [3H]-methylated amino acids.

In Gel Trypsin Digests and Mass Spectrometry

Coomassie-stained gel slices from the 100kDa region of fractionated polypeptides of yeast cell lysates were washed with 50 mM ammonium bicarbonate and destained by incubating in a solution of 50% 50 mM ammonium bicarbonate/50% acetonitrile for 2-4 h until the gel slice became transparent. Slices were incubated in 100% acetonitrile and dried by vacuum centrifugation for 10 min. After incubating the dried slice in a minimal volume of 10 mM DTT in 50 mM ammonium bicarbonate for 1 h at 60 °C to reduce disulfide bond, proteins were alkylated by treatment in 50 mM iodoacetamide in 50 mM ammonium bicarbonate for 45 min at 45 °C. Gel slices were washed by alternating 10 min incubations in 50 mM ammonium bicarbonate and 100% acetonitrile. Slices swelled on ice in a working stock solution of 20 ng/μl of sequencing grade trypsin (Promega, V5111) for 45 min. Digests were performed for 16 h at 37 °C and peptides eluted using 50% acetonitrile/1% trifluoroacetic acid in water. Peptides were dried by vacuum centrifugation and resuspended in 200 μl of 0.1% TFA in water.

Tryptic peptides from the 100 kDa SDS-gel band of wildtype, *efm2Δ*, and *efm3Δ* lysates were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an EASY-nLC 1000 (Thermo Scientific, Waltham, MA) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) and an EASY-Spray nano-ESI source. Peptides were injected onto a 75 μm x 15 cm, 3μ, 100Å PepMap C18 reversed-phase LC column and separated using a linear gradient from 5% solvent B (0.1% formic acid in acetonitrile), 95% solvent A (0.1% formic acid in water) to 50% solvent B in 45 minutes at a constant flow of 300 nl/min. Eluted peptides were analyzed with a top-10 data-dependent acquisition method and identified using Proteome Discoverer (Version 1.4; Thermo Scientific) coupled with MASCOT (Version 2.4.1; Matrix Science, London, UK). Orbitrap MS resolving power was set to 70,000 at m/z 200 for

MS1 and 17,500 at m/z 200 for MS2. Tryptic peptides with up to 1 missed cleavage were searched against the SwissProt *Saccharomyces cerevisiae* database (2013, 7,798 sequences) with dynamic modifications for carbamidomethyl (C), oxidation (M), deamidation (N, Q) monomethyl (K), dimethyl (K), and trimethyl (K). Precursor and product ion mass tolerances were set to 10 ppm and 0.005 Da, respectively. Methylated EF2 peptides identified by MASCOT were manually examined and confirmed from the corresponding MS/MS spectra.

Manually confirmed EF2 peptides with methylated lysine residues were further examined by targeted parallel reaction monitoring mass spectrometry (PRM-MS) to explore the effects of EFM2 and EFM3 deletions on EF2 methylation. Samples (described above) were re-analyzed by a targeted-MS/MS acquisition method using an inclusion list containing the doubly and triply charged mass-to-charge (m/z) values of the manually confirmed EF2 peptides from wild type lysate. Peaks corresponding to methylated EF2 peptides were visualized in Xcalibur Qual Browser software (Thermo Scientific) using precursor -- fragment transitions extracted within 10 ppm mass accuracy. The following transitions (m/z) were used to identify methylated and unmethylated peptides of interest: 286.1920→372.2423 (LVEGLKTMK509R); 281.5201→251.1790 (LVEGLKDMK509R); 390.2060→402.2823 (DDFKDMK613AR); 383.1981→268.1712 (DDFKMMK613AR); 329.7103→446.2609 (LVEGLK); 376.1903→521.3194 (DDFKAR).

Bioinformatic Alignments and Phylogenetic Tree Construction

Whole protein sequences for translocase (EF-2 or EF-G) were aligned using Clustal Omega. A protein-protein BLAST search was performed using *S. cerevisiae* Efm3 (UniProt P47163) or Efm2 (UniProt P32324) as the query. Sequences were aligned using MULTIPLE

Sequence Comparison by Log- Expectation (MUSCLE) in MEGA 6. The evolutionary history was inferred using the Neighbor-Joining method to create phylogenetic trees. The evolutionary distances were computed using the p-distance method. All positions containing gaps and missing data were partially eliminated. In instances where poor homology was found, organisms were eliminated from alignment or replaced by a different representative from the same kingdom.

Translation Inhibitor Assays

Changes in sensitivity to various translation inhibitors were determined using serial dilution spot test growth assays. Briefly, cells were grown at 30 °C in YPD media to an OD600 of approximately 0.5. 1 ml of each culture was centrifuged down at 5000 \times g for 5 min. Cells were washed with water and the pellets were diluted to an OD600 of 0.5. The cells were then diluted in a 5-fold series in water under sterile conditions. 3 μ l of each dilution was spotted onto a 10cm 2% agar plate containing YPD or YPD + antibiotic and incubated at 30° C for 2-5 days. Antibiotics used were cycloheximide (Sigma C7698), puromycin (VWR 97064-280), paromomycin (Sigma P9297), anisomycin (Sigma A9789), tunicamycin (Sigma T7765), and verrucarin A (Sigma V4877). Levels of the drug transporter Pdr5 were measured by Northern blot as previously described (8).

Dual Luciferase Translational Fidelity and Frameshift Assays

The dual-luciferase systems were used as previously described (8,22-24). Stop codon readthrough and amino acid misincorporation reporters and control vectors were generously provided by Dr. David Bedwell and Ming Du (University of Alabama, Birmingham, AL).

Frameshift reporter plasmids were generously provided by Dr. Jonathan Dinman (University of Maryland). All vectors (Table 2) were transformed into wildtype, *efm2* Δ and *efm3* Δ cells by the LiOAc-ssDNA-PEG method. The assay was performed as described with the Dual Luciferase Reporter Assay system (Promega) using a SpectraMax M5 microplate reader.

Structural analysis of Efm3 and related methyltransferases—The structures of Efm3, Efm2, and human FAM86A were modeled using the Protein Homology/analogy Recognition Engine V2.0 (Phyre2). Efm3 modeling was performed using one-to-one threading with METTL21D (VCP-KMT) Chain B from *H. sapiens* (PDB: 4LG1) using the global alignment method with default settings for secondary structure scoring and weight. Efm2 and FAM86A modeling used intensive mode. Structural figures of the catalytic region of these models and crystal structures of other related enzymes were generated in MacPyMol (DeLano Scientific).

Table 2. Vectors used for Dual-Luciferase Assays.

Experimental Purpose	Control vector	Experimental Vector	Source
Amino acid misincorporation	CTY775/luc CAAA	CTY775/luc CAAA FF K529 (AAA to AAT, K \rightarrow N)	Gift from Dr. David Bedwell, University of Alabama
Stop codon readthrough, UAA	CTY775/luc CAAC	CTY775/luc UAAC	
Stop codon readthrough, UAG	CTY775/luc CAGC	CTY775/luc UAGC	
Stop codon readthrough, UGA	CTY775/luc CGAC	CTY775/luc UGAC	
-1 frameshift	pJD375, 0-frame control	pJD376 (L-A virus element)	Gift from Dr. Jonathan Dinman, University of Maryland
+1 frameshift	pJD375, 0-frame control	pJD377 (Ty1)	

RESULTS

Deletion of the EFM3 Gene in S. cerevisiae Results in the Loss of Trimethyllysine in One or More 100 kDa Proteins

An immunoblot-based screen against trimethyllysine residues on polypeptides from whole cell lysates of putative methyltransferase knockout strains was performed (Fig. 1A). When using the Upstate antibody nominally specific for di- and tri-methyllysine residues, distinct bands were detected near 100 kDa and 50 kDa, corresponding to the approximate molecular weights of EF2/EF3 and EF1A, respectively. The patterns were similar in each case with the exception that there was no detectable signal in the 100 kDa band from the extract of the strain with a deletion of the EFM3 (YJR129C) gene (Fig. 1A).

In a separate experiment using deletion strains of known EFMs, we confirmed the loss of 100 kDa immunoreactivity in the *efm3Δ* (*yjr129cΔ*) strain (Fig. 1B). We then stripped and reprobed the membrane with two additional antibodies to methylated lysine residues. A complete loss of immunoreactivity in the 100 kDa region was seen with the Upstate di/trimethyllysine antibody but not with the Abcam dimethyllysine antibody (Fig. 1B). In the latter case, we detected a slight reduction of immunoreactivity in the 100 kDa band. This same reduction was noted in the *efm2Δ* lysate, which suggested that Efm3 might be acting on the same substrates for Efm2, EF2 and/or EF3. A significant loss of signal is also detected at the 50 kDa position in *efm4Δ* (*see1Δ*), which dimethylates lysine 316 on EF1A, confirming the identity of this band.

To confirm that the loss of signal was not due to secondary mutations, the immunoblots were repeated again with gene deletions of the EFM3/YJR129C gene in a- and alpha mating type backgrounds (Fig. 1C). The same loss of signal was observed in both strains.

Due to the potential non-specific binding of the antibodies and varied preference of the different antibody preparations between di- and trimethyllysine species, amino acid analysis was utilized to detect the specific type of methylation lost in the *efm3Δ* strain. *S. cerevisiae* is capable of taking up exogenous methyl donor, AdoMet, from the media. By supplementing cultures with [³H]AdoMet, substrates methylated during incubation incorporate tritiated methyl groups. After in vivo labeling, cells were lysed and the resulting lysates were resolved by SDS-PAGE. The Coomassie-stained bands in the 100 kDa region were excised and acid hydrolyzed into amino acids. The resulting hydrolysate was loaded onto a high-resolution cation exchange column capable of separating methyl derivatives of the amino acids. Analysis of wildtype [³H]-hydrolysates showed presence of [³H]-trimethyllysine and [³H]-dimethyllysine but no [³H]-monomethyllysine (Fig. 2A, left). Hydrolysates from EFM2 deletion strains, a known EF2 dimethylating enzyme (11), were used as a positive control to ensure the excised gel slice contained EF2. [³H]-hydrolysates from *efm2Δ* maintained the presence of trimethyllysine but no detectable dimethyllysine was seen (Fig. 2A, right).

Wildtype and *efm3Δ* cells from both mating type backgrounds were then in vivo labeled and the 100 kDa regions acid hydrolyzed. Analysis of the wildtype [³H]-hydrolysates demonstrated the presence of tri- and dimethyllysine in both mating types (Fig. 2B, top panels). By contrast, [³H]-hydrolysates from *efm3Δ* showed almost complete loss of the trimethyllysine peak (Fig. 2B, bottom panels). To account for variations in the amount of radioactivity loaded onto the columns, one-fifth of the volume of the load (25 μl) was counted for normalization. The fractions of radioactivity for both di- and trimethyllysine relative to the total radioactivity were calculated (Fig. 2C). No significant differences in the relative amount of dimethyllysine were

detected between mating types and knockouts, indicating that Efm3 is specifically responsible for the trimethylated lysine on the protein in this region.

Taken together, these results suggest that EFM3/YJR129C encodes a protein lysine methyltransferase that catalyzes the trimethylation of at least one polypeptide of about 100 kDa. Since this is the approximate size of the polypeptides of EF2 and EF3, we investigated whether EF2/EF3 methylation is altered in the absence of this methyltransferase.

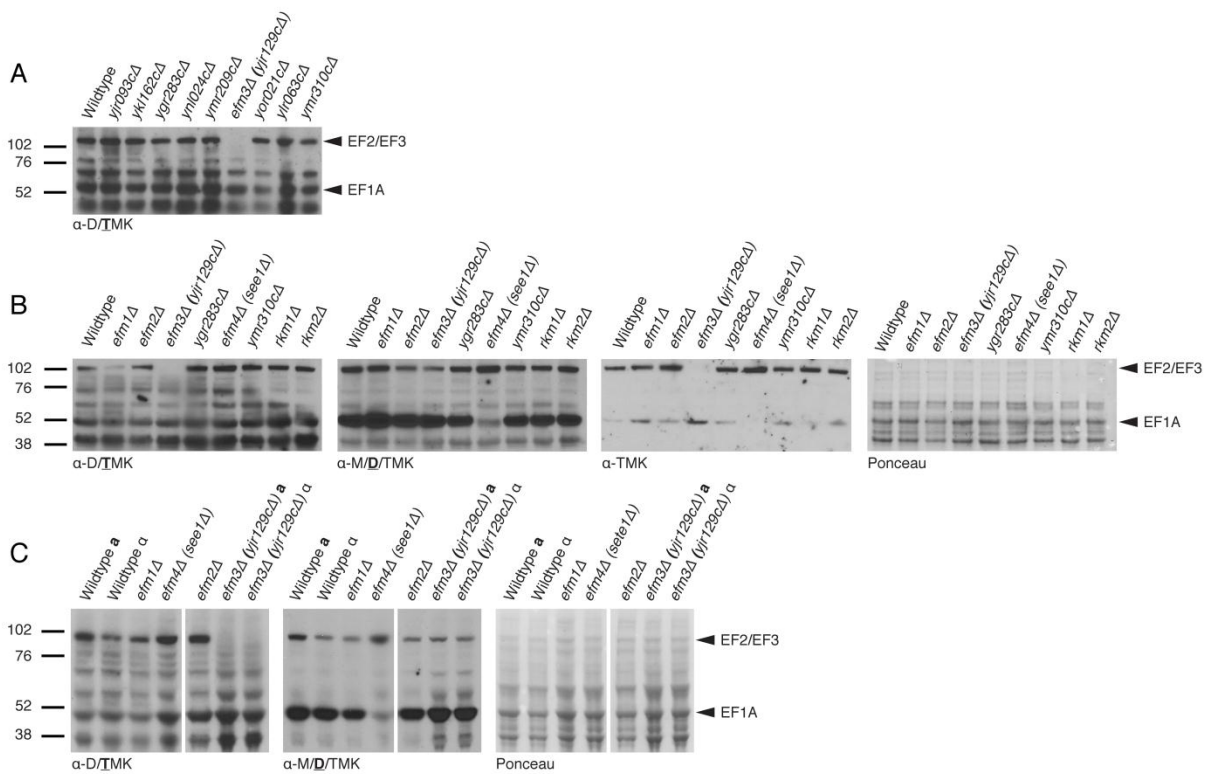
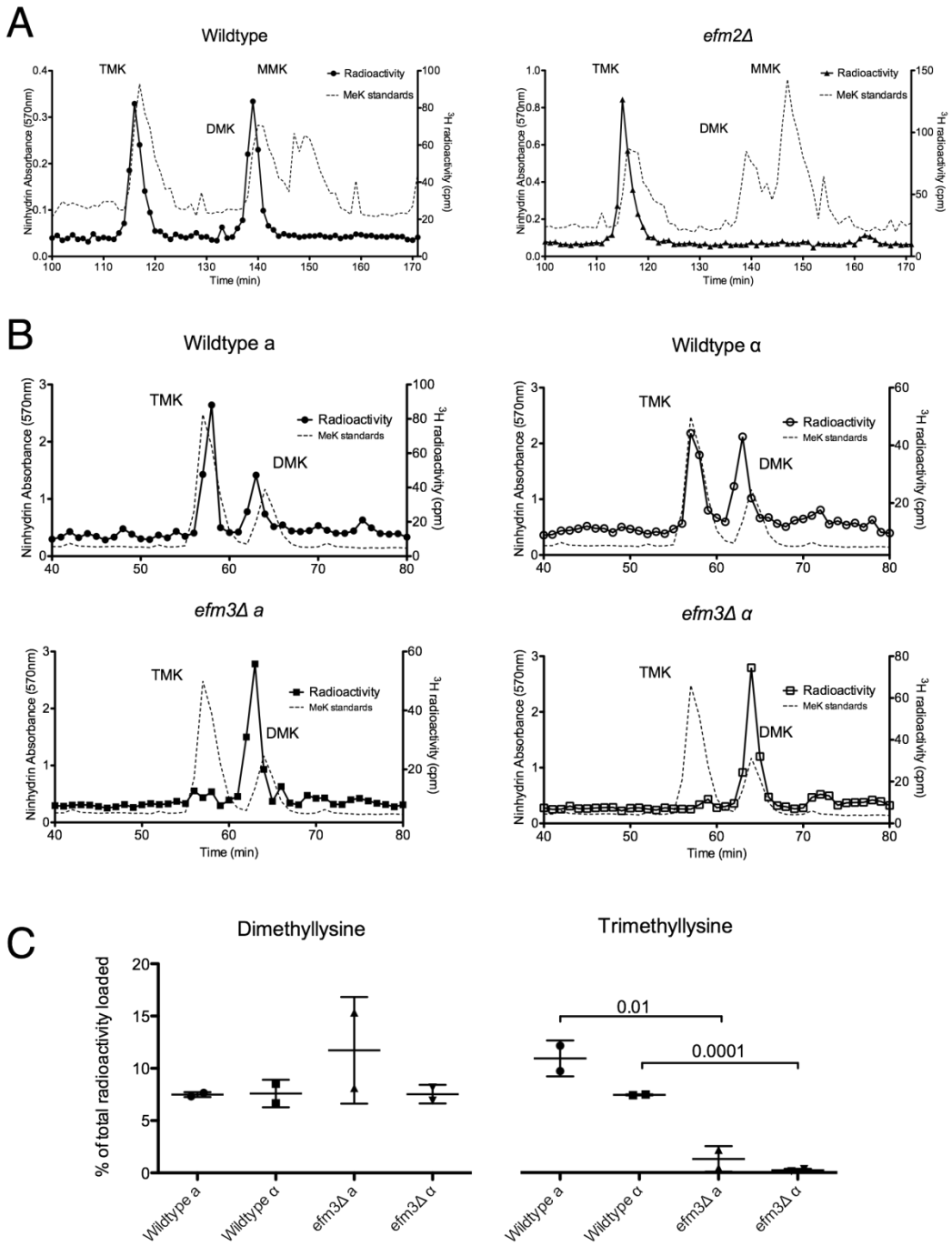


Figure 1. Deletion of *EFM3/YJR129C* results in loss of trimethylated lysines in 100 kDa polypeptides in *S. cerevisiae*. Immunoblots were performed on whole cell lysates as described in “Experimental Procedures” with various antibodies directed against methylated lysine residues. The position of molecular weight markers is shown on the left. (A) Polypeptides from lysates of strains with deletions of candidate methyltransferase genes were probed with anti-di/trimethyllysine (Upstate 07-756). Arrows indicate the distinct signals at 100 and 50 kDa correlating to EF2/EF3 and EF1A, respectively. (B) Polypeptides from known and putative EFM knockout strains were immunoblotted with multiple methyllysine antibodies. The same

membrane was probed, stripped, and re-probed with anti-di/trimethyllysine (left, Upstate 07-756), anti-dimethyllysine (middle left, Abcam Ab7315), anti-trimethyllysine (middle right, Immunechem ICP0602). The Ponceau-stained membrane is shown on the right as a protein loading control. The type of methyllysine recognized by each antibody is indicated below each blot with the bolded and underlined letter representing the strongest specificity. (C) Comparison of wildtype and EFM knockout strains including *efm3Δ* in both mating type backgrounds. All samples were run on the same gel; spaces indicate where non-relevant lanes were removed.

Figure 2. Deletion of EFM2 results in loss of dimethylated lysine and deletion of EFM3 results in loss of trimethylated lysine in 100 kDa polypeptides. Lysates from in vivo radiolabeled cells were fractionated by SDS-PAGE and the 100 kDa gel region was excised. Gel slices were acid hydrolyzed as described in “Experimental Procedures”. The resulting hydrolysates were loaded onto a high-resolution cation exchange column with standards of methylated lysine derivatives. (A) Using a pH 3.8 elution buffer, radiolabelled methylated lysine derivatives were separated. The position of the standards, detected by ninhydrin reactivity, is shown in the dashed line. Due to a tritium isotope effect, the radiolabeled derivatives elute slightly before the non-labeled standards (58). Each trace is representative of three independent experiments. (B) Separation of hydrolysates from radiolabeled wildtype and *efm3Δ* cells in both mating type backgrounds. A pH 4.5 elution buffer was used. The ninhydrin profiles are shown for methylated standards run in a separate experiment due to interference from the large amounts of ammonium ion present in the gel hydrolysates. Each trace is representative of two independent experiments. (C) The amount of di- and trimethyllysine radioactivity as a percentage of the total radioactivity in the hydrolysate is shown with error bars reflecting the standard deviation. P-values from Student's t-test are shown when less than 0.05.



Lysine 509 on EF2 is Unmethylated in the Absence of Efm3

To confirm that Efm3 is acting on an elongation factor, the 100 kDa region on a gel containing non-radioactive lysates was subjected to in-gel trypsin digestion. The resulting peptides were loaded onto a C18 reversed phase column and analyzed by LC-MS/MS on a Q-Exactive Orbitrap Mass spectrometer. A protein sequence search reported EF2 (UniProt P32324) as the main component of the 100 kDa protein band. Known methylations on EF2 peptides LVEGLKR and DDFKAR at K509 (di- and trimethyl) and K613 (mono- and dimethyl) were identified, respectively, with additional manual MS/MS spectra confirmation in wildtype lysate samples (Fig. 3A and 3B). The effect of *efm2* Δ and *efm3* Δ on the methylation status of K509 and K613 was examined using a sensitive targeted mass spectrometry method that scans specifically for EF2 methylated peptides observed in wildtype samples and their corresponding unmethylated variants. Extracted precursor-fragment ion chromatograms specific to each peptide showed a loss of di- and trimethylation at K509 on the LVEGLKR peptide but no effect on K613 methylation in *efm3* Δ samples (Figure 3C). Deletion of EFM2 resulted in a loss of mono- and dimethylation at K613 on peptide DDFKAR without affecting methylation at K509. Further evidence of the effect of Efm2 and Efm3 on EF2 methylation was confirmed by the presence of unmethylated LVEGLK and DDFKAR peptides (not observed in fully methylated wildtype samples) in *efm3* Δ and *efm2* Δ samples, respectively. A similar approach for analyzing the impact of Efm3 also showed the loss of K509 on EF2 (20).

In our experiments, we detected peptides from EF3 but sequence coverage did not include the known methylated sites. Thus, it is possible that Efm2 and/or Efm3 methylates one or more of the three sites on EF3.

Evolutionary Conservation of Efm3 and Efm2 Correlates with the Conservation of their Respective EF2 Methylation Sites

A BLAST search was performed to identify potential homologs of Efm2 and Efm3. The sequences were aligned using MULTiple Sequence Comparison by Log- Expectation (MUSCLE) and a phylogenetic tree was constructed to visually demonstrate the conservation (Fig. 4A and 4C). The K509 and K613 lysine residues and surrounding sequence are highly conserved in Animalia, Plantae and Fungi but less so in Bacteria, Archaea and Protista.

The conservation of these enzymes correlated well with the conservation of the methylation sites (Fig. 4B and 4D); organisms that have homologous enzymes also have the corresponding methylation sites whereas the Protista, Bacteria, and Archaea do not seem to have either the methylation sites or the enzymes. To confirm the similarity of the methylation reactions in yeast and mammals, cytosolic extracts from mouse tissues were immunoblotted with methyllysine antibodies. Although the protein and methylation patterns varied between tissues, each sample tested showed a distinct trimethyllysine band just below the 102 kDa marker (Fig. 4E). This corresponds to the molecular weight of mouse EF2 (95 kDa) and suggests that EF2 in higher eukaryotes may also be similarly methylated.

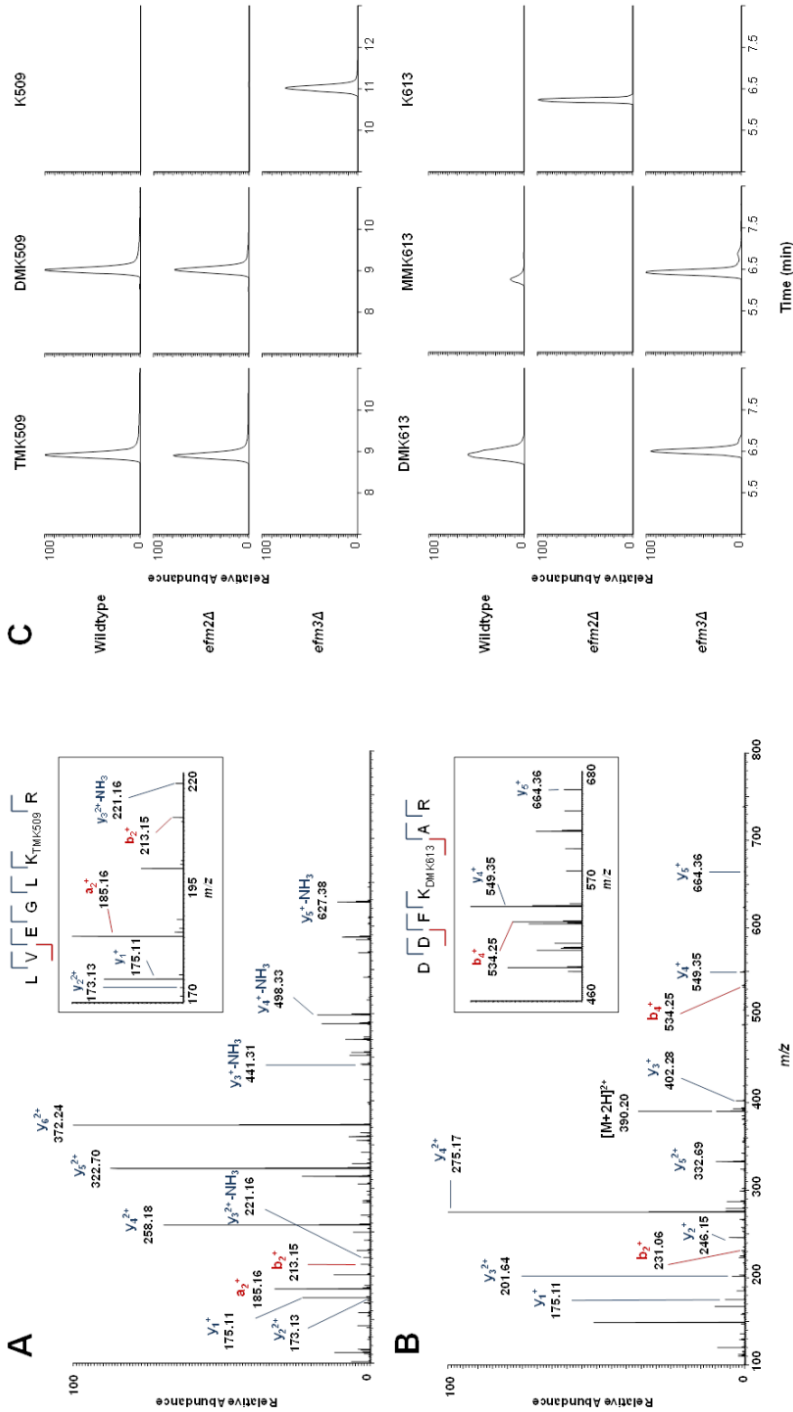


Figure 3. Deletion of *EFM3* results in loss of trimethyllysine 509 on elongation factor 2. The 100 kDa protein bands from wildtype and knockout lysates were excised, in-gel trypsin digested, and analyzed by LC-MS/MS. A Mascot search identified EF2 as the top hit (score = 13763.99) for the 100 kDa band. (A) Known trimethylation at K509 (TMK509) and (B) dimethylation at K613 (DMK613) were identified in EF2 peptides LVEGLK_R and DDFK_A, respectively, in wildtype lysate. Dimethylation at K509 (DMK509) and monomethylation at K613 (MMK613) were also observed in EF2 from wildtype lysate. (C) The effect of *efm2Δ* and *efm3Δ* on the methylation of K509 and K613 was examined by comparison of EF2 from wildtype lysate to knockout lysates. Parallel reaction monitoring mass spectrometry (PRM-MS) with extracted precursor→product ion transitions (within 10 ppm) was used to identify methylated peptides in each sample condition (WT, *efm2Δ*, *efm3Δ*).

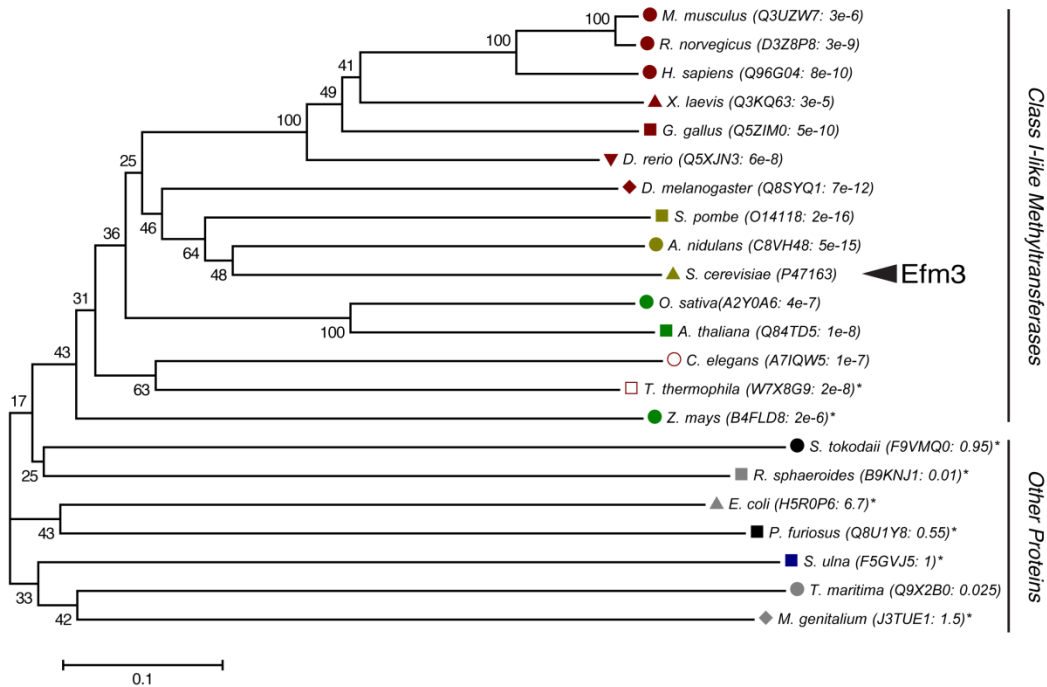
A

	K509	
<i>S. cerevisiae</i>	EVKNANDLPKLV EGLKRLSKSDPCVLT YMSE	524
<i>A. nidulans</i>	EVKNAGDLPKLV EGLKRLSKSDPCVLT MINE	525
<i>S. pombe</i>	EVKNGNDLPKLV EGLKRLSKSDPCVLT CTSE	524
<i>D. melanogaster</i>	EPKNPADLPKLV EGLKRLAKSDPMVQCIIEE	526
<i>C. elegans</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	534
<i>D. rerio</i>	EVM DPADLPKLV EGLKHLAKSDPMLQCIIEE	538
<i>X. laevis</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	540
<i>G. gallus</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	540
<i>H. sapiens</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	540
<i>B. taurus</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	540
<i>M. musculus</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	540
<i>R. norvegicus</i>	EAKNPADLPKLV EGLKRLAKSDPMVQCIIEE	540
<i>T. thermophila</i>	NVKNAGDLPKLV DGLKRLSKSDPLVIC TTEE	519
<i>A. thaliana</i>	QCKVASDLPKLV EGLKRLAKSDPMVVCTMEE	527
<i>Z. mays</i>	QCKVASDLPKLV EGLKRLAKSDPMVLCTMEE	524
<i>O. sativa</i>	QCKVASDLPKLV EGLKRLAKSDPMVLCTIEE	524
<i>A. carterae</i>	KPKDGDLPKLV EGLKRLSKSDPLVVC TTEE	502
<i>T. maritima</i>	EPVTKADEEKL VKALLALSEEDPTLQVRVDK	444
<i>E. coli</i>	EPKTKADQEKMG LALGR LAKEDPSFRVW TDE	452
<i>R. sphaeroides</i>	EPKTKADQEKMG LALARLAAEDPSFRVETDF	458
<i>B. subtilis</i>	EPKSKADQDKMGI ALAKLAEEDPTFR TQTNP	443
<i>M. genitalium</i>	EPKTKADQEKMSI ALSKLAEEDPTFK TFS DP	442
<i>S. tokodaii</i>	EPKNPKDLNKMID ALRKL SIEDPNLLVKINE	434
<i>P. furiosus</i>	EAKNVKDLPR LI EALRQLAKEDPTLHV KIDE	427

Key:

- Animalia**
 - Mammalia
 - Aves
 - ▲ Amphibia
 - ▼ Actinopterygii
 - ◆ Insecta
 - Chromadorea
 - Oligohymenophorea
- Fungi**
 - Eurotiomycetes
 - Schizosaccharomycetes
 - ▲ Saccharomycetes
- Plantae**
 - Magnoliopsida (monocot)
 - Magnoliopsida (eudicot)
- Archaea**
 - Thermoprotei
 - Thermococci
- Bacteria**
 - Thermotogae
 - Alpha proteobacteria
 - ▲ Gamma proteobacterial
 - ◆ Mollicutes
- Protista**
 - Fragilariophyceae

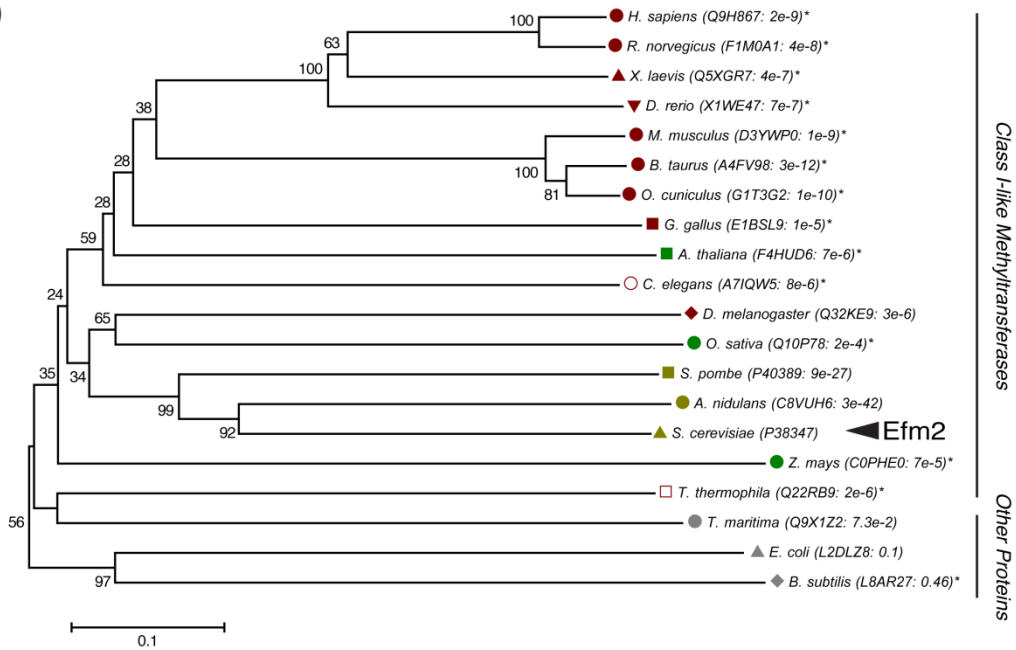
B



C

	K613	
<i>S. cerevisiae</i>	SLAIENGIINPRDDFKARARIMADDYGVWVDT	628
<i>A. nidulans</i>	SKAIEEGKINPRDDFKARARILADEYNWVDT	629
<i>S. pombe</i>	SVAIETGHVNPRDDFKVRRARIMADEFGWVDT	628
<i>D. melanogaster</i>	PEDIIDNGDVSAKDEFKARARYLSEKYDYDVT	630
<i>C. elegans</i>	ADDIEGGTVNARDEFKARAKILAEKYEYDVT	638
<i>D. rerio</i>	AEDIDKGRVTSQEMNARAYLAENYKWEVDT	642
<i>X. laevis</i>	AEDIDKGDVSAKQELKTRARYLAEKYEYDVT	644
<i>G. gallus</i>	AEDIDKGEVSAKQELKQRRARYLAEKYEYDVT	644
<i>H. sapiens</i>	AEDIDKGEVSAKQELKQRRARYLAEKYEYDVA	644
<i>B. taurus</i>	AEDIDKGEVSAKQELKQRRARYLAEKYEYDVA	644
<i>M. musculus</i>	AEDIDKGEVSAKQELKQRRARYLAEKYEYDVA	644
<i>R. norvegicus</i>	AEDIDKGEVSAKQELKQRRARYLAEKYEYDVA	644
<i>T. thermophila</i>	AEDIEKGTINPKDDPKDRAKLLNEKYNWVRT	623
<i>A. thaliana</i>	AEAIDDGRIGPRDDPKIRSKILAEFEFGWDDK	632
<i>Z. mays</i>	AEAIDDGRIGPRDDPKVRSQILSQEFGWDDK	629
<i>O. sativa</i>	AEAIDDGRIGPRDDPKVRSKILSEFEFGWDDK	629
<i>A. carterae</i>	SVLIEAGKAGPKADPKERTKILREQFEWDEN	606
<i>T. maritima</i>	-----	537
<i>E. coli</i>	-----	547
<i>R. sphaeroides</i>	-----	551
<i>B. subtilis</i>	-----	536
<i>M. genitalium</i>	-----	534

D



E

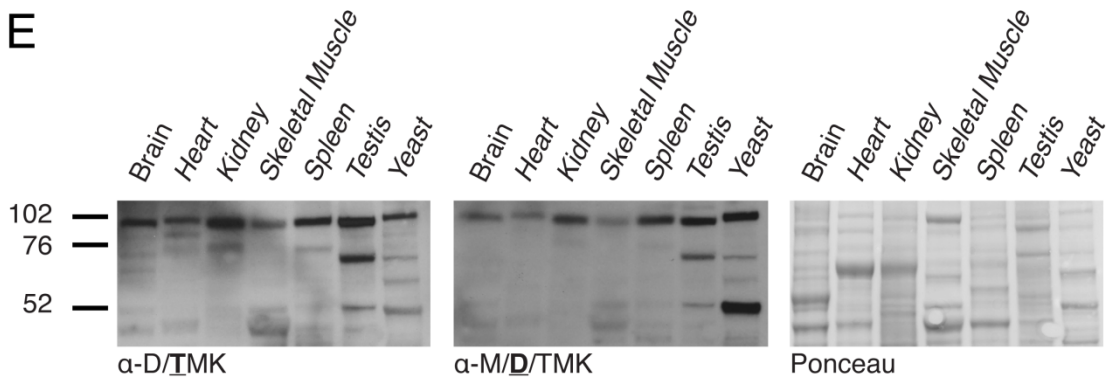


Figure 4. Conservation of EF2 methylation sites and methyltransferases among six kingdoms of life. The region corresponding to (A) the K509 trimethylation site and (C) the K613 dimethylation site in *S. cerevisiae* is shown for representative organisms. The methylated lysine residue is bolded. Aliphatic residues are shown in gray, acidic residues in blue, basic residues in red, polar residues are in green, and aromatic residues in orange. In C, Archaea were removed from alignment due to significant sequence differences. (B,D) Phylogenetic trees depicting evolutionary conservation of (B) Efm3 and (D) Efm2. The UniProt ID of the top ranking alignment for each organism is indicated, along with their respective E-values. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of amino acid differences per site. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All proteins were mutual best hits except those indicated with an asterisk (*). In B, *A. carterae* was removed since no significant homolog to Efm3 was found. *S. ulna* was used as a representative Protista but had no homolog for EF2. In D, Protista, Archaea, *R. sphaeroides*, and *M. genitalium* were completely removed due to no significant homology to Efm2. (E) Polypeptides from cytosolic extracts from various mouse tissues were probed by immunoblotting with anti-di/trimethyllysine antibodies (left panel, Upstate 07-756). The membrane was then stripped and reprobed with anti-dimethyllysine (middle panel, Abcam ab7315). The membrane was Ponceau stained to ensure equal loading (right panel).

Deletion of Efm2 or Efm3 Results in Altered Sensitivity to Translational Inhibitors

To elucidate possible roles of EF2 methylation, wildtype and methyltransferase knockout cells were exposed to a variety of translational inhibitors to look for changes in sensitivity (Fig. 5A). Inhibitors were selected to affect different stages of translation, including those blocking translocation (cycloheximide (25)), inhibiting peptidyl transfer (verrucarin A (26,27)), or inducing premature termination (puromycin, (28,29)). Additional inhibitors were chosen that act

as tRNA structural mimics (anisomycin (30) and paromomycin (31)), or that block initiation via the unfolded protein response (tunicamycin (32,33)).

Both *efm2Δ* and *efm3Δ* cells demonstrated increased sensitivity to verrucarin A, cycloheximide, and tunicamycin. No differences in sensitivity as compared to wildtype were seen for paromomycin, puromycin, and anisomycin. In each case, similar results were found for *efm2Δ* and *efm3Δ*, indicating that both methylation sites are required for resistance to these antibiotics. Given the role of EF2 in translocation, it is not surprising that sensitivity of cells is altered when exposed to inhibitors that block translocation either directly or indirectly.

Pdr5 is a multidrug transporter that can export several translation inhibitors, and changes in its expression have been shown to affect drug sensitivity of various mutant strains (34,35). To confirm that the observed growth phenotypes were not due to variations in PDR5 mRNA expression, the transcript levels of PDR5 were examined by Northern blot. No significant differences were detected between the various strains (Fig. 5B), indicating that the increased sensitivity of the *efm2Δ* and *efm3Δ* strains to verrucarin A, cycloheximide, and tunicamycin is unlikely to be due to variations in drug export efficiencies.

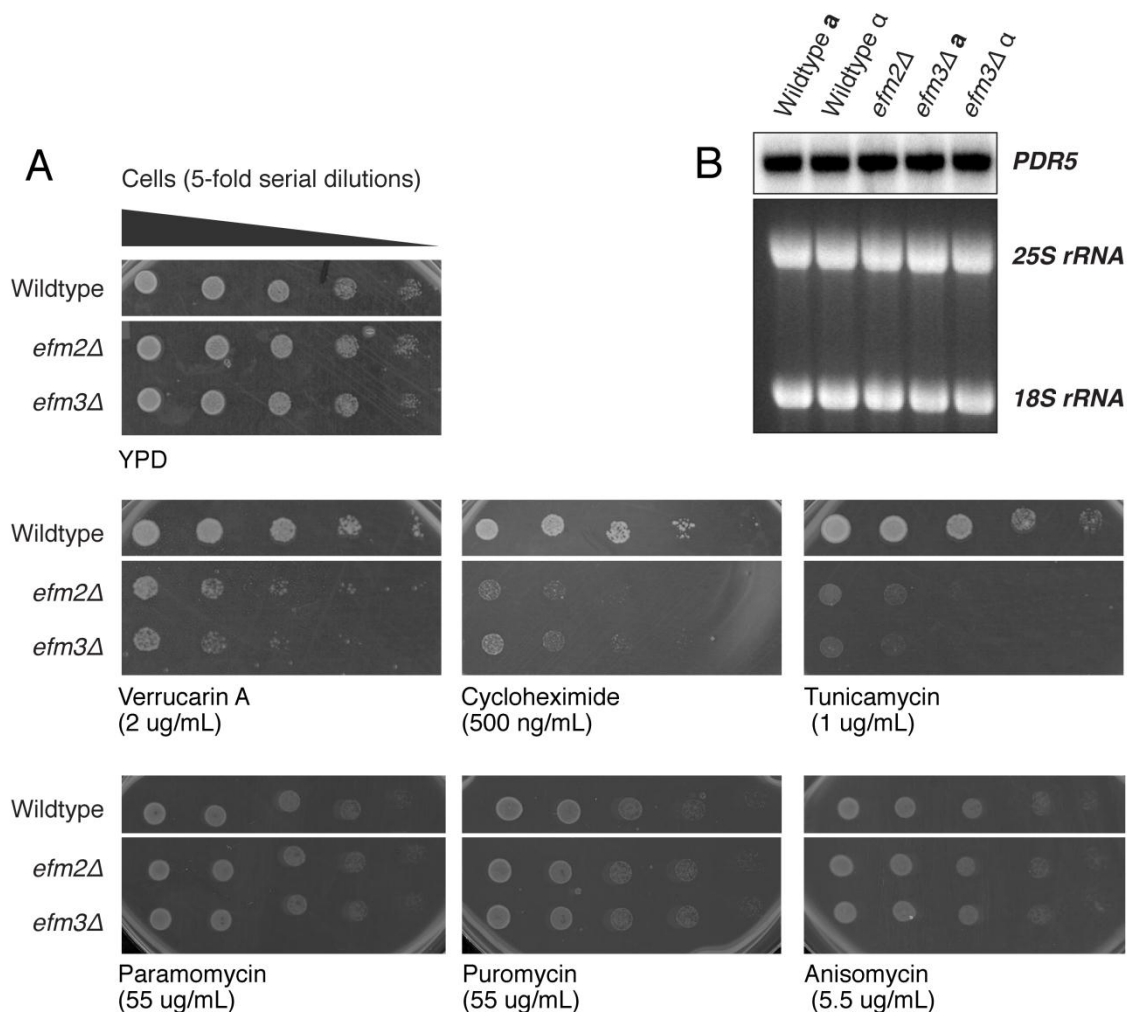


Figure 5. $efm2\Delta$ and $efm3\Delta$ cells have altered sensitivity to translational inhibitors. (A) Wildtype, $efm2\Delta$ and $efm3\Delta$ cells were cultured to OD600 0.5 and five-fold serial diluted in water. 3 μ l from each dilution was spotted onto a YPD agar plate +/- inhibitors at the indicated concentrations. Images shown are representative of at least three individual replicates. Cells shown are on the same antibiotic plate with other tested strains; only strains relevant to this study are displayed. YPD without inhibitor ensures equal cell loading between strains. The number of days each plate was incubated is indicated below each panel. (B) A Northern blot against the multidrug transporter Pdr5 transcript was performed as described (8). The 25S and 18S ribosomal RNAs are shown as loading controls.

Deletion of *EFM2*, but not *EFM3*, Results in Increased Stop Codon Readthrough

Wildtype and knockout strains were transformed with dual-luciferase reporter vectors (22-24). These vectors contain two luciferase genes, renilla and firefly, with one of three types of modifications: an inserted stop codon, an inactivation mutation in the firefly gene, or a frameshift signal between the two genes. Changes in stop codon readthrough, amino acid misincorporation, and programmed frameshift can be measured by the amount of active firefly produced. No significant changes were observed between wildtype and *efm3Δ* cells for any of these assays (Fig. 6). However, *efm2Δ* cells showed a 2- and 3-fold higher stop codon readthrough for UAG and UAA, respectively, although no differences were seen in misincorporation or frameshifting (Fig. 6).

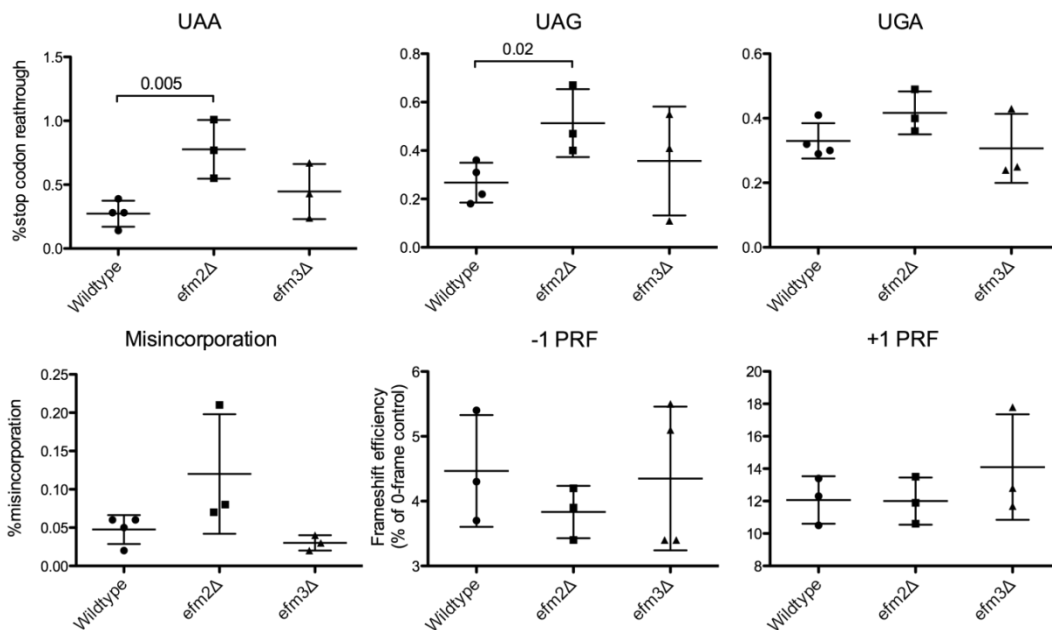


Figure 6. *efm2Δ* but not *efm3Δ* cells have increased stop codon readthrough. Dual luciferase assays were utilized to measure the percent of stop codon readthrough, amino acid misincorporation, and frameshifting. Titles of each panel indicate the stop codon analyzed, the misincorporation of lysine, or the direction of programmed ribosomal frameshift (PRF). Values for three to four replicates with standard deviations are shown as error bars. P-values are displayed where differences were less than 0.05.

Lysine Methyltransferase Structures Share Similarities in the Catalytic Core

The identity of the methyltransferases responsible for four of the methylated lysine residues on the yeast elongation factors is still unknown. In an attempt to narrow down the search for these enzymes, we turned to enzyme structural analysis for potential similarities in known lysine methyltransferases. Efm2 and Efm3 are members of Group J, methyltransferases predicted to have similar substrate types (36). Group J proteins share homology with human Family 16 enzymes, most of which are protein lysine methyltransferases (37-39). Family 16 includes FAM86A, the homolog of Efm3 (20). Here we specifically compared the catalytic centers of modeled Phyre2 structures of Efm2, Efm3, and FAM86A with crystal structures of methyltransferases from the Family 16 and members of the SET domain protein lysine methyltransferases (Fig. 7).

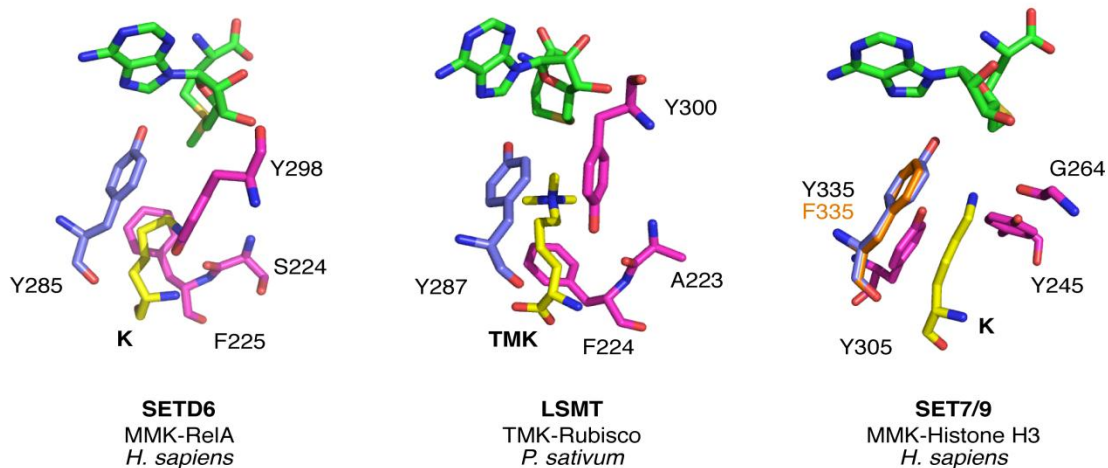
SET domain enzymes differ substantially in sequence and in the overall structure of the AdoMet binding site from Class I methyltransferases (40). Interestingly, these enzymes appear to share a structurally analogous tyrosine or phenylalanine residue in the active site (Fig. 7). In the SETD6 methyltransferase, a Y285A mutation resulted in the loss of catalytic activity, while in the SET7/9 enzyme, a corresponding Y335F mutation reduced AdoMet binding affinity without a large change in the catalytic turnover rate (41,42). Additionally, mutation of the SET7/9 tyrosine to p-aminophenylalanine hindered AdoMet binding by 10,000-fold but only reduced k_{cat} by 35-fold (43). These results suggest two distinct roles of this tyrosine, one of CH•••O hydrogen bonding to the AdoMet methyl group and one of a possible cation- π interaction with the substrate lysine. Importantly, a tyrosine or phenylalanine, part of the previously noted DXX(Y/F) motif of the Family 16 and Group J enzymes, is present and similarly positioned in the catalytic site (Fig. 7) (38). Although there does not appear to be CH•••O hydrogen bonding

between the tyrosine residue and AdoMet, the possibility of cation- π interactions between the aromatic ring and substrate lysine remains. Such cation- π interactions have been previously noted in methyl lysine recognition proteins (44-46).

In the SET domain enzymes shown in Fig. 7, the positive charge on the substrate lysine is balanced by the partial negative charge on a hydroxyl group of a different tyrosine residue and a backbone carbonyl group (47). A similar balance has been noted in human SET8 and SUV4-20H2 (48). In the Class I enzymes described here, the aspartate residue of the DXX(Y/F) motif, shown to be catalytically required in VCP-KMT, appears well positioned for similar charge stabilization of a substrate lysine (Fig. 7) (38). Finally, a substrate binding phenylalanine in SETD6 and LSMT is positioned similarly to a tryptophan in these Class I enzymes. Here, the aromatic residues could also provide substrate stabilization through cation- π interactions (49).

The clear conservation of the DXX(Y/F) motif in Family 16 and Group J led us to ask if other yeast Class I protein lysine methyltransferases evolved a similar substrate binding mechanism. Surprisingly, no analogous motif is found in other types of protein lysine methyltransferases. For example, both Dot1 and Efm4 lack the DXX(Y/F) motif. Thus, the similarity in catalytic structure between SET domain and Group J enzymes may be of limited use in searching for new protein lysine methyltransferases. However, these observations suggest a convergent evolution of lysine recognition from distinct classes of enzymes.

SET Domain Methyltransferases



Class I (Seven-Beta Strand) Methyltransferases - Human Family 16 & Yeast Group J

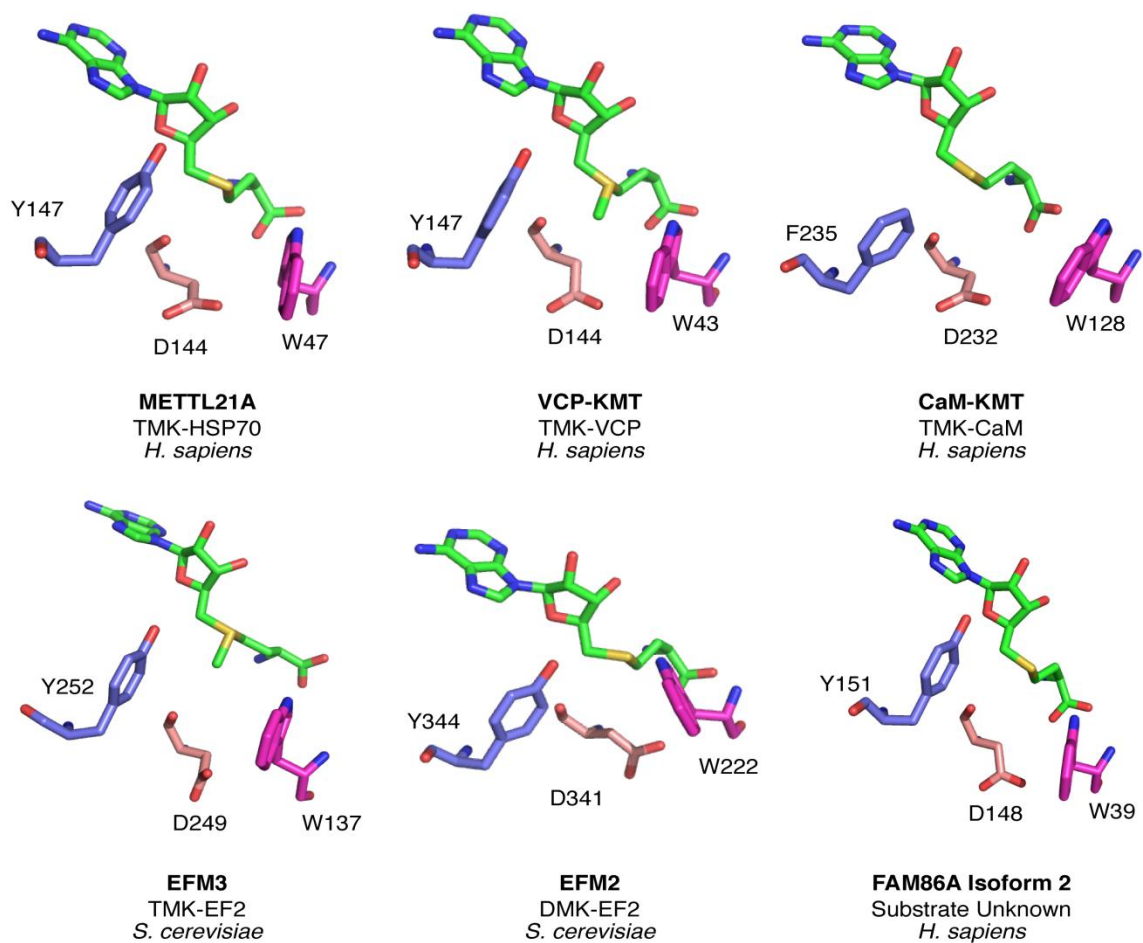


Figure 7. Similarity in the modeled catalytic centers of Efm2 and Efm3 with SET domain lysine methyltransferases and human Family 16 Class I methyltransferases. Top panel: The SET domain enzymes, SETD6 (PDB: 3QXY), LSMT (PDB: 2H2J), and SET7/9 (PDB wildtype: 3M53, Y335F: 4J71) have a catalytically required tyrosine residue (blue) that is positioned between the methyl group and adenine ring of AdoMet (green). Alignment of SET7/9 Y335F showed that the phenylalanine (orange) is positioned almost identically to the wildtype tyrosine. Residues implicated in substrate binding are shown in pink (41,47). The substrate-binding tyrosine (pink) and a nearby side chain carbonyl help balance the full negative charge of the substrate lysine (yellow). The aromatic plane of the tyrosine residue is approximately 4.4 Å, 4.2 Å, and 3.5 Å away (in SETD6, LSMT, and SET7/9, respectively) from the substrate lysine epsilon nitrogen atom, suggesting a cation- π interaction (49). Bottom panels: The catalytic sites on crystal structures of the Class I human METTL21A (PDB: 4LEC), VCP-KMT (PDB: 4LG1), and CaM-KMT (PDB: 4PWY) methyltransferases as well as those of the Phyre2 models of *S. cerevisiae* Efm2 and Efm3, and human FAM86A are displayed in a similar orientation as the three SET-domain enzymes shown above. The Class I enzymes have a tyrosine or phenylalanine residue (blue) oriented similarly as the catalytic tyrosine in the SET enzymes. The aspartate residue (light pink), previously shown to be catalytically required in VCP-KMT (38), could serve a similar purpose as the tyrosine-carbonyl pairings in the SET domain enzymes in stabilizing the substrate lysine. A conserved tryptophan residue (pink) in the Class I enzymes is well suited to form cation- π interactions in a manner similar to the phenylalanine residue in the SET domain methyltransferases.

DISCUSSION

With the identification of both EF2 methyltransferases, it is now possible to examine how these methylation events influence protein synthesis. We assayed cells lacking these methyltransferases for their sensitivity to antibiotics, misincorporation, frameshifting, and stop codon readthrough. Cells lacking either Efm2 or Efm3, exhibited increased sensitivity to several antibiotics affecting translation. While *efm2* Δ and *efm3* Δ cells showed no difference in

misincorporation and frameshifting compared to wildtype, *efm2Δ* displayed increased stop codon readthrough.

EF2 is responsible for coordinating the complex translocation step and maintaining the correct reading frame of the mRNA, yet no effect was observed in frameshifting in the methyltransferase-deficient cells. This is perhaps surprising, given that loss of the diphthamide modification in EF2 has been shown to affect frameshifting (19). The close structural proximity of the diphthamide residue to the methylated K613 residue (50) suggested a similar role. Overall, our results indicate that the methylation of EF2 may help correctly establish connections and contact with the ribosome that, when no longer there, make it more difficult to overcome structural alterations in the presence of translational inhibitors. This is emphasized by the locations of these modifications and the results of this study.

Interestingly, the Efm2 modified K613 residue interacts with helix 33 of the small subunit rRNA, a yeast-specific contact due to an insertion in domain IV (18). This domain inserts directly into the tRNA binding pockets of the ribosome (51) and is thought to mimic the anticodon domain of the A site tRNA. Loss of this methylation by Efm2 could result in loss of contact points and ultimately alter EF2's ability to recognize when termination should occur. This is supported by our translational fidelity data, which demonstrated possible defects in termination and not necessarily elongation. *efm2Δ* cells demonstrated increased stop codon readthrough but no changes in amino acid misincorporation or frameshifting. This is generally an indicator of termination defects (22) and suggests that EF2 may also have a role in termination. In bacteria, EF2 has already been shown to interplay with release and termination factors to stimulate the dissociation of peptidyl-tRNA from the P site (52) and a similar association with termination could be present in yeast. Additionally, the E-values for fungal Efm2 homologs are

significantly better than values across species for Efm2 or Efm3 (Fig. 4D). It is possible that this is a fungal-specific modification necessary to modulate contacts with the ribosome.

It now appears that all of the methyltransferases modifying EF2 have been identified. However, enzymes that modify two of the sites on EF1A and those that modify the three sites of EF3 have not been found. To fully understand why the elongation factors are methylated, it is essential to uncover these enzymes. Recent work has been done to predict substrate category (protein vs. nucleotide vs. small molecule) and correctly predicted Efm3 as a protein methyltransferase (53). However, the specifics of residue type and extent of modification is a much more complex question. Protein lysine methyltransferases are found in both the SET domain and the seven-beta-strand (Class I) families. The SET domain family has been extensively characterized in terms of substrate recognition (48). The Class I methyltransferases, although the largest family, has a wide variety of substrates, making substrate definition more complex. In protein arginine methyltransferases, the recognition of substrates is relatively well understood, with their characteristic post motif-II double-E loop and THW motif (54). For some Class I protein lysine methyltransferases, a similarly positioned post motif-II DXX(Y/F) sequence has been noted (Fig. 7). Our structural analysis comparing two distinct classes of methyltransferases may provide clues regarding the catalytic role of this motif.

Non-histone protein methylation is a largely unexplored yet critical aspect of a multitude of cellular processes including metabolism and cell signaling (7). In the budding yeast, *Saccharomyces cerevisiae*, there are 86 known and putative methyltransferases have been identified with 16 having no known substrates (36,55); of these latter enzymes, six are predicted to have protein substrates (53). Additionally, 40 yeast methyltransferases have human homologs, suggesting that the modifications may be important for a properly functioning cell (56). About

two-thirds of the known yeast enzymes are involved with methylating various components of the translational apparatus (Table 3). Since methylation can regulate, enhance, block, or fine tune interactions, this extensive set of methylation events indicates an intricate system for producing an efficient and accurate protein-making machine as well as suggesting potential mechanisms for its regulation (57). A properly functioning cell is highly dependent on correctly synthesized proteins, giving credence to the idea that fine-tuning the translational apparatus with methylation is essential for optimal cell fitness.

Table 3. *S. cerevisiae* methyltransferases involved in modifying translation components, grouped by substrate type.

<u>rRNA</u>	<u>tRNA</u>	<u>mRNA</u>	<u>Translation Factors</u>	<u>Ribosomal Proteins</u>
Spb1	Trm1	Abd1	Mtq1	Hmt1
Nop1	Trm2		Mtq2	Rmt2
Nop2	Trm3		See1	Ntm1
Rrp8	Trm5		Efm1	Hpm1
Mrm2	Trm7		Efm2	Rkm1
Dim1	Trm8		Efm3	Rkm2
Mrm1	Trm9			Rkm3
Emg1	Trm10			Rkm4
Bmt2	Trm11			Rkm5
Rcm1	Trm13			Sfm1
Bmt5	Trm44			
Bmt6	Ppm2			
Bud23	Gcd14			
	Tyw3			
	Trm140			
	Ncl1			

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FOOTNOTES:

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²The abbreviations used are: EFM, elongation factor methyltransferase; AdoMet, S-adenosylmethionine; [³H]AdoMet, S-adenosyl-L-[*methyl*-³H]-methionine; MMK, monomethyllysine; DMK, dimethyllysine; TMK, trimethyllysine.

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Chapter Three

A New Type of Protein Lysine Methyltransferase Trimethylates Lys-79 of Elongation Factor 1A

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Introduction to the Chapter

The immunoblot screen described in Chapter Two, which successfully identified Efm3, did not demonstrate significant loss of signal in polypeptides of the approximate size of EF1A (50 kDa) in any of the putative methyltransferase knockout strains. It is possible that the antibodies did not recognize EF1A trimethyllysine as they did for EF2 trimethyllysine. I therefore revisited the amino acid analysis screens for loss of radioactive trimethyllysine. Lipson et al. (2010) tested each of the knockouts listed in Table 2-A, including all SET domain enzymes and approximately half of the seven- β -strand enzymes. Table 2-B lists all remaining enzymes that did not have confirmed non-protein substrates prior to 2010. A more recent substrate predication paper indicated that YBR225W, Kar4, Rsm22, and YMR209C were likely RNA methyltransferases. This left only two potential candidates for the EF1A trimethylating enzyme: YLR285W/NNT1 and YGR001C/AML1.

Previous annotation of these enzymes indicated they would not methylate lysine residues. YGR001C was named “Adenine methyltransferase-like 1” and YLR285W had been named “Nicotinamide methyltransferase” based on sequence similarities in other organisms (SGD; Anderson et al. 2003). However, a protein-protein BLAST against yeast using human nicotinamide methyltransferase NNMT as query only extracts YLR285W as a match but the E-value of 2.2. This suggests that there is no statistically significant probability that these proteins are orthologs. Using the yeast YLR285W as query against human proteins does not bring up human NNMT but instead an assortment of protein lysine methyltransferases, making it a prime candidate for an EF1A methyltransferase. YGR001C/AML1 was classified as an N6-adenosine methyltransferase based on a post-motif II sequence (NPPY) although no activity had ever been

established. Additionally, the most recent substrate prediction for AML1 was protein, not nucleotide (Szczepińska et al. 2014).

Amino acid analysis of EF1A from *ylr285A* did not show any change in the levels of methyl lysine but resulted in complete loss of an early eluting radioactive peak (see Chapter Six). Analysis of *ygr001cA* EF1A demonstrated complete loss of the trimethyllysine peak, indicating this enzyme could be responsible for TMK79.

TABLE 3-A

Methyltransferase knockouts tested by Lipson et al. 2010. Protein names are also listed where applicable.

SET Domain Family		Seven Beta Strand Family				
Known	Putative	Known		Putative		
YHR119W	<i>SET1</i>	YKR029C	<i>SET3</i>	YBR034C	<i>RMT1/HMT1</i>	YKL155C
YJL168C	<i>SET2</i>	YJL105W	<i>SET4</i>	YDR465C	<i>RMT2</i>	YKL162C
YHL039W	<i>EFM1</i>	YHR207C	<i>SET5</i>	YBR133C	<i>HSL7</i>	YMR209C
YHR109W	<i>CTM1</i>	YPL165C	<i>SET6</i>	YNL063W	<i>MTQ1</i>	YMR228W
YPL208W	<i>RKM1</i>			YDR140W	<i>MTQ2</i>	YNL024C
YDR198C	<i>RKM2</i>			YDR440W	<i>DOT1</i>	YNL092W
YBR030W	<i>RKM3</i>			YIL064W	<i>SEE1/EFM4</i>	YPL009C
YDR257C	<i>RKM4/SET7</i>			YJR129C	<i>EFM3</i>	
				YIL110W	<i>HPM1</i>	
				YBR271W	<i>EFM2</i>	
				YBR141C	<i>BMT2</i>	
				YDR316W	<i>OMS1</i>	
				YHR209W	<i>CRG1</i>	
				YLR063W	<i>BMT6</i>	
				YLR137W	<i>RKM5</i>	
				YNL022C	<i>RCM1</i>	
				YOR239W	<i>ABP140</i>	
				YBR261C	<i>NTM1</i>	

TABLE 3-B**Methyltransferases with protein or unknown substrates not tested by Lipson et al. 2010**

Seven Beta Strand Family		SPOUT		Other	
Gene name/ Protein Name	Substrate*	Gene name/ Protein Name	Substrate*	Gene name/ Protein Name	Substrate*
YBR225W		YOR021C/SFM1	Rps3 (R)	YGR001C/AML1	
YMR209C				YLR172C/DPH5	EF2 (Dip)
YCL055W/KAR4				YDR410C/STE14	A factor (O)
YKL155C/RSM22					
YLR285W/NNT1					
YDR435C/PPM1	PP2A (O)				
YDL014W/NOP1	Histone H2A (Q) [†]				
YDR440W/DOT1	Histone H3 (K)				

*Substrates listed are for those with confirmed activity. Letter in parentheses indicates residue of methylation (O = carboxyl methyl ester, Q = Gln, K = Lys, Dip = diphthamide)

[†]Nop1 is also a rRNA methyltransferase

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A new type of protein lysine methyltransferase trimethylates Lys-79 of elongation factor 1A

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Abstract

The elongation factors in *Saccharomyces cerevisiae* are heavily methylated and contain a total of ten methylated lysine residues. Four of the elongation factor methyltransferases have been identified (Efm1-4). Here we report the identification of Efm5 (*YGR001C*) which trimethylates Lys-79 on EF1A. We directly show the loss of this modification in *efm5Δ* strains by both mass spectrometry and amino acid analysis. Although Efm5 is not conserved in fungi, it is present in higher eukaryotes, indicating possible unique functions of this modification in certain organisms. Interestingly, Efm5 was initially classified as N6-adenine DNA methyltransferase-like due to the presence of a DPPF sequence in post-Motif II. Further analysis of this motif and others like it demonstrates potential mechanisms for determining the type of atom methylation occurs on.

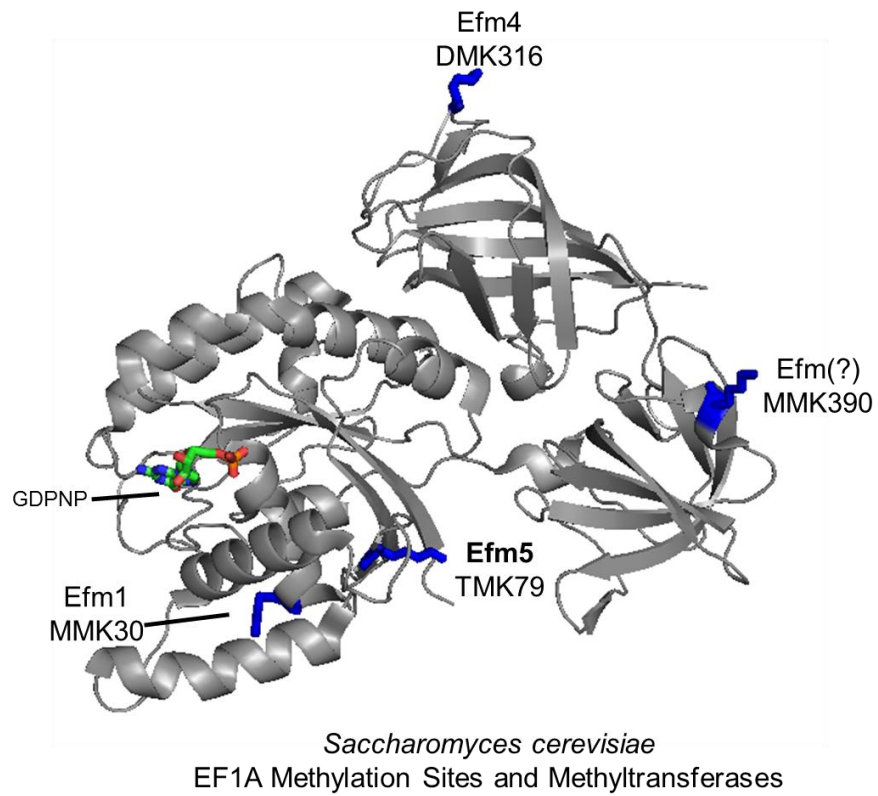
KEYWORDS

S-adenosyl methionine (AdoMet), protein lysine methylation, elongation factor, methyltransferase, translation

GLOSSARY

EFM, elongation factor methyltransferase; AdoMet, *S*-adenosylmethionine; [³H]AdoMet, *S*-adenosyl-L-[*methyl*-³H]-methionine; MMK, monomethyllysine; DMK, dimethyllysine; TMK, trimethyllysine.

Graphical Abstract



Introduction

Elongation factor 1A (EF1A) acts as a molecular spell checker during protein translation by ensuring there are correct codon matches between the incoming amino acylated-tRNA and the mRNA [1]. This highly conserved protein binds a charged tRNA in a GTP-dependent manner and guides it into the ribosome A-site. Once a correct codon match is made, EF1A hydrolyzes its bound GTP resulting in a lower affinity for the tRNA; the tRNA is then released, allowing peptidyl transfer to occur. The GDP-bound EF1A then interacts with its nucleotide exchange factor, EF1B so it can be recharged and the cycle repeats. Intriguingly, *Saccharomyces cerevisiae* EF1A contains five methylated lysine residues that are near critical areas of the enzyme [2]. Monomethyllysine 30 is found in the GTP binding pocket, trimethyllysine 79 is in the nucleotide exchange factor binding pocket and potentially contacts ribosomal components, and dimethyllysine 316, monomethyllysine 390, and the C-terminal lysine alpha-carboxyl methyl ester are located along the tRNA and ribosomal binding interface (PDB: 1F60). Methylation modifications are also abundant in other components of the translational apparatus, including mRNA, tRNA, ribosomal proteins, and release factors [3,4,5,6]. Methyl groups have the potential to block or increase intra- and intermolecular contacts between the translational components [4]. These carefully placed modifications may help EF1A mediate its various interactions with tRNA, other elongation factors, and the ribosome itself.

S. cerevisiae has 86 known and predicted methyltransferases that methylate a wide variety of macromolecules including proteins, nucleotides, lipids, and even small molecules [7]. Generally, these enzymes fall into three major classes based on their structural folds and binding mechanism of the methyl donor S-adenosylmethionine (AdoMet): seven-beta-strand (Class I), SET domain, and SPOUT methyltransferases. The SET domain methyltransferases are classical

protein lysine modifiers and in fact, elongation factor methyltransferase 1 (Efm1) falls into this category. Efm4 is an example of a Class I methyltransferase, a class of enzymes that have a variety of substrate types. Mass spectrometric screens of knockouts of predicted protein methyltransferases yielded the identification of these two EF1A methyltransferases. Efm1 and Efm4 monomethylate Lys-30 and dimethylate Lys-316, respectively [8,9]. The remaining two methyltransferases were not among the 37 enzymes tested in that mass spectrometric screen, indicating that they may be unconventional protein lysine methyltransferases or that more than one enzyme is responsible for catalysis. We therefore broadened our selection of putative methyltransferases to include the minor classification families of methyltransferases.

The protein expressed from ORF YGR001C was initially classified as N6-adenine DNA methyltransferase-like, despite similarity to the Class I enzymes, based on primary sequence and predicted secondary structure from its Hidden Markov Model [7]. This apparent misannotation is most likely due to two deviations in the YGR001C encoded amino acid sequence from that of other Class I methyltransferases. First, the AdoMet binding Motif I sequence from YGR001C differs substantially from the conserved GXGXXG sequence seen in the Class I enzymes utilize to bind AdoMet. Second, it contains a “DPPF” post-Motif II sequence primarily associated with adenine nucleotide methyltransferases [10,11,12]. However, a similar post-Motif II sequence of “(D/E)XX(Y/F)” has recently been indicated as a hallmark of certain protein lysine methyltransferases [13,14].

Here we show that the protein encoded by YGR001C, which we now refer to as EFM5, is required for the trimethylation of lysine 79 on EF1A. Deletion of EFM5 leads to complete loss of trimethyllysine in 50 kDa polypeptides, specifically on the Lys-79-containing peptide of EF1A. Bioinformatic analysis reveals that Efm5 is not as widely conserved as its methylation site,

indicating it may have a role in only a subset of eukaryotes. Lastly, structural analysis of various (D/E)XX(Y/F) post-Motif II's indicate that this motif may generally be associated with N-methylation and nearby active site residues may facilitate the selectivity of macromolecule that is modified.

Methods and Materials

In Vivo Radiolabeling and Amino Acid Analysis

Strains used in this study are listed in Table 1. Yeast culture, radiolabeling, and amino acid analysis were carried out as previously described with some minor changes [13]. After lysates from S-adenosyl-L-[methyl-3H]methionine ([3H]AdoMet) in vivo labeled wildtype and EFM knockout cells were resolved by SDS-PAGE, the Coomassie-stained protein band running just above the 45 kDa marker was excised and placed into a 6 mm x 50 mm glass test tube. 100 μ l of 6N HCl was added to each slice and tubes were placed in a reaction chamber (Eldex Labs, 1163) containing 500 μ l of 6N HCl. Chambers were heated for 20 h in vacuo at 109 °C in a Pico-Tag vapor phase apparatus (Waters). Residual HCl was removed by vacuum centrifugation. Dried gel slices were resuspended in 400 μ l of cation exchange loading buffer (sodium citrate, 0.2 M Na⁺, pH 2.2). Half of the sample was spiked with 2 μ mol of each methyllysine standard (Sigma, N ϵ -methyl-L-lysine hydrochloride 04685, N ϵ ,N ϵ -dimethyl-L-lysine monohydrochloride 19773, N ϵ ,N ϵ ,N ϵ -trimethyllysine hydrochloride T1660) and loaded onto a cation-exchange column (Beckman AA-15 sulfonated polystyrene resin, 0.9 cm inner diameter by 12 cm height) equilibrated with running buffer (sodium citrate, 0.3 M Na⁺, pH 3.85) at 55 °C. Amino acids were eluted in the equilibration buffer at 1 mL/min while collecting 1 min fractions at the

expected elution position of the methyllysine standards. 50 μ l of each fraction was added to a flat-bottom 96-well plate to detect standards by the ninhydrin method. The remainder of each fraction was added to 5 ml of scintillation fluor (Safety Solve, Research Products International) in a 20 ml scintillation vial and counted for three five min cycles using a Beckman LS6500 instrument to detect [3H]-methylated amino acids.

Table 1.
Strains used in this study.

Strain	Genotype	Biological function	Source
	MAT α his3 Δ 1 leu2 Δ 0		Open
BY4741	met15 Δ 0 ura3 Δ 0	Wild type	biosystems
	MAT α his3 Δ 1 leu2 Δ 0		Open
BY4742	lys2 Δ 0 ura3 Δ 0	Wild type	biosystems
	BY4741 background,		Open
<i>efm1Δa</i>	<i>yhl039wΔ</i>	Elongation factor methyltransferase	biosystems
	BY4742 background,		Open
<i>efm1Δ α</i>	<i>yhl039wΔ</i>	Elongation factor methyltransferase	biosystems
	BY4741 background,		Open
<i>efm4Δa</i>	<i>yil064wΔ</i>	Elongation factor methyltransferase	biosystems
	BY4742 background,		Open
<i>efm4Δ α</i>	<i>yil064wΔ</i>	Elongation factor methyltransferase	biosystems
	BY4741 background,	Putative/elongation factor	Open
<i>efm5Δa</i>	<i>ygr001cΔ</i>	methyltransferase	biosystems
	BY4742 background,	Putative/elongation factor	Open
<i>efm5Δ α</i>	<i>ygr001cΔ</i>	methyltransferase	biosystems

In-Gel Trypsin Digests and Mass Spectrometry

Coomassie-stained gel slices from the 50 kDa region of fractionated polypeptides of unlabeled yeast cell lysates were destained and subjected to in gel trypsin digest with sequencing grade trypsin (Promega, V5111) as previously described [13]. Digests were performed for 16 h at 37 °C and the peptides were eluted using 50% acetonitrile/1% trifluoroacetic acid in water.

Peptides were dried by vacuum centrifugation and resuspended in 200 μ l of 0.1% TFA in water. Tryptic peptides from the 50 kDa SDS-gel band of wildtype, *efm1 Δ* , *efm4 Δ* and *efm5 Δ* lysates were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described.

Peptides ions detected by LC-MS/MS were identified using Proteome Discoverer (Version 1.4; Thermo Scientific, Waltham, MA) coupled with MASCOT (Version 2.4.1; Matrix Science, London, UK). Tryptic peptides were searched against the SwissProt *Saccharomyces cerevisiae* database (2013, 7,798 sequences) with following settings: missed cleavages \leq 1, precursor mass tolerance = 10 ppm, product ion mass tolerance = 0.005Da, dynamic modifications for carbamidomethyl (C), deamidation (N, Q), oxidation (M), monomethyl (K), dimethyl (K), and trimethyl (K). Peptides identified with methylations at K30, K79, K316 (along with their un-methylated counterparts) were imputed into an inclusion list and wildtype, *efm1 Δ* , *efm4 Δ* , *efm5 Δ* samples reanalyzed by targeted parallel-reaction-monitoring mass spectrometry (PRM-MS) to confirm the methylation status of EFM mutants. Full MS2 data was used to confirm the identity of each observed peptide.

Bioinformatic Alignments and Phylogenetic Tree Construction

Whole protein sequences for EF1A were aligned using Clustal Omega and the region containing Lys-79 was examined. A protein-protein BLAST search was performed using *S. cerevisiae* Efm5 (UniProt P53200) as the query. Sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) in MEGA 6 as previously described [13].

Phyre2 and Post-Motif II Analysis

The structure of Efm5 was modeled using the Protein Homology/analogy Recognition Engine V2.0 (Phyre2) in one-to-one threading mode with Mtq2 (PDB: 3Q87, chain B) as a template. Coordinates for the model were calculated using the global alignment method with default settings for secondary structure scoring and weight. Structural figures of the catalytic regions of this Phyre2 model as well as crystal structures of related methyltransferases were created using MacPyMol (DeLano Scientific). The crystal structure for VCP-KMT (PDB: 4LG1, chain B) contained two rotamers of I146. As the electron density and difference maps for this structure were unavailable, the rotamer with the most steric hindrance (as observed in PyMol) was removed.

Results

YGR001C/EFM5 is responsible for the presence of trimethyllysine on Lys-79 of EF1A

To identify the remaining EF1A methyltransferases, yeast cells from knockout strains of candidate methyltransferases were *in vivo* radiolabeled with [3H]AdoMet. By supplementing cultures with [3H]AdoMet, substrates methylated during incubation incorporate tritiated methyl groups. The 50 kDa gel slice, corresponding to the molecular weight of EF1A, was excised and acid hydrolyzed. To demonstrate that we were looking at methylation of EF1A, we included the knockouts of known EF1A methyltransferases Efm1 and Efm4. The resulting hydrolysates were loaded onto a high-resolution cation exchange column capable of separating methyl derivatives of the amino acids. Analysis of wildtype [3H]-hydrolysates from both mating type backgrounds showed presence of [3H]-trimethyllysine, [3H]-dimethyllysine, and [3H]-monomethyllysine (Figure 1A, top panels). Relative quantification of the modified residues demonstrated radioactivity of MMK:DMK:TMK as 2:2:3, indicating the presence of two monomethyllysines, one dimethyllysine, and one trimethyllysine (Fig. 1B). This is consistent with previous reports of the methylation of EF1A [2].

Deletion of Efm1 resulted in a 50% reduction in MMK and deletion of Efm4 resulted in almost complete loss of DMK, consistent with their reported activities (Fig. S1 and Fig. 1B) [8]. Residual dimethyllysine in *efm4*Δ is likely due to the presence of partially methylated Lys-79. During the screen, only one deletion, *ygr001c*Δ, resulted in the loss of the TMK radioactive peak (Fig 1A, bottom panels). This was seen in both mating type backgrounds, which indicates that this is not due to a secondary mutation in the yeast genome. These results suggest that YGR001C encodes a protein lysine methyltransferase that catalyzes the trimethylation of at least one

polypeptide of about 50 kDa, most likely EF1A. In some runs, there was residual radioactivity eluting slightly before the trimethyllysine peak (Fig. 1A, asterisk). This peak correlates to a 3-methyl histidine standard and is likely from the closely migrating Rpl3 polypeptide (molecular weight 43.8 kDa) [3,15]. This peak was not present in all runs due to variations in gel slicing that would include or exclude different amounts of Rpl3.

To confirm that Ygr001c is acting on the elongation factor, the 50 kDa region on a gel containing non-radioactive lysates was subjected to in-gel trypsin digestion. The resulting peptides were loaded onto a C18 reversed phase HPLC column and analyzed by LC-MS/MS on a Q-Exactive Orbitrap mass spectrometer. Extracted ion chromatograms demonstrated the presence of all previously reported EF1A methylations (Fig. 2 and Fig. S2) and loss of respective methylations in *efm1Δ* and *efm4Δ* strains (Fig. 2B and 2C). Increases in the abundance of corresponding unmethylated peptides were also observed (data not shown). This experiment also demonstrated that Lys-79 is found both in di- and trimethylated states in wildtype cells, consistent with the presence of low levels of [3H]-dimethyllysine in *efm4Δ* hydrolysates (Fig. S1). Lys-79 was completely unmethylated in *ygr001cΔ* in both mating types (Fig. 2A). We therefore rename YGR001C as Efm5 in continuation of the naming of elongation factor methyltransferases.

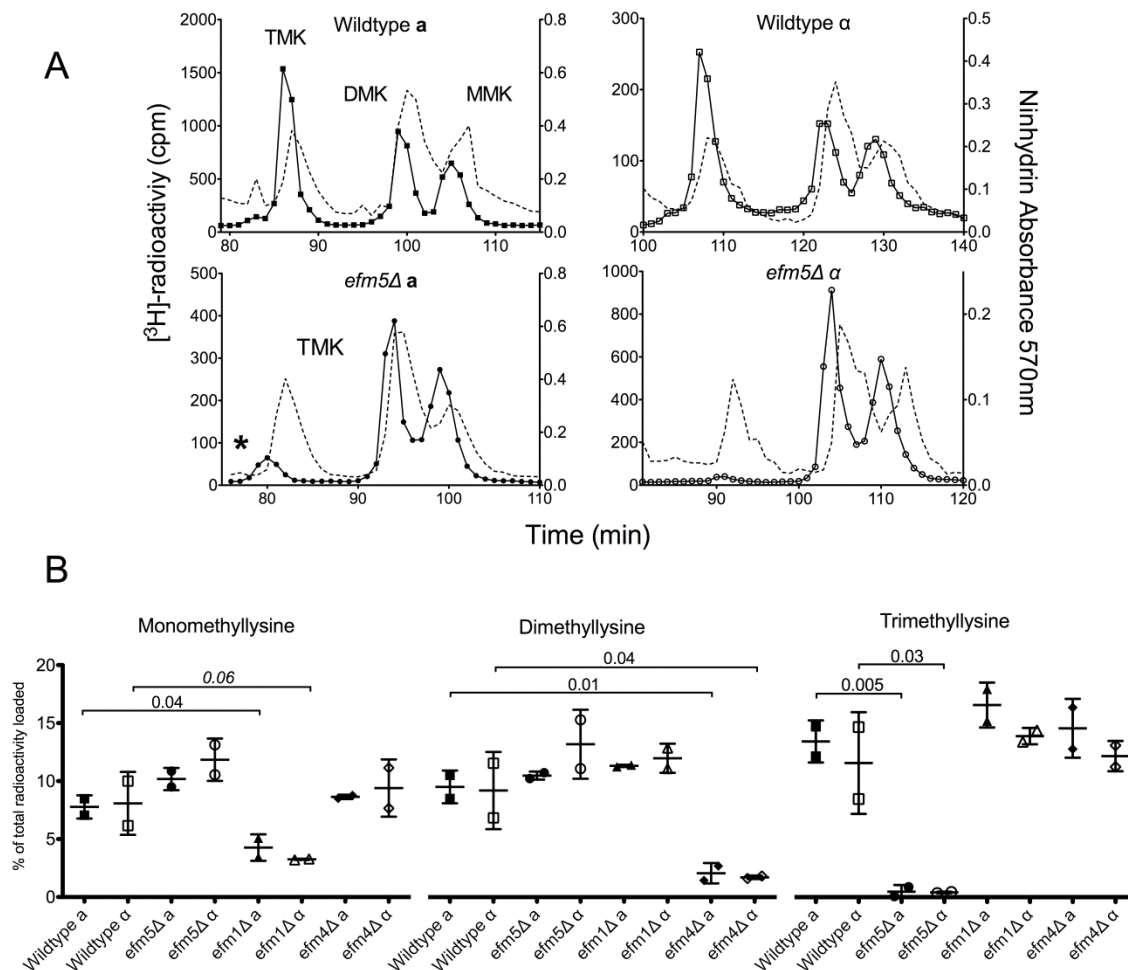


Figure 1. Deletion of EFM5 results in loss of trimethylated lysine in 50 kDa polypeptides. (A) Radiolabelled methylated lysine derivatives from 50 kDa gel slice hydrolysates were separated. The position of the standards, detected by ninhydrin reactivity, is shown in the dashed line and the methylated lysine peaks are labeled (MMK, DMK, TMK). Due to a tritium isotope effect, the radiolabeled derivatives, shown in the solid lines, elute slightly before the non-labeled standards [18]. Separation of hydrolysates from radiolabeled wildtype and *efm5Δ* from both mating type backgrounds are shown. Each trace is representative of at least three independent experiments. The asterisk indicates the presence of radioactive 3-methylhistidine. (B) The amount of mono-, di-, and trimethyllysine radioactivity as a percentage of the total radioactivity in the hydrolysate is shown with error bars reflecting the standard deviation. P-values from Student's t-test are shown. All changes from respective wildtype values were significant with the exception of *efm1Δ* α , indicated by the italicized value, which is likely due to large standard deviation in the wildtype.

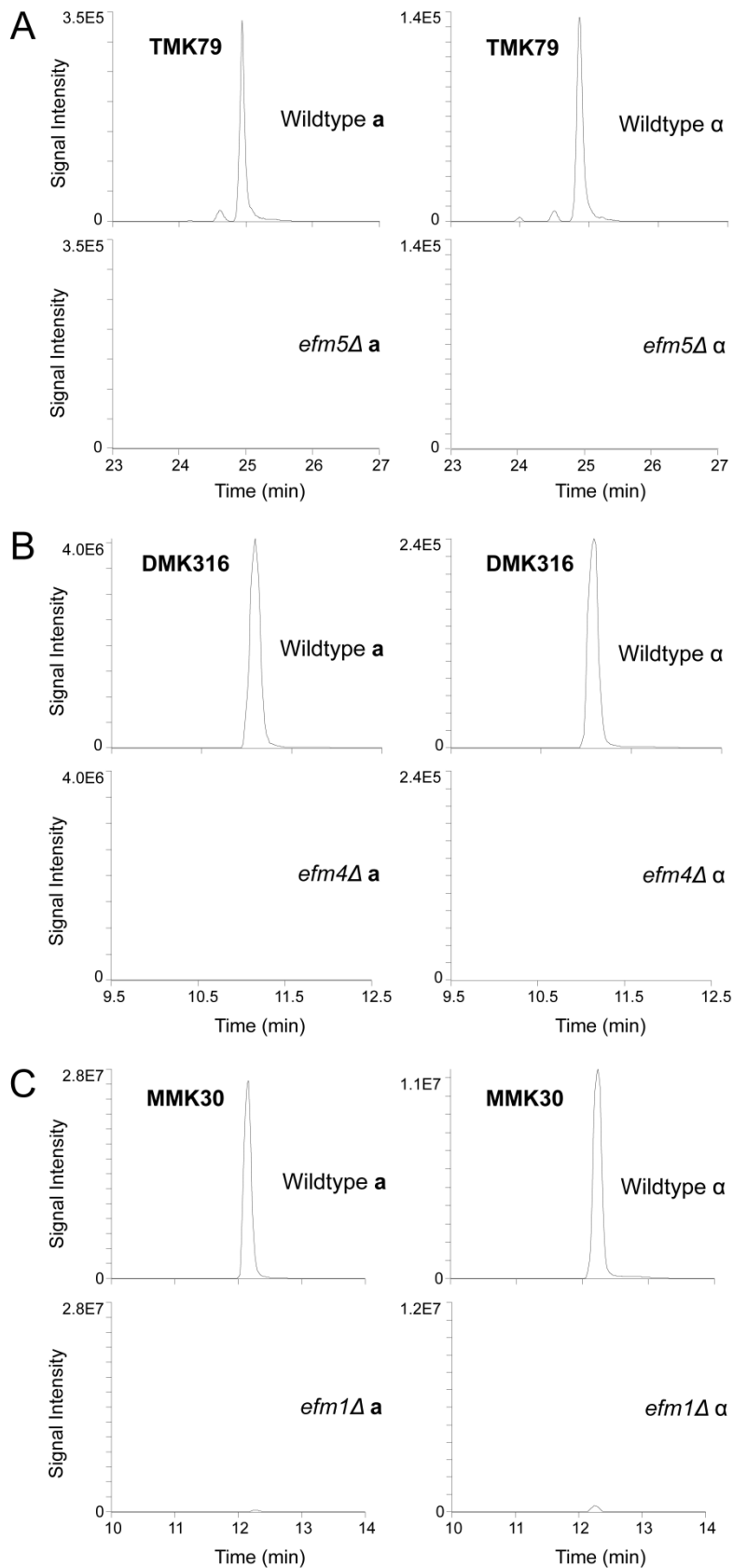


Figure 2. Deletion of *EFM5* results in loss of trimethyllysine 79 on elongation factor 1A. The 50 kDa protein bands from wildtype and knockout lysates were excised, in-gel trypsin digested, and analyzed by LC-MS/MS. Extracted ion chromatograms correlating to the EF1A methylated and unmethylated peptides from wildtype and each EFM knockout strain are shown. (A) Loss of EF1A methylation in *efm5* Δ was visualized by loss of 592.010 m/z (GITIDIALWK_{trimethyl}FETPK) \rightarrow 791.466 m/z precursor \rightarrow product ion transition. (B) Loss of EF1A methylation in *efm4* Δ was visualized by loss of 324.866 m/z (NVSVK_{dimethyl}EIR) \rightarrow 573.371 m/z precursor \rightarrow product ion transition. (C) Loss of EF1A methylation in *efm1* Δ was visualized by loss of 567.811 m/z (STTTGHLIYK_{monomethyl}) \rightarrow 744.440 m/z precursor \rightarrow product ion transition.

Evolutionary Conservation of Efm5 and the Conservation of the EF1A Lys-79 Methylation Site

Trimethylation of Lys-79 has been previously confirmed in other eukaryotes, such as rabbits and brine shrimp [16]. Alignment of EF1A sequences from every kingdom indicates high conservation of this lysine amongst eukaryotes (Fig. S3). Additionally, the lysine is commonly flanked by aromatic residues. A close examination of this region in the *S. cerevisiae* EF1A structure shows these three residues on a small beta strand; the aromatics face the interior of the protein forcing the lysine to face the solvent (PDB: 1F60). Due to this homology, EF1A in higher eukaryotes, such as humans and mice, has also been predicted to be methylated at this site. If these organisms contain methylated EF1A, they should also have the homologous enzyme. A BLAST search was performed to identify potential homologs of Efm5 to determine if the enzyme was similarly conserved. A phylogenetic tree was constructed to visually demonstrate the conservation (Fig. 3). Interestingly, Efm5 is well conserved in higher eukaryotes (e.g. humans and mice), but completely absent in several of *S. cerevisiae*'s fungal relatives. This result indicates that TMK79 may not be essential for EF1A's translational functions but perhaps has more specialized roles in specific organisms. Additionally, the brine shrimp *A. salina* did not appear to have an Efm5 homolog despite the presence of manually confirmed TMK79. However, the genome of this arthropod has not been fully sequenced to date.

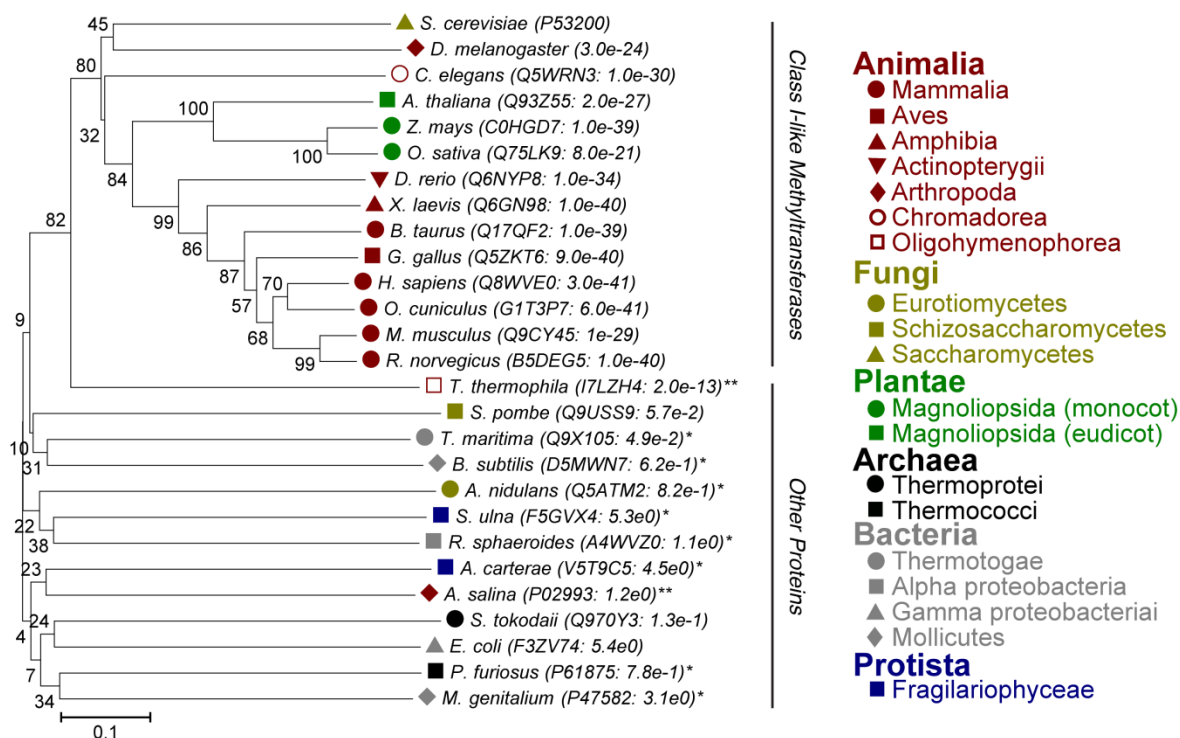


Figure 3. Efm5 is conserved amongst higher eukaryotes. Phylogenetic tree of Efm5 homologs. Accession numbers and E-values are indicated for each protein. All homologs were mutual best hits except those indicated by an asterisk (*). Double asterisk (**) indicates the organism has the methylation site but not the enzyme potentially due to incomplete genome sequences. Proteins with high homology are all predicted methyltransferases; those with low homology are categorized as other proteins.

A closer look at post-Motif II residues reveals similarities between N-methyltransferases.

The apparent misannotation of Efm5 as an adenine methyltransferase due to its DPPF post-Motif II raised questions as to how similar active site amino acids could give rise to very different substrate preferences. We compared the structural location and arrangement of post-Motif II residues from multiple lysine methyltransferases, a DNA methyltransferase, two glutamine methyltransferases and the predicted structure of Efm5 (Fig. 4).

The human Family 16 of protein lysine methyltransferases CAM-KMT, METTL21A, METTL21C, and METTL21D (VCP-KMT) each contain the DXX(Y/F) post-Motif II where the variable XX amino acids are hydrophobic residues that face away from the active site. It has been hypothesized that the aspartate residue helps balance the positive charge on the substrate lysine and the aromatic residue stabilizes the substrate via cation- π interactions. Additionally, each enzyme contains a tryptophan residue, distant in sequence but close in structure, which is relatively planar to the motif tyrosine/phenylalanine. This large aromatic residue could help keep the positive lysine in place for methylation by forming more cation- π interactions on the other side of the lysine and creating a hydrophobic cage.

Mtq2 and Efm5 have post-Motif II sequences NPPY and DPPF, respectively. The double proline hinders the rotational freedom of the protein backbone, limiting the relative conformations of the polar and aromatic sidechains found in this motif. It is possible that the prolines serve to maintain the spatial relationship of the N/D and Y/F residues in the active site to aid in substrate recognition. Additionally, these two enzymes lack the tryptophan seen in the human lysine methyltransferases but have a tyrosine in a similar position. The major difference between the glutamine methyltransferase and the lysine methyltransferase in this set is the replacement of asparagine with aspartate. Asparagine would provide the polar contacts to stabilize a substrate glutamine whereas the aspartate would provide a formal negative charge to balance out a formal positive charge on the substrate lysine. This change from Asp to Asn is found in other glutamine methyltransferases such as the *E. coli* enzyme HemK.

The DNA methyltransferases, DAM and TaqI, contain the classical (D/N)PP(Y/F) motif associated with adenine modifiers. These enzymes appear to vary in the active site more so than the protein N-methylators. TaqI contains a post-Motif II asparagine while DAM has an aspartate.

Like M_{tq2}, a formal negative charge would not be required for stabilizing an adenine and therefore asparagine should suffice in the first position. Interestingly, the biggest deviation of DAM from the lysine modifiers is the absence of the sequentially distant aromatic residue. In its place is a lysine residue that would present a formal positive charge near the active site. TaqI on the other hand, has a nearby phenylalanine. These nearby residues could have more involvement with recognition of the specific DNA sequence than that of the substrate nitrogen.

The importance of the proline-proline motif has been widely studied in the DNA methyltransferases, likely adding to the association of this motif with DNA methylation. The proline backbone of the NPPY motif in TaqI participates in substrate stabilization directly through hydrogen bonding between its backbone carbonyl and substrate adenine [17]. It also confers rigidity in the backbone of this motif, helping to position the motif tyrosine for face-to-face π -stacking interactions with the same adenine. The structurally close Phe-196 rounds out the substrate binding pocket as it is well positioned to form edge-to-face π -stacking interactions. P172A and P172T mutations of phage T4 N6-adenine specific DNA methyltransferase resulted in a 5 to 20-fold increase in K_m for AdoMet binding and a 2 to 4-fold decrease in K_{cat} [11]. This indicates that the rigidity of the (D/N)PP(Y/F) motif may be important for DNA methylation. This requirement seems less important in some of the Class I protein lysine methyltransferases, which feature a conserved smaller hydrophobic residues instead of prolines.

Taken together, this analysis indicates that these similar post-Motif II residues may dictate the atom of methylation for N-methyltransferases. Variations of the motif residues plus the aid of nearby active site residues seems to push the enzyme preference towards adenine, glutamine, or lysine.

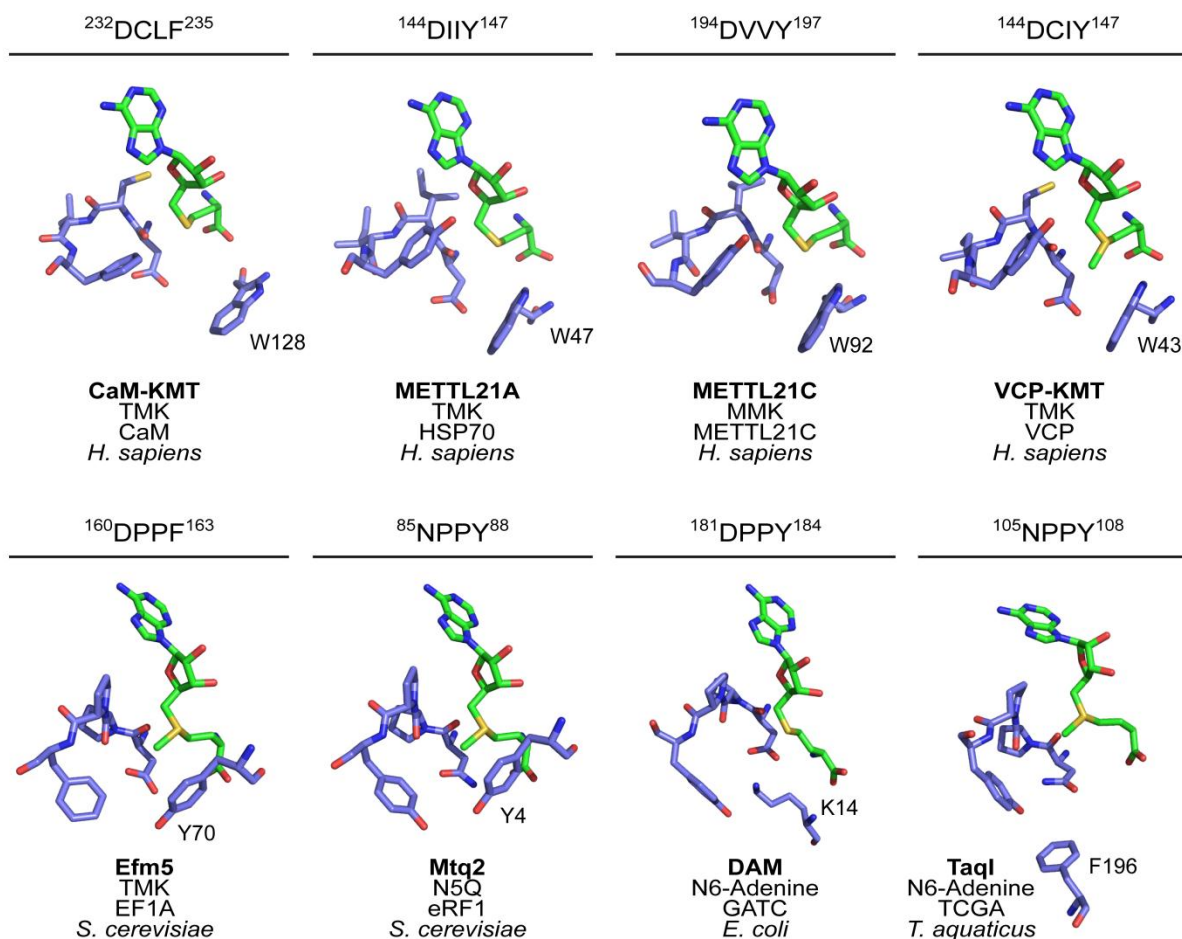


Figure 4. Post-Motif II sequences reveal structural similarities of N-methyltransferases.

The post-Motif II region from the crystal structures of CaM-KMT (PDB: 4PWY), METLL21A (PDB: 4LEC), METTL21C (PDB: 4MTL), METTL21D (PDB: 4LG1), Mtq2 (PDB: 3Q87), DAM (PDB: 2G1P), TaqI (PDB: 2ADM) and that of a model of Efm5 (Phyre²) were compared. Above each structure is the post-Motif II sequence and residue numbers. Below each structure is the enzyme name, the type of methylation, the substrate, and the biological species. AdoMet or AdoHcy is shown in green and the enzyme residues are in purple.

Discussion

Here we have identified a protein encoded by the open reading frame YGR001C that is required for the trimethylation of EF1A. We now designate this protein as elongation factor methyltransferase 5. Deletion of Efm5 led to essentially complete loss of trimethyllysine in 50 kDa polypeptides. Mass spectrometry revealed that trimethylated Lys-79 residue on EF1A is undetectable in the absence of Efm5.

The previous annotation of Efm5 as N-6 adenine DNA methyltransferase-like (AML1) was likely due to its amino acid similarity to DNA methyltransferases containing similar post-Motif II residues. This annotation has now been propagated for the homologs of AML1 in other organisms. There have been other cases of methyltransferases not exhibiting the predicted substrate specificity of enzymes that share amino acid motifs. For example, SPOUT methyltransferases were considered RNA methyltransferases until one member (Sfm1) was shown to generate an omega-monomethylarginine residue on ribosomal protein Rps3. Additionally, the Mtq1 and Mtq2 protein glutamine methyltransferases were also originally annotated as DNA adenine methyltransferases. Interestingly, Mtq1 and Mtq2 also contain a similar post-Motif II (NPPY) as Efm5 (DPPF). In both cases, the unusual proline-proline sequence likely contributed to their misannotation. Interestingly, a recent paper correctly predicted YGR001C as a protein methyltransferase based on information aside from the DPPF motif. There may be additional methyltransferases with unusual post-Motif II sequences that may have novel substrate specificity.

Closer examination of post-Motif II-containing methyltransferases and their respective modifications reveals commonalities that could be linked to N-methylation (Fig. 4). Comparison

of adenine, glutamine, and lysine methyltransferase sequences and structures demonstrated the presence of a (D/E/N)XX(Y/F) motif surrounding the catalytic center. The middle residues seem to be essential in positioning the active site to properly bind the substrate. In the case of proline-proline motifs, the rigidity of the backbone keeps the residues in place. Replacement with smaller hydrophobic residues keep the first and last residues facing the active site but would allow for more flexibility. We previously predicted this motif to be associated with lysine methylation. Now that it is clear this motif extends beyond protein lysine methyltransferases, we have amended our prediction to associate this with recognition of nitrogen atoms regardless of macromolecule type.

The remaining EF1A methyltransferase that monomethylates Lys-390 is still unaccounted for. It is possible that more than one enzyme catalyzes this reaction or that the methods previously used could not detect intact mass changes. A change in 14Da in a 50 kDa protein is not necessarily an easy change to detect. Therefore rescreening putative methyltransferases by the amino acid analysis used here should directly demonstrate a reduction in the presence of MMK as seen for *efm1Δ*. If the change in MMK is too small to detect, screening these mutants in an *efm1Δ* background would exaggerate the MMK loss.

The functional relevance of these modifications remains largely unexplored. Recent work on the elongation factor 2 methyltransferases, Efm2 and Efm3, revealed that these methylations may be required for proper protein synthesis. Deletion of Efm2 led to increased stop codon readthrough and deletion of Efm3 altered frameshifting rates. Deletion of either Efm2 or Efm3 led to increased sensitivity to various translational inhibitors. Investigation into EF1A methylation could reveal similar phenotypes. Alternatively, EF1A has been implicated in various non-canonical functions such as nuclear export of tRNAs, actin binding, and detection of

misfolded proteins. Since EF1A methylation is not completely conserved in all eukaryotes, it is possible that these modifications assist in these accessory functions.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL FIGURES

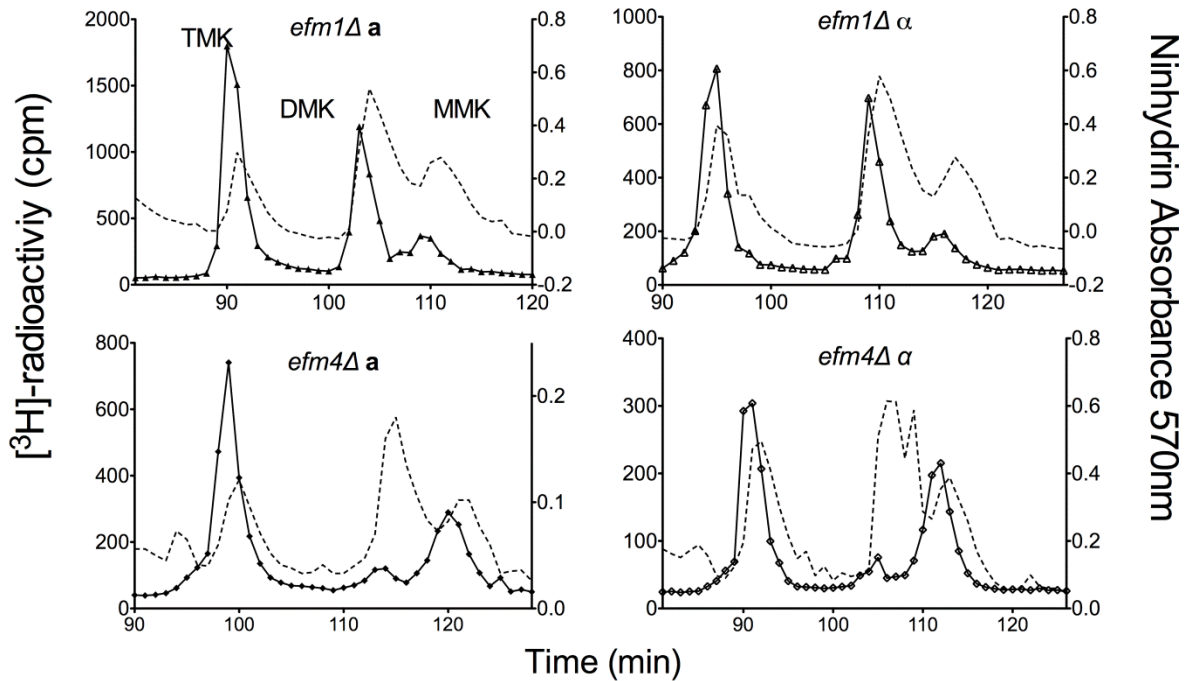
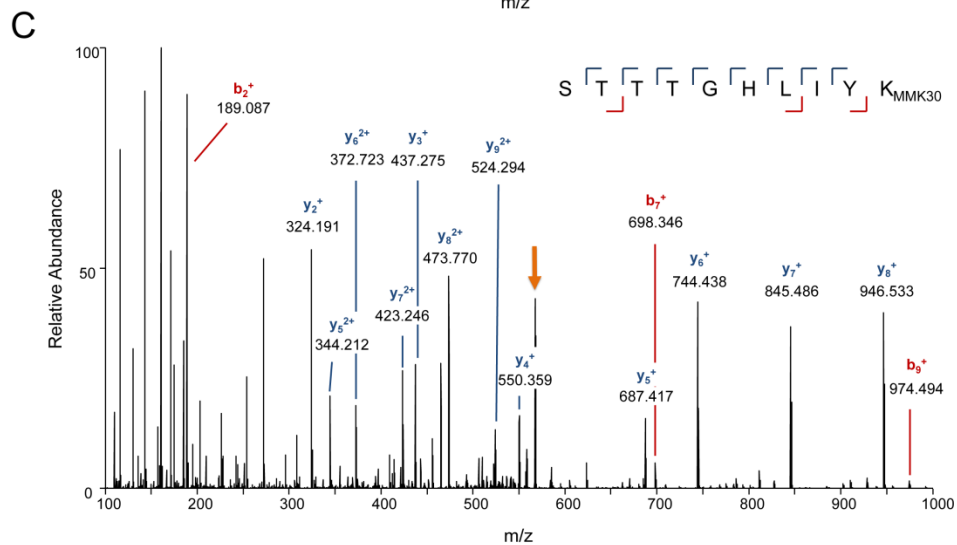
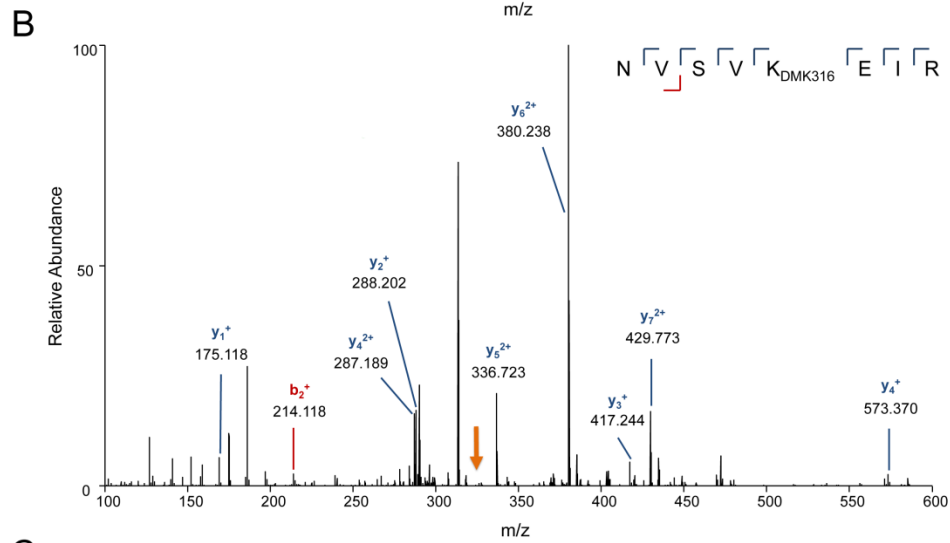
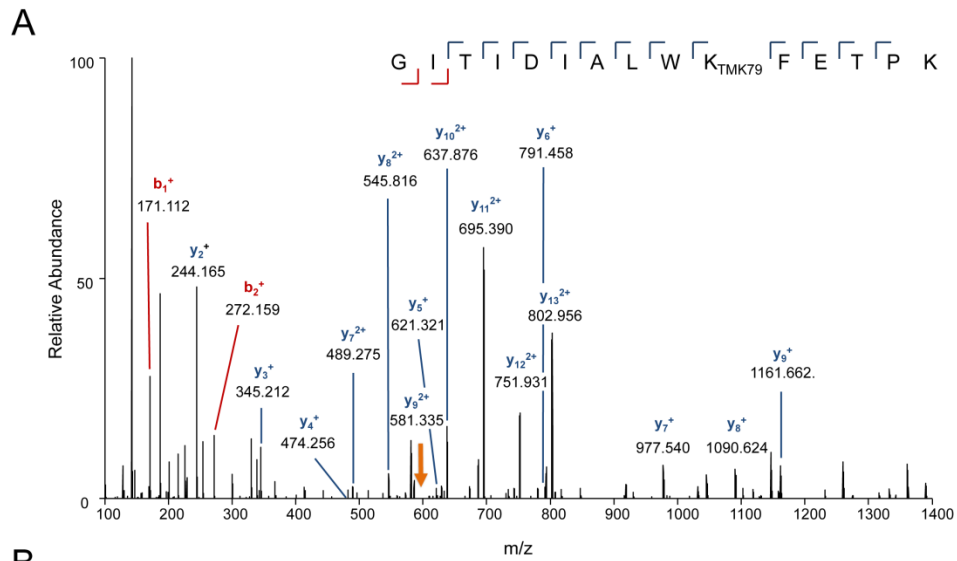


Figure S1. Deletion of *EFM1* and *EFM4* results in loss of methylated lysine residues in 50 kDa polypeptides. The position of the standards, detected by ninhydrin reactivity, is shown in the dashed line and the methylated lysine peaks are labeled (MMK, DMK, TMK). Due to a tritium isotope effect, the radiolabeled derivatives, shown in the solid lines, elute slightly before the non-labeled standards. Separation of hydrolysates from radiolabeled *efm1Δ* and *efm4Δ* from both mating type backgrounds are shown. Each trace is representative of at least three independent experiments.

Figure S2. Mass spectrometric analysis of peptides containing MMK30, TMK79, and DMK316 in the 50 kDa polypeptides of wildtype yeast. (A) MS2 sequence confirmation of TMK79 on EF1A peptide GITIDIALWK_{TMK79}FETPK, (B) DMK316 on EF1A peptide NVSVK_{DMK316}EIR, and (C) MMK30 on EF1A peptide STTTGHLIYK_{MMK30} from yeast mating type **a** samples. The same peptides were also observed for yeast mating type **α** samples. Orange arrows designate the 592.010 m/z [$M+3H$]³⁺, 324.866 m/z [$M+3H$]³⁺, and 567.811 m/z [$M+H$]⁺ precursor ions for panels A, B, and C respectively.



<i>S. cerevisiae</i>	KAERERGITIDIALWKFETPKYQVTVIDAPG	94
<i>A. nidulans</i>	KSERERGITIDIALWKFQTPKYEVTVIDAPG	105
<i>S. pombe</i>	KAERERGITIDIALWKFETPKYNVTVIDAPG	94
<i>D. melanogaster</i>	KAERERGITIDIALWKFETAKYYVTIIDAPG	94
<i>A. salina</i>	KAERERGITIDIALWKFETAKYYVTIIDAPG	94
<i>C. elegans</i>	KAERERGITIDIALWKFETAKYYITIIDAPG	94
<i>D. rerio</i>	KAERERGITIDIALWKFETSKYYVTIIDAPG	94
<i>X. laevis</i>	KAERERGITIDISLWKFQTNRFITIIDAPG	97
<i>G. gallus</i>	KAERERGITIDISLWKFETSKYYVTIIDAPG	94
<i>H. sapiens</i>	KAERERGITIDISLWKFETSKYYVTIIDAPG	94
<i>B. taurus</i>	KAERERGITIDISLWKFETSKYYVTIIDAPG	94
<i>M. musculus</i>	KAERERGITIDISLWKFETSKYYVTIIDAPG	94
<i>R. norvegicus</i>	KAERERGITIDISLWKFETTKYYITIIDAPG	94
<i>O. cuniculus</i>	KAERERGITIDISLWKFETSKYYVTIIDAPG	94
<i>T. thermophila</i>	KAERSRGITIDVTMLKFNTNKYNYTIIDTPG	92
<i>A. thaliana</i>	KAERERGITIDIALWKFETTKYYCTVIDAPG	94
<i>Z. mays</i>	KAERERGITIDIALWKFETTKYYCTVIDAPG	94
<i>O. sativa</i>	KAERERGITIDIALWKFETTKYYCTVIDAPG	94
<i>A. carterae</i>	KEERERGVTIAC TTKEFYTEKWHYTIIDAPG	96
<i>T. maritima</i>	PEEKARGITINITHVEYETEKRHYAHIDCPG	84
<i>E. coli</i>	PEEKARGITINTSHVEYDTPTRHYAHVDCPG	84
<i>R. sphaeroides</i>	PEERARGITISTAHVEYESDTRHYAHVDCPG	79
<i>B. subtilis</i>	PEERERGITISTAHVEYETE TRHYAHVDCPG	85
<i>M. genitalium</i>	PEEKARGITINSAHVEYSSDKRHYAHVDCPG	84
<i>S. tokodaii</i>	KEERERGV TINLTFMRFETKKFFFTIIDAPG	93
<i>P. furiosus</i>	KEERERGITIDVAHTKFETPHRYITIIDAPG	92

Figure S3. Conservation of EF1A trimethylation site. Aligned sequences of the yeast EF1A Lys-79 methylation site indicate a high conservation of this site in other organisms.

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Chapter Four

Searching for Additional Elongation Factor Methyltransferases

Introduction

After the identification of Efm5 in Chapter Three, there remained two unidentified elongation factor methyltransferases. One is responsible for the monomethyl lysine 390 on EF1A and the second for the reversible C-terminal lysine α -methyl ester (Cavallius et al. 1993, Zobel-Thropp et al. 2000, Lipson et al. 2010, Dzialo et al. 2014). As described in the introduction to Chapter Three, Lipson et al. 2010 had tested a significant number of possible enzymes and my bioinformatics analysis only left two candidates: Efm5 and YLR285W. Deletion of those two did not demonstrate any reduction in monomethyl lysine. At this point, essentially every known and putative methyltransferase had been ruled out for the monomethylating enzyme. However, my amino acid analysis of EF1A from *efm1* Δ cells demonstrated slightly more than a 50% reduction in [³H]-mmK (Chapter Three). It was possible that the amount of mmK contributed by Efm1 and this remaining “Efm6” may not be equal. This could have made it difficult for Lipson to detect the loss of monomethylation with intact mass spectrometry. I therefore decided to test several of the predicted protein methyltransferases by amino acid analysis regardless of Lipson’s results.

The C-terminal methyl ester could not be detected by our traditional amino acid analysis due to its labile nature. I therefore tried to adapt the Zobel-Thropp et al. 2000 gel slice base labile assay for use in the BY4742 background. However, the results of this assay were variable enough to preclude identification of a potential methyltransferase. Here I describe the methods I adapted for this assay so that others can potentially optimize this method for success.

Methods

In vivo labeling and amino acid analysis

Labeling and amino acid analysis was performed as described in Chapter Three. Relative quantification of methyl lysine peaks was calculated by doubling the counts found in half of each peak. This was done due to the fact that in some runs, the trimethyl lysine peak overlapped with methyl histidine and in all runs, mono and dimethyl lysine overlap. Therefore, the half used for analysis was the half that touched base line (first half for trimethyl and dimethyl lysine and second half for monomethyl lysine). Results are reported as ratios between the different types of methylation.

In addition to analyzing samples by cation exchange chromatography, hydrolysates were also loaded onto 20 x 20 cm silica thin layer chromatography plates. Standards of methyl lysines were run within each sample and alone (5 nmol each). Plates were placed in chambers pre-equilibrated with 3:1 (v:v) methanol:ammonium hydroxide and then removed once the solvent front reached ~1 cm from the top. The plates were air dried and then sprayed with ninhydrin (0.2% w/v in ethanol) and incubated at 100°C. Grids were drawn on each lane (1 cm wide x 0.5 cm) and each slice was scraped into a microfuge tube and 1 mL of water was added. The tubes were shaken on a vortexer for 1 h and then the slurry was added to 5 mL of scintillation fluor in 20 mL scintillation vials. Vials were counted for 5 min, three times.

In vivo labeling and base labile assays

Cells were labeled [³H]AdoMet as described above and fractionated by SDS-PAGE. Then base labile assays were performed (adapted from Zobel-Thropp et al. 2000). The entire gel

lanes were subjected to slicing. The gel was dried onto 3MM Whatman chromatography paper and then a grid was manually drawn onto the back. Slices were 0.5 cm high x 1 cm wide. Slices were then cut with scissors and the dried gel slice was placed in a microcentrifuge tube. After slicing, 150 μ L of 1.5 M sodium bicarbonate, 0.2 M HCl, 2 M NaOH, or water was added to each tube. Tubes were placed into 20 mL scintillation vials containing 5 mL of scintillation fluid (RPI Safety Solve) without getting the fluor into the microfuge tube. Vials were capped tightly and then incubated for 24 h at 37°C. Vials were then counted for radioactivity that had passed from the microfuge tube into the scintillation fluid via the gas phase as methanol. After counting, vials were uncapped and 1 mL of 30% hydrogen peroxide was added to each microfuge tube. Vials were loosely capped and incubated for 24 h at 37 °C. After incubation, vials were capped tightly, shaken, and then counted for total radioactivity.

Results

YNL024C is potentially an elongation factor 1A methyltransferase

In vivo labeled hydrolysates from 50 kDa polypeptides were subjected to amino acid analysis to assess potential loss of EF1A methylation. Several putative methyltransferase knockout strains were screened with this method (Table 4-1, Figure 4-1). Only one stood out as possibly altering the levels of EF1A methylation. Deletion of YNL024C showed a slight reduction in the monomethyl lysine peak (Figure 4-1). This small change was most obvious when comparing relative levels of each methylation (Figure 4-2). Wildtype EF1A contains one trimethyl, one dimethyl, and two monomethyl lysines and ideally would provide a monomethyl:dimethyl lysine ratio of 1:1 and a trimethyl:monomethyl lysine ratio of 3:2 (or

1.5:1). The actual ratios come very close to this (Figure 4-2). Knocking out EF1A methyltransferases clearly demonstrate strong deviations from these ratios. Deletion of Efm4 results in loss of dmK (Figure 4-1E) and the ratio of mmK:dmK increases significantly (Figure 4-2A). Deletion of Efm1 results in loss of one of the monomethyl lysines (Figure 4-1B) and the ratio of mmK:dmK decreases to 0.4 and tmK:mmK increases to 5.2 (Figure 4-2). Deletion of Efm2 and Efm3 had very slight effects on these ratios, but values depicted here are of one replicate only. Analysis of values from other Chapters in this dissertation demonstrate wildtype mmK:dmK ratios ranging from 0.9-1.3 and tmK:mmK ratios ranging from 1.4-1.8. Given this variability even in wildtype and the fact that Efm2 and Efm3 are EF2 methyltransferases, it was assumed this may be within the margin of error.

In theory, if Efm1 is responsible for exactly half of the monomethyl lysines, the mmK:dmK and tmK:mmK ratios when Efm1 is deleted should be 0.5 and 3, respectively. The deviation seen in various analyses of this strain (0.4 and 5.0) indicates that Efm1 may have a larger contribution to the overall amount of monomethyl lysine found on EF1A. This would also help explain why Lipson et al. 2010 had difficulty detecting loss of the other monomethylation and why the relative loss of methylation in *ynl024cΔ* is so low. This imbalance of methylation between Efm1 and YNL024C is noticeable when analyzing hydrolysates by thin layer chromatography (Figure 4-3). Although the amounts of trimethyl lysine, dimethyl lysine, and methyl histidine appear to be relatively the same, the amount of monomethyl lysine is significantly less in both *efm1Δ* and *ynl024cΔ*. In spite of the results shown by amino acid analysis (Figure 4-2), the TLC result (Figure 4-3) would suggest that YNL024C may catalyze the monomethylation of lysine 390. We attempted to confirm this result by mass spectrometry analysis of tryptic peptides as done in Chapter Three for Efm5. However, we did not obtain

sequence coverage of the K390-containing peptide in wildtype or mutants leaving the identification unconfirmed.

Table 4-1. Methyltransferases screened for loss of EF1A methylation by cation exchange chromatography

Gene Name	Protein Name	Confirmed Substrate
YBR271W	EFM2	Elongation Factor 2
YGR001C	EFM5	Elongation Factor 1A
YGR157W	CHO2	phosphatidylethanolamine
YGR283C		
YHL039W	EFM1	Elongation Factor 1A
YIL064W	EFM4	Elongation Factor 1A
YIL110W	HPM1	Ribosomal Protein L3
YJR073C	OPI3	phosphatidylethanolamine
YJR129C	EFM3	Elongation Factor 2
YLR285W	NNT1	
YMR310C		
YOR021C	SFM1	Ribosomal Protein S2

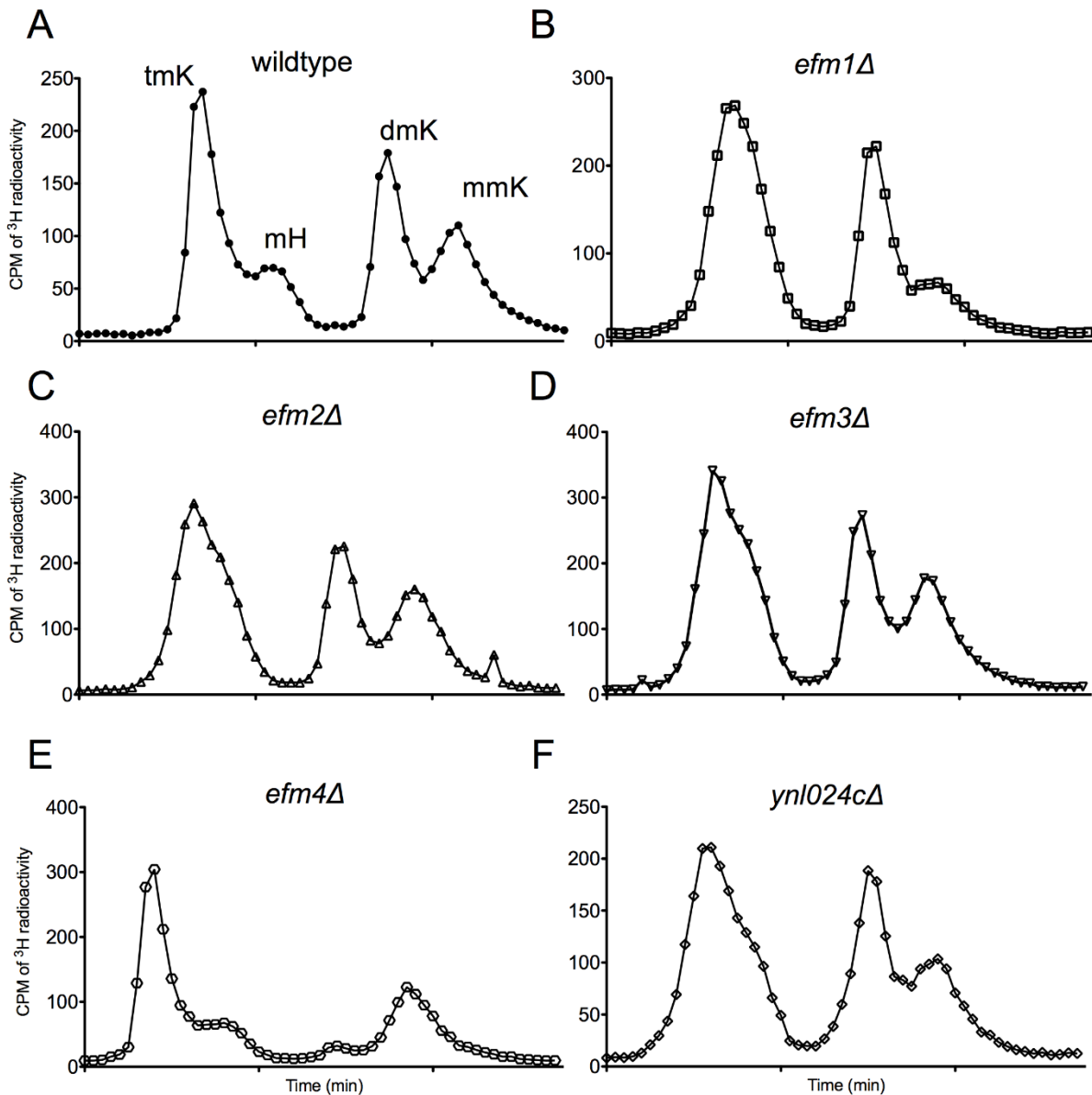


Figure 4-1. Amino acid analysis of 50 kDa polypeptides from various elongation factor methyltransferase knockout strains. Hydrolysates were prepared and subjected to amino acid analysis as described in the Methods section. Shown are representative traces from (A) wildtype, knockouts of (B and E) elongation factor 1 methyltransferases, (C and D) elongation factor 2 methyltransferases, and (F) YNL024C.

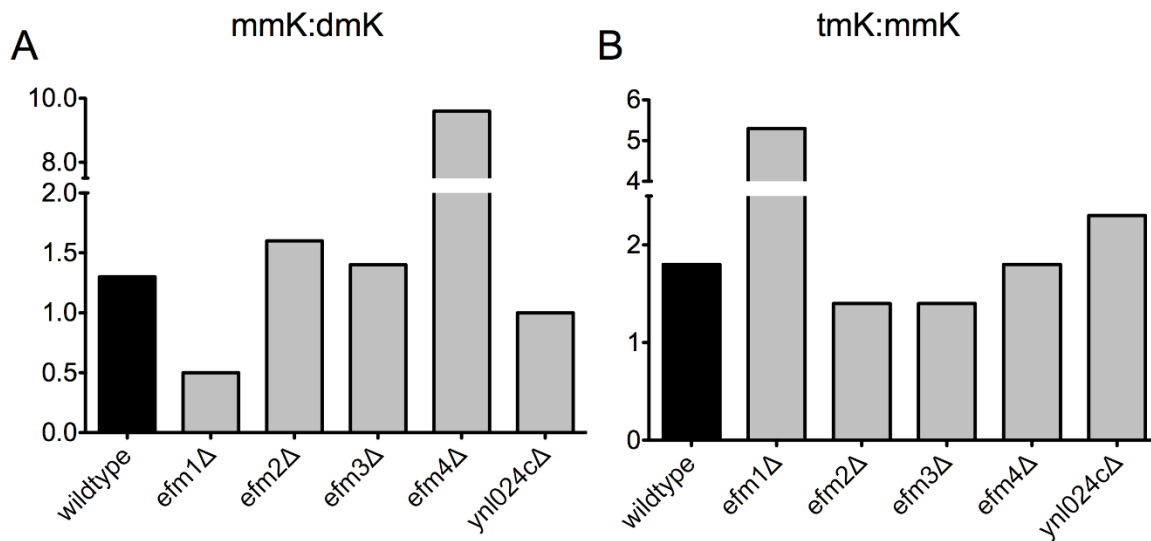


Figure 4-2. Comparison of relative amounts of methyl lysine residues in elongation factor methyltransferase knockout strains. Radioactivity from Figure 4-1 was quantified and values are reported as relative ratios between types of methylation to account for variations in total counts loaded.

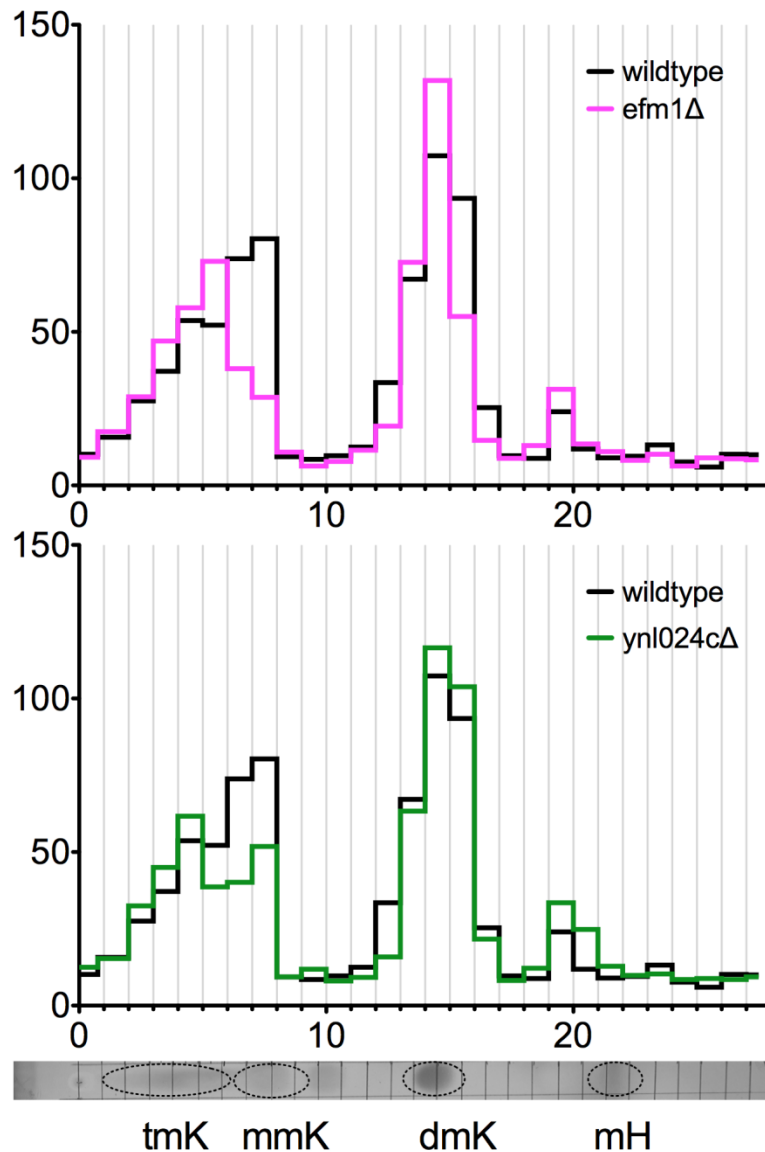


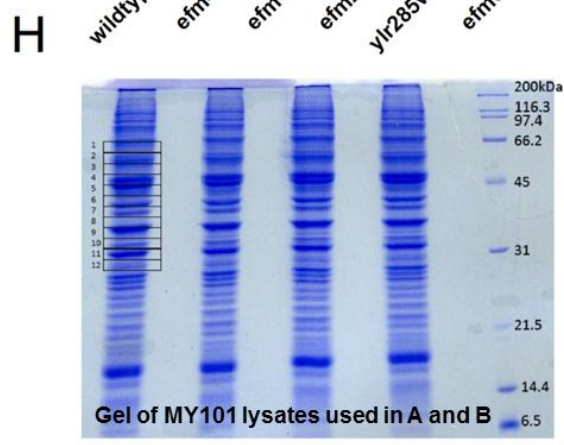
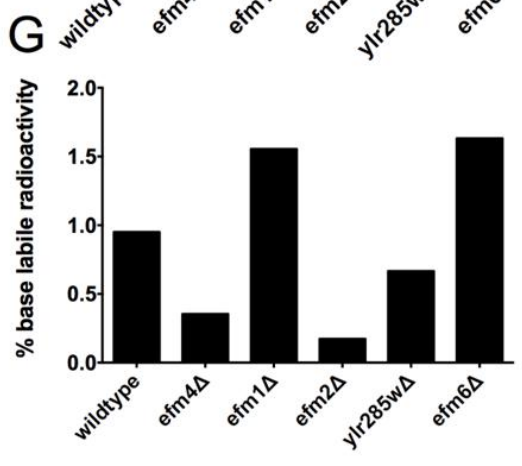
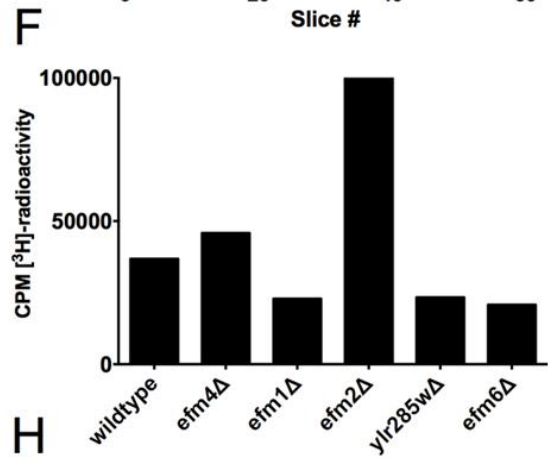
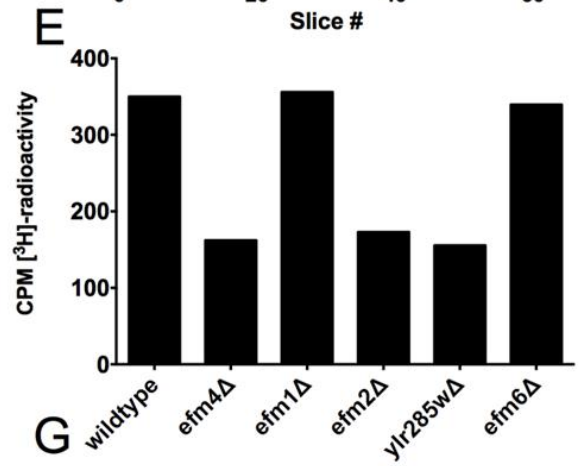
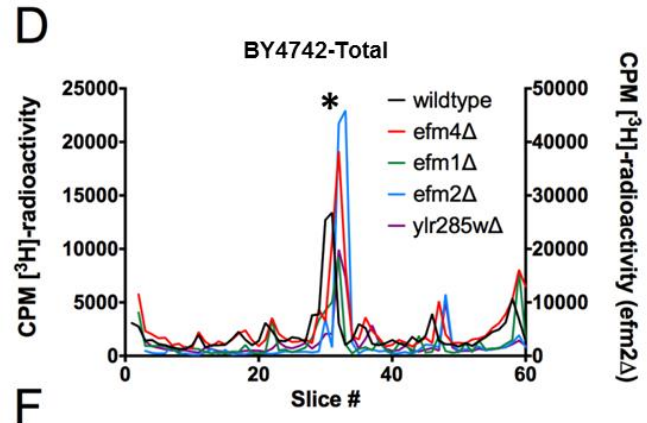
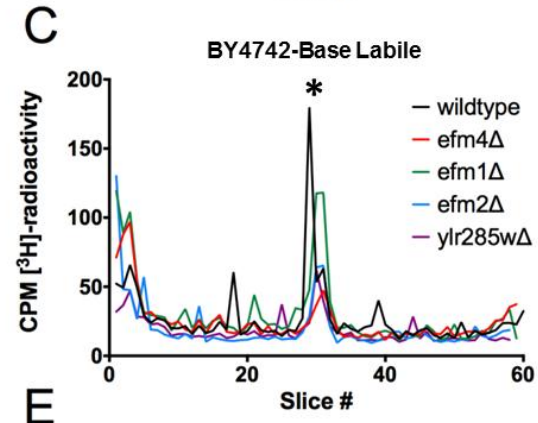
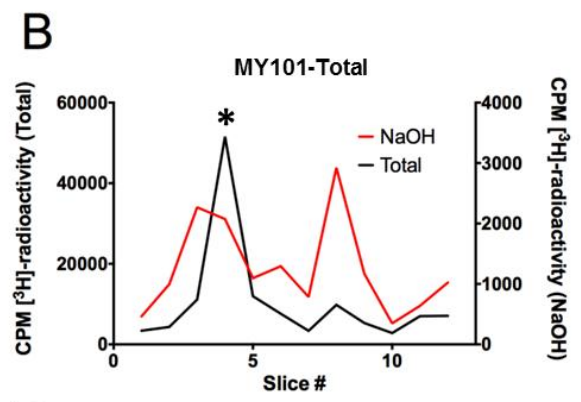
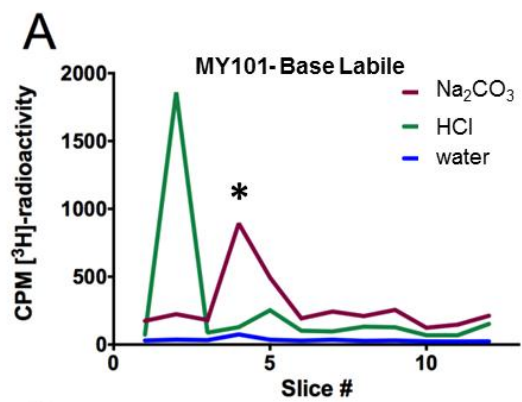
Figure 4-3. Thin layer chromatography of [³H]-labeled 50 kDa hydrolysates from *efm1Δ* and *ynl024cΔ* reveals reduced levels of monomethyl lysine compared to wildtype. The 50 kDa polypeptides from *in vivo* radiolabeled cells were acid hydrolyzed and the resultant hydrolysates were resolved on silica TLC plates using a 3:1 (v:v) methanol:ammonium hydroxide solvent. Approximately 5 nmol of each methyl lysine and methyl histidine standard were loaded with each sample. Plates were gridded and sliced to count for presence of radioactive methyl groups. Top panel: comparison of wildtype to *efm1Δ*. Bottom panel: same wildtype as above compared to *ynl024cΔ*. Below the panels is a representative lane from the wildtype run. Ovals depict positions and sizes of ninhydrin stained standards corresponding to methyl histidine (mH), and the lysines: trimethyl (tmK), dimethyl (dmK), monomethyl (mmK).

Development of base labile assays using the BY4742 yeast strain

I attempted to utilize the base labile assay described in Zobel-Thropp et al. 2000 with the BY4742 strain as the wildtype. This would allow for use of the methyltransferase knockout collection which are all the BY4742 or related background strains. The initial discovery of the C-terminal methyl ester was done using the MY101 strain which lacks the *SAM1*, *SAM2*, and *STE14* genes. These *SAM* genes are responsible for AdoMet synthesis making this strain an AdoMet auxotroph. By growing these cells with [³H]AdoMet, the only source for methyl donor in these cells would be radioactive. Additionally, deletion of *STE14* removes a significant amount of background methylation, making the other methylations more prevalent.

I was able to reproduce the results seen in the Zobel-Thropp paper with the MY101 strain (Figure 4-4A). However, levels of radioactivity were significantly lower in the BY4742 strain and did not demonstrate as high of a level of base labile radioactivity in the 50 kDa range (Figure 4-4B and C). I still attempted to try the entire assay with various knockout strains of elongation factor methyltransferases and several candidate methyltransferases, YLR285W, YNL024C, YBR225W (Figure 4-5). These strains each demonstrated different levels of total radioactivity and base labile radioactivity. It would be important to repeat these experiments to establish whether these changes are reproducible.

Figure 4-4. Base labile assays for detection of the EF1A C-terminal methyl ester. (A and B) Reproduction of the results from Zobel-Thropp et al. 2000. The 50 kDa slice is denoted by the asterisk (*). (C and D) The assay was performed with wildtype and methyltransferase knockout strains in the BY4742 background. The 50 kDa slice is denoted by the asterisk (*). (E and F) Quantification of the amount of radioactivity in the 50 kDa gel slice from C and D, respectively. (G) Relative amounts of base labile radioactivity contained in the gel slice compared to the total. (H) Representative gel of the slicing size from the gel used for the assay in A and B.



Discussion

The initial amino acid analyses of *ynl024Δc* (Figures 4-1 and 4-3) were performed in 2012 but due to the inconsistent reduction in mmK and the unsuccessful attempts to obtain quality mass spectrometry data for the K390-containing peptide, I was unable to fully confirm YNL024C as Elongation Factor Methyltransferase 6 (Efm6). However, in 2015, another group was able to directly demonstrate the methylation capabilities of YNL024C (Jakobssen et al. 2015). This group demonstrated that K390 has relatively low occupancy of methylation, which could account for the very small changes seen by our method upon deletion of YNL024C. Overexpression of YNL024C increased methylation at K390 and in fact resulted in both demethylation and trimethylation.

The apparent low occupancy of this methylation is very intriguing due to evidence that supports our hypothesis that this modification may be required for efficient viral replication. Li et al. 2014 demonstrated that deletion of Efm4, but not Efm1, resulted in decreased viral replication of the tomato bushy stunt virus in *S. cerevisiae*. This paper was very interesting but there were some conflicting interpretations of the data. The results they obtained with EF1A point mutants did not match the phenotype with the EFM knockouts. Mutating K30, 79, and 390 to arginine resulted in an even larger decrease in viral replication than simply deleting Efm4. However, Efm4 methylates K316 and the quadruple point mutant had slightly higher viral replication (11% vs. 24% of wildtype levels). It actually looked like K390 was the cause of the phenotype since the K79R and K30,79R mutants did not demonstrate a reduction. It would be interesting to test *efm6Δ* with this type of viral replication assay to correlate the loss of methylation site with the loss of methylating enzyme. Additionally, it is curious that this mutation could have such a dramatic effect on the replication when methylation seems to be

relatively low under normal conditions. There could be a correlation between viral infection and the presence of the methylated residue.

The C-terminal methyltransferase remains unidentified due to the unstable nature of the modification (Zobel-Thropp et al. 2000) and the variability observed here. Optimizing this assay will require some effort or a novel approach to working with this modification may be required. I put most of my efforts in trying to adapt Zobel-Thropp's base labile method to the BY4742 strain but it would be worthwhile to purify EF1A instead. I tried several times using the protocol from Lipson et al. 2010 but never got it to reproducibly work. Some of my earlier attempts actually isolated breakdown products of EF2 instead of EF1A. Additionally, this required two separate chromatography steps with slightly high pH buffer, which may not keep the methyl ester intact. Instead, the purification described by Zobel-Thropp would be a better option to start with. Purifying EF1A from *in vivo* labeled lysates with CM cellulose cation exchange resin and gravity flow would presumably leave the methyl ester intact. This in-solution EF1A (as opposed to the in-gel EF1A in the gel slicing assays) would allow for testing base lability using the vapor diffusion assay commonly used in our lab for measuring levels of methylated isoaspartyl residues (Patananan et al. 2014). This could then be repeated using candidate methyltransferase knockout strains. Currently, my top candidates for this methylation are PPM1 and PPM2 as they are both capable of carboxymethylation, although PPM2 does this on a nucleotide (Noma et al. 2006). There are also numerous other enzymes that yet to have confirmed substrates if neither of these two enzymes are responsible.

Now that the four internally methylated lysine residues of EF1A have corresponding methyltransferases, I turned my attention to dissecting potential roles of elongation factor methylation (Chapter Five).

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Chapter Five

Phenotype Analysis of Yeast Strains with Multiple Deletions of Elongation Factor Methyltransferases

Introduction

Now that six elongation factor methyltransferases have been identified, the question still remains: why methylate the elongation factors? Altering the methylation state of EF2 via knock out of Efm2 or Efm3 resulted in alterations in drug sensitivity and translational fidelity (Chapter Two). However, preliminary analysis of *efm1Δ*, *efm4Δ*, and *efm5Δ* strains did not demonstrate significant or consistent changes in drug sensitivity (data not shown). This led us to hypothesize that loss of only one methylation site may not be dramatic enough to effect changes in EF1A function; at least not enough to detect with our methods.

I collaborated with Kevin Roy from Guillaume Chanfreau's lab to construct various permutations of EF1A methyltransferase double, triple, and quadruple deletion strains (listed in Table 5-1). Kevin created the deletions in both directions (for example, the *efm1Δefm4Δ* double knockout was created by deleting *efm1* first, then *efm4*, and a second strain was created by deleting *efm4* first, then *efm1*). He confirmed the deletions by PCR and was highly confident that the enzyme genes were deleted. He later constructed, with the help of Jason Gabunilas, point mutations of EF1A and EF2 (Table 5-1). Since both EF1A and EF2 have two genes encoding them, this required first the deletion of one of the genes, followed by transformation of a plasmid containing the mutated gene and subsequent deletion of the second gene.

I first confirmed that the expected methylation was lost in each of the strains utilizing amino acid analysis. Once that was confirmed, I subjected the strains to various assays to determine whether there were any major problems in the elongation cycle. I utilized translational inhibitors, translational fidelity assays, and polysome profiling analysis coupled with glucose starvation to measure elongation rates.

Materials and Methods

*Construction of multiple knockouts and EF1A and EF2 point mutants**

**This method was written by Jason Gabunilas and modified by Maria Dzialo*

All *EFM* and *TEF* mutants were derived from the BY4742 background strain. Deletion mutants created in this study were constructed by transformation of the background strain with PCR products containing an auxotrophic marker or antibiotic resistance cassette as well as 40 base pairs of flanking sequencing around the ORF of interest for efficient homologous recombination. The individual *EFM* ORFs were deleted with the cassettes indicated in Table 5-1. *TEF1* and *TEF2* were deleted with *KAN^R* and *HYG^R*, respectively. Successful transformants were screened by growth on dropout media or antibiotic plates and confirmed by PCR. The *HYG^R*, *KAN^R*, and *HIS3* cassettes share identical promoters and terminators and are thus able to recombine into one-another. Therefore, in order to generate strains with multiple gene knockouts using two or more of these cassettes, strains were propagated on plates maintaining selective pressure for all applicable cassettes.

To clone the *TEF1* intron into pUG23, *TEF1* PCR from genomic DNA using primers that insert *BamHI* (NEB No. R3136) and *SpeI* (NEB No. R0101) restriction sites into the ends of the PCR product. PCR reactions were performed using Phusion Hi-Fi Polymerase (NEB No. M0530) according to the manufacturer's protocol. The PCR product was purified using a microcentrifuge spin column according to the manufacturer's protocol (BioPioneer No. PPP-100). Amplification product and pUG23 vector were digested using *BamHI* and *SpeI* restriction enzymes for 2-16 hours. Restriction digest was done using 1 µl 100X BSA, 10 µl NEBuffer, 1 µl CIP and water. Ligation of insert to vector was then done using T4 DNA Ligase (Life Technologies No. 15224) according the manufacturer's protocol. Plasmids were transformed into

DH5 α competent E.coli cells and plated on LB Agar plates with 100 μ g/mL ampicillin. Screening of colonies for positive clones was done by growing liquid cultures for miniprep (BioPioneer No. CMIP-100) followed by PCR. Positive clones were then confirmed by Sanger sequencing (Laragen Inc.).

Site-directed mutagenesis of plasmid DNA was performed using the QuickChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies No. 200514). Each reaction combined 2.5 μ l of 10X reaction buffer, 10-100 ng of plasmid template, 100 ng of each mutagenic primer, 1 μ l of dNTP mix, 0.75 μ l of QuikSolution reagent, 1 μ l of QuikChange Multi enzyme blend and nuclease-free water to a final volume of 25 μ l. Mutagenesis reactions were performed in a thermocycler according to the manufacturer's instructions. After the reaction cycle, each amplification reaction was mixed with 1 μ l of *DpnI* restriction enzyme and then incubated at 37°C for 1 h to digest the parental dsDNA. *DpnI*-treated DNA was then transformed into XL10-Gold ultracompetent cells per the manufacturer's instructions which were then plated onto LB + 100 μ g/mL ampicillin plates and incubated at 37°C for 16 hours. Bacterial colonies resulting from the transformation were used for plasmid miniprep and plasmids were sequenced by Sanger sequencing (Laragen Inc.) to confirm positive mutagenesis. Mutagenized plasmids were then transformed into yeast cells and screened on -HIS dropout media plates. Yeast plasmid and linear DNA transformations into yeast cells were conducted using the LiAc/SS carrier DNA/PEG method (Geitz and Shiestl, 2007).

To construct the strain expressing the *TEF1* K(30, 79, 316, 390)R mutant, *TEF1* was first deleted with *KAN^R* as described above. Next, the *tef1 Δ ::KAN^R* mutant was transformed with the *TEF1* K(30, 79, 316, 390)R/pUG23 mutagenized plasmid (see above). Finally, *TEF2* was deleted

with *HYG^R* as described above. The plasmid was maintained by growing the strains on –HIS plates in all subsequent steps.

TABLE 5-1. Multiple EFM Knockout Strains constructed for this study

Abbreviated Name	Genotype	Selectable marker
	<i>efm1Δ</i>	<i>HYG^R</i>
	<i>efm4Δ</i>	<i>HIS3</i>
	<i>efm5Δ</i>	<i>KAN^R</i>
	<i>efm6Δ</i>	<i>KIURA3</i>
EF1Aefm1Δ4Δ	<i>efm1Δ efm4Δ</i>	<i>HYG^R HIS3</i>
EF1Aefm1Δ5Δ	<i>efm1Δ efm5Δ</i>	<i>HYG^R KAN^R</i>
EF1Aefm4Δ5Δ	<i>efm4Δ efm5Δ</i>	<i>HIS3 KAN^R</i>
EF1AefmΔΔΔ	<i>efm1Δ efm4Δ efm5Δ</i>	<i>HYG^R HIS3 KAN^R</i>
EF1AefmΔΔΔΔ	<i>efm1Δ efm4Δ efm5Δ efm6Δ</i>	<i>HYG^R HIS3 KAN^R KIURA3</i>
EF2efmΔΔ	<i>efm2Δ efm3Δ</i>	
EF1A K(30, 79, 316, 390)R	<i>tef1Δ::KAN^R, tef2Δ::HYG^R, TEF1 K(30, 79, 316, 390)R/pUG23</i>	
EF1A K(30, 390)R	<i>tef1Δ::KAN^R, tef2Δ::HYG^R, TEF1 K(30, 390)R/pUG23</i>	
EF1A K(79, 316)R	<i>tef1Δ::KAN^R, tef2Δ::HYG^R, TEF1 K(79, 316)R/pUG23</i>	

In Vivo Radiolabeling and Amino Acid Analysis

Radiolabeling and amino acid analysis was performed as described in Chapters Two and Three.

In vitro methylation assays

In vitro methylation was adapted from Lipson et al. 2010. Briefly, unlabeled cells are lysed and the protein concentration is determined for the lysates. Equal amounts of protein from two different lysates are mixed together in the presence of [³H]AdoMet for 1 h at 30°C. The

reactions are stopped with the addition of SDS-PAGE loading buffer followed by boiling for 3 min. The entire sample is resolved by 12% SDS-PAGE and the Coomassie stained gel is incubated with En³Hance. The gel is then dried onto 3MM Whatman paper and exposed to film at -80°C. Films were developed after 1 month exposure.

Translation Inhibitor Assays

Liquid growth curve assays were performed in the presence and absence of various translational inhibitors. Precultures of each strain were used to inoculate 20 mL of YPD to a low OD₆₀₀ so that the OD₆₀₀ would reach 0.1 at approximately 9AM the following day. Cells were then treated with drug or vehicle and the OD₆₀₀ was measured every 1.5 h. Antibiotics tested included anisomycin (3 µg/mL, Sigma, A9789), cycloheximide (35 ng/mL, Sigma, C7968), and paromomycin (4 mg/mL, Sigma P9297). Doubling times were calculated using *Doubling Time* (<http://www.doubling-time.com/compute.php?lang=en>). Results were analyzed as a ratio of doubling times, +drug:-drug.

For tunicamycin, two 10 mL cultures of YPD media for each strain were inoculated to a low OD₆₀₀ and incubated at 30° C to ensure an OD₆₀₀ of 0.20 following morning. The cultures were treated with either DMSO (vehicle) or tunicamycin (500 ng/mL, Sigma, T7765). After one hour the 10 mL cultures were pelleted, washed with 1 mL of pre-warmed YPD, resuspended in 1 mL YPD, and then transferred into a 20 mL culture of pre-warmed YPD. The OD₆₀₀ of the cultures were then measured every hour to establish the growth rate of each strain and condition. The modifications for tunicamycin recovery were adapted from Chen et. al. (2005). Results were analyzed as a ratio of doubling times, +drug:-drug.

Transformation of strains with Dual-Luciferase Reporter Plasmids

Multiple-EFM knockout strains were transformed with the Dual Luciferase Plasmids as described in Chapter Two. The triple EFM knockout, *efm1Δefm4Δefm5Δ*, was used in these assays in place of the quadruple knockout, *efm1Δefm4Δefm5Δefm6Δ*, due to the *KIURA3* selectable marker for *efm6Δ* in this strain which is the same selectable marker used for the fidelity plasmids.

Polysome profile analysis and glucose withdrawal

Polysome profiling was adapted from Al-Hadid et al. (2014) and glucose withdrawal was adapted from Ashe et al. (2000) and Iglesias-Gato et al. (2011). For direct polysome profile analysis, yeast cells were grown in 50mL cultures of YPD until an OD_{600nm} of 0.7. For glucose withdrawal, cells were grown instead in 100mL of synthetic complete media (+glucose). Once the cells reached OD_{600nm} 0.7, the cultures were divided equally; half was directly treated with cycloheximide whereas the other half was briefly washed in sterile water and resuspended in 25mL of synthetic media without glucose added. The cells were incubated for 2 min in the minus glucose and then directly treated with cycloheximide.

To freeze the polysomes, cycloheximide was added to a final concentration of 100 µg/mL and cells were incubated on ice for 10 min. Cells were pelleted and washed twice of ice-cold lysis buffer (10mM Tris-Cl, pH 7.4, 100mM NaCl, 30mM MgCl₂, 100ug/mL cycloheximide, 200ug/mL heparin, 0.1% diethylpyrocarbonate). Cell pellets were stored at -80°C until analysis. Cells were lysed in 1mL of lysis buffer plus a quarter volume of glass beads. Lysis was achieved by vortexing for 30s followed by 30s on ice, twelve times. Crude lysate was centrifuged at 5,000

x g for 5 min at 4°C. The supernatant was transferred to a pre-chilled microfuge tube and cleared again at 12,000 x g for 8 min at 4°C. The absorbance at 260nm was measured using a NanoDrop2000c spectrophotometer (Thermo Scientific) and 10 OD units of the lysate was layered onto an 11-mL 10%-50% (w/v) linearized sucrose gradient. Gradients were linearized overnight at 4°C before lysis using 50mM Tris-Cl, pH 7.0, 50mM NH₄Cl, 12mM MgCl₂, 1mM DTT in Beckman Ultra-Clear centrifuge tubes (344059). Tubes were centrifuged at 37,000 rpm (234,116.4 x g) at 4°C for 3.5 h using a Beckman SW41 swinging bucket rotor. Continuous absorbance of the sucrose gradient at 280 nm was done using an ISCO gradient fractionator (Model 185) with an ISCO Model UA-5 absorbance monitor (sensitivity 0.5 AU/V). The flow rate was set to 1.5 mL/min 234 fluorinert FC-40 as a displacing agent. For digitizing the absorbance readings, an RS-232 equipped voltmeter (Tekpower model TP4000ZC) was connected to the external chart recorder outputs and recorded DC voltage at 1 s intervals using the included software (DMM 238 version 2.0) and then imported into Microsoft Excel for further data processing.

For quantification of peaks, data was imported into Graphical Analysis 3.8.4. Peaks corresponding to the 40S, 60S, and 80S ribosomes were identified and half of each peak was integrated using the GA software (the half chosen always touched baseline). Peaks corresponding to the polysomes were fully integrated using the software. Final data and analysis was performed with GraphPad Prism 6.0.

Results

Point mutation of EF1A methylation sites results in expected loss of methylation on EF1A

The TEF1 point mutants were *in vivo* labeled with [³H]AdoMet and the proteins from the lysates were fractionated by SDS-PAGE. The 50 kDa bands were acid hydrolyzed and the resulting hydrolysates were subjected to amino acid analysis. Additionally, the TEF1-containing pUG23 plasmid was analyzed. Although there is one less copy of EF1A in this strain (i.e. no TEF2 gene is present), the relative amounts of tri-, di-, and monomethyl lysine were comparable to that of a wildtype strain (Figure 5-1A). There appears to be slightly less trimethyl lysine, but overall the pattern is similar to that of the wildtype. Mutation of monomethyl lysine sites K30 and K390 to arginines resulted in a near total loss of radioactive monomethyllysine (Figure 5-1B). Mutating K79 and K316, the trimethyl and dimethyl lysine sites (respectively), to arginine resulted in a near total loss of dimethyl and trimethyl lysine (Figure 5-1B).

Deletion of multiple EFMs results in loss of multiple methyl lysines on EF1A

The multi-EFM knockout strains were *in vivo* labeled with [³H]AdoMet and lysate polypeptides were resolved by SDS-PAGE. The 50 kDa band was acid hydrolyzed and the resulting hydrolysates were subjected to amino acid analysis. For the most part, the combined deletions matched the expected loss of methylated lysines (Figure 5-2). In one case however, there was an unexpected variation that did not directly correlate to the respective deleted enzyme. Deletion of Efm1, Efm4 and Efm5 should have resulted in total loss of trimethyl and dimethyl lysine and approximately half of the monomethyllysine. However, dimethyl lysine remains although at lower levels than wildtype (Figure 5-2D). An additional deletion of Efm6

demonstrated no significant dimethyl or monomethyllysine. It is possible that the EFMs can cross-methylate other sites in the absence of one or more enzymes or that Efm6 is capable of dimethylation as well.

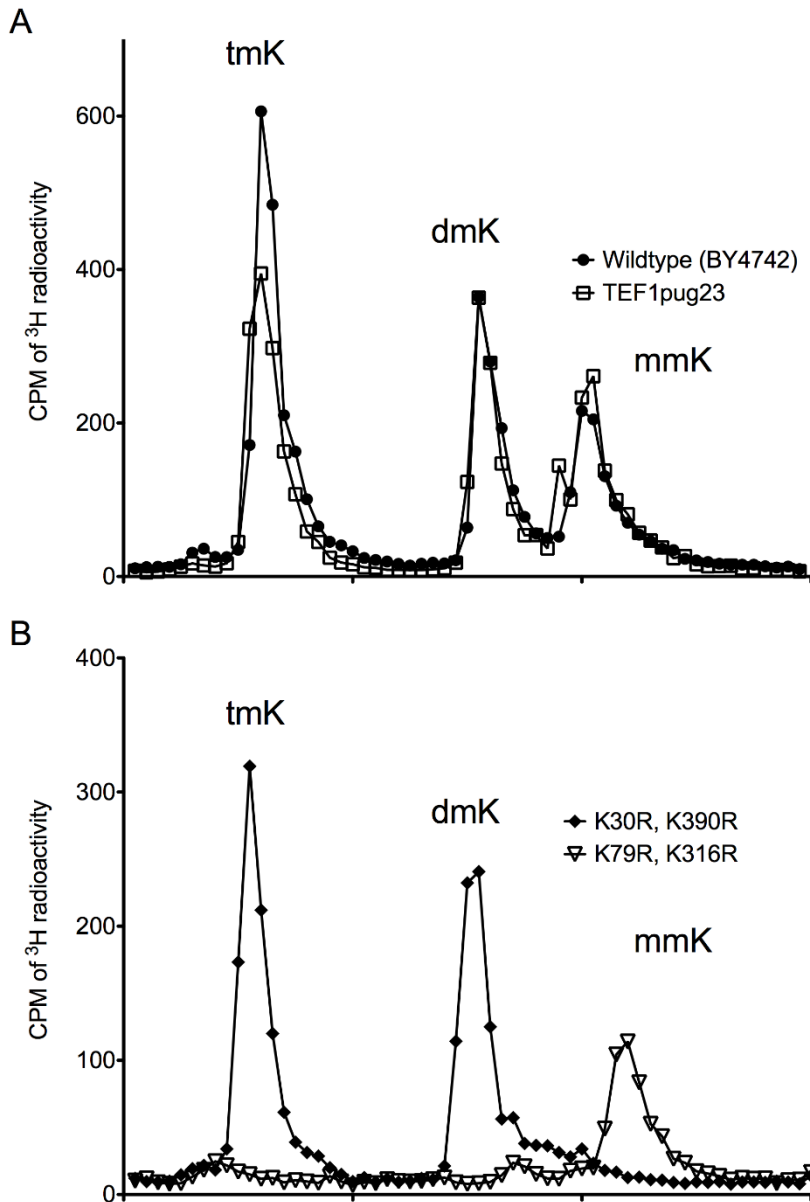


Figure 5-1 Point mutations of known EF1A methylation sites results in loss of corresponding methylation. 50-kDa hydrolysates were subjected to amino acid analysis. (A) Wildtype versus EF1A expression from a plasmid when both TEF1 and TEF2 are deleted. (B) Point mutations of EF1A methylation sites. In both A and B, the x-axis is intentionally left blank due to variations in methyl lysine elution times during different runs. All peaks were aligned to non-radioactive standards run with the hydrolysates to determine the elution times of trimethyl lysine (tmK), dimethyl lysine (dmK), and monomethyl lysine (mmK) (not shown).

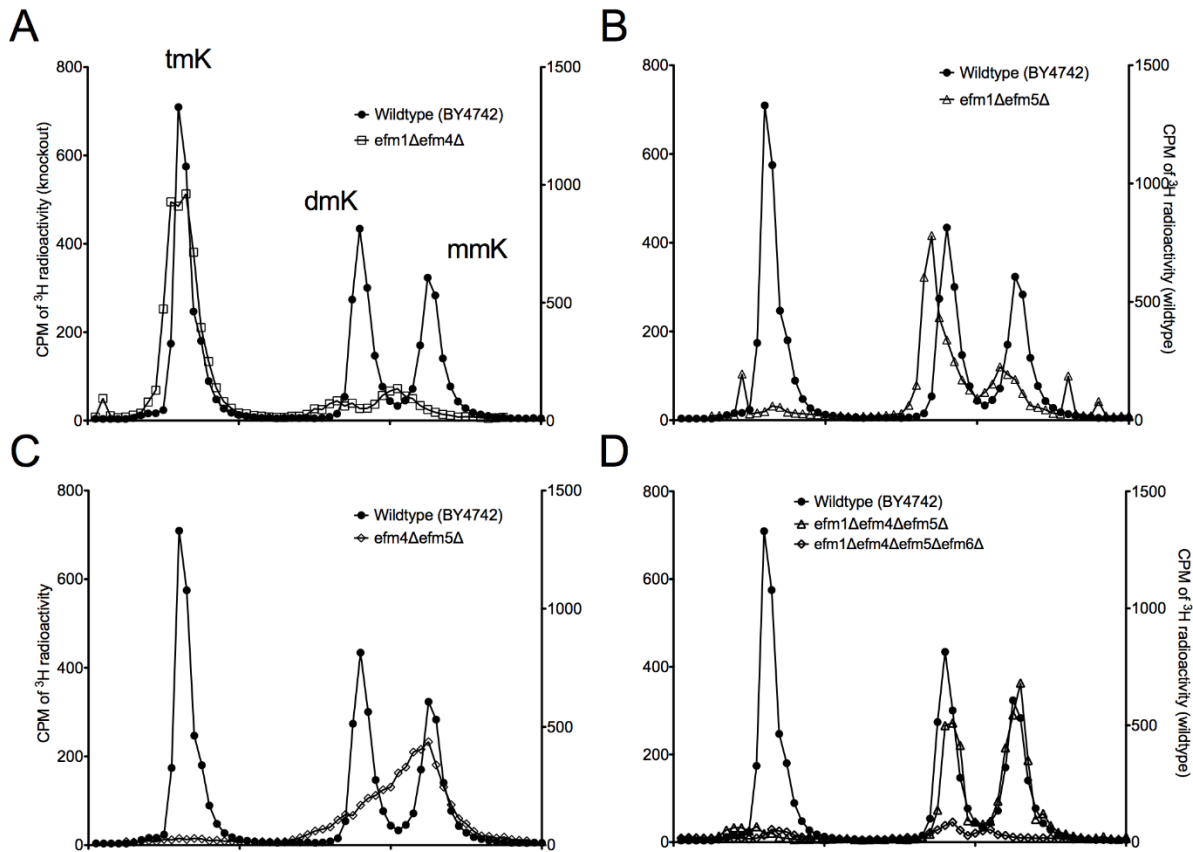
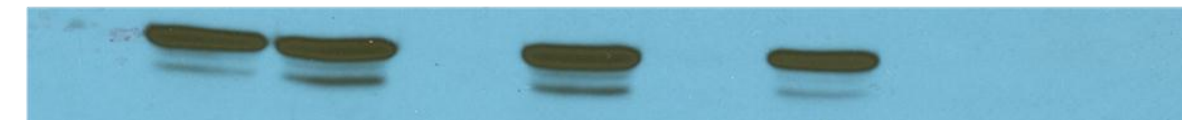
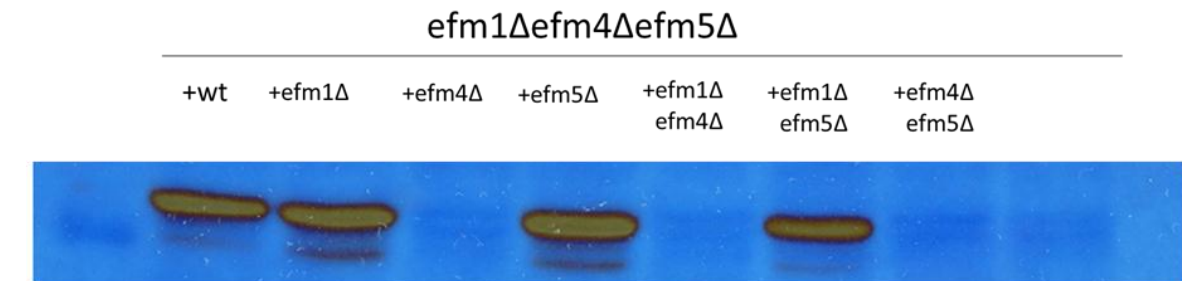
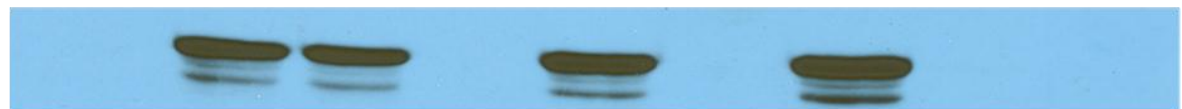
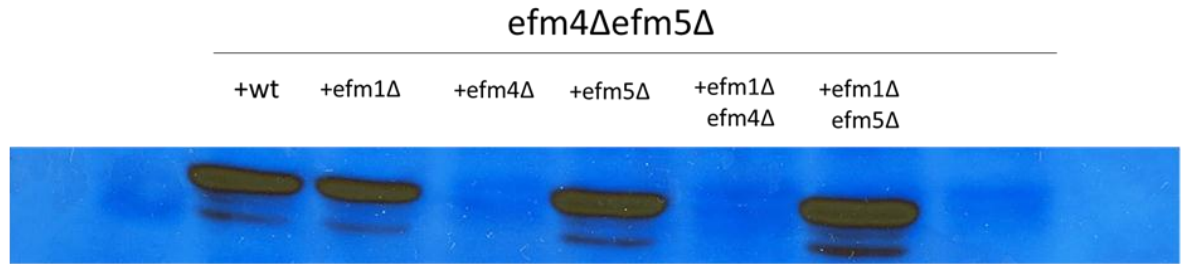
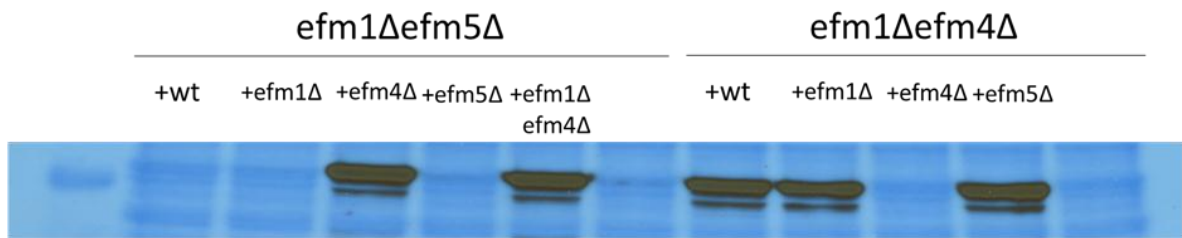
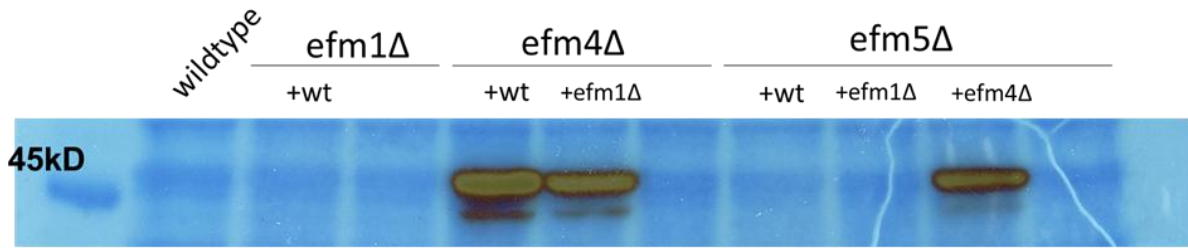


Figure 5-2 Multiple deletions of EF1A methyltransferases results in loss of corresponding methylation. 50-kDa hydrolysates were subjected to amino acid analysis. The same wildtype run is shown in all four panels for comparison to (A) *efm1Δefm4Δ* (B) *efm1Δefm5Δ* (C) *efm4Δefm5Δ* and (D) *efm1Δefm4Δefm5Δ* and *efm1Δefm4Δefm5Δefm6Δ*. In all panels, the x-axis is intentionally left blank due to variations in methyl lysine elution times during different runs. All peaks were aligned to non-radioactive standards run with the hydrolysates to determine the elution of trimethyl lysine (tmK), dimethyl lysine (dmK) and monomethyl lysine (mmK) (not shown).

In vitro methylation assays with mixed lysates reveals that Efm4 may be required for robust methylation of EF1A.

Lipson et al. demonstrated that lysates could be used as enzyme and substrate source when the correct combination was used. She demonstrated that incubating wildtype lysate alone or *efm4Δ* lysate alone with [³H]AdoMet did not result in any radioactive proteins after resolution on SDS-PAGE. However, when equal amounts of wildtype and *efm4Δ* lysates were mixed with [³H]AdoMet, a significant amount of radioactivity is present in the 50 kDa band. I attempted to repeat this with the various other EFM knockout as well as the multiple EFM knockout strains. Interestingly, the only time a signal is visible at 50 kDa is when Efm4 was included in the reaction (Figure 5-3). For example, mixing *efm1Δefm5Δ* lysate with *efm1Δ* lysate does not give rise to any radioactive bands even though Efm5 is essentially added back and its substrate should be blank. However, when *efm1Δefm4Δ* lysate is reacted with *efm1Δ* lysate, a significant band is present at 50 kDa. One hypothesis could be that Efm4 is required for methylation, but this is not reflected in any of the amino acid analysis or mass spectrometry we have presented in Chapters Three, Four, or Five.

Figure 5-3. *In vitro* methylation reactions with mixed lysates from multiple EFM knockouts. Lysates from wildtype, single, double, and triple EFM knockout strains were incubated in equal parts in the presence of [³H]AdoMet. Methylation was observed by fluorography. Shown for each reaction is the 50 kDa range (determined by the 45 kDa marker labeled in the top panel). Each panel shows the overlay of the film with the respective Coomassie-stained gel to demonstrate equal amounts of protein. Additionally the film is shown on its own to clearly show that no detectable signal is seen in any lanes except those containing a mix of samples with *efm4Δ* and Efm4 added back in.



Deletion of multiple EFMs results in dramatic changes in doubling time and sensitivity to translational inhibitors

The inhibitor assays described in Chapter Two were performed using spot tests on agar plates with and without inhibitor. This method is strictly qualitative and initially caused some trouble with the multi-knockouts. There was an obvious slow growth on YPD alone which made interpreting the +drug plates more complex. We therefore switched to liquid growth curves to obtain quantitative results. Optimization and the majority of these experiments were performed by Kyle Travaglini. He determined concentrations for each drug to provide a ratio of approximately 1.5 for wildtype cells. Anisomycin was initially tested at 3 $\mu\text{g}/\text{mL}$ but we later determined that 4 $\mu\text{g}/\text{mL}$ provided a better ratio for wildtype. Subsequently there are not replicates for multiple strains under this concentration of drug.

The first most striking result from these assays was the significant increase in doubling time seen in many of the multiple knockouts (Figure 5-4A). Wildtype cells consistent display a doubling time of 1.5 h whereas *efm1 Δ efm4 Δ efm5 Δ efm6 Δ* have a doubling time of 2 hrs. Even *efm2 Δ efm3 Δ* had a statistically significantly higher doubling time of 1.7 h.

Cycloheximide had no effect on the EF1A methyltransferase knockouts whereas the EF2 methyltransferase double knockout had significantly increased sensitivity to the drug (Figure 5-4B). This is consistent with the results of the individual knockouts that were tested via spot test assays in Chapter Two. Further analysis would need to be done to determine which of the two methylations contributes more to the phenotype or if they are cumulative.

Individual deletion of the EF2 methyltransferase Efm2 and Efm3 in Chapter Two also demonstrated increased sensitivity to tunicamycin. Consistent with that result, our liquid assays demonstrated increased sensitivity in the double knockout (Figure 5-4C). Interestingly, the EF1A

methyltransferase quadruple knockout demonstrated *resistance* to tunicamycin and paromomycin. In fact, in the presence of paromomycin (Figure 5-4D), there is no change in doubling time for the quadruple mutant as shown by a 1:1 ratio in doubling time.

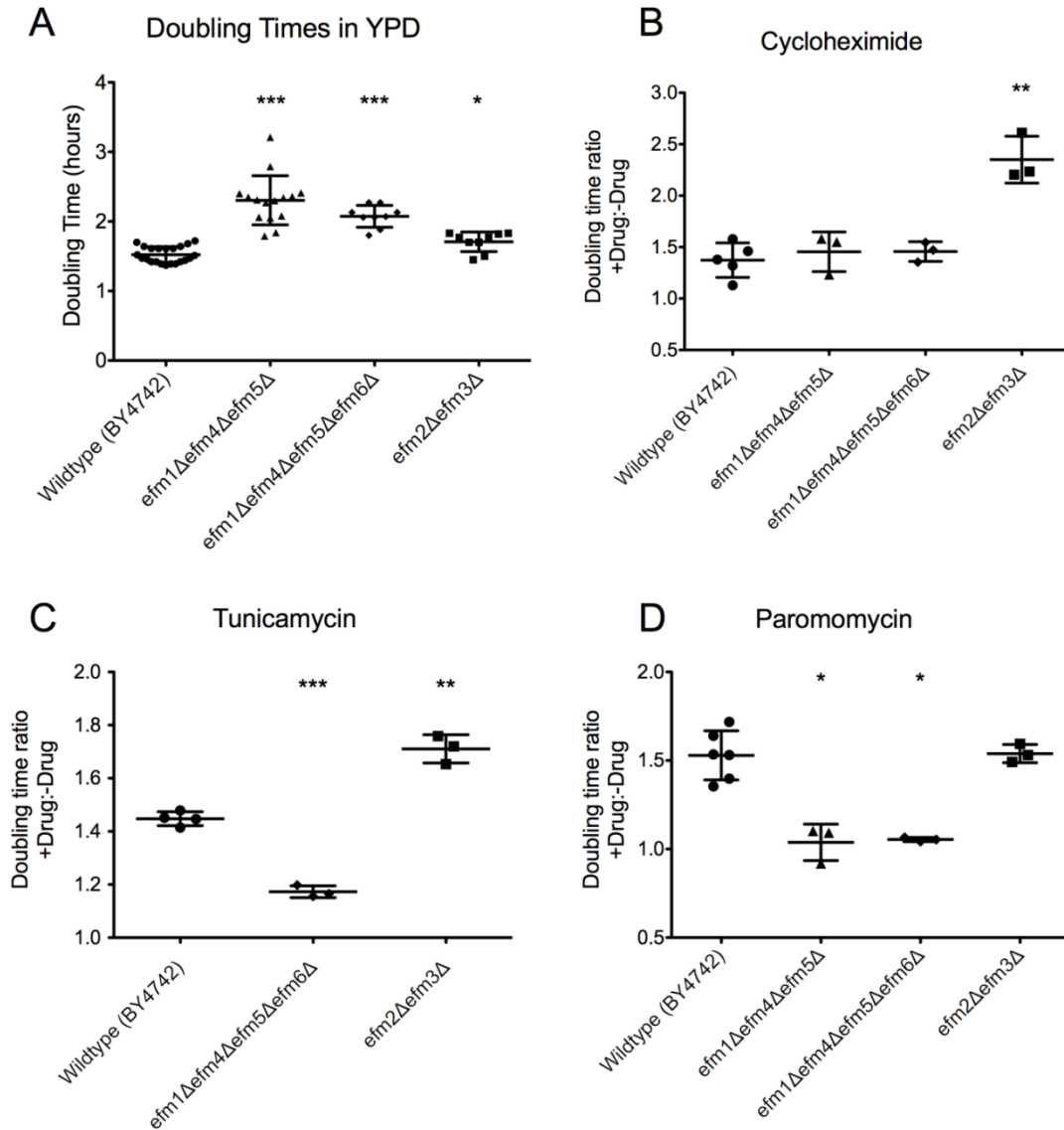


Figure 5-4. Changes to translational inhibitor sensitivity in multiple-EFM deletion strains.

(A) Doubling times of strains in YPD alone. (B-C) Antibiotic sensitivity is determined by comparing a ratio of the doubling time with drug to without drug (i.e. YPD alone) to correct for the dramatic changes in doubling time seen in A. (* $p < 0.001$, ** $p < 0.0005$, *** $p < 0.0001$).

Translational Fidelity Assays with Dual-Luciferase Reporters

All double and triple knockout strains were successfully transformed with the 11 different reporter plasmids required for measuring stop codon readthrough, misincorporation, and frameshifting. I ran into several difficulties with this assay that are described more thoroughly in Chapter Seven (Perspectives). However, preliminary experiments suggest that the *efm1Δ efm4Δ efm5Δ* triple knockout has slightly higher misincorporation of amino acids (Figure 5-5). Based on the difficulties with this assay and the lack of statistical significance ($p = 0.1$), I would only hypothesize that lack of methylation on EF1A does increase misreading and would recommend further tests with this assay to confirm.

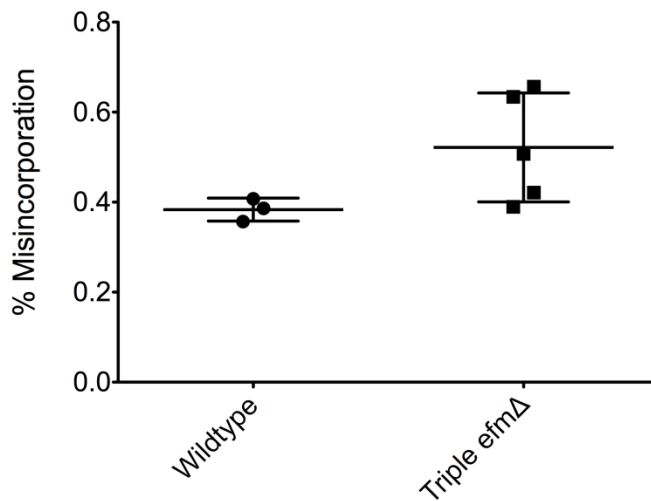


Figure 5-5. Deletion of three EF1A methyltransferases demonstrates no statistically significant change in amino acid misincorporation. Misincorporation was measured using the Dual-Luciferase Reporter plasmids. $p = 0.1$.

Polysome profile analysis reveals no defects in ribosome subunits levels

Polysome profile analysis was performed with wildtype, *efm1Δ*, *efm2Δ*, and *efm4Δ* cells (Figure 5-6A). Further analysis with other strains was not performed due to time constraints and difficulties with the ISCO fractionator. Additionally, Dr. William Munroe, who runs the fractionator, left UCLA and was not running while it was being moved and re-established with

the Biochemistry Department Instrumentation Facility. However, initial analyses demonstrated no significant differences in the 60S:40S or polysome:monosome ratios in these deletions when compared to wildtype (Figure 5-6B).

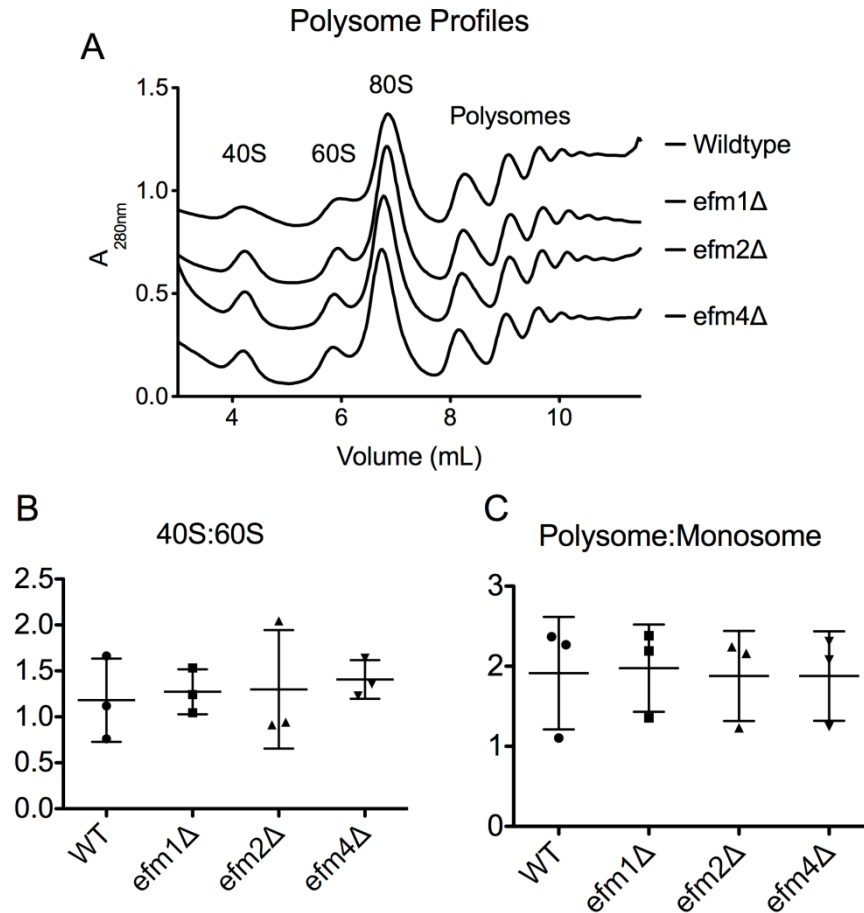


Figure 5-6. Polysome profile analysis of some elongation factor methyltransferase knockout strains. (A) Representative traces of polysome profiles. (B and C) Peaks from three different replicates were integrated using Graphical Analysis 3.8.4 and compared as 60S:40S and polysome:80S ratios. Error bars represent standard deviation. Note: the highest values for the 60:40 ratios and the lowest values for the polysome:monosome ratios are from the same replicate. In that replicate, the polysome peaks were poorly resolved and may have artificially lowered the polysome:monosome ratio.

*Measuring elongation rates indicates possibly reduced elongation rates in *efm4Δ* cells*

It is possible to measure relative elongation rates using polysome profiling after glucose withdrawal. When starved for glucose, yeast cells halt initiation of any new translation (Ashe et al. 2000) but any ribosomes currently translating will continue and eventually “run-off” the mRNA. By freezing the ribosomes at various time points after starvation, the ratio of polysomes to monosomes can provide a relative rate of elongation (Iglesias et al. 2011).

I was able to successfully reproduce this in wildtype yeast (Figure 5-7A) as well as *efm4Δ* and *efm2Δ* cells. In both replicates with *efm4Δ* cells, *efm4Δ* had a larger polysome:monosome ratio after starvation (Figure 5-7B and 8A) indicating a slower elongation rate. This is also evident from the lower % change in ratio after starvation (Figure 5-7C) and the actual polysome profile (Figure 5-8A). *efm2Δ* cells had a very similar polysome:monosome ratio after starvation as well as a similar % reduction (Figure 5-8). However, the starting polysome:monosome ratio before glucose withdrawal was lower than wildtype (Figure 5-7C). This experiment was repeated but due to problems with the ISCO detector, the data were unusable.

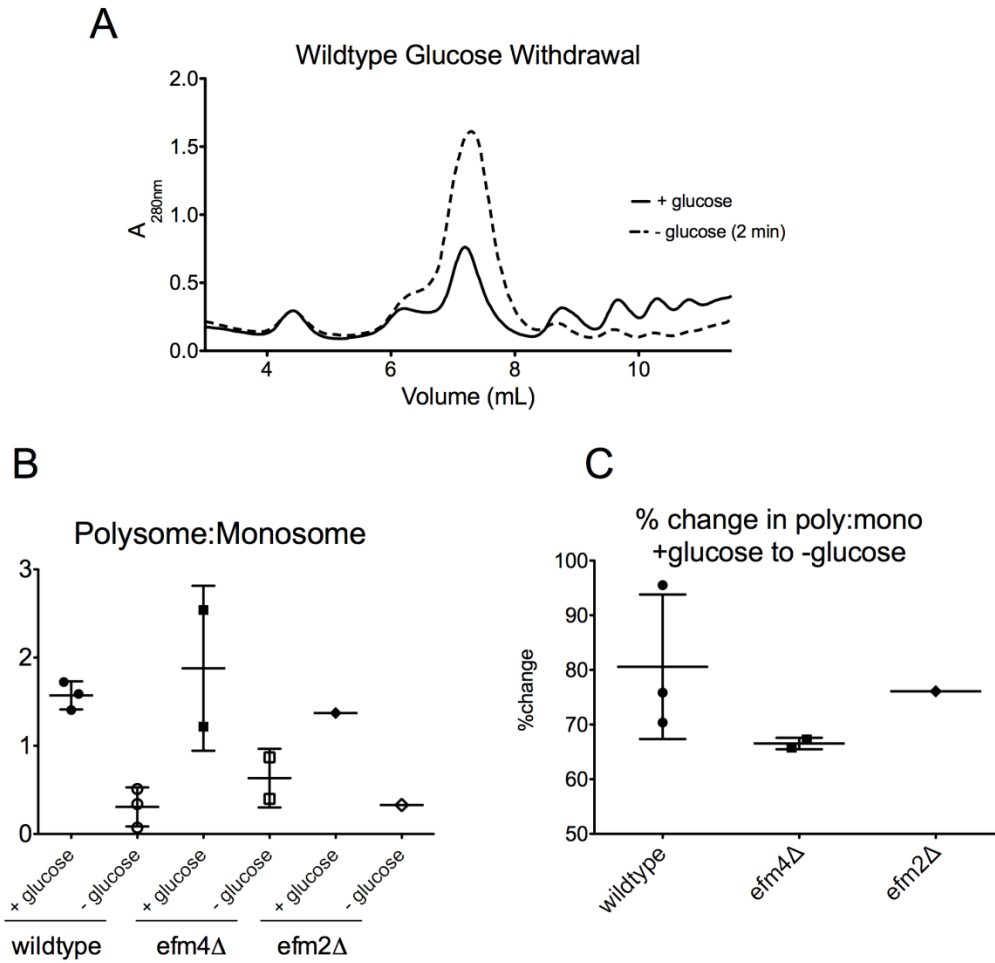


Figure 5-7. Glucose withdrawal causes initiation inhibition and ribosome runoff. (A) polysome profile of wildtype yeast before and after 2 min of glucose starvation. (B and C) Quantification and analysis of data in Figure 5-7. B shows the polysome:monosome ratios for the various starvation experiments performed. C is from the same set of experiments but is depicted as a % reduction in the polysome:monosome ratio after starvation.

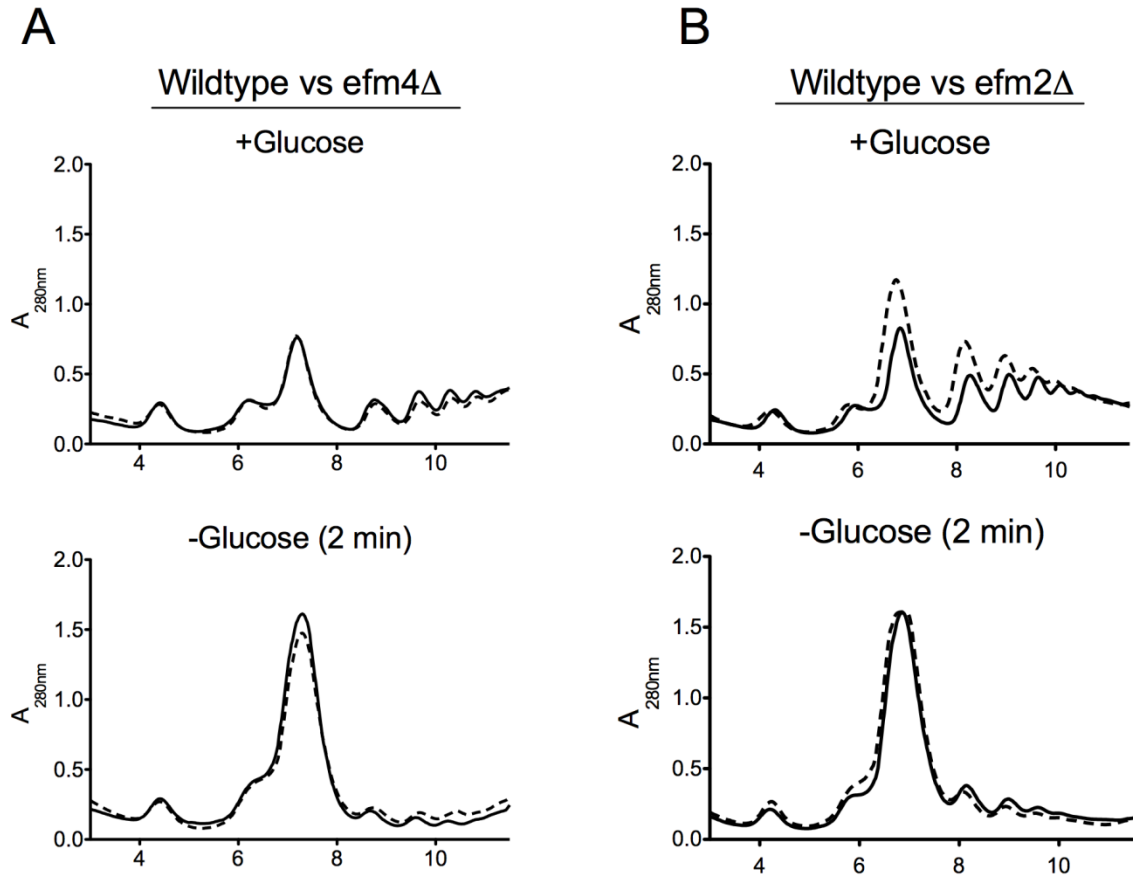


Figure 5-7. Glucose starvation of elongation factor methyltransferase knockouts demonstrate possible changes in elongation rates. (A) *efm4Δ* and (B) *efm2Δ* cells were each analyzed alongside a wildtype control. Peaks were integrated and analyzed using Graphical Analysis 3.8.4. Analysis is shown in Figure 5-6B and C.

Discussion

Deletion of multiple elongation factor methyltransferases in various combinations demonstrated the expected loss of methylation. One surprising result was the comparison of the triple knockout (*efm1Δefm4Δefm5Δ*) versus the quadruple knockout (*efm1Δefm4Δefm5Δefm6Δ*). The triple knockout in theory should have only 50% of the monomethyl lysine remaining as the only modification left should be mmK390. However, equal amounts of monomethyl and dimethyl lysine remained (Figure 5-2). Both peaks are lost when the fourth deletion is added in, indicating that these four enzymes are responsible for all of the methylations. This potentially indicates that Efm6 is capable of dimethylation and furthermore, that it is adding that second methyl group to lysine 316 rather than 390. This is due to the fact that mutating lysine 316 to an arginine results in total loss of dimethyl lysine (Figure 5-1). Construction of other triple knockouts with various combinations would reveal if any other cross-methylation was potentially occurring between these enzymes.

If in fact these enzymes can partially compensate for each other, it could account for the lack of noticeable phenotypes in the single knockouts. An ability to compensate for one another would be beneficial as it would prevent any detrimental effects in the case of a faulty methyltransferase.

This idea is further supported by other findings. As shown in Chapter Four, amino acid analysis of *efm6Δ* (*ynl024cΔ*) does not demonstrate as dramatic a loss of monomethyl lysine as is seen for *efm1Δ* (see Chapter Three). This could be due to the fact that another enzyme is compensating or that the EFMs are not perfectly specific, allowing them to methylate any of the sites. Additionally, Hart-Smith et al. 2014 provided mass spectrometry evidence that Efm1 and Efm4 both were responsible for methylating K30, implicating a cross-talk mechanism could be

in place. As our method of detecting methylated amino acids in this situation is not quantitative, methods such as stable isotope labeling with mass spectrometry would support or refute this idea. Comparing levels of methylation at each of the sites when various combinations of the enzymes are deleted would directly show if other enzymes are compensating for each other.

Individual deletion of Efm2 and Efm3 both showed increased sensitivity to verrucarin A, cycloheximide, and tunicamycin (Chapter Two). Here we wanted to determine if deleting both EF2 methyltransferases would provide a more dramatic phenotype. The double knockout demonstrated significantly increased sensitivity to cycloheximide although at this point, although it is unclear which methyltransferase contributes more to the phenotype or if it is a cumulative effect. Repeating the assay with individual knockouts would quantitatively demonstrate which methylation has a bigger influence. However, it is exciting that sensitivity to cycloheximide and verrucarin A increase as both directly affect the ribosomes ability to translocate. Cycloheximide blocks tRNA exit and verrucarin A prevents peptidyl transfer (Garreau de Loubresse et al 2014; Carrasco et al. 1973). This may indicate that loss of EF2 methylation can reduce its ability to translocate the ribosome. To demonstrate that the loss of methylation rather than the enzyme is responsible for this phenotype, we are currently constructing an EF2 point mutant that will mutate both lysine methylation sites to arginines. Additionally, testing the *efm2Δefm3Δ* strain with the frameshift Dual Luciferase reporter plasmids would reveal any increases in frameshifting. It is also possible that there could be an overall slowdown in elongation upon loss of EF2 methylation. Although there was no obvious reduction in elongation rate in the glucose starvation assay with *efm2Δ* (Figure 5-8), deletion of both enzymes could potentially have a more dramatic effect.

The dramatic increase in doubling time upon deleting multiple EF1A methyltransferases indicates that these cells are not as fit as wildtype yeast. Our current hypothesis is that loss of EF1A methylation increases the number of mistakes EF1A makes and would inevitably cause a cascade of problems that could slow down growth. This hypothesis is further supported by the changes in sensitivity to tunicamycin and paromomycin. Paromomycin induces misincorporation of amino acids (Salas-Marco and Bedwell 2005). If the ribosome is making more mistakes in the absence of elongation factor 1A methylation, the paromomycin may not have an effect. More mistakes would inevitably lead to an increase in the number of misfolded and potentially aggregated proteins. If the increase in misfolded proteins was enough to induce the unfolded protein response, this would also explain the resistance to tunicamycin which induces the UPR (Heifetz et al 1975; Chan et al. 2005; Hiramatsu et al. 2011). If the UPR is already active in these cells, tunicamycin would have little to no effect on them. A simple way to confirm this would be to analyze the splicing of HAC1 mRNA. Hac1 is a transcription factor required for the unfolded protein response (Cox and Walter 1996). Its level is controlled by alternative splicing: when the UPR is activated, Hac1 mRNA is found in two forms with different molecular weights. This makes it possible to monitor relative activation of the UPR (Chawla et al. 2011).

Our initial observations of elongation rates in *efm4Δ* cells indicates a potential slow down in elongation. If translation elongation is struggling and slowing down, it may be connected to the increase in mistakes. It has also been shown previously that mutating EF1A glutamate 317 to a lysine residue can affect translational fidelity (Sandbaken and Culbertson 1988). This mutation increases stop codon readthrough, frameshifting, and sensitivity to paromomycin. Glutamate 317 is adjacent to the Efm4 methylation site K316. If this methylation has a role in maintaining faithful translation, mutating the adjacent residue from a negatively charge to positively charged

residue may have affected methyltransferase recognition or the effectiveness of the methylated residue's interactions.

Although there are numerous follow up experiments to perform to solidify this interpretation, our results directly demonstrate an essential role of the elongation factor methyltransferases. In the absence of one, two, three, or four of these enzymes, there are varying effects on translation elongation as well as translational fidelity. It is likely that these modifications fine-tune the interactions of the elongation factors and the ribosome to ensure proper communication and faithful translation.

As it stands, loss of methylation has only been confirmed in two of the EF1A point mutants, K(30,390)R and K(79,316)R. The quadruple point mutant as well as the double EF2 point mutant (currently being constructed) also need to be checked for loss of methylation. Once that has been accomplished, replicates of the quadruple and *efm2Δefm3Δ* strains in all drugs of interest. Replicates were completed for cycloheximide, paromomycin, and tunicamycin but it would also be worthwhile testing anisomycin (4 μg/mL) as well as finding another drug that directly works on translocation to directly probe at possible problems with EF2.

With the inhibitors that demonstrate significant phenotypes in the mutants, including those seen with cycloheximide, paromomycin, and tunicamycin, should be “dissected”; in other words, determine which deletion has the most dramatic effect on sensitivity by testing each single knockout. When we initially screened in the individual knockouts, we were utilizing the spot test assay which was unreliable and only qualitative. When we switched to liquid growth assays, we started with the multiple knockouts to determine if there was any effect when all were missing. Initial screens of the individual EF1A methyltransferase knockouts indicated that Efm5

was the largest contributor to the paromomycin resistance but this needs to be reproduced. Additionally, the point mutants need to be tested. This will be crucial in an argument that the loss of methylation and not just the enzymes is the cause of any phenotype we observe.

Although there are numerous follow up experiments to perform that would help solidify this interpretation of our data, our results directly demonstrate an essential role of the elongation factor methyltransferases. In the absence of one, two, three, or four of these enzymes, there are varying effects on translation elongation and very likely on translational fidelity. It is likely that these modifications fine-tune the interactions of the elongation factors and the ribosome to ensure proper communication and faithful translation.

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Chapter Six

Analysis of Yeast Nnt1, a Putative Nicotinamide Methyltransferase:

Evidence for a Novel Type of Protein Methylation

Introduction

Methylation has proven to be a diverse and essential modification of many biomolecules. Methyl groups are added to DNA, RNA, lipids, small molecules and protein, illustrating the assortment of potential roles of methylation and the necessary diversity of the enzymes that transfer methyl groups. In the case of *Saccharomyces cerevisiae*, there are 86 predicted methyltransferases based on sequences for the characteristic folds that bind the methyl donor, S-adenosyl-methionine (AdoMet) (Petrossian et al. 2009). Over 80% of these predicted enzymes have at least one confirmed substrate leaving approximately ten that have yet to be classified (Appendix).

To narrow down the candidate substrates for the remaining enzymes, bioinformatic and structural studies have helped predict which type of molecule they will recognize (Wlodarski et al. 2011; Szczepińska et al. 2014 paper). To date, most of the SET domain-containing enzymes are protein lysine methyltransferases (Del Rizzo and Trievel 2011). The SPOUT structural class of methyltransferases typically methylate RNA molecules, although one is a protein arginine methyltransferase (Young et al. 2012). Other protein arginine methyltransferases primarily belong to the seven-beta strand structural family and contain a canonical “double-E loop” that assists in balancing out the positive charge of the substrate arginine. Additional information such as isoelectric point, localization, and expression patterns have been utilized to predict candidate substrates for putative methyltransferases (Szczepinska et al. 2014). These predictions have correctly distinguished protein and RNA methylating enzymes even for some of the “exception” enzymes. For example, the protein-arginine SPOUT methyltransferase was correctly associated as a protein methyltransferase by Szczepińska et al. 2014. Efm5 was initially classified as an Adenine-Like Methyltransferase (AML1) but was predicted by Szczepińska and later confirmed

to be a protein methyltransferase (Dzialo et al. 2014). These outliers led us to examine some of the other predictions and bioinformatics analyses done on the remaining candidate methyltransferases where substrates have not been identified.

One such candidate is YLR285W which was previously annotated as a Nicotinamide N-Methyltransferase (NNT1) based on sequence similarity to the human NNMT (Anderson et al. 2003). However, no study has actually confirmed whether or not YLR285W methylates nicotinamide.

In this study, bioinformatics analysis indicates that YLR285W is potentially an N-protein methyltransferase rather than a nicotinamide methyltransferase. Biochemical analysis of the methylation state of proteins in mutant *ylr285w* Δ yeast cells reveals that this enzyme may in fact be responsible for a novel type of protein methylation that has yet to be fully described. Here we provide several lines of evidence that the substrate of YLR285W is a protein as well as various biochemical descriptions of the modified residue. We have yet to confirm the identity of the substrate identity or on which type of residue this modification is made. The purpose of this paper is to present these data to the scientific community so that others may hypothesize or help us identify this novel methyl species.

Methods

Bioinformatic Analysis

Human nicotinamide N-methyltransferase (NNMT, UniProt P40261) and the human family 16 of methyltransferases (InterPro: IPR019410; Pfam: PF10294) were each subjected to

BLASTp (protein-protein BLAST) against the yeast proteome (taxid:4932). Yeast YLR285W (UniProt Q05874) and the other Group J enzymes (Petrossian and Clarke 2009) were subjected to BLASTp against the human proteome (taxid: 9606). Family 16 consists of ten enzymes: METTL18, METTL20, METTL21A, METTL21B, METTL21C, METTL21D, METTL22, METTL23, CaM-KMT, FAM86 (UniProt O95568, Q8IXQ9, Q8WXB1, Q5VZV1, Q9H867, Q96AZ1, Q9BUU2, Q86XA0, Q7Z624, and Q96G04, respectively). Group J consists of six enzymes: Efm2, Efm3, Efm6, Hpm1, Rkm5, and YLR285W (UniProt P38347, P47163, P53970, P40481, Q12367).

In Vivo Radiolabeling and Amino Acid Analysis

Strains used in this study were from the Yeast Knockout Collection and included BY4742, *ylr285wΔ*, *efm1Δ*, *efm2Δ*, *efm3Δ*, *efm6Δ*. All of the knockout strains are in the BY4742 background and were obtained from OpenBiosystems (now Dharmacon). Yeast culture, radiolabeling, and amino acid analysis were carried out as previously described with some minor changes (Dzialo et al. 2014). After lysates from *S*-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet) *in vivo* labeled wildtype and methyltransferase knockout cells were resolved by SDS-PAGE, the Coomassie-stained protein band running at 50 kDa was excised and placed into a 6 mm x 50 mm glass test tube. 100 μL of 6 N HCl was added to each slice and tubes were placed in a reaction chamber (Eldex Labs, 1163) containing 500 μl of 6 N HCl. Chambers were heated for 20 h *in vacuo* at 109°C in a Pico-Tag vapor phase apparatus (Waters). Residual HCl was removed by vacuum centrifugation. Dried gel slices were resuspended in 400 μl of cation exchange loading buffer (sodium citrate, 0.2 M Na⁺, pH 2.2). Half of the sample was spiked with 2 μmol of each methyllysine standard (Sigma, *N*_ε-methyl-L-lysine hydrochloride 04685, *N*_ε,*N*_ε-

dimethyl-L-lysine monohydrochloride 19773, $N_{\epsilon},N_{\epsilon},N_{\epsilon}$ -trimethyllysine hydrochloride T1660) and loaded onto a cation-exchange column (Beckman AA-15 sulfonated polystyrene resin, 0.9 cm inner diameter by 12 cm height) equilibrated with running buffer (sodium citrate, 0.3 M Na⁺, pH 3.85) at 55 °C. Amino acids were eluted in the equilibration buffer at 1 mL/min while collecting 1 min fractions at the expected elution position of the methyllysine standards. 50 µl of each fraction was added to a flat-bottom 96-well plate to detect standards by the ninhydrin method. The remainder of each fraction was added to 5 mL of scintillation fluor (Safety Solve, Research Products International) in a 20 mL scintillation vial and counted for three five min cycles using a Beckman LS6500 instrument to detect [³H]-methylated amino acids. Where noted in the figure legends, different portions of the SDS-PAGE gel were utilized for acid hydrolysis. Additionally, some experiments utilize a running buffer of a different pH, also noted in the figure legends.

SDS-PAGE Fluorography

In vivo labeled lysates from wildtype, *ylr285wΔ*, the EF1A methyltransferase quadruple knockout (*efm1Δefm4Δefm5Δefm6Δ*), and the EF2 methyltransferase double knockout (*efm2Δefm3Δ*) were loaded onto a 12% polyacrylamide gel (30 x 30 x 0.1 cm). The gel was stained with Coomassie dye and destained overnight. After imaging, gels were washed with water for 1 h and then incubated in EN³HANCE (PerkinElmer Life Sciences) solution for 1 h, followed by a 30 min wash with water. The gel was then dried onto 3 MM Whatman paper under vacuum for 2 h at 80°C, followed by 1 h without heat. The gel was placed with HyBlot Autoradiography film (Denville Scientific) at -80°C. The film was developed after a 1-week incubation.

In-Gel Trypsin Digests and Mass Spectrometry

Coomassie-stained gel slices from the 50 kDa region of fractionated polypeptides of labeled and unlabeled yeast cell lysates were destained and subjected to in gel trypsin digest with sequencing grade trypsin (Promega, V5111) as previously described (Dzialo et al. 2014). Digests were performed for 16 h at 37 °C and the peptides were eluted using 50% acetonitrile/1% trifluoroacetic acid (TFA) in water. Peptides were dried by vacuum centrifugation and resuspended in 200 µl of 0.1% TFA in water. The sample was loaded onto an Econosphere C18 column (Alltech 70071, 5 µm particle, 4.6 mm x 250 mm, 80 Å pore) equilibrated with Buffer A (99.9% water 0.1% TFA). Peptides were eluted with Buffer B (99.9% acetonitrile, 0.1% TFA) at 1 mL/min and 1 min fractions were collected. Aliquots of fractions from labeled samples were counted for radioactivity by Liquid Scintillation Counting and the rest was utilized for further analysis. Fractions from unlabeled samples were subjected to mass spectrometry as previously described for identification of methylated peptides.

o-Phthalaldehyde (OPA) Amino Acid Analysis

Acid hydrolyzed or pronase digested samples were reacted with *o*-phthalaldehyde as previously described (Patananan et al. 2013). Reactions were separated with an Eclipse AAA column (Agilent 993400-902, 5µm particle, 4.6mm x 150 mm) and detected by a Gilson Model 121 fluorometer (excitation and emission filters of 305-395 and 430-470 nm, respectively) set to 0.1 relative fluorescence units. Fractions were collected for detection of radioactive methyl groups by liquid scintillation counting.

Methyl esterification and thin layer chromatography.

Amino acid standards (1 μmol each, except 0.15 μmol for Asp) or radioactive methyl samples from the tryptic digests were added to 10 x 75 mm borosilicate tubes and dried by vacuum centrifugation. The samples were resuspended in 150 μL of 12 N hydrochloric acid:methanol (1:60, v:v) and incubated for at least 40 h in the dark. Reactions were quenched with the addition of 1 mL of water which were immediately dried down vacuum centrifugation. Samples were resuspended in 50 μL of water and 1 μL was loaded onto cellulose thin layer chromatography plates or sample was diluted 1000 fold for analysis by OPA. Silica plates were placed in chambers pre-equilibrated with 4:1:1 (v:v:v) n-butanol:acetic acid:water. The plates were removed when the solvent front reached approximately 1 cm below the top of the plate. Plates were sprayed with ninhydrin (0.2% w/v in ethanol) and incubated at 100°C until spots appeared.

Mass spectrometry of pronase digested samples

Large scale in-gel pronase digestion was adapted from Nwosu et al. 2013. The pronase (Calbiochem 537088) was a kind gift from Hong Nguyen of the Joseph Loo lab at UCLA. Briefly, gel slices were destained using a 50:50 mixture of 50 mM ammonium bicarbonate and acetonitrile as was done for tryptic digestion. After destaining, slices were incubated in 100 mM ammonium bicarbonate (pH 8.0) containing 0.025 $\mu\text{g}/\mu\text{L}$ of pronase for 30 min. Additional ammonium bicarbonate was added to fully cover the gel slices. Digests were incubated at 37°C for at least 16 h. After digestion, the supernatant was removed to a new tube. Digests were further recovered by incubating the gel slices in 5% formic acid in 60% acetonitrile in a sonicator

bath for 30 min. The supernatants were combined with the material from the initial recovery. This step was repeated once more and the digests were dried down by vacuum centrifugation.

Dried samples were resuspended in 0.1% trifluoroacetic acid in water and loaded onto an RP-HPLC column as described above for the tryptic digests. Approximately 25% of each fraction was counted for radioactivity. Fractions containing radioactive methyl groups were dried down by vacuum centrifugation.

Samples were resuspended in water and half was loaded onto a PLRPS column (Agilent) equilibrated with 0.1% formic acid in water. The flow from the LC was split so that ~1/3 of the volume was collected in one minute fractions and the remaining was sent directly to LCQ DecaXP mass spectrophotometer (Thermo). MS1 data was collected to determine parent ions of interest. The second half of each sample was run again this time collecting MS2 data for the selected parent ions.

Production of a radioactive dimethylproline standard.

A dimethylproline standard was created using *in vitro* methylation reactions with the N-terminal methyltransferase (Webb et al. 2010). Ntm1 N-terminally methylates peptides with an XPK recognition sequence. Briefly, 1 µg of recombinant Ntm1 (kind gift from Dr. Qais Al-Hadid) was incubated with 100 µM of a PPKQQLSKY peptide and 0.5 µM [³H]AdoMet in reaction buffer (100 mM NaCl, 100 mM sodium phosphate buffer, pH 7.0) for 1 h at 30°C. Reactions were terminated by the addition of 20 µL of 10% TFA. The methylated peptide was isolated by RP-HPLC and detected by liquid scintillation counting. The fractions containing

methylated peptide were then dried and acid hydrolyzed as described in Chapter Three. This hydrolysate was utilized as the radioactive dimethylproline standard.

Results and Discussion

YLR285W (NNT1) is not the homolog of human NNMT

YLR285W is annotated as a Nicotinamide N-Methyltransferase (NNT1) based on sequence similarity to the human NNMT (Anderson et al. 2003). However, the bioinformatic and biochemical evidence present below suggests that YLR285W is not a nicotinamide methyltransferase.

Although YLR285W is the only protein to be pulled from a BLAST search through the yeast genome utilizing NNMT as query, the E value is 7.0; not a substantial match. An alignment of these two proteins clearly demonstrates this dissimilarity (Figure 6-1). While the AdoMet binding Motifs I and Post-I align, the remaining Motifs II and III do not align using Clustal Omega and needed to be manually aligned. Utilizing YLR285W as a query against the human proteome, the top hits are from a family of protein lysine methyltransferases known as Family 16 (InterPro: IPR019410; Pfam: PF10294). Reciprocal searches with each of these family members extracts a subset of yeast enzymes, Group J, previously grouped together with other substrate predictions (Petrossian and Clarke, 2009). YLR285W comes up several times as the top hit for these human enzymes including METTL20, METTL22, METTL23, and CAM-KMT. Additionally, some of these yeast-human pairs have been shown to be orthologous; Efm3 and FAM86A are mutual best hits and both have been shown to trimethylate elongation factor 2. (Table 6-1)

Many groups have recognized a distinct Post Motif II sequence that is found in every enzyme both in Group J and Family 16 (Table 6-1) (Kernstock et al. 2012, Davydova et al. 2014, Zhang et al. 2014, Dzialo et al. 2014). This “DXXY” motif seems to be characteristic of N-methyltransferases, primarily protein lysine methyltransferases. The only deviation from this DXXY is Hpm1, a histidine methyltransferase, which contains an “EXXY” motif. This is potentially characteristic of a histidine methylator as the closest human homolog, METTL18, also contains EXXY. YLR285W contains “DLVF” at this position, associating it directly with protein N-methyltransferases. This motif missing in human NNMT (Figure 6-1).

New substrate prediction algorithms also predict YLR285W to be a protein methyltransferase. Wlodarski et al. 2011 predicted this enzyme to modify proteins and confirmed this *in vitro*. Combining recombinant YLR285W, lysate from a *ylr285wΔ* strain as substrate source, and [³H]AdoMet, this group demonstrated that YLR285W is capable of methylating a protein at 50 kDa. A 50 kDa substrate plus its connections to other lysine methyltransferases led us to consider YLR285W as a likely candidate for one of the remaining EF1A methyltransferases (three were unidentified at the time).

Hs_NNMT	-----MESG-FTSKDYLSHFNPRDYLEKYYKFGS-----RHSAESQIL
Sc_YLR285W	MSDIESLGEEAGLFEEPEDFLPP-PPKPHFAEYQRSHITKESKSDVKDIKRLVGTSPWL
	<div style="display: flex; justify-content: space-around;"> <u>Motif-I</u> <u>Motif Post-I</u> </div>
Hs_NNMT	KHLLKN--LFKIF---CLDGVKGDLLIDIGSGPTIYQLLSACESFKEIVVTDYSDQNLQ
Sc_YLR285W	GHLLWNAGIYTANHLDSHPelikgkTVLELGAAAALPSVICALNGAQMVVSTDYDPDLM
Hs_NNMT	ELEKWLKK-----EPEAFDWS---PVVTYVCDLEGNRVKGPEKEEKLQAVKQV
Sc_YLR285W	QNIDYNIKSNVPEDFNNVSTEGYIWGNDYSPLLAHIEKIGNN-----GK-----
	<div style="display: flex; justify-content: space-around;"> <u>Motif-II</u> <u>Motif-III</u> </div>
Hs_NNMT	LKCDVTQSQPLGAVPLPPADC-VLSTLCLDAACPDLPYCRALRNLGSLK-PGGFLVIMD
Sc_YLR285W	-----FDLIILSDLVFNHT-----EHHKLLQTTKDLAELKQALVVFS

Hs_NNMT	ALK-----SSYYMIGEQKFSSPLPLGREAVEAAVKEAGYTIEWFEVI-----SQ
Sc_YLR285W	PHRPKLEKDLEFFELAKNEFHLVPQLIE-----MVNWKPMFDEDEETIEVRSR
Hs_NNMT	SYSSTMANNEGLFSLVARKLSRPL 264
Sc_YLR285W	VYAYYLTHEK----- 261

Figure 6-1. Alignment of human NNMT and yeast YLR285W. Human NNMT (UniProt P40261) and *Saccharomyces cerevisiae* YLR285W (UniProt Q05874) were aligned using ClustalOmega. The alignment was adjusted to force-align Motif-II and III. Asterisks (****) indicate the Post-Motif II found in YLR285W and other Group J and Family 16 enzymes.

Yeast Protein	Human Homolog	E-value	% Identity	Substrate	Methylation type	Query Post Motif II	Homolog Post Motif II
Efm2	METTL21D	2.0E-09	29	EF2, K613	DMK	DPIY	DCIY
Efm3	FAM86A	8.0E-10	27	EF2, K509	TMK	DVTY	DVLY
Hpm1	METTL18	3.0E-15	31	Rpl3, H243	3-methylHis	ETIY	ETIY
Rkm5	METTL21A	5.2E+00	37	Rpl1, K46	MMK	DVIY	DIY
Efm6	METTL21A	2.0E-19	31	EF1A, K390	MMK	DCVY	DIY
YLR285W	METTL21C	4.0E-08	27			DLVF	DVY
Human Protein	Yeast Homolog	E-value	% Identity	Substrate	Methylation type	Query Post Motif II	Homolog Post Motif II
METTL21A	Efm6	4.0E-20	31	HSPA1, K561 & HSPA8, K561	TMK	DIY	DCVY
METTL21B	Efm6	1.0E-13	32			DIVY	DCVY
METTL21C	Efm6	3.0E-12	29	METTL21C, K35	MMK	DVY	DCVY
METTL21D	Efm6	5.0E-17	34	VCP, K315	TMK	DCIY	DCVY
METTL23	YLR285W	2.0E-06	25			DVFF	DLVF
FAM86A	Efm3	1.0E-10	27	EF2, K509	TMK	DVLY	DVTY
CaM-KMT	YLR285W	2.6E-02	22	CALM, K116	TMK	DCLF	DLVF
METTL20	YLR285W	1.0E-06	24	ETFBeta, K199 & K202	TMK	DMFY	DLVF
METTL18	Hpm1	6.0E-16	31			ETIY	ETIY
METTL22	YLR285W	2.0E-03	34	KIN, K135	TMK	EVFY	DLVF

Table 6-1. Comparison of Yeast Group J and Human Family 16 methyltransferases. Each member of Group J was used as query in a BLAST search against human proteins. Each member of Family 16 was used as query against yeast proteins. The best hit for each is listed under “homolog” columns with the respective E-values and % identity provided by BLAST results. Known substrates, residue of methylation, and methylation type were taken from UniProt. The Post-Motif II for the query and the homolog are listed.

Yeast enzymes are listed as protein names. Corresponding gene names are as follows: Efm2 (YBR271W), Efm3 (YJR129C), Hpm1 (YIL110W), Rkm5 (YLR137W), Efm6 (YNL024C)

Abbreviations:

Efm = elongation factor methyltransferase, Hpm = histidine protein methyltransferase, Rkm = ribosomal lysine methyltransferase
METTL = methyltransferase-like, EF = elongation factor, Rpl = ribosomal protein large (subunit), HSP = heat shock protein, VCP = valosin containing protein, CALM = calmodulin, ETF = electron transfer flavoprotein, KIN = DNA/RNA-binding protein Kin17

YLR285W is responsible for the presence of a methylated species in a 50 kDa protein

To determine if YLR285W was a possible EF1A methyltransferase, we *in vivo* radiolabeled cells from wildtype and *ylr285wΔ* strains with [³H]AdoMet. Cells were lysed and the polypeptides in 200 μg of lysate were separated by SDS-PAGE. The 50 kDa region from both strains was excised and acid hydrolyzed. Separation of the labeled amino acids demonstrated no major differences in the methyllysine region between wildtype and *ylr285wΔ* (Figure 6-2A). However, we noticed that a radioactive peak that is always seen in the flow through of the column (~5 min elution) disappeared in *ylr285wΔ* (Figure 6-2B). We initially believed this peak to be a [³H]AdoMet breakdown product as it is seen in every run and did not match an elution profile of any of our typically studied methyl-amino acids. We therefore repeated this several times with other various methyltransferase knockout strains and observed the same dramatic reduction only in *ylr285wΔ* (Figure 6-2C).

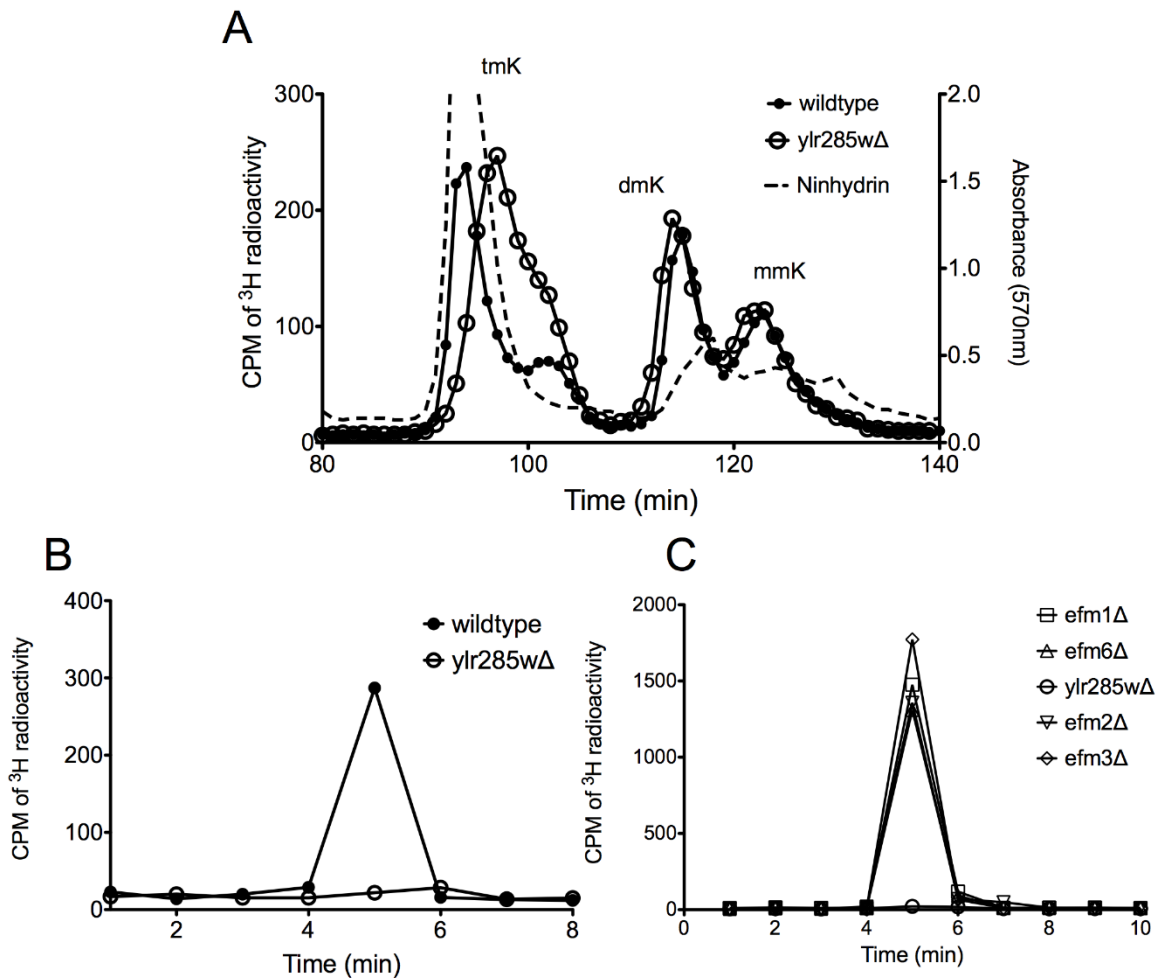


Figure 6-2. Deletion of YLR285W results in loss of an early eluting methyl species. Wildtype and methyltransferase knockout strains were *in vivo* labeled with [³H]AdoMet and the 50 kDa protein was subjected to amino acid analysis. (A) There are no major changes in the relative amounts of methyl lysines in *ylr285wΔ* yeast. (B and C) A radioactive species eluting early from the cation exchange column is greatly reduced in *ylr285wΔ* but not in knockouts of other elongation factor methyltransferases.

To evaluate if this modification is confined to the 50 kDa region, we repeated the amino acid analysis on larger sections of the lysate. Wildtype, *ylr285wΔ*, and a quadruple knockout of EF1A methyltransferases (*efm1Δefm4Δefm5Δefm6Δ*, see Chapter Five for construction of this strain) were tested. Lanes on the SDS-PAGE were divided into four parts, 20-40 kDa, 40-60 kDa, 60-85 kDa, and 85-150 kDa and subjected to acid hydrolysis. Analysis of the early eluting methyl modification demonstrates that the majority of the radioactivity is confined to the 40-60 kDa range (~60% of detectable modification throughout the lysate) with a smaller amount (~30%) found in the 20-40 kDa region (Figure 6-3A). It is currently unclear if the modification found in the lower range is due to breakdown of the major substrate or if there is an additional smaller substrate. Very little (<10%) was found above 60 kDa. This distribution was matched in the EFM quadruple knockout strain. Additionally, there was little to no detectable modification in the *ylr285wΔ* samples in all molecular weight ranges.

To narrow down the polypeptide size of the YLR285W substrate, we divided the 50 kDa band into smaller sections. The large band visible at 50 kDa is actually comprised of three distinct bands only visible if the gel is resolved for longer periods of time and at lower concentrations of protein (Figure 6-3B). These three bands were excised separately from both wildtype and *ylr285wΔ* samples and subjected to amino acid analysis. The majority of methyl lysines (corresponding to EF1A) and the early eluting unknown methyl modification was found in the upper and middle bands (Figure 6-3B). This is reflected in a visualization of the radioactive proteins as seen by SDS-PAGE fluorography of *in vivo*-labeled lysates where a band visualized by fluorography is found only on the upper Coomassie-stained band (Figure 6-3C). This band is significantly reduced in the EFM quadruple mutant and slightly reduced in the *ylr285wΔ* lysates.

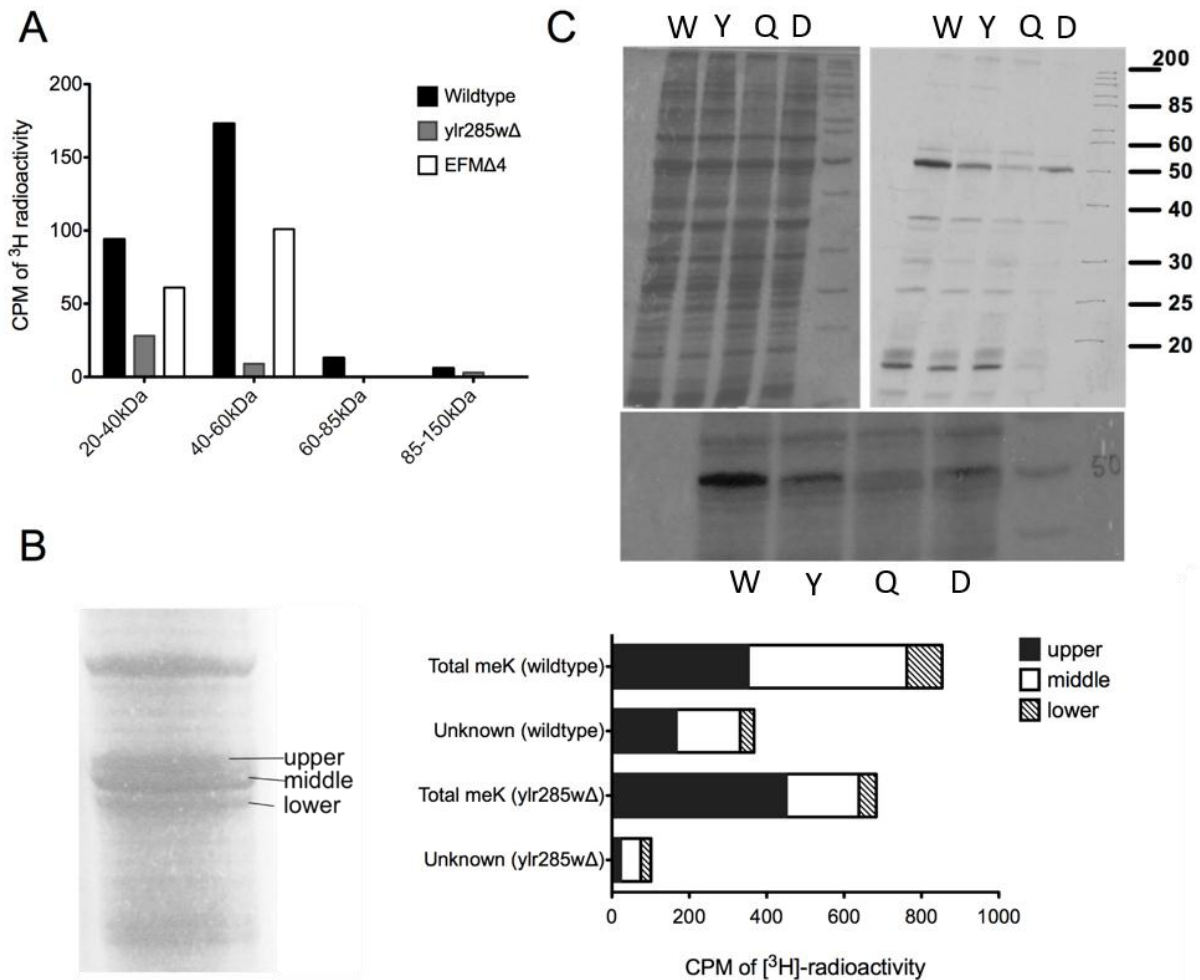


Figure 6-3. The unknown methyl modification is found primarily at 50 kDa on an SDS-PAGE gel. (A) Coomassie-stained, *in vivo*-labeled lysates from wildtype, *ylr285wΔ*, and the EFM quadruple knockout were cut into four 20 kDa ranges and subjected to amino acid analysis. (B) the 50 kDa region on a Coomassie stained gel is comprised of an upper, middle, and lower band each of which was subjected to amino acid analysis (left). Peaks corresponding to the unknown modification and the methyl lysines were quantified for each band (right). (C) Fluorography reveals exact location of potential YLR285W substrate. Top left: Coomassie stained gel, Top right: Fluorograph, Bottom: zoom of 50 kDa region with the fluorograph overlaying the Coomassie gel. W = wildtype, Y = *ylr285wΔ*, Q = EFM quadruple knockout, D = double knockout of EF2 methyltransferases (*efm2Δefm3Δ*).

Tryptic digestion of the 50 kDa band indicates protein association of the unknown modification

In an attempt to isolate this modification still attached to a larger portion of the protein rather than just the amino acid, we subjected the radiolabeled 50 kDa slice to tryptic digestion instead of acid hydrolysis. The resultant peptides were separated by RP-HPLC and the subsequent fractions were counted to track methyl modified peptides. Wildtype peptides consistently demonstrated two major radioactive peaks eluting from the column with several other minor peaks (Figure 6-4A). Only one major change was noted in the *ylr285wΔ* samples, the loss of the first major peak.

We then repeated the digest and separation with some alterations. Only half of each fraction was counted to pinpoint fractions containing radioactivity. The remaining volume was subjected to acid hydrolysis and analysis by cation exchange chromatography and OPA analysis. The chromatography allows us to “map” which type of modification is found in each HPLC fraction (Figures 6-4B and C). The unknown modification mapped to the early HPLC fractions as determined by the 5 min elution on cation exchange (Figure 6-4B). Interestingly, this peak also elutes in the flow through in OPA analysis. The later fractions contained different combinations of the mono-, di-, and tri-methyllysine corresponding to the known EF1A modifications (Figure 6-4C).

We repeated the tryptic digestion with unlabeled samples to potentially identify the peptide and modification via mass spectrometry as described in Dzialo et al. 2014. Analysis of later peptides containing methyl modifications matched up with expected EF1A methylated peptides. For example, fraction 33 demonstrate presence of mmK and dmK (Figure 6-4D) and mass spectrometric analysis revealed the presence of peptides containing mmK30 and peptides

containing dmK316 (Figure 6-S1). Unfortunately, no usable spectra were obtained from the early fraction. We therefore turned to obtaining the modified residue alone to try and identify just the type of modification.

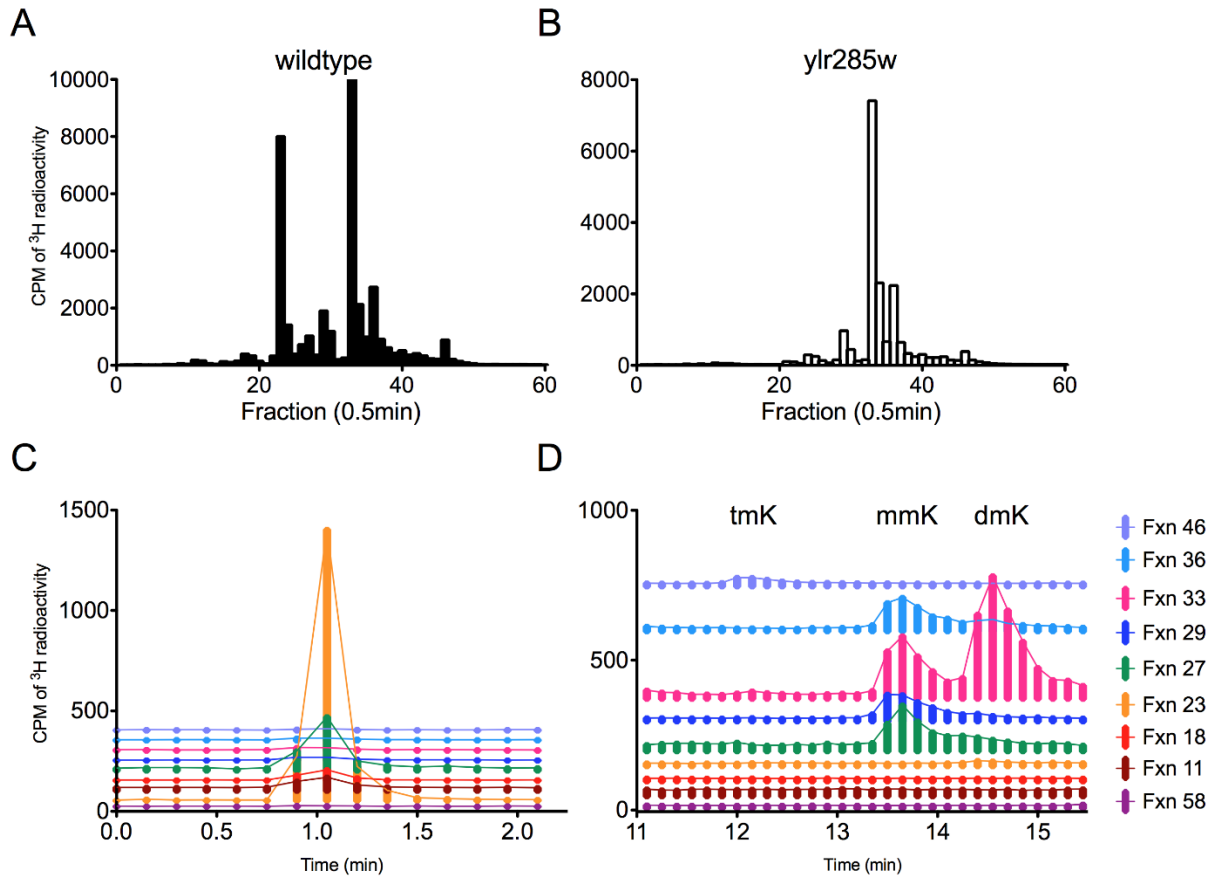


Figure 6-4. HPLC separation of in-gel tryptic digests of the 50 kDa region from *in vivo* labeled lysates. The 50 kDa region from labeled (A) wildtype and (B) *ylr285wΔ* cells were subjected to in-gel tryptic digest. The resultant peptides were separated by RP-HPLC and fractions were analyzed for presence of [³H]-methyl groups. Fractions containing radioactivity were subjected to acid hydrolysis. Resultant hydrolysates were analyzed by OPA analysis to “map” the type of methylated residue to each fraction. (C) the early eluting unknown modification was found primarily in fractions 23 and 27 (with some found in 11 and 18). (D) Methyl lysines were found in the later fractions.

Acid hydrolysis of the unknown modification likely results in a breakdown product containing the methyl group

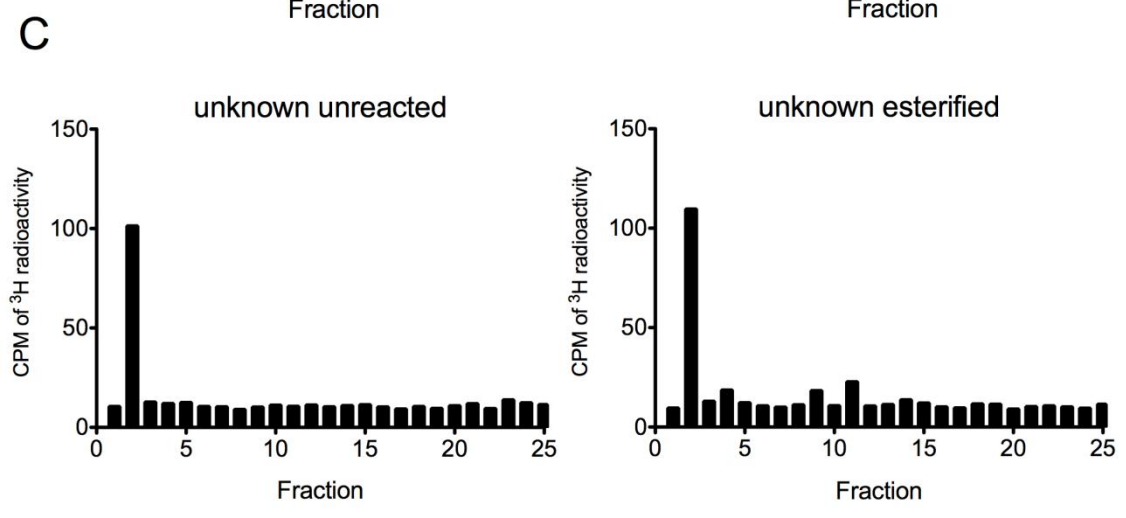
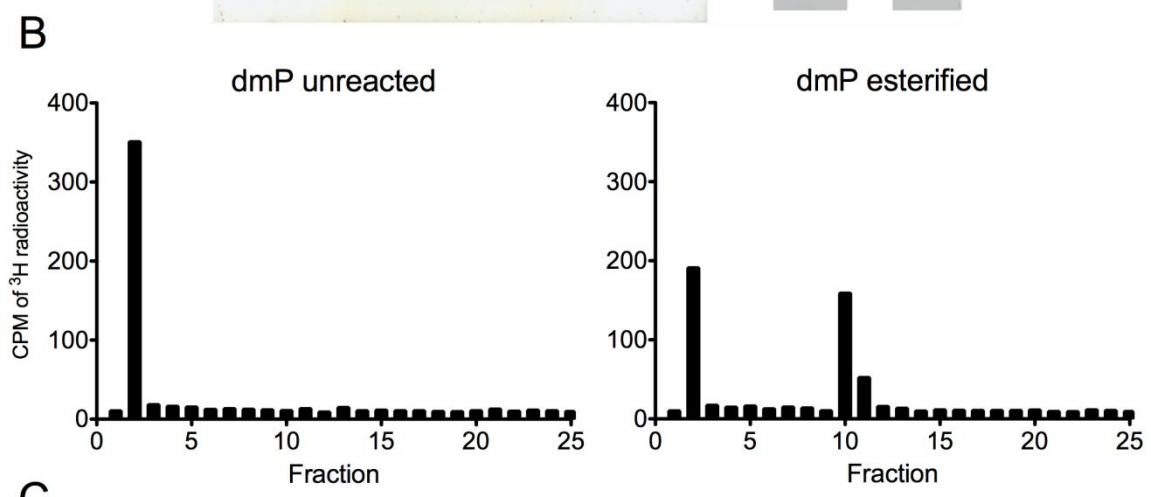
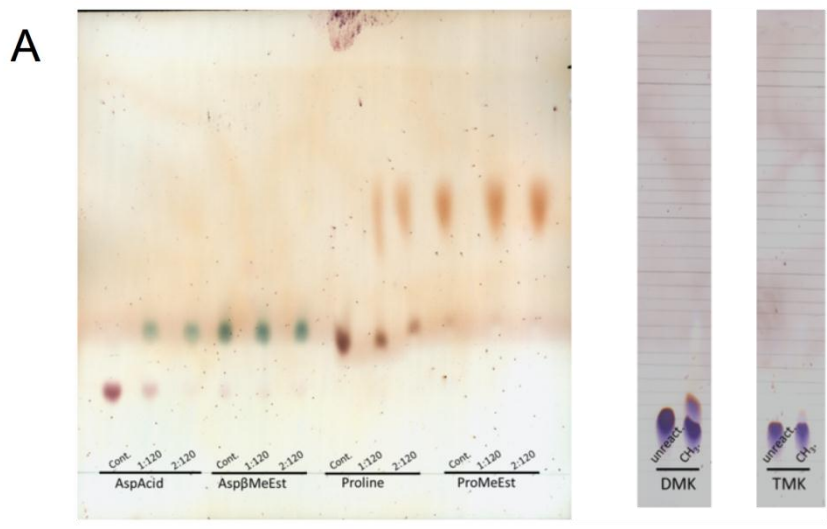
The early elution of the unknown in both cation exchange separations and OPA separations indicated that the modified species was very hydrophilic, possibly with a negative or neutral charge, and that the amino terminus was blocked or absent (OPA reacts with the free amino group of amino acids). We were concerned that this species was not an intact amino acid. To test for the presence of the carboxyl terminus of the amino acid, we attempted to methyl esterify the modified species. We utilized several amino acid standards, both unlabeled and tritiated, to test this method. Reacted and unreacted amino acids were analyzed by thin layer chromatography (for unlabeled standards) and OPA analysis (for tritiated samples – the salt from the acid hydrolysis makes it difficult to analyze by TLC).

Interestingly, not all amino acids were as easily esterifiable as others were. For example, almost all of the proline and aspartic acid standards converted to their methyl ester counterparts whereas dimethyllysine and trimethyllysine only reacted very little (Figure 6-5A). Approximately 45% of a radioactive DMP standard was converted to a methyl ester form (Figure 6-5B). There is potentially a correlation with charge, where positively charged amino acids have more difficulty reacting but negative or neutral amino acids work better with this method. The acid hydrolyzed unknown modification had almost no detectable change in retention after the methyl esterification reaction. In some replicates, there would be small amounts of radioactivity found in later fractions (Figure 6-5C, top) but this was not consistent between replicates (Figure 6-5C, bottom) indicating that this species is highly positive or no carboxyl group is present. This left us with two options for the identity of the YLR285W product – either the modification is in

fact on a different type of molecule, or the acid hydrolysis is causing a secondary chemical reaction resulting in a breakdown product that contains the tritiated methyl group.

To prevent any secondary acid-catalyzed reactions, peptides obtained from the tryptic digestion and RP-HPLC separation were subjected to pronase digestion. We then subjected each fraction to various types of chromatography. After pronase digestion, the radioactivity from the unknown modification fraction no longer eluted early from the cation exchange column but shifted to approximately 42 minutes, prior to the methyllysines (Figure 6-6A). Additionally, the radioactive species now reacted with OPA and was 100% methyl esterifiable (Figures 6-6B and C). This indicates that with pronase digestion, the amino and carboxyl group remained associated with the methylation but acid hydrolysis causes a breakdown of this modified species.

Figure 6-5. The acid hydrolyzed form of the unknown modification does not have an amino group or a carboxyl group. (A) Amino acid standards (0.15 μmol of Asp and Asp β methyl ester, 1 μmol of Pro, Pro-methyl ester, dmK, and tmK) were subjected to methyl esterification with 0:120, 1:120, or 2:120 (v:v) of HCl:methanol for 40 hrs. The reaction was quenched with water and dried down, and resuspended in 50 μL of water. The samples were loaded onto cellulose plates and placed in chambers with 4:1:1 (v:v:v) n-butanol:acetic acid:water. Standards were visualized with ninhydrin. When no HCl is added, no methyl esterification occurs to the unesterified standards. Increasing HCl levels increases present of the methyl ester form. The positively charged dmK and tmK had very little conversion to the methyl ester form. (B) Methyl esterification of the radioactive dmP standard was performed as done in (A) except samples were analyzed using OPA separation. Even though dmP cannot react with OPA, the addition of the methyl group changes the hydrophobicity and the retention of the molecule. (C) Methyl esterification of the unknown was performed and analyzed as in (B). This molecule does not react with OPA (as seen in Figure 6-4) but also has no change in retention after esterification indicating no reaction occurred.



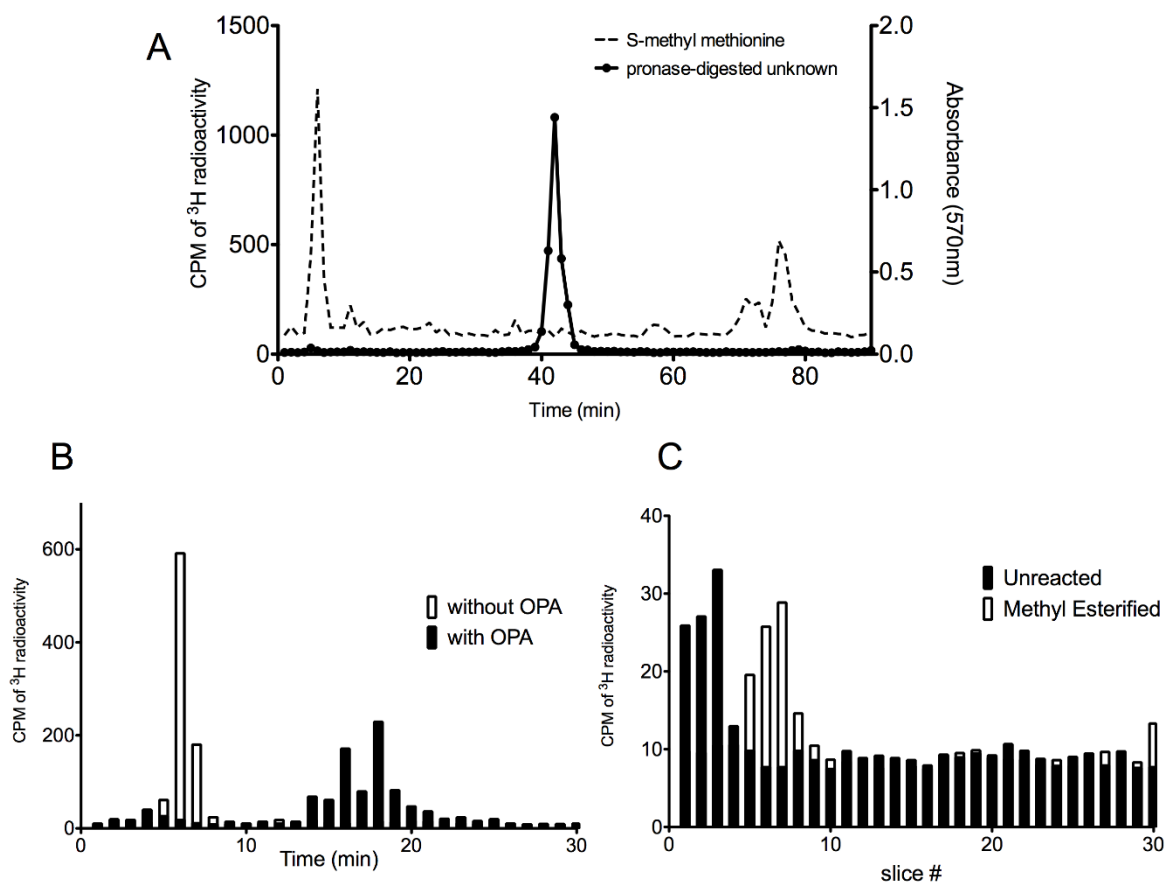
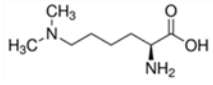
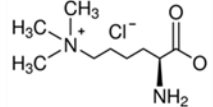
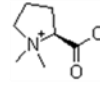
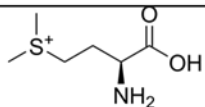
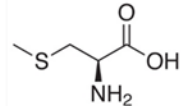


Figure 6-6. Pronase digestion of unknown-containing tryptic peptides demonstrates presence of intact amino and carboxyl groups. After tryptic digestion and HPLC separation of radioactive 50 kDa polypeptides, fractions containing the unknown modification were subjected to pronase digestion instead of acid hydrolysis. (A) The radioactivity now elutes at 42 min off of the cation exchange amino acid analysis column. (B) The unknown is now slightly retained on the hydrophobic Eclipse AAA column and can react with OPA. (C) The unknown can now be methyl esterified as seen by change in retention on cellulose thin layer chromatography plates.

Exploring other possible methylated amino acids

Proteins are commonly methylated on arginine and lysine residues. We explored several other candidates and biochemically analyzed them alongside the unknown methyl species (Table 6-2). The retention factor of methyl-cysteine matches the acid hydrolyzed unknown in two different TLC solvents as well as the elution time on cation exchange at pH 4.25. However, at pH 3.25, methyl-cysteine elutes at 10 minutes while the unknown remains at 5 min. Dimethylproline (dmP) matches the elution time of the unknown at pH 3.25, the OPA elution time (dmP has a blocked N-terminus and therefore cannot react with OPA), but only matches one of the retention factors by TLC. Additionally, dmP is methyl esterifiable, with approximately 50% of the sample reacting. When compared to the pronase digested unknown, DMP no longer matches the chemical characteristics. We also considered *S*-methyl methionine (SMM). This modification was ruled out for two reasons. First, it elutes off of cation exchange at 75 min, long after the elution of the pronase digested unknown. Second, acid hydrolysis of [*methyl*-³H]-SMM or [*methyl*-¹⁴C]-SMM did not fully convert to the early elution time. Although some of the SMM was initially found in the 5 min fraction before hydrolysis, we expected full conversion based on there being no other radioactive peak corresponding to the intact SMM in the hydrolyzed sample. (Table 6-2 and Figure 6-7)

Table 6-2. Biochemical characteristics of various methylated amino acids and the unknown “mystery” modification.

Residue	TLC Rf 1	TLC Rf 2	OPA elution	Methyl esterify-able?	Elution on cation*	Structure
Mystery Modification – Acid Hydro	0.6	0.6	1 min (FT)	No	5 min (FT)	?
Mystery Modification – pronase	0.14	0.1	18 min	Yes! ~100% Rf2 = 0.2	42min	?
DMK (dimethyl lysine)	0.5	0.1	12.5 min	~30%	~100min	
TMK (trimethyl lysine)	0.2	0.1	10.5min	<5%	~100min	
DMP (dimethyl proline)	0.5	0.4	1 min	~45%	5 min	
S-methyl methionine	<i>nt</i>	<i>nt</i>	<i>nt</i>	<i>nt</i>	~75min	
Methyl cysteine	0.6	0.6	<i>nt</i>	<i>yes</i>	5 min (@pH4.25) 10 min (@pH 3.25)	

nt = not tested; TLC conditions: (1) 3:1 methanol:ammonium hydroxide (2) 4:1:1 acetic acid:water:n-butanol; *notes regarding cation exchange chromatography – methyl lysine elution times vary between 100-130 depending on pH (3.8-5.5); the acid hydrolyzed unknown always 5 min regardless of pH; runs for the pronase digested unknown and the s-methylmethionine were run with pH 4.5.

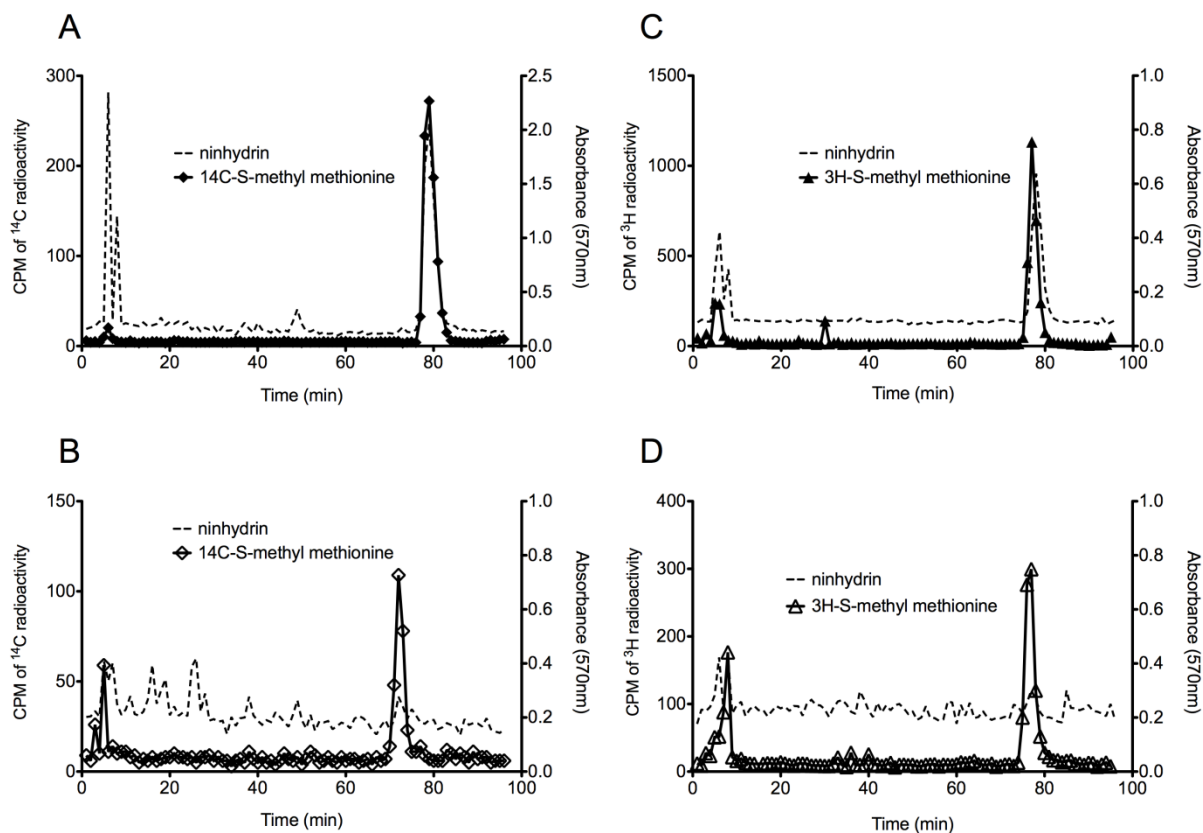


Figure 6-7. Amino acid analysis of radioactive *S*-methylmethionine standards. [^{14}C]SMM and [^3H]SMM were loaded onto a cation exchange column equilibrated with 0.3 M sodium citrate, pH 4.5 before (A and C) and after acid hydrolysis (B and D). The hydrolysis resulted in slightly higher levels of the early eluting breakdown product. However, in sample runs of wildtype hydrolysate, there is no peak that would correlate with the later eluting SMM peak.

Isolation of the modification via in-gel nonspecific proteolysis with pronase

We attempted to purify a significant amount of the unknown modified species but circumventing several steps of chromatography. We produced a large amount of lysate from each strain (wildtype, EFM Δ 4, and *ylr285w* Δ) and loaded over 5 mg onto three individual SDS-PAGE gels. The 50 kDa bands were excised and destained using the trypsin digestion protocol. We then

subjected the slices to in-gel pronase digestion. This method was adapted from Nwosu et al. 2013 which was able to isolate short, glycosylated peptides in this way. We separated the peptides by RP-HPLC and tracked loss of the unknown or the methyl lysines by radioactivity. The corresponding peaks were subjected to nano-LC mass spectrometry.

The methyl lysine-containing fractions demonstrated elution of the methyl derivatives in the 20-25 min range. There is a promising peak with m/z 107 (Figure 6-8B) which is found in extracted ion chromatograms for wildtype and the EFM quadruple knockout (Figure 6-8A and C). It is also found in the *ylr285w* Δ but at lower levels, which is consistent with the residual amount of radioactivity we also see in this strain (Figure 6-8D). However, the modified species seems to elute in the flowthrough and therefore decent spectra were not obtained. We will therefore be attempting to use a mixed bed HPLC column and rerun the sample for cleaner spectra. Parent ions were identified for the methyl lysine-containing samples which are being prepared for secondary runs for obtaining MS2 spectra.

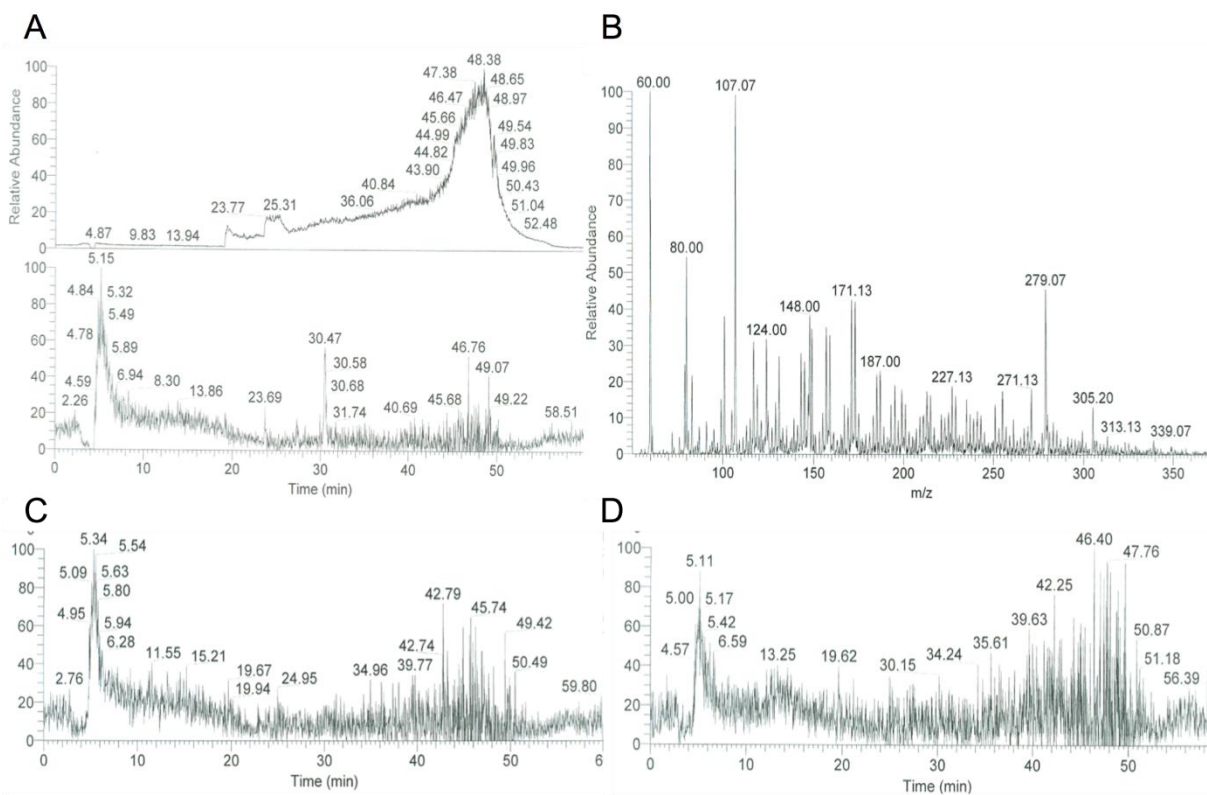


Figure 6-8. Mass spectrometric analysis of the unknown-containing pronase-digested HPLC fraction from wildtype, the EFM quadruple knockout, and *ylr285wΔ*. (A) Total ion chromatogram (top) and extracted ion chromatogram for the 107 m/z peak for the wildtype sample (B) mass spectrum of the 4-6 min range of the wildtype sample. (C) Extracted ion chromatogram for the 107 m/z from the EFM quadruple mutant and (D) *ylr285wΔ*.

Exploring the possibility that the unknown modification is the unusual diphthamide modification on elongation factor 2.

We considered the possibility that we were observing the diphthamide modification on elongation factor 2. Diphthamide is a unique modification on a histidine residue that involves several methylation and methylation-like modifications during its biosynthesis (Lin et al. 2014). The currently accepted biosynthetic pathway requires seven enzymes (Dph1-7). Dph1-4 perform

the first step by transferring a 3-amino-3-carboxypropyl (ACP) radical from *S*-adenosyl-methionine to the imidazole of the histidine residue. Next, Dph5 trimethylates the amino and carboxylate groups on the molecule. Dph7 then de-methylates the carboxyl group and Dph6 performs an amidation of the carboxyl group (Schaffrath et al. 2014).

Although histidine is a positively charged residue and should have been retained on the cation exchange column, we knew from our analysis that the initial acid hydrolyzed unknown was likely a breakdown product of the intact modified residue. Additionally, mass spectrometry analysis from EF1A purification mentioned in Chapter Four and data base searches for identifying peptides in Chapter Three identified breakdown products of EF2 at 50 kDa despite its molecular weight of 93 kDa. Lastly, the unusual ability for DPH5 to perform an O- and N-methylation led us to wonder whether or not a second methyltransferase could be involved.

To test this theory, we used amino acid analysis with deletion strains of the seven diphthamide synthesis genes (*DPH1*, *DPH2*, *DPH5*, and *DPH7*). If in fact we were looking at the diphthamide, deletion of *DPH1* or *DPH2* should have completely knocked out the modification and deletion of *DPH5* should have significantly lowered it. *DPH7* deletion should have had very little effect or potentially even slightly increase the levels of radioactivity.

Initial analysis of *dph1Δ* and *dph2Δ* showed promising results (Figure 6-9A). The total amount of radioactivity in the early eluting peak decreased in both mutants. When *dph5Δ* and *dph7Δ* were compared to wildtype, the reduction was not as dramatic (Figure 6-9B). The results with *dph1Δ* and *dph2Δ* indicate that the modification is not likely diphthamide. All four enzymes are required for the addition of the ACP molecule so in theory the radioactivity should have

disappeared. This should be repeated to ensure this is the case and perhaps even in a double mutant of Dph1 and Dph2 or a quadruple deletion of Dph1-4

Loss of the diphthamide modification results in increased frameshifting. This unusual modification directly relates to our overarching hypothesis that methylation in translation fine-tunes interactions to ensure proper protein synthesis. It would be worthwhile to find the peak corresponding to diphthamide in the EF2 (90 kDa) gel slice and determine if it also present in the EF1A (50 kDa) region due to breakdown. This would ensure that we are not mistaking the diphthamide for any of our methyl amino acids. It would also help firmly rule out diphthamide as the unknown modification. This would also provide an opportunity to explore the unusual ability for Dph5 to perform both an O- and N-methylation. Lastly, identifying one or two DPH mutants that completely eliminate or only partially synthesize diphthamide would provide an interesting and useful control for the phenotype analysis of elongation factor methylation.

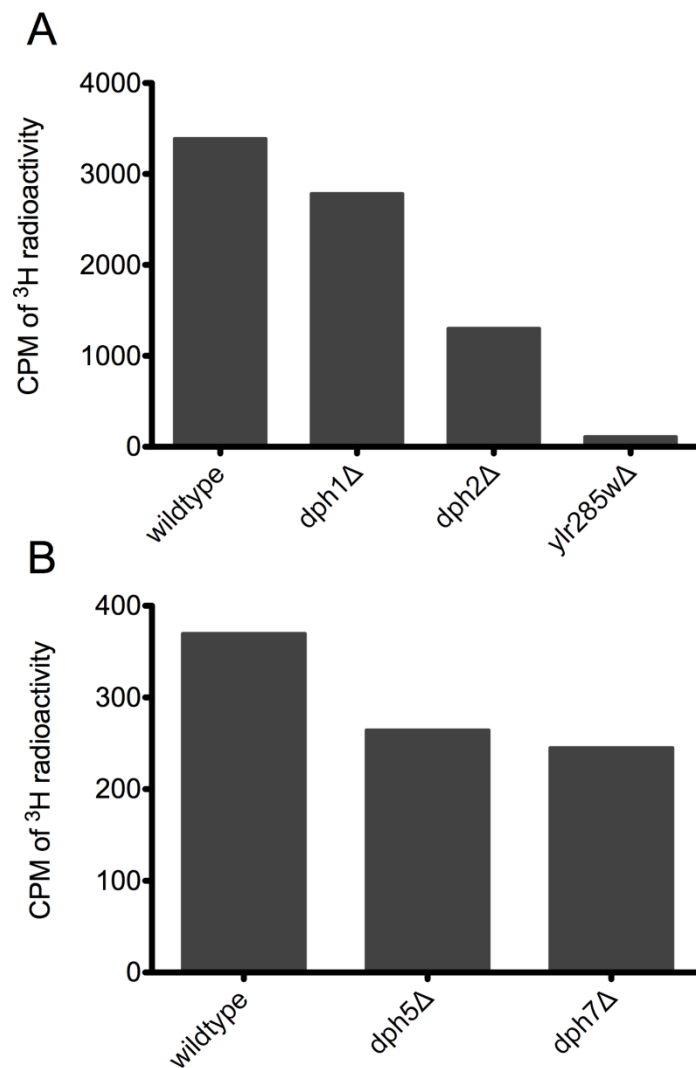


Figure 6-9. Deletion of diphthamide synthesis enzymes results in decreased, but not loss of, the unknown modification. Deletions of *DPH1*, *DPH2*, and *DPH7* were analyzed by amino acid analysis. Top: amino acid analysis by cation exchange chromatography. Bottom: amino acid analysis by OPA.

Final thoughts on the unknown modification

Although the unknown modification or substrate is not yet identified, significant progress has been made in understanding the biochemical characteristics of this modification. Additionally, the current status of the pronase-digest mass spectrometry experiments have given some promising leads. Attempting the experiment again with the mixed-bed column should

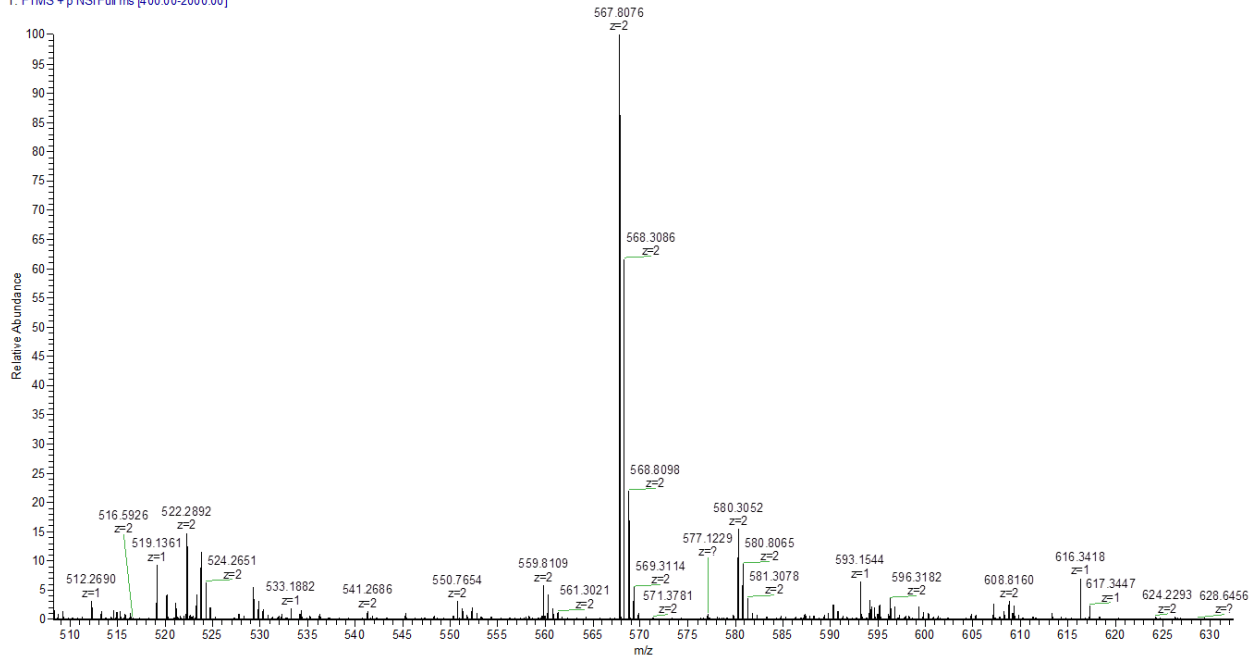
allow the modification to be retained for production of quality mass spectra. Additionally, we plan to test this with positive and negative ion mode to ensure we catch it.

It may also be worth trying 2-dimensional gel electrophoresis with the *in vivo* labeled lysates to determine if this modified protein separates from EF1A. This may also provide a way to identify the substrate. On a similar note, 2-dimensional TLC of the pronase digested sample could help identify the residue or at least provide additional information about its chemical characteristics. 2D-TLC has been used in the past to identify other methyl amino acids (Ohba et al. 1979) and could provide relevant information.

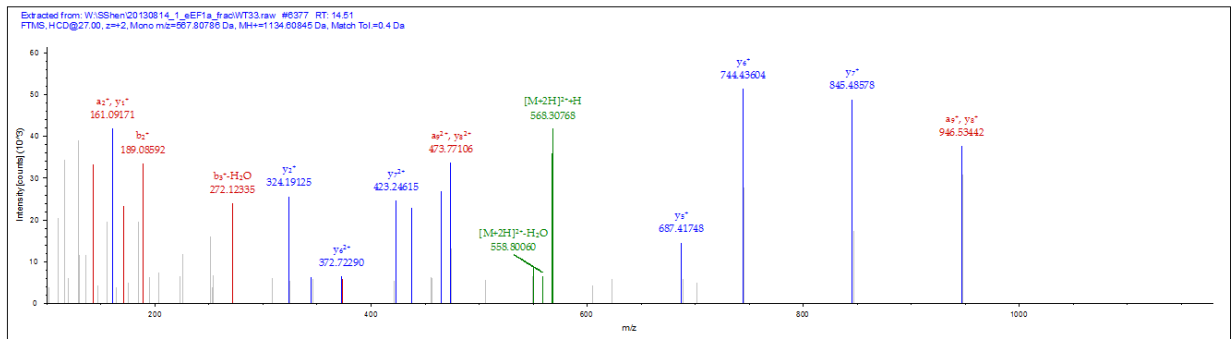
Overall, this Chapter describes significant findings in the realm of methylation. First, that YLR285W is very likely a protein methyltransferase, not the nicotinamide methyltransferase. Second, we have clearly found an unusual, likely novel, type of modification. Once identified, this will further our understanding of the diversity of protein methylation.

Supplemental Figures

Figure 6-S1. Identification of methylated EF1A peptides from fraction 33 of the tryptic digest. (A and B) MS1 and MS2 corresponding to the monomethyl lysine 30 peptide on EF1A. (C and D) MS1 and MS2 corresponding to the dimethyl lysine 316 peptide on EF1A.

ASTTTGHLIYK_(MMK30) MS1 – 567.8076 (M+2H⁺)WT33 #5982-6488 RT: 13.80-14.71 AV: 46 NL: 1.60E7
T: FTMS + p NSI Full ms [400.00-2000.00]**B**STTTGHLIYK_(MMK30) MS2

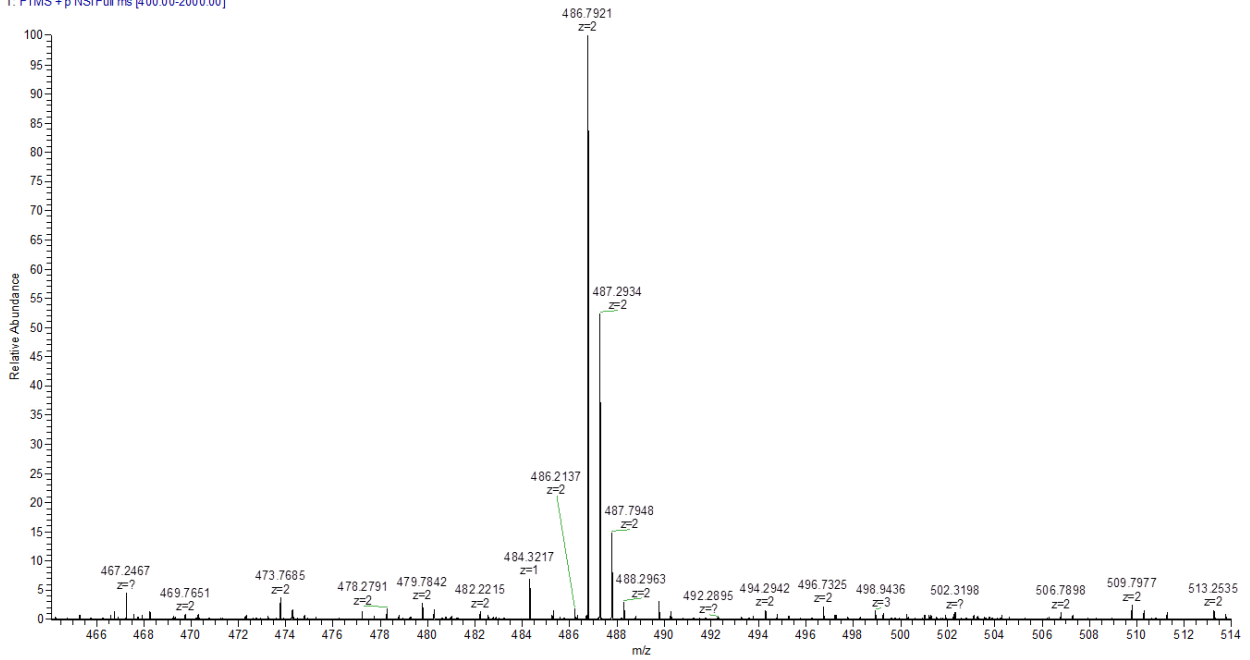
#1	a ⁺	a ²⁺	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	60.04439	30.52583	88.03931	44.52329	S			10
2	161.09207	81.04967	189.08699	95.04713	T	1047.58334	524.29531	9
3	262.13975	131.57351	290.13467	145.57097	T	946.53566	473.77147	8
4	363.18743	182.09735	391.18235	196.09481	T	845.48798	423.24763	7
5	420.20890	210.60809	448.20382	224.60555	G	744.44030	372.72379	6
6	557.26781	279.13754	585.26273	293.13500	H	687.41883	344.21305	5
7	670.35188	335.67958	698.34680	349.67704	L	550.35992	275.68360	4
8	783.43595	392.22161	811.43087	406.21907	I	437.27585	219.14156	3
9	946.49927	473.75327	974.49419	487.75073	Y	324.19178	162.59953	2
10					K-Methyl	161.12846	81.06787	1



C

NVSVK_(DMK316)EIR MS1 – 486.7921 (M+2H⁺)

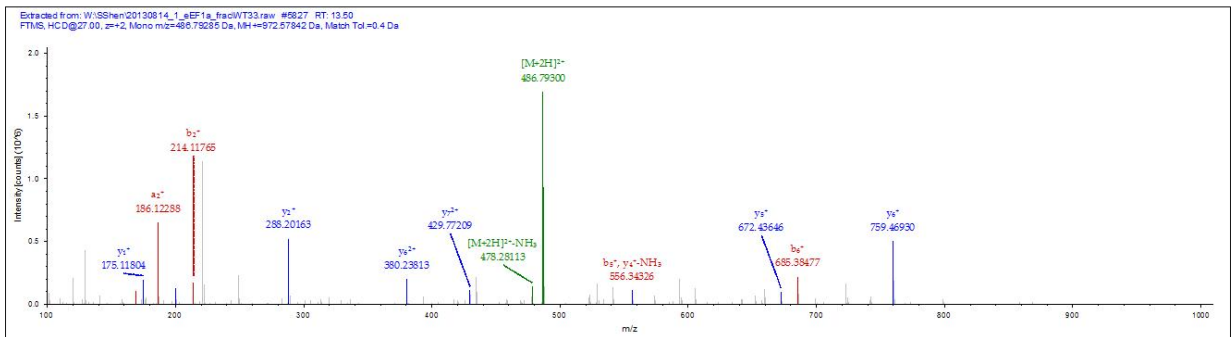
WT33 #5706-6043 RT: 13.30-13.88 AV: 30 NL: 1.89E7
T: FTMS + p NSIFull.ms [400.00-2000.00]



D

NVSVK_(DMK316)EIR MS2

#1	a ⁺	a ²⁺	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	87.05529	44.03128	115.05021	58.02874	N			8
2	186.12371	93.56549	214.11863	107.56295	V	858.54077	429.77402	7
3	273.15574	137.08151	301.15066	151.07897	S	759.47235	380.23981	6
4	372.22416	186.61572	400.21908	200.61318	V	672.44032	336.72380	5
5	528.35043	264.67885	556.34535	278.67631	K-Dimethyl	573.37190	287.18959	4
6	657.39303	329.20015	685.38795	343.19761	E	417.24563	209.12645	3
7	770.47710	385.74219	798.47202	399.73965	I	288.20303	144.60515	2
8					R	175.11896	88.06312	1



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Chapter Seven

Conclusions and Perspectives for Future Studies

Protein methylation in translation has been given significantly increased amount of attention by the scientific community. In preparation for writing this dissertation, I was curious about the historical origins of elongation factor methylation. Methylation of the prokaryotic equivalent of EF1A, EF-Tu, was first identified *E. coli* in the late 1970s (Ames and Nikaido 1979). Within a few years, methylation of EF-Tu was found in various prokaryotes and eukaryotes, including *Saccharomyces cerevisiae* (Hiatt et al. 1982, Coppard et al. 1983, Cavallius et al. 1993). Many of these papers proposed that methylation of these proteins could be associated with nucleic binding activities or even as a response to environmental stimuli. However the methyltransferases responsible for any of these modifications were not identified until much later and we are still only scratching the surface of the functional ramifications of methylating translational components. In this dissertation I describe the identification of two novel elongation factor methyltransferases, evidence supporting the identity of elongation factor methyltransferase Efm6, preliminary characterization of elongation factor methylation, and a novel type of protein methylation.

Chapters Two and Three described the identification of Efm3 and Efm5, which trimethylate EF2 and EF1A, respectively. Chapter Four provides evidence for the identify of Efm6 which was later confirmed by another group just this past year. Efm3 was a relatively quick discovery in the sense that methylation of EF2 was only described in 2012 and by 2014 both of its methyltransferases were confirmed. The methyltransferases for EF1A have taken significantly longer time between discovery of the sites and the enzymes, one of which is still unknown. This is likely due to the slightly unusual character of the EF1A methyl lysines and its enzymes. Monomethyl lysine 390 of EF1A is at relatively low occupancy, making it difficult to

determine if the methylation is really absent or just too low to detect. The C-terminal methyl ester rapidly turns over and its base lability limits the use of our conventional methods for detecting loss of methyl lysines. Efm5 was incorrectly annotated as an adenine methyltransferase which likely prevented others from looking more closely at it. However, I believe these complications and intricacies indicate a very complex system for maintaining proper EF1A function.

Chapter Five explores this possibility by probing at large scale effects of loss of the elongation factor methyltransferases. My initial analysis definitively demonstrates that translation is not working at 100% efficiency in the absence of several elongation factor methyltransferases. Slow growth and the altered sensitivity to inhibitors indicate that something is wrong with the system. Currently our evidence points to increased levels of synthesis errors which in turn activate the unfolded protein response. Repeating these assays with the point mutants and furthering the investigation with the translational fidelity assays and perhaps the Hac1 analysis for induction of the UPR will be crucial in confirming this hypothesis.

For anyone that decides to continue work in this area, I offer some advice. I would highly recommend proceeding with caution when it comes to the translational fidelity assays. After a while, the transformed cells seem to “age” and the colonies start to turn a brownish color on the plates. Additionally, these older transformants do not seem to work as well as freshly transformed. I have prepared glycerol stocks for most of the strains I transformed but my advice would be to freshly transform each time. I would also recommend that this be performed one plasmid at a time. In other words, transform all strains of interest (I recommend no more than three or four) with the same set of reporter plasmids. Each type of assay (misincorporation, three different stop codons, frameshift) has a corresponding control plasmid. So for example,

transform wildtype, the EF1A quadruple point mutant, and the EF2 double point mutant with the “A” and “H” plasmids for misincorporation. Once those replicates have been completed, move onto another set (“B” and “G”, “C” and “F”, “D” and “E”). The frameshift assay requires three transformants (0 frame, -1, and +1). Be sure to use very fresh substrates and perform technical replicates to ensure you are lysing efficiently.

After seeing the results in Li et al. 2014 on the effects of mutating K390 on viral replication, I attempted to start a communication with and potential collaboration with this group. I sent them several knockout candidates for the K390 methyltransferase (including *ynl024cΔ*) with the understanding that they would send me their point mutants for us to check for loss of methylation. I checked in with the group one month after sending the strains and they had not tested them yet. I checked back three months later regarding their assays and sending me the point mutants but never heard from them again. We then decided to drop it and construct our own point mutants with the help of Dr. Kevin Roy here at UCLA.

It would be worth trying to find a different lab capable of performing this type of assay, ideally someone on campus. Regardless of the incorrect initial interpretation, Li et al. 2014 clearly demonstrated a role for the K390 in viral replication. As this site is part of the tRNA binding region, it could be involved with interacting with the viral RNA. Additionally, mmK390 has been shown to be at relatively low occupancy (Jakobssen et al. 2015). I am curious as to whether or not the levels of occupancy change in the presence of the virus. With the identification of Efm6, it would now be possible to alter the levels of K390 methylation *in vivo* by overexpressing Efm6 and determine if viral replication is enhanced.

Lastly, Chapter Six described the discovery of a novel type of methylation catalyzed by Ylr285w. This finding provides us with a unique opportunity to expand our understanding of protein methylation.

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Appendix: All known and putative methyltransferases in *Saccharomyces cerevisiae*

All of the information contained in this Appendix was obtained from the *Saccharomyces Genome Database* in 2015. For each open reading frame (ORF), I have given the protein name(s), the methyltransferase structural fold class, the predicted substrate type according to Szczepińska et al. 2014, the confirmed substrate type (if applicable), the substrate and the residue of methylation, and the type of modification made. They are listed in alphabetical order by ORF.

ORF	Protein Name	MT Class	Predicted Substrate Type*	Confirmed Substrate Type	Substrate	Type of Methylation
YBL024W	TRM4	Seven Beta Strand	RNA	tRNA	several	m5C (cytosine)
YBR030W	RKM3	SET	Protein	Protein	Rpl42a/b K40	monomethyl lysine
YBR034C	HMT1/RMT1	Seven Beta Strand	Protein	Protein	hmRNPs, Rps2, histone H3 and H4	monomethyl and asymmetric dimethyl arginine
YBR061C	TRM7	Seven Beta Strand	RNA	tRNA	tRNA-Phe, tRNA-Trp, tRNA-Leu	2'-O-ribose at positions C32 and N34 of tRNA anticodon loop
YBR133C	HSL7	Seven Beta Strand	Protein	Protein		monomethyl and asymmetric dimethyl arginine
YBR141C	BMT2	Seven Beta Strand	RNA	rRNA	25S rRNA, position 2142	m1A (adenine)
YBR225W		Seven Beta Strand	RNA			
YBR236C	ABD1	Seven Beta Strand	RNA	mRNA	GpppN terminus of capped mRNA	
YBR261C	TAE1/NTM1	Seven Beta Strand	Protein	Protein	Rpl12/Rps25 N-terminal proline	dimethyl proline
YBR271W	EFM2	Seven Beta Strand	Protein	Protein	EF2 K613	dimethyl lysine
YCL054W	SPB1	Seven Beta Strand	RNA	rRNA	G2922 in large subunit rRNA	Gm (guanosine)
YCL055W	KAR4	Seven Beta Strand	RNA			
YCR047C	BUD23	Seven Beta Strand	RNA	rRNA	18S rRNA, position 1575	Gm (guanosine)
YDL014W	NOPI	Seven Beta Strand	RNA	Protein	Histone H2A, Q105	methyl glutamine
YDL112W	TRM3	SPOUT	Protein	tRNA	tRNAs, position 18	2'-O-ribose (guanosine)
YDL200C	MGT1	DNA/RNA-binding 3 helical bundle	RNA	DNA		6-O-methylguanine
YDL201W	TRM8	Seven Beta Strand	RNA	tRNA	tRNA, position 46	N-methyl guanine
YDR083W	RRP8	Seven Beta Strand	RNA	rRNA	21S rRNA, position 645	m1A (adenine)
YDR120C	TRM1	Seven Beta Strand	RNA	tRNA	tRNAs	N2,N2-dimethylguanosine
YDR140W	MTQ2	Seven Beta Strand	Protein	Protein	Sup45 (eRF1)	methyl glutamine
YDR198C	RKM2	SET	Protein	Protein	Rpl12a/b, K3	trimethyl lysine
YDR257C	RKM4/SET7	SET	Protein	Protein	Rpl42a/b, K55	monomethyl lysine
YDR316W	OMS1	Seven Beta Strand	RNA			

ORF	Protein Name	MT Class	Predicted Substrate Type*	Confirmed Substrate Type	Substrate	Type of Methylation
YDR410C	STE14	Transmembrane	Protein	Protein	a-factor and RAS, CAAX motif	Carboxyl methylation
YDR435C	PPM1	Seven Beta Strand	Other	Protein	PP2A	Methyl Ester
YDR440W	DOT1	Seven Beta Strand	RNA	Protein	Histone H3, K79	trimethyl lysine
YDR465C	RMT2	Seven Beta Strand	Protein	Protein	Rpl12, R67	δ -monomethyl arginine
YER091C	MET6	TIM beta/alpha-barrel	Other	Small molecule	Homocysteine	methyl ester
YER175C	TMT1	Seven Beta Strand	Other	Small Molecule	3-isopropylmalate	wybutosine (modified guanosine)
YGL050W	TYW3	SSo0622-like	RNA	tRNA	Phe-tRNA	
YGL136C	MRM2	Seven Beta Strand	RNA	rRNA	21S mitochondrial rRNA, position 2791	2'-O-ribose methylation (uracil)
YGL192W	IME4	Seven Beta Strand	RNA	mRNA	mRNAs	N6-methyl-adenosine
YGR001C	AML1/EFM5	Seven Beta Strand	Protein	Protein	EF1A, K79	trimethyl lysine
YGR157W	CHO2	Transmembrane	Other	Lipid	phosphatidylethanolamine	forms phosphatidylcholine (with OPI3)
YGR283C		SPOUT	RNA			
YHL039W	EFM1	SET	Protein	Protein	EF1A, K30	monomethyl lysine
YHR070W	TRM5	Seven Beta Strand	RNA	tRNA	cytoplasmic and mitochondrial tRNAs, position 37	mIG
YHR109W	CTM1	SET	Protein	Protein	Cytochrome C, K72	trimethyl lysine
YHR119W	SET1	SET	Protein	Protein	Histone H3K4	trimethyl lysine
YHR207C	SET5	SET	Protein	Protein	Histone H4: K5, K8, K12	monomethyl lysine
YHR209W	CRG1	Seven Beta Strand	Other	Small Molecule	cantharidin	
YIL064W	SEE1/EFM4	Seven Beta Strand	Protein	Protein	EF1A, K316	dimethyl lysine
YIL096C	BMT5	Seven Beta Strand	RNA	rRNA	25S rRNA position 2634	m3U
YIL110W	HPM1	Seven Beta Strand	Protein	Protein	Rpl3, H243	3-methyl histidine
YJL105W	SET4	SET	Protein			
YJL125C	GCD14/TRM61	Seven Beta Strand	RNA	tRNA	tRNAs, position 58	m1A
YJL168C	SET2	SET	Protein	Protein	Histone H3K36	trimethyl lysine
YJR073C	OPI3	Transmembrane	Other	Lipid	phosphatidylethanolamine	forms phosphatidylcholine (with CHO2)
YJR129C	EFM3	Seven Beta Strand	Protein	Protein	EF2, K509	trimethyl lysine

ORF	Protein Name	MT Class	Predicted Substrate Type.*	Confirmed Substrate Type	Substrate	Type of Methylation
YKL155C	RSM22	Seven Beta Strand	RNA			
YKL162C		Seven Beta Strand	RNA			
YKR029C	SET3	SET	Protein			
YKR056W	TRM2	Seven Beta Strand	RNA	tRNA	tRNAs, position 54	m5U
YKR069W	MET1	Tetrapyrrole methylase	Other	Small Molecule	uroporphyrinogen III	involved in biosynthesis of siroheme
YLL062C	MHT1	TIM beta/alpha-barrel	Other	Small Molecule	Homocysteine	
YLR063W	BMT6	Seven Beta Strand	RNA	rRNA	25S rRNA, position 2843	m3U
YLR137W	RKM5	Seven Beta Strand	Other	Protein	Rpl1a/b, K46	monomethyl lysine
YLR172C	DPH5	Tetrapyrrole methylase	Other	Protein/small molecule	EF2, H699	performs trimethylation during diphthamide biosynthesis
YLR186W	EMG1	SPOUT	RNA	rRNA	18S rRNA, position 1191	methyl pseudouridine
YLR285W	NNT1	Seven Beta Strand	Protein			
YML008C	ERG6	Seven Beta Strand	Other	Lipid	zymosterol, position C-24	convert zymosterol to fecosterol in ergosterol biosynthesis pathway
YML014W	TRM9	Seven Beta Strand	RNA	tRNA	tRNA anticodons	2, 5-methoxycarbonylmethyluridine and 5-methoxycarbonylmethyl-2-thiouridine
YML110C	COQ5	Seven Beta Strand	Other	Small Molecule	2-hexaprenyl-6-methoxy-1,4-benzoquinone	involved in ubiquinone biosynthesis
YMR209C		Seven Beta Strand	RNA			
YMR228W	MTF1	Seven Beta Strand	Protein			
YMR310C		SPOUT	RNA			
YNL022C	RCM1	Seven Beta Strand	RNA	rRNA	25S rRNA, position 2278	m5C (cytosine)
YNL024C	EFM6	Seven Beta Strand	Protein	Protein	EF1A, K390	monomethyl lysine
YNL061W	NOP2	Seven Beta Strand	Protein	rRNA	25S rRNA, position 2870	m5C (cytosine)
YNL063W	MTQ1	Seven Beta Strand	RNA	Protein	Mrf1, Q	methyl glutamine
YNL092W		Seven Beta Strand	Other			Automethylation
YOL093W	TRM10	SPOUT	RNA	tRNA		
YOL096C	COQ3	Seven Beta Strand	Other	Small Molecule	tRNAs, position 9	m1G

ORF	Protein Name	MT Class	Predicted Substrate Type*	Confirmed Substrate Type	Substrate	Type of Methylation
YOL124C	TRM11	Seven Beta Strand	RNA	tRNA	tRNAs, position 10	m2G
YOL125W	TRM13	Seven Beta Strand	RNA	tRNA	tRNAs, position 4	2'-O-methyl
YOL141W	PPM2/TYW4	Seven Beta Strand	Protein	tRNA	tRNA-Phe	wybutosine (modified guanosine)
YOR021C	SFM1	SPOUT	Protein	Protein	Rps3, R146	monomethyl arginine
YOR074C	CDC21	Thymidylate synthetase	Other	Small Molecule		
YOR201C	MRM1	SPOUT	RNA	rRNA (mito)	21S rRNA	2'-O-methylguanosine
YOR239W	ABP140	Seven Beta Strand	Protein	tRNA	tRNA-Thr and tRNA-Ser, position 32	3mC
YPL030W	TRM44	Seven Beta Strand	RNA	tRNA	tRNA-Ser	2'-O-methyl
YPL157W	TGS1	Seven Beta Strand	RNA	mRNA	m(7)G cap structure of snRNAs, snoRNAs, and telomerase TLC1 RNA	trimethyl guanosine, forms m(2,2,7)G
YPL165C	SET6	SET	Protein			
YPL208W	RKM1	SET	Protein	Protein	Rpl23 K106, K110 and Rps18 K48	dimethyl lysine on L23, monomethyl lysine on S18
YPL266W	DIM1	Seven Beta Strand	RNA	rRNA	18S rRNA, 3'-terminal loop	m6(2)Am6(2)A dimethylation
YPL273W	SAM4	TIM beta/alpha-barrel	Other	Small Molecule	convert AdoMet to methionine	