

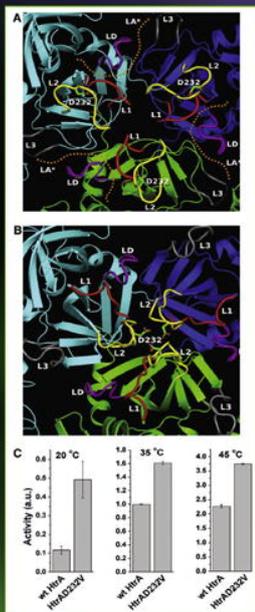


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Two novel methyltransferases acting upon eukaryotic elongation factor 1A in *Saccharomyces cerevisiae*

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ABSTRACT

Eukaryotic elongation factor 1A (eEF1A) is an abundant cytosolic protein in *Saccharomyces cerevisiae* and is well conserved amongst species. This protein undergoes multiple posttranslational modifications, including the N-methylation of four side chain lysine residues. However, the enzyme(s) responsible for catalyzing these modifications have remained elusive. Here we show by intact protein mass spectrometry that deletion of either of two genes coding for putative methyltransferases results in a loss in mass of eEF1A. Deletion of the YHL039W gene, a member of the SET domain subfamily including cytochrome c and ribosomal protein lysine methyltransferases, results in an eEF1A mass loss corresponding to a single methyl group. Deletion in the YIL064W/SEE1 gene, encoding a well conserved seven beta strand methyltransferase sequence, has been shown previously to affect vesicle transport; in this work we show that deletion results in the loss of two methyl group equivalents from eEF1A. We find that deletion of thirty-five other putative and established SET domain and seven beta strand methyltransferases has no effect on the mass of eEF1A. Finally, we show that wild type extracts, but not YIL064W/SEE1 mutant extracts, can catalyze the S-adenosylmethionine-dependent *in vitro* methylation of hypomethylated eEF1A. We suggest that YHL039W (now designated EFM1 for elongation factor methyltransferase 1) and YIL064W/SEE1 encode distinct eEF1A methyltransferases that respectively monomethylate and dimethylate this protein at lysine residues.

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Introduction

Posttranslational modifications are found throughout eukaryotic cells, playing structural roles and contributing to the regulation of protein function. Great progress has been made recently in the understanding of protein lysine methylation, particularly since the discovery of numerous methyltransferases responsible for these modifications as well as demethylases that can reverse at least some of the modification reactions [1–7]. While the focus to date has largely been on the role of histone lysine methylation in transcriptional control, there are a number of additional non-histone substrates acted upon by lysine methyltransferases [1,3–6,8,9]. For a number of these modified proteins, the identity of the methyltransferase, as well as the function methylation imparts, remains to be discovered.

We have been interested in the methyl modification of proteins involved in translation, and more specifically in the enzymes that catalyze these modifications. There are a number of translation-associated proteins that have been observed to be modified by lysine methylation, including several ribosomal proteins [8,9]. The functions of these lysine modification reactions remain obscure. One of the most highly methylated proteins at lysine

residues is the well conserved translational affiliated protein eEF1A¹ [8,10–12]. Perhaps best known for its role in escorting tRNA to the ribosomal A-site in a GTP-dependent manner, eEF1A has additionally been described as having multiple moonlighting functions including interactions with the cytoskeleton [13–15]. Phosphorylation has been determined to modify some of the activities of mammalian eEF1A, typically resulting in a stimulatory effect [14,15]. The impact of methyl modification on the functions of eEF1A is more poorly understood [10,12,16]. However, it is known that some methyl modifications of eEF1A are well conserved from simple eukaryotes like *Saccharomyces cerevisiae* all the way to humans [11]. The equivalent bacterial GTP-dependent translation factor EF-Tu is lysine trimethylated, though at a residue 12 positions C-terminal to the strictly conserved trimethyl lysine residue of eukaryotes [11,17]. Nevertheless, the methyltransferase species that are responsible for eEF1A modification have proven elusive; none have been identified to date.

We have focused our efforts on identifying the methyltransferase(s) responsible for eEF1A methylation in *S. cerevisiae* with the hope that this work can provide a foundation for understanding the functional role of the methylation reactions in this and other eukaryotes. We screened deletion mutants of putative methyl-

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¹ Abbreviations used: eEF1A, eukaryotic elongation factor 1A; [³H]AdoMet, S-adenosyl-L-[methyl-³H]methionine; AdoMet, S-adenosyl-L-methionine.

transferases of both the seven beta strand and the SET domain families to identify potential catalysts for eEF1A methyl modification. In the past, we have used *in vivo* radiolabeling techniques to identify methyltransferase-substrate pairs [18]. However, due to multiple methylated sites, these techniques were not useful in determining the enzymes acting upon eEF1A. In this study we took an approach using intact protein mass spectrometry to analyze protein modifications [19]. We obtained intact mass values for chromatographically purified eEF1A at high enough resolution to observe the 14 Da changes that occur due to loss of methylation in a mutant strain. Using these techniques, we have identified two novel proteins involved in methylating eEF1A in *S. cerevisiae*.

Materials and methods

Yeast strains

With the exception noted below, all *S. cerevisiae* strains were obtained from the Saccharomyces Genome Deletion Project and included the parent “wild type” strains BY4741 and BY4742 as well as the Δ YHL039W and Δ YIL064W/*see1* gene deletion strains in both of these backgrounds. The Δ set1 gene deletion strain was a gift from Drs. Renee Chosed and Sharon Dent at the MD Anderson Cancer Center (Houston, TX) along with its corresponding parent strain, KT1112. A complete list of strains screened for catalysis of eEF1A methylation is given in Supplemental Table 1.

Isolation of cytosolic proteins

Cells were grown at 30 °C in YPD media (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) to an optical density of 0.5–1.0 at 600 nm. The cells were subsequently harvested by centrifugation at 4 °C for 5 min at 5000g. Cell pellets were combined with 1.5 g of baked zirconium glass beads (Biospec Products; Bartlesville, OK) in 3 ml buffer A (20 mM Tris HCl, 15 mM Mg acetate, 60 mM KCl, 1 mM DTT, 1 mM PMSF, and proteinase inhibitors from the Roche Proteinase Inhibitor Cocktail Tablet with 1 mM EDTA) and submitted to ten repetitions of one min of vortexing followed by one min at 0 °C. Samples were fractionated as described previously [18]. Briefly, lysates were centrifuged at 4 °C first at 12,000g for 5 min and then 20,000g for 15 min in a Beckman JA-17 rotor. The final centrifugation was performed at 100,000g for 2 h at 4 °C in a Beckman Ti-65 rotor. The approximately 4 ml of supernatant containing the cytosolic fraction was stored at –80 °C pending further protein separation.

Column purification of eEF1A

Isolation of eEF1A was achieved by use of a pair of ion exchange columns in a manner similar to the one described by Lopez-Valenzuela et al. [20]. Specifically, the total volume of each cytosolic sample (approximately 4 ml) was individually loaded onto a 5 ml HiTrap Q HP anion exchange column (GE Healthcare) that had been equilibrated with buffer A (5 mM NaCl, 20 mM HEPES, 5% glycerol, 1 mM DTT, 1 mM EDTA, pH 8) and was then washed with an additional 5 ml of buffer A. The total flow-through containing eEF1A was next loaded at 2 ml/min onto a 5 ml HiTrap SP HP cation exchange column equilibrated in buffer A and the column subsequently washed with buffer A at 5 ml/min for 5 min. To elute eEF1A an increasing salt gradient of 0–50% buffer B (1 M NaCl, 20 mM HEPES, 5% glycerol, 1 mM DTT, 1 mM EDTA, pH 8) run at 5 ml/min over 15 min was used and 1.5 ml fractions collected. All of these steps were performed at 4 °C. Purified eEF1A fractions were identified by the presence of a single 49 kDa polypeptide band on SDS gel electrophoresis and were monitored by UV absorbance at 280 nm.

Intact mass determination by coupled liquid chromatography–mass spectrometry

The intact mass of eEF1A was analyzed using a PLRP-S polymeric column with pore size of 300 Å, bead size of 5 µm, and dimensions of 150 × 1.0 mm (Polymer Laboratories, Amherst, MA) coupled to an Applied Biosystems Q-Star Elite instrument based on the protocol described previously [21]. Here, the column was equilibrated with 5% buffer B (0.05% trifluoroacetic acid in acetonitrile) and 95% buffer A (0.05% trifluoroacetic acid in water) and maintained at 50 °C for the duration of the run. The samples were eluted at 50 µl/min with 5 min at equilibration conditions, followed by a 55 min gradient from 5% to 60% B, and finally a 5 min gradient from 60% to 100% B. Afterwards, the column was returned to equilibration conditions over 5 min. Data was evaluated and intact masses calculated using Analyst software (Applied Biosystems, Foster City, CA). The peak containing eEF1A was found at approximately 42 min.

In vitro methylation of eEF1A

Cells were grown at 30 °C in YPD media (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) to an optical density of 0.5–0.8 at 600 nm. The cells were subsequently harvested by centrifugation at 4 °C for 5 min at 5000g. Cell pellets were lysed by combining with 0.5 g glass beads in 1 ml phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, and proteinase inhibitors from the Roche Proteinase Inhibitor Cocktail Tablet with 1 mM EDTA, pH 7.4) and submitted to seven rounds of vortexing and ice treatment as described above. After recovering the lysate, the beads were washed with an additional 0.25 ml phosphate-buffered saline which was then combined with the original aliquot. The lysate was centrifuged at 4 °C first at 12,000g for 15 min and then 17,000g for 10 min, with the supernatants retained. The protein concentration was determined by precipitating an aliquot using 10% trichloroacetic acid by the Lowry method with bovine serum albumin as a standard.

For the *in vitro* assay, a total of 40 µg of lysate protein (wild type, Δ YHL039W, Δ YIL064W, or a 1:1 mixture of wild type and mutant) was combined in 0.1 M sodium phosphate buffer (pH 7.0) and incubated with 0.66 µM of the radiolabeled methyl donor S-adenosyl-L-[methyl-³H]methionine [³H] AdoMet; 78.0 Ci/mmol) for 60 min at 30 °C. Reactions were stopped by the addition of an equal volume of 2× SDS running dye (180 mM Tris-HCl, pH 6.8, 4% SDS, 0.1% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and subsequently heated at 100 °C for 3 min. Samples were then loaded onto a 12.6% SDS polyacrylamide gel in the Laemmli buffer system [22] and run for 3 h at 30 mA. Afterwards the gels were rocked for 1 h submerged in Coomassie Brilliant Blue dye and then de-stained overnight to enable visualization of protein bands. The wet gel lanes were chopped into ten 2 mm slices surrounding the 42.7 kDa ovalbumin marker band, and each slice placed in an individual 1.5 ml polypropylene microcentrifuge tube with 1 ml of 30% H₂O₂. The tubes were then inserted into vials containing 5 ml of Safety-Solve Complete Counting Mixture (Research Products International, Mt. Prospect, IL), caps were loosely put on, and left to incubate 24 h at 37 °C. After incubation, the caps were tightened and vials shaken vigorously to mix the samples so that total radioactivity could be counted by a Beckman LS6500 scintillation counter.

Results

YHL039W is required for monomethylation of eEF1A

It has previously been established that eEF1A is lysine methylated in *S. cerevisiae* at four sites including two non-conserved

monomethylation sites at residues 30 and 390, one conserved dimethylated site at residue 316, and one conserved trimethylated site at residue 79 [11]. Evidence has also been presented for the substoichiometric modification of the C-terminal lysine residue at its alpha-carboxyl group [23]. Therefore, we initiated our screen using deletion mutants of genes encoding known and putative methyltransferases of the *S. cerevisiae* SET domain family, a family of enzymes that appears to specifically catalyze the methylation of protein lysine residues. Previous work has shown that the *S. cerevisiae* SET domain family of methyltransferases can be further grouped into two subfamilies [24]. The first subfamily contains the Set1 and Set2 proteins known to methylate histone H3 at lysine residues 4 and 36, respectively [25,26]. Although no substrates have yet been identified for Set3–Set6, Set3 has been found to be a part of a histone deacetylation complex so is also linked to transcription [27]. The second subfamily contains identified ribosomal lysine methyltransferases Rkm1–Rkm4 as well as the cytochrome *c* lysine methyltransferase Ctm1 [9,24]. Recently, Set1 was shown to have lysine methyltransferase activity for an additional substrate, the kinetochore protein Dam1 [28]. With this study illustrating that SET domain lysine methyltransferases may have multiple substrates, we chose to include all known and putative *S. cerevisiae* SET domain methyltransferases in our screen for enzymes catalyzing eEF1A lysine methylation.

To determine the enzyme(s) responsible for catalyzing the methylation of eEF1A, we purified eEF1A from wild type yeast cells and from deletion mutants in the same background for the twelve known and putative SET domain methyltransferases previously identified [24]. Cells from each strain were lysed, the cytosolic fraction purified by centrifugation, and eEF1A purified by three steps of column chromatography as described in Materials and methods. The final reverse phase HPLC column was linked directly to the mass spectrometer and samples were injected using an electrospray source, allowing for intact mass measurement. The predicted average mass of the eEF1A polypeptide is 50,032 Da. With the expected N-terminal acetylation and the seven internal methyl groups the expected mass is 50,172 Da. For the wild type sample, we observed a broad peak with an apex indicating the predominant species has a mass of 50,162 Da (Fig. 1, top panel). It is not clear at this point whether the smaller experimental value reflects measurement error (in this case of about 200 ppm) or the presence of some species lacking one or more methyl groups. The C-terminal methyl ester is not observed in our preparations, presumably due to hydrolysis during the purification steps, and its mass is not included in the calculated mass above.

Analysis of eleven of the twelve SET domain family member deletion strains showed mass spectra effectively identical to that of the wild type (data not shown). However, we observed a loss of approximately 14 Da from the total eEF1A mass in the Δ YHL039W deletion strain (Fig. 1, middle panel). This suggests that the gene product encoded by YHL039W is responsible for monomethylating eEF1A. These results for the Δ YHL039W deletion strain in the BY4742 background were confirmed in an independently derived Δ YHL039W deletion strain in the BY4741 background (data not shown). Based on the fact that all SET domain methyltransferases to date modify lysine residues, and on the absence of non-lysine stoichiometric modifications of eEF1A, we suggest that the protein product of the YHL039W gene monomethylates eEF1A and designate it elongation factor methyltransferase 1 (Efm1).

YIL064W is required for dimethylation of eEF1A

While the SET domain family methyltransferases may exclusively act on protein lysine substrates, members of the larger seven beta strand methyltransferase family have also been observed to methylate lysine residues. For example, in *S. cerevisiae* the seven

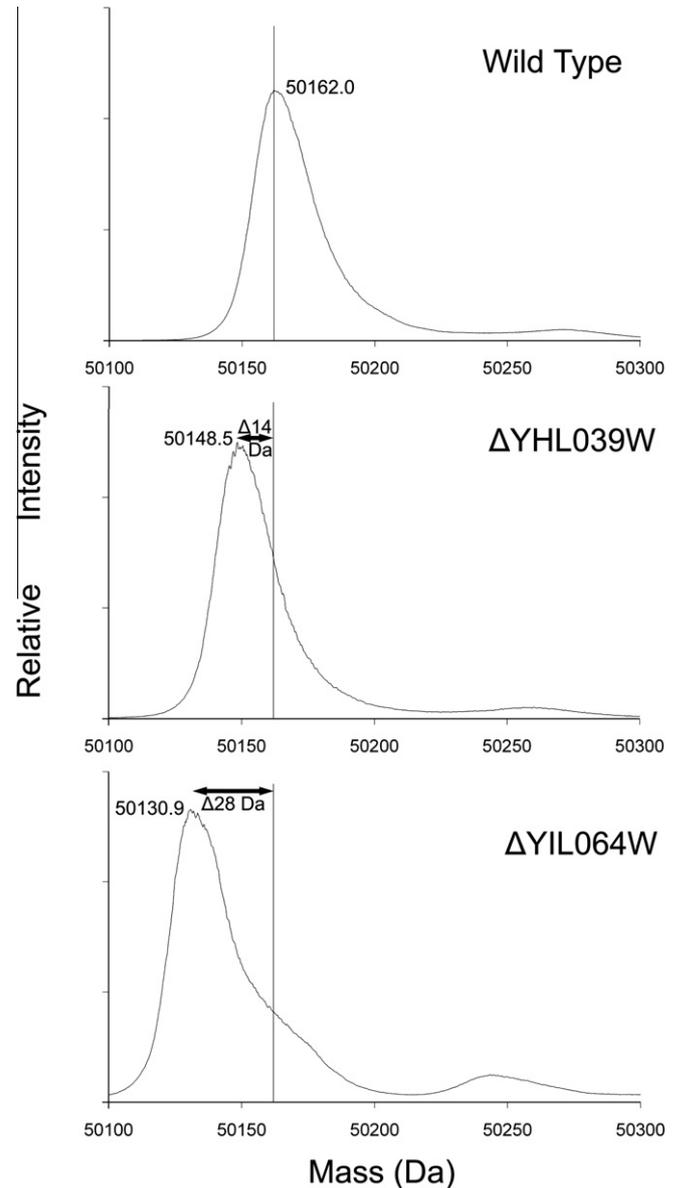


Fig. 1. Loss of eEF1A methylation in two mutants, Δ YHL039W and Δ YIL064W. The intact masses of wild type (top panel), Δ YHL039W (middle panel), and Δ YIL064W (lower panel) eEF1A were determined as described in Materials and methods. Strains shown are in the BY4742 background. Arrows indicate the loss of methyl groups based on the shift of the peak's apex.

beta strand methyltransferase Dot1 modifies lysine 79 of histone H3 [29]. With this in mind, we expanded our screen for eEF1A methyltransferases to include putative seven beta strand methyltransferases identified from a recent bioinformatic analysis [30]. Supplemental Table 1 shows the set of 19 putative seven beta strand methyltransferase deletion strains analyzed, as well as the known seven beta strand lysine methyltransferase Dot1, the known glutamine methyltransferases Mqt1 and Mqt2, and the three known protein arginine methyltransferases Rmt1, Rmt2, and Hsl7.

With one exception, the mass spectra of eEF1A obtained from all of these deletion strains did not deviate significantly from that of the wild type strain (data not shown). However, in the Δ YIL064W/*see1* deletion strain, we found a loss of approximately 28 Da from the intact mass of eEF1A as compared to wild type (Fig. 1, bottom panel). This suggests that YIL064W/*See1* is a seven beta strand lysine methyltransferase that catalyzes the addition of two methyl groups to eEF1A.

boxes). Likewise, control samples for the mutant-only lysates showed no radiolabel incorporation for eEF1A (Fig. 2, open circles, for ΔYIL064W/see1; data not shown for ΔYHL039W). However, in samples where wild type lysate containing the active protein YIL064w/See1 was combined with lysate from the ΔYIL064W/see1 deletion strain (containing eEF1A lacking the modification dependent upon the YIL064W/See1 protein), we observed an incorporation of the radiolabel consistent with eEF1A methylation (Fig. 2, closed circles). The only difference in the lysates is the presence or absence of YIL064W/See1, further supporting the identity of this protein as an eEF1A methyltransferase. Similar results were obtained using extracts from the independent ΔYIL064W/see1 deletion and wild type strains from the BY4742 background (data not shown).

Interestingly, the corresponding lysate combination for YHL039W did not yield *in vitro* methylated eEF1A (data not shown). Since the folded eEF1A may a poor substrate for this enzyme, we additionally tried *in vitro* assays using urea-treated lysate from the ΔYHL039W delete strain. This would theoretically denature hypomethylated eEF1A and make the YHL039W site more available to the functional enzyme present in the wild type lysate. However, we were still unable to observe any counts above background (data

not shown). It is possible that YHL039W modification occurs co-translationally and that even the denatured mature protein is not a preferred substrate for the methyltransferase.

Site specificity and amino acid sequence conservation of eEF1A methyltransferases Efm1 and See1

The only known stoichiometric modifications of yeast eEF1A are the monomethylation of lysine 30, the trimethylation of lysine 79, the dimethylation of lysine 316, and the monomethylation of lysine 390 [11]. Since we observed a loss of 14 Da upon deletion of Efm1, this suggests that the site of action of this enzyme is one of the two monomethylated lysine residues of eEF1A. Neither of the monomethylation sites of eEF1A in *S. cerevisiae* appears to be conserved in higher eukaryotes [11]. It appears as if Efm1 may be an enzyme specific to yeast, as we find closely related species in *Candida albicans* and *Schizosaccharomyces pombe*, but not in higher organisms (Fig. 3). In contrast, the dimethylation at lysine 316 is conserved among eukaryotes, although the corresponding residue in higher eukaryotic organisms is found in a trimethylated rather than a dimethylated state [11]. This conservation is reflected in the wide distribution of proteins in eukaryotes with amino acid

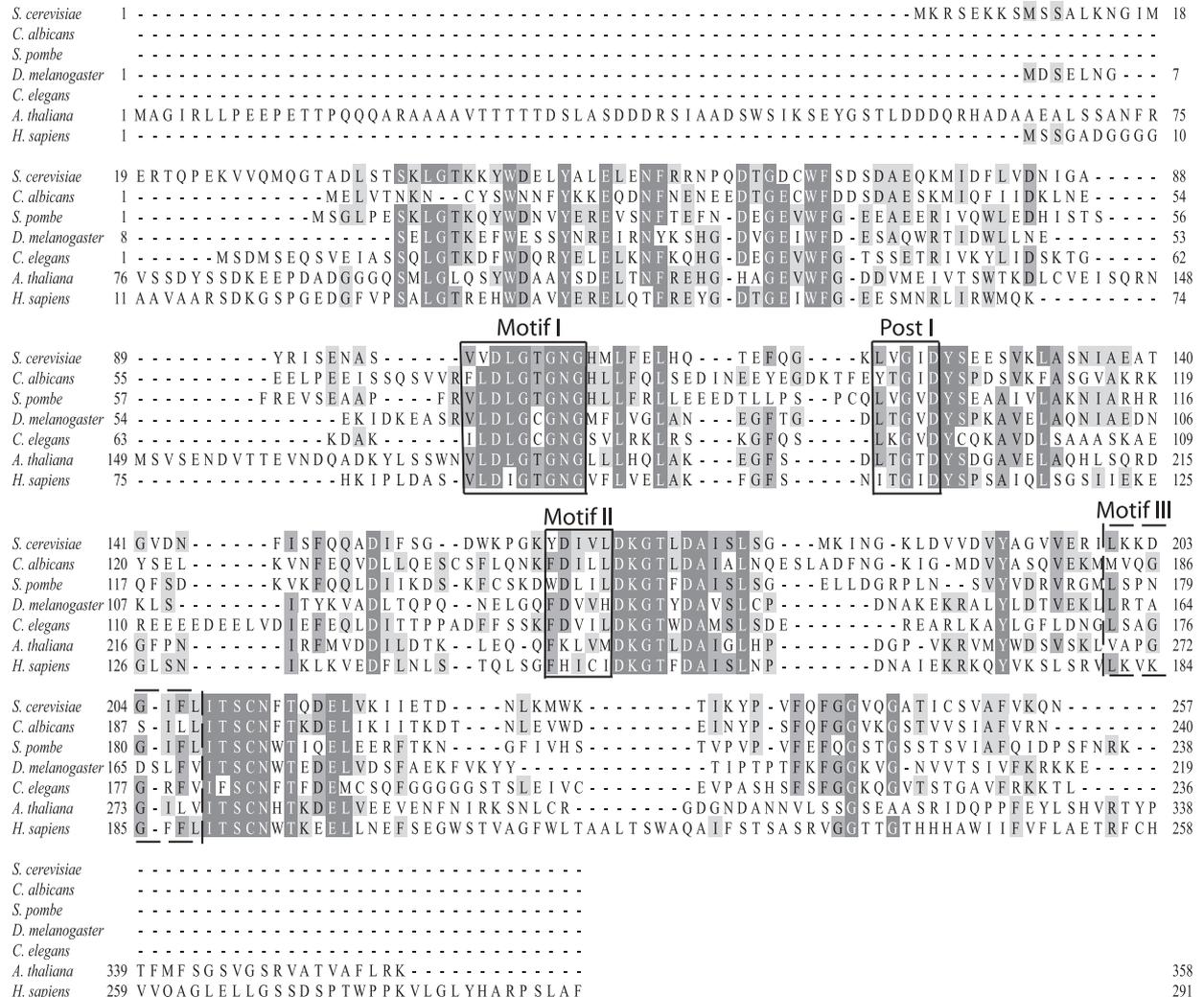


Fig. 4. YIL064W is well conserved in nature. The amino acid sequence of YIL064W was compared to the non-redundant protein database using the BLAST algorithm to obtain expect values. Amino acid sequence alignments determined by CLUSTALW2 are shown for *S. cerevisiae* (NCBI ID: NP_012200), *C. albicans* (NCBI ID: XP_715004, expect value 2e–49), *S. pombe* (NCBI ID: NP_595254, expect value 3e–37), *D. melanogaster* (NCBI ID: NP_608733, expect value 8e–31), *C. elegans* (NCBI ID: NP_500612, expect value 1e–25), *A. thaliana* (NCBI ID: NP_176841, expect value 3e–21), and *H. sapiens* (NCBI ID: NP_997719, expect value 2e–23). Boxes identify the proposed motifs of the conserved AdoMet binding domain.

Table 1
Translation/cytochrome *c* SET domain subfamily in *S. cerevisiae*.

Methyltransferase		BLAST expect value	Substrate	Extent of methylation	Site
Efm1	YHL039W	–	eEF1A K30? eEF1A K390?	Mono Mono	TGHLIY-K-CGGIDK KLEDHP-K-FLKSGD
Rkm1	YPL208W	3.00e–174	Rpl23 K105 Rpl23 K109	Di Di	GVIANP-K-GEMKGS NPKGEM-K-GSAITG
Rkm2	YDR198C	4.00e–86	Rpl12 K3	Tri	MPP-K-FDPNEV
Rkm3	YBR030W	7.00e–77	Rpl42 K40	Mono	SLFAQG-K-RRYDRK
Rkm4	YDR257C	7.00e–100	Rpl42 K55	Mono	GFGGQT-K-PVFHKK
Ctm1	YHR109W	1.00e–19	Cytochrome <i>c</i> K72	Tri	EYLTNP-K-KYIPGT

sequence similarity to YIL064W/See1 (Fig. 4). The apparent ortholog of YIL064W/See1 in humans is METTL10, a protein of as yet unknown function containing a seven beta strand AdoMet binding domain.

We compared the monomethylation sites of eEF1A to those of other substrates of SET domain methyltransferases (Table 1). While we found little or no similarity in the sequence surrounding lysine 30, we noted that lysine 390 was preceded by a proline residue that also preceded a methylated lysine residue in the ribosomal proteins Rpl23 and Rpl12 as well as cytochrome *c*. Furthermore, lysine 390 in eEF1A and the methylated lysine 3 in Rpl12 are both followed by a phenylalanine residue. These considerations suggest that the SET domain methyltransferase Efm1 modifies lysine 390, although this needs to be experimentally confirmed. It is important to note that the overall native structure of at least one substrate protein for SET domain methyltransferases has been observed to be required for substrate recognition and this may be a determining factor rather than, or in addition to, the primary sequence [31].

Discussion

In this work we have identified two yeast genes required for eEF1A methylation. Previous work found no phenotypic change or *in vitro* translational defects when the four internal lysine methylation sites of eEF1A were changed to arginine residues [12]. Additionally, strains with deletion mutants in either gene are viable and no phenotype has been observed to date for *EFM1* mutants. However, mutant studies of *SEE1* have suggested a role for this protein in vesicle trafficking, particularly in early endocytotic transport [32]. With the observation that eEF1A can associate with filamentous actin and microtubules [13–15], we suggest that eEF1A methylation may modulate the cytoskeletal interactions involved in vesicle trafficking.

The ortholog of See1 in humans is METTL10, a protein that has already been annotated in the UniProt database as having a seven beta strand AdoMet binding domain. Additionally, METTL10 has been recently identified as being a target for mitotic phosphorylation at serine 21 in a [pS/pT]-P cyclin-dependent kinase recognition site [33]. The extent of phosphorylation at this site increased 7.3-fold in mitotic arrest, suggesting a regulatory control.

At this time, we have no evidence for the functional role of the Efm1 methyltransferase. Unlike See1, the distribution of Efm1 in nature is narrow and appears to be limited to fungal species. The two best BLAST matches of Efm1 with human proteins are putative SET domain family proteins SETD3 and SETD4 (Fig. 3). However, these species are unlikely to have the same function because BLAST searches with these latter proteins demonstrate comparable similarity to yeast ribosomal methyltransferases. Additionally, human SETD4 has significantly better matches to *S. pombe* (NCBI ID: NP_588349) and *C. albicans* (NCBI ID: XP_722076) proteins that are distinct from the Efm1 orthologs shown in Fig. 3. Interestingly, the identification of Efm1 now completes the yeast translation/

cytochrome *c* SET domain subfamily, including members that modify ribosomal proteins and cytochrome *c* [9,24,31,34].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.05.023.

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