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Elongation factor methyltransferase 3 – A novel eukaryotic lysine methyltransferase

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Here we describe the discovery of *Saccharomyces cerevisiae* protein YJR129Cp as a new eukaryotic seven-beta-strand lysine methyltransferase. An immunoblotting screen of 21 putative methyltransferases showed a loss in the methylation of elongation factor 2 (EF2) on knockout of YJR129C. Mass spectrometric analysis of EF2 tryptic peptides localised this loss of methylation to lysine 509, in peptide LVEGLKR. *In vitro* methylation, using recombinant methyltransferases and purified EF2, validated YJR129Cp as responsible for methylation of lysine 509 and Efm2p as responsible for methylation at lysine 613. Contextualised on previously described protein structures, both sites of methylation were found at the interaction interface between EF2 and the 40S ribosomal subunit. In line with the recently discovered Efm1 and Efm2 we propose that YJR129C be named elongation factor methyltransferase 3 (Efm3). The human homolog of Efm3 is likely to be the putative methyltransferase FAM86A, according to sequence homology and multiple lines of literature evidence.

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1. Introduction

The methylation of proteins is emerging as one of the most widespread protein post-translational modifications [1]. It predominantly occurs on lysine and arginine residues, but has also been documented on glutamine, asparagine, cysteine, histidine and glutamate residues [2]. Lysine methylation is known to affect a wide variety of biological functions by modifying histone proteins, and more recently, non-histone proteins [3,4]. On histones, lysine methylation regulates transcription by facilitating docking of chromatin remodelling proteins and complexes, in order to bring about either transcriptional silencing or activation [3]. More generally, lysine methylation is known to modulate protein–protein interactions and thereby also affects the function of non-histone proteins [4,5].

The first lysine methyltransferases (KMTs) to be discovered were SET domain methyltransferases, however upon the discovery of Dot1 in *Saccharomyces cerevisiae*, which monomethylates histone H3 at lysine 79 [6], it became evident that seven-beta-strand (or Class I) methyltransferases were also able to catalyse lysine methylation. Since then, a number of other seven-beta-strand KMTs have been discovered in both yeast and human. In yeast these are See1, which dimethylates elongation factor 1α at lysine 316, Efm2 which dimethylates elongation factor 2 at lysine 613, and Rkm5 which monomethylates the ribosomal protein Rpl1 at lysine 46 [7–9]. In human these are DOT1L, which monomethylates histone H3 at lysine 79, CaM-KMT, which trimethylates calmodulin (CaM) at lysine 115, VCP-KMT, which trimethylates Valosin-containing protein (VCP) at lysine 315, HSPA-KMT, which trimethylates a number of Hsp70 proteins at a conserved lysine, and METTL22, which trimethylates KIN17 at lysine 135 [10–14]. The catalytic core of seven-beta-strand methyltransferases contains alternating β-strands and α-helices which form a seven-stranded beta sheet surrounded by α-helices [15]. In terms of sequence there are four key motifs that define seven-beta-strand methyltransferases: I, Post-I, II and III [15]. Motifs I and Post-I are involved in binding S-adenosyl L-methionine (AdoMet or SAM), the methyl donor, while Motifs II and III have been proposed to bind the substrate [16].

In recent years, there has been an intense search for new protein methyltransferases. Sequence-based studies, focused on the presence of the seven-beta-strand, SET domain or SPOUT methyltransferase motifs, have predicted the presence of about 30 putative methyltransferases in *S. cerevisiae* [17,18]. The specificity of seven-beta-strand methyltransferases remains challenging to predict, yet these studies have classified putative methyltransferases into those most likely to methylate proteins, as opposed to RNA, fatty acids and lipids, or small molecules. Antibody-based studies of protein methylation patterns, in conjunction with the single gene knockout of methyltransferases, have proven useful for the discovery of putative new enzymes [8,19]. *In vitro* methylation

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assays can then validate these methyltransferases [12–14,19,20]. This direct validation of activity is important as simple knockout analysis of putative methyltransferases may only unveil secondary effects. Ultimately, tandem mass spectrometry is required to map the site specificity of methyltransferases. This allows unequivocal enzyme-substrate relationships to be built, for incorporation into methylproteome networks [5].

Elongation factors 1α and 2 are known to be methylated in eukaryotes [7,8,21]. In particular, we previously reported that *S. cerevisiae* elongation factor 2 (EF2), which catalyses the translocation step of translation elongation, is trimethylated at lysine 509 and dimethylated at lysine 613 [22]. Methylation of lysine 613 was found to be removed in knockout of *EFM2*, but methylation of lysine 509 was not found to change in any knockouts of elongation factor-associated methyltransferases *SEE1*, *EFM1* or *EFM2* [22].

Here we describe the discovery and validation of *S. cerevisiae* protein YJR129Cp as a new eukaryotic seven-beta-strand lysine methyltransferase. Through knockout analysis with antibodies and *in vitro* methylation analyses, we show that the enzyme methylates elongation factor 2 on lysine 509, in the tryptic peptide LVEGLKR. We also confirm that Efm2p is responsible for the dimethylation of EF2 at lysine 613. Interestingly, both sites of methylation are found at EF2 interaction interfaces with the 40S ribosomal subunit. In line with the recently discovered and named Efm1 and Efm2, both which methylate elongation factors, we propose that YJR129C be named elongation factor methyltransferase 3 (Efm3). We finally show that there are multiple lines of literature evidence to suggest that the human homolog of Efm3 is the putative methyltransferase FAM86A.

2. Materials and methods

2.1. Yeast strains, double knockout generation and antibody-based screens

Yeast strains used are shown in Table 1. The double knockout of *EFM2* and YJR129C was generated by deleting *EFM2* from the YJR129C single knockout, by use of a hygromycin B resistance cassette amplified from plasmid pFA6a–hphNT1, according to [23]. Yeast was grown in YEPD at 30 °C to mid-log (~0.8 OD_{600}) before harvest and protein extraction as per [8], except that lysates were not subjected to ultracentrifugation. SDS–PAGE and immunoblotting were performed as per [24] with the following antibodies: anti-trimethyllysine (Immunechem, ICP0601), 1:2000 in 1% BSA overnight; anti-Nε-methyllysine (Immunechem, ICP0501), 1:1000 in 2% BSA overnight; anti-PentaHis HRP-Conjugated antibody (Qiagen, 34460), 1:10,000 in blocking buffer provided overnight.

2.2. Recombinant protein production and in vitro methylation

Efm2p and YJR129Cp were made recombinantly in *Escherichia coli* (Rosetta DE3) by cloning genes into plasmid pET15b (Novagen); expression and lysis was as per [25], with the exception that expression was for 4.5 h at 25 °C. The gene *EFT1* (which codes EF2) was cloned into plasmid pAG426GAL-ccdB, with addition of a C-terminal 6×His-Tag, and transformed into yeast strain Δ*EFM2*ΔYJR129C. EF2 was overexpressed as per [26] and lysis was by bead-beating cell pellets (BioSpec BeadBeater) for 30 s three times in binding/wash buffer 1 (50 mM sodium phosphate buffer, 500 mM NaCl, 40 mM imidazole, 20% (v/v) glycerol, 0.25% (v/v) Triton X-100, 10 mM β-mercaptoethanol, pH 8) with EDTA-free protease inhibitor (Roche). Resultant lysates (from either *E. coli* or *S. cerevisiae*) were clarified at 21,000*g* for 30 min at 4 °C. A 1 mL Ni–NTA Superflow Cartridge (Qiagen) charged with Ni^{2+} was used to purify the 6×His-Tagged proteins as per manufacturer instructions.

For *in vitro* methylation reactions, purified EF2 was incubated with purified Efm2p and/or purified YJR129Cp in the presence of 50 μM AdoMet in *in vitro* methylation buffer (50 mM HEPES–KOH, 20 mM NaCl, 1 mM EDTA, pH 7.4) at 30 °C for 1 h. No enzyme was added for the negative control. Ten microlitres of 6× SDS buffer (350 mM Tris–Cl pH 6.8, 30% (v/v) glycerol, 10% (v/v) SDS, 0.6 M DTT, 0.012% (w/v) bromophenol blue) was added to stop reactions, which were then boiled for 10 min, subjected to SDS–PAGE and immunoblotted with anti-trimethyllysine or anti-PentaHis HRP-Conjugated antibodies. Alternatively, for mass spectrometry, gels were fixed in 25% (v/v) isopropanol/10% (v/v) acetic acid for 10 min, and then stained with Bio-Safe™ Coomassie (BioRad).

2.3. Methylation site analysis by mass spectrometry

Samples for mass spectrometry were prepared according to [27]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) using collision-induced dissociation was performed on an Orbitrap Velos or Velos Pro as described [27]. Targeted data acquisition was performed with an inclusion list containing the theoretical *m*/*z* values of every potential EF2 doubly- and triply-charged lysine-methylated tryptic peptide (with two missed cleavages), within the range of 350–2000*m*/*z*. Peptides associated with K509 and K613 methylation of EF2, i.e. mono-, di- and tri-methylated forms of LVEGLKR, and mono- and di-methylated forms of DDFKAR, respectively, were identified by comparing observed *m*/*z* values, LC retention times and MS/MS spectra to synthetic $^{13}C(6)$ $^{15}N(4)$ C-terminal arginine labelled versions of these peptides (SpikeTides TQL, JPT, Germany). All peptide identifications were confirmed following previous criteria [22] (for example see Fig. S1). Unmodified forms of LVEGLKR, DDFKAR, and the proteotypic EF2 peptide AGIISAAK, were identified from Mascot sequence database searches (v2.3, Matrix Science) following previous procedures [22]. Relative abundances for methylated and unmethylated peptide ions of interest were monitored using extracted ion chromatograms (XICs) [22]. XICs were obtained using Thermo Xcalibur 2.2 SP1.48; mass ranges were set as the theoretical *m*/*z* for the monoisotopic peak of the peptide ion of interest ±10 ppm. MS data were manually interrogated to confirm that no co-eluting peptide ions contributed to the XICs derived from the peptide ions of interest.

2.4. Analysis and comparison of methyltransferase sequences

YJR129C ortholog search was performed using BLASTp (NCBI) using default parameters against the Swiss-Prot database. Pairwise protein sequence alignment between YJR129C and FAM86A was

Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Source
Wild type (BY4741)	*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	EUROSCARF
ΔEFM2	Δ*EFM2*::kanMX4 in BY4741	EUROSCARF
ΔYJR129C	ΔYJR129C::kanMX4 in BY4741	EUROSCARF
Δ*EFM2*ΔYJR129C	Δ*EFM2*::hphNT1, ΔYJR129C::kanNMX4 in BY4741	This study
Δ*EFM2*ΔYJR129C, overexpressing EF2	Above, with the pAG426GAL-*EFT1*-6×His-Tag plasmid	This study

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